Biology of myelofibrosis and possible drug targets
“The true nature of stem cells can be learned only by discovering how they are regulated”

Spradling et al., Nature, 2001
Hematopoietic stem cell niche

**Endosteal niche:**
Osteoblasts, stroma cells. Function: HSC dormancy

**Vascular niche:**
Granulocytes, megakaryocytes. Function: barrier (controlled release into bloodstream of erythroid, platelets and mature granulocytes)

A subtle balance between the two types of microenvironmental compartments maintains hematopoietic homeostasis - an accurate equilibrium between HSC dormancy, activation, and differentiation.
Tight balance is maintained through cell intrinsic and extrinsic mechanisms

**Extrinsic mechanisms:**
- cytokines
- chemokines
- adhesion molecules
- negative regulators (osteopontin)
- proteases (MMP-9, cathepsin K)
- hormones (PTH, PGE2)
- sympathetic nerves
- oxygen status
- calcium concentration
- circadian rhythm

**Intrinsic mechanisms:**
- signalling molecules
  - cytoplasmic
  - nuclear, incl. transcription factors
- epigenetic mechanisms
  - DNA/histone modif.
  - microRNAs

**HSC**

**Ang-1** - tie2
**SCF** - c-kit
**Flt3-L** - Flt3
**TPO** - mpl
**Wnt** - Frizzled
**Jagged/Delta** - Notch

**Cadherins**
- VCAM - VLA
- ICAM - LFA
- CXCL12 - CXCR4
- HA - CD44

**Quiescence**
**Self-renewal**
**Expansion**

**Cell-cell adhesion migration**

**PTH, PGE2**

**Nerves**
Main actors of complex regulatory signaling

- Combined cell-intrinsic regulatory mechanisms (Tie2/angiopoietin-1, Notch/Jagged, Frizzled/Wnt, etc),

- Adhesion molecule interactions (VLA4, VLA5, CD44, N-cadherin, etc),

- Extracellular matrix components and environmental elements such as calcium, oxygen concentration, hormones (parathyroid hormone), bone morphogenic proteins (BMP-2/-4), cytokines (FGF-4, SCF, VEGF, TPO, etc), and chemokines (CXCL12, IL8, etc).

Alterations of this balance or regulatory signalling can lead to uncontrolled cellular proliferation and, ultimately, to the promotion of leukemias and MPDs.
Myelofibrosis is a stem cell and its niche disease

- Primary myelofibrosis (PMF) is a chronic hematologic malignancy characterized by splenomegaly, leukoerythroblastosis, cytopenias, teardrop poikilocytosis, marrow fibrosis, extramedullary hematopoiesis, increased marrow microvessel density, and constitutive mobilization of HSC and HP.

- 1. Clonal hematopoiesis is a sign of involvement of the hematopoietic stem cells.

- 2. Myelofibrosis, osteosclerosis and neoangiogenesis indicate the profound modifications of the stroma within bone marrow and spleen.

- 3. Disequilibrium between endosteal and vascular niches favors the proliferation of stem cells including hematopoietic, mesenchymal (?), and endothelial stem cells together with their mobilization from the bone marrow to the blood.
Myelofibrosis is a complex disease
I. Molecular HSC clone events

- The molecular mechanism leading to the transformation of HSC clone is not known.
- The discovery of Jak2 V617F mutation in 2005 has changed understanding of the pathogenesis of the disease. However, Jak2 V617F syndromes exhibit various clinical features like PV, ET or PMF. Cases negative for this mutation have the same disease phenotype. It strongly suggests that other acquired events are required for the development of PV, ET and PMF in Jak2 negative cases. It is likely, that Jak2 V617F is the next step of HSC transformation process and contributes but not causes the disease phenotype.
- Candidate event required for development of PMF might be mutations in trombopoietin receptor MPL. The presence of activating mutations affecting MPL (MPLW515L and MPLW515K) is in agreement with this assumption. However, frequency of MPL mutations is 5-7%. Moreover, 80% MPL mut+ cases also harbor Jak2 V617F.
- JAK2V617F and MPLW515 mutations affect cytokine signaling, survival, and cell-cycle transition, through a constitutive activation of JAK-STAT pathway.
- Experimental models have shown that myeloproliferative diseases could not result simply from hematopoietic cell-intrinsic defects (altered Jak-STAT signalling), but are heavily influenced by alterations and/or mutations in the hematopoietic microenvironment.
The Jak kinases normally function through their interaction with cytokine receptors that lack intrinsic kinase activity. Receptor-bound ligand complex is not able itself to transduce signal. Such complex activates Jak kinase located underneath of cell membrane and ligand produced signal is transduced into the cell resulting in adequate cell response.

EPOR, MPL and GCSFR are homodimeric type I cytokine receptors that are expressed on cells of the erythroid, megakaryocytic and granulocytic lineages.
The Jak kinases have seven homologous domains (JH1–7), including the catalytic kinase domain (JH1) and a catalytically inactive pseudokinase domain (JH2). The JH2 domain serves an autoinhibitory function similar to the juxtamembrane domain of receptor tyrosine kinases. The JAK2V617F point mutation results in a single amino acid substitution within the JH2 domain, abrogates autoinhibition and results in constitutive kinase activity. The JAK2V617F protein has constitutive kinase activity, that is it is constitutively phosphorylated - JAK2V617F is a gain-of-function mutation.
MPL mutations

Homodimeric trombopoietin receptor MPL, containing conserved C residues and WSXWS motifs. W515 mutations localize to the transmembrane domain of MPL and abrogates autoinhibition. MPLW515L/K mutant thrombopoietin receptors are able to phosphorylate wild-type JAK2 in the absence of thrombopoietin, and result in the activation of signalling pathways downstream of JAK2.

MPL mutations – initiating event?
Cytokines normally bind cytokine receptors, which results in JAK2 phosphorylation. Activated Jak2 phosphorylates a transcription factor STAT, which migrates into nucleus, where induces transcription of genes, required for the cell proliferation and survival.

Similarly, other signalling pathways are activated - mitogen activated protein kinase (MAPK) signalling proteins, and the phosphotidylinositol 3-kinase (PI3K)–Akt pathway resulting in activation of respective gene transcription.

The JAK2V617F mutant kinases bind cytokine receptors and are phosphorylated in the absence of ligand, and lead to ligand-independent activation of downstream signalling pathways.
II. CD34+ cells

A number of in vitro/in vivo studies indicate that JAK2V617F and MPL mutational events originate in a cell capable of generating both myeloid and lymphoid cells such as the multiipotent HSC.

In animal models transplanted PMF CD34+ cells generate myeloid and B cells that are clonal (marked by JAK2V617F). The differentiation program of PMF CD34+ cells following transplant is also remarkably different from normal CD34+ cells: PMF cells produce greater numbers of CD34+, CD33+ (myeloid lineage), and CD41+ (MK and platelets) cells but fewer CD19+ B cells (results in lymphopenia).

The phenomenon was named as predisposition to produce greater numbers of MK. It was proved in vitro by incubating PMF CD34+ cells in the presence of stem cell factor and thrombopoietin (TPO). PMF CD34+ cells displayed a greater proliferative capacity and ability to produce greater numbers of MK that were resistant to apoptosis due to overexpression of the antiapoptotic factor Bcl-XL. The MK hyperplasia in PMF is determined by: (1) an increased ability of CD34+ cells to generate MK and (2) the accumulation of MK Bcl-XL.
III. Mobilization of CD34+ and endothelial cells

- PMF is defined by unique clinical feature – HSC/HPC trafficking. Indeed, the huge mobilization of hematopoietic progenitors from bone marrow to spleen and liver is illustrated by an up to 200-fold increase in the circulating CD34+ cell count.

- Endothelial progenitor cell mobilization predominates during the prefibrotic phase of PMF, while HSC/HPC mobilization occurs in more clinically advanced phases of the disease.

- PMF stem cell mobilization results from an impairment of their adherence to bone marrow stroma allowing them to leave their niches, to enter the circulation, and to colonize the spleen and liver where primitive endothelial cells built up a new vascular niche attracting HSCs and supporting their homing. It results in extramedullary hematopoiesis.
III. Mobilization of CD34+ and endothelial cells

- Several proteolytic pathways play a role in abnormal cytokine-mediated stem cell mobilization.

1. Proteins released by activated neutrophils cleave vascular adhesion molecule-1 (VCAM-1) expressed by stromal cells, leading to the disruption of a key adhesive interaction between VCAM-1 and very late antigen-4 (VLA-4) expressed by HSC/HPC. The concentration of soluble VCAM-1, a degradation product of VCAM-1, is elevated in the plasma of patients with PMF and is correlated with the absolute numbers of CD34+ cells in the peripheral blood of patients with PMF.

2. The interaction between stromal cells, endothelial cells, and osteoblast-derived stromal cell derived factor-1 (SDF-1) and the CXC chemokine receptor-4 (CXCR-4) expressed by HSC/HPC. CXCR-4 expression by CD34+ cells is downregulated and plasma SDF-1 levels are elevated, which disrupt SDF-1/CXCR-4 interactions leading to CD34+ cell mobilization.

3. Proteases, including neutrophil elastase, soluble matrix metalloproteinase-9 (MMP-9) and cell bound MMP-9 have been shown to play a role in the constitutive mobilization of CD34+ cells that occurs in patients with PMF.
Myeloproliferation

1. The presence of mutations in the JAK2 and MPL genes associated with an inappropriate activation of JAK/STAT proteins that participate in the proliferation and hypersensitivity of HSC/HP to growth factors,

2. The multipotency of the hematopoietic clonal cell with myeloid and lymphoid differentiation, although an absolute lymphopenia has been described in peripheral blood;

3. The hypersensitivity of hematopoietic progenitors to growth factors, resulting in the overproduction of one or more of the mature elements of blood and the progressive dominance of clonal (myelo)hematopoiesis over normal polyclonal hematopoiesis;

4. An involvement of the megakaryocytic (MK) lineage, with hyperplasia and dysplasia resulting in an excessive production of several cytokines and chemokines;
IV. Myelofibrosis, angiogenesis and osteosclerosis

- One of the hallmarks of PMF is profound marrow fibrosis. Collagen type 3 (reticulin) and collagen type 1 (COL-1) are the predominant extracellular components of marrow fibrosis in PMF.

- These matrix components are produced by marrow fibroblasts that do not belong to the malignant clone. This deposition of collagen is a result of the release of fibrogenic cytokines by MK and monocytes derived from the malignant stem cell population. TGF-β is a critical cytokine in the development of marrow fibrosis by increasing matrix biosynthesis and reducing the rate of matrix degradation.

- TGF-β upregulates osteoprotegerin expression. Upregulated osteoprotegerin, a secreted inhibitor of bone resorption, play a role in the development of osteosclerosis in PMF by disrupting osteoblast production.

- Platelet factor 4 (PF4), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF) activate mesenchymal cells, leading to myelofibrosis, and endothelial cells, contributing to angiogenesis.
1. Unknown molecular event $\Rightarrow$ 2. Clonal hematopoietic stem cells proliferation and generation of differentiated cells (medullar hyperproliferative stage) $\Rightarrow$ 3. Dystrophic megakaryocytes produce or release growth factors and proteases within the bone marrow environment $\Rightarrow$ 4. Myelofibrosis and an imbalance between endosteal and vascular niches $\Rightarrow$ 5. Imbalance favor proliferation of stem cells (SCs) including hematopoietic, endothelial, and likely mesenchymal stem cells (HSCs, ESCs, and MSCs, respectively) and their mobilization through blood $\Rightarrow$ 6. Higher number of circulating SCs and terminal bone marrow aplasia $\Rightarrow$ 7. These SCs colonize the spleen in which newly created or reinitialized vascular niches favor their homing and differentiation $\Rightarrow$ 8. The splenomegaly/hepatomegaly due to initiation of extramedullar hematopoiesis.
Possible drug targets

PMF is a complex disease in which the pathological mechanism involves pathological hematopoietic stem cell clone. A remissions in young patients following hematopoietic cell transplantation support the assumption that genetic lesions of HSCs take part in the genesis of the disease.

1. First therapy target – pathological hematopoietic stem cell clone.
   a) SCT
   b) Jak2 inhibitors.

Some speculation – extramedullary hematopoiesis occurs in two organs – spleen and liver – that were used for hematopoiesis in embryonic stages. It shows plasticity of hematopoietic niches – the niche may be reinitiated. It raises the opposite question - whether the altered adult hematopoietic niche may be regenerated?

The possibility exists that during allogeneic transplantation, the normal donor hematopoietic cells permit the recipient microenvironment to recover and function normally.

2. Second therapy target – to recover hematopoietic niche:
   a) SCT
   b) Compounds, affecting cellular and non-cellular components of hematopoietic niche, including various inhibitors and antibodies or soluble receptors, inhibiting production and immunomodulating activity of fibrogenic factors, angiogenic cytokines (TGF-β or VEGF), etc.
Possible drug targets

- Anti-VEGF
- VEGF Trap
- VEGF R inhibitors
- Antiangiogenic and cytokine modulators

- Fibrosis inhibitors
- MMP inhibitors

Bone remodelling agents

Cytokines / Chemokines

GFs and GFR inhibitors
Targeting pathologic clone – Jak2 inhibitors

\[ N\text{-tert-Butyl-3-\{5-methyl-2-\{4-(4-methyl-piperazine-1-yl)-phenylamino\}pyrimidin-4-ylamino\}-benzenesulfonamide} \]

<table>
<thead>
<tr>
<th></th>
<th>JAK2</th>
<th>FLT3</th>
<th>RET</th>
<th>JAK3</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC_{50} (nM)</td>
<td>6</td>
<td>25</td>
<td>17</td>
<td>169</td>
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</table>
### Jak2 inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (nanomolar)</th>
<th>Stage of development</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Lestaurtinib</td>
<td>3</td>
<td>NA</td>
<td>Phase II</td>
</tr>
<tr>
<td>Ruxolitinib</td>
<td>2.8</td>
<td>4.5</td>
<td>Phase III Approved by FDA</td>
</tr>
<tr>
<td>TGI01348/5AR302503</td>
<td>105</td>
<td>3 (19 vs. JAK2 V617F)</td>
<td>Phase II</td>
</tr>
<tr>
<td>XL019</td>
<td>124</td>
<td>195</td>
<td>Halted</td>
</tr>
<tr>
<td>SBI1518/Pacitinib*</td>
<td>1.7</td>
<td>0.75</td>
<td>Phase II/III</td>
</tr>
<tr>
<td>CYT387</td>
<td>11</td>
<td>155</td>
<td>Phase I/I</td>
</tr>
<tr>
<td>LY2781544</td>
<td>NA</td>
<td>NA</td>
<td>Entering Phase II</td>
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<tr>
<td>INCB028050</td>
<td>5.8</td>
<td>5.7</td>
<td>Phase II†</td>
</tr>
<tr>
<td>INCB16562</td>
<td>2.5</td>
<td>0.3</td>
<td>Preclinical</td>
</tr>
<tr>
<td>NVP-BSK805</td>
<td>31</td>
<td>0.48</td>
<td>Preclinical</td>
</tr>
<tr>
<td>R723</td>
<td>&gt;1000</td>
<td>24</td>
<td>Preclinical</td>
</tr>
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</table>

IC₅₀, 50% inhibitory concentration; FDA, Federal Drug Administration; NA, not available.

*Values expressed as KD (dissociation constant).

†Tested only in rheumatological diseases, not in myeloproliferative neoplasms.
### Table 2: Results of Janus family of tyrosine kinase inhibitors in patients with myelofibrosis

<table>
<thead>
<tr>
<th></th>
<th>INCB018424</th>
<th>CEP-701</th>
<th>TG10138</th>
<th>CYT387</th>
<th>SB1518</th>
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<tbody>
<tr>
<td><strong>Selectivity</strong></td>
<td></td>
<td></td>
<td>JAK2/FLT3 [44]</td>
<td>JAK1/JAK2 [45,46]</td>
<td>JAK2/FLT3 [47]</td>
</tr>
<tr>
<td><strong>Improvement in</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>splenomegaly</td>
<td>Phase 1–2 [55**]:</td>
<td>CI in 52%</td>
<td>CI in 47%</td>
<td>CI in 45%</td>
<td>CI in 45%</td>
</tr>
<tr>
<td></td>
<td>CI in 19%</td>
<td>Phase 2 [50]:</td>
<td>Phase 1/2 [51]</td>
<td>Median decrease of 6.4 cm</td>
<td>57% with 25% reduction in volume by MRI</td>
</tr>
<tr>
<td></td>
<td>35% reduction by imaging in 42 and 29%</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Alleviation of constitutional symptoms</strong></td>
<td></td>
<td>Early satiety (46%)</td>
<td>Fatigue (25%)*</td>
<td>Pruritus</td>
<td>Symptom improvement in 40–65%</td>
</tr>
<tr>
<td>Phase 2 not reported</td>
<td>Phase 1/2 not reported</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46% improved symptom score</td>
<td></td>
<td>Cough (37%)*</td>
<td></td>
<td>Bone pain</td>
<td></td>
</tr>
<tr>
<td><strong>Decrease in JAK2 V617F allele burden</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase 1–2 [55**]:</td>
<td>Phase 2 [50]:</td>
<td>16 of 20 patients with median decrease of 6.1%</td>
<td>Not reported</td>
<td>Not reported</td>
<td></td>
</tr>
<tr>
<td>13% after 12 cycles</td>
<td>no significant change</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Adverse effects</strong></td>
<td></td>
<td>Myelosuppression</td>
<td>Myelosuppression</td>
<td>Myelosuppression</td>
<td>First dose effect*</td>
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<tr>
<td>Myelosuppression</td>
<td>Gastrointestinal</td>
<td>Gastrointestinal</td>
<td>Gastrointestinal</td>
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<tr>
<td><strong>Unique effects</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Phase 1–2 [55**]:</td>
<td></td>
<td>Phase 2 [50]:</td>
<td></td>
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<tr>
<td>Reduction in level of pro-inflammatory cytokines</td>
<td></td>
<td>CI in anemia in 14%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*CI, clinical improvement; NS, night sweats.

**In complete resolution.

*bTransient lightheadedness, hypotension.
### Table III. Potential agents or modalities to partner Janus kinase (JAK) inhibitors.

<table>
<thead>
<tr>
<th>Class</th>
<th>Molecule/modality</th>
<th>In vitro study</th>
<th>Clinical Trial as sole agent</th>
<th>Clinical Trial with JAK inhibitor Planned or underway</th>
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</thead>
<tbody>
<tr>
<td>Immunomodulators</td>
<td>Pomalidomide</td>
<td></td>
<td>□</td>
<td>□</td>
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<tr>
<td></td>
<td>Lenalidomide</td>
<td></td>
<td>□</td>
<td>□</td>
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<tr>
<td>mTOR inhibitors</td>
<td>Everolimus</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Hypomethylating agents</td>
<td>Azacitidine</td>
<td>□</td>
<td>□</td>
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<tr>
<td></td>
<td>Decitabine</td>
<td></td>
<td>□</td>
<td>□</td>
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<tr>
<td>Histone deacetylase inhibitors</td>
<td>Givinostat</td>
<td>□</td>
<td>□</td>
<td>□</td>
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<tr>
<td></td>
<td>Panobinostat</td>
<td>□</td>
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<tr>
<td>BCL2 inhibitors</td>
<td>Obatoclax mesylate</td>
<td>□</td>
<td>□</td>
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<tr>
<td>α-Interferon</td>
<td></td>
<td></td>
<td>□</td>
<td>□</td>
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<tr>
<td>BCL-XL inhibitors</td>
<td>ABT-737</td>
<td>□</td>
<td>□</td>
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<td>HSP90 inhibitors</td>
<td>PU-H71</td>
<td></td>
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<tr>
<td>Androgenic steroid</td>
<td>Danazol</td>
<td></td>
<td>□</td>
<td>□</td>
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<tr>
<td>Stem cell transplant</td>
<td>SCT</td>
<td>□</td>
<td>□</td>
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</table>
Targeting recovery of niche

**Inhibitors of NFκB**
Activation of NFκB induce TGF-β production in monocytes and MK of patients with PMF. The NFκB pathway is activated in CD34+ cells from patients with PMF. The transcription factor NFκB regulates the activation of hundreds of genes involved in hematopoiesis. Bortezomib indirectly impairs NFκB activation by inhibition of TGF-β production. Leucocytosis and thrombocytosis reduction in dose-dependent manner. Reduction of the degree of constitutive mobilization of HSC and degree of splenomegaly.

**TGF-β Inhibitors**
Specific antibodies or soluble receptors that can compete with cellular receptors for ligand binding have been created to reduce the biological effects of TGF-β. Although marrow fibrosis was reduced in mice receiving the adenoviral-encoded soluble receptors, the mice still developed a lethal MPD characterized by leukocytosis, thrombocytosis, and constitutive stem cell mobilization.
**Anti-VEGF Therapy**

PMF is characterized by a profound increase in marrow microvessel density which is associated with increased plasma vascular endothelial growth factor (VEGF) levels. Thalidomide and lenalidomide have potent antiangiogenic and cytokine modulatory activity. Significant response rates have been reported with the use of both agents (~25%). Bevacizumab is a humanized monoclonal neutralizing antibody directed against VEGF, which is used to inhibit tumor-related angiogenesis.

**Inhibitors of Bcl-xL**

PMF is characterized by a profound MK hyperplasia. MK from patients with PMF are characterized by upregulation of the antiapoptotic factor Bcl-xL. Small compounds and interfering RNAs (siRNAs) directed against BcL-xL have been shown to inhibit cell growth and induce apoptosis of a variety of solid tumors. Might be useful for the treatment of PMF.
Inhibitors of Proteases
Neutrophil elastase and MMP-9 have been implicated in altering interactions between PMF CD34+ HSC/HPC with VCAM-1 and SDF-1, respectively, within the marrow microenvironment, leading to constitutive CD34+ cell mobilization and the development of extramedullary hematopoiesis. A variety of MMP-9 inhibitors as well as small-molecule inhibitors of neutrophil elastase are currently under evaluation.

Bone remodeling agents
Bisphosphonates (zoledronic acid) - the antiproliferative, antiapoptotic, and bone-remodeling effects – in combination with statins.
Statins inhibit macrophage activation and secretion of various proteolytic enzymes, including metalloproteinases. They also down-regulate the expression of urokinase-type plasminogen activator receptor (u-PAR), which modulates the bone marrow proteolytic environment.
Statins and zoledronic acid are promising therapeutic agents that deserve to be tested in PMF patients
Chromatin-Modifying Agents

High rate of methylation (for instance negative regulatory of JAK2 activation/phosphorylation including SHP-1, SOCS-1 and SOCS-3) in PMF shows that epigenetic silencing of genes may play an important role in the pathogenesis of this disorder.

A hypomethylating agent, decitabine, has also been shown to induce expression of TGF-β receptors that have been silenced by hypermethylation and has the potential to restore sensitivity of PMF HSC/HPC to TGF-β. Exposure of PMF CD34+ cells to 5azaD/TSA resulted in a reduction of the proportion of JAK2V617F+ HPC in 83% of the patients studied and the reduction in the proportion of homozygous HPCs in 50% of the patients. 5azaD/TSA treatment also led to a dramatic reduction in the number of HPC that contained chromosome abnormalities.

Treatment of PMF CD34+ cells with 5azaD/TSA also resulted in the upregulation of CXCR4 expression by CD34+ cells and restoration of their migration in response to SDF-1.