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Cancer Stem Cells in Multiple Myeloma

Sabino Ciavarella, Annalisa Milano, Annalisa Savonarola,
Oronzo Brunetti, Franco Dammacco and Franco Silvestris
University of Bari "A. Moro" /Department of Biomedical Science and Clinical Oncology
Italy

1. Introduction

Over the last few years, the hypothesis that rare stem cell-like cells are responsible for tumor initiation and maintenance, namely the cancer stem cell (CSC) theory, has remarkably changed the way to approach cancer biology. The scientific relevance of this theory is based, in fact, not only on the existence of hierarchically organized, self-renewing malignant progenitor cells within each tumor, but also on the assumption that their drug resistance may account for the high frequency of relapse that renders incurable most tumors despite their treatment with specific cytotoxic drugs. The view that CSCs may descend from normal stem cells finds its historical background in studies on acute myeloid leukemia where the leukemic clone is organized according to a defined hierarchy in which the majority of tumor cells rapidly proliferate, while quiescent "leukemic stem cells" resembling early hematopoietic progenitors remain as a potential reservoir of cancer propagation due to their chemoresistance to specific cytotoxic protocols.

Recently, however, a large number of studies have turned the CSC theory into more than an idea, since clear experimental evidence has demonstrated the existence of CSCs in a number of tumors. Indeed, brain (Singh et al., 2004), intestinal (O'Brien et al., 2007) and breast cancers (Al-Hajj et al., 2003) have been shown to arise from rare cells, organized in a precise hierarchy resembling that of stem cells, which are capable of generating a differentiated progeny by repetitive asymmetric divisions. In most of these tumors, however, the earliest event driving the malignant transformation remains elusive and the original cell is far from being defined, since both histological and cell-surface marker profiles of the bulk tumor cells do not necessarily resemble those of the lineage-related stem cell. These observations, moreover, have been interpreted to suggest that CSCs could derive either from normal stem cells or from committed progenitor cells that have acquired the ability to self-renew due to specific genetic mutations (Visvader, 2011).

Similar controversies have arisen with hematological malignancies of both myeloid and lymphoid origin, including multiple myeloma (MM). In fact, differently from acute leukemia, in the initial phases of most chronic lymphoproliferative disorders, neoplastic cells do not resemble early hematopoietic stem cells, but rather cellular elements in late maturation phases, as clearly demonstrated in chronic myeloid leukemia (Jamieson et al., 2004). In MM, malignant plasma cells are characterized by unique patterns of immunoglobulin (Ig) and surface antigen expression, thus suggesting that the tumor bulk is exclusively composed of terminally differentiated cells with high proliferation potential.

However, the evidence that the majority of MM cells are quiescent and that, similarly to their normal counterpart, they lack long-lasting proliferation potential both in vitro and in vivo (Dewinko et al., 1981) implies that tumor growth is actually dependent on a more restricted subset of clonogenic and self-renewing lymphoid cells. Thus, based on the observation that both blood and bone marrow (BM) from MM patients contain lymphoid elements with a hypervariable region Ig gene repertoire identical to that of bulk tumor cells, it has been hypothesized that part of the hierarchical model of the lymphoid lineage maturation may recur also in MM (Pilarski & Jensen, 1992; Billadeau et al., 1993). The functional heterogeneity of tumor cells in MM provides the rationale for exploring the coexistence of bulk plasma cells and putative tumor stem cells resembling both phenotype and biological characteristics of B lymphocytes, consistent with the similarity observed between hematopoietic progenitors and tumor cells in leukemia. To date, several studies have highlighted the existence of putative myeloma CSCs that share with normal adult stem cells a number of molecular and functional aspects, including their resistance to conventional treatments (Agarwal & Matsui, 2010). Therefore, although issues such as CSC origin, phenotype and potential pathogenetic role in MM are still being debated, investigating MM CSCs may provide new molecular targets for treatment of this still incurable disease.

2. Implication of the CSC theory in MM pathogenesis: from "clonotypic B cells" to putative MM CSCs

Major clinical manifestations of MM, including anemia, skeletal impairment, hypercalcemia and renal failure, are related to the progressive accumulation of malignant plasma cells within BM. Moreover, similarly to normal plasma cells, malignant plasma cells produce Ig as the monoclonal component commonly detectable in serum and urine of MM patients. But as early pathogenetic events, including key oncogenic mutations, occur during B cell maturation, MM is regarded as a B cell malignancy rather than a plasma cell neoplasm.

Normal plasma cells derive from "post-germinal center B cells" (PGBCs) that have completed a program of somatic hypermutations following the Ig heavy chain (IgH) switching in lymph nodes, and undergone the subsequent maturation in circulating "memory B cells" that migrate to BM, where the stromal microenvironment drives their terminal differentiation at both morphological and functional level (Johnson et al., 2005). In this context, a recent model of myelomagenesis (Anderson & Carrasco, 2011) postulates that malignant plasma cells derive from B cells of the lymph node post-germinal center, since they display an identical pattern of hypermutated Ig genes within their clone. It is thus conceivable that a PGBC likely undergoes a first oncogenic mutation, giving rise to a clone of premalignant B cells expressing identical Ig genes and capable of migrating to BM. Therefore, the asymptomatic phase of MM development, namely monoclonal gammopathy of undetermined significance (MGUS), may be sustained by the emergence within BM of only a limited number of malignant plasma cell clones producing monoclonal Ig. Subsequent changes within marrow milieu, including induction of angiogenesis, development of cytokine-based paracrine signalling loops, and acquisition of additional genetic mutations by tumor cells, are thought to promote further growth of malignant plasma cells, thus definitely mediating the switch from MGUS to MM.

Early investigations revealed that peripheral blood of both MGUS and MM patients contains a large fraction of circulating B cells at a late stage of differentiation, as proven by their

minor CD19 and CD20 expression (Jensen et al., 1991). Although morphologically heterogeneous, these cells display identical Ig gene rearrangements in support of their monoclonal derivation and, of note, expressed the same type of either kappa or lambda light chain mRNA of BM plasma cells (Jensen et al., 1992). In parallel studies, these "clonotypic B cells" (CBCs) were found to even express typical plasma cell antigens, such as the plasma cell membrane glycoprotein-1 (PCA-1) and CD38, variable levels of CD11b β2-integrin, CXCR4 and CXCR5, pivotal molecules for the homing to BM, while lacking CD34 as typical marker of the hematopoietic stem cell subset (Jensen et al., 1992). Subsequent investigations, moreover, have demonstrated that the number of CBCs is variable among patients with an average value of 65% of peripheral B cells, although they equally express identical IgH rearrangements as parental BM myeloma cells (Bergsagel et al., 1995), that has been confirmed by gene sequencing analyses (Berenson et al., 1995; Szczepec et al., 1998). Thus, in order to identify the actual differentiation state of circulating CBCs, a number of studies were aimed at assessing the CD45 expression level, since normal B cells usually lose CD45 antigen as their maturation progresses. CBCs display different patterns of CD45 expression, depending on their location, ranging from higher levels when they reside in proximity to secondary lymphoid organs to minor expression in either BM or peripheral blood (Jensen et al., 1992). Interestingly, in a single previous report, clonal circulating B cells from MM patients were found to resemble the memory B cell phenotype (Rasmussen et al., 2004), thus suggesting that CBCs could represent an ongoing differentiating population (from PGBCs to plasmablasts) in MM, and that they may play a pivotal role in MM pathogenesis. These cells may originate in peripheral lymphoid system and, once having undergone key oncogenic mutations, migrate toward the BM to complete their differentiation into malignant plasma cells (Pilarski & Jensen, 1992).

In spite of their malignant nature, myeloma cells seem to resemble their normal counterpart at both phenotypic and functional level. Examination of BM biopsies from MM patients commonly reveals the infiltration of cells expressing the typical mature plasma cell marker CD138. They appear relatively quiescent (Kyle & Rajkumar, 2004), and, similarly to their terminally differentiated normal counterpart, display low proliferative index (Robillard et al., 2005). Other early experiments using murine MM cells showed that only a small proportion of malignant plasma cells are able to form single cell-derived colonies either in vitro or in vivo (Bergsagel & Valeriote, 1968; Canc Res; Park et al., 1971), thus emphasizing that both clonogenic and proliferation potential are restricted to a small fraction of neoplastic cells. Subsequent observations underscored that, similarly to murine MM, human malignant plasma cells possess low colony-forming capacity and only rare cells within tumor clone give rise to colonies in vitro (Hamburger & Salmon, 1977).

Evidence of a functional heterogeneity in MM is also supported by other clinical observations. Today, treatment of MM with steroids, alkylating drugs, immunomodulatory agents, proteasome inhibitors and stem cell transplantation (SCT) procedures induces improvement of median survival of patients but does not provide potential for cure, due to the high rate of MM relapse after treatment. Taken together, these observations suggest that only a small fraction of tumor cells possess the capability of initiating tumor growth and, similar to stem cell behavior within normal tissues, this small fraction is able to rebuild and maintain the original MM clone after therapy.

CBCs obtained from peripheral blood of patients with MM have been shown to successfully engraft into immunocompromised mice and induce major clinical features of human MM, such as osteolytic lesions and the production of a serum monoclonal component (Pilarski et

al., 2000). These cells, moreover, possess slight sensitivity to current anti-MM agents. It has also been demonstrated that the majority of monoclonal B cells from patients with MM show high levels of P-glycoprotein which is involved in the multi-drug resistance, and that they increase its expression after chemotherapy (Pilarski & Belch, 1994). High levels of circulating CBCs have been found during transient post-treatment remissions, and patients undergoing disease progression or relapse showed larger fractions of CBCs than those responding to treatment as well as untreated patients (Bergsagel et al., 1995). Furthermore, given the high occurrence of disease relapse after auto-SCT, it is conceivable that even high-dose chemotherapy regimens used during conventional SCT procedures is not enough to eliminate CBCs or, alternatively, that CBCs are being collected during leukapheresis and, once transplanted to patient, are able to drive the tumor relapse.
Taken together, these observations support a new model of MM pathogenesis in which CBCs, as clonogenic, self-renewing, and drug resistant cells belonging to the original malignant cell clone, may also represent the malignant stem cell population in MM (Fig. 1).

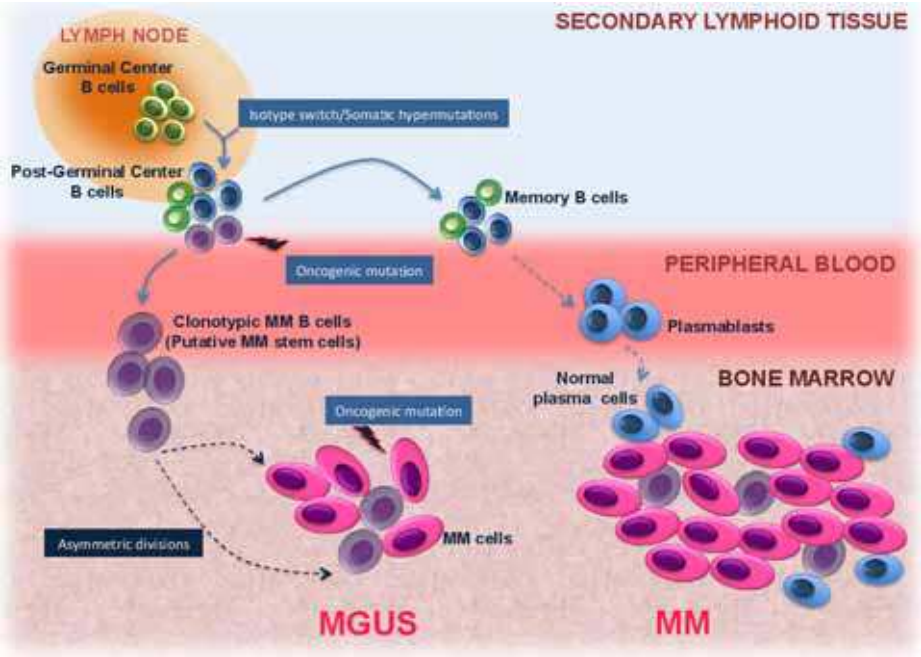

Fig. 1. Cancer stem cells in a proposed model of MM pathogenesis

In the germinal center of lymph nodes, healthy B lymphocytes physiologically undergo antigen selection and give rise to different clones of post-germinal B cells that have successfully completed sequential rounds of somatic hypermutations and Ig gene recombination. Each post-germinal B cell clone may produce memory B cells possessing consistent pattern of hypermutations within their own clone. These cells progress along their differentiation course and migrate, as plasmablasts, to BM where the stromal environment induces their terminal maturation into functional plasma cells capable of secreting Ig. A first oncogenic mutation may hit a post germinal center B cells, giving rise to

a clone of pre-malignant circulating B lymphocytes expressing identical Ig genes (clonotypic MM B cells). These cells, however, may retain their stem cell properties (self-renew and differentiation) and, once migrated to BM, divide by asymmetric divisions that can generate malignant plasma cells and a reservoir of MM stem cell fraction (MGUS). Presumably, the malignant plasma cell population may acquire additional mutations and expand by symmetric divisions. Other BM factors, including neoangiogenesis and formation of a variety of paracrine signalling loops with stromal BM components, may further support MM cell growth, whereas a little proportion of clonotypic MM stem cells with tumor-initiation capacity, persist within tumor mass.

2.1 Biological properties of MM CSCs: searching for the putative myeloma stem cell phenotype

In a number of both hematologic and solid malignancies, including acute myeloid leukemia and brain tumors, identification of CSCs was accomplished by searching the lineage-specific antigens of respective progenitors (Bonnet & Dick, 1997; Singh et al., 2004), and by their capacity to engraft into murine immunodeficient mice and recapitulate the disease. Similarly, based on the accredited theory that bulk malignant myeloma cells arise from asymmetric divisions of rare elements belonging to the B cell lineage, it has been prospectively assumed that the MM CSC population should lack CD38 and CD138 antigens, and display high self-renewal and myelomagenic potential both in vitro and in vivo.

In the first animal model engrafted with primary human myeloma cells, BM specimens from MM patients were directly injected in fetal bone chips subcutaneously implanted in SCID mice (Yaccoby et al., 1998). This model resembled several features of human MM, including the occurrence of the circulating monoclonal component, hypercalcemia and the typical MM-associated bone disease. Subsequent studies revealed that the tumor bulk is the effect of the growth of plasma cells expressing CD38, while lacking CD45 and showing high self-renewing capacity as proven by their ability to engraft into the secondary SCID-hu recipient. On the contrary, no proliferation was ascribed to the cell fraction containing $CD19^+$ B cells (Yaccoby et al., 1999). These data, however, were in apparent contrast with the knowledge that, at least in vitro, the clonogenic frequency of primary malignant cells was as low as 0.1% (Hamburger & Salmon, 1977), although it is conceivable that microenvironmental factors within the SCID-hu bone chips might support the growth of $CD38^+$ plasma cells. On the other hand, peripheral blood-derived $CD19^+$ clonotypic B cells from a patient with progressive MM were shown to successfully engraft into immunodeficient mice and produce tumor bulks mostly composed by $CD19^+$/ $CD138^+$ malignant cells (Pilarski et al., 2002). These findings were in agreement with the previous observation showing that NOD/ SCID mice are good recipients for the engraftment of circulating B cells from MM patients developing myeloma masses predominantly composed of $CD38^+$ and $CD19^+$ cells (Pilarski et al., 2000). The latter studies also emphasized that clonotypic B cells, rather than $CD138^+$ malignant plasma cells, are directly involved in tumor formation, although the proof that clonogenic B cells have self-renewal potential is still missing, since no serial transplantation has been performed in secondary animal hosts.

Based on these findings, initial studies comparing the ability of highly $CD138^+$ myeloma plasma cells with $CD138^-$ cells to form colonies in vitro revealed that cells lacking typical plasma cell markers showed higher clonogenic frequency and easy undergoing to serial passages in vitro. Accordingly, $CD138^+$ cells were unable to engraft into NOD/ SCID mice

when intravenously injected, whereas cells with B cell phenotype effectively engrafted in mice and produced tumor burdens of mature CD138+ plasma cells secreting the monoclonal Ig. In the same study, moreover, single cell-derived colonies capable of in vivo myelomagenesis were shown to contain variable amounts of cells expressing CD45, CD19, CD20 and CD27 as typical surface antigens of the memory B cells (Matsui et al., 2004). The concept that cells expressing markers of memory B cells share exclusive properties with normal stem cells further supported the derivation of the malignant plasma cell from a post-germinal cellular element. Consistently, although unable to undergo multilineage differentiation due to their committed state, memory B cells physiologically maintain the capacity to self-renew even during their maturation, thus ensuring the immune memory as a crucial requirement of adaptive immunity. Other independent groups, previously involved in exploring the role of B cells in MM development, showed that the gene repertoire encoding the monoclonal Ig component mostly occurred in B cells from both BM and peripheral blood rather than in mature plasma cells (Billadeau et al., 1993; Bakkus et al., 1994), albeit such a clonogenic B cell population can rarely be detected in primary bioptic specimens (0.1% of all tumor cells) (Chen & Epstein, 1996; Rasmussen, 2001).

In line with this view, blood and BM specimens from MM patients were shown to contain a CD138-/ CD19+/ CD20+/ CD27+ cell fraction with higher clonogenic capacity as compared to the CD138+ cell counterpart. More interestingly, this memory B cell-like subpopulation was found to share identical Ig gene rearrangement as the malignant plasma cells and, unlike these, effectively engraft into NOD/ SCID mice that develop plasmacytomas mostly composed of CD138+ cells clonally related to the injected cells by similar Ig light chain restriction (Matsui, 2004). In addition, small subsets of CD138-/ CD19+/ CD20+/ CD27+ cells harvested from BM of the mice were successfully injected in secondary recipients that in turn developed MM (Matsui et al., 2008). These findings, in agreement with previous evidence of circulating memory B cells in MM patients (Rasmussen et al., 2004), suggest that the CD138-/ CD19+/ CD20+/ CD27+ B cell subset may represent the putative CSC clone in MM (Clarke et al., 2006).

Despite these convincing data, many questions regarding the actual phenotype of the CSCs in MM remain unanswered, and new observations make this issue rather controversial. At present, several in vivo reproductions of MM have successfully utilized immunodeficient mice injected with CD138+ malignant plasma cells (Yaccoby & Epstein, 1999). Although empiric, these observations emphasize the ability of phenotypically mature tumor cells by themselves to recapitulate MM in vivo. In addition, further studies underscore the absence of MM cells clonal derivation from respective B cell precursors, since CD19+ cells from different patients were shown to express no tumor-specific genetic mutations (McSweeney et al., 1996). Finally, malignant plasma cells display high plasticity and are capable, after long-term interaction with other components of the BM microenvironment, to reprogram their gene array as well as to turn back to a more immature phenotype including low levels of CD19 but lacking both CD38 and CD138 molecules. In addition, these cells resemble the plasmablastic morphology, thus supporting a "de-differentiation" theory (Yaccoby, 2005) rather than the existence of a subset of CSCs within the MM bulk during early phase of its development. Fig. 2 shows the detection of putative CSCs in a representative bone biopsy from a patient with MM. As can be seen, in the context of a myeloma bone lesion including CD138 stained cells (black arrows), we found a minimal number of cells with typical plasmablast morphology and lacking the CD138 antigen that infiltrate a tumor mass mostly composed by CD138+ plasma cells.

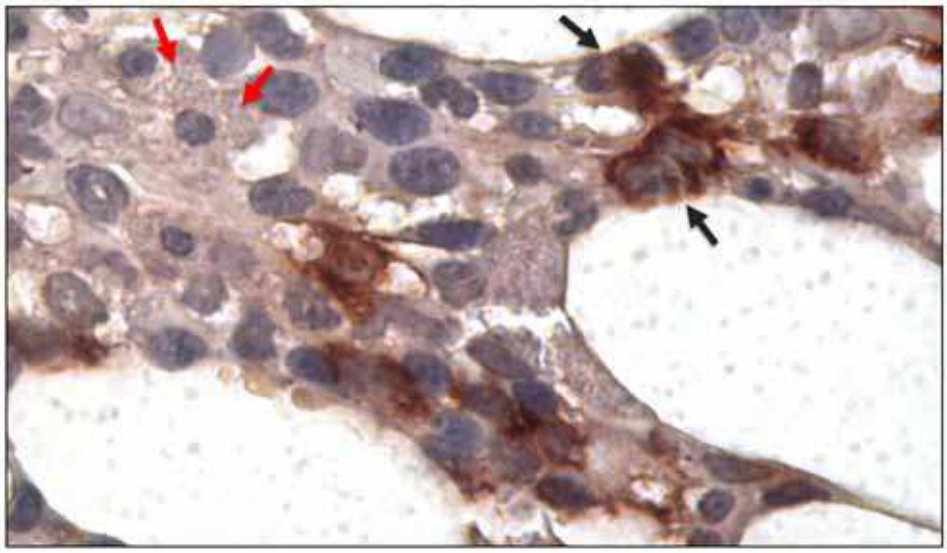

Fig. 2. Representative immunohistochemical detection of CD138+ malignant plasma cells within MM bone marrow biopsy

MM bone marrow section showing high infiltration of cells with mature plasma cell morphology and intense positivity to CD138 staining (black arrows) that stand close to cellular elements with plasmablastic appearance lacking CD138 (red arrows).

2.1.1 Functional properties of MM CSCs

The property to undergo asymmetric divisions and give rise to more differentiated cells retaining high proliferative behavior is not the unique functional hallmark that MM CSCs share with their normal progenitor cells. In fact, similarly to stem cells of either leukemia or brain tumors (Lapidot et al., 1994; Singh et al., 2004), putative MM-initiating cells are resistant to toxic chemical injury, thus accounting for the common risk of disease relapse following conventional treatments. Long-term proliferating CD138- cells, rather than both their CD138+ counterpart and myeloma cell lines, were shown to be slightly influenced in their survival and clonogenic capacities by exposure to common anti-MM agents, such as Dexamethasone, Lenalidomide and Cyclophosphamide (Matsui et al., 2008). Similarly, investigation in our laboratory demonstrated that primary CD138- malignant plasma cells incubated with different concentration of the proteasome inhibitor Bortezomib reach higher survival rates as compared with the control CD138+ U-266 myeloma cell line (Fig. 3).

Cells were cultured for 48 hours with increasing concentrations of Bortezomib and evaluated by MTT assay for their viability. CD138- MM cells show higher survival rates (red curve) as compared with CD 138+ U-266 cells (blue line).

Both normal stem cells and cancer cells have been intensively investigated for their drug resistance (Fletcher JI et al., 2010). A few biological processes are commonly claimed to explain their scarce sensitivity to most cytotoxic agents and are typical of some tumors, including MM. When long-term cultured, in fact, putative MM CSCs predominantly stand in the G0-G1 cell cycle phase (Matsui et al., 2008), thus explaining why they appear poorly

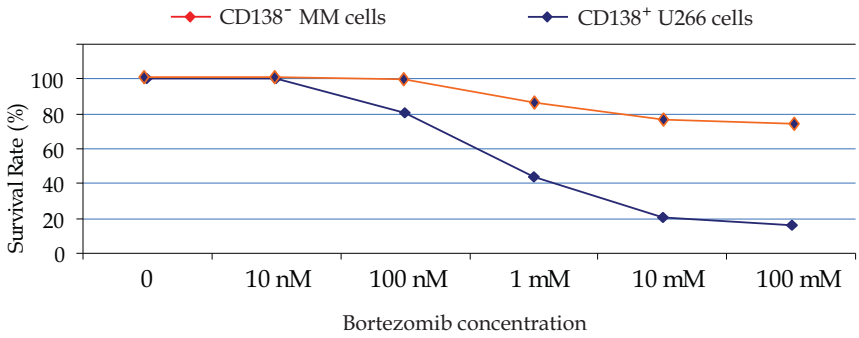

Fig. 3. Bortezomib responsiveness of CD138- MM cells and CD138+ U-266 MM cells

responsive to DNA-damaging compounds, such as alkylating agents. However, the drug resistance in MM CSCs seems to be mediated by additional mechanisms, such as the over-expression of the ATP-binding cassette family of drug transporters (ABCG2/ BCRP), as well as the high levels of intracellular detoxification enzymes such as the aldehyde dehydrogenase (ALDH) (Matsui et al., 2008). Interestingly, each of these properties is currently used for cytometric assessment of CSCs for distinguishing the putative stem cell fraction within normal (Goodell et al., 1996) and tumor tissues (Sussman et al., 2007). In MM, as well as in other tumors, the capacity to export the nuclear dye Hoechst 33342, that identifies the so-called "side population" (SP), was recently evaluated on several CD138+ plasma cell lines resulting in the detection of a small proportion of SP cells lacking CD138 molecules within each cell line. These cells, moreover, expressed high levels of ALDH, emphasizing that the putative drug resistant myeloma stem cells exhibit a more immature phenotype than CD138+ bulk malignant plasma cells (Matsui et al., 2008). On the other hand, MM cell lines stained by Hoechst 33342 showed to mostly contain distinct SP subsets that, however, were variably expressing CD138. Interestingly, these SP fractions were found to lack CD19, CD20 and CD27 surface molecules, thus arguing against previous findings that emphasized the memory B cell phenotype of MM CSCs (Jakubikova et al., 2011).

2.1.2 Molecular aspects of MM CSC biology

Given their high clonogenicity, self-renewal and drug resistance, MM CSCs have been suspected to share exclusive pathways and molecular signals with normal adult stem cells. Wnt, Notch and Hedgehog (Hh), the most conserved developmental pathways in humans, are involved in normal stem cell self-renewing and differentiation following injury and, although dormant in most human tissues, they are aberrantly triggered in a variety of human cancers (Taipale et al., 2001; Ruiz I Altaba et al., 2002). In particular, other than being involved in cancer development (Duman-Scheel et al., 2002), Hh plays a pivotal role in CSC biology of different malignancies, including breast cancer, chronic myeloid leukemia and MM (Liu et al., 2006; Zhao et al., 2009; Agarwal et al., 2010). The surface receptor patched (PTCH), a transmembrane molecule, provides the starting point of Hh signalling cascade, whereas three different ligands, namely Sonic, Indian and Desert, bind PTCH in mammalians. Once bound, PTCH de-represses a seven-transmembrane smoothened (SMO) protein which, in turn, regulates the activity of three GLI proteins that enhance the transcription of several cell cycle regulator genes (Duman-Scheel et al., 2002). Mutations of PTCH or SMO can thus

enhance the pathway activity and underlay the development of significant percentages of basal cell carcinomas and medulloblastomas (Gailani et al., 1996; Raffel et al., 1997). Interestingly, both human myeloma cell lines and primary MM cell preparations overexpress some Hh signalling components, while CD138- MM stem cells from established tumor cell lines show higher levels of Sonic-Hh (SHh) signalling activity with respect to their differentiated counterpart (Peacock et al., 2007). Moreover, treatment with Sonic ligand results in a relevant expansion of MM CSC subsets and improves their clonogenic capacity by inhibiting the cell differentiation. By contrast, inhibition of SHh pathway in MM CSCs reduces their attitude to form colonies in vitro, thus suggesting their progressive differentiation. These data imply that a constitutive deregulation of SHh may contribute to the maintenance of undifferentiated CSC clones in MM, whereas other findings revealed that stromal cells, in the context of MM microenvironment, produce Hh ligands and may strongly impact on the survival of MM CSCs within their niches (Dierks et al., 2007).

MM CSCs exhibit other molecular aspects typical of normal stem cells. MM CSCs from both in vitro established MM cell lines and patients have been shown to possess high telomerase activity (Brennan et al., 2010). CD138- cells from commercial cell lines, in fact, showed higher levels of telomerase activation than their differentiated counterpart, thus emphasizing the crucial role of telomerase activity in regulating several biologic processes, such as cellular senescence and apoptosis in normal adult progenitor cells as well as in growth and maintenance of most human cancers (Shay & Bacchetti, 1997). MM CSCs have also been described to overexpress mRNAs of OCT4, NANOG and SOX2, the major regulators of self-renewal and pluripotency of embryonic stem cells (Yu et al., 2009). In particular, OCT4 drives the major transcriptional networks during the embryonic development and, at the same time, the formation of pluripotent stem cells in the mammalian embryos (Nichols J, et al. 1998). In this context, we have detected OCT4 in CD138- cells from the BM of patients with MM. As seen in Fig. 4, this transcription factor was dramatically overexpressed in these cells and, thus, it was interpreted as a marker of their stemness.

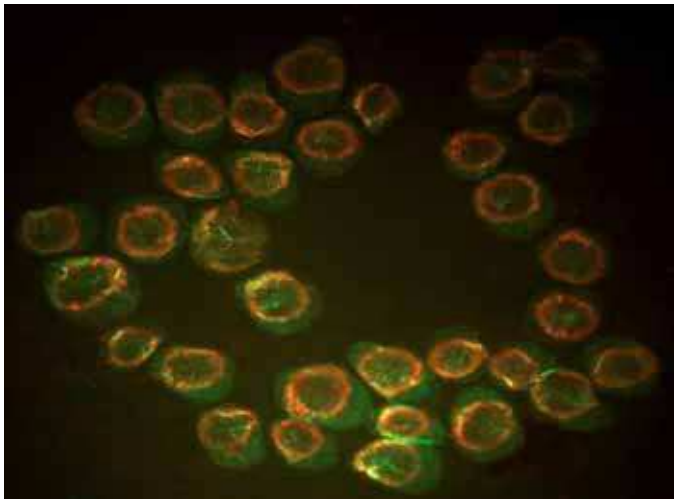

Fig. 4. Immunofluorescence analysis of OCT4 expression in MM stem cells

Fluorescence microscopy image showing a colony of putative MM stem cells stained for both cytoskeletal actin and OCT4 by falloidin (green) and anti-OCT4 PE-conjugated antibody (red), respectively. The typical ovoid shape of these cells is marked by the homogeneous distribution of cytoskeletal actin, whereas all nuclei are strongly positive for OCT4. The OCT4 signal is particularly intense at the nuclear periphery and originates red rings along nuclear contours.

Telomerase activity in MM CSCs, but not in bulk $CD138^+$ plasma cell fraction, was described to parallel the expression of embryonic stem cell genes as Notch and Hh (Brennan et al., 2010). Inhibition of telomerase activity significantly strengthens its role in biology of MM CSCs, since long-term treatment with Imetelstat, a specific competitor of telomerase reverse transcriptase, dramatically reduces both the colony-forming ability of MM CSCs and their engraftment into immunodeficient mice. On the other hand, short-term telomerase inhibition fails to induce telomere shortening in MM CSCs, but relays with a relevant down-regulation of OCT4, NANOG and SOX2 expression, resulting in parallel decrease of their clonogenicity. Therefore, it is becoming clear that specific genes and transcriptional factors driving normal stem cell fate may also affect the functions of MM CSCs and, at the same time, provide potential therapeutic targets in MM.

2.2 Targeting MM CSCs: Implication in MM treatment

For many years, the alkylator-based treatment schedules have represented the most used options for MM, due to their ability to induce prompt responses and symptom improvement (Oken et al., 1997). However, the median survival of patients treated with conventional drugs rarely exceeds 3 years and the disease commonly relapses or shortly progresses after treatment. On the other hand, either autologous or allogeneic hematopoietic stem cell transplantations induce extension of the disease-free survival, but not of the overall survival, in a small group of patients (Bensinger, 2002; Koreth et al., 2007),while a high rate of mortality may occur in allogenically transplanted patients. Based on their effectiveness in inducing significant prolongation of disease-free survival, novel classes of agents, including immunomodulatory compounds, such as Thalidomide and Lenalidomide, the proteasome inhibitor Bortezomib, and new alkylating molecules such as Bendamustin (Cheson, 2010; Lonial et al., 2011), have recently entered into the spectrum of anti-MM treatments, although they also fail to provide long-lasting remissions and their real improvement of overall survival is still to be accurately assessed.

These clinical observations further support the "CSC hypothesis" in MM, suggesting that tumor burden may be actually composed of a biologically heterogeneous cell population containing two subsets of cancer cells with different drug responsiveness, namely: i) terminally differentiated plasma cells with short doubling time and chemotherapy sensitivity; ii) quiescent stem cells with intrinsic drug resistance and the ability to self-renew that drive both disease relapse and progression. In this view, the development of strategies aimed at selectively eradicating the stem cell-like cell population offers a real perspective to achieve long-term remissions in MM. Thus, these novel strategies include: i) targeting specific pathways of CSCs, such as SHh pathway; ii) inhibition of the telomerase activity; and iii) induction of CSC differentiation (Tab. 1).

2.2.1 Targeting developmental signalling pathways

Given the exclusive role of SHh pathway in the pathobiology of MM CSCs, development of specific inhibitors of SHh signalling components, such as PTCH, SMO and GLI proteins, has

been strongly focused on by researchers over the past few years. Cyclopamine, a plant-derived alkaloid with natural antagonism against SMO, was the first anti-SHh drug successfully tested to inhibit growth of some lung, SNC and prostate cancer models, both in vitro and in vivo (Vestergaard et al., 2006; Bar et al., 2007; Mimeault et al., 2010). In line with the CSC theory, these studies describe the contribution of SHh to the initiation and expansion of aberrant cell subsets of poorly differentiated, clonogenic progenitors underlying renewal and growth of the respective tumor burdens. An interesting study, performed on both MM cell lines and patients' BM specimens, revealed the occurrence of MM tumor stem cells expressing higher levels of SHh pathway components than their more differentiated counterpart. In addition, this study showed that inhibition of SHh by cyclopamine selectively impairs the function of MM CSCs in terms of clonogenic potential, while enhancing their tendency to differentiate (Peacock et al., 2007). Thus, cyclopamine may provide a useful therapeutic approach in humans and may be primarily considered as a helpful tool to verify the CSC hypothesis in MM.

Therapeutic agent	Mechanism of action	Biologic effect	*References*
Cyclopamine	Hh pathway antagonism	Apoptosis	*Peacock et al., 2007*
GRN163L (Imetelstat)	Telomerase inhibition	Telomere shortening Cell cycle arrest Apoptosis	*Shammas et al., 2008* *Brennan et al., 2010*
Rosiglitazone Retinoic acid	PPARγ binding RAR binding	Cell cycle arrest Cell differentiation	*Huang et al., 2009*
Rituximab	CD20 targeting	Growth inhibition Apoptosis	*Matsui et al., 2008*

Abbreviations: Hh: Hedgehog; PPARγ: Peroxisome proliferator-activated receptor gamma; RAR: Retinoic acid receptor

Tab. 1. List of potential therapeutic agents targeting CSCs in MM

At present, the first trial aimed at defining the anti-cancer effects of SHh inhibition in humans included GDC-0449, a small-molecule inhibitor with a mechanism of action similar to cyclopamine, that showed a significant therapeutic effect in 55% of 33 patients with basal cell carcinoma (BCC) (Von Hoff et al., 2009). These data have been recently confirmed in subsequent studies indicating that the drug exerts a considerable though variable anti-tumor effect in patients bearing different metastasizing solid tumors such as BCC and medulloblastoma (Lorusso et al., 2011). The mutable therapeutic outcome of GDC-0449, however, may be ascribed to the lower clinical doses used with respect to in vitro preclinical studies, as well as to the high probability that both BCCs and medulloblastomas may hold mutations in their SHh pathway components (Gibson et al., 2010). Nevertheless, most human cancers, including MM, have been found to accumulate no SHh pathway mutations, thus providing the rationale for testing GDC-0449 in novel clinical studies. On the other hand, distinct agents targeting SMO or other components of the SHh signalling pathways,

such as the GLI transcription factors (Gould et al., 2011; Hyman et al., 2009), may provide alternative strategies to target CSCs in experimental tumor models, including MM (Fig. 5).

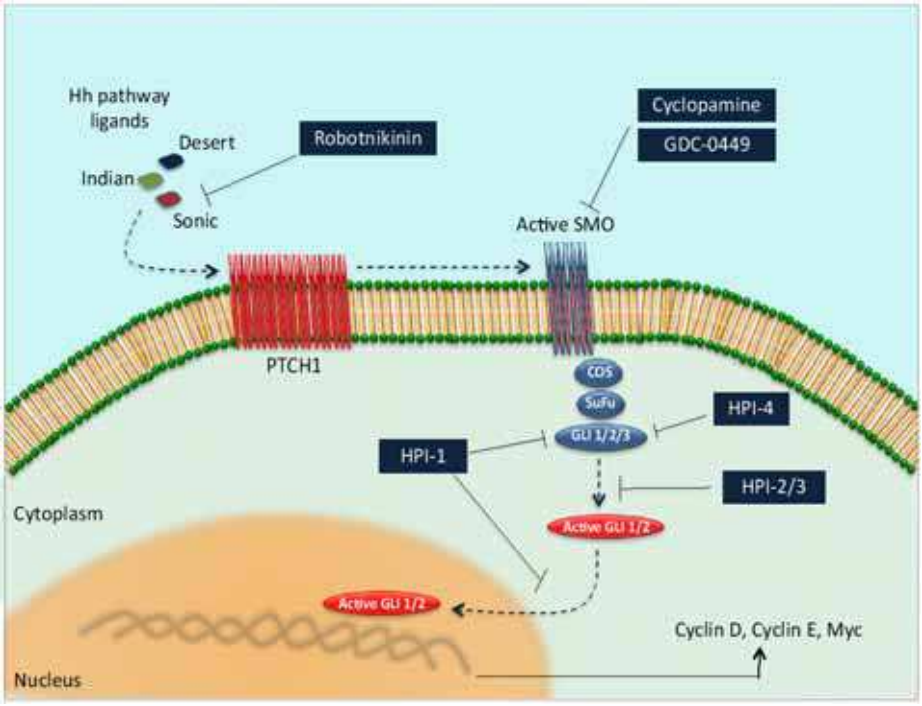

Fig. 5. Preclinical agents targeting Hedgehog signalling pathway

The first step of Hedgehog (Hh) pathway activation, that is the binding of Hh ligands (Sonic, Desert and Indian) to the receptor patched 1 (PTCH1), includes the activation of the transmembrane protein smoothened (SMO), which in turn enables the release from the SMO protein complex, including the Costal (COS) and Suppressor of fused (SuFu) proteins, of GLI 1 and GLI 2 proteins, which translocate to the nucleus where they activate the transcription of major cell cycle regulator genes (Cyclin D, Cyclin E, Myc). Novel agents inhibiting the Hh signalling pathway include natural compounds such as Cyclopamine which targets SMO, and other small synthetic molecules. Among these, Robotnikinin targets the extracellular Sonic ligand, GDC-0449 inhibits SMO activation, whereas the Hh Protein Inhibitors (HPI) 1-4 interfere with the SMO-dependent activation of GLI proteins or with their nuclear translocation.

2.2.2 Inhibition of CSC telomerase activity

Similarly to most human tumor cells (Kim et al., 1994), myeloma cells express high levels of telomerase activity (Wu et al., 2003) that correlate with both disease severity and prognosis (Shiratsuchi et al., 2002). Several in vitro and in vivo studies, in fact, demonstrate that the inhibition of telomerase activity in MM cells considerably affects their survival and growth (Shammas et al., 2004). In fact, the new-generation telomerase inhibitor GRN163L

(Imetelstat), used either alone or in combination with Hsp90 inhibitor 17AAG, exerts a powerful apoptotic effect on MM cells both in vitro and in a mouse model of MM (Shammas et al., 2008). Moreover, identification of high telomerase activity as a MM CSC requirement to self-renewal and clonal expansion may represent a new rationale for evaluating the effect of Imetelstat in this tumor. Interestingly, long-term treatment with Imetelstat induces progressive reduction of telomere length in MM CSCs, resulting in a consistent inhibition of their capacity to form colonies in vitro as well as of their engraftment into immunodeficient mice. On the other hand, shorter incubation of these cells with the drug dramatically impairs their clonogenic growth, although this effect is apparently not mediated by significant shortening of the telomere length, but by a differentiation-dependent mechanism (Brennan et al., 2010). These findings provide the experimental proof that telomerase inhibiting strategies are useful anti-CSC therapeutics in MM. However, further studies are necessary to elucidate the molecular mechanisms of the telomerase inhibitors in MM CSCs.

2.2.3 Induction of CSC differentiation

Emerging evidences support the perspective that induction of CSC differentiation may provide a valid alternative to induce stem cell clone exhaustion within tumors. In a variety of solid and hematologic cancers, in fact, treatment with molecules involved in the stem cell maturation have been shown to exert significant inhibition of tumor growth (Garg, 2009). For instance, retinoic acid is well known to induce a therapeutic "differentiation syndrome" in acute promyelocytic leukemia (Rogers & Yang, 2011), whereas bone morphogenetic proteins have been shown to exert similar effect on brain CSCs, thus restraining their tumorigenic potential (Chirasani et al., 2010). Also, certain chemical compounds have been experienced as anti-cancer therapeutics due to their pro-differentiation potential. The anti-inflammatory acetaminophen, for example, has been found to induce both morphologic and functional differentiation of breast CSCs, and to impair their tumor-initiating ability in vivo (Takehara et al., 2011).

Recent observation strengthens the correlation between the differentiation and anti-tumor activity exerted in vitro by molecules as rosiglitazone and retinoic acid in MM (Huang et al., 2009). These data, moreover, are conceptually in line with previous observations that terminal differentiation of MM cells, triggered by interferon-alpha in association with interleukin-6, results in both arrest at G1 cell cycle phase and impairment of their clonogenic proliferation (Matsui et al., 2003). Therefore, the CSC hypothesis further supports the rationale for clinical differentiation therapy in MM. Inhibition of MM CSC developmental pathways or telomerase activity by specific compounds results in a down-modulation of genes involved in maintaining CSCs in undifferentiated state (Peacock et al., 2007), although further studies are needed to elucidate the specific mechanisms of action of this therapy. On the other hand, the findings that gene expression in myeloma tumor cells is highly regulated by epigenetic modifications driving their proliferation, differentiation and survival (Sharma et al., 2010), have supported the development of new therapeutic strategies. For instance, histone deacetylase and DNA methylation inhibitors are under intensive investigation as potential tools to counterbalance epigenetic, rather than genetic, modifications in both cancer cells and CSCs (Kim et al., 2011; Zhang et al., 2010; Hagemann et al., 2011). Several in vitro and in vivo studies have achieved encouraging data regarding the usefulness of epigenetic therapies in MM (Niesvizky et al., 2011), although no experimental data so far have focused their potential in eradicating MM CSCs.

Novel therapeutic perspectives against CSCs have stemmed from the observation that certain types of cancer possess specific microRNA expression patterns, and that microRNAs (miRNAs) are crucially involved in the regulation of stem cell differentiation even by modulating *OCT4*, *NANOG* and *SOX2* coding sequences (Hatfield & Ruohola-Baker, 2008; Santarpia et al., 2009). In fact, the targeting of specific miRNAs has been shown to effectively antagonize growth of glioma CSCs (Moore & Zhang, 2010), whereas the therapeutic use of miRNA inhibiting specific cellular mRNAs such as Notch-1 and -2, resulted in glioma stem cell differentiation (Guessous et al., 2010). A recent study exploring the miRNA expression patterns in patients with MM, showed that selected miRNAs were specifically altered in patients as compared to healthy subjects and, in particular, miRNA-15a and -16 exerted interesting anti-MM activity both in vitro and in vivo (Roccaro et al., 2009).

2.2.4 Alternative molecular targets of MM CSCs

Additional cellular mechanisms may account for the drug resistance of MM CSCs, thus providing new potential therapeutic targets. Several processes, such as increased DNA-repairing capacity, alteration of cell cycle checkpoints, overexpression of ABC transporters and functional defects of apoptotic pathways, have been suspected to influence the CSC biology, although very little is known on their relative contribution to MM CSC biology and no data concerning their usefulness as therapeutic targets are currently available.

A special interest is presently being reserved for developing strategies to induce death by apoptosis in CSCs. Different natural human proteins involved in immunosurveillance have been shown to trigger apoptosis in a wide variety of tumor cell types (Abe et al., 2000) and, particularly, the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was demonstrated to promptly induce apoptosis in CSCs from a few solid tumors (Loebinger et al., 2010; Sussman et al., 2007). New experimental data, however, indicate that CSCs are mostly resistant to TRAIL-induced apoptosis as a consequence of their deregulated apoptotic machinery. TRAIL effectively kills differentiated myeloma cells (Labrinidis et al., 2009), but little is known concerning the sensitivity of MM CSCs to the apoptosis inducer. In this context, only a single report has shown that these cells display both minor levels of TRAIL receptors and TRAIL resistance in vitro. However, the combinatory treatment with Doxorubicin dramatically enhances their sensitivity to the ligand, resulting in complete and lasting eradication of tumor cells in a mouse model of MM (Vitovski et al., 2011).

On the other hand, recent data emphasize the central role of microenvironmental factors, such as stromal cytokines and chemokines, neovascularization and oxygen tensions, in ensuring survival, differentiation and chemoresistance of CSCs within their "niche" (Borovski et al., 2011). Therefore, targeting the major components of the "CSC niche", at both cellular and molecular level, is an attractive perspective to eradicate CSCs, with particular regard to MM whose development and progression are mediated by tight interactions between malignant cells and tumor microenvironment. As a matter of fact, MM CSCs isolated from both cell lines and patient BM samples have been shown to display high expression and functional activity of ABCG2 transporter and, interestingly, increase their viability and tumorigenic potential when co-cultured with bone marrow stromal cells (BMSC). In this single study, moreover, treatment with Lenalidomide exerted a significant reduction of both viability and clonogenicity of MM CSCs, and this effect was particularly evident in CSCs co-cultured with BMSCs. These data emphasize the role of stromal environment in the maintenance of MM CSC compartment and suggest that

immunomodulatory compounds disabling interactions between CSCs and their niche are potential therapeutic tools to eradicate CSCs in MM (Jakubikova et al., 2011).

3. Conclusion

New evidence emphasizes the hypothesis that circulating CBCs with clonogenic potential and properties similar to normal memory B cells are pathogenetically relevant in MM and may be considered tumor stem cells in this malignancy. To investigate this, based on the assumption that putative MM stem cells express markers of normal B cell development, including CD19 and CD20 molecules, two clinical trials have been aimed at evaluating the efficacy of the anti-CD20 monoclonal antibody Rituximab in MM patients, but, beyond the expected reduction of circulating B cells, no curative effect was observed, suggesting that this therapy fails to eradicate the self-renewing tumor cell fraction in MM (Treon et al., 2002; Zojer et al., 2006). Moreover, though the development of drug resistance in MM CSCs is favoured by the BM environment, which may account for the clinical uselessness of Rituximab (Basak et al., 2009), these preclinical results are in line with early studies suggesting that all clonal malignant plasma cells are highly capable of self-renewal in vitro as well as initiating MM growth in vivo (Yaccoby et al., 1998).

In addition, other experimental studies clearly deny the existence of clonotypic MM B cells as an effect of the absence of similar genetic alterations between B cells and tumor plasma cells in MM patients (McSweeney et al., 1996). Therefore, there is no definitive proof that these B cells compose the tumor-propagating compartment in MM and, similarly to other human cancers (Dalerba et al., 2007; O'Brien et al., 2007), several questions concerning their exact phenotype remain unanswered.

The discrepancies in the MM CSC phenotype include the excessive variability of cell isolation methods used by different research groups, whether based on positive or negative selections or on the derivation of tumor cells from BM or peripheral blood. In addition, both the clinical stage of individual patients and any previous treatments may also affect the biology of isolated MM cells and their tumorigenic precursors. Not only the discrepancies in defining the exact phenotype of MM CSCs, but also conflicting results on their functional properties have contributed to generate skepticism in the scientific community regarding the real existence of stem cells in this disease. The clonogenic potential of putative MM stem cells has been examined by a variety of both in vitro and in vivo assays, and different types of MM mouse models have been used to assess the self-renewing capacity of CSCs, thus resulting in potential discrepancies among experimental results from different research groups. Particularly in the in vivo experimental approaches, distinctly different modalities and sites of cell injection, as well as the different microenvironmental factors among the different MM animal models, may profoundly impact on the ability of specific cell types to home, survive, grow and self-renew in vivo, thus reflecting the inconsistency of the results. Therefore, improvements in discriminating both advantages and limitations of the specific assays used to assess the phenotype and the functional properties of MM CSCs are necessary, so that they can provide important knowledge in defining their actual role in MM biology.

At the same time, new clinical strategies aimed at achieving long-term outcomes in MM patients should be based on the current knowledge in the biology of CSCs, and one of the major challenges of clinicians and researchers involved in the field of MM CSCs will hopefully be the translation of the CSC theory into a clinical evaluation of the efficacy of

novel anti-MM therapeutics. In fact, the development of new agents targeting MM CSCs to be used in combination with both conventional and new-generation compounds represents a useful tool to test the CSC hypothesis in this tumor and obtain relevant results for many other types of cancer.

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Over the last thirty years, the foremost inspiration for research on metastasis, cancer recurrence, and increased resistance to chemo- and radiotherapy has been the notion of cancer stem cells.The twenty-eight chapters assembled in Cancer Stem Cells - The Cutting Edge summarize the work of cancer researchers and oncologists at leading universities and hospitals around the world on every aspect of cancer stem cells, from theory and models to specific applications (glioma), from laboratory research on signal pathways to clinical trials of bio-therapies using a host of devices, from solutions to laboratory problems to speculation on cancersâ€™ stem cellsâ€™ evolution. Cancer stem cells may or may not be a subset of slowly dividing cancer cells that both disseminate cancers and defy oncotoxic drugs and radiation directed at rapidly dividing bulk cancer cells, but research on cancer stem cells has paid dividends for cancer prevention, detection, targeted treatment, and improved prognosis.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821