Stromal SPARC contributes to the detrimental fibrotic changes associated with myeloproliferation whereas its deficiency favors myeloid cell expansion

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In myeloid malignancies, the neoplastic clone outgrows normal hematopoietic cells toward BM failure. This event is also sustained by detrimental stromal changes, such as BM fibrosis and osteosclerosis, whose occurrence is harbinger of a dismal prognosis. We show that the matricellular protein SPARC contributes to the BM stromal response to myeloproliferation. The degree of SPARC expression in BM stromal elements, including CD146+ mesenchymal stromal cells, correlates with the degree of stromal changes, and the severity of BM failure characterizing the prototypical myeloproliferative neoplasm primary myelofibrosis. Using Sparc−/− mice and BM chimeras, we demonstrate that SPARC contributes to the development of significant stromal fibrosis in a model of thrombopoietin-induced myelofibrosis. We found that SPARC deficiency in the radioresistant BM stroma compartment impairs myelofibrosis but, at the same time, associates with an enhanced reactive myeloproliferative response to thrombopoietin. The link between SPARC stromal deficiency and enhanced myeloid cell expansion under a myeloproliferative spurring is also supported by the myeloproliferative phenotype resulting from the transplantation of defective Apcmin mutant hematopoietic cells into Sparc−/− but not WT recipient BM stroma. Our results highlight a complex influence of SPARC over the stromal and hematopoietic BM response in myeloproliferative conditions. (Blood. 2012;120(17):3541-3554)
plays a major role in the maintenance and restoration of tissue homeostasis.\textsuperscript{19} SPARC has been implicated in tissue remodeling during embryogenesis, wound healing, immune responses,\textsuperscript{20} cancer stromatogenesis, and epithelial-to-mesenchymal transition.\textsuperscript{19,21}

In myeloid malignancies, SPARC (5q31.3–32) deletion has been associated with 5q– MDS,\textsuperscript{22} and low or absent SPARC expression has been reported in a subset of AML.\textsuperscript{23} Conversely, SPARC overexpression characterizes CML cells displaying resistance to imatinib in vitro, suggesting a role in myeloid clone progression.\textsuperscript{24}

Herein, we investigated the role of SPARC in the BM stromal alterations associated with myeloproliferation. Altogether, our results suggest that stromal SPARC plays a key role in sustaining the detrimental fibrosis that occurs in myeloid malignancies and reveal SPARC’s influence on the BM hematopoietic response in myeloproliferative conditions.

Methods

Patient samples

BM trephine biopsies (BMBs) of patients with myeloid malignancies were selected from the archives of the Human Pathology Section, University of Palermo. Ten consecutive AML cases, 10 MDS, 12 essential thrombocytemia, 10 polycythemia vera (PV), and 56 primary myelofibrosis (PMF) cases diagnosed between December 2008 and January 2011, according to the 2008 WHO classification criteria,\textsuperscript{3} were included. Ten BMBs from patients with reactive thrombocytosis and 6 BMBs of patients with Hodgkin lymphoma without marrow involvement were used as controls. Only representative BMBs\textsuperscript{6} collected at the time of diagnosis were included. For all of the cases, the following presenting data were retrieved from the patients’ medical records: age, sex, hemoglobin levels (Hb), peripheral blood (PB) leukocyte count (white blood cell count), and platelet count (PLT; Tables 1 and 2). All of the procedures were in accordance with the Declaration of Helsinki.

Mice

BALB/cAnNCrl mice, 8-10 weeks old, were purchased from Charles River Laboratories. The CNCr.129S(B6)–Sparctm1Hwe mice were developed in our animal facility as previously described.\textsuperscript{25} Apc\textsuperscript{min} mice (B6, Ly5b) were a kind gift of Dr Antonio Sica (Fondazione Istituto Clinico Humanitas). Chimeric Sparc\textsuperscript{−/−} > wild-type (WT; Thy-1b > Thy-1a), WT > Sparc\textsuperscript{−/−} (Thy-1a > Thy-1b), WT > WT(Thy-1a > Thy-1b), Sparc\textsuperscript{−/−} > Sparc\textsuperscript{−/−} (Thy-1b > Thy-1b), Apc\textsuperscript{−/−} > WT (Ly5b > Ly5a), and Apc\textsuperscript{−/−} > Sparc\textsuperscript{−/−} (Ly5b > Ly5a) mice were obtained by BM transplantation as previously reported.\textsuperscript{26} Engraftment was verified 6-8 weeks after BM transplantation by FACS analysis of PB mononuclear cells according to Thy-1a (BD Biosciences) and Thy-1b (BD Biosciences) or Ly5a (BD Biosciences) expression. The animal experiments were authorized by the Institutional Ethical Committee for Animal Use.

Table 1. Presenting clinical and laboratory features of myeloid neoplasm and control cases

<table>
<thead>
<tr>
<th></th>
<th>AML</th>
<th>MDS</th>
<th>ET</th>
<th>PV</th>
<th>PMF</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
<td>10</td>
<td>10</td>
<td>12</td>
<td>10</td>
<td>24</td>
<td>16</td>
</tr>
<tr>
<td>Mean age, y (range)</td>
<td>56.1 (31-77)</td>
<td>60.8 (36-80)</td>
<td>64.3 (30-80)</td>
<td>58.5 (34-85)</td>
<td>65.5 (40-86)</td>
<td>52.5 (21-85)</td>
</tr>
<tr>
<td>Sex, %</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Male</td>
<td>27.2</td>
<td>50</td>
<td>83.3</td>
<td>30</td>
<td>58.3</td>
<td>43.7</td>
</tr>
<tr>
<td>Female</td>
<td>72.8</td>
<td>50</td>
<td>16.7</td>
<td>70</td>
<td>41.7</td>
<td>56.3</td>
</tr>
<tr>
<td>Sex, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>80 (10-100)</td>
<td>68 (60-90)</td>
<td>43 (35-60)</td>
<td>83 (40-100)</td>
<td>75 (50-100)</td>
<td>48 (25-85)</td>
</tr>
<tr>
<td>Fibrosis grade†</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Grade 0</td>
<td>0</td>
<td>0</td>
<td>33.3</td>
<td>0</td>
<td>8.4</td>
<td>50</td>
</tr>
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<td>Grade 1</td>
<td>70</td>
<td>40</td>
<td>66.6</td>
<td>60</td>
<td>45.8</td>
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</tr>
<tr>
<td>Grade 2</td>
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<td>50</td>
<td>0</td>
<td>20</td>
<td>20.8</td>
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<tr>
<td>Grade 3</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>20</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>WBC count, × 10(^9)/L*</td>
<td>63.8 (1.6-420)</td>
<td>3.2 (1.2-5.2)</td>
<td>8.2 (4.1-13.2)</td>
<td>11.4 (4.9-22.6)</td>
<td>12 (2.3-51.5)</td>
<td>9.1 (2.2-14.6)</td>
</tr>
<tr>
<td>Hb, g/L*</td>
<td>83.6 (64-95)</td>
<td>88 (62-142)</td>
<td>130.6 (102-146)</td>
<td>160 (100-210)</td>
<td>122 (81-150)</td>
<td>128 (88-159)</td>
</tr>
<tr>
<td>PLT count, × 10(^9)/L*</td>
<td>52.1 (16-146)</td>
<td>107.8 (19-264)</td>
<td>737.8 (411-942)</td>
<td>292.9 (126-494)</td>
<td>463.5 (13-1218)</td>
<td>305.9 (143-762)</td>
</tr>
</tbody>
</table>

*Values are mean (range).
†Fibrosis grade is reported as median (%) of each grade. Fibrosis is evaluated according to Thiele et al.\textsuperscript{7}

Table 2. Presenting clinical and laboratory data of the 56 PMF cases

<table>
<thead>
<tr>
<th>Sex, no. (%)</th>
<th>Prefibrotic/early fibrotic (grade 0/1)*</th>
<th>Advanced fibrotic (grade 2/3)*</th>
<th>P†</th>
<th>Low/Intermediate-1 risk‡</th>
<th>Intermediate-2/ high risk‡</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>9 (29)</td>
<td>14 (56)</td>
<td>14 (30.4)</td>
<td>9 (90)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>22 (71)</td>
<td>11 (44)</td>
<td>32 (69.6)</td>
<td>1 (10)</td>
<td></td>
<td></td>
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<tr>
<td>Age (y) at diagnosis*</td>
<td>63 (25-86)</td>
<td>65 (44-86)</td>
<td>.86</td>
<td>62 (25-88)</td>
<td>71 (57-79)</td>
<td>.1</td>
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<tr>
<td>Hb, g/L</td>
<td>131.6 (82-172)</td>
<td>111 (80-164)</td>
<td>.0003</td>
<td>127.7 (87-172)</td>
<td>98 (80-127)</td>
<td>.0004</td>
</tr>
<tr>
<td>WBC, × 10(^9)/L</td>
<td>9.6 (3.8-15)</td>
<td>11.9 (2.8-51.5)</td>
<td>.67</td>
<td>10.3 (3.8-22.1)</td>
<td>12.1 (2.8-51.5)</td>
<td>.2</td>
</tr>
<tr>
<td>PLT, × 10(^9)/L</td>
<td>662.7 (200-1218)</td>
<td>401 (49-1031)</td>
<td>.004</td>
<td>605 (121-1218)</td>
<td>304 (490-753)</td>
<td>.01</td>
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<tr>
<td>Cellularity, %</td>
<td>60.8 (20-90)</td>
<td>71.6 (30-100)</td>
<td>.02</td>
<td>62.9 (20-100)</td>
<td>78 (50-90)</td>
<td>.008</td>
</tr>
<tr>
<td>SPARC* stromal cells × HPF</td>
<td>3.9 (0.5-15.9)</td>
<td>6.7 (2.4-15.7)</td>
<td>.0003</td>
<td>4.7 (0.5-15.9)</td>
<td>7.3 (1.4-15.7)</td>
<td>.004</td>
</tr>
<tr>
<td>CD146* stromal cells × HPF</td>
<td>6 (1-12.2)</td>
<td>10.2 (3.8-23.5)</td>
<td>.0004</td>
<td>7.5 (1-23.5)</td>
<td>9.3 (3-21)</td>
<td>.4</td>
</tr>
<tr>
<td>MVD × HPF</td>
<td>5.8 (1-12.2)</td>
<td>9.9 (2.8-18.6)</td>
<td>.00001</td>
<td>6.5 (1.6-18.6)</td>
<td>8.5 (3-14.8)</td>
<td>.1</td>
</tr>
</tbody>
</table>

*Values are mean (range). HPF (×400).
†Fibrosis was evaluated according to Thiele et al.\textsuperscript{7}
‡According to Mann-Whitney U test.
§According to Cervantes et al.\textsuperscript{31}
**In vivo recombinant TPO treatment**

Recombinant murine thrombopoietin (TPO; PeproTech) or sterile saline was injected intraperitoneally into WT, Sparc<sup>−/−</sup>, and chimeric mice once daily (500 μg/kg) for 10 or 14 consecutive days. After treatment, PB was collected and analyzed for Hb, PLT, and circulating Gr1<sup>−</sup>CD11b<sup>+</sup> myeloid cells. At the same time point, the mice were killed and their spleen, femurs, and tibias were collected for histopathologic/immunophenotypical and FACS analyses and for BM colony formation assays.

**Histopathology and immunohistochemistry**

Histopathologic analysis was performed on formalin-fixed, paraffin-embedded specimens as previously reported. Four-micrometer-thick sections were routinely stained with hematoxylin and eosin, Gomori reticulin, and Masson trichrome stain. The degree of BM fibrosis on human BMBs was semiquantitatively assessed on Gomori-stained sections according to the European Consensus Grading System, which ranges from 0 (normal BM fiber content) to 3 (diffuse and dense increase in reticulin with extensive intersections of coarse collagen bundles, associated with osteosclerosis). A similar semiquantitative system was also adopted for BM fibrosis evaluation on Gomori-stained mouse BM sections.

Immunohistochemistry was performed using the streptavidin-biotin-peroxidase complex method as previously reported. The antibodies adopted for immunohistochemistry are listed in supplemental Methods.

The slides were analyzed under a Leica-DM2500 optical microscope (Leica Microsystems) using Leica-TCS-SP5 laser scanning confocal microscope (Nikon). Immunofluorescence was performed using the streptavidin-biotin-peroxidase complex method as previously reported. The antibodies used are detailed in supplemental Methods.

**Isolation, culture, and treatment of human and murine BM-MSCs**

Human BM mesenchymal stromal cells (BM-MSCs) were obtained from aspirations of normal BM of patients undergoing hip replacement or Hodgkin lymphoma staging. The BM-MSC cultures were established from plastic-adherent BM cell fractions as detailed in supplemental Methods. Murine BM-MSCs were obtained from the trabecular fraction of femurs and tibias of WT and Sparc<sup>−/−</sup> mice as previously reported, and cultures were established as detailed in supplemental Methods.

In vitro experiments on human and murine BM-MSCs were performed using cells between the second and fifth passages.

**Statistical analysis**

Continuous and categorical variables were compared using the Mann-Whitney U test. Correlation between variables was assessed using Pearson and Spearman coefficients. Analysis of the effects of TPO treatment and Sparc genotype on BM hematopoiesis was performed by 2-way ANOVA. Data were analyzed with the Statistical Package for the Social Sciences Version 13.0 (IBM) software.
Results

SPARC stromal expression characterizes myeloid malignancies with BM stroma alterations and correlates with signs of BM failure

Because of the pleiotropic role of SPARC in tissue remodeling, we investigated whether its expression had any role in the stromal alterations associated with myeloid malignancies. Initially, we immunohistochemically tested SPARC expression in BMBs from 66 patients with different types of myeloid neoplasms and variable degrees of associated stromal changes, namely, BM fibrosis and/or osteosclerosis, and in 16 control BMBs. The clinical characteristics of these patients are summarized in Table 1.

In control cases and in myeloid neoplasms without associated stromal changes, such as essential thrombocytopenia, early PV, and prefibrotic PMF, SPARC expression was confined to MKs (Figure 1A black arrows; supplemental Figure 2A). By contrast, in cases with significant stromal alterations, such as overt PMF, MDS with fibrosis (MDS-F), and acute panmyelosis with myelofibrosis, SPARC expression was also identified in spindle to stellate BM stromal cells intermingling with hematopoietic cells, branching around vessels or forming an intricate meshwork (Figure 1A green arrows; supplemental Figure 2A).

We subsequently investigated whether stromal SPARC expression correlated with histopathologic and laboratory variables of clinical significance in myelofibrotic myeloid neoplasms5 using PMF as a prototypical model. The number of SPARC-expressing...
stromal cells was assessed in 56 consecutive cases of PMF and correlated with the following variables: BM cellularity, degree of BM fibrosis, MVD, age, sex, Hb, complete PB counts, and PMF prognostic score according to Cervantes et al. In addition, a correlation was made with the number of CD146-expressing BM-MSCs, which we have reported to increase proportionally with the degree of stromal changes that occur along PMF progression (supplemental Figure 3). The clinical and laboratory features of these PMF cases are summarized in Table 2.

We found that the number of SPARC+ stromal cells correlated significantly with the degree of BM fibrosis (rho = 0.583, P = .01), MVD (rho = 0.550, P < .0001), and the number of CD146+ BM-MSCs (rho = 0.632, P < .00001), reflecting the entity of stromal disarrangement (Table 2; Figure 1B). Moreover, the number of SPARC+ stromal cells was higher in PMF patients belonging to a higher prognostic risk group (low/intermediate-1 vs intermediate-2/high, P = .04), and correlated with signs of BM hematopoietic failure, such as low Hb levels (rho = −0.429 vs Hb level, P = .002) and thrombocytopenia (rho = −0.539 vs PLT count, P < .00001; Table 2; Figure 1B). The significant difference in the mean number of SPARC+ stromal cells per HPF among cases with different degrees of BM fibrosis was also confirmed in PV, MDS, and AML (supplemental Figure 2B).

These data indicate a correlation between SPARC stromal expression and the establishment of relevant BM fibrotic changes that parallel the progressive failure of normal BM hematopoiesis in PMF.

**SPARC expression marks CD146+ BM-MSCs and is associated with the osteoblastic milieu expansion underlying osteosclerotic progression**

CD146+ BM-MSCs are the mesenchymal precursors of BM stromal cells, including fibroblasts, BM reticular cells, adipocytes, and osteoblasts. In human BM, BM-MSCs display a spindle to stellate branching morphology and subendothelial distribution. The concurrent changes in SPARC+ cells and CD146+expressing BM-MSCs in PMF cases, and the morphology and distribution of several SPARC+ stromal cells mirroring those of CD146+ cells (Figure 2A black arrows), prompted us to test whether CD146+ BM-MSCs were a source of SPARC in myeloid neoplasms with stromal alterations. Double immunofluorescence analysis for SPARC and CD146 in 30 cases of myeloid neoplasms with variable degrees of stromal fibrosis (range, 0-3, including 18 cases of PMF, 8 cases of PV, and 4 cases of MDS) showed that, aside from MKs and some stromal cells that were marked by SPARC but not CD146 (Figure 2B white arrows), SPARC expression overlapped that of CD146 in BM-MSCs displaying subendothelial localization (Figure 2B yellow arrows).

The BM stromal alterations associated with myelofibrotic myeloid malignancies may progress toward osteosclerosis (supplemental Figure 4), which could reflect an imbalance in the differentiative fate of BM-MSCs at the bifurcation between the osteoblastic and adipocytic differentiation, a checkpoint in which SPARC is involved (supplemental Figure 5). We thus focused on the expression of SPARC and key ECM components of the osteoblastic niche, namely, collagen type I and fibronectin, in advanced PMF cases undergoing osteosclerotic progression. Expression of collagen type I and fibronectin, which was confined to the endosteal edge of the hematopoietic parenchyma in PMF cases without relevant stromal alterations (Figure 2C arrows), spread to the intertrabecular areas in cases with severe fibrosis and osteosclerosis (Figure 2C). Interestingly, in these cases, SPARC colocalized with collagen type I in sclerotic foci in situ (Figure 2D), as assessed by double immunofluorescence, and exhibited strong expression in osteoblasts at sites of osteosclerotic bone formation (Figure 2E arrows).

The in situ association between SPARC and foci of new bone formation was in line with the whole transcriptome GEP analysis that we performed on a panel of human samples representative of BM hematopoietic and mesenchymal cells (supplemental Table 1), including samples of BM-MSCs committed to osteoblastic differentiation. The list of SPARC best correlates (204 unique genes with Pearson correlation r² > 0.95; supplemental Table 2) was significantly enriched in key genes involved in cell adhesion/motility and communication (eg, TGFβ1 and PDGFRA), and ECM composition (supplemental Tables 2 and 3). Notably, among these, we found ECM genes involved as critical regulators of the osteoblastic milieu, such as COL1A2, COL3A1, COL5A1/A2, COL12A1, and FN (supplemental Figure 6).

Altogether, these results associate SPARC with advanced osteosclerotic modifications, which may develop in myeloproliferative malignancies as a result of skewed BM-MSC differentiation dynamics.

**Stromal SPARC is required for BM fibrotic changes accompanying TPO-induced reactive myeloproliferation**

To investigate the relevance of SPARC in the induction of BM fibrotic changes associated with myeloproliferation, we adopted a murine experimental model of myelofibrosis. In mice, reactive myeloproliferation and associated stromal fibrosis could be induced by recombinant TPO treatment, as previously reported. Thus, WT and Sparc−/− mice were treated intraperitoneally with TPO at a daily dose of 500 μg/kg for 10 or 14 days, killed, and analyzed for signs of myeloproliferation, and associated BM stroma alterations.

WT and Sparc−/− mice both developed myeloproliferation in the BM after 10 days of TPO treatment (Figure 3A). Myeloproliferation was paralleled by extramedullary splenic hematopoiesis with red pulp expansion (Figure 3B), PB thrombocytosis, and anemia (supplemental Figure 7A). BM histopathology and differential hematopoietic cell counts on BM sections highlighted that, differently from saline-treated controls, TPO-treated mice were characterized by increased BM cellularity with expansion of granulocytes and immature myeloid cells, and by MK hyperplasia with marked pleomorphism and cluster formation (Table 3; Figure 3A,C). Comparable results were obtained by 14 days TPO treatment (not shown).

In TPO-treated WT mice, myeloproliferation was associated with the development of moderate to severe stromal fibrosis and osteosclerotic foci (Figure 3A inset). Interestingly, none of the TPO-treated Sparc−/− mice displayed severe fibrosis or signs of osteosclerosis (Figure 3A). According to the histopathologic grading of BM fibrosis, which ranged from 0 to 3, the mean score of WT TPO-treated mice was 2.2 (SD, 0.76) compared with 0.8 of Sparc−/− mice (SD, 0.84; P = .013), whereas control marrows from both WT and Sparc−/− saline-treated mice scored 0 (Figure 3D). These in vivo results demonstrate that the absence of SPARC hampers the development of stromal fibrosis associated with TPO-induced reactive myeloproliferation.

To determine the cellular source of SPARC, either hematopoietic or stromal, relevant to the formation of BM stromal fibrosis, BM chimeras were generated in which WT or Sparc−/− mice were either donors or hosts, as well as Thy-1 (CD90) congenic. Because of the low Thy-1 expression in murine BM-MSCs, to verify whether BM stromal cells were substituted or not by BM transplantation,
(CxB6)F1 mice expressing H-2Kb-d MHC class I were transplanted with marrow from B6 mice expressing H-2Kb to follow MHC class I genotype in BM-MSCs. Flow cytometric analysis revealed that almost all of the nonhematopoietic CD44+ and CD146+ cells (gated on the CD45.2- population) expressed the host H-2Kb-d MHC class I molecule (supplemental Figure 8A), thus confirming that BM mesenchymal cells remained of host origin after transplantation. Six weeks after BM transplantation, WT > WT, Sparc−/− > WT, WT > Sparc−/−, and Sparc−/− > Sparc−/− chimeras were evaluated for reconstitution with donor hematopoietic cells and treated with high-dose TPO as detailed in “Methods.” In all of the chimeras, TPO treatment induced significant myeloproliferation in the BM and spleen (Figure 4A-B; Table 4), thrombocytosis, and anemia (supplemental Figure 7B). The Sparc genotype of the radioresistant stroma was determinant for the development of myeloproliferation-related stromal changes. Indeed, although TPO-treated BM chimeras with host WT stroma showed marked fibrosis associated with myeloproliferation (Figure 4A, arrows), those with Sparc−/− recipient stroma developed slight or no fibrosis, regardless of the genotype of the BM donor. Specifically, the mean histopathologic scores of BM fibrosis for TPO-treated WT > WT and Sparc−/− > WT chimeras were 2.6 (SD, 0.55) and 2.8 (SD, 0.45), respectively, compared with 0.8 (SD, 0.55) of WT > Sparc−/− and 0.6 (SD, 0.89) of Sparc−/− > Sparc−/− chimeras (WT > WT vs WT > Sparc−/−, P = .004; Sparc−/− > WT vs Sparc−/− > Sparc−/−, P = .001; Figure 4C). The phenotype of such chimeras was consistent with the in situ Sparc expression detected by immunohistochemistry (supplemental Figure 8B).

These results confirm that SPARC contributes to the induction of BM stromal changes driven by myeloproliferation and identify BM stromal cells as the relevant source of SPARC in this setting.
Because chimeras with a Sparc<sup>−/−</sup> stroma failed to develop significant stromal alterations, we tested whether WT and Sparc<sup>−/−</sup> BM-MSCs respond differently to the prototypical epithelial-to-mesenchymal transition inducer TGF-β1 in vitro. BM-MSCs from WT and Sparc<sup>−/−</sup> mice, whose phenotype is reported in supplemental Figure 9, were starved for 24 hours in serum-free medium before the addition of rTGF-β1. After 48 hours, confocal microscopy analysis of SPARC expression and collagen type I deposition revealed that rTGF-β1 treatment induced up-regulation of SPARC and concurrent collagen type I fiber deposition in WT BM-MSCs (Figure 5A top panels), whereas Sparc<sup>−/−</sup> MSCs were refractory to TGF-β1-induced collagen deposition (Figure 5A bottom panels).

Table 3. Differential hematopoietic cell counts on BM sections in control and TPO-treated WT and Sparc<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th></th>
<th>WT saline</th>
<th>Sparc&lt;sup&gt;−/−&lt;/sup&gt; saline</th>
<th>WT TPO</th>
<th>Sparc&lt;sup&gt;−/−&lt;/sup&gt; TPO</th>
<th>P value of treatment</th>
<th>P value of host SPARC genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>81 ± 12.3</td>
<td>92.8 ± 11.9</td>
<td>143.4 ± 19.9</td>
<td>163.8 ± 7.0</td>
<td>&lt;.0001</td>
<td>.0208</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>6.6 ± 2.1</td>
<td>13.4 ± 3.5</td>
<td>8.4 ± 2.9</td>
<td>7.6 ± 2.5</td>
<td>.9315</td>
<td>.3508</td>
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<tr>
<td>Immature myeloid cells</td>
<td>26.2 ± 6.3</td>
<td>35.8 ± 9.1</td>
<td>52 ± 7.6</td>
<td>68 ± 9.1</td>
<td>&lt;.0001</td>
<td>.0208</td>
</tr>
<tr>
<td>Erythroid cells</td>
<td>69 ± 15.7</td>
<td>68.4 ± 13.6</td>
<td>65 ± 8.3</td>
<td>41.8 ± 6.9</td>
<td>.0101</td>
<td>.0375</td>
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<td>Megakaryocytes</td>
<td>10.2 ± 2.8</td>
<td>13.4 ± 2.2</td>
<td>54.4 ± 8.9</td>
<td>65.8 ± 8.7</td>
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<td>.0226</td>
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<tr>
<td>Lymphoid cells</td>
<td>29.8 ± 7.9</td>
<td>40.6 ± 12.2</td>
<td>50.8 ± 9.1</td>
<td>60.8 ± 7.1</td>
<td>.0001</td>
<td>.075</td>
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Data have been analyzed with a 2-way ANOVA. *P value expresses how the treatment (TPO) and the Sparc host genotype affect the variables.
supporting that BM-MSCs intrinsically require SPARC for a proper fibrotic response. WB analysis confirmed the induction of SPARC up-regulation by TGF-β1 in WT BM-MSCs and showed that WT and Sparc<sup>−/−</sup> BM-MSCs differed in the cellular content of the mature collagen form (Figure 5B-C), which was only detectable in WT cells. Notably, TGF-β1, whose expression in WT cells paralleled SPARC modulation, was basally more expressed in Sparc<sup>−/−</sup> BM-MSCs and was slightly up-regulated after exogenous rTGF-β1 stimulation (Figure 5B), suggesting that defective collagen type I maturation and deposition occurring in the absence of SPARC were not the result of impaired TGF-β1 synthesis.

Therefore, the defective collagen matrix deposition characterizing Sparc<sup>−/−</sup> BM-MSCs is responsible for the reduced fibrosis of TPO-treated Sparc<sup>−/−</sup> recipient chimeras, as also supported by

![Figure 4. BM stromal cells are the relevant source of SPARC in the development of experimental BM fibrosis. BM chimeras were obtained by transplanting WT and Sparc<sup>−/−</sup> mice with either WT or Sparc<sup>−/−</sup> BM cells to obtain WT > WT, WT > Sparc<sup>−/−</sup> > WT, WT > Sparc<sup>−/−</sup>, and Sparc<sup>−/−</sup> > Sparc<sup>−/−</sup> BM chimeras. Chimeras were treated with either recombinant TPO to induce myeloproliferation and fibrosis or medium alone as a control (n = 5 mice per group, per experiment). The data shown represent 1 experiment of the 3 performed. (A) Histopathologic analysis of BM samples from TPO-treated BM chimeras. Hematoxylin and eosin staining shows signs of myeloproliferation and clustering (insets). Original magnifications ×100, insets ×200. Scale bars represent 200 μm. (B) Histopathologic analysis of the splenic architecture of control and TPO-treated chimeric mice showing that the splenic parenchyma of treated mice is characterized by effacement of the white pulp (WP) because of the expansion of the red pulp (RP) with prominent megakaryocytic hyperplasia and clustering (insets). Original magnifications ×400. Scale bars represent 50 μm. (C) Gradation of BM fibrosis in TPO-treated BM chimeric mice was performed on Gomori-stained BM sections according to a 4-grade semiquantitative scoring system (see "Histopathology and immunohistochemistry"). The mean grade of fibrosis is significantly higher in TPO-treated chimeric mice with a WT recipient than in those with Sparc<sup>−/−</sup> recipients. **P < .01.

Table 4. Differential hematopoietic cell counts on BM sections in control and TPO-treated chimeric mice

<table>
<thead>
<tr>
<th></th>
<th>WT &gt; WT saline</th>
<th>WT &gt; Sparc&lt;sup&gt;−/−&lt;/sup&gt; saline</th>
<th>WT &gt; Sparc&lt;sup&gt;−/−&lt;/sup&gt; &gt; WT saline</th>
<th>WT &gt; Sparc&lt;sup&gt;−/−&lt;/sup&gt; &gt; Sparc&lt;sup&gt;−/−&lt;/sup&gt; saline</th>
<th>WT &gt; WT TPO</th>
<th>WT &gt; Sparc&lt;sup&gt;−/−&lt;/sup&gt; TPO</th>
<th>WT &gt; Sparc&lt;sup&gt;−/−&lt;/sup&gt; &gt; WT TPO</th>
<th>WT &gt; Sparc&lt;sup&gt;−/−&lt;/sup&gt; &gt; Sparc&lt;sup&gt;−/−&lt;/sup&gt; TPO</th>
<th>P value of treatment</th>
<th>P value of host SPARC genotype</th>
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<tbody>
<tr>
<td>Neutrophils</td>
<td>89.6 ± 7.63</td>
<td>113.8 ± 13.0</td>
<td>114.0 ± 8.3</td>
<td>122.2 ± 5.4</td>
<td>138.7 ± 15.3</td>
<td>164.33 ± 19.3</td>
<td>156.2 ± 19.9</td>
<td>182 ± 8.13</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
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<tr>
<td>Eosinophils</td>
<td>4.2 ± 1.8</td>
<td>3.0 ± 1.2</td>
<td>3.4 ± 1.1</td>
<td>4 ± 1.2</td>
<td>12.1 ± 3.2</td>
<td>10.5 ± 1.4</td>
<td>13.1 ± 2.9</td>
<td>12.3 ± 2.0</td>
<td>&lt;.0001</td>
<td>2443</td>
</tr>
<tr>
<td>Immature myeloid</td>
<td>31.6 ± 7.2</td>
<td>37.6 ± 9.8</td>
<td>41 ± 7.1</td>
<td>46.2 ± 4.1</td>
<td>46.3 ± 4.7</td>
<td>56.2 ± 5.5</td>
<td>44.3 ± 5.4</td>
<td>63.1 ± 6.6</td>
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<td>0.0004</td>
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<td>Erythroid cells</td>
<td>66.8 ± 10.3</td>
<td>69.2 ± 7.2</td>
<td>69.2 ± 5.1</td>
<td>68.8 ± 6.1</td>
<td>52.7 ± 9.5</td>
<td>40 ± 4.9</td>
<td>38.7 ± 7.5</td>
<td>33.5 ± 10.1</td>
<td>&lt;.0001</td>
<td>1241</td>
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<tr>
<td>Megakaryocytes</td>
<td>11.6 ± 3.6</td>
<td>11.6 ± 3.2</td>
<td>12.8 ± 2.4</td>
<td>14 ± 1.6</td>
<td>49 ± 2.3</td>
<td>62.3 ± 3.4</td>
<td>53 ± 13.0</td>
<td>74.8 ± 5.6</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
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<tr>
<td>Lymphoid cells</td>
<td>44.6 ± 10.2</td>
<td>52.0 ± 13.8</td>
<td>54.6 ± 10.3</td>
<td>54.8 ± 8.7</td>
<td>46.8 ± 5.0</td>
<td>54.5 ± 4.9</td>
<td>34.5 ± 7.4</td>
<td>28 ± 7.6</td>
<td>&lt;.0001</td>
<td>.0405</td>
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Data have been analyzed with a 2-way ANOVA. P value expresses how the treatment (TPO) and the Sparc host genotype affect the variables.
in situ defective expression of collagen type I in the presence of conspicuous TGF-β1 expression (Figure 5D-E). TGF-β1 was more expressed in the stroma of Sparc−/− recipient chimeras, than in WT counterparts (Figure 5E, arrows), which was consistent with the in vitro WB analysis of TGF-β1 expression on WT and Sparc−/− BM-MSCs.

**SPARC deficiency in the BM stroma associates with an enhanced myelopoietic response to TPO**

The analysis of BM chimeras, although crucial for dissecting the role of hematopoietic cell-derived and stroma-derived SPARC in BM fibrosis, uncovered that stroma-derived SPARC may affect myelopoiesis under a myeloproliferative stress, such as TPO treatment.

Indeed, TPO-induced reactive myeloproliferation in chimeras with a Sparc−/− stroma was characterized by increased granulopoiesis with enrichment of immature myeloid precursors, and by a higher degree of megakaryocytic hyperplasia with dysmegakaryopoiesis (ie, micromegakaryocytes with small hypolobulated nuclei) compared with chimeras with a WT stroma (Table 4; Figure 6A-B). Accordingly, a significant increase in myeloid colony forming units (CFU-GM; Figure 6C-D; WT > Sparc−/− vs WT > WT, P = .038; Sparc−/− > Sparc−/− > WT, P = .020) and an increase in the fraction of GMP BM hematopoietic precursors...
Figure 6. SPARC deficiency in the BM stroma associates with an enhanced myelopoietic response to TPO. (A) Histomorphologic analysis performed on hematoxylin and eosin-stained BM sections from TPO-treated mouse chimeras shows that mice with Sparc−/− stroma (WT > Sparc−/−, Sparc−/− > Sparc−/−) have significantly higher numbers of immature granulocyte precursors (green arrows) and highly atypical/dysplastic MKs (red arrows) compared with chimeras having WT stroma (WT > WT, Sparc−/− > WT). Four representative sections (1 per group) of the 20 evaluated are shown. Original magnifications ×400. Scale bars represent 50 μm. (B) Differential hematopoietic cell counts (mean ± SD) performed on BM sections of TPO-treated chimeric mice showing the different expansion of myeloid cell fractions of mice with a Sparc−/− or WT BM stroma. In each BM sample, the number of neutrophils, eosinophils, morphologically immature myeloid cells, erythroid cells, MKs, and lymphoid cells was counted of 10 HPFs. *P < .05. **P < .01. Data are relative to counts performed on 2 different BM sections, per mice, per group. (C-D) Hematopoiesis in chimeric mice was analyzed using a clonogenic colony culture assay. BM cells from chimeric mice were seeded in Methocult M3434 for 10 days, and the colonies that formed were scored and counted of 10 HPFs. *P < .05. **P < .01. The data represent 1 experiment of the 3 performed with 5 mice per group. (E) Hematopoietic progenitor cell numbers expressed as a percentage (mean ± SD) of total BM, showing the significant increase of GMP precursors in TPO-treated mice with Sparc−/− stroma compared with those with WT counterparts. *P < .05. The data represent 1 experiment of the 3 performed with 5 mice per group. (F) Flow cytometric analysis of Gr1+/CD11b+ circulating myeloid cells performed on the PB mononuclear cells of mouse chimeras. *P < .05. **P < .01. The data represent 1 experiment of the 3 performed with 5 mice per group. (G) Morphologic analysis of hematoxylin and eosin-stained BM smears from WT recipients, a higher frequency of bandform stage immature granulocytes and pseudo-Pelger-Huet figures (Figure 6G) were detected, further supporting the enhanced myelopoietic response to TPO in the absence of stromal SPARC.

The different support of Sparc−/− BM mesenchymal cells to myelopoiesis also emerged by in vitro coculture experiments in which Lin− hematopoietic cells from WT mice were cocultured with either WT or Sparc−/− BM-MSCs in the presence of stem cell factor and TPO. After 7 days of coculture, hematopoietic cells were analyzed by FACS for expression of Gr1 and F4/80, which marked the fractions of Gr1+/F4/80− granulocytes, Gr1−F4/80− monocytes, and Gr1 F4/80 double-positive or double-negative populations. In the presence of Sparc−/− BM-MSCs, a significantly higher fraction of Lin− cells was driven toward granulopoiesis compared with that of Lin− cells cocultured with WT BM-MSCs or cultured
alone (supplemental Figure 10B; \( P = .0284 \)), indicating that the absence of SPARC in BM mesenchymal cells results in the favored expansion of specific myeloid cell populations.

**Sparc deficiency in the BM microenvironment induces features of a myeloproliferative disorder in the presence of Apc\(^{min}\) mutant hematopoietic cells**

The observation of an enhanced myelopoietic response to TPO in the presence of a Sparc\(^{−/−}\) stroma prompted us to further investigate the influence of stromal Sparc deficiency over myeloproliferation. To this aim, BM cells from 8-week-old Apc\(^{min}\) mutant mice were transplanted into WT or Sparc\(^{−/−}\) recipients. Apc\(^{min}\) BM cells were adopted because Apc\(^{min}\) mice display altered HSC function and defective quiescence and develop an MDS/MPD phenotype in the presence of HSC-extrinsic factors.\(^{38,39}\)

Eight weeks after transplantation, chimeras were evaluated for reconstitution and myelopoiesis. Apc\(^{min} >\) Sparc\(^{−/−}\) chimeras, but not Apc\(^{min} >\) WT chimeras, displayed features of a myeloproliferative disorder, which included BM myeloid hyperplasia with increased granulopoiesis and enrichment in morphologically immature myeloid cell clusters, as assessed by BM histopathology and immunohistochemistry for Grl (Figure 7A) and by differential BM hematopoietic cell count analysis (Figure 7B). FACS immunophenotypic characterization of BM precursors of Apc\(^{min} >\) Sparc\(^{−/−}\) chimeras revealed a significant increase of the Lin<sup>−</sup> c-kit<sup>−</sup> precursors, and in particular of the GMP fraction compared with Apc\(^{min} >\) WT chimeras (Figure 7C-D; \( P < .05 \)). The hypergranulopoiesis of Apc\(^{min} >\) Sparc\(^{−/−}\) mice was also highlighted by PB FACS analysis, which revealed an expanded Gr1<sup>−</sup>CD11b<sup>+</sup> circulating myeloid cell fraction (Figure 7E). The BM of Apc\(^{min} >\) Sparc\(^{−/−}\) chimeras showed increased numbers of circulating mature granulocytes and immature or blast-like myeloid cells (Figure 7F-G). Excess myelopoiesis in Apc\(^{min} >\) Sparc\(^{−/−}\) chimeras was also detected in the spleen by histopathologic analysis, which revealed red pulp hyperplasia and partial effacement of the normal splenic architecture (Figure 7H), and by FACS analysis showing the increase of myeloid populations (supplemental Figure 1I).

These results support the involvement of SPARC in the crosstalk between hematopoietic cells and the stromal microenvironment and suggest the contribution of SPARC stromal deficiency to the induction of local conditions favorable to myeloproliferation.

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**Discussion**

Myeloproliferative conditions are paralleled by BM stroma remodeling events that variably involve modifications of the ECM, ranging from the slight changes of nonfibrotic disorders to the deep stromal disarrangement of osteosclerosis.\(^{6}\) Stromal modifications may cast different influences on expanding myeloid populations and residual hematopoietic components. In this context, the functions of ECM proteins are poorly understood. In this study, we described a role for SPARC, a nonstructural protein of the ECM, as a stromal factor involved in the BM response to myeloproliferation. We investigated SPARC expression in myeloid malignancies with different degrees of associated stromal changes. SPARC was confined to hematopoietic cells in cases devoid of stromal fibrotic alterations, while being extensively expressed in stromal cells in myelofibrotic cases. In the prototypical setting of PMF, increased stromal SPARC expression correlated with the degree of stromal modifications, with a higher prognostic risk score, and with features reflective of impaired hematopoiesis, which implicated stromal SPARC in the detrimental scenario caused by BM fibrosis development and progression.

As a collagen chaperon involved in ECM assembly, SPARC has been investigated in fibrotic conditions, including pulmonary,\(^{25}\) hepatic,\(^{40}\) and renal fibrosis,\(^{41}\) as well as cardiac infarction.\(^{42}\) In these pathologic settings of inflammation-driven fibrosis, SPARC is expressed by stromal cells and hematopoietic immune cells, and its function is dependent on the cellular source. In bleomycin-induced lung fibrosis, SPARC produced by stromal and inflammatory cells promote collagen deposition and the resolution of inflammation, respectively.\(^{25}\)

In myelofibrotic myeloid malignancies, stromal remodeling is not merely triggered by inflammation, although elevated pro-inflammatory cytokine levels have been described,\(^{43}\) but rather it is sustained by the self-activity of the myeloid clone. Neoplastic myeloid cells synthesize a plethora of mediators, including TGF-\(β\), PDGFs, VEGFs, bFGF, and IGFs, which have been variably implicated in the pathogenesis of myelofibrotic changes.\(^{6}\) It is conceivable that these factors, sensed by BM mesenchymal cells, could be responsible for the induction of SPARC stromal expression during myeloproliferation-related BM remodeling. Accordingly, the analysis of genes whose expression is significantly correlated with that of SPARC in BM hematopoietic and stromal cells highlighted the remarkable correlation between SPARC and some of the abovementioned axes (supplemental Table 2). These included PDGFA and TGF\(B1\) (\( r^2 = 0.96 \) and \( r^2 = 0.96 \), respectively; supplemental Figure 12A), both implicated in the pathogenesis of myelofibrotic myeloproliferative neoplasms.\(^{44,45}\) SPARC may directly interact with PDGFs to regulate their binding to cognate receptors.\(^{36}\) In addition, a reciprocal regulation between SPARC and TGF-\(β\)1 in stromal cells emerges from in vitro and in vivo evidence,\(^{32,47}\) including our results showing TGF-\(β\)-induced up-regulation of SPARC in murine BM-MSCs. Therefore, SPARC expression in the BM stroma of myeloid neoplasms may be induced by clone-derived mediators, such as PDGFs and TGF-\(β\), in an attempt to restore homeostasis in the BM milieu. The persistence of myeloproliferation, which limits tissue normalization, would eventually divert the effects of SPARC expression toward detrimental fibrosis.

The role of SPARC in myelofibrosis development was investigated using a model of TPO-induced myelofibrosis in WT, Sparc\(^{−/−}\) mice, in which SPARC proved to be required for the BM stromal remodeling triggered by reactive myeloproliferation. TPO experiments on BM chimeras, in which hematopoietic cells were of donor origin whereas stromal cells remained of host genotype, identified BM stromal cells as the relevant source of SPARC for the development of myeloproliferation-associated fibrosis. Moreover, in vitro stimulation of murine BM-MSCs with TGF-\(β\)-1 showed defective collagen fiber deposition in Sparc\(^{−/−}\) MSCs, which is a probable mechanism for the reduced fibrosis of TPO-treated chimeras retaining the host Sparc\(^{−/−}\) stroma.

SPARC inhibition by RNA interference has been reported to effectively attenuate fibrosis in different cellular contexts, suggesting a possible application of this approach to fibrotic diseases.\(^{40,48}\) A major caveat to the adoption of SPARC inhibition strategies in myeloproliferation-related BM fibrosis may come from our results showing that in the absence of stromal SPARC the myeloproliferative response to TPO was enhanced. Prompted by this observation, we investigated whether the selective SPARC deficiency in the BM stroma could favor myeloid cell expansion in the presence of a
genetically determined HSC defect linked with altered myelopoiesis, such as the one characterizing *Apc<sup>min</sup>* mice. In the *Apc<sup>min</sup>* model, the enhanced HSC function and the defective quiescence resulting from increased Wnt-β-catenin signaling led to the development of an MDS/MPD phenotype, which requires the concurrence of HSC-intrinsic and microenvironmental factors.
Therefore, the Apcmin represented a suitable experimental setting to investigate the influence of a SPARC-deficient microenvironment on MDS/MPD-prone HSC with preserved reliance on stromal signals. Transplantation of Apcmin BM cells into Sparc−/− recipients resulted in a myeloproliferative disorder classified in mice as “myeloproliferation-nonreactive,” which was different from the MDS/MPD observed in Apcmin mice, underlining the driving contribution of the stromal environment to the outcome of the myeloproliferative spur. The myeloproliferative phenotype of Apcmin > Sparc−/− chimeras consisted of BM hypergranulopoiesis with increased numbers of circulating mature granulocytes and myeloid precursors and was not observed in Apcmin > WT chimeras, further indicating that SPARC deficiency in the BM stroma may represent a condition favoring myeloid expansion under a myeloproliferative stress. Recently, Siva et al have reported that SPARC is dispensable for murine hematopoiesis both at the steady state and under the stress induced by acute hemolysis or BM transplantation, suggesting that SPARC deficiency does not alter the hematopoietic potential of the BM and that its contribution to the 5q− MDS phenotype may be subordinated to that of other molecular events that are not recapitulated in Sparc−/− models. We obtained similar results in the BM transplantation setting, in which Sparc−/− recipients were reconstituted without difference from WT recipients, even in the presence of low numbers of donor Lin− cells (ie, 2 × 10⁵ cells, data not shown). This suggests that a true myeloproliferative spur complementing the total body irradiation is necessary to bring out the role of SPARC in myeloproliferation. Indeed, the enhanced myeloproliferative response of mice with Sparc−/− BM stroma to TPO stimulation and to Apcmin hemopoietic cell transplantation indicates that the downstream effects of stromal SPARC synthesis, including collagen fibers assembly and deposition, may affect the control exerted by the BM stroma/HSC interaction over myelopoiesis.

The complex role of stromal SPARC in myeloproliferative conditions emerging from our experimental results is coherent with the homeostatic nature of matricellular proteins critically involved in tissue repair and normalization. Some of these proteins, such as thrombospondin-I and osteopontin, whose role in the pathogenesis of myelofibrotic myeloid neoplasms has been postulated, share with SPARC detrimental effects on the BM stroma homeostasis related to their up- or down-regulation in hematologic malignancies. Merging together our results from in situ expression analyses on human myeloid neoplasms and in vivo mouse models, we could hypothesize that the BM stroma response to myeloproliferative stimuli is entwined with the status of stromal SPARC expression. Increased or defective expression of SPARC in the BM stroma may exert different, yet detrimental, effects by fostering the development of fibrotic changes or inducing a flawed stromal niche permissive for the deregulated myeloid expansion, respectively. A possible limit to the generalization of this latter event related with defective stromal SPARC expression is represented by the evidence that in our models of TPO-induced reactive myeloproliferation and Apcmin > Sparc−/− transplantation the role of the stromal microenvironment was pivotal to the determination of the myeloproliferative phenotype. Indeed, this frame might not fit models of myeloproliferative disorders, such as those related with JAK2 or MPL gain-of-function mutations, in which hematopoietic cell-intrinsic defects are sufficient to determine the full-blown myeloproliferation in the absence of a recognized stromal contribution. In this light, a role for BM stroma-derived SPARC may be envisaged in the early stress response of the BM microenvironment to myeloproliferation. Variations in SPARC stromal expression may allegedly be accomplices in the loss of BM hematopoietic homeostasis associated with stroma disarrangement and/or altered myelopoiesis.

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Authorship

Contribution: C.T. and S.S. conceived the study and designed the research; C.G., P.P.P., G.C., C.C., C.M., S.M., M.G., A.C., A.M.F., and A.O. gathered data, performed the experiments, and analyzed the results; C.T., S.S., P.P.P., K.S., A.O., and M.P.C. wrote the manuscript; and S.A.P. and M.P.C. supervised the project and revised the manuscript.

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Stromal SPARC contributes to the detrimental fibrotic changes associated with myeloproliferation whereas its deficiency favors myeloid cell expansion

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