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REVIEW

Identify multiple myeloma stem cells: Utopia?

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Abstract

Multiple myeloma (MM) is a hematologic malignancy of monoclonal plasma cells which remains incurable despite recent advances in therapies. The presence of cancer stem cells (CSCs) has been demonstrated in many solid and hematologic tumors, so the idea of CSCs has been proposed for MM, even if MM CSCs have not been define yet. The existence of myeloma CSCs with clonotypic B and clonotypic non B cells was postulated by many groups. This review aims to focus on these distinct clonotypic subpopulations and on their ability to develop and sustain MM. The bone marrow microenvironment provides to MM CSCs self-renewal, survival and drug resistance thanks to the presence of normal and cancer stem cell niches. The niches and CSCs interact each other through adhesion molecules and the interplay between ligands and receptors activate stemness signaling (Hedgehog, Wnt and Notch pathways). MM CSCs are also supposed to be responsible for drug resistance that happens in three steps from the initial cancer cell homing microenvironment-mediated to development of microenvironment-independent drug resistance. In this review, we will underline all these aspects of MM CSCs.

Key words: Bone marrow microenvironment; Cancer stem cells; Multiple myeloma; Stem cells niche; Stemness

Core tip: Multiple myeloma is a still incurable malignancy. Several study about multiple myeloma cancer stem cells showed their ability of self-renewal, survival and drug resistance. Besides, these cells are able to initiate and develop tumor when transferred into mice recipients. So understanding multiple myeloma cancer stem cells mechanisms become important to design new efficient targeting strategies for multiple myeloma. The aim of this review is to elucidate the state of art about multiple myeloma cancer stem cells and their critical role in maintenance of disease.

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MULTIPLE MYELOMA

Multiple myeloma (MM) is the second most common hematologic malignancy after non-Hodgkin lymphoma. It is characterized by uncontrolled proliferation of malignant plasma cells (PCs) that infiltrate in the bone marrow (BM), although small numbers of MM cells can be encountered in the peripheral blood circulation.

The first effect of MM is on the bone, but the blood and the kidneys are also involved. In bone marrow, malignant PCs induce damages in two ways: (1) proliferating cells form clusters disrupting the physiological structure of bone and causing osteolytic lesions; and (2) MM cells secrete not only cytokines and growth factors (GFs) that promote tumor progression and survival aging on MM cells themselves and on BM stromal cells (BMSCs) but also PCs secrete high amounts of monoclonal paraprotein (M protein) an abnormal immunoglobulins (Igs). Tumor cells interfere with hematopoiesis inducing a reduction in the number of white blood cells, condition known as leukopenia and increase the risk of infection. Moreover the decrease in red blood cells results in anemia, and low platelets level (thrombocytopenia) reduces normal blood clotting. Finally, the Bence Jones proteinuria, free light chain κ and, more important, λ, induces interstitial nephropathy and kidney failure because of their precipitation in distal tubules and collecting ducts[1].

MM is usually preceded by a pre-malignant stage termed monoclonal gammopathy of undetermined clinical significance (MGUS) which progresses to overt MM at a rate of 0.5% to 3% per year[2].

MGUS is characterized by a low number of PCs in BM and it isn't related to organ damages. MGUS has an increasing prevalence with age, affecting early 6% of over 60 years, but no treatment is indicated in these patients. Many studies evidence that patients with MGUS have a risk approximately 1% per year to develop to myeloma or to other related diseases[3].

Another stage is represented by smouldering multiple myeloma (SMM). In these patients, the tumour burden is higher than in MGUS; they have a higher risk of progression to symptomatic myeloma and they require therapy. The current care for SMM patients is the monitoring disease and in case of progression, the treatments are recommended[4].

MM it's still incurable despite the implementation of novel therapies and the great part of patients relapse even if initially they response to therapy. This is due to the presence of clonogenic cells inducing the so-called undetectable minimal residual disease (MRD)[5].

BONE MARROW MICROENVIRONMENT AND THEIR PRECURSOR CELLS

MM progression is strongly supported by bone marrow microenvironment. A complex and mutual interactions between PCs and BMSCs support tumor cells growth, migration, survival, differentiation, drug resistance and angiogenesis[6,7].

MM microenvironment is composed by extracellular matrix (ECM) proteins such as laminin, vitronectin, fibronectin, collagen, and by a large number of different stromal cells: fibroblasts, osteoblasts/osteoclasts, endothelial cells (ECs) and endothelial progenitor cells (EPCs), cells of immune system, hematopoietic and mesenchymal stem cells (HSCs and MSCs). During cancer progression, tumor cells are able to modify the surrounding stroma to build a promoting microenvironment from whose take advantages. The interplay between PCs and BMSCs is mediated by several cytokines, receptors and adhesion molecules[8].

Plasma cells

The majority of PCs is identified in the bone marrow and a critical role in B cells development is played by stromal cells. Indeed they get in touch and secret cytokines and growth factors needed to B cells maturation. The early stages of B cells proliferation depend on the interplay of PCs with stromal cells through vascular cell adhesion molecule-1 (VCAM-1) and thanks to growth factors such as interleukin-7 (IL-7), stem cell factor (SCF) and CXC chemokine ligand 12 (CXCL12)[9].

During the development of B cells, within BM, the B cell precursors became independent from interaction with microenvironment and from cytokines secreted by stromal cells, and in the final stage, immature B cells, located near to the central sinus, lose expression of CXC chemokine receptor-4 (CXCR4), CXCL12 receptor, and they are released from marrow. Therefore, entering in the central sinus, immature B cells migrate to the spleen, where they complete their development becoming naïve mature B cell. Then, they may pick up antigen within the tissue, alternatively, B cells may return to bloodstream and they continue to look for antigen activating themselves. The activation of B cells induces the differentiation into memory B cells, PCs or plasmablasts that will move to the BM.

First, plasmablasts and PCs to stromal cell and derived factors (*e.g.,* IL-6), for their survival, besides chemokines appear responsible for plasmablast entry into BM as well as PCs retention. PCs express CXCR4 on their surface and migrate towards CXCL12, produced by stromal cells and sinusoidal ECs in the BM, showing that PCs express membrane antigen required for their localization in the BM.

The most specific PCs surface marker is Syndecan-1 (CD138), that has been shown to bind fibronectin, collagen and basic fibroblast growth factor (b-FGF); PCs express the adhesion molecules CD44 and very late antigen-4 (VLA-4) the presence of several interplays between PCs and stroma.

Myeloma PCs express on their surface antigens such as $\alpha_v\beta_3$, VLA-4, LFA-1 MPC-1, CD54, CD56 which allow interaction with BMSCs and stimulate the production of IL-6, RANK ligand (RANKL), insulin-like growth factor, tumor necrosis factor alpha (TNFα), vascular endothelial growth factor (VEGF), stromal cell-derived factor (SDF-1) promoting their proliferation[10-12].

During maturation, PCs acquire specific lineage antigens and lose early markers, thus, BM PCs exhibit CD138, the survival factor Bcl-2, adhesion molecules such as VLA-4 and the chemokine receptor CXCR4, while they lose the B cell phenotype [CD19, CD20, CD22, human leukocyte antigen-DR (HLA-DR), Pax-5] and the death receptor CD95[13].

Cancer associated fibroblasts

Fibroblasts represent the main component of BM stroma. Several fibroblasts, activated by cytokines and growth factors circulating in the microenvironment such as FGF and transforming growth factor-beta (TGF-β), are called cancer associated fibroblasts (CAFs). Markers identifying CAFs are alpha smooth muscle actin (alpha SMA), fibroblast activation protein, fibroblast-specific protein-1, Thy-1, desmin, and S100A4 protein[14]. CAFs are able to promote cancer cell growth and to increase the invasiveness of cancer and stromal cells through cell-cell interactions, the production of pro-invasive cytokines, chemokines and inflammatory factors. This interplay between PCs and CAFs may serve as direction for cancer migration, breaking of the adjacent ECM and basement membrane, which represents the first step for cancer cells escape into the blood system[15,16]. Furthermore, CAFs act also in immune responses producing pro-inflammatory cytokines and chemokines which attract immune cells such as macrophages, neutrophils, and lymphocytes to cancer region[17].

Tumor-associated macrophages

Macrophages moved towards cancer bulk attracted by chemotactic factors and here they differentiate into tumor-associated macrophages (TAMs) favoring tumor progression[18]. Macrophages allow tumor cells to escape immune-surveillance creating a particular microenvironment characterized by chronic inflammation and immune tolerance allowing cancer to escape immune-surveillance[19]. Besides TAMs release a number of factors such as VEGF, hepatocyte growth factor (HGF), matrix metalloproteinase-2 (MMP-2), IL-8 which influence ECs behavior. Our group showed that BM macrophages in patients with active MM contribute to build neovessels through vasculogenic mimicry[20,21]. TAMs preserve their own CD14 and CD68 lineage markers, indicating that they do not trans-differentiate into ECs, but only adapt themselves functionally, phenotypically and morphologically to ECs, under VEGF and FGF-2 stimulation produced by PCs[22]; moreover macrophages secrete themselves VEGF and FGF-2, inducing BM angiogenesis[8]. Finally, BM monocytes and macrophages from patients with MM are able to form capillary-like structures through vasculogenesis *in vitro* and contribute to vasculogenic mimicry *in vivo*[23].

Osteoblasts and osteoclasts

In patients with MM the physiological balance between bone resorption and bone formation is often altered as results of a disregulation of osteoclast and osteoblast activity, resulting in the formation of osteolytic lesions, accompanied by bone pain[24]. Many factors are involved in osteoclast activation, including macrophage inflammatory protein-1α (MIP-1α), IL-3, and IL-6 and receptor activator of NF-κB ligand (RANKL)[25]. RANK is a transmembrane receptor expressed by osteoclast cells; the interaction of PCs with BMSCs induce an increase of RANK ligand (RANKL) expression which binds its receptor expressed by osteoclast precursor cells, promoting their differentiation by NF-κB and Jun-N-terminal kinase pathways[26]. A great contribution to bone destruction is also due to the inhibition of osteoclast apoptosis by RANKL and to the suppression of osteoblast activity. Besides osteoblasts are important in this mechanisms cause they are able to supports MM cells growth and survival thanks to the secretion of IL-6[27,28].

Endothelial cells and angiogenesis

Tumor ECs are more different from that of healthy vessels. They have an higher proliferation rate, in according to the enhanced angiogenesis that typically contributes to tumor progression. MM ECs highly express antigens such as VEGFR-2 and Tie/Tek, FVIII-RA, CD31 and VE-cadherin and their activity depends on growth factors circulating in BM microenvironment. They have an unusual shape and they are very permeable thanks to the presence of fenestrae, vesicles and a discontinuous basement membrane and they participate to the formation of new vessels with tumor cells capable to form mimic vessels. Angiogenesis plays a critical role in tumor progression important for tumor growth, invasion and metastasis not only in solid malignances but also in haematological malignances, such as MM. Angiogenesis is a multistep process which occurs in the switch from the avascular to the vascular phase in MGUS patients and leads to the transition to MM. Our group demonstrated an increase of angiogenesis in BM biopsies from patients with active MM compared with MGUS patients which suggest the passage from an avascular phase in MGUS or SMM to a vascular phase in active MM. The angiogenic switch is accompanied by mutations in PCs which acquire an angiogenic phenotype secreting growth factors (VEGF, FGF, HGF and others), and stimulating chemotaxis and proliferation of stromal cells[21].

EPCs and BM vasculogenesis

Vasculogenesis was first described as a phenomenon occurring in early embryogenesis, and was believed to not occur in adult tissues. In 1997, Moschetta *et al*[29] purified a population of circulating cells that showed characteristics typical of ECs as well as progenitor cells, and identified these cells as "endothelial progenitor cells" (EPCs). In humans, EPCs are identified by the expression of VEGFR-2, CD34, VE-cadherin, CXCR4, CD31, CD133, CD105, CD144, CD106, and CD117 (c-Kit). Mobilization of EPCs may occur in response to factors secreted by ischemic tissues and by inflammatory and tumor cells and it results in the generation of new vessels. When EPCs are recruited to tumor sites, they differentiate in mature ECs, sustain neovessel formation *via* paracrine secretion of

proangiogenic growth factors and they integrate into the nascent vessels which are a mosaic of ECs, EPCs, tumor cells and macrophages[29].

Many studies indicate that BM-derived circulating EPCs can take part to tumor angiogenesis and to sustain tumor cells proliferation. A great amount of EPCs have been found in the BM of patients with active MM compared with treated MM, MGUS, or healthy people, highlighting the increased angiogenic activity in MM patients. Besides, Ria *et al*[30] showed for the first time that EPCs had phenotypic and functional characteristics of the mature endothelium. In the MM BM microenvironment, they postulated that PCs and inflammatory cells recruit EPCs into tumor site, they induce their differentiation into ECs and they contribute directly in the formation of new vessels thus contributing to tumor vasculature.

STEM CELLS IN MULTIPLE MYELOMA

The implication of B cells in the pathogenesis of MM has been investigated by many groups because normal and myeloma PCs arise from their differentiation. The rearrangement of immunoglobulin gene and their resulting antibody allows to understand the different relationships between different clones in B cell tumors. Sequencing of immunoglobulin genes of MM PCs has underlined the presence of somatic hypermutation without intraclonal variation suggesting that MM arises from a post germinal center B cell compartment[31]. Already in many tumors it has been shown the existence of cancer stem cells (CSCs) or cancer-initiating cells[32-34]. While CSCs markers differ from one to another, their peculiar characteristics are common, such as self-renewal, tumorigenesis and drug resistance. Therefore, these stemness abilities are useful for identifying the MM stem cells. The idea of CSCs model bases on the concept that cancers are similar to hematopoietic system with an asymmetric division where CSCs should maintain cancer cells population.

The possible existence of MM CSCs was first postulated by Drewinko *et al*[35] that demonstrated the presence of a small population of MM cells with the capability of self-renewal in experiments with MM cell lines and primary cell lines from patients with MM. Then, Hamburger *et al*[36] and Pilarski *et al*[37] showed, respectively *in vitro* and *in vivo*, the capacity for self-renewal of MM primary lines. Finally, the onset of relapse in some patients after treatment gives the idea that, maybe, MM CSCs really exist.

Many studies underline the presence of different subpopulations able to give arise to MM: clonotypic B cell, clonotypic non B cell and side population (SP) cell.

Clonotypic B cells

MM cells are more functionally and phenotypically heterogeneous population. Within the tumor it has been identified a mature normal cell population and a minor one which is able to form tumor after transplantation into susceptible recipients in contrast to the first one. Matsui *et al*[38] demonstrated that clonotypic B cells are capable to give rise to monoclonal immunoglobulin-secreting PCs *in vitro*. In particular, $CD19^+$ B cells, isolated from MM patients, showed the capacity to form a new tumor in xenograft models implying the existence of cells with self-renewal ability. Moreover they found that $CD138^-CD34^-$ cells were able to form colonies *in vitro,* like $CD138^+$ PCs *in vivo*, which present the same intracellular immunoglobulin light-chain restriction as MM patients.

Normal B cells maintain long-term immunologic memory thanks to the ability for self-renewal as well as the clonotypic B cells of MM lead to belief that myeloma PCs may arise from this compartment. Indeed Matsui *et al*[39] studying the $CD138^-$ clonogenic myeloma cells, found that only cells which co-express CD19 and CD27 cell membrane antigen, typical of memory B cells, were able to form colony *in vitro*. They also reported that $CD19^+CD27^+CD138^-$ cells isolated by peripheral blood of MM patients engrafted NOD/SCID (Non-obese diabetic/severe combined immunodeficiency) mice and gave rise to mature $CD138^+$ MM PCs secreting M protein. $CD19^+$ B cells isolated by these engrafted mice were able to induce MM in other recipient mice, underlining their self-renewal potential. These results mark that MM-initiating cells with tumorigenic ability, localized in clonotypic post-germinal B center, may differentiate and build up again the bulk of MM cells. Moreover, Boucher *et al*[40] showed that $CD34^+CD19^+$ immature B cells and $CD34^-CD19^+$ mature B cells, but not $CD34^+CD19^-$ cells, harvested from MM patients form colonies, suggesting that undifferentiated clonotypic B cell may present MM-initiating ability.

More recently, Kirshner *et al*[41] evaluated a novel *in vitro* 3D stromal culture system to study typical properties of BM microenvironment, in which results that tumor growth derived from clonotypic B cells. Pilarski *et al*[37] demonstrated that cells from the peripheral blood of patients with late-stage of MM or from patients with minimal residual disease, or cells mobilized through G-CSF, engrafted NOD/SCID mice. Engrafted mice presented high levels of circulating M protein and bone lesions as in patients with myeloma; besides these tumor cells could be transplanted successfully into secondary recipients indicating self-renewal ability. Indeed, Chaidos *et al*[42] found that the amount of circulating clonotypic B cells correlates with disease progression.

Clonotypic B cells play an important role in MM disease because they are also detected in MM patients with complete remission, becoming potential source for MM-initiating cells which could relapse.

Clonotypic non B cells plasma cells

Although all the evidences that clonotypic B cells could be MM CSCs, many studies demonstrate the clonogenic potential of non-B cell plasma cell population in MM.

First experiments demonstrating clonogenic ability of non-B cells was realized by Yaccoby *et al*[43]. They successfully induced human MM disease by intraosseous transplantation of $CD38^{++}CD45^-$ human cells in SCID mice implanted with rabbit femurs (SCID-rab mice)

or with human fetal bone fragments in SCID-hu mice creating a humanized microenvironment[44]. In these models, the rabbit or human implanted bone fragments promote MM growth within the bone with several clinical aspects of MM including lytic bone lesions, hypercalcemia and circulating M protein. While, in the same work, Yaccoby *et al*[43] demonstrated that $CD38^{-}CD45^{+}$ peripheral blood B cells weren't able to engraft into SCID-hu mice as well as $CD19^{+}$ B cells did not allow the xenograft in SCID-rab mice. But PCs regained from SCID-hu models were successfully transferred to secondary and tertiary recipients to produce MM disease with the clinical symptoms. In contrast, plasma cell-depleted BM cells did not induce MM disease in these models.

Hosen *et al*[45] studied the clonogenic MM plasma cells in terms of CD138 negativity in SCID-rab mice. Phenotypic $CD138^{-}CD19^{-}CD38^{++}$ PCs isolated from MM patients were engrafted in SCID-rab mice developing MM disease; moreover $CD138^{+}$ PCs from patients were also able to induce MM in mice, although more slowly than $CD138^{-}$ cells.

Recent studies carried out by Kim *et al*[46] tested the clonogenic potential of plasma cells in relation with BM microenvironment using NOD/SCID/common cytokine receptor γ chain-deficient (NSG) and recombinase-activating gene 2/common cytokine receptor γ chain-deficient (RAG2-/-) mice. The results of this work underlined the ability of only differentiated $CD138^{+}CD38^{+}$ cells, risen from patients, to repopulate of B lineage cells in human bone-bearing mice but no engraftments were detected in human bone-free mice. Besides, serial transfer of the disease to secondary recipients were possible. In these models, completely differentiated MM PCs enriched MM-initiating cells contrary to B cells. All these data showed that MM PCs can induce MM *in vivo* even in the absence of $CD19^{+}$ B cells.

Finally, Paino *et al*[47] evaluated the presence and function of $CD20^{+}$ putative MM stem cells population in several MM cell lines. In the RPMI8226 cell line they found a small subpopulation of $CD20^{dim+}$ that was not essential for CD17-SCID mice engraftment. Moreover, $CD20^{dim+}$ cells didn't differentiate into $CD20^{-}$ cells, even if $CD20^{-}$ cells can differentiate into $CD20^{dim+}$ cells, suggesting a sequential differentiation order. All these outcomes showed that CD20 isn't a marker related to MM CSCs.

SP population

Moreover, the SP phenotype is characteristic of stem cells in various normal tissues as described by Challen *et al*[48] SP cells show a strong ABC (ATP-binding cassette) transporter activity resulting in high ability to efflux dyes as Hoechst 33342, a substrate for the ABC transporter ABCG2, also known as breast cancer resistance protein 1. SP cells have characteristics of stem cells such as the ability of self-renewal, expression of stem cell-like genes and resistance to chemio- and radio-therapy.

Loh *et al*[49] detected SP cells in both MM cell lines and primary MM cells. Also Jakubikova *et al*[50] studied SP fraction in the same lines too. Their research showed that SP population is mainly presented in both $CD138^{+}$ and $CD138^{low}$ without any correlation between the lack of CD138 expression and amount of this fraction. Indeed they demonstrated that SP is composed of cells highly proliferating and with tumorigenic ability. Besides, they also showed that SP cells were susceptible to lenalidomide treatment in a dose- and time-dependent manner, in contrast, other authors displayed that lenalidomide results ineffective against other possible MM CSCs subpopulations (clonotypic $CD19^{+}$ cells)[39].

Interestingly, IMIDs reduced SP cells rate in co-culture with BMSCs, invalidating interplay between CSCs and the BM microenvironment; even if they did not affect ABC transporter function[51].

Bone marrow stem cells

Bone marrow is a source of different tissue-specific stem cells, in fact BM hosts Bone marrow hematopoietic stem cells (BM-HSCs) and Bone marrow mesenchymal stem cells (BM-MSCs). Probably in the BM a universal adult progenitor exists and its phenotype could be modified by the local environment. Stem cells may move from BM to another tissue through the blood circulation and can differentiate into various types of stromal cells[52]. A large part of BMSCs is composed by HSCs, important for the homeostasis of blood system, giving rise to all blood cells (lymphocytes, erythrocytes, monocytes, granulocytes and platelets). The distribution of HSCs in the BM is well-organized: the majority of HSC are located within the endosteal region, while progenitors and mature cells are principally sited in the central marrow area in proximity to the central marrow vessels[53,54]. Most HSC express the CD34 antigen, an integral membrane glycoprotein that functions as a regulator of hematopoietic cell adhesion to BMSCs[55]. Antigens such as CD90, CD117, and CD133 are also expressed by HSCs[56]; moreover they express CD90, CD117, and CD133[56] but not CD38, CD45RA, CD71, HLA-DR, or any other lineage-specific antigen according to their immaturity[57]. Following injury HSCs are mobilized from the BM niche and they start proliferating to supply new mature cells.

The BM mesenchymal stroma is essential for the normal functioning of HSCs, ensuring their renewal and differentiation, creating an ideal microenvironment and contributing to the formation of the HSC niche[58]. The first evidence of MSCs was reported by Cohnheim on "mesenchymal precursor cells" defining those cells as fibroblastoid, adherent, and extravasated cells at sites of tissue injury. In the early 1990s, these cells began to be known as "mesenchymal stem cells", cause they exhibit multipotential differentiation and self-renewal capacity[59]. MSCs isolated from the adult BM have the ability to differentiate into osteoblasts, adipocytes, chondrocytes *in vitro* and to heterotopic osseous tissue when transplanted *in vivo*. MSCs express a large variety of surface markers including CD29, CD44, CD49a-f, CD51,

CD73, CD105, CD106, CD166 and they don't express typical hematopoietic lineage receptors such as CD11b, CD14, CD45[60]. Wallace *et al*[61] compared BM-MSCs from patients with myeloma at diagnosis and normal donors to further examine the role of the bone marrow stroma in myeloma. They established that BM-MSCs from myeloma showed the same expression of adhesion molecules and of integrin, such as VLA-2, VLA-4, VLA-5, β1, L-selectin, and CD44 and were negative for VLA-2, β2, and β3; however they had a weaker expression of VCAM-1 and fibronectin compared with normal BMSCs. Adhesion and migration cell abilities depend on the expression of adhesion receptors and on the type and concentration of ECM proteins. BM-MSCs low expression of cellular fibronectin, may cause localization and retention of malignant PCs in the BM because PCs lines show to move on fbronectin. Besides BM-MSCs express an intracellular RHAMM, a receptor for hyaluronan-mediated motility which induces the migration of several kinds of cells such as smooth muscle cells, fibroblasts, neuronal cells, and leukocytes. RHAMM activation results in a transduction signal which leads to cellular mitosis. Finally this study showed an over-expression of cytokines and growth factors by BM-MSCs, including IL-6 critical for PCs proliferation, survival and resistance. The analysis of mesenchymal cells in myeloma underlines the importance of BMSCs in myeloma cell growth and progression.

STEM CELL NICHE

Stem cells are located in a specific microenvironment defned niche. The niche and stem cells interact each other through adhesion molecules that activate molecular signals able to ensure stemness. For the frst time, the idea of a stem cell niche was suggested by R. Schofeld in 1978 for the HSC in BM[62]. Schofeld called "stem cell niche" the cellular environment, retaining stem cells. Until the stem cell restrains in the niche, its differentiation is avoided and so they replicate indefinitely as stem cell. Many groups investigate several types of stem cells and respective niches.

Osteoblastic niche

In human, hematopoiesis occurs in BM, where microenvironment creates optimal conditions for the HSCs maintenance and differentiation. Quiescent HSCs have been recognized near to the endosteal surface of the BM, in the trabecular bone, forming a source of HSC that can induce hematopoiesis after tissue damage. Lo Celso *et al*[63] showed that after transplantation, HSCs were localized closest to the endosteum and that as differentiation progressed, they moved to different locations[63]. There are many pathways and molecules involved in the maintenance of stemness and stem niche. HSCs and osteoblasts interact through adhesion molecules, as N-cadherin (N-cdh) that is considered the most important cell-cell adhesion molecule expressed by many cancer cells. Vandyke *et al*[64] showed that expression of N-cadherin in primary MM cell lines correlates with poor prognosis; in fact it has been demonstrated that N-cdh regulates HSCs proliferation[64]. Sadler *et al*[65] proved that, inhibiting N-cadherin through a neutralizing antibody, the interaction between MM cells and stroma is prevented and it is induced the proliferation of HSCs compartment. Thus, stem cells are quiescent (G_0 stage) and they are not going to enter into the cell cycle until they received stimulations. Arai *et al*[66] found that osteoblasts express angiopoietin-1 (Ang-1) that binds Tie2 (tyrosine kinase receptor) expressed by BM-HSCs. This bond induces the activation of β1-integrin and N-cadherin contributing to the close interaction between osteoblasts and stem cells necessary to ensure the maintenance of the stem cell and self-renewal capability.

The niche size is regulated by bone morphogenetic protein (BMP) signaling through its binding to osteoblasts receptor type IA (BMPRIA)[67]; besides the stimulation by PTH (parathyroid hormone)/PTHrP(parathyroid hormone-related peptide) receptors produces high levels of Jagged 1, a Notch ligand, which recruit more HSCs in the niche. A study highlight Osteopontin (Opn) as another important osteoblasts receptor; in fact in these work knock-out mice showed that transplanted HSCs were randomly distributed within BM, whereas HSCs should be usually located at the endosteal region. This data suggests that Opn is involved in the interplay between HSCs and osteoblastic niche and it decreases HSCs proliferation[68]. Furthermore, c-Myc is another regulator of HSCs fate: c-Myc deficient mice have a higher amount of HSCs in BM and their differentiation was maintained by interactions mediated by adhesion molecules between HSC and the niche, such as N-cadherin and integrins. Contrary, overexpression of c-Myc induces HSCs differentiation[69]. Also ECM and its components play an important role in osteoblastic niche. Glycosaminoglycan hyaluronic acid (HA), is the major component of the ECM and its receptor, CD44, is a pleotropic transmembrane protein presented by several cell types, including the HSCs and progenitors. HA is important in supporting and regulating hematopoiesis.

Osteoblasts are sited in endosteal area where there is a high calcium concentration, so Adams *et al*[70] supposed that on HSCs a sensor for calcium exists and it contributes to their homing and localization in the BM. They demonstrated this in CaR-deficient mice, showing that even if they had a healthy amount of primitive HSCs in the circulation and in spleen, they had very few of these cells in the BM[70].

Vascular niche

Recently, vascular niche, was found within the sinusoidal vessels in BM or spleen. The presence of a vascular niche is confirmed by HSCs migration to the vascular region of the BM after injury, restoring hematopoiesis. So the vascular niche encourages proliferation, differentiation and the release in the bloodstream of HSCs[71]. HSCs were identifed as $CD150^{+}CD244^{-}CD48^{-}$ cells, and many of them were in sinusoidal endothelium[72], near to cells that express high level of CXCL12[73]. Vascular niche is

also important not only in maintain HSCs but also in the regulation of the various phases of hematopoietic processes within BM, such as mobilization, migration, and differentiation of HSCs. So the discover of the vascular niche make the attention on its crucial role in blood system and hematopoiesis.

Kopp *et al*[74] showed that the only translocation of megakaryocyte progenitors near vascular sinusoids of BM was able to induce megakaryocyte maturation and platelet production. This process dependes on chemokines such as SDF-1 and bFGF that enhance expression of adhesion molecules, as VLA-4 on megakaryocytes and VCAM-1 on BMECs. Besides, the breakdown of VE-cadherin-mediated adhesion BMEC makes in vascular niche incapable to induce megakaryocyte differentiation and to release platelet in peripheral blood. This focuses on the importance of ECs structural integrity for HSCs differentiation and homing. BM-ECs of sinusoidal vessels represent a barrier between BM stroma and the peripheral blood circulation, suggesting that they are implied not only in hematopoiesis but also in stem cells mobilization and homing[74].

The different functions of osteoblastic and vascular niches aren't yet known. A difference between the two niches is the oxygen level: vascular niche presents a higher oxygen level than osteoblastic niche. In this conditions, cells resume their cell cycle, undergoing mitosis[75].

Furthermore, a new model of hematopoiesis is proposed: in osteoblastic niche, in hypoxic environment, the HSCs are in G_0 stage. Under stimulation of cytokine such as G-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-7, IL-3, IL-12, SCF, and ft-3 ligand and chemokines like IL-8, Mip-1 and SDF-1, HSCs could move to the vascular niche, undergoing differentiation and supplying cells of bloodstreams[76]. When no longer HSCs needed, they could return from the vascular niche to the osteoblastic one, where they revert to the G_0 stage again. The movement of the HSCs between osteoblastic and vascular niches appears to be necessary for well-balanced hematopoiesis[77].

Cancer stem cell niche

It has been proposed that as well normal stem cells niche exists, as CSCs niche could exist and interplay with the cancer niche may have a similar role in differentiation, proliferation and self-renewal capability of tumor cells. The interaction between the niche and CSCs prevents their differentiation in response to chemokines and growth factors as happens for HSCs in the osteoblastic niche. Increasing evidence proved that factors secreted by the cancer microenvironment regulate cancer cells. The niche is able to anchor stem cells in their appropriate microenvironment through adhesion molecules, such as cadherin and β-catenin. In addition, recent data support the role of the vascular niche in initiating metastasis; MMPs family molecules are involved in the process of cancer migration and also integrins have been reported to be associated with tumor cell homing and mobilization in which SDF1 and CXCR4 play an essential roles. For metastatic process, tumor cells must reduce cell-cell contacts and migrate to distant sites. Thus, these molecules, involved in stem cells mobilization from the niche, represent possible targets to block tumorigenesis, cancer progression and metastasis[78]. Evidences showed that factors derived from tumor stroma niche are able to regulate cancer cells and to direct their diffusion, in fact genetic studies have shown that stromal cells are altered in many tumors and supports cancer progression. Specialized microenvironments of BM-ECs are needed for the homing and engraftment of both normal HSCs and cancer cells[79]. There are several possible hypothesis for CSCs niche: (1) CSCs may not require specifc niche for survival and are able to survive in the healthy stem cell niche; (2) a distinct CSCs niche may be necessary for their activation and CSCs may dependent on this tumor-niche for expansion; or (3) an inhibitory niche for CSCs could exists which provides factors that induce differentiation or death; (4) CSCs may provide signals that activate an otherwise quiescent niche, thus signals from the CSCs could result in amplifcation of an activated niche that already exists; and (5) CSCs may be niche independent and they may have acquired the ability to provide themselves with the necessary factors for expansion and self-renewal[80].

PATHWAYS IN MM STEM CELLS

Many signaling that preserve physiological stem cells, are also involved in CSCs maintaince and self-renew. Three pathways results most activated in cancer stem cells: Hedgehog (Hh), Wnt and Notch.

Hedgehog signaling plays a crucial role during embryonic development, and it regulates cell proliferation, migration and differentiation. Hh signal transduction involves three ligands *i.e.*, Sonic (SHh), Indian (IHh) and Desert (DHh) which bind the cell surface antigen Patched (PTCH). Hh pathway normally inhibits the Protein Smoothened SMO, a trans-membrane receptor with a high homology with coupled G protein. When ligands bind PTCH, SMO is de-repressed and regulates the activity of 3 GLI proteins which act as transcriptional regulators. In particular, GLI1 induces the expression of cell cycle regulator cyclin D1, inducing mitosis; GLI2 can act as positive or negative transcriptional regulator depending on post-trascriptional and translational modifcation; whereas GLI3 is a negative downstream effector of Hh and down-regulates genes transcription. Altered Hh signaling has been found in many human tumors. In myeloma, *PTCH, SMO* and *GLI1* were over-expressed in both human cell lines and primary MM PCs compared to normal PCs and B cells[81].

Wnt pathway is an ancient and conserved signaling which regulates cell fate determination, migration and differentiation. In MM Wnt pathway is activated by the interplay of BM microenvironment. Wnt stimulation by bond with Frizzled (Fz) activates several intra-cellular pathways, including the canonical Wnt/β-catenin way and the non-canonical Wnt/Ca^{2+} one. Komiya *et al*[82]

showed that genetic modifications of Wnt signaling induce alterations in development and function of several organs, in particular, down-regulation of Wnt pathway supports proliferation of both MM cell lines and primary patient samples. Finally, the inhibition of Wnt pathway by small molecule inhibitor disruptes the maintenance of MM cells both *in vitro* and *in vivo*. This result encourages the development of Wnt-targeted inhibitors for MM therapy[82,83].

Notch signaling is an evolutionary pathway highly conserved both in vertebrates and in invertebrates. Notch pathway is involved in various neoplastic processes such as tumor angiogenesis, EMT, metastasis.

Mammals express four Notch transmembrane receptors (Notch-1, -2, -3, -4) and five ligands named Jagged1, Jagged2, Delta-like 1 (DLL1), DLL3, DLL4 which mediate Notch activation (cell to cell) in trans and inhibition (on the same cell) in cis. Activation of Notch receptors consists in three proteolytic cleavage which result in the formation of "notch transcriptional complex" (NTC) inducing the expression of many genes related to differentiation and survival, including hairy/enhancer of split (HES), the family of helix-loop-helix transcription factors, cyclin D, c-Myc[84]. In MM, Notch activation advances cell proliferation and MM progression. Agarwal *et al*[81] searched the presence of Notch on BM clonotypic B cells from MM patients and found great expression of Notch that underlines Notch signaling implication in MMSCs.

DRUG RESISTANCE

Drug resistance remains an important complication to the cure of most cancers. During therapies cancer bulk initially response, but, over time, cancer stem cells may become drug resistant, thus therapies fail to eradicate them. So CSCs have not only the classic capacities of self-renewal and proliferation, but also are more resistant to chemo- and radiotherapy[85]. But, in MM patients, drug resistance mechanisms that could arise during treatments, not explain completely MRD onset, though the BM microenvironment present components that can induce drug resistance and could reduce drug activity such as cytokines, stromal cells and ECM compounds.

Drug resistance development consists of three phases: (1) Cancer cells homing to the protective microenvironment represented by BM; (2) Initial microenvironment-mediated drug resistance; and (3) Development of microenvironment-independent resistance and acquired drug resistance.

Cancer cells homing to the protective microenvironment represented by BM

Cytokines and chemokines are more important in hematopoietic cells homing within BM. The major axis is the CXCR4/SDF-1 one, that promotes myeloma cells migration and homing to BM *in vivo* and *in vitro*[86]. SDF-1 or CXCL12 is constitutively expressed by BMSCs and it is the main source of chemokines in adult. It retain hematopoietic cells and progenitors for growth and differentiation and impounds mature B cells to BM. CXCR4 (CD184) is a G-coupled cell surface receptor and can be expressed by normal and malignant cells. The bond CXCR4/SDF-1 mediates cell survival, adhesion and migration contributing to tumor progression in several cancers. Moreover VLA-4-mediated adhesion to ECM (in particular to fibronectin and collagen) enhances the drug resistance in MM[87]. The inhibition of this pathway not only blocks tumor homing and engraftment, but also avoids cell adhesion-mediated drug resistance and cells retain in BM decreasing VLA-4 expression.

Initial microenvironment-mediated drug resistance

In this phase, tumor cells acquire a soluble factor- and/or cell adhesion-mediated drug resistance (CAM-DR) *i.e., de novo* drug resistance. The BM collects GFs and cytokines necessary to maintain cellular homeostasis. Among these GFs, IL-6 is essential to MM. The binding to its receptor on target cells induces STAT3 signaling that inactivates Fas-mediated apoptosis by up-regulation of Bcl-Xl with anti-apoptotic effect. Besides Frassanito *et al*[88] supported that myeloma clones secreting IL-6 were more resistant to both spontaneous and drug-induced apoptosis than non-IL-6 secreting clones that were sensible. Furthermore Voorhees *et al*[89] showed that blockage of IL-6 signaling in MM cell lines increases their sensibility to bortezomib.

Interaction between tumor cells and BMSCs is more complex than adhesion of integrin to ECM compounds alone because it involves other pathways and signaling events activated by adhesion molecules. In drug resistance development, cell-cell and cell-ECM interacts are more important and integrin-mediated adhesion to ECM compounds and stromal cells induces pathways that regulate proliferation, migration, and survival of normal hematopoietic cells. But integrins are also important in the tumorigenesis and integrin expression patterns are altered in tumor cells.

The role of VLA-4 has been investigated by several groups in mediating *de novo* drug resistance in hematopoietic malignancies. In these cases, CAM-DR is leaded by the interaction of cancer cells to ECM components and/or BMSCs *via* integrin $\alpha4\beta1$. Damiano *et al*[6] demonstrated that drug resistant MM cells up-regulate some integrins as $\alpha4$, $\beta1$ and $\beta7$ while, when drug-sensible myeloma cells are seeded on fbronectin, a reversible *de novo* drug resistance phenotype was observed.

Thus, CAM-DR is characterized by non-transcriptional mechanisms into drug resistant cells. Adhesion-mediated survival and drug resistance pathways induced by soluble factors contribute to MRD allowing the development of more complex drug resistance mechanisms caused by the selective pressure of chemotherapy. Also the study of *de novo* drug resistance mechanisms from CAM-DR explains how tumor microenvironment promotes drug resistance onset.

Development of microenvironment-independent resistance and acquired drug resistance

Into BM microenvironment, cancer cells can survive to chemotherapies activity, resulting in MRD. In particular,

under continuous pressure of chemo-treatments, tumor cells acquire intrinsic genetic and epigenetic changes that lead to drug resistance phenotype without extracellular stimuli as soluble factor- and/or cell adhesion-mediated drug resistance. Furthermore, acquired drug resistance is mediated by intrinsic changes at the transcriptional level while *de novo* drug resistance is mediated by post-transcriptional mechanisms[90]. A possible solution to destroy MM CSCs is targeting self-renewal and drug resistance signals specifically activated in CSCs: Hedgehog (HH), Wnt, and Notch pathways[91,92].

For example, Nefedova *et al*[93] showed Notch1 receptors expression on MM cell lines that stimulates their adhesion to BMSCs, which express the membrane Notch ligand Jagged. The binding induces up-regulation of $p21^{Cip1/WAF1}$ that encourages growth inhibition and protection from drug-induced apoptosis.

Indeed, the existence of a drug resistant sub-clone, which may be composted by CSCs and may expand during therapy, may represent the reason of tumor treatments fail[94]. Most important, Peacock *et al*[95] affirmed that overexpression of RARα2 provided MM CSCs ($CD138^-$ MM cells) drug resistance by activation ABCC3 gene through stem cell related pathways Hh (hedgehog) and Wnt. In effect, MM CSCs express also some functional markers such as drug efflux pumps (ABCC3), ALDH1 and RARα2 which have been associated with clonogenic potential and resistance to chemotherapy contributing to drug resistance and relapse in MM patients[39].

Moreover, MM CSCs also express a telomerase which has a fundamental role in controlling normal stem cell functions and cancer drug resistance. Inhibitors of telomerase activity block MM CSCs clonogenic potential *in vitro* and *in vivo*, triggering differentiation of $CD138^-$ cells into $CD138^+$ cells and decreasing the number of $ALDH1^+$ cells[96].

CONCLUSION

Taken all together, these above studies provide strong evidence for existence of well-defined stem/progenitor cells possessing the three prominent features common to CSCs in all cancers: self-renewal, proliferation and drug resistance[97].

Because MM remains still incurable, the idea of the MM CSCs has been suggested to explain the ability of self-renewal, survival and drug resistance of some myeloma cells. To evaluate this hypothesis, several groups use clonotypic B and non B cells and SP cells and transfer them into mice models with opposite results due to the different ability of BM microenvironment to sustain their survival inducing MM. Interestingly, there are many resemblances between the HSCs and MM CSCs about extracellular and intracellular receptors and signalings which can be employed to improve targeted therapy. So stemness pathways such as Hedgehog, Wnt and Notch, constitutively activated by the interaction between MM CSCs and their niche could represent new potential targets for MM treatment. The comprehension of interactions between MM CSCs and the BM microenvironment will enable us to establish the necessary reasons for MM CSCs maintenance and avoidance from therapies inducing drug resistance[98].

However, despite the introduction of novel therapy against MM, it still remains incurable. This shows the necessity to further elucidate causes of MM onset and implement innovative strategies in order to improve patient outcomes and reduce the rate of relapse.

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