Published in final edited form as:

J Cell Physiol. 2010 May ; 223(2): 460–470. doi:10.1002/jcp.22055.

EVIDENCE FOR ORGAN-SPECIFIC STEM CELL MICROENVIRONMENTS

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Abstract

The X-linked *Gata1*^{low} mutation in mice induces strain-restricted myeloproliferative disorders characterized by extramedullary hematopoiesis in spleen (CD1 and DBA/2) and liver (CD1 only). To assess the role of the microenvironment in establishing this myeloproliferative trait, progenitor cell compartments of spleen and marrow from wild-type and *Gata1*^{low} mice were compared. Phenotype and clonal assay of non-fractionated cells indicated that *Gata1*^{low} mice contain progenitor cell numbers 4-fold lower and 10-fold higher than normal in marrow and spleen, respectively. However, progenitor cells prospectively isolated from spleen, but not from marrow, of *Gata1*^{low} mice expressed colony-forming function in vitro. Therefore, calculation of cloning activity of purified cells demonstrated that the total number of *Gata1*^{low} progenitor cells was 10–100-fold lower than normal in marrow and >1,000-times higher than normal in spleen. This observation indicates that *Gata1*^{low} hematopoiesis is favored by the spleen and is in agreement with our previous report that removal of this organs induces wild-type hematopoiesis in heterozygous *Gata1*^{low/+} females (Migliaccio et al, Blood 114:2107,2009). To clarify if rescue of wild-type hematopoiesis by splenectomy prevented extramedullary hematopoiesis in liver, marrow cytokine expression profile and liver histopathology of splenectomised *Gata1*^{low/+} females were investigated. After splenectomy, the marrow expression levels of TGF- β , VEGF, osteocalcin, PDGF- α and SDF-1 remained abnormally high while *Gata1*^{low} hematopoiesis was detectable in liver of both CD1 and DBA/2 mutants. Therefore, in the absence of the spleen, *Gata1*^{low} hematopoiesis is supported by the liver suggesting that treatment of myelofibrosis in these animals requires the rescue of both stem cell and microenvironmental functions.

Keywords

Gata1; primary myelofibrosis; microenvironment; extramedullary hematopoiesis

INTRODUCTION

Primary myelofibrosis (PMF) is a myeloproliferative neoplasm (Hoffman, 2000; Jacobson et al., 1978; Tefferi, 2000; Tefferi et al., 2007) characterized by distinct abnormalities in megakaryocyte (MK) development which include reduced levels of Gata1 expression (Vannucchi et al., 2005), increased proliferation with retarded maturation (Ciurea et al., 2007), abnormally high p-Selectin localization on the demarcation membrane system and increased pathological neutrophil emperipolesis (Schmitt et al., 2000). It is assumed that these MK abnormalities, by altering the growth factor milieu of the microenvironment, lead to fibrosis, neo-vascularization of the marrow and bone formation (Hoffman and Xu, 2006). The alterations of the microenvironment have been suggested to dislodge the stem/progenitor cells from their niches in the marrow, resulting in increased stem/progenitor cell trafficking and hematopoiesis in extramedullary sites, including the spleen (Migliaccio et al., 2008). Splenomegaly, one of the consequences of extramedullary hematopoiesis in the spleen, is associated with numerous clinical complications of PMF (Cervantes et al., 2007). Therefore, splenectomy is recommended as a palliative treatment strategy for symptomatic splenomegaly refractory to other treatments (Cervantes et al., 2007). Whether the involvement of the spleen in the pathogenesis of PMF is secondary to hematopoietic failure in the marrow or whether the spleen, by providing a specific microenvironment, plays an active role in the progression of the disease, is debatable. Since splenectomy involves a substantial risk in patients with PMF, the role of the spleen in the development of the disease has not been systematically addressed in humans.

Gata1 is essential for appropriate erythroid and megakaryocytic differentiation (Orkin and Zon, 2008; Pang et al., 2005). In mice, the expression of Gata1 in MK can be experimentally reduced by ablating sequences upstream to the gene that regulate its expression in MK (Guyot et al., 2006; McDevitt et al., 1997; Vyas et al., 1999). Mice lacking these sequences, i.e. carrying the hypomorphic Gata1^{low} mutation, are born anemic and thrombocytopenic and die soon after birth (McDevitt et al., 1997; Vyas et al., 1999). The mutation, however, is not lethal in other genetic backgrounds (CD1 and DBA/2) that efficiently activate erythropoiesis in the spleen in response to erythroid stress¹⁶. In these strains, the mutant mice recover from their anemia at 1-month of age by developing splenomegaly (Martelli et al., 2005; Vannucchi et al., 2001). These mice, however, remain thrombocytopenic because of severe abnormalities in MK maturation (Centurione et al., 2004). These abnormalities are identical to those observed in the MK of PMF patients (Centurione et al., 2004; Schmitt et al., 2000). It is therefore not surprising that, with age, Gata1^{low} mutants develop myelofibrosis (MF), a phenotype that closely resembles the human disease including, in the CD1 background, extramedullary hematopoiesis in liver (Vannucchi et al., 2002).

Recently, we have demonstrated that the spleen and marrow microenvironment selectively support growth of Gata1^{low} and wild-type stem cells, respectively (Migliaccio et al., 2009). Splenectomy is lethal for hemizygous Gata1^{low/0} mice [Gata1 is on the X chromosome (Zon et al., 1990)] while favoring hematopoiesis from stem cells expressing the wild-type allele in the marrow, eliminating many features of MF, in heterozygous Gata1^{low/+} females (increasing blood platelet counts and reducing fibrosis and bone formation) (Migliaccio et al., 2009). The aim of the present study was to quantify the size of Gata1^{low} stem/progenitor compartments in the spleen, to identify the mechanisms that support proliferation of these cells in this organ and to clarify whether the prevalently wild-type hematopoiesis observed in the marrow of heterozygous Gata1^{+/-} females, in the absence of the spleen, restores the cytokine expression profile in this organ preventing stem/progenitor cell trafficking and development of extramedullary hematopoiesis in liver. In Gata1^{low} mice, the majority of stem/progenitor cells capable of forming colonies in vitro were found in the spleen. In addition, progenitor cells from the spleen expressed uniquely high Gata2 levels that were

likely responsible to rescue the Gata1^{low} defect (Huang et al., 2009). However, after splenectomy, the cytokine expression profile of the marrow of heterozygous Gata1^{low/+} females remained abnormally high and Gata1^{low} hematopoiesis was observed in the liver even in strains (DBA/2) in which this trait had not been observed before (Martelli et al., 2005). Therefore, Gata1^{low} hematopoiesis is not stem cell autonomous but requires the permissive microenvironment of spleen and liver, suggesting that treatment of this animal model of myelofibrosis requires rescue of both stem cell and microenvironmental functions.

MATERIALS AND METHODS

Mice

Gata1^{low} mice were obtained by breeding 5th generation Gata1^{low/0} males (Martelli et al., 2005) with CD1 females (Charles River, Calco, Italy). The mice used in the experiments were represented by 5th generation Gata1^{low/0} males and by the heterozygous Gata1^{low/+} female and wild-type male offspring of these crossings. Heterozygous Gata1^{low/+} DBA/2NCRBR females were used in selected experiments. Mice were housed for up to 2 years under good animal care practice conditions in the animal facilities of Istituto Superiore Sanità. All the experiments were performed with sex- and age-matched mice under protocols approved by the institutional animal care committee.

Splenectomy

Gata1^{low/+} females (Martelli et al., 2005) were anaesthetized with xylazine (10 mg/kg, Bayer, Milan, Italy) and ketamine (200 mg/kg, Gellini Farmaceutics, Latina, Italy) i.p. one day following food withdrawal. The spleen was removed after double ligation of the splenic artery and vein. The muscle, peritoneum and skin were closed in separate layers using sterile 5-0 absorbable suture. Animals received the analgesic butorphanol s.c. (5 mg/kg/day, Intervet Italia Srl, Milan, Italy) for 4 days post-surgery. Groups of at least 3 mice were sacrificed 3, 6–8 and 9–10 months post splenectomy for further analyses.

Immuno-histochemistry

Livers were fixed in 10% (v/v) phosphate-buffered formalin (Sigma, St. Louis, MO, USA). Paraffin embedded, 2.5–3 μ M sections were prepared and stained with hematoxylin-eosin or incubated with an anti-CD45 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then stained with avidin-biotin immunoperoxidase (Vectastain Elite ABC Kit; Vector laboratories, Burlingame, CA, USA), as described by the manufacturer and then counterstained with hematoxylin-eosin. Samples not incubated with the primary antibody, or incubated with non-immune IgGs, served as negative controls. Histopathological evaluations were performed with a DM RB microscope (Leica LTD, Heidelberg, Germany) set in a transillumination mode and equipped with a Coolsnap videocamera in order to capture computerized images (RS Photometrics, Tucson, AZ, USA).

Flow cytometry and cell sorting

The cells analyzed in these experiments were mononuclear cells (MNC) of the liver and marrow and light density cells (LDC) of the spleen. LDC were isolated by centrifugation over a mouse-specific Ficoll cushion ($\rho < 1.083$, Histopaque-1083, Sigma-Aldrich, St Louis, MO, USA). Cells were suspended in Ca⁺⁺ Mg⁺⁺-free phosphate buffered saline supplemented with 1% (v/v) bovine serum albumin, 2 mM EDTA, 0.1 % NaN₃ and incubated for 30 min on ice with 1 μ g/10⁶ cells of phycoerythrin (PE)-conjugated CD117 (anti-cKit), CD71 and CD61 and fluorescein isothiocyanate (FITC)-conjugated anti-Sca1, CD34, TER119 and CD41 (all from PharMingen, San Diego, CA) (Migliaccio et al., 2009). Dead cells were excluded by propidium iodide staining (5 μ g/mL, Sigma) and non-specific

signals were excluded with appropriate isotype controls (PharMingen). Cell fluorescence was analyzed with the ARIA cell sorter (Becton Dickinson, Franklin Lakes, NJ). In selected experiments, cells in the prospective stem cell (CD117^{pos}/Sca1^{pos}) (Spangrude et al., 1988), progenitor cell (CD117^{pos}/CD34^{high} and CD117^{pos}CD^{low}) (Akashi et al., 2000; Na Nakorn et al., 2002), erythroblast (CD71^{pos}/TER^{pos}) (Socolovsky et al., 2001) and megakaryocytic (CD41^{pos}/CD61^{pos}) (Migliaccio et al., 2009) gates were isolated by sorting and shown to be >90% pure by reanalysis.

Progenitor cell counts

The progenitor cells in hematopoietic tissues from representative normal and Gata1^{low/+} littermates were assayed by plating cells of the marrow (2×10⁴ MNC/mL), blood (2 μL/mL), spleen (2×10⁴ LDC/mL) and liver (10⁵ MNC/mL) in standard methylcellulose cultures (0.9% w/v) containing fetal bovine serum (30% v/v, Sigma), rat stem cell factor (SCF, 100 ng/mL), mouse interleukin-3 (IL-3, 10 ng/mL) (from Sigma) and human erythropoietin (EPO, 2 U/mL; Boehringer Mannheim, Mannheim, Germany). Prospectively isolated progenitor cells were cultured under conditions of limiting dilution (100 cells/mL). The cultures were incubated at 37 °C in a humidified incubator containing 5% CO₂ in air and hematopoietic colonies scored after either 8 or 15 days. BFU-E-, CFU-GM- and CFU-GEMM-derived colonies were scored individually but results expressed as total colony numbers for simplicity. The numbers of colonies (Table I and II) were then used to calculate the size of the progenitor cell compartments in the marrow and spleen according to the following algorithms:

Marrow MNC:

$$\frac{[\text{number of colonies per dish}]}{[\text{number of plated MNC}]} \times [\text{number of MNC in femur}] \times 3.3$$

Marrow purified cells:

$$\frac{[\text{number of colonies per dish}]}{[100]} \times [\text{frequency of progenitor cells}] \times [\text{number of plated MNC}] \times 3.3$$

Spleen LDC:

$$\frac{[\text{number of colonies per dish}]}{[\text{number of plated LDC}]} \times [\text{number of LDC in spleen}]$$

Spleen purified cells:

$$\frac{[\text{number of colonies per dish}]}{[100]} \times [\text{frequency of purified cells}] \times [\text{number of LDC in spleen}]$$

These calculations considered the number of cells present in a femur to correspond to 3.3% of total marrow cells of a mouse (Migliaccio et al., 2003).

RNA isolation and semi-quantitative and quantitative RT-PCR analysis

Total RNA was prepared by lysing bone marrow or purified cells directly into a commercial guanidine thiocyanate/phenol solution (Trizol, Gibco BRL, Paisley, UK). RNA (1 μg) was reverse transcribed with 2.5 μM random hexamers using the superscript kit (Invitrogen, Milan, Italy). Osteocalcin, TGF-β1, PDGFα and VEGF cDNAs were quantified by

semiquantitative RT-PCR based on increasing amplification cycles (20, 25, 30 and 35 cycles, indicated by triangles on the top of the figure), as described (Vannucchi et al., 2002). In this analysis, gene expression was quantified spectrophotometrically on the basis of the optical density of the amplified test bands compared to the amount of concurrently amplified Actin product, and expressed as ratio. SDF-1 and Gata1 expressions were quantified by Real Time RT-PCR using pre-developed assays (Applied Biosystem, Foster City, CA, USA) (Migliaccio et al., 2008). GAPDH cDNA was concurrently amplified as housekeeping control. Reactions were performed in a ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using the following parameters: 40 cycles of a two-step PCR program at 95°C for 15 sec. and 60°C for 60 sec., after an initial denaturation/activation step at 95°C for 10 min. The SDS software was used to analyze and calculate the cycle threshold (CT). Quantitative normalization of cDNA was obtained by the ΔC_t method (ΔC_t = target gene Ct - GAPDH Ct) and the levels of mRNA expressed as $2^{-\Delta C_t}$. Samples were assayed in triplicate and appropriate negative and positive controls were included in each assay.

Statistical analysis

Results are presented as mean ($\pm$ SD) of those observed in at least 3 independent experiments with 3 mice per experimental group. Statistical analysis was performed by analysis of variance (Anova test) using Origin 3.5 software for Windows (Microcal Software Inc., Northampton, MA). Differences between experimental groups were considered statistically significant with $p < 0.05$ -0.01.

RESULTS

The marrow from Gata1^{low} mice contains modest numbers of prospectively isolated progenitor cells with hemopoietic activity in vitro

The effect of the hypomorphic Gata1^{low} mutation on the size of progenitor cell compartments in the marrow was assessed by comparing total progenitor cell numbers calculated on the basis of frequencies of hematopoietic colonies generated in cultures by MNC and by prospectively isolated progenitor cells (Table I,II and Figure 1). Progenitor cells were phenotypically defined as cells that bind CD117 (the antibody that recognizes cKit, the receptor for stem cell factor) (Besmer, 1991), in combination with the stem cell marker Sca1 (Spangrude et al., 1988), or CD34, an antigen that distinguishes megakaryocytic/erythroid restricted (MEP, CD117^{pos}/CD34^{low}) and common myeloid (CMP) and granulo/monocytic restricted (GMP) (both CD117^{pos}/CD34^{high}) (Akashi et al., 2000; Na Nakorn et al., 2002) progenitor cells.

As described (Vannucchi et al., 2002), due to fibrosis, the femur from Gata1^{low} mice contained lower numbers of MNC than the femur from wild-type animals (5.2 vs 18×10^6 cells, respectively) (Table I). MNC from Gata1^{low} marrow generated numbers of hematopoietic colonies slightly lower (and statistically different only at day 15) than those observed in the corresponding wild-type cultures (Table I). In addition, in Gata1^{low} mice, the frequencies of phenotypically defined progenitor cells in the marrow were also normal (Figure 1A and Table II). Therefore, by colony assay on MNC and by phenotype analyses, the difference in progenitor cell compartments in the marrow of Gata1^{low} and wild-type littermates were modest and reflected mainly decreased cellularity due to fibrosis.

Progenitor cells prospectively isolated from the marrow of wild-type mice generated numerous hematopoietic colonies in culture (average cloning efficiency 0.85–32% at day 8 and 14–77% at day 15) (Table II and Figure 1) confirming that these phenotypes predict hematopoietic activity in vitro (Akashi et al., 2000; Na Nakorn et al., 2002; Spangrude et al., 1988; Vannucchi et al., 2002). By contrast, progenitor cells prospectively isolated from the

marrow of Gata1^{low} mice had poor cloning efficiency (0.1–14% at day 8 and 0.1–15% at day 15) (Table II). Therefore, in the mutant mice, progenitor cell phenotype/function correlations were lost in the marrow (Figure 1A). Calculations based on these results indicated that the size of Gata1^{low} progenitor cell compartments in the marrow is 10–100-times lower than normal (Figure 1A).

The spleen from Gata1^{low} mice contains the majority of prospectively isolated progenitor cells with hemopoietic activity in vitro

The effect of the hypomorphic Gata1^{low} mutation on the size of progenitor cell compartments in the spleen was also assessed (Figure 1 and Table I and II). Progenitor cells were phenotypically defined according to the same criteria used to characterize cells from marrow (Akashi et al., 2000; Na Nakorn et al., 2002; Spangrude et al., 1988).

The spleen from Gata1^{low} mice was bigger than normal and contained 7-fold more LDC than the spleen from wild-type littermates (4.6 vs 31×10⁶ LDC, respectively) (Table I). LDC from the spleen of Gata1^{low} mice generated 3–4-times more hematopoietic colonies than the corresponding wild-type cells. Therefore, by calculation, the spleen from Gata1^{low} mice contained 10-times more progenitor cells than the spleen of wild-type mice (1.7–2.4×10⁵ vs 0.6–1.1×10⁴ progenitor cells, respectively) (Figure 1B). Similarly, the frequencies of phenotypically defined progenitor cells in the spleen of Gata1^{low} mice were 10-times greater than normal (Figure 1B and Table II). According to these data, the difference in progenitor cell content of the spleen from Gata1^{low} and wild-type littermates was modest.

Progenitor cells prospectively isolated from the spleen of wild-type mice generated low numbers of colonies in vitro (average cloning efficiency <1%, Table II). Therefore, the spleen of wild-type mice contained barely detectable numbers of progenitor cells capable to form colonies under conditions of limiting dilution (<100 progenitor cells/spleen) (Figure 1B) confirming that in wild-type mice the vast majority of progenitor cell activity is prospectively isolated from marrow. By contrast, in the case of Gata1^{low} mice, progenitor cells prospectively isolated from the spleen generated great numbers of colonies (cloning efficiency 15–68% at day 8 and 50–100% at day 15). Therefore, the size of progenitor cell compartments in the spleen calculated on the basis of colony numbers generated by purified cells and LDC were similar, indicating that in these mutant mice progenitor cell phenotype/function correlations were conserved in the spleen (Figure 1B).

In conclusion, in Gata1^{low} mice, the vast majority (2-log more than in the marrow) of progenitor cell activity was prospectively isolated from spleen.

Progenitor cells from the spleen of Gata1^{low} mice express high levels of Gata2

The function of hematopoietic progenitors is determined by appropriate levels of transcription factor expression. Hematopoietic progenitor cell maturation is sequentially controlled by Gata2 and Gata1 (Orkin and Zon, 2008). The proteins encoded by these two genes are very similar and may exert redundant functions (Ferreira et al., 2007). Since the stage specificity of the function of these two proteins is ensured by their expression levels (Arinobu et al., 2007), retention of Gata2 expression with maturation has been proposed as the mechanism that rescues Gata1^{low} progenitor cells (Huang et al., 2009). To confirm this hypothesis, the levels of Gata1 and Gata2 expressed by progenitor cells (CD117^{pos}/CD34^{high}, CMP/GMP, and CD117^{pos}/CD34^{low}, MEP) prospectively isolated from the spleen and marrow of wild-type and Gata1^{low} mice were compared by quantitative RT-PCR (Figure 2). The levels of expression were standardized to those expressed by the corresponding cell populations isolated from the marrow of wild-type animals. In the case of wild-type mice, both MEP and CMP/GMP purified from the spleen expressed low levels of

Gata1 but only MEP expressed high levels of Gata2 (>18-fold higher than those expressed by MEP purified from the marrow). Therefore, the poor cloning efficiency of wild-type cells prospectively isolated from the spleen was associated with low levels of Gata1 expression. In the case of Gata1^{low} mice, MEP and CMP/GMP purified both from the marrow and from the spleen expressed reduced levels of Gata1 (Figure 2). However, progenitor cells purified from the spleen expressed high levels of Gata2 (>14-times the levels of expression in cells from wild-type marrow).

These results indicate that low levels of Gata1 expression is a property of progenitor cells purified from the spleen (wild-type and Gata1^{low} alike) and that high levels of Gata2 expression may represent at least one of the mechanisms that rescue the function of Gata1^{low} progenitor cells in this organ.

Splenectomy does not normalize the abnormally high cytokine expression profile of the marrow of Gata1^{low/+} females

Extramedullary hematopoiesis in MF is thought to be, at least partially, mediated by increased stem/progenitor cell trafficking (Hoffman and Xu, 2006) induced by the high levels of growth factors (TGF- β , osteocalcin, VEGF, PDGF α and SDF-1) released in the marrow microenvironment by stromal cells activated by immature Gata1^{low} MK (Lataillade et al., 2008; Migliaccio et al., 2008). The observation that removal of the spleen favours wild-type hematopoiesis including the formation of Gata1^{pos} MK, in the marrow of heterozygous Gata1^{low/+} females (Migliaccio et al., 2009), suggested studies to compare the levels of TGF- β , osteocalcin, VEGF, PDGF α and SDF-1 expressed in the marrow of untreated and splenectomised Gata1^{low/+} females (Figure 3). As expected, the marrow from untreated Gata1^{low/+} females expressed all the cytokines analysed at levels significantly higher than those expressed by wild-type marrow. Removal of the spleen did not reduce the levels of cytokine expression by the marrow and significantly increased the expression of osteocalcin (at 3 and 6 month) and VEGF (at 9-months) (Figure 3).

These results indicate that the activated stromal cells of the marrow from Gata1^{low/+} females remain activated even when Gata1^{low} MKs are no longer formed in this organ.

In the absence of the spleen, Gata1^{low} progenitor cells colonize the liver

The high levels of cytokines produced in the marrow after splenectomy suggested that removal of the spleen may not halt stem/progenitor cell trafficking and may even favour development of extramedullary hematopoiesis in liver. To test this hypothesis, the presence of progenitor cells in the blood and of extramedullary hematopoiesis in the liver of heterozygous Gata1^{low/+} females after splenectomy were investigated (Figure 4 and 5). These experiments included mice carrying the mutation in backgrounds permissive (CD1) and not (DBA/2) for development of this trait (Martelli et al., 2005), as well as wild-type mice as control.

By colony assay, hematopoietic progenitor cells were detectable in blood (5 progenitor cells/ μ L) and liver (10–20 progenitor cells/ 2×10^4 cells) of CD1 and DBA/2-Gata1^{low/+} females after splenectomy but not in blood from splenectomised wild-type mice. Therefore, in Gata1^{low} mutants progenitor cell trafficking persisted after splenectomy.

As previously published (Martelli et al., 2005), at 15–18-months of age, the liver parenchyma of untreated CD1-Gata1^{low} mice, but not that of DBA/2-Gata1^{low} mice, contained numerous hemopoietic cell clusters recognizable by hematoxylin-eosin staining (Figure 4). By the more sensitive CD45 immunostaining method, numerous hematopoietic cells (CD45^{pos}) were detected in the parenchyma of untreated CD1-Gata1^{low} mutants while few were present in liver of untreated DBA/2-Gata1^{low} mice (Figure 4). Removal of the

spleen did not induce hematopoiesis in the liver parenchyma of wild-type mice. By contrast, massive numbers of hematopoietic foci were observed in the liver of both CD1- and DBA/2-Gata1^{low/+} mice after splenectomy (Figure 4).

The extent of extramedullary hematopoiesis in the liver of DBA/2 mutants was quantified by FACS analyses (Figure 5A). At 15–19 months of age, the liver of untreated DBA/2 Gata1^{low/+} mutants contained negligible numbers of cells expressing the phenotype of stem/progenitor cells (CD117^{pos}, 0.7%), MK (CD61^{pos}/CD41^{pos}, 1.3%) and erythroblasts (CD71^{pos}/TER119^{pos}, 1.9%) (Figure 5A). By contrast, in the corresponding splenectomized littermates, the frequency of stem/progenitor cells, MK and erythroblasts rose to 6.1, 6.7 and 10.2%, respectively.

By colony assay, both well hemoglobinized and poorly hemoglobinized bursts were observed in cultures of liver cells from the mutant mice after splenectomy (Figure 5B), suggesting that the liver of these animals contained both Gata1⁺ and Gata1^{low} progenitor cells. Since genetic markers for clonal analyses of hematopoietic colonies in mice are not available, the progenitor cell type, Gata1⁺ and/or Gata1^{low} that colonized the liver of these females and its contribution to hematopoiesis in this organ was inferred from the levels of Gata1 expressed by cells prospectively isolated from the liver of untreated and splenectomized CD1-Gata1^{low/+} mice (Figure 5C). The levels of Gata1 expressed by the corresponding populations purified from the marrow of untreated wild-type, Gata1^{low} and Gata1^{low/+} mice served as comparison. In the case of the marrow, all the Gata1^{low} cell populations expressed levels of Gata1 significantly lower than those expressed by the corresponding wild-type cells. Cells purified from Gata1^{low/+} females expressed instead Gata1 levels intermediate between the wild-type and Gata1^{low} ones, indirectly confirming that in the presence of the spleen both wild-type and Gata1^{low} progenitor cells contribute to hematopoiesis in the marrow of heterozygous females²⁰. Also, cells purified from the liver of untreated CD1-Gata1^{low/+} females expressed levels of Gata1 intermediate between values expressed by wild-type and Gata1^{low} cells. By contrast, CD117^{pos} cells purified from the liver of splenectomized CD1-Gata1^{low/+} females expressed levels of Gata1 similar to those expressed by wild-type marrow cells, suggesting that the majority of them expressed the Gata1⁺ allele (Figure 5C). Erythroid cells from the liver of these females expressed levels of Gata1 similar to those expressed by cells purified from the marrow of untreated Gata1^{low/+} mice and therefore, were likely to be derived both from Gata1⁺ and Gata1^{low} progenitor cells. MK purified from the liver of these splenectomized animals, however, expressed levels of Gata1 as low as those expressed by MK purified from the marrow of Gata1^{low} mice (Figure 5A), indicating that megakaryocytopoiesis in the liver was mostly Gata1^{low}.

These results indicate that, although the majority of progenitor cells in the liver of splenectomized heterozygous Gata1^{low/+} females express the Gata1⁺ allele, both Gata1⁺ and Gata1^{low} progenitor cells contribute to erythropoiesis and only Gata1^{low} progenitor cells contribute to megakaryocytopoiesis in the liver after splenectomy.

DISCUSSION

The results presented in this study indicate that the spleen plays an important role in the Gata1^{low} mouse model of MF by rescuing the hematopoietic functions of Gata1^{low} stem/progenitor cells and by trapping these cells, possibly preventing colonization of additional extramedullary sites. Although calculations based on colony numbers in cultures from MNC indicated modest differences in progenitor cell compartment sizes between marrow and spleen of wild-type and Gata1^{low} littermates (Figure 1), calculations based on colony numbers generated by prospectively isolated cell populations indicated that Gata1^{low} progenitor cells capable to form colonies in vitro were localized mainly in spleen (Figure 1

and Table I and II). Differences in estimates of progenitor cell populations determined by the two assays reflect the fact that progenitor cell phenotype/function correlations were lost in the marrow of *Gata1^{low}* mice (Figure 1). This loss of correlation was associated with low *Gata1* expression (Figure 2). Also *Gata1^{low}* progenitor cells from the spleen expressed low levels of *Gata1*. These cells, however, unexpectedly expressed levels of *Gata2* 14-fold higher than normal. These results suggest that in the spleen *Gata1^{low}* hematopoiesis is rescued, at least in part, by microenvironmental cues (the nature of which remains to be identified) which allow retention of *Gata2* expression with maturation.

In contrast to the extensive knowledge obtained in recent years on microenvironmental control of hematopoiesis in the marrow (Adams and Scadden, 2006; Moore and Lemischka, 2006; Papayannopoulou and Scadden, 2008; Yin and Li, 2006), much less is known about the microenvironmental properties of the spleen. Spleen hematopoiesis, in fact, was actively considered in the past (Curry et al., 1967; Jandl et al., 1965; La Pushin and Trentin, 1977) but few studies (Slayton et al., 2002; Yanai et al., 1991) have analyzed the hematopoietic role of this organ in recent years. A recent publication has suggested BMP4 as an extramedullary-specific cue whose expression is specifically involved in activation of spleen erythropoiesis under conditions of stress (Perry et al., 2009). BMP4, however, has also been identified as a growth factor that regulates hematopoiesis in the marrow (Goldman et al., 2009). It is possible then that other pathways, such as the retinoblastoma pathway, may cooperate with BMP4 in determining the *Gata1^{low}* specificity of the microenvironmental cues. In fact, the concept that microenvironmental cues could be permissive for the development of myeloproliferative disorders was developed on the basis of the phenotype expressed by mice in which either the retinoblastoma gene (Walkley et al., 2007a; Walkley and Orkin, 2006), or the gene encoding the γ subunit of the retinoic acid receptor (Walkley et al., 2007b), was inactivated. These mutants experience profound myeloproliferation with increased numbers of stem/progenitor cells in bone marrow, increased levels of stem/progenitor cell trafficking and extramedullary hematopoiesis in the spleen. Transplantation experiments, however, demonstrated that the myeloproliferation trait segregates with the genotype of the host microenvironment and not with that of the hematopoietic stem cells of the donor suggesting that these mutations may alter the ability of the extramedullary microenvironment to support stem cell proliferation. Further studies are necessary to clarify whether microenvironmental cues that rescue *Gata1^{low}* hematopoiesis in the spleen are represented by BMP4 and/or the retinoblastoma pathway or other factors still to be identified.

Splenectomy favoured wild-type hematopoiesis in the marrow of heterozygous *Gata1^{low/+}* mice (Migliaccio et al., 2009) but did not halt stem/progenitor cell trafficking and promoted development of extramedullary hematopoiesis in the liver even in DBA/2 mutants (Figure 4 and 5) that would not normally develop this trait (Martelli et al., 2005). The observation that splenectomy restored maturation of *Gata1^{pos}* MK in the marrow (Migliaccio et al., 2009), but did not reduce the high cytokine expression profile of this organ (Figure 3), suggests that marrow stromal cells, once activated by *Gata1^{low}* MKs, remain activated even in the absence of *Gata1^{low}* MKs, perhaps explaining why splenectomy did not halt stem/progenitor cell trafficking. In addition to cytokines, activated stromal cells of PMF patients (Hoffman and Xu, 2006) and *Gata1^{low}* mice (Centurione et al., 2004) express high levels of the metalloprotease MMP9. Recently it has been suggested that MMP9 forms with other as-yet unidentified proteins a supramolecular structure defined as “the invadosome” that mediates extramedullary infiltration by leukemic cells (Stefanidakis et al., 2009). It is conceivable that MMP9 is organized into an invadosome-like complex also in PMF patients and in *Gata1^{low}* mice contributing to stem/progenitor cell infiltration in extramedullary sites. The fact that stromal cells of *Gata1^{low/+}* females remained activated after splenectomy suggests that these cells, in addition to cytokines, are likely to produce great levels of “invadosome” explaining

why removal of the spleen facilitated infiltration of other organs (the liver) by stem/progenitor cells (wild-type and *Gata1*^{low} alike). The failure of wild-type stem/progenitor cells to generate erythroid and megakaryocytic cells in the liver of splenectomized *Gata1*^{low/+} mutants may then reflect again the fact that the microenvironmental cues of the extramedullary sites favors maturation of *Gata1*^{low} progenitor cells. Although additional studies are required to assess whether an invadosome-like structure is involved in extramedullary hematopoiesis in the *Gata1*^{low} model of PMF, our results are consistent with the recent hypothesis that the spleen in patients with PMF might also actively participate in the development of disease manifestations by selectively interacting with subpopulations of cells belonging to the malignant clone (Lane et al., 2009; Lataillade et al., 2008). In fact, splenectomy in patients with PMF has also been associated with a higher incidence of development of extramedullary hematopoiesis in liver and higher rate of blast transformation (Cervantes et al., 2007). Further evidence for such a role of the spleen in the biology of PMF has recently been provided by observations made during the initial clinical trials with JAK2 inhibitors in patients with PMF. The administration of these drugs led to a dramatic rapid reduction of spleen size without affecting other haematological parameters, clearly indicating the existence of a subpopulation of malignant cells that might preferentially reside in the spleen (Verstovsek et al., 2007a; Verstovsek et al., 2007b).

In conclusion, these results support the notion that the spleen plays an important role in the development of MF in *Gata1*^{low} mice by providing a permissive microenvironment for the mutant stem/progenitor cells. However removal of this organ restored stem/progenitor cell function but not microenvironmental function in *Gata1*^{low/+} females resulting in development of hematopoiesis in extramedullary sites. These results suggest that treatment of MF in *Gata1*^{low} mice requires restoration of both hematopoietic and microenvironmental functions of the marrow.

Acknowledgments

This study was supported by Ministero per la Ricerca Scientifica and Alleanza sul Cancro, Italy, the National Cancer Institute, grant no. P01-CA108671 and NY STAR program, USA.

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Bone Marrow

Figure 1A

Spleen

Figure 1B

Figure 1. A,B. In Gata1^{low} mice, the majority of colony forming activity can be prospectively isolated from the spleen

Total numbers of progenitor cells present in marrow (A) and spleen (B) from wild-type and Gata1^{low} mice calculated on the basis of colony numbers grown from marrow MNC and spleen LDC or from CD117^{pos}Sca1^{pos}, CD117^{pos}CD34^{high} and CD117^{pos}CD34^{low} cells prospectively isolated from the two organs (algorithms used for calculations are described in Materials and Methods). *,**p<0.05-0.01 respect to wild-type.

Figure 2. Progenitor cells (CD117^{pos}CD34^{high} and CD117^{pos}CD34^{low}) prospectively isolated from the spleen of Gata1^{low} mice express great levels of Gata2
 Gata1 and Gata2 mRNA levels were normalized with those expressed by the corresponding wild-type marrow cells considered as 1. *, § p<0.05 with respect to values from wild-type and Gata1^{low} marrow cells, respectively.

Figure 3. Splenectomy does not restore the cytokine expression profile of marrow from Gata1^{low/+} females

A) Semiquantitative RT-PCR TGF-β, VEGF, Osteocalcin and PDGF-α of marrow from untreated wild-type mice and untreated or splenectomized Gata1^{low/+} females. B) Quantification of growth factor expression in marrow from wild-type (shaded-area) and untreated or splenectomized Gata1^{low/+} females. *(p<.01) with untreated values.

Figure 4. Removal of the spleen promotes development of extramedullary hematopoiesis in the liver of *Gata1^{low/+}* females

Hematoxylin-eosin and CD45-immunostaining of liver sections from splenectomized wild-type and untreated or splenectomized CD1- and DBA/2-*Gata1^{low/+}* females. Arrows indicate MKs dispersed in the parenchyma. Magnification 20× and 40× in panels and inserts.

Figure 5. Both wild-type and Gata1^{low} progenitor cells colonize the liver of Gata1^{low/+} females after splenectomy

A) Flow-cytometry of liver cells from untreated and splenectomized DBA/2-Gata1^{low/+} females. B) Erythroid colonies generated by liver cells. C) Gata1 levels in cells purified from untreated and splenectomized CD1-Gata1^{low/+} females. $p < .001$ with wild-type(*)- and Gata1^{low/+}(§)-marrow cells and untreated Gata1^{low/+}-liver cells(†), respectively.

Table 1

Total cell number and cloning efficiency of femur and spleen from wild type and Gata1^{low} mice.

Mice	Bone Marrow			Spleen		
	MNC/femur	No colonies/2×10 ⁴ MNC		LDC/spleen	No colonies/2×10 ⁴ LDC	
		Day 8	Day 15		Day 8	Day 15
Wild-Type	18.0±4.5×10 ⁶	163.0±13.5	183.1±8.5	4.6±0.7×10 ⁶	30±12	49.5±15.3
Gata1 ^{low}	5.2±0.9×10 ⁶ **	125.4±13.1	151.7±7.2 *	31.3±12.6×10 ⁶ **	112.0±11.9**	158.3±5.5**

Results represent the mean (±SD) of at least three independent experiments.

Values statistically different from wild-type controls are indicated by * (p<.05) and***(p<.001).

Frequency and cloning efficiency under conditions of limiting dilution of progenitor cells prospectively isolated from the marrow and spleen of wild-type and Gata1^{low} littermates.

Table II

Cell Phenotype	Bone Marrow			Spleen		
	Frequency (% of MNC)	No colonies/100 cells plated		Frequency (% of LDC)	No colonies/100 cells plated	
		Day 8	Day 15		Day 8	Day 15
Wild-Type						
CD117 ^{pos} /Sca1 ^{pos}	2.7±0.7	31.5±6.7	77.35±5.0	0.13±0.08	0.25±0.1	0.35±0.1
CD117 ^{pos} /CD34 ^{low}	5.2±1.9	0.85±0.4	14.2±3.8	0.10±0.02	0.0±0.0	0.0±0.0
CD117 ^{pos} /CD34 ^{high}	3.4±0.5	21.6±2.7	57.1±4.4	0.09±0.05	0.15±0.1	0.25±0.1
Gata1 ^{low}						
CD117 ^{pos} /Sca1 ^{pos}	4.1±2.1	3.9±0.9	7.7±2.9 [*]	1.24±0.37 [*]	38.8±12.5 [*]	53.0±10.6 [*]
CD117 ^{pos} /CD34 ^{low}	1.2±0.1	0.15±0.1 ^{**}	0.1±0.2 ^{**}	2.36±0.58 [*]	15.3±7.1 [*]	50.1±21.7 [*]
CD117 ^{pos} /CD34 ^{high}	3.0±1.0	14.1±2.9	20.7±3.4 ^{**}	2.18±0.06 ^{**}	68.1±15.2 [*]	100.2±31.3 [*]

Results represent the mean (±SD) of at least three independent experiments. Frequency are expressed as percent of total mononuclear cells (MNC) for the marrow and light-density cells (LDC) for the spleen.

Values statistically different are indicated by * (p<.05) and ** (p<.001).