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Altered SDF-1/CXCR4 axis in patients with primary myelofibrosis and in the Gata1^{low} mouse model of the disease

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Abstract

Objective—To assess whether alterations in the SDF-1/CXCR4 occur in patients with primary myelofibrosis (PMF) and in Gata1^{low} mice, an animal model for myelofibrosis, and whether these abnormalities might account for increased stem/progenitor cell trafficking.

Materials and Methods—In the mouse, SDF-1 mRNA levels were assayed in liver, spleen and marrow. SDF-1 protein levels were quantified in plasma and marrow and CXCR4 mRNA and protein levels were evaluated on stem/progenitor cells and megakaryocytes purified from the marrow. SDF-1 protein levels were also evaluated in plasma and in marrow biopsy specimens obtained from normal donors and PMF patients.

Results—In Gata1^{low} mice, the plasma SDF-1 protein was 5-times higher than normal in younger animals. Furthermore, SDF-1 immuno-staining of marrow sections progressively increased with age. Similar abnormalities were observed in PMF patients. In fact, the plasma SDF-1 levels in PMF patients were significantly higher (by 2-fold) than normal ($p < 0.01$) and SDF-1 immuno-staining of marrow biopsy specimens demonstrated increased SDF-1 deposition in specific areas. In two of the patients, SDF-1 deposition was normalized by curative therapy with allogeneic stem cell transplantation. Similarly to what already has been reported for PMF patients, the marrow from

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Authors' contributions

Fabrizio Martelli, Maria Verrucci, Alessandro Maria Vannucchi, Hongyu Ni, Yi Jiang, and Betty Nakamoto performed experiments. Anna Rita Migliaccio, Giovanni Migliaccio, Ming Xu, Thalia Papayannopoulou and Ronald Hoffman, designed research, analyzed the data and wrote the paper.

All of the authors have read the manuscript, concur with its content and state that the data have not been submitted anywhere else for publication.

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Gata1^{low} mice contained fewer CXCR4^{pos}CD117^{pos} cells and these cells expressed low levels of CXCR4 mRNA and protein.

Conclusion—Similar abnormalities in the SDF-1/CXCR4 axis are observed in PMF patients and in the Gata1^{low} mice model of myelofibrosis. We suggest that these abnormalities contribute to the increased stem/progenitor cell trafficking observed in this mouse model as well as patients with PMF.

Keywords

Gata1; SDF-1; megakaryocytes; primary myelofibrosis

INTRODUCTION

Primary myelofibrosis (PMF) is characterized by constitutive mobilization of CD34⁺ hematopoietic stem/progenitor cells, as well as endothelial progenitor cells^{1–3}. Increased stem/progenitor cell trafficking has also been described in the Gata1^{low} mouse model of this disease. Mice harboring the hypomorphic Gata1^{low} mutation, that impairs expression of this gene in megakaryocytes⁴, are born thrombocytopenic^{5,6}. The megakaryocytes in the marrow from these mutants present the same morphological abnormalities as those observed in the marrow of PMF patients^{7–9}. The mutants slowly develop with age all the hallmarks of the disease, including increased stem/progenitor cell trafficking and extramedullary hematopoiesis in liver^{10,11}. The disease is associated with a profound disruption of the structural architecture of the marrow. We hypothesized that the abnormal cell trafficking observed both in the patients and in the mouse model might be the consequence of stem/progenitor cell dislodgment due to structural alterations of the stem cell niche. Alternatively, anatomical abnormalities may not be sufficient to alter the balance of events that result in the retention of stem/progenitor cells within the marrow. Other parameters, such as changes in chemokine levels^{10,12–14} and/or disruption of adhesive interactions favoring retention of stem/progenitor cells within the marrow¹⁵, may be operational.

SDF-1 is a chemokine primarily produced by the liver¹⁶. In addition to the liver, SDF-1 is produced in the marrow by several cell types, including osteoblasts¹⁷. Recent genetic studies have demonstrated that osteoblasts are critical cellular elements for stem cell maintenance and development^{18–20}. Stem cells are believed to reside in close proximity to the endosteal surface of the bone, where osteoblasts are also located^{21,22}. Interactions between SDF-1, produced by the osteoblasts²¹, and its receptor, CXCR4, expressed by the stem/progenitor cells^{23,24}, is thought to play a major role in stem cell trafficking in embryogenesis^{17,24} and in maintaining the stem cells within their niche in the marrow^{25,26}. A great deal of evidence indicates that alterations in SDF-1 concentrations in marrow and blood result in stem/progenitor cell mobilization. In fact, under steady-state conditions, the levels of soluble SDF-1 present in the blood of younger animals are low. SDF-1 is very sensitive to protease degradation^{27–30}.

A significant source of SDF-1 in the blood and marrow is present within megakaryocytes and platelets. These cells, in fact, express CXCR4^{31,32} and are capable of capturing SDF-1, which is stored within their cytoplasm and released in response to external stimuli³³. The concentration of soluble SDF-1 in the blood, however, increases in association with many of the conditions that induce stem/progenitor cell mobilization. Both sustained elevations of SDF-1 in NOD/SCID mice delivered by an adenoviral vector delivery system^{34–36}, and chronic administration to the animals of SDF-1 peptide analogs^{37,38}, result in mobilization of stem/progenitor cells. In addition, increased plasma SDF-1 levels are also observed during mobilization induced by sulfated glycans³⁹. Although G-CSF-induced stem/progenitor cell mobilization is not associated with increased levels of SDF-1 in the blood, this treatment reduces the levels of SDF-1 in the marrow and alters CXCR4 expression by stem/progenitor

cells^{40,41} by inducing a proteolytic environment⁴². By contrast, increased SDF-1 production is observed in the marrow following DNA induced damage and has been suggested to improve bone marrow recovery and facilitate stem cell engraftment post-transplantation⁴³. On the other hand, inactivation of CXCR4 function with neutralizing antibodies impairs stem/progenitor cell engraftment in the bone marrow³⁵.

The aim of this study was to determine whether abnormalities in the SDF-1/CXCR4 axis exist and might play a role in stem/progenitor cell trafficking in PMF. To achieve this goal, we analyzed the expression of SDF-1 (both mRNA and protein) in tissues from Gata1^{low} mice during different stages of the disease. The results provide evidence for abnormalities in the SDF-1/CXCR4 axis both in the mouse model of the disease and in patients with PMF. In the latter, it was possible to directly attribute these abnormalities to the defective PMF stem cell clone by comparing results obtained in two patients before and following curative therapy with stem cell transplantation. Overall, alterations in the SDF-1/CXCR4 axis observed with disease progression may at least participate in, if not be primarily responsible for, abnormal stem/cell progenitor cell trafficking in PMF.

MATERIALS AND METHODS

Mice

Mice with the hypomorphic Gata1^{low} mutation^{5,10} were bred at the Istituto Superiore di Sanità and maintained under good animal care practice conditions. Wild type littermates at the Gata1 locus were used as controls. Mice were divided into two age groups: 8–9-month (younger adult mice), i.e. at the time when they presented increased stem/progenitor cell trafficking, and 12–23-month (older adult mice), i.e. when they had developed extramedullary hematopoiesis in the liver (^{10,11} and Table 1). All the experiments were performed according to protocols approved by the Institutional Animal Care Committee.

Hematological parameters

Blood was collected from the retro-orbital plexus into ethylen-diamino-tetracetic acid-coated microcapillary tubes (20–40 µL/sampling). Platelet (plt) counts were determined manually¹².

Histology and immunohistochemistry of murine tissues

Mouse tissues were fixed in 10% (v/v) phosphate-buffered formalin (Sigma, St, Louis, MO, USA), paraffin embedded and cut into 2,5–3 µM section according to standard procedures. SDF-1β (Rb anti-rat SDF-1b, Torrey Pines Biolabs, Huston, TX) staining was done in frozen sections using the ABC/Complex/HRP kit from Dako (Carpinteria, CA), stained with 0.05% diaminobenzidine and counterstained with Mayer's Hematoxylin. All the preparations were stained at the same time and under the same conditions. Negative controls were represented by preparations incubated with irrelevant antibodies (data not shown). Slides were permanently mounted and photomicrographs were taken with a Nikon COOLPIX995 digital camera with an adapter fitting into the eyepiece of an Olympus BH-2 microscope with a 20X objective (Images were transferred directly to the computer and Photoshop 7.0 was used to adjust brightness, contrast and color balance).

Progenitor cell determination

The frequency of progenitor cells was determined by plating either total blood (2 µL/mL), or the mononuclear cell fraction of marrow (2×10⁴ cells/mL) or liver (5×10⁴ cells/mL) from representative normal and GATA-1^{low} littermates in standard methylcellulose cultures (0.9% w/v) containing fetal bovine serum (30% v/v, Sigma) and recombinant growth factors [rat stem cell factor (SCF, 100 ng/mL), mouse interleukin-3 (IL-3, 10 ng/mL), granulocyte-colony

stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) (50 ng/mL each) (all from Sigma) and human erythropoietin (EPO, 2 U/mL; Boehringer Mannheim, Mannheim, Germany)]¹¹. The cultures were incubated at 37 °C in a humidified incubator containing 5% CO₂ in air and colonies derived from more or less mature progenitor cells scored at day 8.

Flow cytometry analysis and cell purification

Cells were incubated for 30 min on ice with fluorescein isothiocyanate (FITC)-CXCR4 in combination either with phycoerythrin (PE)-CD34 and allophycocyanin (APC)-CD117 (that recognizes c-Kit) or biotinylated-streptavidin-CD61 and phycoerythrin (PE)-CD41. All the antibodies were purchased from PharMingen (San Diego, CA, USA) and used at a concentration of 1 µg/10⁶ cells. In addition to the Pharmingen antibody, a polyclonal purified rabbit anti-rat CXCR4 (Partec, Münster, Germany) followed by a FITC-conjugated goat antirabbit IgG (Immunotech, Miami, FL) was also used. Cell fluorescence was analyzed with a FACS ARIA (Becton Dickinson, Franklin Lakes, NJ). Non-specific fluorescent signals were gated out with appropriate fluorochrome-conjugated isotype controls and dead cells were excluded by propidium iodide staining. Cells in the stem/progenitor cell (CD117^{pos}) or megakaryocyte (CD61^{pos}) gates were purified by sorting, as previously described^{44,45} and were >90% pure upon FACS reanalysis.

SDF-1 ELISAS

Bone marrow washes were prepared by gently flushing the cavity of two femurs with 400 µL of phosphate buffered saline containing 0.1% Tween. Blood was collected from the retro orbital plexus of the animals with a heparin-coated Pasteur pipette and platelet-poor plasma prepared by centrifugation at 5000 rpm (platelet contamination of the samples <9 %). SDF-1 was measured with a commercial ELISA kit (R&D Systems, Inc. Minneapolis, MN, USA).

RNA isolation and quantitative RT-PCR

Total RNA was prepared from mononuclear cell fractions of marrow, spleen and liver following direct lysis with Trizol (Gibco BRL). Total RNA was also prepared from the entire femur by pulverizing the organ with a pestle under liquid nitrogen before dissolution in Trizol. RNA (1 µg) was retrotranscribed with random primers using the SuperScript III kit (Invitrogen Life Technologies, Bethesda, MD). Gene expression was quantified with a Taqman PCR kit (PE Applied Biosystems, Foster City, CA) using the ABI PRISM 7300 Sequence Detection System (Applied Biosystem). GAPDH was also quantified in each reaction. Quantitative values were obtained from the threshold cycle number (Ct), by subtracting the average Ct of the target gene from that of Gapdh and expressed as $2^{-\Delta Ct}$.

Human Subjects

The normal donors (13), patients with PMF (27) and polycythemia vera (PV, 10) included in this study have been previously described⁴⁶. Two of the patients with PMF underwent allogeneic stem cell transplantation and were studied again 12 months after the transplant⁴⁷. At the time of the analyses, the transplanted PMF patients had normocellular bone marrows with few reticulin fibers and near normal number of CD34⁺ in peripheral blood.

Human Immuno-histochemistry

Bone marrow biopsies were fixed with zinc formalin, decalcified and embedded in paraffin. For immunochemical staining, the tissue sections were deparaffinized in xylene and hydrated in graded alcohol. Heat-induced antigen retrieval was performed with citrate buffer for 20 minutes at 95 degrees in a steamer. The sections were then stained with a monoclonal anti-human SDF-1 antibody at 1:200 dilution (R&D Systems, Inc., Minneapolis, MN, USA),

followed by incubation with a peroxidase-labeled polymer conjugated to a goat antimouse immunoglobulin. Bound antibody was stained with a histostainer (DAKO) using a peroxidase detection kit with the chromogen 3,3'-diaminobenzidine tetrahydrochloride. The slides were counterstained with Light Green. All staining procedures were performed utilizing an isotypic antibody as a negative control.

RESULTS

Levels of SDF-1 mRNA in the femurs of Gata1^{low} mice are reduced with disease progression

Although SDF-1 mRNA was detectable in all organs analyzed, the major site of SDF-1 mRNA production in wild type mice was the liver. In fact, levels of SDF-1 mRNA in the liver were 1-log higher than those in the marrow and 2-logs higher than those in the spleen (Figure 1A). No difference between levels of SDF-1 mRNA expressed by the liver and the spleen was observed in wild type and Gata1^{low} littermates, regardless of the age of the animals (Figure 1A, and analyses not shown). In contrast, marrow cells from Gata1^{low} mice expressed levels of SDF-1 mRNA that were significantly ($p < 0.01$) lower than those expressed by normal mice.

Since osteoblasts, the cells primarily responsible for producing SDF-1 in the marrow, are poorly represented in cells recovered from the bone cavity, the levels of SDF-1 mRNA in the entire mouse femur were analyzed (Figure 1B). Levels of SDF-1 mRNA in the entire femur from wild type littermates, indicated by shaded area in Figure 1B, did not change with age (analyses not shown). In contrast, the levels of SDF-1 mRNA present in the entire femur from Gata1^{low} mice significantly decreased with age and was lower than those of wild type littermates (Figure 1B).

Adult Gata1^{low} mice contain elevated peripheral blood levels of soluble SDF-1 protein

The amount of SDF-1 present in the marrow cells of wild type and Gata1^{low} mice was generally low (< 100 – 400 pg/mL) (Figure 2). Marrow washes from Gata1^{low} mice contained levels of SDF-1 that were not statistically significantly greater than normal mice (Figure 2A). There was also no difference in the SDF-1 content of marrow washes from younger and older Gata1^{low} animals (Figure 2A).

In the blood, SDF-1 is present as a soluble protein or within platelets. Approximately 2 ng of soluble SDF-1 was detected in ~ 1 mL of blood from wild type mice (Figure 2B). By contrast, approximately ~ 350 pg of SDF-1 was detected per 10^5 platelets. The average number of platelets in the blood is $7.5 \times 10^5/\mu\text{L}$ and the total blood volume of a mouse is ~ 3 mL^{48,49}. Based on these assumptions, we calculated that ~ 24 μg of SDF-1 was present in the platelets (1000 times more than the soluble form) (Table II).

The levels of soluble SDF-1 in the plasma of older Gata1^{low} mice were similar to normal controls (~ 8 ng), but the levels in the plasma of younger (≤ 9 month) mutant mice were 4-times higher than normal (~ 24 μg , $p < .01$, Figure 2B and Table II). No difference was observed in the SDF-1 content of platelets prepared from wild type and Gata1^{low} mice, at all ages studied (Figure 2C). Since the blood from Gata1^{low} mice contains 5-times fewer platelets than normal controls (Table 1), the amount of SDF-1 within platelets in the mutant mice is, therefore, estimated to be 5-times lower than normal (Table II).

Increased levels of SDF-1 protein by immuno-histochemistry in the marrow of Gata1^{low} mice with disease progression

SDF-1 was detectable in marrow sections from wild type mice (Figure 3A). The staining was localized within megakaryocytes and did not increase with age (Figure 3A). SDF-1 staining was also localized within megakaryocytes in marrow sections from younger Gata1^{low} mice.

The intensity of the staining of the mutant megakaryocytes, however, was greater than that of the corresponding normal cells (Figure 3A). The SDF-1 staining of marrow sections from older animals (>10-month) was clearly stronger than that of wild type animals and younger mutant mice (Figure 3A). In this case, SDF-1 staining was not only localized within the megakaryocytes, but was also associated with structures that resemble collagen fibers (Figure 3A).

Also the spleen sections of Gata1^{low} mice reacted more strongly with the SDF-1 antibody than those from normal animals (Figure 3b). In this case, the staining was localized within the megakaryocytes of younger mutant mice and both in megakaryocytes and extracellular areas of older mice (Figure 3B). No difference was observed in SDF-1 staining of liver sections from wild type and Gata1^{low} mice.

Stem/progenitor cells from the marrow and the blood of Gata1^{low} mice express lower levels of CXCR4 than normal controls

The frequency of megakaryocytes (CD41^{pos}/CD61^{pos} cells) in the marrow of Gata1^{low} mice is 3–4-times greater than normal (Figure 4A and ^{10,45}). Six percent of both wild type and Gata1^{low} megakaryocytes expressed CXCR4 on their surface (Figure 4A). No difference was observed in the intensity of CXCR4 expressed (calculated as AFI) by megakaryocytes from the marrow of wild type and Gata1^{low} littermates.

Stem/progenitor cells present in the marrow of normal mice express CD117⁵⁰ and are further divided by the CD34 marker, into specific cell subpopulations^{51,52}. CD117 expression identifies also stem/progenitor cells in the marrow of mice with the Gata1^{low} mutation^{11,44}. CD34, however, is not an effective marker for identifying functional subpopulations from Gata1^{low}CD117^{pos} cells⁴⁵. Therefore, for the purpose of this study, the levels of CXCR4 expression were analyzed on the entire CD117^{pos} cell population present in the marrow of wild type and Gata1^{low} littermates.

The frequency of stem/progenitor cells (CD117^{pos}) in the marrow of Gata1^{low} mice was similar to that of normal animals (Figure 4B, Table I and ^{11,44,45}). However, because of a 5-fold reduction in marrow cellularity observed in these mice with disease progression, the total number of stem/progenitor cells in the marrow was calculated to be 5-fold lower than normal (Table I and ^{11,44}). CXCR4 was detectable in ~70% of the CD117^{pos} cells present in the marrow of wild type mice (Figure 4B, C). Since wild type CD117^{pos}CXCR4^{pos} cells purified by sorting retained the sorting profile upon re-analyses (Figure 5A), these cells represent a distinct subpopulation of the normal stem/progenitor cells present in the marrow. In contrast, both the frequency (~40%), and the level of expression (AFI/cell = 430 vs 710 in mutant and wild type cells, respectively), of CXCR4 expressed by the CD117^{pos} cells in the marrow of Gata1^{low} mice were significantly lower than in wild type animals (Figure 4B, C).

The levels of CXCR4 mRNA expressed by megakaryocytes (CD61^{pos}), by the entire stem/progenitor cell population (CD117^{pos}) and by the subpopulation of stem/progenitor cells expressing CXCR4 (CD117^{pos}CXCR4^{pos}) purified from the marrow of wild type and Gata1^{low} littermates are shown in Figure 5A–B. CXCR4 mRNA was expressed at comparable levels by CD61^{pos} (megakaryocytes) cells purified from the marrow of wild type mice and Gata1^{low} littermates; (Figure 5B). By contrast, CD117^{pos}CXCR4^{pos} cells purified from the marrow of Gata1^{low} mice expressed levels of CXCR4 mRNA significantly lower than those expressed by wild type CD117^{pos}CXCR4^{pos} (Figure 5B).

In conclusion, the CD117^{pos} cells in the marrow of Gata1^{low} mice express less CXCR4 than their wild type counterpart in terms of frequency of CXCR4^{pos} cells and the amount of surface receptor and mRNA expressed by CXCR4^{pos} cells.

To further characterize the trafficking abnormalities of the Gata1^{low} mice, we compared the frequency of progenitor cells in the blood of younger and old Gata1^{low} and wild type littermates. These experiments also evaluated the frequency of erythroblasts (Ter119^{pos}/CD71^{pos}) and of progenitor cells in the liver, which was used as a control for development of extramedullary hematopoiesis. Since it has been suggested that the proteolytic marrow microenvironment of PMF might lead to trafficking by cleaving surface proteins on stem/progenitor cells, the frequency of progenitor cells in the blood was determined both by functional assay flow cytometrically (CD117^{pos} cells). The expression of CXCR4 on the surface of the circulating CD117^{pos} cells was analyzed as well. As previously described¹², the blood from Gata1^{low} mice contains greater numbers of progenitor cells than the blood from wild type littermates (Table 1 and Figure 4B). The contour plots of the CXCR4 staining of the CD117^{pos} cells in the blood and in the marrow of Gata1^{low} mice were identical (Figure 4B). By contrast, wild type CD117^{pos} cells present in the blood express lower levels of CXCR4 than CD117^{pos} cells present in the marrow (⁵³ and Figure 4B).

PMF patients have higher blood levels of SDF-1 higher than normal

SDF-1 levels ranging from 800 to 1800 pg/mL were detectable in the plasma of normal volunteers. However, plasma levels of SDF-1 in PMF patients were increased ($p < 0.01$, Figure 6). This increase in plasma SDF-1 levels was not unique to patients with PMF, since the plasma of patients with PV also contained significantly higher levels of SDF-1 than controls ($p < 0.05$, Figure 6).

The excessive expression of SDF-1 in the marrow of PMF patients is restored by marrow transplantation

We assessed SDF-1 expression in BM biopsy specimens of both normal subjects (5) and PMF (10) patients using immunohistochemical staining. In two PMF patients, SDF-1 expression was analyzed before and 12 months after successful allogeneic stem cell transplantation. In the 5 normal BM biopsies examined, SDF-1 was present primarily along the osteoblasts lining the BM endosteal region and endothelial cells lining both the small and large blood vessels including the periarterial regions as well as the blood capillaries of the bone (Figure 7A). By contrast, the extent of and intensity of SDF-1 staining was significantly increased in each of the 10 PMF BM biopsy specimens examined (Figure 7A). The staining for SDF-1 was present in the dilated BM sinusoids that characterize PMF, around the vessel walls within the BM which were increased in PMF, and within the extracellular matrix. The increased SDF-1 deposition was seen in BM biopsies from patients with the prefibrotic forms of PMF as well as those with marked BM fibrosis (Figure 7A). The increased distribution of SDF-1 was no longer observed in two patients who had received curative treatment with allogeneic stem cell transplants (Figure 7B). These data indicate that the increased distribution of SDF-1 in PMF is a consequence of an abnormal clone and can be normalized by the replacement of this malignant clone with normal HSC and their progeny.

DISCUSSION

Disruption of the SDF-1/CXCR4 axis has been reported to lead to stem cell mobilization in several animal models (see ^{18–21}). The comparison of data obtained using an animal model and by studying patients with PMF provides evidence that alterations in CXCR4 expression or SDF-1 levels may be, at least partially, responsible for the abnormal stem/progenitor cell trafficking observed in PMF. The majority (~90%) of SDF-1 protein detected in the blood from wild type animals was present within platelets (Figure 2). The SDF-1 content of the blood platelets from Gata1^{low} mice was normal. However, since these mice have 3-times fewer platelets than normal in the blood, the overall content of platelet-associated SDF-1 in the circulation of these animals was calculated to be lower than normal (Table I). By contrast, the

levels of soluble SDF-1 in the blood of younger Gata1^{low} mice were 4-fold higher than normal (Figure 2). A two-fold increase in the amount of soluble SDF-1 present in the blood was also observed in patients with PMF (Figure 6). These increases were not specific to this myeloproliferative disorder as high levels of SDF-1 were also observed in the plasma of patients with PV (Figure 6). The elevation of SDF-1 observed in the blood of the animal model and of the PMF patients were modest (~2–4-fold) (Figure 2 and 6) when compared to the levels required to experimentally induce stem/progenitor cell mobilization in NOD/SCID mice (25-fold)^{34,35} and are, therefore, unlikely to be responsible for a gradient driven mobilization of stem/progenitor cells.

Both in wild type and Gata1^{low} mice, the major source of SDF-1 mRNA is the liver (Figure 1A). Significant levels of SDF-1 mRNA, however, were also expressed by cells flushed from the bone cavity and in the entire femur (i.e. marrow cells plus osteoblasts). The levels of SDF-1 expressed by the cells in the marrow from Gata1^{low} mice (at all ages) were significantly lower than normal while those expressed by the entire femur progressively decreased with age. These results may reflect a progressive reduction with age of specific cell populations, possibly represented by the hemopoietic cells, responsible for producing SDF-1 in the femur. In fact, it has been reported that human stem/progenitor cells and megakaryocytes produce SDF-1 mRNA^{54,55}. However, CD117^{pos} cells (wild type and Gata1^{low} cells alike) express negligible levels of SDF-1 mRNA ($2^{-\Delta Ct} < 2 \times 10^{-4}$) while some SDF-1 mRNA expression ($2^{-\Delta Ct} < 1 \times 10^{-3}$) was observed in CD61^{pos} cells from normal mice but not in those from Gata1^{low} mice.

Marrow sections both from Gata1^{low} mice (Figure 3A and ^{7,10}) and from PMF patients⁸ are characterized by an increased frequency of megakaryocytes which react strongly with the SDF-1 antibody (Figure 3 and 7). In the marrow of Gata1^{low} mice, the immunostaining was mainly localized within the megakaryocytes (Figure 3A). The fiber deposition observed in older animals was also associated with strong SDF-1-staining of the extracellular matrix (Figure 3A). These high levels of SDF-1 detected by immuno-histochemistry are not in contradiction with the near normal levels of SDF-1 detectable by ELISA in marrow washes from the same animals (Figure 2A). It is possible, in fact, that the mild detergent solution used to wash the marrow was incapable of removing SDF-1 from the extracellular matrix. High levels of SDF-1 immunostaining were also observed in marrow megakaryocytes of PMF patients (Figure 7A). Indirect proof that increased SDF-1 staining both of the altered megakaryocytes and of the extracellular matrix was due to the abnormal PMF clone is provided by the observation that SDF-1 staining of marrow biopsies from 2 PMF patients that had undergone curative allogeneic stem cell transplantation was normal (Figure 7B). Since wild type and Gata1^{low} megakaryocytes expressed similar levels of CXCR4 (both mRNA and protein, Figure 5), the increased SDF-1 uptake of Gata1^{low} megakaryocytes revealed by immunostaining is not due to increased numbers of receptors on the surface. Megakaryocytes from Gata1^{low} mice and from PMF patients express, however, the same intrinsic abnormalities (i.e. higher frequency and higher retention in the marrow because of maturation retardation^{6–8,56} and increased localization near the endosteum, in close spatial relationship with the osteoblasts⁵⁷. All these factors can contribute to the increased megakaryocyte up-take of SDF-1. Since production of SDF-1 in the marrow of Gata1^{low} mice is not higher than normal (Figure 1), entrapment of SDF-1 within the increased number of mutant megakaryocytes reduces the amount of SDF-1 available for other cells in the marrow. Furthermore, since SDF-1 is very sensitive to protease degradation^{27–30} and both the full length and its proteolytic products⁵⁸ are recognized by immunostaining, it is possible that the high levels of SDF-1 observed in the marrow both of Gata1^{low} mice and PV patients represent proteolytically cleaved SDF-1. In summary, it is possible that, in spite of high levels of SDF-1 immunostaining, the marrow of the animal model and of the PMF patients is deprived of functional SDF-1 because of increased SDF-1 uptake by the high numbers of defective megakaryocytes and presence of inactive degradation products of SDF-1 bound to the extracellular matrix.

Stem/progenitor cells purified from the marrow of Gata1^{low} mice expressed reduced levels of CXCR4 mRNA and barely detectable levels of CXCR4 antigen on their surface (Figure 4 and 5). Low levels of CXCR4 expression were also reported in mobilized human CD34⁺ cells in PMF patients⁵⁹. The biological consequences of reduced CXCR4 expression on these cells were identified by experiments showing that the CD34⁺CXCR4⁻ cells from PMF patients had reduced capability to migrate in vitro toward a gradient of SDF-1⁶⁰. The fact that reduced CXCR4 expression is implicated in abnormal cell trafficking in PMF is further suggested by the inverse correlation existing between CXCR4 expression and the number of circulating CD34⁺ cells that has been recently reported⁵⁹.

The mechanism responsible for reduced CXCR4 expression by stem/progenitor cells in the animal model and in the PMF patients remains unclear. It has been suggested that it may be due to increased cleavage of the extracellular domain of CXCR4 because of the proteolytic environment created by the pathological neutrophil emperipoiesis within the megakaryocytes¹⁵. In this context, the normal levels of CXCR4 expressed by the megakaryocytes present in the marrow of Gata1^{low} mice are of interest. If proteolytic mechanisms are responsible, one might suggest this is not the case in mutant mice. On the other hand, since mRNA levels were decreased, it is likely that altered CXCR4 expression on the stem/progenitor cells is a direct consequence of the mutation that induces the formation of the PMF clone. This hypothesis is supported by the transcriptosomal analysis of the genes abnormally expressed in PMF that has indicated that reduced CXCR4 mRNA expression on CD34⁺ cells from PMF patients is directly associated with the presence of the V617F JAK2 mutation⁶¹, the genetic abnormality recently demonstrated to be frequently associated with myeloproliferative disorders^{62–64}. These observations are similar to the dose dependent alteration of CXCR4 expression on stem/progenitor cells induced by p210, the BCR-ABL mutation determining chronic myelogenous leukemia^{65,66}. The availability of the Gata1^{low} model should allow one to demonstrate whether altered CXCR4 expression in the stem/progenitor cells is a direct consequence of the mutation responsible for the development of PMF and, if so, gaining a greater understanding of the molecular mechanism that determines the altered CXCR4 expression on the abnormal PMF clone.

In conclusion, these results suggest that abnormal stem/progenitor cell trafficking in PMF is accompanied by down regulation of CXCR4 expression by the stem/progenitor cells by a mechanism yet to be defined.

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Figure 1A-B

Figure 1. Reduced expression of SDF-1 mRNA in the bone marrow of *Gatal1^{low}* mice
A. Levels of SDF-1 mRNA in cells from the marrow, the spleen and the liver of wild type (grey bars) and *Gatal1^{low}* (light grey bars) littermates, as indicated. Results are expressed in $2^{-\Delta Ct}$, and are presented as mean ($\pm$ SD) of independent determinations, each one with a different mouse, the number of which is indicated in parenthesis on the top of each column. Results obtained with animals at different ages were pooled. Values statistically different ($p < 0.01$) from controls are indicated by **. **B.** The levels of SDF-1 mRNA in the entire femur of *Gatal1^{low}* mice with disease progression. Each determination was performed in duplicate and results are expressed in $2^{-\Delta Ct}$. The age of *Gatal1^{low}* mice analyzed is indicated on the X axis, and each dot represents an individual mouse. The levels of SDF-1 mRNA in the entire

femur of the wild type littermates did not change with age and are indicated by the shaded area [mean($\pm$ SD) of 5 independent determinations].

Figure 2. The plasma from younger (≤ 9 month-old) $Gata1^{low}$ mice contains significantly higher SDF-1 levels than those present in the plasma of wild type littermates

A. SDF-1 levels in marrow washes of $Gata1^{low}$ mice and of wild type littermates. Mutant mice were divided into two age groups (younger, ≤ 9 month-old, and older, ≥ 12 month-old) while control mice were 9–13-month-old. The number of animals analyzed in each group is indicated in parenthesis. Each triangle represents the mean of replicate determinations on the plasma of an individual mouse. (–) indicates the mean for each experimental group. The SDF-1 levels observed in the three age groups were not statistically different. **B. SDF-1 levels in the plasma of $Gata1^{low}$ mice and of wild type littermates.** The mice were divided in the same experimental groups described in A. Each determination was performed in duplicate and results

are presented as mean ($\pm$ SD) of independent determinations. The number of mice analyzed per experimental group is indicated in parenthesis. ** and § indicate values statistically different ($p < 0.01$) from wild type and younger mutant mice, respectively. **C. SDF-1 levels in platelets purified from the blood of *Gata1*^{low} mice and of wild type littermates.** Each determination was performed in duplicate and results are presented as mean ($\pm$ SD) of independent determinations. The number of mice analyzed per experimental group is indicated in parenthesis.

Figure 3A

Figure 3B-C

Figure 3. Increased SDF-1-immunostaining of marrow and spleen sections from Gata1^{low} mice with age

SDF-1-immunostaining of representative sections from marrow (A), spleen (B) and liver (C) of younger (7-month) and older (10–12-month) wild type (top panels) and Gata1^{low} (bottom panels) littermates are presented as indicated. Slides were counterstained with hematoxylin-eosin. Similar results were obtained with sections from at least 4 additional mice per experimental point, for a total of 14 animals. Arrows and arrowheads indicate representative megakaryocytes and stromal fibers, respectively. Magnification 40X.

Figure 4A-B

Figure 4C-D

Figure 4. Marrow megakaryocytes (CD41^{pos}/CD61^{pos}) from Gata1^{low} mice express normal levels of CXCR4 while marrow stem/progenitor cells (CD117^{pos}) from the same mice express reduced levels of CXCR4. On the other hand, CD117^{pos} cells from the marrow and blood of Gata1^{low} mice have a similar CXCR4 flow cytometric profile

Flow cytometric determinations of cells expressing CXCR4 among the CD41^{pos}/CD61^{pos} (megakaryocytes) and CD117^{pos} (stem/progenitor cells) cells of the marrow and of marrow and blood of representative wild type and Gata1^{low} mice are presented in A and B, respectively. In the case of the marrow, CXCR4 expression is presented both as a contour plot and as histogram in order to highlight the population profiles and the mean fluorescence intensity, respectively. The numbers within the quadrants indicate the frequency of the different cell

populations while those near the arrows indicate the average fluorescence intensity (AFI) per cell. Isotype controls for CXCR4 analyses are presented in grey while isotype controls for CD41/CD61 and CD34/CD117 analyses are not shown. The mean ($\pm$ SD) of independent determinations of the frequency and levels (AFI) of CXCR4 expressed by the cells are presented in C and D, respectively. In these graphs, results obtained with wild type and Gata1^{low} mice are indicated in dark and light grey, respectively. The number of mice analyzed per experimental point is indicated in parenthesis while values statistically different ($p < 0.05$ or 0.01) from wild type controls are indicated with * and **. b.d. = below detection.

Figure 5A-B

Figure 5. In the marrow from *Gata1*^{low} mice, megakaryocytes (CD61^{pos}) express normal levels of CXCR4 mRNA while the CXCR4^{pos} fraction of the stem/progenitor cells (CD117^{pos}/CD34^{neg} and CD34^{pos}) express levels of CXCR4 mRNA lower than normal

Representative gates used to purify the different populations and re-analyses of the sorted cells for purity are presented on the left. Wild type CD117^{pos} cells sorted on the basis of high CXCR4 expression retained their antigenic profile upon re-analysis. In contrast, >60% of the cells sorted as CXCR4^{pos} from *Gata1*^{low} mice resulted CXCR4^{neg} upon re-analysis. Mean ($\pm$ SD) CXCR4 mRNA levels, expressed as $2^{-\Delta Ct}$, of independent determinations, each one in duplicate, are presented on the right. Results obtained with wild type and *Gata1*^{low} littermates are indicated by grey and light grey bars, respectively. The number of mice analyzed per experimental point

is indicated in parenthesis. Values statistically different ($p < 0.01$) from those expressed by wild type CD117^{pos} cells are indicated with § while those different from wild type CD117^{pos}/CXCR4^{pos} cells are indicated with **.

Figure 6. The plasma from PMF and PV patients contains SDF-1 levels significantly higher than those present in the plasma of normal donors

Each symbol represents the value observed in a different subject and (–) indicates the mean SDF-1 content in each experimental group. The N values indicate the number of subjects analyzed per group.

Normal Controls

PMF Patients

Figure 7A

PMF Pre-transplant

PMF Post-transplant

Figure 7B

Figure 7. A. SDF-1 immunohistochemistry of bone marrow sections from two normal controls and 2 PMF patients

Sections were stained with a monoclonal anti-human SDF-1 antibody and then were counterstained with Light Green. Results are representative of those obtained from 2 different normal controls, and 2 different PMF patients (Magnification 200X).

Figure 7B SDF-1 immunohistochemistry of marrow sections from a PMF patient before and after allogeneic bone marrow transplantation. Bone marrow biopsy sections were stained with a monoclonal anti-human SDF-1 antibody and then were counterstained with Light Green (Magnification X200).

Number of platelets (Ptl) in the blood and frequency of stem/progenitor cells in the blood, femur and liver from wild type and Gata1^{low} littermates. Gata1^{low} mice were divided into adult and old animals, as indicated. The presence of erythroblasts (TER119^{pos}CD71^{pos}) in the liver is reported as sign of extramedullary hematopoiesis.

Table 1

Mice	Blood			Femur				Liver	
	Ptl (×10 ⁶ /μL)	CD117 ^{pos} (%)	CFC/μL	BM cells (×10 ⁶)	CD117 ^{pos} /CD34 ^{pos}		CFC	Ter119 ^{pos} /CD71 ^{pos} (%)	CFC ×10 ⁻⁵ L cells
					(%)	Total (×10 ⁶)			
Wild Type (9)	0.71±0.09	< 0.2	0.08±0.08	20.5±0.8	3.1±0.3	0.85±0.1	490±67	< 2	0.9±0.6
Gata1 ^{low} (≤ 9 months)(9)	0.30±0.01 [*]	3.9±1.2 [*]	1.9±0.1 [*]	6.0±0.6 [*]	2.6±0.3	0.21±0.06 [*]	645±65	6.5±0.5 [*]	12±1.8 [*]
Gata1 ^{low} (≥12 months) (7)	0.18±0.04 [*]	3.5±0.8 [*]	3.4±0.6 [*]	8.6±1.5 [*]	2.8±0.4	0.28±0.07 [*]	457±127	11.4±2.8 [*]	27±5 [*]

* Stem/progenitor cells were evaluated both by flow cytometry (CD117^{pos} and CD117^{pos}/CD34^{pos}) and by colony assay. Their total numbers in the femur is also presented. BM = bone marrow; L = liver. The number in parenthesis indicates the number of mice analyzed in each experimental group. Values statistically different (p< .05) from wild type controls are indicated.

Table II
Absolute values of soluble and Ptl-associated SDF-1 in the blood of Wild Type and Gata1^{low} mice. Gata1^{low} mice were divided into adult and old animals, as indicated.

Mice	Body Weight (gr)	Total Blood			
		Volume (mL)	Ptl numbers (× 10 ⁶)	Soluble SDF-1 (ng)	Ptl-associated SDF-1 (µg)
Wild Type (9)	40.6 ± 3.3	3.2 ± 0.3	2542 ± 121	7.8 ± 0.9	24 ± 16
Gata1 ^{low} (≤ 9 months) (9)	34.2 ± 4.3	2.7 ± 0.3	806 ± 31 *	20.7 ± 3.9 *	3.1 ± 0.02
Gata1 ^{low} (≥ 12 months) (7)	38.7 ± 2.7	3.1 ± 0.3	567 ± 131 *	8.0 ± 2.9	3.9 ± 0.5

* The number in parenthesis indicates the number of mice analyzed in each experimental group. Values statistically different (p<.01) from wild type controls are indicated.
The total volume of blood was calculated according to the index 80 mL/Kg, as recommended by (48,49).