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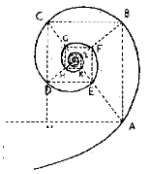

DOTTORATO IN MEDICINA MOLECOLARE

E TRASLAZIONALE

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Role of Jagged in multiple myeloma-associated angiogenesis

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RIASSUNTO

Il mieloma multiplo è una neoplasia delle plasma cellule ancora incubabile, caratterizzata dall'accumulo di cellule tumorali nel midollo osseo. Durante la progressione dalla fase di Gammopatia monoclonale a significato incerto (MGUS) a mieloma multiplo diverse modifiche avvengono sia nelle plasma cellule sia nel microambiente del midollo osseo. È stato infatti dimostrato che le cellule di mieloma presentano la deregolazione della via di segnalazione di Notch causata dall'aumento dell'espressione dei ligandi Jagged1 e Jagged2. L'aumentata espressione di Jagged2 si riscontra anche durante la fase di MGUS, invece, Jagged1 risulta iper-espresso solo nella fase di mieloma multiplo. I ligandi Jagged possono attivare la via di segnalazione di Notch nelle cellule vicine, inducendo quindi un'attivazione di Notch non solo tra le stesse cellule di mieloma ma anche nelle cellule del microambiente midollare quali osteoclasti e cellule stromali.

Un'altra importante caratteristica della progressione del mieloma multiplo è l'aumento dell'angiogenesi nel midollo. L' angiogenesi è una caratteristica comune e peculiare di molti tumori poiché è fondamentale per la sopravvivenza e la crescita del tumore ed inoltre è sfruttato come via preferenziale per la formazione di metastasi. Diverse evidenze indicano che l'angiogenesi si sviluppa di pari passo con la progressione del mieloma multiplo. Infatti, le cellule endoteliali di pazienti affetti da mieloma mostrano un differente fenotipo rispetto a quelle derivanti da pazienti affetti da MGUS o rispetto a cellule sane. L'endotelio derivante da soggetti con mieloma multiplo ha un'aumentata capacità migratoria e organizzativa in saggi condotti *in vitro* ed inoltre esprime in misura maggiore fattori angiogenici.

L'angiogenesi è un evento complesso controllato da diversi fattori solubili e da diverse vie di segnalazione. Tra questi il fattore di crescita dell'endotelio vascolare (VEGF) e la via di segnalazione di Notch svolgono un ruolo fondamentale nella formazione e nell'organizzazione dei vasi di nuova formazione.

Da queste premesse iniziali si sviluppa questo lavoro che mira a studiare il ruolo dei ligandi Jagged1 e Jagged2 nella stimolazione dell'angiogenesi associata al mieloma multiplo. Sono stati considerati tre eventi associati alla progressione dell'angiogenesi che sono: l'adesione delle cellule endoteliali ad una matrice extracellulare, la migrazione e l'organizzazione di strutture simil-tubulari. Per questo studio sono state utilizzate tre linee cellulari umane di mieloma multiplo nelle quali l'espressione dei ligandi Jagged1 e 2 è stata silenziata con il conseguente decremento dell'attivazione della via di segnalazione di Notch.

L'effetto del silenziamento dei ligandi Jagged è stato studiato da due diversi punti di vista. È stata valutata la modulazione del potenziale angiogenico dei fattori solubili rilasciati dal mieloma multiplo usando il *medium* condizionato delle cellule di mieloma. Tutti i saggi effettuati mostrano che il silenziamento dei Jagged nelle cellule di mieloma causa un decremento del potenziale angiogenico dei *medium* condizionati. Inoltre, abbiamo valutato la presenza e l'eventuale modulazione di VEGF ed abbiamo dimostrato che i ligandi Jagged e quindi la via di segnalazione di Notch, è in grado, nelle cellule di mieloma, di regolare la produzione di VEGF che è uno dei principali fattori che regolano il processo di angiogenesi.

Inoltre, abbiamo valutato l'eventuale attivazione di Notch nelle cellule endoteliali mediate contatto diretto con le cellule di mieloma esprimenti o meno i ligandi Jagged.

Presumendo che il contatto diretto tra cellula di mieloma e cellula endoteliale avvenga nel momento in cui i vasi neoformati raggiungono la massa tumorale e che questo influisca sull'organizzazione vascolare, abbiamo sviluppato un saggio di co-coltura tra cellule di mieloma e cellule endoteliali per valutare l'effetto dei ligandi Jagged nella modulazione dell'organizzazione endoteliale.

I risultati ottenuti indicano che i ligandi Jagged hanno un ruolo essenziale nel promuovere l'organizzazione dell'endotelio. Per avere ulteriore conferma di questo effetto, lo stesso saggio è stato condotto sfruttando una forma solubile di Jagged1. In tal modo abbiamo potuto escludere l'influenza derivante dai fattori solubili rilasciati dalle cellule di mieloma e il dato così ottenuto conferma il ruolo centrale di Jagged1 nella stimolazione diretta dell'endotelio di cui promuove l'organizzazione.

Nella seconda parte del lavoro, abbiamo valutato se l'interazione tra mieloma e cellula stromale fosse in grado di aumentare il potenziale angiogenico dello stroma. Infatti, diverse evidenze indicano che le cellule di mieloma hanno la capacità d modellare il comportamento delle cellule circostanti incluse le cellule stromali di midollo, aumentando la loro capacità di sostenere il tumore promuovendone la crescita, la sopravvivenza e inducendo lo sviluppo di meccanismi di resistenza farmacologica. Abbiamo deciso di studiare in particolare le cellule stromali poichè esse sono tra le maggiori produttrici di fattori solubili pro-tumorali alcuni dei quali coinvolti nella progressione dell'angiogenesi come il VEGF.

Per questo scopo è stato sfruttato un sistema di co-cultura tra la linea cellulare umana di stroma midollare, HS5, e le 3 linee cellulari mieloma silenziate o meno per i ligandi Jagged. Il potenziale angiogenico dei *medium* condizionati delle co-culture, confrontato con quello derivante dalla coltura singola di cellule stromali, è stato valutato nei tre saggi di adesione, motilità e organizzazione endoteliale.

I nostri risultati confermano che I ligandi Jagged del mieloma attivano nello stroma la via di segnalazione di Notch e stimolano una maggiore produzione di VEGF sia rispetto alla coltura singola che rispetto alla co-cultura in assenza dei Jagged.

Il *medium* condizionato derivante dalla co-cultura in presenza di un'attiva segnalazione di Notch induce un aumento nell'adesione, nella motilità e nell'organizzazione dell'endotelio, caratteristiche che si riducono notevolmente nella stimolazione con *medium* condizionato derivante da co-culture in assenza dei jagged.

L'effetto pro-angiogenico dei ligandi Jagged è ulteriormente stato confermato utilizzando un modello embrionale di *Zebrafish*. Questo modello ha il vantaggio di essere di facile manipolazione e permette di ottenere una elevata riproducibilità; inoltre, risulta essere meno oneroso rispetto ad un modello murino che comporta tempi di sperimentazione più lunghi. Nel nostro modello di *Zebrafish*, l'iniezione di cellule di

mieloma esprimenti i ligandi stimola la formazione di numerose ramificazioni vascolari dal plesso di vasi intestinali preesistenti. Al contrario, l'assenza dei Jagged nel mieloma riduce notevolmente questo effetto.

Infine, abbiamo cercato di ricapitolare i risultati ottenuti *in vitro* ed *in vivo* per verificare se il meccanismo pro-angiogenico attivato dai ligandi Jagged potesse essere efficace anche nei pazienti affetti da mieloma multiplo. L'ultima parte del presente lavoro si concentra sulla valutazione dell'effetto dei Jagged nell'angiogenesi tumorale in biopsie umane di midollo osseo.

Sono stati identificati 3 gruppi di pazienti con mieloma multiplo in base al loro grado di infiltrazione: i pazienti con infiltrazione di cellule di mieloma minore del 20% sono stati classificati come basso grado; con infiltrazione dal 21% al 50% come grado medio e con infiltrazione superiore al 51% come grado alto.

Le analisi immunoistochimiche hanno mostrato che Jagged1 ed HES6 aumentano parallelamente al grado di infiltrazione delle cellule di mieloma, all'espressione di VEGF e alla quantità di nuovi vasi (cellule positive al marcatore CD34). Questi risultati sono coerenti con le precedenti evidenze ottenute sia *in vitro* che *in vivo* che indicano che i ligandi Jagged svolgono un ruolo pro-angiogenico.

Inoltre, le analisi sulle biopsie di midollo sembrano suggerire che l'effetto angiogenico è limitato ad uno dei due ligandi, in particolare Jagged1, poiché Jagged2 presenta alti livelli di espressione in tutti i gradi di infiltrazione e non mostra alcuna correlazione con l'espressione di VEGF e con la presenza di vasi.

In conclusione, questo studio mostra il ruolo centrale della via di segnalazione di Notch nell'attivazione dell'angiogenesi tumorale mediata dalla iper-espressione dei ligandi Jagged espressi dal mieloma e suggerisce che il ruolo principale è svolto da Jagged1. Ciò conferma ulteriormente il ruolo chiave dei ligandi Jagged espressi dalla cellula di mieloma nel riprogrammare il microambiente circostante per promuovere la progressione del tumore stesso.

In particolare, questo lavoro attribuisce ai ligandi Jagged un ruolo anche nella stimolazione dell'angiogenesi, che si aggiunge ad altri effetti tra cui il supporto alla crescita tumorale, l'induzione delle lesioni ossee e lo sviluppo di resistenza a trattamenti farmacologici contribuendo così a fornire un razionale per l'uso di ligandi Jagged come bersagli terapeutici promettenti nel trattamento di pazienti con mieloma multiplo.

ABSTRACT

Multiple myeloma (MM) is an incurable malignancy of plasma cells which accumulate within the bone marrow (BM). During progression from monoclonal gammopathy of undetermined significance (MGUS) to active MM, different modifications occur in plasma cells and BM microenvironment. Among the others, MM cells display Notch pathway dysregulation due to the up-regulation of two of its ligands, Jagged1 and Jagged2. Up-regulation of Jagged2 occurs during MGUS phase, while Jagged1 is upregulated overt MM. Jagged ligands may trigger Notch signaling in the nearby cells, therefore leading to the aberrant activation of Notch pathway within MM cells and also in the surrounding BM resident cells. Up to now, the outcome of this activation has been studied in the same tumor cells, osteoclasts and bone marrow stromal cells (BMSCs).

Another key feature of MM progression is the increased levels an angiogenesis. Angiogenesis is a hallmark of different tumors and a crucial event for tumor growth and survival, moreover it represents a preferential way for metastasis formation. Previous evidences indicate that angiogenesis develops in accordance with MM progression. Indeed, endothelial cells (ECs) from MM patients display a different behavior if compared with ECs from MGUS patients or healthy ECs. Indeed, MMECs show increased capability to organize, migrate and express angiogenic factors.

Angiogenesis is a complex event controlled by many soluble factors and signaling pathways. Among them vascular endothelial growth factor (VEGF) and Notch signaling play crucial roles in guiding new vessels formation and stabilization.

Starting from these premises, this work aims to study the role of MM-derived Jagged1 and Jagged2 in modulating ECs behavior focusing on three events occurring during the angiogenic process. The events are EC adhesion to the extracellular matrix, migration and tube-organization. Three human multiple myeloma cells lines (HMCLs) have been used in which the expression of Jagged1 and Jagged2 has been knockdown with a subsequent Notch pathway downregulation.

The effect of Jagged silencing has been studied under two different points of view. The modulation of the angiogenic potential of MM-derived soluble factors has been evaluated by stimulating ECs with conditioned media (CM)

All performed assays show that Jagged silencing in MM cells decreases the CM ability to stimulate EC adhesion, motility and tube organization. We focused our attention to the molecular player released in the CM and found that MM-derived Jagged ligands are able to modulate MM-derived VEGF, a key angiogenic factor.

We also assessed if MM-derived Jagged could activate the angiogenic Notch signaling in the adjacent ECs by direct cell-cell contact. We reasoned that direct interaction between MM cells and ECs could occur when new vessels reach MM cells, and specifically in the phase of vessel network organization, therefore we set

up co-culture system of ECs with HMCL and assessed the effect of Jagged silencing in MM cells. Results indicated that Jagged was essential for HMCLs to promote tube organization. To provide a definitive confirmation of the role of Jagged in this process as activator of Notch signaling in ECs through direct contact, tube formation assay was performed using a soluble form of Jagged1, thereby excluding the effect of angiogenic factor release from ECs. Our results indicated that the only stimulation with Jagged1 is sufficient to induce an angiogenic effect promoting EC tube organization.

In the second part of this work, we explored the possibility that MM-derived Jagged could promote of the angiogenic potential of BMSCs, specifically in the stimulation of the three steps of angiogenesis analyzed before. Indeed, several evidences indicate that MM cells can shape the surrounding cell behavior, including BMSCs, potentiating their ability to enhance MM proliferation, survival and drug resistance. We focused our attention on BMSCs since these cells secrete many soluble pro-tumor factors, some of which are involved in angiogenesis, such as VEGF.

To this aim, we set up a co-culture system of HS5, a BM stromal cell line, and HMCLs silenced or not for Jagged ligands and analyzed the angiogenic potential of the conditioned medium.

Our results indicate that MM-derived Jagged trigger Notch pathway in BMSCs as well as the secretion of VEGF; on the contrary the effect was lost when Jagged1 and 2 were silenced in HMCLs.

Consistently, the conditioned medium of BMSCs stimulated with MM cells increased adhesion, motility and tube organization in ECs, while angiogenesis was not supported if BMSCs were cultured with HMCL silenced for Jagged ligands.

The angiogenic effect of MM-derived Jagged has also been confirmed exploiting an embryo zebrafish model of MM. This model allows a simpler and more replicable transplantation and results less expensive compared to a mouse model which requires longer time for development and results acquirement. The injection of MM-expressing Jagged stimulates sprouting angiogenesis from the sub-intestinal plexus while the absence of Jagged strongly impairs this effect.

Finally, we tried to recapitulate the principal results obtained in vitro and in vivo to verify if the pro-angiogenic mechanism activate by MM-derived Jagged could be effective also in MM patients. Thereby, the last part of this work is focused on the evaluation of Jagged effect on tumor angiogenesis in human BM biopsies. To address this issue, three groups of patients were identified according to their MM infiltration grade: Low grade for infiltration lower than 20%; medium grade for infiltration from 21% to 50%; High grade for infiltration higher than 51%.

Immunohistochemical analyses showed that Jagged1 and HES6 increased in parallel with MM cell infiltration grade, VEGF expression and the amount of new vessels (CD34+ cells). These results are consistent with previous in vitro and in vivo evidences indicating that Jagged ligands play an angiogenic role by stimulating

the angiogenic potential of both MM cells and BMSCs. In addition, the analysis on BM biopsies further suggests that angiogenic effect is restricted to one of the two ligands, Jagged1, since Jagged2 is expressed at high levels in all BM biopsies at all the infiltration grades and does not show correlation with VEGF expression or new vessel formation.

In conclusion, this study shows the central role of Notch pathway activation in myelomatous BM to activate tumor angiogenesis mediated by the up-regulation of Jagged in MM cells and suggests that a main role is played by Jagged1. These results further support the key role of MM-derived Jagged ligands in the pathological communication through which MM cells may reprogram the surrounding microenvironment to further promote tumor progression. Specifically, this work adds angiogenesis to other effects previously reported, including MM cell growth, bone destruction, intrinsic and BMSC-induced drug resistance, and thereby contributes to provide further evidence for the use of Jagged ligands as promising therapeutic targets in the treatment of MM patients.

INDEX

INTRODUCTION

1. THE NOTCH SIGNALING PATHWAY

Notch is a single-pass transmembrane receptor belonging to a signaling pathway highly conserved during the evolution and active in different biological processes. The evidence of the Notch gene existence backs to genetic studies on *Drosophila melanogaster*. J. Dexter (1914) and T.H. Morgan (1917) observed a mutant fruit fly with notched wings. The Notch gene owes its name to the notched phenotype of *Drosophila*.

Nowadays, it is well established that, in mammals, Notch signaling is one of the crucial pathways during both development and adulthood in orchestrating cells differentiation, tissue homeostasis regulation and stem cell maintenance (1). The central role of Notch pathway is also confirmed by the great amount of diseases associated to mutations of the different Notch isoforms, including Alagille syndrome (2, 3) and Tetralogy of Fallot (4) carrying Jagged1 mutation, spondylocostal dysostosis with Dll3 mutations, Aortic valve disease with mutated Notch1 (5, 6). Diseases with adult onset such as Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), display Notch3 mutations (7), as well as solid (8) and hematological malignancies (1, 9). Alagille syndrome, Aortic valve disease and CADASIL show autosomal dominant inheritance.

Regarding mammals, Notch signaling pathway counts 4 receptor isoforms (Notch1 to 4) and two classes of ligands: Delta-like (Dll1, 3 and 4) and Jagged family (Jagged1 and Jagged2). The *trans*-interaction between the ligand, expressed by the signal-sending cell, and the receptor, on signal-receiving cell, leads to two proteolytic cleavages (on sites S2 and S3) on receptor intracellular portion and this event releases the intracellular portion of Notch (ICN). It translocates into the nucleus where it acts s as transcriptional factor and modulates target genes expression.

1.1 Structure of Notch receptors

Notch receptors (Figure1A) are single-pass transmembrane proteins composed by different domains: from N-terminal, in the extracellular portion, there are the Epidermal growth factor (EGF)-like repeats. This domain is responsible for ligand interaction and it varies among the isoforms for the different number of repeats (29-36). Within this domain, repeats 11-12 are involved in *trans*-interaction, thus in signaling activation; while, repeats 24-29 lead to signaling inhibition due to their involvement in *cis*-interaction. EGF-like repeats are followed by a Negative Regulatory Region (NRR) which consists of Lyn-Notch repeats (LNR) and a Heterodimerization domain (HD). NRR avoids Notch activation in absence of ligand bond (10); it prevents erroneous activation, hampering the first proteolytic cleavage, in site S2, by maintaining a specific conformation which change only after ligand bond. The extracellular portion is linked by the Transmembrane domain (TM) to the C-terminal intracellular part which consists of RBPj (Recombination Signal Binding Protein

for Immunoglobulin Kappa J Region) association module (RAM) domain, 3 Nuclear Localization Signals (NLS) divided by 7 ankyrin repeats (ANK) and ends with a motif rich in proline, glutamic acid, serine and threonine known as PEST sequence. RM domain is the key part of the receptor for its transcriptional activity. Thus, RAM domain is able to recognize the mammalian transcriptional factor RBPj and to promote proteins complex assembly in collaboration with ANK repeats, which are fundamental to recruit other nuclear proteins to form the activated transcriptional complex. The NLS domains are responsible for trafficking into the nucleus and PEST sequence regulates receptor stability. This sequence is associated with protein with short half-life (11) and is a signal peptide for protein degradation by polyubiquitination and subsequent proteasomal degradation (12).

1.2 Structure of Notch pathway ligands

The two families of ligands (Figure1B), Delta-like and Jagged, are both single-pass transmembrane proteins and display extracellularly, an N-terminal portion involved in receptor binding and activation (13). Dll and Jagged ligands differ in the extracellular portion for the number of EGF repeats: Jagged ligands differ for the higher number of repetition respect to Dll ligands, and for the cysteine rich region (CR) which is absent in Dll ligands.

Except of Dll3, the intracellular part of the remaining ligands contains all lysine residues which work for ligand signaling activity and ligands Jagged1, Dll1 and 4, display also a PDZL (PSD-95/Dlg/ZO-1)–ligand motif required for interaction with cytoskeleton. This domain is important to induce in receiving cells key effects including cell migration (14, 15), cell adhesion (16), and oncogenic transformation (17).

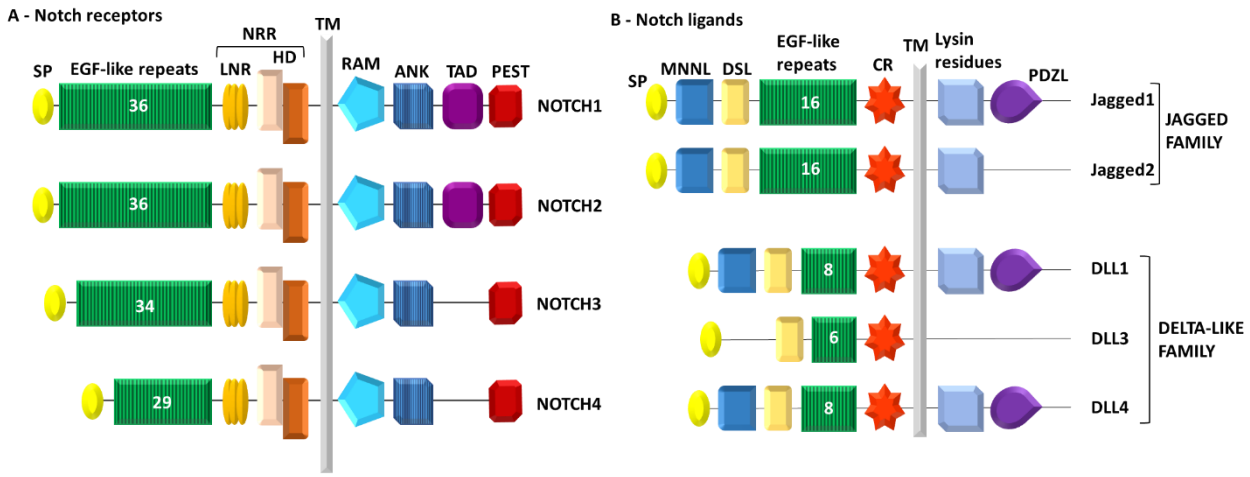


Figure1-Notch signaling pathway: receptors and ligands domains. A) Notch receptors and B) Notch ligands belonging to Jagged and delta-like families with their domains. ANK, ankyrin repeats. CR, cysteine rich region. DSL, Delta/Serrate/LAG-2 domain. EGF, epidermal growth factor. HD, heterodimerization domain. LNR, Lin-Notch repeats. MNNL, Notch ligand N-terminal domain. NRR, Negative Regulatory Region. PDZL, [(PSD-95/Dlg/ZO-1)–ligand motif]. PEST, proline(P),glutamic acid(E),serine(S) and threonine (T) domain; RAM, Rbpj- association module domain; SP, signal peptide; TAD, transactivation domain; TM, transmembrane domain.

1.3 Notch signaling pathway activation: intracellular Notch as transcriptional activator

The engagement of Notch receptor on signal-receiving cells by its ligand expressed by sensing cells leads to conformational changes, which exposes the extracellular S2 site. As illustrated in Figure2, this site is recognized by A Disintegrin and metalloproteinase 10 (ADAM10) which is able to generate a truncated form still anchored to the plasma membrane. The truncated Notch receptor is subsequently recognized and cleaved by the γ-secretase complex, releasing ICN. The γ-secretase complex is composed by different subunit proteins (18) and recognizes the second cleavage site, S3, located among TM and RAM and its proteolytic activity releases ICN from cell membrane (10). ICN can translocate into the nucleus where recognizes RBPj on sequences of target genes (10). RBPj acts as repressor in collaboration with other proteins including histone deacetylase (HDAC), histone demethylase KDM5A (19) and (20) KyoT2. (20). Once arrived into the nucleus, ICN is able to remove the co-repressors; in this way, a tertiary complex is formed together with Master mind-like protein (MALM) which binds to co-activators such as the histone demethylase LSD1 and PHF8, histone acetyltransferase, PBAF nucleosome remodeling complex subunit BRG1 and AF4p12 (21) leading to Notch target genes transcription.

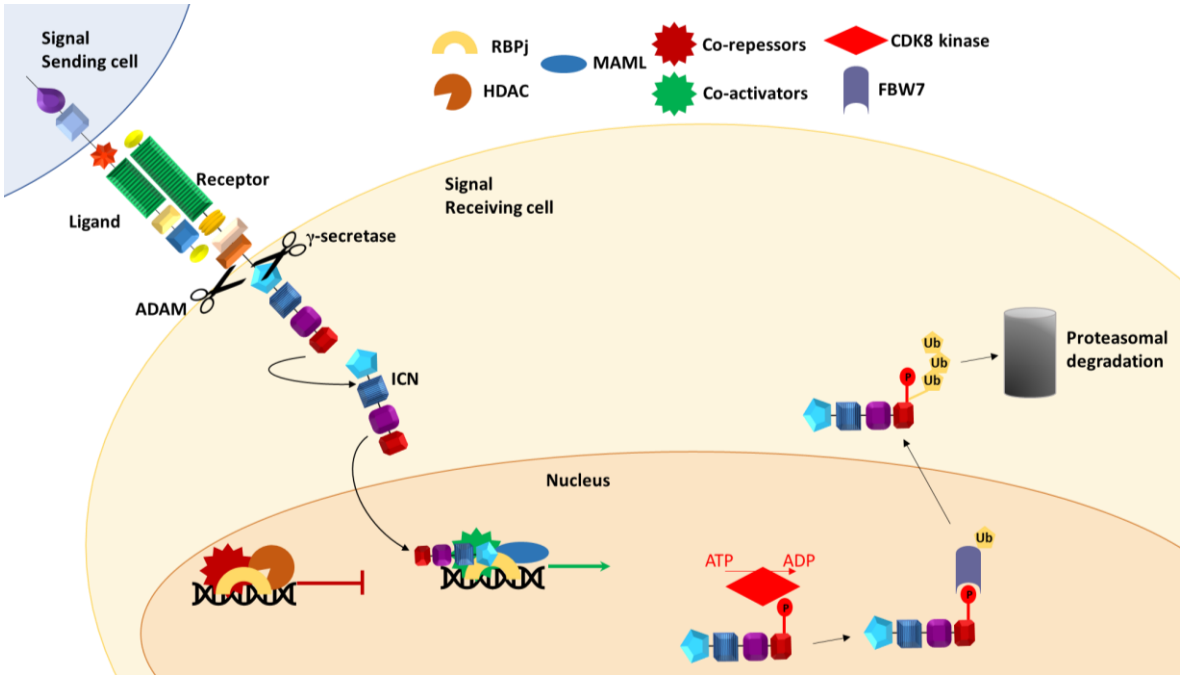


Figure2-Notch signaling pathway activation: receptors and ligands engagement brings to cleavages of Notch receptor at site S2 by ADAM and at site S3 by γ-secretase. The double cleavage leads to ICN released and translocation into the nucleus where it binds to RBPj, removes co-repressors which include HDAC and recruit co-activators and MAML. The tertiary complex ICN-RBPj-MAML acts as transcriptional activator on Notch target genes. ICN is modified with phosphorylation by CDK8, subsequent ubiquitination by FBW7 and is brought to proteasomal degradation.

Notch target genes can be ubiquitous or tissue-specific and include basic helix-loophelix (bHLH) transcriptional modulators as the Hairy and enhancer of split (*HES*) family of genes (22) and Hairy-related transcription factor (*HEY*) genes (22), that act as transcriptional repressors involved in multipotent maintenance during development and adulthood cell fate decision (22). Other important Notch targets are: NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) (23); the positive regulator of cell cycle Cyclin D1 (24). The negative regulator p21 which inhibits Cyclin-dependent Kinases (CDKs) (25); the transcription factor c-Myc, a well-known proto-oncogene overexpressed in many tumors such as leukemias and lymphomas (26) and the phosphatidylinositol-3,4,5- trisphosphate 3-phosphatase, Phosphatase and tensin homolog (PTEN), a common tumor suppressor protein that inhibits AKT activation. Mutations in PTEN are associated to increased cell proliferation and reduction of cell death (27). After gene transcription, Notch is degraded by a cascade mechanism. Indeed, ICN is phosphorylated by CDK8 Kinase (28) on PEST domain that is recognized by E3 ubiquitin ligase FBW7. This protein catalyzes ubiquitin addition directing ICN to proteasomal degradation (29).

1.4 The Notch signaling pathway in cancer: oncogene or tumor suppressor?

It is well known that the Notch signaling pathway plays a crucial role in development and adulthood homeostasis and that it is a master regulator of self-renewal and differentiation in many tissues and cell

types. During fetal and adult hematopoiesis, Notch signaling pathway is tightly regulated. In particular it is well known its involvement in T- and B- lymphocyte maturation. Notch1 receptor is essential in T-cell lineage commitment and Notch2 is a fundamental receptor in marginal zone B-cells differentiation within the spleen (30). Moreover, Notch signaling is strongly involved in hematopoietic stem cells (HSCs) generation, vascular development and arterial vessels density. Angiogenesis is strongly correlated with HSCs development as HSCs arise from dorsal aorta and umbilical arteries (31). Another role of Notch pathway concerns stromal cells. Indeed, within the bone marrow (BM), Notch signaling is activated in HSCs thank to the interaction with the bone marrow stromal cells (BMSCs) that express Notch ligands allowing thereby regulating HCSs maintenance and/or differentiation in mature cell subpopulations (31).

Due to its involvement in lymphoid and myeloid cells generation, it is not surprising that Notch pathway is often related to hematological malignances. These can be divided into precursor-derived and mature subtypes depending on the cell of origin. The first subtypes are characterized by aggressive progression with rapid cell proliferation rate while the second generally display slow development and partial maturation phenotypes (32). In this wide spectrum of malignances, Notch can be recognized as oncogene but, in some cases, as tumor suppressor pathway.

T-acute lymphoblastic leukemia (T-ALL) is characterized by T progenitor cells clonal expansion and in 60% of all cases Notch pathway displays mutations mainly in *Notch1* gene. A low percentage of patients (involved in translocation t(7;9) with TCRB locus (33) and resulted in constitutive pathway activation. Another kind of mutation occurs in PEST domain that loses its function due to nonsense or truncating mutations that do not allow ICN proteasomal degradation through FBXW7 (34, 35).

In acute myeloid leukemia (AML) the role of Notch pathway is not deeply characterized but it seems to be a tumor suppressor pathway because it is silenced in patients primary samples and its reactivation brings to cell cycle arrest and apoptosis induction in AML cells (36).

In mature B-cells neoplasms such as Hodgkin lymphoma and Burkitt lymphoma, chronic lymphocytic leukemia (B-CLL) and multiple myeloma (MM), Notch pathway plays an oncogenic role (31). Indeed, receptors and ligands overexpression leads to aberrant signaling and pathway activation. Moreover, these types of cancer may induce Notch pathway activation in normal cells of the tumor microenvironment leading to pro-tumoral modifications that involve stromal and endothelial cells (31).

2. MULTIPLE MYELOMA ONSET

Multiple Myeloma (MM) is a B-cells malignancy that represents the 13% of all hematological tumors. This type of cancer displays a late onset during adulthood and the average age at diagnosis is around 66 years (37). Although recent progresses in discovering novel drugs, MM is still an incurable disease with a survival

rate of 49% at 5 years (http://seer.cancer.gov/csr/19752015). MM displays symptoms that are fatigue and bone disease that is characterized by bone pain and osteolytic skeletal lesions. Other symptoms are anemia and immunodeficiency, and decreased renal function related to immunoglobulin (Ig) aberrant production and subsequent precipitation.

MM is characterized by clonal expansion of malignant antibody-producing plasma cells within the BM. MM is a progression phase deriving from an asymptomatic preliminary stage known as monoclonal gammopathy of undetermined significance (MGUS) (38), as shown in Figure3. MGUS is totally asymptomatic and shows low level of cancer cells infiltration in the BM, low M protein in the circulation and the absence of bone lesions (38). The asymptomatic expansion of clonal plasma cells can be detected by clinical exams assessing the percentage of clonal B-cells (<10% in MGUS).

Overt MM can be subdivided into two classes, smoldering multiple myeloma (SMM), still asymptomatic, and active MM. During the progression from MGUS to SMM and MM, malignant plasma cells interact with BM microenvironment that provide an important contribution to tumor maintenance and survival.

Finally, MM cells can become totally independent from BM microenvironment and migrate toward extra-medullary sites (plasmacytoma) or diffuse in the circulation (plasma cell leukemia-PCL). PCL can be classified as primary or secondary. Secondary PCL occurs I patients with relapsed or refractory MM. While, primary PCL occurs as *de novo* tumor. The incidence in Europe is estimated as 0.04/100,000 persons per year, ranging between 2-4% of MM patients (39).

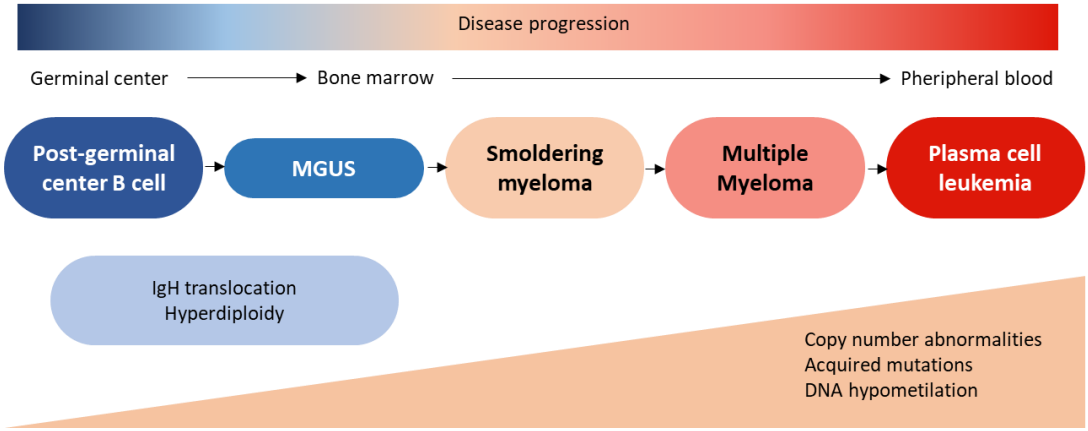


Figure3-Initiation and progression of Multiple myeloma. Disease arise from post-germinal center B-cells and develops in the asymptomatic MGUS, which start to acquire genetic mutations such as translocation that involve IgH locus. From MGUS phase to MM plasma cells reside within bone marrow where undergo clonal expansion. During the last phase called plasma cell leukemia, malignant cells escape from BM and start circulating in blood. During progression, tumor cells increase number of genetic alterations including novel mutation of important signaling pathway.

2.1 Pathogenesis of Multiple Myeloma and therapies

All the stages of MM progression are characterized by genetic mutations in MM plasma cells resulting in alterations of fundamental signaling pathways (40).

Genetic modifications that occur in malignant plasma cells during MM progression, exploiting a peculiar feature of B cells maturation process: genomic rearrangement of Immunoglobulin heavy chain. Heavy chain is rearranged in the germinal center of secondary lymphoid organs following antigen presentation by B cells to activated T cells. This crosstalk triggers T cell signaling mediated by soluble cytokines or ligand-receptor interaction that induce the activation of heavy chain rearrangement in B cells allowing the Immunoglobulin class switch that initially results in the secretion of IgG immunoglobulins (41, 42). After this rearrangement, B cells undergo cell cycle arrest and remain within lymph node as memory B cells or migrate to the BM as long-lived secretory plasma cells.

In MM the tumorigenic process gets advantage of the activation of genomic rearrangement resulting in key translocations. It is plausible that the first translocation can affect the cell cycle machinery since healthy plasma cells within the BM display low proliferative rate, while malignant plasma cells show increased proliferative capability. This set of translocations involves cyclin D family members and IgH locus at 14q32 (43). One of the most frequent translocation is t(11,14) (q13;q32) observed in approximately 18% of MM patients (44), followed by t(4,14)(p16;q32) with 13-15% prevalence (45, 46). Other few common translocations that involve IgH locus are t(14;16)(q32;q23), t(6;14) and t(14;20)(q32;q11) with a frequency lower than 5% (47). All the above mentioned translocations cause the dysregulation of cyclin D. Indeed, t(11;14)(q13;q32) directly induces the overexpression of cyclin D1 (48). t(6;14)(p21;q32) translocation induces cyclin D3 gene at 6p21 (49). t(4;14)(p16;q32) induces the overexpression of MMSET, an histone methyltransferase which modifies the methylation profile of the whole genome increasing the amount of H3K36me2; this, in turn, causes the abnormal expression of cyclin D2 (50, 51). Finally, the translocations t(14;16)(q32;q23) and t(14;20)(q32;q11) cause the upregulation of the Maf family members C-MAF and MAFB that acts as transcription factors increasing cyclin D2 transcription (50, 51).

Hyperdiploidy is a second mechanism occurring during malignant transformation from MGUS to MM and observed in up to 55% of MM patients. In 10% of MM cases, hyperdiploidy occurs together with translocation involving 14q locus (52).

A feature that characterized MM stage compared to MGUS is the increased accumulation of other genetic alterations. There are two oncogenic pathways involved in this process, Ras and Myc. The frequency of point mutations in K-Ras and N-Ras during MGUS to MM progression increases from 7% to 24-27% in MM indicating a correlation between proliferation and the frequency of point mutation (53, 54). During the last stage of PCL it is common a mutation of NFκB which is constitutively activated (55) and another characteristic involves p53 that is inactivated due to e.g. deletion of 17p13 locus.

At diagnosis, MM patients are divided into groups in relation to age, category of risk, presence of comorbidities factors or other features that could impair the effect of the treatment.

For patients at first diagnosis the most used protocol consists in an initial treatment with three classes of compounds that are corticosteroids (dexamethasone), proteasome inhibitors (bortezomib) and immunomodulators (Lenalidomide), known as "VRd" protocol, followed by autologous hematopoietic stem cells (HSC) transplantation. Due to high risk of toxicity or fatal complication of HSC transplantation, this protocol is used in patients younger than 65 years. After "VRd" protocol, patients undergo treatments to induce the release of stem cells in the circulation. After stem cells collection, a high dose chemotherapy is used to kill tumor cells. The final step of this protocol is the injection of autologous stem cells back to patients to allow BM regeneration. This protocol is not always applicable, and it is not a conclusive therapy, nevertheless it increases the overall survival. Daratumumab is another drug used in combination with lenalidomide and dexamethasone in people who have already received one prior therapy.

The older, non-transplantable group of patients are treated with "VRd" regimen for longer periods.

For MM treatment, different classes of drugs have recently been released. Melphalan is often used as chemotherapeutic agent; beside thalidomide and its derivative lenalidomide, pomalidomide has been recently released as immunomodulatory agent; finally, another proteasome inhibitor, carfilzomib, has been added to bortezomib.

Despite novel drug and novel protocols, MM patients often develop resistance to treatment due to intrinsic and extrinsic mechanisms (56). Indeed, genetic alteration and/or the supportive role of BM microenvironment may be at the basis of drug resistance mechanisms that cause therapy failure.

The clinical need to overcome drug resistance in MM gave origin to different studies to identify novel strategies that target cell signaling or tumor microenvironment for relapsed or refractory patients.

Among therapies that target cell signaling, HDAC inhibitors promote cell cycle arrest and tumor cells apoptosis by regulating the expression of different signaling pathways interfering with epigenetic mechanisms(57). Another example of molecular targeted therapy is the use of PI3K/AKT/mTOR inhibitors. Phosphatidylinositol 3-kinase (PI3K) mediates proliferative and anti-apoptotic effects in MM cells and display an increased activity in relation to disease progression (58); PI3K activates AKT that, in turn, phosphorylates different downstream targets involved in cell cycle progression, cell growth and apoptosis resistance (59). One of the well-known downstream targets of AKT is the mammalian target of rapamycin (mTOR) that controls cellular growth. mTOR is composed by two multi-subunits complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 activation leads to increase in mRNA translation, thus proteins synthesis and induces proliferation while, mTORC2 regulates cytoskeleton and can work upstream of AKT inducing its phosphorylation (57). Inhibition of mTORC1 can induces a mTORC2 negative feedback loop which

brings to AKT phosphorylation (60). Different drugs targeted PI3K/AKT/mTOR pathway have been tested in combination with other treatments such as bortezomib administration or lenalidomide and dexamethasone therapy. A phase I study demonstrates that the combined therapy is well tolerated by patients and bring to an increased overall response rate (57).

Other promising targetable pathways are Wnt and Notch. These signaling pathways are commonly activated in normal stem cells and can be exploited also by cancer stem cells for maintenance and survival. Thus, drugs targeting these molecular cascades aim to hit tumor cells and cancer stem cells to decrease the percentage of relapsed patients. Moreover, both pathways affect tumor microenvironment. Wnt pathway activity is associated to osteolytic lesions in MM since Wnt activation is triggered by MM cells to promote osteoclast differentiation by suppressing osteoblast (OBL) maturation. As a result, inhibiting Wnt signaling pathway can limit the asymmetry between OBL and osteoclasts (OCL), reducing bone lesions (57).

Notch signaling pathway is active in MM cells and supports proliferation and survival. Notch signaling pathway inhibition by γ-secretase inhibitors shows promising results *in vitro* (57) but the ubiquitously expression of Notch in different tissues can bring to toxicity above all at intestinal level in human.

In MM, BM microenvironment has a fundamental role in supporting MM progression, survival and drug resistance (DR). Thus, it is not surprising that novel strategies against MM target microenvironmental features. These include hypoxia and angiogenesis, among the others. Hypoxia contributes to MM progression and DR development. Hypoxia inducible factor 1α (HIF-1α) is overexpressed in MM cells and its inhibition leads to reduction of tumor burden and bone destruction *in vivo* through a mechanism that involves also inhibition of tumor proliferation and angiogenesis.

Angiogenesis is a second mechanism that can be targeted in MM because it plays a crucial role in disease progression and dissemination. Angiogenesis is controlled by pro- and anti-angiogenic factors and their imbalance toward pro-angiogenic stimuli activate the “angiogenic switch” during the progression from an “avascular phase” of MGUS to a “vascular phase” in active MM (61). Among anti-angiogenic drugs, Bevacizumab is a monoclonal antibody direct against Vascular endothelial growth factor (VEGF), one of the most prominent angiogenic stimulus. Bevacizumab inhibits VEGF action and is mostly used in solid malignancies. It has recently been tested in MM patients in combination with Thalidomide therapy but with disappointing results because of combination therapy leads to similar results obtained with thalidomide in single therapy (57, 62).

2.2 Notch pathway in Multiple Myeloma

The dysregulation of Notch signaling pathway in MM plays a crucial role in tumor progression. Indeed, Notch pathway results dysregulated in many MM patients due to different mechanisms, that still need to be fully elucidated.

In MM cells both receptors and ligands result dysregulated. Indeed, Notch1 and Jagged1 are overexpressed during tumor progression through still unidentified mechanisms. Notch2 activation is associated to the translocation t(14;16)(q32;q21), occurring in approximately 6%. This translocation results in the hyper-expression of the transcription factors C-MAF and MAFB transcription factors that, in turn, trans-activate Notch2(63). Finally, Jagged2 level may be increased by three different possible mechanisms: promoter hypo-methylation, altered expression of Skeletrophin that is a ubiquitin-ligase necessary for Jagged2 activity and loss of SMRT/NCoR2 corepressor that causes the acetylation of Jagged2 promoter and the subsequence increase of Jagged2 transcription (64).

Dysregulation of Notch receptors and ligands may have different outcomes: first of all, it may cause the aberrant activation of Notch signaling pathway in the same tumor cells. This effect stems from the instauration of homotypic interactions among adjacent MM cells. On the other side, tumor cell-derived Jagged ligands may trigger Notch signaling in BM resident cells such as e.g. BM stromal cells (BMSCs) by heterotypic interactions.

The importance of Notch signaling pathway in MM cells has been confirmed by the evidence that its inhibition causes a decrease in MM cells proliferation and increases apoptotic cells and sensitivity to drugs (65, 66). Moreover, Notch activation in MM cell can also increase their ability to migrate in the BM (66), the numbers of osteolytic bone lesions generation (67), development of drug resistance (65) and thus in disease relapse.

The Notch-mediated communication of tumor cells with surrounding healthy cells can contribute to the development of pro-tumoral mechanisms in MM. It has been demonstrated that BMSC-derived Dll1 expression can activate Notch signaling pathway by engagement of Notch2 in MM cells. This interaction causes the overexpression of proteins involved in drug resistance indicating that BMSCs are actively involved in MM cells maintenance (68, 69). Another important communication activity reported is mediated by MM-derived Jagged2 that is able to trigger Notch activity in MM cells themselves (70) resulting in the release a tumor supportive cytokines, including interleukin-6 (IL6), VEGF and Insulin-like growth factor 1 (IGF1) (71, 72). IL6 is one of the major players involved in MM cells growth and it is also implicated in drug resistance instauration *in vitro* (71); VEGF has a proliferative effect on MM cells and activates the angiogenic process in endothelial cells(73); IGF1 supports MM cells growth and are involved in resistance to Bortezomib(74). Finally, Notch signaling affects bone disease development by stimulating the release of osteoclastogenic soluble factors including RANKL (75). RANKL bound to RANK receptor in osteoclast (OCL) progenitors activates NFκB pathway that in turn results in the increase of Notch2 receptor expression. Notch2 interacts with Jagged ligand overexpressed by MM cells activating Notch pathway in OCL progenitors (75). Moreover, it has been demonstrated that Notch pathway activation in osteoblasts (OBLs) inhibits their differentiation. This effect has been observed also in *in vivo* mouse model in which a Notch inhibitory treatment with γ-secretase inhibitors re-activate OBL maturation (76).

3. ENDOTHELIAL CELLS AND ANGIOGENESIS

Within vasculature development, it is possible to recognize two distinct processes, the vasculogenesis and the sprouting angiogenesis. Vasculogenesis is a *de novo* formation process that consists in endothelial cell organization to generate primordial vascular plexus from which veins, arteries and capillaries are remodeled (77). Sprouting angiogenesis, or angiogenesis consists in sprouting and branching of new vessels from pre-existing vessels (77, 78). Angiogenesis sustains tissues growth and function being the way to provide nutrients and oxygen to growing cells and becomes a fundamental process during adulthood in response to increased metabolic tissue demand, inflammation and in pathological conditions such as cancer.

Angiogenesis often occurs when environmental stimuli such as chemokines ad growth factors are produced by hypoxic tissues that need nutrients and oxygen; these stimuli bring endothelial cells (ECs) to modify their quiescent state, become active, migrate and proliferate toward source which produce the attracting factors organizing a network of vessels.

Tumor angiogenesis is a process that characterizes development and progression of solid tumors in which tumor mass displays a hypoxic core. Hypoxic tumor core induces vessels to sprout and attract them; moreover new vessels network represents one of the principal ways for tumor to disseminate and reach through the circulation distant body districts contributing to metastasis formation (78, 79). Recently, different evidences have reported that also in hematological malignancies angiogenesis is stimulated and plays a critical role during disease progression.

3.1 New vessel formation: Tip and Stalk cells and the role of VEGF and Notch pathway

During the stimulation of angiogenesis, ECs undergo to molecular and cellular modifications that induce cell movement, proliferation and cells organization in order to create new sprouting vessels. In this process it is possible to identify two different types of ECs named “tip” and “stalk” cells. These two subtypes of ECs differ at molecular level for gene expression profile (80) and at cellular level for their functions. As illustrated in cartoon in Figure4, tip cells show the formation of long dynamic filopodia which sprout toward an attractive source, that in most cases produces different soluble molecules including VEGF-A. VEGF-A creates a gradient which attracts the tip cells thank to the expression of VEGF Receptor 2 (VEGFR2) on ECs (80).

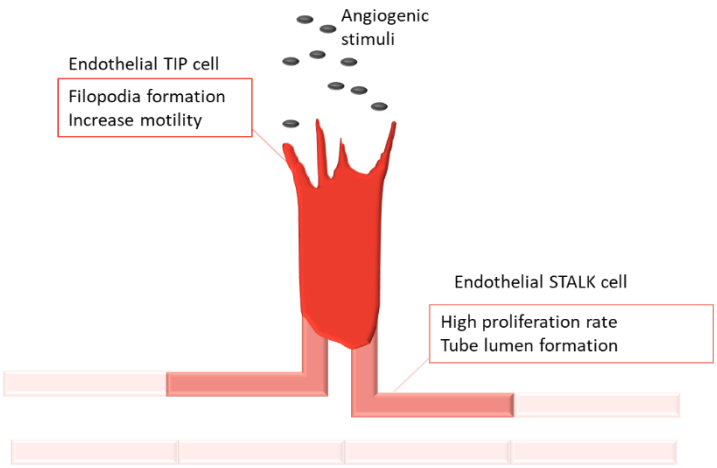


Figure4-Tip and Stalk endothelial cell. Tip cells are cell type able to response to angiogenic stimuli and that drive neo vessel formation. Tip cells are characterized by filopodia formation and a motile behavior. Stalk cells are those cells that form the new vessels proliferating and creating the lumen.

Indeed, tip cells are characterized by the expression of VEGFR2, not expressed in stalk cells, and this characteristic gives to tip cells the ability to guide the newly forming vessel toward the angiogenic source. Stalk cells do not display sprouting process neither migratory capability nor filopodia protrusions, but stalk ECs are characterized by a higher proliferative rate and are deputed to form the vascular lumen (80).

The crosstalk between tip and stalk cells gives rise to an organized vessels tree that has the main function to re-establish optimal levels of nutrients and oxygen within tissues. VEGF signaling pathway is the driving molecular mechanism that converts quiescent state of ECs into active one and is the first molecular player driving ECs differentiation in tip or stalk phenotype (81). Notch pathway plays a key role in EC phenotypes assignment through two of its ligands, Jagged1 and Dll4 (81). As shown in Figure5, also Notch pathway displays a particular expression pattern within tip and stalk ECs. After VEGF stimulation, tip ECs upregulate Dll4 increasing its expression. This event leads to Notch-mediated interaction with stalk cells in which Notch2 receptor is cleaved bringing to ICN translocation into the nucleus and thus Notch pathway activation. Moreover, stalk ECs increase Jagged1 expression that affect Notch activation within tip cell, acting as negative regulator of Notch pathway in adjacent tip cells.

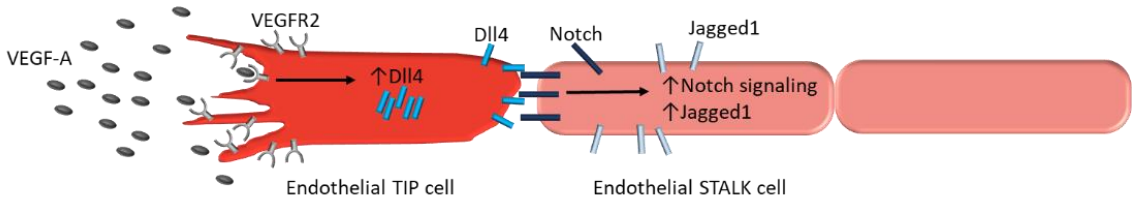


Figure5-VEGF and Notch signaling pathway cooperation in tip and stalk endothelial cells selection. Tip cells respond to VEGF-A stimulation thank to their expression of VEGFR. The activation of VEGF signaling pathway leads to upregulation of Dll4 in tip cells that are able to interact with Notch2 in stalk cells This interaction induces the activation of Notch signaling pathway in stalk cells that upregulate Jagged1 expression. Dll4 activation induce Notch inhibition in tip cells.

Tip cell phenotype is characterized by filopodia and by the gene expression of Dll4, VEGFR2, VEGFR3, Platelet derived growth factor β (PDGFβ) and C-X-C chemokine receptor type 4 (CXCR4) (82). VEGFR2 enables tip ECs to respond to VEGFA gradient guiding the angiogenic process toward the source of angiogenic stimuli. Concerning the role of stalk cells, they take direct contact with the tip cell that influences their gene expression profile and the subsequent proteins expression. Stalk ECs are characterized by the instauration of adherent junction among themselves and interact with extracellular matrix (ECM) proteins to ensure stability of newly formed sprout and form lumen of the new vessels (82).

The importance of VEGFA-VEGFR2 axis has been highlighted *in vivo*, exploiting mouse models knockout (KO) for VEGF ligands or other members of the VEGF signaling pathway. Indeed, the VEGF-A KO mouse model dies between embryonic days 8,5 (E8,5) and E10,5 (82, 83).The same condition is reported for Dll4 KO mouse indicating that both VEGF-A and Dll4 are fundamental for angiogenesis. About the importance of Notch signaling pathway in angiogenesis other evidence come from Notch1 or Jagged1 KO mice which die around E10 and, as occurs for VEGF signaling pathway KO mice, display vascular disfunction and remodeling abnormalities (82). The involvement of Notch pathway in the tip/stalk phenotype decision is related also to the action of FRINGE. FRINGE is a glycosyltransferase involved in post-translational modification of EGF-like repeats within Notch receptors extracellular portion (84). FRINGE action can regulate Notch activation in response to ligand interaction, increasing Dll-mediated activation and reducing it upon Jagged binding (85, 86). It has been shown that loss of FRINGE leads to the acquisition of hybrid tip/stalk cell phenotype associated to small blood vessels development typical of tumoral angiogenesis (87).

3.2 Tumoral angiogenesis

The involvement of Notch signaling pathway in angiogenesis and in EC homeostasis in clear also due to human hereditary diseases in which mutations of Notch family members induce vascular disorders. Indeed, Notch3 mutation in CADASIL (7) and Jagged1 mutation in Alagille syndrome (3) induce abnormalities in cardiovascular system. The balance between VEGF and Notch pathways is often deregulated in tumoral

microenvironment in which angiogenesis plays a fundamental role for tumor cells. Tumoral angiogenesis represents the main process that supports tumor growth and survival and new blood vessels formation is the preferential way for metastasis formation.

Within a tumoral microenvironment, angiogenesis is a complex process which involves different players such as tumor cells, endothelial cells, tumor-surrounding cells (as stromal cells and cells belonging to immune system) and secreted factors (88). As for physiological angiogenesis, the tumoral one depends from the production of pro- and anti-angiogenic factors. In tumoral microenvironment, an imbalance toward angiogenic factors leads to the so-called angiogenic switch (89) that corresponds to a transition of endothelial cells from a quiescent to an active state (89). The angiogenic switch arises from the necessity of tumor cells for nutrients and oxygen. Thus, hypoxic microenvironment activates hypoxia inducible factor (HIF) which induces transcription of angiogenesis-related genes including VEGF (88). This condition modifies the balance between anti- and pro-angiogenic factors stimulating angiogenesis. VEGF is the major and well-known angiogenic factor that stimulates ECs to organize a novel vessels network and in tumoral microenvironment the resulted networks often appears disorganized and with an increased permeability (88).

Also in tumoral angiogenesis, Notch pathway plays a key role thus, it is involved in tip/stalk cells phenotype decision and is often deregulated in tumor cells. In this context many studies focus on Dll4-Notch engagement as key molecular interaction in tumor-associated angiogenesis (88). Indeed, it has been shown that Dll4 inhibition in cancer cell line shows antitumoral activity in xenograft mouse model (88) and mice treated with Dll4 inhibitors show overgrowth of tumoral vessels that are not able to support tumor growth due to hyper-sprouting and subsequent dysfunctional perfusion capability of vessels (90). *In vitro* inhibition of Dll4 in ECs causes cell cycle arrest, increased apoptosis and reduction of capability to organize tubes on Matrigel (91).

Dll4 on ECs dialogues with adjacent cells engaging Notch1 receptor. Indeed, during physiologic angiogenesis VEGF-A upregulates Dll4 in tip cells that activates Notch pathway in stalk cells through Notch1 receptor. Stalk cells in an autocrine manner, negatively regulate VEGFR2 decreasing their capability to response to VEGF-A stimulation. It has been shown that Nocth1 deletion in ECs causes increasing tip cells formation (91). Thus, interaction between Dll4 and Notch1 can regulates tip/stalk cells phenotype decision.

On the other way, Jagged1 stimulation of ECs leads to fewer tip cells formation and increases branches (88). The opposite role covered by Dll4 and Jagged1 suggests a reciprocal regulation of these two ligands during physiologic angiogenesis and development of functional vessels network. Indeed, Benedito *et al*. showed that mice with Jagged1 overexpression in ECs display enhanced angiogenesis (92). The balance between Dll4 and Jagged1, that involves also the action of FRINGE (as reported in first chapter), is often lost during tumor progression, associated to the generation of a disorganized network of thick vessels in which tip and stalk cell phenotypes seem to be not well distinguishable. Different works show that Dll4 inhibition leads to the formation of vessels with low perfusion unable to support tumor growth (80, 93). On the contrary, Dll4-mediated activation of Notch pathway in ECs reduces tumor angiogenesis but develops vessels with higher diameter and higher perfusion that well sustain tumor growth (80). Jagged1 antagonizes Dll4 action, thus,

the overexpression of Jagged1 induces the stabilization of vessel wall enhancing tumor growth showing a proangiogenic function within tumoral microenvironment(94).

Due to the uncertain condition within tumoral microenvironment and the frequent dysregulation of Notch pathway in tumoral contexts, it has been also proposed the existence of an intermediate phenotype called tip/stalk that induces the formation of new sprouts, but cells display lower migration capability and fewer filopodia (87). This hybrid tip/stalk cells phenotypes can be seen as an advantage for tumor cells because it confers higher plasticity to ECs and induces fast and irregular vessel branches that better supply oxygen and nutrients to tumor cells (87). As shown in Figure6, the new vessels network formed by ECs with hybrid phenotype appears chaotic with excessive number of small vessels with many sprouts and branches and this is in line with the aberrant vasculature observed in many different tumors.

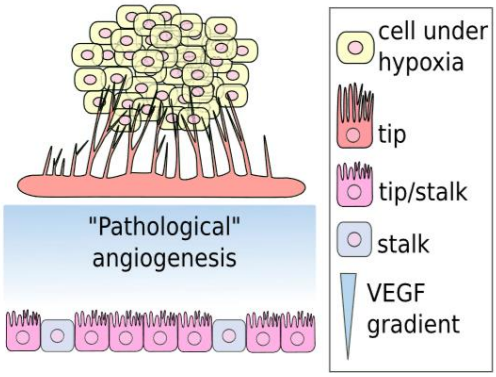


Figure6 -Hybrid endothelial cells phenotype. The picture represents the hybrid tip/stalk cells phenotype that can develop during tumoral angiogenesis. This pathological vasculature is characterized by a great number of small blood vessels that can rapidly reach and nourish growing tumor mass (Adapted from Boareto *et al.* (87)).

4. BONE MARROW MICROENVIRONMENT IN MULTIPLE MYELOMA

During last years, different works have highlighted the importance of microenvironment in healthy tissues homeostasis and above all in tumor development. The reciprocal influence between tumoral and surrounding cells is a fundamental aspect in cancer progression. MM cells settle in the BM that represent MM microenvironment. Here, malignant plasma cells tightly interact with the different types of surrounding cells and with non-cellular components. Well represented cell types are BMSCs, a fibroblast-like cells, ECs, often in a quiescent state before MM stimulation, hematopoietic stem cells (HSCs), progenitor cells, immune cells and cells deputed to remodeling bones (OBL and OCL). Concerning the soluble factors, the ECM provides mechanical support to cells and tissues and its composition changes depending on resident cells or in relation to specific events such as inflammation or tumor onset. BM ECM is mainly composed by proteins such as collagens, laminins, fibronectin produced in higher amount by resident stromal cells and by trapped soluble factors that can be released upon matrix degradation induced by the enzymatic activity of proteases.

Several component within the BM microenvironment can act to stimulate tumor cells and support tumor cell survival and proliferation, motility, invasion and drug resistance (95).

An important feature of MM cells is the ability to shape the surrounding microenvironment, educating BM resident cells to acquire a pro-tumoral behavior. For instance, MM cells can induce the production of BMSC-derived soluble factors that in turn promote tumor growth. Among the main factors released by BMSC are tumor necrosis factor α (TNF-α), VEGF, MMPs, Angiopoietin-1, IL6 and IGF1. In MM, TNF-α is a cytokine that plays a dual role, by stimulating MM cell proliferation and production of BMSC-derived IL6. Of note, IL6 is a key cytokine in MM cell biology (56). Finally, VEGF is the primary pro-angiogenic molecules mainly produced by BMSCs (96).

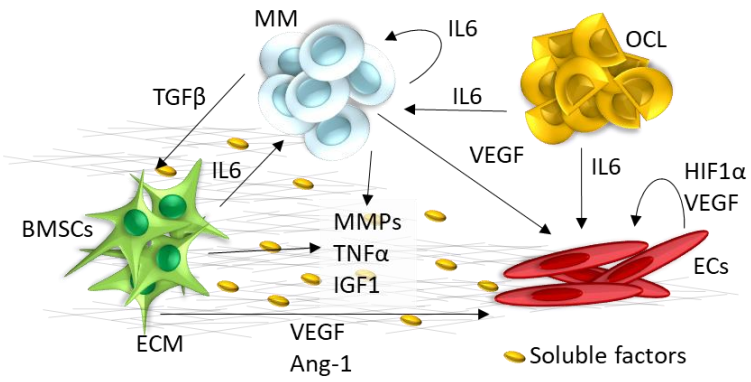


Figure7-Bone marrow microenvironment within multiple myeloma. Some of the principal cellular players in BM microenvironment are BMSCs, MM cells, OCL and ECs. The other fundamental component is the ECM in which many soluble factors are trapped and are used as signal molecules by cells. The complex microenvironment is due to a cross-talk among all cell types that reside within BM and that create a dense network of communication through direct contact and above all through released soluble factors.

4.1 Angiogenesis in multiple myeloma

As indicated in Figure3, during MGUS to MM progression, malignant plasma cells homing within the BM where they expand. Moreover, it has been described that disease progression is associated with increased angiogenesis that correlates with poor prognosis. Indeed, several previous works highlight that MM patients-derived bone marrow endothelial cells (BMECs) acquire a tumorigenic behavior in the presence of MM cells, but not with MGUS cells indicating that angiogenesis is a peculiar event that discriminates the asymptomatic from the symptomatic stage of disease progression (97, 98).

MM cells can stimulate ECs through soluble factors. IL6 is the major MM-promoting factor that is involved in angiogenesis stimulation due to its capability to induce VEGF production by MM cells and BMSCs (99, 100).

VEGF is one the most important angiogenic factor and its production is up-regulated in myeloma and stromal cells upon MM-BMSCs direct contact (101). Another factor involved in MM-related angiogenesis is FGF2. It is detectable in BM of MM patients (72) and Bisping and colleagues have shown that FGF2 induces a dose-dependent increase of BMSC-derived IL6 (72).

In this context, BMECs display a pro-tumoral behavior that plays a role in supporting MM cells. BMECs from MM patients produce higher amount of soluble factors including CXC chemokines such as CXCL8 (IL8), CXCL12 (stromal derived growth factor 1 α - SDF1α) and CCL2 than quiescent and healthy ECs (102). These chemokines bind to receptors expressed on MM cells and stimulate proliferation and chemotaxis (102) that are fundamental features of tumor progression.

Angiogenesis development which occurs during progression from MGUS to MM and the remodeling of BM microenvironment are also confirmed exploiting Vk*MYC transgenic mouse model that may fully recapitulate the progression along the MM stages. This mouse model carries a dysregulation of MYC induced by its sporadic activation by exploiting the physiological somatic hypermutation that occurs in B cells in germinal center (103). This mouse model well recapitulates features of MM disease progression including serum M-spike, progressive organs damage and the angiogenic switch. Indeed, it has been shown that Vk*MYC BM microenvironment has higher levels of angiogenic factors including VEGF that correlate with serum M-spike quantification, as compared to age-matched wild type mice (103).

4.2 Bone marrow stromal cells-mm interaction

BMSCs plays a crucial role in supporting MM cells and MM is able to shape BMSCs machinery in order to develop a pro-tumoral environment. The interaction between BMSCs and MM cells derives from cell to cell contact mechanism and a cross talk through soluble factors. Adhesion of tumor cells to BMSCs activates many pathways including Notch that causes the upregulation of proteins related to cell cycle and apoptosis (104). Stroma-myeloma interaction leads to the creation of a paracrine loop in which cell-cell contact induces the upregulation of stromal-derived IL6 that, as mentioned before, is the principal pro-tumoral cytokine and stimulates VEGF production (73). MM cells-derived Jagged ligands over-expression is exploited to interact with BMSCs in which Notch pathway is up-regulated. This heterotypic interaction leads also to stromal up-regulation of some soluble factors including IL6 and IGF1 that promote MM proliferation and survival (65, 105). BMSCs after MM-derived “education” modify their proteins expression pattern increasing the production of Angiopoietin-1 (ANG1), VEGF, basic FGF (bFGF), platelet-derived growth factor (PDGF) and hepatocyte growth factor (HGF) (106). BMSCs are also involved in MM protection from drug-induced apoptosis. Our group has recently demonstrated that BMSCs prevent MM drug-mediated apoptosis and this mechanism is mediated by Notch. This evidence highlights the importance of Notch signaling pathway in cell-cell crosstalk in MM microenvironment.

AIM OF THE WORK

Multiple myeloma (MM) is a still incurable plasma cells malignancy that represents 13% of hematologic malignancies. Its progression starts in the asymptomatic phase named MGUS and progresses to active MM characterized by malignant plasma cells accumulation within the bone marrow (BM).

During disease progression, MM cells increase genome instability that indirectly results in the increased activity of the Notch signaling pathway. This pathway regulates the differentiation choices during embryonal and adult tissues development by mediating the communication between adjacent cells. In MM cells, Notch signaling activation is mainly due to Jagged1 and Jagged2 over-expression that may also trigger Notch signaling in the neighboring stromal cells.

Tumor angiogenesis is key in the progression from MGUS to MM, since it provides oxygen and nutrient supply and an escape way for MM cell dissemination; the role of Notch pathway in angiogenic sprout is widely recognized. Therefore, the evidence of the dysregulation of Jagged1 and Jagged2 in MM cells prompted us to verify if MM cell-derived Jagged1 and Jagged2 can play a role in supporting MM-associated angiogenesis, by triggering the angiogenic potential of Notch signaling in ECs.

Accordingly, this work aims to investigate the role of MM cell-derived Jagged1 and Jagged2 in angiogenesis promotion. To address this issue, we studied three principal events that underlie new vessels formation as illustrated in Figure8, i.e. EC adhesion to the extracellular matrix, EC motility and tube organization.

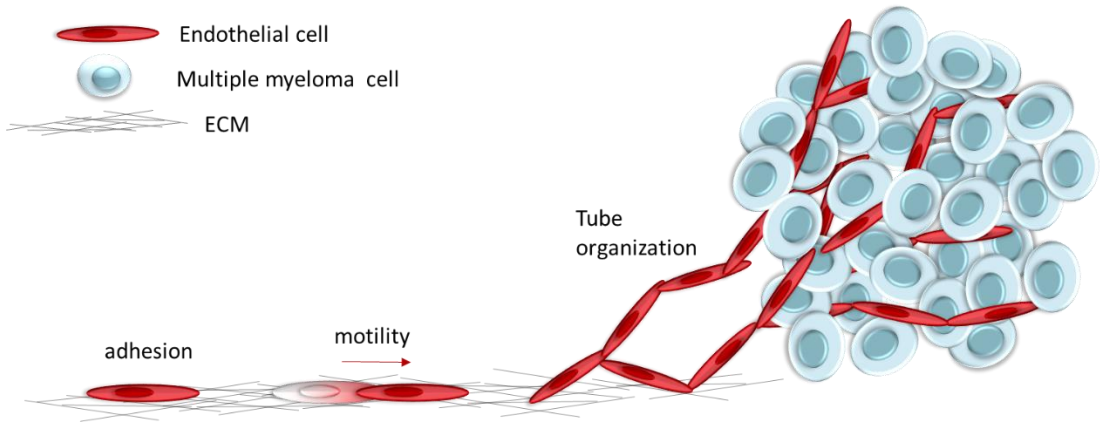


Figure8-Schematic representation of the three events considered to study MM-derived Jagged1 and Jagged2 effects on endothelial cells. ECM: extracellular matrix

Moreover, the evidence that MM cells are able to modify the behavior of healthy cells of tumor microenvironment and that bone marrow stromal cells (BMSCs) are one of the major producers of soluble angiogenic factors, prompted us to investigate the possible involvement of Notch signaling activation induced by MM-derived Jagged in enhancing the pro-angiogenic potential of BMSCs.

MATERIALS AND METHODS

1. CELL LINES

Human multiple myeloma cells (HMCLs) were RPMI8226 (ATCC® CCL-155™), OPM2 (ACC-50) and U266 (ATCC® TIB-196™) cell lines and were cultured in RPMI1640 (Lonza) medium supplemented with 10% FBS (fetal bovine serum) (Euroclone), 100U/ml P/S (penicillin/streptomycin) (Microgen) and 2mM L-glutamine (Microgen). Cell lines were seeded at $3X10^5$ cells/ml every 48h. HPAECs (human pulmonary arterial endothelial cells) (ATCC® PCS-100-022™) were cultured in Vascular Basal medium (ATCC® PCS-100-030™) supplemented with Endothelial cells Growth Kit-VEGF (ATCC® PCS-100-041™) following manufacturer instruction. ECs were seeded in 0,2% gelatin pre-coated dishes at a final concentration of $3X10^3$ cells/cm^2 when confluence reach 80%. The human bone marrow stromal cell line HS5 (ATCC® CRL-11882™) was previously infected with pGIPZ vector carrying GFP protein (GFP^+HS5). This cell line was cultured in DMEM (Lonza) supplemented with 10% FBS, 100U/ml P/S and 2mM L-glutamine and puromycin 1µg/ml every 48h and was used for co-culture experiments with MM cell lines. Phoenix cell lines was cultured in supplemented DMEM. NIH3T3 (ATCC® CRL-1658™) were used to mimic stromal compartment in co-culture experiments to discriminate stromal contribution by qRT-PCR. This cell line was culture as GFP^+HS5 cell lines without puromycin.

1.2 HMCLs infection with inducible lentiviral vector

1.2.1 Virus production and collection

For virus production Phoenix-Ampho cell line was seeded in T25 flask at $2,5X10^5$ cells/ml in a final volume of 4 ml. The day after cells were transfected using $CaCl_2$ method. A mix was prepared freshly with reagents reported in Table1, solution was vigorously mixed and equal volume of HeBs (HEPES buffered saline solution) was added. After incubation 3' RT (room temperature), it was added to 3ml of fresh medium on Phoenix cells. 24h after transfection medium was completely changed and virus was collected 2 days later. For virus collection, medium was centrifuges at 800 RPM (revolution per minute) 4' and filtered with 0,45nm filter (BD); it was used fresh or stored at -80°C.

pTRIPZ vector	600ng/mL
Packaging mix	15µL
$CaCl_2$ 2M	62µL add drop by drop at the end
H_2O	To volume
Final volume	500µL

Table1: reagents used for CaCl2 transfection method on Phoenix Ampho

1.2.2 HMCLs infection

HMCLs were seeded in 6-well plate at $2X10^6$ cells/well. To infect cells, solution containing virus (1,5 ml/well) was mixed with Polybrene 10µg/ml and IL-6 20ng/ml. After 48h medium was changed and puromycin 1µg/ml was added for infected cells selection.

Two cell lines were obtained and were named HMCLs^{shSCR} and HMCLs$^{shJ1/2}$ (reported in section Results as HMCLs^{SCR} and HMCLs$^{J1/2KD}$). Infected HMCLs were cultured with puromycin 1µg/ml every 48h and shRNA induction were stimulated with doxycycline 3mg/ml added daily for 72h. Conditioned medium (CM) was collected at 72h and mixed in ratio 1:1 RPMI1640 supplemented with 2%FBS, 100U/ml P/S, 2mM L-glutamine for experiments on ECs.

1.3 HMCLs transfection and co-culture experiments

HMCLs were transfected using two siRNAs (short interfering RNAs) direct against Jagged1 and Jagged2 at final concentration of 50nM. Cells were seeded in 24-well plate at $1,5X10^5$ cells/well and were transfected with RNAi Lipofectamine (Thermo Fisher Scientific) following manufacturer instruction. HMCLs transiently transfected were named HMCLs^{siSCR} and HMCLs$^{siJ1/2}$ (reported in section Results as HMCLs^{SCR} and HMCLs$^{J1/2KD}$). Transfection protocol were repeated twice, at day1 and day3. Tranfected cells were collected 8h after transfection for co-culture system with HBMSCs until day5 (schematic protocol was shown in table2). For co-culture GFP$^+$HS5 cell line was seeded at day2 to obtain 1:1 ratio with myeloma cells, when co-culture started. Cells were maintained 2 days and then CM was collected for experiments with ECs. Same procedure was followed for co-culture with NIH3T3 cell line and at day5 stromal cells were used for RNA extraction and qRT-PCR.

	Day1	Day2	Day3	Day5
Morning	HMCLs siRNA transfection		HMCLs siRNA transfection	HMCLs collection for qRT-PCR
8h after transfection		$HS5^{GFP+}$ (or NIH3T3) cells seeding in 24-well plate	HMCLs and stromal cells co-culture	NIH3T3 collection for qRT-PCR
				CM collection from co-culture with $HS5^{GFP+}$

Table2: scheme of the protocol used for HMCLs J1 and J2 silencing with siRNAs and co-culture system with HBMSCs and with NIH3T3.

2. ADHESION ASSAY

HPAEC cells were seeded in 6-well plate at $3X10^3$ cells/cm^2. After 24h medium was changed with CM from $HMCLs^{shSCR}$ and $HMCLs^{shJ1/2}$ cultures or from co-cultures, HPAECs were incubated for 24h and a black 96-well plate was coated with 100 µg/ml fibronectin diluted in 0,005M Tris-HCl pH 7.4 O/N 37°C. The day after HPAECs were stained with 5µM Calcein AM 1h at 37°C and were seeded on fibronectin at $2,5X10^4$ cells/well in Fresh-RPMI1640 for 1h in incubator. Fluorescence intensity was read at EnSight Multimode Plate Reader (Perkin Elmer).

3. WOUND HEALING ASSAY

HPAECs were seeded in 48-well plate in order to have a confluent well after 48h. When cells reach confluence, medium was substituted with CM from $HMCLs^{shSCR}$ and $HMCLs^{shJ1/2}$ cultures or from co-cultures and wound was done using a p200 tip. After 24h incubation, HPAECs were washed once with 1XPBS, stained with Blue Comassie and photos were acquired at Zeiss PriomoVert microscope (Zeiss) at 4X magnification. To analyze images, wound area and wound edges distance (calculated as mean distance of 3 pre-decided points) were measured using ImageJ software.

4. MATRIGEL ASSAY

Matrigel reduced (Corning) was defrost O/N on ice at 4°C. The day after, Matrigel was dispensed in a 96-well plate 50µl/well and incubated 1h at 37°C. HPAECs were seeded on Matrigel-coated well at $2x10^3$ cells/well for every experimental condition. Photos were acquired after 24h incubation using Zeiss PrimoVert microscope at 4X magnification. For the analysis, numbers of area, numbers of Branch points and length between two branch points (length arm) were measured by ImageJ software from 3 different photos of each well.

4.1 Matrigel assay with HMMC co-cultured system

For this kind of experiments HMCLs^{shSCR} and HMCLs$^{shJ1/2}$ were used. HMCLs were treated with doxycycline for 72h, harvested and seeded at 4x10^4 cells/well with endothelial cells in ratio endothelium: myeloma = 1:2. Photos were acquired after 24h after HMCLs removal.

4.2 Matrigel assay with conditioned media

HPAEC cells were seeded as reported above using CM from HMCLs^{shSCR} and HMCLs$^{shJ1/2}$ cultures to study myeloma contribution and with CM from HBMSCs-HMCLs^{siSCR} or HBMSCs-HMCLs$^{siJ1/2}$ co-cultures to study stromal contribution in modulating endothelial cells behavior. Photos were acquired after 24h incubation.

4.3 Matrigel assay with Jagged1

For this assay, HPAECs were seeded in Fresh-RPMI1640 with GFs from Endothelial cells Growth Kit-VEGF (condition named positive control), without GFs (negative control) and with soluble Jagged 1 at final concentration of 10µg/ml. Cells were incubated 24h and photo were acquired at Zeiss PrimoVert.

5. FLOW CYTOMETRY INTRACELLULAR STAINING

To carry out flow cytometry experiments, BD FACSVerse was used and experiments analyzed with FACSuite Software (BD, San Jose, CA). Volumes are indicative for 10^6 cells.

Cells were fixed with 100µl of 4%PFA, vortexed and incubated 20' at 4°C. Samples were diluted with 500µl 1XPBS and centrifuged 1500 RPM 5'. After supernatant discharged, pellet was resuspended in 100µl of a solution of 0,2% saponin together with antibody (α-hVEGF PE-conjugated antibody R&D system). Sample with isotype matched antibody was used as control. Primary antibody was incubated 40' 4°C. After centrifugation at 1500 RPM 5', samples were resuspended in 1X PBS and acquired at cytometer.

6. RNA EXTRACTION AND qRT-PCR FOR GENE EXPRESSION ANALYSIS

RNAs were extracted from NIH3T3 cells after 48h culture alone or with HMCLs^{SCR} or HMCLs^{KD} to evaluate VEGF modulation in stromal compartment, from HMCLs^{SCR} or HMCLs^{KD} after 5 days from first transfection and from HMCLs^{shSCR} and HMCLs$^{shJ1/2}$ after 72h of doxycycline induction to confirm Jagged 1 and Jagged2 silencing and subsequent notch signaling pathway downregulation.

6.1 RNA extraction

Total RNA was extracted by TRIzoL® reagent (Sigma-Aldrich). Protocol is optimized for 5X10^5 cells:

- wash cells one with 1X PBS and centrifuge at 1000 RPM 5'
- Lyse cells with 200ul of TRIzol® Reagent.
- Incubate samples for 5' RT
- Add 1/5 (v/v) of chloroform and mix by inversion to homogenize solutions
- Incubate for 15' RT

- Centrifuge at 12000g for 15’ at 4°C.
- Collect aqueous phase into a new tube
- Add ½ (v/v) of 100% isopropanol
- Incubate at RT for 10’
- Centrifuge at 12000g for 10’ at 4°C
- Remove supernatant and wash pellet with 75% ethanol
- Centrifuge at 7500g for 5’ at 4°C and discard supernatant and repeat wash
- discard supernatant and dry samples 5’ RT
- Re-suspend the RNA pellet in RNase-free water
- Proceed to downstream application, or store at –20°C.

RNA was quantified by Nanodrop (ThermoFisher) using 1 µl of RNA and following manufacture’s instruction. RNA was considered usable for gene expression analysis only with 260/280 and 260/230 ratios higher then 1,8 and 1,99.

6.2 Reverse transcription

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

The reaction was settled as follow:

- 1µg RNA
- 1µl of Random primers (25ng/µl)
- H_2O DEPC up to 10µl
- Sample was heated at 65°C for 5’.

The mix was prepared as follow:

- 4µl of 5x RT Buffer
- 4µl dNTPs 10mM (2,5mM each)
- 1µl of RevertAid M-MuLV Reverse Transcriptase (200 U/µl)
- 1µl of H2O DEPC

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

6.3 Semi-quantitative Real-Time PCR

Gene target expression was obtained using the ΔCt method; GAPDH was used as housekeeping gene. Differences in gene expression between two different conditions, e.g. scrambled vs silenced, were determined using the ΔΔCt method. In detail, was applied the following formula $2^{(-\Delta\Delta Ct)}$:

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

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

- ΔΔCt: represents the difference between the ΔCt of the treated sample (in this case the silenced sample) and the ΔCt of the control sample (scrambled sample).

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

The reaction mix for a 96 well plate is the following (final volume of 15 µl):

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

Primers sequences are reported in Table3.

hGAPDH	ACA GTC AGC CGC ATC TTC TT	AAT GGA GGG GTC ATT GAT GG
h18S	GTA ACC CGT TGA ACC CCA TT	CCA TCC AAT CGG TAG TAG CG
hJagged1	TCC GCC TGG CCG AGG TCC TAT	GCC CTG GTT CTG CTT CGG CGT
hJagged2	CCG GCC CCG CAA CGA CTT TT	CCT CCC TTG CCA GCC GTA GC
hHes1	GAT GCT CTG AAG AAA GAT AGC TCG	GTG CGC ACC TCG GTA TTA AC
hHes6	ATG AGG ACG GCT GGG AGA	ACC GTC AGC TCC AGC ACT T
hVEGF	GGG CAG AAT CAT CAC GAA GT	TGG TGA TGT TGG ACT CCT CA
mGAPDH	TTG GCC GTA TTG GGC GCC TG	CAC CCT TCA AGT GGG CCC CG
mHes5	GGC TCA CCC CAG CCC GTA GA	TCG TGC CCA CAT GCA CCC AC
mVEGF	CAC TGG ACC CTG GCT TTA CT	GCA GTA GCT TCG CTG GTA GA

Table3: Primers sequences used for qRT-PCR

7. ELISA ON HMCLs FOR VEGF-A DETECTION

HMCLs were seeded and conditioned media was collect at 72h of induction with doxycycline. Media were stored at -80°C. VEGF-A ELISA kit (Thermo Scientific) was used to detect human VEGF-A in culture media. ELISA was performed following manufacturer instruction.

8. ZEBRAFISH INJECTION

Zebrafish transgenic embryos TG(fli1a:EGFP) were injected with 72h-induced RPMI8226^{shSCR} or RPMI8226$^{shJ1/2}$. HMCLs were stained with CM-Dil (Molecular Probes-Invitrogen) dye, following manufacturer instruction and were injected in 48h post-fertilization (48hpf) embryos into the sub-peridermal (perivitelline) space, close to the developing sub-intestinal venous plexus (SIV). 24h post-injection (24hpi), photos were acquired and total length of neo-vessels (sprouts) from SIV were measured using ImageJ software.

9. IMMUNHISTOCHEMISTRY ON HUMAN BONE MARROW SAMPLES

Human bone marrow samples were collected, and consecutive sections were cut at microtome 3µm thick. Sections were dewaxed, and antigen sites were unmasked using 0.01M citrate buffer pH6 through heat. Slides were incubated with hydrogen peroxide 10' followed by primary antibodies incubation as reported in Table4. For signal detection Novolink™ Max Polymer detection system (Leica) was used following manufacturer instruction. Nuclei are stained with hematoxylin and slides were mounted with coverslip gasses. Kappa light chain and CD34 were stained using Dako OMINS automated platform.

Antibody	Company	Concentration	Dilution	Incubation time	Incubation temperature
Jagged1	R&D system AF1277	0.2 mg/ml	1:100	1h	RT
Jagged2	Santa Cruz Sc-293433	0.1µg/µl	1:200	O/N	4°C
HES6	Abcam ab66461	100µl	1:300	O/N	4°C
VEGF-A	Santa Cruz Sc-152g	200µg/ml	1:800	1h	4°C

Table4: IHC primary antibodies

10. STATISTICAL ANALYSIS AND PERCENTAGE VARIATION AMONG HMCLs EXPERIMENTAL CONDITIONS

All statistical analyses of *in vitro* assays were performed on at least three independent experiments. Statistical analysis on experiments with HMCLs include two groups and were performed with one-tailed Student's t-test; experiments involving BMSCs include 3 or more groups and therefore were evaluated with one-way ANOVA with Tukey's post-test. Statistical analyses were performed using GraphPad Prism 6 software.

For *in vivo* experiments, the minimum size of each group was determined on *a priori* power analysis for a one-way ANOVA with an alpha=0.05 with G-power 3.2 software. The *in vivo* experiments involved 20 embryos divided into two groups injected with scrambled or silenced cells.

Percentage variations among experimental conditions were calculated as follow. For assays with HMCLs scrambled (SCR) and HMCLs knock down (KD) conditions the formula used is: (KD-SCR)/SCRx100. For assays with stromal cells alone (ALONE), co-culture of stromal cells with $HMCLs^{SCR}$ (SCR) and co-culture of stromal cells with $HMCLs^{J1/2KD}$ (KD) conditions two different formulas are used to evaluate differences between ALONE and SCR condition (SCR-ALONE)/ALONEx100 and between SCR and KD conditions (KD-SCR)/SCRx100.

RESULTS

1. MULTIPLE MYELOMA CELL-DERIVED JAGGED LIGANDS AFFECT ENDOTHELIAL CELLS BEHAVIOR

The over-expression of Jagged1 and Jagged2 in multiple myeloma (MM) causes Notch signaling aberrant activation both in MM cell and also in the surrounding microenvironment. Notch signaling pathway contributes to MM cell proliferation, prevents apoptosis and induces the instauration of drug resistance. Moreover, during tumor cell progression from MGUS to MM a massive remodeling of vessels network occurs. This angiogenic switch causes an increase in microvascular density. The process is fundamental for MM growth and survival, moreover new vessels represent a preferential route for metastasis dissemination and the formation of new bone lesions.

Although the hyper-expression of Jagged1 and Jagged2 induces a pro-tumor behavior in different cell populations of the tumor microenvironment such as BMSCs (107) and osteoclasts (75), it is not clear whether MM-derived Jagged1 and Jagged2 can play a role in promoting angiogenesis by affecting endothelial cell behavior.

To study if Jagged ligands may stimulate the angiogenic switch, I have used an inhibitory approach through RNA interference in order to knock down ligands expression in three different human MM cell lines (HMCLs), RPMI8226, U266 and OPM2. For the first set of experiments, HMCLs have been silenced exploiting an inducible lentiviral vector as reported which allows ligands expression temporally controlled by doxycycline administered to cell culture media every 24h.

1.1 Effect on Notch signaling pathway activation

Jagged1 and 2 (J1/2) RNA interference have been obtained on three different HMCLs: RPMI8226, U266 and OPM2. Stable cell lines have been obtained by transducing them with the lentiviral vector pTRIPZ, which contains two short hairpin RNAs (shRNAs), directed against Jagged1 and Jagged2, or the respective scrambled sequences. From now on, the obtained cell lines will be reported as HMCLs$^{J1/2KD}$ or HMCLs^{SCR}. pTRIPZ carries an inducible promoter which may be turned on by administering doxycycline every 24h. After 72h of treatment with doxycycline, I have verified the inhibitory effect of J1/2 shRNAs on the levels of gene expression of the two inhibited ligands and the Notch transcriptional targets HES1 and 6 by semiquantitative real time PCR. As shown in Figure 9, both Jagged ligands are down regulated after 72h of doxycycline treatment along with HES1 and HES6, indicating that Notch signaling pathway has been effectively inhibited in the HMCLs$^{J1/2KD}$. Although Jagged silencing induces a light reduction in HMCLs growth, due to block of cells in G0 phase, and increase apoptotic cells (data not shown), HMCLs$^{J1/2KD}$ and HMCLs^{SCR} was used in equal number for experiments with endothelial cells.

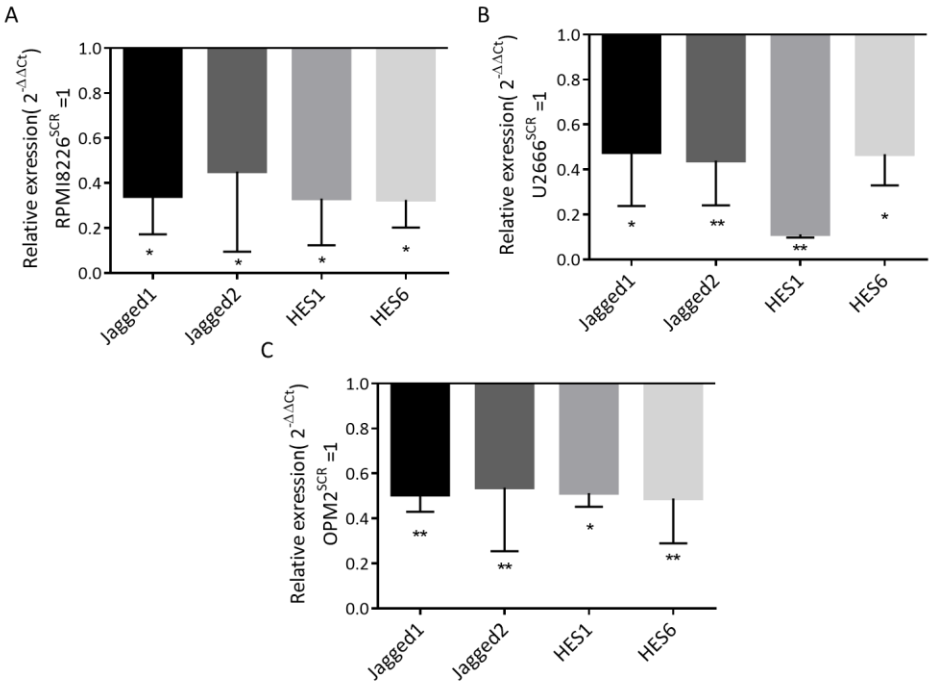


Figure9-Jagged1 and Jagged2 silencing in MM cells lines affects the expression of the Notch target genes HES1 and HES6. (A) RPMI8226; (B) U266; (C) OPM2. Cell lines are treated with doxycycline to stimulate the expression of two short hairpin RNAs (shRNAs) direct against Jagged 1 and jagged2 transcripts (scrambled sequences are used as control). Data are representing as Relative expression variation using GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) for normalization; reported values are calculate as 2-ΔΔCt +/-SD of three independent experiments. Statistical analysis is carried out by One-tailed t test, * is for $p \leq 0.05$; ** for $p \leq 0.01$; *** for $p \leq 0.001$.

1.2 Effect mediated by myeloma-derived soluble factors on endothelial cell adhesion to extracellular matrix

As reported in the Introduction section, to form a complex vascular network, endothelial cells (ECs) undergo different modifications.

This work is focused on three different processes that characterize ECs during the passage from a quiescent state to an active and pro-angiogenic behavior: adhesion, migration and organization of tube-like network.

Initially, I have evaluated the adhesion to the proteins of the extracellular matrix (ECM). This is the first step during which ECs modify the expression of their adhesion molecules in order to anchor to different substrates. This modification can be induced through a stimulation mediated by soluble factors released by tumor cells. Specifically, HMCLs produce a great variety of stimuli acting on surrounding microenvironment and able to influence EC capability to adhere to ECM protein (108).

Since Jagged ligands overexpression may activate Notch signaling pathway in HMLCs and, in turn, this can modify MM cell proteins expression pattern(109), I have evaluated if MM-derived Jagged ligands can influence EC adhesion induced by different soluble factors released in conditioned media, focusing in this chapter on the general angiogenic properties of conditioned media.

At this purpose, conditioned media from HMCLs$^{J1/2KD}$ or HMCLs^{SCR} were used to study the modulation of ECs adhesion to a fibronectin coating. ECs have been treated for 24h with HMCLs-derived conditioned media. After Calcein AM fluorescent staining, ECs are seeded on black 96-well plate coated with fibronectin and fluorescence intensity of vital adherent cells has been evaluated after 1h.

As shown in Figure10, a different basal adhesion capability of ECs can be observed when they are stimulated with CM from different HMCLs, this is likely due to a different pattern of soluble factors produced by HMCLs affecting cell adhesion. More importantly, conditioned media from HMCLs$^{J1/2KD}$ cause a decrease in EC adhesion to fibronectin compared to HMCLs^{SCR}. The decrease of percentage of adherent cells between HMCLs^{SCR} and HMCLs$^{J1/2KD}$ are: 46.4% for RPMI8226 cells; 11.9% for U266 cells; 34.3% for OPM2 cells.

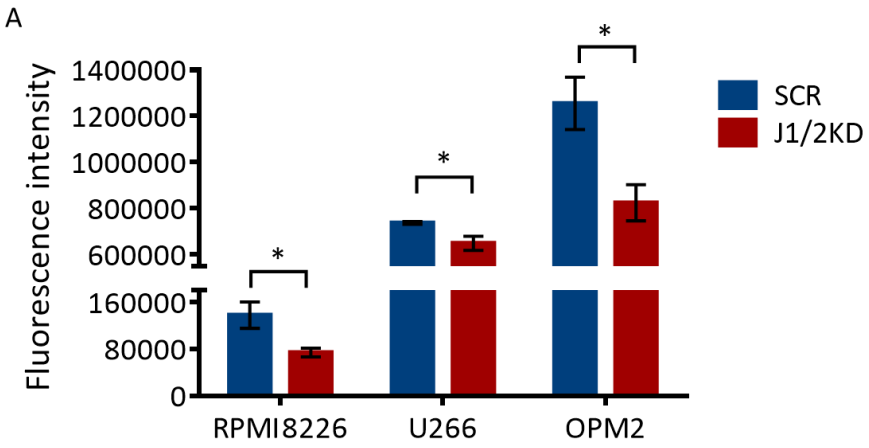


Figure10-Adhesion modulation of ECs treated conditioned media from HMCLs^{SCR} and HMCLs$^{J1/2KD}$. Endothelial cells are stained with Calcein and the intensity of adherent fluorescent cells is detected using a microplate reader. Statistical analysis has been carried out by One-tailed t test, * is for $p \leq 0.05$.

These data indicate that Jagged-mediated activation of Notch signaling pathway stimulates HMCLs to produce soluble factors that can modulate EC adhesion to fibronectin. It should be noted that the ability of the CM from the different HMCLs is differently influenced by Jagged1 and 2 KD, indeed U266 cell line displays a lower difference between scrambled and KD conditions, possibly duo to the production of a reduced amount of adhesive factors under the control of Notch pathway.

1.3 Effect mediated by myeloma-derived soluble factors on endothelial cells motility

Following EC adhesion, the angiogenic process involves ECs movement toward the angiogenic source. This migration is possible thank to the presence of two different types of ECs, tip and stalk cells, which are characterized by a different migratory behavior. Tip cells show filopodia and a marked motility, differently from stalk cells, that represent a proliferative component (82).

As reported in Background section, Notch pathway, together with VEGF, plays a crucial role in the induction of the tip/stalk destiny. Therefore, I have hypothesized that the dysregulation of Notch activity associated to MM cells may contribute to tumor angiogenesis.

To study if Jagged ligands play a role in increasing MM-derived soluble factors stimulating ECs motility, human pulmonary artery endothelial cells (HPAECs) have been plated onto a 48-well plate upon confluence of HPAECs, a wound of 1.48 mm^2 has been done and the 48h-conditioned media from $HMCLs^{SCR}$ or $HMCLs^{J1/2KD}$ have been used to stimulate HPAEC migration for 24h. Images have been acquired through optical microscopy (4x); wound areas and wound edges have been analyzed by ImageJ software.

As shown in Figure11, images (from A to F) demonstrate that conditioned media obtained by $HMCLs^{SCR}$ positively influence EC motility if compared to conditioned media obtained from $HMCLs^{J1/2KD}$. Indeed, the lower level of migration results in an increased wound area in the $HMCLs^{J1/2KD}$ respect to $HMCLs^{SCR}$ of 64.2% for RPMI8226, U266 89% for U266, OPM2 568% for OPM2. Similarly, also the distances between wound edges are increased of 59.5% in RPMI8226, 68.6% in U266; 733% forOPM2.

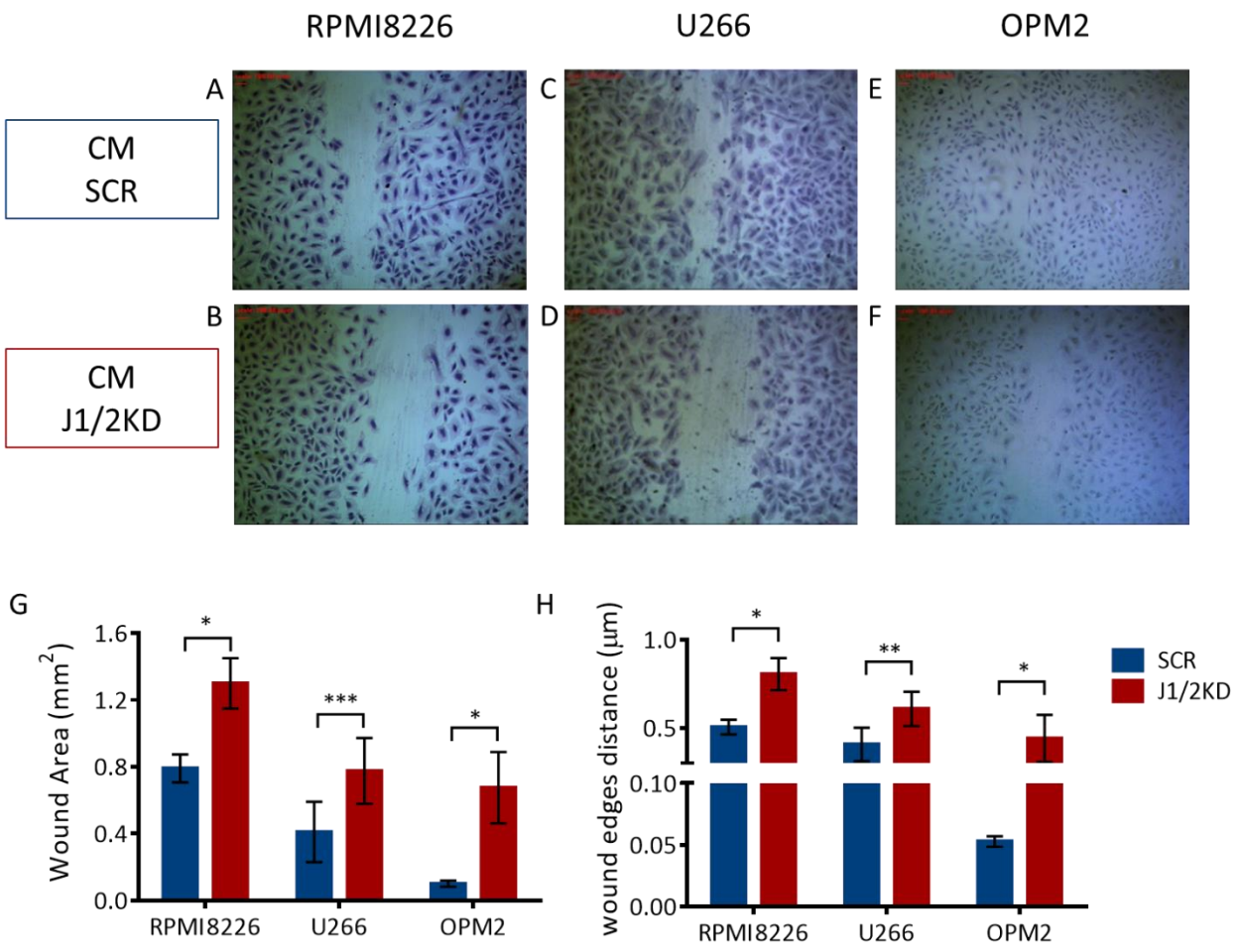


Figure11-Wound healing assay. HPAECs treated for 24h with CM from RPMI8226^{SCR} (A) and RPMI8226$^{J1/2KD}$ (B), from U266 ^{SCR} (C) and U266$^{J1/2KD}$ (D) and from OPM2 ^{SCR} (E) and OPM2$^{J1/2KD}$ (F). Photo pictures at 4X magnification are representative of the obtained results. Graphs show measurements of (G) wound area and (H) wound edges distances (3 measures for each of 3 replicates in each experiment) calculated with ImageJ software. Statistical analysis of three independent experiments is carried out by One-tailed t test, * is for $p \leq 0.05$; ** for $p \leq 0.01$; *** for $p \leq 0.001$.

It should be noted that decrease in the ability of the CM to stimulate the migration of HPAEC is obtained in OPM2$^{J1/2KD}$. We hypothesize that the effect of Jagged ligands knockdown can be emphasized in OPM2 cells since they are characterized by a higher capability to induce EC motility.

1.4 Endothelial cells organization in a grid-like structure

The last step of angiogenesis that I will explore is the ability of ECs to generate a network of vessels toward the source of the angiogenic stimulus.

We reasoned that ECs might be stimulated to organize an interconnected grid by tumor-derived soluble factors and direct contact between ECs and MM cells. Thereby, we investigated the possible role of MM cell-

derived Jagged in ECs organization trying to distinguish between the two possible mechanisms by setting up an *in vitro* tube formation assay on Matrigel-coated wells. This type of assay can recapitulate endothelial cell capability to organize a complex vessels network in a 2D cell system. Matrigel is a mixture of secreted extracellular proteins from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells that can influence cell behavior improving different processes including cell organization. Matrigel used for these experiments is depleted of growth factors in order to avoid any possible interference with EC behavior.

1.4.1 Overall contribution of multiple myeloma cells

A preliminary evaluation of the cumulative effect of Jagged on the release of MM cell-derived soluble factors or the direct contact between MM cells and ECs has been performed by using co-culture systems including HPAECs with HMCLs^{SCR} or HMCLs$^{J1/2KD}$ in a ratio 1:2 maintained for 24h (for details see Materials and Methods). The EC organization obtained in the two experimental conditions displays differences that can be measured using three different parameters: number of areas, number of branch points (BPs; a point from which at least three ramifications take origin) and average length of the arms (arm is tube-like structure between two BPs).

As shown in Figure12, myeloma cells are placed mainly in contact with HPAECs, which in turn, arrange an endothelial network. A quantitative analysis of number of areas, of branch points and length of the arms are summarized in graphs G-H-I. The percentage differences between HMCLs^{SCR} and HMCLs$^{J1/2KD}$ are as follows. number of areas decreases in HMCLs$^{J1/2KD}$ respectively of 33.1% for RPMI8226; 39.2% for U266; 46.2% for OPM2; number of BP decreases of 39.1% for RPMI8226; 43.2% for U266; 55.1% for OPM2; average arm length increases of 33.1% for RPMI8226; 35.6% for U266; 33.2% for OPM2.

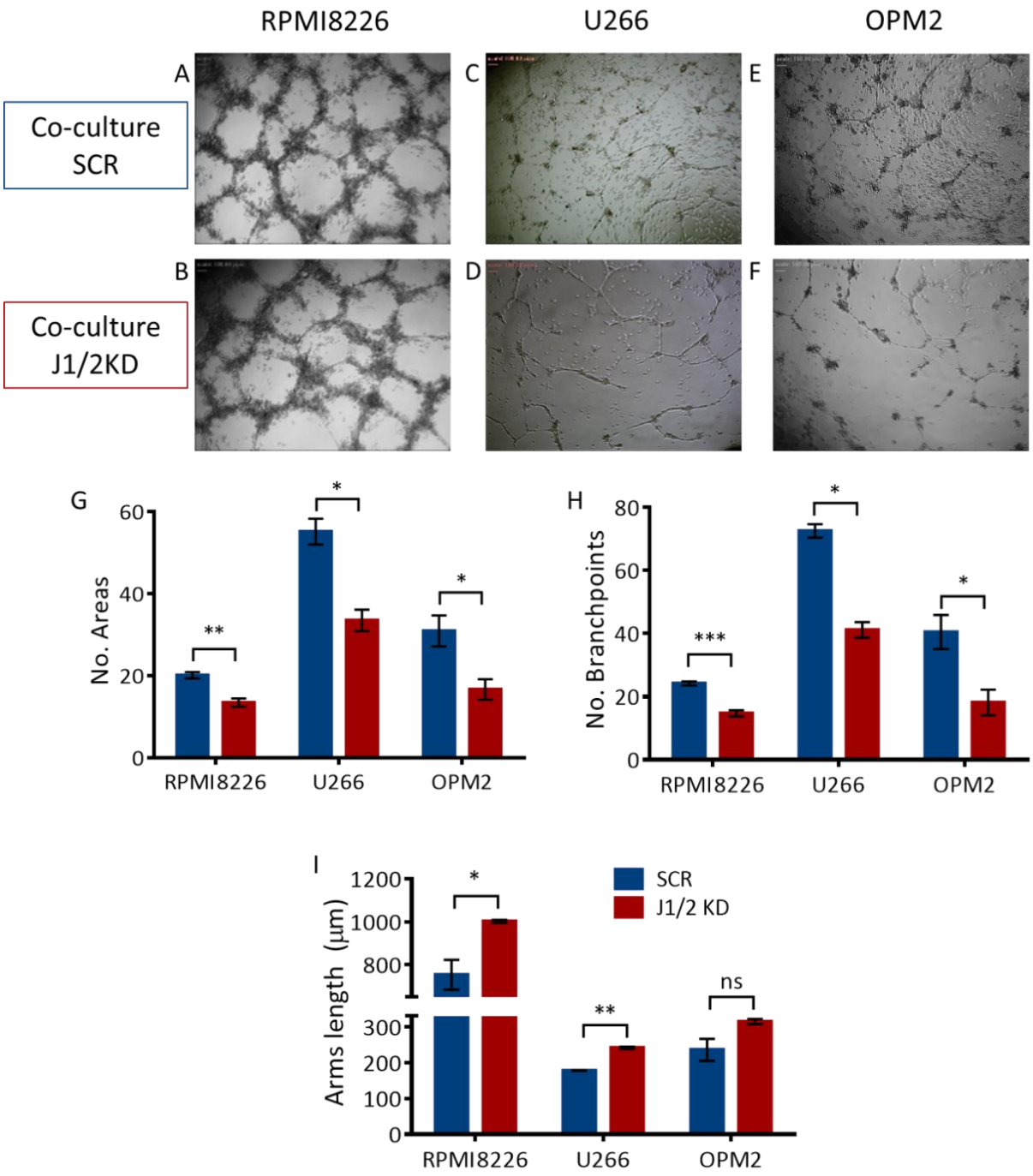


Figure12-Tube formation assay performed on Matrigel with a co-culture system of HMCLs and HPAECs. 4X magnification images of HPAECs co-cultured with (A) RPMI8226^{SCR} and (B) RPMI8226$^{J1/2KD}$, (C) U266^{SCR} and (D) U266$^{J1/2KD}$ and (E) OMP2^{SCR} and (F) OPM2$^{J1/2KD}$ are shown. Graphs show quantification of (G) number of areas, (H) number of branch points and (I) average length of arms. Statistical analysis of three independent experiments is carried out by one-tailed t test, * is for $p \leq 0.05$; ** for $p \leq 0.01$; *** for $p \leq 0.001$.

The reduced ability of HMCLs$^{J1/2KD}$ to determine a bidimensional EC organization strongly suggests that Jagged ligands can contribute to stimulate vessels network organization. Nonetheless, the use of this co-culture system does not allow us to distinguish the specific contributions of soluble factors or direct cell-cell contact. These will be assessed in the next sections.

1.4.2 Involvement of multiple myeloma-derived soluble factors

To exclusively assess the effect of MM cell-derived Jagged on EC organization mediated by the release of tumor-derived soluble factor, we have analyzed the outcome of EC stimulation induced by conditioned media from HMCLs^{SCR} or HMCLs$^{J1/2KD}$ in a tube formation assay.

Thereby, the tube formation assay has been carried out by stimulating HPAECs for 24 h with a 48h-HMCLs-derived CM. In Figure13, images illustrate ECs organization after 24 h-culturing on Matrigel with CM from HMCLs^{SCR} or HMCLs$^{J1/2KD}$ (from A to F). HMCLs^{SCR}-derived CM stimulates HPAECs to organize a grid, as a network of tube-like structures.

The percentage difference between HMCLs^{SCR} and HMCLs$^{J1/2KD}$ are: No. of areas are reduced in HMCLs$^{J1/2KD}$, i.e. 29.7% in RPMI8226, 37% in U266; 40.7% in OPM2; No. of BPs are reduced of 35.2% in RPMI8226; 41.7% in U266; 44.6% in OPM2; consistently arm length is increased of 35% in RPMI8226, 31.6% in U266; 20% in OPM2 cells.

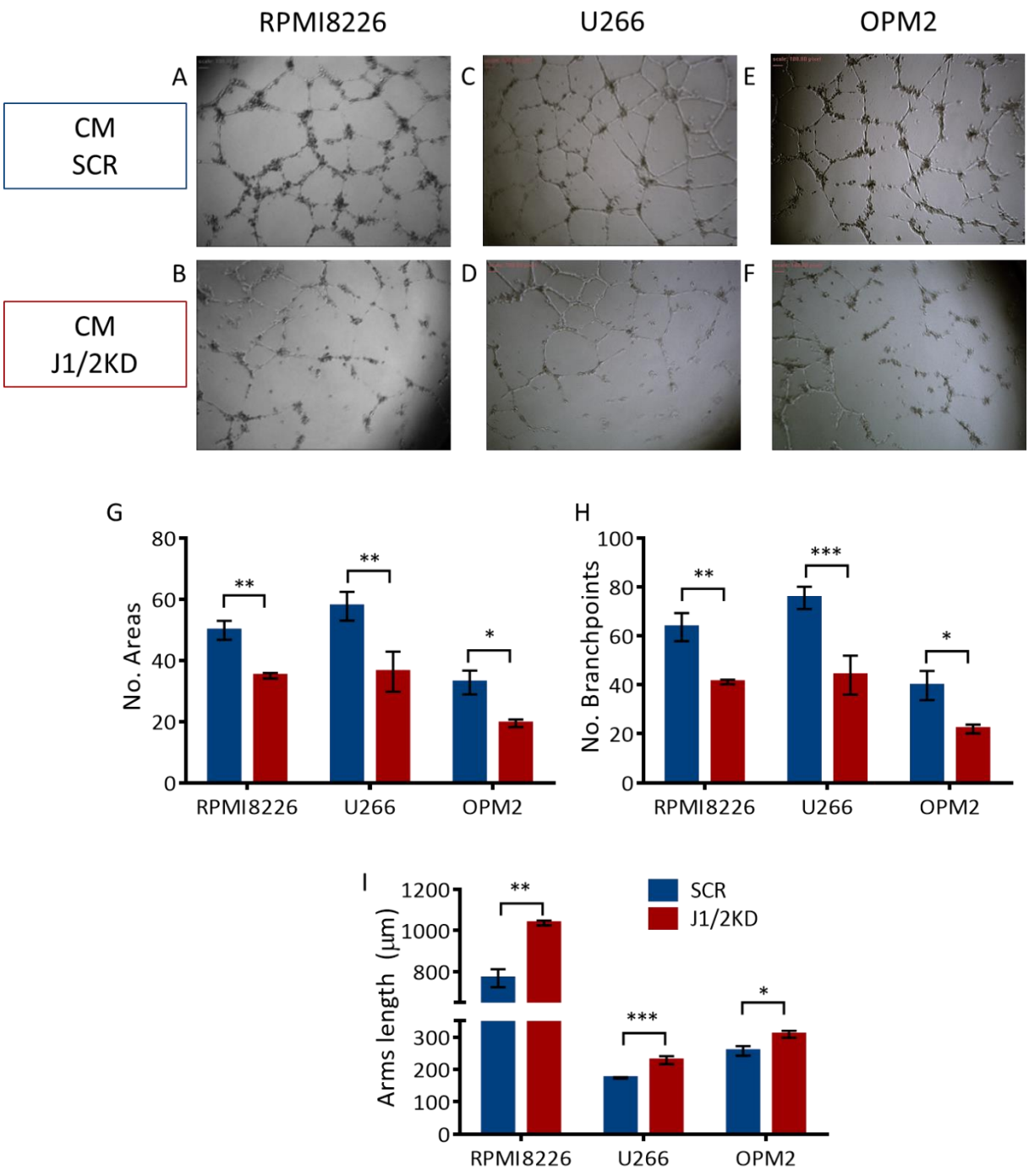


Figure13-Matrigel assay with HMCL-derived conditioned media. Photos at 4X magnification of ECs treated with CM from RPMI8226^{SCR} (A) and RPMI8226$^{J1/2KD}$ (B), from U266 ^{SCR} (C) and U266$^{J1/2KD}$ (D) and from OPM2 ^{SCR} (E) scrambled and OPM2$^{J1/2KD}$ (F). Graphs show quantification of (G) number of areas, (H) number of branch points and (I) average length of arms. The difference between HMCLs^{SCR} and HMCLs$^{J1/2KD}$ has been measured through: No. of areas, No. of BPs, average arm length. Statistical analysis is carried out by one-tailed t test, * = $p \leq 0.05$; ** for $p \leq 0.01$; *** for $p \leq 0.001$.

Overall, the analysis of this phase of the angiogenic process indicates that the absence of Jagged ligands in MM cells can interfere with MM-ability to release soluble factors involved in the organization of the new vessels.

1.4.3 Effect of Notch signaling downregulation on the production of multiple myeloma-derived VEGF

The evidence that Jagged1 and 2 are able to stimulate MM cells to release angiogenic factors prompted me to assess the possible involvement of the main angiogenic factor, VEGF, also recognized as a Notch transcriptional target (82). At this purpose, I have investigated if HMCLs^{SCR} and HMCLs$^{J1/2KD}$ expressed different amount of VEGF mRNA, by using qRT-PCR.

As reported in Figure14, panel A, a decreased Notch signaling in HMCLs$^{J1/2KD}$ is associated to the negative modulation of VEGF expression. Indeed, the downregulation of J1/2 and the subsequent Notch pathway silencing (data shown in Figure9) bring to a lower production of VEGF mRNA in all HMCLs. I also assessed the possible variation of secreted VEGF protein by ELISA on CM of HMCLs^{SCR} and HMCLs$^{J1/2KD}$. As expected, the amount of secreted protein is reduced in two cell lines after Jagged silencing, i.e. U266 and OPM2. On the contrary, unexpectedly the CM of RPMI8226$^{J1/2KD}$ contains higher levels of VEGF if compared to RPMI8226^{SCR}. I hypothesized that the reduced amount of VEGF in the CM of RMPI8226 could be due to the possible binding of VEGF to its receptor. To address this issue, I analyzed the expression of VEGFR1 and VEGFR2 in HMCLs at basal level and after Jagged1 silencing. As reported in Figure 14C, RPMI8226^{SCR} express both VEGFR1 and VEGFR2, while RPMI8226$^{J1/2KD}$cells show a reduced expression of VEGFR1, while VEGFR2 is unchanged. The levels of VEGFR1 and 2 expressed by OPM2 and U266 cells are very low/undetectable (data not shown). These data suggest that the higher levels of VEGF secreted by RPMI8226^{SCR} may not be detected in the CM due to its binding to higher levels of VEGFR1. Consistently, the lower expression of VEGFR1 and VEGFR2 in U266 and OPM2 cell lines does not reduce the level of VEGF secreted in the medium. To confirm that Jagged KD in RPMI8226 cells affects their ability to produce VEGF, we reasoned that the amount of intracellular VEGF protein could be a more reliable measure of VEGF produced RPMI8226 cells. Thereby, we have measured the intracellular VEGF by flow cytometry in RMPI8226 cells. Results in figure 14 D show that RPMI8226$^{J1/2KD}$cells express approximately 50% VEGF in comparison with RPMI8226^{SCR} cells.

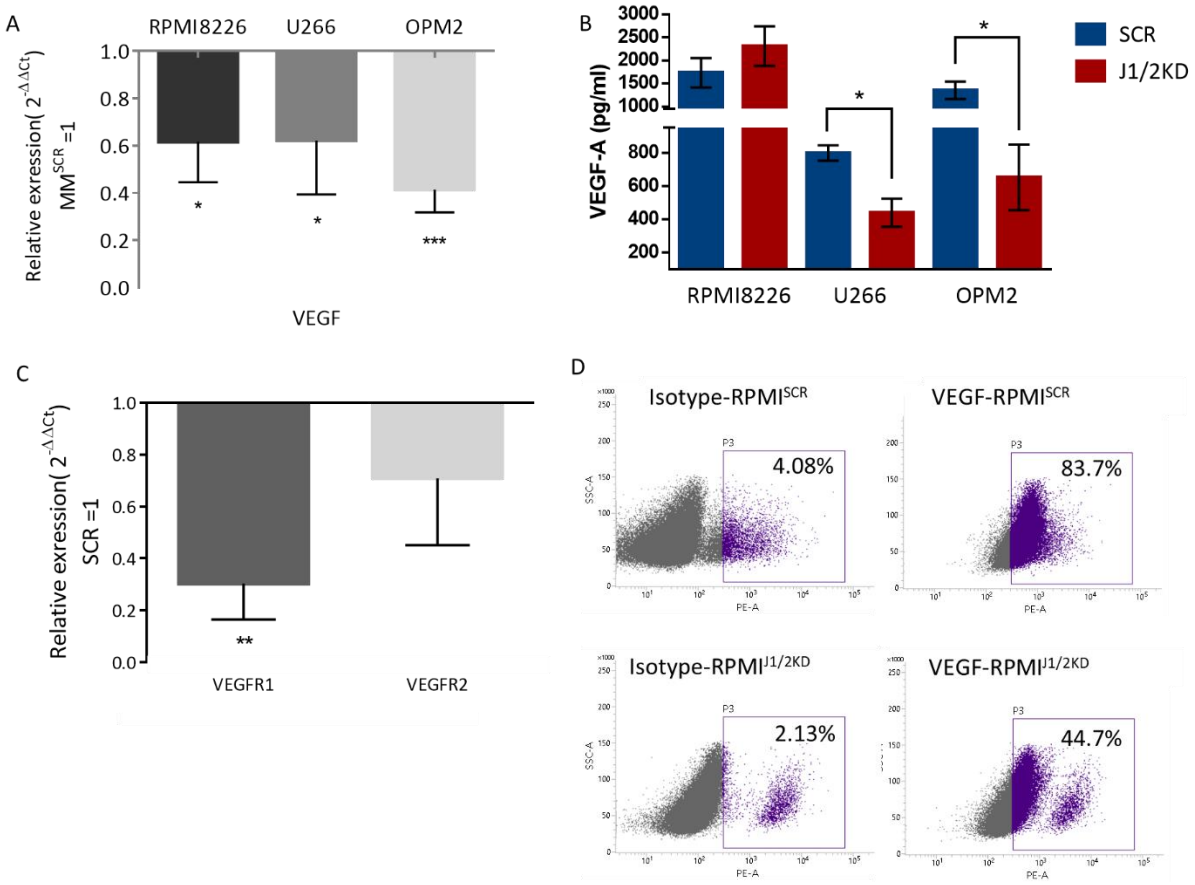


Figure14 - Modulation of VEGF expression in HMCLs (RPMI8226, U266 and OPM2 cell lines) knockdown for Jagged1 and Jagged2. (A) Analysis by qRT-PCR. Data are represented as relative expression variation using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for normalization; reported relative expression values are calculate as $2^{-\Delta\Delta Ct}$ +/-SD. (B) ELISA for VEGFA detection within CM from HMCLs. (C) Modulation of VEGFR1 and 2 in relation to Jagged silencing in RPMI8226 cell line by qRT-PCR. Data are represented as relative expression variation using GAPDH for normalization; reported relative expression values are calculate as $2^{-\Delta\Delta Ct}$ +/-SD. (D) Representative flow cytometry analysis of VEGF expression in RPMI8226 cell line scrambled or knocked down for Jagged. Statistical analysis is carried out by One-tailed t test, * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

In conclusion, these results indicate that in the studied myeloma cell models the expression levels of Jagged ligands correlates with Notch activation and VEGF secretion, suggesting a direct or indirect transcriptional regulation of the main angiogenic factor in MM, VEGF, by Notch.

1.4.4 Myeloma cell-derived Jagged triggers Notch signaling in endothelial cells

The above reported results indicate that evidence that HMCLs$^{J1/2KD}$ can release a reduced amount of angiogenic factors thereby negatively affecting three different steps of tumor angiogenesis. On the other side, we reasoned that HMCL-derived Jagged might further contribute to tumor angiogenesis by triggering the angiogenic Notch signaling on the nearby ECs. To confirm this hypothesis, I evaluated Notch target genes modulation in ECs after 24h co-culture HMCLs^{SCR} and HMCLs$^{J1/2KD}$. As reported in Figure15, Jagged1 and Jagged2 silencing in myeloma cells induces a down-regulation of HES1 and HEY1 genes in endothelial cells.

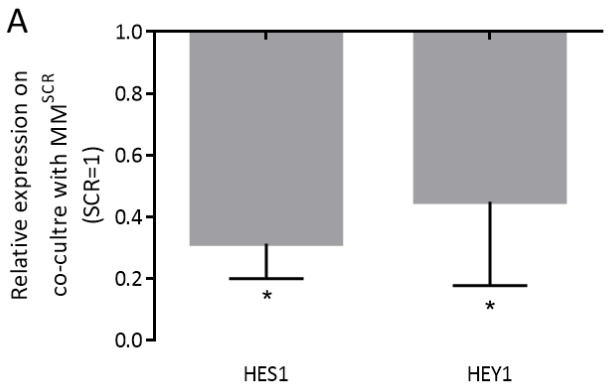


Figure15-Effect of MM cell-derived Jagged ligand on Notch activation in endothelial cells. Analysis by qRT-PCR of HES1 and HEY1 expression in endothelial cells after 24h co-culture with scrambled or silenced myeloma cells. Data are represented as relative expression variation of HPAEC co-cultured with silenced HMCLs respect to HPAEC co-cultured with scrambled HMCL, calculated as $2^{-\Delta\Delta Ct}$ +/-SD. GAPDH has been used for normalization. Statistical analysis is carried out by One-tailed t test, * = $p \leq 0.05$.

This result indicates that myeloma-derived Jagged may trigger Notch activation in endothelial cells when these cells are in contact.

1.4.5 Direct effect of Jagged ligand in modulation of tube-formation capability

We further confirmed at functional level that MM cell-derived Jagged have a role in angiogenesis stimulated by the direct contact between MM cells and ECs. We reasoned that the generation of an organized network of vessels can be stimulated also by a direct contact between ECs and MM cells, and possibly involves the activation of Notch signaling triggered by MM cell-derived Jagged engaging Notch receptor on adjacent ECs.

In order to distinguish if MM cell-derived Jagged ligands may stimulate ECs organization by cell-cell contact, tube formation assay has been performed by culturing HPAECs (detailed in Materials and Methods) in presence or absence of 10ng/ml soluble Jagged1 ligand and compared with the medium with or without angiogenic soluble factors (i.e. VEGF, IGF1, EGF and bFGF).

Quantitative analysis of areas, branch points and length of the arms is reported in Figure16. Results show that the use of Jagged1 stimulates tubes formation by HPAECs respect to the condition without Jagged1 and angiogenic factors (negative control), and similarly to the condition with angiogenic soluble factors. Variations in HPAECs stimulation obtained without or with Jagged1 are an increase in the number of areas of 28.1% and number of BP of 36.8%, and a decrease in the average arm length of 22.7%. These results show that Jagged1 alone may contribute to stimulate tubes formation.

On the whole, we can conclude that MM cell-derived Jagged ligands contribute to stimulate ECs to organize a tube-like network through two different mechanisms: a) triggering Notch signaling in the same MM cells

that, in turn, release soluble angiogenic factors; b) directly triggering Notch receptor activation and its pro-angiogenic signaling in adjacent ECs.

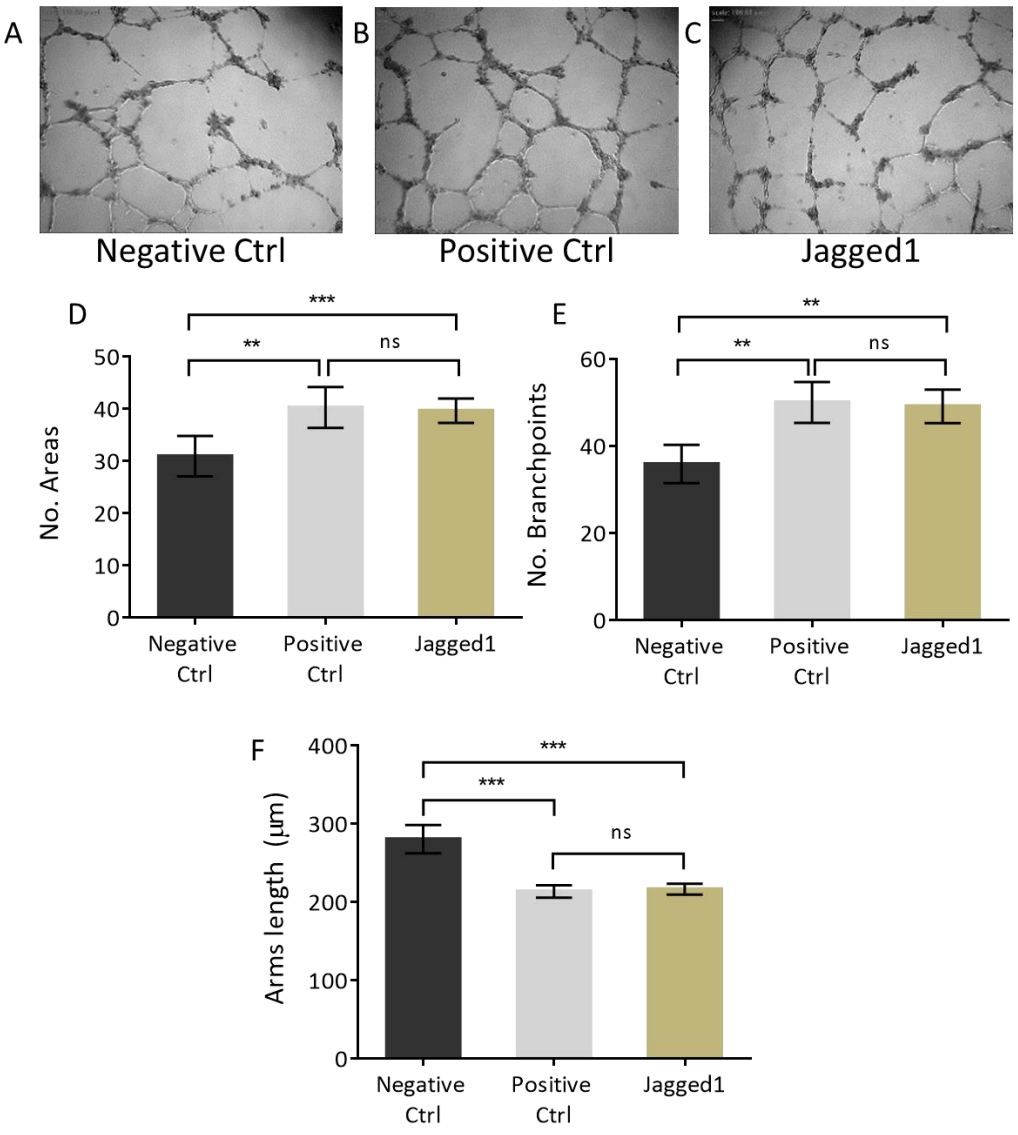


Figure16-Tube formation assay with soluble Jagged1. Images are a 4X magnification of HPAECs cultured (A) without angiogenic factors (negative Crtl), (B) with angiogenic factors (positive Crtl) and (C) with soluble Jagged1 in medium without angiogenic factors. Graphs show quantification of (D) number of areas, (E) number of branch points and (F) average length of arms. Statistical analysis is carried out by one-way ANOVA with Tukey post hoc test, ** = $p \leq 0.01$; *** = $p \leq 0.001$; ns = non-significant.

2. MYELOMA CELL-DERIVED JAGGED1 AND 2 INCREASE BONE MARROW STROMAL CELL ANGIOGENIC POTENTIAL

In myelomatous BM microenvironment, BMSCs play a crucial role in supporting not only MM progression in terms of pro-survival signals (72) and protection from drug induced apoptosis (69) but can also influence EC

compartment triggering tumor-associated angiogenesis. Furthermore, several evidences indicate that HMCLs can stimulate the pro-tumoral behavior of stromal cells and that this modulation may be mediated by Notch signaling pathway (65, 75, 95). Notably, BMSCs are able to produce high amount of angiogenic soluble factors including VEGF (110). To evaluate if myeloma-derived Jagged can influence BMSCs angiogenic potential, a co-culture system of myeloma cells and BMSCs in ratio 1:1 for 48h has been exploit and CM have been collected and used to assess their effect on the 3 steps of angiogenesis previously investigated: adhesion to fibronectin, wound healing and tube formation assays. The used ratio of 1:1 between MM cells and BMSCs is comprised in the range observed in 15 human bone marrow samples of MM patients analyzed by flow cytometry spanning from 1 : 0.5 to 1 : 1.46 (MM cells: stromal cells).

2.1 Myeloma cell-derived Jagged1 and 2 increase bone marrow stromal cells angiogenic potential

To test whether myeloma-derived Jagged1 and 2 can determine Notch pathway activation in BMSCs as well as the production of stromal-derived VEGF, a co-culture system has been set up including HMCLs and the stromal compartment mimicked by NIH3T3 fibroblasts or the BMSC line HS5, stably transfected with GFP, $HS5^{GFP+}$.

For these experiments Jagged1 and Jagged2 have been silenced in HMCLs ($HMCLs^{J1/2KD}$) by using two short interfering RNAs (siRNAs), the corresponding scrambled siRNA was used as control ($HMCLs^{SCR}$). HMCLs underwent a double round of transfection (every 48h) with siRNAs in order to obtain an optimal downregulation. Transfected HMCLs have been co-cultured for 48h with stromal cell lines.

Specifically, murine NIH3T3 cells have been used to assess the variation of VEGF mRNA of stromal origin thank to the use of primers specific for murine VEGF. All the values have been normalized to basal condition obtained by culturing NIH3T3 cells alone. mRNA variation is calculated as $2^{-\Delta\Delta Ct}$ by setting alone condition as 1.

HMCLs have been cultured with $HS5^{GFP+}$ cell line to study their ability to increase VEGF protein of stromal origin, this could be distinguished by that of tumoral origin thank by flow cytometry. At this purpose, co-cultured cells were fixed and stained with anti-VEGF antibody conjugated with phycoerythrin (PE) fluorochrome and intracellular VEGF was selectively quantified within GFP^+ cells gate (HS5).

In Figure17 graphs (from A to C) show the modulation of VEGF and HES5 gene expression assessed by RT-PCR after co-culturing $HMCLs^{SCR}$ and $HMCLs^{J1/2KD}$ with NIH3T3 cells. Results indicate that $HMCLs^{SCR}$ induce an increase of VEGF and HES5 gene expression in NIH3T3 cells from 2 to 7-fold for HES5 and from 1 to 4 fold for VEGF if compared to NIH3T3 cultured alone. This increase is completely reverted when NIH3T3 cells are cultured with $HMCLs^{J1/2KD}$ indicating that Jagged ligands expressed on MM cells can influence BMSC promoting Notch signaling pathway activation in stromal cells and increasing the production of stromal-derived VEGF.

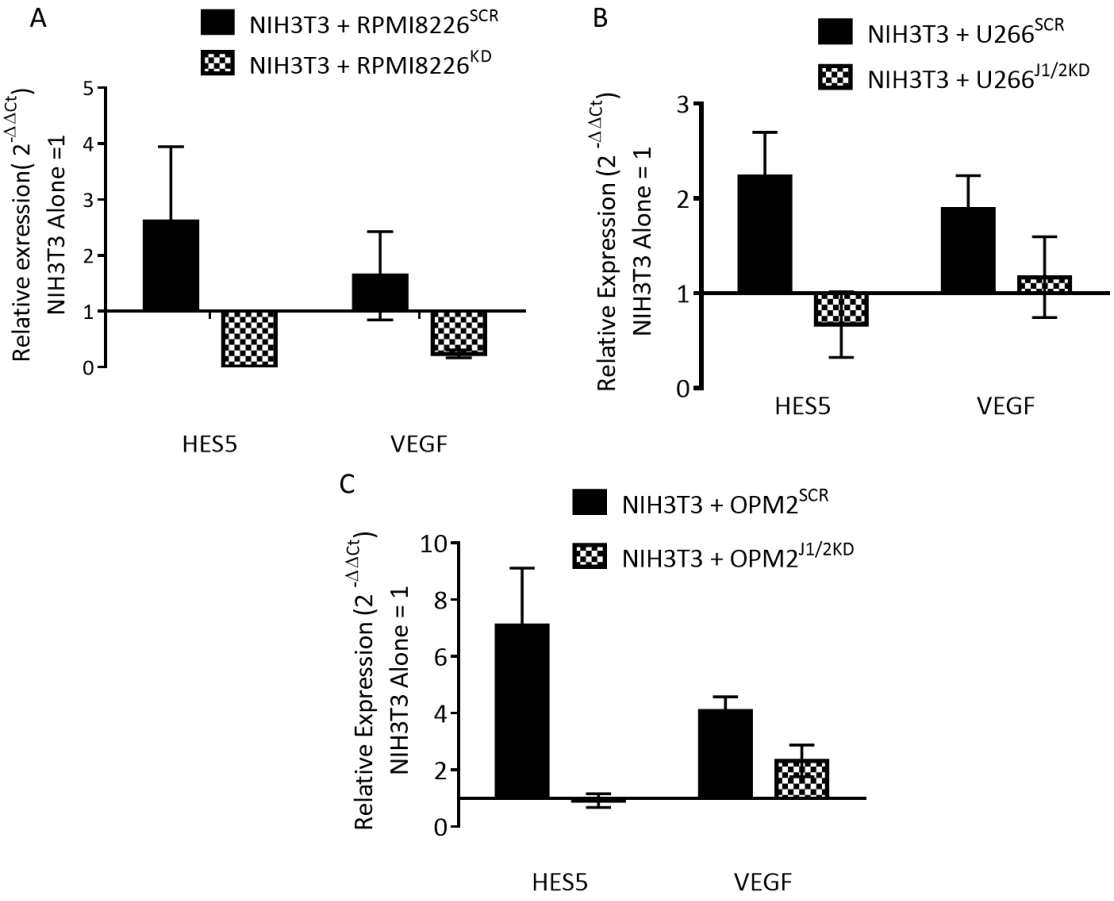


Figure17-Effect of MM cell-derived Jagged ligand on BMSC expression of VEGF and HES5. Analysis by qRT-PCR of stromal-derived VEGF and HES5 expression after 48h co-culture of NIH3T3 murine fibroblasts with (A) RPMI8226, (B) U266 and (C) OPM2 cells transfected with scrambled siRNAs or siRNAs directed against Jagged1 and Jagged2. Data are represented as relative expression variation of NIH3T3 cells co-cultured with HMCLs respect to NIH3T3 cells cultured alone, calculated as $2^{-\Delta\Delta Ct}$ +/-SEM. GAPDH has been used for normalization.

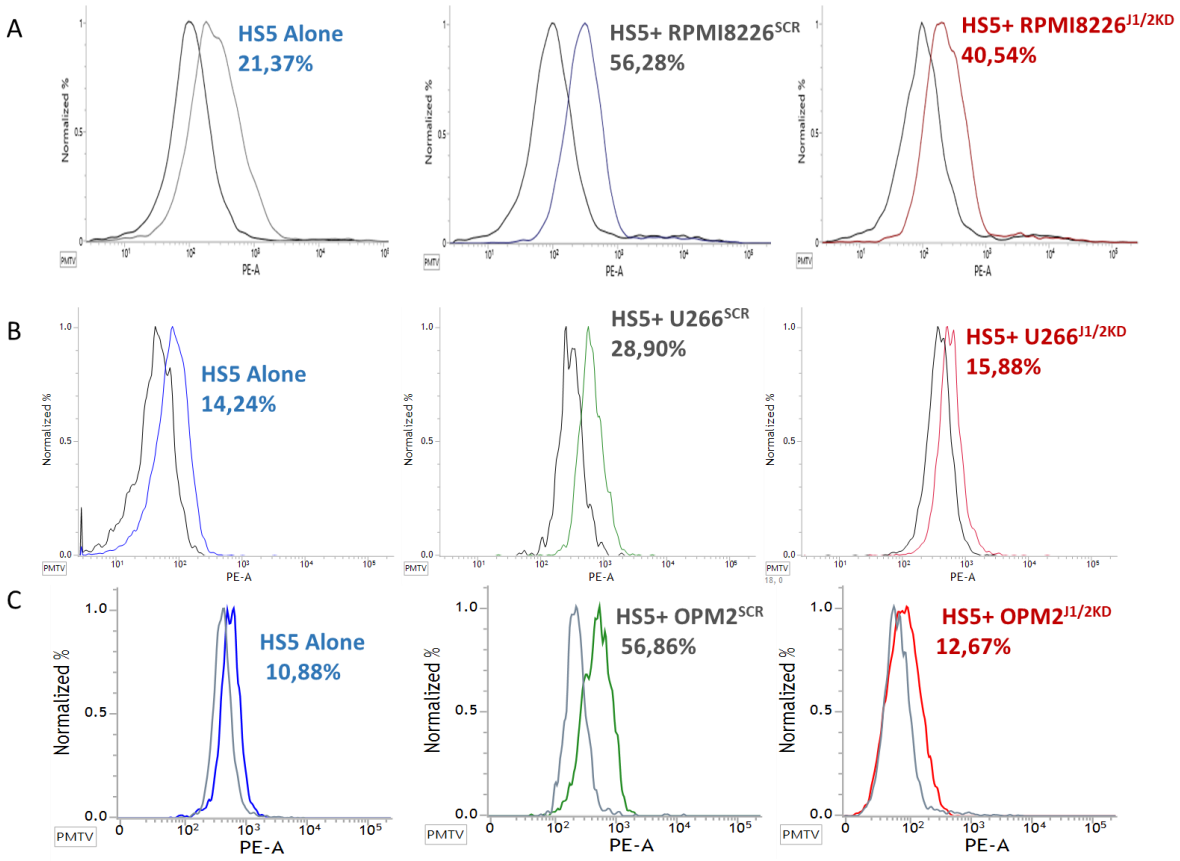


Figure18-Protein expression of stromal-derived VEGF after co-culture of $HS5^{GFP+}$ stromal cells with (A) RPMI8226, (B) U266 and (C) OPM2 scrambled or J1/2KD. Histograms display protein expression level of VEGF analyzed by flow cytometry in $HS5^{GFP+}$ stromal cell alone (blue lines), in co-culture with $HMCLs^{SCR}$ (green lines) and with $HMCLs^{J1/2KD}$ (red lines), and isotype-matched control (grey lines). Histograms are representative of three independent experiments with comparable results.

The flow cytometric analysis of the variation in VEGF protein expression induced in $HS5^{GFP+}$ cell line by HMCLs treated as reported before showed analogous results.

Histograms in Figure18 are representative of three different experiments and show changes in VEGF protein expression. These results show that stromal-derived VEGF is positively regulated by myeloma cells: RMI8226 cells increase HS5 cell-mediated production of VEGF from 21.3% to 56.2%, U266 from 14.2% to 28.9% and OPM2 from 10.8% to 56.8%. Furthermore, this upregulation is totally reverted when HS5 are stimulated by $HMCLs^{J1/2KD}$ carrying lower levels of Jagged ligands.

On the whole, these data show that HMCLs can increase BMSCs pro-angiogenic potential thank to the expression of Jagged ligands and the consequent activation of Notch signaling in BMSCs.

2.2 Effect on bone marrow stromal cell-mediated stimulation of endothelial cell adhesion to extracellular matrix

The BM microenvironment is characterized by different stimuli provided by different resident cell types (99). This prompted us to investigate if MM cells may stimulate also BMSCs increasing their potential to favor to ECs adhesion, motility and organization into a tubular grid.

To this aim, we set up an experimental approach to evaluate if HMCLs-expressing Jagged ligands can stimulate BMSCs to release higher amount of angiogenic factors and if the overexpression of Jagged ligands in HMCLs may play a role. Thereby, three culturing conditions have been arranged to discriminate the effects induced on ECs by BMSCs (Alone), BMSCs in co-culture with $HMCLs^{SCR}$ and BMSCs with $HMCLs^{J1/2KD}$. After 48h, CM have been collected and used to perform adhesion, motility and tube formation assay on HPAECs as reported above. For adhesion assay, after Calcein AM staining, ECs are seeded on fibronectin coated 96-well plate and fluorescence intensity of vital adherent cells is evaluated after 1h.

Figure19 illustrates the results of the adhesion assay. CM of co-culture systems with $HMCLs^{SCR}$ stimulates HPAEC adhesion ability. If compared to the effect induced by CM of BMSCs alone. Indeed, there is an increase in fluorescence intensity ranging from 20.9% of OPM2 cell line, 23.2% of U266 cell line, to 24.4% of RPMI8226 cells. The increased adhesive abilities of HPAEC stimulated by CM of BMSCs co-cultured with $HMCLs^{SCR}$ are completely lost when HPAECs are stimulated with the CM of BMSCs co-cultured with $HMCLs^{J1/2KD}$, suggesting that Jagged ligands silencing in HMCLs negatively influences BMSC capability to stimulate HPAEC adhesion. Indeed, we observed a decrease in adhesion capability expressed as percentage variations between the two conditions of, respectively, 21.7% for RPMI8226 cells, 24.1% for U266 cells and 20.2% for OPM2 cell line.

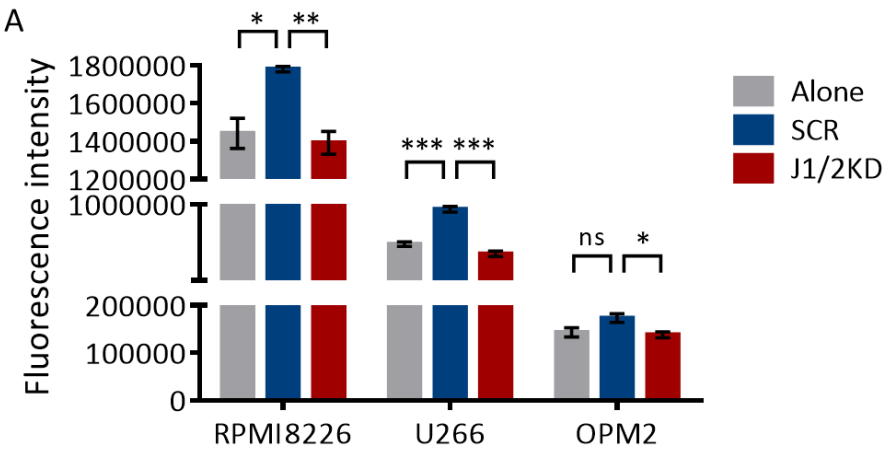


Figure19- Modulation of adhesion capability of ECs through CM derived from BMSCs conditioned by MM cells. HPAECs are stimulated with CM from HS5 cells cultured alone (alone), with HMCLs^{SCR} (SCR) or HMCLs$^{J1/2KD}$ (J1/J2KD). The ability of calcein stained HPAECs to adhere to a fibronectin layer has been measured as florescence intensity +/- SD. Statistical analysis has been carried out by one-way ANOVA with Tukey post hoc test, *= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

Overall, these results show that Jagged ligands expressed by HMCLs are crucial in stimulating BMSC pro-angiogenic potential measured as increase of ECs adhesion capability.

2.3 Effect on bone marrow stromal cell-mediated stimulation of endothelial cell motility

To understand whether MM-derived Jagged1 and 2 may contribute to stimulate another feature of BMSC angiogenic potential, we have analyzed also the modulation of ECs motility by performing a wound healing assay in the presence of the conditioned media from BMSC cultured alone, with HMCLs^{SCR} or with HMCLs$^{J1/2KD}$.

As shown by images in Figure20 the migration of HPAECs treated with CM from HS5 cells co-cultured with HMCLs^{SCR} (B, E, H) are able to replenish the wound more effectively if compared to the treatment with CM from HS5 cultured alone (A, D, G). Areas increase approximately of 43.1% for treatment with CM from co-cultured HS5-RPMI8226, 34.5% for CM from co-cultured HS5-U266 and 61.6% for CM from co-culture HS5-OPM2. For distance between wound edges percentages of variation are: 36.2% for CM from co-cultured HS5-RPMI8226, 31.8% for CM from co-cultured HS5-U266 and 62.2% for CM from co-cultured HS5-OPM2.

Moreover, it is evident that when HPAECs were treated with CM obtained from the co-cultures of HS5 cells with HMCLs$^{J1/2KD}$ (C, F; I), CM ability to stimulate EC migration was severely hampered as demonstrated by measurement. Indeed, wound areas increase of 91.3% with CM from co-culture including RPMI8226$^{J1/2KD}$, 67.7% with CM from co-culture with U266$^{J1/2KD}$ and 137% with CM including co-culture with OPM2$^{J1/2KD}$ cells. Consistently, distances of wound edges are reduced of 74% when using the CM derived from HS5 co-cultured

with RPMI8226 cells, of 69.1% when using HS5 with U266 cells and of 146% in the case of HS5 with OPM2 cells.

These results indicate that Jagged ligands expression in HMCLs stimulate BMSCs to produce soluble factors able to increase EC motility.

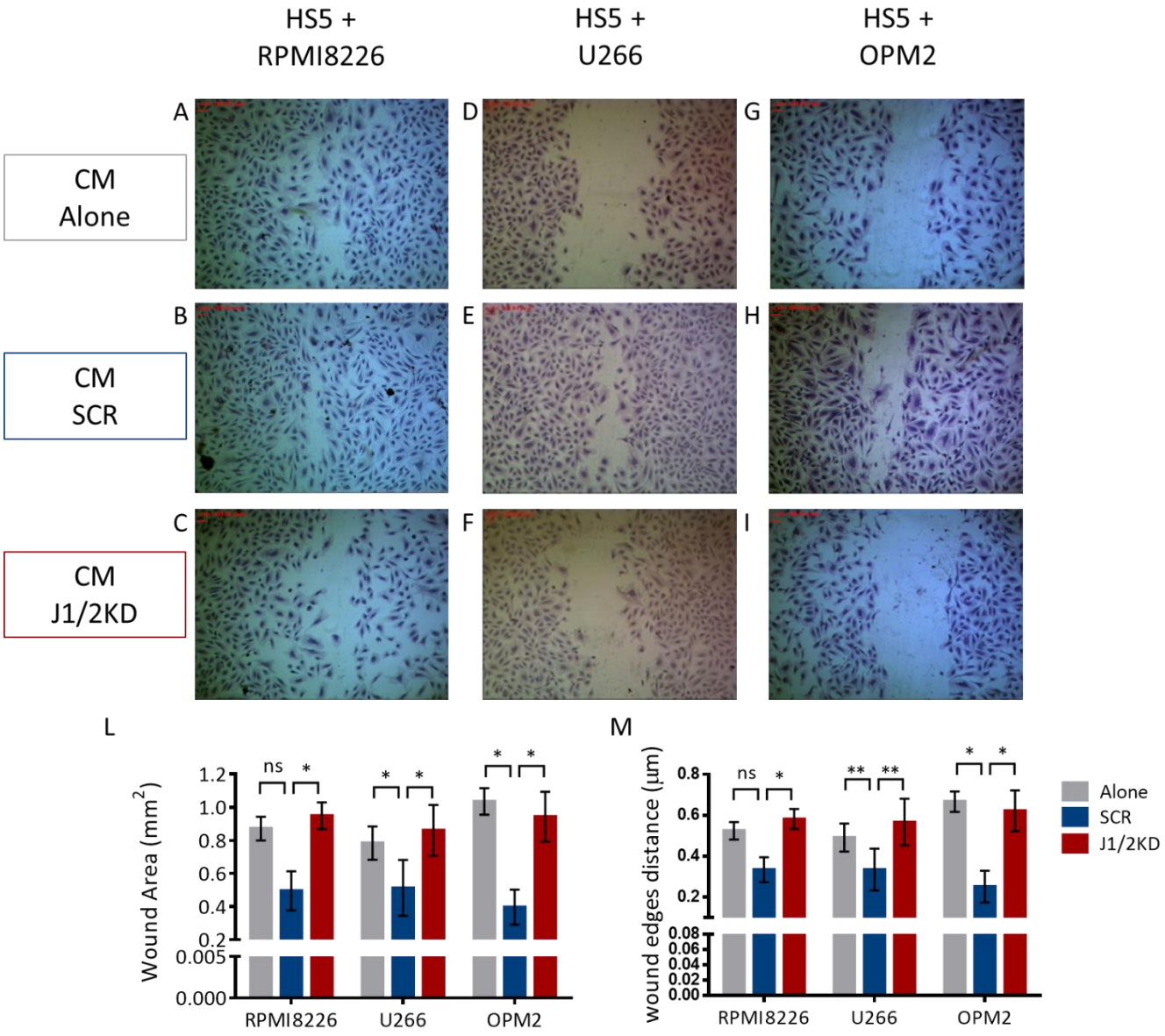


Figure20-Wound healing assay with CM from BMSCs culture systems. Representative images of ECs treated for 24h with CM coming from (A-D-G) HS5 cell lines cultured alone, co-cultured with (B) RPMI8226^{SCR}, (E) U266 ^{SCR} and (H) OPM2^{SCR} (SCR) and HS5 cell line co-cultured with (C) RPMI8226$^{J1/2KD}$, (F) U266 $^{J1/2KD}$ and (I) OPM2 $^{J1/2KD}$ (J1/2KD). Graphs show mean +/- SD of (L) wound area and (M) wound edges distance calculated with ImageJ software. Statistical analysis of three independent experiments is carried out by one-way ANOVA with Tukey post hoc test, * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

2.4 Effect on bone marrow stromal cell-mediated stimulation of endothelial cell ability to organize a grid-like structure

The last step in angiogenesis that we analyzed involves ECs capability to organize a complex network. This assay was performed using CM from BMSCs cultured alone or in the presence of $HMCLs^{SCR}$ or $HMCLs^{J1/2KD}$ and analyzing the EC ability to form a tube-like structure on a Matrigel layer as reported before.

Figure21 shows ECs organization on Matrigel coated wells; after stimulation with CM from HS5 cultured alone (panel A, D and G) ECs form a network of tubes. When CM is derived from co-culture of HS5 with $HMCLs^{SCR}$, the newly formed network appears more complex, with a higher number of connections among cells indicating a role of the activated Notch signaling in increasing ECs capability to generate a well-organized network (Figure21 B, E and H).

Comparing HPAEC organization when stimulated with CM from HS5 cultured alone or with $HMCLs^{SCR}$. I observed the following percentage of variations: number of areas increase of 55.9% if CM was from HS5 cells and RPMI8226, 22.4% with U266 and 51.3% with OPM2. Following the same sequence, the number of BPs increased of respectively 73.4%, 38.5% and 69.8%, and the arms lengths decreased of, respectively, 24.2%, 19.3% and 28.8%, indicating a reduction in EC grid complexity.

The involvement of Jagged-mediated Notch signaling activation was further confirmed by the loss of the effect observed when ECs were stimulated with CM from co-cultures of HS5 with $HMCLs^{J1/2KD}$. In this case HPAECs showed a reduced grid of connections (Figure21 C, F and I) very similar to the effect of CM from HS5 cell cultured alone. Specifically, the number of areas formed by HPAECs was reduced of 58.4% when treated with CM from HS5 cells co-cultured with $RPMI8226^{J1/2KD}$, of 26.7% when CM were from HS5 and $U266^{J1/2KD}$ and 32.8% when CM derived from HS5 and $OPM2^{J1/2KD}$ cells. The number of BPs display the same behavior, following the same sequence the reduction was of, respectively, 67.2%, 34.5% and 40.1%, on the contrary arm length was respectively longer of 40.9%, 31.7% and OPM2 40.6%.

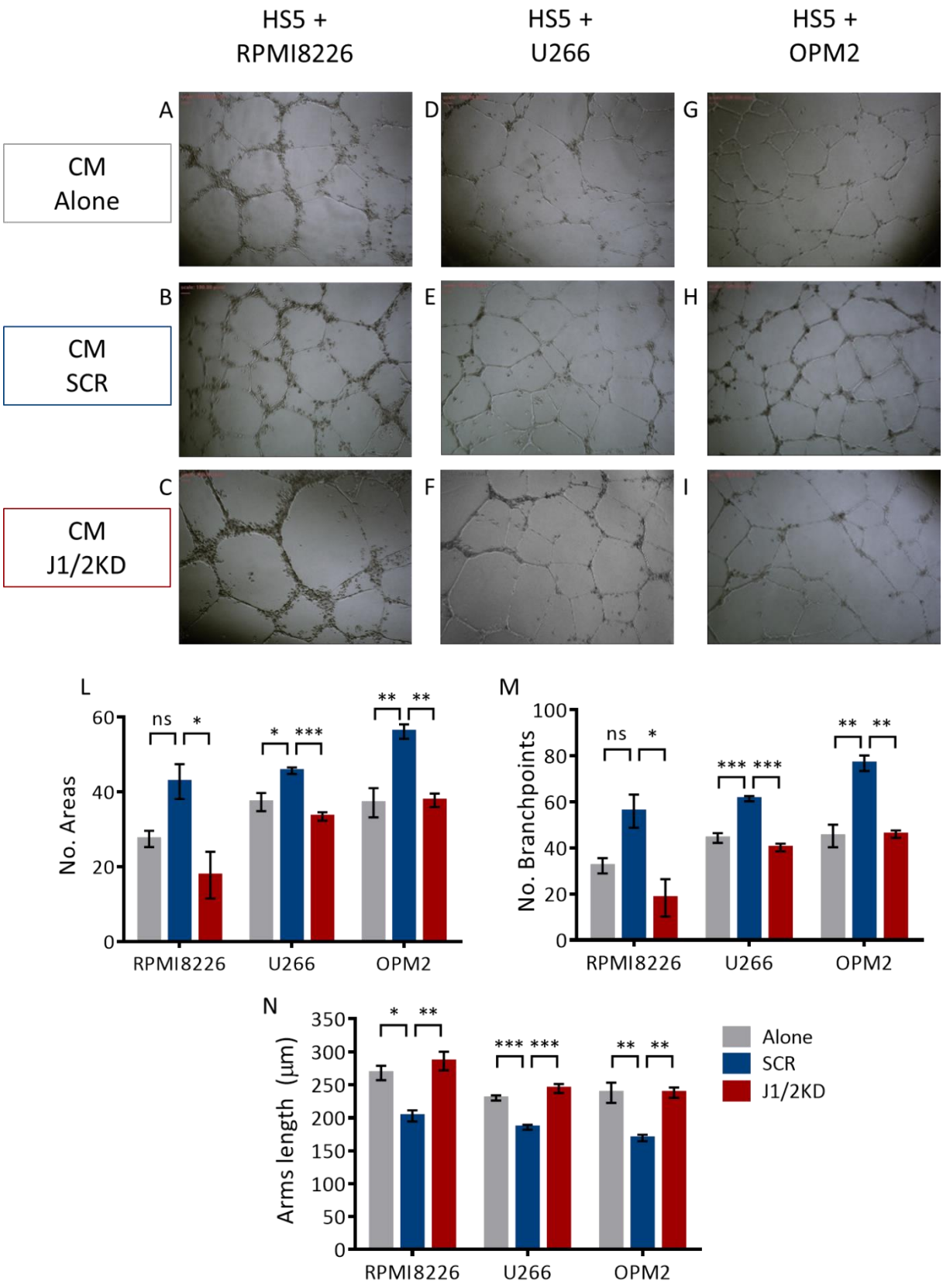


Figure21-Tube formation assay with BMSCs-derived CM. Pictures (4X magnification) of HPAECs treated for 24h with CM from (A-D-G) HS5 cell line cultured alone, HS5 cell line co-cultured with, (B) RPMI8226^{SCR}, (E) U266^{SCR} and (H) OPM2^{SCR}, and HS5 cell line co-cultured with (C) RPMI8226$^{J1/2KD}$, (F) U266$^{J1/2KD}$ and (I) OPM2$^{J1/2KD}$. Graphs show quantification of (L) number of areas, (M) number of branch points and (N) average length of arms. Statistical analysis of three

independent experiments was carried out by one-way ANOVA with Tukey post hoc test, * = $p \leq 0.05$; ** = $p \leq 0.01$; ***=$p \leq 0.001$.

Results obtained from this assay are in accordance with previous assays and together show that MM cells contributes to modulate EC behavior acting also through BMSCs.

Moreover, the angiogenic potential of stromal cells may be stimulated by MM cell-derived Jagged ligands through the increased production of VEGF. The interruption of the Jagged-mediated crosstalk between MM cells and BMSCs causes a reduction in EC stimulation underlying the central role played by Notch in the crosstalk between MM cells and the surrounding microenvironment.

3. ZEBRAFISH IN VIVO MODEL CONFIRMS MULTIPLE MYELOMA J1/2 ROLE IN SPROUTING STIMULATION

To confirm *in vitro* results in an *in vivo* model of MM-induced angiogenesis, we investigated if Jagged1 and 2 expressed on MM cells promote angiogenesis a zebrafish embryo model.

At this purpose, transgenic zebrafish embryos TG(fli1a:EGFP) 48h post-fertilization (48hpf) have been injected into the sub-peridermal space, close to the developing sub-intestinal plexus (SIV) with RPMI8226^{SCR} or RPMI8226$^{J1/2KD}$ cells pre-treated with the CellTracker CmDil, a red fluorescent dye, to evaluate possible changes in the sprouts arising from SIV. In this model, blood vessels constitutively express the GFP.

24h post-injection (24hpi), embryos have been analyzed by fluorescence microscopy at 100X magnification, sprouts length has been quantified by ImageJ software after images acquisition.

Figure22 shows two representative images of fish embryos injected with RPMI8226^{SCR} (Figure22A) or RPMI8226$^{J1/2KD}$ cells (Figure22B). RPMI8226^{SCR} cells induce endothelial sprouts formation from SIV move toward the injected myeloma cells. A similar but less intense effect was induced by RPMI8226$^{J1/2KD}$ cells that induced angiogenesis less efficiently.

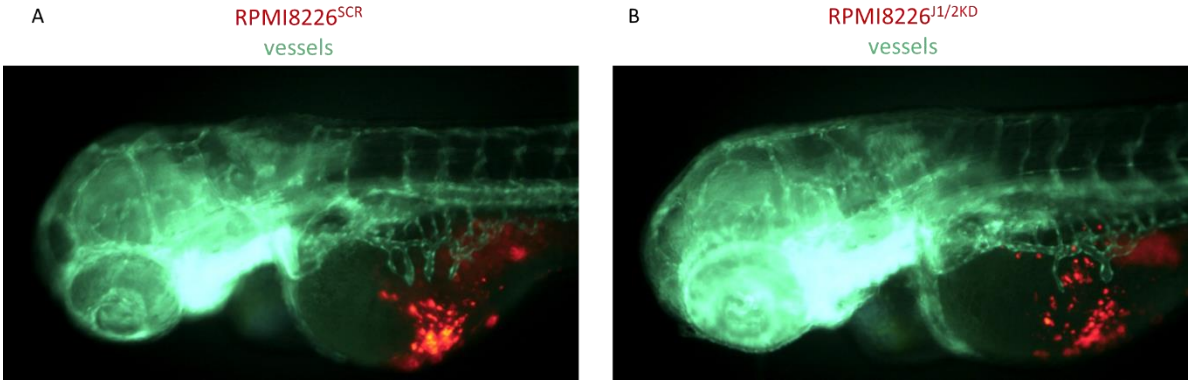


Figure22-HMCLs stimulation of vessel sprouting in a transgenic zebrafish model is dependent by Jagged1 and 2 expressions. Two representative pictures of (A) RPMI8226^{SCR} or (B) RPMI8226$^{J1/2KD}$ cells stained with the fluorescent CmDil vital dye (red) in a TG(fli1a:EGFP) zebrafish embryos with GFP expressing vessels (green).

Figure23 shows images acquired in green channel in order to exclusively better display vessels to compare their growth into the three experimental groups composed by fish injected with: PBS (negative control of the experimental procedure) (Figure23A), RPMI8226^{SCR} cells (Figure23B) and RPMI8226$^{J1/2KD}$ cells (Figure23C). As expected, fish embryos belonging to PBS injected group did not display any sprouts from developing SIV. Fish embryos injected with RPMI8226^{SCR} cells showed the generation of sprouts from SIV (Figure23B), confirming myeloma cell ability to induce sprouting angiogenesis, while new vessel formation diminished in embryos injected with RPMI8226$^{J1/2KD}$ cells, (Figure23C) indicating that Jagged expression in MM cells is crucial for angiogenesis stimulation.

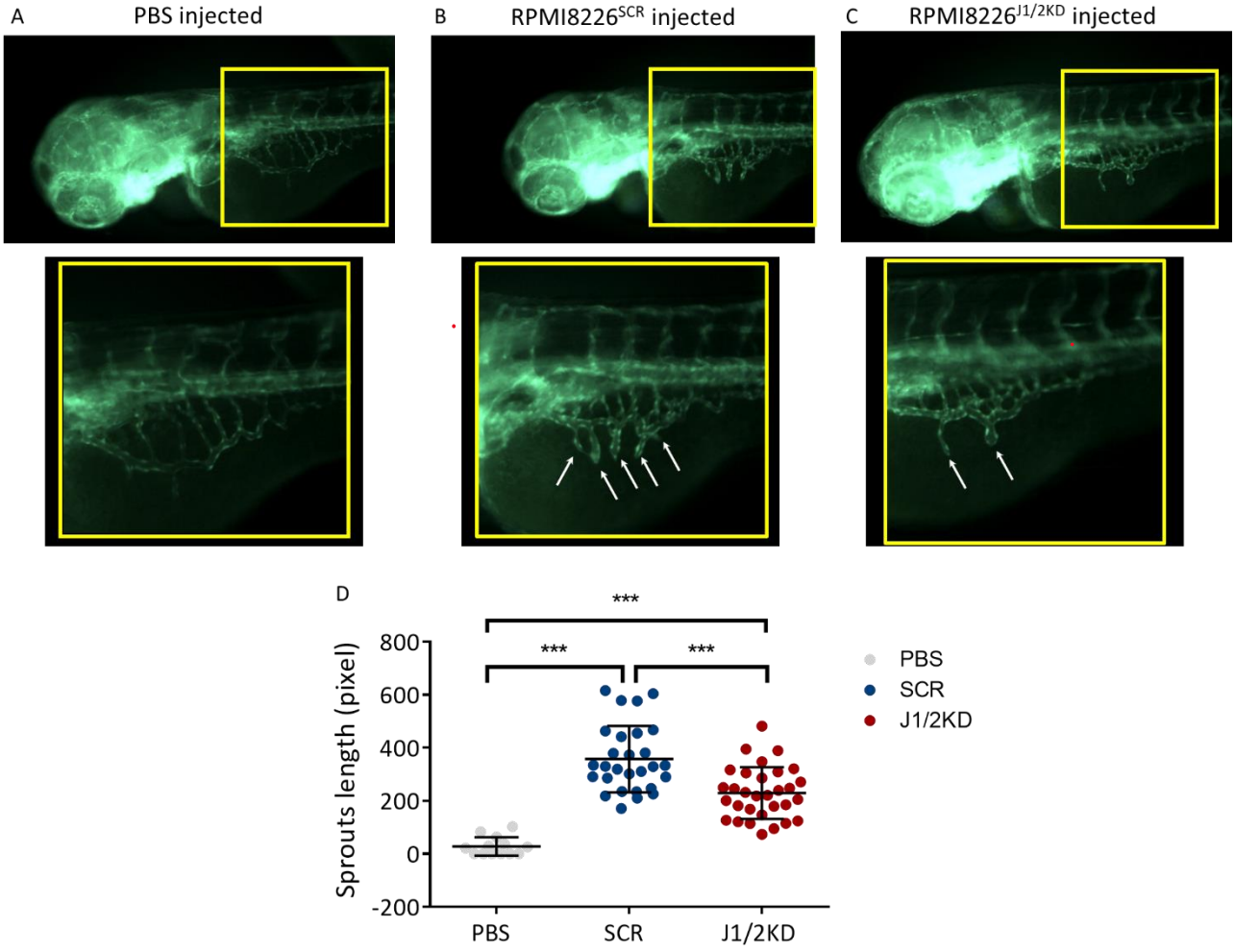


Figure23-J1/2 expression in HMCLs stimulates vessels sprouting in transgenic zebrafish model. Photos of zebrafish injected with (A) PBS used as experimental procedure control, (B) RPMI8226^{SCR} cells and (C) RPMI8226$^{J1/2KD}$ cells. Photos show a single channel (green) that stain vessels. White arrows indicate sprouts that arise from SIV 24hpi. Graph (D) shows sprouts length measurement at 24hpi and statistical analysis of three independent experiments is carried out by One-way ANOVA with Tukey post hoc test, *** for $p \leq 0.001$.

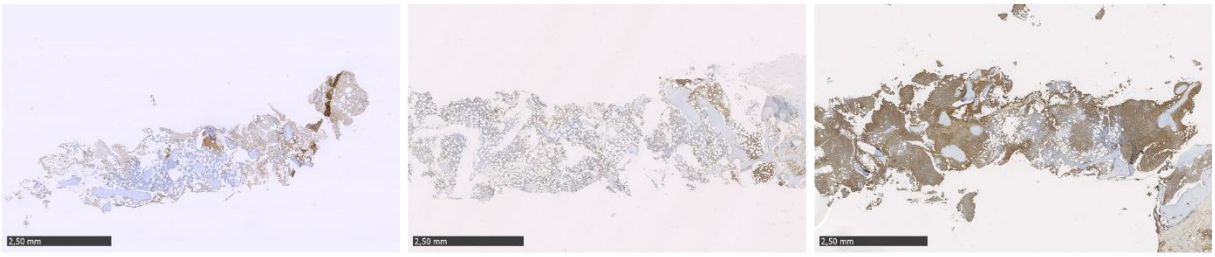
Photo pictures have been analyzed using ImageJ software and data are plotted as total length of sprouts. PBS injected group displays a negligible mean value of total sprout length of 33.26 pixels. The mean value of sprout length induced by RPMI8226^{SCR} cells is 357.62 pixels and that of RPMI8226$^{J1/2KD}$ cells of 229.83 pixels. The percentage decrease in the angiogenic sprout length between RPMI8226^{SCR} and RPMI8226$^{J1/2KD}$ cells is 35.7% indicating that Jagged1 and Jagged2 contribute to myeloma-associated angiogenesis.

4. CORRELATION ANALYSIS IN PATIENTS' BONE MARROW BIOPSIES BETWEEN JAGGED EXPRESSED IN MULTIPLE MYELOMA CELLS AND TUMOR ANGIOGENESIS

To confirm that *in vitro* and *in vivo* results reported above could recapitulate the molecular mechanism active in MM patients, bone marrow biopsies (BOM) from MM patients were analyzed to verify if a correlation exists between MM cell infiltration, the expression of Jagged1 and Jagged2 and the formation of new vessels.

To address this issue, MM patients' BOMs with different infiltration grades (5 patients for each infiltration grade) were stained with antibodies for the CD34 to identify ECs, Jagged1 and Jagged2 to evaluate Notch ligands distribution; HES6 to assess Notch signaling activation and VEGF-A to evaluate the presence of the major angiogenic factor explored in the previous *in vitro* study.

First of all, to assess the infiltration grade BOMs from patients collected at the Unit of Pathology of San Paolo Hospital were stained for κ/λ light chain (DAB-brown) to evaluate MM cells percentage in relation to non-tumor cells. Three stages were identified according to MM cell infiltration percentage: up to 20% has been considered as low infiltration grade (Low), from 21% to 50% as medium infiltration grade (Medium) and more than 51% as high infiltration grade (High). In Figure 16 three representative pictures show examples of different levels of MM cells infiltration.



Kappa light chain – Low infiltration | Kappa light chain – Medium infiltration | Kappa light chain – High infiltration

Figure24-MM cells infiltration after κ/λ light chains staining. BM aspirate from MM patient with (A) 10% infiltration defined as low infiltration, (B) 25% infiltration defined as medium infiltration and (C) 90% infiltration defined as high infiltration. Nuclei are stained with hematoxylin (Blue). Scale bar 2.5mm

For every infiltration grade, 5 different patients were further analyzed for the other selected markers in order to evaluate their distribution in relation to MM cell distribution and percentage of infiltration. Biopsies have been stained with antibodies for Jagged1 and Jagged 2, HES6, CD34 and VEGF-A. In Figure25 a complete panel for each marker is reported.

Patients with low infiltration (Figure25D) displays fewer CD34+ cells as compared to the other with a higher grade of infiltration (Figure25E and F). Moreover, CD34 staining appears more intense at high levels of infiltration as compared to low and medium indicating a role of MM cell infiltration in inducing of a well-established network of vessels.

Jagged ligands expression is correlated to the range of MM cells infiltration. Additionally, Jagged1 (Figure25 from G to I) generally displays a lower expression level respect Jagged2 that shows a more intense staining on equal grade of infiltration (Figure25 from H to M).

HES6 (Figure25 from O to Q) represents a measure of Notch pathway activity. Consistently, it follows a similar modulation of Jagged ligands, indicating an increased Notch pathway activation with higher MM cell infiltration, consistently with the evidences shown in this and other works (75) and a causal correlation between Jagged expression and Notch pathway activation.

Finally, VEGF-A (Figure25 from R to T) shows an increased expression in BOMs with high infiltration grade compared to low and medium grade. Interestingly, VEGF-A signal does not always overlap with κ chains staining indicating that it can be produced by different BM resident cells.

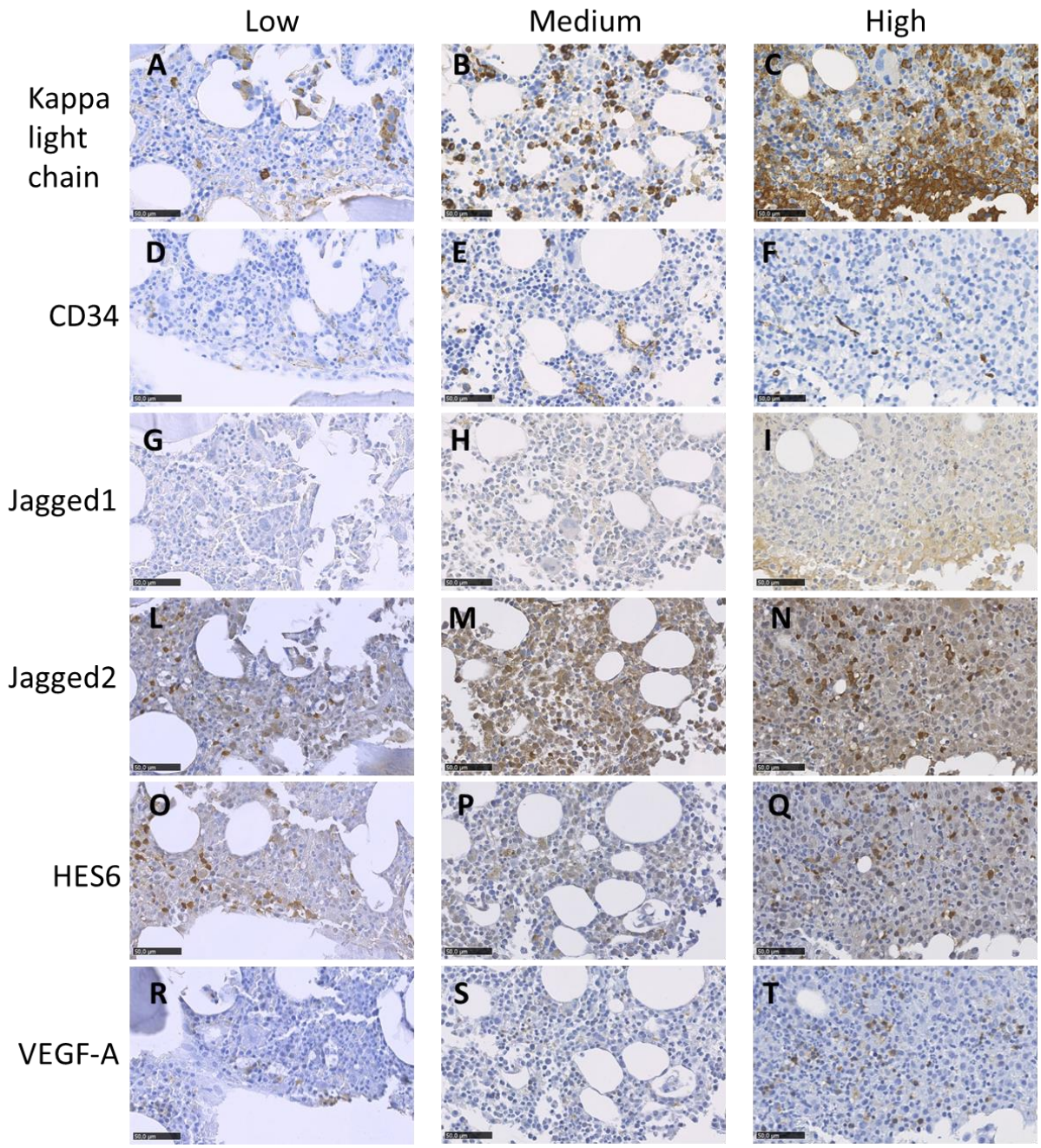


Figure25- Representative pictures of MM patients' biopsies at different grades of infiltration with the all evaluated markers. (A-B-C) Kappa light chain; (D-E-F) CD34; (G-H-I) Jagged1 ligand; (L-M-N) Jagged 2 ligand; (O-P-Q) HES6; (R-S-T) VEGF-A; All markers are stained with DAB (brown), nuclei with hematoxylin (blue). Photos A, D, G, L, O and R comes from BM aspirate of patients with low MM cell infiltration. Photos B, E, H, M, P and S are from medium infiltration percentage. Photos C, F, I, N, Q and T from high infiltration percentage patient. Scale bar 50µm.

Concerning the relationship between MM cells and angiogenesis, the immunohistochemical analysis of patients with medium infiltration percentage also indicates that MM cells are arranged in clusters corresponding to CD34+ cells elongated and better organized as reported in Figure26. On the other side there

are no specific signals corresponding to VEGF-A. I speculated that VEGF-A plays an earlier role, that could explain its lack in zone where vessels are already developed.

Consistently with this hypothesis, in areas with fewer MM cell infiltration (Figure27A and D), the staining for CD34 appears less intense and vessels do not seem to be completely developed (Figure27B and E). In the same areas VEGF-A shows higher expression level (Figure27C and F) indicating that angiogenesis stimulation is an ongoing event.

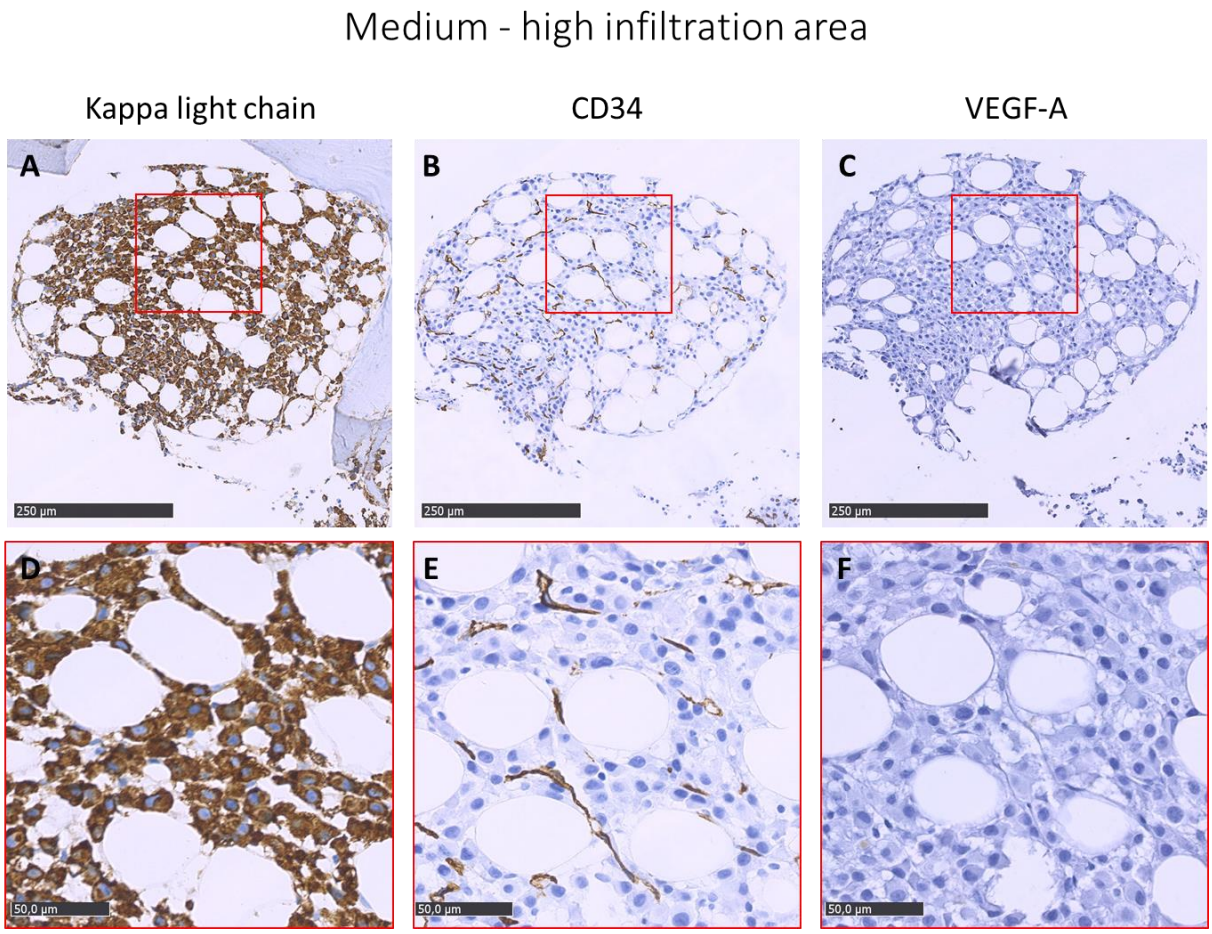


Figure26- Patients with medium infiltration percentage: focus on area with MM cells cluster. MM cells stained for Kappa light chain (A); ECs stained for CD34 (B) and VEGF-A (C). Nuclei are stained with hematoxylin (blue). Inset of red square with higher magnification of Kappa light chain (D), CD34 (E) and VEGF-A (F). in relation to MM cell cluster CD34 marker appears as dark brown and vessel are elongated. No signal derives from VEGF-A. Scale bar A, B and C 250µm; D, E and F 50µm.

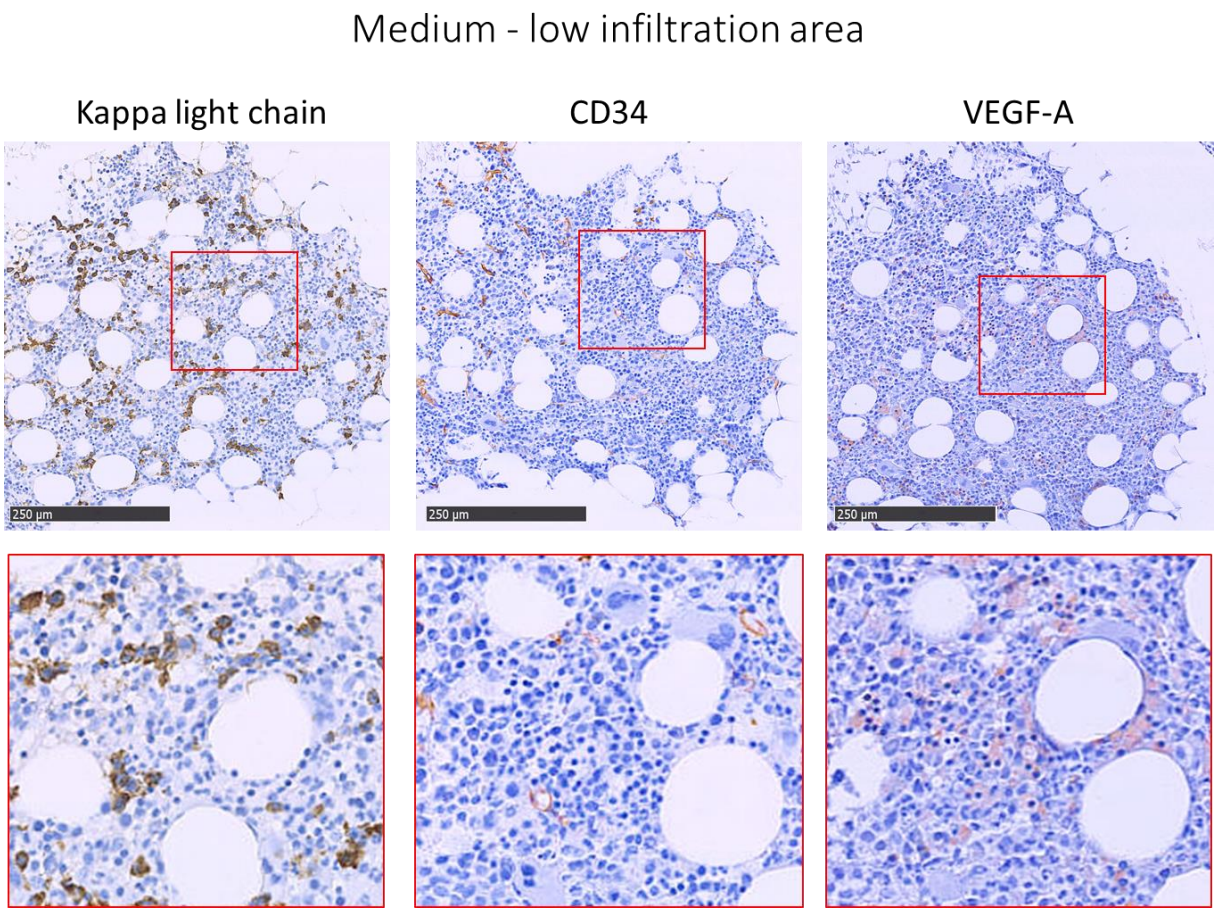


Figure27- Patients with medium infiltration percentage: focus on area without MM cells cluster. MM cells stained for Kappa light chain (A); ECs stained for CD34 (B) and VEGF-A (C). Nuclei are stained with hematoxylin (blue). Inset of red square with higher magnification of Kappa light chain (D), CD34 (E) and VEGF-A (F). Scale bar of A, B and C 250µm; D, E and F inset of red square.

Another confirmation stems from on the analysis of BOMs with high infiltration of MM cells. As shown in Figure28, the high level of infiltration of these areas does not allow to identify MM cells clusters. But analyzing areas with different levels of cellularity, as reported in Figure28A, B and C, it is possible to observe that those surrounded with red square (Figure28D, E and F) do not display CD34+ cells but show high level of VEGF-A expression, while areas marked with blue squared area display high expression of CD34+ ECs and almost the absence of VEGF-A+ cells.

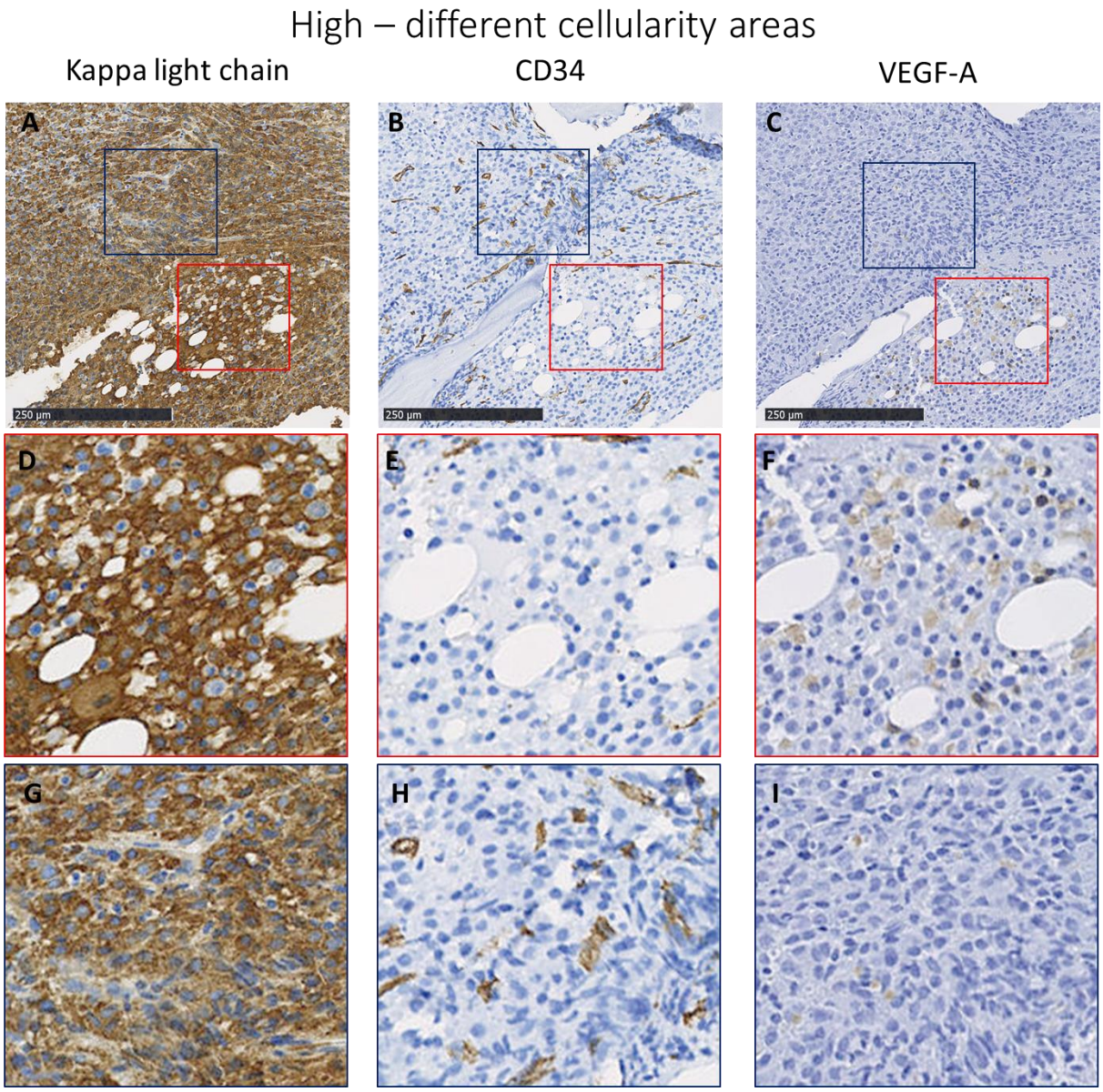


Figure28- Sequential biopsy sections of patients with high MM cells infiltration. MM cells are identified by antibody anti-Kappa light chain (A, D and G); ECs are stained with anti-CD34 (B, E, and H) and coloration for VEGF-A is reported, too (C, F and I). Nuclei are stained with hematoxylin (blue). In A, B, and C insets of red square of area with low cellularity. Inset of blue square of area with high cellularity. Scale bar of A, B and C 250µm.

A more general evaluation of the immunohistochemistry results was obtained by a microscopically analysis in which a score has been attributed in proportion to the number of cells expressing the marker in analysis (from 1 to 3). This analysis clearly indicates that Jagged1 and HES6 expressions increase together with MM cell infiltration percentage.

Indeed, the scoring of markers indicate that Jagged1 (Figure29A) displays an increase during MM cell infiltration, while Jagged2 is always expressed at high levels (Figure29B) and does not show any modification in relation to MM cell infiltration grade, suggesting that it can be dysregulated in early stage of MM

progression. HES6 (Figure29C) shows a statistically significant increase between medium and high infiltration grade indicating that the increase of Notch pathway activation could be a late event associated with MM cell accumulation within the BM, possibly due to the increased Jagged1 expression.

Similarly to Jagged1, the expression of the angiogenic marker VEGF-A (Figure29D) and of the EC marker CD34 displays the same trend of MM cell infiltration grade.

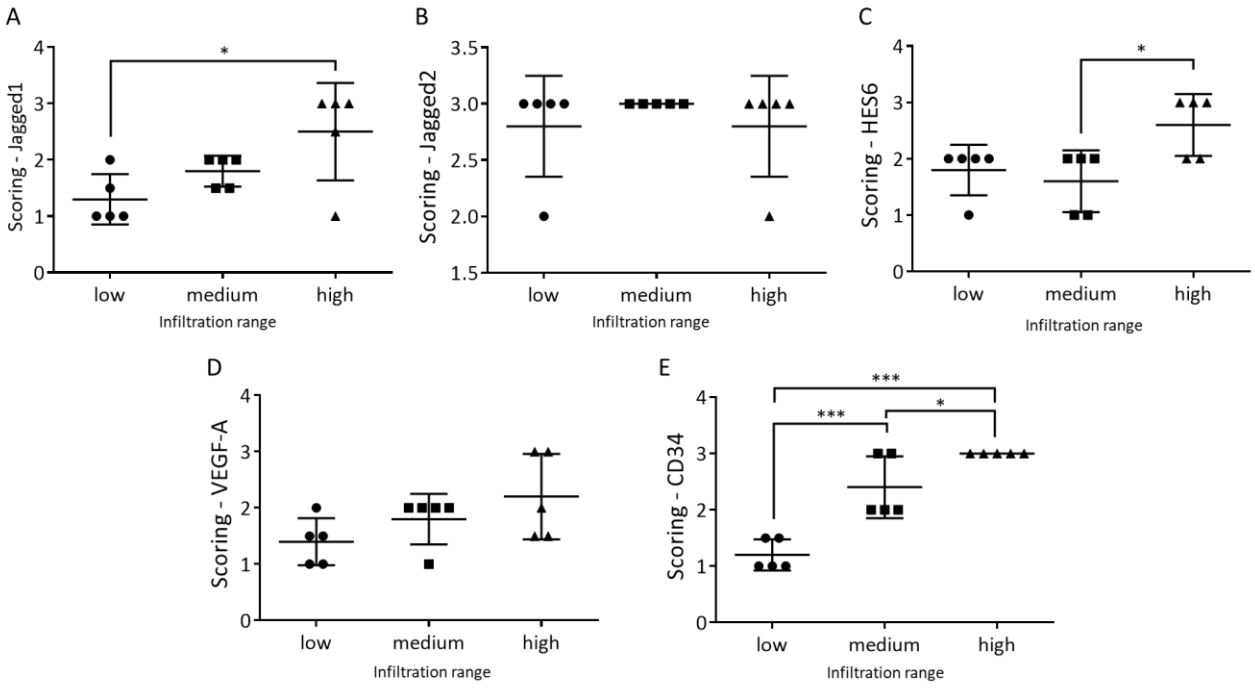


Figure29- Scoring of analyzed markers in BOMs of MM patients. Graphs show the score of Jagged1 (A), Jagged2 (B) HES6 (C), VEGF-A (D) and CD34 (E) related to patients at the different grade of infiltration. For each BOM, we have analyzed 3 fields. The score indicates the proportion of cells expressing a specific marker. Statistical analysis on 5 patients for each grade of infiltration is carried out by one-way ANOVA with Tukey post hoc test, * = $p \leq 0.05$; *** = $p \leq 0.001$.

On the whole, these results are consistent with *in vitro* and *in vivo* evidences indicating a key role of Jagged ligands in the angiogenic switch induced by MM cell infiltration. More specifically, these results confirm the role of Jagged1, while the effect of Jagged2 in MM-associated angiogenesis needs to be better elucidated.

DISCUSSION AND CONCLUSION

MM is a plasma cells malignancy characterized by the accumulation of tumoral cells within the BM (56). The development of this tumor includes an initial benign phase of MGUS that evolves in MM characterized by bone lesions accumulation and tumor angiogenesis (95). Angiogenesis is a hallmark of tumor progression and is a fundamental process for tumor growth and survival by providing oxygen and nutrients and creating a preferential way for tumor cell dissemination. Thereby this work aims to explore the mechanisms activated by MM cells to induce tumor angiogenesis during disease progression with a final purpose to provide rational molecular targets for an anti-angiogenic and anti-tumor therapeutic approach.

Notch pathway plays a key role in driving the differentiation choices by regulating cell-cell communication and, regarding the study scope of this work, it is also involved in angiogenesis regulation (89, 106, 111). Indeed, Notch receptors together with Dll ligands control EC differentiation and proliferation in new vessels formation process (82), in a tight interplay with VEGF pathway (82). Angiogenesis is a complex process coordinated by soluble factors and direct cell-to-cell contact and involves the direct effect of tumor cell on ECs, but also the ability of tumor cells to stimulate the angiogenic potential of surrounding healthy cells in the microenvironment, i.e. BMSCs.

MM displays Notch pathway dysregulation caused by the over-expression of Jagged1 and Jagged2 ligands that correlates with disease progression (95). The Notch pathway is involved in MM cell proliferation , survival (95) and drug resistance (65). Moreover, it plays a crucial role in MM crosstalk with surrounding BM resident cells including osteoclasts (75) and BMSCs (112).

This work aims to investigate the role of MM-derived Jagged ligands in stimulating the angiogenic potential of tumor microenvironment by directly affecting EC behavior or indirectly boost the angiogenic potential of BMSCs.

This study stems from previous evidences obtained by the laboratory where I attended my PhD program and other groups. Indeed, previous studies have clarified that ECs from MGUS or MM patients display different behaviors. Vacca and collaborators have demonstrated that ECs derived from MM patients (MMECs) display a potentiated angiogenic phenotype (113). Indeed, MMECs increase the expression of VEGFR2 and bFGFR2 consistently with a better response to the angiogenic factors VEGF and bFGF. Additionally, MMECs ameliorate their capability to develop an organized grid of tubes in Matrigel assay and induce higher numbers of vessels in chick embryo chorioallantoic membrane (CAM)-gelatin sponge assay. This indicates that MMECs acquire an increases ability to support tumor growth and dissemination (98).

In a previous collaborative work with Vacca's group, we have demonstrated that this altered behavior of MMECs may be, at least in part, ascribed to high levels of Notch signaling. As a matter of fact, Notch1 and

Notch2, together with the Notch targets Hey1 and HES1 are upregulated in ECs from MM patients respect those from MGUS. In addition, we demonstrated that *in vitro* Notch activation in ECs is triggered by Jagged ligands expressed on MM cells by a direct cell-to-cell mechanism (paper in publication on Neoplasia).

It is well-documented that during physiologic and tumor angiogenesis Notch contributes in vessels formation upon VEGF stimulation (82). In this molecular circuit, VEGF triggers VEGFR2 that upregulates Dll4. Dll4 increase is essential to start the sprouting of a new vessel, since it drives the differentiation choice toward tip cell. On the contrary, through the process of lateral inhibition, high level of Dll4 turns off Notch expression in tip cells, activates it in the adjacent stalk cells and downregulates VEGFR2, thereby allowing only tip cells to respond to VEGF stimulation. Thus, Dll4 acts as negative regulator of angiogenesis abrogating VEGF stimulation through VEGFR2 (114, 115).
In contrast to Dll4, Jagged1 exerts a positive regulatory role in angiogenesis thank to its capability to play an antagonist role on Dll4-Notch axis (92, 94). Indeed, it has been shown that a decoy peptide selectively able to inhibit Jagged1-mediated activation of Notch1 causes the reduction of EC angiogenic activity (116). This evidence suggests that MM-derived Jagged might be play a key role in promoting MM-associated angiogenesis.

In the light of these evidences, my thesis work therefore aims to dissect the effect of MM-derived Jagged in driving angiogenesis focusing on the direct stimulation of ECs or the strengthening of the angiogenic potential of BMSCs.
Concerning the first point we reasoned that the effect of MM-derived Jagged on EC angiogenesis could be dual: 1) Jagged ligands may trigger Notch signaling within the same MM cell boosting the secretion of tumor-derived angiogenic factors, or 2) MM cell-derived Jagged may trigger the angiogenic Notch signaling within the EC resulting in new vessels formation.
I explored *in vitro* three subsequent steps of the angiogenetic process. EC adhesion to ECM, cell migration and organization of a tube network reasoning that the first two steps occurring in the initial phase could be triggered by soluble stimulation form MM cells, while the third and later step could depend either on soluble factor released by MM cells or on the direct contact from between EC and MM cells.
Results show that the MM-derived Jagged ligands activate Notch signaling within the same tumor cells boosting their ability to release soluble factor in the conditioned medium that potentiate EC adhesion, motility and tube organization. By contrast Jagged silencing strongly reduces these effects.
Moreover, it has been shown that the pro-angiogenic effect of MM-derived Jagged can be mediated not only by the increased release of soluble factors from MM cells, but EC mediated tube formation may also exploit a direct cell-to-cell contact. This is consistent with the hypothesis that, when ECs belonging to the new sprouted vessels come in contact with MM cells, MM-derived Jagged may trigger Notch signaling in ECs resulting in the strengthening of their organization ability. In particular, I have demonstrated this ability using

soluble Jagged1. We cannot exclude that Jagged 2 may play a similar role, but it should be noted that immunohistochemical analysis on patients' biopsies indicates that Jagged1 expression is associated to increased microvessels density, while Jagged2 expression is constitutively expressed at high levels in MM patients independently from the angiogenic switch.

To complete this picture, I also evaluated the contribution of MM-derived Jagged in the secretion of angiogenic factors by tumor cells. In consideration of the central role played by VEGF a key angiogenic factor produced by MM cells, I further investigated whether MM-derived Jagged could modulate VEGF expression in MM cells. Through an inhibitory approach I demonstrated that the expression of Jagged resulted in the activation of Notch signaling in MM cells together with increased VEGF expression, by contrast Jagged silencing and the subsequent Notch pathway down-regulation significantly decreased VEGF gene transcription as well as VEGF protein expression. Thereby, in the complex relationship between VEGF and Notch, if VEGF inhibits Notch transcription via Dll4-mediated lateral inhibition, we demonstrated that the activation of Notch signaling triggered by Jagged ligands results in VEGF increased expression. This positive regulation of VEGF has been confirmed in another work, as a matter of fact, it has been demonstrated that a co-culture system of MM-BMSCs through the engagement of Jagged2 and Notch2 induces increase in VEGF production and secretion (109).

As anticipated this work takes also in consideration the effect of MM-derived Jagged on the angiogenic potential of BMSCs basing our work hypothesis on the evidences that MM cells can shape surrounding BM cells exploiting Notch pathway. Indeed, Jagged-mediated interaction with Notch-expressing BMSCs or OCLs causes Notch target gene upregulation and modification in BM resident cells-released factors (75, 112). We have observed a similar effect for the release of angiogenic factors.

Our results indicate that, when BMSCs are co-cultured with MM cells, their ability to release soluble angiogenic factor is increased and the effect is, at least in part, dependent upon the expression of Jagged. Indeed, Jagged1 and 2 silencing in MM cells abrogate their ability to stimulate co-cultured BMSCs to release a conditioned medium with potentiated angiogenic properties. In particular, we have explored the effect of the conditioned medium obtained by these co-culture systems for their ability to stimulate the same three steps of the angiogenic process studied above. Also, in this case, we could assess that at least part of the increased angiogenic potential of BMSCs stimulated by MM cells expressing Jagged is mediated by VEGF. As a matter of fact, although BMSCs alone is able to produce VEGF, the gene expression level of this angiogenic factor was increased in BMSCs when stimulated by MM cells and resulted in increased level of protein. Moreover, this effect was totally reverted upon Jagged silencing in MM. Together these data further confirm the central role of MM-derived Jagged ligands in shaping angiogenesis also through an action mediated by BMSCs.

Also the role of MM cells in shaping microenvironment toward an angiogenic supportive behavior was confirmed by another work demonstrating that BMSCs was stimulated by MM cells to increase the expression of other angiogenic factors including angiopoietin, FGF, PDGF and HGF (99). It has also been shown that the forced expression of ICN1 in a stromal cells line promoted their ability to induce modifications in ECs behavior that show enhanced ability to form grid-like structure(117). *Dao* and collaborators used HUVECs and aortic ring model to assess the effect of stromal-derived CM on ECs tube organization and sprouting capability. They showed that overexpression of Notch1-ICN in stromal cells increases ECs capability in *in vitro* assays and they have verified that this effect is mediated by a VEGF by the use of SU5416, a VEGFR2 inhibitor (117).

To extend our results to a more complex situation, I confirmed MM-derived Jagged role in stimulating sprouting angiogenesis by using an *in vivo* zebrafish embryo model on MM. Embryo zebrafish model of MM results a useful system that recapitulates the complexity of a complete organism with a rapid development and simple use and manipulation. This animal model allows a simpler and more replicable transplantation and it results less expensive compared to a mouse model which requires longer time for development and results acquirement. Finally, zebrafish model shows similar cytokines pattern and similar pathways as mouse model and human being supporting the use of zebrafish as pre-clinical model.

Results obtained after xenotransplanting the RPMI8226^{SCR} cells and the corresponding silenced RPMI8226$^{J1/2}$ in zebrafish embryos clearly indicated that MM-derived Jagged1 and 2 increase the sprouting vessels at the levels of SIV suggesting that also in a more complex system, MM cells promote angiogenesis.
It is possible to hypothesize that also in this *in vivo* model, the effect of MM-derived Jagged could be mediated by VEGF, indeed the blockade of VEGFR2 by SU5416 inhibitor reverts the effect (119). Moreover, the same group has demonstrated that the introduction of human VEGF into embryos considerably increases SIV formation (119).

Finally, to demonstrate that the picture draft through *in vitro* and *in vivo* findings was also representative of the angiogenic sprouting induced by MM localized in the myelomatous bone marrow of MM patients, I have tried to recapitulate the main results previously obtained through an immunohistochemical analysis of BM biopsies from MM patients.

The correlation between MM and Notch pathway have been widely demonstrated exploiting human samples (107). Similarly, it has been demonstrated that MM progression correlates with angiogenesis increase (98).

The immunohistochemistry on human biopsies shows a correlation among MM infiltration, Notch pathway activation, VEGF modulation and new blood vessels formation. Interestingly, only Jagged1 increases together with MM infiltration grade, VEGF expression and CD34^{+} endothelial cells, while Jagged2 does not show any modulation in relation to MM cells infiltration grade. This result suggests that Jagged1 is essential for the

angiogenic switch, while Jagged2 is unrelated. This is in line to what is reported by different papers demonstrating that the upregulation of Jagged2 can occurs in the earlier benign phase of MGUS (64, 107) when angiogenesis has not been activated yet, while Jagged1 upregulation occurs only in MM phase characterized by the angiogenic switch (120).

Moreover, IHC analysis also indicate that specific areas characterized by the presence of a mature vessels show a decreased signal of VEGF. On the contrary, higher expression of VEGF is detected close to shorter vessels or simply few endothelial still not organized in vessels. These evidences are consistent with a complex regulation of VEGF, positively regulated by Notch activation, but also by hypoxia-induced HIF1α. Thereby, following VEGF secretion and the promotion of new vessels formation, as expected, new vessels release oxygen and nutrients, re-establishing normoxyc conditions with the consequent degradation of HIF1α and decrease transcription of VEGF.

In conclusion, this work confirms the central role of Jagged in MM also regarding tumor angiogenesis. This, together with other evidences previously obtained in this laboratory showing that Jagged1 and Jagged2 overexpressed in MM cells are key in pharmacological resistance (121), bone disease (75), tumor cell proliferation (112), indicate that Jagged ligands could be exploited as therapeutic targets for novel drugs. Indeed, the laboratory where I attended my PhD program recently provided the proof of concept that the interaction between Jagged ligands and Notch receptors can be disrupted by using novel small molecules (122). The use of small molecules interrupting only Jagged-mediated activation of Notch can overcome the gastrointestinal toxicity related to the use of Notch pan-inhibitors such as γ-secretase inhibitor (123) as recently demonstrated by Kangsamaksin and coworkers (124, 125).

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SCIENTIFIC PRODUCTS

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