DEVELOPMENT OF MYELOFIBROSIS IN MICE
GENETICALLY IMPAIRED FOR GATA-1 EXPRESSION (GATA-1\textsuperscript{low} mice)

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ABSTRACT

The phenotype induced by the GATA-1<sub>low</sub> (neoδHS) mutation is here further characterized by analyzing the hemopoietic system during the aging (up to 20 months) of a GATA-1<sub>low</sub> colony (135 mutants and 40 normal littermates). The mutants expressed normal hematocrit values (Hct=45.9±4.0) until 12 months but became anemic from 15 months on (Hct= 30.9±3.9, p<0.05). The appearance of anemia was associated with several markers of myelofibrosis such as the presence of 1) tear drop poikilocytes and progenitor cells in the blood, 2) collagen fibers in the marrow and in the spleen and 3) hemopoietic foci in the liver. Semiquantitative RT-PCR analysis showed that growth factors genes which have been implicated in the development of myelofibrosis (such as osteocalcin, TGF-β1, PDGF and VEGF) were all expressed in the marrow from the mutants at higher levels than in corresponding normal tissues. The GATA-1<sub>low</sub> mutants experienced a slow progression of the disease since the final exitus was not observed until at least 15 months with a probability of survival more favorable than that of W/W<sup>v</sup> mice concurrently kept in the animal facility (p<0.001, by Kaplan-Meier analysis). In conclusion, impaired GATA-1 expression may contribute to the development of myelofibrosis and the GATA-1<sub>low</sub> mutants may represent a suitable animal model for the human disease that may shed light into its pathogenesis.

INTRODUCTION

Idiopathic myelofibrosis (IM), also known as agnogenic myeloid metaplasia, is a myeloproliferative disorder of clonal origin of unknown etiopathology characterized by fibrotic degeneration of the marrow and extensive extramedullary hemopoiesis in the spleen...
and liver (1-3). The fact that human IM is often associated with alterations of megakaryocytopoiesis, such as thrombocytopenia and accumulation of megakaryocytes (Mk) in the tissues (1-3), has prompted the hypothesis that the disease may be the consequence of chronic activation of bone marrow stromal cells by growth factors, such as TGF-β1, PDGF, VEGF and FGF-A, released by Mk in the microenvironment (4, 5). In mice, the number of Mk in the marrow can be altered by manipulating the in vivo levels of thrombopoietin (TPO), the growth factor which specifically regulates megakaryocytopoiesis (6). Mice expressing high levels of human TPO, either because transgenic for the human gene (7) or because transplanted with bone marrow cells infected with a retrovirus containing human TPO (8), express high levels of marrow Mk and blood platelet counts. Both animal models rapidly develop IM (within 2-3 months) (9-11) further supporting the hypothesis for the involvement of Mk in the etiopathology of the human disease. However, in the 14 cases of human IM analyzed so far, the high number of Mk in the marrow was not associated with autocrine TPO production or with mutations in the gene for the TPO receptor (Mpl) (12). This observation indicates that, unlike the experimentally-induced murine disease, human IM might not be the consequence of alterations in the TPO/Mpl pathway leaving the cause for the increased numbers of Mk observed in these patients still to be ascertained.

GATA-1 is a transcription factor which exerts a well established role in erythroid and megakaryocytic cell differentiation (13). Definitive proof for its importance in hemopoiesis came from genetic manipulation of the expression of GATA-1 in mice. In fact, mice expressing ectopic levels of the gene show an increased rate of erythroid differentiation (14) while those lacking the gene by targeted mutation die in utero as the consequence of severe anemia (15, 16). Instrumental to determining the role of GATA-1 in megakaryocytopoiesis has been the generation of mutant mice with reduced GATA-1 expression (17, 18). These mice
are anemic but, unlike the deletion mutants, survive for longer periods during gestation up to the stage when alterations in megakaryocytopoiesis are detectable. One of these mutants, the mutant in which the first enhancer (DNA hypersensitive site I) has been replaced with a neo cassette and which lacks the distal GATA-1 promoter (neo\(\delta\)HS mouse or GATA-1\(^{\text{low}}\) mutant), is born both thrombocytopenic (18, 19) and anemic (20). The few animals that survive adulthood recover from their anemia at 3-4 weeks of age but remain thrombocytopenic all their life (21 and this manuscript) because of a block in the maturation of Mk into pro-platelets which results in the accumulation of Mk in the marrow and spleen (18, 21). The GATA-1\(^{\text{low}}\) adult mice can be, therefore, considered as a model to study the effects of chronic increases in the Mk population within tissues in the absence of alterations in the TPO/Mpl signalling pathways.

To further confirm the hypothesis that development of IM may result from accumulation of Mk in the hemopoietic tissues, it was investigated whether the GATA-1\(^{\text{low}}\) mice would develop the disease with age. Indeed, early IM traits (presence of reticulinic fibers in the marrow and spleen) could be detected since 10 months of age. However, a frank IM picture (anemia, occurrence of tear drop poikilocytes and progenitor cells in the blood, fibrotic degeneration of the marrow and spleen and active hemopoiesis in the liver) developed only after 15 months. These results indicate that Mk accumulation in the marrow, as consequence of impaired GATA-1 expression, may contribute to the pathogenesis of IM.

**MATERIALS AND METHODS**

**Mice.** The GATA-1\(^{\text{low}}\) colony (21) was bred according to standard genetic protocols at the animal facilities of the Istituto Superiore di Sanità starting from two genetically modified
animals (17) (one female and one male) kindly provided for this study by Dr. S. Orkin. The original neoδHS couple (of mixed C57BL/6-SV 129 background) generated a single male offspring that was crossed with a CD1 female (Charles River, Calco, Italy). Their female offspring was back-crossed with the father until a line of homozygous mutant mice (of mixed C57BL/6-SV 129/CD1 background) was obtained. All of the littermates were genotyped by PCR at birth for the absence of the deleted GATA-1 sequence. Littermates whose genotype contained the GATA-1 region were considered as wild type and used as negative controls. Other mice used in this study were represented by WBB6F1-W/Wv (W/Wv) female mice, purchased at 6 weeks of age from Jackson Laboratory (Bar Harbor, ME, USA). All the animals were housed for up to 2 years in the animal facilities of the Istituto Superiore di Sanità under humane conditions. All the experiments were performed with sex- and age-matched mice under protocols approved by the institutional animal care committee.

**Hematological blood parameters.** Blood was collected from the retro-orbital plexus into ethylen-diamino-tetracetic acid (EDTA)-coated microcapillary tubes (20-40 µL/sampling). Hematocrit (Hct), white cell and platelet counts were determined manually. Differential counts of white blood cells were performed on May-Grumwald-Giemsa stained slides, counting at least 100 cells.

**Immunohistochemical analysis.** Samples of liver, spleen and bone marrow were routinely fixed in 10% (v/v) phosphate-buffered formalin; bones were decalcified using standard techniques with acidified EDTA. Fixed tissues were paraffin embedded and 2.5-3 µM sections were prepared for hematoxilin-eosin staining or Gomori’s reticulum staining (22). For the search of extramedullary foci of hematopoiesis, liver, lung, and kidney samples were serially cut at 100 µM intervals and at least 25 sections/organ/mouse were examined and their sequence numbered. Immunohistochemical staining with a rat monoclonal antibody
directed against murine CD45 (Ly-5; Pharmingen, San Diego, CA, USA) was performed on numbered livers sections immediately preceeding or following those stained with hematoxilin-eosin in which foci of extramedullary hematopoiesis had been discovered. CD45-positive cells were identified with the avidin-biotinylated horseradish peroxidase system (ABC Staining system; Santa Cruz Biotechnology Inc, Santa Cruz, CA) using diaminobenzidine (DAB) as a substrate.

**Progenitor cell counts.** The frequency of progenitor cells (colony-forming unit, erythroid, CFU-E; burst-forming unit, erythroid, BFU-E; colony-forming unit, granulomacrophagic, CFU-GM; pure (p) or mixed (m) colony forming unit, megakaryocytic, CFU-Mkp and CFU-Mkm) was analyzed by culturing high density mononuclear cells (0.25-1.0x10^5 cells/plate) of selected organs (marrow, spleen, liver) from wild-type and mutant littermates under specific culture conditions. In the case of peripheral blood, 10 µL of whole blood were directly plated per mL of culture as described (23). Briefly, cultures were established in standard methylcellulose culture (0.9% w/v) in the presence of fetal bovine serum (FBS, 30% v/v, Hyclone, Logan, Utah, USA) and of a combination of recombinant growth factors including rat stem cell factor (SCF, 100 ng/mL Amgen, Thousand Oaks, CA; USA), mouse interleukin 3 (IL-3, 10 ng/mL; Genetic Institute, Cambridge, MA, USA) and either human erythropoietin (EPO, 2 U/mL; Boehringer Mannheim, Mannheim, Germany) for BFU-E growth or human granulocyte-colony stimulating factor (G-CSF, 50 ng/mL; Neupogen, Dompè Biotech, Milan, Italy) and murine granulocyte-macrophage colony stimulating factor (GM-CSF, 50 ng/mL; a gift of Dr. K. Kaushansky) for CFU-GM growth (24). The growth of CFU-E-derived colonies was stimulated with EPO alone (2 U/mL) (25). The growth of CFU-Mk-derived colonies was obtained in collagen-supplemented cultures (MegaCult™-C; Stem Cell Technologies, Vancouver, BC, Canada) stimulated with the
combination of recombinant cytokines including murine IL-3 (25 ng/mL), IL-6 (100 ng/mL; Sigma), IL-11 (50 ng/mL; Sigma) and human TPO (50 ng/mL; Amgen). The cultures were incubated at 37°C in a humidified incubator containing 5% CO2 in air and scored after 3 days (for CFU-E-derived colonies) or 7 days (for CFU-GM-, and BFU-E-derived colonies) of culture under an inverted microscope according to standard morphological criteria. Megakaryocytic colonies and isolated megakaryocytes were counted at day 7 on dehydrated and acetone-fixed collagen cultures stained for acetylcholinesterase (AChE) for 6 hrs, followed by methyl-green nuclear counter-staining, as described (26). Megakaryocytic colonies (≥ 5 cells) were considered pure or mixed when containing only AChE+ cells or both AChE+ and AChE− cells, respectively.

**Flow cytometry analysis and megakaryocyte purification.** Bone marrow cells were flushed from femurs in Ca++ and Mg++-free PBS supplemented with 1% (v/v) bovine serum albumin, 2 mM EDTA and 0.01% NaN₃, and labeled on ice with the erythroid specific phycoerythrin (PE)-conjugated Ter-119 (Ly-76) (Pharmingen, San Diego, CA) monoclonal antibody and the megakaryocytic-specific fluorescein isothiocyanate (FITC)-conjugated 4A5 antibody as described (27). Cells incubated with the corresponding irrelevant isotype-matched antibodies were used for gating non–specific fluorescence, and dead cells were excluded by propidium iodide staining (5 µg/mL) (Sigma St Louis, Mo, USA). Megakaryocytes were purified from the spleen as described (27). Briefly, monocellular spleen suspensions were prepared by cutting the spleens into small fragments in 5 mL of Ca++ and Mg++-free PBS containing 10% (v/v) FBS, EDTA 5mM, and theophylline 2 mM, and by passing the fragments through progressively smaller needles. Spleen light density mononuclear cells were isolated by centrifugation over Lympholyte-M (ρ=1.0875 g/mL; Cedarlane Lab, Hornby, Canada) at 800 x g for 20 min. at room temperature, washed twice in Ca++ and Mg++-free
PBS, and adherent cells were first removed by two cycle of adherence to plastic at 37°C for 1 hr. The non-adherent fraction was then enriched for megakaryocytic cells by immunomagnetic selection: briefly, cells were incubated with FITC-conjugated 4A5 rat monoclonal antibody (±1 µg/10^6 cells) for 30 min. On ice, washed twice with PBS containing 0.5% (w/v) bovine serum albumin and 2 mM EDTA and finally suspended in 80 µL of buffer and 20 µL of MACS microbeads conjugated with a monoclonal mouse anti-fluorescein antibody (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). The cell suspension was washed twice with labeling medium and loaded on a MS^+/RS^+ MiniMacs column placed in a MACS separator. 4A5-negative cells were recovered in the effluent fraction, while the 4A5-positive cells were flushed out of the column. The purity of the sorted fractions was evaluated by re-analysis of the fluorescent cells with the cytometer. Cell aliquots concurrently incubated with irrelevant isotype-matched antibodies were included in each analysis as a negative control.

**RNA isolation and semiquantitative RT-PCR analysis.** Total RNA from femurs was prepared by freezing the bones, carefully cleaned from the soft tissues, in liquid nitrogen and then grinding them with mostar and pestle directly into a commercial guanidine thiocyanate/phenol solution (Trizol, Gibco BRL, Paisley, UK). Total RNA (1 µg) was reverse transcribed at 42 °C for 30 min. in 20 µl of 10 mM Tris-HCl, pH 8.3, containing 5 mM MgCl₂, 1 U RNAse inhibitor, 2.5 U Moloney Murine Leukemia Virus reverse-transcriptase and 2.5 µM random hexamers (all from Perkin-Elmer, New Jersey, USA). Gene expression was analyzed by amplifying reverse-transcribed cDNA (2.5 µL) in the presence of the specific sense and anti-sense primers (100 nM each) described in Table I. The reaction was performed in 100 µL of 10 mM Tris-HCl, pH 8.3, containing MgCl₂ (2 mM), dNTP (200 µM each), 0.1 µCi of [α-32P]dCTP (specific activity 3,000 Ci/mmol, Amersham Italia, Cologno Monzese, England).
Italy) and 2 U AmpliTaq DNA polymerase. Primers specific for $\beta_2$-microglobulin (50 nM each) were added to each amplification after the first 10 cycles as a control for the amount of cDNA used in the reaction (33). PCR conditions were as follows: 60 sec at 95 °C, 60 sec at 60 °C, and 60 sec at 72 °C. All of the reactions were done using a GeneAmp 9700 Perkin-Elmer thermocycler and were analyzed in the linear range of amplification defined by preliminary experiments to be between 20-35 cycles for osteocalcin, TGF-β1, PDGF, FGF-A and VEGF and 20-30 cycles for $\beta_2$-microglobulin. Positive (RNA from adult marrow) and negative (mock cDNA) controls were included in each experiment. Aliquots (20 µL) were removed from the PCR mixture after amplification at different cycle numbers, and the amplified bands separated by electrophoresis on 4% polyacrylamide gel. Gels were dried using a Biorad apparatus (Hercules, CA, USA) and exposed to Hyperfilm-MP (Amersham) for 2 hours at -70 °C. All procedures were done according to standard protocols (34).

**Statistical analysis.** Statistical analysis of the data was performed by analysis of variance (Anova test) using Origin 3.5 software for Windows (Microcal Software Inc., Northampton, MA). The probability of mice to survive over time was calculated by the Kaplan-Meier method with censored data using the SigmaStat 2.0 program for Windows (SPSS, Erkrath, Germany)(23). Animals were censored from the survival curve when sacrificed for experimental purposes. The survival probability curves obtained for the GATA-1\textsuperscript{low} and the W/W\textsuperscript{v} mice were compared by the Mann-Whitney Rank sum test.

**RESULTS**

**Changes in the hematological parameters of GATA-1\textsuperscript{low} mice with age.**

The blood values in progressively older GATA-1\textsuperscript{low} and wilde-type littermates are summarized in Table II. The mice were divided into three age groups (4-8, 8-12 and 15-20
months of age) for convenience. In the normal littermates, small but not statistically significant variations in the blood cell counts occurred during the aging of the animals (Table II). The Hct (50.1±3.9 vs 45.3±3.1 at 4-8 vs 15-20 months of age, respectively) and the white cell counts (8.1±4.3 vs 6.7±2.3) slightly decreased with age while the platelet counts slightly increased (728±135 vs 1,060±189). In contrast, age-matched GATA-1\textsuperscript{low} animals underwent significant changes of hematological parameters with age. In particular, the number of circulating red cells decreased significantly at 15-20 months age (Hct 47.2±2.3 vs 30.0±3.9, p<0.05) (Table II) when tear drop poikilocytes (Figure 1) and erythrocytes with Howell-Jolly bodies and polychromatophilia were detectable in the blood. The frequency of Howell-Jolly body-positive erythrocytes increased from <0.1% at 12 months to 1-2% at 15 months of age. Although the total number of circulating white cells did not significantly change with age, the differential counts revealed changes in the relative cell distributions. There was a progressive increase in the percentages of neutrophils which became statistically significant at 15-20 months and a corresponding decrease in the percentages of lymphocytes. Furthermore immature myeloid cells were released in the circulation of the mutant mice starting from 8 months of age (Table II). No variations were observed during aging of the mutant mice in terms of platelet counts that remained significantly lower than normal all through life-span (Table II). The morphology of the circulating platelets remained also abnormally large and dysplastic (Figure 1).
Figure 1

In the blood of the wild type animals, the number of circulating progenitor cells did not significantly change with age remaining at the lower limit of detection in all the experimental groups (<10 CFC/10 µL, Table III). In contrast, the number of progenitor cells (of all types) circulating in the blood from the GATA-1<sup>low</sup> mice increased by 2-10 fold at 8-12 months and remained higher than normal in older mice (15-20 months). The bigger changes were observed for the CFU-E, detected at all ages in the blood from the mutants, that reached
a peak concentration as high as 114±13 CFU-E per 10 µL of blood at 8-12 months of age (Table III).

**Development of myelofibrosis in the GATA-1<sup>low</sup> mice with age.**

The myelofibrotic degeneration of the marrow and spleen of the GATA-1<sup>low</sup> mice with age is documented in Figure 2 and 3, respectively.

Hematoxilin-eosin examination of bone marrow sections from 1 month-old mutant shows the great prevalence of Mk in the marrow without any sign of alterations detectable in the intracellular space by Gomori’s staining (Figure 2A). As shown by FACS analysis (Figure 2D), the marrow of 1-month old GATA-1<sup>low</sup> animals contains ≥7-times more Mk cells than the marrow from their normal littermates (8.6 vs 1.3%, respectively).

Starting from 12 months of age, there was a progressive reduction of the space available in the marrow for hemopoietic cells due to the simultaneous increase of bone trabeculae and of intercellular matrix. The massive presence of newly-formed bone trabeculae in the femoral cavity led, by 18 months, to a decrease in the space available for hemopoietic cells (see the hematoxilin-eosin staining in the left panels of Figure 2A). In the oldest mice, the increased osteogenesis eventually occluded the femoral cavity as evident in the cross-sections of the femur diaphysis that show the thickness of the cortical region with the interconnecting bone trabeculae occluding the lumen (Figure 2B). The progressive accumulation of fibers in the intercellular space of the marrow from the GATA-1<sup>low</sup> mutants was also documented by Gomori’s silver staining (Figure 2A, right panels). The fibers changed from fine and diffuse reticulin fibers at 6-months of age to gross collagen fibers from 12 months on and ultimately filled almost completely the femoral cavity.
The reduction of the space available in the marrow for hemopoietic cells was associated with a reduction in total marrow cellularity (Figure 2C). While the cellularity of the marrow from the wild-type mice increased with age, that of the mutant animals, that was slightly but significantly lower than normal already in the 4-8 months age group, was further reduced by 2-fold in the 15-20 months-old group.

![Figure 2 A](Image)

**Figure 2 A**
Changes were specifically observed in the frequency of progenitor cells in the marrow from the GATA-1\textsuperscript{low} mice with age. In fact, while no consistent change was found to be associated with age in normal mice, a significant (2-3-fold) decrease was observed in the frequency of all types of progenitor cells in the GATA-1\textsuperscript{low} mice with age (Table III) which, if adjusted for the reduction in total marrow cellularity, correspond to a 4-fold reduction in total number of progenitor cells per femur.

**Figure 2 B,C**
Figure 2D

The accumulation of reticulin fibers with age was also documented by Gomori’s staining in the GATA-1\textsuperscript{low} spleen (Figure 3A). Additional proof for the fibrotic degeneration of this organ came from the fact that the size of the already massive spleen of the mutant mice further increased with age (Figure 3B) but the total spleen cellularity - although remaining significantly higher than that of the spleen from the normal mice - decreased with age (Figure 3C). Therefore, the increase in spleen weight observed in the GATA-1\textsuperscript{low} mice at 15-20 months of age was due to accumulation of collagen fibers (Figure 3). The total number of cells in the spleen was found to be reduced for the first time at 8-12 months of age when the highest number of CFU-E was present in the circulation (Figure 3 and Table III).

No changes were observed in the frequency of progenitor cells in the spleen of the wild-type littermates with age (Table III) while, in the spleen of the GATA-1\textsuperscript{low} mice, only the frequency of the myeloid progenitors (CFU-GM) remained constant. Those of the
erythroid progenitors (BFU-E, CFU-E), although remaining higher than in normal animals, decreased 2-3-fold over time. On the other hand, the frequency of megakaryocytic progenitors (CFU-Mkp and CFU-Mkm), which had been already higher than normal in the 4-8 months group (21), further increased up to 62±6 – 80±15 per 10^5 cells at 15-20 months. However, because of the overall decrease in the spleen cellularity (Figure 3B), the total number of progenitor cells of all types in the spleen actually decreased with age.

Figure 3
The liver as site of extramedullary hematopoiesis in the old Gata-1^low mice.
As expected, the hematoxylin-eosin staining of fetal liver shows a hemopoietic organ with abundant Mk in its parenchyma (Figure 4A) but no sign of active hemopoiesis was detectable in the liver of the GATA-1<sup>low</sup> mice after birth (1-12 months of age) (Figure 4A and results not shown). However, by 18 months, foci of hematoipoiesis were recognized again within the liver parenchyma of the mutant animals as confirmed by CD45-immuno-staining of the consecutive liver section (insert in the right panel of Figure 4A). These foci were never found in liver sections obtained from age-matched wild-type mice (results not shown).

The progressive involvement of the liver as a site of active hemopoiesis in the old GATA-1<sup>low</sup> mice was further confirmed by the analysis of progenitor cells in this organ (Figure 4B). Until 4-8 months of age, progenitor cells were detectable in the liver of the mutant mice at frequencies very low and similar to those found in the liver of normal mice of all age (35 and results not shown). However, starting from 8-12 months, significant numbers of progenitor cells were detected in the liver of the mutants (0.35% of all the cells, Figure 4B). The frequency of these cells further increased (0.8% of all the liver cells) in the 15-20 months old mice group. CFU-E were among the most frequent progenitor cells in the liver of old animals.

Foci of hematoipoiesis were not detectable in sections of lungs and kidneys of the mutant or normal littermates at any age (results not shown).
Figure 4

RT-PCR analysis of the expression of TGF-β, PDGF, FGF-A, VEGF and osteocalcin by the femur and by isolated Mk from GATA-1<sup>low</sup> and wild-type mice.

Using RNA prepared from the entire femur of wild-type animals, cDNA fragments specific for TGF-β, PDGF, and VEGF were amplified at detectable levels only at the highest PCR cycles (Figure 5A). On the other end, cDNA fragments for osteocalcin were never amplified even when the reaction was extended up to 40 cycles (Figure 5A and results not shown). These results confirm that these genes are expressed at low levels by preparations of unfractionated bone and marrow cells from adult wild-type mice. In contrast, with RNA prepared from GATA-1<sup>low</sup> femurs, cDNA fragments for osteocalcin were readily amplified.
and amplification of cDNA fragments for TGF-β, PDGF, and VEGF was detectable even after few cycles (Figure 5A). These results indicate that all these genes are expressed at higher levels in the femur of the mutant mice than in that of the normal ones. Since fragments for FGF-A were barely amplified at the highest PCR cycles using RNA from both normal and GATA-1\textsuperscript{low} femurs, it is not possible to draw any conclusion on its relative levels of expression in the GATA-1\textsuperscript{low} vs the wild-type femur.

**Figure 5**
To clarify whether the high expression of growth factors known to be produced by Mk in the GATA-1\textsuperscript{low} femur was due only to its increased Mk content (18, 21 and Figure 2A) or also to altered gene expression by the mutated cells, Mk were purified (> 90%, see the FACS dot plots in Figure 5B) with standard immuno-affinity techniques from the spleen of normal and mutant littermates and the RNA extracted from them used to analyze the levels of expression of TGF-β, PDGF, FGF-A and VEGF by semi-quantitative RT-PCR (Figure 5A, right panels). \(\beta_2\)-microglobulin was amplified with similar kinetics using RNA extracted from both GATA-1\textsuperscript{low} and normal Mk. cDNA fragments for TGF-β1, PDGF and VEGF were all amplified less efficiently with RNA from GATA-1\textsuperscript{low} Mk than from the corresponding wild-type cells. Once again, amplification of the cDNA for FGF-A was observed only at the highest PCR cycles, making impossible to draw any conclusions on its relative levels of expression in normal and mutant cells.

**Survival of the GATA-1\textsuperscript{low} mice with age.**

The Kaplan-Meier analysis of the probability of survival of the GATA-1\textsuperscript{low} mice with age is shown in Figure 6. Mice sacrificed for experimental purposes were censored from the analysis. It is difficult to calculate the percent of survival of the GATA-1\textsuperscript{low} mice at birth because only 30% of the pregnancies induced (as established by the presence of the vaginal plug 24-48 hrs after mating) resulted in viable pups. Once the pups were born, however, they were highly vital since only 9, out of 178 GATA-1\textsuperscript{low} mutants born alive, died within the first 15 days of life (Figure 6). Very few (6) mice died from 15-days to 13-month of age. The majority of the deaths (10 over a total of 25 natural death recorded) occurred between 14\textsuperscript{th} and 20\textsuperscript{th} months of age with only 9 animals alive after 20-months. Since normal littermates in numbers sufficient for survival studies were not available, the probability for survival of the
mutant mice was compared with that of mice harboring the W/W\textsuperscript{v} mutation, a mild genetic defect that, as the GATA-1\textsuperscript{low} mutation, causes anemia and mast cell deficiency (36 and manuscript in preparation). The GATA-1\textsuperscript{low} mice had a probability of survival over time significantly higher than that of the W/W\textsuperscript{v} mice (50\% of mice dead at 20 vs 14 months of age, respectively, p<0.001 by Rank Sum Test).

![Kaplan-Meier Analysis of Survival](image)

**Figure 6**

**DISCUSSION**

This paper describes that mice harboring the GATA-1\textsuperscript{low} mutation, unlike their normal littermates, develop at 15 months of age an IM-like disease characterized by the presence of 1) anemia (Table II), tear drop poikilocytes (Figure 1) and progenitor cells (Table III) in the blood, 2) collagen fibers in the marrow (Figure 2) and in the spleen (Figure 3) and 4)
hemopoietic foci in the liver (Figure 4). The phenotype was expressed by both hemizygotes males and homozygote females with an apparent penetrance of 100%. These results suggest that the development of the disease in these mice may be the direct consequence of the GATA-1\textsuperscript{low} mutation.

Although the GATA-1 gene has been identified since 1989 as a pivotal gene in erythroid differentiation (37), genetic GATA-1 alterations have not been found associated with human pathologies for long time. In fact, the extreme severity of the phenotype expressed by GATA-1 mutatations experimentally-induced in mice had suggested that alterations of in this gene possibly occurring in humans would go undetected because of their lethality. Most recently, GATA-1 mutations altering either the FOG-binding or the DNA-binding domain of the protein have been described to be associated with human inherited diseases which are characterized by alterations in both the megakaryocytic and the erythroid differentiation pathway, such as dyserythropoietic anemia (38) and X-linked thrombocytopenia (39, 40) and thalassemia (41).

IM is a clonal disease that can be experimentally transmitted in mice (10) and cured in mice (9) and men (42) by bone marrow transplantation and which is associated with Mk accumulation in the marrow and thrombocytopenia as well as anemia in the blood. These facts suggest that IM may be caused by gene defects that impair the stem cell ability to differentiate along both the Mk and the erythroid pathway. TPO and its receptor, Mpl, the two genes most likely to cause defective Mk differentiation (6), have not been found altered in 14 IM patients carefully analyzed for this purpose (12). Therefore the search for the genetic defect involved in the development of IM is still open. On the basis of 1) the fact that IM, as the human genetic diseases associated with GATA-1 mutations described so far (38 – 41), presents defective differentiation both of the megakaryocytic and the erythroid pathway, 2)
the slightly higher incidence of IM in males that in females [0.73 and 0.40 new diagnoses every year per 100,000 males and females, respectively (43) which indicate that its cause may be partially X-linked] and 3) the phenotype of the GATA-1^{low} mice described in this paper, we suggest that, at least in some of the cases, human IM may result from altered regulation of GATA-1 gene expression and/or from other mutations in the GATA-1 functional pathway. Current experiments are underway to verify this hypothesis.

Several animal models have been developed in the recent years for the study of IM. The major difference found between the IM traits expressed by the GATA-1^{low} mice and those expressed by other models for the disease is represented by the rate of the progression toward its final exitus that was slow in the GATA-1^{low} mice but very rapid in the other models. In fact, wild-type mice transplanted with bone marrow cells infected with a TPO-containing retrovirus develop a fatal IM syndrome within 2.5-3 months and the final exitus was recorded approximately seven months from the transplant (9, 10). Furthermore, mice transgenic for the TPO gene survive only shortly after birth (7). In contrast, the GATA-1^{low} mice manifested their first IM symptoms, i.e., reticulin fibers in the marrow and in the spleen (Figure 2 and 3) and alterations in the frequencies of progenitor cells in the various organs (Table III and Figure 4), at 8-12 months of age while a clear picture of IM was not observed until 15 months (Figure 1, 2 and 3). However, very few of the GATA-1^{low} mice survived more than 2-4 months after the first sign of the disease had became manifested (Figure 6). The slow appearance of the first IM traits followed by a rapid final exitus has strong analogies with the human disease. In fact, human IM is usually diagnosed in the second half of life but, when the first sign of the disease became manifested, the final exitus occurs within 4.5 years (3). In contrast with human IM that may evolve into acute leukemia, leukemic cells and growth factor independent hemopoietic progenitors were never detect in tissues from GATA-
1^\text{low} \text{ mice (ref. 21 and data not shown). However the transformation of an abnormally proliferating stem/progenitor cell pool represents the final step of disease progression which may not have the time to occur within the two year life span of a mouse. Serial transplantation in congenic mice will be required to clarify whether the GATA-1^\text{low} \text{ stem cell pool will undergo transformation with time.}

The GATA-1^\text{low} \text{ mice express in their marrow and spleen 10-times more Mk cells than those present in normal tissues already at 1 month of age (17, 18, 20 and Figure 2) but manifest the first signs of fibrosis only 11 months later. In contrast, the TPO over-producing mice develop the disease only 2-3 months after their marrow Mk content had increased by 2-fold (6-9). To clarify this difference, we compared by semi-quantitative RT-PCR the levels of expression of genes encoding growth factors associated with the development of IM by the femur and by Mk cells of GATA-1^\text{low} \text{ mice and of their normal littermates. Osteocalcin and TGF-\beta1, PDGF, VEGF were all found to be expressed at higher levels in the femur of the GATA-1^\text{low} \text{ mice than in that of the normal littermates but the levels of expression of the Mk specific genes were not as high as expected on the basis of the absolute Mk content of their marrow (Figure 5). Indeed, Mk purified from the spleen of the mutants expressed lower levels of TGF-\beta1, PDGF and VEGF as compared to the corresponding cells from normal spleens (Figure 5) indicating that the GATA-1^\text{low} \text{ mutations not only impairs the capacity of Mk to differentiate into pro-platelets (17, 18) but also their capacity to express certain growth factors. The presence of so many but functionally impaired Mk in the tissue of these mice may, then, underlay their slow progression toward IM. Alternatively, causes other than accumulation of Mk cells in the marrow may contribute to the development of IM in the GATA-1^\text{low} \text{ animals. In fact, increased number of Mk in the marrow is a necessary but not sufficient condition for the development of the disease in mice. Wild-type (9) and SCID (10)
mic e that over-express the human TPO by retrovirus-mediated gene transduction develop IM but NOD-SCID mice infected with the same virus (10) and a transgenic line in which the TPO over-expression was targeted to the liver (6) do not. Since all of these mice express similarly high levels of TPO in the serum and of Mk in the marrow, genetic components different from those that regulate Mk cells and that are probably linked to those involved in the regulation of the monocyte-macrophages may represent important co-factors in the development of the disease in mice.

In conclusion, the GATA-1\textsuperscript{low} mutants represent a new animal model for human IM and the observed association between a poor capacity of the mutated Mk cells to produce growth factors with a low morbidity of the disease may provide insights into possible treatment strategies for humans.
ACKNOWLEDGEMENTS

The authors gratefully thank Dr. Stuart Orkin for providing the GATA-1<sup>low</sup> mouse model, for continuous support and for critical reading of the manuscript and Prof. Giuliano D’Agnolo for encouragement. The authors also thank Luca Marsilli for assistance with the breeding program, Andrea Beni for help in cell cultures, and Eugenio La Torre for technical assistance.
REFERENCES


LEGEND TO FIGURES

Figure 1. Presence of tear-drop poikilocytes in the blood of 18 months old GATA-1<sup>low</sup> mice. May-Grünwald-Giemsa staining of representative blood smears obtained from GATA-1<sup>low</sup> mice at the age of 6 (upper panel, as negative control) and 18 (lower panel) months of age. The void arrows indicate the dismorphic platelets characteristic of the blood of the Gata-1<sup>low</sup> mice (18, 21). The solid arrows indicate the tear drop poikilocytes which circulate in the blood from these mutants since 15 months of age. Similar results were observed in blood smears from at least 4-6 mice. In particular, a total of 18 mice were analyzed in the 12-18 months old age-group. (Original magnification 100x).

Figure 2. Development of myelofibrosis in the bone marrow of the GATA-1<sup>low</sup> mice.

A. Representative hematoxylin-eosin (left column) and Gomori’s silver (right column) staining of longitudinal sections of femurs from progressively older (1 to 18 months) GATA-1<sup>low</sup> mice. Representative stainings of the corresponding sections from an 18 months old wild-type mouse are also shown for comparison (bottom panels). (Original magnification: left column, 20x in all cases, except for the hematoxylin-eosin staining at 1 month of age that is 40x to evidentiate the massive presence of Mk shown by the arrows; right column, 40x). Similar results were observed in at least 4 mice per age group. In particular, a total of 12 mice were analyzed for the 12-18 months old age-group.

B. Hematoxylin-eosin staining of representative transversal sections of femur diaphysis from 18-months old wild-type (left) and GATA-1<sup>low</sup> (right) mice.
Similar results were observed in at least 3 mice per experimental group.

C. Total number of mononuclear cells (TCN) in the femur of progressively older wild-type (white bars) and GATA-1<sub>low</sub> (grey bars) mice. The mice were arbitrarily divided into the same age groups used in Table II and III. The results are presented as the mean (± SD) of four to eight separate determinations per age group for a total of 12 wild-type and 22 GATA-1<sub>low</sub> mice analyzed. **, p<0.01 with respect to the values observed in the age matched wild-type group.

D. Flow cytometry analysis of the expression of TER-119 and 4A5 in bone marrow cells harvested from wild-type (on the right) and GATA-1<sub>low</sub> (on the left) animals at 1 month of age. The bone marrow from the GATA-1<sub>low</sub> animals contain 6-7 fold more 4A5 cells than the marrow from the wild-type animals.

**Figure 3. Development of myelofibrosis in the spleen of GATA-1<sub>low</sub> mice.**

A. Gomori’s reticulum staining of representative spleen sections from GATA-1<sub>low</sub> mice at 1, 12 and 18 months of age that show the progressive appearance of fibers with age. The corresponding staining of a representative section from one 18-months old wild-type mouse is also presented for comparison (original magnification 20x). Similar results were obtained in at least 3 independent experiments per age group. In particular, a total of 12 mice were analyzed for the 12-18 months old age-group.

B. Weight (in mg) of the spleen from wild-type (white bars) and GATA-1<sub>low</sub> (grey bars) littermates arbitrarily divided into progressively older age groups (the same as used in Table II). The results are presented as the mean (±SD) of at least 4
independent determination per experimental group for a total of 12 wild-type and 18 mutant mice analyzed. **, indicates values in the GATA-1\textsuperscript{low} age groups which are significantly (p<0.01) different from that found in normal controls of the same age.

C. Total cellularity (TNC) of the spleen from wild-type (white bars) and GATA-1\textsuperscript{low} (grey bars) littermates arbitrarily divided into progressively older age groups. The same animals as those presented in panel B. See legend to panel B for further details.

Figure 4. The liver as an extramedullary site of hematopoiesis in old GATA-1\textsuperscript{low} mice.

A. Hematoxilin-eosin staining of liver sections from a 15-days post-coitum (pc) GATA-1\textsuperscript{low} fetus (left panel) or from adult GATA-1\textsuperscript{low} mice at 12 (middle panel)- or 18 (right panel)-months of age. The insert in the left panel presents the CD-45 immuno-histochemical staining of the liver section immediately following that stained with hematoxilin-eosin. A total of 6 mice were independently analyzed in the 12-18 months old age-group. (Original magnification: 20x for the left panel, 10x for the middle and right panel and 40x for the insert)

B. Frequency of hemopoietic colonies (BFU-E, CFU-E, CFU-GM and CFU-Mk, as indicated) per 10\textsuperscript{5} liver cells from GATA-1\textsuperscript{low} mice. The animals were arbitrarily divided into the same age groups used in Table II. The results are presented as the mean (±SD) of 3 independent experiments for a total of at least 4 mice analyzed per experimental group.
Figure 5

A. RT-PCR expression of Mk (TGF-β1, PDGF, FGF-A and VEGF)- and osteoblast (osteocalcin)-derived growth factors in the whole femurs (left panel) and in Mk cells (right panel) isolated from the spleens of wild type and Gata-1^{low} mice. β₂-microglobulin was amplified as positive control while mock-extracted RNA was amplified as negative control (not shown). Each product was amplified for increasing number of cycles within the linear portion of the respective amplification kinetics (21, 24 and 27 cycles for β₂-microglobulin and 25, 30 and 35 cycles for all the other genes), as indicated by the triangle on the top of the panel. Similar results were obtained in two additional experiments.

B. Flow cytometry re-analysis for purity of the Mk cells isolated by immunomagnetic selection from the spleens of wild-type (left) and GATA-1^{low} (right) mice as described in Materials and Methods and used for the RT-PCR shown in A.

Figure 6. Kaplan-Meier analysis of the probability of survival with age for GATA-1^{low} (squares) and W/W^{v} (triangles) mice. For the GATA-1^{low} mice, the probability of survival was calculated starting with 178 mutants born in the facility over a period of three years. Because the birth of the animals was not synchronous and animals were sacrificed for experimental purposes, the number of mice used to calculate the survival curve changes with the age and includes a total of 147 animals up to 13 months and 31 animals from 14 months on. In the case of the W/W^{v} mice, the survival probability was calculated on a cohort of 204 mice, 50 of which were censored because sacrificed for other experiments. The two survival curves are statistically different (p<0.001) by Mann-Whitney Rank sum test.
Table I. Oligonucleotide primers used for the reverse-transcriptase polymerase chain reaction analysis presented in the study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>5' primer</th>
<th>3' primer</th>
<th>Product</th>
<th>Ref</th>
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</thead>
<tbody>
<tr>
<td>β2-microglobulin</td>
<td>TGCTATCCAGAAAACCCCTC</td>
<td>GGACGTCTCAATTCGTACTG</td>
<td>258 bp</td>
<td>28</td>
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<tr>
<td>TGF-β1</td>
<td>GCTGCCTTCAGAGATTAAA</td>
<td>TTGCTGTACTGATGGTCAG</td>
<td>552 bp</td>
<td>29</td>
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<tr>
<td>PDGF-A</td>
<td>GCTCAGGTGAGGTTAGAGG</td>
<td>CACGGAGGAGAACAAGAC</td>
<td>210 bp</td>
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<tr>
<td>VEGF</td>
<td>TTACTGCTGTACCTCCACC</td>
<td>ACAGGACGCTTTGAAGATG</td>
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<tr>
<td>FGF-A</td>
<td>TCAGCTCTTAGCAGACA</td>
<td>GCCCACTTCAGGACCCCAAG</td>
<td>398 bp</td>
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<td>Osteocalcin</td>
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<td>GGTCCCTAAATAGTGATACCG</td>
<td>795 bp</td>
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Table II. Hematological parameters of age-matched wild-type and GATA-1<sup>low</sup> mice.

<table>
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<tr>
<th>Age (months)</th>
<th>Wild-Type</th>
<th>GATA-1&lt;sup&gt;low&lt;/sup&gt;</th>
<th>Wild-Type</th>
<th>GATA-1&lt;sup&gt;low&lt;/sup&gt;</th>
<th>Wild-Type</th>
<th>GATA-1&lt;sup&gt;low&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>4 - 8</td>
<td>Hct (%)</td>
<td>50.1±3.9</td>
<td>47.2±2.3</td>
<td>48.5±3.4</td>
<td>45.9±4.0</td>
<td>45.3±3.1</td>
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<tr>
<td></td>
<td>Platelet</td>
<td>728.0±135.0</td>
<td>118.0±45.0**</td>
<td>1,300.0±270.0</td>
<td>113.0±35.0**</td>
<td>1,060±189.0</td>
</tr>
<tr>
<td></td>
<td>count (x10&lt;sup&gt;9&lt;/sup&gt;/L)</td>
<td>8.1±4.3</td>
<td>4.5±2.8</td>
<td>5.0±1.6</td>
<td>8.0±2.5</td>
<td>6.7±2.3</td>
</tr>
<tr>
<td></td>
<td>White cell</td>
<td>18.0±5.0</td>
<td>25.0±5.0</td>
<td>32.0±12.0</td>
<td>54.0±18.0</td>
<td>39.0±9.0</td>
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<tr>
<td></td>
<td>count (x10&lt;sup&gt;9&lt;/sup&gt;/L)</td>
<td>74.0±12.0</td>
<td>63.0±13.0</td>
<td>60.0±9.0</td>
<td>36.0±11.0</td>
<td>52.0±7.0</td>
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<tr>
<td></td>
<td>Lymphocytes</td>
<td>5.0±3.0</td>
<td>10.0±5.0</td>
<td>4.0±2.0</td>
<td>6.0±3.0</td>
<td>5.0±2.0</td>
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<tr>
<td></td>
<td>Monocytes</td>
<td>3.0±2.0</td>
<td>2.0±1.0</td>
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<tr>
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<td>Eosinophils</td>
<td>0</td>
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<td>0</td>
<td>1.0±1.0</td>
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<tr>
<td></td>
<td>Basophils</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.0±1.0 **</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Immature myeloid cells</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.0±1.0 **</td>
<td>0</td>
</tr>
<tr>
<td>N. of mice</td>
<td>11</td>
<td>25</td>
<td>12</td>
<td>12</td>
<td>3</td>
<td>8</td>
</tr>
</tbody>
</table>

*, p = <.05; **, p = <.001. Immature myeloid cells were represented by metamyelocytes, myelocytes, and occasional promyelocytes.
Table III. Frequencies of progenitor cells in the tissues from progressively older wild-type (WT) and GATA-1<sup>low</sup> (KD) Littermates

<table>
<thead>
<tr>
<th>AGE</th>
<th>4-8 months</th>
<th>8-12 months</th>
<th>15-20 months</th>
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<tr>
<td></td>
<td>WT</td>
<td>KD</td>
<td>WT</td>
</tr>
<tr>
<td>SPLEEN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFU-E</td>
<td>28±6</td>
<td>233±28**</td>
<td>34±9</td>
</tr>
<tr>
<td>BFU-E</td>
<td>15±3</td>
<td>110±21**</td>
<td>21±6</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>40±5</td>
<td>56±16</td>
<td>36±11</td>
</tr>
<tr>
<td>CFU-Mkp</td>
<td>5±1</td>
<td>6±2</td>
<td>12±3</td>
</tr>
<tr>
<td>CFU-Mkm</td>
<td>2±1</td>
<td>25±5*</td>
<td>3±1</td>
</tr>
<tr>
<td>Single Mk cells</td>
<td>7±4</td>
<td>89±14*</td>
<td>11±6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>BONE MARROW</td>
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<tr>
<td>CFU-E</td>
<td>87±20</td>
<td>381±45**</td>
<td>59±19</td>
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<tr>
<td>BFU-E</td>
<td>149±33</td>
<td>165±33</td>
<td>90±12</td>
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<tr>
<td>CFU-GM</td>
<td>165±25</td>
<td>145±17</td>
<td>187±34</td>
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<tr>
<td>CFU-Mkp</td>
<td>22±4</td>
<td>4±1*</td>
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<td>CFU-Mkm</td>
<td>10±3</td>
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<td>7±3</td>
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<tr>
<td>Single Mk cells</td>
<td>17±9</td>
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<td>PERIPHERAL BLOOD</td>
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<td>CFU-E</td>
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<tr>
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<tr>
<td>CFU-Mkm</td>
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</tr>
<tr>
<td>Single Mk cells</td>
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<td>0</td>
</tr>
</tbody>
</table>

The results are presented as number of colonies per 10<sup>5</sup> mononuclear cells (either from marrow or spleen) and per 10µL of peripheral Blood. Mean (±SD) of at least 3 separate experiments performed in duplicate for a total of 3-5 mutants and 3 normal littermates analyzed per each age group. Values in the GATA-1<sup>low</sup> mice statistically different from those observed in the corresponding wild-type animals are indicated by * (p<0.05) and ** (p>0.01). Individual progenitor cells are indicates as: colony-forming unit, erythroid, CFU-E; burst-forming unit, erythroid, BFU-E; colony-forming unit, granulo-macrophagic, CFU-GM; pure (p) or mixed (m) colony forming unit, megakaryocytic, CFU-Mkp and CFU-Mkm.
DEVELOPMENT OF MYELOFIBROSIS IN MICE GENETICALLY IMPAIRED FOR GATA-1 EXPRESSION (GATA-1 low mice)

Alessandro M Vannucchi, Lucia Bianchi, Cristina Cellai, Francesco Paoletti, Rosa A Rana, Rodolfo Lorenzini, Giovanni Migliaccio and Anna R Migliaccio