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Review Article

Megakaryocyte Contribution to Bone Marrow Fibrosis: many Arrows in the Quiver

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Abstract. In Primary Myelofibrosis (PMF), megakaryocyte dysplasia/hyperplasia determines the release of inflammatory cytokines that, in turn, stimulate stromal cells and induce bone marrow fibrosis. The pathogenic mechanism and the cells responsible for progression to bone marrow fibrosis in PMF are not completely understood. This review article aims to provide an overview of the crucial role of megakaryocytes in myelofibrosis by discussing the role and the altered secretion of megakaryocyte-derived soluble factors, enzymes and extracellular matrices that are known to induce bone marrow fibrosis.

Keywords: Megakaryocyte, Bone marrow, Fibrosis, Myeloproliferative neoplasms, Platelets, Transforming growth factor-β.

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Introduction. Bone marrow (BM) fibrosis is characterized by increased deposition of reticulin fibres and in some cases collagen fibres.[1] There are a number of hematologic and non-hematologic disorders that are associated with increased BM fibrosis. In particular, reticulin fibres are composed by type III collagen and may be evident in many benign situations, including autoimmune and granulomatous diseases, and different tumors, such as lymphoid neoplasms, myelodysplastic syndromes, and acute myeloid leukemia. On the contrary, collagen fibres, are composed of type I collagen and appear to be characteristic of the advanced phases of myeloproliferative neoplasms (MPN), such as primary myelofibrosis (PMF) or secondary myelofibrosis (MF) arising from a pre-existing diagnosis of polycythemia vera (PV) or essential thrombocythemia (ET).[1] The content of BM fibres in routine sections of trephine biopsies of patients is usually demonstrated by histochemical staining using silver impregnation for reticulin fibres or by trichrome stains for collagen fibres.

Recent evidence has shown that the amount of BM reticulin often exhibits no correlation to disease severity, while the presence of collagen fibres is often associated with more severe disease and a poorer prognosis.[2] The exact pathogenesis of BM fibrosis is not fully understood. Aberrant tyrosine kinase signaling is a common hallmark in

MPNs and has been shown to represent a key driver of the disease. The three currently recognized driver mutations in PMF are JAK2 (Janus kinase 2; located on chromosome 9p24), CALR (calreticulin; located on chromosome 19p13.2), and MPL (myeloproliferative leukemia virus oncogene; located on chromosome 1p34). The JAK2 V617F substitution of a valine for a phenylalanine destabilizes the JH2 domain of JAK2 and causes loss of the auto-inhibitory activity of this domain.[3] The most common MPL mutations, W515L (tryptophan-to-leucine substitution) and W515K (tryptophan-to-lysine substitution), cause both cytokine-independent growth and hyper-TPO sensitivity.[4] JAK2V617F and MPL 515 mutations are present in about 50% and 5% of PMF cases respectively, resulting in permanent activation of the JAK/STAT signaling pathways and conferring *in vitro* altered response of mutated clones to thrombopoietin (TPO) and other cytokines.[3,5] In 2013 type 1 mutations (52-bp deletion) and type 2 mutations (5-bp insertion) were discovered in the Calreticulin gene. These mutations determine a constitutive activation of the MPL receptor through abnormal interaction with mutated calreticulin.[6,7]

Of the myeloproliferative disorders, PMF has the worst overall prognosis and morbidity.[8] Despite many significant advances in the treatment of this disease, many aspects of its origin and progression remain poorly understood. While recent understanding, on the pathogenic mechanisms in hematopoietic stem cells (HSCs), provides an explanation for myeloproliferation, several pieces of evidence clearly demonstrate that other processes are involved in this disease than the simple uncontrolled growth of mutant cells. In addition to BM fibrosis, the malignant stem cells exit from the BM as the disease progresses, and relocate in other hematopoietic organs, mostly the spleen and the liver.[9] This leads to the enlargement of the spleen and liver that is characteristic of this disease, causing significant morbidity.[10] In PMF the pathogenesis of myelofibrosis appears to be intimately linked with megakaryocyte (Mk) proliferation and differentiation.[11] Mks have the primarily function to generate and release platelets in close proximity of BM vasculature,[12] but they have also been shown to be involved in the control of BM homeostasis through the generation of signals that regulate HSC self-renewal and quiescence,[13,14] or differentiation of others BM cell niche, such as plasma cells[15] or osteoblasts.[16]

In PMF clusters of immature and necrotic Mks, surrounded by fibrotic areas in the BM, suggests that improper or premature release of their neat cargo of intracellular proteins unleash the uncontrolled and disseminated fibrotic reaction driven by BM stromal cells.[17] Noteworthy, Mks are very rich in cytokines, growth factors, cross-linking enzymes and extracellular matrix proteins (ECM) that are known to directly cause tissue fibrosis by stimulating stromal cells to produce collagen or that physically participate to ECM remodeling and BM scarring. Thus, here we will review what is known about the potential contribution of Mks to the onset and progression of BM fibrosis.

Megakaryocyte cargo in physiological thrombopoiesis. Mks are unique, polyploid hematopoietic cells that are found only in mammals, responsible for everyday production and release of millions of platelets into the bloodstream.[18]

Megakaryopoiesis is mainly driven by TPO, although this cytokine may be dispensable for terminal megakaryocyte maturation *in vitro*.[19] During the early stages of their differentiation, Mks become polyploid through repeated DNA replication and endomitotic cycles without cytokinesis.[20] At the end of maturation, the Mk cytoplasm becomes very specialized with the development of a complex system of membranes, called the demarcation membrane system (DMS), and three different types of granules including lysosomes, dense granules and α-granules.[20] Proteins contained in the α-granules (specialized secretory granules) can be synthesized or endocytosed.[21] As in other cells, the cell-specific proteins are synthesized by ribosomes on the rough endoplasmic reticulum and then packaged via the Golgi apparatus into nascent granules.[22] Mk α-granules are the most abundant secretory organelles and contain a large variety of adhesive proteins, such as β-thromboglobulin, CXCL4 (platelet factor 4, PF4), thrombospondin, fibronectin (FN), von Willebrand factor (vWF) and P-selectin. In addition, anti-angiogenic factors, endostatin and angiostatin, and pro-angiogenic factors such as VEGF and SDF-1α, are involved in the regulation of lymphatic system development and vascular integrity promoted by α-granule.[23,24] Mk α-granules contain additional growth factors for vascular repair, such as Platelet Derived Growth Factor (PDGF), Transforming Growth

Factor-β1 (TGFβ1), Epidermal Growth Factor (EGF) and Insulin Growth Factor (IGF).[25] Furthermore, members of the metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP) family, which are important factors in angiogenesis and tissue remodeling, are also stored in Mk cytoplasm.[26] In addition, these granules have been shown to contain several plasma proteins, such as fibrinogen,[27] Factor V, albumin and immunoglobulin,[28] which are not synthesized by the cell and are, therefore, endocytosed. Lastly, the pro-coagulant factors II, V, XI and XIII, high molecular weight kininogens, the anti-fibrinolytic factors plasminogen activator inhibitor-1, α2-antiplasmin and carboxypeptidase B2, anti-coagulant factors, for example, antithrombin, protein S, C1-inhibitor, TFPI and protease nexin 2 and pro-fibrinolytic proteins such as plasminogen and plasmin have been all localized in α-granules.[25]

Differently from α-granules, secretory lysosomes contain distinct acid hydrolases, such as beta- hexosaminidase, heparanase, elastase, and cathepsin D and E.[29] Finally, Mk dense granules store small bioactive molecules, while α-granules are predominantly protein-packed. The cargo comprises nucleotides (e.g., ADP, ATP), polyphosphates, Ca^{2+} and Mg^{2+} cations, but also neurotransmitters and hormones, such as serotonin and histamine.[30]

How do megakaryocytes target and deliver their protein cargo within bone marrow milieu?

The constitutive *in vitro* secretion of both α- and dense granules-derived bioactive molecules by developing Mks has been previously demonstrated.[31,32] These functional experiments revealed that secretion of intracellular Mk products governs autocrine mechanisms that sustain cell development and platelet release.[33-36] On the contrary, the physiological *in vivo* relevance of these autocrine loops has been less explored. However, recent data shed new light on the *in vivo* involvement of Mks in maintaining BM homeostasis though the controlled release of targeted stimuli.[37] To this regard, conditional ablation of Mks in mice resulted in increased BM HSC frequency and cycling, suggesting that Mks normally restrain HSC proliferation through the production of CXCL4 and TGF-β.[13,14] Conversely, Mk production of fibroblast growth factor-1 (FGF-1) is thought to play a key role in supporting HSC and osteoblast expansion recovery following myeloablative therapy.[16] Therefore, these studies raise the fundamental question: how is Mk compound exocytosis regulated during physiologically or pathologically BM functions? Differently from Mks, platelet activation is at the heart of the control of vascular integrity.[38] During circulation, platelets are reactive to various stimuli and release the materials stored in their specific granules.[39] The extrusion of storage granules' content to the platelet's environment occurs according to regulated secretion events: movements of granules, apposition and fusion of granules and plasma membranes.[40] This 'release reaction' is a key step of primary hemostasis, but it participates also in inflammation, atherosclerosis, antimicrobial host defense, wound healing, angiogenesis, and malignancy.[41] Our current understanding of Mk/platelet secretion at the molecular level is still insufficient to explain how the careful balance between all the bioactive molecules released from granules, under certain activation conditions, is achieved. One of the hypotheses is that Mk/platelet granules are not uniform and may be differentially packaged and thus released in a segregated manner following specific stimuli.[42]

To this regard, Ma et al. observed that platelet stimulation with specific protease-activated receptor- 1 (PAR-1) or PAR-4 agonist resulted in the preferential release of VEGF or endostatin (anti- and pro-angiogenic factors, respectively).[24,43] More recently, a super-resolution immunofluorescence co-localization analysis of 15 platelet α-granule cargoes failed to confirm any functional co- clustering of these proteins.[44] Moreover, Zingariello et al. demonstrated by immunoelectron microscopy that P-selectin and von Willebrand factor (vWF) are co-localized within the same intracellular α-granules in immature Mks. The two proteins, however, are not co-localized in α- granules of mature Mks after wild type mice treatment with TPO.[45] These results suggested that P- selectin and vWF are associated in the Mk cytoplasm at early stages of maturation but that they are routed into separate anti-angiogenic or pro-angiogenic α-granule subtypes as these cells mature. Thus, the differential association of vWF and P-selectin with anti-angiogenic or pro-angiogenic factors suggest that a mechanism regulating the sequential release of different α-granule subtypes may be involved in Mk/platelet function during tissue repair.[45] Interestingly, Mks derived from the GATA-1^{low}

mice, which harbors a hypomorphic mutation that blocks Mk maturation and displays BM fibrosis, showed reduced levels of expression of vWF and displaced P-selectin on the demarcation membrane system.

Further, the loss of alpha granules within BM Mks in a mouse model of Gray Platelet Syndrome (GPS) induced a myelofibrotic phenotype.[46] In PMF patients, ultrastructural abnormalities and variety in Mk-granules were reported by Thiele et al. more than 25 years ago.[47] Platelets derived from MPN patients showed several qualitative abnormalities, including decreased alpha granules and mitochondria and also alterations of the dense and tubular canalicular system.[48] Reduced levels of ADT, ATP and serotonin content in dense granules and lower content of beta-thromboglobulin (BTG) and platelet factor 4 (PF4) in alpha granules were also described.[49,50] Thus, aberrant assembly and secretion of Mk granules represent a potential mechanism of BM fibrosis progression **(Figure 1)**.

A second intriguingly hypothesis is that intracellular Mk/platelet products may be delivered in the surrounding space by extracellular vesicles (EVs). EVs are membrane-enclosed structures of varying size (50-10,000 nm) released from cells to mediate both local and distant intercellular communication.[51] EVs of various shapes and sizes have been demonstrated in several body fluids, with substantial variation in their structure, content and function.[52] There are three main types: exosomes (50-100 nm), microparticles (200 nm-1 μm) and apoptotic bodies. Protein, lipid and RNA components contribute to cell-cell crosstalk at a short distance, in a paracrine or endocrine manner via the bloodstream. In addition, they may transfer surface receptors from one cell to another and deliver proteins, mRNA, bioactive lipids, and even whole organelles (e.g., mitochondria) into target cells.[53] It has been reported that two mechanisms used by target cells to integrate EVs are cell endocytosis and membrane fusion.[53] Recent studies of EVs in the BM have shown that these vesicles serve to regulate hematopoiesis, participate in immune cell activation, and hemostasis.[54,55] Several lines of evidence suggest that EVs are involved in regulating BM function during homeostasis and in response to injury, but also that hematological malignancies such as leukemia, multiple myeloma or viral infections can exploit EVs trafficking to reinforce tumor growth, chemotherapeutic resistance, invasion and metastasis.[56,57] Platelet-derived vesicles were first identified by electron microscopy over 50 years ago, but the definition of their features and activities have only become a major focus of interest in recent years.[58] Platelet-derived microparticles (PMPs) are released from the platelet surface and are distinguished from platelet exosomes, which are derived from endocytosis and released from multivesicular endosomes.[59] PMPs may directly stimulate other cells (e.g., hematopoietic cells, lymphocytes and endothelium),[60] transfer platelet expressed receptors (e.g., CD41 or CXCR4) to the surface of other cells,[61] and, in some situations, transfer mRNA, proteins, and even infectious particles to the target cells. Interestingly, in healthy donors, the majority of circulating CD41+ PMPs do not express surface activation marker CD62P, suggesting that they do not originate from activated platelets.[62] In a very elegant study, Flaumenhaft et al. report that a significant number of circulating CD41+ MPs in healthy individuals are derived directly from Mks.[63] Authors first demonstrated via electron microscopy of spontaneous formation of Mk-derived MPs (MkMPs) from cultured murine Mk and that these MkMPs were different from PMPs. However, a functional role for MkMPs was not revealed until a recent study, which documented a novel biological role of MkMPs that are able to induce HSC differentiation towards the Mk lineage without exogenous TPO stimulation.[64] In this paper, Jiang et al. demonstrated that MkMPs, which are distinct from Mk exosomes, target HSC with high specificity since they have no effect on other BM-resident cells, such as mesenchymal stem cells, endothelial cells or granulocytes. They showed that both endocytosis and membrane fusion were responsible for the delivery of MkMP cargo to HSCs, and that MkMPs attached to and entered HSCs preferentially through their uropods, with CD54, CD11b, CD18 and CD43 being involved in target-cell recognition.[64] Aside from the role of EVs in the Mk-HSC crosstalk in the BM under physiological conditions, we can speculate that EV trafficking may also play a distinct role in deregulated hematopoiesis during fibrotic progression **(Figure 1)**. Interestingly, increased MP generation under high shear stress has been reported in platelets in the presence of TPO,[65] a main trigger of BM fibrosis in human and mice.

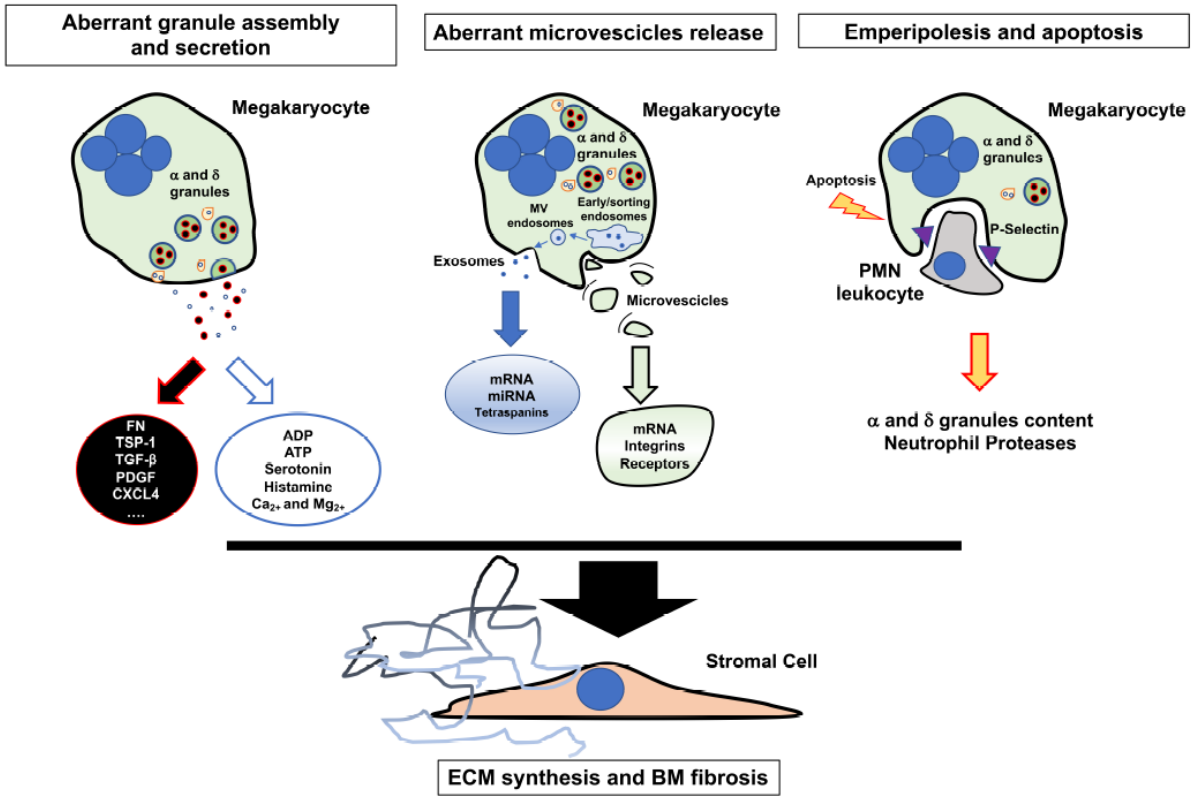

Figure 1. Schematic representation of potential mechanisms for aberrant release of Mk content in bone marrow fibrosis.

Megakaryocyte-derived pro-fibrotic cytokines in bone marrow fibrosis. In MPNs different mutations lead to myelofibrosis. The most frequent driver mutation in MPNs, *JAK2* V617F, is found in 50-60% of PMF as well as 50-60% of ET, but in almost all cases of PV.[66] It is now clear that the clinical phenotype of myelofibrosis is a consequence both of primary clonal myeloproliferation and secondary inflammation, characterized by profound changes to BM stromal compartment and an atypical cytokine storm.[67] Several evidences argue for an impaired microenvironment in association with inflammation rather than one single genetic trigger: 1) MPNs (PV, ET, and PMF) are all characterized by a significant change in the cytokine production mirrored by increased plasma levels of several inflammatory cytokines (e.g., IL1, IL2, IL6, IL8, IL12, TNFα, and IFNγ), growth factors (e.g., GM-CSF, G-CSF, HGF, PDGF, and EGF), and angiogenic factors (e.g., VEGF);[68] 2) clinical evidences that chronic inflammation is responsible of the constitutional symptoms which negatively affect the quality of life of MPN patients;[69] 3) clinical use of JAK inhibitors has confirmed that functional symptoms and splenomegaly in patients were concomitant with a significant increase in the plasma levels of many cytokines.[70]

In PMF, Mks and monocytes are supposed to be the main source of reactive cytokines that force fibroblast proliferation, fibrotic evolution, neoangiogenesis, and osteosclerosis.[11] Several lines of evidence obtained both from studies of patients or murine models are in favor of a crucial role of Mk in myelofibrosis induction: 1) all driver mutations in MPNs result in overproduction of abnormal Mks by hyperactive Jak2/Stat signaling;[71] 2) mice bearing a human JAK2V617F gene restricted exclusively to the Mk lineage develop many of the features of MPNs;[72] 3) patients and rats treated with TPO mimetics show BM fibrosis (usually reversible);[73] 4) high and persistent TPO production by transduced hematopoietic cells in mice results in a fatal myeloproliferative disorder that has a number of features in common with human PMF;[74] 5) impaired expression of the transcription factor GATA-1, involved in erythroid and megakaryocytic differentiation, results in the development of myelofibrosis;[75] 6) patients with GPS, a rare macro-thrombocytopenia with agranular Mk/platelets, manifest myelofibrosis and splenomegaly.[76] On the contrary, contribution of monocytes is less clear. Monocytes from patients with PMF were reported to be spontaneously

activated and to secrete abnormally TGF-β1,[77] however, TPO overexpression inducing BM fibrosis in NOD/SCID mice, which harbor impaired mononuclear phagocyte functions, led to controversial data.[78,79] Thus, based on the potential role of inflammation during fibrotic progression, we below describe the involvement and features of individual main Mk- derived cytokines/chemokines in the context of myelofibrosis **(Table 1)**.

Platelet-Derived Growth Factor (PDGF). PDGF is one of the first growth factors that has been implicated in the role of Mk in development of BM fibrosis.[80] PDGF is produced by Mks in the BM and is physiologically carried to the circulation in the α-granules of the platelets to act at the site of tissue injury as a mediator of tissue repair.[81] PDGF receptors (PDGFRs) are members of the membrane tyrosin-kinase family, composed of the two subunits PDGFRα and PDGFRβ, which form homo- or heterodimers. In the context of tissue repair, the PDGF/PDGFR axis not only enhances the replication, survival, and migration of myofibroblasts but also modulates the production and release of several pro- and anti-inflammatory mediators in fibrotic diseases.[82] Interestingly, PDGF has been reported to increase the expression of the collagen cross-linking enzyme, Lysil Oxidase (LOX), which in turn, oxidizes the PDGF receptor on smooth muscle cells, fibroblasts, and Mks, enhancing the proliferation signaling from this cytokine.[83,84] Ultimately, this loop has the potential to further boost the fibrotic phenotype.[85]

Increased levels of PDGF in plasma and urine from patients with MPNs have been reported.[86,87] Further, Mks and erythroid precursors contained increased levels of immunohistochemically detectable PDGF in BM biopsies of PMF patients.[88] The expression of members of the PDGF system in BM cells derived from PMF patients has been also investigated by real-time RT-PCR.

Increased expression of PDGFs could be demonstrated to be a feature of advanced fibrosis in PMF that is not demonstrable in the pre-fibrotic phase of the disease.[88]

Differently from their ligands, up-regulation of both PDGFRs during fibrotic progression is more controversial. In normal BM PDGFRα appeared in endothelial and endosteal cells in addition to strong labeling in Mks and platelets. In contrast, PDGFRβ subunit marked perisinusoidal stromal cells and adventitial fibrocytes of the larger vessels. However, in PMF patients, Bedekovics et al. found that PDGFRβ expression closely correlates with the grade of MF, while this was not evident for PDGFRα.[90] On the contrary, Bock et al. reported a strong up-regulation of the PDGFRα in patients with advanced myelofibrosis.[89]

Recently, the involvement of the PDGF/PDGFR axis in BM fibrosis has been definitively proven. Conditional deletion of the PDGFR-α gene and inhibition of PDGFRα by imatinib in leptin receptor+ stromal cells was shown to suppress their expansion and to ameliorate BM fibrosis in mice.[91]

Transforming Growth Factor-β (TGF-β). Among the abnormally expressed cytokines in PMF, TGF-β1 has received attention due to its critical role in inducing fibrosis not only in BM, but also in other organs.[92] TGF-β occurs in 3 isoforms: TGFβ1, TGFβ2 and TGFβ3. TGFβ1 is the most abundant of all these isoforms and platelets, Mks and monocytes cells are sources of TGF-β production.[93] TGFβ1 is secreted as latent protein and is stored in the extracellular matrix. Reactive oxygen species, proteases, integrins and thrombospondin-1 (TSP-1), convert the inactive latent complexes to the active forms.

Once activated, TGFβ-1 induces BM fibrosis on one hand, by increasing the synthesis of types I, III and IV collagen, FN, proteoglycans and tenascin;[94] while on the other hand, by decreasing matrix degradation through down-regulation of metalloproteinases (MMPs), particularly MMP3, and up- regulation of tissue inhibitors of metalloproteinase (TIMP), particularly TIMP-1.[95] However, effects of TGF-β1 are not restricted to the stromal compartment and TGF-β1-mediated changes to the BM niche remain to be fully elucidated. It is well known that TGF-β has direct effects on hematopoietic cells by negatively regulating granulocyte, erythroid, Mk and macrophage progenitor proliferation.[96] Further, Erba et al. showed that release and activation of TGF-β1 by Mks and platelets, forced endothelial cells from the BM microvasculature of PMF patients, and mouse model of PMF, to acquire a mesenchymal phenotype through Endothelial Mesenchymal Transition (EndMT), during the development of fibrosis.[97]

Table 1. List of Mk-derived cytokines implicated in bone marrow fibrosis progression.

Soluble factor	Mechanism of action	Evidences from mice models	Patient specimens	Ref.
Transforming Growth Factor-β (TGF-β)	- Increases the synthesis of types I, III and IV collagen, FN, proteoglycans and tenascin in stromal cells. - Down-regulation of metalloproteinases (MMPs) and up-regulation of tissue inhibitors of metalloproteinase (TIMP). - Favor Endothelial Mesenchymal Transition (EndMT).	- Significantly increased in the extracellular fluid of the BM, plasma and platelet extracts in the TPO^{high} and the $GATA\text{-}1^{low}$ mice. - Homozygous TGF-β1 null mice are protected from reticulin deposition after TPO overexpression.	- Quantitative alterations of TGF-β and its receptors in Mk, platelets, and CD34+ progenitor cells from MPN patients. - Aberrant TGF-β signaling in BM and spleen of PMF patients.	31, 89-96
Platelet Derived Growth Factor (PDGF)	- Enhances the replication, survival, and migration of myofibroblasts but also modulates the production and release of several pro- and anti-inflammatory mediators.	- Conditional deletion of the PDGFR-α gene and inhibition of PDGFR-α by imatinib in leptin receptor+ stromal cells suppress their expansion and ameliorate BM fibrosis in mice	- Increased levels of PDGF in plasma and urine from patients with MPNs - Up regulation of PDGFRs and PDGF in BM biopsies of PMF patients.	78-79, 81-85
Fibroblast Growth Factor (FGF)	Basic FGF is a potent angiogenic factor (Folkman & Klagsbrun, 1987); moreover, it is a mitogen for human bone marrow stromal cells Basic FGF is a potent angiogenic factor (Folkman & Klagsbrun, 1987); moreover, it is a mitogen for human bone marrow stromal cells - Sustains angiogenesis and is a potent mitogen factor for stromal cells.	N/A	- Increased expression in circulating Mks and platelets of PMF patients.	125
Vascular Endothelial Growth Factor (VEGF)	- Angiogenic factor	N/A	- Significantly higher level of VEGF in plasma samples of MPN patients.	126
Thrombospondin	- Anti-angiogenic factor - TGF-□ activator	- Tsp-1-null/TPO^{high} mice develop a myelo-proliferative syndrome with higher grade compared to wild type mice.	- Significantly overexpressed in BM biopsies of PMF patients when compared to controls.	128-129
CXCL4 (PF4)	- Induce activation and migration of myofibroblasts	N/A	- No correlation between BM fibrosis and plasma levels or the platelet content of CXCL4 in MPN patients.	107-108
Macrophage Inflammatory Protein 1α and 1β	- Pro-inflammatory chemokines	- Increased levels in Mks of $NBEAL2^{-/-}$, a GPS mouse model which display BM fibrosis.	- Increased in plasma samples of PMF patients.	116-117
Interleukin-8	- Stimulates neutrophil chemotaxis - Sustains neo-angiogenesis - Negative regulator of Mk proliferation	N/A	- IL-8 level is strongly increased in the serum and plasma of patients with PMF	63, 120
Lipocalin-2 (LCN2)	- LCN2 generates increased reactive oxygen species, leading to increased DNA strand breaks and apoptosis of normal, but not PMF, CD34(+) cells. - Increases generation of osteoblasts and fibroblasts, but not adipocytes, from mesenchymal stem cells.	N/A	- Increased in plasma samples of PMF patients.	124

Secreted phospho-protein 1 (Spp1)	- SPP1 promotes fibroblasts and mesenchymal stromal cells proliferation and collagen production	N/A	- SPP1 plasma levels are significantly higher in PMF compared with ET and PV patients. - Higher SPP1 plasma levels in PMF patients correlate with a more severe fibrosis degree and a shorter overall survival	127
Bone morphogenic proteins (BMPs)	- BMP-1 is an activator of latent TGF-β and processor of collagen precursors.	N/A	- Expression of BMP1, BMP6, BMP7, and BMP-receptor 2 are significantly increased in advanced stages of myelofibrosis compared with controls	122-123
Oncostatin M	- Stimulates growth of fibroblasts and microvascular endothelial cells and induces the production of angiogenic and profibrogenic cytokines (HGF, VEGF, and SDF-1) in BM fibroblasts.	N/A	- OSM mRNA levels are increased in the BM of patients with MPNs compared to control patients	114-115

Not surprisingly, several groups reported on quantitative alterations of TGF-β and its receptors in Mk, platelets, and CD34+ progenitor cells from MPN patients and concluded that TGF-β was involved in myelofibrosis and myeloproliferation.[31,98-100] In addition to quantitative alterations, Ciaffoni et al., recently demonstrated abnormalities in TGF-β1 signaling genes in the marrow and spleen of PMF patients.[101] These alterations included genes of TGF-β1 signaling, cell cycling, Hedgehog and p53 signaling and suggested a non-canonical TGF-β1 signaling in marrow identifying, for the first time, autoimmunity as a possible cause of BM fibrosis in PMF.[101] Among the genes that predict the activation of the non-canonical TGF-β signaling, expression level of the Jun gene was increased in BM of PMF patients. Interestingly, over-expression of Jun was sufficient to induce myelofibrosis, severe fibrosis in multiple organs and steatohepatosis in mice.[102]

Moreover, the involvement of TGF-β in *in vivo* mouse model has been deeply investigated. TGF-β was significantly increased in the extracellular fluid of the BM, plasma and platelet extracts in two widely MF studied mouse models, which include the TPO^{high} and the GATA-1^{low} mice.[103,104] To test directly the impact of TGF-β1 in the pathogenesis of MF, BM stem cells from homozygous TGF-β1 null (TGF-β1(-/-)) and wild-type littermates were infected with a retrovirus encoding the murine TPO protein and engrafted into lethally irradiated wild type hosts for long-term reconstitution. Differently from wild type mice, none of the mice repopulated with TGF-β1(-/-) cells showed deposition of reticulin fibres at any time during the follow-up.[105] Consistently with patient data, alterations of TGF-β1, Hedgehog, and p53 signaling pathways were identified in the BM of GATA- 1^{low} mice model.[106] Inhibition of TGF-β1 signaling in these mice by an inhibitor of the tyrosine kinase activity of TGF-β1 receptor type I, led to restoration of normal Mk development, reduced fibrosis, neoangiogenesis, and osteogenesis in the BM.[106] Based on these consistent observations, TGF-β inhibition has become a potential therapeutic strategy to decrease BM fibrosis in MPNs and is also being investigated in several clinical and experimental scenarios.[107]

Cxcl-4 (Pf-4). Cxcl-4, (C-X-C motif) ligand 4 (CXCL4) (also known as platelet factor 4 [PF4]), is one of the most abundant protein in the α-granules of Mk/platelets (estimated micromolar concentration), together with CXCL7.[108] This 70 a.a., cationic, lysine-rich, 7.8-kDa chemokine, is mainly synthesized by Mks, and comprises 2%-3% of the releasate from agonist-activated platelets. Once secreted, CXCL4 avidly binds to glycosaminoglycans, but only a splice-variant of the human chemokine receptor CXCR3 (CXCR3B), which is not present in mice, and LDLR90 have been identified as high-affinity receptors.[109] In contrast, the specific receptor for CXCL4 has not yet been identified in mice. However, in some circumstances, CXCL4 can interact with other chemokines (e.g. CCL5) and thereby modulate their effects on target cells. A central role of platelet-derived CXCL4 was demonstrated in solid organs. *In vivo*, mice lacking CXCL4 are significantly protected from severe liver fibrosis, demonstrating the pro-fibrotic phenotype of this chemokine and that its effects in mice are indeed mediated by other receptors than CXCR3.[110] In addition, it was shown that CXCL4 is secreted not only by activated platelets, but also by plasmacytoid dendritic cells and fibroblasts in systemic sclerosis.[111] Thus, these studies further involve Mks/platelets to pro- inflammatory and pro-fibrotic programmes in fibrosis. Interestingly, more than 30 years ago, Burstein et al. linked CXCL4 to myelofibrosis, by suggesting that abnormal Mks stimulate the proliferation of fibrosis-driving fibroblasts though the release of CXCL4.[112] However, no correlation was seen between BM fibrosis and plasma levels or the platelet content of CXCL4 in the same study.[112] Recently, Schneider et al. using a mouse model with genetic fate tracing *in vivo*, provided evidence that Gli1+ cells are key players in the initiation and progression of BM fibrosis and that Mk-derived CXCL4 was necessary and sufficient to induce the migration of Gli1+ stromal cells and their myofibroblastic differentiation.[113] In these experiments, CXCL4 was shown to induced myofibroblast differentiation of Gli1+ cells comparable to induction with TGF-β, a known stimulus for differentiation of MSCs into myofibroblasts.[113]

Other cytokines. Oncostatin M (OSM), is a pleiotropic cytokine belonging to the interleukin-6 (IL-6) family.[114] Produced mainly by activated T cells and monocytes, OSM can elicit different biological effects, depending on the cell type.

OSM acts through two types of receptors. The type I OSM receptor is composed of gp130 and the leukemia inhibitory factor (LIF) receptor β-subunit (LIFR), and the type II OSM receptor is composed of gp130 and the OSM-specific receptor β- subunit (OSMR).[115] OSM has emerged as an important cytokine in the control of hematopoiesis. Transplantation experiments with OSM-deficient mice have shown that OSM stimulates stromal cells as well as hematopoietic progenitors and is required for the proper generation and maintenance of microenvironment in the BM.[116,117] Noteworthy, BM Mks express substantial amounts of OSM,[118] and OSM has been reported to behave as a megakaryocytic maturation factor *in vitro* and to augment platelet production *in vivo.*[119] Within the context of myelofibrosis, JAK2 V617F mutation promotes expression of OSM in neoplastic myeloid cells and, consequently, OSM mRNA levels are increased in the BM of patients with MPNs compared to control patients.[120] Mechanistically, OSM secreted by JAK2V617F+ cells stimulated growth of fibroblasts and endothelial cells by sustaining the production of angiogenic and pro-fibrogenic cytokines.[120]

Aberrant packaging of α-granule-specific proteins is supposed to trigger myelofibrosis in patients with GPS. Using a Nbeal2$^{-/-}$ murine model of GPS, Guerrero et al. demonstrated that BM Mks from these mice were enriched in a restricted set of chemokines transcripts, namely CCL3 and CCL4, which encode macrophage inflammatory protein (MIP) 1α and 1β, respectively, well-known pro- inflammatory chemokines increased in PMF.[46] A peculiar role for MIP 1α in sustaining osteoblasts proliferation in MPN mice model has been also proposed.[121]

Interleukin 8 (IL-8) is a member of the family of chemokines related by a CXC motif. It binds to CXC chemokine receptor 1 (CXCR1) and 2 (CXCR2).[122] It is produced by several cell types, including Mks[123] and exhibits many biological functions in inflammation, HSC proliferation and mobilization and neo-angiogenesis. Increased levels of IL-8 were found in serum[124] and plasma[68] of patients with PMF. Additionally, IL-8 and its receptors were reported to be involved in PMF-altered Mk growth.[124] Finally, rodents lack a direct homologue of IL-8, but the chemokines CXCL1/KC, CXCL2/MIP-2, and CXCL5-6/LIX are regarded as functional homologues of IL-8.[125]

Finally, Mks were repeatedly identified as the main cellular source of an increasing list of cytokines, which show higher plasma levels in PMF patients, and that are individually involved in the promotion of myelofibrosis. This list further includes bone morphogenic proteins (e.g. BMP-1[126], BMP-2, -4, and -6[127]), Lipocalin-2 (LCN-2),[128] Fibroblasts Growth Factor (FGF),[129] Vascular Endothelial Growth Factor (VEGF),[130] Secreted Phospho Protein-1 (SPP1)[131] and Thrombospondin- 1 (TSP-1).[132,133]

Megakaryocyte expression of extracellular matrices and cross-linking enzymes in bone marrow fibrosis. Deregulated extracellular matrix (ECM) dynamics in terms of amount, composition and topography is a hallmark of BM fibrosis.[1] This in turn potentiates the oncogenic effects of growth factor signaling pathways and alters cell behaviors during fibrosis progression. ECM components are not solely expressed by stromal cells, several evidences suggest that Mks may directly influence the biochemical properties and architecture of BM ECM both in physiological and pathological conditions.[134] It is known that Mks can secrete various ECM components which are supposed to sustain Mk maturation and platelet release by creating a regulatory niche within the BM environment.[135] Mks express different collagen types (e.g., III, IV), glycoproteins (e.g., Fibronectin and Thrombospondin) and proteoglycans. Interestingly, TPO has been recently recognized as a pivotal regulator of this new Mk function, by inducing TGF-β1 release and consequent activation of TGF-β downstream signaling pathways, both *in vitro* and *in vivo*.[136] This activation led to a dose dependent increase of ECM component synthesis by Mks, which was reverted upon incubation with JAK and TGF-β1 receptor specific inhibitors.[136]

In parallel with ECM secretion, Mks express several modifiers of ECM structure. Factor XIII-A is synthesized by Mks and both protein and mRNA are packaged into the cytoplasm of forming platelets.[137] Factor XIII-A belongs to transglutaminases, a class of calcium ion-dependent enzymes that catalyze an acyl transfer reaction in which y-carboxamide groups of peptide-bound glutaminyl residues are acyl donors and primary amine including the ε-amino group of peptide-bound lysyl residues, are acyl acceptors. By this reaction, transglutaminases catalyze the formation of ε-(y- glutamy1) lysine linkages between proteins. Thus, based on these properties,

the potential role of FXIII-A in the BM environment may consist in the cross-link of extracellular fibrillar FN matrix with collagen.[138,139]

LOX is a copper-dependent amine oxidase that catalyzes oxidative deamination of lysine and hydroxylysine residues on collagen and elastin, leading to cross-linking within these proteins and changes in ECM elasticity. Eliades et al., detected LOX expression in diploid-tetraploid Mks, but scarce traces in polyploid Mks and identified a peculiar role for this enzyme in BM fibrosis.[84] They found that in the GATA-1^{low} mouse model, which is characterized by increased frequency of low ploidy Mks and extensive matrix of fibres, LOX was abundantly expressed by low ploidy Mks. More importantly, administration of β-aminopropionitrile (a LOX inhibitor) to the GATA-1^{low} mice inhibited the progression of myelofibrosis. Consistently, human platelets and Mks from patients with PMF overexpress LOX and show higher adhesion to collagen that is dependent on LOX activity.[140]

In addition to cross-linking enzymes, also ECM degradation directly impacts cell behavior and migration. Metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases and function in remodeling the ECM by its ability to degrade and cleave ECM components with wide substrate specificities.[141] Once activated, the MMPs are subject to inhibition by the tissue inhibitors of metalloproteinases (TIMPs) that bind MMPs non-covalently and counteract their proteolytic activity.

Mks synthetize several MMPs, particularly gelatinases MMP-2 and MMP-9.[142] Moreover, transcripts for MMP-1, 11, 14, 15, 17, 19, 24 and 25 have also been identified.[26] Conversely, biosynthesis of TIMPs 1-4 in Mks/platelets intervenes in excessive tissue remodeling.[143] It is suggested that BM fibrosis in PMF results from enhanced TIMP and decreased MMP activities. In particular, TIMP-1 (both the total, complex and the free form) is significantly increased in MPNs, while MMP-3 is significantly decreased, and levels of MMP-2 and MMP-9 are not different from control values.[95,144] Further, membrane type 1-MMP (MMP-14) was found overexpressed by up to 80-fold in advanced stages of fibrosis, and Mks and endothelial cells were unmasked as the major cellular source.[145] By contrast, a significantly higher expression of neutrophil collagenase (MMP-8) was encountered in the pre-fibrotic stages of PMF. Although the JAK-STAT signaling pathway is directly involved in the regulation of genes encoding MMPs, the altered expression of MMPs seem not influenced by the *JAK2* mutation status but predominantly related to the stage of disease.[145]

Direct Megakaryocyte-cell interactions in the context of bone marrow fibrosis. In addition to secretory events, one more pathophysiological mechanism operating in the development of myelofibrosis is the abnormal interaction of Mks with cell components of the BM (**Figure 1**). Selectins (CD62L, CD62P) and Mk glycoproteins (CD41a, CD42b) were demonstrated to mediate Mk-fibroblast interactions in human BM and to increase fibroblast growth.[146]

Abnormalities in mesenchymal stem cells derived from PMF patients were reported to alter the ability of these cells to support Mk differentiation *in vitro*.[147-149] Further, a pathological interaction, between polymorphonuclear (PMN) leukocytes and Mk, correlated with MF development, has been also proposed.[17] Emperipolesis is the random passage of the different types of BM cells through Mk intracellular space. The phenomenon is strongly increased in BM of patients with MPN disorders.[150] Schmitt et al., first showed both in the BM of patients with PMF, and in the TPO^{high} murine model, abnormal subcellular P-selectin distribution, which appeared to correlate with excessive and pathological emperipolesis of PMN leukocytes within Mk.[150] This abnormal interaction was considered the main cause of the destruction of Mk storage organelles and leakage of α-granular contents into the BM microenvironment.[151] As in patients, a similar pathologic neutrophil emperipolesis was detected in the GATA-1^{low} mouse model of myelofibrosis.[152] In BM Mk of these mice, P-selectin, although normally expressed, was found frequently associated with the demarcation membrane system (DMS) instead of within granules. In addition, pathologic Mks were surrounded by myeloperoxidase-positive neutrophils, some of which appeared in the process to establish contact with Mks by fusing their membrane with those of the DMS. Quantification of this process revealed that 34% (in BM) of GATA-1(low) Mks contained 1 to 3 neutrophils embedded in a vacuolated cytoplasm. The neutrophil-embedded GATA-1(low) Mks

displayed morphologic features compatible with those of cells dying from para-apoptosis, confirming the hypothesis that emperipolesis sustains myelofibrosis by driving the release of fibrogenic Mk cytokines and neutrophil proteases in the BM microenvironment.[152] Moreover, abnormal localization of P-selectin in Mks and platelets, induced by the GATA-1(low) mutation, was further involved in the pathological interactions of circulating platelets with leucocytes, responsible for the increased presence of thrombosis seen in these mice,[153] as well as, in the promotion of extramedullary hematopoiesis.[154] Consistently, high rate of emperipolesis is detectable in BM biopsies of patients with GPS, a rare inherited bleeding disorder characterized by deficiency of platelet α-granules, macrothrombocytopenia and marrow fibrosis.[155]

Is the pro-fibrotic role of Megakaryocytes/ platelets restricted to the bone marrow? New discoveries in the field of thrombopoiesis and platelet roles have revealed unprecedented features of the Mk/Platelet lineage that open new avenues in the study of these cells, particularly in diseased conditions. Bioactive mediators, stored in platelets, have been implicated in fibrotic conditions that target solid organs, rather than BM (**Table 2**).

A large amount of experimental evidence implies that platelets participate in the liver fibrotic process mainly by releasing pro- fibrotic mediators. Using mice carrying a Mk/platelet-specific targeted conditional deletion of the *TGF-β1* gene ($PF4^{Cre}Tgf\beta1^{f/f}$), Ghafoory et al. demonstrated that platelet TGF-β1 deficiency decreases liver fibrosis in a mouse model of carbon tetrachloride (CCl_4)-induced liver injury.[156] However, there is also evidence that platelets under certain circumstances may have a protective role against liver fibrosis. To this regard, thrombocytopenic mice, with selective disruption of the anti-apoptotic gene Bcl-xL, were shown to be more prone to liver fibrosis by bile duct ligation compared to their wild type counterparts.[157] The authors, suggested that the anti-fibrotic Hepatocyte Growth Factor (HGF) released from activated platelets in liver, attenuated the expression of collagen in hepatic stellate cells, the key cell type in liver fibrosis.[157] Additionally, Mk-specific disruption of the *TGF-β1* gene resulted in mice protection from cardiac hypertrophy, fibrosis, and systolic dysfunction in response to transverse aortic constriction, suggesting that platelet profibrotic behavior is not solely restricted to the liver.[158] Similarly, evidence has been accumulated implicating platelets in the pathogenesis of interstitial lung fibrosis in several animal models. Piguet et al. found that trapping of platelets in contact with the alveolar endothelium of the lungs after bleomycin injection was increased and correlated with the deposition of collagen.[159] The authors suggested that this could represent not only a simple correlation but also a potential pathological mechanism that links platelets and pulmonary fibrosis. Interestingly, in a recent study platelets were shown to promote acute lung injury through the massive release of the Wnt/β-catenin inhibitor Dickkopf-1 (Dkk-1) from their α-granules, leading to increased expression of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) on the surface of alveolar epithelial cells (AECs) and abnormal macrophage/neutrophils interaction with AECs.[160] In addition to the direct involvement of platelets in fibrosis of solid organs, the potential contribution of Mks to organ fibrosis still needs to be uncovered. It is becoming increasingly clear that Mks transfer unique genetic codes to platelets, and that environmental changes can alter

Table 2. Megakaryocytes/Platelets contribution to organ fibrosis.

Organ	Role in fibrosis	Mechanism	Experimental model	Ref.
Liver	Pro-fibrotic	Release of TGF-β1	Platelet specific TGF-β1 deficiency in a mouse model of carbon tetrachloride (CCl4)-induced liver injury	152
Liver	Anti-fibrotic	Release of HGF	Platelet specific Bcl-xl deficiency in a mouse model of bile duct ligation	153
Heart	Pro-fibrotic	Release of TGF-β1	Mk-specific TGF-β1 deficiency in a mouse model of transverse aortic constriction	154
Lung	Pro-fibrotic	Trapping into alveolar endothelium	Bleomycin induced pulmonary fibrosis	155
Lung	Pro-fibrotic	VCAM-1 and ICAM-1 upregulation in AECs due to Dkk-1 platelet release	2-hit acute lung inflammation model with a moderate dose of lipopolysaccharide followed by a high tidal volume mechanical ventilation	156

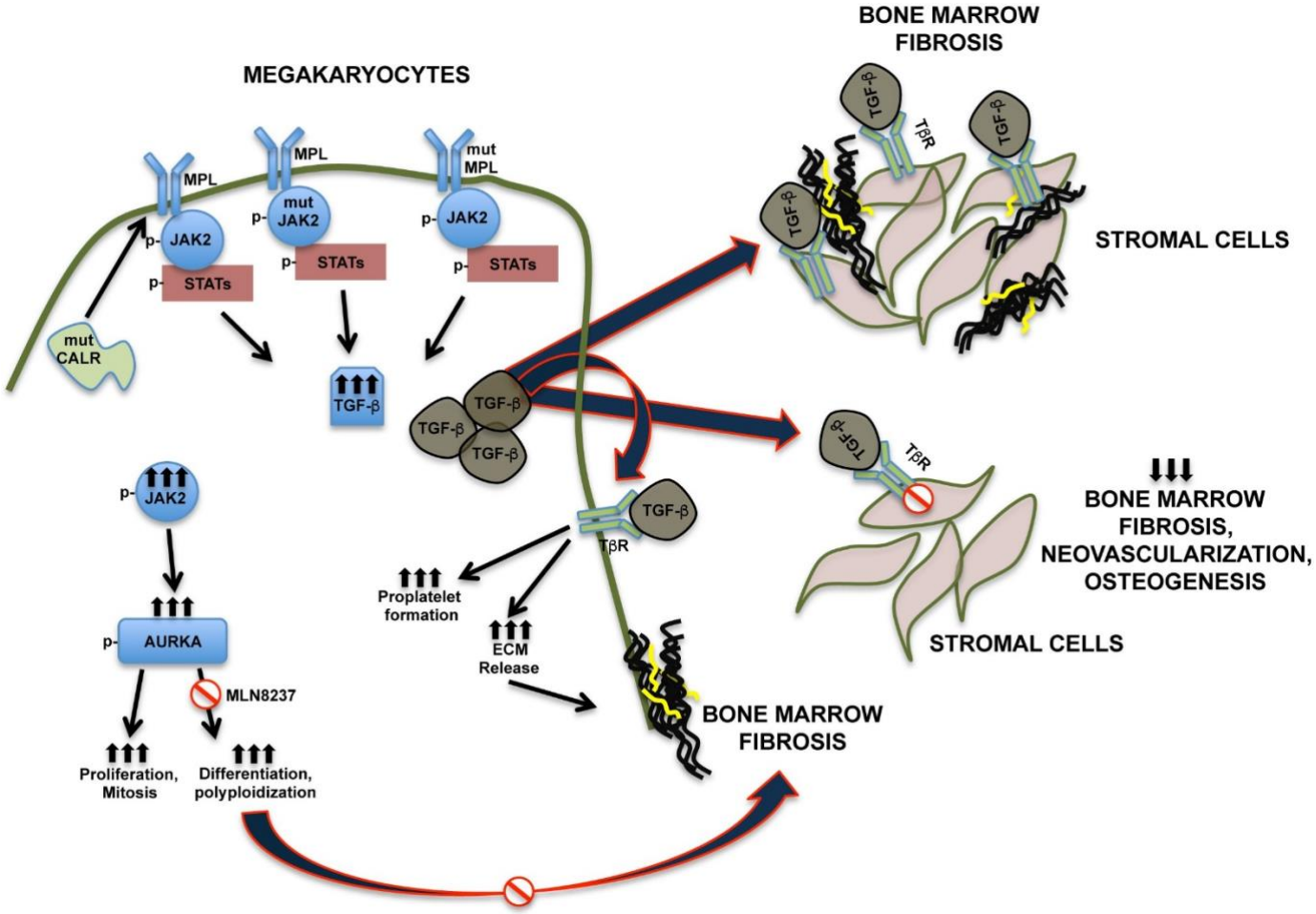

Figure 2. Schematic representation of potential novel mechanisms for MPN pathogenesis.

transcriptional, translational, and post-translational processes in Mks, affecting the genetic code of platelets in circulation.[161] Change in Mk-platelet transcriptional axis is a dynamic process, especially in disease situations, and can rapidly affect the platelet repertoire of messenger RNAs (mRNAs), microRNAs (miRNAs), and proteins that contribute to their primary and alternative functions. Freishtat et al., revealed for the first time, that a *de novo* transcriptome is imparted to platelets by BM residing Mks during sepsis.[162] Septic Mks produce platelets with acutely altered mRNA profiles, and these platelets mediate lymphotoxicity via the potent cytotoxic serine protease, granzyme B.[162] Similarly, in the context of cancer, Mks of tumor-bearing mice endocytose circulating thrombospondin-1 (TSP-1) and increase its synthesis to produce platelets with elevated levels of TSP-1, one of the most potent angiogenesis inhibitors. These TSP-1-enriched platelets were shown to adhere to tumors and to act as potent inhibitors of angiogenesis and cancer growth.[163] Thus, similar changes may occur in fibrotic conditions, but this has not been demonstrated yet.

Conclusions. In this review, we summarized the involvement of the Mk lineage in the development of BM fibrosis. We now know that, in addition to genetic triggers, BM fibrosis is sustained by the intramedullary release of cytokines that are responsible for the abnormal activation of stromal cells, resulting in extensive deposits of reticulin and collagens. Mks are supposed to constitute the main source of these reactive cytokines. Abnormal Mk differentiation, apoptosis and emperipolesis were all proposed as major mechanisms for the enhanced release of cytokines with a fibrogenic potential. Unfortunately, mechanisms underlying Mk secretion, their relationships with other BM lineages and their functional activities in physiological conditions as well as during myelofibrosis progression, are not well understood to date. The first attempt to directly target the Mk lineage was shown, recently, to revert the disease in both Jak2V617F and MPLW515L mice models. Using a small molecule, the AURKA inhibitor MLN8237, that induce Mk polyploidization, differentiation, and subsequent apoptosis, the Crispino's group demonstrated that the pharmacological induction of Mk maturation was

beneficial in terms of reduced burden of immature Mks and amelioration of PMF features, including BM fibrosis.[164] Thus, developing drugs able to re-establish Mk normal function may represent a new strategy to treat the disease and, at the same time, to understand its pathogenic mechanisms (**Figure 2**).

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