Bone marrow niche dysregulation in myeloproliferative neoplasms

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ABSTRACT

The bone marrow niche is a complex and dynamic structure composed of a multitude of cell types which functionally create an interactive network facilitating hematopoietic stem cell development and maintenance. Its specific role in the pathogenesis, response to therapy, and transformation of myeloproliferative neoplasms has only recently been explored. Niche functionality is likely affected not only by the genomic background of the myeloproliferative neoplasm-associated mutated hematopoietic stem cells, but also by disease-associated 'chronic inflammation', and subsequent adaptive and innate immune responses. 'Cross-talk' between mutated hematopoietic stem cells and multiple niche components may contribute to propagating disease progression and mediating drug resistance. In this timely article, we will review current knowledge surrounding the deregulated bone marrow niche in myeloproliferative neoplasms and suggest how this may be targeted, either directly or indirectly, potentially influencing therapeutic choices both now and in the future.

Introduction

'Philadelphia chromosome negative' myeloproliferative neoplasms (MPN) are a group of relatively rare hematologic diseases characterized by a clonal proliferation of blood cells, most commonly secondary to acquired hematopoietic stem cell (HSC) mutations that directly or indirectly induce upregulation of the JAK-STAT pathway. The 2016 World Health Organization consensus recognizes the following categories under the MPN classification: chronic myeloid leukemia *BCR-ABL*$^{+}$, chronic neutrophilic leukemia, essential thrombocythemia (ET), polycythemia vera (PV), primary myelofibrosis (PMF) (which includes both the prefibrotic/early stage and overt fibrotic stage), chronic eosinophilic leukemia not otherwise specified, and MPN-unclassifiable.[1] Recent analyses estimate the incidence rates of the classical 'Philadelphia negative'-MPN PV, ET and PMF as 0.7-2.6 cases, 0.34-1.7 cases and 0.1-1.0 cases per 100,000 patients-per-year, respectively.[2] Median age at diagnosis is variable, estimated at between 69-76 years for PMF, 65-74 years for PV, and 64-73 years for ET, although MPN has been described in many younger patients and can manifest at any age.[3] Regarding clinical features, these disorders produce a markedly heterogeneous clinical phenotype. For example, in PMF, patients may range from those lacking any discernible symptomatology to those describing debilitating constitutional symptoms, abdominal discomfort due to splenomegaly, bone pain, and symptomatic anemia, amongst others. The most common complications linked to MPN are thrombotic and hemorrhagic events and an inherent risk of leukemic transformation that is dependent upon the underlying MPN phenotype; this risk is higher for PMF (estimated at a range of 10-20% in the first 10 years from diagnosis) and much lower for both PV (2.3%) and ET (1%).[4] These figures reflect historical data, and it is likely that with the move away from consecutive cytotoxic therapeutic approaches, blastic transformation rates may well be lower.

Following the pivotal reports in 2005 by four different research groups concerning the prevalence of the acquired somatic mutation *JAK2* V617F in MPN, knowledge of the mutational landscape continues to expand.[5-8] The *JAK2* V617F mutation is present in approximately 98% of PV patients, and has an estimated incidence in ET and MF of 50% and 60%, respectively. Mutations in the thrombopoietin receptor (*MPL*) are described in approximately 3% of ET and 5-8% of MF cases, whereas mutations in calreticulin (*CALR*) are evident in 25% of ET and 30% of MF

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patients.[9-11] Up to 20% with ET and up to 15% of patients with PMF lack detectable mutations in these three genes, as assessed by conventional assays; such patients are termed 'triple negative'.[12-14] Lastly, comprehensive genomic analyses have revealed the presence of additional mutations that can appear before, simultaneously, or following the so-called 'driver mutations' (*JAK2*, *CALR* and *MPL*) in PMF and can affect a wide-array of key genes, such as those involved in epigenetic regulation (*TET2*, *ASXL1*, *EZH2*), splicing (*SRSF2*, *U2AF1*), and cellular signaling (*SH2B3*, *PIAS3*), some of which also affect prognosis.[15]

Multiple factors contribute to the dynamic complexity of the bone marrow niche in MPN, such as the inherent increase in pro-inflammatory cytokines, skewed adaptive and innate immune responses, and 'cross-talk' between the normal and mutated-HSC, endosteal and vascular niches and extracellular matrix. In this review, we will summarize current knowledge concerning bone marrow niche composition in health and how it differs in MPN. Likewise, as we gain further understanding of these dynamics, we will explore what potential there is for therapeutic intervention specifically targeting the niche to provide clinical benefit.

Overview of the bone marrow niche in health

It is evident that much remains to be elucidated concerning the dynamic BM microenvironment, both in normal physiological and disease states. Traditionally, the niche is conceived as being divided into individual compartments with bi-directional 'cross-talk' between the well-defined spatially organized HSC, multiple surrounding permissive cells, and the extracellular matrix (Figure 1). This concept was first delineated by Lord *et al.* and Schofield more than 40 years ago.[16,17] The accumulated evidence demonstrates that multiple additional factors can influence, either directly or indirectly, this niche, such as microenvironmental oxygen tension variations, sympathetic nervous system activity, and endocrine signaling such as the estrogen pathway.[18,19]

Simplistically, the endosteal niche, which is highly vascularized, is considered to be where the 'potent and primitive' HSC reside, rich in long-term (LT)-HSC. The pivotal paper by Nilsson *et al.* demonstrated that, following HSC 'transplantation' in mice, HSC 'homed' to the endosteum, with subsequent maintenance and promotion of HSC development.[20] Later studies by Celso *et al.* and Xie *et al.* showed similar results.[21,22] This niche is formed predominantly by osteoblasts (which mainly line the endosteal bone surface), osteoclasts, and a specific osteoblastic subpopulation known as spindle-shaped N-cadherin$^+$ osteoblasts (SNO cells). Within the niche, both BM mesenchymal stem cells (BMSC) and the N-cadherin$^+$ cell population play an indispensable role in HSC maintenance. Each of these cell populations and their interactions with each other (and with the HSC population) ultimately determines maintenance and proliferation of the hematopoietic stem/progenitor cell pool and downstream lineage differentiation.

Osteoblasts are derived from multipotent BMSC where-

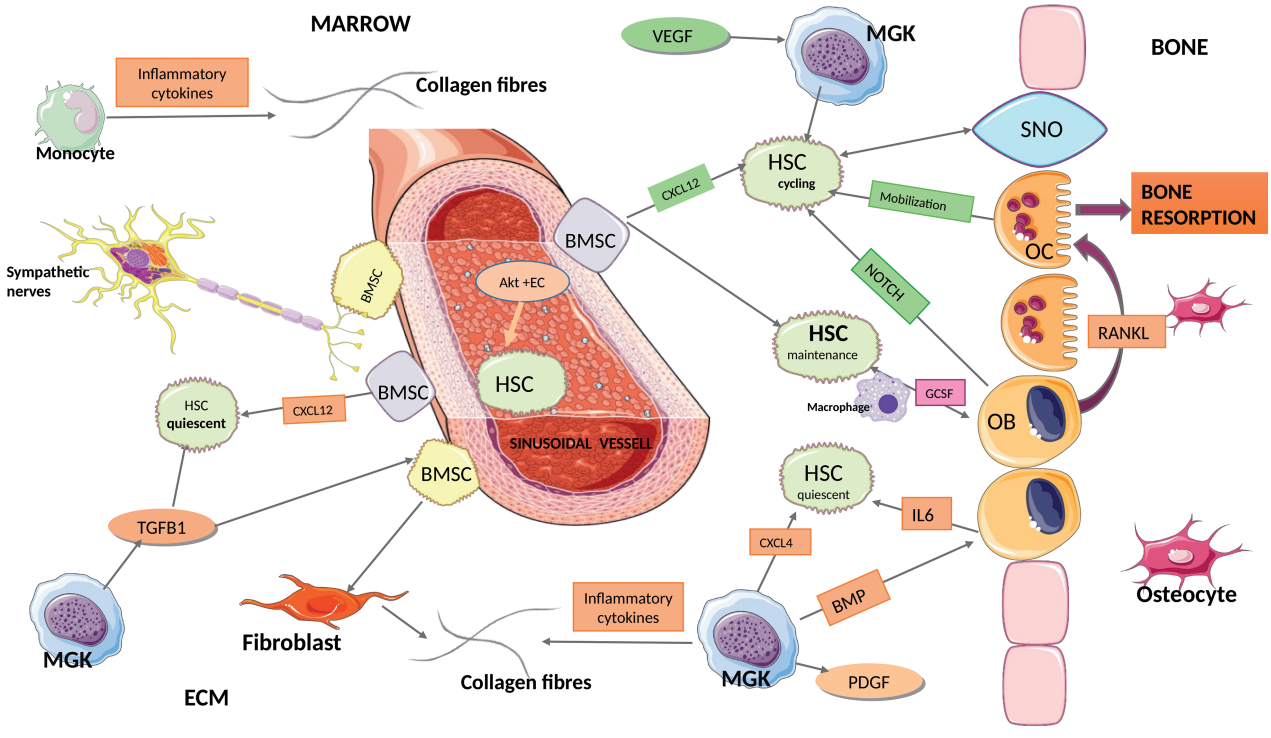

Figure 1. Hematopoietic stem cell (HSC) cycling is regulated by osteoclast (OC), osteoblast (OB) by NOTCH expression and spindle-shaped N-cadherin$^+$ osteoblast (SNO) cells. Different bone marrow mesenchymal cells (BMSC) participate in HSC regulation, such as CXCL12-abundant reticular (CAR) cells that stimulate HSC cycling by producing CXCL12. On the other hand, megakaryocytes (MGK) are activated by vascular endothelial growth factor (VEGF) and participate in the activation of HSC cycling. HSC maintenance is regulated by a reciprocal communication between OB and HSC. CAR cells also participate in this regulation. Finally, HSC quiescent is regulated by both the bone marrow niche (BMN) and the extracellular matrix (ECM), thus, OB and MGK interact in this control. The growth factor TGFb1 and, indirectly, the platelet-derived growth factor (PDGF) (by activating MGK) would participate in the HSC quiescent. Nestin$^+$ BMSC regulate the CXCL12 production and the sympathetic nerves contribute to BMN functionality. EC: endothelial cell.

as osteoclasts originate from CD34+ hematopoietic cells.[23] Early work showed that the two populations were functionally interdependent, e.g. osteoblasts constitutively expressed G-CSF and CD34+ hematopoietic cells enhanced IL-6 production by osteoblasts hence stimulating further investigations into these interactions.[24,25] It is accepted that, in general, osteoblast functionality plays an important role in HSC maintenance, in particular with regard to HSC trafficking. Regulatory roles depend upon osteoblastic differentiation stage, whereby the immature osteoblast progenitor population influences HSC maintenance/proliferation and the mature osteoblasts modulate their differentiation.[26] In murine models, Calvi *et al.* demonstrated that osteoblastic cells influenced HSC functional capacity through NOTCH activation, and it was suggested that HSC are located in close physical proximity to SNO cells, although the role of N-cadherin in these 'cell-cell' interactions remains under debate.[27,28] Multiple soluble factors derived from the osteoblast population play a role in HSC pool fate, including CXCL12, angiopoietin-1 and osteopontin, in addition to multiple other cytokines/chemokines.[29] CXCL12 is a CXC chemokine produced by stromal cells, the major source is from BMSC but also by osteoblasts influenced by circadian oscillations and there is evidence that CXCL12/CXCR4 signaling plays a pivotal role in modulation of HSC trafficking.[30] In addition, the acidic matrix glycoprotein osteopontin is produced by pre-osteoblasts and osteoblasts and negatively regulates both HSC pool 'size' and egress.[27,31] Of note, osteoblasts play an additional role in T lymphopoiesis, whereby DLL4 on the cell surface is pivotal for the production of 'thymic seeding' T progenitors.[32]

As introduced above, osteoclasts derived from monocyte-macrophage lineage cells in the presence of receptor activator of nuclear factor-κB-ligand and macrophage colony-stimulating factor play multiple regulatory roles within the niche in addition to their bone resorption characteristics.[33] Kollet *et al.* demonstrated that, through endosteal component degradation, osteoclasts can promote HSC mobilization.[34] However, the literature also includes contrasting evidence concerning their exact role within the HSC niche, which is most likely context dependent. For example, using the osteopetrotic 'OC/OC'-murine model, absence of functional osteoclasts induced a defective HSC niche with increased mesenchymal precursors, impaired osteoblast development, and resultant aberrant HSC homing.[35] However, Miyamoto *et al.* evaluated hematopoietic activity in three murine models without osteoclasts and showed that HSC mobilization was, in fact, similar, or indeed higher, than that of wild-type animals, suggesting that osteoclasts are not essential for HSC mobilization.[36] Lastly, there is a great deal of cross-talk and interdependency between the osteoblast and osteoclast populations. It has also been shown that osteoclasts can act as antigen presenting cells and activate both CD4+ and CD8+ T cells.[37]

The vascular niche is the other pivotal component of the bone marrow niche and broadly encompasses thin-walled sinusoidal blood vessels, arterioles, transition zone vessels, endothelial cells (that also produce *CXCL12*), stromal elements, fibronectin, and collagen. Functionally, interactions between these perivascular elements determine both HSC dormancy/expansion and migration properties. By way of example, Akt activation in endothelial cells following mTOR recruitment induces upregulation of specific angiocrine factors which promotes expansion of cells with LT-HSC repopulation capacity.[38] Cell-cell contact also appears key. For example, E-selectin expression by endothelial cells in the vascular niche can regulate HSC dormancy and HSC proliferation.[39] Moreover, the vascular niche provides an environment rich in multiple pro-inflammatory chemokines/cytokines, which contribute to niche maintenance. The so-called 'hypoxic-gradient' plays a major role in spatial HSC location within the vascular niche. In this way, quiescent HSC preferentially locate to small arterioles, unsheathed by rare NG2-pericytes, predominantly found in the endosteal bone region. HSC tend to exhibit a strong hypoxic profile, promoting quiescence, irrespective of localization.[40,41] Likewise, in those situations whereby the sinusoids are under stress induced by, for example, myeloablative chemotherapy or irradiation, the endosteal niche becomes an important host of HSC and promotes quiescence.[42]

Bone marrow mesenchymal stem cells encompass a diverse group of cells with multipotent differentiation and self-renewal properties indispensable for HSC maintenance. BMSC can interact in a pleotropic fashion with HSC including direct cell-cell interaction and by the altered production of cytokines and cell markers.[43] CXCL12-abundant reticular cells are a subpopulation of BMSC that produce *CXCL12* and regulate the maintenance and quiescence of the HSC pool. Multiple other cell types, outside the remit of this review, contribute to the regulation of the niche including macrophages which modulate the CXCL12 pathway promoting HSC retention, and monocytic-lineage cells which regulate osteoblasts, facilitate HSC mobilization and also encourage a pro-inflammatory cytokine environment.[31] Concerning the role of the sympathetic nervous system, Mendez-Ferrer *et al*. demonstrated that circulating HSC and their progenitors exhibit marked circadian fluctuations regulated by noradrenaline secretion by the sympathetic nervous system.[30,44] Adrenergic signals *via* the beta-(3)-adrenergic receptor mediate downregulation of CXCL12 and there is a close association between so-called Nestin+ BMSC and adrenergic nerve fibers of the SNS with resultant regulation of HSC functionality and egress. This neuro-hematopoietic axis is exploitable as a therapeutic target, as will be discussed later.

With regard to the extracellular matrix (ECM), this is a non-cellular space that supports the integrity, proliferation and 'elasticity' of the entire bone marrow niche. It acts as a pivotal HSC 'regulator' and ECM-related components critically determine the functionality of HSC lodged within its confines.[45] The 'core matrisome' is a complex structure that consists of up to 300 protein components, enzymes, and growth factors (e.g. *TGFβ1*, *PDGF* and *VEGF*), and overall functionally drives maintenance of the HSC pool.

Bone marrow niche / extracellular matrix disruption in myeloproliferative neoplams

Myeloproliferative neoplasm-associated bone marrow niche homeostasis is disrupted on many levels which collectively can promote the proliferation, survival and migration of mutated MPN HSC. As described by Mead and Mullaly, both 'host' and extrinsic factors can influence MPN HSC behavior, and as the malignant clone expands,

this favors MPN HSC growth over normal HSC expansion.[46] Mullally *et al.* have described that *JAK2*V617F-LT-HSC are capable of initiating and promoting the disease, giving a clonal advantage to dominate the niche against WT cells. In addition, mutated LT-HSC could induce fibrotic changes in the bone marrow niche in WT transplanted mouse models.[47] Furthermore, Lundberg *et al.* proved elevated *JAK2* expression levels impact negatively on the repopulation capacity of LT-HSC and will promote the disease expansion.[48] Finally, acquisition of other mutations, such as *TET2* deletions in *JAK2* V617F-LT-HSC, gives a clonal advantage favoring the disease progression.[49]

Regarding the osteoblast-osteoclast axis, it is clear that aberrant functionality of the endosteal osteoblastic niche plays an important role in MPN maintenance and progression. For example, it has been shown in murine models that osteoblast expansion is functionally altered in MPN and promotes the development of fibrosis.[50] Over time, disease-driven remodeling of the endosteal niche occurs, leading to a self-reinforcing 'leukemia-niche' with impaired normal hematopoiesis. Several mechanisms, as suggested by the authors, are implicated in dysregulated osteoblastic expansion, such as overstimulation of MSC driving production of functionally impaired osteoblasts, resultant direct 'cell-cell contact' with mutated MPN HSC, and up-regulated production of TPO, CCL3, TNF-β and Notch, thus inducing a chronic state of 'inflammation'.[51,52] Expression of *CXCL12*, essential for controlled HSC mobilization, as discussed above, is reduced due to this abnormal osteoblast functionality. Moreover, Spanoudakis *et al.* recently showed that monocytes derived from *JAK2* V617F (heterozygote)-MPN cells had enhanced osteoclast-formation ability compared to wild-type monocytes. An enriched osteoclast environment additionally favors MPN-associated mutated cell population proliferation and survival.[53] Collectively, these findings highlight the importance of the osteoblast-osteoclast axis and its disruption in MPN and how this may be therapeutically exploited.

Clonal-MPN cells additionally have the capacity to disrupt the finely balanced vascular niche. By way of example, *JAK2*-mutated endothelial cells promote the abnormal proliferation and survival of mutated-HSC whilst inhibiting normal HSC functionality. This occurs secondary to alterations within the CXCL12 and stem cell factor pathways. Vascular endothelial growth factor (VEGF), produced by both the endothelial cells and the 'mutated'-HSC, supports neo-angiogenesis and increases both survival and proliferation of these HSC. Therefore, a self-reinforcing vascular niche also develops as a favorable environment for MPN mutated-HSC.[54] Hypoxia-induced signaling also appears to influence HSC behavior by encouraging quiescence and influences long-term repopulating activity.[55] Utilizing transgenic MPN-murine-models, it has been shown that downregulation of HIF-1α induces an enhanced MPN phenotype reflected by increased leukocytosis and significant splenomegaly.[56]

Importantly, BMSC appear pivotal to the development and maintenance of the MPN phenotype. BMSC promote the abnormal expansion of osteoblasts as inflammatory 'myelofibrotic' cells; a conversion mediated by dysregulation of inflammatory signaling pathways with excess production of *TGF-β1*, *Notch*, IL-6, IL-1β and TNF-β and secondary to direct contact between the clonal MPN-HSC and BMSC.[57] Schneider *et al.* has demonstrated that Gli1$^+$-BMSC participate in the activation of myelofibroblasts.[58] Ultimately, the overproduction of inflammatory 'myelofibrotic cells' contributes to progressive BM fibrosis observed in the advanced stages of these diseases.[31] At the same time, excessive osteoblast production perpetuates clonal-MPN cell proliferation.[50] Ramos *et al.* recently demonstrated that BMSC derived from MPN patients (mainly PV and ET) present an altered gene and immunophenotypic expression profile compared to those derived from healthy donors. In PV, BMSC show an overexpression of genes involved in cell differentiation and migration such as *MYADM*, *Angiopoietin-1* expression and decreases in *CXCL12*; that are associated with 'cross-talk' between the mutated-HSC and BMSC.[59] Angiopoietin-1 participates in both angiogenesis and the quiescence of the HSC. Osteoblast production of angiopoietin-1 facilitates interaction with Tie-2, resulting in increased adhesion of HSC to osteoblasts within the niche.[60]

More recently, other studies have explored the neuro-hematopoietic axis, demonstrating that the sympathetic nervous system influences bone marrow niche regulation. Arranz *et al.* elegantly showed that a local neuropathy occurs in MPN-BM, with a reduction in both Nestin$^+$ BMSC and CXCL12 expression and promotion of *JAK2*$^+$ HSC expansion. The relationship, if any, between this local neuropathy and the patient's symptomatology and phenotype is still not clear, although it has been described as a possible therapeutic target, as discussed below.[51,61] Lastly, an increased understanding of the role of estrogen signaling is emerging. In normal HSC, it has been shown that estrogen receptor stimulation *in vivo* led to an increased proliferation of quiescent LT-HSC and tamoxifen induced apoptosis of short-term HSC and multipotent progenitors. In chronic MPN, *JAK2*-mutated murine models, tamoxifen led to preferential restoration of apoptosis in mutated-HSC.[62]

Regarding the ECM, clonal-HSC demonstrate dysregulated 'cross-talk' with augmented levels of cytokines and growth factors within the ECM, enhancing both disease establishment and progression. In MF, there is an intensified deposition of ECM components. Thus, highly fibrogenic *TGFβ1* activates fibrosis deposition by two main routes: (i) skewing BMSC activation towards fibroblastic and osteoblastic genesis; and (ii) an augmented production of collagen. Moreover, *TGFβ1* levels are intimately linked to megakaryocytic activity.[63] Additional growth factors such as PDGF (platelet derived growth factor) and VEGF play a pivotal role in this unbalanced ECM-MPN marrow niche communication. *PDGF* promotes fibrogenesis by activating both megakaryocytes and fibroblasts whereas VEGF contributes towards megakaryocytic maturation and migration.

Other relevant modifiers of the MPN-associated ECM are matrix metalloproteinases (MMP) and Lysyl Oxidase (LOX).[64] In MPN, Wang *et al.* demonstrated downregulation of MMP, supporting the accumulation of ECM substances. Focusing on MF, this study demonstrated decreased MMP3 levels which inversely correlated with increased fibrosis and enhanced expression of tissue inhibitors of the metalloproteinases.[65] Both *MMP2* and *MMP9* are highly expressed in MPN patients and are reduced after treatment with JAK inhibitors.[66] LOX is a potent regulator of fibrogenesis and is involved in collagen cross-linking. Previous studies have demonstrated a link between deregulated megakaryocytic production of PDGF, TGF-β1 and IL-1β and augmented LOX activity,

with resultant collagen accumulation in MF.[64] Tadmor *et al.* demonstrated that, in MF, all LOX members genes are activated compared to the pattern seen in either ET or PV; postulating that this occurred during fibrogenesis. Of interest, *LOXL1* was only expressed in MF, suggesting a relationship with advanced fibrosis.[67]

In summary, it is evident that the bone marrow niche is profoundly dysregulated on multiple, yet interacting levels, in MPN (Table 1). Mutated-MPN-HSC activate a cascade of dysregulated signaling and abnormalities in multiple key players across the niches, compromising functionality of both the osteoblastic and vascular niches and ECM. Consequently, these irregularities promote the abnormal proliferation inherent to these disease states. Although our knowledge of the MPN-associated dysregulated niche has increased in recent years, further studies are required to help understand how this niche can be successfully targeted in therapy.

Direct or indirect targeting of the bone marrow niche in myeloproliferative neoplasms: is there a role?

To date, the only curative treatment for MF remains allogeneic stem cell transplantation, although this is not a feasible option for many due to age, risk profile, co-morbidities, or lack of a suitable donor.[68] Many clinicians have familiarity with the JAK1/JAK2 inhibitor (JAKi) ruxolitinib (Novartis, Switzerland), currently the only licensed agent in MF; which has demonstrated improvement in disease-related symptomatology, induced reductions in spleen size, and prolonged the overall survival (OS) in many MF patients.[69,70] Of note, ruxolitinib has also been used in both PV and ET, particularly in the setting of hydroxycarbamide resistance or intolerance.[71-74] Many other agents have entered the clinical trial arena to address the multiple unmet needs, particularly when individuals are failing or become intolerant of standard therapies, including novel JAKi, BET-inhibitors, BCL-2 inhibitors, HDAC inhibitors, telomerase inhibitors, and MDM2 inhibitors.[75-81] Regarding novel JAKi, pacritinib (which is also a FLT3 inhibitor) has been investigated in MF patients with thrombocytopenia showing improvements in splenic responses within both the PERSIST-I and -II studies.[82,83] The drug was on clinical hold from 2016 due to concerns regarding cardiac toxicity, but following the Food and Drug Administration (FDA) review and removal of the clinical hold, the PAC203 study has now fully recruited and further studies are planned. Momelotinib, a JAK1/2 inhibitor, demonstrated anemia and transfusion responses in both the SIMPLIFY-1 and 2 clinical trials but it failed to meet the pre-defined clinical end points, although some patients demonstrated symptom, spleen and anemia responses.[84,85] This agent will be compared on a randomized basis to danazol in the upcoming MOMEMTUM study. Fedratinib (Inrebic®, Celgene, USA) is a more selective JAKi than ruxolitinib; both JAKARTA-1 and 2 trials showed this agent to have significant efficacy in MF

Table 1. Bone marrow niche in health and myeloproliferative neoplasm.

	Bone marrow niche in health	Bone marrow niche in MPN
Endosteal niche: Osteoblasts, Osteoclasts and spindle-shaped N-cadherin$^+$ osteoblast cells	• Maintenance, proliferation and differentiation of HSC. • **Osteoblasts**: - Interact with CD34$^+$HSC by expressing GSCF and IL6. - Regulate HSC trafficking by expression CXCL12, angiopoietin-1 and osteopontin. • **Osteoclasts**: - Regulatory role. - Bone resorption. - Promote HSC mobilization. • **SNO cells**: - Cell-cell contact with HSC.	Self-reinforcing of clonal cells. - **Osteoblasts**: - Abnormal OB expansion due to overstimulation by BMSC. - Overproduction of inflammatory cytokines. - Promotion of fibrogenesis. - Reduction in CXCL12 expression. - **Osteoclasts**: - Abnormal stimulation by JAK2 positive monocytes. - Favoring survival of clonal HSC. - **SNO**: - No clear role described yet.
Vascular niche: sinusoidal blood vessels, endothelial cells, stromal elements, fibronectin and collagen	• Regulate HSC migration . • Expression of e-selectin by endothelial cells. • Production of inflammatory chemokines and cytokines. • Regulation of hypoxia status . • **BMSC-CAR cells express CXCL12**- maintenance and quiescence HSC. • **Macrophage-** modulate CXCL12 pathway. • **Monocytes**- regulate osteoblasts, promote pro-inflammatory cytokine environment.	• Alteration CXCL12 pathway: upregulated in JAk2$^+$ endothelial cells, downregulated BMSC- promotes expansion mutated HSC. • Clonal endothelial cells support neo-angiogenesis by VEGF production. • Increase survival mutated HSC. • Alteration of HIF-1α and **hypoxia status**. • BMSC promote expansion of osteoblasts by cell contact and excessive TGFβ1, Notch and cytokines. • Overproduction of **inflammatory markers** produce fibrosis.
Sympathetic nervous system	• Noradrenaline secretion regulate HSC circulation and functionality .	• Local **neuropathy** by reduced expression of Nestin$^+$ and CXCL12 promoting HSC expansion.
Extracellular matrix	• Integrity, proliferation and elasticity of BMN. • Presence of growth factors (TGFβ-1, PEGF, VEGF) to maintain HSC.	• Increase **cytokines** and **growth factor** levels (TGFβ-1, PEGF, VEGF) promotes fibrogenesis. • VEGF contributes to MK maturation and migration. • Decrease of MMP and increase of LOX favoring fibrosis.

SNO: spindle-shaped N-cadherin$^+$ osteoblasts; HSC: hematopoietic stem cells; BMSC: bone marrow mesenchymal cells; VEGF: vascular endothelial growth factor; PDG: platelet-derived growth factor; TGFβ1: transforming growth factor beta; HIF-1α: Hypoxia inducible factor 1-alpha; MK: megakaryocytes; MMP: matrix metalloproteinases; LOX: Lysyl Oxidase.

Table 2. Therapies targeting directly or indirectly the bone marrow niche.

Drug	Target	Disease/update results	Reference	
PACRITINIB	JAK2/FLT3 inhibitor	MF with thrombocytopenia Spleen responses 18% volume reduction ≥ 35% – PERSIST I & II trials	82 83	Clinical trials ongoing
MOMELOTINIB	JAK1/2 inhibitor	MF Anemia and transfusion responses in addition to spleen and symptoms: - SIMPLIFY 1 (momelotinib *vs* ruxolitinib): 66.5% transfusion independent at week 24. 26.5% reduction of spleen volume ≥ 35% - SIMPLIFY 2 (momelotinib *vs* BAT): 7% spleen volume ≥ 35%	 84 85	Planned MOMENTUM Study
FEDRATINIB	Selective JAK 2 inhibitor	MF (ruxolitinib resistant or intolerant) Spleen response and symptoms improvement. - JAKARTA-1: reduction spleen volume ≥ 35%, 36% (400mg) and 40% (500-mg compared with placebo. - JAKARTA-2: Second line study 55% of patients achieved spleen volume ≥ 35%. Recent hold due to Wernicke's encephalopathy removed. Approved by FDA in 2019.Fibrosis grade-reduction in 44% (8/18) patients after cycle 6.	 86 87 99	Clinical trials ongoing/ planned
NAVITOCLAX	BCL-2 inhibitor	MF failed ruxolitinib. Clinical trial ongoing in combination with ruxolitinib	No data published available yet	Clinical trial ongoing
PANOBINOSTAT	Histone deacetylase inhibitor	MF -combination with ruxolitinib 36% achieved overall response by IWG-MRT Median spleen reduction was 34% 6.8% decrease in JAK2 allele burden	119	Clinical trial ongoing – expansion phase.
IMETELSTAT	Telomerase inhibitor	MF and ET. - MF clinical trial: Pilot- 33 MF patients- complete and partial response 7(21%) median response 18m. Bone marrow fibrosis reversal in 4 with CR. Molecular response 3 / 4 patients. - Phase-II study: OS 19.9 months in low dose and 29.9 months in higher dose. 93% patients discontinued study (25% due to adverse events). Update data compared with real world showed OS was 30.69 months. Significant myelosuppression and hepatic toxicity in some. ET clinical trial: 16/18 (89%) achieve complete hematological response. And 7/8 molecular response with allele burden reduction between 15-66%. Bone marrow fibrosis reduction of at least 1 grade was described in 4/6 (67%)	 92 93 94 91	Recruitment suspended
IDASANUTLIN	MDM2 inhibitor	PV, ET and MF Alters the MDM2/p53 interaction. PV/ET clinical trial: 58% response on monotherapy and 50% for combined therapy after 6 cycles. Combined with BET inhibitor in MF: Reduction of hematopoietic colony formation CD34+ in MF. Reduction in pro-inflammatory cytokines (decreased the levels of IL-8 in MF MNC by 50% (P=0.0003))	95 96	Clinical trial ongoing
BET inhibitor		Combined with ruxolitinib in MF MF- inhibition of the NF-KB pathway, reduction of inflammatory cytokines. Reduction of bone marrow fibrosis.	120	Clinical trial ongoing

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RUXOLITINIB	JAK inhibitor	MF Association between reduction in fibrosis grade and cytokines reduction (AUC=0.85939, *P*=0.0134)	69,97	Single agent studies closed
TAMOXIFEN	Estrogen receptor antagonist	MPN Reduction of JAK2 and CALR allele burden ≥50% at 24 weeks mutation	No data published as yet.	Clinical trial ongoing
PRM-151	Recombinant human pentraxin-2 analogue	MF Fibrosis grade reduced in 25 patients by ≥ 1 initial study. In extension phase, improvements in 71% and 44% of individuals with grade 2&3 fibrosis at baseline. Updated results showed 28% decrease in fibrosis.	100 101 102	Study closed
IPI926	Hedgehog inhibitor	MF No improvements in fibrosis	103,104	Study discontinued
SONIDEGIB (LDE225, NOVARTIS, SWITZERLAND)	SMO receptor antagonist	MF in combination with ruxolitinib Spleen and symptoms responses- 65% of pts achieved a ≥ 50% reduction spleen and 9 pts had resolution of splenomegaly. Reduction of bone marrow fibrosis	105	-
PIRFENIDONE	Antifibrotic agent	MF *In vitro*- reduced both fibroblast activity and ECM components *In vivo*- minimal clinical benefits.	106	-
FRESOLIMUMAB	Monoclonal antibody against TGF-β	MF No relevant changes in fibrosis	107	Clinical trial ongoing
GALUNISERTIB (LY2157299)	TGF-β receptor I kinase	MF Reductions in fibrosis in murine models (*P*=0.02)	108	-
SIMTUZUMAB	Monoclonal antibodies against the Lysyl oxidase like-2	MF- monotherapy and combination with Ruxolitinib Reduced fibrosis score at 24 weeks in 36.7%. Overall limited efficacy	109	-
AZACITIDINE DECITABINE	Hypomethylating agents	MF- high risk and accelerated/blastic phase. Combined with ruxolitinib- 57% fibrosis reduction and spleen responses observed. MF-Blastic phase Increased OS.	110 121	Clinical trial ongoing
MIRABEGRON	Oral β-3 adrenergic agonist	MPN- *JAK2* V617F positive Increase in Nestin$^+$ BMSC (week 24 was 3.52/mm^2 [95%CI: 1.65-5.39]) Mild reduction in fibrosis 1.0 (interquartile range 0–3) to 0.5 (interquartile range 0–2) (*P*=0.01)) Modulation of megakaryocyte clustering	112	
BEVACIZUMAB	Anti-VEGF agent	MPN No significant benefit	113	Study closed
VATALANIB	Anti-VEGF receptors	MF 3% CR and 17% clinical improvement 3/7 patients have bone marrow fibrosis reduction	114	-
BORTEZOMIB	Proteasome inhibitor indirectly inhibits HIF1- α	MF 9/15 patients reduced in the bone marrow vessel density.	118	-

MF: myelofibrosis; CR: complete response; OS: overall survival; PV: polycythemia vera; ET: essential thrombocythaemia; MPN: myeloproliferative neoplasm; CI: confidence interval; MNC: mononuclear cells; BMSC: bone marrow mesenchymal stem cells; VEGF: vascular endothelial growth factor; HIF1-α: hypoxia-inducible factor.

patients, either JAKi naïve or those with resistance or intolerance to ruxolitinib in terms of spleen response and improvement of symptoms.[86,87] The FDA placed the drug on hold due to several cases of Wernicke's encephalopathy, but after further investigations, this clinical hold has now been removed and the agent has recently been approved by the FDA in both the first-line and successive treatment settings in MF.[88]

Multiple alternative pathways are being investigated as potential therapeutic targets for MPN patients; ongoing trials are investigating the use of BET inhibitors, PI3K inhibitors, HDAC inhibitors, BCL-2 inhibitors and MDM2 inhibitors, to name only a few (Table 2). BCL-2 protein inhibitors (BH3-mimetics) have already been investigated in lymphoid and other myeloid disorders.[89] Curiously, these agents trigger remarkable megakaryocytic and

mature platelet apoptosis.[90] A phase II clinical trial of the BCL-2 inhibitor navitoclax in combination with ruxolitinib in MF patients is ongoing and results are awaited with interest. Other emerging agents indirectly targeting the marrow niche are the telomerase inhibitor, imetelstat (GRN163L, Geron, USA), which has been investigated in both ET and MF patients and initial clinical results are encouraging.[91,92] Unfortunately, subsequent studies in MF revealed limited overall spleen responses, significant myelosuppression, and in some patients, hepatic toxicity; therefore, recruitment for the study was suspended (Geron Corporation, June 7 2017, press release). However, the data presented in ASH-2018 showed complete and partial responses in 21% cases, with bone marrow fibrosis reversal in four cases with complete response (CR); OS after 27.4 months of treatment was 19.9 months in the low-dose therapy arm and 29.9 months in the higher-dose therapy arm.[93] Unfortunately, 93% of patients discontinued the study, and of these, 25% were due to adverse events. Recent data presented at the EHA congress 2019 demonstrated that when survival data from the 9.4 mg/kg imetelstat-cohort, from the phase-II trial in ruxolitinib relapsed/refractory higher-risk MF, was compared to 'real world' data for this group of patients treated with best alternative therapy (BAT), there was a potential OS advantage (30.69 months in the imetelstat group, HR 0.35 months, *P*<0019); although this was an unweighted analysis and had inherent comparative limitations.[94] Geron plans to conduct an up-dated phase II trial meeting with the FDA to determine if there is a regulatory path forward for imetelstat in MF in 2020.

MDM2 inhibitors alter the MDM2/p53 interaction, in order to restore p53 functionality/activity. Preliminary data from early phase studies in PV and ET demonstrated favorable clinical responses. Mascarenhas *et al.* recently presented the results of a phase I study in which 13 *JAK2*-mutated PV/ET patients were treated with idasanutlin and combined with pegylated interferon if a partial response was not achieved following cycle 6. Responses were robust: 58% for the monotherapy cohort and 50% for the combination therapy cohort after 6 cycles, with a median treatment duration of 16.8 months.[95] Two multinational clinical trials are currently open investigating the efficacy and safety of the MDM2 inhibitor KRT-232 for ruxolitinib-failure/intolerant MF patients and poorly controlled PV patients. Recently, Lu *et al.* presented early data from a combinatorial study of an MDM2 antagonist and BET inhibitor in MF patients.[96] This combination reduced hematopoietic colony formation by MF-CD34$^+$ cells and targets the microenvironment by reducing the pro-inflammatory cytokine milieu. Results of this particular combinatorial approach are eagerly awaited, as is the combination of a BET inhibitor with JAKi. Lastly, as introduced above, another potential niche pathway target is the estrogen-signaling axis. Inhibition of estrogen-signaling has recently been explored in the TAMARIN trial, investigating clinical benefits and molecular responses induced by the concomitant administration of tamoxifen to patients with MPN established on treatment (excluding interferon).

Historically, the exact relationship between BM fibrosis and clinical outcome/prognosis in MF has been somewhat unclear. An important question is: does improvement in BM fibrosis correlate with improved overall symptom/spleen burden and OS? This has not been comprehensively studied in the clinical trial setting, particularly in the longer term. Kvasnicka *et al.* recently examined the effects of long-term ruxolitinib therapy on BM cytomorphology and fibrosis in 68 patients compared to 192 matching patients with BAT.[97] Compared to baseline reticulin fibrosis grade, ruxolitinib, in contrast to BAT, was associated with augmented odds of fibrosis grade stabilization or improvement and decreased odds of a worsening of reticulin fibrosis. Furthermore, this was often associated with higher degrees of reduction in spleen size. Similar effects have also been noted in a much smaller cohort of patients treated with fedratinib.[98,99] Collectively, these data suggest a possible disease-modifying effect, at least in a subset of those patients undergoing JAKi therapy, which evidently requires a longer duration of drug exposure. Novel therapies such as PRM151, a recombinant human pentraxin-2 analog, have also demonstrated promising findings following reductions in BM fibrosis in some patients with MF. In the first stage of the clinical trial, 27 patients with either primary or secondary MF and ≥ grade 2 reticulin fibrosis were due to receive PRM-151 ± ruxolitinib for 24 weeks; 20 completed therapy.[100] In general, the agent was well tolerated, both alone and with JAKi, with no evidence of myelosuppression. Improvements in symptoms and modest reductions in splenomegaly in some were observed and 11 out of 25 patients evaluated had a reduction in BM fibrosis by ≥ 1 grade. A total of 18 patients were in the open label extension, all of whom received a monthly infusion of PRM-151 at 10 mg/kg, treated for up to 35 cycles (140 weeks).[101] A total of 50% were also receiving ruxolitinib. A similar percentage of patients experiencing reductions in spleen size and improvements in total symptom score (TSS) were seen in both the combination and monotherapy arms. Improvements in reticulin grade was observed in 71% and 44% of those with Grade 2 and 3 marrow fibrosis at baseline, respectively. Recent results presented at the EHA 2019 by Verstovsek *et al.* showed that BM fibrosis decreased at any time point in 28% of patients, and 16-29% patients had a ≥50% reduction in transfusion requirement or hemoglobin improved >10 g/L for 12 consecutive weeks.[102]

Other therapeutic agents have been developed during recent years to specifically target the BM fibrosis and/or relevant pathways in MPN, but with limited success. Inhibitors of hedgehog signaling, important in both primitive and definitive hematopoiesis, cellular proliferation and survival, have been studied both as monotherapies and in combination with ruxolitinib in MF.[103] IPI926, an oral hedgehog-inhibitor, was studied as a monotherapy in MF; however, no significant improvements in fibrosis were reported and the study was discontinued.[104] The SMO-inhibitor sonidegib (LDE225, Novartis, Switzerland) has been investigated in combination with ruxolitinib in a phase-Ib/II study and demonstrated spleen and symptom responses in a minority of patients, and, in some patients, reductions or stability in BM fibrosis.[105] Schneider *et al.* have showed that Gli1$^+$ mesenchymal cells are involved in the fibrosis pathogenesis of MF. The investigators have used GANT61, an inhibitor of Gli1 transcription factor that regulates the hedgehog signaling pathway, in MF murine models and demonstrated reductions in both the fibrosis and the malignant clone. These results suggest a possible new target in reducing marrow fibrosis in MF.[58]

Pirfenidone, an established antifibrotic agent, showed promising results *in vitro* by reducing both fibroblast activ-

ity and ECM components; however, a phase-II study in MF failed to show significant clinical benefits.[106] A study with a monoclonal antibody against TGF-β (fresolimumab) is currently ongoing in MF, although preliminary results have not described any relevant changes in fibrosis.[107] Finally, an inhibitor of the TGF-β receptor-I-kinase, galunisertib (*LY2157299*) has been shown to induce reductions in fibrosis in MPN murine models.[108] Monoclonal antibodies against LOL-2 (simtuzumab) have been tested either as monotherapy or in combination with ruxolitinib in a phase-II study with overall limited efficacy in MF,[109] despite the promising *in vitro* results. Hypomethylating agents such as azacitidine (5-Aza) and decitabine have been investigated in high-risk MF patients and accelerated/blastic phases of the disease. A combined clinical trial with ruxolitinib and 5-Aza is currently ongoing and recent published results have demonstrated marrow fibrosis reductions in 57% of the total cohort (31 cases) at 24 months in addition to acceptable spleen responses.[110] Further research is required to determinate the impact of these hypomethylating agents, with particular attention to the MPN marrow niche.

Therapeutic modulation of the neuro-HSC niche in MPN, introduced above, has recently been explored. Drexler *et al.* report on a phase II trial of an oral β-3 adrenergic agonist (mirabegron) in 39 patients, many of whom had a long duration of disease, with a *JAK2* V617F-mutated MPN who underwent treatment for up to six months. BM core analysis in 20 of the enrolled patients showed increases in Nestin$^+$ BMSC in a proportion of patients (but not in those also receiving hydroxycarbamide); several showed mild reductions in fibrosis and modulation of the characteristic megakaryocyte clustering.[111,112] Although the study end points of a >50% reduction in *JAK2* allelic burden or sustained reductions in splenomegaly were not reached, these intriguing data highlight the potential therapeutic avenues of targeting this neuro-HSC axis in MPN.

With regard to aberrant upregulation of cytokines in MPN, ruxolitinib and other JAKi have been shown to decrease levels of many pro-inflammatory cytokines, including both VEGF and PDGF, as discussed above.[69] The Myeloproliferative Disorders Research Consortium conducted a phase II trial of the anti-VEGF agent (bevacizumab) in 13 patients, 11 of whom were evaluable, to assess if a potential disease modification could be achieved. The dosing strategy was 15 mg/kg intravenously every 21 days; none of the patients demonstrated significant benefits. This lack of response coupled with toxicity led to the premature closure of the study; the authors commented that different dosing strategies may be required.[113] Other drugs have been developed to target VEGF-receptors, like the tyrosine kinase inhibitor vatalanib, but with modest results.[114] Lastly, control or regulation of the marrow hypoxia status could be a potential goal in the management of MPN in view of the key role of oxygen regulation pathways in the pathogenesis and maintenance of these disorders. HIF-1α is essential for HSC maintenance, as discussed. However, in the MPN environment, it participates in both angiogenesis and promotion of suppressor genes, aiding clonal cell adaptation to a hypoxic environment.[115,116] Therefore, targeting HIF1-α has been explored in recent years in both solid and non-solid cancers.[117] Bortezomib, a proteasome inhibitor extensively used in plasma cell dyscrasias, indirectly inhibits HIF-1α in MF patients as demonstrated by Barosi *et al.*[118] Although, to date, few published studies have focused on targeting hypoxia and HIF-pathways in MPN, it remains an attractive area of research.

Conclusions

As our knowledge expands, the complex and dynamic structure of the bone marrow niche in both health and disease is being constantly refined. It is apparent that, in MPN, the mutated-HSC disrupts the harmony of the bone marrow niche, promoting a self-reinforcing environment that facilities their proliferation at the expense of normal hematopoiesis. Furthermore, the MPN-niche can confer therapeutic resistance and potentiate disease progression towards blastic phase disease. Besides the potentially curative procedure of allogeneic stem cell transplantation, attempts to target various components of the MPN-niche have led to variable results and often a lack of sustained clinical benefit. Given the complexity, it is, therefore, increasingly apparent that combinatorial or sequenced therapeutic strategies will be required. As our appraisal of niche dysregulation grows, and we learn more from the current therapeutic trials discussed above, more rational niche-targeted treatment strategies will ultimately be developed.

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