Graded Levels of GATA-1 Expression Modulate Survival, Proliferation, and Differentiation of Erythroid Progenitors*

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Transcription factor GATA-1 plays an important role in gene regulation during the development of erythroid cells. Several reports suggest that GATA-1 plays multiple roles in survival, proliferation, and differentiation of erythroid cells. However, little is known about the relationship between the level of GATA-1 expression and its nature of multifunction to affect erythroid cell fate. To address this issue, we developed in vitro embryonic stem (ES) culture system by using OP9 stromal cells (OP9/ES cell co-culture system), and cultured the mutant (GATA-1.05 and GATA-1-null) and wild type (WT) ES cells, respectively. By using this OP9/ES cell co-culture system, primitive and definitive erythroid cells were developed individually, and we examined how expression level of GATA-1 affects the development of erythroid cells. GATA-1.05 ES-derived definitive erythroid cells were immature with the appearance of proerythroblasts, and highly proliferated, compared with WT and GATA-1-null ES-derived erythroid cells. Extensive studies of cell cycle kinetics revealed that the GATA-1.05 proerythroblasts accumulated in S phase and expressed lower levels of p16INK4A than WT ES cell-derived proerythroblasts. We concluded that GATA-1 must achieve a threshold activity to achieve selective activation of specific target genes, thereby influencing the developmental decision of an erythroid progenitor cell to undergo apoptosis, proliferation, or terminal differentiation.

Transcription factor GATA-1 recognizes conserved GATA motifs ((T/A)GATA(A/G)) in the regulatory regions of many genes encoding erythroid-restricted proteins, such as globins, heme biosynthetic enzymes, membrane proteins, and transcription factors (1, 2). To analyze the in vivo function(s) of GATA-1, GATA-1-deficient mice were generated (3). Disruption of primitive erythropoiesis caused GATA-1 homozygous null mutant embryos to die by embryonic day (E)11.5, demonstrating that GATA-1 is required for primitive erythropoiesis. This early demise precluded the possibility of analyzing the role of GATA-1 in definitive erythropoiesis. To experimentally circumvent this impediment, chimeric mice were derived using GATA-1-null ES cells, and this confirmed that the null mutant cells did not contribute to the mature definitive erythroid pool (4). Thus, GATA-1 is required for the terminal differentiation of both primitive and definitive erythroid progenitors.

We previously utilized an erythroid promoter-specific loss-of-function (knockdown) strategy to generate a GATA-1-hypomorphic (GATA-1.05) allele (5). The GATA-1 gene is X-linked, and in GATA-1 hemizygous hypomorphic males (GATA-1.05/Y), GATA-1 mRNA was detected at ~5% of the normal levels. Hence, we termed these mice GATA-1.05 (6). GATA-1.05/Y embryos exhibited a defective maturation of primitive erythroid cells and died by E12.5 (6), which, like GATA-1–/– embryos, precluded the analysis of the function of GATA-1 in definitive erythropoiesis.

Impaired primitive and definitive erythropoiesis in both GATA-1-null and hypomorphic mutant embryos resulted in the generation of extremely limited numbers of erythroid progenitors that could be used for further cytological and molecular analyses. Additionally, we suspected that defective erythropoiesis in the mutant embryos could cause secondary growth retardation, which would in turn affect later hematopoietic development. Under such circumstances where cell-autonomous as well as non-cell-autonomous deficiencies could contribute to the phenotype, it becomes difficult to determine conclusively in vivo how different quantitative levels of GATA-1 may affect the developmental decisions available to an erythroid progenitor cell.

The generation of homogenous erythroid populations from ES cells (7) is a useful experimental tool for analyzing the definitive erythropoiesis in instances where gene-targeted mutation leads to embryonic death prior to the onset of definitive erythropoiesis. In a two-step ES cell in vitro differentiation method, the ES cells are cultured in methylcellulose medium containing stem cell factor and erythropoietin (Epo) (7). Alternatively, if ES cells are co-cultured with OP9 stromal cells, they differentiate into a hematopoietic cell population that consists of erythrocytes, neutrophils, macrophages, mast cells, megakaryocytes, and lymphoid cells (8). Subsequently,
Nakano et al. (9) developed the ES/OP9 cell co-culture system, in which two waves of erythroid (primitive and definitive) cell production were detected after either 6 or 14 days of induction, respectively. Consequently, the ES/OP9 cell co-culture system reflects not only primitive, but also definitive, erythropoiesis in vivo and is a useful tool for dissecting in vitro the functional role of any molecule of interest during erythroid development.

Here, we report that both differentiation and apoptosis are inhibited in GATA-1-definitive erythroid cells. Although GATA-1/primitive definitive erythroid cells are similarly arrested in differentiation, they, unlike the GATA-1.05 cells, preferentially undergo apoptosis. Hence, we propose that although normal levels of GATA-1 promote terminal differentiation, the low level of intracellular GATA-1 is insufficient to block the primitive progenitor proliferation but is sufficient to prevent apoptosis. In contrast, the complete absence of GATA-1 favors an apoptotic response. In this way, graded levels of transcription factor GATA-1 modulate multiple facets of erythroid cell physiology, including survival, proliferation, and differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—E14 ES cells were maintained on embryonic fibroblast cells and kept undifferentiated in the presence of recombinant leukemia inhibitory factor (1000 units/ml, ESgro, Chemicon International). OP9 cells were cultured as described previously (8). After the harvest of ES cells from OP9 feeder cells by trypsinization, 7 × 10⁶ cells were plated onto subconfluent OP9 cells grown in α-minimum essential medium supplemented with 10% FBS, mouse vascular endothelial growth factor (10 ng/ml, Peprotech), and human bone morphogenic protein-4 (5 ng/ml, R&D systems). After 4 days of co-culture, ES cells were trypsinized and replated onto fresh OP9 cells in α-minimal essential medium supplemented with 10% FBS, Epo (2 units/ml; generous gift from Chugai Pharmaceutical), and stem cell factor (50 ng/ml). Cobblestone-like colonies, termed CFU-OP9, were confirmed by RT-PCR. Floating cells were removed from 11-day ES/OP9 cell cultures confirmed to be primitive and definitive erythroid cells from days 6–8 and 11–14, respectively (11). Therefore, WT, GATA-1.05, and GATA-1-null ES cells were cultured on OP9 cells for 11 days, and is a useful tool for dissecting in vitro the functional role of any molecule of interest during erythroid development.

**Construction of Retrovectors and Virotransduction**—Plasmid for murine stem cell virus internal-ribosomal entry site-enhanced green fluorescent protein (MSCV-IRES-EGFP) was kindly provided by Dr. Akiko Kume. Murine GATA-1 and p16INK4A cDNAs were independently ligated into the BamHI and EcoRI restriction sites of MSCV-IRES-EGFP. Phoenix-Eco virus was generated using the packaging cell lines PHAGE-L packaging cells (Clontech) in the presence of 8 μg/ml Polybrene for 2 days. The cultures were washed once with washing buffer, and non-adherent cells were washed with washing buffer, and non-adherent cells were removed from 11-day ES/OP9 cell cultures to discard the virus in the presence of 8 μg/ml Polybrene (Sigma). After 4 days, FACS analysis for enhanced green fluorescent protein fluorescence was performed to ensure that the cultures used in this study were >1 × 10⁶ infectious particles/ml.

To establish retroviral packaging cell lines, supernatant from transduced Phoenix-Eco cells was harvested and used to infect PT67 cells (Clontech) in the presence of 8 μg/ml Polybrene. After 2–4 days, the brightest green fluorescent protein-expressing cells were sorted using a FACS Vantage and expanded in culture. The expression of GATA-1 and p16INK4A in NIH3T3 cells infected with viral supernatants was confirmed by RT-PCR. Floating cells were removed from 11-day ES/OP9 co-culture, and adherent cells on OP9 stromal cells were trypsinized. After a 1-h incubation on the dish to remove stromal cells, non-adherent cells were replated onto fresh OP9 cells and co-cultured in the presence of the viral supernatant with 4 μg/ml Polybrene for 2 days. The cultures were washed once with washing buffer, and non-adherent cells were selected with 10% FBS containing Epo and stem cell factor. The number of colonies that developed on OP9 stromal cells was scored. Floating cells were harvested and cytospin samples were stained with May-Grunwald-Giemsa to verify the differentiation stage of erythroid cells.

**RESULTS**

**Definitive Erythroid Differentiation of GATA-1.05 and GATA-1-null ES Cells**—We previously reported that differentiation in GATA-1.05/Y primitive and definitive erythroid cells was blocked at different stages thereby implicating different requirements for GATA-1 levels during erythroid development in distinct hematopoietic organs, such as the yolk sac and fetal liver (6). However, there is no clear molecular explanation for how varied GATA-1 levels might differentially affect the development of the primitive and definitive erythroid lineages. To address this question, ES cells carrying WT, GATA-1.05, and GATA-1-null alleles were separately co-cultured with OP9 feeder cells, and the resultant differentiated primitive and definitive erythroid cells were harvested for further analyses.

Morphological and gene expression analyses confirmed that ES cells cultured on an OP9 feeder layer differentiated into primitive and definitive erythroid cells from days 6–8 and 11–14, respectively (11). Therefore, WT, GATA-1.05, and GATA-1-null ES cells were cultured on OP9 cells for 11 days,
and the non-adherent fractions were then subjected to flow cytometric analyses using anti-c-Kit and TER-119 antibodies (Fig. 1, A and B). Mature definitive erythroid cells (c-Kit/TER119<sup>+</sup>) are most abundant in GATA-1 mutant ES cell culture, whereas c-Kit/TER119<sup>+</sup> mature erythroid cells (striped) were highly represented in the WT ES cell culture. (*, p < 0.01; APC, allophycocyanin. C, non-adherent, definitive erythroid cells from each ES cell cultures were scored on day 11 of co-culture using trypan-blue dye exclusion. GATA-1-null ES cells gave the lowest cell number (black column). *, p < 0.01. D, number of adherent cells in ES cell cultures counted on day 11. Two independent GATA-1.05 ES cell cultures (gray) had ~2-fold more cells than the WT or GATA-1-null culture. (*, p < 0.01. E, after 11 days of co-culture, adherent hematopoietic cells on OP9 cells were trypsinized and replated on new OP9 feeder cells. CFU-OP9 colonies were counted 5 days later. Proliferative potential was, again, highest in the GATA-1.05 (G1.05) ES cells. (*, p < 0.01. FSC, forward scatter; SSC, side scatter.

Elevated Apoptosis in GATA-1-null Erythroid Cells—May-Grünwald-Giemsa staining revealed that WT ES cells after 6 days in co-culture contained primitive erythroid cells with orthochromatic cytoplasm and large nuclei, whereas immature blast cells with large, prominent nuclei and polychromatic cytoplasm were present in cultures that developed from both kinds of GATA-1 mutant ES cells (Fig. 2, A–C). Uptake of trypan blue dye (indicating dead or dying cells) and nuclear fragmentation (apoptotic intermediates) were most frequently

FIG. 1. Characteristics of definitive erythroid cells differentiated from WT or mutant GATA-1 ES cells. A and B, flow cytometric analysis of WT, GATA-1.05, and GATA-1-null (G1-null) definitive erythroid cells using hematopoietic cell surface markers. c-Kit/TER119<sup>+</sup> erythroblasts (hatched) are most abundant in GATA-1 mutant ES cell culture, whereas c-Kit/TER119<sup>+</sup> mature erythroid cells (striped) were highly represented in the WT ES cell culture. (*, p < 0.01; APC, allophycocyanin. C, non-adherent, definitive erythroid cells from each ES cell cultures were scored on day 11 of co-culture using trypan-blue dye exclusion. GATA-1-null ES cells gave the lowest cell number (black column). *, p < 0.01. D, number of adherent cells in ES cell cultures counted on day 11. Two independent GATA-1.05 ES cell cultures (gray) had ~2-fold more cells than the WT or GATA-1-null culture. (*, p < 0.01. E, after 11 days of co-culture, adherent hematopoietic cells on OP9 cells were trypsinized and replated on new OP9 feeder cells. CFU-OP9 colonies were counted 5 days later. Proliferative potential was, again, highest in the GATA-1.05 (G1.05) ES cells. (*, p < 0.01. FSC, forward scatter; SSC, side scatter.

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observed in the GATA-1-null ES cell culture (Fig. 2, D and F).

WT ES cells, after 11 days in co-culture, contained definitive erythroid cells at various differentiation stages, whereas immature blast cells with large, prominent nuclei were the dominant species recovered in both of the GATA-1 mutant ES cell cultures (Fig. 2, G–I). Using benzidine staining to distinguish terminally differentiated erythroid cells, we noted that the benzidine-positive population was significantly reduced in both GATA-1 mutant, but not in GATA-1/+ ES cell cultures (WT, 48.7 ± 14.9%; GATA-1.05, 8.1 ± 1.8%; GATA-1-null, 0.4 ± 0.1%). Furthermore, at day 11 of differentiation both trypan blue dye uptake and nuclear fragmentation were observed at the highest frequency in the GATA-1-null ES cell culture (Fig. 2, J–L), indicating unusually high apoptotic activity in GATA-1−/− erythroid progenitors.

Taken together, these data indicate that although the low GATA-1 level (5% in the GATA-1.05 mutant cells) is sufficient to avert apoptosis, higher GATA-1 expression levels are necessary to induce terminal erythroid maturation. This observation is consistent with the previous report that GATA-1 could act as a survival factor in committed erythroid cells (12), although the GATA-1 expression level was not determined in that study. Given the intriguing differences observed in ES cells that express graded levels of GATA-1 when exposed to differentiation stimuli, we investigated the activity of candidate GATA-1 target genes during erythroid proliferation versus differentiation.

Bcl-xL Is Highly Expressed in GATA-1.05 ES Cell-derived Definitive Erythroid Progenitors—It has been reported that Epo cooperates with GATA-1 to stimulate Bcl-xL gene expression and to maintain erythroid survival, and that Bcl-xL is essential for normal erythroid differentiation (13). Thus, Bcl-xL seems to be a critical downstream effector of GATA-1 and/or Epo-mediated signals. We therefore investigated the expression of several Bcl-2 family members, including Bcl-xL, in primitive and definitive erythroid populations recovered from ES/OP9 cultures (Fig. 3).

Although Bcl-2 mRNA was absent in GATA-1-null primitive erythroid cells, it was present at similar levels in definitive erythroid cells differentiated from all three types of ES cells. Bcl-xL expression was not detected in primitive erythroid cells (at day 6) from both GATA-1 mutant ES cell cultures. Bcl-xL expression was also not found in GATA-1-null definitive erythroid cells. Remarkably, however, a slightly higher than normal Bcl-xL mRNA level was observed in GATA-1.05 definitive erythroid cells. These findings strongly suggest that Bcl-xL, but not Bcl-2, is important for cell survival during definitive erythroid differentiation and that low levels of GATA-1 may be adequate for inducing ROM, expression to protect against apoptosis.

In contrast, the expression of apoptotic inducers, such as Bax, remained constant during primitive and definitive erythroid differentiation. The stabilization and accumulation of the tumor suppressor protein, p53, has also been shown to contribute to apoptosis. We therefore examined p53 accumulation in GATA-1-mutant erythroid cells. Lower p53 expression was detected in primitive erythroid cells from both GATA-1.05 and GATA-1-null cells compared with WT cells, underscoring the possibility that the apoptosis observed in primitive erythroid GATA-1-null cells is independent of p53, as reported previously (12).

Erythroid Cells Derived from GATA-1 Mutant ES Cells Accumulate in S Phase—It has been reported that forced expression of GATA-1 alters the length of cell cycle segments (14) and that especially high levels of GATA-1 were found to lengthen S phase in NIH3T3 cells (15). In addition, Cullen et al. (16) showed that GATA-1 activity in mouse erythro leukemia cells was low in G1 phase, peaked in mid-S phase, and then dimin-
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Fig. 3. Expression profiles of genes involved in apoptosis in erythroid cells expressing varying GATA-1 levels. Total RNA isolated from primitive and definitive erythroid cells harvested from day 6 and day 11, respectively, of co-culture was analyzed by RT-PCR (lower (A) and higher PCR cycles (B)). Compared with WT-definitive erythroid cells, Bcl-xL expression was undetected in GATA-1-null (GATA-1-null) definitive erythroid cells, but was higher in the GATA-1.05 (GATA-1.05) cell equivalents. HPRT, hypoxanthine quanine phosphoribosyl transferase; ddw, deionized distilled water.

GATA-1 but Not p16INK4A Induces Differentiation of GATA-1.05 ES Cell-derived Erythroid Progenitors—To determine the role of p16INK4A and GATA-1 in definitive erythroid maturation, GATA-1.05 erythroid progenitors were infected with retroviruses carrying p16INK4A or GATA-1 cDNAs (Fig. 5A). Adherent GATA-1.05 erythroid progenitor cells were harvested at day 11 and reseeded onto fresh OP9 cells together with supernatant containing no virus or containing viruses with wild type or modified genomes. We then scored the number of CFU-OP9, which represented immature cell populations. GATA-1- and p16INK4A virus-infected cells were suppressed in colony formation (Fig. 5B), consistent with previous reports of cell cycle arrest (15, 20).

Morphological examination of the non-adherent fractions from GATA-1- and p16INK4A cultures indicated the presence of more mature erythroid cells as compared with control cultures infected with no or vector-alone virus (Fig. 5C). Compared with the vector alone (Fig. 5C, striped column), the frequency of proerythroblast and basophilic erythroblasts diminished (gray column), whereas the polychromatophilic and orthochromatophilic erythroblasts populations increased in the GATA-1-infected culture.

In contrast to GATA-1-infected cells, p16INK4A-infected cells did not undergo extensive erythroid maturation (Fig. 5C), whereas erythroid-specific aminolevulinate synthase mRNA, a marker of terminal erythroid differentiation, was slightly up-regulated in p16INK4A-infected cells compared with the vector control (Fig. 5D). These results suggest that p16INK4A induces, albeit marginally, erythroid differentiation. Interestingly, p16INK4A expression was up-regulated in GATA-1-infected erythroid cells, but the reciprocal was not seen in that GATA-1 did not increase in p16INK4A-infected cells suggesting that GATA-1 regulates, directly or indirectly, p16INK4A expression.

To examine whether GATA-1 directly activates p16INK4A gene transcription, we constructed a reporter plasmid bearing 3.9 kbp of the 5′-promoter proximal sequences from the murine p16INK4A gene, and co-transfected it with a GATA-1 expression plasmid into QT6 cells. Despite the presence of three GATA consensus sequences within the 3.9-kbp DNA fragment, forced expression of GATA-1 failed to enhance reporter gene activity significantly (data not shown). However, co-transfection of FOG-1 cDNA along with GATA-1 cDNA increased the reporter activity to ~2.5-fold compared with the control vector transfection (data not shown). Thus, although the increase of reporter gene expression was not so significant as compared with the previously observed GATA-1-mediated increase of the reporter gene expression (for instance see Ref. 21), nonetheless these data further support the contention that the p16INK4A gene is
one of the targets of GATA-1-mediated transcriptional regulation. The relatively weak induction might be because of the presence of multiple regulatory influences within the 3.9-kbp genomic region. Alternatively, the 3.9-kbp fragment used in this experiment might be missing some important motifs cooperating with GATA-1 for the regulation of p16INK4A gene.

Expansion of c-Kit-positive Cells in the Fetal Livers of GATA-1.05/0.05 Mutant Embryos—Because the GATA-1 gene is subjected to random inactivation of the X chromosome (i.e. lyonization), chimeric GATA-1 expression occurs in the somatic tissues of GATA-1.05 heterozygous (GATA-1.05/0.05) female mice. To gain insight into the effect of GATA-1 on definitive erythroid maturation in vivo, we examined the fetal livers of GATA-1.05/0.05 mice by flow cytometry (Fig. 6). Although the majority of E12.5–E14.5 fetal liver cells from WT embryos were c-Kit-TER-119+ (Fig. 6A), the frequency of erythroid (TER-119+) cells decreased with increasing gestational age (E12.5, 80%; E13.5, 52%; E14.5, 14%) in the liver GATA-1.05/0.05 cells. Additionally, the frequency of non-erythroid-committed (c-Kit-TER-119-) progenitor cells increased (68%) in E14.5 GATA-1.05/0.05 fetal livers (Fig. 6B). These findings are consistent with the data obtained from GATA-1.05 ES cell differentiation in vitro (Fig. 1A).

Expression of the transferrin receptor (CD71) and TER-119 marker in the fetal livers were also analyzed. Most TER-119+ erythroid progenitors expressed CD71 in WT fetal livers (92%); however in E13.5 and E14.5 GATA-1.05/0.05 fetal livers, the expression of CD71 was gradually lost, coincident with loss of TER-119+ cells (18%). We also examined the expression of CD44, which is an adherent cell surface marker on immature erythroid cells, and found that the frequency of TER-119-CD44- cells increased in E14.5 fetal livers of GATA-1.05/0.05 embryos (81%), whereas the majority of WT fetal liver cells stained positive with TER-119 and CD44 (82%), indicating that immature TER-119-negative proerythroblasts were predominant in E14.5 GATA-1.05/0.05 fetal livers. May-Grunwald-Giemsa staining revealed that the GATA-1.05/0.05 fetal livers were populated by immature erythroblasts, substantiating the conclusions from previous flow cytometric analyses (data not shown).
DISCUSSION

In the present study, we found that immature GATA-1.05 erythroid cells are highly proliferative and accumulated in S phase because of the down-regulation of p16INK4A. GATA-1-null definitive erythroid cells also accumulate in the S phase and p16INK4A mRNA is down-regulated as well. However, unlike GATA-1.05 erythroblasts, GATA-1-null erythroid progenitors undergo apoptosis as a consequence of low Bcl-xL content. Thus, these results demonstrate that low versus no GATA-1 expression alters the developmental outcome of erythroid progenitors. This notion is summarized in Fig. 7.

Cell cycle exit is a prerequisite for terminal erythroid differentiation. It was previously reported that forced expression of GATA-1 accelerates erythroid differentiation along with the suppression of cell proliferation (22), whereas overexpression of the transcription factor during mouse erythroleukemia cell differentiation shows the opposite effect (14). Ectopic expression of human GATA-1 by retroviral infection into NIH3T3 fibroblasts results in reduced growth rates by prolonging S phase and altering the proliferative growth response to serum (15). These reports suggest that GATA-1 participates in the cell cycle control. Physical association of GATA-1 with Rb has been suggested to play a role in this process (14), but it has not been reported which cell cycle-associated molecules might be subjected to the actions of GATA-1 at the mRNA level, in particular during erythroid development. The results shown here suggest that p16INK4A is involved in suppressing cell proliferation.

Although p16INK4A inhibits colony formation on OP9 cells, p16INK4A is unlikely to affect profoundly the erythroid differentiation. INK4A gene targeting resulted in the increased proliferation of T cell lineage in the thymus and spleen (23). It is also noteworthy that INK4A heterozygote and homozygote mice are tumor-prone and develop a wide spectrum of cancers, particularly after exposure to chemical carcinogens (22). These results strongly suggest that GATA-1.05 or GATA-1-null erythroid progenitors may acquire genetic lesions at a higher frequency (see below).

Bcl-xL-deficient mice die at E13 because of severe anemia with apoptosis of definitive erythroid cells (24). However, primitive erythroid cells in Bcl-xL−/− mice were not so drastically underrepresented (25), suggesting that a discriminatory anti-apoptotic mechanism might be employed during primitive versus definitive erythroid differentiation in the mutant mice. In the ES/OP9 cell co-culture system, primitive erythroid cells appeared on day 6 and upon the analysis of anti-apoptotic gene expression in primitive erythroid cells, Bcl-xL mRNA was found to be expressed in WT primitive erythroid cells, but not in both GATA-1.05 and GATA-1-null cells. Because the number of primitive erythroid cells in GATA-1 mutants (both 1.05 and

FIG. 5. Forced expression of GATA-1 promotes erythroid differentiation in definitive GATA-1.05 erythroid cells. A, outline of the experimental scheme. B, colony-forming ability of definitive erythroid cells infected with retroviruses containing no (vector), GATA-1, or p16INK4A cDNA. Colony counts from GATA-1.05-adherent cells (4 × 10^3 (4K), 2 × 10^4 (20K), and 1 × 10^5 (100K)) seeded onto OP9 cells (*, p < 0.05). C, distribution of erythroid cells at distinct differentiation stages in cultures that were exposed to no, wild type, GATA-1-, or p16INK4A-containing viruses. Data were from average of at least three experiments. (***, p < 0.05, mean ± S.E.). D, increase of endogenous p16INK4A mRNA observed after overexpression of GATA-1 in the GATA-1.05-adherent cells. After retroviral infection, cells expressing green fluorescent protein were sorted and analyzed by RT-PCR. Two independent experiments are shown. G3PDH, glyceraldehyde-3-phosphate dehydrogenase; DDW, deionized distilled water.
null) on day 6 represented one-third or one-fifth of the number of WT primitive erythroid cells, respectively (data not shown), these results suggest that distinct mechanisms for promoting progenitor cell survival may be operative in primitive versus definitive erythroid cells.

Bcl-2 has been shown to prevent apoptosis triggered by various stimuli including oxidative stress, chemotherapeutic drugs, viral infections, and growth factor deprivation (26). Therefore, Bcl-2 was one of the candidates mediating the anti-apoptotic function of GATA-1. Importantly, however, erythro-
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Erythropoiesis proceeds normally in postnatal Bcl-2-null mutant mice (27), and erythroid progenitors in which Bcl-2 is forcibly expressed maintain Epo dependence in vitro (28), implying that Bcl-2 is not essential for normal erythroid maturation. Because the expression of both Bcl-2 and Bcl-xL was severely diminished in the GATA-1-null primitive erythroid cells, this might be the explanation for the differential response to differentiation induction between GATA-1.05 and GATA-1-null ES cell-derived primitive erythroid cells (Figs. 1 and 2).

GATA-1.05/X mice develop normally but begin to die after about 5 months of age (5). Our extensive cohort analysis has proved that these mice develop two types of overt leukemia; one type is c-Kit-positive non-lymphocytic leukemia, whereas the other is CD19-positive B cell leukemia (29). Both type of mice display anemia and thrombocytopenia with marked splenomegaly. Histological analyses clearly demonstrated that in the former type of leukemic mice proerythroblasts and megakaryocytes accumulated in the spleen, indicating that both erythroid and megakaryocytic lineages were severely affected by the lowered expression of GATA-1.

Interestingly, it was reported that the majority of hemizygous GATA-1.05−/− female mice were anemic at birth but recovered postnatally (3). It has been suggested that clonal selection for erythroid cells expressing GATA-1 occurs in vivo. We also examined the number of GATA-1.05/X neonates that survived postnatally despite their usually anemic appearance. As a result, we found that 14 of 54 neonates from a GATA-1.05/X versus WT crossing were GATA-1.05/X genotype, which was close to the expected Mendelian ratio. The anomalous c-Kit+ cells in GATA-1.05/X embryos probably disappeared after birth and/or a small population of normal erythroid progenitor cells proliferate vigorously to compensate for the precipitously diminishing erythroid progenitor pool (Fig. 6), as reported in the GATA-1.05−/− female mice (3). However, it is still unclear how echythropoiesis proceeds normally in postnatal GATA-1.05/X mice or how leukemic changes arise in the adult GATA-1.05/X mouse spleen (5, 29). Analyses of GATA-1.05/X mice during embryogenesis may provide insights into the origin of the expanded population of proerythroblasts in the GATA-1.05/X enlarged adult spleen. In addition, a more precise study of the time course leading to the appearance of splenomegaly or severe anemia may shed light on the mechanism(s) underlying the unusual expansion of proerythroblasts in GATA-1.05/X mice.

GATA-1 mutations are frequently found in acute megakaryoblastic leukemia patients with Down’s syndrome (30–35). A premature stop codon is introduced into the coding region encoding the GATA-1 N-terminal activation domain, which results in a truncated GATA-1 protein. Although it is still unclear one hypothesis that must be entertained in the light of our results in a truncated GATA-1 protein. This finding is consistent with the weakened activity of aberrant GATA-1 and GATA-2 expression, which was ~50-fold higher than that in WT, was observed in GATA-1-deficient proerythroblasts (40). Thus, it will be of interest to decipher the cellular mechanisms that govern the coordinated regulation of GATA-1 and GATA-2 during the various stages of erythroid development in vivo. This in turn would help us understand the ontogeny of the leukemogenic changes that occur in GATA-1.05/X mice.

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Graded Levels of GATA-1 Expression Modulate Survival, Proliferation, and Differentiation of Erythroid Progenitors

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