



Derepression of the DNA Methylation Machinery of the *Gata1* Gene Triggers the Differentiation Cue for Erythropoiesis

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ABSTRACT GATA1 is a critical regulator of erythropoiesis. While the mechanisms underlying the high-level expression of GATA1 in maturing erythroid cells have been studied extensively, the initial activation of the *Gata1* gene in early hematopoietic progenitors remains to be elucidated. We previously identified a hematopoietic stem and progenitor cell (HSPC)-specific silencer element (the *Gata1* methylation-determining region [G1MDR]) that recruits DNA methyltransferase 1 (Dnmt1) and provokes methylation of the *Gata1* gene enhancer. In the present study, we hypothesized that removal of the G1MDR-mediated silencing machinery is the molecular basis of the initial activation of the *Gata1* gene and erythropoiesis. To address this hypothesis, we generated transgenic mouse lines harboring a *Gata1* bacterial artificial chromosome in which the G1MDR was deleted. The mice exhibited abundant GATA1 expression in HSPCs, in a GATA2-dependent manner. The ectopic GATA1 expression repressed *Gata2* transcription and induced erythropoiesis and apoptosis of HSPCs. Furthermore, genetic deletion of *Dnmt1* in HSPCs activated *Gata1* expression and depleted HSPCs, thus recapitulating the HSC phenotype associated with GATA1 gain of function. These results demonstrate that the G1MDR holds the key to HSPC maintenance and suggest that release from this suppressive mechanism is a fundamental requirement for subsequent initiation of erythroid differentiation.

KEYWORDS *Gata1* gene regulation, hematopoietic stem and progenitor cell, erythropoiesis

The transcription factor GATA1 plays a key role in erythroid and megakaryocytic cell differentiation and in the differentiation of basophilic, eosinophilic, and mast cell lineages (1–8). For erythroid differentiation, GATA1 expression increases in stages, from common myeloid progenitors (CMPs) to megakaryocyte-erythroid progenitors (MEPs), and reaches a peak at the proerythroblast (ProEB) stage (9, 10). Earlier studies identified a series of *cis*-acting regulatory elements within the *Gata1* gene that participate in the elaborate transcriptional regulation of the gene (11, 12). Based on both *in vivo* and *in vitro* studies, *Gata1* gene expression during erythropoiesis has been reported to require promoter-proximal CACCC motifs, a palindromic double-GATA motif (dbG) approximately 650 bp upstream of the transcription start site (TSS), and a GATA motif in the *Gata1* hematopoietic enhancer (G1HE), 3.7 kb upstream of the TSS (10, 13–15).

GATA2 is abundantly expressed in hematopoietic stem and progenitor cells (HSPCs) (16–22). During commitment to the erythroid-megakaryocytic lineages, GATA2 acts to induce *Gata1* gene expression, while at the same time, the enhanced level of GATA1

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represses *Gata2* gene expression. This exchange of GATA2 for GATA1 has been referred to as GATA factor switching (23–28). One intriguing observation is that while GATA2 is abundantly expressed in HSPCs, the *Gata1* gene is expressed only at a low level. This observation suggests that a certain predominantly repressive mechanism controls the transcription of the *Gata1* gene in HSPCs. In this regard, we previously identified a *cis*-acting silencer element located between the G1HE and dbG elements that may play a pivotal role in *Gata1* gene repression in HSPCs. We also found that DNA methyltransferase 1 (Dnmt1) is recruited to the element and participates in *Gata1* gene methylation (29). We refer to this silencer element as the *Gata1* methylation-determining region (G1MDR).

While the mechanisms maintaining the high level of *Gata1* expression in maturing erythroid lineage cells have been studied extensively, the initial activation of the *Gata1* gene in early hematopoietic progenitors remains to be fully understood. As mentioned above, in HSPCs, *Gata1* expression is repressed to a very low level, even in the presence of abundant GATA2. In light of the GATA factor switching mechanism, we surmise that certain cues that release the *Gata1* gene from the silencing machinery may serve as the central mechanism that initiates *Gata1* gene activation. In this regard, several lines of evidence are worthy of attention. First, Dnmt1 is strongly recruited to the G1MDR to maintain DNA methylation of the *Gata1* locus in HSPCs, and deletion of the G1MDR selectively abrogates *Gata1* gene repression in HSPCs, which is associated with an increase of GATA2 occupancy in the *Gata1* gene enhancer (29). Second, demethylation of the *Gata1* enhancer and promoter around the G1MDR is associated with the enhancement of gene expression during erythropoiesis (29). Based on these observations, we hypothesized that derepression of Dnmt1-G1MDR-mediated repression is the key molecular mechanism that triggers the initial activation of *Gata1* gene expression in HSPCs, which subsequently leads HSPCs to differentiate toward the erythroid lineage.

To address this hypothesis, we generated three transgenic mouse lines that carry modified *Gata1* bacterial artificial chromosome (BAC) DNAs in which the G1MDR was deleted to express either wild-type GATA1, a GATA1-estrogen receptor fusion protein (G1ERT²), or a Cre-estrogen receptor fusion protein (CreERT²) in HSPCs. The mouse lines harboring these constructs individually are referred to as MG-G1, MG-G1ERT², and MG-CreERT², for minigene-*Gata1*, minigene-*Gata1ERT*², and minigene-*CreERT*², respectively. We found that MG-G1 and MG-G1ERT² transgenic mice exhibited aberrant *Gata1* gene expression in HSPCs, in a GATA2-dependent manner. The release of *Gata1* from repressive regulation by means of this strategy provoked skewed hematopoiesis toward erythroid differentiation and concomitantly induced HSPC apoptosis. Importantly, the genetic deletion of *Dnmt1* in HSPCs in MG-CreERT² mice activated *Gata1* gene expression and recapitulated the hematopoietic phenotype of MG-G1 and MG-G1ERT² transgenic mice. Taken together, these results demonstrate that the Dnmt1-G1MDR complex plays a key role in the repression of the *Gata1* gene for HSPC maintenance and suggest that release from this repression is critical for subsequent *Gata1* gene activation for erythroid commitment and differentiation.

RESULTS

G1MDR-mediated *Gata1* repression is crucial for hematopoietic homeostasis and perinatal survival. To address the consequences of release from G1MDR-mediated *Gata1* gene repression in HSPCs on subsequent hematopoietic differentiation, we generated a transgenic mouse line (MG-G1) carrying a *Gata1* BAC that retained a *Gata1* minigene lacking the G1MDR (Fig. 1A). Because the deletion of the G1MDR abrogated *Gata1* repression in HSPCs (29), the MG-G1 transgenic mice had a 3.3-fold higher level of *Gata1* mRNA in the fetal liver Lin⁻ Sca1⁺ c-Kit⁺ (LSK) fraction (representing HSPCs) than that for the comparable wild-type LSK cells (Fig. 1B). We found that all MG-G1 embryos exhibited severe anemia at embryonic day 17.5 (E17.5) (Fig. 1C).

In our initial experiment, we crossed MG-G1 male mice with wild-type female mice and recovered 47 neonates with live births. However, we identified only four MG-G1

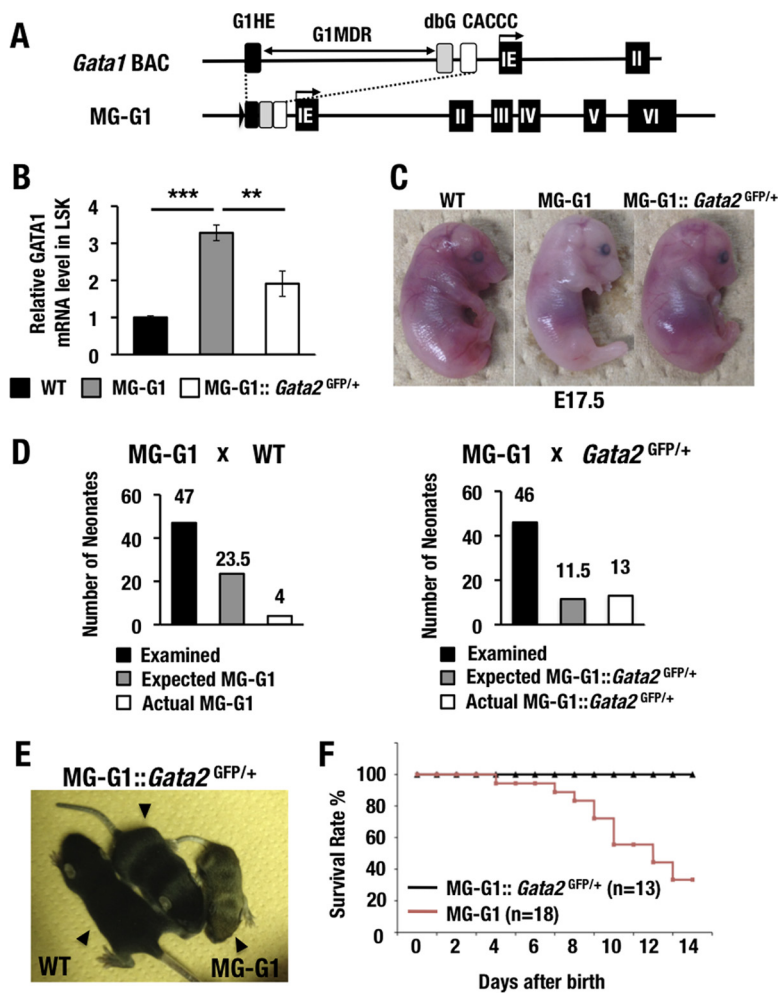


FIG 1 GATA2 transactivates GATA1 in the HSPCs of MG-G1 mice, and haploid insufficiency of the *Gata2* gene rescues MG-G1 mice from perinatal lethality. (A) Structure of MG-G1 BAC DNA. The G1MDR sequence was deleted in the context of *Gata1* BAC DNA. The three regulatory elements of the *Gata1* gene, shown as black (G1HE), gray (dbG), and white (CACCC) rectangles, were combined to generate MG-G1. IE, erythroid cell-specific 1st exon of the *Gata1* gene. (B) *Gata1* mRNA levels in LSK cells from E14.5 livers. Note that MG-G1 embryos showed 3.3-fold more *Gata1* mRNA but that the increase was abrogated by *Gata2* haploid insufficiency. WT, wild type. (C) Pale appearance of an MG-G1 embryo compared to that of a wild-type littermate embryo at E17.5. The anemic appearance was rescued by the reduction of GATA2 abundance. (D) Genotyping results for neonates born from crosses of MG-G1 and wild-type mice (left) or MG-G1 and *Gata2*^{GFP/+} mice (right). The total numbers of neonates examined, the Mendelian expected numbers, and the actual numbers of neonates born alive are shown. (E) Retarded growth of MG-G1 neonates was rescued by *Gata2* haploid insufficiency. (F) Kaplan-Meier survival curves for MG-G1 and MG-G1::Gata2^{GFP/+} mice after birth. The numbers of mice examined in this cohort were 18 and 13 for MG-G1 and MG-G1::Gata2^{GFP/+} mice, respectively. Data shown are the means ± SD for three mice. **, *P* < 0.01; ***, *P* < 0.001 (unpaired Student's *t* test).

transgenic neonates among these 47 live births, which was far fewer than expected from the Mendelian frequency (Fig. 1D, left panel). We repeated this experiment on a larger scale (*n* = 18 MG-G1 mice) and found that successfully delivered MG-G1 mice showed severe growth retardation (Fig. 1E). Approximately 70% of the MG-G1 neonates died of anemia within 2 weeks of birth (Fig. 1F). Taken together, these data indicate that G1MDR-mediated *Gata1* gene repression in HSPCs is crucial for the maintenance of HSPC homeostasis in the fetal mouse liver.

Haploid *Gata2* deletion rescues MG-G1 mice from lethality. Since it has been shown that GATA2 activates *Gata1* expression in erythroid and megakaryocytic progenitors (30), we were interested in the relationship between G1MDR-mediated repression and GATA2-mediated activation of *Gata1* gene expression in HSPCs. In this regard,

we previously demonstrated that GATA2 occupancy at the critical *Gata1* gene enhancer and upstream regulatory element in the proximity of the G1MDR (i.e., G1HE and dbG, respectively) is significantly increased in HSPCs when the G1MDR is deleted (29). Therefore, one plausible possibility is that upon removal of G1MDR-mediated repression, GATA2 binds to *Gata1* and activates *Gata1* gene expression.

To address this possibility, we next crossed MG-G1 mice with *Gata2*^{GFP/+} mice that heterozygously lacked *Gata2* expression (16). As expected, the *Gata2* haploid deletion significantly reduced the MG-G1-directed GATA1 expression in the fetal liver LSK fraction (Fig. 1B). In agreement with the reduction of GATA1 expression, the anemic appearance of MG-G1 E17.5 embryos was nicely rescued by the *Gata2* haploid insufficiency (Fig. 1C, right panel), and the live birth rate of MG-G1 pups was significantly improved. In fact, 13 MG-G1::*Gata2*^{GFP/+} pups were born among 46 littermates (28.3%) from the intercross between MG-G1 and *Gata2*^{GFP/+} mice, which is close to the expected ratio from Mendelian frequency analysis (Fig. 1D, right panel). The size of the 12-day-old MG-G1::*Gata2*^{GFP/+} pups was comparable to that of their wild-type littermates, while the MG-G1 littermates exhibited a small body size (Fig. 1E, middle mouse). A Kaplan-Meier analysis demonstrated that the postnatal survival rate of the MG-G1::*Gata2*^{GFP/+} mice was also rescued to a normal level (Fig. 1F). These results indicate that GATA2-mediated activation of *Gata1* gene expression is elaborately counterbalanced by the G1MDR-mediated repression of gene expression in HSPCs. We surmised that removal of G1MDR-mediated repression or demethylation of the G1MDR triggers the GATA2-mediated transcriptional activation of the *Gata1* gene.

Derepression of *Gata1* results in HSPC depletion and enhanced erythropoiesis.

As the perinatal lethality precluded closer analyses of adult MG-G1 mice, we examined fetal liver cells for the phenotypes of hematopoietic differentiation. We found that MG-G1 embryos exhibited a severe reduction of fetal liver cellularity from E13.5 to E17.5 (Fig. 2A). Flow cytometry of the fetal liver cells demonstrated that the percentages of LSK cells (Fig. 2B and C), CMPs, and granulocyte-monocyte progenitors (GMPs) (Fig. 2D and E) were significantly decreased, while the percentages of MEPs (Fig. 2D and E) and ProEBs (Fig. 2F and G) were slightly increased. The percentages of Mac1/Gr1⁺ myeloid and B220⁺ B-lymphocytic cells were also lower (Fig. 2H and I). These results demonstrate that release from G1MDR-mediated repression induced GATA1 expression in HSPCs, which directed their differentiation toward erythroid lineages, at the expense of myeloid and lymphoid differentiation, and induced depletion of HSPCs in the fetal liver.

Ectopic GATA1 expression exhausts the HSC population and skews differentiation toward the erythroid lineage. As the lethality of the MG-G1 embryos largely obscured the analysis of adult hematopoiesis, we generated another line of G1MDR-deleted mice to circumvent this difficulty. To this end, we generated a new mouse line harboring a tamoxifen-inducible GATA1-ER^{T2} fusion protein expressed under the regulation of the *Gata1* MG-G1 BAC (MG-G1ER^{T2}) (Fig. 3A). The MG-G1ER^{T2} transgenic mice had a 12-fold higher level of *Gata1* mRNA in adult bone marrow LSK cells than that in wild-type control LSK cells in the absence of tamoxifen (Fig. 3B). In contrast, the *Gata2* haploid insufficiency significantly reduced GATA1 expression (Fig. 3B), supporting the notion that transcription of the *Gata1* gene in the MG-G1ER^{T2} construct is also dependent on the abundance of GATA2. Importantly, without tamoxifen administration, there was no difference in the LSK cell populations between MG-G1ER^{T2} and wild-type mice, indicating that the estrogen receptor (ER) in the GATA1-ER^{T2} fusion protein in MG-G1ER^{T2} mice was intact and not leaky (data not shown).

To induce the GATA1 activity of GATA1-ER^{T2}, we injected tamoxifen intraperitoneally five times and analyzed bone marrow cells 7 and 14 days after the first tamoxifen administration (Fig. 3A). MG-G1ER^{T2} mice with vehicle administration were used as the control. We found that the number of lineage-negative bone marrow mononuclear (BM MNC L⁻) cells, which includes HSPCs, gradually diminished after the tamoxifen treatment (Fig. 3C). For each of the HSC and hematopoietic progenitor fractions, the MG-G1ER^{T2} mice showed dramatic reductions in the percentages of LSK and CMP cells

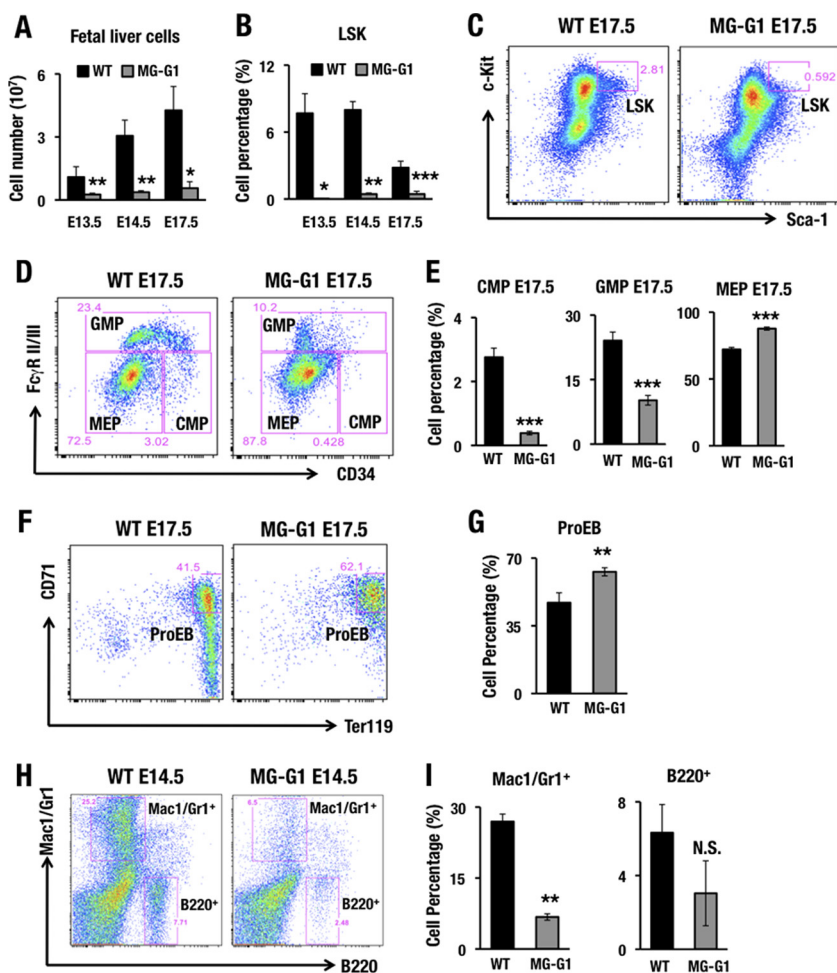


FIG 2 MG-G1 BAC transgenic mice exhibit enhanced erythropoiesis and HSPC depletion during the embryonic stage. (A) Fetal liver cellularity in E13.5, E14.5, and E17.5 embryos. Note that MG-G1 fetal livers showed a significant reduction in the hematopoietic cell population compared to that in wild-type fetal livers. (B) Percentages of LSK cells in E13.5, E14.5, and E17.5 fetal livers of MG-G1 and littermate control embryos. (C and D) Flow cytometry analysis of LSK cell (C) and progenitor (D) fractions. (E) CMP, GMP, and MEP percentages in E17.5 fetal livers of MG-G1 and littermate control embryos. (F) Flow cytometry analysis of proerythroblasts from WT and MG-G1 E17.5 fetal livers. (G) Percentages of CD71⁺ Ter119⁺ proerythroblasts in E17.5 livers. (H) Flow cytometry analysis of Mac1/Gr1⁺ and B220⁺ cells from WT and MG-G1 E14.5 fetal livers. (I) Percentages of Mac1/Gr1⁺ and B220⁺ cells. Data shown are the means ± SD for three to six mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (unpaired Student's *t* test). N.S., not significant.

following tamoxifen treatment (Fig. 3D to F). In contrast, the percentages of MEPs and ProEBs in the MG-G1ERT² mice were significantly increased after tamoxifen administration (Fig. 3D and F to H).

The LSK fraction is known to represent a heterogeneous cell population containing long-term HSCs (LT-HSCs; CD150⁺ CD48⁻), short-term HSCs (ST-HSCs; CD150⁻ CD48⁻), and multipotent progenitors (MPPs; CD48⁺) (31). The percentage of LT-HSCs was increased, while that of ST-HSCs was decreased, within the LSK fraction of the tamoxifen-treated MG-G1ERT² bone marrow cells (day 7) (Fig. 3I). More remarkably, the absolute numbers of LT-HSCs, ST-HSCs, and MPPs were dramatically reduced after GATA1 induction (day 7) (Fig. 3J). Thus, consistent with the results observed with the MG-G1 transgenic fetal livers, these results demonstrate that derepression of the *Gata1* gene in HSPCs increases erythroid differentiation and depletes the HSPC population in the adult bone marrow.

Aberrant GATA1 expression directs erythroid differentiation but reduces hematopoietic colony-forming activity. To examine whether aberrant GATA1 expres-

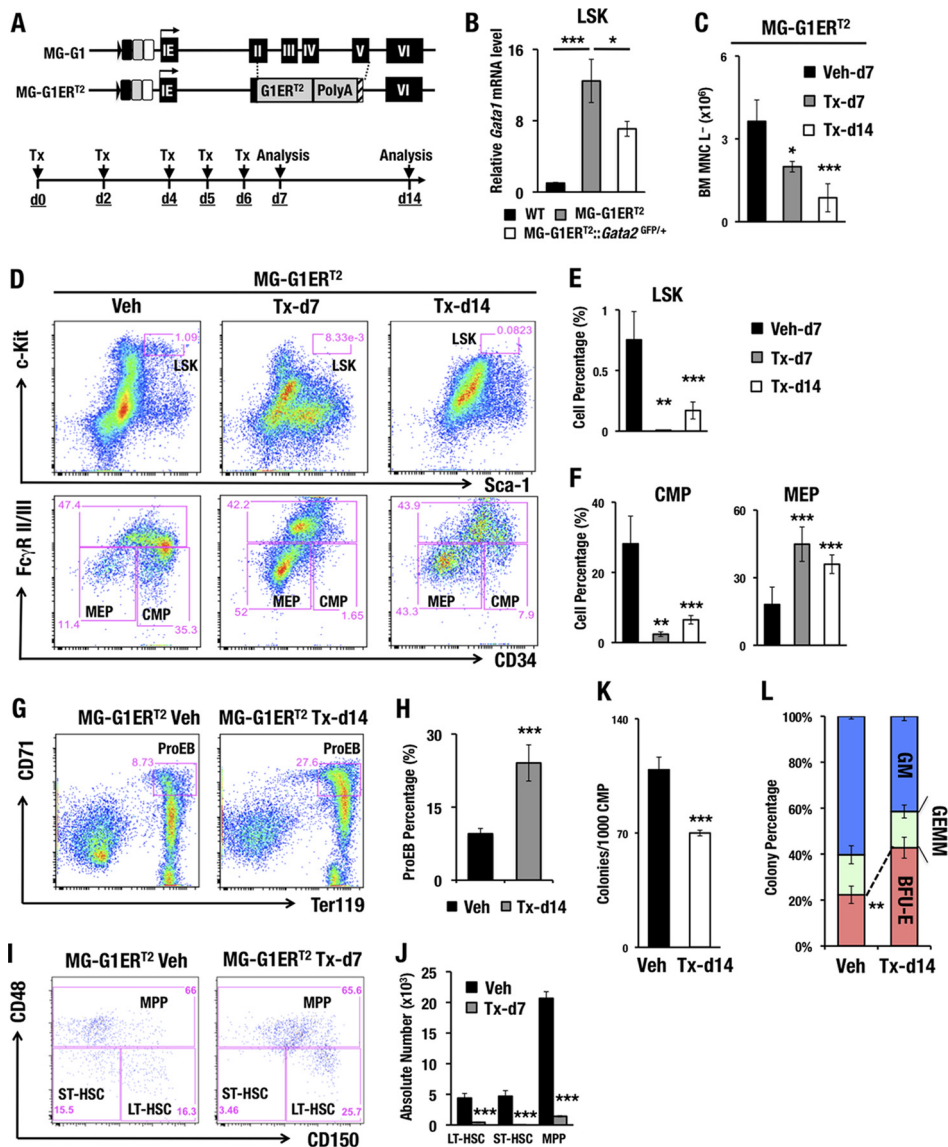


FIG 3 Increased erythropoiesis and HSPC depletion in the adult bone marrows of MG-G1ERT² mice upon tamoxifen treatment. (A) Structure of the MG-G1ERT² BAC transgenic construct. The G1ERT² fusion cDNA was integrated by homologously replacing the 2nd to 5th exons of the *Gata1* gene in the MG-G1 BAC DNA. (Top) Transgenic mice were generated using the MG-G1ERT² BAC construct. (Bottom) Tamoxifen (Tx) was injected intraperitoneally into the mice on days 0, 2, 4, 5, and 6. The analysis was performed on days 7 and 14. (B) *Gata1* mRNA levels in LSK cells from WT, MG-G1ERT², and MG-G1ERT²::*Gata2*^{GFP/+} bone marrows from adult mice without tamoxifen treatments. (C) Number of bone marrow lineage-negative mononuclear cells (BM MNC L⁻) in vehicle (Veh)- or tamoxifen-treated MG-G1ERT² mice at day 7 or 14. (D) Flow cytometry analysis of LSK cells (upper panels) and progenitors (lower panels) in MG-G1ERT² mice treated with vehicle (left) or with tamoxifen for 7 days (middle) or 14 days (right). (E and F) Percentages of LSK cell (E) and CMP and MEP (F) populations. Note that after tamoxifen treatment, the percentages of LSK cells and CMPs were significantly reduced, while that of MEP was increased. (G) Flow cytometry analysis of proerythroblasts from MG-G1ERT² mouse bone marrow 14 days after vehicle or tamoxifen treatment. (H) Percentages of CD71⁺ Ter119⁺ proerythroblast cells. (I) LT-HSC, ST-HSC, and MPP populations were separated by means of flow cytometry from LSK-gated adult bone marrow cells from MG-G1ERT² mice 7 days after vehicle (left) or tamoxifen (right) treatment. (J) Absolute numbers of the LT-HSC, ST-HSC, and MPP subsets. Note the significant decreases in LT-HSC, ST-HSC, and MPP populations in LSK-gated MG-G1ERT² mouse bone marrow cells. (K and L) Colony number per 1,000 CMP cells (K) and percentages of CFU-GM, CFU-GEMM, and BFU-E colonies (L) from vehicle- or tamoxifen-treated MG-G1ERT² mice. Data shown are the means ± SD for three to seven mice. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 (unpaired Student's *t* test).

sion changes the erythroid differentiation properties and/or perturbs the progenitor number, we performed CFU assays by exploiting CMP cells recovered from MG-G1ERT² bone marrow that was isolated 14 days after tamoxifen treatment. We employed the MethoCult GF M3434 culture system and identified burst-forming unit–erythroid

(BFU-E), CFU-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM), and CFU-granulocyte, macrophage (CFU-GM) colonies according to their distinct morphologies. We found that the total colony-forming activity of the MG-G1ERT² cells was reduced to 64% compared to that of vehicle-treated control cells (Fig. 3K). Among the remaining colonies, the percentage of BFU-E was significantly increased, while the percentage of CFU-GM was decreased and that of CFU-GEMM was unchanged (Fig. 3L). These results support the notion that elevated GATA1 activity in HSPCs skews hematopoiesis toward the erythroid lineage.

GATA1 dysregulation evokes erythropoiesis- and apoptosis-related gene signatures. To comprehensively elucidate the molecular alterations that occur under conditions of GATA1 dysregulation in HSPCs, we next performed deep RNA sequencing (RNA-Seq) of the LSK fraction, which was purified from bone marrows from surviving adult MG-G1 mice ($n = 3$ mice, each approximately 3 months of age). The RNA-Seq data demonstrated statistically significant upregulation of 429 genes and downregulation of 360 genes in MG-G1 LSK cells compared to the gene expression in wild-type LSK cells (Fig. 4A). Upregulated genes included *Hba* (α -globin), *Alas2* (erythroid-type 5-aminolevulinic synthase), and *Slc4a1* (Band3), while downregulated genes included *Gata2*.

To verify the RNA-Seq results, we also examined the gene expression profiles of several related genes by real-time quantitative PCR (RT-qPCR). In this verification, we utilized fetal liver LSK cells for comparison to the RNA-Seq results obtained from the adult bone marrow LSK cells. Consistent with the results of the RNA-Seq analysis, the RT-qPCR analysis detected significant upregulation of *Hba* and *Hbb* (β -globin) but downregulation of *Gata2* and *Kit* in the MG-G1 fetal liver LSK cells (Fig. 4B). Gene set enrichment analysis (GSEA) of the RNA-Seq data demonstrated significant enrichment of gene signatures affiliated with erythroid differentiation (Fig. 4C, upper panel). Interestingly, we also found significant enrichment of gene signatures affiliated with apoptosis (in the Kyoto Encyclopedia of Genes and Genomes [KEGG]) in the MG-G1 LSK cells (Fig. 4C, lower panel). Twenty-four genes among all the significantly affected genes were likely to have an apoptosis-related function by gene annotation analysis with DAVID (Database for Annotation, Visualization and Integrated Discovery) (Fig. 4D). These results show agreement with the observation that derepression of *Gata1* gene expression in HSPCs of MG-G1 and MG-G1ERT² mice directed hematopoietic differentiation toward the erythroid lineage and HSPC depletion.

Aberrant GATA1 induction represses *Gata2* expression in bone marrow HSPCs.

During the erythroid lineage differentiation, GATA1 represses *Gata2* gene transcription; this phenomenon is known as GATA factor switching (23–27). Consistent with this notion, we found that aberrant GATA1 activation in both fetal liver and adult bone marrow LSK cells markedly repressed *Gata2* gene expression (Fig. 4A and B).

To further confirm the reproducibility and to elucidate the significance of GATA1-mediated repression of *Gata2* in HSPCs, we employed *Gata2*^{GFP/+} mice to monitor GATA2 expression in adult bone marrow LSK cells by flow cytometry (16). For this purpose, MG-G1ERT²::*Gata2*^{GFP/+} mice were treated with tamoxifen five times and analyzed 12 days after the initial tamoxifen treatment. We found that the green fluorescent protein (GFP) intensity in LSK cells was significantly reduced after the aberrant GATA1 activation by tamoxifen in MG-G1ERT²::*Gata2*^{GFP/+} mice (Fig. 4E), confirming that abundant GATA1 represses *Gata2* gene transcription in HSPCs. Because GATA2 is responsible for *Gata1* gene activation in MG-G1 (Fig. 1B) and MG-G1ERT² (Fig. 3B) LSK cells, the repression of *Gata2* transcription by GATA1 in the same cells of these mice demonstrated that the ectopic GATA factor switching indeed occurred in the absence of the G1MDR. These lines of evidence thus indicate that the G1MDR is the molecular key to preventing dysregulation of GATA factor switching in HSPCs.

***Gata1* gene derepression induces apoptosis in HSPCs.** Because GSEA indicated that enhanced GATA1 expression in MG-G1 LSK cells is associated with apoptosis, and since this effect is consistent with our observation that HSC and progenitor cells were

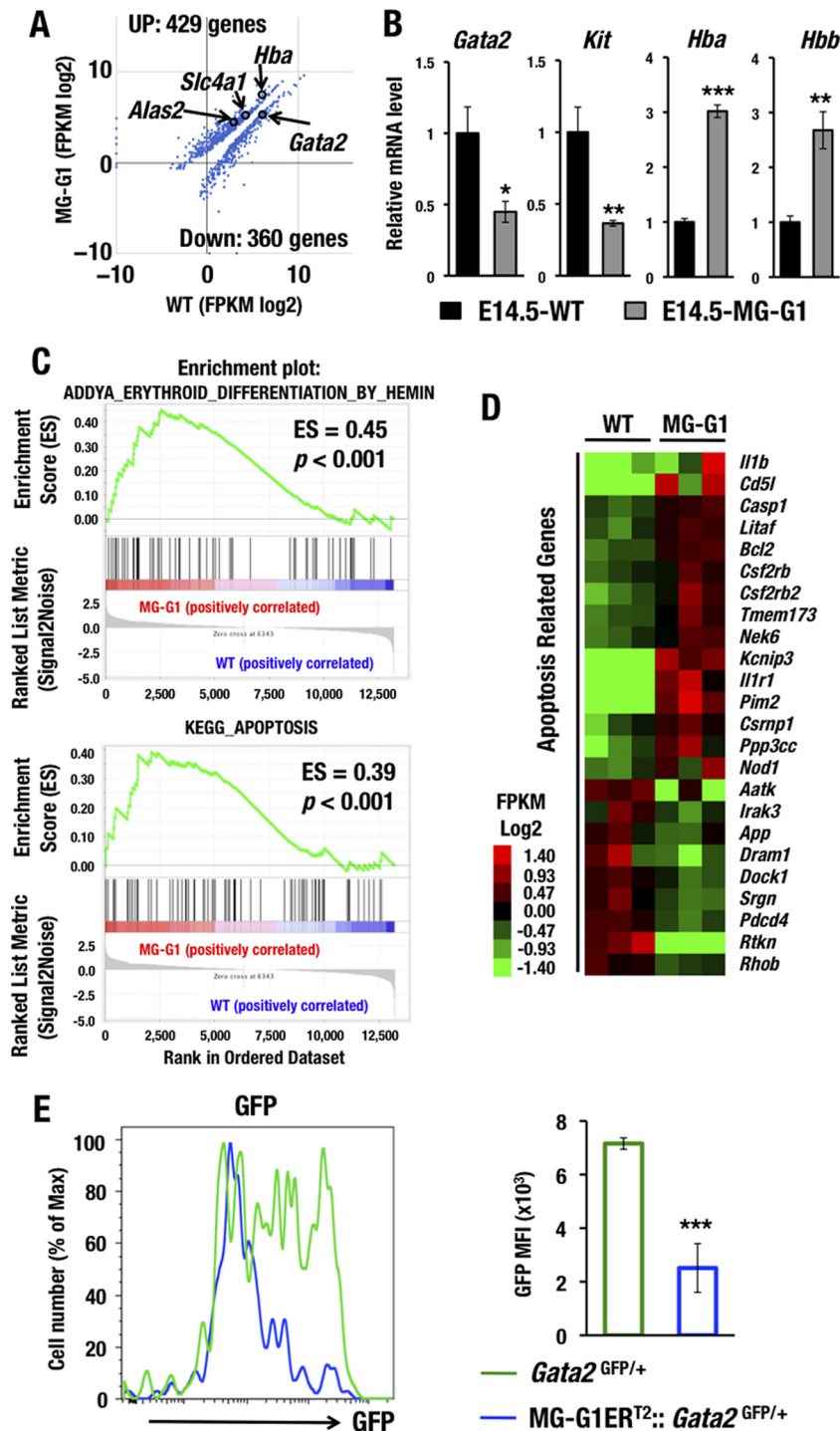


FIG 4 GATA1 dysregulation changes the HSPC gene signature to erythroid differentiation and apoptotic cell death. (A) Scatterplots comparing transcript levels (in fragments per kilobase of exon per million fragments [FPKM]) in wild-type (x axis) and MG-G1 (y axis) mice. Only data for significantly changed genes are shown (*P* < 0.05). (B) mRNA levels of GATA1 target genes as assessed by manual RT-qPCR using E14.5 LSK cells from livers of wild-type and MG-G1 embryos. (C) GSEA of erythroid differentiation- and apoptosis-related data sets for all RNA-Seq reads of LSK RNAs from MG-G1 and wild-type littermate adult bone marrow cells. (D) Heat map showing relative expression levels of apoptosis-related genes in MG-G1 LSK cells compared to wild-type LSK cells. Heat map colors indicate normalized expression levels. (E) GFP mean fluorescence intensities (MFI) in LSK-gated fractions from *Gata2*^{GFP/+} and MG-G1ERT2::*Gata2*^{GFP/+} mice after tamoxifen treatment. Note the decrease of GFP MFI in MG-G1ERT2::*Gata2*^{GFP/+} mice. Data shown are the means ± SD for three mice. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 (unpaired Student's *t* test).

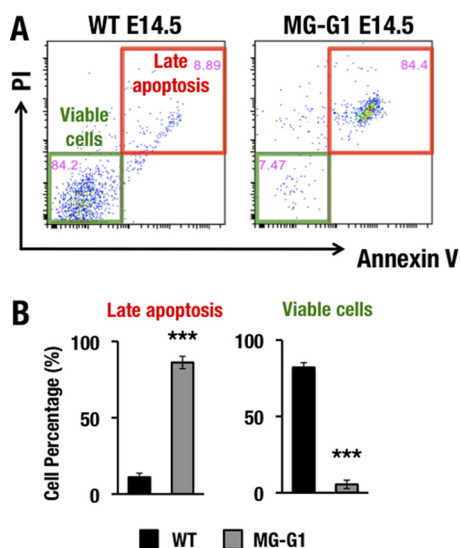


FIG 5 *Gata1* gene derepression induces HSPC apoptosis in the MG-G1 fetal liver. (A) PI and annexin V staining of LSK-gated cells from wild-type (left) and MG-G1 (right) E14.5 fetal livers. Note that the late apoptosis fraction was significantly increased in LSK-gated cells from MG-G1 fetal livers, while viable cells were predominant in LSK-gated cells from wild-type fetal livers. (B) Percentages of late apoptotic (PI⁺ annexin V⁺) and viable (PI⁻ annexin V⁻) LSK cells in wild-type and MG-G1 fetal livers. Data shown are the means \pm SD for three mice. ***, $P < 0.001$ (unpaired Student's *t* test).

depleted in MG-G1 mice, we next approached the issue of whether the elevated GATA1 expression in HSPCs actually participates in the program of apoptotic cell death. For this purpose, we examined the cell viability status of the LSK cell population in the livers of E14.5 MG-G1 embryos by annexin V-propidium iodide (PI) staining. In wild-type embryos, the vast majority of LSK cells were distributed in the annexin V⁻ PI⁻ population, whereas only a small number of LSK cells underwent late apoptosis, corresponding to the annexin V⁺ PI⁺ population (Fig. 5A and B). In MG-G1 embryos, the percentage of viable LSK cells was dramatically reduced, from 82% \pm 3% to 5% \pm 2%, whereas the percentage of late apoptotic LSK cells was significantly increased, from 11% \pm 3% to 86% \pm 4% (data are means \pm standard deviations [SD] for three independent analyses; a representative analysis is shown in Fig. 5A). These results demonstrate that aberrant GATA1 activity induces HSPCs to undergo apoptosis in MG-G1 embryos.

High-level GATA1 activity induces apoptosis in LT-HSCs, ST-HSCs, MPPs, and CMPs but prevents apoptosis in MEPs. To clarify whether abrogation of G1MDR-mediated *Gata1* repression renders HSPCs vulnerable to apoptosis in adult mouse bone marrow, we examined LSK cell viability in the bone marrows of adult MG-G1ERT² mice. Consistent with the results of the MG-G1 embryonic fetal liver analysis described in the previous section, the vast majority of LSK cells were viable annexin V⁻ PI⁻ cells (81% \pm 3%) in the absence of tamoxifen treatment (Fig. 6A and B). However, 7 days after the tamoxifen treatment, the percentage of viable cells was significantly decreased, to 20% \pm 3% of the LSK cell population, while the percentages of annexin V⁺ PI⁺ late apoptotic cells and annexin V⁺ PI⁻ early apoptotic cells were both significantly increased, from 10% \pm 0.5% to 33% \pm 4% and from 7% \pm 3% to 40% \pm 3%, respectively (data are means \pm SD for three independent analyses; a representative example is shown in Fig. 6A). These results indicate that the majority of LSK cells in MG-G1ERT² mouse bone marrow underwent apoptosis upon the induction of GATA1 activity.

To further address the consequences of aberrant GATA1 activity for the viability of cell subsets during erythroid differentiation, we stained bone marrow LT-HSCs, ST-HSCs, MPPs, CMPs, and MEPs with annexin V. In the tamoxifen-treated MG-G1ERT² mice, the percentages of annexin V⁺ apoptotic cells were significantly increased for the LT-HSC,

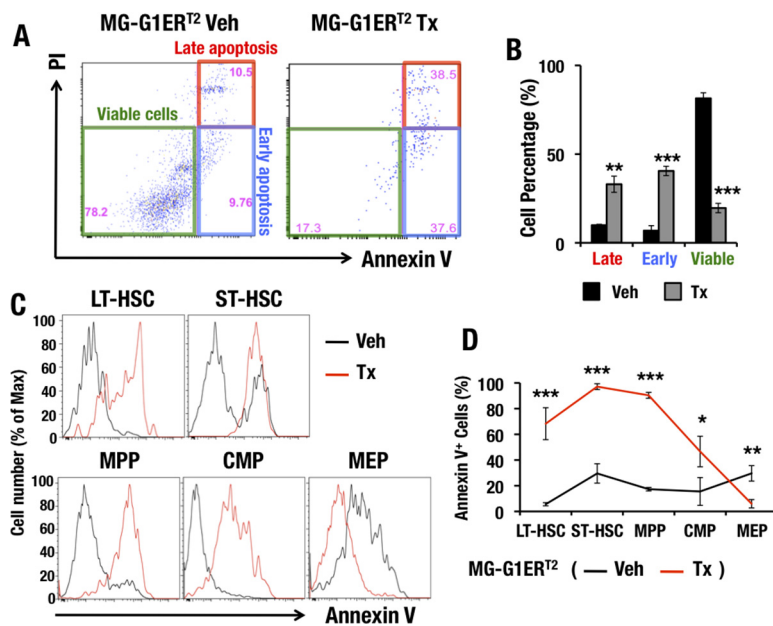


FIG 6 Ectopic GATA1 activation induces HSPC apoptosis but prevents programmed cell death of erythroid and megakaryocytic committed cells. (A) PI and annexin V staining of LSK-gated cells from MG-G1ER^{T2} mouse bone marrow after vehicle treatment (left) or 7 days of tamoxifen treatment (right). (B) Percentages of late apoptotic, early apoptotic, and viable adult bone marrow LSK cells. (C) Flow cytometry analysis of annexin V staining in LT-HSCs, ST-HSCs, MPPs, CMPs, and MEPs from MG-G1ER^{T2} mouse bone marrow 7 days after vehicle or tamoxifen treatment. (D) Percentages of annexin V⁺ apoptotic cells based on the data from panel C. Data shown are the means \pm SD for three or four mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (unpaired Student's t test).

ST-HSC, MPP, and CMP subsets (Fig. 6C and D). In contrast, the percentage of annexin V⁺ cells was significantly reduced in MEP cells from tamoxifen-treated MG-G1ER^{T2} mice (Fig. 6C and D). Thus, our results demonstrate that the aberrant GATA1 activity observed by deletion of the G1MDR rendered HSCs and CMPs vulnerable to apoptosis, while it prevented MEPs from initiating programmed cell death.

The vast majority of HSPCs are labeled in MG-CreER^{T2}::R26T transgenic mice.

To further clarify *Gata1* gene regulation in HSPCs, we generated two lines of MG-CreER^{T2} transgenic mice (Fig. 7A). We bred MG-CreER^{T2} mice with Rosa26 flox-Stop-flox tdTomato reporter mice (MG-CreER^{T2}::R26T) and examined the recombination efficiency of the inducible Cre recombinase. MG-CreER^{T2}::R26T mice were administered tamoxifen and then subjected to analysis 14 days after the first administration (Fig. 7A). In this analysis, we used R26 Δ T mice (Rosa26 flox-Stop-flox tdTomato reporter mice with a constitutive stop codon deletion) as a positive control in which all cells expressed tdTomato fluorescence, while wild-type C57BL/6 mice served as a negative control. We found that 87.5% and 73.4% of LSK cells from lines 1 and 2, respectively, of MG-CreER^{T2}::R26T mice showed tdTomato fluorescence after the tamoxifen treatments (Fig. 7B). These fluorescence percentages were comparable to those observed in the positive-control R26 Δ T mice, in which 88.2% of LSK cells were tdTomato⁺. These results indicate that MG-CreER^{T2} efficiently activated Cre activity in the majority of LSK cells. The vehicle-treated MG-CreER^{T2}::R26T mice and the wild-type negative-control mice had no tdTomato-positive cells (Fig. 7B, right panel), indicating the absence of Cre recombinase activity without prior tamoxifen treatment.

Dnmt1 plays a key role in repressing *Gata1* gene expression in HSPCs.

Dnmt1 is recruited to the G1MDR and participates in the maintenance of DNA methylation at the *Gata1* locus in HSPCs (29). Therefore, we attempted to further dissect this mechanism by employing a *Dnmt1* conditional knockout mouse line (32). We crossbred the MG-CreER^{T2} mice (line 1) with *Dnmt1*^{fl/fl} and generated *Dnmt1*^{fl/fl}::MG-CreER^{T2} mice. We called these mice *Dnmt1*-CKO mice and used them to elucidate the consequences of

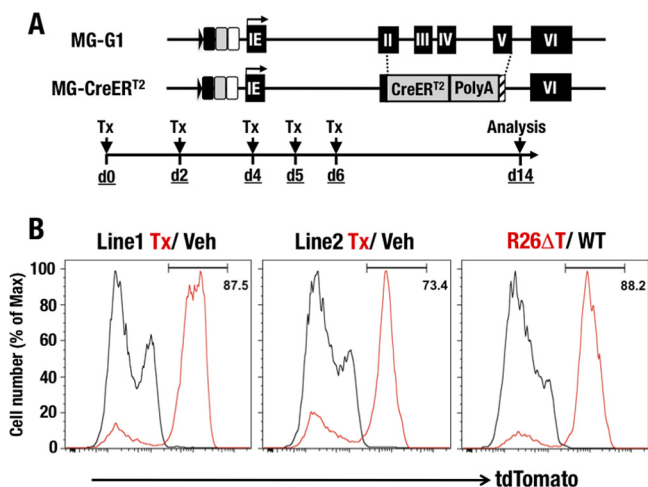


FIG 7 Generation of MG-CreER^{T2} mice. (A) Structure of the MG-CreER^{T2} BAC transgenic allele. The CreER^{T2} fusion cDNA was integrated by homologously replacing the 2nd to 5th exon regions of the *Gata1* gene in the MG-G1 BAC DNA. Tamoxifen was administered to the mice by intraperitoneal injection on days 0, 2, 4, 5, and 6. Analysis was performed on day 14. (B) tdTomato histograms confirming Cre activity in the LSK fractions of R26T::MG-CreER^{T2} and control mice. (Left) R26T::MG-CreER^{T2} line 1 (red line, tamoxifen; black line, vehicle). (Middle) R26T::MG-CreER^{T2} line 2 (red line, tamoxifen; black line, vehicle). (Right) R26ΔT positive control (red line) and WT negative control (black line).

Dnmt1 deletion in HSPCs. We exploited straightforward MG-CreER^{T2} mice with tamoxifen treatment as a negative control.

In the *Dnmt1*-CKO mice, *Dnmt1* mRNA expression in bone marrow LSK cells was reduced more than 60% 14 days after tamoxifen administration (Fig. 8A). Consistent with our hypothesis, the *Gata1* mRNA abundance increased significantly upon *Dnmt1* deletion (Fig. 8A). To clarify whether the *Gata1* mRNA induction was associated with the gene enhancer demethylation in *Dnmt1*-CKO mice, we performed bisulfite sequencing using DNA samples isolated from bone marrow LSK cells of *Dnmt1*-CKO mice and control MG-CreER^{T2} mice. We designed primers that specifically amplified the dbG enhancer from the endogenous *Gata1* locus. Notably, the methylation level of the dbG enhancer was significantly reduced, from 100% in control LSK cells of MG-CreER^{T2} mice to approximately 33% in LSK cells of *Dnmt1*-CKO mice (Fig. 8B), which shows agreement with the increase of *Gata1* expression in LSK cells from *Dnmt1* conditional knockout mouse bone marrow.

In the previous section, we showed that ectopic GATA1 expression led to HSPC depletion in MG-G1 mice and tamoxifen-treated MG-G1ER^{T2} mice. Consistent with the increase of GATA1 expression in the *Dnmt1* conditional knockout mice, the percentage of LSK cells was significantly decreased in the bone marrows of *Dnmt1*-CKO mice (Fig. 8C and D). To elucidate whether the LSK cell depletion in *Dnmt1*-CKO mouse bone marrow was associated with the induction of apoptosis, we examined the viability of these cells. *Dnmt1*-CKO LSK cells harbored significantly increased percentages of early and late apoptotic cells, but the viable cell population in *Dnmt1*-CKO LSK cells was dramatically diminished compared to that in the MG-CreER^{T2} control mice (Fig. 8E and F). Thus, the genetic *Dnmt1* ablation caused an increase of *Gata1* expression, which recapitulated the HSPC deficiency in the MG-G1 and tamoxifen-treated MG-G1ER^{T2} mouse models. These results strongly support our contentions that *Dnmt1* represses *Gata1* gene expression in HSPCs through methylation of the G1MDR for HSPC maintenance and that G1MDR demethylation and GATA2-mediated activation of *Gata1* gene expression are prerequisites for the start of erythroid differentiation.

DISCUSSION

In this study, we showed the essential requirement of the G1MDR *cis*-element as the key regulator of *Gata1* gene suppression for HSPC maintenance. As summarized in the

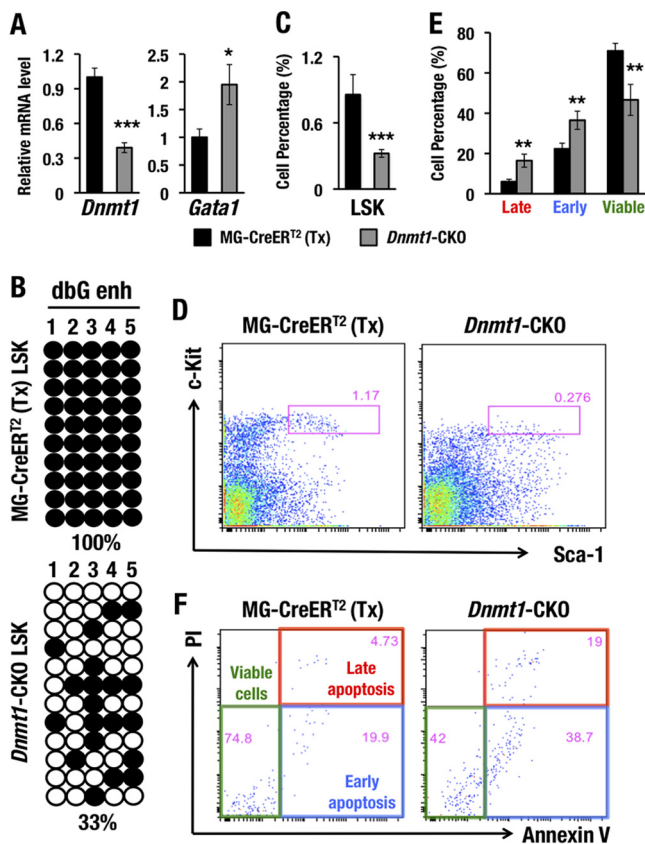


FIG 8 *Dnmt1* deletion induces *Gata1* expression and subsequently depletes HSPCs. (A) *Dnmt1* and *Gata1* mRNA levels in the LSK fractions from MG-CreER^{T2} and *Dnmt1*-CKO mouse bone marrows 14 days after tamoxifen treatment. (B) Bisulfite sequencing of the dbG enhancer (dbG enh) in LSK cells from MG-CreER^{T2} and *Dnmt1*-CKO adult mouse bone marrows 14 days after tamoxifen treatment. (C and D) LSK percentages (C) and LSK flow cytometry (D) for MG-CreER^{T2} and *Dnmt1*-CKO mice after tamoxifen treatment. (E and F) Late apoptotic, early apoptotic, and viable LSK cell percentages (E) and annexin V-PI staining of LSK-gated cells (F) for MG-CreER^{T2} and *Dnmt1*-CKO adult mouse bone marrows 14 days after tamoxifen treatment. Note that conditional knockout of *Dnmt1* induced GATA1 expression and reduced the total LSK population and the number of viable LSK cells. Data shown are the means \pm SD for three to five mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (unpaired Student's *t* test).

top panel of Fig. 9, the G1MDR recruits Dnmt1 and methylates DNA at the *Gata1* locus, which prevents *Gata1* gene activation by GATA2 in HSPCs. For the initiation of erythroid differentiation (Fig. 9, middle panel), Dnmt1- and G1MDR-mediated methylation of the *Gata1* locus is gradually downregulated, which facilitates gene activation by GATA2. Subsequently, increased GATA1 expression represses *Gata2* gene expression, and GATA factor switching occurs. In this way, erythroid differentiation begins. However, if the G1MDR-mediated *Gata1* gene repression is completely abrogated in HSPCs, as is the case in MG-G1 mice or tamoxifen-treated MG-G1ER^{T2} mice (Fig. 9, bottom panel), excess GATA1 provokes accelerated erythropoiesis and HSPC apoptosis. Thus, this study demonstrates that Dnmt1-G1MDR-mediated *Gata1* gene repression plays a crucial role in HSPC maintenance and suggests that derepression of Dnmt1-G1MDR repression is the critical mechanism that initiates *Gata1* gene expression and promotes erythroid commitment and differentiation of HSPCs.

We found in this study that the aberrant GATA1 expression in HSPCs observed on deletion of the G1MDR induces erythropoiesis that is coupled to apoptosis. We surmised that HSPC apoptosis might be due to the induction of apoptosis-related genes by GATA1. In this regard, it is interesting that erythroid lineage cells tend to undergo apoptosis and that erythropoietin signaling prevents this apoptosis (33, 34). The expression of the erythropoietin receptor is usually upregulated after the BFU-E stage

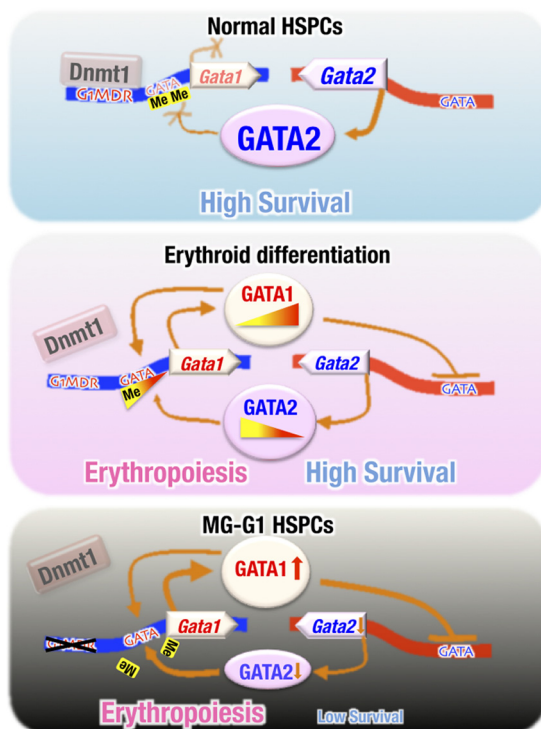


FIG 9 Dnmt1 and the G1MDR *cis*-acting repressor element maintain the HSPC balance between erythropoiesis initiation and cell survival. (Top) Under normal conditions, G1MDR-mediated Dnmt1 recruitment and methylation of the *Gata1* gene enhancer and the upstream promoter regions repress *Gata1* gene expression in HSPCs, which maintains HSPC homeostasis. (Middle) During erythroid differentiation, the Dnmt1-G1MDR-mediated DNA methylation of the *Gata1* enhancer is gradually reduced (in contrast to the total loss caused by G1MDR deletion). The decreased methylation allows GATA2 to initiate *Gata1* transcription gradually during the initiation of erythropoiesis. The progressively enhanced level of GATA1 then transactivates the expression of the *Gata1* gene itself (autoregulation) and represses *Gata2* gene expression, thus directing the progress of erythropoiesis. (Bottom) Upon deletion of the G1MDR, the epigenetic repression of *Gata1* gene expression is completely abrogated such that GATA2 is able to fully transactivate *Gata1* gene expression in HSPCs. The aberrant GATA1 activation represses the *Gata2* gene and accelerates HSPC depletion through inducing erythroid differentiation and apoptosis.

of erythropoiesis (34). Therefore, we envisaged that full *Gata1* gene activation needed to be balanced by the enhancement of erythropoietin signaling in the erythroid lineage-committed progenitors; without erythropoietin signaling, for instance, in the case of MG-G1 mice (Fig. 9, bottom panel), HSPCs head toward apoptosis. These observations suggest that *Gata1* gene expression must be coupled with enhanced antiapoptotic signaling to maintain erythroid homeostasis. Indeed, we observed a decrease of the *c-Kit* expression level in MG-G1 LSK cells (Fig. 4B). The *Kit* gene is a well-known target that is negatively regulated by GATA1 and plays a crucial role in HSPC survival (35, 36). The decrease of *c-Kit* expression may also be involved in the induction of HSPC apoptosis.

During erythroid differentiation, GATA2 activates *Gata1* gene expression, while GATA1 activates *Gata1* gene expression but represses *Gata2* gene expression to make the switch of GATA factors from GATA2 to GATA1 (23–28). In this study, we addressed the mechanisms of GATA factor switching. Our results demonstrate that G1MDR-mediated DNA methylation is the molecular key to initiating GATA factor switching.

We surmise that this research opens the doors to a number of intriguing projects. For instance, the mechanism that initiates enfeebling of the Dnmt1 activity at the G1MDR and demethylation of the *Gata1* gene enhancers is the immediate target of our future study, as it is crucial for the understanding of the molecular basis of GATA factor switching at the initiation of erythroid differentiation. In this regard, the following observations may be pertinent. The 3'-end sequence of the G1MDR contains a con-

sensus binding site for the E2F transcription factor, and a substitution mutation of the binding site abrogates the repressor activity of the G1MDR (29). This E2F activity is particularly intriguing because Dnmt1 is known to form a complex with E2F and to repress target gene expression (37). These observations suggest that negative regulation of the *Gata1* gene in HSPCs may be based on a G1MDR-E2F-Dnmt1 complex. We surmise that the E2F expression pattern or activity may be changed during erythroid differentiation, which consequently reduces Dnmt1 recruitment and removes DNA methylation at the G1MDR.

Myelodysplastic syndrome (MDS) is a hematopoietic stem cell disorder characterized by ineffective blood cell production, which can progress to acute myeloid leukemia (AML) at a high frequency (38). Increased apoptotic death of CD34⁺ hematopoietic progenitors has been assumed to be one of the mechanisms underlying the hematopoietic defect in early-stage MDS patients (39). One clinical report found that GATA1 mRNA expression had increased approximately 10-fold in the CD34⁺ progenitors of MDS patients (40). In this study, we found that ectopic increases of GATA1 expression in HSCs led them to apoptosis and subsequent depletion. Based on these lines of evidence, we surmise that our current observation may be relevant to clinical observations on MDS patients, such that an enhanced level of GATA1 in CD34⁺ MDS cells may be critically involved in the pathology of MDS through activation of MDS cell apoptosis.

In summary, we found that Dnmt1-G1MDR-mediated *Gata1* repression is crucial for HSPC survival and that derepression of the *Gata1* gene following Dnmt1-G1MDR-mediated repression is an important mechanism for promoting the initiation of GATA factor switching, which subsequently drives the HSPC differentiation toward the erythroid lineage.

MATERIALS AND METHODS

BAC modification and generation of BAC transgenic mice. A mouse *Gata1* BAC clone (RP23-443E19) was homologously recombined with the targeting vector which was used to modify the MG-GFP BAC in our previous study (29). The correctly targeted BAC was then subjected to Neo excision to generate the MG-G1 BAC (Fig. 1A). The MG-G1 BAC was used as a parental clone to generate MG-G1ER^{T2} and MG-CreER^{T2} BACs (Fig. 3A and 7A). The targeting vector includes a 2-kb 5' homologous region (containing the DNA region from the first intron to right before the *Gata1* starting ATG in the second exon) and a 1.3-kb 3' homologous region. G1ER^{T2}-polyA-Neo or CreER^{T2}-polyA-Neo was inserted into the endogenous *Gata1* initiating ATG by recombination. The correct clone was then used for Neo excision to generate MG-G1ER^{T2} and MG-CreER^{T2} BAC clones. BAC transgenic mice were generated as described previously (10, 29). Mouse experimental procedures were approved by the Institutional Animal Experiment Committee of Tohoku University.

Flow cytometry analyses. Flow cytometry was performed on a FACSAria II flow cytometer (BD Biosciences). Mononuclear cells were prepared by use of Histopaque (Sigma), and lineage-positive cells were removed using a mouse hematopoietic lineage biotin panel (eBioscience) and Dynabeads M-280 streptavidin (Invitrogen). Apoptotic analysis was performed with a BD Pharmingen APC annexin V kit (BD Biosciences) according to the manufacturer's instructions. PI-negative cells were used for all flow cytometry experiments except for the apoptosis analysis. The following flow cytometry antibodies were used: c-Kit-allophycocyanin (APC) (eBioscience), c-Kit-APC-eFluor780 (eBioscience), CD34-fluorescein isothiocyanate (FITC) (eBioscience), CD34-APC (R&D), FcR-phycoerythrin (PE) (BD Biosciences), Sca1-FITC (BD Biosciences), Sca1-PE (BD Biosciences), Sca1-PE-Cy7 (Biolegend), Ter119-PE (eBioscience), CD71-FITC (eBioscience), CD41-FITC (BD Biosciences), CD61-PE (BD Biosciences), CD150-BV421 (Biolegend), and CD48-PE (Biolegend) antibodies.

CFU assay. One thousand CMP cells were isolated and cultured in 3 ml MethoCult GF M3434 (Stemcell Technologies) for 7 days. Colonies were determined by morphology according to the instructions in the product manual.

RT-qPCR. Cells were directly sorted into Isogen or Isogen-LS (Nippon Gene). RNA extraction, cDNA synthesis, and quantitative PCR were performed as described previously (41). The primer sequences are listed in Table 1.

Tamoxifen administration. Tamoxifen (Sigma) was dissolved in corn oil at 20 mg/ml and administered intraperitoneally.

RNA sequencing. Lin⁻ Sca1⁺ c-Kit⁺ (LSK) cells were isolated from wild-type or MG-G1 adult bone marrow in triplicate by flow cytometry, and RNA was extracted. Seventy-five nanograms of total RNA was used to generate an RNA library. Isolation of poly(A)-tailed RNA and library preparation were performed using a SureSelect strand-specific RNA sample prep kit (Agilent Technologies). The quality and quantity of the libraries were assessed by the quantitative MiSeq (qMiSeq) method (42). The libraries were sequenced using a NextSeq500 system (Illumina) for 86 cycles of single reads.

TABLE 1 Primer and probe sequences used for genomic PCR and RT-qPCR

Primer use and target	Sequence		
	Sense primer	Antisense primer	Probe
Genotyping			
MG	GGTCTCAAATGGAAGCCTGA	TGGAGTCCAGTGTCCAGTCA	
Chloramphenicol acetylase (BAC transgene)	CAGTCAGTTGCTCAATGTACC	ACTGGTGAAACTCACCCA	
GFP	CTGAAGTTCATCTGCACCACC	GAAGTTGTA CTCCAGCTTGTGC	
ERT ²	GACAGGGAGCTGGTTCACAT	AGAGACTTCAGGGTCTGGA	
Cre	ACGTTACCCGGCATCAACGT	CTGCATTACCGGTCGATGCA	
<i>Dnmt1</i> flox	GCCAGTTGTGTACTGGAAA	GAGTGTGTGTTCCGTTCTCCA	
R26T reporter	GGCATAAAGCAGCGTATCC	CTGTTCTGTACGGCATGG	
qRT-PCR with SYBR green			
Hba	CCCGGTGCCTTGTCTGCT	GGGTGAAATCGGCAGGGTGG	
Hbb	TGTGGGGAAAGGTGAACGC	CATGGGCCTTCACTTTGGC	
c-Kit	TGACAAATTCACCTCAAAGTG	CCCCTTTCTTAAGGAGGTGAC	
GATA2	ACCTGTGCAATGCCTGTGGG	TTGCACAACAGGTGCCCGCT	
GATA1	CAGAACCAGGCTCTCATCC	TAGTGCAATTGGGTGCCTGC	
<i>Dnmt1</i>	CACCTTTGGTGTGCTCCAG	CGCAAACACATGCAGAGG	
qRT-PCR with TaqMan			
rRNA	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT	TGCTGGCACCAGACTTGCCCTC

RNA-Seq data analysis. TopHat (43) was used to map the RNA-Seq data, and Cufflinks, version 2.1.1 (44), was used to quantify the expression level of each gene, in fragments per kilobase of exon per million fragments (FPKM), using default parameters. The differentially expressed genes were identified using Cuffdiff, version 2.1.1, with a threshold *q* value of 0.05. Pathway analysis of the gene expression data was performed using Gene Set Enrichment Analysis (GSEA) software (45).

Bisulfite sequencing. LSK cells were isolated from MG-CreER² or *Dnmt1*-CKO adult bone marrow 14 days after tamoxifen treatment. Bisulfite treatment and sample recovery were performed by use of a MethylEasy Xceed kit (Genetic Signatures) according to the manufacturer's instructions. The sequences of the primers used for nested PCR amplification of the dbG enhancer were as follows: dbG 1st sense primer, AAAATACATACACTTCTTAATACTTC; dbG 1st antisense primer, TTATTTGGGTGTTATTTAGTT TTT; dbG 2nd sense primer, AGTTTAAAGATAGTTTGTATTG; and dbG 2nd antisense primer, ACATACAC TTCTTAATACTT.

Accession number(s). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through accession number [GSE85750](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE85750).

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