

Multiple myeloma mesenchymal stromal cells: Contribution to myeloma bone disease and therapeutics

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which clonal plasma cells proliferate and accumulate within the bone marrow. The presence of osteolytic lesions due to increased osteoclast (OC) activity and suppressed osteoblast (OB) function is characteristic of the disease. The bone marrow mesenchymal stromal cells (MSCs) play a critical role in multiple myeloma pathophysiology, greatly promoting the growth, survival, drug resistance and migration of myeloma cells. Here, we specifically discuss on the relative contribution of MSCs to the pathophysiology of osteolytic lesions in light of the current knowledge of the biology of myeloma bone disease (MBD), together with the reported genomic, functional and gene expression differences between MSCs derived from myeloma patients (pMSCs) and their healthy counterparts (dMSCs). Being MSCs the progenitors of OBs, pMSCs primarily contribute to the pathogenesis of MBD because of their reduced osteogenic potential consequence of multiple OB inhibitory factors and direct interactions with myeloma cells in the bone marrow. Importantly, pMSCs also readily contribute to MBD by promoting OC formation and activity at various levels (*i.e.*, increasing RANKL to OPG expression, augmenting secretion of activin A, uncoupling ephrinB2-EphB4 signaling, and through augmented production of Wnt5a), thus further contributing to OB/OC uncoupling in osteolytic lesions. In this review, we also look over main signaling pathways involved in the osteogenic differentiation of MSCs and/or OB activity, highlighting amenable therapeutic targets; in parallel, the reported activity of bone-anabolic agents (at preclinical or clinical stage) targeting those signaling pathways is commented.

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Key words: Mesenchymal stromal cells; Multiple myeloma; Osteolytic lesions; Myeloma bone disease; Bone-directed therapy; Bone-anabolic drugs

Core tip: In multiple myeloma, bone marrow mesenchymal stromal cells (MSCs) primarily contribute to associated osteolytic lesions because of their defective differentiation to mature osteoblasts. Importantly,

Abstract

Multiple myeloma is a hematological malignancy in

these MSCs also contribute to myeloma bone disease by enhancing osteoclast formation and activity through various mechanisms (*i.e.*, increasing the receptor activator of nuclear factor- κ B ligand/osteoprotegerin ratio, augmenting activin A secretion, uncoupling ephrinB2-EphB4 signaling and because of heightened production of Wnt5a). In addition, we overview signaling pathways involved in the osteogenic differentiation of MSCs or osteoblast activity and comment on the reported activity of bone-anabolic agents (preclinical or clinical stage) to restore bone homeostasis in myeloma patients.

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MESENCHYMAL STROMAL CELLS: *IN VITRO* AND *IN VIVO* PROPERTIES

Mesenchymal stromal cells

Bone marrow (BM)-derived mesenchymal stromal cells (MSCs) were initially described by Friedenstein *et al.*^[1] in the late 60-s as adherent cells of fibroblastic morphology with the ability to differentiate into osteogenic cells, although it was later demonstrated that these cells also have chondrogenic and adipogenic differentiation potential^[2]. They were initially named as Colony Forming Unit-Fibroblasts^[3], but soon they were referred to as MSCs, term that gained general acceptance^[4]. Instead, the International Society for Cellular Therapy (ISCT) recommends the term “mesenchymal stromal cells” for MSCs^[5] and published several years ago a number of minimal definition criteria for these cells^[6], which are indicated in Table 1.

MSC isolation, characterization and *in vitro* expansion

BM-derived MSCs may be isolated from mononuclear cells obtained after density-gradient centrifugation of BM aspirates and subsequent adherence to tissue culture plasticware. Since their proportion in a normal BM sample is really low (between 0.01% and 0.0001% of nucleated cells)^[7], for most applications MSCs need to be *in vitro* expanded. The standard culture medium is based on Dulbecco's Modified Eagle Medium or α -Minimum Essential Medium with 10% of fetal bovine serum, although the latter can be replaced by platelet lysate or a commercial concentrate of growth factors^[8]. The expansion medium is replaced twice a week and thus non-adherent cells are removed. After two or three passages, the primary culture contains more than 95% of MSCs, and these cells are then used for most experiments^[9].

According to the ISCT definition criteria^[6] (Table 1), an immunophenotypic study is mandatory to evaluate the

Table 1 Minimal criteria for mesenchymal stromal cell definition (International Society for Cellular Therapy)

	Positive (> 95% +)	Negative (< 2% +)
Adherence to plastic surfaces in standard culture conditions		
Immunophenotype	CD105 CD73 CD90	CD45 CD34 CD14 or CD11b CD79a or CD19 HLA-DR
<i>In vitro</i> differentiation to osteoblasts, adipocytes and chondroblasts (demonstrated by appropriate staining of cell cultures)		

positivity for at least CD73, CD105 and CD90 and negativity for HLA-DR and hematopoietic antigens (CD45, CD34, CD19 or CD79 α , CD14 or CD11b).

Differentiation and immunomodulatory properties of MSCs

Upon *in vitro* culture with the appropriate differentiation media, MSCs are able to differentiate into osteogenic, adipogenic and chondrogenic phenotypes^[10]. This multilineage differentiation ability into mesodermal cell types is another definition criteria established by the ISCT^[6] (Table 1), and is the basis for evaluating the therapeutic potential of MSCs in a number of clinical trials, especially for treating musculoskeletal diseases^[11].

Being this property important, the range of diseases in which MSCs are of potential use has widely expanded when these cells demonstrated to display potent immunomodulatory and anti-inflammatory effects both *in vitro* and *in vivo*^[12]. In this regard, MSCs are able to reduce the activation of both T cells^[13] and B cells^[14], and to increase the number of T-regulatory cells^[15]. In addition, MSCs inhibit the maturation of dendritic cells and their capacity to process and present antigens^[16]. MSCs also reduce neutrophil activation and proliferation of natural killer cells^[17,18], thus regulating innate immune system responses. For these reasons, MSCs are currently being evaluated for the treatment of several immune-mediated diseases.

MULTIPLE MYELOMA AND THE BONE MARROW MICROENVIRONMENT. MYELOMA-ASSOCIATED BONE DISEASE

Multiple myeloma and the bone marrow microenvironment

Multiple myeloma (MM) is a hematological malignancy resulting from the clonal expansion of plasma cells in the BM. Diagnostic criteria of symptomatic myeloma include the presence of at least 10% BM myeloma cells and of monoclonal protein in serum and/or urine, together with myeloma-related end-organ or tissue damage (including hypercalcemia, renal dysfunction, anemia, immunodeficiency and bone destruction)^[19]. In fact, almost 80% of myeloma patients develop osteolytic lesions, which are responsible for some of the most devastating characteris-

tics of the disease. In most (if not all) cases, symptomatic myeloma is preceded by sequential asymptomatic stages of monoclonal gammopathy of undetermined significance (MGUS) and smoldering myeloma^[20], with increasing BM plasmocytosis and monoclonal component as well as augmented risk of progression to active MM (around 1% per year for MGUS patients and 10%-20% for patients with smoldering myeloma)^[21]. MM accounts for more than 1% of all cancers, with an incidence of 33400 new myeloma cases and 20300 deaths in the European Union 27 in 2012^[22].

During last decade, substantial advances both in the knowledge of the biology of the disease and in the use of more effective molecular-targeted novel agents and combinatorial regimens^[23,24] have led to improved responses and survival rates (median survival has increased from 3 to over 6 years^[25]). It is expected that advances in remaining controversies of the pathophysiology of the disease^[25] together with novel therapies currently in development and testing, may further improve myeloma survival in coming years, with future therapeutic aims rather focusing on increasing long-term remission rates and improving the quality of life of myeloma patients.

The biological behavior and clinical outcome in MM is partly dependent on the genomic and epigenetic abnormalities of myeloma cells^[26]. Compelling evidence has accumulated, however, supporting a critical role of the BM microenvironment in the pathogenesis and progression of the disease^[27-31]. MM cells establish complex interactions with other cellular components of the BM milieu [MSCs, osteoclasts (OCs), osteoblasts (OBs) and osteoprogenitor cells, endothelial cells, adipocytes, immune cells-dendritic cells, macrophages, T cells-], with components of the extracellular matrix (ECM) (*e.g.*, laminin, collagen, proteoglycans, glycosaminoglycans), and also with secreted soluble factors (cytokines, chemokines and growth factors). These interactions have bidirectional consequences: on the one hand, interactions of MM cells mainly with MSCs and OCs lead to activation of multiple cellular signaling pathways on myeloma cells [phosphatidylinositol 3-kinase/AKT, Janus kinase/signal transducer and activator of transcription 3, Ras/Raf/mitogen-activated protein kinase/extracellular signal-related kinase, nuclear factor- κ B (NF κ B)] which support their proliferation, survival, migration and even resistance to therapeutic agents (reviewed in^[27,31,32]). On the other hand, myeloma cells perturb the BM homeostasis causing anemia, immunosuppression, and uncoupling of the bone remodeling process leading to the development of osteolytic bone lesions characteristic of the disease.

Myeloma bone disease: Mechanisms of OC activation and OB inhibition

Myeloma bone disease (MBD) is characterized by the presence of osteolytic lesions that result in skeletal-related events (SREs) including severe bone pain, osteopenia, diffuse osteoporosis, focal lytic lesions, pathological fractures and spinal cord compression^[33,34]. Of importance, MBD

not only severely affects the quality of life of MM patients, but is also linked to poor prognosis, shorter overall survival and progression-free survival^[35,36]. This highlights the need of bone-targeted supportive treatments in addition to anti-myeloma therapy, which may reduce the risk of bone complications in MM patients. In addition, accumulating evidences of preclinical and clinical studies support the notion of an intimate relationship between tumor growth and the development of MBD, being one the necessity and consequence of the other^[37]. Since many of the dysregulated factors involved in the pathophysiology of osteolytic lesions also promote MM growth and survival, it is expected that effective interventions on MBD would secondarily lead to myeloma inhibition^[38].

Clinical observations, histomorphometric studies and measurements of serum/urine bone metabolism markers^[39-41] showed that uncoupled bone remodeling in MM was associated to both increased bone resorption (with increased number and activity of OCs) and almost absent bone formation (impaired OB formation and function). Only recently, many of the cellular and molecular interplayers involved in the pathophysiology of MBD have been identified and excellently reviewed^[34,37,38,42,43]. Next, we summarize the main factors and molecular mechanisms leading to the enhanced OC activation and suppressed OB function in MM.

Enhanced OC differentiation and resorptive activity

Enhanced OC formation from myeloid precursors and OC hyperactivation is primarily mediated through increased production of multiple "OC-activating factors (OAFs)" both by MM cells and other cells from the BM microenvironment {*e.g.*, receptor activator of NF- κ B ligand (RANKL), CCL3 [also known as macrophage inflammatory protein-1 α (MIP-1 α)], activin A, interleukin-3 (IL-3), IL-7, IL-1 β , IL-6 and CCL20^[44]; for reviews of cellular origin of OAFs, see^[37,45]; also see Figure 1}.

RANKL: RANKL is a member of the tumor necrosis factor (TNF) superfamily expressed as a transmembrane protein by BM MSCs and OBs, and by T lymphocytes as a soluble form; whether MM cells are producers of RANKL is still a controversial issue^[46,47]. Instead, the receptor of RANKL, RANK, is expressed on the surface of OCs and OC precursors. RANK-RANKL signaling has been shown to play an essential role in OC formation, activation and survival preventing OC apoptosis^[48,49]. Osteoprotegerin (OPG) is a soluble glycoprotein secreted by MSCs and OBs which acts as a decoy receptor for RANKL, neutralizing its activity and thus inhibiting osteoclastogenesis and bone resorption^[50]. Myeloma cells induce MSCs and OBs in the BM to upregulate the expression of RANKL and to reduce the expression of OPG, leading to increased RANKL/OPG ratios. In addition, MM cells may also sequester OPG through its binding to syndecan-1 (CD138), which is subsequently internalized and degraded^[51]. Furthermore, T lymphocytes in MM also overexpress TNF-related apoptosis inducing

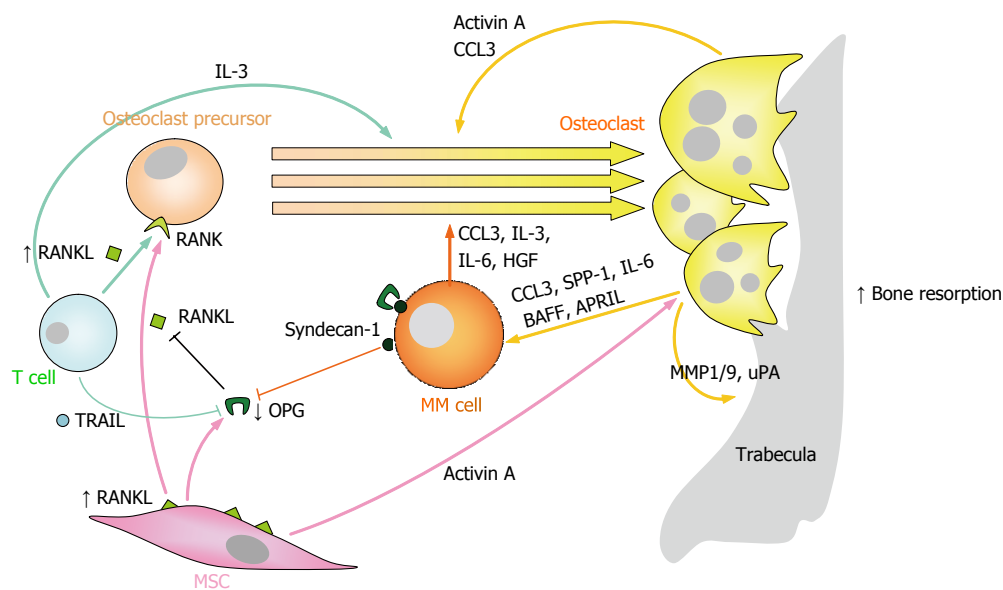


Figure 1 Enhanced osteoclast formation and resorption partially mediates the development of myeloma bone disease. Numerous “OC-activating factors” produced by multiple myeloma cells and other cells in the bone marrow microenvironment (including RANKL, CCL3, activin A, IL-3, HGF and IL-6) readily promote OC differentiation from OC precursors and/or stimulation of OC resorptive activity. In MM, the RANKL/OPG ratio is clearly favored towards RANKL, both because of increased expression of RANKL on MSCs and T lymphocytes, and because of reduced expression of OPG by MSCs and inactivation of OPG through binding to TRAIL or syndecan-1 on the surfaces of myeloma cells. On the other hand, OCs produce several factors (e.g., IL-6, CCL3, BAFF and APRIL) which promote the growth and survival of multiple myeloma cells. RANKL: Receptor activator of NF κ B ligand; NF κ B: Nuclear factor- κ B; CCL3/MIP1 α : Macrophage inflammatory protein 1- α ; IL-3/6: Interleukin 3/6; HGF: Hepatocyte growth factor; OPG: Osteoprotegerin; RANK: Receptor activator of NF κ B; TRAIL: Tumor necrosis factor-related apoptosis inducing ligand; BAFF: B-cell-activating factor; APRIL: A proliferation-inducing ligand; SPP-1: Osteopontin; MMP1/9: Matrix metalloproteinase 1/9; uPA: Urokinase plasminogen activator; MSCs: Mesenchymal stromal cells; OC: Osteoclast; MM: Multiple myeloma.

ligand (TRAIL), which binds and neutralizes OPG, further reducing its OC inhibitory activity^[52]. Whereas in physiological conditions the RANKL/OPG ratio tightly regulates OC function for adequate bone remodeling, in MM it is clearly favored towards RANKL, promoting osteoclastogenesis and bone destruction^[53,54]. Thus, the RANKL/OPG axis constitutes an important target for the treatment of MBD.

CCL3: The CCL3 (MIP-1 α) chemokine is mainly produced by both myeloma cells and OCs, and functions as a major osteoclastogenic and OC survival factor, both directly and indirectly by enhancing the osteoclastogenic activity of RANKL and IL-6^[55,56]. Interestingly, CCL3 has been found to have pleiotropic roles in MM, also inducing growth, survival and chemotaxis for malignant plasma cells^[57] and, as will be commented later, inhibition of OB differentiation^[58,59].

Activin A: Activin A is a transforming growth factor (TGF) β family member identified as a key component of MBD, having a dual role in stimulating OC formation and activity^[34,60] and as an inhibitor of OB differentiation^[61,62]. MSCs and OCs are the main sources of activin A and interacting myeloma cells further upregulate its expression in MSCs^[61].

Other factors promoting OC formation and activity: IL-3 is majorly produced by activated T lymphocytes and by myeloma cells, and may stimulate OC formation and resorption directly or by further augmenting that

of RANKL and CCL3^[63,64]. Other chemokines such as IL-7, tumor necrosis factor α (TNF α) and IL-1 β indirectly stimulate osteolytic processes, inducing RANKL expression on BM stromal cells (TNF α and IL-1 β)^[65] and by circulating T cells (IL-7)^[66]. Several other OAFs are secreted by myeloma cells and/or stromal cells [e.g., hepatocyte growth factor (HGF)^[67,68], IL-6^[69], IL-8^[70], and vascular endothelial growth factor (VEGF)^[71]], or by dendritic cells, Th17 1 lymphocytes, osteocytes and megakaryocytes in the BM milieu [(e.g., IL-17, IL-11) reviewed in^[37,72]], which further increase the gradient of osteoclastogenic factors in focal lesions and contribute to OC production and activity.

At the same time, OCs readily promote MM cell survival and growth by physical cell-cell contact and by the release of several soluble factors [including IL-6, CCL3, osteopontin, B-cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL)], and thus creating a vicious circle between bone lesions and tumour expansion (reviewed in^[30,37]).

On the other hand, myeloma-OC interactions may directly contribute to bone matrix degradation *via* secreted metalloproteinases 1/9 and urokinase-type plasminogen activator from OCs^[73]. Besides, some myeloma cells may acquire resorbing capabilities and degrade bone^[74,75], and dendritic cells in the BM may transdifferentiate to bone-resorbing OCs after myeloma interaction^[76], further contributing to enhanced resorption.

Suppression of osteoblastogenesis and OB function

Myeloma-induced suppression of osteoblastogenesis

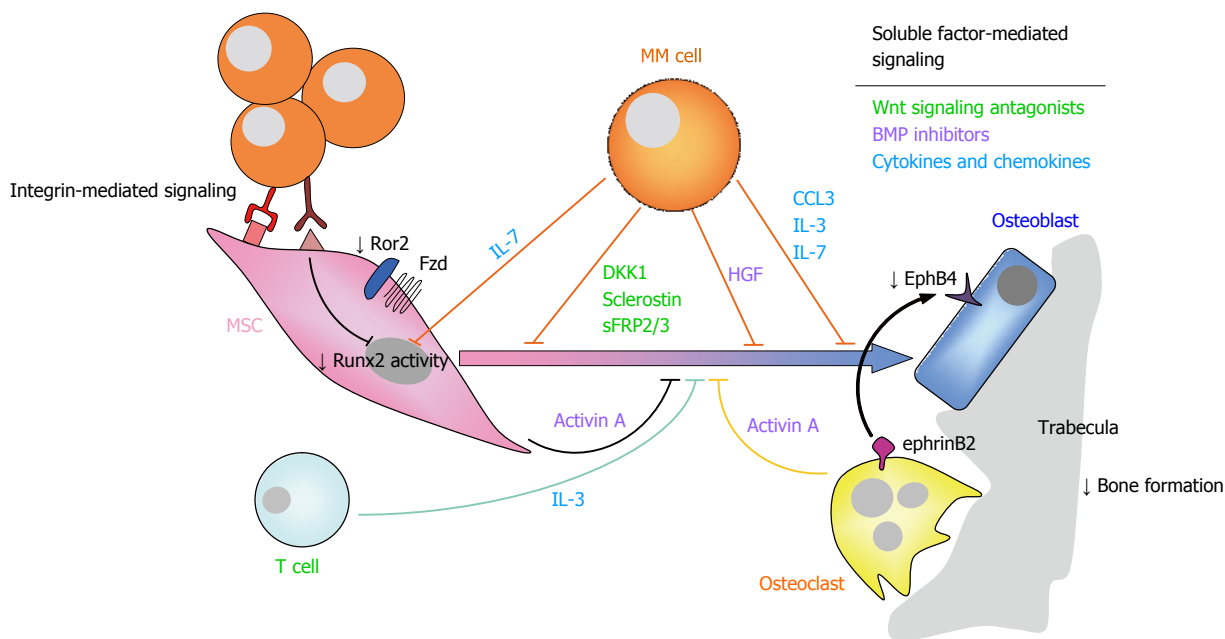


Figure 2 Suppression of osteoblastogenesis and osteoblast function in multiple myeloma is also involved in the pathophysiology of myeloma bone disease. Myeloma-induced OB suppression is partially mediated by direct cell to cell contact interactions with MSCs, leading to reduced activity of the Runx2/Cbfa1 transcription factor, and to inhibition of non-canonical Wnt5a signaling due to decreased expression of Ror2 in pre-OBs. In addition, soluble factors produced by myeloma cells and cells in the bone marrow microenvironment, such as Wnt signaling antagonists (e.g., DKK1, sclerostin, sFRP-2/3), BMP inhibitors (activin A, TGFβ, HGF), cytokines and chemokines (such as IL-7, TNFα, IL-3, CCL3) and apoptotic factors also contributed to inhibition of osteogenic differentiation and function. Finally, reduced ephrinB2-EphB4 signaling (from OCs to OBs) because of diminished EphB4 expression in MSCs, further contributes to impaired OB differentiation. Ror2: Receptor tyrosine kinase-like orphan receptor 2; DKK1: Dickkopf-1; sFRP-2/3: Secreted frizzled related protein-2/3; TGFβ: Transforming growth factor β; HGF: Hepatocyte growth factor; IL-7/3: Interleukin 7/3; ephrinB2: ephrin-B2 ligand; EphB4: Eph receptor B4; MSCs: Mesenchymal stromal cells; OB: Osteoblast; OC: Osteoclast; CCL3/MIP1α: Macrophage inflammatory protein 1-α; TNFα: Tumor necrosis factor α; BMP: Bone morphogenetic protein; MM: Multiple myeloma.

and OB activity is exerted both by functional inhibition of existing OBs as well as by impaired differentiation of MSCs into mature OBs. This is in accord with the findings of a significant reduction in the number of active OBs in BM biopsies^[77] and extremely low serum markers of osteoblastogenesis (such as osteocalcin and OPG) in patients with active osteolytic lesions as compared to myeloma patients not having bone lesions^[45].

In recent years, many of the molecular mediators underlying suppression of OB differentiation and function in MM have been identified, involving both direct cellular interactions and soluble factors (Figure 2).

Soluble factors contributing to OB suppression include inhibitors of the two major signaling pathways governing osteoblastogenesis [*i.e.*, Wnt and bone morphogenetic protein (BMP) signaling pathways], several cytokines and chemokines, as well as MM-induced apoptotic factors for OBs.

Wnt signaling antagonists (Dickkopf-1, sclerostin, secreted Frizzled-related proteins-2 and 3): MM cells secrete Dickkopf-1 (DKK1)^[78] and sclerostin^[79], both inhibiting Wnt canonical signaling and thus OB differentiation because of direct binding to the low-density lipoprotein receptor-related protein (LRP)5/6 co-receptor in osteoprogenitor cells^[80]. Sclerostin is also produced by osteocytes, mediating osteocyte-OC communication necessary for bone homeostasis^[81]. Interestingly, both DKK1

and sclerostin further increase the RANKL/OPG ratio on MSCs and osteoprogenitor cells by upregulating the expression of RANKL and reducing that of OPG, thus indirectly enhancing OC differentiation and activity^[79,82]. A direct stimulatory effect of sclerostin on OC formation has also been reported^[83]. Other Wnt antagonists produced by primary cells and MM cell lines are the secreted Frizzled-related proteins-2 and 3 (sFRP2 and sFRP3), which bind directly to secreted Wnt ligands and at least sFRP3 has been associated to the extent of MBD at diagnosis^[84-86].

BMP inhibitors (activin A, TGFβ, hepatocyte growth factor): The BMP is another major molecular pathway involved in osteogenesis, in which members of the TGFβ superfamily of cytokines (BMPs, activin A, TGFβ) bind to heterodimeric receptors to activate Smad proteins, which may directly regulate the expression of osteoblastogenic genes as transcription factors (e.g., DLX5-distal-less 5) or indirectly *via* Runx2/Cbfa1^[87]. Although some ligands (e.g., BMP2) directly stimulate osteogenesis through this pathway, others (such as activin A and TGFβ) have opposite effects. Activin A is produced by OCs and by MSCs after interaction with myeloma cells, and in addition to its commented pro-OC effect, it inhibits OB differentiation *via* Smad2-dependent DLX5 downregulation^[61]. Similarly, TGFβ also downregulates DLX5^[88], and inhibits OB differentiation. The HGF is produced by MM cells and is found at high concentrations in the BM of my-

eloma patients^[89]. It has been shown to promote proliferation of human MSCs keeping cells in an undifferentiated state, and to inhibit BMP-induced Smad translocation, thus inhibiting OB formation.

Other cytokines and chemokines: Other cytokines and chemokines may additionally contribute to suppression of OB activity (*e.g.*, IL-7, TNF α , IL-3 and CCL3). IL-7 is produced by malignant plasma cells and partially mediates Runx2/Cbfa1 decreased activity in MSCs interacting with myeloma cells, and reduces the expression of OB markers^[77,90]. The pro-inflammatory cytokine TNF α inhibits the expression of both Runx2/Cbfa1 and Osterix transcription factors^[91,92] and increases the expression of sclerostin in OBs^[93]. Interestingly, IL-7 and TNF α effects on osteoprogenitor cells in MM were found to be partly mediated by increased levels of the Gfi1 transcriptional repressor of Runx2/Cbfa1^[94]. IL-3 is majorly secreted by T lymphocytes (but also by myeloma cells) and besides its commented activity on OC formation and activation^[63], it indirectly inhibits basal and BMP2-induced OB differentiation by stimulating CD45⁺ monocyte-macrophages^[95,96]. In addition to the commented activity of CCL3 related to its pro-OC activity and in support of MM growth, CCL3 has been shown to inhibit OB differentiation and function through CCR1^[58].

MM-induced apoptosis on OBs: MM-induced apoptosis on OBs and osteocytes may also account for OB suppression in MM. OBs from myeloma patients with extensive osteolytic lesions have been shown to overexpress the Fas Cell Surface Death Receptor, death receptors DR4/5 and receptors to TRAIL, and to promptly undergo apoptosis when co-cultured with myeloma cells^[97,98]. Similarly, pre-osteocytes of patients with active bone disease in co-culture with myeloma cells showed increased apoptosis and upregulated expression of IL-11, thus increasing their pro-osteoclastogenic properties^[99].

In addition to soluble factors, direct contact interactions of myeloma and pre-OBs further contribute to OB suppression in MBD leading to reduced activity of Runt-related transcription factor 2/core-binding factor Runt domain α subunit 1 (Runx2/Cbfa1), which is a critical transcriptional regulator of OB differentiation^[77]. Blocking of very late antigen 4 (VLA4)-vascular cell adhesion molecule 1 (VCAM1) interactions with a neutralizing anti-VLA4 antibody reduced the inhibitory effect on Runx2/Cbfa1 activity, thus making these adhesion molecules partially responsible for the inhibition of OB differentiation and function^[77]. Further, long-term inhibition of Runx2/Cbfa1 and Osterix in pre-OBs seems to be mediated by overexpression of the transcriptional repressor 4EBP1^[100].

Only recently, Giuliani *et al.*^[101] identified another mechanism of myeloma-induced OB impairment through contact interactions. Although canonical Wnt signaling is known to play a critical role in osteoblastogenesis^[87], the non-canonical Wnt5a ligand has been

shown to mediate the osteogenic differentiation of BM human MSCs through activation of co-receptor receptor tyrosine kinase-like orphan receptor 2 (Ror2)^[102,103]. Myeloma cells were found to inhibit the expression of Ror2 when in co-culture interaction with pre-OB cells, therefore inhibiting non-canonical Wnt5a signaling and the osteogenic differentiation of MSCs^[101].

Finally, suppressed osteoblastogenesis in MM is further mediated by dysregulation of cell surface molecules involved in OB-OC communication (*i.e.*, ephrinB2-EphB4). Bidirectional signaling between the cell-surface molecules ephrin ligands and Eph receptors controls numerous processes including OB-OC communication^[104]. Specifically, MSCs and OBs express both ephrinB2 and EphB4, whereas OC precursors mainly express ephrinB2. The ephrinB2-EphB4 signaling (from OCs to OBs) stimulates OB differentiation and leads to new bone formation; on the other hand, EphB4-ephrinB2 signaling (from OBs to OCs) blocks OC differentiation^[105]. MSCs from myeloma patients have reduced expression of both ephrinB2 and EphB4 due to interacting myeloma cells, which results in reduced osteogenic differentiation as compared to dMSCs, and in stimulation of osteoclastogenesis^[105].

A secondary consequence of suppression of OB differentiation is that it renders an excess of MSCs/immature OBs in the BM which would enhance OC activation due to higher expression of RANKL, activin A and reduced secretion of OPG, as compared to mature OBs^[106,107]. These MSCs/immature OBs pool would also further support myeloma progression and survival by providing higher levels of cytokines and growth factors than mature OBs.

COMPARISON BETWEEN MSCs FROM MM PATIENTS AND HEALTHY DONORS. CONTRIBUTION OF pMSCs TO MYELOMA BONE DISEASE

A number of studies have compared MSCs derived from the BM of newly diagnosed myeloma patients (pMSCs) and those from healthy donors (dMSCs) (reviewed in^[108]), in an attempt to gain insight into their role in the pathophysiology of MM and MBD. Although MSCs from both origins similar adipogenic and chondrogenic potential, pMSCs functionally and genetically differ from their healthy counterparts. A summary of main similarities and differences found between BM-derived pMSCs and dMSCs is shown in Table 2.

Relative to the contribution of MSCs to the pathogenesis of MBD, and despite some opposite results within groups, several features of pMSCs readily reflect their reduced osteogenic potential as compared to their healthy counterparts (*e.g.*, reduced expression of bone formation markers and critical transcription factors in OB differentiation-Runx2/Cbfa1, Osterix and TAZ)^[109]; reduced expression and activity of early OB marker alkaline phosphatase (ALP)^[110]; reduced matrix mineralization under

Table 2 Main similarities and differences between bone marrow mesenchymal stromal cells from myeloma patients and mesenchymal stromal cells from healthy donors

Study	Assay	Description
Similarities		
Adipogenic differentiation	Oil O Red staining	Both pMSCs and dMSCs showed accumulation of lipid-rich vacuoles ^[109,194]
Chondrogenic differentiation	Toluidine Blue staining	Chondrogenic differentiation potential was similar between pMSCs and dMSCs ^[109]
Differences		
Chromosomal alterations	CGH arrays, FISH	pMSCs did not carry the genomic abnormalities detectable in their correspondent myeloma cells ^[111,194,195] . Only pMSCs showed several non-recurrent chromosomal gains and losses (> 1 Mb size) and “hot-spot” regions with discrete (< 1 Mb) genomic alterations ^[195]
Gene expression profiling	Gene expression microarray	Among 145 differentially expressed genes between pMSCs and dMSCs, 46% accounted for tumor-microenvironment cross-talk. Functional assignment revealed their implication in tumor-support (<i>e.g.</i> , GDF15), angiogenesis (<i>e.g.</i> , ANGPTL4, PAI1, SCG2), and contribution to bone disease (<i>e.g.</i> , NPR3, WISP1, EDG2) ^[111] . Even a distinct transcriptional pattern was found associated to the occurrence of bone lesions in pMSCs ^[113]
Immunophenotype	Flow cytometry	Although few significant differences in cell surface marker expression were found between dMSCs and pMSCs, the latter expressed reduced VCAM1 and fibronectin ^[196] , and higher ICAM1 ^[197] compared to dMSCs
Bone formation markers	qPCR, WB	Expression of bone formation markers (<i>i.e.</i> , osteocalcin and osteopontin), master transcription factors of osteogenic differentiation (<i>i.e.</i> , Runx2/Cbfa1 and Osterix) and TAZ (a Runx2/Cbfa1 transcriptional co-activator) was lower in pMSCs than in dMSCs ^[109]
Expression and secretion of growth factors/cytokines/chemokines	RT-PCR, ELISA	Compared to dMSCs, pMSCs showed increased expression of IL-1 β ^[111] , IL-3 ^[112] , IL-6 ^[111,112,194,198] , IL-10 ^[199] , BAF β ^[199] , GDF15 ^[111,198] , TNF α ^[112] , TGF β 1 ^[112,198] , DKK1 ^[111,121,198] , RANKL ^[112] , AREG ^[111] , and decreased expression of TGF β 2, TGF β 3 and FasL ^[112]
Senescence profile	β -gal staining, propidium iodide DNA staining, qPCR	pMSCs showed an early senescence state compared to dMSCs, as assessed by increased expression of senescence-associated β -galactosidase, increased cell size and accumulation of cells in S phase ^[198]
Immunoability	Co-cultures of MSCs and lymphocytes or PBMCs	pMSCs exhibited reduced efficiency to suppress T-cell proliferation compared to that of dMSCs ^[112,194,198]
Angiogenic potential	qPCR, ELISA, tube formation assay	Angiogenic factors (bFGF, HGF and VEGF) were elevated in the CM of pMSCs compared to dMSCs. Besides, CM from pMSCs significantly promoted proliferation, chemotaxis and capillary formation of HUVECs compared to dMSCs ^[200]
Controversial points		
Proliferation rate	Cell density, CFU-F	Whereas some studies did not find differences in CFU-F number and cell density between dMSCs and pMSCs ^[111] , others found a deficient proliferative potential in pMSCs which could be partly explained by the reduced expression of receptors for several growth factors ^[121]
ALP expression and activity	BCIP-NBT staining and pNPP hydrolysis	ALP expression/activity did not differ between MSCs from both origins ^[111] , whereas other authors found it was significantly reduced in pMSCs compared to dMSCs, with lowest levels in pMSCs from patients with bone lesions ^[110]
Matrix mineralization	Alizarin Red and Von Kossa staining	Some groups have reported a significant reduction of matrix mineralization by pMSCs relative to dMSCs ^[110,111,198] , although others have not observed those differences ^[121,194]
Hematopoietic stem cell support	Long-term co-cultures	Some authors reported that the ability to support the growth of hematopoietic stem cells did not differ between dMSCs and pMSCs ^[111,194] , whilst others found that pMSCs better supported CD34 ⁺ progenitor expansion ^[198]

pMSCs: Mesenchymal stromal cells from myeloma patients; dMSCs: Mesenchymal stromal cells from healthy donors; CGH: Comparative genomic hybridization; FISH: Fluorescence *in situ* hybridization; qPCR: Quantitative PCR; WB: Western blot; RT-PCR: Reverse transcription-PCR; PBMC: Peripheral blood mononuclear cells; CM: Conditioned media; HUVECs: Human umbilical vein endothelial cells; CFU-F: Colony forming unit-fibroblast assay; BCIP-NBT: Bromo-chloro-indolyl-phosphate and nitro blue tetrazolium staining; pNPP: p-nitrophenyl phosphate; ALP: Alkaline phosphatase; IL-3: Interleukin-3; TNF α : Tumor necrosis factor α ; TGF β 1: Transforming growth factor β 1; DKK1: Dickkopf-1; RANKL: Receptor activator of NF κ B ligand; NF κ B: Nuclear factor- κ B.

osteogenic conditions^[110,111]; increased expression of OB inhibitory factors (DKK1, IL-3, IL-1 β , TGF β)^[111,112], and a discernible gene expression signature for pMSCs with or without osteolytic lesions^[113]. These characteristics on pMSCs are likely the consequence of myeloma cell interactions and exposure to multiple soluble OB inhibitory factors and microenvironment conditions (*e.g.*, hypoxia)^[114] as occurring in the BM milieu of myeloma

patients. Most of these studies have been conducted in MSCs after *in vitro* expansion and, in the case of pMSCs, after long-term absence of interaction with myeloma cells; thus, the presented differences between dMSCs and pMSCs may have been retained *in vitro* likely by epigenetic mechanisms.

Importantly, pMSCs not only contribute to MBD because of their reduced osteogenic potential, but also

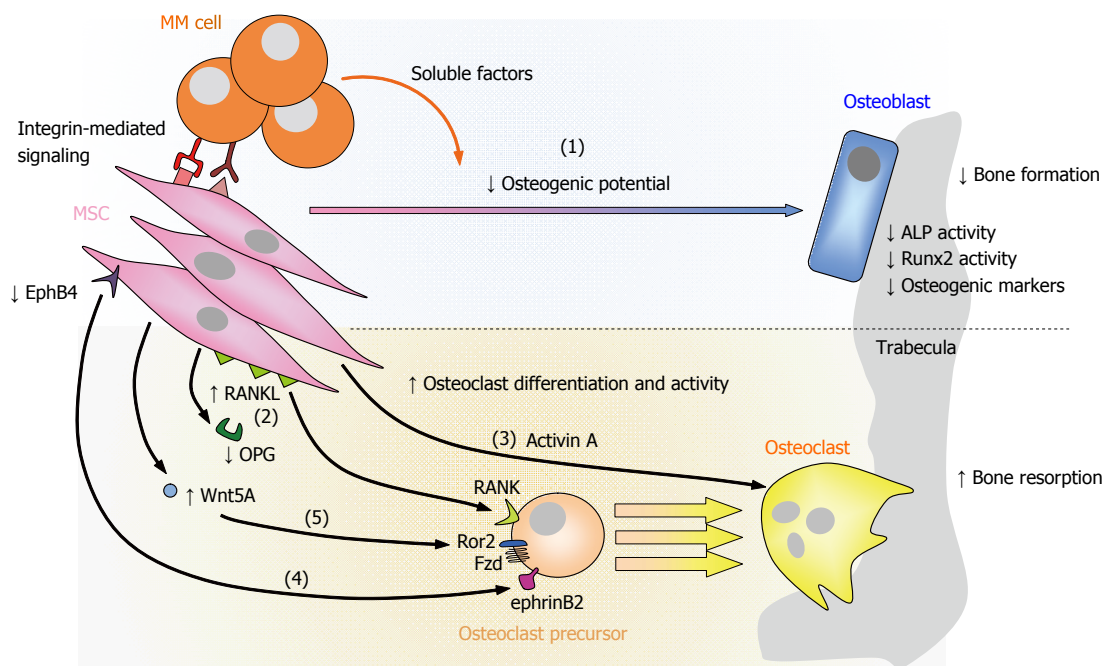


Figure 3 Contribution of mesenchymal stromal cells to myeloma bone disease. In MM, MSCs contribute to the development of osteolytic lesions not only because of their reduced osteogenic potential [(1)], but also because they promote OC differentiation and hyperactivation at various levels: pMSCs upregulate the expression of RANKL and reduce that of OPG [(2)]; pMSCs augment the secretion of activin A [(3)]; diminished EphB4-ephrinB2 signaling from pMSCs/OBs to OCs allows osteoclastogenesis [(4)]; increased Wnt5a secretion by pMSCs interacting with myeloma cells enhances RANK expression in OC precursors through Ror2, ultimately increasing their sensibility to RANKL [(5)]. RANKL: Receptor activator of NF κ B ligand; NF κ B: Nuclear factor- κ B; OPG: Osteoprotegerin; RANK: Receptor activator of NF κ B; EphrinB2: Ephrin-B2 ligand; EphB4: Eph receptor B4; Ror2: Receptor tyrosine kinase-like orphan receptor 2; MSCs: Mesenchymal stromal cells; MM: Multiple myeloma; OC: Osteoclast; OB: Osteoblast; ALP: Alkaline phosphatase; MM: Multiple myeloma.

because they ultimately lead to the differentiation and/or activation of OCs at various levels (Figure 3): (1) increased RANKL/OPG ratio: interacting myeloma cells upregulate the expression of RANKL in MSCs, whereas the expression of OPG is reduced, thus favouring osteoclastogenesis and OC activation through RANKL-RANK signaling; (2) augmented secretion of activin A: interaction with myeloma cells leads to increased secretion of activin A in MSCs *via* adhesion-mediated c-Jun N-terminal kinase (JNK) activation^[61]. Besides inhibiting OB differentiation, increased activin A levels would stimulate OC formation and activity^[60]; (3) diminished expression of ephrinB2 and EphB4 in pMSCs: myeloma cells reduce the expression of both ephrinB2 and EphB4 in pMSCs^[105] thereby dysregulating the ephrinB2/EphB4 signaling between OCs and OBs. Besides an impaired OB differentiation (due to reduced ephrinB2-EphB4 signaling from OCs to OBs), diminished EphB4-ephrinB2 signaling (from OBs to OCs) would no longer prevent OC formation, allowing increased osteoclastogenesis; and (4) increased Wnt5a production by MSCs: Wnt5a has been identified as a myeloma growth factor being overexpressed by myeloma plasma cells and pMSCs (as compared to their healthy counterparts)^[115]. Interestingly, we have found the upregulated expression of Wnt5a in MSCs after interaction with myeloma cells (our unpublished results), which would further contribute to its enhanced production in the BM. Recently, a link between Wnt5a and increased osteoclastogenesis has been found by identification of signaling between Wnt5a (secreted

by OB-lineage cells) and the membrane Ror2 receptor (expressed on OC precursors), leading to upregulated RANK expression in the latter and increased sensitivity to RANKL^[116]. Accordingly, myeloma interacting-MSCs would heighten the production of Wnt5a which in turn would increase OC formation and activity.

Thus, both a reduced osteogenic capacity and a hyperstimulation of OCs^[68] at various levels constitute the two major contributions of MSCs to the development of MBD. Other contributions of MSCs, such as modification of ECM components (in relation with retention of OC-activating and OB-inhibiting factors or growth factors) have not been addressed here, but would likely participate in the pathophysiology of the disease.

PATHOPHYSIOLOGY OF IMPAIRED OSTEOGENIC DIFFERENTIATION OF pMSCs: THERAPEUTIC OPPORTUNITIES BASED ON MSC TARGETING

In this section, we will review the major signaling pathways involved in OB differentiation and OB function [*e.g.*, Wnt, Notch, BMP and CCL3 signaling, ephrin-Eph axis, unfolded protein response (UPR)]; at the same time, we also discuss potential therapies targeting members of these pathways in order to restore OB differentiation and activity in patients with MBD.

Bisphosphonates (BPs) are the current mainstay for

Table 3 Therapeutic targets, bone anabolic drugs and preclinical/clinical studies in the context of myeloma bone disease or other bone diseases

Drug	Mechanism of action	Signaling pathway	Cell target	Preclinical studies	Phase of clinical trials
BHQ880	Neutralizing anti-DKK1 antibody	Wnt	MSC, MMPC	[122,123,125]	II ^[126,127]
Romosozumab (AMG785)	Neutralizing anti-sclerostin antibody		MSC	[79,83]	II (postmenopausal osteoporosis) ^[129]
LiCl	GSK3 β inhibitor		MSC, MMPC	[131]	NA
DAPT	γ -secretase inhibitor	Notch	MSC, MMPC	[110,142]	NA
GSI15			OC, MMPC	[139]	
Bortezomib and second generation PIs	Proteasome inhibitor	UPR	MSC, OC, MMPC	[179,180,201]	Bortezomib and carfilzomib: Approved Oprozomib: I / II Ixazomib: III
RAP-011 (mouse)	Decoy receptor neutralizing	BMP	MSC, OC, MMPC	[61,62]	II ^[153,154]
Sotatercept/ACE-011 (human)	activin A				
SB431542	TGF β inhibitor		MSC	[150]	NA
Ki26894			MSC		
MLN3897	CCR1 antagonists	CCL3	MSC, OC, MMPC	[58,160]	NA
CCX721 (mouse)			OC, MMPC	[157]	II (rheumatoid arthritis) ^[161]
CCX354-C (human)					

MSC: Mesenchymal stromal cell; MMPC: Multiple myeloma plasma cell; OC: Osteoclast; NA: Not applicable; DKK1: Dickkopf-1; GSK3 β : Glycogen synthase kinase 3 β ; DAPT: N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; TGF β : Transforming growth factor β ; UPR: Unfolded protein response; BMP: Bone morphogenetic protein; CCR1: Chemokine (C-C Motif) receptor 1; CCL3/MIP1 α : Macrophage inflammatory protein 1- α .

the treatment of bone complications in MM patients, generally administered as supportive therapy in addition to anti-myeloma agents. BPs are pyrophosphate analogs with great affinity for mineralized matrix surfaces, causing inhibition of OC function and OC apoptosis^[117]. Second generation nitrogen-containing BPs now in use, such as pamidronate and zoledronic acid, have been shown to be superior to previous BPs and to more effectively reduce the incidence of SREs^[33]. However, adverse side-effects after long BP treatment (*i.e.*, osteonecrosis of the jaw, kidney failure and accumulation of bone microfractures^[34,118]) and eventual progress of bone disease under treatment^[33], has prompted preclinical and clinical studies for the use of alternate bone anabolic agents which may achieve a more efficacious and improved management of MBD (Table 3).

Wnt signaling

Role in MBD: Wnts are a family of 19 secreted glycoproteins that trigger several pathways involved in cell fate determination, proliferation, migration and polarity, both in embryogenesis and regeneration of adult tissues. Specifically, Wnt signaling in MSCs is critical for OB differentiation and hence, for bone metabolism^[119]. Literature categorizes this pathway in canonical or non-canonical, depending on the requirement of β -catenin or not, respectively. In the Wnt canonical pathway and in the absence of Wnt stimulation, cytoskeletal β -catenin is phosphorylated by a multi-protein destruction complex and undergoes ubiquitin-mediated degradation in the proteasome. Upon binding of canonical Wnt ligands to a Frizzled (Fzd) receptor and a LRP co-receptor, the destruction complex is inhibited allowing β -catenin to translocate into the nucleus where it interacts with T-cell factor/lymphoid enhancer factors to activate transcription

of target genes involved in osteoblastogenesis. On the other hand, the two better characterized Wnt non-canonical pathways are the planar cell polarity and the Wnt/Ca²⁺ pathways, mainly implicated in cell polarity and cell migration mediated by cytoskeletal-actin rearrangements^[119].

Several secreted factors may negatively regulate canonical and non-canonical Wnt signaling: DKK1-4 and sclerostin directly bind to the LRP5/6 co-receptor limiting its availability to Wnt ligands; on the other hand, sFRP1-5 or Wnt inhibitory factor 1 (Wif1), directly bind to Wnt ligands, preventing their functional association with Fzd receptors. Since Wnt signaling plays such a critical role in the osteogenic differentiation of MSCs, alterations in this pathway may lead to skeletal disorders as observed in MBD. In fact, newly diagnosed MM patients showed elevated DKK1^[78], sclerostin^[120] and sFRP3^[84] levels compared to that of healthy donors both in BM and peripheral blood plasma, correlating with the presence of bone lesions.

Although malignant plasma cells are the main source of these Wnt antagonists in the BM^[78,79,84-86], pMSCs secreted higher DKK1 levels than their healthy counterparts^[111,121]. Similarly, sclerostin was found to be produced by OBs derived from pMSCs co-cultured with a MM cell line, further contributing to suppression of OB differentiation and function in MBD^[83]. Even though non-canonical Wnt signaling is not usually associated to osteogenic functions, Wnt5a ligand seems to be at least partially responsible for the osteogenic differentiation of MSCs in the BM^[102,103]. As we commented before, this pathway was inhibited in MSCs from myeloma patients, due to downregulation of Ror2 co-receptor expression^[101].

Therapeutic approaches: Given that Wnt inhibition (by DKK1, sclerostin and sFRPs) has been involved in the

development of osteolytic lesions, modulation of Wnt signaling by different approaches constitutes a potential clinical strategy in MBD.

BHQ880 is a humanized monoclonal antibody against DKK1, which has been shown to reverse the hampering effects of this Wnt inhibitor on OB formation. Treatment with anti-DKK1 or BHQ880 therapy prevented OB suppression and reduced the development of osteolytic lesions in *in vivo* studies with mouse models of murine/human MM^[122-125]. Furthermore, BHQ880 showed an anti-myeloma effect, overcoming the growth advantage conferred by MSCs to MM cells in co-culture through downregulation of cell adhesion and IL-6 production by MSCs^[123], which was also corroborated in *in vivo* models^[122,123,125]. A phase I / II study of BHQ880 in relapsed or refractory MM patients with or without BPs besides standard chemotherapy (NCT00741377), assessed the bone anabolic efficacy of this DKK1 inhibitor through an increase in bone mineral density and regulation of bone metabolism markers^[126]. Ongoing phase II studies of BHQ880 in untreated patients with high risk smoldering myeloma (NCT01302886), have reported increased vertebral strength but no anti-MM activity^[127].

Inhibition of sclerostin by monoclonal antibodies has been explored in different bone disorders, leading to increased bone formation, bone mass and bone strength in preclinical models in mouse, rats and monkeys (reviewed in)^[128]. The development of romosozumab (AMG785), a humanized monoclonal antibody to sclerostin, has allowed its translation to clinical trials. In phase I studies, romosozumab was administered to healthy men and postmenopausal women resulting in a dose-related increase in bone formation markers, a decrease in bone resorption markers, and significantly increased bone mineral density at the lumbar spine and total hip^[129]. A phase II trial is currently ongoing to compare the efficacy of romosozumab with alendronate and teriparatide in the treatment of postmenopausal women with low bone mineral density (NCT00896532). In the MM setting, *in vitro* assays with neutralizing anti-sclerostin antibodies restored OB function as assessed by increased expression of bone formation markers and transcription factors Fra-1, Fra-2 and JunD, modulation of the unbalanced OPG-RANKL ratio and accumulation of β -catenin^[79,83].

Wnt3a administration was also shown to enhance Wnt signaling on OB progenitors, and promoted bone formation and attenuated MM growth in a myeloma SCID-hu mouse model^[130]. Inhibition of glycogen synthase kinase 3 β (GSK3 β), a serine-threonine kinase involved in the phosphorylation of β -catenin for proteasome degradation has also been explored. GSK3 β inhibitors such as lithium chloride^[131] ameliorated the development of MBD and inhibited tumor growth in a disseminated 5TGM1 mouse model of MM, despite some concerns about the possibility that this strategy may stimulate myeloma growth^[132].

Notch signaling

Role in MBD: Evolutionarily conserved Notch signaling

plays an important role during embryonic and postnatal life by regulating cell fate determination, proliferation, differentiation and apoptosis in a spatio-temporal manner^[133]. Notch is a family of four (Notch1-4) transmembrane receptors activated by single-pass membrane ligands (Jagged1-2 and Delta like-1/3/4). Upon Notch-ligand interactions, the γ -secretase complex cleaves the Notch intracellular domain, which then translocates to the nucleus to regulate the transcription of target genes, including Hairy enhancer of split (Hes) and Hes-related to YRPW motif^[133]. Notch signaling plays a key role in skeletal development and remodeling maintaining MSCs in an undifferentiated stage by suppressing OB differentiation (directly repressing Runx2/Cbfa1 activity^[134] or inhibiting Wnt/ β -catenin pathway^[135]). However, once Notch signaling is activated in MSCs, it stimulates early osteoblastic proliferation^[134] leading to the maintenance of an immature OB pool. Considering the well-established role of Notch in osteogenic differentiation, dysregulation of this pathway is associated with human diseases affecting the skeleton. In this sense, alterations in Notch signaling have been reported in pMSCs^[110], which maintain high gene expression levels of some Notch signaling molecules (*e.g.*, Notch1 receptor and the transcription factors Hes1 and Hes5) as compared to dMSCs, which suggests an inhibitory role of these molecules in OB differentiation.

On the other hand, it has also been reported that activation of Notch signaling may regulate osteoclastogenesis depending on the ligands and receptor isoforms involved. Notch1 and Notch3 are able to suppress OC differentiation and activity *via* ligand-mediated receptor activation^[136], whereas Notch2 is upregulated during RANKL-induced osteoclastogenesis and enhances OC formation through increased NFATc1 expression^[137].

Therapeutic approaches: To date, Notch signaling blockade has focused on inhibition of the γ -secretase complex, the intramembrane-cleaving protease with a growing list of protein substrates, including Notch receptors and the amyloid precursor protein involved in Alzheimer's disease^[138]. Treatment with N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a γ -secretase inhibitor (GSI), restored the osteogenic ability of MSCs both *in vitro* (by increasing the gene expression of bone formation-related markers, ALP activity and matrix mineralization) and *in vivo* (as assessed by increased OB cell number at the endocortical surface in naive mice)^[110]. However, GSI treatment failed to stimulate OB formation in a MM model, probably due to the lack of activity over MM cells^[110]. Looking at the OC compartment, treatment with the Notch inhibitor GSI15 blocked MM cell-induced activation of OCs, reinforcing evidences for the use of GSIs as a therapeutic option in MBD^[139]. Other preclinical studies performed with GSIs (GSI-XII, MRK003, DAPT) in the myeloma setting prevented MM cell migration, proliferation, clonogenic ability, resistance to apoptosis, angiogenesis as well as tumor growth *in vitro* and in a SCID-human model of MM^[140-144]. Moreover, it has been found that combined

treatment of GSIs and established anti-MM drugs (such as bortezomib^[145], melphalan, doxorubicin^[144]), or other agents such as ABT-737^[143] or Akt1/2 inhibitors^[141], results in a synergistic cytotoxic effect on myeloma cells. In this sense, combining Notch inhibitors with anti-MM drugs holds promise as a valuable therapeutic approach for the treatment of both MM and MBD.

BMP signaling (activin A and TGF β)

Role in MBD: Activin A is a TGF β superfamily member mainly secreted by BM-derived MSCs from myeloma patients and OCs^[61]. MM cell lines and primary MM plasma cells secrete very low or undetectable levels of activin A, but co-culture with MSCs induces the secretion of activin A in the latter *via* JNK pathway activation^[61]. Activin A binds to the serine/threonine kinase activin A receptor, type II A (ActR II A), which recruits and phosphorylates the receptor type I B (ActR I B), leading to phosphorylation of cytoplasmic Smad2/3 proteins. This complex associates with Smad4, which translocates into the nucleus and controls gene expression^[146]. Activin A has several roles in the development of osteolytic lesions: it enhances OC formation and activity (inducing nuclear translocation of NF κ B and RANK expression in OC precursors)^[60], inhibits OB differentiation (*via* Smad2-mediated DLX5 downregulation)^[61] and alters the extracellular matrix maturation phase^[147]. Accordingly, high levels of circulating activin A correlate with extensive bone disease and inferior survival^[148].

TGF β is abundantly deposited in the bone matrix and the enhanced bone resorption in MM bone lesions causes a marked increase in the release and activation of this factor^[149,150]. Although TGF β enhances the recruitment and proliferation of OBs progenitors, it potently suppresses later phases of OB differentiation, maturation and matrix mineralization^[149,150].

Therapeutic approaches: Sotatercept (ACE-011) or RAP-011 are chimeric proteins derived from the fusion of the extracellular domain of ActR II A and the Fc domain of human IgG1 or murine IgG2a, respectively. These proteins sequester ligands of ActR II A (activin A among others), interfering with Smad signaling and restoring the uncoupled bone remodeling.

Treatment of MSCs with RAP-011 increased OB differentiation, even in the presence of MM cells, by rescuing DLX5 expression^[61]. The bone anabolic effect of RAP-011 could be translated to the *in vivo* setting on a SCID-hu model of MBD. RAP-011 treatment prevented bone destruction and reduced MM tumor burden^[61], providing the basis for clinical testing in myeloma patients suffering from severe bone disease. Similar results were obtained in healthy and ovariectomized mice^[151], in murine models of osteolytic disease induced by MM cells and breast cancer cells^[62], and in non-human primates^[152].

The human counterpart of RAP-011, sotatercept, has been evaluated in phase II studies in MM patients with osteolytic lesions receiving a regimen of melphalan,

prednisone and thalidomide (NCT00747123); after sotatercept treatment, patients showed an increase in bone formation markers (bone-specific ALP), improvement in osteolytic lesions, reduction of bone pain and myeloma burden^[153]. Other studies of sotatercept in combination with lenalidomide and dexamethasone in patients with relapsed and/or refractory myeloma are currently recruiting participants (NCT01562405). In accordance with these studies, a phase I trial of sotatercept in postmenopausal women has evidenced a bone anabolic and anti-resorptive effect, as observed by sustained increase in bone formation markers (bone-specific ALP) and decrease in bone resorption markers (CTX and TRACP-5b)^[154].

Relative to TGF β , pharmacological inhibition of the TGF β type I receptor kinase (T β R I), SB431542 and Ki26894, potently enhanced OB differentiation *in vitro*, releasing MSCs from their differentiation arrest and facilitating the formation of terminally differentiated OBs^[150]. *In vivo* administration of these agents showed anabolic and anti-catabolic effects on bone, in parallel with suppression of MM cell growth^[150,155]. Therefore, TGF β appears to be an important therapeutic target in MBD.

CCL3 signaling

Role in MBD: CCL3 (MIP1 α) is a chemokine mainly secreted by OCs and MM cells, which binds to G-protein-coupled receptors CCR1 and CCR5. Both chemokine receptors are expressed in MM cells, MSCs/OBs and OCs^[58,156], being CCR1 the major receptor on OC precursors and mature OCs^[157]. The CCL3 pathway is not only involved in the survival, growth and migration of MM cells^[156], but CCL3 also readily contributes to the imbalance between bone formation and bone resorption by enhancing OC formation^[158] and hampering OB function^[58]. BM plasma CCL3 levels were found to be elevated in MM patients, correlating directly with the extent of MBD and inversely with survival^[159] and osteocalcin expression^[58].

Therapeutic approaches: Preclinical *in vitro* and *in vivo* studies have been performed either targeting CCL3 (anti-sense construct to human CCL3^[56] and neutralizing antibody against CCL3^[57]), or the CCR1 receptor (small-molecule CCR1 antagonists MLN3897^[160] and CCX721^[157]). These treatments reduced myeloma tumor burden and prevented osteolysis, thus providing a strong rationale for the clinical evaluation of these compounds in the treatment of MBD.

Therapeutic strategies towards the inhibition of the CCL3 pathway have mainly focused on their effect on the OC compartment^[56,57,157,160], although there is also preclinical evidence of the anabolic effect of the CCR1 inhibitor MLN3897 in osteogenic differentiation^[58]. In the latter study, *in vitro* CCR1 inhibition suppressed CCL3-induced ERK activation and restored both Osterix and osteocalcin expression in OBs differentiated from a human stromal cell line; in the SCID-hu murine model of MM, treatment with MLN3897 reduced tumor burden,

decreased OC number and increased both the trabecular bone area and the percentage of osteocalcin-positive area in the trabeculae^[58]. These studies set the stage for development of clinical trials to assess the effects of CCR1 inhibitors in MM. CCX354, the human structural analog of CCX721, is currently in phase II studies for rheumatoid arthritis, exhibiting clinical activity with a good safety and tolerability profile^[161].

Eph/ephrin signaling

Role in MBD: Another example of a bidirectional signaling pathway capable of regulating both osteoblastic and osteoclastic lineages is the one mediated by Eph receptors and ephrins (Eph receptor-interacting ligands). There are two classes of ephrins: the B class (ephrin B1 to B3), which are ligands for EphB tyrosine kinase receptors (B1 to B6), and the A class (ephrin A1 to A5), which are ligands for glycosylphosphatidylinositol-anchored EphA receptors (A1 to A10)^[162]. Eph-ephrin complexes signal bidirectionally to orchestrate several cellular processes including immune regulation, neuronal development and cancer metastasis. The Eph/ephrin system is expressed by BM microenvironment cells (including OBs and OCs), and growing evidence point out the pivotal role of this pathway in the control of normal and pathological bone remodeling^[163]. Specifically, the ephrinB2/EphB4 axis has been involved in bone homeostasis: reverse signaling through ephrinB2 ligand (expressed by OCs and MSCs/OBs) limits OC activity, whereas forward signaling through EphB4 receptor (expressed by MSCs and OBs) enhances OB differentiation^[104]. Dysregulation of Eph/ephrin function may also contribute to other bone pathological conditions such as osteoarthritis, rheumatoid arthritis or osteosarcoma^[163].

In the MBD context, Pennisi *et al.*^[105] have found reduced levels of ephrinB2 and EphB4 in MSCs from MM patients as compared to their healthy counterparts, and also in OBs/OCs of myelomatous bones compared to non-myelomatous bones. In co-culture experiments, MM cell lines markedly downregulated EphB4 receptor and ephrinB2 ligand in human MSCs, thus confirming a MM cell-induced imbalance of ephrinB2/EphB4 signaling in the MSC-OB lineage^[105].

In addition to the EphB4/ephrinB2 axis, OB-OC, OB-OB and OC-OC interactions through other ephrins and Eph receptors do in fact occur and participate in bone homeostasis. For example, it has been reported that OC-derived ephrinA2/EphA2 interaction enhanced OC differentiation *via* reverse signaling, whereas ephrinA2 inhibited osteoblastogenesis through OB-derived EphA2 receptor *via* forward signaling, contributing to the transition phase of bone remodeling from bone formation to bone resorption^[164]. Future studies about the expression/function of A class ephrins/Eph in MSCs from MM patients may thus unravel new governing mechanisms of impaired OB differentiation in MBD.

Therapeutic approaches: The dual role of EphB4/

ephrinB2 signaling in the OB/OC compartment is especially attractive as a therapeutic approach in MBD, since its activation is able to promote both OB differentiation and function and attenuate OC formation and bone resorption. Pennisi *et al.*^[105] performed experiments with two chimeric proteins (ephrinB2-Fc and EphB4-Fc) in an attempt to induce forward and reverse signaling in MSCs and OC progenitors respectively, and to observe their effects on OB/OC differentiation. Treatment of MSCs with ephrinB2-Fc induced forward signaling (as assessed by phosphorylation of the EphB4 receptor), and increased osteocalcin expression and matrix mineralization of OBs under osteogenic conditions^[105]. On the other hand, EphB4-Fc treatment had an inhibitory effect in OC progenitors (as checked by phosphorylation of ephrinB2 and downregulated expression of NFATc1 and reduced numbers of TRAP⁺ OCs), but no effect in MSCs. In the same line of reasoning, both ephrinB2-Fc and EphB4-Fc treatments in the SCID-hu model of MM, increased bone formation and OB number, but only EphB4-Fc reduced the number of OCs^[105] (since no expression of EphB4 was found in the OC lineage). These results supported the notion that activation of either forward or reverse EphB4/ephrinB2 signaling affects bone remodeling, resulting in increased bone formation. Moreover, the anti-myeloma effect of ephrinB2-Fc and EphB4-Fc treatments was evaluated in myelomatous bones, as assessed by the area of myeloma infiltration and the human Ig monoclonal component; however, only EphB4-Fc-treated SCID-hu mice showed a reduction in tumor burden. Since no effect was found for EphB4-Fc on MM cells *in vitro*, the anti-myeloma activity of this molecule was probably due to its modulatory effects on the BM environment (inhibition of osteoclastogenesis and neo-vascularization and stimulation of OB activity)^[105]. In this sense, upregulation of the endogenous expression of EphB4 in pMSCs or osteoprogenitor cells of myeloma patients (*e.g.*, by Wnt3a administration-since EphB4 receptor is a Wnt signaling target-or directly by EphB4-Fc treatment) could restore coupling of bone homeostasis and simultaneously reduce MM tumor burden in MM patients with bone affection.

Unfolded protein response pathway

Role in MBD: The endoplasmic reticulum (ER) is a membranous compartment present in eukaryotic cells which controls the synthesis, folding and trafficking of proteins to be secreted, as well as calcium storage and synthesis of membranes^[165]. Increased load of unfolded or misfolded proteins within the ER triggers a sophisticated mechanism known as the UPR, in an attempt to re-fold those proteins and to allow cellular adaptation to the imbalance in the protein folding homeostasis, referred as ER stress. Briefly, when unfolded proteins accumulate in the lumen of the ER, three coordinated pathways are activated by the transmembrane ER stress-sensor proteins, namely: PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme

1 (IRE1). The activation of these ER sensor proteins leads to the induction of a battery of transcription factors [orchestrated by ATF4, ATF6 and X box-binding proteins (XBP1s)] to promote the transcription of ER chaperone proteins and folding enzymes to increase the protein folding capacity of the ER, as well as proteins controlling the ER-associated degradation machinery, a mechanism by which misfolded proteins are retro-translocated into the cytosol for degradation by the proteasome. Alternatively, prolonged or severe exposure to ER stress may result in the cell undergoing apoptosis^[165-167].

Although ER stress often arises in pathological situations, specialized secretory cells such as hepatocytes, insulin-producing β cells of the pancreas, plasma cells and connective tissue cells (fibroblasts, chondrocytes and OBs) are particularly sensitive to ER stress induction in their normal development and function^[167]. Therefore, ER stress is essential during osteoblastogenesis through the three arms of the UPR: IRE1-XBP1s (promoting Osterix transcription)^[168], PERK-ATF4 (increasing osteocalcin and bone sialoprotein expression)^[169] and ATF6 (enhancing osteocalcin expression)^[170].

On the other hand, a recent study showed that MSCs from MM patients displayed elevated mRNA and protein levels of endogenous XBP1s (an active transcription factor involved in the clearance of unfolded/misfolded proteins) compared with dMSCs, suggesting that the IRE1-XBP1s pathway is activated in pMSCs^[171]. Experiments with overexpression of XBP1s in MSCs led to an increase in IL-6 and RANKL secretion and VCAM1 expression, which translated into an enhanced *in vitro* ability of MSCs to support MM cell growth and OC formation^[171]. Future studies exploring the expression and role of the other components of the UPR in MSCs would be of particular value for disrupting the protective effects of the MM microenvironment on tumor cell growth and bone destruction.

Therapeutic approaches: Plasma cells seem to be exquisitely sensitive to their core protein handling machinery due to the large amounts of immunoglobulins that these cells produce and secrete. The ubiquitin-proteasome pathway, linked to the UPR response to discard misfolded proteins, has become a potential drug target for the treatment of several tumors including MM^[172]. Bortezomib was the first-in-class proteasome inhibitor (PI) introduced in the clinical practice with a significant benefit in terms of anti-myeloma response rate and overall survival in both front-line and relapsed/refractory settings^[23]. Moreover, bortezomib not only reduces myeloma tumor burden, but directly restrains the progression of MBD, clinically evidenced by changes in bone turnover markers and radiologic data favouring bone healing^[173,174]. The beneficial impact of bortezomib on bone metabolism is not merely secondary to its anti-myeloma activity, but rather this agent directly targets the OC and MSCs/OB populations, both hampering osteoclastogenesis and OC resorption and promoting osteoblastogenesis and

OB function^[175-177].

A next-generation of PIs, including peptide boronic acid analogs (delanzomib and ixazomib), peptide epoxyketones (carfilzomib and oprozomib) and a β -lactone compound (marizomib) are have been developed to address the shortcomings of bortezomib treatment with the aim of retaining or improving bortezomib efficacy^[178]. Our group has investigated the potential bone anabolic and anti-resorptive effects of three of these second-generation PIs (*i.e.*, carfilzomib, its orally bioavailable analog oprozomib and ixazomib) in preclinical models of MM^[179,180]. *In vitro* studies evidenced that the three PIs were able to promote osteoblastogenesis and OB function (as assessed by augmented expression of bone formation markers, increased ALP activity and enhanced bone matrix mineralization), and to inhibit OC formation and resorption (through disruption of RANKL-induced NF κ B signaling together with reduced expression of integrin α V β 3 and F-actin ring disruption)^[179,180]. These effects were subsequently corroborated *in vivo*, since the three PIs provided a marked benefit in associated bone disease, sustained by bone anabolic and anti-resorptive activities^[179,180].

Moreover, the UPR was identified as a crucial pathway affected by PI-treatment of MSCs and osteoprogenitors resulting in enhanced osteoblastogenesis. Treatment of a BM-derived mesenchymal stromal cell line with PIs led to increased protein levels of the ER stress sensor IRE1 α . IRE1 α knockdown by siRNAs significantly diminished PI-enhanced mineralized bone formation, thus underscoring the crucial role of IRE1 α in the promotion of OB activity by these agents^[180]. In the same line, Nakamura *et al*^[181] recently reported a critical role for other ER stress mediator, ATF4, in bortezomib-mediated osteoblastogenesis, and suggested the optimization of a dose regimen for PI-treatment in order to obtain a maximal bone anabolic response (lower doses) avoiding the induction of pro-apoptotic pathways in the MSC-OB lineage (higher doses). It is thought that the adaptive threshold for myeloma plasma cells and OBs is quite different, since UPR induced by PIs (at the same range of doses) results in a cytotoxic effect in MM cells^[172] whereas promotes OB differentiation on mesenchymal precursors^[179].

Other therapeutical approaches on OB differentiation and function

Inhibition of tyrosine kinases: Several studies showed that the tyrosine kinase inhibitor imatinib mesylate directly promoted OB differentiation and stimulated osteogenic gene expression and mineralization, majorly by inhibiting PDGFR function on osteoprogenitors^[182,183]. This partially explained the increased trabecular bone volume and bone mineral density of long-term imatinib treated patients^[182]. As expected, subsequent studies with dasatinib, a second generation tyrosine kinase inhibitor with more potency and broader target profile, also evidenced enhanced OB differentiation from mesenchymal

precursors and promotion of OB activity both *in vitro* and *in vivo*^[184-187]. Preclinical anti-myeloma and anti-angiogenic efficacy of dasatinib was also reported, but attained at higher concentrations than those required for the bone anabolic effect of this drug, and which were cytotoxic for mesenchymal osteoprogenitors and OBs^[52,187]. Therefore, the latter suggests that if dasatinib is to be used for the treatment of MBD, it should be administered in combination with another anti-myeloma agent.

MSC cytotherapy for MBD: MSCs have been considered as excellent candidates for cytotherapy studies due to their immunoprivileged nature, their ability to migrate to damaged and tumor tissues, together with their capacity to differentiate to several mesenchymal lineages^[188]. Some concerns have been raised, however, for the use of MSCs in the treatment of MBD since interacting BM MSCs have been shown to support the proliferation, survival, migration and chemotherapeutic resistance of MM cells^[27,30,31,189]. When genetically-modified human MSCs overexpressing OPG were administered to a model of medullary myeloma with associated bone disease, they reduced OC activation and restored bone volume^[190]. Moreover, human placenta or BM derived MSCs were intrabone or systemically administered in the severe combined immunodeficiency (SCID)-rab model, and found to promote bone formation, prevent MM-induced bone disease and tumor growth^[191,192].

Specific delivery of RNAi-based anabolic therapy: The use of siRNA-based bone anabolic therapies in the clinic has been hampered by lack of specific targeting to bone-formation surfaces. The (AspSerSer)₆ has been found to be a targeting moiety for bone formation sites *in vivo*, due to its great affinity to lowly crystallized hydroxyapatite and amorphous calcium phosphonate. Systemic administration of (AspSerSer)₆-labeled liposomes containing osteogenic siRNAs has been shown to be an effective therapeutic approach in a model of osteoporosis^[193] and its use may also be explored in MM to promote OB function.

CONCLUSION

In conclusion, MSCs from myeloma patients are important contributors to the development of osteolytic lesions because of their reduced osteogenic potential and because they also promote OC differentiation and/or activity at various levels (increased RANKL/OPG ratio, augmented activin A secretion, uncoupled ephrinB2/EphB4 axis and because of increased Wnt5a production). We have reviewed current therapeutic approaches targeting components of signaling pathways involved in the osteogenic differentiation and maintenance of OB activity. It is likely that due to the multifactorial character of MBD, combinations of both anti-resorptive and bone-anabolic agents may be required for an effective restoration of bone homeostasis and for an additional anti-myeloma

benefit.

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