The hypomorphic Gata1<sup>low</sup> mutation alters the proliferation/differentiation potential of the common megakaryocytic-erythroid progenitor

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Recent evidence suggests that mutations in the Gata1 gene may alter the proliferation/differentiation potential of hemopoietic progenitors. By single-cell cloning and sequential replating experiments of prospectively isolated progenitor cells, we demonstrate here that the hypomorphic Gata1<sup>low</sup> mutation increases the proliferation potential of a unique class of progenitor cells, similar in phenotype to adult common erythroid/megakaryocytic progenitors (MEPs), but with the “unique” capacity to generate erythroblasts, megakaryocytes, and mast cells in vitro. Conversely, progenitor cells phenotypically similar to mast cell progenitors (MCPs) are not detectable in the marrow from these mutants. At the single-cell level, about 11% of Gata1<sup>low</sup> progenitor cells, including MEPs, generate cells that will continue to proliferate in cultures for up to 4 months. In agreement with these results, trilineage (erythroid, megakaryocytic, and mastocytic) cell lines are consistently isolated from bone marrow and spleen cells of Gata1<sup>low</sup> mice. These results confirm the crucial role played by Gata1 in hematopoietic commitment and identify, as a new target for the Gata1 action, the restriction point at which common myeloid progenitors become either MEPs or MCPs. (Blood. 2007;109:1460-1471)

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Introduction

Among the GATA family of transcription factors,1 Gata1 exerts a specific role in the control of erythroid,2 megakaryocytic,3,4 eosinophil,3 and mast<sup>6</sup> cell differentiation. Genetic alterations of this gene, however, are not only associated with X-linked inherited erythroid or megakaryocytic disorders, but are also found in acquired myeloproliferative disorders. Each mutation is associated with a specific abnormality; point mutations that abrogate the ability of the amino-terminal zinc finger domain of the protein to bind either DNA or Fog1, a partner of Gata1, are found in inherited disorders.2,7 On the other hand, frame shift and splice mutations encoding GATA1s, a protein lacking the amino-terminal domain, are associated not only with impaired inherited erythropoiesis,11,12 but are also found in patients with megakaryocytic leukemia in Down syndrome,13,14 in newborns with transient myeloproliferative syndromes,15 and in one adult patient with megakaryocytic leukemia.16 These observations suggest that, in addition to its effect on terminal differentiation, Gata1 might control the biologic properties of hematopoietic progenitor cells, predisposing them to accumulate secondary mutations in a multistep leukemogenic process. However, direct proof for a possible function of Gata1 in progenitor cells has not been provided as yet.

We had previously described that hematopoietic tissues from mice carrying the hypomorphic Gata1<sup>low</sup> mutation contain high numbers (~10%) of “unique” progenitor cells that generate colonies composed of erythroblasts, megakaryocytes, and mast cells.6 Predicted by the stochastic model of hematopoietic commitment,17 such a trilineage progenitor has not been isolated prospectively as yet from the marrow of normal mice. In fact, antigenic profiling has prospectively divided normal murine progenitors into myeloid- and mast cell-restricted. The myeloid-restricted ones are further divided into granulomonocytic progenitors (GMPs), megakaryocytic-erythroid progenitors (MEPs), and common myeloid progenitors (CMPs).18 GMPs correspond to cells previously defined, by functional clonogenesis, as colony-forming cells that generate in 7 days granulocytic, monomacrophagic and granulomonocytic colonies (CFU-Gs, CFU-Ms, and CFU-GMs). MEPs, on the other hand, include cells once functionally defined as those that generate megakaryocytic or erythroid colonies either in 3 days (CFU-MK<sub>S<sub>day</sub>3</sub> and CFU-Es) or 7 days (CFU-MK<sub>S<sub>day</sub>7</sub> and BFU-Es). CMPs were functionally defined as multilineage progenitor cells, that is, those that generate colonies of multiple lineages after 12 to 15 days either in vitro (CFU-mix) or in vivo (spleen colony-forming cells, CFU-S<sub>S<sub>day</sub>12</sub>). Mast cells are localized in extramedullary sites where they engage themselves in the process of allergic response and in the immune reaction against parasites.19,20 As all the other hematopoietic cells, they derive from progenitor cells present in the marrow (and in the spleen) of the mouse. The marrow mast cell-restricted progenitor cells (MCPs) are characterized by the phenotype Lin<sup>-</sup>c-Kit<sup>-</sup>Sca-1<sup>-</sup>-Ly6<sup>c</sup><sup>-</sup>-FceRI<sup>-</sup>-CD27<sup>+</sup>-β7<sup>+</sup>+T1<sup>-</sup>/ST2<sup>-</sup>.21 MCPs normally complete their...
maturation in extramedullary sites through a pathway that involves first up-regulation of c-Kit and T1/ST2 expression (c-Kit high/T1/ST2 high) and, then, induction of the expression of tissue-restricted mast cell proteases (MMCPs) and of the receptor that binds with high affinity the Fc portion of IgE (FcεRI). In contrast with most hemopoietic lineages, mastocytic cells retain extensive proliferative activity until completely mature. MCPs give rise in vitro to mast cell colonies within 7 days of culture. On the other hand, CMPs generate MCPs, in addition to GMPs and MEPs, both in vivo and in vitro.21

The aim of this study was to clarify the role of Gata1 in hematopoietic commitment by identifying the antigenic profile and proliferation potential of the Gata1low progenitors giving rise to trilineage colonies. First, we compared the number and function of mast cells generated in bone marrow-derived mast cell cultures (BMMCs) seeded with marrow from Gata1low and wild-type (positive controls) mice. Heterozygous W/Wmice (ie, expressing the W, truncated, and Ws, kinase defective, form of c-Kit27), that do not express mast cells in vivo but whose marrow generates defective mast cells in vitro, were used as negative controls. Next, we examined the frequency of CMPs, MEPEs, and MCPs in marrow and spleen from wild-type and Gata1low littersmates. The different populations were also isolated and their differentiation and proliferation potential characterized under conditions of limiting dilution followed by single cell reconstituting. Our results confirm that mast cell differentiation is defective in Gata1low mice (decreased differentiation and increased proliferation). The defect includes the ability to generate, with high frequency, factor-dependent trilineage cell lines. In Gata1low mice, the frequency of cells with the antigenic profile of CMPs, MEPEs, and GMPs was normal in marrow and markedly increased in spleen, whereas those with the MCP profile were not detectable. However, mutant cells isolated according to the MEP phenotype, had the “unique” property to generate mast cells and their precursors in 7 days of culture, in addition to erythroblasts and megakaryocytes. Furthermore, the progeny of about 10% of mutant MEPEs could be propagated in culture, as single cells, with 95% efficiency, for up to 4 months. In comparison, the progeny of wild-type MEPEs became extinguished in 7 to 14 days. These results indicate that cells with the phenotype corresponding to myeloid, but not those corresponding to mastocytic, progenitors are detectable in tissues from Gata1low mice. However, in these mice, the mast cell-generating activity is abnormally acquired by MEPEs, which, therefore, are antigenically, but not functionally, equivalent to wild-type MEPEs. These results indicate that the Gata1low mutation also targets cells at the restriction point between CMPs and either MPEs or MEPEs.

**Materials and methods**

**Mice**

Mice carrying the hypomorphic Gata1low mutations (ie, deletion of the first enhancer and of the distal promoter of the gene) were bred at the Istituto Superiore di Sanità. Transgenic μLCR-hGATA1 males and females (carrying the hGATA1 cDNA under the control of a μLCR linked to the β-globin promoter), were crossed with homozygous Gata1low females. Because Gata1 is on the X chromosome whereas μLCR-hGATA1 is autosomal, 50% of the male Gata1low offspring will carry the transgene. Offspring were genotyped by polymerase chain reaction (PCR) at birth, as described, and μLCR-hGATA1 Gata1low males used as negative controls. WB6F1A/Wmice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed under good animal care practice conditions and all the experiments were performed with 3- to 4-month-old males, under protocols approved by the institutional animal care committee.

**Prospective isolation of hematopoietic progenitors**

Myeloid (CMP and MEP) and mast cell (MCP) progenitors were purified according to modifications of procedures described by Akashi et al18 and Chen et al.21 Briefly, mononuclear cell suspensions were labeled with the biotinylated rat antimonoclonal antibodies CD3, CD45R/B220, CD19, CD127 (PharMingen, San Diego, CA). Mature (Lin⁻) cells were removed by binding to sheep anti-rat IgG–conjugated magnetic beads (Dynabeads M-450; Dynal Biotech, Oslo, Norway). The cells eluted from the beads (Lin⁺) were divided in 2 aliquots. One aliquot was processed for purification of myeloid progenitors by staining with fluorescein isothiocyanate–FITC–CD34, allophycocyanin (APC)–CD117 (c-Kit), phycoerythrin (PE)–CD16/CD32 (FcεRI/I; PharMingen), and streptavidin-Cy7–PE (Caltag Laboratories, Burlingame, CA). The second aliquot was used for purification of MCPs by staining with FITC–T1/ST2 (BD Biosciences, St. Paul, MN), PE–CD117, and streptavidin-Cy7–PE. Cells labeled with fluorochrome-conjugated isotype antibodies (PharMingen) were used to gate nonspecific signals; dead cells were excluded by propidium iodide staining (5 μg/mL, Sigma, St Louis, MO). Cell analysis and sorting were performed with the FACS Aria (Becton Dickinson, San Jose, CA) equipped with 3 air-cooled and solid state lasers (488 nm, 633 nm, and 407 nm).

**Flow cytometry**

Cells were suspended in Ca²⁺⁺/Mg²⁺⁺-free phosphate-buffered saline (PBS) containing 0.5% (vol/vol) bovine serum albumin (BSA; Sigma) and 2 mM EDTA. Mast cells were recognized by labeling with PE–CD117, FITC–CD34, FITC–T1/ST2, and FITC–anti-FcεRIs (MAR-1; eBioscience, San Diego, CA). Other mature phenotypes analyzed were represented by PE–TER119/FITC–CD71 (erythroblasts), PE–CD61/FITC–CD41 (megakaryocytes), PE–Gr1/FITC–Mac3 (monocytes and granulocytes), and PE–CD11c/FITC–B220 (neutrophils and B cells). Purified anti-CD16/ CD32 (FcγRII/II) was included, as blocker, in all the analyses. Unless stated otherwise, antibodies were from PharMingen and incubated at a concentration of 1 μg/10⁶ cells for 30 minutes on ice. Nonspecific signals and dead cells were excluded, respectively, by appropriate fluorochrome-conjugated isotype and propidium iodide staining. Cell fluorescence was analyzed with the FACS Aria (Becton Dickinson).

**Liquid cultures of prospectively isolated progenitor cells**

MPEs, CMPs, and MCPs (1 × 10⁵ cells/mL) were cultured for 6 to 21 days in Iscove modified Dulbecco medium (IMDM; Gibco, Invitrogen, Carlsbad, CA) supplemented with fetal calf serum (FCS, 10% vol/vol; Sigma), 7.5 × 10⁻⁵ M β-mercaptoethanol (Sigma), penicillin, and streptomycin sulfate (50 U/mL, Gibco), and glutamine (2 mM, Gibco). Single-cell cloning experiments were performed by limiting dilution, that is, by resuspending the cells as 3 cells/mL and culturing 100 μL of this solution in each of a 96-well plate.31 Cultures were stimulated either with rat SCF (100 ng/mL; Amgen, Thousand Oaks, CA) and murine IL-3 (10 ng/mL, PeproTech, London, United Kingdom) or with a cocktail containing rat SCF (10 ng/mL), murine IL-3 (10 ng/mL), and GM-CSF (10 ng/mL; PeproTech) and human FLT3-ligand (10 ng/mL; PeproTech), IL-11 (10 ng/mL; PeproTech), thrombopoietin (TPO, 50 ng/mL; PeproTech), c-kitl, and thrombopoietin (EPO, 3 U/mL; Hoffmann-La Roche, Basel, Switzerland).34 The cultures were incubated at 37°C in a humidified incubator containing 5% CO₂ in air and cell growth and phenotype analyzed 6 to 7, 14, and 21 days later. The proliferation potential of the progenitor cell progeny was evaluated by randomly selecting 10 wells and by culturing their content under conditions of limiting dilution. The wells were scored 7 days later for sign of cell proliferation and subcloned again as long as growth was detected.
BSA (1.5% wt/vol), glutamine (2 mM), penicillin and streptomycin sulfate (50 U/mL), SCF (100 ng/mL), and IL-3 (10 ng/mL), as described. Every 3 to 4 days, cells were counted and the cultures replenished with fresh medium to maintain cell density at 2.5 × 10^7/mL.

**Culture of the SN cell lines**

The SN cell lines were obtained from Gata^low^ BMMCs and passed twice a week in IMDM supplemented with FBS (20% vol/vol), BSA (1.5% wt/vol), glutamine (2 mM), penicillin and streptomycin sulfate (50 U/mL), SCF (100 ng/mL), and IL-3 (10 ng/mL).

**Immunohistochemistry**

Cytocentrifuged cell preparations (3 × 10^5-10^6 cells/smear) were stained either with May-Grünnwald Giemsa (Sigma), benzidine, acetyl cholinesterase, or Alcian blue, according to standard protocols.

**RNA isolation and RT-PCR analysis**

Total RNA was prepared with TRIzol (Gibco), using glycogen (20 µg/L; Hoffmann-LaRoche), as carrier. For semiquantitative analysis, RNA (1 µg) was reverse transcribed with random hexamers and Moloney murine leukemia virus reverse transcriptase (RT, Perkin-Elmer, Downers Grove, IL) and cDNA was amplified with the specific sense and antisense primers described in Table S1 (available on the Blood website; see the Supplemental Materials link at the top of the online article). The reaction was performed in 100 µL 10 mM Tris-HCl, pH 8.3, containing MgCl2 (2 mM), dNTP (200 µM each), 0.1 µCi (0.037 MBq) [α³²P]dT (specific activity 3000 Ci/mmol, Amersham Italia, Cologno Monzese, Italy) and 2 U AmpliTaq DNA polymerase. Primers specific for β₂-microglobulin (50 nM each) were added to each amplification after the first 10 cycles as control for the amount of cDNA. The reactions were done with the GeneAmp 2400 thermocycler (Perkin-Elmer) and analyzed in the linear amplification range defined by preliminary experiments. Positive (RNA from 32D35 and 32D thermocycler (Perkin-Elmer) and analyzed in the linear amplification range expressed as 2^-ΔCt.

**Serotonin-release assay**

Cells (1 × 10^6/mL) were incubated with 5-hydroxy-[³H]tryptamine trifluoroacetate ([³H]-serotonin, 2 µCi/mL [0.074 MBq]); 84.0 CI/mmol, Amersham) for 6 hours at 37°C, washed twice, and incubated again, at a concentration of 20 × 10^6/mL, for 1 hour on ice either with 10 µg/mL monoclonal mouse anti-DNP-IgE (clone SPE-7, Sigma) or medium alone. After washing, cells were divided into aliquots (0.4 × 10^6/50 µL) and stimulated, for 15 minutes at 37°C, with either medium alone, 2 µg/mL monoclonal rat anti-mouse IgE (R35-72, PharMingen), 1 µg/mL DNP-human serum albumin (Sigma), or 1 µg/mL ionomycin (Sigma). Reactions were terminated with 50 µL cold Hanks balanced salt solution (Sigma), cells removed by centrifugation and levels of [³H]serotonin in supernatants measured with the Packard 1600TR liquid scintillator counter (Perkin Elmer). Total [³H]-serotonin incorporation was determined by lysing unstimulated cells with 1% Triton X-100 (Sigma). The amount of [³H]-serotonin released by the cells was calculated as counts per minute in the supernatant ± counts per minute of total incorporation × 100 (see also Rodewald et al. 2^ΔCt).

**Statistical analysis**

Statistical analysis was performed by analysis of variance (ANOVA test) using Origin 6.1 software for Windows (Microcal Software, Northampton, MA).

**Results**

Hematopoietic tissues from Gata^low^ mice contain cells with the antigenic profile of myeloid progenitors but lack those with the mastocytic progenitor profile

Normal progenitor cell subclasses were defined according to the antigenic profiles summarized in Figure 1A. Cells with MCP, MEP and CMP phenotype were detected, at the expected frequencies, in marrow from wild-type animals (Figure 1B; Table 1). The fact that these phenotypes discriminate different populations was confirmed by the observation that the sorted cells retained their phenotype on reanalysis (> 80% purity in all cases; Figure 1B). Lin^- c-Kit^ cells from marrow (and spleen) of Gata^low^ mice were prospectively divided by CD34-CD16/CD32 staining into populations similar to those observed in wild-type tissues (Figure 1B; Table 1) that, once sorted, retained their phenotype on reanalysis (> 80% purity). In contrast, T1/ST2 staining failed to label Lin^- c-Kit^- marrow (and spleen) cells from Gata^low^ mice (Figure 1B; Table 1). To prove that T1/ST2^- cells were indeed absent in Gata^low^ tissues, Lin^- c-Kit^- cells (myeloid progenitors [MPs]) were artificially divided into those in the third lower (MP^low^) and third higher (MP^high^) portion of the T1/ST2 profile (Figure 1B). The profile of the 2 populations overlapped on reanalysis (< 60% pure), confirming that T1/ST2^- cells are rare among Gata^low^-Lin^- c-Kit^- cells.

The levels of Gata1 expressed by progenitor cells prospectively isolated from Gata^low^ and wild-type marrow are compared in Figure 1C. The wild-type cells expressing the highest and lowest Gata1 levels were MEPs (2^-ΔCt = 0.1 ± 0.05) and CMPs (2^-ΔCt = 0.014 ± 0.006, 1 log lower), respectively. CMPs and MEPS isolated from Gata^low^ mutants expressed levels of Gata1 lower than those expressed by the corresponding normal cells. Surprisingly, the Gata1^- cells in which Gata1 expression was reduced the most were represented by CMPs (2^-ΔCt = 0.10 ± 0.01 × 10^-2, 1 log reduction) and not MEPS (2^-ΔCt = 0.023 ± 0.001, 4-fold reduction; Figure 1C). In addition, the expression of Fog1 was similar in wild-type CMPs and MEPS (2^-ΔCt = 0.10-0.11, in both) and decreased by 50% in wild-type MCPs (2^-ΔCt = 0.06 ± 0.01, P < 0.05). Also Gata1^-CMCs and MEPS expressed similar Fog1 levels, but the values were significantly higher than those expressed by wild-type cells (2^-ΔCt = 0.13-0.14, P < 0.05).

In conclusion, whereas MCPs were barely detectable, CMPs and MEPS were identified in hematopoietic tissues from Gata^low^ mice and expressed levels of Gata1 about 0.5 to 1-log lower and of Fog1 about 50% higher than the corresponding wild-type cells.

**The altered proliferation and differentiation observed in BMMCs from Gata1^- mice is permissive to the establishment of factor-dependent cell lines**

In BMMCs seeded with either wild-type or W/W^v^ cells, the cell number increased regularly (by 1 log) during the first 3 weeks to remain constant at later time points (Figure 2A). In contrast, in BMMCs from Gata1^- mice, the cell number increased regularly up to the fourth to fifth week and was significantly higher than in wild-type and W/W^v^ BMMCs after 25 to 30 days (Figure 2A).

In wild-type and W/W^v^ BMMCs, few (5%-10%) cells expressed the mature c-Kit^high^FceRI^+ mast cell phenotype by day 7 (Figure...
Table 1. Frequency of progenitor cells prospectively isolated from marrow and spleen of Gata1low and wild-type littermates

<table>
<thead>
<tr>
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<th>GMPs, %</th>
<th>CMPs, %</th>
<th>MEPs, %</th>
<th>MCPs, %</th>
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</thead>
<tbody>
<tr>
<td><strong>Wild type</strong></td>
<td></td>
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<tr>
<td>BM</td>
<td>36.7 ± 3.4</td>
<td>34.1 ± 0.7</td>
<td>20.8 ± 0.7</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>Spleen</td>
<td>BD</td>
<td>BD</td>
<td>BD</td>
<td>BD</td>
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<tr>
<td><strong>GATA-1low</strong></td>
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</tr>
<tr>
<td>BM</td>
<td>32.8 ± 3.9</td>
<td>35.5 ± 3.7</td>
<td>15.5 ± 1.5</td>
<td>&lt; 0.1*</td>
</tr>
<tr>
<td>Spleen</td>
<td>18.3 ± 6.3</td>
<td>26.6 ± 3.8</td>
<td>34.1 ± 6.0</td>
<td>&lt; 0.1*</td>
</tr>
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</table>

GMPs are defined as CD16/CD32+/CD43-, as CD16/CD32-/CD43+, as CD16/CD32-/CD43-, and as CD16/CD32-/CD43-; MEPs, as CD16/CD32+/CD43-, and as CD16/CD32+/CD43-; and MCPs, as T1/ST2-. All the cells are c-Kit+. Results are presented as the mean ± SD of at least 3 experiments per group of mice.

BM indicates bone marrow; BD, below detectable levels.

*Values statistically different (P < 0.05) from those observed in wild-type littermates.

2B-C) but almost all (90%-100%) of them were c-KithighFcεRI+ by day 21 to 26. In contrast, in BMMCs from Gata1low mice, although many (35%) of the cells were c-Kithigh already by day 7, many (20%) of the c-Kit+ cells remained FcεRI- until day 21 to 26 (Figure 2B-C). Differences were observed in level of antigen expression between cells obtained after 26 days in BMMCs from different mouse groups. As expected,32 W/Wv BMMCs expressed less c-Kit and FcεRI than the corresponding wild-type cells (mean fluorescence intensity/cell: c-Kit = 31 ± 1 versus 192 ± 22, P < 0.01, and FcεRI = 7 ± 1 versus 18 ± 1, P < 0.05, respectively). On the other hand, Gata1low BMMCs expressed levels of c-Kit similar to those expressed by normal cells (mean fluorescence intensity/cell = 265 ± 17) but levels of FcεRI receptor similar to those expressed by W/Wv cells (mean fluorescence intensity/cell = 8 ± 1). Expression profiling confirmed the immature nature of day 26 Gata1low BMMCs. In fact, they expressed consistently more Gata1, Gata2, MTF, and MMC-6 (earlier marker)39 but less MC-CIA (a serosal maturation marker)39 than the corresponding wild-type cells (Figure 2D). Interestingly, these cells expressed NFE2, a gene involved in the control of megakaryocytic-erythroid, rather than mastocytic, differentiation40 (Figure 2D). BMMCs from all the mice groups investigated were capable of incorporating and releasing serotonin after IgE-xIgE stimulation already by day 7.
separate experiments, per group of mice, performed in duplicate. Values observed in Gata1low of cells obtained in BMMCs seeded with marrow from wild-type, Gata1low, and W/Wv up to day 26.

Figure 2. Gata1low BMMCs are characterized by high proliferation rates and generate mast cell precursors as early as day 7, but the cells remain partially immature up to day 26. (A) BMMCs from Gata1low mice generate more cells and for longer time than BMMCs from wild-type littermates and W/Wv mice. Time-course analysis of BMMCs seeded with marrow cells from wild-type, Gata1low, and W/Wv mice are presented in the top, middle, and bottom panel, as indicated. The dotted lines indicate the regular demipopulation necessary to feed the cultures. Results are presented as mean (± SD) of at least 6 independent cultures per experimental animal. Asterisk indicates values statistically different (P < .001) between BMMCs seeded with Gata1low and wild-type, or W/Wv cells. (B) Representative flow cytometric analysis for c-Kit and FcεRI expression of cells obtained in BMMCs seeded with marrow from wild-type, Gata1low, and W/Wv mice, as indicated. Cells were analyzed either at day 7, 21, or 26 of culture. Negative controls were represented by cells labeled with irrelevant antibodies and are not presented for convenience. Similar results were obtained in at least 3 independent experiments per group of mice (see panel C). (C) Frequency of c-Kithigh (on the left) and FcεRIhigh (on the right) cells in BMMCs seeded with bone marrow from wild-type (large hatched bars), Gata1low (tight hatched bars), and W/Wv (tight hatched bars) mice. Cells were analyzed either at day 7, 21, or 26 of culture, as indicated on the x-axes. Results are presented as the mean (± SD) of at least 3 independent experiments per group of mice. Values observed in Gata1low BMMCs and statistically different from those obtained in the corresponding cultures with wild-type and W/Wv cells are indicated with a single asterisk (P < .05) and double asterisks (P < .01), respectively. (D) Semiquantitative RT-PCR analysis for the expression of mast cell specific genes (MMCP-6, MC-CPA, and MITF) as well as of Gata1, Gata2, and NFE2 in cells obtained after 26 days in BMMCs seeded with marrow from wild-type, Gata1low, or W/Wv mice, as indicated. The Gata1low c-Kithigh cells were divided into immature (FcεRI−) and mature (FcεRI+) cells by sorting (> 95% purity on reanalysis, not shown). c-KithighFcεRI− cells from wild-type and W/Wv BMMCs could not be isolated because of their infrequency. The triangle on top of the panels indicates increasing numbers of cycles. Similar results were observed in 2 additional experiments per experimental group; n.d. indicates not done. (E) Time-course analysis of serotonin uptake (on the top) and release (on the bottom) by cells obtained in BMMCs from wild-type (large hatched bars), Gata1low (tight hatched bars), and W/Wv (tight hatched bars) mice. Cells were analyzed either at 7, 21, or 26 days of culture, as indicated on the x-axes. Levels of serotonin are expressed as cpm/105 cells. The total serotonin incorporated was measured by lysing BMMCs in Triton. Serotonin release was induced by IgE-stimulation. Positive and negative controls were represented by cells stimulated with IgE alone, medium + αlgE; medium + HSA + DNP and ionomycin and are not shown for clarity. Results are presented as the mean (± SD) of at least 3 separate experiments, per group of mice, performed in duplicate. Values observed in Gata1low BMMCs and statistically different from those obtained in the corresponding cultures with wild-type and W/Wv cells are indicated with a single asterisk (P < .05) and double asterisks (P < .01), respectively.

In all the cases, however, maximal levels of serotonin were released by cells obtained by day 21. Those released by Gata1low cells remained significantly lower than those released by the other 2 cell types at all time points (Figure 2E).

The proliferation observed after the first 3 weeks in Gata1low BMMCs prompted us to ask whether these cells had become cell lines. To test this hypothesis, Gata1low, and wild-type as control, BMMCs were regularly replenished with fresh medium and growth factors for 2 additional months. Whereas wild-type cultures became extinguished within 1 month, Gata1low cells were maintained and, by the end of the 2 months, cultured as single cells in 96-well plates. One week later, cell proliferation was observed in about 30% of the wells and the cells in 10, randomly selected, wells transferred into flasks. These cultures, once replenished with fresh medium and growth factors, could be regularly passed for 6 additional months. At this time point (9 months from the beginning of the Gata1low BMMCs), the cells in each culture were considered as clonal cell lines. Because similar results were obtained in 2 additional experiments, a total of 30 individual lines were established over the course of the study.

Ten of 30 Gata1low BMMC lines were characterized in terms of morphology, growth factor requirement, and antigenic profile, and 3 of them were also analyzed by expression profiling and serotonin-releasing activity. Because results obtained with different clones were similar, only those obtained with the representative SN1 cell line are presented (Figure 3). SN1 cells were large with few cytoplasmic granules reactive with Alcian blue (5%-10%), acetyl cholinesterase A (5%; Figure 3A), and benzidine (0.1%; not shown).

These cells failed to survive in the absence of growth factors (Figure 3B). Few of them (20%-30%) survived in the presence of...
either SCF, IL-3, or TPO alone. For optimal proliferation, they required the presence of both SCF and IL-3. In their presence, a doubling time of about 24 hours was observed (Figure 3B).

SN1 cells expressed 10 times more surface c-Kit, similar levels of CD34 and Sca1, and lower levels of FcRRI than the original Gata1low mast cells from which they had derived (Figure 3C). Consequently, although capable of some (~8%) serotonin release after IgE/IgE stimulation, their serotonin-releasing activity remained modest (Figure 3D). Some SN1 cells expressed the erythroid TER119 (21% ± 4%) or megakaryocytic 2D5 (61% ± 3%) markers, with 15% of them expressing both (not shown). Because almost all SN1 cells expressed FcRRI (Figure 3C), erythroid, megakaryocytic, and mastocytic surface markers were coexpressed in about 15% of the cells. By molecular profiling, SN1 cells expressed mast cell specific genes as well as erythroid (β-globin) and megakaryocytic (GpIa and GpIIb)–specific genes (Figure 3E). Interestingly, they expressed high levels of NFE2, Gata2, and Gata1. This last result indicates a different Gata1 regulation in these cells as compared with that of erythroid Gata1low progenitors. The levels of Gata1 expressed by SN1 cells are higher than those expressed by normal hematopoietic cells and resemble those found in MEL cells.61 As in the case of these erythroleukemic cells, such a high level of Gata1 expression was not sufficient per se to restore proper terminal differentiation of SN1 cells. In conclusion, BMMCs seeded with marrow (and spleen, not shown) cells from Gata1low mice are characterized by increased proliferation and decreased cell differentiation and consistently generate 3-lineage growth factor–dependent cell lines.

Gata1low MEPs generate mast cells, and their precursors, in BMMC cultures

The fact that mast cells, although defective, were present in skin and peritoneum of Gata1low mice, as well as in BMMCs seeded with their marrow (Migliaccio et al68 and this report), indicated that the mastocytic activity of Gata1low stem cells was not impaired. The fact that MCPs were not detectable in Gata1low tissues implies, then, that a cell different from an MCP acquired MCP function in these mutants. To identify which of the Gata1low progenitor cells...
acquired mast cell-generating activity, cells prospectively isolated from tissues of wild-type and \textit{Gata1}\textsuperscript{low} littermates were cultured in BMMCs and the cultures analyzed 7 days later for presence of mast cells (c-Kit\textsuperscript{high}/FcεRI\textsuperscript{+}) and of their precursors (c-Kit\textsuperscript{high}/T1/ST2\textsuperscript{high}, Figure 4; Table S2).

Numerous cells (≈300-500-fold increase with respect to day 0) were generated in 7 days of culture by all of the prospectively isolated progenitor cells (wild-type and \textit{Gata1}\textsuperscript{low} alike), with the exception of wild-type MPs that grew poorly (Figure 4A). As expected, wild-type MEPs and CMPs generated mostly erythroid and megakaryocytic cells (Table S2 and Suda et al., 17 Akashi et al., 18 and Nakorn et al.\textsuperscript{19}), and few (1%-2%) mast cells and their precursors. These cells represented, instead, more than 7% to 8% of the progeny of wild-type MCPs (Table S2; Akashi et al.\textsuperscript{18}). In contrast, MEPs and CMPs isolated from \textit{Gata1}\textsuperscript{low} animals consistently generated many (>20%) c-Kit\textsuperscript{high}/FcεRI\textsuperscript{+} and c-Kit\textsuperscript{high}/T1/ST2\textsuperscript{high} cells, in addition to erythroblasts and megakaryocytes (Figure 4B; Table S2).

The mastocytic nature of cells present among the progeny of \textit{Gata1}\textsuperscript{low} MEPs was confirmed by functional assay (c-Kit\textsuperscript{high}/T1/ST2\textsuperscript{high} cells; Table S3) and expression profiling (c-Kit\textsuperscript{high}/FcεRI\textsuperscript{+} cells; Figure 4C). c-Kit\textsuperscript{high}/T1/ST2\textsuperscript{high} cells, derived from \textit{Gata1}\textsuperscript{low} MEPs, generated, after 14 days of BMMCs, more c-Kit\textsuperscript{high}/FcεRI\textsuperscript{+} cells than the corresponding progeny of wild-type MCPs (fold increase = 97- and 600-fold, \(P < .01\), respectively; Table S3).

On the other hand, c-Kit\textsuperscript{high}/FcεRI\textsuperscript{+} cells generated by the various wild-type progenitor cells had different expression profiling (Figure 4C). Those generated by MCPs and CMPs expressed robust levels of the late MC-CPA marker and low level of expression of the early MMCP-6 marker.\textsuperscript{24,39} Wild-type CMP-derived c-Kit\textsuperscript{high}/FcεRI\textsuperscript{+} cells expressed also high levels of MMCP-7, a protease usually not expressed by BMMC-derived mast cells.\textsuperscript{24} In contrast, mast cell-specific genes were poorly expressed by c-Kit\textsuperscript{high}/FcεRI\textsuperscript{+} cells derived from wild-type MEPs. In comparison, c-Kit\textsuperscript{low}/FcεRI\textsuperscript{+} cells generated by all of the \textit{Gata1}\textsuperscript{low} progenitors, including MEPs, expressed a homogenous profile, characterized, as that of wild-type CMP-derived cells, by robust expression of all the mast cell-specific genes analyzed, including MMCP-7.

To further confirm that \textit{Gata1}\textsuperscript{low} MEPs, but not wild-type MEPs, expressed mast cell-generating activity, the progeny of both cells was cultured for additional 14 to 21 days (Figure S1). High numbers of mast cells were consistently detected in cultures seeded with \textit{Gata1}\textsuperscript{low} MEPs but not in those seeded with the corresponding wild-type cells.

In conclusion, \textit{Gata1}\textsuperscript{low} MEPs generate numerous mature and precursor mast cells in BMMC cultures.
Altered differentiation/proliferation potential of single Gata1\textsubscript{low} MEPs

To confirm that Gata1\textsubscript{low} MEPs generate mast cells and to determine the extent of the abnormal proliferation of their progeny, prospectively isolated Gata1\textsuperscript{low}, and wild-type as controls, MEPs were cultured as single cells, using the limiting dilution protocol. The morphology and cloning potential of their progeny was then evaluated at weekly intervals (Figure 5A).

Approximately 15% of single wild-type MCPs proliferated by day 7, giving rise to about 6 \times 10\textsuperscript{3} cells by day 14 of culture (Figure 5B). In all the cases, the progeny had mast cell morphology (Figure 5C). Also 15% of single Gata1\textsubscript{low} MEPs proliferated under these conditions, generating about 6 \times 10\textsuperscript{3} to 10\textsuperscript{5} cells by day 7 to 14 (Figure 5B). The morphology of the Gata1\textsubscript{low} MEP-derived progeny included mast cells, in addition to megakaryocytes and erythroblasts, by day 7 (Figure 5C). Starting from day 14, the progeny of selected wells was harvested, and recultured again under conditions of limiting dilution at weekly intervals. The replating efficiency of wild-type MCP-derived cells declined over time from 5% at day 14 to undetectable levels at day 21. These cultures were discontinued. In contrast, the number and replating efficiency of the Gata1\textsubscript{low} MEP-derived progeny increased over time. By the third to fourth passage, each Gata1\textsubscript{low} cell generated about 8 to 10 \times 10\textsuperscript{5} cells/wk, with a cloning efficiency of about 30%. When corrected for the limiting dilution factor (0.3 cells/well), a cloning efficiency of 30% indicates that all the cells had proliferated (Figure 5D; Table S4). The progeny of Gata1\textsubscript{low} MEPs could be propagated as single cells for at least 4 additional months. The design of the replating experiments allows tracking of the proliferation potential of the progeny of individual MEPs. The tracking indicates that about 11% of single Gata1\textsubscript{low} MEPs have the potential to generate cells capable of growth with 100% efficiency after 4 passages (Table S4).

Ectopic expression of \textit{hGATA1} restores the functions of Gata1\textsubscript{low} MEPs

To prove that the differentiation/proliferation alterations expressed by Gata1\textsubscript{low} MEPs are a direct consequence of reduced Gata1
expression, we analyzed the biologic properties of MEPs purified from marrow of μLCRhGATA1 Gata1low/0 males (Figure 6). MEPs purified from μLCRhGATA1/Gata1low/0 mice expressed, as those purified from their Gata1low littersmates, low levels of mGata1 (Figure 6). These cells, however, also expressed hGATA1 (Figure 6). Once again, MEPs from Gata1low littersmates generated many (~13%) mast cells (c-KithighFcεRI+). In contrast, those isolated from μLCRhGATA1/Gata1low mice, as those isolated from wild-type mice, generated few (<2%) mast cells. Furthermore, these cells expressed low levels of mast cell-specific genes (Figure 6). Last, but not least, the progeny of μLCRhGATA1/Gata1low MEPs, as that derived from wild-type MEPs, did not proliferate beyond 15 to 21 days of culture.

Discussion

It is shown here that the marrow and spleen from Gata1low mice contain cells expressing the antigenic profile of wild-type GMPs and MEPs, and MEPs but not those resembling wild-type MCPs (Figure 1B). The frequency of CMPs and MEPs is normal in marrow and increases, by several fold, in spleen of mutant mice (Table 1). The hypomorphic mutation reduces by 0.5 to 1-log marrow and increases, by several fold, in spleen of mutant mice (Figure 1B). The frequency of CMPs and MEPs but not those resembling wild-type MCPs contain cells expressing the antigenic profile of wild-type GMPs.

Physiologic manipulations that would alter commitment by targeting a transcription factor have not been reported so far. The observation that Fog1 represses FceRI expression suggests that Fog1 might represent a repressor of mastocytopoiesis. To test this hypothesis, we compared the level of expression of Fog1 in wild-type and Gata1low MEPs. However, the mutant cells expressed values of Fog1 significantly higher, and not lower than, those expressed by wild-type MEPs. Such a higher expression in Gata1low MEPs, though not providing any clue for their high mastocytopoietic activity, is consistent with their robust capacity to generate megakaryocytes in cultures (Table S2).

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Several mutations in hematopoiesis affect the end stage of erythroid, megakaryocytic, and mast cell differentiation. We propose that the *Gata1* mutation, being associated with myeloid and B-cell leukemia, may act at the restriction point between hematopoietic stem cells (HSCs) and CMPs and common lymphoid progenitors (CLPs). The *Gata1* mutation affects cells at the restriction point between CMPs and MEPs and MCPs (this report). On the other hand, the *Gata1* mutation is associated with development of thrombopoietin-dependent cell lines and has been suggested to affect the restriction point between MEPs and CFU-Mk<sup>high</sup> and CFU-E<sup>low</sup>. Modified from Stachura et al<sup>59</sup> with permission.

Recently, it has been proposed a transcription factor–based model for lineage commitment according to which lineage-specific expression is determined by the balance between the relative concentrations of transcription factors for alternative lineages.<sup>46,47</sup> This model is based on the observation that single hematopoietic progenitors activate transcription factor gene expression in random combinations and before the stage when their fate is fixed.<sup>45</sup> This model is also supported by results on forced transcription factor expression on cell fate. In fact, forced *Gata1* expression favors generation of erythroid cells in multiple models of stem cell commitment (in vivo, in mouse transplantation models<sup>29,61</sup> and in vitro, avian myeloid cells<sup>27</sup>). The observation, however, that *Gata1*<sup>null</sup> embryonic stem cells do generate proerythroblasts,<sup>32,39</sup> although apoptotic,<sup>55</sup> in vitro, has been considered for a long time as a proof that *Gata1*, although required for erythroid maturation, is dispensable for commitment. By showing that *Gata1*<sup>low</sup> MEPs (and CMPs) are capable of differentiating into mast cells, in addition to erythroblasts and megakaryocytes, we demonstrate for the first time that the level of *Gata1* expression in the progenitor cell compartments determine the lineage toward which their progeny will be committed. As counter-proof, forced expression of *μLcrR6GATA1* restored the function of *Gata1*<sup>low</sup> MEPs (Figure 6). These results reinforce the concept that *Gata1* is a key element, not only for maturation, but also for commitment toward erythroid, megakaryocytic, and mast cell lineage.

The results presented here, and an overview of published results that did not formally involve analysis of prospectively isolated cells, not only confirm the transcription factor-based model for lineage specification<sup>46,47</sup> but, as suggested in Figure 7, indicate that the extent of *Gata1* reduction might determine the level, in the hematopoietic hierarchy, of the cell targeted by the mutation. In fact, the *Gata1*<sup>low</sup> mutation affects bipotent megakaryocytic/erythroid cells (the PEM?) that, although maturation defective, have such high proliferation activity to generate, both in culture and in the liver of chimeric mice, TPO-dependent megakaryocytic cell lines.<sup>59</sup> As shown here, the hypomorphic *Gata1*<sup>low</sup> mutation affects cells at the restriction point between CMPs and MEPs or MCPs. On the other hand, the hypomorphic *Gata1*<sup>0.5</sup> mutation, which induces in chimeric mice either myeloid (at 2 months) or B-cell (at 1 year) leukemia,<sup>58</sup> might affect cells at the restriction point between myeloid and B-cell development. Because the levels of *Gata1* expression in *Gata1*<sup>0.5</sup> progenitor cells are unknown, the model cannot be completed with the hypothesis that the extent of *Gata1* expression impairment is inversely correlated with the spectrum of progenitor cells affected.

The increased proliferation potential of mice carrying the *Gata1*<sup>0.5</sup> mutation is associated with development of leukemia.<sup>58</sup> Furthermore, mice carrying the *Gata1* mutation, which is associated with transient myeloproliferative disorders of newborns,<sup>13-15</sup> have increased proliferation capacity of a “unique” fetal stem/progenitor cell extinguished in adult life.<sup>57</sup> These results suggest that altered *Gata1* expression, by increasing progenitor cell proliferation, may predispose to leukemia by favoring accumulation of secondary mutations. Although *Gata1*<sup>low</sup> mice do not develop leukemia, they develop a syndrome similar to idiopathic myelofibrosis (CD1) or essential thrombocytopenia (DBA/2), depending on background,<sup>29,61</sup> 2 diseases that may evolve to leukemia in humans.<sup>52</sup> The high number (∼11%) of *Gata1*<sup>low</sup> MEPs that generate factor-dependent cell lines suggest that these mice, if exposed to stimuli such as SCF or IL-3 (or both), that increase MEP proliferation, might develop leukemia as well. Further studies will be necessary to clarify this point.

In conclusion, these results confirm the crucial role played by *Gata1* in hematopoietic commitment and identify as new target for *Gata1* activity the restriction point between CMPs and MEPs or MCPs.

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**Authorship**

Contribution: B.G. designed the experiments, performed the cell purifications, the cultures, the serotonin release assays, prepared
the data for publication, and wrote the manuscript; M.S. designed the experiments and supervised cell purification; F.M. performed cytofluorimetric analysis and cell sorting; G.A. performed quantitative RT-PCR analysis; A.M.V. established and characterized the SN cell lines and discussed the results; G.M. analyzed the data and discussed the results; S.H.O. analyzed the data and wrote the manuscript; and A.R.M. designed research, analyzed the data, and wrote the paper.

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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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References


The hypomorphic $\text{Gata1}^{\text{low}}$ mutation alters the proliferation/differentiation potential of the common megakaryocytic-erythroid progenitor

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