

Early block to erythromegakaryocytic development conferred by loss of transcription factor GATA-1

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Transcription factor GATA-1 is essential at multiple stages of hematopoiesis. Murine gene targeting and analysis of naturally occurring human mutations demonstrate that GATA-1 drives the maturation of committed erythroid precursors and megakaryocytes. Prior studies also suggest additional, poorly defined, roles for GATA-1 at earlier stages of erythromegakaryocytic differentiation. To investigate these functions further, we stimulated *Gata1*⁻ murine embryonic stem-cell-derived hematopoietic cultures with thrombopoietin, a multistage cytokine. Initially, the

cultures generated a wave of mutant megakaryocytes. However, these were rapidly overgrown by a unique population of thrombopoietin-dependent blasts that express immature markers and proliferate indefinitely. Importantly, on restoration of GATA-1 function, these cells differentiated into both erythroid and megakaryocytic lineages, suggesting that they represent bipotential progenitors. Identical cells are also present in vivo, as indicated by flow cytometry and culture analysis of fetal livers from *Gata1*⁻ chimeric mice. Our findings indicate that

loss of GATA-1 impairs the maturation of megakaryocyte-erythroid progenitors. This defines a new role for GATA-1 at a relatively early stage of hematopoiesis and provides potential insight into recent discoveries that human *GATA1* mutations promote acute megakaryoblastic leukemia, a clonal malignancy with features of both erythroid and megakaryocyte maturation. (Blood. 2006;107:87-97)

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Introduction

Generation of a diverse blood-cell repertoire from hematopoietic stem cells (HSC) is coordinated by transcription factors that control developmental decisions, survival, and proliferation. These actions are critical to normal hematopoiesis, as illustrated by numerous transcription factor mutations that cause inherited and acquired cytopenias and leukemias in mice and humans. One interesting and important example is GATA-1, a double zinc finger nuclear protein that was discovered through its binding to a core GATA consensus motif in many hematopoietic gene regulatory elements (reviewed in Weiss et al¹ and Ferreira et al²). Enforced expression of GATA-1 in multipotential precursors influences lineage commitment.¹⁻⁴ Targeting of the *Gata1* gene in mice demonstrates essential requirements for erythroid, megakaryocytic, eosinophilic, and mast cells at various developmental stages.⁵⁻¹⁰ For example, without GATA-1, recognizable lineage-committed erythroblasts and megakaryocytes form but fail to mature normally.

Mutations in the X-linked human *GATA1* gene are associated with disorders of erythrocyte and megakaryocyte development. Germ line missense mutations within the amino (N)-terminal zinc finger that impair DNA binding or disrupt interaction with the cofactor FOG-1 cause X-linked thrombocytopenia and anemia with accumulation of dysplastic erythroblasts and megakaryocytes in hematopoietic tissues.¹¹⁻¹⁵ Somatic mutations in the *GATA1* gene contribute to the development of transient myeloproliferative

disorder (TMD) and acute megakaryoblastic leukemia (AMKL) associated with Down syndrome (DS, trisomy 21).¹⁶⁻²²

The close relationship between *GATA1* mutations, myeloid leukemia, and DS illustrates a new pathway to malignancy. TMD, which occurs in about 10% of infants with DS, is characterized by the accumulation of clonally derived myeloid blasts in the circulation and hematopoietic tissues, including liver, a major source of hematopoiesis in the late fetus and newborn (reviewed in Gurbaxani et al²¹ and Hitzler and Zipursky et al²³). Although TMD usually resolves spontaneously, about 20% of previously affected infants later develop full-blown AMKL within the first few years of life. Remarkably, both TMD and AMKL blasts associated with DS all contain somatic mutations in the first coding exon of *GATA1*. These mutations disrupt the normal reading frame of *GATA1* but permit the production of GATA-1 short (GATA-1s), a naturally occurring variant protein that lacks an N-terminal acidic transcriptional activation domain.²⁴ In several individual patients followed serially, identical *GATA1* mutations were present in TMD and AMKL that followed years later. These findings suggest that *GATA1* mutations combined with trisomy 21 are an early event in a multistep malignant transformation process.

How *GATA1* mutations contribute to TMD and AMKL is incompletely understood. *Gata1*⁻ megakaryocytes are developmentally blocked and divide excessively.¹⁰ In contrast, fetal

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Pax5, *C/EBPA* (*Cebpa*), *GPIb-a* (*Gp1ba*), *GPIb-b* (*Gp1bb*), and *Pf4*.³⁹ *Ahsp* (*Eraf*) primers were forward (CAC AGG ATC CAT GGC CCC TTT TCA GAG CAA TAA) and reverse (TAT GAG GAG GGC AGT GTA TTG CTT).

Flow cytometry

Flow cytometry was performed using a FACSCalibur or LSRII flow cytometer and analyzed with CellQuest Pro software (Becton Dickinson, San Jose, CA). Lineage depletion was performed using the Lineage Cell Depletion Kit (Miltenyi Biotec GmbH, Auburn CA) supplemented with biotin anti-mouse Ly6A/E (Sca-1; 1:250; Becton Dickinson) and biotin anti-mouse IL7R α (1:250; Becton Dickinson) followed by 2 rounds of magnetic separation on an autoMACS Separator (Miltenyi Biotec GmbH). The sources and dilutions of antibodies used for flow cytometry are as follows: Becton Dickinson, PE anti-mouse Ly6A/E (Sca-1; 1:100), biotin anti-mouse Ly6A/E (Sca-1; 1:250), biotin anti-mouse IL7R α (1:250), PE anti-mouse CD117 (cKit; 1:100), APC anti-mouse CD117 (cKit; 1:250), FITC anti-mouse CD41 (1:100), PE anti-mouse CD45R (B220; 1:100), PE anti-mouse CD11b (Mac1; 1:100), PE anti-mouse Ly-76 (Ter119; 1:100), APC anti-mouse Ly-76 (Ter119; 1:100), PE anti-mouse Ly6C (Gr1; 1:100), biotin anti-mouse CD9 (1:100), biotin anti-mouse CD229.1 (Ly9.1; 1:250), PE anti-mouse CD45.1 (1:100), streptavidin PerCP (1:250), streptavidin APC (1:250), FITC anti-CD229.1 (Ly9.1; 1:100), and PE anti-mouse CD41 (1:100); EMFRET Analytics (Würzburg, Germany), anti-mouse PE-GPIb α (1:20); eBioscience (San Diego, CA), APC-Cy7 anti-mouse CD117 (cKit; 1:100), and PE-Cy7 anti-mouse CD16/32 (Fc γ R; 1:100).

Cell proliferation/viability

Cell proliferation was assessed by counting trypan blue (Sigma Aldrich)-excluding cells on a hemocytometer and by counting 7-aminoactinomycin D (7-AAD; Sigma Aldrich) excluding cells with a flow cytometer.

Additional proliferation studies of G1ME cells were performed using the membrane dye, PKH-26.^{40,41} Five \times 10⁶ cells were stained with 12 μ M PKH-26 (Sigma-Aldrich) at day 0 of retroviral infection. Deconvolution of histogram distributions into generations was performed by ModFit LT software Proliferation Wizard model (Verity Software House, Topsham, ME).

Results

Expansion of hematopoietic blasts from *Gata1*⁻ ES-cell in vitro differentiation cultures

Normal hematopoietic development is recapitulated by in vitro differentiation of ES cells.⁴² Previously, we used this approach to show that GATA-1 is required for survival and maturation at the proerythroblast stage of hematopoiesis.^{6,7} For the current studies, we examined *Gata1*⁻ hematopoiesis using described protocols to generate megakaryocytes from ES cells in vitro^{33,34} (Figure 1A). We cultured ES cells on the stromal line OP9 to generate definitive multipotential hematopoietic precursors.^{43,44} After 5 days, we added Tpo, a cytokine that stimulates hematopoietic stem cells, multipotential progenitors, and megakaryocytes⁴⁵ (Figure 1A). The cultures were maintained on OP9 cells with Tpo as the only added cytokine, and differentiated hematopoietic progeny were serially enumerated and examined by histologic staining. By day 12, excessive cell proliferation was evident in the *Gata1*⁻ cultures, which were expanded about 5-fold relative to wild-type cultures (Figure 1B). At this time point, both wt and *Gata1*⁻ cultures contained an obvious population of megakaryocytes. These were recognized by their characteristic large size and multinuclearity (Figure 1C, top), DNA content greater than 4N (45%-50% of the population), increased forward and side scattering profile on flow cytometry, and staining with AChE, a rodent megakaryocyte marker (75%-80% of the population) (not shown). The *Gata1*⁻ megakaryocytes were distinctly different from the wt ones. Specifically, mutant megakaryocytes were more numerous, smaller, and stained more weakly with AChE than their wt counterparts (not shown), consistent with previous observations that *Gata1*⁻ or deficient megakaryocytes are developmentally arrested and proliferate excessively.^{10,24,46} Megakaryocytes can be identified by their

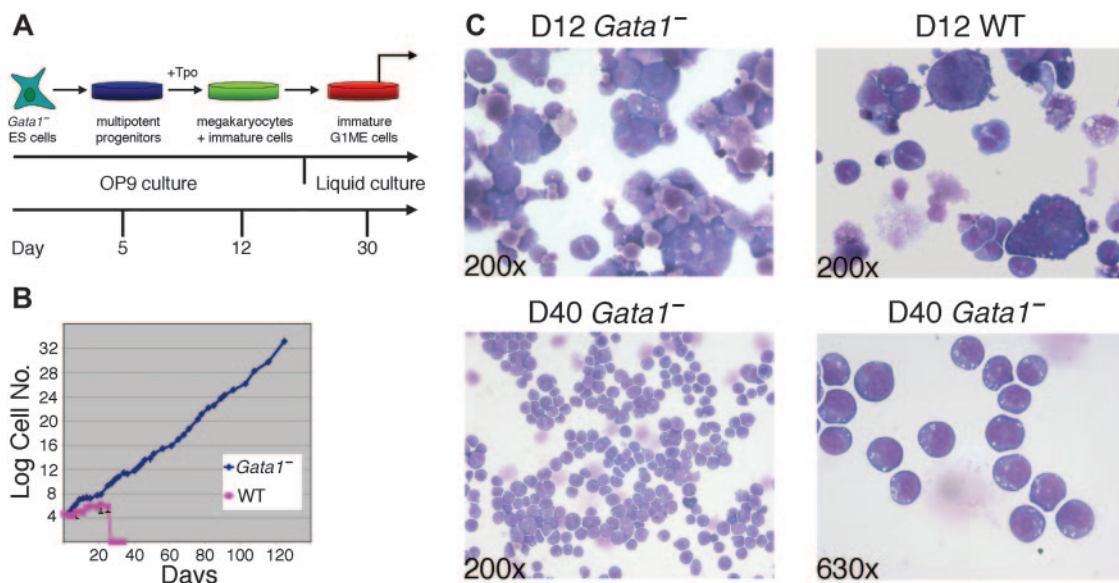


Figure 1. Selective expansion of immature hematopoietic blasts from *Gata1*⁻ ES-cell in vitro differentiation cultures. (A) Summary of the culture method used. wt or *Gata1*⁻ ES cells were cultured on OP9 stromal cells for 5 days to generate multipotential hematopoietic progenitors. These were expanded and differentiated further on OP9 cells in the presence of Tpo. At about 3 weeks, nonadherent cells were removed from the OP9 stromal layer and transferred to liquid culture with Tpo. (B) Proliferation of nonadherent cells in differentiation cultures from *Gata1*⁻ and wt ES cells. Cumulative cell numbers are plotted against time. One of 3 representative experiments is shown. (C) Morphology of cells after various times in culture. May-Grünwald-Giemsa stain. The blast cells derived from *Gata1*⁻ cultures are shown at day 40. We refer to cells at day 40 as GATA-1 megakaryocyte-erythroid (G1ME) cells because they exhibit erythromegakaryocytic potential, as demonstrated in Figure 3. Original magnification of top (day 12), \times 200; bottom left (day 40), \times 200; bottom right (day 40), \times 630. Photographs were taken by using a microscope (Axioskop 2; Carl Zeiss) equipped with a color digital camera (AxioCam; Carl Zeiss).

high forward and side scatter profiles in flow cytometry.³⁴ Using these gating criteria, both wt and *Gata1*⁻ megakaryocytes expressed CD41 and were negative for myeloid and erythroid lineage markers (not shown). However, wt megakaryocytes expressed GPIb, whereas *Gata1*⁻ megakaryocytes did not (not shown), consistent with recently reported gene profiling studies.²⁶

After about 3 weeks, cultures derived from wt ES cells stopped dividing, whereas *Gata1*⁻ cultures continued to proliferate (Figure 1B). The mutant cells were transferred to liquid culture without OP9 stroma and monitored for several months. By 4 to 5 weeks, mutant cultures were composed of a monomorphous population of blast cells with an undifferentiated morphology, high nuclear-cytoplasmic ratio, homogeneous nuclear staining pattern, and prominent nucleoli (Figure 1C, bottom). These continued to divide in a Tpo-dependent fashion (not shown) for more than 6 months. Similar cell-growth kinetics as illustrated in Figure 1B occurred in 3 independent experiments. These immature *Gata1*⁻ cells arose reproducibly in culture without growth lag or crisis, indicating that they derive directly from loss of GATA-1 and not from random genetic events acquired during cell culture. After several months in culture, the karyotype was normal. As described in the next section, these blasts exhibit both erythroid and megakaryocytic potential. Therefore, from this point, we refer to them as G1ME cells, for GATA-1⁻ megakaryocyte-erythroid.

To further characterize G1ME cells and to assign them to a specific stage in hematopoiesis, we analyzed their mRNA expression (Figure 2A) and cell-surface immunophenotype (Figure 2B). The cells were negative for mature lineage markers, including beta major globin mRNA and Ter119 (erythroid), Gr1 (granulocyte), Mac1 (macrophage), and B220 (B cell). G1ME cells also did not express markers for early lymphoid precursors (*Gata3* and *Pax5* mRNAs protein, and *Il7r*) or committed granulocyte/macrophage/monocyte precursors (GM-CSFR, G-CSFR, and C/EBP α mRNAs). The cells were Sca1⁻, cKit⁺, CD41⁺ and expressed mRNAs encoding the transcription factors FOG-1, GATA-2, and PU.1. Together, these findings are consistent with an early stage of

erythromegakaryocytic maturation.^{1,47,48} *Gata2* mRNA may be expressed abundantly because its gene is repressed directly by GATA-1.^{7,49} G1ME cells also express the cell-surface tetraspanin CD9, which marks the commitment of bipotential megakaryocyte-erythroid precursors (MEPs) to unilineage megakaryocyte precursors (MKPs) in adult mice.⁴⁷ Consistent with this, there was low-level expression of the megakaryocytic marker GPIb α . From these RT-PCR and cell-surface immunophenotyping analyses, in combination with their morphologic features, it seemed most likely that G1ME cells represented early committed megakaryocyte precursors that are distinct from the recognizable megakaryocytes generated from *Gata1*⁻ ES cells (Figure 1C) and fetal liver of GATA-1-deficient mice.^{10,24} However, studies described in the next section indicate that G1ME cells have both erythroid and megakaryocytic potential.

Erythromegakaryocytic maturation of G1ME cells

To characterize G1ME cells functionally, we assessed their developmental potential after GATA-1 complementation by retroviral transfer (Figure 3). We used MIGR1, an MSCV-based retroviral vector in which GATA-1 is linked to a GFP cDNA via an internal ribosome entry site (Figure 3A), which permits tracking of infected cells by their GFP fluorescence.³⁷ As expected, G1ME cells, or those infected with MIGR1 empty vector, expressed no GATA-1 (Figure 3B), whereas the protein was detected in cells infected with GATA-1 virus. Considering that the GATA-1 virus transduced 30% to 40% of G1ME cells (not shown), their level of GATA-1 protein expression was similar to that of MEL cells (Figure 3B). This suggests that GATA-1 was not massively overexpressed in transduced G1ME cells, although the endogenous GATA-1 protein level in wild-type progenitors that approximate G1ME cells is not known.

After infection, cells were cultured in multiple cytokines that support a broad range of hematopoietic maturation. These included Tpo, Epo, KL, IL-3, IL-11, GM-CSF, and MCSF. Three days after

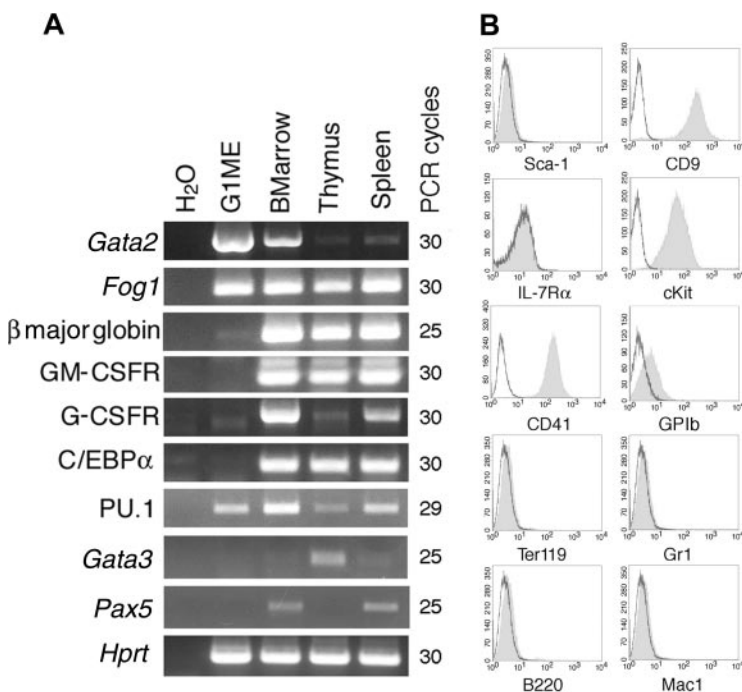
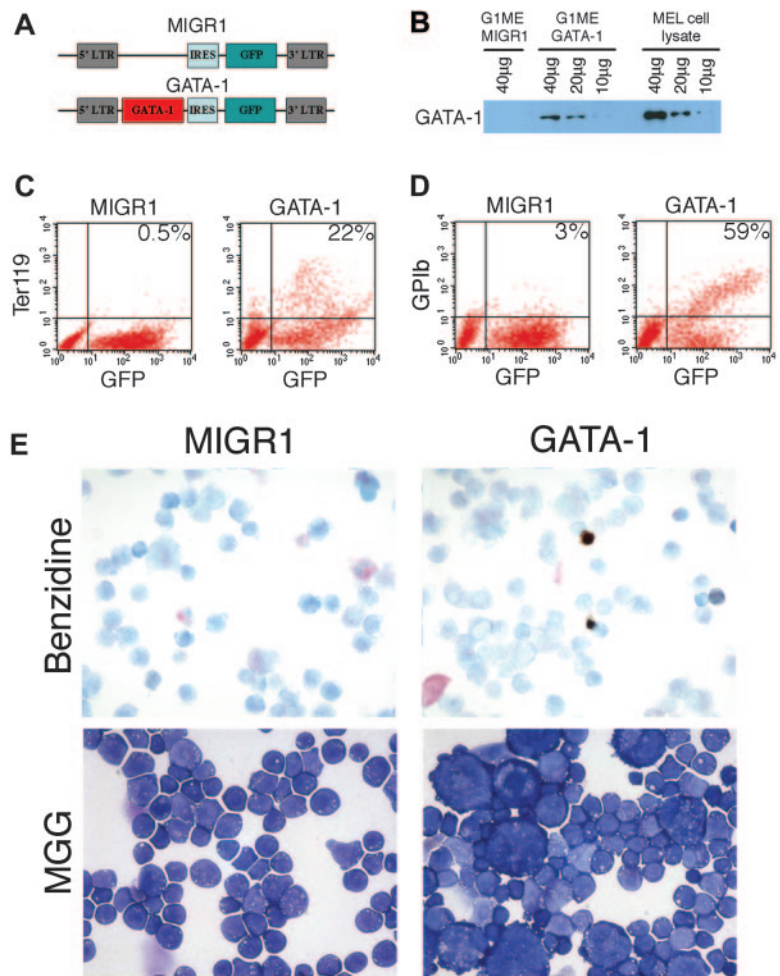


Figure 2. Gene expression and cell-surface phenotype analyses of G1ME cells. (A) RT-PCR analysis. Bone marrow (BMarrow), spleen, and thymus were analyzed in parallel as controls. GM-CSFR indicates granulocyte macrophage colony-stimulating factor receptor; G-CSFR, granulocyte colony-stimulating factor receptor; *Hprt*, hypoxanthine phosphoribosyl transferase. PCR cycle numbers for each primer pair are shown at the right side of the panel. (B) Cell-surface marker phenotype assessed by flow cytometry. Antibody-stained cells are shown in filled curve. Open curves denote staining with appropriate isotype control antibodies. Murine bone marrow and/or thymus were used as positive controls (not shown).

Figure 3. GATA-1 induces erythromegakaryocytic maturation of G1ME cells. (A) Retroviral constructs used for gene rescue. The MIGR1 vector encodes green fluorescent protein (GFP) linked to an internal ribosome entry site (IRES). MIGR1-GATA-1 also contains the full-length coding region of murine GATA-1 cDNA. For panels B to E, cells were analyzed 3 days after retroviral transduction. Viral infection efficiencies assessed by flow cytometry for GFP expression were about 65% for MIGR1 and 35% for MIGR1-GATA-1. (B) GATA-1 protein expression in transduced cells determined by Western blotting. The amount of whole-cell lysate analyzed in each lane is indicated. MEL cell lysate was analyzed in parallel for comparison (last 3 lanes). (C) Expression of the erythroid-specific surface marker Ter119. Percentages in panels refer to fraction of GFP⁺ cells expressing Ter119. Approximately 65% of MIGR1- and 30% of GATA-1-transduced cells were GFP⁺. (D) Expression of the terminal megakaryocyte maturation marker GPIb. Percentages in panels refer to fraction of GFP⁺ cells expressing GPIb. (E) Morphology of cells after GATA-1-induced maturation. The top panels show benzidine staining for hemoglobin, with dark brown benzidine-positive cells visible only in the GATA-1-rescued sample. From the flow cytometry results for Ter119 in panel 3C, roughly 6% of cells are expected to be benzidine positive. The bottom panels show May-Grünwald-Giemsa (MGG) staining with large multinucleated megakaryocytes occurring specifically in the GATA-1-rescued sample. Original magnification, $\times 400$. Photographs were taken by using a microscope (Axioskop 2; Carl Zeiss) equipped with a color digital camera (AxioCam; Carl Zeiss).



infection, cells were analyzed by flow cytometry for expression of mature lineage markers. Induction of erythroid and megakaryocytic maturation by GATA-1 was evidenced by the expression of Ter119 and GPIb, respectively (Figure 3C-D). Erythroid maturation was further evidenced by the appearance of benzidine-staining cells, a marker for hemoglobin (Figure 3E, top), and induction of erythroid-specific genes, including β major globin and *Ahsp*⁵⁰ (Figure 4B). Morphologic signs of megakaryocytic maturation included the formation of large cells with multilobular nuclei (Figure 3E, bottom right) and characteristic ultrastructural features, including multivesicular bodies (α -granule precursors), dense granules, α -granules, and platelet demarcation membranes (Figure 4A).⁵¹⁻⁵³ GATA-1-transduced G1MEs had a ploidy greater than 4N (27% of GATA-1-transduced cells were $> 4N$, whereas only 2% of MIGR1-transduced cells were), and had a distinct scatter profile (large and granular) when compared with MIGR1-transduced G1ME cells. Numerous mature megakaryocyte/platelet expressed genes including GPIb- α , GPIb- β , and *Pf4* were also induced (Figure 4B).

Flow cytometry indicated that platelet and erythroid markers were not coexpressed on the same cells (Supplemental Figure S1A-B, available at the *Blood* website; see the Supplemental Figures link at the top of the online article). Additionally, cells that stained with benzidine were always small, distinguishing them from the much larger megakaryocytes (Figure 3E, compare top and bottom panels). Hence, distinct erythroid and megakaryocytic maturation occurred in separate cells. Using the intensity of GFP expression in transduced cells as a surrogate marker for GATA-1

protein level, it appears that optimal erythroid maturation occurs at intermediate GATA-1 levels, whereas megakaryocytic maturation is optimal at higher levels (compare Figure 3C with 3D). These findings are in accord with previous studies demonstrating that lineage fate decisions are GATA-1 dose dependent.² Hence, the differentiation of normal bipotential erythromegakaryocytic precursors may be influenced by GATA-1 level and activity, although this requires further investigation.

Cells infected with MIGR1 vector grew at about the same rate as uninfected cells (Figure 4C). In contrast, uninfected cells rapidly outgrew cells infected with GATA-1. This was not due to increased cell death, because the proportion of viable cells was identical in G1ME populations transduced with GATA-1 or vector (Supplemental Figure S2A). In addition, a membrane-dye proliferation assay showed that the rate of cell division was reduced specifically in GATA-1-transduced G1ME cells but not controls (Supplemental Figure S2B). Together, these data indicate that GATA-1 inhibits cell division as part of normal maturation, and are consistent with the antiproliferative activities of GATA-1 described previously.^{26,29,54,55} Despite culture of G1ME cells in a broad range of supportive cytokines, we never observed signs of maturation into granulocyte, macrophage, lymphoid, or mast-cell lineages before or after rescue with GATA-1, either by morphology or by flow cytometry analysis using the lineage markers Gr1, B220, Fc ϵ RI, and Mac1 (not shown). Therefore, our findings indicate that GATA-1-rescued G1ME cells are restricted to erythromegakaryocytic differentiation.

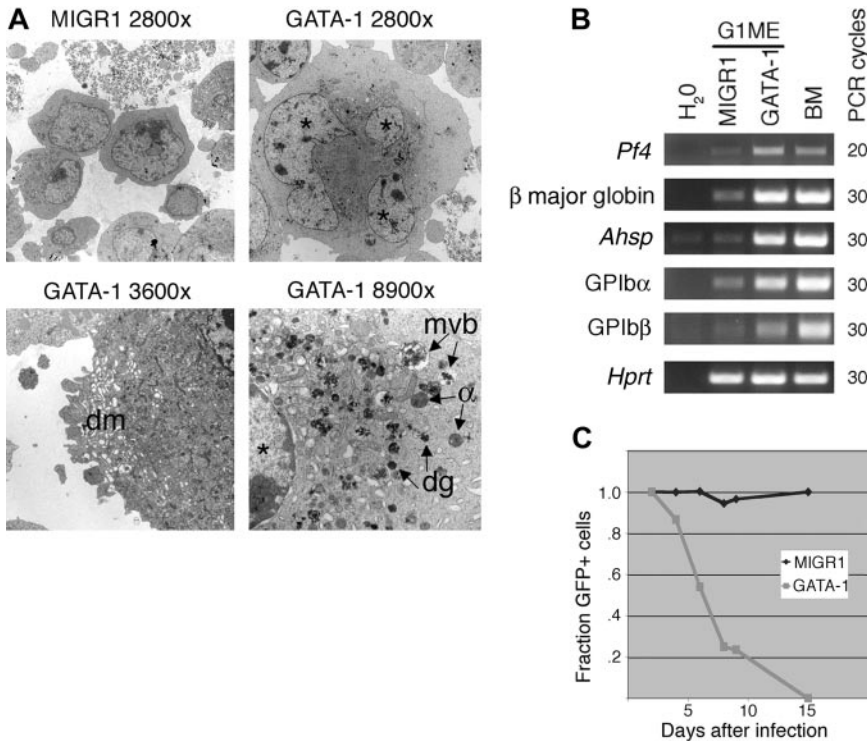


Figure 4. Erythromegakaryocytic maturation of G1ME cells. (A) Transmission electron microscopy demonstrating features of megakaryocytic maturation. Control (MIGR1-transduced) cells (top left) are small and lack features of differentiation. GATA-1-rescued cultures contain a population of large cells with megakaryocytic features, including dense chromatin around the periphery of the nuclei, large multilobed nuclei (*), multivesicular bodies (mvb), dense granules (dg), α granules (α), and a developing platelet-membrane demarcation system (dm). Original magnifications are indicated. Photographs were taken by using a microscope (208S transmission electron; Philips) equipped with a Hamamatsu digital camera (Hamamatsu, Japan) and analyzed with visualization software (Advanced Microscope Techniques). (B) RT-PCR analysis showing GATA-1 induction of both erythroid and megakaryocyte genes. Erythroid genes are β major globin and *Ahsp*. Megakaryocyte genes are platelet factor 4 (*Pf4*) and glycoprotein (GP) I α and I β . PCR cycle numbers for each primer pair are shown at the right side of the panel. (C) GATA-1 induces proliferation arrest as part of terminal maturation. Transduced cells were analyzed by flow cytometry for GFP expression. The fraction of GFP+ cells was normalized to 1.0 at 2 days after retroviral infection and then followed over time. GFP-expressing cells transduced by vector grew normally, whereas GATA-1-expressing cells were outgrown by noninfected cells.

Two possibilities could account for our gene complementation studies in G1ME cells. First, individual cells could exhibit bipotential megakaryocyte erythroid differentiation. Second, the cells could contain a mixture of committed erythroid and megakaryocyte precursors that undergo unilineage maturation after GATA-1 rescue. To distinguish these possibilities, we generated clonal lines from single cells by limiting dilution and tested their differentiation patterns after GATA-1 complementation (Table 1). Ten clonal lines tested all showed bipotential erythromegakaryocytic maturation, at proportions similar to what was observed in the original polyclonal parental G1ME line. Hence, G1ME cells represent developmentally blocked, immortalized progenitors with megakaryocytic and erythroid potential, similar to MEPs. Interestingly, these cells exhibit somewhat different properties than the MEP population identified previously in adult murine bone marrow⁴⁷ (see "Discussion").

Table 1. Analysis of GATA-1–induced erythromegakaryocytic maturation in 10 randomly selected G1ME-cell clones derived from *Gata1*[−] ES-cell differentiation

Clone no.	Ter119 positive, %		GPIb positive, %	
	MIGR1	GATA-1	MIGR1	GATA-1
1	3	24	13	61
2	5	17	18	71
3	4	18	7	56
4	1	19	11	55
5	4	19	13	77
6	3	15	8	75
7	5	18	15	77
8	2	17	11	63
9	1	10	7	62
10	3	19	10	61

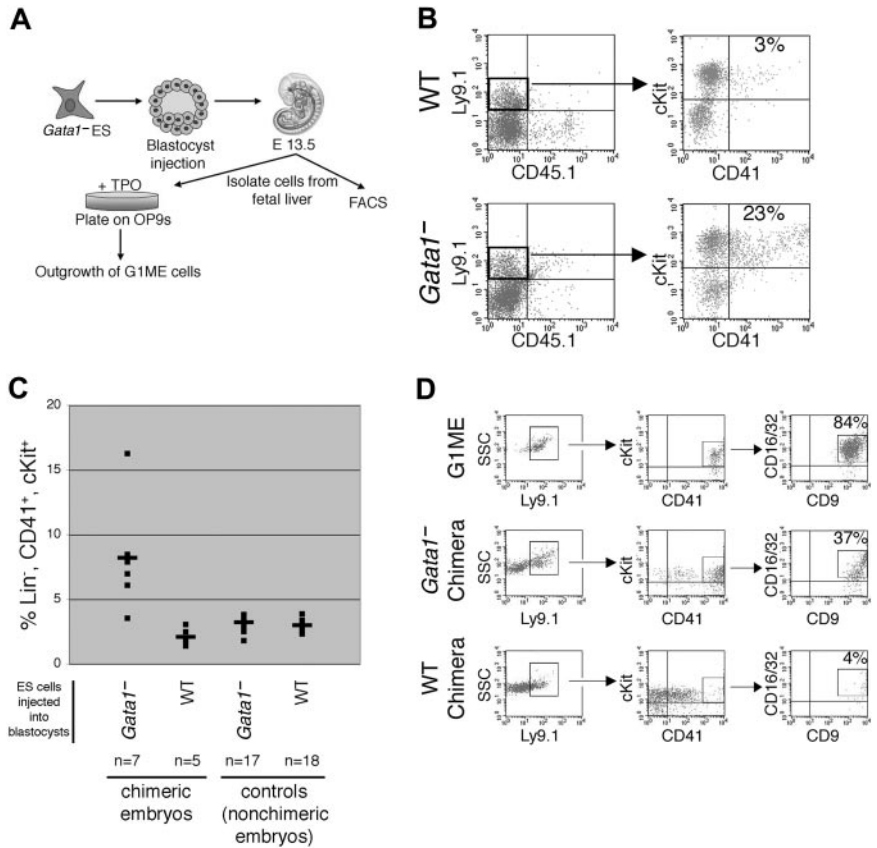
G1ME cells were maintained in liquid culture with Tpo. Individual clones were isolated by limiting dilution, expanded, and then infected with GATA-1 retrovirus or MIGR1 control. Ter119 and GPIb expression was assessed by flow cytometry, as described in Figures 3 and 7.

Arrested *Gata1*[−] megakaryocyte-erythroid progenitors in fetal liver

The isolation of G1ME cells specifically from *Gata1*[−] ES cells differentiated in vitro suggests a block to hematopoiesis at the MEP stage. To investigate whether this block also occurs in vivo, we analyzed fetal liver hematopoiesis in chimeric embryos prepared from blastocysts injected with *Gata1*[−] or control wt ES cells (Figure 5A). We chose to analyze this stage of hematopoietic ontogeny because in vitro differentiation of ES cells is believed to recapitulate stages of hematopoiesis that occur in the developing embryo.⁴² We used the polymorphic markers Ly9 and CD45 to distinguish donor ES-cell–derived from host-derived hematopoiesis. Specifically, hematopoietic cells generated from donor ES cells (strain 129) are Ly9.1⁺, CD45.1[−]. Host hematopoietic cells are Ly9.1[−], CD45.1⁺ (not shown). First, we used flow cytometry to analyze chimeric fetal livers for cells resembling the G1ME surface phenotype (Figure 5B-D). Fetal livers with cells contributed by *Gata1*[−] ES cells contained a significantly expanded population of donor-derived (Ly9.1⁺) hematopoietic cells that were lin[−], CD41⁺, and cKit⁺, similar to G1ME cells (Figure 5B). This expanded population was specific to chimeric embryos generated from *Gata1*[−] ES cells and not observed in wt chimeric fetal livers or nonchimeric fetal livers containing only host-derived cells (Figure 5C). Next, we lineage depleted fetal liver cells, expanded them in culture for 4 days on OP9 stroma with Tpo, and reanalyzed them using a more extensive panel of markers used previously to characterize early erythromegakaryocytic development.^{47,48} In the *Gata1*[−] chimeric livers, there was an approximate 10-fold expansion of Ly9.1⁺, lin[−], CD41⁺, cKit⁺, CD16/32(Fc γ R)^{lo}, CD9⁺ progenitors, the same immunophenotype as G1ME cells derived from in vitro ES-cell differentiation (Figure 5D). After only a few days in culture, the analogous population from fetal liver constituted a significant proportion of the total cells, reflecting their

Figure 5. Development of G1ME cells in *Gata1*⁻ chimeric embryos.

(A) Experimental approach. Chimeric embryos were prepared by injecting *Gata1*⁻ or wt ES cells into wt host blastocysts. Fetal liver hematopoietic cells from day 13.5 chimeric embryos were analyzed by FACS or cultured on OP9 cells with Tpo according to the same conditions used to derive G1ME cells from ES cells (Figure 1). The polymorphic cell-surface marker Ly9.1 was used to facilitate tracking of ES-cell donor-derived hematopoietic cells. Donor ES cells (strain 129) express Ly9.1 and CD45.2 (not shown), whereas host (C57/BL6) blastocyst-derived cells express Ly9.2 and CD45.1 (not shown). (B) Flow cytometry analysis of E13.5 fetal livers. *Gata1*⁻ chimeric embryos contain an expanded population of donor-derived (Ly9.1) cells that are lineage negative, CD41⁺, and cKit⁺, identical to the surface phenotype of G1ME cells derived from in vitro differentiation of *Gata1*⁻ ES cells, as described in Figure 1. Representative studies of fetal livers from wt and *Gata1*⁻ chimera are shown. The wt chimeric fetal liver was 30% Ly9.1⁺, and the *Gata1*⁻ chimeric fetal liver was 23% Ly9.1⁺. (C) CD41⁺ cKit⁺ cells as a percentage of Lin⁻ fetal liver cells. As indicated on the x-axis, embryos prepared from *Gata1*⁻ or wt ES-cell-injected blastocysts were termed chimeric only if donor-derived Ly9.1 hematopoietic cells were detected in fetal livers. Levels of chimerism ranged from 2% to 23% in *Gata1*⁻ chimeric animals, and 30% to 47% in wt chimeric fetal livers. (D) Additional cell-surface marker expression comparing G1ME cells (top) and E13.5 chimeric fetal livers (middle and bottom). Fetal livers from *Gata1*⁻ or wt chimeric embryos were analyzed 4 days after expansion on OP9 stroma. All cells shown are Lin⁻, IL7R α ⁻, and Sca1⁻. Left panels show the detection of donor cells (Ly9.1⁺). Middle panels show cKit and CD41 expression in Ly9.1⁺ cells; these are analyzed further for Fc γ R and CD9 expression in the right panels. Percentages in the right panels refer to Lin⁻, IL7R α ⁻, Sca1⁻, CD9⁺, Fc γ R^{lo}, CD41⁺, and cKit⁺ cells within the Ly9.1⁺ population.



relatively high proliferative capacity and growth advantage in the conditions used. Together, these findings demonstrate that *Gata1*⁻ stem cells give rise to a significantly expanded population of G1ME-like cells in the fetal liver. Note that relative to wt controls, the *Gata1*⁻ chimeric fetal livers were not visibly enlarged and did not contain significantly increased total cell numbers (see “Discussion”).

We assessed the proliferative capacity and longevity of wt and *Gata1*⁻ fetal liver-derived hematopoietic cells in culture under conditions identical to those used for in vitro differentiation of ES cells, as described in “Materials and Methods” and in Figure 1. After 12 days, chimeric fetal livers from wt and *Gata1*⁻ ES cells

contained numerous recognizable megakaryocytes (Figure 6A, first 2 panels). By day 40, cells within the *Gata1*⁻ cultures were predominantly CD41⁺, cKit⁺, exhibited a monomorphous blast morphology, and were Tpo dependent for proliferation and survival, similar to G1ME cells derived from in vitro differentiation of *Gata1*⁻ ES cells (Figure 6A right panel, and not shown). In contrast, no live cells were present in cultures of nonchimeric or wt ES-cell-chimeric fetal livers. These findings were reproduced in 7 *Gata1*⁻ chimeric fetal livers and 40 negative controls (Table 2). Thus, G1ME-like blast cells with extensive proliferative capacity were derived exclusively from fetal livers containing *Gata1*⁻ hematopoietic progenitors.

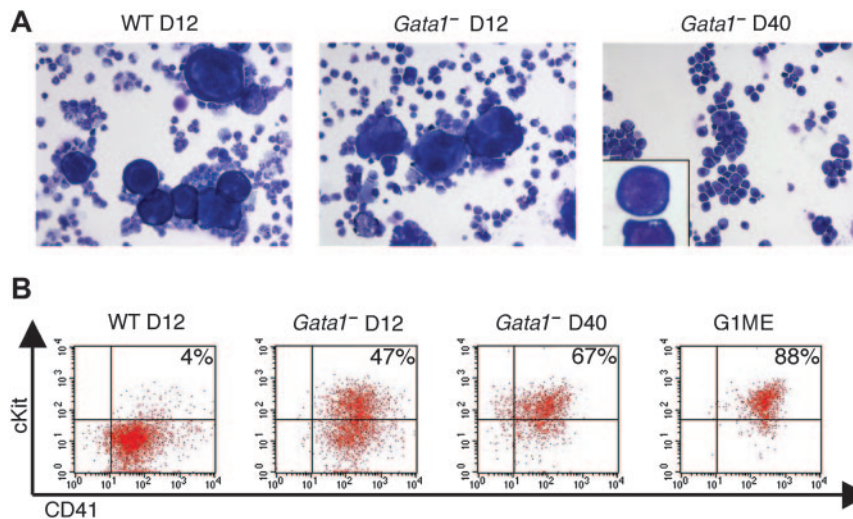


Figure 6. Expansion of fetal liver-derived G1ME cells in long-term culture. (A) Cell morphologies at various times in culture (May-Grünwald-Giemsa stain). Chimeric fetal livers were cultured on OP9 stroma with Tpo, according to the strategies in Figures 1 and 5. At day 12, a population of large, multinucleated megakaryocytes was visible in both wt and *Gata1*⁻ cultures. By day 40, only the *Gata1*⁻ cultures were growing and contained predominantly immature blasts (right) with morphology similar to ES-cell-derived G1ME cells (compare with Figure 1C). Original magnification, $\times 200$; inset, $\times 630$. Photographs were taken by using a microscope (Axioskop 2; Carl Zeiss) equipped with a color digital camera (AxioCam; Carl Zeiss). (B) Flow cytometry analysis of the cultures in panel A. The hematopoietic blasts from *GATA1*⁻ chimeric fetal liver cultures are predominantly cKit⁺ CD41⁺. These cells are also Ly9.1⁺ (data not shown), indicating that they are *Gata1*⁻ donor ES cell-derived.

Table 2. Long-term viability of E13.5 fetal liver-cell cultures in culture

Fetal liver	Viability at day 40, no./total
<i>Gata1</i> ⁻ ES chimeric	7/7
wt ES chimeric	0/5
Nonchimeric	0/35

Fetal liver cells from *Gata1*⁻ or wild-type (wt) chimeric embryos were harvested at E13.5 and cultured in Tpo according to conditions described in Figures 1 and 5.

Next, we tested the developmental capacity of fetal liver-derived G1ME cells by assessing their maturation after complementation with GATA-1–encoding retrovirus. We observed distinctive erythroid and megakaryocytic maturation, both in bulk populations of rescued fetal liver G1ME cells (Figure 7A-C) and in 5 of 5 clonal lines generated by limiting dilution (Table 3). In each of these fetal liver lines, the ratio of megakaryocyte-erythroid maturation was similar to that observed in GATA-1–rescued G1ME lines generated from ES cells. G1ME lines derived from *Gata1*⁻ chimeric fetal livers behaved identically to those generated from in vitro differentiation of ES cells in all respects, including cell morphology, surface immunophenotyping, gene-expression profiles, and the capacity for GATA-1–induced erythromegakaryocytic maturation (Figure 7; Table 3; not shown). Together, our data demonstrate that loss of GATA-1 causes developmental arrest and proliferation of an early Tpo-dependent hematopoietic progenitor with erythroid and megakaryocytic potential. This abnormal progenitor, termed G1ME, is present in ES differentiation cultures and in embryos.

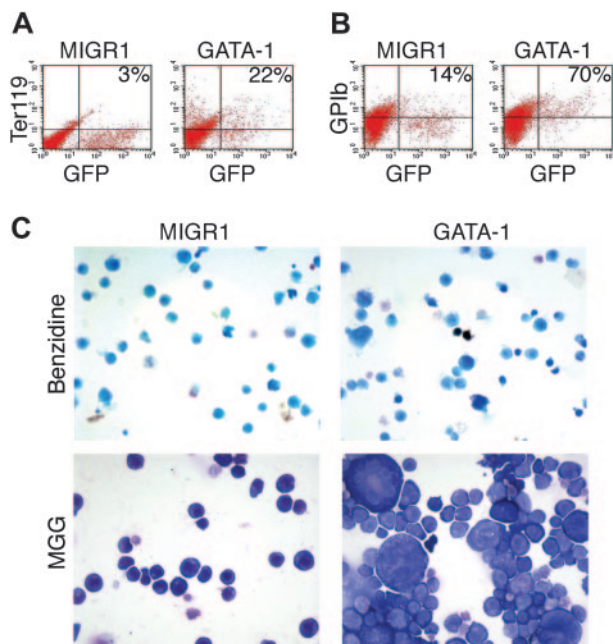


Figure 7. GATA-1 complementation induces erythromegakaryocytic maturation in *Gata1*⁻ fetal liver chimera–derived G1ME cells. Cells were transduced with GATA-1 or control retrovirus and analyzed for maturation as described in Figure 3. (A) Ter119 expression after rescue by GATA-1. Percentages in top panels refer to fraction of GFP⁺ cells expressing Ter119. (B) GPIb expression after rescue by GATA-1. Percentages in panels refer to fraction of GFP⁺ cells expressing GPIb. (C) Cell morphology after retroviral expression; (top) benzidine stain for hemoglobin, (bottom) May-Grünwald-Giemsa staining. Original magnification, $\times 400$. Photographs were taken by using a microscope (Axioskop 2; Carl Zeiss) equipped with a color digital camera (AxioCam; Carl Zeiss).

Table 3. Analysis of GATA-1-induced erythromegakaryocytic maturation in 5 randomly selected G1ME clones derived from E13.5 *Gata1*⁻ chimeric fetal livers

Clone No.	Ter119 positive, %		GPIb positive, %	
	MIGR1	GATA-1	MIGR1	GATA-1
1	8	18	31	72
2	10	31	20	63
3	5	22	14	70
4	8	30	18	50
5	11	37	33	77

G1ME cells derived from E13.5 fetal livers were cultured in the same conditions that were used to derive G1ME cells from *Gata1*⁻ ES cells, as described in Figure 1. Fetal liver G1ME clones were isolated by limiting dilution, expanded, and then infected with GATA-1 retrovirus or MIGR1 control. Ter119 and GPIb expressions were assessed by flow cytometry, as in Figures 3 and 7.

Discussion

GATA-1 is critical for normal red blood cell and platelet formation; in its absence, committed erythroid precursors are blocked in maturation and undergo apoptosis, whereas megakaryocytes mature inefficiently and proliferate excessively.^{7,10,56} Previous studies also hint at important but poorly understood functions for GATA-1 at earlier points in the development of these 2 lineages.^{21,29-31,57,58} Here, we report that *Gata1*⁻ ES cells give rise to an abnormal blast population with high-proliferative capacity that undergoes erythromegakaryocytic maturation when GATA-1 is restored. These cells, termed G1ME, were detected in chimeric embryos and cultured in vitro for more than 6 months. G1ME cells grown from primary ES cells or *Gata1*⁻ chimeric fetal liver reproducibly expanded logarithmically in vitro without lag or crisis, indicating that their derivation stemmed directly from absent GATA-1. Our results illustrate a new block to hematopoiesis caused by loss of GATA-1 and provide an important basis for interpreting hematopoietic abnormalities that arise from reduced GATA-1 expression or altered protein function.

G1ME cells approximate an early stage of erythromegakaryocytic development

The capacity for G1ME cells to undergo megakaryocytic and erythroid maturation exclusively suggests that they are arrested at the MEP stage of hematopoiesis, although other interpretations are possible. Enforced GATA-1 expression can induce erythromegakaryocytic differentiation by reprogramming common myeloid (CMPs), granulocyte/monocyte (GMPs), and common lymphoid progenitors (CLPs).¹⁻⁴ However, markers that distinguish murine CMPs, GMPs, or CLPs are not expressed in G1ME cells (Figure 2). The constellation of cell-surface proteins and transcription factors expressed by these cells most closely approximates MEPs or MKPs.^{47,48} In regards to the latter, CD9 expression is believed to mark the maturation of MEPs to unipotent MKPs.⁴⁷ However, we found that G1ME cells express CD9 strongly, yet exhibit bilineage differentiation on rescue by GATA-1. Several interpretations can explain this apparent contradiction. First, CD9 was defined as a marker for megakaryocyte commitment in adult bone marrow. G1ME cells derive from fetal tissues, which contain different hematopoietic populations than adults.^{25,59,60} Hence, it is possible that G1ME cells represent a distinct fetal progenitor in which expression of CD9 occurs at a transitional stage from MEP to MKP when erythroid potential is retained. Second, G1ME cells may represent committed MKPs that are reprogrammed to a partial erythroid fate by ectopic GATA-1 expression. Finally, loss of

GATA-1 may generate an abnormal bipotential CD9⁺ progenitor that does not occur during normal hematopoiesis. Aberrant progenitors with unique signatures of gene expression and developmental potential are observed frequently in leukemias arising from altered transcription factor function.⁶¹ In any case, loss of GATA-1 generates abnormal, immortalized, Tpo-dependent blasts with the capacity for both erythroid and megakaryocytic maturation.

Multiple blocks to *Gata1*⁻ hematopoiesis

The most likely interpretation for the current findings is that GATA-1 is required for normal maturation of an MEP-like progenitor, particularly in light of prior studies that also support this possibility.^{21,29-31,57,58} This role for GATA-1 is different than its previously demonstrated requirements in the maturation of lineage-committed erythroblasts and megakaryocytes. According to the classic model for hematopoiesis, CMPs give rise to MEPs, which then differentiate into committed erythroid and megakaryocyte precursors (Figure 8A). In the context of this model, the block to MEP maturation demonstrated in this study must be partial, because recognizable erythroblasts and immature megakaryocytes are generated from *Gata1*⁻ hematopoietic stem cells in ES-cell

cultures and in mice^{5,7,10,46} (see also Figure 1C). However, recent evidence suggests that alternate developmental pathways for erythromegakaryocytic maturation also exist.⁶² For example, MEPs could also arise from hematopoietic stem cells independent of CMPs. If MEPs are formed simultaneously from parallel independent pathways, then one of these could be selectively and fully blocked by loss of GATA-1, with mutant erythroblasts and megakaryocytes generated through alternative mechanisms (Figure 8B).

Dosage effects of GATA-1

Compared with complete ablation, partial loss of GATA-1 function tends to be more effective at promoting the accumulation of mutant hematopoietic cells in vivo. Mice that are heterozygous for an X-linked *Gata1* hypomorphic mutation contain a population of GATA-1-deficient progenitors generated by lyonization of the intact allele. It is believed that low-level GATA-1 expression permits survival but not maturation or proliferation arrest of the mutant progenitors, supporting the development of late-onset myelodysplasia, myelofibrosis, and leukemias.^{29-32,57} In contrast, these disorders are not observed to occur in female *Gata1*^{+/-} mice, which presumably contain a population of GATA-1-null progenitors generated by lyonization.^{5,31} Moreover, although our findings show that arrested G1ME-type cells are present in fetal liver of *Gata1*⁻ chimeric embryos, there is no organ enlargement or obvious accumulation of blasts detected histologically (not shown). Therefore, the expansion of arrested *Gata1*⁻ G1ME blasts in vivo is somehow restricted, perhaps by limiting cytokines and/or a suboptimal microenvironment. It is also possible that the early block to erythromegakaryocytic maturation described here is limited to fetal progenitors, resembling the unique population that is sensitive to the growth-promoting effects of GATA-1s.²⁵

Implications for TMD/AMKL

Recent studies suggest that the N-terminal-deleted form of GATA-1 (GATA-1s) associated with TMD/AMKL in Down syndrome acts as a dominant oncoprotein that drives megakaryocyte proliferation but retains the ability to promote terminal maturation.^{25,26} However, TMD/AMKL leukemic stem cells likely derive from a multipotential progenitor, prior to the development of recognizable megakaryocytes.⁶³⁻⁶⁵ One model to incorporate these findings is that additional abnormalities, including increased dosage of one or more genes on chromosome 21 and other unknown mutations, are required to confer a differentiation block that cooperates with the mitogenic activities of GATA-1s to promote malignant transformation. G1ME cells, which express no GATA-1, resemble TMD/AMKL blasts in several respects, including their fetal origin, erythromegakaryocytic developmental potential, and extensive self-renewal capacity. Given these similarities, it is possible that critical functions of GATA-1 related to MEP maturation are deficient in TMD/AMKL. In this case, mutations in *GATA1* that promote TMD/AMKL are predicted to act through at least 2 mechanisms: by producing an altered protein, GATA-1s, which acts dominantly to promote cell proliferation, and as hypomorphic alleles that fail to adequately promote GATA-1-dependent functions related to MEP maturation. The latter defect may stem from qualitative abnormalities in GATA-1s, failure to express the protein to adequate levels, or both. In considering this model, it is important to note that although loss of selected GATA-1 functions may contribute to maturation arrest of early erythromegakaryocytic progenitors in TMD/AMKL, it is not sufficient, because these

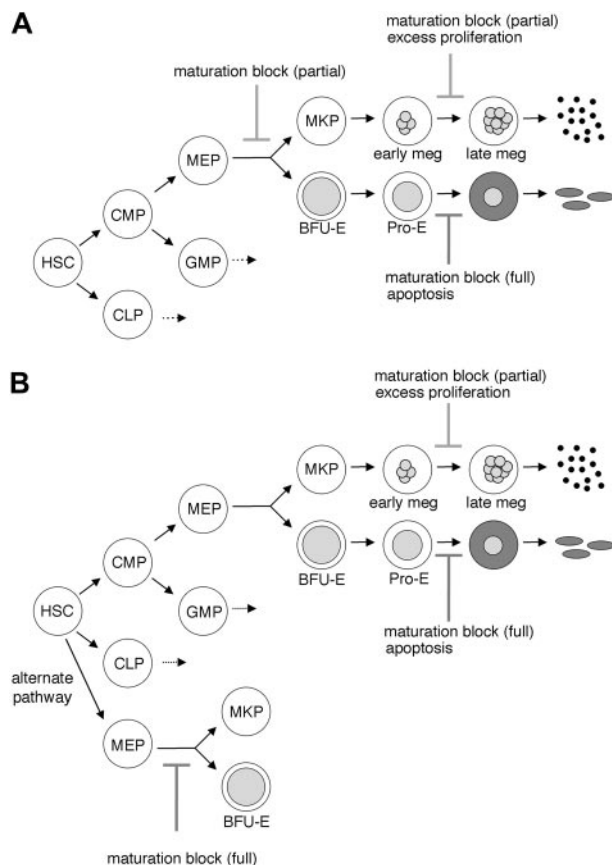


Figure 8. Models for GATA-1 actions in MEPs. (A) The classic model for hematopoiesis in which MEPs derive from the CMP. Loss of GATA-1 generates recognizable, developmentally arrested megakaryocytes and committed erythroid precursors. Therefore, the earlier stage block to differentiation of *Gata1*⁻ MEPs must be partial, because some downstream progeny are produced. GATA-1 is also required for eosinophil and mast-cell development (not shown). (B) Newer models for hematopoiesis indicate that alternate pathways may exist for MEP production. If multiple pathways exist simultaneously, then loss of GATA-1 may cause a complete block to one of these pathways with mutant erythroblasts and megakaryocytes generated through coexisting ones. HSC indicates hematopoietic stem cell; BFU-E, erythroid burst-forming unit; Pro-E, proerythroblast; meg, megakaryocyte; and CLP, common lymphoid progenitor.

progenitors do not accumulate to pathologic levels in *Gata1*^{+/-} embryos, *Gata1*⁻ chimeric embryos, or the corresponding adult progeny (see discussion at “Dosage effects of GATA-1”).

A new tool for the study of erythromegakaryocytic development

Several years ago, we generated a developmentally arrested *Gata1*⁻ proerythroblast line, termed G1E, that exhibits GATA-1–dependent erythroid maturation.⁵⁵ G1E cells provide a useful tool for examining various GATA-1–dependent aspects of erythropoiesis.⁶⁶ Compared with G1E cells, the G1ME cells described here are distinctly different in their mode of derivation, cytokine requirements, gene-expression profiles, and developmental potential. In particular, their capacity to undergo erythromegakaryocytic maturation places them at an earlier stage of hematopoiesis. Little is known about the mechanisms that control the maintenance and differentiation of MEPs. The current findings demonstrate a role for GATA-1 in these processes. Moreover, the ability to expand pure cultures of G1ME cells virtually indefinitely provides a new tool for investigating MEP and early megakaryocyte biology.

Note added in proof: In agreement with the current study, Kuhl et al⁶⁷ published a paper showing that megakaryocyte erythroid progenitors are significantly expanded in the fetal livers of mice with a hypomorphic *Gata1* allele.

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