Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE BIOMEDICHE

Ciclo XXVI

Settore Concorsuale di afferenza: 06/D3

Settore Scientifico disciplinare: MED/15

THE SEARCH FOR MULTIPLE MYELOMA STEM CELLS: MOLECULAR CHARACTERIZATION AND SELF-RENEWAL MECHANISMS INVOLVED IN THE DISEASE PERSISTENCE

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Esame finale anno 2014

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1. MULTIPLE MYELOMA

Multiple myeloma (MM) is a neoplastic plasmacell disorder that is characterized by clonal proliferation of malignant plasma cells in the bone marrow microenvironment, the presence of monoclonal immunoglobulin (Ig) in the blood or urine, and associated organ disfunction (1). It is a genetically complex disease that is becoming more common in today's ageing population, and it accounts for approximately 1% of neoplastic diseases and 13% of hematologic cancers. Worldwide, approximately 86,000 patients will be diagnosed each year with myeloma, which, in many areas, makes it the second most common hematologic malignancy, while about 63,000 patients die every year from disease-related complications. In the United States, due to an aging populace, it is anticipated that the number of cases of myeloma will grow by 57% between 2010 and 2030 (2), ranking myeloma behind only stomach and liver cancer in the rate of growth of new cases. In Italy, the disease is more prevalent in men than women, and approximately 2,200 patients will be diagnosed each year, and the risk of developing this cancer in their lifetime ranges from 3.6 per thousand women (one woman out of 275) to 5.2 per thousand of men (one man out of 191).

1.1 THE B CELL IMMUNE RESPONSE

To better get insight into MM pathogenesis, it is important to understand how B cells develop. A virgin B cell that encounter antigen is able to generate a low-affinity plasma cell or stimulates its migration to a germinal centre. In the germinal centre, affinity maturation occurs and is mediated through two processes: somatic hypermutation and antigen selection. Subsequently, class switch recombination occurs, leading to the development of immunoglobulin (Ig) isotypes (4). Once this process is complete, the plasmablast leaves the germinal centre and migrates to the bone marrow where it becomes a long-lived plasma cell that produces antibody. The machinery that is necessary to generate these physiological DNA rearrangements can malfunction, leading to mutations in crucial oncogenes and tumour suppressor genes, and malignant change. Key challenges for a plasma cell include switching off cellular characteristics that are no longer required, such as cell cycling, activating programmes that are essential for antibody production, and undergoing apoptosis if they do not find a receptive niche in the bone marrow. Failure to complete these programmes correctly could potentially leave active cellular processes, which may result in the features of myeloma. The key transcription factors underlying this coordinated differentiation process are also shown in the figure below (Fig. 1). BCL-6, B cell lymphoma 6; BLIMP1, B lymphocyte-induced maturation protein 1; CIITA, MHC class II transactivator; ID3, DNA-binding protein inhibitor ID3; PAX5, paired box gene 5; XBP1, X box-binding protein 1 (3).

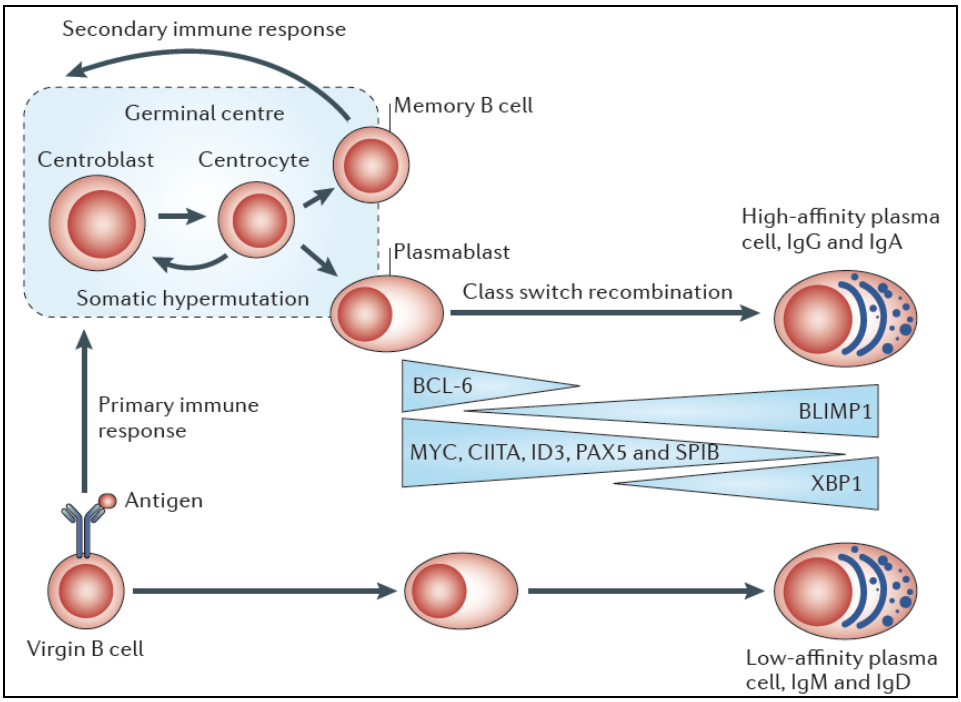

Fig. 1 B cell development in Multiple Myeloma

1.2 INITIATION AND PROGRESSION OF MYELOMA: THE EVOLUTION OF THE DISEASE

Myeloma evolution is thought to start from a monoclonal gammopathy of undetermined clinical significance (usually known as MGUS) that progresses to smoldering myeloma and, finally, to symptomatic myeloma . MGUS is an indolent, asymptomatic condition that transforms to myeloma at a rate of 1% *per annum*. Smoldering myeloma lacks clinical features; by contrast, symptomatic myeloma has various clinical features that are collectively referred to as calcium, renal, anaemia and bone abnormalities (CRAB), which provide an indication that treatment is required (5). Later in the disease progression, the myeloma plasma cells become able to migrate from bone marrow to extramedullary sites. It is thought that the passage through these different states requires the acquisition of genetic aberrations that lead to the development of the biological hallmarks of MM. It has been proposed that the ancestral deregulated cell belongs to the MGUS clone; however, subsequent to the development of sufficient genetic alterations, it acquires a clonal advantage, expands and evolves. This clonal evolution is through the branching pathways that are typically associated with Darwin's explanation of the origin of species. Along the evolution from MGUS to myeloma, these processes lead to the development of several ecosystems, which correspond to the clinically recognized phases of disease. At the end of this evolutionary process, at the stage of plasma cell leukaemia (PCL), the clone is proliferative and it is able to escape from the bone marrow; it expands rapidly and leads to patient's death. Cells at this stage are almost fully genetically altered, and the precursor subclones is under-represented, due to the competition for the access to the bone marrow stromal niches, and they

might be eradicated by more aggressive clones (6-14). In evolutionary terms, this phase of disease could be considered to be initiated by a migration and founder effect whereby a cell that is able to survive and grow in the peripheral blood is faced with no competition, thus limiting its clonal expansion (3). All these fundamental concepts are resumed in the figure blow (Fig.2).

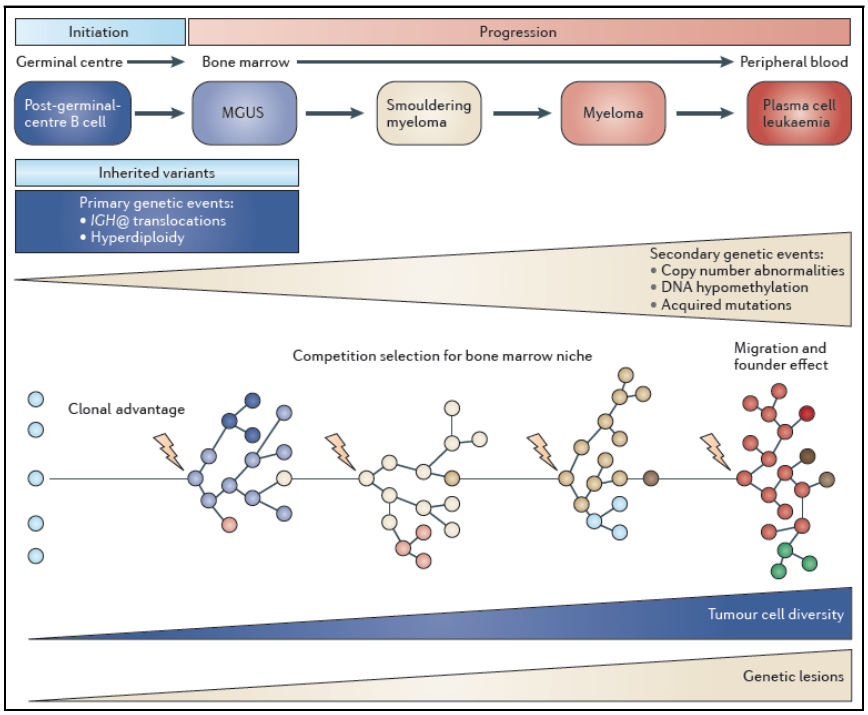

Fig. 2 Myeloma initiation and progression follows a Darwinian evolution

1.3 GENETIC BASIS OF MULTIPLE MYELOMA

In the classical view of the initiation and progression of MM, an initiating hit is required to immortalize a Multiple Myeloma initiating cell. Such a cell is then able to acquire additional genetic hits over time, mediated via 5 major type of alterations: translocation, loss of heterozygosity, gene amplification/deletion, mutation, or epigenetic changes. The acquisition of additional hits further deregulates the behavior of the MM initiating cell, leading to the clinically recognized features of MM (5). The basic premise underlying these interactions is that multiple mutations in different signalling finally lead to the alteration of the intrinsic plasma cell biology, modifying it in a way that generate the features of MM.

1.3.1 Translocations

At the cytogenetic level, the MM genome is recognized as being complex (6,9). The study of chromosomal translocations generated by aberrant class-switch recombination showed that several oncogenes, including cyclin D1 (*CCND1*), *CCND3*, *MAF*, *MAFB,* fibroblast growth factor receptor 3 (*FGFR3*), the MMSET domain (*MMSET*; also known as *WHSC1*), are placed under the control of the strong enhancers

of the heavy chain Ig (*IGH*) loci, leading to their deregulation (15). Deregulation of the G1/S transition is a key early molecular event in MM, and the consistent deregulation of a D-group cyclin was first noted as a consequence of studying the t(11;14) and t(6;14) translocations, which deregulate cyclin D1 and cyclin D3, respectively (5). Overexpression of a D-group cyclin independent from translocation can also occur, and in the t(14;16) is modulated via *MAF*, which up-regulates *CCND2* by binding directly to its promoter. Patients with the t(4;14), which translocates *FGFR3* and *MMSET* to the *IGH* enhancers, also overexpress cyclin D2, but in this case the underlying mechanism is uncertain (15). Other *IGH* translocations are observed in MM and, in contrast to the class-switch recombination– driven events, tend to occur in the advanced phases of disease. The gene typically deregulated by such events is *MYC*, whose deregulation may lead to a more aggressive disease phase. Translocations outside of the Ig gene loci can also occur and constitute an important mechanism leading to gene deregulation that has not been yet fully explored (5). However, it is known that such translocations can range from single to multiple events per patient, but no recurrent events deregulating a single crucial gene have yet been identified (16).

1.3.2 Copy number alterations

The frequency and recurrent nature of interstitial loss of copy number and loss of heterozygosity suggests that the minimally deleted regions contain tumor-suppressor (TS) genes that are driver events (17,18). Most TS genes require the inactivation of both alleles and have been identified either by the study of homozygous deletions or through the integration of mutational analysis with copy number status (20). Examples of relevant TS genes include *FAM46C*, *DIS3*, *CYLD*, baculoviral IAP repeat containing protein 2 (*BIRC2*; also known as cIAP1), *BIRC3*, and TNF receptor associated factor 3 (*TRAF3*) (6,9,10). Deregulation of the G1/S transition by overexpression of a D-group cyclin is a key early molecular abnormality in MM. Conversely, also important events are the loss of a negative cell-cycle regulator, the down-regulation of *CDKN2C* by loss of chromosome 1p32, and the inactivation of *CDKN2A* by methylation (9,21). Inactivation of *RB1* also affects this checkpoint and may occur as a result of loss of chromosome 13, which is present in 58% of cases of MM; however, homozygous loss and mutational inactivation of this gene is infrequent (6). Other important regions of loss of heterozygosity include 11q, the site of the *BIRC2* and *BIRC3* genes; 16q, the site of *CYLD*; and 14q32, the site of *TRAF3* (17-19). All of these genes played in the NF-κB pathway, indicating that iperactivation of NF-κB signaling is important in MM. The other major set of recurrent genetic aberrations seen in MM is the hyperdiploidy, associated with the gain of the odd-numbered chromosomes, including 3, 5, 7, 9, 11, 15, 19, and 21. Interstitial copy number gain associated with increased gene expression or with activating mutations in oncogenes represents another set of “driver” genes that can lead to MM progression. A classic example is the amplification of 1q, which potentially harbors more than one relevant oncogene; for example, CDC28 protein kinase 1B (*CKS1B*), acidic leucine rich nuclear phosphoprotein 32 family member E (*ANP32E*), *BCL9*, and *PDZK1* (9).

1.3.3 Mutations

There are approximately 35 nonsynonymous mutations per case in MM (6,22); this value is intermediate between the one observed in the genetically simpler acute leukemias (8) (23), and the one observed in the more complex epithelial tumors, such as lung cancer (540) (24). There are few recurrently mutated genes in MM and, in general, they affect known oncogenes. However, a few novel genes have been identified (*FAM46C* in 13% of cases, *DIS3* in 11% of cases) and as the numbers of samples analyzed increases, the incidence of recurrent genes will undoubtedly increase as well. This observation is consistent with a hypothesis in which deregulation of pathways is pathogenically important, rather than the deregulation of a specific gene. Examples of deregulated pathways include the frequent deregulation of the NF-κB pathway, and strategies targeting this pathway upstream of mutated genes may fail if the presence of activating mutations is not taken into account. The observation that the ERK pathway is frequently deregulated (*NRAS* in 24% of cases, *KRAS* in 27% of cases, and *BRAF* in 4% of cases), suggests the need for a novel treatment strategy targeting this pathway. Moreover, deregulation of the PI3K pathway is also important in MM, but in contrast to the RAS pathway, the PI3K pathway is not frequently mutated (6). The frequency of these events makes MM a good model system in which to evaluate targeted inhibitors of the RAS and PI3K pathways.

1.3.4 Epigenetic changes

Despite the recent evidencies on the genetics of MM, little is known about the epigenetic changes leading to disease progression and their impact on therapy resistance. DNA can be modified by different modifications. In particular, methylation of cytosine residues in CpG dinucleotides and, in addition, chromatin structure may be modified via histone modifications such as methylation, acetylation, phosphorylation,and ubiquitination. Both DNA and histone modifications can play a part in modulating gene expression. The most important epigenetic change relevant to the pathogenesis of MM is global hypomethylation and gene-specific hypermethylation during the transformation of monoclonal gammopathy of undetermined significance to MM. The most relevant DNA methylation change is seen in the 15% of patients with the t(4;14) translocation, who have increased gene-specific hypermethylation compared with other cytogenetic subgroups. This subgroup enhanced *MMSET* overexpression, which encodes a histone methyltransferase and transcriptional repressor. *MMSET* mediates histone 3 lysine 36 (H3K36) dimethylation, and its deregulation leads to global changes in histone modifications that promote cell survival, cell-cycle progression, and DNA repair. Other chromatin modifiers are also deregulated in MM, including *UTX*, a histone demethylase, *MLL*, *KDM6B*, and *HOXA9*, and the full importance of these modifications needs other validations (25-28)

1.4 MODEL OF MOLECULAR PATHOGENESIS

Based on the current knowledge on MM biology, a model for the molecular pathogenesis of MM has been proposed (Fig. 3). Chromosome alterations appears to identify two different, but perhaps overlapping, pathways of pathogenesis: Non-hyperdiploid tumors and Hyperdiploid tumors. In approximately 40% of the tumors, a primary chromosome translocation results in the dysregulated expression of an oncogene and direct or indirect *CCND* dysregulation. Like the primary *IGH* translocations, trisomies of chromosomes 3, 5, 7, 9, 11, 15, 19, and 21, are already present at the initial identified stage of tumorigenesis, and define subtypes of MM with distinctive clinical (eg, bone disease, heavy-chain subtypes, and prognosis), molecular (eg, types of *CCND* expressed and associated mutations), pathological (eg, morphology and CD expression), and cytogenetic (ploidy) features. A second "genetic hit" leading to subsequent transformation from MGUS to MM may be mediated by activation of *MYC*, mutation of *KRAS*, or del(13). The MYC pathway may be further dysregulated by late rearrangements, often involving an Ig locus. Activating mutations of the NFκB pathway and inactivating mutations of *TP53* are associated with extramedullary migration of disease, and inactivation of *CDKN2C* and *RB1* with increasingly proliferative disease (29).

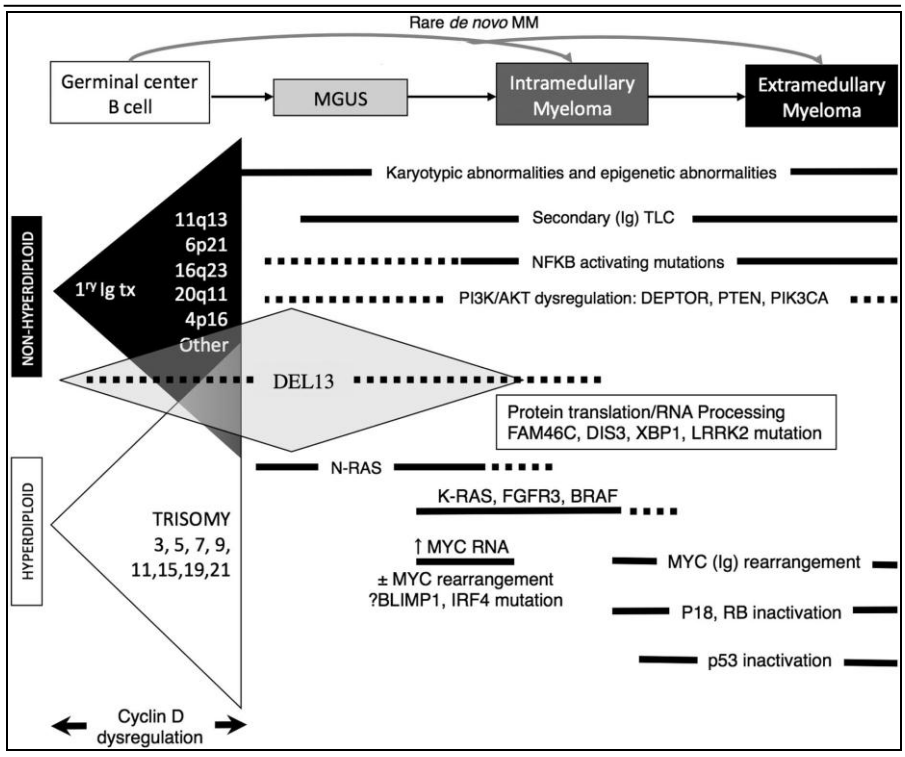

Fig. 3 Model of molecular pathogenesis of Multiple Myeloma

1.5 THERAPEUTIC STRATEGIES IN MULTIPLE MYELOMA TREATMENT

A number of advances over the past decade have dramatically improved patient outcomes. Among these are the advent of novel chemotherapeutics, including the immunomodulatory agents thalidomide, lenalidomide, and pomalidomide, and the proteasome inhibitors (30) bortezomib and carfilzomib. All of these drugs have garnered regulatory approvals and are used in patients with newly diagnosed, relapsed, or relapsed/refractory disease and have contributed to a doubling of the median overall survival.

1.5.1 IMiDs

Thalidomide and its derivatives represent the class of antineoplastic compounds called ImmunoModulatory Drugs (IMiDs). The efficacy of these agents in MM and other hematologic malignancies is attributed to their immunomodulatory, antiinflammatory, and antiangiogenic properties. IMiDs target tumor cells directly by inducing cytotoxicity and indirectly by interfering with components of the bone marrow microenvironment that promote MM progression (31).

Thalidomide, initially introduced in Germany in 1957 as a sedative, was withdrawn from the market in 1961, when it was linked to severe fetal malformations. The discovery of its activity in patients with MM renewed the interest in thalidomide (32). Thalidomide induces apoptosis of MM cells and down-regulates the expression of several cytokines involved in cell proliferation and survival, such as TNFα, IL-6, and VEGF. However, its precise mechanism of action has not been fully understand (33). Although the molecular target of thalidomide has yet to be detected, a recent study showed that cereblon (CRBN), a protein encoded by a candidate gene for mental retardation, binds thalidomide and mediates its teratogenicity (34). Clinically, the use of thalidomide in relapsed/refractory MM is associated with response rates (RR) ranging from 25% to 65% (31). The main side effects of thalidomide include sedation, peripheral neuropathy, bradycardia, hypotension, constipation, and venous thromboembolism.

The second- generation thalidomide analogues lenalidomide and pomalidomide (CC4047) are IMiDs developed to enhance the anticancer properties and reduce the adverse effects associated with thalidomide. Lenalidomide proved effective in refractory patients including those who had relapsed following thalidomide treatment (35). Furthermore, lenalidomide generated superior response rates along with progression-free and overall survival compared to thalidomide in newly diagnosed patients (36). Pomalidomide elicited responses in 47% of patients who had received three or more previous regimens, including lenalidomide (37). More clinical studies investigating the activity of pomalidomide are needed, but these data suggest that pomalidomide is clinically effective in advanced MM, even when the disease is refractory to other IMiDs.

1.5.2 Proteasome Inhibitors

The proteasome is a multi-subunit, cylinder-shaped protein complex that degrades ubiquitinated proteins. Plasma cells are terminally differentiated B-cells that are specialized for the secretion of immunoglobulins. The increased protein load associated with this task lowers the threshold for proteotoxic stress and increases the susceptibility of plasma cells to toxic misfolded/unfolded proteins that trigger proapoptotic signals of the unfolded protein response (UPR) and endoplasmic reticulum (ER) stress response (38). Additionally, plasma cell differentiation is accompanied by a dramatic decrease in expression of the proteasome(39). Taken together, these cellular characteristics are thought to make plasma cells particularly sensitive to inhibitors of the proteasome. In addition, the proteasome regulates the expression of proteins and cytokines that promote MM growth and angiogenesis, and inhibit apoptosis, such as Nf-kB (40).

The proteasome inhibitor bortezomib was developed for the treatment of MM based on this rationale, and it has been widely recognized as a remarkable clinical success. Bortezomib received accelerated approval from the U.S. Food and Drug Administration (FDA) in 2003, after clinical efficacy was demonstrated in refractory MM (41). Bortezomib was later approved for first-line treatment of MM. Despite the efficacy of bortezomib, MM cells invariably develop resistance to it.

Carfilzomib, a second generation proteasome inhibitor, has shown efficacy against bortezomib-resistant MM cell lines and primary patient samples in vitro (42), and has also exhibited promising activity in patients (43). The activity of carfilzomib against bortezomib-resistant MM cells may be due to its pharmacological profile, which differs from bortezomib. Both drugs inhibit the same proteasomal subunit (20S chymotrypsin-like b5 subunit), but only carfilzomib does so irreversibly.

1.5.3 Toward a targeted therapy in MM

Targeted treatment based on the presence of a specific molecular lesion predictive for response to that treatment is the likely way forward so that we can achieve personalized cancer care for MM patients. The best illustration of this approach is the treatment of the t(4;14) subtype of MM, which has been associated with poor prognosis. In the table below (Tab.2, Fig. 4), are listed novel therapeutic agents that are currently in clinical development for the treatment of MM along with the associated clinical protocol.

Molecular feature	Current detection method	Future detection method	Potential targeted treatment
t(4;14) Overexpression of *MMSET* and *FGFR3*	FISH	Targeted NGS, RQ-PCR, GEP	Proteasome inhibitors (?), MMSET inhibitors, FGFR3 inhibitors, MEK inhibitors
t(14;16), t(14;20) Overexpression of *MAF* or *MAFB*	FISH	Targeted NGS, RQ-PCR, GEP	MEK inhibitors
ISS/FISH high risk Combination of t(4;14) or t(14;16)/t(14;20), del(17p) and/or gain(1q)	FISH, SSCP	Targeted NGS, RQ-PCR + FISH/SSCP, GEP + FISH/SSCP	Treatment intensification, novel drugs
BRAF V600E mutation	Various (SSCP, Sanger sequencing)	Targeted NGS	BRAF inhibitors
Unfavorable GEP	GEP	Validated GEP signature, GEP-derived RQ-PCR (?)	Novel inhibitors targeting overexpressed genes, eg, AURKA inhibitors
Absence of unfavorable features	FISH	Targeted NGS, RQ-PCR + FISH, GEP + FISH	Combinations of established agents, innovative maintenance strategies

RQ-PCR indicates real-time quantitative PCR.

Tab. 2 Novel therapeutic agents in clinical development for Multiple Myeloma's targeted therapy

1.5.3.1 Therapeutic mAbs

The introduction of the mAb rituximab has revolutionized the clinical care of B-cell lymphomas. However, the search for a clinically efficacious mAb for patients with MM has been less challenge thus far (45). *Rituximab* is a monoclonal antibody against the B-cell specific membrane protein CD20. The rationale for this therapeutic strategy was that CD20 is expressed in 10–15% of MM plasma cells (46). Unfortunately, the use of rituximab provided no clinical benefit in a phase II study of ten patients with MM (47), and it did not demonstrate significant clinical activity in a cohort of 14 patients selected for CD20-expressing MM (48).

Interleukin 6 (*IL - 6*) is known to play an important role in growth, differentiation, and survival of normal and malignant plasma cells. The transforming potential of IL-6 is underscored by the fact that IL-6 overexpressing transgenic mice show accelerated development of malignant plasmacytomas (49). Monoclonal antibodies against IL-6 have been developed, and they have been used with success against Castleman's disease, a rare B-cell lymphoproliferative disorder (50), but clinical experience in MM is currently lacking.

Elotuzumab has shown anti-MM activity both *in vitro* as well as in an *in vivo* MM xenograft model (51). Many monoclonal antibodies are currently being evaluated in clinical trials for MM, with different targets and strategies. Some of these antibodies are designed to target surface proteins of plasma cells (e.g., CD38, CD56) or MM growth factors (e.g., IL-6), while others have been coupled to cytotoxins or chemotherapy agents.

1.5.3.2 Agents Directed Against Dysregulated Translocation Products

The t(11;14) (q13;q32) chromosomal translocation is a known translocation of MM. The t(11;14) translocation juxtaposes the IgH locus with the*CCND1* gene (also called *BCL1*), which leads to overexpression of the *CCND1* gene product cyclin D1. Cyclin D1 associates with cyclin-dependent kinase 4 (CDK4) to form a catalytically active complex that drives progression through the G1/S phase of the cell cycle. Subsequently, up-regulation of cyclin D1 as a result of the t(11:14) translocation has been implicated in the uncontrolled proliferation of MM plasma cells (52,53). The selective targeting of cyclin D1 in one model was accompanied by compensatory up-regulation of cyclin D2 and demonstrated only modest inhibition of MM cell proliferation in vitro (54). By contrast, targeting CDK4 kinase activity with the small molecule inhibitor P276-00 was a more potent strategy for inhibiting growth of MM cells in vitro and tumors in vivo (55). Flavopiridol is a broad spectrum CDK inhibitor with activity against CDK1, CDK2, CDK4, CDK7, and CDK9. CDK9 is a key regulator of transcription as it functions as a subunit of the P-TEFb (*P*ositive- *T*ranscription *E*longation *F*actor b) complex, which phosphorylates the carboxyterminus of RNA polymerase II, a signaling event that releases the enzyme into the elongation phase of transcription (56). Targeting CDK9 is a promising strategy for the treatment of MM.

The t(4;14) (p16;q32) chromosomal translocation is also common in MM, and is associated with worse prognosis (72). At least some recent clinical trial data support frontline treatment with proteasome inhibitors for this subtype and, in view of the characteristic oncogene profile, it is the optimum group in which to address the potential role of FGFR3 and MMSET inhibitors. This is a good example of how, by characterizing the biology of an initiating lesion thought to be present in 100% of cells, we can begin the development of novel targeted treatments. The realization that MMSET is a member of a family of oncogenes with H3K36me2 transferase activity raised the possibility of targeting this activity as a therapy for MM. Therefore, the crystal structure of the MMSET protein is currently being resolved and this information is being used in specific structure-function– based drug design approaches to specifically inhibit the activity of this enzyme. This translocation may induce overexpression of the FGFR3 gene, which encodes for a receptor tyrosine kinase. All cases overexpress MMSET, a histone methyltransferase, but about one third of cases do not overexpress FGFR3 (58). Moreover, FGFR3 amplification may occur even in the absence of t(4;14) (59). These mutations produce a constitutively active receptor, which exhibits ligand-independent dimerization and autophosphorylation. NF449, a novel compound that antagonizes FGFR3 signaling, was found to be active against MM in vitro (60). Anti-FGFR3 agents, such as CHIR-258, a small-molecule inhibitor of multiple receptor tyrosine kinases including FGFR3 (61), and PRO-001, an FGFR3- specific mAb (62), showed activity in mouse models of MM. In view of these promising results, anti-FGFR3 agents are currently being evaluated in clinical trials for FGFR3-expressing MM.

1.5.3.3 Targeting the MM Tumor Microenvironment

The role of the tumor microenvironment in the development, progression, and resistance of various tumor types to therapy is well recognized (63). In MM, the impact of tumor microenvironmental factors such as hypoxia, angiogenesis, and interactions between MM and bone marrow stromal cells have become an important consideration for understanding disease progression, resistance to treatment, and have been incorporated into novel therapy screening approaches (64). For instance, bone marrow angiogenesis has been implicated in MM disease development, as it progressively increases along the spectrum of plasma cell dyscrasias, from monoclonal gammopathy of undetermined significance (MGUS) to smoldering myeloma, and advanced MM (65).

Malignant plasma cells not only secrete vascular endothelial growth factor (VEGF), a soluble protein that stimulates the growth of new blood vessels, but they can also express its receptors, VEGFR-1 and VEGFR-2 (66,67). After the availability and success of the anti-VEGF monoclonal antibody bevacizumab in the clinical practice against several types of solid malignancies (68), anti-angiogenic therapy was tested in MM, although the results with this strategy have been disappointing. In a phase II trial of vandetanib (formerly ZD6474), a small molecule receptor tyrosine kinase inhibitor of both VEGFR and epidermal growth factor receptor (EGFR), no responses were found among 18 patients with relapsed MM (70). Similarly, no clinical responses were observed in another phase II trial of 21MM patients with the use of pazopanib, a multi-targeted receptor tyrosine kinase inhibitor of VEGFR-1, VEGFR-2, VEGFR-3, PDGFR- a / b , and c-kit (68). The fact that not a single response was observed among a total of 66 MM patients in three different clinical trials using anti-angiogenic drugs casts doubt that this therapeutic strategy will be further explored in MM.

1.5.3.4 Other Targeted Therapies

Several targeted therapies were initially judged as promising in the treatment of MM based on preclinical evidence or their scientific rationale, yet clinical trials using these agents failed to demonstrate their utility in humans. We report the following selected experiences:

- *Tipifarnib*, a farnesyltransferase inhibitor, was administered to 43 patients with advanced MM, at a dose of 300 mg PO bid for 3 weeks every 4 weeks. The most common side effect was fatigue (66%). Although 64% of patients had disease stabilization, no complete nor partial responses were observed (69).

- *Oblimersen* is an antisense drug (a short sequence of RNA which hybridizes with and inactivates a specific mRNA, preventing the formation of the protein) blocking the Bcl-2

oncogene. Despite its activity in other hematologic malignancies, a phase III randomized study that included 224 patients found no clinical benefit of in MM (70).

- *Etanercept* is a tumor necrosis factor (TNF) alpha-neutralizing agent. It is a soluble protein engineered by fusing part of the TNF-receptor with the Fc portion of an IgG antibody. Treatment with etanercept produced no response among ten patients with refractory MM (71) .

- *Imatinib* is a tyrosine kinase inhibitor that blocks the activity of c-Abl, c-Kit, and PDGF receptors. The development of imatinib has been the most successful achievement of molecular biology applied to hematological malignancies. In a phase II trial of imatinib in 28 patients with refractory/relapsed MM, no responses were observed. Of note, 52% of cases had positive c-kit staining (71).

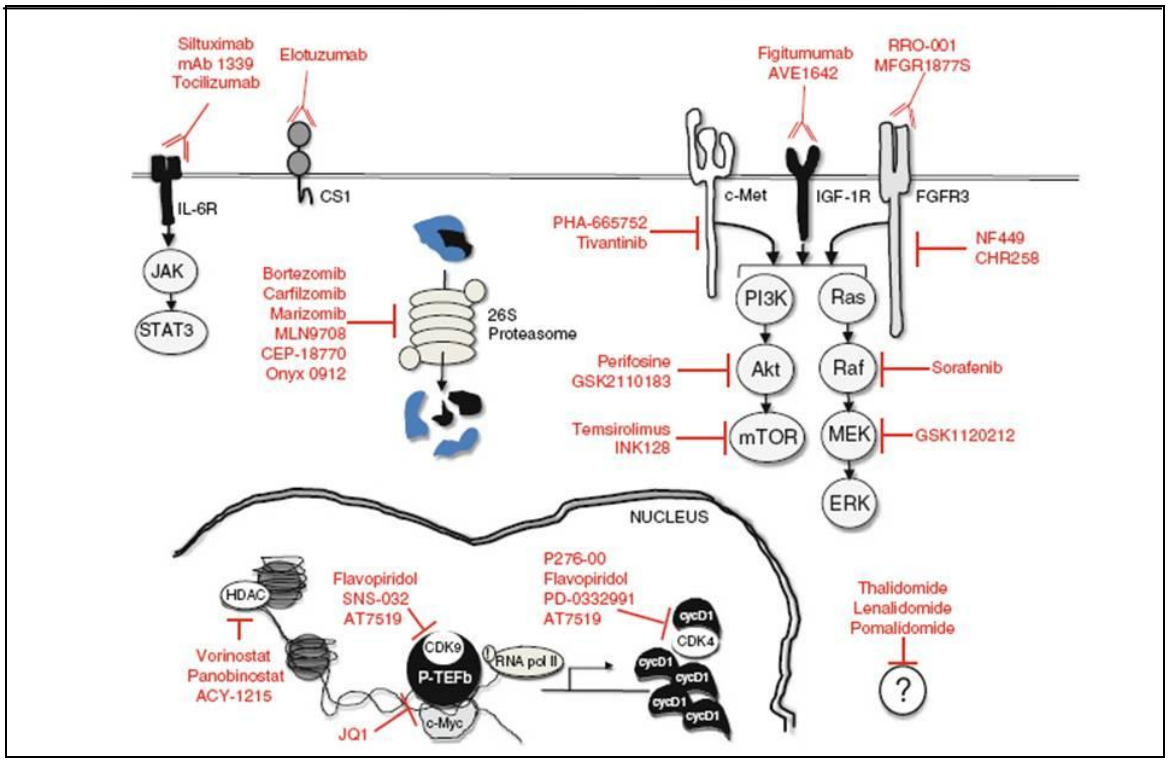

Fig. 4 Potential molecular targets in Multiple Myeloma along with the target-specific agents

2. CANCER STEM CELL

The consensus definition of “cancer stem cell” is cell within a tumor that possesses the ability to self-renew and to cause the different lineages of cancer cells that include the tumor. Cancer stem cells can thus only be defined by experiment based on their capacity to recapitulate the generation of a growing tumor. The numerous published studies used various terms, such as ‘‘tumorigenic cell’’ and ‘‘tumor-initiating cell’’ to describe putative cancer stem cells. A self-renewing cell division results in one or both daughter cells that have essentially the same ability to replicate and generate differentiated cell lineages as the parental cell. Moreover, they have the ability to undergo a symmetrical self-renewing cell division, that carry to the formation of identical daughter stem cells that have the self-renewal capacity, or an asymmetrical self-renewing cell division, resulting in one stem cell and one more differentiated progenitor cell. In addition, it is thought that stem cells may divide symmetrically to form two progenitor cells, which could lead to stem cell depletion. Promoting this form of division would be a way to deplete the cancer stem cell population and may constitute an alternative strategy to inducing cell death to fight cancer.

2.1 THE CANCER STEM CELLS HYPOTHESIS AND ITS IMPLICATIONS

The cancer stem cell hypothesis is at the center of a rapidly evolving research field that may play a pivotal role in changing the point of view on cancer. In the cancer stem cell model of tumors, there is a small subset of cancer cells, the cancer stem cells, which constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and maintain the tumor. These cancer stem cells have the capacity to both divide and expand the cancer stem cell pool and to differentiate into the heterogeneous non-tumorigenic cancer cell types that in most cases appear to constitute the bulk of the cancer cells within the tumor (Fig.5). If cancer stem cells are relatively resistant to treatment that have been developed to eradicate the rapidly dividing cells within the tumor that constitute the majority of the non-stem cell component of tumors, then they are unlikely to be curative and relapses would be expected. If correct, the cancer stem cell hypothesis would require that we rethink the way we diagnose and treat tumors, as our objective would have to turn from eliminating the bulk of rapidly dividing but terminally differentiated components of the tumor and be refocused on the minority stem cell population that fuels tumor growth (73).

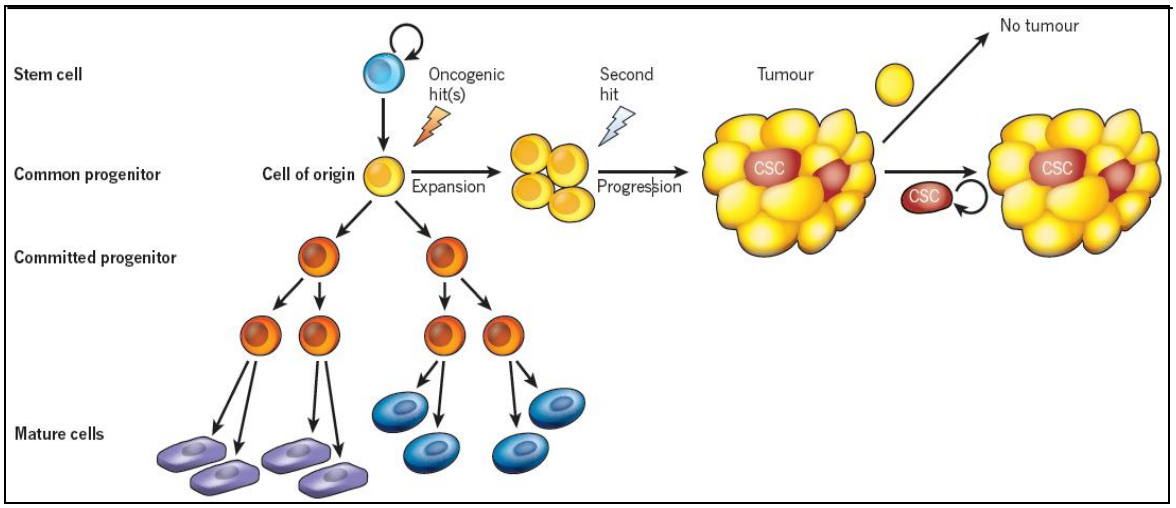

Fig. 5 The Cancer Stem Cells hypothesis

Cells within the tumor often seem to correspond to different stages of development. Epithelial cancers, for example, typically contain cells exhibiting divergent nuclear morphologies and differentiation features. Prevailing explanations for the observed tumor cell heterogeneity include influences of the microenvironment and genomic instability that generate the genetic and epigenetic changes, which prevent faithful and accurate replication and transmission of stable genotypes and phenotypes. Such instability could also explain why tumors typically contain a subset of cells that are refractory to most treatments. However, an alternative emerging concept is that malignant cell populations may reflect the continuing works of perturbed differentiation processes. Inherent to such a model is the formation of malignant populations consisting of a developmentally defined hierarchy of heterogeneous phenotypes derived from a small subset of ‘‘cancer stem cells” (74).

2.2 EVIDENCES OF CANCER STEM CELLS IN SOLID TUMORS

Evidence for the existence of cancer stem cells in solid tumors has been more difficult to obtain for several reasons. Cells within solid tumors are more difficult to obtain, and functional assays suitable for detecting and quantifying normal stem cells from many organs have not yet been developed. Therefore, the cell surface markers required to enriched such cells have not been identified.

There has been some important work in this field recently, including the demonstration that single mouse mammary cells can be transplanted and recapitulate a complete mammary gland. Cells have also been isolated from human breast tumors that can cause breast cancer in NOD/SCID mice through serial transplantations, suggesting a capacity for self-renewal. These cells were CD44+CD24- in eight of nine patients and established tumors in recipient animals when as few as one hundred cells were transplanted, whereas tens of thousands of breast cancer cells with a various marker set do not induce tumors. Brain

tumor stem cells that can produce serially transplantable brain tumors in NOD/SCID mice have also been isolated from human medulloblastomas and glioblastomas. These cells can be isolated by sorting for CD133+, a marker found on normal neural stem cells, and the transplantation of one hundred CD133+ tumor cells was sufficient to initiate the growing of a tumor in recipient animals. In contrast, no mice injected with the negative population developed brain tumors. More recently, cells have been isolated from human prostate cancer patients that can produce serially transplantable prostate tumors in NOD/SCID mice (75).

Together, these studies reveal that only a small subset of cells in several different tumor types have the ability to form the tumor in such transplant assays. These data are consistent with the cancer stem cell hypothesis. Nonetheless, caution needs to be exerted when interpreting transplantation assays. It is crucial to note that although many studies have clearly identified the lineage in which the cancer initiates, the unique cell type in the hierarchy in which transformation occurs remains elusive in most cases. Nevertheless, in mouse models of intestinal and prostate tumours, it seems clear that the cancers originate in a bona fide stem cell that is capable of self-renewal and multilineage differentiation.

2.3 CANCER STEM CELLS IN HAEMATOLOGICAL MALIGNANCIES

In different leukaemias, both normal stem and committed progenitor cells have been implicated as cellular targets of transformation Human cells fulfilling the properties expected of drug-resistant cancer stem cells were initially isolated from blood cancers.

For human AML, cancer stem cells were defined as those cells capable of recapitulating human AML cell populations in irradiated transplanted non obese diabetic (NOD)/severe combined immunodeficient (SCID) mice. The AML stem cells owing this property were found to display a CD34+CD38- cell surface phenotype, similar to that typical of normal human primitive hematopoietic progenitors. This suggested that the AML stem cells may have originated from normal stem cells rather than deriving from more committed progenitors, although as will be discussed, this may not necessarily be the case for all cancer stem cells.

In chronic myeloid leukaemia (CML) — one of the first disorders to be defined by a dominant genetic mutation — the long-term haematopoietic stem cell containing the *BCR–ABL* mutation has been established as the cell of origin by *in vivo* clonality studies in humans (76). Although the HSC maintains the chronic phase of the disease, analysis of samples from patients in blast crisis, has indicated that consequent genetic events occurring in downstream precursor cells give rise to leukaemia stem cells, highlighting the dynamic state of the tumorigenesis process (77). For mouse models of CML, only *BCR–ABL* targeted to HSCs, but not to committed progenitor cells, induced myeloproliferative disease (78), consistent with findings for human CML. Although HSCs generally appeared more susceptible to transformation than

committed progenitors, a self-renewal pathway seemed to be reactivated in the cells during development of disease.

2.4 IMPLICATIONS FOR CANCER THERAPY

The cancer stem cell hypothesis posits that cancer stem cells are a minority population of self-renewing cancer cells that fuel tumor growth and remain in patients after different treatment. The hypothesis predicts that effective tumor elimination will require obtaining agents that can target cancer stem cells while sparing normal stem cells. Experimental studies in human AML suggests that, compared with the bulk population of leukemic blasts, the leukemia stem cells are relatively resistant to conventional therapies. Although it has been speculated in solid tumors that conventional agents kill the non-tumorigenic cancer cells while sparing the cancer stem cells, this has not been proven. The moving target nature of cancer stem cells may present a challenge in the clinic.

To obtain effective introduction of new therapies, physicians will require methods of determining the type (or types) of cancer stem cells present in a given patient's tumor. It is important that agents directed against cancer stem cells discriminate between cancer stem cells and normal stem cells. This will require identification of realistic drug targets unique to cancer stem cells. The identification of such targets and the development of anticancer agents will require a fuller understanding of normal stem cell biology as well as the genetics and epigenetics of tumor progression. There is some indication that such an approach can be successful. For example, stem cells isolated from AML patients display differences from normal hematopoietic stem cells. There has also been some success identifying agents effective against leukemia stem cells. Conventional anthracycline agents show synergy with proteasome inhibitors against AML stem cells, reducing viability in vitro dramatically. The novel agent parthenolide, isolated from Mexican medicinal plants and shown to be a potent nuclear factor-nB inhibitor, promotes apoptosis of AML stem cells and inhibits tumor development in NOD/SCID mice. Mutation of the Janus-activated kinase 2 (JAK2) kinase is found in many patients with the blood disorder Polycythemia Vera, and JAK2 inhibitors display efficacy against the cancer stem cells from these patients, although individual responses vary significantly (73). In Tab. 3 are showed potential cancer stem cell self-renewal pathway inhibitors. Among the others, Hedgehog and Notch signaling inhibitors are already included in phase I and II study in patients with refractory MM.

Drug	Company / Institute	Pathway	Indication	Combination drug	Phase
LGK974	Novartis Pharmaceuticals	Wnt	melanoma, breast neoplasms, lobular carcinoma		I
PRI-724	Prism Biolab Corp	Wnt	advanced solid tumors		I
Resvaratrol	Univ of California, Irvine	Wnt	colon cancer		I / II
Diclofenac + Vitamin D3	Maastricht Univ Medical Center	Wnt + HH	basal cell carcinoma		III
RO4929097	Cancer Institute of New Jersey / NCI	Notch	multiple myeloma and plasma cell neoplasm after autologous stem cell transplant	melphalan	II
RO4929097	M.D. Anderson Cancer Center / NCI	Notch	lung cancer after front-line chemotherapy		II
RO4929097	Albert Einstein College of Medicine of Yeshiva Univ / NCI	Notch	cutaneous melanoma		II
RO4929097	Children's Oncology Group / NCI	Notch	brain and central nervous system tumors leukemia, lymphoma, unspecified childhood solid tumor	dexamethasone for all indications	I / II
PF-04449913	Pfizer	HH	solid tumors, unspecified hematological malignancies		I
PF-04449913	Pfizer	HH	chronic myeloid leukemia	dasatinib, bosutinib	I
LEQ506	Novartis Pharmaceuticals	HH	advanced solid tumors, recurrent or refractory medulloblastoma		I
IPI-926	Infinity Pharmaceuticals	HH	metastatic pancreatic cancer, recurrent head and neck cancer conventional chondrosarcoma	gemcitabine, cetuximab	I / II I II
GDC-0449	Roche Pharma AG Genentech	HH	various indications including MM**	various interventions**	I / II

**From clinicaltrials.gov July 2011: http://clinicaltrials.gov/ct2/results?term=gdc-0449

Tab. 3 Cancer stem cell self-renewal pathway inhibitors

3. MULTIPLE MYELOMA CANCER STEM CELLS

Neoplastic plasma cells are the hallmark of multiple myeloma. Like other hematologic malignancies, the vast majority of myeloma plasma cells appear mature and quiescent (5). This suggests that functional heterogeneity may exist within myeloma and the potential for clonogenic growth is restricted to a minority population of cells. The normal counterpart of myeloma cells are terminally differentiated plasma cells that lack substantial replicative capacity. Instead, these cells arise from the maturation of B cells. Therefore, it is possible that aspects of the hierarchical nature of normal B-cell and plasma cell development is maintained in multiple myeloma, similar to the relationship observed between relatively immature hematopoietic and neural stem cells in myeloid leukemias and brain tumors, respectively. Recent evidences have identified cells expressing the identical immunoglobulin idiotype and gene rearrangements as the neoplastic plasma cells within the blood and bone marrow of patients that phenotypically display a wide range of B-cell maturation. However, the role of these cells in the pathogenesis of the disease has been unclear and a point of controversy.

3.1 PHENOTYPIC HETEROGENEITY IN MULTIPLE MYELOMA

Unique immunoglobulin idiotype and gene sequences provide a highly specific means to establish clonal relationships in B-cell malignancies and have permitted the phenotypic heterogeneity of tumor cells to be studied within individual patients. The established relationship between normal B cells and plasma cells prompted identifications that myeloma plasma cells were derived from clonotypic B cells. Early evidence that peripheral blood lymphocytes were clonally related to myeloma plasma cells was provided by studies in which anti-idiotype antibodies formed against M protein were found to identify phenotypic B cells in the peripheral blood and bone marrow of patients with multiple myeloma (79-81). Subsequent studies examine the clonal relationship between plasma cells and B cells at the molecular level by means e ability to sequence tumor-specific immunoglobulin heavy chain gene sequences. Utilizing allele-specific oligonucleotide-based polymerase chain reactions, a number of studies have demonstrated that clonal cells expressing B-cell, rather than plasma cell, antigens could be found at varying frequencies within the bone marrow or peripheral blood of myeloma patients (82-88). Genetic, phenotypic, and functional studies have suggested that these clonotypic cells are equivalent to memory B cells. The analysis of immunoglobulin heavy chain gene sequences in myeloma demonstrates extensive somatic hypermutation without evidence of intraclonal variation suggesting that they arise from a post-germinal center compartment (89-91). Furthermore, clonotypic immunoglobulin gene rearrangements have been found within cells displaying a pre-switched isotype and in cells expressing surface CD19, CD27, and lacking CD38 consistent with memory B cells (82,92,93). The clinical relevance of circulating clonotypic cells has been controversial, but studies have suggest that these cells persist after systemic therapy (94-98). Functional evidence that these cells may give rise to myeloma plasma cells has been provided by evidence that earlier B cells can differentiate

into immunoglobulin secreting–plasma cells in vitro (100,101). The clonogenic growth potential of clonotypic B cells has also been studied both in vitro and in vivo. These cells have been found to engraft immunodeficient non-obese diabetic severe combined immunodeficiency mice and give rise to clonal plasma cells that recapitulate bone disease and monoclonal immunoglobulin production similar to the clinical disease (101-103). Furthermore, using an *in vitro* clonogenic assay, has been shown that colony formation was not a property of cells expressing the characteristic surface antigen CD138 that is expressed by myeloma and normal plasma cells (104). Instead, cells lacking CD138 but expressing B-cell surface antigens formed tumor colonies that could be serially replated. Similarly, CD138+ plasma cells were not able of engrafting non obese diabetic severe combined deficiency mice whereas CD138- cells, later defined to phenotypically resemble memory B cells, produced disease in immunodeficient mice (103,104). These results contrast with those obtained by detecting the growth of primary myeloma specimens within severe combined immunodeficiency mice implanted with human or rabbit bone fragments (105-107). In these models, CD138+ plasma cells that were implanted into the ectopic bone can proliferate and be re-transplanted into secondary recipients, whereas earlier B cells lack engraftment potential. The reasons for these controversies are not clear but they likely represent the intrinsic differences between the animal models used, similar to studies examining the growth of human leukemias (108). It is possible that the barriers of xeno-transplantation also play a role in these discrepancies and the characterization of clonogenic cells from a number of unique mouse models of myeloma might clarify this issue (109-112).

3.2 STEM CELLS PROPERTIES OF MULTIPLE MYELOMA STEM CELLS

The ability of multiple myeloma stem cells to self-renew and give rise to differentiated effectors (ie, plasma cells) are two properties they share with normal adult stem cells. Another property of normal stem cells is resistance to toxicities, and the continual risk of relapse among patients treated with standard therapies suggest that myeloma stem cells should also be relatively drug resistant. Resistance to standard cytotoxic agents and ionizing radiation has been demonstrated for leukemic and brain tumors stem cells, respectively (113-115). In multiple myeloma, several novel agents have been recently approved for clinical use. The ability of these drugs to produce disease responses seen as decreased mature cell compartments and suggest that they lack the ability to impact long term outcomes. Furthermore, it appears that myeloma stem cells display properties common to normal stem cells, such as expression of membrane-bound drug transporters, intracellular detoxification enzymes, and quiescence. Thus, the chemoresistance of cancer stem cells might be mediated by multiple processes similar to those protecting normal stem cells.

Matsui et al. (104) recently reported the results of *in vitro* studies apparently confirming the existence of so-called ‘‘MM stem cells’’ at the B lymphocyte level of differentiation (Fig.6). The authors showed that human MM cell lines contain a small (about 5%) subpopulation that lack CD138 expression. They performed serial replating experiments *in vitro* with MM cell lines and MM cells isolated from clinical

samples. CD138- cells isolated from the cell lines were shown to undergo significantly greater clonogenic expansion than CD138+ cells after serial replating. Similarly, CD138-MMcells from clinical BM samples gave rise to colonies and could be successfully replated, whereas CD138+ cells did not. In contrast to CD138+ cells, CD138- MM cells from human BM successfully engrafted longterm into NOD/SCID mice, indicating their potential for self-renewal. CD138- MM stem cells isolated from cell lines expressed CD19 and CD20 molecules characteristic of B lymphocytes. In addition, depletion of CD19-, CD22-, and CD20- cells from the population of CD138- MM cells obtained from clinical samples significantly decreased their clonogenic growth. Therefore, it is reasonable to assume that MM stem cells are CD19+, CD20+, CD22+, and CD138- and express clonal immunoglobulin light chains, consistent with the findings of previous studies on clonotypic MM B cells.

In contrast to MM CD138+ plasma cells, the clonogenic MM CD138- B cells were further revealed to be highly resistant to clinical antimyeloma agents in vitro (104). This is related, at least partially, to their high drug efflux capacity and intracellular drug detoxification activity, as shown in Hoechst efflux and Aldefluor assays. Cells with these characteristics have been found in the PB of MM patients as well. Therefore, it is reasonable to assume that circulating clonotypic B cell populations represent MM stem cells, and that the relative resistance of such cells to chemotherapy leads to relapse following chemotherapy and SCT. On the other hand, some earlier studies have suggested that cells with properties of MM stem cells reside within malignant plasma cell fraction (105). Yaccoby and Epstein (106) used a model of humanized SCID mice (SCID-hu) with human bone implant and sorted plasma cells based on their CD38++CD45- phenotype from the BM and blood of MM patients. Interestingly, they showed that only plasma cells could engraft into the human bone implant and produce xenogenic myeloma in recipient animals. In contrast, the remaining fractions, regardless of B lymphocyte content, did not produce MM. Moreover, the circulating plasma cells obtained from human blood appeared to grow more avidly in the SCID-hu hosts compared with their bone marrow counterparts. This suggests that these cells represent a subpopulation of the plasma cells in the bone marrow. Importantly, the MM plasma cells could engraft only human bone implants, not murine organs. They also could disseminate the disease to another implant, meaning that a fraction of them circulated within the murine model. These results have been argued by Matsui et al., since they reached the opposite conclusion, suggesting that the human BM environment used in the SCID-hu model could selectively enhance the proliferation of a more differentiated plasma cell fraction, as it does in the AML blast cell population. If this were the case, then the methodology of Yaccoby and Epstein would not provide reliable data for determining the MM stem cell phenotype.

The similarities between normal adult stem cells and myeloma cancer stem cells suggest that specific signaling pathways conserved between these cell types can serve as therapeutic targets. For instance, highly conserved signaling pathways are required for axial patterning during embryonic

development, such as Notch, Wnt, and Hedgehog. Accumulating data have demonstrated that these pathways play a role in regulating normal stem cells and the pathogenesis of a wide variety of human cancers, including multiple myeloma. Recently, a role for these developmental pathways in regulating cancer stem cells has begun to emerge. Aberrant activation of Hedgehog signaling has been identified in multiple myeloma and may have distinct biologic effects on plasma cells or stem cells (116). Within plasma cells, this pathway primarily mediates survival similar to a number of other signal transduction pathways. In contrast, the Hedgehog signaling pathway appears to regulate the fate decisions of myeloma cancer stem cells. Pathway activation by ligand results in the expansion of immature myeloma cells, whereas the inhibition of signaling utilizing a ligand-neutralizing monoclonal antibody or antagonists of the positive mediator of pathway signaling, smoothened, induces plasma cell differentiation.

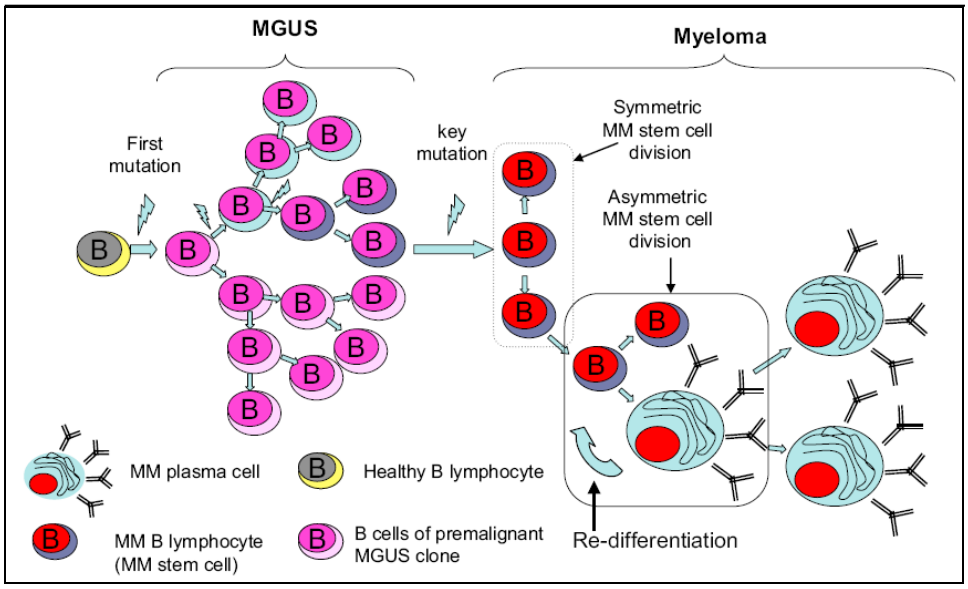

Fig. 6 B cells during development from MGUS to MM

3.3 THE CLINICAL TRANSLATION OF MULTIPLE MYELOMA STEM CELLS HYPOTHESIS

A major challenge in translating the cancer stem-cell hypothesis will be evaluating the efficacy of novel therapeutic strategies generated from basic studies of clonogenic myeloma cells. Traditional measures of response have relied on the serial measurement of serum and urine M proteins and bone marrow plasmacytosis, and these criteria have evolved over time as investigators have sought to more stringently define complete remissions based on the belief that the total disappearance of detectable tumor burden correlates with overall survival benefit. In the initial WHO criteria, complete remissions were defined by resolution of the serum or urine M protein. A negative immunofixation test was subsequently added by the European Group for Blood and Marrow Transplantation to the criteria required to achieve a complete remission followed by normalization of the serum free light chain assay and absence of clonal

plasma cells by immunohistochemistry in the recently proposed uniform response criteria (118,119). However, neither the magnitude nor the kinetics of the response have been shown to impact overall survival, although the use of molecular techniques to define prognostic patient categories may identify subsets of patients in which this relationship holds true (120,121). These parameters are not likely to be helpful in assessing early changes in myeloma stem cells since they evaluate changes in the burden of malignant plasma cells. Therefore, the best indicators of response to multiple myeloma stem-cell–targeted approaches at present may be progression-free and overall survival, but these end points have typically required large numbers of patients and prolonged periods of follow-up. New trial designs that incorporate novel end points will be needed to study myeloma stem-cell–targeted therapies. One potential strategy is to incorporate these approaches with existing therapies to determine whether they prevent tumor re-growth and prolong the duration of remissions after cytoreduction with chemotherapeutic or novel agents. This approach could be studied in a randomized phase II design comparing the primary end point of response duration between patients receiving induction therapy alone with those additionally administered a myeloma stem-cell–directed therapy. The demonstration of a significant difference in conjunction with evidence for the specificity of these approaches would lend support to the clinical importance of eradicating myeloma stem cells. Thus, a secondary end point could be correlative laboratory studies directly assessing multiple myeloma cancer stem cells that might include the use of serial clonogenic assays. Furthermore, the quantitative detection of circulating myeloma stem cells by flow cytometry utilizing combinations of surface antigen expression and functional stem-cell assays may be used to monitor cells during treatment.

4. HEDGEHOG SIGNALLING

4.1 HEDGEHOG SIGNALLING IN CANCER

The orderly process of development depends upon well orchestrated signals. These signals transform a single cell into a complex multicellular organism. Incredibly, in spite of the complex end result, the transformation retains few types of signals, including Wnt, Notch, transforming growth factor-b, fibroblast growth factor and Hedgehog (Hh). These secreted protein signals direct rearrangement of cells by motility and adhesion changes, cell proliferation, epithelial-to-mesenchymal transitions and the cell fate determination. The processes used to build organs and tissues during development are highly relevant to cancer. Recent evidence suggests that tumors used normal developmental pathways for their own growth; by activating a single transduction signal, tumors can grow, recruit a blood supply and migrate adjacent tissues. Between others, Hh signaling contributes to normal organ development and the reawakened Hh cascade drives the initiation, growth, invasion and maintenance of tumors associated with these organ systems (122).

Hh signaling was first described in the context of cell fate determination and patterning of the fruit fly, Drosophila melanogaster. The core components of the Hh pathway that were identified in Drosophila are conserved in mammals. The basic signaling cascade consists of a series of repressive interactions, with each protein holding the next in check, until the transcription of a still mostly unknown array of target genes is affected. In the absence of Hh pathway activity, these target genes are actively repressed. When secreted Hh binds to its receptor Patched (Ptc/PTCH), the inhibition of Smoothened (Smo/SMOH), a G-protein-coupled receptor that activates downstream intracellular components of the pathway, by Ptc is relieved. The transcription of target genes (including Ptc and Gli) is subsequently activated by the Gli family of transcription factors (Fig. 7) (124).

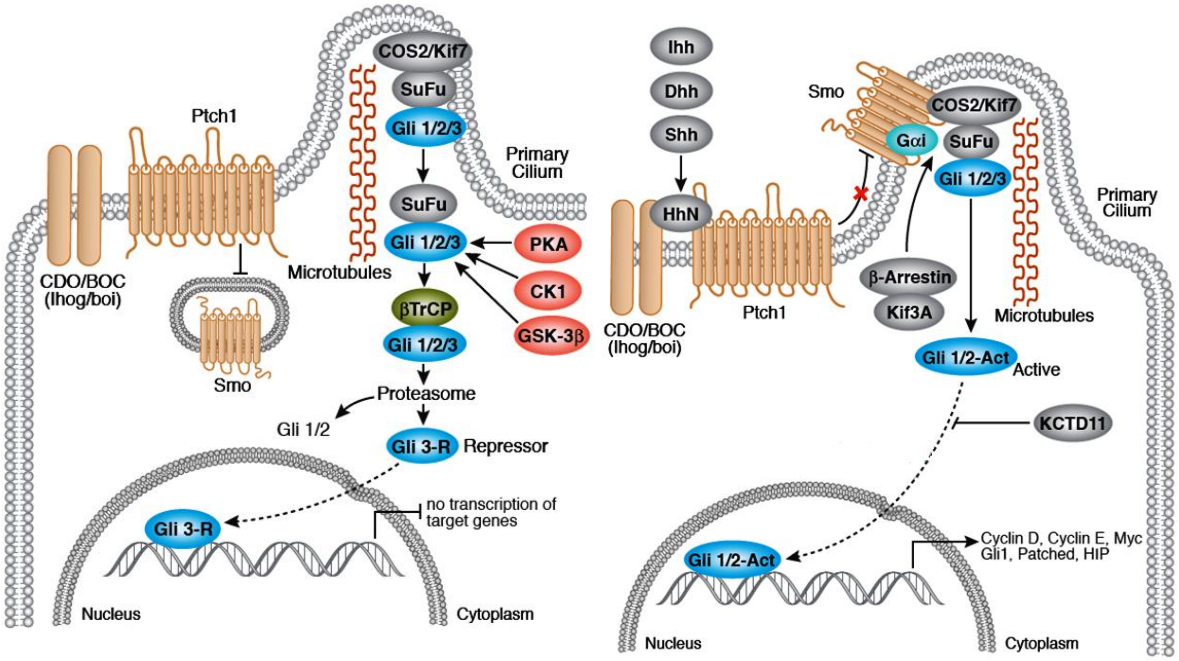

Fig. 7 Hedgehog pathway: OFF and ON state

The resultant genetic program forms and organizes many tissues and organ systems during embryogenesis. The role Hh plays in the growth of tumors can be classified according to how the pathway is activated. These mechanisms include loss-of-function mutations in inhibitory proteins such as Ptc1, gain-of-function mutations in positive regulators such as Smo and overexpression of the Hh ligands, leading to autocrine or paracrine activation of the pathway and renewal of cancer stem cells. Hh signaling was first linked to cancer when a mutation in PTCH, the gene encoding PATCHED1, was found to cause Gorlin syndrome, a rare genetic disorder characterized by tumor formation in the skin (basal cell carcinoma, BCC), cerebellum (medulloblastoma, MB) and soft tissue (rhabdomyosarcoma, RMS). Loss of one copy of PTCH is sufficient to cause the syndrome; germline mutation of both copies is presumed fatal based on mouse models. In the tumors, both copies of the gene are often inactivated. In the late 1990s, most sporadic BCCs were found to have hyperactivated Hh signaling. Subsequently, mutations in other Hh pathway components, including hyperactivating mutations of SMO and loss-of-function mutations in Suppressor of fused (SUFU), have been discovered in BCC. Activating Hh pathway mutations can cause sporadic MB. As is typical in other developmental pathways, activating components of the pathway are potential proto-oncogenes that promote tumor growth when overactive, whereas restraining components of the normal pathway are tumor suppressors that allow tumor growth if they are damaged.

Following initial findings that the Hh pathway plays a role in rare cancers, aberrant Hh pathway activation has been observed in numerous other cancers. It has been speculated that Hh plays a role in tumors responsible for over a third of cancer deaths. Development provides a context for understanding

tumorigenesis; as Hh signaling plays a different role in the formation of each tissue, the effect of abnormal Hh signaling might have different implications for each type of cancer. It is possible to classify tumors by when Hh becomes important for the neoplastic process.

4.2 HEDGEHOG SIGNALLING IN MULTIPLE MYELOMA

Recent findings has demonstrated that Hedgehog pathway was also involved in Multiple Myeloma disease. In particular, it has been showed the hedgehog signaling regulated the maintenance of CD19+ CD138- cells isolated from MM cell lines and clinical BM samples. Hh stimulation was not required by terminally differentiated CD19-CD138+ plasma cells, however (116). The Hh ligand promoted expansion of MM stem cells without their differentiation. On the other hand, the Hh pathway blockade, although having little or no effect on malignant plasma cell growth, markedly inhibited clonal expansion, accompanied by terminal differentiation of purified MM stem cells.

Hh signaling in multiple myeloma exacerbate its function by the inhibition of multiple myeloma CSC and the induction of terminal plasma cells differentiation of multiple myeloma CSC as indicated by the expression of CD138 (Fig. 8). Multiple modes of signaling seem to be active in multiple myeloma. Experimental data suggest that differentiated plasma cells can produce the ligand necessary for CSC survival and proliferation. Blocking signaling leads to CSC differentiation. Normal bone marrow stromal cells can also produce ligand and signal to myeloma cells to support their growth and survival. A possible role for tumor-to-stroma paracrine signaling may also take occur.

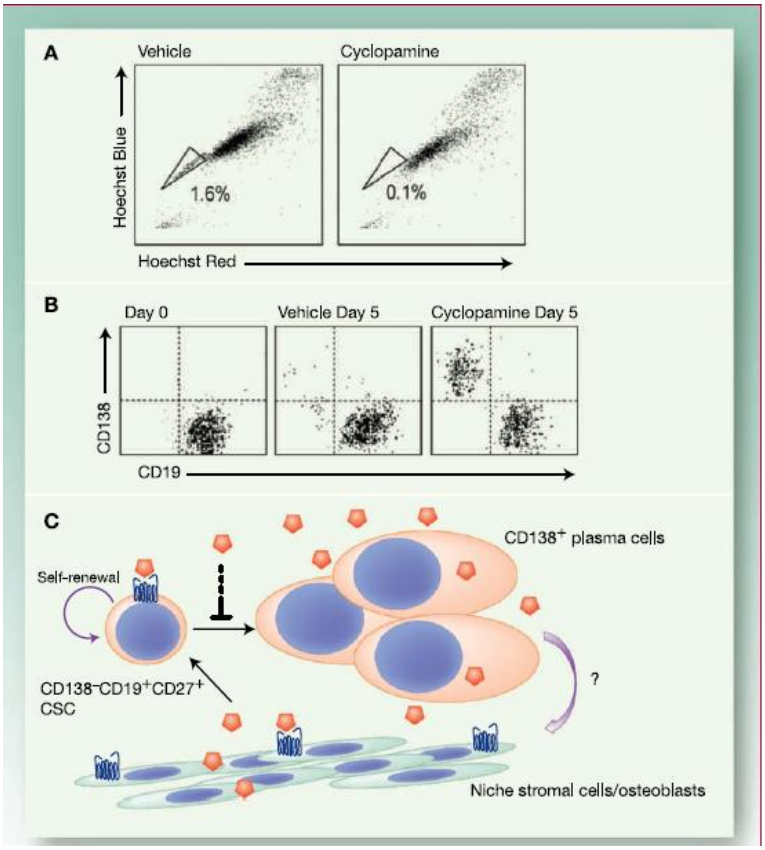

Fig. 8 A,B: Hedgehog pathway inhibition. C: proposed model of activation of the signaling

In contrast to these findings, the group of Blotta et al. (123) have published data that supporting a role of Hh pathway also in CD138+ PCs, and showed canonical as well as non canonical mechanisms leading to its activation in MM. They demonstrated that CD138+ PCs from MGUS patients have a significant up-regulation of Hh-activating genes, such as *Smo*, *Ptch1*, and *Gli2* compared with CD138+ PCs from healthy persons. This supports a role of Hh pathway in malignant transformation of PCs and in the pathogenesis of the precursor condition MGUS. In contrast, they observed a significant down-regulation of Hh-repressor genes, such as *Ptch2* and *Gli3* in MM PCs compared with their normal cellular counterpart. In addition, MM tumor cells overexpress *Gli1/Gli2* and *Ptch1*, which is itself a Gli1 target gene, suggesting a Gli-dependent Hh activation. These data suggest that Hh activity may be induced in MM cells in a Smo-independent manner. Finally, they found a relatively reduced *Hh*-gene expression in MM cell lines and PCL, a more advanced and BM-independent disease, suggesting a critical role for stroma derived Hh signals consistent with a paracrine model of Hh pathway in MM. Despite the *Hh*-gene expression, Hh-protein analysis in MM cell lines revealed that Shh ligand is expressed at significant levels in both CD138+ as well as CD138- cells. The CD138- MM cell population probably also includes the side population, which is a CD138low subpopulation with stem cell properties. Matsui et al. have previously reported predominant effect and activity of Hh signaling in CD138- MM stem cells, although we observed that CD138+ cells are also

susceptible to inhibition by NVP-LDE225. Various molecular explanations can be considered for this observation, including a lower level or differential expression of some Hh genes in CD138+ MM cells versus CD138low side population. Moreover, a wide spectrum of genetic alterations have been described in MM and are differently associated with MM disease initiation and progression; disease stage and previous treatment may also impact the biology of the cancer and hence Hh signaling in MM cells. Therefore, it is possible that MM represents a number of biologically distinct disease, each containing different initiating cells. These factors may all contribute to the reported differences. They did not find correlation between Shh expression and Hh responsiveness, suggesting the absence of link among expression level and functional activity; therefore, autocrine and/or paracrine mechanisms can both contribute to Hh activation. Importantly, MM cell lines strongly coexpress Ptch1 and Smo receptors, suggesting their potential Hh responsiveness. They confirmed Smo-dependent Hh signaling in MM using an Smo inhibitor NVPLDE225,16,17 which decreased MM cell viability in a range of 3-5 Min the majority of MM cell lines tested. This was associated with specific down-regulation of Gli1 and/or Ptch1, hallmarks of cell response to the Hh pathway. In the remaining MM cell lines, despite Smo expression, Gli1 and/or Ptch1 down-regulation was not observed after treatment, suggesting lack of correlation between Smo expression level and functional protein activity. Importantly, in those MM cell lines not responding to Smo inhibitor, Gli1 nuclear localization indicates that the Hh pathway is constitutively activated, suggesting that alternative, non canonical and Gli-dependent mechanisms may contribute to Hh signaling activation in MM. Therefore, the combination of Gli-modulating agents with Smo inhibitors may provide an alternative and more effective strategy for Hh inhibition. NVP-LDE225 similarly inhibited Gli1 nuclear translocation in MM cells with Smo-dependent Hh activity. Cytotoxicity of NVPLDE225 was also observed in primary MM cells, whereas no toxicity has been observed in PBMCs from healthy persons, suggesting a specific antitumor activity and a favorable therapeutic index. Finally, in vitro as well in vivo studies showed antitumor activity of NVPLDE225 in combination with bortezomib, demonstrating that NVP-LDE225 can potentiate the efficacy of well-established anti-MM agents. In conclusion, their findings suggest that both canonical (Smo-dependent) and non canonical (Smo-independent) mechanisms are crucial regulators of Hh activation in MM cells. Canonical and noncanonical pathways probably work in parallel, with a possible crosstalk between them. Directed analyses of the non canonical Hh signaling as well as a better understanding of all the mechanisms contributing to the noncanonical Hh activation, such as genetic mutations, ciliary protein overexpression, crosstalk between Hh signaling and unrelated pathways, and MM-BMSCs interactions, are therefore needed (Fig. 9).

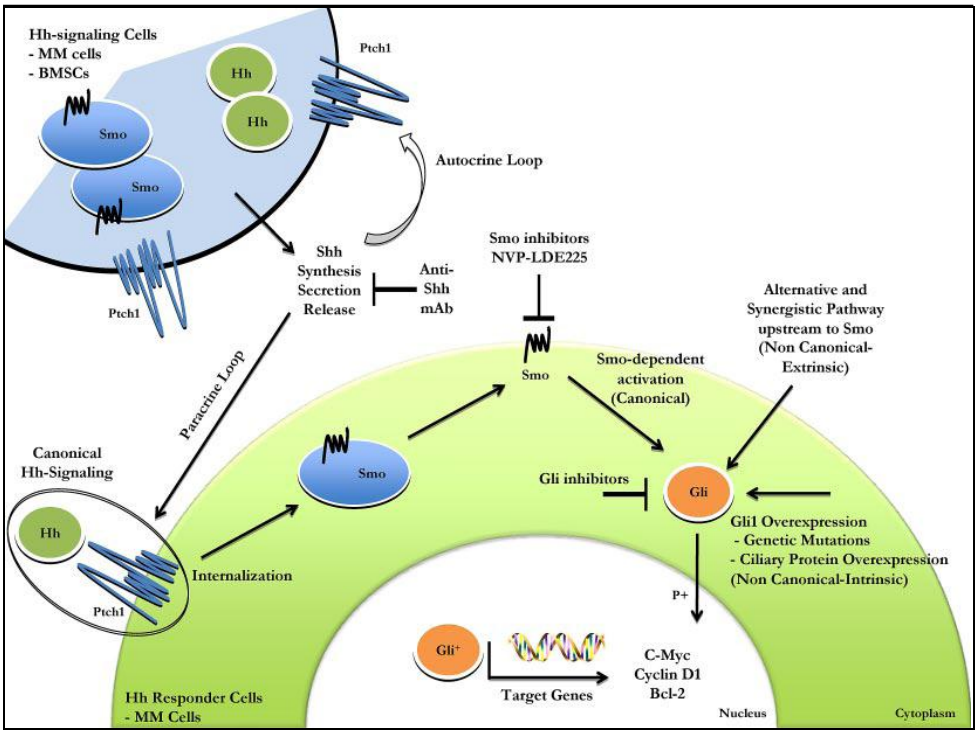

Fig. 9 Crosstalk between HH signaling cells and HH responder cells

5. AIM OF THE STUDY

Increasing data suggest that within an individual tumor, human cancers initiation, relapse, and progression might be driven by specific cell populations,. However, inconsistencies emerged in precisely defining the phenotypic markers that are reliably able to identify these “cancer stem cells” in nearly every human malignancy studied to date. In Multiple Myeloma, although plasma cells phenotypically characterize the disease, recent studies suggested that these cells lack significant proliferative capacity and instead, arise from clonogenic cells that resemble memory B cells.

Aim of the study was to molecularly characterize the putative clone of Multiple Myeloma Stem Cells in order to identify specific alterations, like Copy Number Alterations and modulated expression of genes, which might unequivocally distinguish these type of cells in Multiple Myeloma environment. This might lead to better define whether these “initiating cells” might be as pathogenic as plasma cells, as well as whether they might contribute to Multiple Myeloma pathogenesis.

To this aim, the following tasks have been considered:

- **Analysis of the *in vitro* clonogenic capacity of CD138- cells**: flow cytometric analysis of the different cell subpopulations in MM cell lines, RPMI-8226 and NCI-H929. Enrichment of the CD138 negative population by depletions of the CD138 positive fraction. Plating of CD138 negative and CD138 positive cells on methylcellulose and different concentrated media in order to evaluate the best growth conditions. Scoring of colonies.

- **Isolation of the putative Multiple Myeloma Stem Cells clone**: enrichment of the different cell fractions from BM and PBL by means immunomagnetic beads. In particular, CD138 positive cells are separated by positive selection by using a specific antibody anti-CD138; subsequently, B cells are isolated by depletions of the uninterested populations by means a cocktail of antibodies directed versus all non-B cells (T cells, NK cells, monocytes, dendritic cells, granulocytes, platelets, and erythroid cells); finally, memory B cell fractions are enriched by positive selection by using specific antibodies anti-CD27. Flow-cytometric analysis pre and post separation are conducted in order to evaluate the purity of the selected cell fractions.

- **Analysis of the clonal relationships between immature and plasma cells**: amplification of the VDJ rearrangement in the CD138 positive neoplastic clone by using a set of seven consensus primers derived from framework-1 region or six consensus primers derived from the IgH leader as forward primers, and a consensus primer derived from the joining region as the reverse primer. An approximately 300-bp band, corresponding to the VDJ gene rearrangement, is gel-purified and directly sequenced by traditional Sanger sequencing, using the VH primer

corresponding to the VH family used in the VDJ rearrangement. Identification of the CDRII and CDRIII regions and design of a couple of sequence-specific primers. Testing of the sequence-specific primers on CD138 positive fractions and, then, in all the different cellular subpopulations of the same patient in order to track the clonal relationship.

- **Genomic characterization of Multiple Myeloma Stem cells**: SNP Array analysis of Memory B cells and CD138 positive cells derived from BM and PBL of the same patient. Copy number analysis in order to evaluate specific alterations of Multiple Myeloma stem cells, like gains, losses or LOH, by direct comparison with a pool of blood samples from healthy individual. Aberrations included in genomic variants described in DGV – Database of Genomic variants are excluded in order to understand possible mechanisms strictly associated with the pathogenicity of these cells.

- **Analysis of the alterated pathways in Multiple Myeloma cellular subsets**: Gene expression profiling of B cells and CD138 positive cells derived from MM patients. In order to finely characterize the biology of the immature population, and to define the basic genetic characteristics that distinguish these two cell populations, the comparison need to be made with the normal counterpart of these cells. Analysis on the levels of expression of different mechanisms of self-renewal. Evaluation of the phenotype associated to the response to therapy.

6. PATIENTS AND METHODS

6.1 PATIENTS

For the purposes of this project, B cells and B memory cells were obtained from BM and PBL samples of 133 patients which have been enrolled in different trials; in particular. 92 out of 133 have been enrolled in the HOVON95-EMN02 trial, a randomized phase III study to compare Bortezomib, Melphalan, Prednisone (VMP) with high dose Melphalan followed by Bortezomib, Lenalidomide, Dexamethasone (VRD) consolidation and Lenalidomide maintenance in patients with newly diagnosed MM. Baseline characteristics of these 92 patients are resumed in the table below (Tab. 4).

PATIENTS CHARACTERISTICS	EMN02 (n=92)
Median age	72,6
Sex, %	
Male	56
Female	44
ISS, %	
ISS 1	24
ISS 2	52
ISS 3	24
Ig heavy chains, %	
IgG	82
IgA	18
IgD	0
Ig light chains, %	
K	70
L	1
Not known	29
Median β_2-microglobulin, mg/L	4
Median hemoglobin, g/dL	10,5
Median Albumin, g/L	34
Median creatinine, µM	96
Median LDH, U/L	293

Tab. 4 Baseline clinical characteristics of patients included in the study

We aimed to study the MM immature cells in newly diagnosed patients and, to this purpose, we collected biologic material mainly at diagnosis but, whenever it has been possible, we also collected cell

fractions obtained at different disease phases, as showed in Tab. 5, in order to get insight into the behavior of these immature cells during disease progression.

CELL FRACTIONS	SOURCE	TOTAL	DISEASE PHASE			
			MGUS	DIAGNOSIS	COMPLETE REMISSION	RELAPSE
B CELLs	**BM**	16	1	11	4	0
B CELLs	**PBL**	85	0	74	2	7
B MEMORY CELLs	**BM**	23	3	11	0	9
B MEMORY CELLs	**PBL**	48	3	36	0	9

Tab. 5 Samples obtained during different disease phases

In synthesis, B memory cell fractions were obtained both from the PBL (36) and the BM (11) collected from 47 patients at diagnosis. On the contrary, B cell fractions were isolated both from the BM (16 patients) and the PBL (85 patients). This, is mainly due to the poor amount of cells in the samples (B memory cells: <6% of total sample), confirmed by the flow citometry analysis. Indeed, for most samples we encountered a lot of difficulties in order to obtained enough cells to isolate good quality and quantity nucleic acids. . Moreover, whenever it has been possible, we aimed at characterized , the immature cells and the respective mature neoplastic clone obtained from the same patient, in order to enlighten every possible differences in their biology in the tumor framework. This has led to perform molecular analyses only in a small series of samples (Tab. 6).

CELL FRACTIONS	SOURCE	TOTAL (pts)	TOT CELLS (*10^6)	SAMPLES AVAILABLE for molecular studies (>500.000 cells)	DNA/RNA isolation
B CELLs	BM	16	0,49 (0,07 - 1,1)	25% (4)	4
B CELLs	PBL	85	1,21 (0,02 - 9)	40% (34)	15
B MEMORY CELLs	BM	23	0,17 (0,03 - 0,87)	17% (4)	4
B MEMORY CELLs	PBL	48	0,25 (0,02 - 1)	8% (4)	4

Tab. 6 Samples characteristics

In particular, we profiled by gene expression B cells fractions of 14 patients; for 8 of them, we also performed the gene expression profile of the CD138+ neoplastic clone. To validate the results, an expression dataset obtained from 122 newly diagnosed MM patients (unpublished data), as well as data obtained from public repositories, have been used. SNP array experiments were performed on 4 patients at diagnosis; , in each patient, genomic aberrations of 4 cellular populations have been characterized, ie the

CD138+neoplastic clone from BM and PBL and the CD138-19+27+ B memory cell clone from BM and PBL. Deletions and amplification were subsequently validated in a larger set of 94 138+ SNP array experiments. VDJ sequencing was performed on the 4 patients, already analyzed by SNP array. A complete list of the molecular analysis performed is resumed in the table below (Tab. 7).

CELL FRACTIONS	SOURCE	MOLECULAR ANALYSIS		
		GEP	SNP	VDJ sequencing
B CELLs	BM	/	6	/
B CELLs	PBL	14	/	/
B MEMORY CELLS	BM	/	4	10
B MEMORY CELLS	PBL	/	4	10
PLASMACELLS	BM	131	94	10
PLASMACELLS	PBL	/	4	10

Tab. 7 Molecula analysis performer

6.2 METHODS

6.2.1 Sample collection and enrichment

Bone marrow (BM) and peripheral blood (PBL) samples for molecular studies were obtained during standard diagnostic procedures. Written informed consent was obtained from each patient. Plasmacells were purified from mononuclear BM and PBL cells obtained by Ficoll-Hypaque density gradient centrifugation using anti-CD138 micro beads on an AutoMacs Magnetic Cell Separator (MACS system, Miltenyi Biotec, Auburn, CA). Subsequently, we isolated B cells from CD138- cell fractions by means a cocktail of antibodies that depletes all non-B cells. Non-B cells (T cells, NK cells, monocytes, dendritic cells, granulocytes, platelets, and erythroid cells) were labeled with a cocktail of biotinylated CD2, CD14, CD16, CD36, CD43, and CD235a (glycophorin A) antibodies. These cells were subsequently magnetically labeled with Anti-Biotin MicroBeads for depletion. Highly pure B cells have been obtained by depletion of magnetically labeled non-B cells. From B cells enriched fraction we then isolated by positive selection the B memory populations by means anti-CD27 micro beads. All the cell fractions isolated were then stored at −80 °C in guanidium thiocyanate, until use (Miltenyi Biotech).

2.2 Immunophenotypic evaluation

The purity of positively selected plasma cells was assessed by flow cytometry and was ≥90 % in all cases. Moreover, we assessed the absence of any CD138+ cell in the negative fractions and we evaluate the presence of CD19 and CD27 markers before and after separation (Miltenyi Biotech).

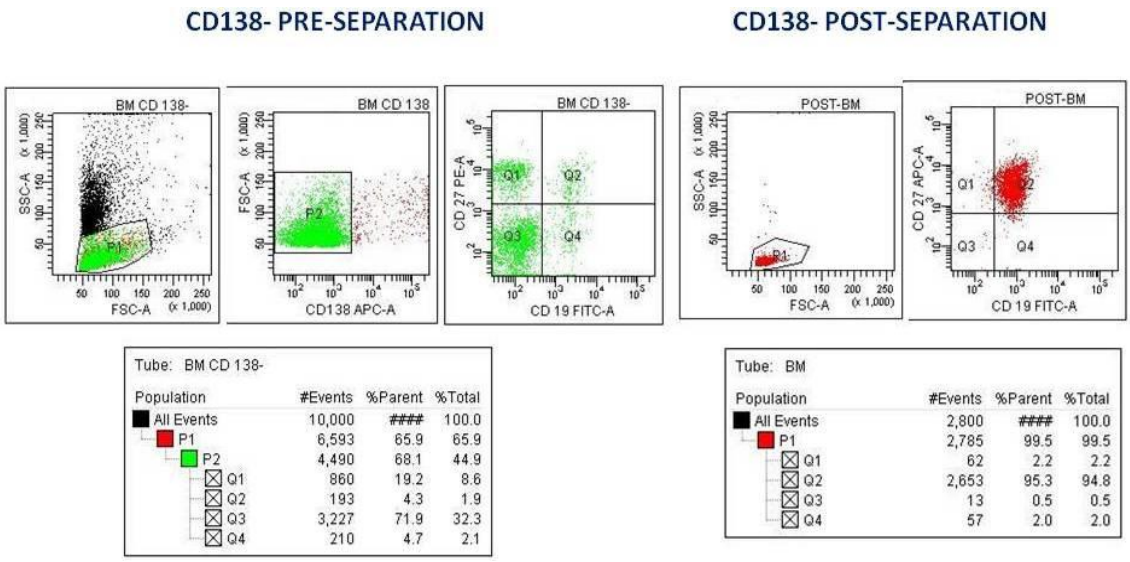

Fig. 10 Immunophenotypic evaluation pre- and post-separation

6.2.3 Nucleic acids isolation

Total DNA was obtained from each sample by Maxwell 16 LEV Blood DNA kit (Promega).Total RNA was obtained from each sample by the RNeasy® kit (Qiagen, Valencia, CA) extraction procedure: the RNeasy® Mini kit was used for more than 5×10^5 cells, the RNeasy® Micro kit for less than 5×10^5 cells. To measure concentration and purity of RNA, a NanoDrop ND-1000 spectrophotometer was used (NanoDrop Technologies, Wilmington, DE), which require only 1 µL of undiluted sample for assessment of concentration; purity of the extracted RNA was based on the 260/280 and the 260/230 O.D. ratios, as calculated and displayed by the NanoDrop spectrophotometer.

6.2.4 Clonogenic assay

Clonogenic assay were conducted as described in Matsui et al. (125). We used RPMI-8226 and NCI-H929 cell lines (DSMZ, Germany); clonogenic growth was evaluated by plating cells (1000 cells/mL for cell lines or $1x10^5$ to $5x10^5$ cells/mL for clinical specimens) in 1 mL 1.2% methylcellulose, 30% bovine serum albumin (BSA), 10_4 M 2-mercaptoethanol, and 2 mM L-glutamine. Methylcellulose cultures assessing clinical MM growth also contained 10% lymphocyte conditioned media as a source of growth factors.

Samples were plated in quadruplicate onto 35-mm2 tissue culture dishes and incubated at 37°C and 5% CO2. Colonies consisting of more than 40 cells were scored at 7 days for cell lines and 14 to 21 days for MM colonies from clinical samples.

6.2.5 Qualitative analysis of immunoglobulin gene rearrangement

VDJs were amplified, starting from genomic DNA or total cDNA, depending on sample availability. Briefly, 1 mg of genomic DNA or 1 mL of total cDNA (1/50th of the RT reaction) was amplified using a set of seven consensus primers derived from framework-1 region or six consensus primers derived from the IgH leader as forward primers, and a consensus primer derived from the joining region as the reverse primer (126). The reaction was carried out for 30 cycles (denaturation at 94°C for 30 seconds, annealing at 61°C for 40 seconds, and extension at 72°C for 50 seconds), with a final extension of 7 minutes. PCR products were analyzed by electrophoresis on 3% agarose gel. An approximately 300-bp band, corresponding to the VDJ gene rearrangement, was gel-purified and directly sequenced by traditional Sanger sequencing, using the VH primer corresponding to the VH family used in the VDJ rearrangement. Sequencing analysis was performed using the FASTA program at the European Molecular Biology Laboratory Web site (www2.ebi.ac.uk/fasta3/). CDRII and CDRIII regions were identified and a couple of sequence-specific primers was designed (forward on CDRII and reverse on the CDRIII region). Primers were purchased by PRIMM. First, primers were tested on the CD138+ neoplastic clone and, then, we repeated the same PCR reactions including the cDNA of all the different cellular populations. The amplification products were checked by electrophoresis on 3% agarose gel.

6.2.6 SNP array

SNP Array procedure was performed in all the samples in which sufficient and high quality DNA was obtained. In particular, a total of 500 ng was the minimum amount of DNA required to conduct the analysis. Total genomic DNA (500 ng; 250 ng each enzyme) was digested with Nsp I and Sty I restriction enzymes and ligated to adaptors that recognize the cohesive 4 bp overhangs. All fragments resulting from restriction enzyme digestion, regardless of size, were substrates for adaptor ligation. A generic primer that recognizes the adaptor sequence was used to amplify adaptor-ligated DNA fragments. PCR conditions were optimized to preferentially amplify fragments in the 200 to 1,100 bp size range. PCR amplification products for each restriction enzyme digest were combined and purified using magnetic beads. The amplified DNA, if we obtained a minimum of 450 ng/µl, was then fragmented, labeled and hybridized to the array. The Genome-Wide Human SNP Array 6.0 contains more than 906,600 single nucleotide polymorphisms (SNPs) and more than 946,000 probes for the detection of copy number variation. SNPs on the array are present on 200 to 1,100 base pairs (bp) Nsp I or Sty I digested fragments in the human genome, and were amplified using the Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0. SNPs on the SNP Array 6.0 were screened in more

than 500 distinct samples, including 270 HapMap samples and separate diversity samples. Approximately 482,000 SNPs were derived from the previous-generation Mapping 500K and SNP 5.0 Arrays. The remaining 424,000 SNPs included tag SNP markers derived from the International HapMap Project. These novel markers have better representation of SNPs on chromosomes X and Y, mitochondrial SNPs, SNPs in recombination hotspots, and new SNPs added to the dbSNP database after completion of the GeneChip® Human Mapping 500K Array Set. This array contains a total of 946,000 non-polymorphic copy number probes. These probes—744,000 originally selected for their spacing and 202,000 selected based on known copy number changes reported in the Toronto Database of Genomic Variants (DGV)—enable to detect de novo copy number changes and perform association studies by genotyping both SNP and known copy number polymorphism (CNP) loci (as reported by McCarroll, *et al*.). The median inter-marker distance over all 1.8 million SNP and copy number markers combined is less than 700 bases.

6.2.7 Gene expression profiling

To perform the gene expression profile experiments the GeneChip® 3' IVT Express Kit has been used, according to the manifacturer's instructions; briefly, the method is based upon linear RNA amplification and employs T7 *in vitro* transcription technology. The total RNA was reverse transcribed to synthesize first-strand cDNA. This cDNA was then converted into a double-stranded DNA template for transcription. *In vitro* transcription synthesized aRNA and incorporated a biotin-coniugated nucleotide (cRNA is also known as amplified RNA or aRNA). The aRNA was then purified to remove unincorporated NTPs, salts, enzymes, and inorganic phosphate. Fragmentation of the biotin-labeled aRNA prepared the sample for hybridization onto GeneChip 3' expression arrays. The GeneChip® Human Genome U133 Plus 2.0 Array (HG-U133 Plus 2.0 Array) analyzes the relative expression level of more than 47,000 transcripts and variants, including more than 38,500 well characterized genes and UniGenes. Moreover, offers an additional 9,900 probe sets, representing approximately 6,500 new genes, compared to the previous generation HG-U133 Set. Comprised of more than 54,000 probe sets and 1,300,000 distinct oligonucleotide features.

6.2.8 Data analysis

SNP array results were processed by ChAS (Chromosome Analysis Suite 2.0, Affymetrix)in order to evaluate the copy number alterations in each sample. The software use an optimal reference that includes 380 microarrays which were run as part of a larger set of microarrays by nine operators processing at least 48 unique samples in two rounds each, with randomization of the placement of sample DNAs across the PCR plates and randomization of the reagents and instruments used. The source DNA includes:

- 284 HapMap samples including at least one replicate of each of 270 HapMap samples: 90 from each of the Yoruban, Asian, and Caucasian ethnic groups, from cell-line derived DNAs from the Coriell Institute of Medical Research;

- 96 DNA samples from blood of phenotypically healthy male and female individuals obtained from BioServe Biotechnologies.

The biologic interpretation of CNAs was attained by using Panther analysis.

In order to analyze the gene expression data, the CEL files corresponding to both the B and the CD138+ cells have been downloaded at http://www.ncbi.nlm.nih.gov (GEO, Gene Expression Omnibus; accession number: GSE12453, Brune V et al, *J Exp Med* 2008 (128) and were directly compared to their neoplastic counterparts, by means of Partek Genomic® software. All samples were normalized and analyzed using the bioconductor function for Robust Multi-Array Analysis (129), in which perfect match intensities were background adjusted and normalized by means of quantile-quantile normalization. Differentially expressed genes were then analyzed by means of GeneGo® software.

7. RESULTS

7.1 CLONOGENIC CELLS RESIDES IN THE CD138- COMPARTMENT

Recent studies suggested that both human and murine MM cell lines are able to heterogeneously express several MM-related cell surface antigens, in particular syndecan-1 CD138 antigen. Therefore, we analyzed the expression of CD138 in 2 distinct human MM cell lines, RPMI 8226 and NCI-H929. As expected, the majority of cells in both lines expressed high levels of CD138; however, distinguishable CD138- cells populations, representing approximately 2% to 6% of total cells, respectively, can be identified in both analyzed cell lines (Fig. 11).

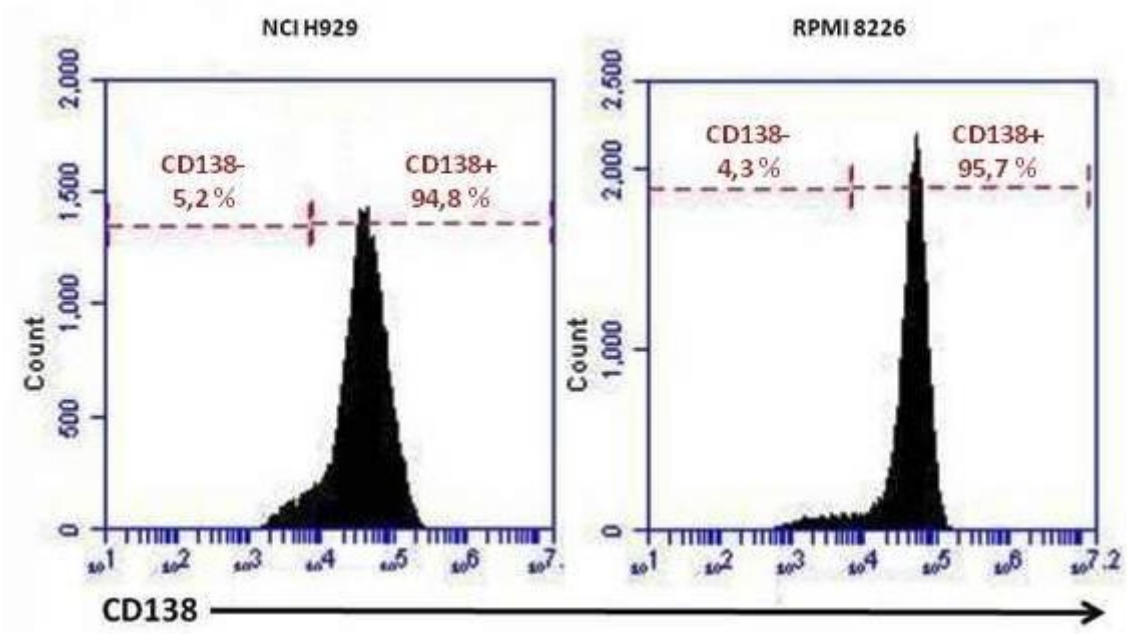

Fig. 11 Flow citometry analysis on MM cell lines

To evaluate the clonogenic capacity of these subpopulations, the CD138+ and CD138- cells fractions have been enriched from both cell lines, by means immunomagnetic beads and a methylcellulose colony formation assay was set up for each subpopulation. We set up the best conditions for CD138- growth, represented by methylcellulose with growth factors-enriched conditioned media (Fig. 12). Interestingly, after 2 weeks culture, the number of colonies grown from the CD138- plated cells was higher, as compared to that obtained from the CD138+ cells (4 vs 1).These data, even if preliminary, suggest that CD138- cells hold an higher clonogenic potential, as compared to CD138+ cells; to validate this hypothesis it could be important to evaluate a) whether these immature cell fraction might actually include the MM cancer stem

cells and b) which were the possible mechanisms, allowing the maintenance and persistence of this side population during MM disease.

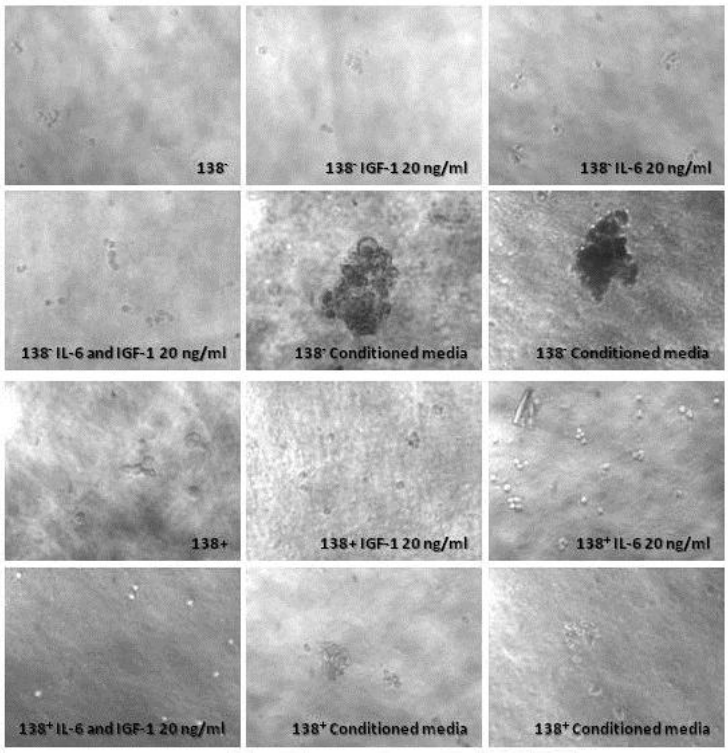

Fig. 12 Colonies observed in different growth conditions

7.2 VDJ REARRANGEMENT IS RESTRICTED TO MEMORY B CELL CLONES BUT NOT IN AN EXCLUSIVE WAY

To identify the VH family used in the patient-specific VDJ gene rearrangement, the VDJ regions of 4 MM patients were amplified either with a set of seven VH family-specific primers, together with a JH-consensus primer. We have been able to obtain the VDJ patient-specific rearrangement only 10 out of 18 patients, due to the lack of suitable samples; the direct sequencing of the amplified rearrangements allowed to identify the monoclonal VDJ rearrangement in 4 patients, whereas 6 patients gave rise to polyclonal PCR bands. Patients' specific primers were designed over the CDR2 (forward) and the CDR3 (reverse) regions; the sensitivity and specificity of our assay was tested as previously described.

The patients' specific VDJ amplification, performed for each patients on the different isolated cell fractions showed that the same rearrangement was present in the CD138+ cells, obtained both from BM and PBL. The same patient-specific primers were tested also on the B memory cells obtained from the same patients, in order to evaluate whether these cells might be clonally related to the neoplastic clone, thus showing that the immature cell populations and the neoplastic clone shared the same rearrangement , in

the circulating as well as in the bone marrow cell fractions. Consistent with previous report (101-102), the same rearrangement was observed also in the CD138-19+27- cells that we used as a negative control, fading away the hypothesis that the specific VDJ rearrangement was exclusively owned by the CD138+ neoplastic clone and the putative stem cell clone (Fig. 13).

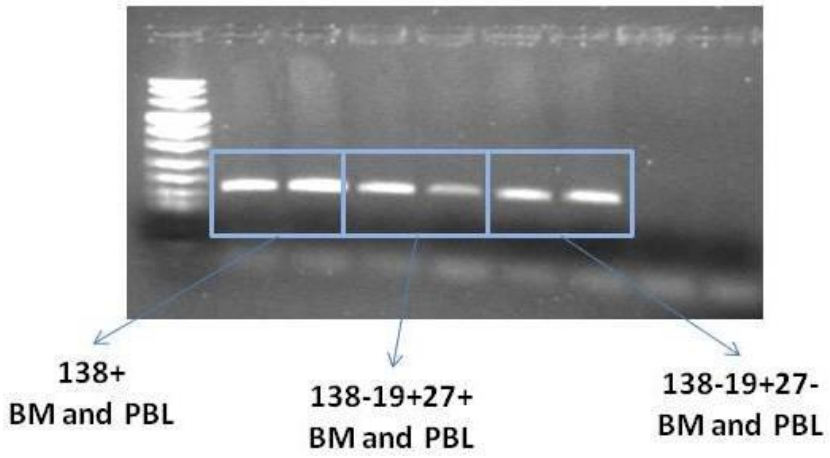

Fig. 13 VDJ rearrangement analysis on different cell fractions

7.3 MOLECULAR CHARACTERISTICS ASSOCIATED TO MULTIPLE MYELOMA HETEROGENEITY: A COPY NUMBER ANALYSIS

7.3.1 Genomic instability of the neoplastic mature clone

Whereas to date the genomic instability, which characterize the MM plasma cells, has extensively been described, very few data are available regarding the molecular landscape of the CD138 negative cell compartment, where presumably the putative MM cancer stem cells resides.

To explore these issues, we analyzed the genomic profile of 4 different cell populations (the CD138+ cells from BM and PBL, and the CD138-19+27+ cells from BM and PBL) obtained from 4 different newly diagnosed patients, by using a high throughput technology for genomic analysis, as a SNP array. This small series of samples was intended as “training set”, and the observed molecular aberrations were subsequently monitored in a larger series of 90 CD138+ SNP array-profiled samples..

We first observed that, in all analyzed patients, the CD138+ neoplastic clone was characterized by an extended genomic instability, proven by the presence of several macroalterations. Due to the small number of analyzed samples, we were not able to perform a statistical analysis; the complete set of macroalterations is resumed in the following table (Tab. 8)

PATIENTS	n. SNP	CELLS	CNAs
1	197	CD138+ BM	Del1p, Amp3, monosomy chr4, del8p, Del10p
2	105		Hyperdiploidy of chr 5,7,9,11,19, Del16q
3	193		Amp1q, Amp19p
4	149		Del1p, Amp1q, Del6q, Del12p, Del13q, Del14q

Tab. 8 Set of macroalterations observed in CD138+ cells

Interestingly, we showed that all the macroalterations observed in the CD138+ cells obtained from BM were shared also by the circulating CD138+ cell fractions,, as shown by the karyoviews displayed in the figure below (Fig. 14).

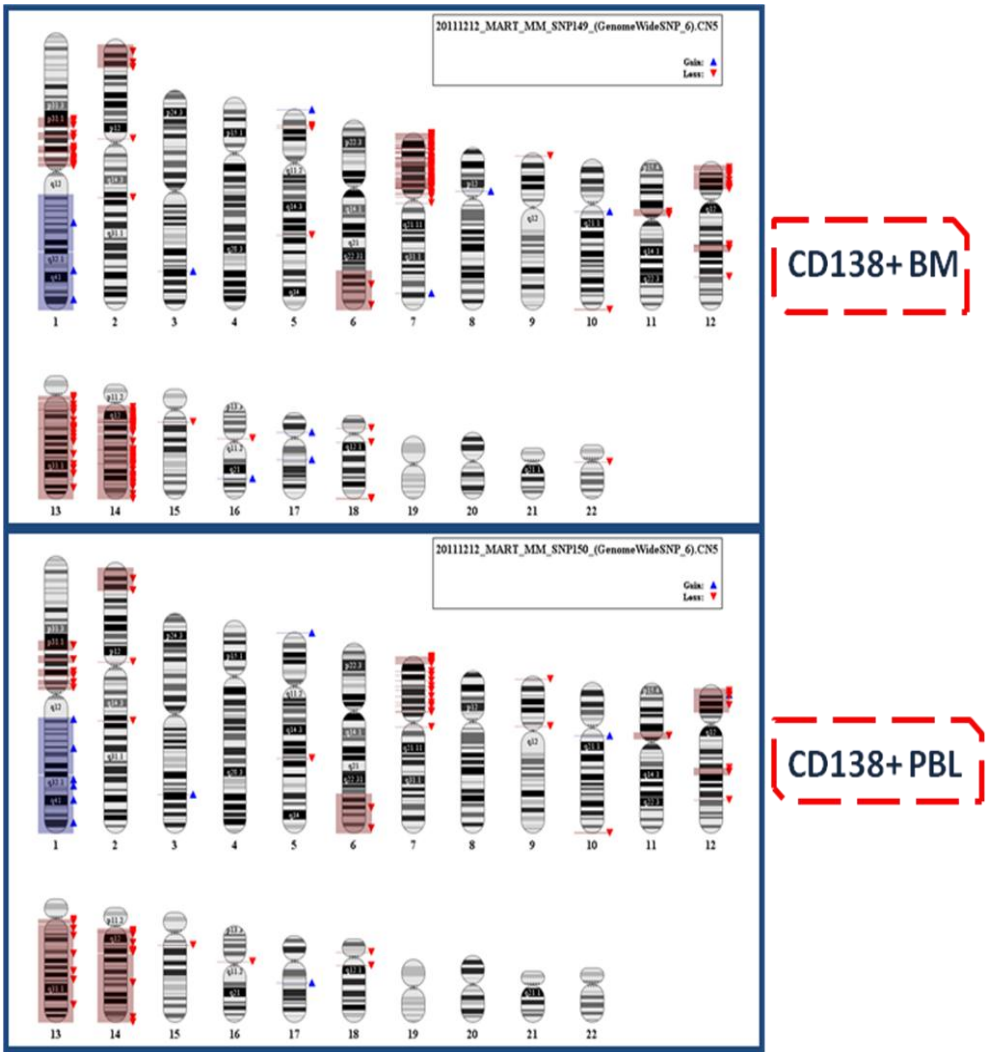

Fig. 14 Karyoview of CD138+ from BM and PBL of one selected patient

7.3.2.Inside the genome of Multiple Myeloma Stem Cells

The genomic background of the putative stem cells clone was as well evaluated by copy number alterations analysis. Notably, B memory cell clones lack any previously reported macroalterations, whereas they carry an extended number of microalterations, as shown in the carioviews displayed in figure (Fig. 15).

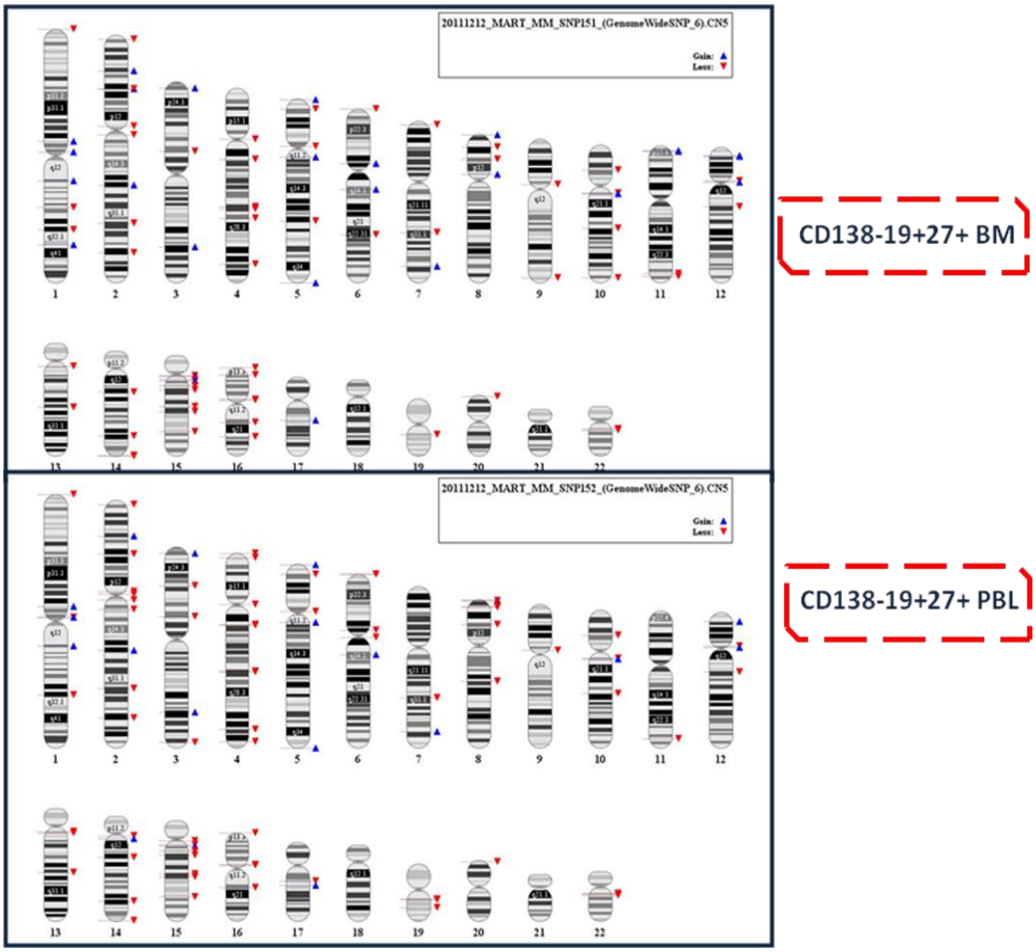

Fig. 15 Karyoview of CD138-19+27+ from BM and PBL of one selected patient

7.3.2.1 Microdeletion on chromosome 14

Among the numerous microalterations observed in the CD13-19+27+ cells, we first focused on that observed on the telomeric region of the chromosome 14 q arm. This chromosome 14 region is known to play a crucial role in MM pathogenesis, since it carries the IgH locus, which is known to be involved in most primary translocations, as t(4;14), t(11;14), t(16;14) and t(6;14).

In any analyzed B memory cell fraction, we observed the presence of a microdeletion on chr 14q32.33 (minimal deleted region = 410 Kb, see table 9), which includes the following 5 genes:

- *JAG2*, a Notch pathway's ligand, critical for self-renewal of myeloma cells;
- *BRF1*, which encodes one of the three subunits of the RNA polymerase III transcription factor complex; this complex plays a central role in transcription initiation, regulates ES cells development and acts as tumor suppressor;
- *PACS2*, which is involved in trafficking acid cluster-containing ion channels to distinct subcellular compartments; it has a propapoptotic rôle and its knockdown increased clonogenic cell survival;
- *NUDT14*, an UDP-glucose pyrophosphatases, overexpressed in tumors;
- *BTBD6*, a cytoplasmatic mRNA processing body.

Chromosome 14	Copy Number	lenght	markers	CN state	SNP	CNV
	Deletion	414	224	1	53	171

Tab. 9 Microdeletion on chromosome 14q32.33

Similarly, a deleted region on chromosome 14 was also observed in the corresponding CD138+ cell fraction obtained from the same patient, even if the extension of the deletion either is higher or regards the whole chromosome 14 q arm. Moreover, the CD138+ cells of 2 out of the 4 analyzed patients carry also an IgH translocation (patients n.4: t(4;14); patient n. 3: t(11;14)), as evaluated by FISH analysis.

Of note, the transcriptional down-regulation of the genes located within the microdeletion was observed, by analyzing the gene expression data.

Interestingly, a microdeletion on 14q32.33 has been previously associated with a pediatric syndrome (135), suggesting that the deletion extension might lead to different developmental disorder and mental retard (136).

7.3.2.2 Microalterations: losses and amplifications

A series of microalterations have been observed exclusively in the B memory clone: an average of 32 amplifications and 16 losses were observed for any given analyzed sample, whose dimensions span between 81 and 122 Kb, for amplifications and losses respectively. Each alterations were well represented by an average of 50 marker per region (Tab. 10).

VARIATION	NUMBER	DIMENSION (Kb)	MARKERS
AMPLIFICATION	32	122	60
LOSS	16	81	40

Tab. 10 Microalterations of Multiple Myeloma stem cells

A list of genes affected either by losses (a total of 17) or amplifications (a total of 46) was obtained by excluding all affected regions described in the DGV (Database of Genomic Variant, Toronto) (Tab 11); these genes are presumably most related to MM pathogenesis.

LOSS	**AMPLIFICATION**		
AKT3	*ADCY2*	*MPPED1*	*TTTY3*
AMY2A	*BACE2*	*MUC16*	*UGT2B17*
AUTS2	*CASP7*	*MYOD1*	*UNC5B*
ERBB4	*CDY1*	*NBPF1*	*WNT4*
ETS1	*CFHR3*	*NETO1*	*WWOX*
FAM90A7P	*CHST1*	*PCBP3*	*XIAP*
GLIS3	*CLIC5*	*PDGFRA*	*KANSL1*
LCE1E	*CPA6*	*PPFIBP2*	*KCNC1*
LIG1	*DAB2IP*	*PPYR1*	*KRAS*
MACROD2	*DYSF*	*PRSS2*	*KRT6A*
MCPH1	*ECE1*	*RBFOX1*	*LMO1*
MGAM	*EPHB1*	*RXFP3*	*MASP1*
MRGPRX1	*FAM72B*	*SIRPB1*	*TENM2*
PDXDC1	*GALNT2*	*SLIT1*	*TGIF2LX*
PKNOX2	*GSTM2*	*STRA6*	
POTEG	*IRX1*		
RGS13	*JAK1*		

Tab. 11 Genes involved in microalterations

The Panther analysis tool was used to explore the biologic significance of genes included in the above mentioned list and already described as being involved in cancer development, thus showing that deleted genes are mainly involved in DNA repair mechanisms and in the transcriptional regulation, whereas amplified genes are mainly involved in the negative regulation of apoptosis and in the angiogenesis (Fig. 16).

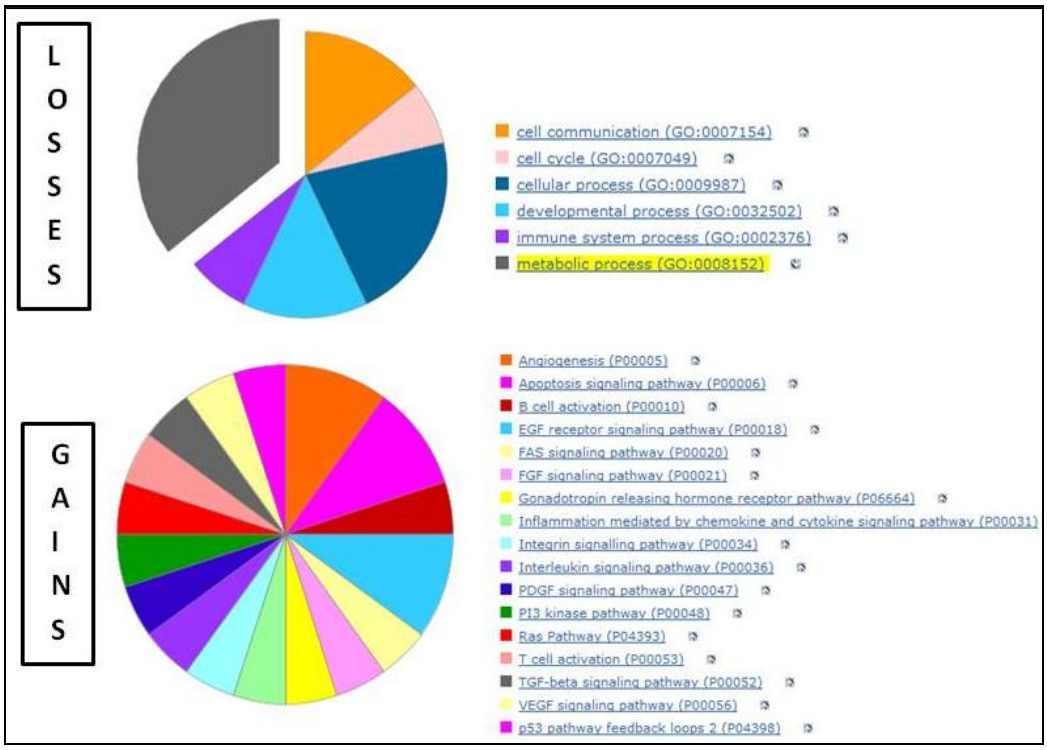

Fig. 16 Biological processes affected by microalterations

In particular, genes included in the list comprise *XIAP*, *WWOX* and *KRAS*, which have been already described as being involved in MM pathogenesis. XIAP is the best characterized and the most potent direct endogenous caspase inhibitor, and is considered a key actor in the control of apoptotic threshold in cancer cells. Importantly, it has been shown that *XIAP* knockdown by RNA interference enhances drug sensitivity and decreases tumor formation in NOD/SCID mice (131). *WWOX* is a well known tumor suppressor gene involved in apoptosis. In MM, numerous studies confirmed that alterations, and in particular del(16q), is important to determining the clinical outcome of MM patients; furthermore, the gene spans in the *FRA16D* chromosomal fragile site that have been proposed to have a determining role in cancer-associated DNA instability . This is consistent with a protective role for normal *WWOX* gene, where aberrant expression, as a result of breakage at the associated fragile site, could contribute directly to cancer progression (132-133). Finally, KRAS is a Kirsten ras oncogene homolog from the mammalian ras gene family, and encodes a protein that is a member of the small GTPase superfamily. Its role in MM is well known, because a single amino acid substitution is responsible for a frequently detected activating mutation. Indeed, NRAS and/or KRAS mutations were found in 54.5% of MM at diagnosis, and in 81% at the time of relapse, justifying thei important function in MM disease (134).

7.3.2.3 LOH regions

Besides the presence of deletions and amplifications, the copy number analysis of SNP data highlighted the presence of Loss Of Heterozigosity (LOH) regions, as well. LOH is a common occurrence in cancer, where it indicates the absence of a functional tumor suppressor gene (TS) in the lost region. Although most people remain healthy with such a loss, since one functional gene is functional, on the not affected chromosome , nevertheless the remaining copy of the tumor suppressor gene might be inactivated, e.g. by a point mutation, thus leaving any tumor suppressor gene to protect the normal functionality of the genome.

We showed that each patient is characterized in the B memory cell clones by an average of 65 LOH regions whose dimension is higher than 1 Mb, containing an average number of 257 markers (Tab. 11). Interestingly, the LOH regions are shared by the B memory cell clone and the CD138+ neoplastic clone.

VARIATION	NUMBER	DIMENSION	MARKERS
LOH	65	>1 Mb	257

Tab. 11 LOH regions in Multiple Myeloma stem cells

The Tumor Suppressor Gene Database (TSgene) analysis tool was employed to investigate the TS gene which might be included in these regions; we set up a list of 106 TS genes already known to be involved in MM and in other hematological malignancies. The list includes 23 TS gene already described in MM pathogenesis, 30 TS gene already described in leukemia pathogenesis, 10 TS gene already described in chronic lymphocytic leukemia pathogenesis, 7 TS gene already described in chronic myeloid leukemia pathogenesis and 36 TS gene already described in lymphoma pathogenesis. Of the TS genes included in the above-mentioned list, 8 were located in the LOH regions described in the group of samples analyzed here; of these, *CDKN2C*, and *TP53* are known to be included in cytoband frequently affected by deletions in MM disease and are known to be associated to poor prognosis (Tab. 12).

Data overall suggest the relevance of LOH regions in MM precursor cells, suggesting a possible involvement of particular TS genes in MM pathogenesis, whenever mutated or altered by epigenetic changes along the B cell development.

TS Gene	CHR
CDKN2C	1p32
RASSF1A	3p21.3
TP53	17p13.1
ST13	22q13.2
NF1	17q11.2
CEACAM1	19q13.2
PPP2R4	9q34
CTDSPL	3p21.3

Tab. 12 TS genes in LOH regions

7.4 ALTERATED PATHWAYS IN MULTIPLE MYELOMA CELLULAR SUBSETS: A GENE EXPRESSION ANALYSIS

To analyze the transcriptome of the putative clone of MM initiating cells, gene expression profile experiments were performed on CD19+ B cells and the following comparison among gene profiles were analyzed:

- MM B cells versus healthy donors B cells (8 vs 5 samples), to obtain a fine characterization of the immature population biology;
- MM B cells versus CD138+ cells, obtained from the same patients (8 vs. 8 samples), to define the basic genetic characteristics distinguishing these two cell populations;
- MM CD138+ cells vs healthy donors CD138+ cells (130 vs 5 samples), to identify the main altered pathways and to confirm already published data.

We have not been able to perform the gene expression analysis of B memory cells, due to the low amount of cells/nucleic acids. We thus decided to analyze the B cell compartment, since the VDJ rearrangement analysis confirmed the clonal relationship between CD19+ cells and the neoplastic clone,

7.4.1 The biology of MM circulating B cells

We first compared the gene expression profiles of 11 MM B cells patients versus 5 healthy donors, to obtain a fine characterization of the immature population biology. An unsupervised hierarchical clustering was able to highlight the differential expression of 11,480 probes sets between the two compared groups (fold change: <-2;>2; FDR: 0,05; p-value: <0,05). A gene enrichment analysis identified the whole set of genes acting as transcription factors, receptors and secreted ligands in MM circulating B cells

(Tab. 13). Three pathways, identifying the biology of MM precursor cells, were shown to be the most significative ($p<0,05$): the Wnt pathway, the Nf-kB pathway and the TGF-beta signaling. Whereas the predominant role of the Nf-kB pathway and the TGF-beta signaling might suggest the possible incipient role of inflammation processes in MM B cells, which might lead and contribute to disease progression, the deregulated expression of genes involved in the Wnt pathway might suggest a possible concomitant activation of other interacting pathways, involved in self-renewal processes.

Transcription factors		**Receptors**		**Secreted proteins**	
Edges IN	**Edges OUT**	**Edges IN**	**Edges OUT**	**Edges IN**	**Edges OUT**
c-Jun	Lef-1	alpha-10/beta-1 integrin	alpha-10/beta-1 integrin	Fibronectin	DKK1
c-Myc	SLUG	alpha-2/beta-1 integrin	alpha-2/beta-1 integrin	WNT	Laminin 1
Fra-1	Tcf(Lef)	alpha-3/beta-1 integrin	alpha-3/beta-1 integrin	WNT5A	WNT
ITF2	TCF7 (TCF1)	CD44	LRP5	BAFF(TNFSF13B)	WNT5A
Lef-1	TCF7L2 (TCF4)	LRP5	LRP6	IL-1 beta	Angiopoietin 3
MITF	NF-AT2(NFATC1)	LRP6	BAFF-R	IL-12 alpha	APRIL(TNFSF13)
NRSF	NF-kB	BAFF-R	BCMA(TNFRSF17)	IL-12 beta	BAFF(TNFSF13B)
PPAR-beta(delta)	NF-kB1 (p50)	BCMA(TNFRSF17)	TACI(TNFRSF13B)	IL-6	ENA-78
SLUG	RelA (p65 NF-kB subunit)	CD21	TIE2	MIP-1-beta	Epo
Tcf(Lef)	C/EBPalpha	CD23	TLR4	TNF-alpha	GRO-1
TCF7 (TCF1)	C/EBPbeta	CD69	Epo receptor		IL-8
TCF7L2 (TCF4)	CREB1	CD86	LIF receptor		LIF
NF-AT2(NFATC1)	ER81	ICAM1			TGF-beta 1
NF-kB	SMAD2	TACI(TNFRSF13B)			VEGF-A
NF-kB1 (p50)	SMAD3	TIE2			
RelA (p65 NF-kB subunit)	SMAD4	TLR4			
c-Fos	SP1	Epo receptor			
C/EBPalpha		LIF receptor			
C/EBPbeta					
CREB1					
ER81					
SMAD2					
SMAD3					
SMAD4					
SP1					

Tab. 13 Principal alterated pathways in MM B cells

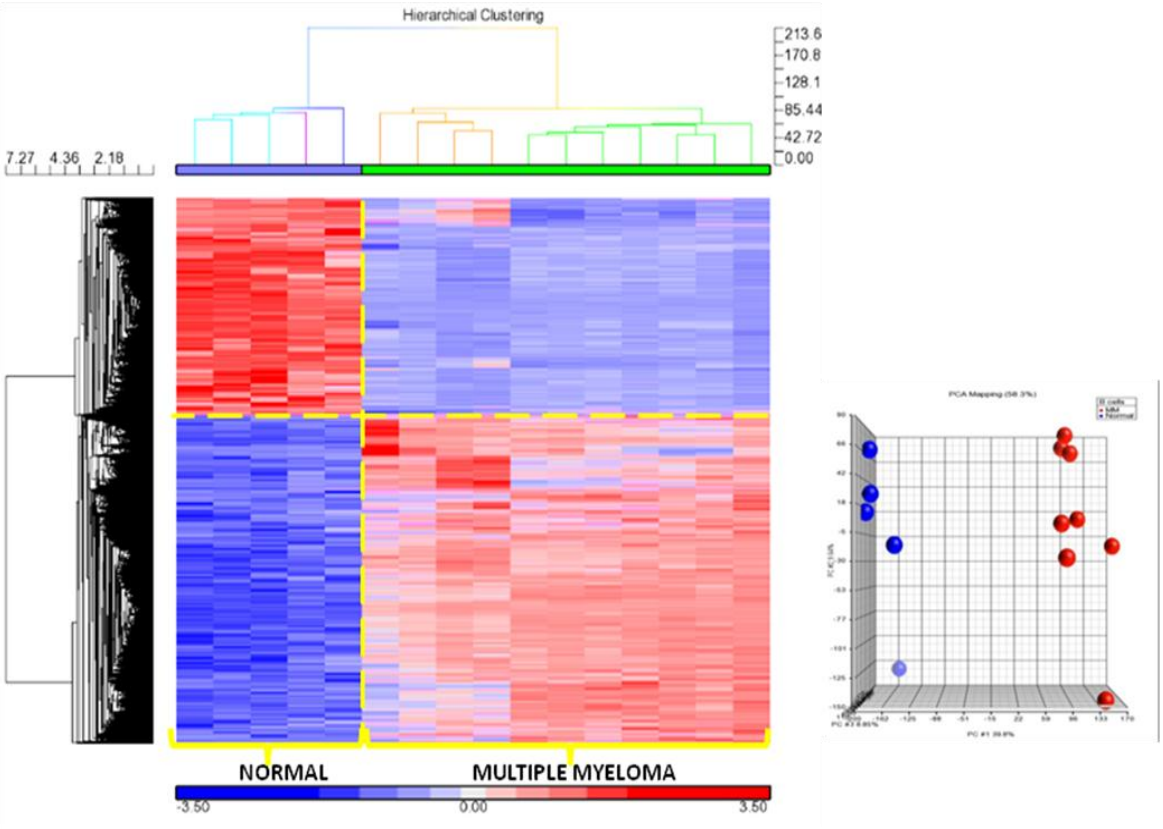

Fig. 17 Hierarchical clustering and PCA analysis of MM B cells vs normal B cells

The hierarchical clustering of MM vs normal B cells highlighted the presence of two distinct subsets of samples, with slightly different transcriptional profiles (subtype 1 and subtype 2) (Fig. 17). In detail, the subtype 1 samples showed an elevated upregulation of the PKA signaling, which suggest a tight interconnection both with Nf-kB pathway and with TGF-beta signaling, as well as the involvement in the regulation of cell cycle, proliferation and cell survival. These preliminary data need to be more deepened in a larger series of samples, in order to understand the role of the differentially affected pathways shown here; nevertheless, data support the hypothesis that MM heterogeneity originates during the early development stages of B cell differentiation.

7.4.2 Principal differences between B cells and CD138+ cells

To define the basic genetic characteristics distinguishing B and CD138+ cells, we than compared the gene profiles of 8 MM B cells versus 8 MM CD138+ cells, obtained from paired samples. An unsupervised hierarchical clustering was able to discriminate the differential expression of 8,131 probes sets between the two groups (fold change: <-2;>2; FDR: 0,05; p-value: <0,05) (Fig. 19). The main affected pathways, modulated in B cells with respect to the CD138+ cells are those involved in the oxidative phosphorylation, the remodeling of cytoskeleton and mechanisms of cell adhesion. This might be related to the different

environments, where are located the two types of cells : B cells circulate in the peripheral blood, whereas the plasma cells are confined to the bone marrow. Basically, plasma cells are more quiescent but also particularly activated in order to create connections with the hypoxic microenvironment, as suggested by the deregulations of the above-mentioned pathways.

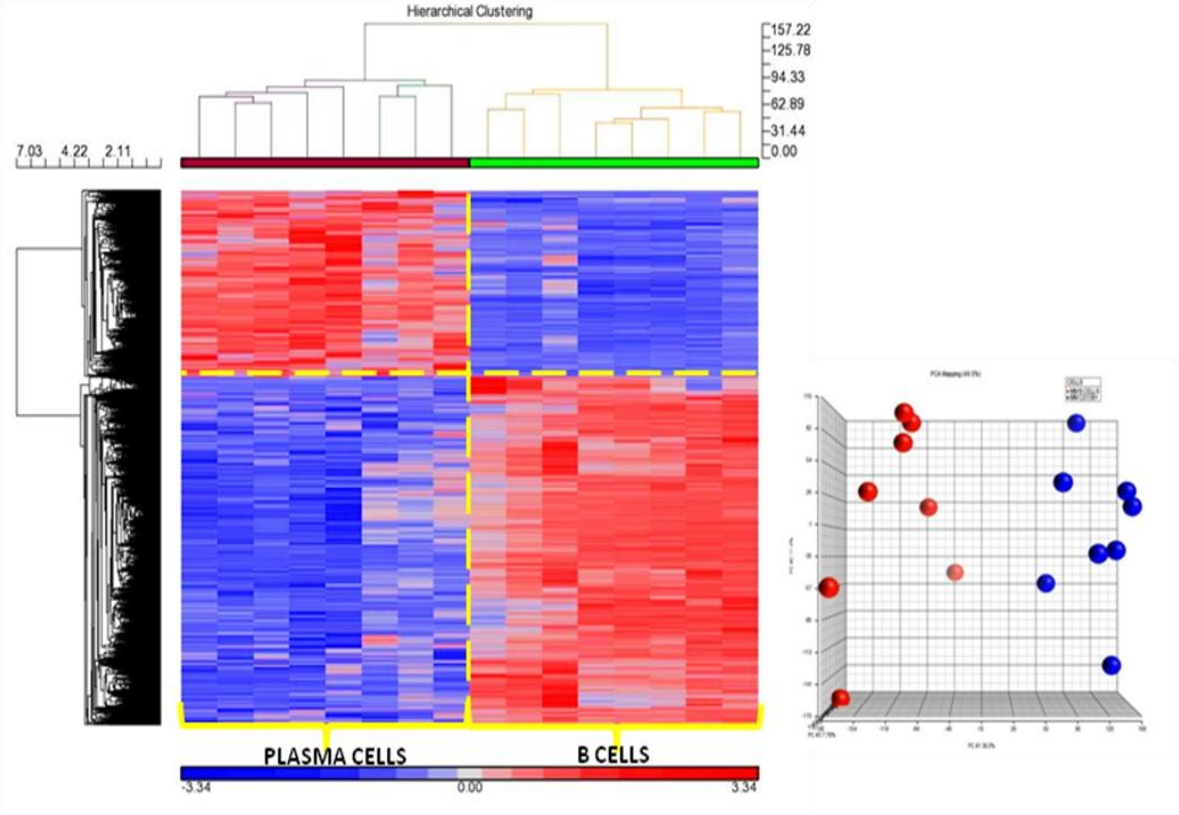

Fig. 18 Hierarchical clustering and PCA analysis of MM B cells vs MM plasma cells

7.4.3 The Hedgehog pathway in MM circulating B cells

The Hedgehog pathway role in the maintenance of myeloma stem cells in MM disease has been recently described by Matsui et al. (116). Our data regarding the comparison of B cells transcriptome obtained from MM and healthy donor samples showed a complete shut-off of the HH pathway in the MM B cells, supported by the significant downregulation of ligands and transcription factors involved in the pathway (Tab. 14).

GENE	B cells	
	log	p-value
SHH	-1,2	1,34E-05
IHH	-1,59	0,0033252
DHH	-0,48	0,0361883
SMO	-0,72	0,0039467
PTCH1	-0,67	5,61E-06
GLI1	-1,2	0,0011098
GLI2	-0,9	0,0043925
GLI3	-1,37	0,0003816
SUFU	-1,47	0,0001061

Tab. 14 HH gene expression in MM B cells

We thus explored the possibility that alternative self-renewal mechanism might be active in this cell compartment, showing that Notch signaling resulted significantly iper-activated. Indeed, several genes, like *NOTCH2* and *JAG1*, which are the receptor and the ligand of Notch pathway, respectively resulted over expressed. Similarly, we also observed the possible overexpression of Wnt signaling, due to the overexpression of β-catenin gene . (Tab. 15).

Notch pathway genes	log ratio	p-value
HES1	0,80	0,043694
NUMB	1,87	1,14E-05
NOTCH2	2,69	8,51E-09
JAG1	0,16	0,195631
DLL1	2,07	0,000377
EP300	2,58	1,82E-07

Wnt signalling genes	log ratio	p-value
WNT6	1,11	0,004437
LEF1	0,13	0,002848
APC	1,64	0,000223
DKK4	0,27	0,001617
FZD1	0,14	1,52E-06
CTNNB1	2,78	2,05E-06

Tab. 15 Notch and Wnt signalling genes expression in MM B cells

7.4.4 The Hedgehog pathway in CD138+ MM cells

The role of HH pathway has been recently explored also in CD138+ mature plasma cells (123), showing that both canonical and non-canonical Hedgehog pathway participated in MM pathogenesis; the study demonstrated that Hedgehog pathway was strongly over-expressed in MGUS and in MM patients, whereas it resulted down-regulated in the more advanced and BM-independent phase of the disease, known as plasma cell leukemia.

Our data, obtained from the comparison of the gene profiles of 122 newly diagnosed MM patients versus 5 healthy donors showed, on the contrary, that all principal ligands and transcription factors involved in Hedgehog pathway were down-regulated, whereas PTCH1 was over expressed, thus indeed confirming the shut-off state of HH pathway, since when PTCH1 is over expressed, SMO will be turned off and the pathways will be inactivated (Tab. 16).

GENE	Plasma cells	
	log	p-value
SHH	-0,688649	0,010458
IHH	-2,02261	0,00344565
DHH	-0,227152	ns
SMO	-0,854101	0,00030387
PTCH1	1,96993	0,00170606
GLI1	-0,192111	ns
GLI2	-1,32087	7,27E-13
GLI3	-0,851626	0,0117799
SUFU	-1,71999	0,00015385

Tab. 16 HH gene expression in MM plasma cells

7.4.5 Analysis of the phenotype associated to the resistance to therapy

Recent evidences have gathered increasing attention on the CD138 negative compartment in MM patients, because it has been proposed that the resistance to therapy is intrinsic in this cell populations.

Based on this studies, we explored if in B cells, as a precursors of the mature neoplastic clone, are characterized by a particular phenotype possibly associated with the resistance to therapy. Several genes possibly involved in mechanisms related to drug resistance have been shown to be deregulated, when comparing the gene profiles of MM versus healthy donors B cells. In particular, we examined the status of the endoplasmatic reticulum stress in our series of samples. Notably, *IRE1α-XBP1* genes are affected by a significative down-regulation that presumably bring to an attenuation of the unfolded protein response. In the figure below, all the genes participating in the signaling are represented with the respective level of expression (Fig. 19).

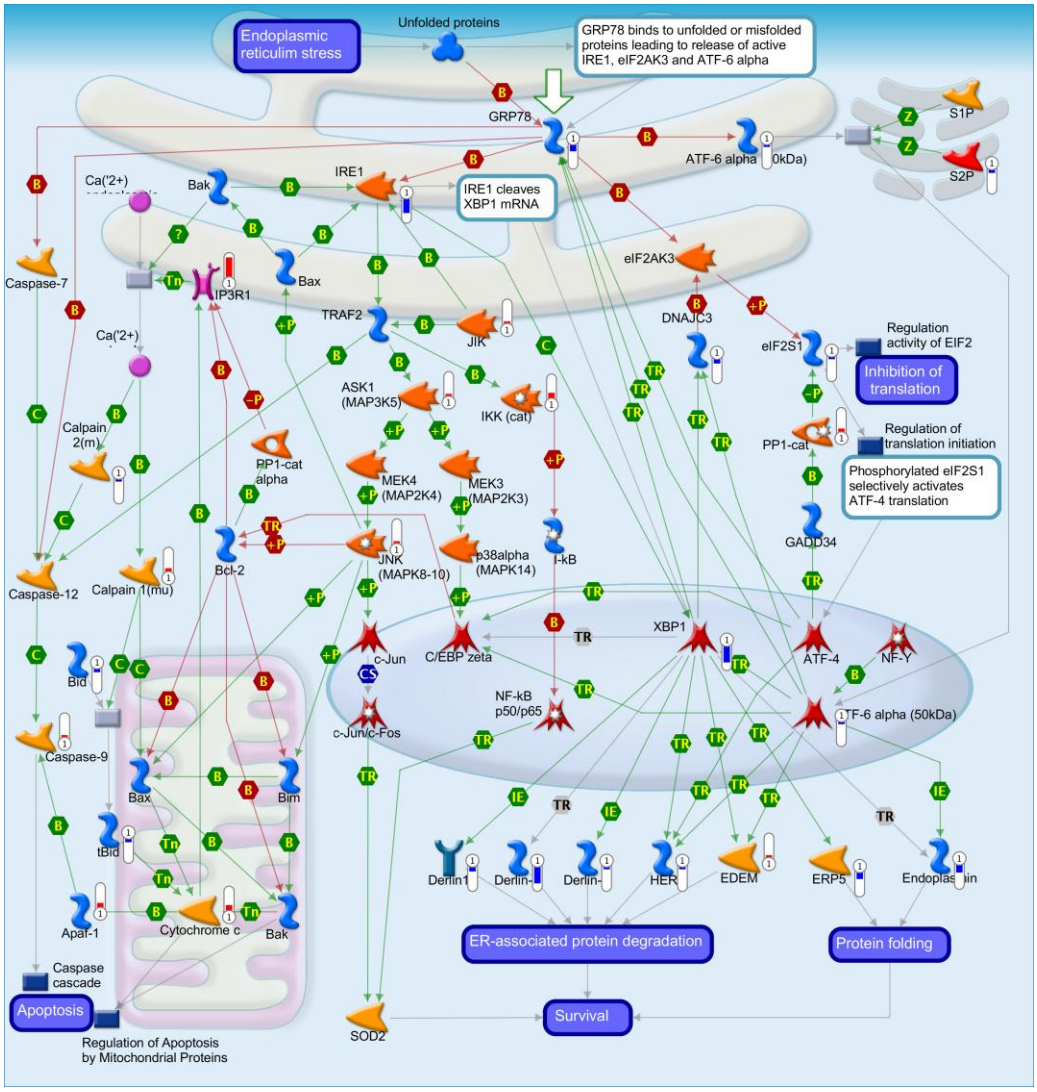

Fig. 19 The endoplasmatic reticulum stress response pathway

Moreover, we investigated if there were other possible mechanisms that played in order to confer to this immature precursor a resistance phenotype. By focused our analysis on mechanisms of drug sensibility and resistance, we identified a series of genes which are strongly overexpressed. In particular, *ABCA1*, *ABCC1*, *MDR1* and *ABCG2* are known to be involved in the metabolism associated to drugs intake because they functioned as a membrane transporter or ion channels (Tab. 17). Their upregulation was also confirmed in the comparison of gene expression profiles between 8 MM B cells versus 8 MM CD138+ cells, as a demonstration that this gene signature is specific of MM B cells.

GENES	Signal	p-value
ABCA1	3,70	0,005556
ABCC1	2,38	5,19E-05
MDR1	16,68	1,08E-06
ABCG2	4,55	0,006743

Tab. 17 Genes associated to the resistance to therapy

Our data suggested that the maturation status of MM heterogeneity is an important characteristic to be evaluated, because it may represent a common mechanism that possibly unifies a spectrum of genetic changes within MM tumors that escape theraupeutic pressures. Moreover, the inability of proteasome inhibitors to address these non-secretory tumor cell progenitors is mainly linked to the attenuation of the unfolded protein response, laying the groundwork for a possible introduction of IRE1α in MM treatment scenario.

GENE	Log	
Beta-catenin	7,70	
SMAD4	7,33	
TCF4	6,78	
CD86	5,76	
SMAD2	4,27	
SP1	4,22	
NFKB1	4,17	
CREB1	3,71	
NFATC1	3,39	
ILK	3,34	
REST	3,21	
GSK3B	2,90	
SHC1	2,50	B CELL vs NORMAL
IL12A	2,49	
PPARD	2,41	
TRAF6	2,27	
TNFSF13B	2,25	
RELA	2,10	
SMAD3	2,05	
TNFRSF13C	2,01	
ETV1	-2,00	
ANGPTL1	-2,08	
LIF	-2,13	
TNFSF13	-2,20	
EPO	-2,51	
LEF1	-3,05	
WNT5A	-6,23	

GENE	Log	
STAT1	4,5471	
EVI2A	3,86011	
SEC62	3,76931	
JAK2	2,87092	
CREB1	2,86937	SUB1 vs SUB2
ZNF274	-2,25214	
IL6ST	-2,55316	
DLL1	-5,31315	

GENE	Log	
CXCL5	8,95899	
TGFB1	6,55403	
Beta-catenin	5,38985	
SMAD3	5,18889	
EGF	4,78836	
PGF	4,09132	
PPARD	4,05908	
NFATC1	3,80602	
JAK1	3,66042	
CREB1	3,57716	B CELL vs 138
STAT5A	2,72793	
RAF1	2,53008	
PDGFA	2,15827	
SHC1	-2,11121	
CEBPB	-3,86615	
EPOR	-3,89446	
CREM	-4,39194	
IL8	-9,06383	
VEGFA	-12,9632	
DKK1	-15,485	
IGF1	-51,1827	

GENE	Log	
IL8	55,8914	
IGF1	45,3582	
CD86	6,51422	
ETV1	6,22746	
CREM	3,99101	
SMAD4	3,70236	
MAP3K7	3,54808	
TRAF6	3,41543	
NR3C1	3,28038	
GNAS	3,27952	
IL1B	3,0718	
VEGFA	2,76945	
FOS	2,60829	
CCL2	2,38899	
TGFB1	2,35924	
SHC1	2,12109	
IL6	2,02568	
CD40	2,02505	
TCF7L2	1,86946	
PTK2B	1,77911	
SMAD3	1,75576	
CREB1	1,71338	
IL17RC	1,59223	
IL12A	1,53545	
TNFRSF1B	1,52778	138 vs NORMAL
CEBPD	1,52734	
CXCL1	1,49834	
TLR4	1,45324	
Fibronectin	1,39821	
NTS	1,37037	
PPARG	1,27952	
IL2	1,24874	
CD69	1,22425	
TLR2	1,14926	
MYD88	1,11979	
E2F1	1,05263	
CCL11	1,02559	
DEFB4A	1,00521	
IFNB1	-1,00686	
IL17F	-1,09407	
TNF	-1,11416	
IL12B	-1,12532	
FSHB	-1,14599	
CSF2	-1,34224	
PPARD	-1,34496	
IL17RA	-1,55491	
EPO	-1,6507	
CSF3	-1,70089	
EPOR	-1,84637	
CD80	-2,24294	
IKBKB	-6,47862	

8. DISCUSSION

Although so far Multiple Myeloma has been described as a neoplasm of the mature CD138 positive plasma cell , recent data have shed the light on the important role played also by the CD138 negative cells compartment . Indeed, over the last years the role of any different stage of B cell differentiation, starting from B cell to the complete differentiated plasma cell, has been highlighted, in contributing to the disease development and, most importantly, in representing a reservoir, which might reconstitute the neoplastic clone, throughout the clinical course of the disease. Moreover, in the wake of the cancer stem cell hypothesis, it has been postulated the existence of a cancer stem cell population also in Multiple Myeloma, which might resides in the CD138 negative compartment and might phenotipically resemble the memory B cells. Up to now, the role of these cells is however still unclear and it represents a point of controversy.

The aim of this project has been to molecularly characterize the putative clone of Multiple Myeloma stem cells, in order to identify peculiar alterations, like Copy Number Alterations and modulated expression of genes, which might unequivocally identify these cells in the context of Multiple Myeloma microenvironment.

To this purpose, we first evaluated the clonogenic potential of this cell compartment. Our results showed that the CD138- cell fraction obtained from two different Multiple Myeloma cell lines displayed a higher clonogenic potential, as compared to the CD138+ mature plasma cells; indeed, the number of colonies observed after two weeks of culture was higher in the CD138- as compared to the CD138+ plate. These data support the idea that the side myeloma-initiating cell population consist of B-lineage immature cells, lacking CD138 marker. Further analyses are required, in order to evaluate the ability of these colonies to recapitulate the tumor development, in terms of cells phenotype and activity. Moreover, it will be important to test whether the clonogenic potential of CD138- cells will be maintained under the selective pressure of specific molecules, like self-renewal inhibitors.

One of the hallmarks of the human B cell lineage consists in the ability to rearrange the germ-line immunoglobulin DNA to generate antibody diversity: this represents an essential prerequisite for the production of a functional and efficient repertoire. While this mechanism is essential to prevent infections, it also represents the “Achilles heel” of the B cell lineage, occasionally leading to malignant transformation of these cells by translocation of proto-oncogenes into the immunoglobulin loci.

The study of the configuration and rearrangements of the Ig gene locus has contributed extensively to our understanding of the natural history of myeloma. Indeed, immunoglobulin gene rearrangement molecular analysis is currently part of the routine clinical management, since it represents one of the best methods to monitor high risk patients and to predict disease relapse.

In order to evaluate the clonal relationships existing between the mature plasma cells and the immature clones, object of our study, we employed the IgH VDJ gene rearrangement as a patient-specific

marker of clonality. This allowed to track the specific rearrangement between the various B cell differentiation stages that precede the mature plasma cells.

We showed that the memory B cells and the terminally differentiated neoplastic plasma cells shared the same VDJ rearrangement; nevertheless, the same VDJ qualitative assay tested on the CD27-negative fraction highlighted the presence of the same VDJ gene rearrangement observed in the other cell fractions, as well. Our data are in agree with previously reported ones and confirmed the clonal relationships existing between the quiescent plasma cells and the B memory clone; in addition to that, we also showed that the same VDJ rearrangement is shared between different stages of B cell differentiation. This need to be taken into account, when performing minimal residual disease analyses, since the cell population we track by means of the VDJ molecular marker is actually much more heterogeneous than what we have expected so far. Indeed, it might include not only the terminally differentiated plasma cells, but also more immature cells, which do not harbor the CD138 antigen, and which might temporarily precede the stage of established neoplasm.

Once we have confirmed the restriction of clonogenic cells to the CD138- compartment, as well as the sharing of the same IgH VDJ patient-specific rearrangement of the neoplastic CD138+ clone, we projected to characterize the genomic and genetic background of 19+27- cells of the 138- compartment, by means of high-throughput technology, in order to highlight the presence of specific alterations, which might contribute to the disease development and progression.

To this purpose, for each patient included in the study, we compared the genomic profiles of the CD138+ cells andthe B memory cells, obtained either from BM or PBL.

We first showed that the genomic complexity, that quite exclusively distinguish the bone marrow mature CD138+ plasma cells, is perfectly mirrored by the circulating CD138+ plasma cells. The presence of circulating CD138+ plasma cells, identical to the bone marrow CD138+ clonal cells might be related to the tumor spread typical of the more advanced disease phases; nevertheless, it is worth of note that since diagnosis, most MM patients do carry potentially pathogenic plasma cells in the peripheral blood; it cannot be excluded that these cells, while temporary preceding the CD138+ cells located in the bone marrow, are actually designated to “feed” the more mature neoplastic clone.

Unlike mature CD138+ cells, memory B cells display a much more simple genomic landscape, where, any macroalterations can be detected, whereas several typical micro-alterations can be enumerated; this is actually expected, as being consistent with an immature state of these cells,

Among others, the microdeletion located on the terminal portion of the long arm of chromosome 14 seemed quite intriguing: indeed, it suggests that these immature cells already carry a chromosomal transforming event. Physiologically, normal cells tolerated continuous translocations that occurs in genome because they promptly activated mechanisms of DNA repairs. Thus, rare aberrant mutations and transloca-

tions that are capable of pushing a cell towards subsequent malignancy can be generated as a result of a normal physiological process. These rearrangements are tolerated because in most individuals they improve immune function and, as a consequence, the ability to reproduce and pass on genes to the next generation. Therefore, it seems that the price of an effective immune system protecting from infections throughout life is a background rate of B cell tumours and myeloma, particularly later in life. In this respect it is perhaps not surprising that in >3% of individuals over the age of 60 there is evidence of a clonal expansion of plasma cells in the form of MGUS3.. As myeloma is a cancer diffuse among aged people, the finding that the micro deletion is present in memory B cells possibly proved the initial event of breakage of tolerance observed in the early phase of the disease.

Overall, the analysis of the prevalent microalterations observed in memory B cells, either losses or amplifications, let suppose that these cells might be characterized by altered DNA repair and transcriptional regulation mechanisms, as well as negative regulation of apoptosis and angiogenesis. These data suggest that in this particular subset of cells, a complex series of events take place, which finally culminate in the well-established genomic complexity, usually observed in plasma cells.

From this point of view, particularly interesting are the *XIAP*, *WWOX* and *KRAS* genes amplifications, since they have already been reported to be involved in the pathogenesis of myeloma; this again supports theidea that memory B cells do actually play an important role in the initiating events, leading to myeloma establishment.

Finally, copy number analysis suggested also the involvement of tumor suppressor genes included in the observed LOH regions. The match between the presence of LOH regions in memory B cells and of an inactivated tumor suppressor gene in the CD138+ mature clone might finally demonstrate the mechanisms underlying myeloma pathogenesis, from B to plasma cells. Obviously, more studies in a larger series of samples are needed, in order to validate these preliminar results.

Studies performed to analyze the gene profiles of the immature cells as compared to the mature clone confirmed the already observed differences between these two related compartments.

We were able to show an early stratification of patients, according to their B cell transcriptome profiles; this suggests that the intrinsic heterogeneity typically observed in myeloma takes place during the B cell lineage differentiation, quite earlier with respect to the final stage of mature plasma cell.

Recently it has been shown that the Hedgehog pathway is strongly involved in maintaining the stem cell population in Multiple Myeloma (116). This pathway is also known to be iperactivated in several solid tumors, as well as in hematological malignancies. Based on these findings, we focused on the identification of self-renewal pathways, which might be possibly related to the biology of the B cell subset.

First, we showed an overall down regulation of Hedgehog pathway in myeloma immature B cells, as compared to B cells from healthy donors, ascribable to the down regulation of both ligands, receptors and transcription factors. Conversely, in these cells, the up regulation of Notch and Wnt signaling might be

involved in the proliferation processes, which recapitulate the tumor evolution. These data need to be confirmed by *in vitro* assays, in order to evaluate the effective activation of these signaling at protein level; moreover it would be important to demonstrate also that the inhibition of these pathways is able to compromise the self-renewal of these cells.

We then analyzed the gene profiles of the mature cell compartments, by comparing the transcriptome of CD138+ plasma cells obtained from MM patients and healthy donors. Indeed, it has been recently reported that Hedgehog signaling is activated in CD138+ plasma cells in different disease stages, starting from MGUS up to plasma cell leukemia, and that a decreasing gradient of expression of Hedgehog genes can be highlighted along the disease course. This suggests that this pathway might have a relevant role in malignant transformation and disease progression (123). Our results were in contrast with these findings, since all genes related to HH pathway resulted down regulated, whereas PTCH1, which is the receptor that activates SMO, resulted overexpressed, which finally confirms the overall off state of the pathway. We thus concluded that Hedgehog pathway seems to be not as much relevant in our context, as it has been previously reported, whereas both the activation of proliferation and the growth potential of myeloma B cells might be attributed to the up regulation of Notch and Wnt signaling, which are known to finally control the cell renewal and the cell fate in several biological contexts

We finally tried to explain our results in the clinical context. Indeed, it has been recently shown that in Multiple Myeloma mechanism of resistance to therapy are mainly activated in the immature CD138- cell compartment (137). We thus looked for the expression at diagnosis of a particular “phenotype” possibly related to therapy resistance.

The expression of genes involved in drug metabolism was evaluated in B cells, thus showing a significant over expression of genes known to played an important role in drug resistance, as *ABCA1*, *ABCC1*, *MDR1* and *ABCG2*. In addition to that, also the *IRE1α-XBP1* axis resulted attenuated, thus confirming the idea that the circulating myeloma B cells might be actually able to resist to the present treatment strategies employed for Multiple Myeloma therapy.

9. CONCLUSIONS

The study presented here represents the first attempt to delineate an extensive molecular characterization of Multiple Myeloma Stem cells. Specific genomic alterations have been highlighted in these immature cells, which might explain their specific role in the pathogenesis of myeloma. Gene expression analysis detected the over expression of both Notch and Wnt signaling, which might explain the ability of these cells to sustain the tumor; moreover, a peculiar phenotype of these cells was described, which might account for a general mechanism associated to the resistance to different therapeutic regimens.

Based on these evidences, it seems increasingly important to not consider any more Multiple Myeloma as a disease of the fully mature CD138+ plasma cell; on the contrary more attention should be devoted to the CD138 negative compartment, since it actually might play an important role in the pathogenesis of myeloma. Indeed, our results showed that the well-known myeloma heterogeneity is not only restricted to the plasma cell compartment, but it also belongs to the more immature B cell compartment.

These observations actually might be explained in the context of the recently proposed “clonal tide” theory of a clonal evolution of multiple myeloma. Indeed, it seems likely that each myeloma multiple genetic subclone - which respond to treatment with “clonal tides” modality – includes a range of progenitors that markedly contribute to intra-tumor diversity and to the ability of myeloma tumor to endure treatment. Since tumor progenitors might contribute to treatment failure in multiple myeloma, further investigations in a larger series of patients are warranted, in order to confirm our findings related to specific genomic alterations and molecular mechanisms active in multiple myeloma stem cells. This might drive to more specific therapies, targeted on the immature compartment, possibly able to produce a more durable control of the disease.

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