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REVIEW

The secret life of a megakaryocyte: emerging roles in bone marrow homeostasis control

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Abstract Megakaryocytes are rare cells found in the bone marrow, responsible for the everyday production and release of millions of platelets into the bloodstream. Since the discovery and cloning, in 1994, of their principal humoral factor, thrombopoietin, and its receptor c-Mpl, many efforts have been directed to define the mechanisms underlying an efficient platelet production. However, more recently different studies have pointed out new roles for megakaryocytes as regulators of bone marrow homeostasis and physiology. In this review we discuss the interaction and the reciprocal regulation of megakaryocytes with the different cellular and extracellular components of the bone marrow environment. Finally, we provide evidence that these processes may concur to the reconstitution of the bone marrow environment after injury and their deregulation may lead to the development of a series of inherited or acquired pathologies.

Keywords Megakaryocytes (Mk) · Bone marrow (BM) · Vascular niche · Endothelial cells · Osteoblasts · Fibrosis · Myeloproliferative diseases · Thrombopoietin (Thpo) · Extracellular matrices (ECMs)

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Introduction

Bone marrow (BM) is a complex tissue protected by bones and dedicated to the production of white, red blood cells and platelets [1]. Packaged into the cavity of bones, the BM is a tridimensional network of branching sinusoids surrounding islets of hemopoietic cells immersed in a mesh of extracellular matrix (ECM) components and soluble/humoral factors [2–5]. Mechanisms of single cell differentiation and transit to the bloodstream must be tightly regulated to satisfy physiological blood cell requests and supplies. To this regard, recent insights in the characterization of BM structure and function has allowed the identification of specialized microenvironment implicated in the direction of hemopoietic stem cell (HSC) quiescence, differentiation or mobilization [6, 7]. Specifically, cell composition and localization has let to discriminate a peri-vascular niche mainly composed of endothelial, CXCL12^{+} reticular cells and Nestin^{+} mesenchymal stromal cells [8, 9] from an endosteum-associated niche occupied by osteoblasts and osteolineage cells and NG2^{+} (CSPG4) pericytes [10–12]. Despite the fact that a coordinated crosstalk and connection between these niches is indispensable for efficient HSC differentiation and BM function, the anatomical definition of those districts as separated or overlapping entities is still debated [13]. Within this context, megakaryocytes (Mks) migrate, while differentiating from HSCs, from the endosteal to the vascular niche where platelet are shed into the bloodstream [14, 15]. Mks are historically associated with the vascular BM niche as they were detected adjacent to marrow sinusoids ex vivo as well as in vivo by intra-vital microscopy fluorescence [16–18]. However, ex vivo analysis of Mk localization in murine BM demonstrated that these cells can be located in the different anatomical portion and

associated with endosteum, arterioles and sinusoids niche of the BM [5, 19]. The main Mk function within BM vascular niche is platelet production. During this process a giant endomitotic Mk converts into thousands platelets through a highly controlled formation of long, branching protrusions, designated proplatelets, comprised of platelet-sized swellings connected by thin cytoplasmatic bridges [14, 20]. The proplatelet model of platelet biogenesis has been identified both in vitro as well as in vivo [21, 22]. Nevertheless, how proplatelets are extended through sinusoidal endothelial cells and signals that regulate terminal Mk maturation remain poorly understood. Importantly, it is known that positioning of Mks in close proximity of BM sinusoids relies on chemokines that are released by several BM cell types, such as endothelial and perivascular stromal/mesenchymal cells. Stromal-Derived Factor-1 (SDF-1) was the first chemokine implicated in Mk redirection to the vascular niche [23]. Although produced by both endosteal osteoblasts and vascular endothelial cells, SDF-1 seems to regulate Mk maturational chemotaxis through engagement of its specific receptor CXCR4 (also known as CD186) [24]. Moreover, the elegant work by Avecilla and colleagues definitely assessed the importance of chemokines, including SDF-1 and fibroblast growth factor-4 (FGF-4) in restoring thrombopoiesis Thrombopoietin$^{-/-}$ (Thpo$^{-/-}$) and Myeloproliferative Leukemia Protein$^{-/-}$ (Mpl$^{-/-}$) mice by enhancing vascular cell adhesion molecule-1 (VCAM-1)- and very late antigen-4 (VLA-4)-mediated localization of CXCR4^{+} Mks to the vascular districts, thus promoting their survival, maturation and platelet release [25]. Consistently, both administration of SDF-1 and stabilization of endogenous SDF-1 were demonstrated to increase Mk-vasculature association and thrombopoiesis [26]. In particular, in the setting of radiation injury, dynamic fluctuations in marrow SDF-1 distribution spatially and temporally correlate with variations in Mk vascular niche positioning [26]. Recently, also angiogenic signals have been involved in Mk relocation to the vascular niche. A novel pro-inflammatory vascular endothelial growth factor receptor 1 (VEGFR1)-mediated pathway was seen to stimulate the maturation and up-regulation of CXCR4 on Mks, leading to their redistribution to sinusoids, thereby enhancing platelet production in vivo [27]. Therefore, the picture of Mks as static vascular single cells within BM vascular districts has evolved in dynamically and maturing cells that may change their location under specific stimuli, as, for example, during regenerative hemopoiesis. Despite this understanding, still little is known about the exact role of Mks in the BM beside being platelet progenitor cells.

Here we will review what is known about the reciprocal interaction of Mks with the BM environment in terms of cell function, BM homeostasis and related pathologies.

Mk interaction with the different cellular components of the bone marrow environment

While migrating between BM endosteal and vascular niches, Mks interact with different cellular components. Several studies have demonstrated that Mks establish interactions with both stromal lineages arising from the mesenchymal stem cells as well as lineages of hemopoietic origin. In some cases, these interactions are supported by strong in vivo evidences and come to light as reciprocal regulatory connections, while others emerged as weak interactions that have been demonstrated only in vitro. Therefore, in the next section of this review we will discuss the most studied Mk-BM cell interaction divided on the basis of their belonging to the BM connective or hemopoietic tissues (Table 1).

Mk-BM stromal cell interactions

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are a rare population of multipotent stem cells that constitute an important component of the hemopoietic niche in the BM. They are clonogenic, self-renewing cells that can differentiate into tissues of mesodermal origin, in particular osteoblasts (OBs), adipocytes and chondrocytes [28]. MSCs are able to recapitulate and organize a functional hemopoietic niche when transplanted in heterotopic sites [29], underling their importance as regulators of BM physiology and homeostasis.

The contribution of Mks to MSCs in vivo is an unexplored field. On the contrary, many evidences implicated MSCs as important regulator of Mk function. Several studies showed that MSCs express and secrete a broad array of cytokines and soluble factors [30, 31], among these Interleukin-6 (IL-6), Interleukin-11 (IL-11), Stem Cell Factor (SCF) and Leukemia Inhibitory Factor (LIF) that represent important modulators of Mk development and maturation [32–35].

An early report published by Cheng et al. [36] showed that BM expanded MSCs are able to support megakaryocytic differentiation in vitro. In this paper the authors indirectly identified the association of MSCs and Mks in human BM using a co-purification assay. They observed Mk-like cells expressing the CD41 marker in MSCs cultures isolated from BM and assumed that if MSCs and Mks are in direct contact inside the BM niche, it would be possible to co-purify them. Indeed, by using anti-CD41 conjugated magnetic beads, they were able to observe the appearance of fibroblast-like colonies after 2 weeks of culture in MSC medium, while just few colonies were

Table 1 Summary of megakaryocytes-cell interaction within bone marrow

	In vitro evidences	In vivo evidences	Interaction score	Suggested references
Osteoblasts	– Increase of osteoblast proliferation in co-culture system	– Thpo^{HIGH} mice or mice injected with Thpo display osteosclerosis – Increase of osteoblast proliferation after total body irradiation	○○○○	[38, 43, 46, 57, 58, 63–65]
Osteoclasts	– Inhibition of osteoclastogenesis in co-culture system	– GATA1^{LOW}and NF-E2 K.O. mice display reduced osteoclastogenesis and increase in bone mass density	○○○	[50, 51, 63–65, 68]
Mesenchymal stem cells	N/A	N/A	○	
Adipocytes	N/A	N/A	○	
Endothelial cells	– Increase survival and angiogenesis in co-culture systems	– Mks deployed of Trombospondin 1 and 2 increase bone marrow microvascular density	○○	[86]
Hemopoietic stem cells	– Increased proliferation in co-culture systems	– Control of HSC quiescence in steady state hemopoiesis – Increase expansion after chemotherapy treatment	○○○○	[18, 90, 91]
Plasma cells	N/A	– Mice injected with Thpo display increase plasma cells generation in immune response – Reduced plasma cells response in c-MpI K.O mice	oo	[98]
Monocytes macrophages	N/A	N/A	○	

In this table are listed the in vitro and in vivo evidences that reported a megakaryocyte contribution to the single bone marrow cellular components from the literature. An arbitrary score between 1 and 4 (empty dots) meaningful of strong or weak interactions and a selection of suggested references are also provided

Legend of score: ○ no evidences of interaction available, ○○ weak interaction, ○○○ moderate interaction, ○○○○ strong interaction

obtained using a control IgG or an anti-CD14 antibody. Moreover when co-cultured with CD34^{+} cells from BM, MSCs were able to maintain hemopoietic progenitors and support Mk differentiation in absence of exogenous cytokines. Interestingly proplatelet formation was also observed in the co-cultures and platelet-sized particles expressing CD41 and CD62 and capable of a partial activation were recovered from the supernatant. Cheng et al. found also that expanded MSCs expressed Thpo transcript, but were not able to measure the secreted protein in the supernatant, suggesting that other factors may be involved.

A more recent study performed by our group further confirmed these results. To reproduce the cellular interactions inside the BM osteoblastic niche, we differentiated human Mks on confluent monolayers of MSCs or MSC-derived OBs. Though both the co-culture systems yielded the same amount of Mks, a significant difference in the maturation levels was observed, with a higher number of large, polyploid cells in the MSCs/Mks co-cultures and an increased proplatelet formation compared to the OBs/Mks co-cultures [15].

These data suggest that MSC interaction plays an important role in an advanced stage of Mk development rather than acting on early progenitors. In addition to secreting cytokines, MSCs can directly interact with hemopoietic cells and Mks through the expression of cell adhesion molecules like Intercellular Adhesion Molecule-1 (ICAM-1) and 2, Vascular Cell Adhesion Molecule-1 (VCAM-1) and E-Selectin [37]. Indeed, we confirmed that BM MSCs express VCAM-1 and demonstrated that anti-bod- mediated inhibition of VLA-4/VCAM-1 binding in MSCs/MKs co-cultures resulted in a decreased proplatelets formation compared to IgG-treated controls. However, since our system was dependent on IL-6, IL-11 and Thpo administration, it was not possible to determine the contribution of MSCs secreted factors to Mk maturation [15].

It is hence conceivable that direct cell/cell interaction and paracrine-secreted cytokines act in concert to assist Mk terminal differentiation and platelet production inside the BM niche. Some evidences reinforcing these results come from in vivo studies of MSCs/HSCs co-transplantation.

In 2003 Angelopoulou et al. [38] demonstrated that co-transplantation of human-derived MSCs and HSCs in NOD/SCID mice reinforced myelopoiesis and megakaryocytopoiesis. The authors showed that MSCs were able to enhance HSCs engraftment into irradiated mice and skew the hemopoiesis toward myeloid and megakaryocytic lineages. This was particularly evident when HSCs were transplanted at a limiting dose and resulted in a higher percentage of human Mks in the transplanted animal's BM and an increased number of circulating platelets.

In addition to their role in regulating Mk maturation, MSCs can direct their migration inside the BM by secreting SDF-1. This chemokine is produced in the BM by endothelial cells, OBs and perivascular mesenchymal progenitors cells. The latter include CXCL12-abundant reticular (CAR) cells and Nestin^{+} stromal cells, two subtypes of early mesenchymal progenitors cells that retain the potential to differentiate toward OBs and adipocytes in vitro [9, 39].

All these evidences underlie a fundamental role for MSCs in orchestrating Mk development and function within the BM, providing the cellular, chemical and physical stimuli to support their terminal maturation and migration.

Osteoblasts/osteoclasts

Bone remodelling dynamics result from the activity of two distinct cell types, osteoblasts (OBs), that represents the bone forming cells and osteoclasts (OCs) that resorb bone. Many in vitro and in vivo evidences clearly stated a role of Mks in modulating the replication and differentiation of both those cells, with significant outcomes for human pathologies. Specifically, four different mouse models, characterized by megakaryocytosis and osteosclerosis, indicated a positive effect of the megakaryocytic lineage in bone cell dynamics [40–48]. Further, in vitro studies demonstrated that Mks express many factors responsible of OC development inhibition (Osteoprotegerin (OPG), RANKL, Transforming Growth Factor-β1 (TGF-β1), Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF), IL-10, IL-13) [49–58] as well as factors known to modulate osteoblastogenesis and bone formation [49, 59, 60]. The role of Mks on OBs appears dual as they were demonstrated to enhance both their proliferation and differentiation [47, 50, 61]. Proliferation seemed to be mediated by cell–cell contacts, while more conflicting results were proposed about the role of Mks on OB differentiation. Direct cell–cell contacts, promoting Mk-mediated OB expansion, were dependent on the engagement of integrin signalling. To this regard, the fibronectin integrins α4β1 and α5β1 and the glycoprotein GPIIb (CD41) were demonstrated to play a critical role in Mk-induced OB proliferation, as assessed by neutralizing antibody studies [62]. Recently, Cheng et al. [63] demonstrated the importance of the non receptor tyrosine kinase Pyk2, a downstream mediator of integrin signalling, in Mk-induced OB proliferation. Although the increase in Pyk2 expression, obtained co-culturing Mks and OBs, was not related to β1 integrin activation, this up-regulation resulted critical for Mk-mediated OB expansion, both in vitro and in vivo, as demonstrated by the reduced responsiveness to Mk stimulation of Pyk2$^{-/-}$ OBs with respect to wild type. The importance of direct cell–cell interactions and β1 signaling in Mk-mediated proliferation of OBs were further confirmed by the same group in a recent work demonstrating a β1 dependent reduction of p53 and Rb cell cycle regulators upon Mk-OB interaction [64]. Moreover, direct contact between Mks and OBs in the regulation of skeletal homeostasis implies the communications via gap junctions. Gap junction-mediated intercellular communications were shown to be involved in the negative regulation of Mk-mediated OB proliferation by gap junction pharmacological inhibition [61].

Studies on the impact of Mks on OB differentiation were unexpectedly divergent. Ciovacco and colleagues, showed in vitro the inhibition of OB differentiation when co-cultured with Mks, in terms of type I collagen, osteocalcin and alkaline phosphatase expression and OB mineralization [65], Bord and colleagues showed, by in vitro studies, that Mks promoted OB differentiation as assessed by an increase in type I collagen and osteoprotegerin production [50]. However, divergences in these results may be ascribed to differences in culture timing. Interestingly, the increase in OB differentiation, described by Bord et al., was paralleled by a suppression of RANKL expression, highlighting an indirect negative effect of Mks on OC formation.

Mk-mediated effects on OBs appeared clearly dependent on cell–cell interactions, while the less characterized effects on OCs seemed to be mediated by the release of soluble factors, as demonstrated by the effect of Mk-conditioned media on OC culture. Specifically, Kacena et al. [55] demonstrated that Mks were able to release molecules with a molecular mass comprised between 10 and 50 kDa that inhibited OCs development and that seemed to be unrelated to the already known OC inhibitory factors (Interferon-γ (IFN-γ), TGF-β1, OPG, IL-4, IL-10).

While the Mk-mediated effects on osteoblastogenesis and osteoclastogenesis were independent on the developmental stage of megakaryocytic maturation, Mk number and longevity resulted critical for the outcomes on bone cells [65]. These results were confirmed in mouse models characterized by dysmegakaryopoiesis and osteosclerosis. As an example, mice overexpressing or repeatedly injected with the major Mk growth factor, Thpo, showed an

important increase of Mk number at all stages of differentiation in the BM. The phenotype manifested in these mice was a myelofibrotic syndrome with associated osteosclerosis. Importantly, these mice presented high plasmatic levels of two Mk-secreted growth factors previously reported to influence bone cells, TGF-β1 and platelet-derived growth factor (PDGF) [40–44]. The involvement of these molecules in supporting an osteosclerotic phenotype was further confirmed by Chagroui et al. [66], who demonstrated that in the absence of TGF-β1, Thpo overexpressing mice failed to exhibit the myelofibrotic/osteosclerotic syndrome. The same group demonstrated that also overexpression of OPG was critically involved in the development of the osteosclerotic phenotype in $Thpo^{high}$ mice, although no dependency between TGF-β and OPG secretion was found [67]. The Thpo/megakaryocytic lineage axis has been further claimed to be fundamental in osteoblastic niche expansion and thus in the engraftment of donor HSCs, in post total body irradiation (TBI). In this work Olson et al. [69], confirmed previous results of a SDF-1 dependent Mk relocation to the endosteal osteoblastic niche after TBI, which resulted concomitant to the marked expansion of endosteal OBs [68]. Furthermore, TBI-induced Mk migration was demonstrated to be dependent on MPL signalling and on the CD41-mediated adhesion, while OB proliferation required Mk-secreted PDGF-BB.

Two mouse models, extensively studied to elucidate Mk influence on bone cells homeostasis, were the GATA-1 and NF-E2 deficient mice [45–48, 70, 71]. GATA-1 and NF-E2 are key transcription factors in the regulation of Mk development as their genetic ablation in mice resulted in marked megakaryocytosis, paradoxically thrombocytopenia and a significant increase in bone mass density detectable after 4 months after birth. Although these mice exhibited high levels of OPG, the reduction in OC development and function was not associated to the high bone mass [47]. Nevertheless the increase of Mk number played a central role in new bone mass formation in GATA-1 and NF-E2 deficient mice. Further, the direct contact between Mks (either mutant or wild type) and OBs was required to induce OB proliferation [47]. Recently Meijome et al. [72], studied the influence of GATA-$1^{-/-}$ Mks in the rescue of bone alterations in $OPG^{-/-}$ mice, characterized by reduced trabecular and cortical bone due to increased osteoclastogenesis. This study demonstrated that, although GATA-$1^{-/-}$ × $OPG^{-/-}$ mice still present the osteoporosis of the cortical bone they rescue trabecular bone parameters, suggesting that Mks can locally enhance trabecular bone volume but that they cannot access cortical bone to inhibit osteoclastogenesis.

Finally, the Platelet Type-von Willebrand Disease (Pt-vWD) mouse model developed by Suva et al. [48], harboring a gain of function mutation of the specific von Willebrand Factor (vWF) receptor GpIbα, showed a phenotype that mirrored the human disease, including platelet dysfunction and impaired hemostasis. Further, these mutant mice presented thrombocytopenia, a spleen specific megakaryocytosis and a high bone mass phenotype detectable at two months after birth. In this model the bone alteration appeared to be due to the decrease in OC number with consequent bone breakdown that was attributed to the Mk/platelet dysfunction. However, the exact mechanism by which spleen megakaryocytosis affects osteoclastogenesis remains to be elucidated.

Adipocytes

Adipose tissue occupies considerable part of adult BM, but its role has been for long time controversial and poorly understood. Initially thought to be just a "filler" of the BM cavity, in the last years this position has been revisited and fat contribution to BM physiology and homeostasis has been brought to light. As reported by Naveiras et al. [73] the amount of adipose tissue in the BM inversely correlates with the presence of hemopoietic progenitors and active hemopoiesis. Using an engineered strain of "fatless" mice, the authors showed that BM engraftment and hemopoietic recovery after irradiation was faster compared to wild-type mice. The same effect was obtained by pharmacological inhibition of Peroxisome Proliferator-Activater Receptor-γ (PPAR-γ) in wild-type mice, collectively indicating a repressive effect of adipocytes on hemopoiesis.

Adipose tissue secretes a discrete number of cytokines and adipose-derived hormones that can influence the immune system activity and energy metabolism. In addition to IL-6, whose role in Mk differentiation is critical, other factors like adiponectin and leptin have been described to influence Mk development and function, though complete evidence is still missing. Two groups reported adiponectin receptor expression on platelets, Mks and megakaryocytic cell lines [74, 75]. Specifically, Kato et al. [74] observed accelerated thrombus formation in adiponectin knock-out (K.O.) mice after laser injury. This effect was reverted by adenovirus-mediated adiponectin restoration, indicating its possible antithrombotic role. A less defined function of adiponectin in Mk development has been described so far. Sun et al. [75] reported a decreased Mk differentiation and megakaryocytic colony formation in liquid or semisolid assays, respectively, when adiponectin was added to the media. In addition, in liquid assays this effect was evident only when adiponectin was added at the beginning of the culture, therefore, it is not possible to exclude an effect of this factor on early hemopoietic progenitors rather than on committed cells.

Leptin has been shown to enhance ADP-mediated platelet activation [76], but it's not able to induce activation alone, indicating that it may act as a positive modulator of platelets function. The same effect was described in the megakaryoblastic cell line MEG-01 where leptin administration was shown to enhance ADP-induced cytosolic calcium (Ca^{2+}) in a synergistic manner [77]. High adipokines doses, comparable to the one measured in obese patients were also shown to produce insulin resistance in megakaryocytic cell lines and primary cells [78].

Despite adipocytes have been demonstrated to be central player in hemopoiesis homeostasis, their specific contribution to Mk and other lineages differentiation is still unclear and new studies are required to get further insights.

Endothelial cells

Mks mainly reside in the vascular district within BM environment and platelet shedding is thought to happen by a direct extravasation of proplatelet through sinusoid endothelium [17]. Sinusoidal endothelium is uniquely specialized to support hemopoiesis and unlike other vessels, it is devoid of pericytes and surrounded by specialized SDF-1 secreting reticular cells. BM sinusoids can be specifically identified by their expression of Vascular Endothelial Growth Factor Receptor-3, while endothelial cells of arterioles uniquely express Sca-1 [79]. Many evidences have highlighted an important role of endothelial cells in the regulation of Mk functions, in terms of differentiation, development and release of platelets [23–25, 80, 81]. On the contrary, few studies have examined the functional role of Mks in the regulation of BM vasculature development or recovery, despite Mks represent an important reservoir of bioactive hemopoietic and angiogenic factors. Technical limitations have slow down these studies both in vivo and in vitro. As an example, long-term endothelial cultures have been established form different tissues, while BM endothelial cells have been isolated using different technique [82–85], but they always showed in vitro the phenotypic and functional features of microvascular cells. Further, endothelial cells and Mks are considered "sister cells", both deriving from the common stem cell for hemopoietic/endothelial lineages, the hemangioblasts [86]. Therefore, co-expression of several developmental regulators has made difficult the investigation of their functional role in mediating Mk/endothelial cells interaction. However, in vitro studies have attempted to elucidate the mechanisms involved in Mk-endothelial cell contact. Mohle and colleagues demonstrated that ex vivo-generated human Mks isolated from BM express and synthesize VEGF and that, in co-culture with BM endothelial cells can maintain endothelial layer in serum-free medium [87]. Further, Thpo was demonstrated to induce the active release of VEGF by Mks in vitro, confirming a potential role of Mks in supporting angiogenesis [88]. Recently, human Mks were shown to foster cell differentiation and colony formation of endothelial progenitor cell (EPCs) [89]. Moreover, in vivo models clarified some aspects of this connection in conditions of regenerative hemopoiesis. In 2006 Kopp et al. [90] showed that thrombospondins (TSP) act as an important angiogenic switch. Generation of TSP1 and TSP2 double KO mice (TSP-Double KO) demonstrated that these mice were characterized by a faster recovery of BM vasculature after myelosuppression than wild-type mice. Further, TSP-DKO Mks were more proficient in supporting the generation of new vessel within Matrigel with respect to wild-type Mks. Finally, evidences of Mk-endothelial cell interdependence come from patients affected from prolonged isolated thrombocytopenia (PT), a serious complication after allogeneic HSC transplantation characterized by lower Mk content within BM. Patients BM exhibited remarkable decreases in cellular elements of the BM vascular microenvironment, including endothelial cells and peri-vascular cells [91]. Even though endothelial cells and Mks are closely associated within BM sinusoids in vivo, yet precise mechanisms of interactions are still missing.

Mk-hemopoietic tissue interactions

Hemopoietic stem cells

Previous research on the cellular components that contribute to the regulation of the HSC niches has been focused on the role of OBs, reticular stromal cells, endothelial cells and nerve cells [92]. However, the hypothesis that Mks can directly regulate HSCs was confirmed in several studies demonstrating that Mks produce and secrete significant amount of IL-6, which has been shown to dramatically expand spleen colony forming unit in vivo [56, 93]. Furthermore, Kirouac et al. [94] proposed that the self-renewal of umbilical cord blood-derived HSCs is regulated by a coupled positive–negative intercellular circuit composed of Mk-derived stimulatory growth factors (VEGF, PDGF, Epidermal Growth Factor and serotonin) and monocyte-derived inhibitory factors.

Nilsson's group was the first to explore the possible role of Mks in maintaining mature HSCs in their niche. They found, by both in vivo and in vitro approaches, that transplanted HSCs preferentially lodge within two cells of mature Mks in murine BM and that HSCs isolated from the mice endosteum showed a significant increase in cell proliferation while in co-culture with mature sorted Mks. Furthermore, this endosteal HSC progeny was shown to retain HSC potential and to maintain long-term multi-

lineage reconstitution capacity when transplanted into lethally ablated recipients. The mechanisms of increased HSC proliferation was not Mk contact dependent, but could be recapitulated with media supplemented with two factors identified in Mk-conditioned media: insulin-like growth factor binding protein-3 (IGFBP-3) and insulin-like growth factor-1 (IGF-1) [19]. The role of Mk as HSC-regulating niche cells was further confirmed by Zhao and colleagues [95]. In this study the authors showed that Mks are the major source of TGF-β1in the BM and maintain HSC quiescence under homeostatic conditions through TGF-β signaling. In particular, authors generated a *Pf4-cre* induced *DTR* (encoding diphtheria toxin receptor) mice (*Pf4-cre*; *iDTR*) in which Mks are rendered sensitive to diphtheria toxin. Removal of Mks after diphtheria toxin injection resulted in the increase of absolute numbers of short-term HSCs and multipotent progenitor cells. On the contrary, under conditions of chemotherapeutic conditions, when rapid expansion of HSCs is required, Mks were demonstrated to promote HSC expansion through FGF-1 production. Conditional deletion of FGF-1 in Mks in the *Pf4-cre*$^{+}$; *Fgfr1*$^{flox/flox}$ mice led to reduced Mk expansion and HSC regeneration after 5-fluorouracil challenge. Contemporary, Bruns and colleagues, intercrossed *Cxcl4-cre* mice with iDTR animals. Also in this mouse model, selective in vivo Mks depletion using inducible diphtheria toxin receptor expression resulted in specific loss of HSC quiescence with a marked expansion of functional HSCs. However, in this work Mk impact on HSC function was attributed to Mk expression of a regulator of HSC cell cycle activity, CXCL4, also known as Platelet Factor 4 (Pf4) [96].

Plasma cells

Long-lived Antibody-secreting Plasma cells (PCs) represents an example of mature hemopoietic cells that exhibit their own niche within BM. They are critical for long-term protection post-infection or immunization and persist for decades throughout the body, namely at sites of inflammation, but predominantly in the BM compartment [97]. A complex orchestration guides these cells to exit the germinal centers of lymphoid organs and migrate toward the BM, a privileged location where they receive additional signals required for their final differentiation and prolonged survival [98]. There is strong evidence that the longevity of BM PCs is dependent on environmental factors [99]. Different cells type displayed preferential contacts with PC and expressed putative PC survival factors, such as OBs, reticular cells and monocytes [100, 101]. The finding that only approximately 30 % of BM PCs colocalize with Mks suggested a transient contact between these mature cells and led Winter and colleagues to explore the possibility of Mks as cells producing important factors, such as IL-6 and APRIL (a proliferation inducing agent), in supporting PC growth and survival [102]. The authors demonstrated that injection of Thpo increased megakaryopoiesis and allowed the accumulation and persistence of a larger number of PCs generated in the course of a specific immune response in wild-type mice. On the contrary, *c-mpl*$^{-/-}$ mice, which exhibited reduced megakaryopoiesis, showed an impaired PC response in the BM. Together, these results demonstrate that Mks constitute a functional component of the PC niche in this tissue, thus establishing a novel link between PC memory and megakaryopoiesis.

Monocytes/macrophages

Few experimental evidences have highlighted a possible interaction between these two mature cell lineages deriving from the HSC. Data available suggest a prevalence of macrophage effects on thrombopoiesis rather than a reciprocal interaction. Landoni et al. [103] suggested that BM and spleen macrophages-derived signaling regulates negatively the Mk compartment as liposomal-encapsulated clodronate treatment provoked enhancement of megakaryopoiesis characterized by a dose-dependent increase in the number of BM and spleen Mks that was inversely correlated to the macrophage count in these organs. Furthermore, higher dose of liposomal-encapsulated clodronate induces enhanced thrombopoiesis in mice as demonstrated by an increased frequency of reticulated platelets and an improvement in the total platelet count two days later. D'Atri et al. [104] have recently showed in vitro that resting macrophages release soluble factors that sustain Mk growth, cell ploidy, a size increase, proplatelet production, and platelet release. Specifically, stimulation of macrophages with lipopolysaccharide triggered the secretion of cytokines that exerted opposite effects together with a dramatic switch of CD34^{+} commitment to the megakaryocytic lineage toward the myeloid lineage. Those effects seemed to be IL-8-dependent as neutralization of this cytokine partially reversed the inhibition of Mk growth.

Mk contribution of soluble factors and extracellular matrices within BM environment

Beyond cell–cell contacts, hemopoiesis is regulated by soluble factors and extracellular matrix components [105, 106]. Interestingly, mature Mks in the BM, present three different types of secretory granules in their cytoplasm: the alpha-granules, dense granules and lysosomes [107]. While dense granules predominantly contain serotonin, adenosine

diphosphate (ADP) and polyphosphates, and lysosomes are fulfilled of enzymes, α-granules comprise more than eight hundred proteins, including hemostatic factors (vWF), angiogenic factors (VEGF), anti-angiogenic factors (endostatin, Platelet Factor 4), growth factors (EGF, PDGF, FGF, SDF-1α), metalloproteinases (MMP2, MMP9), necrotic factors (TNFα, TNFβ) and Extracellular Matrix components [108]. Delivery of these growth factors and cytokines represent the most intuitive way by means of which Mk might support and regulate cell function around the BM niches. However, while secretion of platelet granules is a key event in normal response to vascular damage in physiology, the mechanisms of Mk release of granule content are less clear. In general, what we know on Mk granule release come from studies on platelets and only hypothesis can be formulated on the ability of Mks to set an exocytic control of the external environment. Therefore, several questions still need to be addressed such as: are all α-granule cargoes, synthesized by Mks, completely delivered to nascent platelets in vivo or some of them are actively released to finely regulate the surrounding environment by an autocrine/paracrine manner? Which mechanisms are exploited by Mks to control the kinetics of granule release? Are differentially packaged α-granule cargoes released during physiologic hemopoiesis or in conditions of BM injury? Despite this paucity of knowledge, in 2008 Italiano and colleagues demonstrated that angiogenic and anti-angiogenic proteins were segregated into different sets of α-granules in Mks and platelets and that the selective release of those granules led to the regulation of angiogenesis [109]. Similar mechanisms were identified for the coagulation factors vWF and fibrinogen [110] as well as for inhibitors of matrix MMPs [111]. Moreover, pro-fibrotic factors of Mk α-granule derivation, such as TGF-β and PDGF, are critically involved in the onset of BM diseases and will be discussed in depth in the next sections of this review. Beside cytokines and growth factors, an intriguing research field concerns the mechanisms of ECM rearrangement by Mks both in physiological conditions and disease. Growing evidences demonstrated that Mks differently interact with the BM extracellular environment during their maturation and positioning in the BM niche [112]. ECM components constitute a dynamic structure that is continuously degraded and deposited. Interestingly, Mks possess collagenase activity, as they express several MMPs, such as MMP-2, MMP-9, MMP-14, MMP-24, MMP-25 [113], assemble podosomes that rearrange the ECM through proteolytic activity [114] and express several ECM components such as fibronectins, laminin isoforms, type IV collagen, nidogen and entactin [5, 115, 116]. In addition, our group has recently demonstrated that expression of many of those ECMs is not related to the physiological production of platelets but is increased concomitantly with the regeneration of BM environment following myelosuppression [5].

The BM extracellular microenvironment is composed of structural fibrils and extracellular matrix components. The most common structural fibrils in the BM are collagen, reticulin, laminin and fibronectin [4, 5, 117–119], embedded in a matrix of glycosaminoglycans and glycoproteins, most of which have not been well characterized. Moreover, a wide variety of benign and malignant disorders are characterized by an increase in BM stromal fibers [120]. These fibers are commonly composed solely of reticulin fibers but may also include collagen fibers. Immunohistochemical detection of reticulin with silver-based stains and collagen with trichrome stains represent routine stains performed on BM biopsy specimens in diagnostic laboratories. Two slightly different, but widely supported grading scales for these stains have been created [121, 122]. The original Bauermeister scheme [121] has been simplified into a five grade system, while, the more recent Thiele scale includes only four categories [122]. Both of these grading scales take into account both the type of fibers seen (reticulin or collagen) and the overall amount of fibrosis. Interestingly, when an increase in BM reticulin staining does occur, it is usually accompanied by an increase in BM Mks, often morphologically atypical.

The contribution of Mks to BM fibrosis remains only partially understood and it is likely that cytokines, growth factors, cross-linking enzymes and ECMs might all be involved. The pivotal role of Mks in the onset of BM fibrosis associated with hematological malignancies will be discussed in the following section of this review. However, new features of Mks as pro-fibrotic cells have emerged. As an example, a linkage between BM fibrosis and lysyl oxidase (LOX) derived from low ploidy Mks has been recently described [123]. LOX is a copper-dependent enzyme that cross-links collagen or elastin by oxidative deamination of peptidyl lysine or hydroxylysine and peptidyl lysine residues, respectively, and contributes to the accumulation of ECMs by promoting intrapeptide and interpeptide chain cross-linking [124]. Interestingly, LOX is highly expressed in low ploidy, proliferating Mks, while mature Mks have a scarce expression of this enzyme. Consistently, LOX is also abundant in the GATA-1^{low} mouse model with pathologically high levels of low-ploidy Mks associated with an extensively fibrotic matrix. Inhibition of LOX enzymatic activity in GATA-1^{low} mice by β-aminopropionitrile (BAPN), inhibited the progression of myelofibrosis, demonstrating for the first time the potential role of LOX derived from low ploidy Mks in sustaining BM fibrosis [123].

Megakaryocytes and diseases

Germline mutations in genes encoding for different proteins relevant for the regulation of Mk biology have been described to dramatically affect platelet count, thus resulting in human diseases known as inherited thrombocytopenias. Several different forms have been identified so far and classified according to the presence or absence of other inherited defects associated with the low peripheral blood platelet numbers [125]. The clinical phenotype of these patients can vary widely depending on the type of mutation. In some cases the pathology is recognized early in infancy, for example in Jacobsen syndrome, the thrombocytopenia is associated with physical growth retardation and characteristic facial dysmorphism, while congenital amegakaryocytic thrombocytopenia always evolves into BM aplasia. However, in other forms, the clinical phenotype becomes evident in adulthood, such as in thrombocytopenia associated with sitosterolemia (STSL), which may display anemia, tendon xanthomas and atherosclerosis [126].

Thrombocytopenia is the only common feature of all inherited thrombocytopenias, thus indicating that, regardless of funding mutation, any alteration in proteins involved in the control of specific Mk function may result in defective platelet formation. Our knowledge of the cellular mechanisms of altered platelet production in many of these pathologies has greatly expanded in recent years due to the opportunity to culture human Mks differentiated from patients' hemopoietic progenitor cells in vitro. To this regard, we have demonstrated that Mks from MYH9-Related Disease patients, carrying mutations in the MYH9 gene encoding for the heavy chain of non-muscle myosin IIA, show a significant defect in proplatelet branching and completely lose the physiologic suppression of proplatelet extension exerted by interaction with type I collagen, thus suggesting that a premature platelet release within BM may contribute to the appearance of thrombocytopenia [127, 128]. Moreover, in heterozygous Bernard-Soulier syndrome type Bolzano, mutations of the membrane glycoprotein GPIbα determine a profound reduction in Mk ability to form proplatelet on different ECM components, such as fibrinogen and vWF, with altered distribution of cytoskeletal tubulin and increased size of proplatelet tips, consistent with the increased diameters of peripheral blood platelets [129]. Consistently, Mks from patients with the homozygous Bernard-Soulier syndrome are completely unable to extend proplatelets in vitro [130]. The key role for membrane glycoproteins in regulating Mk interaction with components of the extracellular environment that sustain platelet formation is further confirmed by the finding that a constitutive activation of $\alpha_{IIb}\beta_3$-mediated outside-in signaling negatively influences proplatelet formation and severely impairs cell adhesion because of altered actin distribution and consequent defective stress fibers assembly [131]. The relevance of cytoskeleton contractility in the regulation of platelet formation and released is also evident in a recently described macrothrombocytopenia caused by mutations in the alpha-actinin 1 gene (ACTN1), that occurs within the actin-binding domain of alpha-actinin 1 that cross-links actin filaments into bundles [132]. In vitro transfection of Mks with disease-associated ACTN1 variants causes a disorganized actin-based cytoskeleton that is responsible for the abnormal proplatelet formation with proplatelet branches displaying few large tips [133]. Beside cytoskeleton and glycoproteins, a fine regulation of biochemical signaling is also important to ensure proper platelet formation. In familial thrombocytopenia 2 (THC2) point mutations in the 5′ UTR of ankyrin repeat domain 26 (ANKRD26) are responsible for the constitutive expression of this gene in the late stages of Mk maturation, when is normally silenced [134]. This leads to increased signaling via the Thpo pathway that in turn is responsible for impaired proplatelet formation. Interestingly, the main signaling molecule which appears to be deregulated is the Mitogen Activated Protein Kinase Extracellular Related Kinase (ERK), given that its selective inhibition completely rescues the in vitro defect [134]. Importantly, a feature of ANKRD26 Mks is the presence of a large amounts of PaCSs, cytoplasmic structures with selective immunoreactivity for polyubiquitinated proteins and proteasome, normally detected in solid cancers and their pre-neoplastic lesions, suggesting a link of these structures with oncogenesis [135]. Interestingly, ANKRD26 patients are exposed to an increased risk of leukemia, but the mechanisms explaining the relationship between dysmegakaryopoiesis and leukemia have not been yet investigated.

Overall, these mutations specifically affect proplatelet formation, the final stage of Mk differentiation, while preserving HSC commitment, polyploidization and maturation, thus suggesting that Mk participation in BM homeostasis is not significantly influenced. One exception is the gray-platelet syndrome (GPS), in which α-granules are reduced or absent in mature Mks because of mutation in the NBEAL2 gene [136]. The associated bleeding syndrome is usually mild to moderate, but can be also severe. In most patients thrombocytopenia is accompanied by high risk of developing evolutive myelofibrosis and splenomegaly with a profound change of BM architecture that dramatically compromise hemopoiesis [137]. Mks usually appear highly vacuolated; but their number is often normal and all the other organelles are normally present [138]. The major hypothesis for the development of BM fibrosis is that proteins stored inside Mk α-granule, such as PDGF and TGF-β1, are normally synthesized, but spontaneously

released from Mk cytoplasm into the marrow because of deregulated storage, thus promoting ECM synthesis and deposition. Interestingly, a GPS murine model was recently shown to recapitulate the typical platelet phenotype observed in patients including splenomegaly and myelofibrosis [139]. This model demonstrated that expression of macrophage inflammatory protein (MIP) 1α and 1β is significantly increased in GPS Mks. These well-known pro-inflammatory chemokines are increased also in primary myelofibrosis, a myeloproliferative disease characterized by BM fibrosis and splenomegaly as well. Actually, beside inherited mutations, it has been described that also numerous somatic genetic alterations, usually acquired by the HSCs, may affect Mk differentiation and platelet formation [140, 141]. Specifically, the appearance of a mutate hemopoietic clone, that can escape the proliferation arrest, leading to the abnormal accumulation of BM Mks, is the typical feature of polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), three diseases belonging to the family of the Philadelphia-negative classical myeloproliferative neoplasms (MPNs) [142–145]. BM from PV patients presents neoplastic proliferation and maturation of erythroid, megakaryocytic and granulocytic elements. Mks are increased in number and display characteristic morphological abnormalities, such as hyperlobated nuclei. Histology of BM from ET patients usually reveals marked Mk hyperplasia. There are frequently giant polyploidy Mks, but the overall morphology is fairly normal. Mks from ET patients have an increased tendency to form high-branched proplatelet, in vitro [146]. Consistently, ET is often discovered incidentally in asymptomatic patients because of the abnormally high platelet count, which may be cause of thrombotic complications. BM biopsies of individuals with PMF are characterized by important Mk hyperplasia and dysplasia, with hypolobated and cloud-like Mk nuclei. Platelet count may differ between the onset and the progression of the disease, from high to low numbers [147], and also platelet formation has been described to present several structural alterations in vitro [146]. Only PMF patients show increased deposition of reticulin and collagen fibers. A condition that progressively drives reactive BM fibrosis, which in turn is the major responsible for the final BM failure, with consequent splenomegaly because of the appearance of extramedullary hemopoiesis. A myelofibrotic phenotype may be secondary to ET or to an early/prefibrotic myelofibrosis state, characterized by the absence of relevant reticulin fibrosis in BM, but with evident Mk proliferation and dysplasia [148]. The close link among these different diseases is not surprising, given the evidence that they all share the same molecular pathogenesis, even if for a little percentage of patients the causing mutation remains still elusive. Nearly 95 % of PV and 50 % of ET and PMF present a somatic point mutation in the gene encoding for the Janus Kinase-2 (JAK2) (JAK2V617F). This genetic alteration, which was the first to be characterized, is selectively acquired by the myeloid compartment, and leads to production of a protein with kinase activity that is constitutively activated [149, 150]. JAK/STAT signaling, together with Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3 K)/Akt and Ras/ERK pathways, are the major determinant of Thpo-mediated control of megakaryopoiesis and Mk polyploidisation upon interaction with its receptor, c-Mpl [151, 152]. It has been shown that enhanced activation of JAK/STAT signaling promotes HSC proliferation and contributes to render them hypersensitive to Thpo [153]. However, why a single mutation contributes to different clinical outcomes is still unknown. One of the possible explanations is the difference in the allelic burden [154, 155]. Recently, different knock-in mouse models of the mutant JAK2V617F, have been created. Interestingly, all of them developed an MPN that is closely reminiscent of human PV. However, loss of wild-type JAK2 allele in homozygous JAK2V617F mice results in markedly increased white blood cells, neutrophils, reticulocytes and platelets in the peripheral blood, together with accelerated BM fibrosis and significantly larger spleen size, suggesting that loss of wild-type cells increases the severity of the MPN [156]. However, it is becoming clear that although HSCs harboring JAK2V617F mutation promote MPN, additional components maybe necessary for disease progression. To this regard, it has been recently described that loss of the Thpo receptor significantly ameliorates MPN development in JAK2V617F transgenic mice, thus suggesting that c-Mpl expression is a fundamental trigger of MPNs development [157]. Moreover, JAK2 mutations may occur in ET or PMF patient in concomitance with Lymphocyte Specific Protein (LNK) mutations [158–160], which can be acquired either early or late during the clonal evolution of the disease [161]. Loss-of function mutations of LNK lead to further activation of JAK/STAT signalling given that the protein normally down-regulates this pathway upon c-Mpl activation [162–164]. Of note, mice with a complete loss of LNK display many features in common with human MPN, including splenomegaly, thrombocytosis and extramedullary hemopoiesis [162, 163, 165].

The importance of Thpo signaling in the pathogenesis of MPNs is confirmed by the description of activating mutations in gene encoding for c-Mpl in about 10 % of JAK2-negative ET and PMF patients, but not PV [166–168]. c-Mpl mutation is significantly associated with thrombocytosis [169, 170], and severe anemia [171], and cells form these patients display spontaneous formation of Mk colonies [172, 173]. Mice engineered to permanently express high doses of Thpo, through retroviral infection, develop a

myeloproliferative syndrome, characterized by hyperproliferative Mks and thrombocytosis, with associated spleen and BM fibrosis, while injections of high doses of Thpo have been used to induce in mice and rats a myelofibrotic phenotype associated with thrombocytosis and increased number of BM Mks [42, 44, 174, 175]. Consistent with these evidences, therapeutic treatment with Thpo mimetics in humans, beside the rescue of platelet count to normal levels in thrombocytopenic patients, may determine the development of BM fibrosis as side effect [176, 177].

The last identified somatic mutations responsible for about 25 % of all PMF and ET, and some JAK2-negative PV, involves the gene encoding for calreticulin, CALR [178–180]. CALR mutations are mutually exclusive with mutations in both JAK2 and c-Mpl [178, 179], indicating that CALR is the second most frequently mutated gene in myeloproliferative neoplasms. CALR, is a multifunctional protein that binds with high-capacity Ca^{2+} ions that must be restrained into the endoplasmic reticulum (ER) [181]. All the mutations that have been described so far, affect a region of the gene that encodes for the C-terminal peptide containing a negatively charged calcium-binding domain, and the ER retention motif. The resulting mutant proteins share a novel amino acid sequence containing positively charged amino acids, thus probably affecting Ca^{2+} binding capacity, and loose the reticulum retention motif, suggesting possible protein dislocation. All patients with mutated CALR display thrombocytosis, but thrombosis-free survival is significantly longer compared with other mutations [178, 179]. Recently, a fine regulation of intracellular Ca^{2+} signaling has been described as a major trigger of both proplatelet formation and Mk interaction with ECM components that sustain Mk motility, through the activation of the mechanism of store operated Ca^{2+} entry (SOCE) [182]. These data, together with the finding that Mks express the highest amount of mutated CALR among BM cells [183], suggest that altered Ca^{2+} homeostasis may contribute to this phenotype. Interestingly, it has been shown that CALR is actively involved in the modulation of SOCE dynamics [184, 185], and mice that present constitutive activation of SOCE, exclusively in BM Mks, resemble many of the alterations of PMF patients, especially fibrosis and splenomegaly [186]. Moreover, it has been demonstrated that the same mechanisms is up-regulate in human endothelial cells from PMF patients [187]; however insight into the role of Ca^{2+} signaling in human Mk in the pathogenesis of PMF and ET are currently missing. Importantly, in an effort to try to understand the pathogenic mechanisms promoted by CALR mutations, it has been demonstrated that, as in the case of JAK2 and c-Mpl mutations, JAK/STAT signaling is over-activated in these patients [178, 188]. An important finding that, taken together with data from JAK2 and c-Mpl mutated patients, definitively suggests that deregulated JAK/STAT signaling is a common future of nearly all PMF and ET regardless of founding driver mutation and clinical diagnosis [188].

A driving role for Thpo signaling in affecting BM homeostasis is confirmed also in acute megakaryoblastic leukemia (AMKL), another disease with impaired Mk maturation and a profound alteration of BM organization due to variable degree of fibrosis [141, 189, 190]. AMKL affects three major subgroups of patients: adults (adult AMKL), infants with down syndrome (DS-AMKL), and children without DS (non-DS paediatric AMKL). Interestingly, nearly all patients with AMKL present thrombocytopenia, although some may have thrombocytosis. Morphologically, BM histopathology shows the presence of uniform blasts, mainly of the Mk lineage, with presence of mature dysplastic Mks and only few residual foci of granulo and erythropoiesis. Constitutive activation of signaling associated with JAK/STAT cascade has been described in a significant proportion of adult AMKL, thus suggesting that, as in the case of myelofibrosis, alterations of biochemical pathways relevant for Mk differentiation result in AMKL phenotype. The molecular basis for the constitutive activation of this signaling cascades is due to mutations in genes encoding for c-Mpl and JAK [190, 191]. Moreover, several studies reported that activating mutations in Fms-like tyrosine kinase 3 (FLT3), as well as c-KIT, are associated with adult AMKL [192, 193].

Interestingly, activating mutations in FLT3, JAK2, JAK3 and c-Mpl can be present also in child with trisomy 21 in DS-AMKL [141, 194, 195]. However, the major cause of DS-AMKL is due to acquired somatic mutations in the hemopoietic transcription factor gene GATA1 preventing the synthesis of a full-length protein, but allowing the synthesis of a shorter variant (GATA-1 s) with altered trans-activation capacity [191, 196] that has been described to increase the proliferation of Mk progenitors and to represses final Mk differentiation [197, 198]. Interestingly, GATA-1^{low} mice model usually develop a hematological picture that features human myelofibrosis [46, 59, 199] with Mk progenitors exhibiting striking hyper-proliferative rates, both in vivo and in vitro [200]. This phenotype is almost similar to that observed in wild-type mice subject to high concentration of Thpo [201]. Actually, mice treated with Thpo show a decrease of GATA-1 content in Mks, thus further strongly supporting the strict relationship between Mk and extrinsic (forced expression of Thpo, $Thpo^{high}$ mice) or intrinsic (reduced GATA-1 expression, GATA-1^{low} mice) factors in the development of myelofibrosis [201]. Conversely, expression of a mutant GATA-1 s protein in a knock-in mouse model of the disease is able to induce developmental stage-specific defects in yolk sac and fetal liver Mk progenitors, but is not sufficient to induce AMKL per se [197]. Therefore, this genetic

mutation may not be sufficient to induce AMKL. Consistently, most DS-AMKL patients with constitutional trisomy 21 and GATA1 mutations present a transient myeloproliferative disorder at birth, with spontaneous remission and absence of further malignant disease in most instances [200] and humans with inherited mutant alleles fail to develop leukemia [202]. These observations indicate that there are multigenic contributions to the development of AMKL. To this regard it has been demonstrated that trisomy 21 alone provides a proliferative stimulus of fetal megakaryopoiesis and erythropoiesis [203]. In fact, cells harboring trisomy 21 overexpress the transcription regulator ERG which strongly cooperates with the GATA1 s mutated protein and interferes with Akt signaling molecule to immortalize Mk progenitors, thus contributing to aberrant megakaryopoiesis [204]. Accordingly, a fine regulation of Akt activation is required to guarantee an efficient platelet production [205, 206], and defective regulation of this pathway is associated with abnormal Mk proliferation [206].

Together, all these data suggest that, independently from the founding mutation, any signals that impair c-Mpl downstream transduction cascades may result in alteration of BM Mks with a significant tendency to develop fibrosis. But how aberrant megakaryopoieisis can be linked with the BM fibrosis which finally causes the ineffective hemopoiesis? Besides BM a variety of organs including lung, liver, kidney, intestine, heart, skin may develop fibrotic disease. It is currently assumed that in all of these organs the underlying mechanism of fibrotic reactions, leading to progressive organ imbalance, involves the over-stimulation of resident stromal cells, namely mesenchymal cells and myofibroblast, to produce and deposit collagens and other ECM components. The major cytokines known to have a role in mediating the fibrotic process are PDGF and TGF-β1 [207, 208].

TGF-β1 is the first member identified of pleiotropic cytokines involved in normal tissue repair and development. Although expressed by all cell types, is particularly abundant in platelets. TGF-β1 activity is regulated by proteases that convert the latent complex into an active form, thus inducing the expression of both extracellular matrix components (collagen type I and fibronectin) and their cognate receptors on target cells and its sustained production induces fibrosis in numerous organs, including myelofibrosis [66, 209].

The role of TGF-β1 and its downstream signaling are suggested by the evidence that development of myelofibrosis in wild-type mice treated with high doses of Thpo is associated with high TGF-β1 content in extracellular fluids of both BM and spleen [201], and that Gata1^{low} mice Mks show increased TGF-β1 content [210]. Moreover, TGF-β1 deficient mice over-expressing Thpo are fibrosis resistant as compared to wild-type mice [66], and inhibition of TGF-β1 signaling is able to rescue the phenotype of Gata1^{low} mice, reducing fibrosis/neovascularization/osteogenesis and increasing hemopoiesis in BM with its concomitant reduction within spleen [210]. In parallel to these evidences, it has been also reported that Mk from PMF and AMKL patients synthesize and release higher amount of both latent and bioactive TGF-β1 [211–214], and that this behavior is highly correlated with BM fibrosis grade [213].

Moreover, PDGF is a potent mitogen for cells of mesenchymal origin, including myofibroblasts; [215, 216]. Interestingly, PDGF expression is increased in BM Mks from patients with myeloproliferative disorders [217] and PMF patients display a significant association between BM fibrosis and aberrant expression of PDGF within BM [218]. Moreover, AMKL Mks secrete higher amount of PDGF and the highest PDGF secretion is associated with more severe fibrosis [219]. Thus, it is possible to assume that aberrant pro-fibrotic cytokines released by Mks of patients with BM fibrosis may be the major determinant for the appearance of this phenotype.

Both PDGF and TGF-β1 are stored in Mk α-granules and are constitutively released by BM Mks during the course of their physiological maturation [49, 220, 221]. Actually, it has been demonstrated that both these cytokines may exert an autocrine effect on Mks by regulating both megakaryopoiesis and platelet production [212, 222, 223]. Further, Thpo has been shown to promote α-granules release in human platelets [224]. Platelets and Mks share the same activating mechanisms, thus is seems reasonable to hypothesize that an over-stimulation of JAK/STAT signaling, as a consequence of the different driving alterations described, may be a major determinant of fibrosis by promoting the release of Mk pro-fibrotic cytokines that acting on stromal cells induces the excessive collagens and ECM components production.

Perspectives and concluding remarks

Research in recent years has made many progress in understanding the unique process of proplatelet formation and platelet shedding by Mks. Despite all these efforts, little is known about the signals that regulate these events and the interactions of Mks with the different components of the BM environment. In this context, the most recent research has ascribed to Mks and platelets new and variegated functions beside their roles in platelet production and hemostasis. Mks can sense different biological and physical stimuli and regulate different cellular processes accordingly. As an example, Mks can participate in angiogenesis by modulating and secreting their granule content [109], in bone formation and in matrix reorganization by

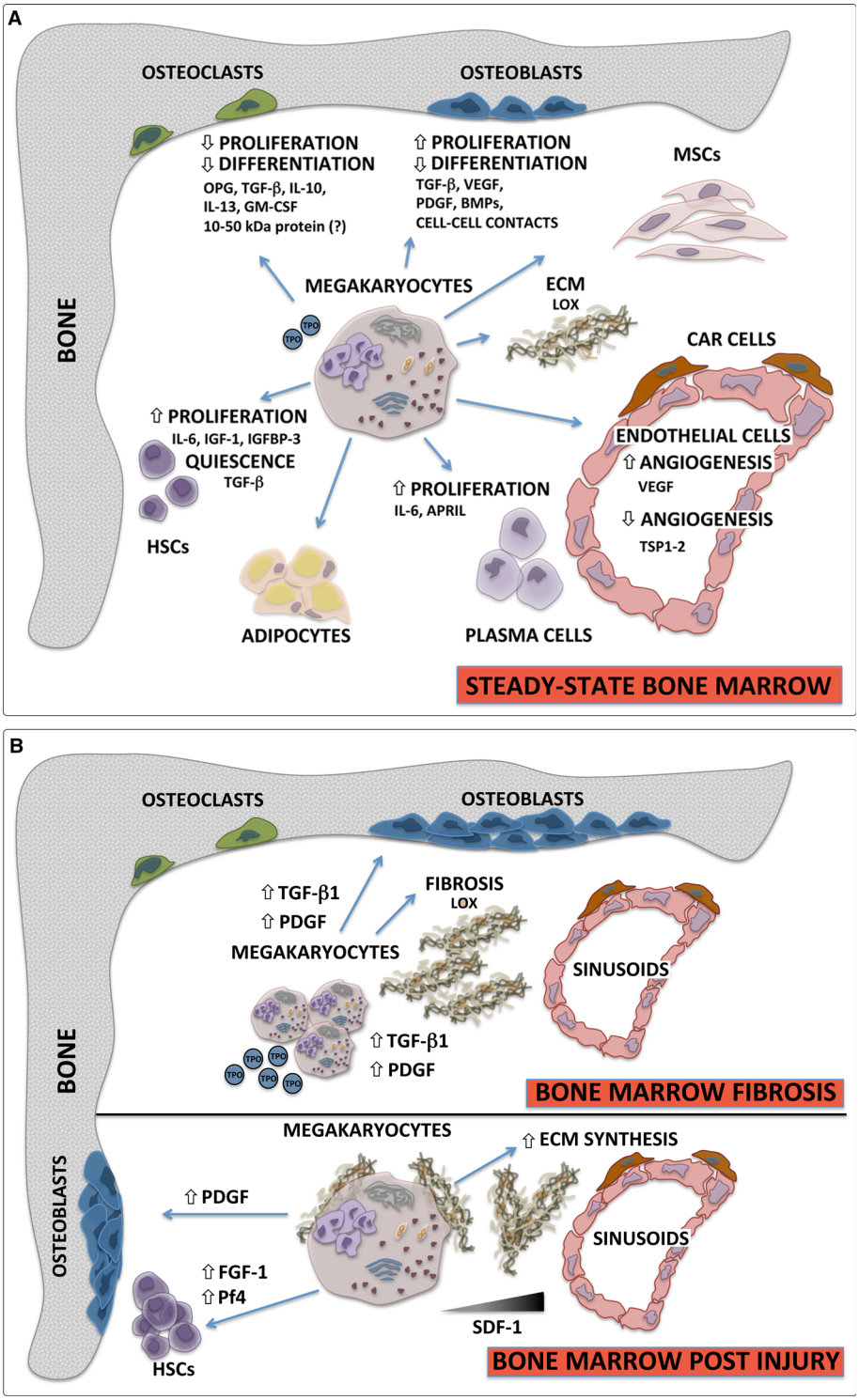

Fig. 1 Schematic representation of Mk contribution to the bone marrow environment in **a** bone marrow steady state **b** bone marrow fibrosis and injury

releasing soluble factors, ECMs and MMPs [68, 113, 225]. All these discoveries prompt a central role of Mks in the regulation of BM homeostasis in steady state and "stressed" hemopoiesis as well as in the development of pathologies, such as myeloproliferative neoplasms, where alterations in Mk morphology and function are associated to BM fibrosis (Fig. 1). To this regard there are still two major questions to be answered: how the synthesis of ECM components by Mks is regulated? and in which conditions this process is activated? It is known that both ECM composition and structure in the BM affect Mk function and platelet release. Moreover, in vivo Mks are surrounded

by a peri-cellular matrix mainly composed by type IV collagen, laminin and fibronectin with a fibrillar organization [5]. These ECM components may complex with growth factors and regulate their activity or constitute themselves autocrine regulatory factors by activating biochemical and/or mechanical signals upon binding to their receptors [225, 226]. Alterations of these processes, like in BM fibrosis, may lead to impaired modulation of the different Mk functions with one function prevailing on the others (e.g. Mk proliferation observed in PMF patient BM biopsies) [144]. How Mk and stromal-derived ECM components interact and reciprocally regulate their expression and function are still to be understood. As an example, we have demonstrated that Mks release and organize fibronectin upon adhesion to type I collagen and not fibrinogen through activation of the transglutaminase factor XIII [225]. Interestingly, we have recently demonstrated that intracellular calcium signaling is fundamental for Mk interactions with the different ECM components of the BM [182]. These data find an obvious link with the newly discovered mutation in CALR in myeloproliferative neoplasm [178, 179]. Here alterations in Ca^{2+} signaling may lead to abnormal interaction of Mks with BM ECM components with consequences in Mk functions and matrix homeostasis and remodeling. Interestingly, both Mk interaction with the BM environment and proplatelet formation are powered by cytoskeleton forces [227]. Specifically, the Rho-Rock-myosin axis regulates Mk contractility, interaction with type I collagen in the BM and inhibition of proplatelet formation [128]. Activation of this axis is dependent on the environment stiffness as demonstrated by the recovery of proplatelet formation on softer type I collagen [228, 229]. Thus, the reciprocal regulation of Mks and BM is also dependent on the mechanics of both cells and environment. Similarly, proplatelet formation occurs in the proximity of BM sinusoids and is regulated by the coordinate activity of different factors of that district. Here ECM is mainly composed by softer and non-fibrillar components (e.g., type IV collagen, fibronectin) that are known to promote both Mk differentiation and proplatelet formation [112]. Proplatelet elongation depends on microtubule remodeling mainly driven by dynein-facilitated microtubule sliding [230].

On the top of these mechanisms a reciprocal regulation exists between Mks and all the other cellular components of the BM environment. Some interactions are well known, as for example Mks and OB/OC reciprocal regulation, and some are still to be completely understood, as the link between endothelial cells, adypocytes or monocytes/macrophages and Mks. All these are important points to be clarified as it is important to understand how Mks extend proplatelets through the vascular wall, as well as how Mk differentiation is regulated by the different composition in BM adypocytes that occurs during aging or diseases and, finally, how inflammation may impact platelet release.

Research on BM has been impaired by technical difficulties in obtaining an intact organ without bone decalcification in mouse models or BM biopsies in human. Multiphoton intravital microscopy has been used to visualize platelet production in vivo in murine BM [17, 18]. Although this live imaging technique has strengthened the physiological role of proplatelet formation during both steady state thrombopoiesis or thrombocytopenic conditions, it raised new questions on the roles of hydrodynamic forces in platelet morphogenesis or on the events that lead to proplatelet morphogenesis in peripheral blood.

For this reason, lately the study has been focused on the creation of ex vivo BM models to extrapolate new insight on BM function in vivo. To this regard we have recently proposed a 3D model, entirely made by silk biomaterial, in which we have recapitulated the vascular niche environment for functional human platelet production [231]. By this model we believe to be able to dissect the role of the different cellular, molecular and physical components of the BM in directing platelet production and, in reverse order, the role of Mks in modulating BM homeostasis. The advantage of our model is the use of silk biomaterial. Silk is a naturally derived, biocompatible and tunable biomaterial with outstanding optical properties [232], which can be prepared in a range of material formats and processed entirely in aqueous systems allowing the incorporation of labile compounds without loss of bioactivity [233]. Silk biomaterial has been proven to have non-thrombogenic features, while supporting functional platelet production. The importance of 3D tissue systems has grown substantively in recent years, moving from 2D and towards more complex and relevant biomaterials. A few years ago Matsunaga et al. [234] proposed a 3D system for platelet production using a three-phase culture system. More recently, different 3D models were described for hematopoietic stem cell migration and proliferation using co-culture systems [235] and for platelet production [231, 236]. Lately, different models have been developed in the attempt of mimicking the bone marrow niches to study hemopoiesis [237], to support platelet production by iPS cell-derived Mks [238].

In conclusion, Mks actively interact with the different components of the BM both physically and molecularly. By this connection both platelet production and BM homeostasis are regulated. Many aspects of these fascinating processes have still to be clarified and this will be possible only by joining different efforts and expertise in a multidisciplinary approach.

We apologize to those whose work was not included in this review due to space limitations.

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