Induction of hyperproliferative fetal megakaryopoiesis by an N-terminally truncated GATA1 mutant

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Two GATA1-related leukemias have been described: one is an erythroleukemia that develops in mice as a consequence of diminished expression of wild-type GATA1, whereas the other is an acute megakaryoblastic leukemia (AMKL) that arises in Down syndrome children as a consequence of somatic N-terminal truncation (ΔNT) of *GATA1*. We discovered that mice expressing the shortened GATA1 protein (ΔNTR mice) phenocopies the human transient myeloproliferative disorder (TMD) that precedes AMKL in Down syndrome children. In perinatal livers of the ΔNTR mutant mice, immature megakaryocytes accumulate massively, and this fraction contains cells that form hyperproliferative megakaryocytic colonies. Furthermore, showing good agreement with the clinical course of TMD in humans, ΔNTR mutant mice undergo spontaneous resolution from the massive megakaryocyte accumulation concomitant with the switch of hematopoietic microenvironment from liver to bone marrow/spleen. These results thus demonstrate that expression of the *GATA1/Gata1* N-terminal deletion mutant *per se* induces hyperproliferative fetal megakaryopoiesis. This mouse model serves as an important means to clarify how impaired GATA1 function contributes to the multi-step leukemogenesis.

Introduction

Numerous examples have established the generality of multi-step models of leukemogenesis, and often the balance of homeostatic cellular regulation by disruption of normal transcriptional activity forms the basis for such pathogenesis. GATA1 has been implicated in the control of cellular proliferation, in the inhibition of apoptosis and in the stimulation of differentiation in both the erythroid and megakaryocytic lineages (Weiss & Orkin 1995; Dubart *et al.* 1996).

As the mouse *Gata1* gene is located on the X chromosome, either *Gata1* knockout ($Gata1^{-/Y}$) or *Gata1* knockdown ($Gata1^{G1.05/Y}$, in which the expression of GATA1 is reduced to 5% of endogenous level) loss of function mutant embryonic males die *in utero* of impaired primitive erythropoiesis. In contrast, heterozygous knockout and knockdown female mice ($Gata1^{-/X}$ and $Gata^{G1.05/X}$, respectively) survive gestation and are fertile, although females of both genotypes show varying degrees of anemia as a consequence of random X chromosome inactivation (Fujiwara *et al.* 1996; Takahashi *et al.* 1997). We reported earlier that $Gata1^{G1.05/X}$ mice often developed acute proerythroblastic leukemia, whereas $Gata1^{-/X}$ mice did not (Shimizu *et al.* 2004a). Molecular analyses revealed that the residual 5% of wild-type GATA1 protein that is present in $Gata1^{G1.05/Y}$ blood cells was sufficient to protect erythroid progenitors from apoptosis, but was not enough to properly regulate proliferation and erythroid differentiation (Pan *et al.* 2005). Therefore, immature erythroid precursors abundantly accumulated in $Gata1^{G1.05/X}$ mice, hypothetically permitting increased susceptibility for possible acquisition of a second genetic hit that

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would be required for erythroid leukemic transformation (Shimizu *et al.* 2004a).

Down syndrome (DS) is a genetic disorder characterized by cells bearing all or part of an extra chromosome 21. DS children frequently develop a transient myeloproliferative disorder (TMD), followed by the development of acute megakaryoblastic leukemia (AMKL) harboring a somatic mutation in the human *GATA1* gene; this mutation generates a form of GATA1 that lacks the N-terminal (NT) 83 amino acids (Shimizu *et al.* 2008). Although blast cells in TMD patients are not readily distinguishable from those produced in AMKL (Karandikar *et al.* 2001), the clinical courses of TMD and AMKL are morbidly distinct. TMD is a self-correcting disorder (Zipursky 2003), whereas AMKL is a potentially lethal disease that exhibits no spontaneous remission. Recent evidence suggests that acquisition of the *GATA1* mutation leading to TMD occurs *in utero* (Ahmed *et al.* 2004), and AMKL blasts are clonally evolved from TMD blasts because of the acquisition of additional mutation(s) after years of clinical latency (Hitzler *et al.* 2003). Critically, this specific *GATA1* mutation is observed neither in non-megakaryoblastic leukemias in DS patients nor in DS-unrelated megakaryoblastic leukemias (with one exception) (Greene *et al.* 2003; Harigae *et al.* 2004), strongly implicating a direct role for the truncated GATA1 protein in the leukemogenesis that develops in DS patients.

We hypothesized that modeling of the human truncation mutation in mice might provide novel insights into human TMD and AMKL, because the GATA1 NT domain function is apparently important in these disorders. A previous report showed that immature megakaryocytic progenitors in the yolk sac and early fetal liver of knock-in mice that expressed a mutant GATA1 protein lacking amino acids 3 to 63 (and not 1–83) developed a hyperproliferative phenotype, but also that these aberrant cells disappeared from fetal blood by E14.5 (Li *et al.* 2005). The same phenotype was detected in conditional knockout mice in which *Gata1* exon 2 (which encodes the wild-type start codon) was deleted (Li *et al.* 2005). As this phenotype differs substantially from human TMD, the contribution of the GATA1 NT (83 amino acids) to human TMD pathogenesis remained obscure. Therefore, we adopted a transgenic complementation/rescue approach to more critically assess NT domain function.

In the present study, we generated mice that were rescued from GATA1-deficient lethality by transgenic expression of a GATA1 cDNA lacking the NT 83 amino acids (ΔNT-G1) (Shimizu *et al.* 2001), importantly generating a protein that is identical to the mutant GATA1 found in human TMD and DS-AKML. We found that complementation of germ-line *Gata1*-deficiency with transgenic lines in which the N-terminally truncated form of GATA1 was forcibly expressed led to hyperproliferative fetal megakaryopoiesis that mimicked human TMD.

Results

Generation of ΔNTR mice

To explore the contribution of the GATA1 NT 83 amino acids to the development of TMD and DS-AMKL, we analyzed transgenic mice harboring transgenic ΔNT-G1 ($Tg^{\Delta NT\text{-}G1}$) in two different *Gata1*-deficient genetic backgrounds. We previously established transgenic mouse lines in which either wild-type GATA1 (WT-G1) or ΔNT-G1 were expressed under the transcriptional control of the *Gata1* hematopoietic regulatory domain (*G1-HRD*), thereby restricting transgene expression exclusively to those tissues in which GATA1 is normally expressed (Onodera *et al.* 1997; Nishimura *et al.* 2000; Shimizu *et al.* 2001). Because the NT domain of GATA1 is important for fetal erythropoiesis, ΔNT-G1 expression at levels comparable with the wild-type protein was insufficient to rescue *Gata1*-imposed lethal deficiency, whereas $Tg^{WT\text{-}G1}$ lines rescued even when expressed at lower than haploid equivalent levels (Shimizu *et al.* 2001). In contrast, greater than wild-type abundance of ΔNT-G1 expression was able complement the *Gata1*-deficient lethal anemia (Fig. 1a). Therefore, to avoid experimental complications that might arise from any hypothetical dyserythropoietic phenotypes in these animals, we compared the $Tg^{\Delta NT\text{-}G1}$ and $Tg^{WT\text{-}G1}$ lines that both expressed abundant GATA1 protein by crossing to *Gata1* germ-line mutants to generate variously rescued animals. For the sake of simplicity, we refer to null-mutant animals that have been rescued by transgenic expression of either wild-type GATA1 as $G1R^{null}$ (genotype $Gata1^{-/Y}$: $Tg^{WT\text{-}G1}$) or by ΔNT-GATA1 as ΔNTR^{null} ($Gata1^{-/Y}$: $Tg^{\Delta NT\text{-}G1}$) males, and we refer to $Gata1^{G1.05/Y}$ males or $Gata1^{G1.05/-}$ females rescued by the same two transgenes as G1R or ΔNTR, respectively.

To verify the accumulation of transgenic WT-G1 or ΔNT-G1 in the hematopoietic cells of rescued mice, total cell lysates were prepared from the fetal livers of G1R, ΔNTR and wild-type mice between embryonic days 17.5 (E17.5) and E18.5.

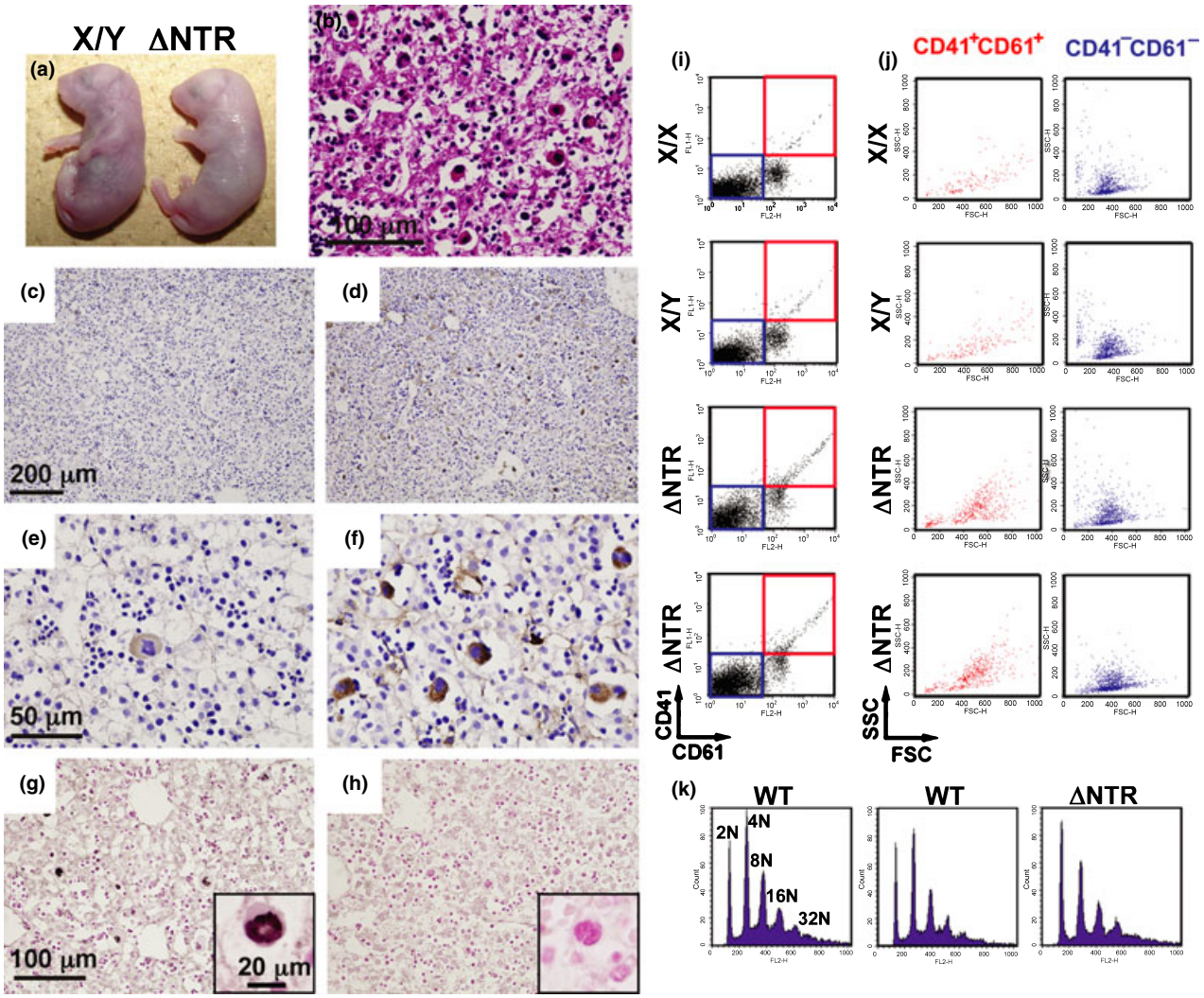


Figure 1 Abnormal megakaryocytosis in ΔNTR embryos. (a) Appearance of E18.5 ΔNTR embryos and their wild-type littermates (X/Y). Note that these two genotype mice are indistinguishable. We evaluated more than 20 ΔNTR embryos at E15.5, but after this stage the ΔNTR embryos were recovered from anemia and indistinguishable from the wild-type embryos (data not shown). (b) Hematoxylin and eosin-stained section of E18.5 ΔNTR liver shows accumulation of mononuclear or binuclear large cells with more than 10 μm diameter. (c–h) Immunostaining of E18.5 wild-type (c, e and g) and ΔNTR (d, f and h) liver sections of with anti-CD41 antibody (c–f) or N6 anti-GATA1 antibody (g and h). Note the megakaryocytes that show intense CD41 staining, but negative staining for GATA1 in E18.5 ΔNTR livers. Representative megakaryocytes in high magnification were shown in inset. (i and j) Flow cytometric analysis of P0 liver cells. $CD41^{+}CD61^{+}$ cells are abundant in ΔNTR embryos [(red rectangle in (i)]. Note that $CD41^{+}CD61^{+}$ cells are accumulated $FSC^{low}SSC^{low}$ fraction in rescued embryos, whereas distribution of $CD41^{-}CD61^{-}$ cells [blue rectangle in i)] is comparable that in the wild-type pups (j). (k) DNA ploidy assay of $CD41^{+}$ cells in E18.5 livers. The low-ploidy megakaryocytes are dominant in a ΔNTR embryo (right panel), whereas the frequency of higher ploidy megakaryocytes is higher in pooled liver MNCs from wild-type embryos (two left panels). More than 10 000 CD41-positive cells/sample recovered from four wild-type embryo samples (five embryos/sample) and three rescued embryos samples (one embryo/sample) were utilized for the assay, and representative data are shown.

The expression of GATA1 derived from the transgenes was evaluated by immunoblotting using two different anti-GATA1 antibodies: N6 recognizing the NT domain of GATA1 (Ito *et al.* 1993) and C20 recognizing the C-terminus of the protein (Fig. S1a).

As expected, both N6 and C20 antibodies recognized WT-G1 in G1R animals, whereas only C20 reacted with the foreshortened GATA1 protein in ΔNTR mice. These results indicate that the residual 5% of wild-type *Gata1* mRNA present in ΔNTR mice (from the *Gata1*$^{G1.05}$ allele) negligibly contributes to the total detectable GATA1 protein in rescued animals (Fig. S1b). Unexpectedly, the C20 antibody also detected a short form of GATA1 (which has a comparable molecular weight to that of ΔNT-G1) in G1R mice, whereas only trace levels of the short form of GATA1 were observed in wild-type embryos. We suspect that the second in-frame AUG codon in *Gata1* mRNA is utilized as an alternative initiation codon, leading to the generation of ΔNT-G1 protein in G1R animals, as previously reported (Calligaris *et al.* 1995).

NT domain contributes to megakaryocyte proliferation

We previously showed that transgenic expression of ΔNT-G1 at levels greater than wild-type GATA1 abundance led to rescue of *Gata1*$^{G1.05/Y}$ mutants from early embryonic lethality (Shimizu *et al.* 2001). The ΔNTR embryos displayed obvious anemia at E13.5 with marked perturbation of erythroid differentiation, whereas no anemia was observed in their counterpart G1R embryos (Fig. S2a–c). These embryos invariably recovered from the anemia by E15.5, and they could no longer be distinguished visually from their wild-type littermates (Fig. S2d). These results indicate that greater than diploid abundant expression of GATA1 protein that lacks the NT domain is adequate to rescue the erythroid GATA1 lethal deficiency, but is still insufficient for full restoration of GATA1 function in hematopoiesis throughout embryogenesis.

To dissect the NT domain function, we conducted serial analyses of embryonic hematopoiesis in these genetically rescued embryos. At E18.5, macroscopic anemia was not observed in ΔNTR mice (Fig. 1a) and flow cytometry failed to reveal significant differences between ΔNTR mutants and their wild-type littermates, except for a moderately lower level of Ter119 expression (Fig. S2e). In contrast, small megakaryocytic cells harboring discretely segmented single or double nuclei were abundant in the ΔNTR fetal livers (Fig. 1b). These cells strongly expressed CD41, whereas relatively weaker CD41 expression was observed in the multi-lobulated megakaryocytes that are normally detected in wild-type fetal livers (Fig. 1c–f). Immunostaining with the N6 antibody revealed no reactivity to fetal liver cells in ΔNTR mice (Fig. 1g,h), indicating that the abundantly accumulated immature megakaryocytes lack detectable expression of wild-type GATA1.

Flow cytometry revealed that the number of CD41^{+} CD61^{+} megakaryocytic cells is significantly higher in the livers of neonatal ΔNTR and ΔNTR^{null} embryos compared with those of G1R, G1R^{null} and wild-type controls (Fig. 1i, Fig. S3a,b and data not shown). CD41^{+} CD61^{+} megakaryocytic cells in rescued embryos were accumulated in the FSC^{low}SSC^{low} fraction (Fig. 1j). The frequency of low-ploidy CD41^{+} megakaryocytes was relatively high in the livers of E18.5 ΔNTR embryos compared with their wild-type counterparts (Fig. 1k). Although the average ploidy was reduced, a considerable number of high-ploidy CD41^{+} megakaryocytes were also recovered in rescued animals (Fig. 1k), suggesting that there are progenitor cells in ΔNTR embryos that do not have the megakaryocyte maturation defects. In accord with previous *in vitro* analyses in which recombinant retroviruses were used to infect progenitors with ΔNT-G1 (Kuhl *et al.* 2005), the present data support the contention that the NT domain of GATA1 is necessary to keep proliferation of immature megakaryocytic progenitors in the homeostatic condition, but not for their terminal differentiation *in vivo*.

Megakaryocytic cells accumulated in ΔNTR embryos express erythroid markers

We found that hematopoiesis in ΔNTR pups was perturbed in both the erythroid and megakaryocytic lineages. Platelet counts were higher in the rescued animals (Fig. 2a). Cytological examination of peripheral blood smears revealed the presence of abundant platelets in ΔNTR pups (Fig. 2b), and megathrombocytes were occasionally observed (Fig. 2b, insert). Immature megakaryocyte-like cells exhibiting hypogranularity and cytoplasmic vacuoles with emperipolesis were also observed (data not shown). Denatured cells were frequently detected on the blood smears (Fig. 2b, arrowhead), which could be derived from CD41^{+} megakaryocytic cells (see below) that tend to be destroyed by manipulation.

Surprisingly, blast-like cells (which varied in diameter from 12 to 15 μm) were also observed in the ΔNTR blood smears. These cells with a relatively large nuclear to cytoplasmic ratio had a round to irregular nuclear outline containing a few nucleoli

(a)

Mouse type	Embryo number	Hemoglobin (g/dL)	Hematocrit (%)	Red blood cell count (X10^4/mL)	White blood cell count (X10^2/mL)	Platelet count (X10^4/mL)
Rescued	11	12.4 ± 1.7**	38.1 ± 5.4†	351 ± 54†	78 ± 39*	133.4 ± 21.7††
Wild type	19	14.8 ± 2.1	44.6 ± 5.7	438 ± 56	56 ± 19	46.0 ± 5.7

(* : < 0.05, ** : < 0.005, † : < 0.005, †† : < 0.0001)

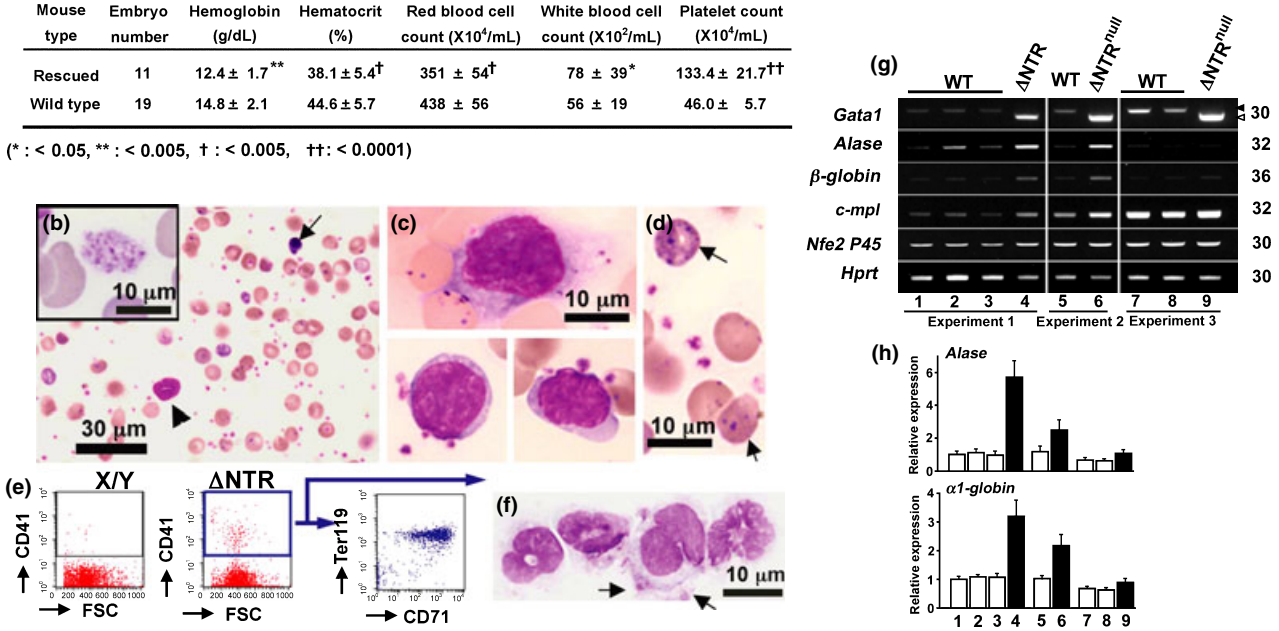


Figure 2 Features of CD41$^+$ cells in perinatal rescued embryos. (a) Hematopoietic indices of rescued [ΔNTR^{null} (n = 7) and ΔNTR (n = 4)] P0 pups. (b–d) Abnormal peripheral blood cells in the blood smears of ΔNTR pups stained with Wright-Giemsa. (b) Nucleated red blood cell (arrow) and denatured cell (arrowhead) are present in the peripheral blood of rescued pups. Note that platelets with normal appearance are abundant. Megathrombocytes also exist as shown in the inset. (c) Blast-like cells with prominent Golgi area and poorly developed cytoplasmic granulation are also found in the rescued pups. (d) Higher magnification micrograph revealed the presence of atypical red blood cells with multiple Howell-Jolly bodies and basophilic stippling (arrows). (e) Flow cytometric analysis of MNCs in the peripheral blood of P0 ΔNTR embryo. Note that the CD41$^+$ cells also express the erythroid marker Ter119. (f) Morphological analysis of accumulated CD41$^+$ cells in the peripheral blood of rescued pups. These cells resemble megakaryocytic progenitors with cytoplasmic fragmentation (arrows). Note that the appearance of nuclei is quite similar to the naked nucleus observed in (b). (g) RT-PCR expression analysis of erythroid- and megakaryocyte-specific genes in CD41$^+$CD61$^+$ cells from E18.5 livers. Samples 1–4 (experiment 1), samples 5 and 6 (experiment 2), and samples 7–9 (experiment 3) were recovered from the same litter. *Hprt* is amplified as an internal control. Amplification cycles are shown in the right side of the figure. Black arrowhead indicates the migration position of the endogens *Gata1* amplicon, whereas white arrowhead indicates the position of ΔNT-G1 transgene derived amplicon. Note that, as 5′-region of *Gata1* cDNA coding for the NT domain was deleted, the transgene-derived PCR product is shorter than that derived from the endogenous gene. (h) Quantitative RT-PCR analyses on *Alase* (*Alas2*; upper panel) and *a1-globin* (lower panel) genes. Sample numbers in the bottom of panel corresponds to those in panel G. Open bars represent wild-type embryos, whereas solid bars represent ΔNTR embryos. Data are shown relative to the expression of wild-type embryo shown as sample number 1.

and a basophilic cytoplasm; a few also exhibited cytoplasmic budding (Fig. 2c**)**. Furthermore, dysplastic red blood cells and unclassified cells were also observed in perinatal rescued blood (Fig. 2d, arrows), suggesting that even abundant ΔNT-G1 protein does not fully compensate for the functional deficits of GATA1 in either the perinatal erythroid or megakaryocytic lineages.

To more thoroughly characterize the megakaryocyte phenotype in the rescued pups, we examined surface markers of mononuclear cells (MNC) isolated from the peripheral blood of neonatal (P0) pups. CD41$^+$ cells in these pups were more abundant than in wild-type littermates (Fig. 2e). CD41$^+$ cells in the rescued pups resembled promegakaryoctyes or intermediate stage megakaryocytes, possessed lobulated nuclei, cytoplasmic granulation and cytoplasmic fragmentation (Fig. 2f**)**. Surprisingly, these CD41$^+$ cells also expressed erythroid surface markers (Fig. 2e, far right). In accord with this observation, CD41$^+$ CD61$^+$ cells in rescued fetal livers expressed erythroid-specific mRNAs [e.g. erythroid 5-aminolevulinate synthase (ALAS-E) and β-globin] more abundantly than those in wild-type littermates did

(Fig. 2g). Megakaryocytic genes, *c-mpl* and *Nfe2 p45*, were also expressed at higher level in $CD41^+$ $CD61^+$ cells from rescued embryos than in the cells from wild-type littermates. We also analyzed expression levels of two erythroid genes, *Alas2* (*Alase*) and *α1-globin*, by quantitative PCR. The results were consistent with the semi-quantitative RT-PCR analysis (Fig. 2h). Although expression levels showed considerable variations among litters throughout these analyses, the erythroid and megakaryocytic gene expressions in $CD41^+$ $CD61^+$ cells were consistently higher in the cells from rescued embryos than in the cells from wild-type littermates, demonstrating that enforced expression of ΔNT form GATA1 provoked abnormal accumulation of cells expressing both erythroid and megakaryocytic markers.

Hyperproliferative megakaryocyte progenitors accumulate in ΔNTR embryos

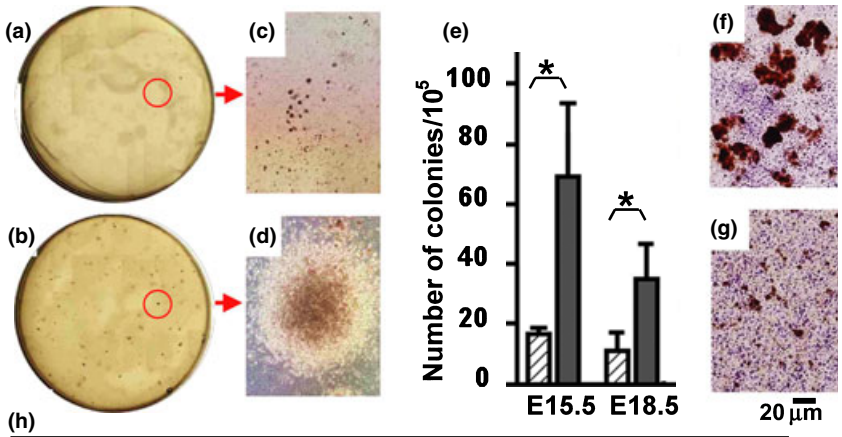
As the NT domain of GATA1 had been shown important for growth control in immature megakaryocytes (Kuhl *et al.* 2005), we surmised that the elevated number of $CD41^+$ cells detected in the livers and peripheral blood of rescued embryos could be a consequence of improperly regulated megakaryopoiesis. Recently, it was reported that a unique population of megakaryocytes with a hyperproliferative phenotype appeared transiently in the yolk sacs and early fetal livers of mice harboring the GATA1 ΔNT mutation (Li *et al.* 2005). To examine the hematopoietic proliferative phenotype of ΔNTR embryos, we carried out *in vitro* differentiation assays utilizing fetal livers from embryos of several different ages with low concentrations of thrombopoietin (Tpo; 5 ng/mL) to match the conditions applied in a previous report (Li *et al.* 2005). We found, in contrast to those data, that cells recovered from E12.5 and E15.5 ΔNTR livers formed abnormally large colonies that stained with AchE (Fig. S3c–g). Colonies developed from E15.5 fetal liver cells were smaller than those from E12.5 livers. However, under these same culture conditions we were unable to detect any hyperproliferative colonies in cells prepared from E18.5 fetal livers (data not shown).

As definitive megakaryocytic progenitors have been reported to display different cytokine sensitivity from primitive progenitors (Xu *et al.* 2001; Tober *et al.* 2007), we tested additional cytokine combinations for late fetal liver-derived megakaryocyte colony formation. We carried out colony assays using media containing high-concentration of Tpo (50 ng/mL) in combination with IL-3 and IL-6. Under these conditions, cells from E15.5 and E18.5 ΔNTR embryonic liver formed megakaryocytic colonies (Fig. 3a–d). Importantly, the number of $AchE^+$ colonies larger than 0.1 mm diameter had now markedly increased in the liver cells recovered from both ΔNTR embryos compared with those recovered from wild-type embryos (Fig. 3e). Of particular interest, higher magnification examination of the cultures revealed that, although colonies developed from the wild-type embryos contained a small number of mature megakaryocytes that strongly stained with AchE (Fig. 3c), most of the colonies observed in the culture developed from ΔNTR fetal livers contained numerous smaller cells that were either only weakly positive or negative for AchE (Fig. 3d). Those cells were CD41-postive and morphologically resembling to immature megakaryocytes with indented nuclear outline and cytoplasmic budding (Fig. S3h–k).

We also examined CFU-Mk by seeding E18.5 fetal liver cells into a collagen-based dish in the presence of high concentrations of Tpo plus IL-3 and IL-6. After 8 days of incubation, the clusters were stained with AchE and colonies that contained more than 10 $AchE^+$ cells were scored as positive. In this CFU-Mk assay, cells from wild-type embryos generated large mature megakaryocytes (Fig. 3f), whereas numerous smaller cells were detected in ΔNTR embryo cell cultures (Fig. 3g). The number of CFU-Mk in E18.5 ΔNTR fetal livers was comparable with that from wild-type livers (Fig. 3h), indicating that the colony-forming megakaryocyte progenitors had not increased in the ΔNTR embryos. In contrast, the number of erythroid colony-forming units (CFU-E) was reduced in ΔNTR fetal livers (Fig. 3h). These results thus demonstrate that the hematological abnormalities observed in the ΔNTR pups are caused, at least in part, by altered proliferation of erythroid/megakaryocytic progenitor cells.

Relationship between TMD-like abnormality and GATA1 NT truncation

The hematopoietic abnormality observed in perinatal ΔNTR pups strikingly resembles that of DS-associated TMD. Approximately 10% of DS children experience a clonal expansion of megakaryoblasts tightly correlated with somatic mutation of the *GATA1* locus (Zipursky 2003). In the vast majority of these cases, the blast cells and associated abnormalities in platelet number and morphology disappear spontaneously during the first 3 months of life.



(h)

Genotype	No. of embryos	Fetal liver cell (X 10^5)		
		CFU-E	CFU-Mk	CFU-GM
Wild type	3	102.8 ± 13.8	66.1 ± 9.8	479.2 ± 53.5
Rescued	4	39.9 ± 13.0	73.3 ± 25.9	417.3 ± 13.1

(CFU-E, erythroid colony-forming unit; CFU-Mk, megakaryocytic colony-forming unit, CFU-GM, granulocyte/macrophage colony-forming unit)

Figure 3 Hyperproliferative megakaryocytic progenitors in rescued embryos during late gestation. (a–d) Representative megakaryocytic colonies in semi-solid culture. E18.5 wild-type (a) and ΔNTR (b) livers were cultured for 6 days and AchE activity was measured. Representative megakaryocytic colonies under higher power magnification of panels a and b are shown in panels C and D, respectively. Colonies composed of a few mature megakaryocytes were observed in the wild-type embryos (c), whereas colonies containing a large number of smaller cells were observed in the rescued embryos (d**)**. (e) The number of hyperproliferative $AchE^+$ colonies significantly increased in ΔNTR embryos at E15.5 and E18.5. The hatched bars represent wild-type embryos and the filled bars represent ΔNTR embryos. Each group contains 4–6 embryos ($P < 0.005$). (f and g) Character of megakaryocytes in the colonies. Large, mature megakaryocytes stain strongly for AchE in the wild-type embryos (f), whereas a greater number of smaller cells, either stained or unstained with AchE, are present in the rescued embryos (g). (h) Counting of hematopoietic colony assays starting with cells recovered from E18.5 liver of ΔNTR rescued embryos ($n = 4$).

To begin to investigate the phenotypic course of the hematopoietic disorder(s) observed in the rescued newborn mice, we first examined the peripheral blood indices. By 5 days of age leukocytosis in the P5 rescued pups disappeared, but thrombocytosis was still detectable (data not shown). In contrast, but in agreement with a previous report showing that only minor abnormalities in adult megakaryopoiesis were detected in patients bearing a mutation that produces ΔNT-G1 (Hollanda *et al.* 2006), the number of splenic megakaryocytes in P0 and P5 rescued mouse pups was comparable with wild-type animals (Fig. 4). The $CD41^+$ $CD61^+$ cells in the rescued embryo livers were gradually disappeared along with the decline of liver hematopoiesis (Fig. 4b,c). *In vitro* culture assay confirmed the transient nature of this hyperproliferative megakaryocytosis (Fig. S4a). The number of $CD41^+CD61^+$ megakaryocytes in both the bone marrow and spleen disappeared coincident with the loss of any detectable peripheral blood abnormalities by the time the rescued pups reached 3 weeks of age (Fig. S4b,c).

These observations demonstrate that a mutation resulting in the production of an N-terminally truncated GATA1 molecule provoked a transient but self-rectifying hyperproliferative disorder in the livers during the postnatal period in a mouse genetic background that is the equivalent of disomic human chromosome 21. Based on these results, we conclude that in the mouse, a TMD-like phenotype is not associated with a chromosome imbalance (as it appears to be in human DS patients), but only with the presence of a specifically altered GATA1 protein. This central observation suggests that human DS-TMD may be unlinked to trisomy 21, but could be directly related to GATA1 NT truncation.

Discussion

GATA1 governs proper development of erythroid and megakaryocytic progenitors through precise regulation of at least three different phases of cellular maturation: regulation of proliferation, inhibition of

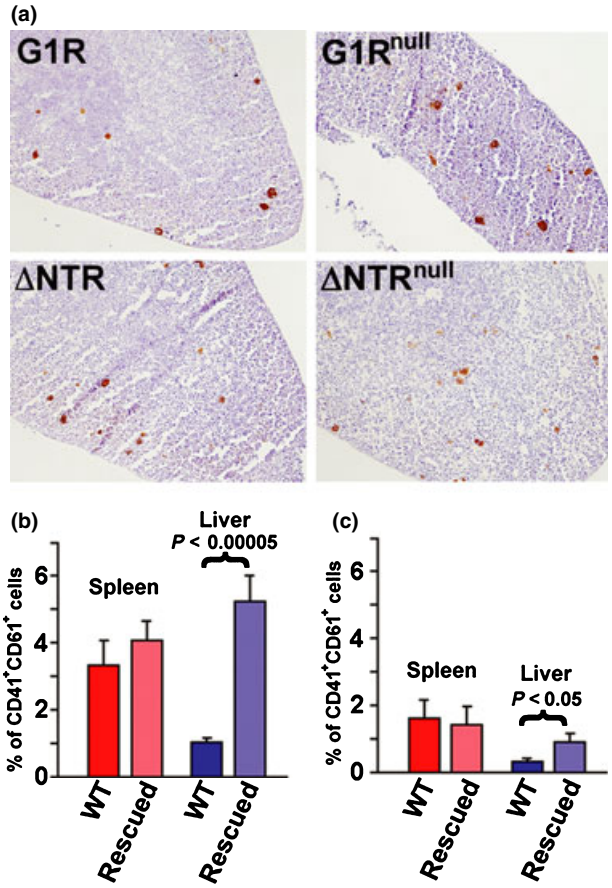


Figure 4 Megakaryopoiesis in postnatal hematopoietic organs. (a) The numbers of megakaryocytes in spleens of ΔNTR and ΔNTR^{null} P5 pups (lower panels) are comparable with the numbers in G1R and $G1R^{null}$ pups (upper panels). Megakaryocytes were visualized by AchE staining. (b and c) Frequency of megakaryocytes in the spleens and livers of postnatal pups. Frequency of $CD41^+CD61^+$ megakaryocytes in the spleens (red bar) and livers (blue bar) of P0 [ΔNTR (n = 6) and wild type (n = 3); (b)] and P5 [ΔNTR (n = 4) and wild type (n = 3); (c)] pups, respectively, were analyzed by flow cytometry.

apoptosis and promotion of differentiation. A GATA1 knockdown mutation that leads to diminished GATA1 expression at 5% of wild-type levels results in the accumulation of immature erythroid progenitors in affected animals (Shimizu *et al.* 2004a). We report here that this genetically engineered mouse, when combined with an N-terminally truncated GATA1 protein (ΔNTR) through transgenic rescue, resulted in abnormal accumulation of $CD41^+$ megakaryocytic progenitors. Therefore, we propose that defective GATA1 function in the mouse provokes leukemia or promotes a preleukemic condition in two distinct cell populations: one is in a precursor to $c\text{-}Kit^+CD71^+$ erythroid leukemia cells and the other is in a distinct cell that leads to transient megakaryoblast expansion that closely resembles human DS TMD.

To date, two types of GATA1-related leukemias have been reported (Shimizu *et al.* 2008). One is the $c\text{-}Kit^+CD71^+$ erythroid leukemia that develops in mice as a consequence of deficient *expression* (reduced abundance) of full-length GATA1 (Shimizu *et al.* 2004a), whereas the other is AMKL that arises in DS children as a consequence of *structural* change in GATA1 protein (Wechsler *et al.* 2002). As summarized in Fig. 5, these GATA1-related leukemias bear a few of striking similarities in their pathogenesis. First, the enfeebled GATA1 function provokes abnormal accumulation of immature progenitors that constitute the basis of pathogenesis. As *Gata1* is on the X-chromosome, either the wild-type or $Gata1^{G1.05}$ mutant allele is randomly inactivated in female mice, and although hematopoiesis in general is supported by cells that express the wild-type *Gata1* allele, erythroid and megakaryocytic progenitors derived from cells that express the $Gata1^{G1.05}$ allele abnormally accumulate (Shimizu *et al.* 2004a). Similarly, in the present study, we found abnormal accumulation of megakaryocytic progenitors in ΔNTR and ΔNTR^{null} embryos. Consistent with the observation in mice, human *GATA1* mutations that lead to NT domain deletion were found in the blasts of virtually all TMD patients (Wechsler *et al.* 2002; Greene *et al.* 2003; Hitzler *et al.* 2003; Rainis *et al.* 2003), suggesting that loss of the NT domain is necessary to provoke aberrant accumulation of the megakaryocytic progenitors in humans. In contrast, accumulation of erythroid progenitors does not occur in DS patients bearing the ΔNT mutation (Wechsler *et al.* 2002; Hitzler *et al.* 2003; Rainis *et al.* 2003), despite the fact that both human TMD cases and ΔNTR mice display erythroid abnormalities in addition to the megakaryocytic phenotype. These results suggest that the quantitative and qualitative deficits of GATA1 are directly linked to the changes in the regulation of gene expression that lead to the accumulation of lineage-specific progenitors.

Second and perhaps most importantly, GATA1 dysfunction alone does not appear to be sufficient for the development of megakaryocytic leukemia. A GATA1 knockdown line of mice that virtually lack GATA1 expression in megakaryocytes were reported to show aged-related myelofibrosis and hyperprolifer-

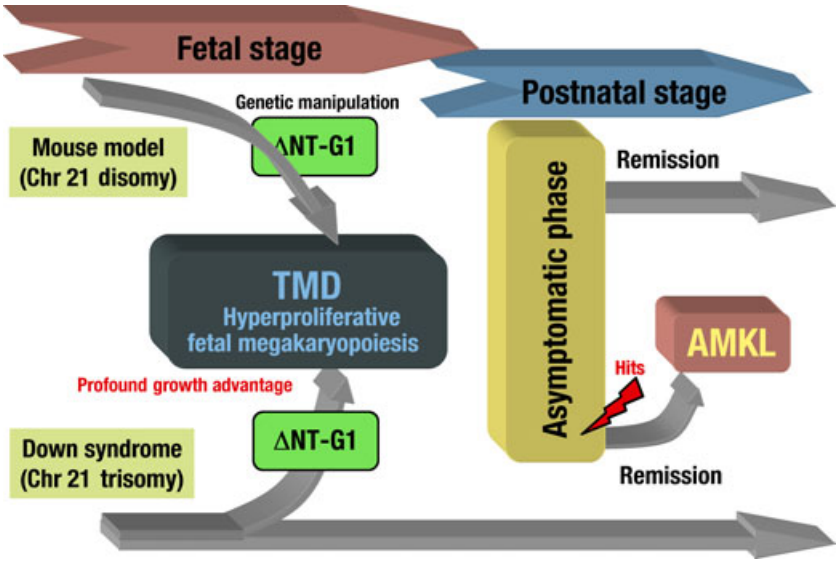


Figure 5 Model for the contribution of GATA1 mutation to the pathogenesis of DS-AMKL. During fetal liver hematopoiesis, either genetically engineered (mouse) *Gata1* or spontaneously acquired (human) *GATA1* gene mutations provide a growth advantage for megakaryocytic progenitors, which is independent of chromosome 21 dosage (TMD stage). However, because progenitors in the spleen and bone marrow are insensitive to this GATA1 mutation and begin to accumulate, abnormal megakaryocytic progenitors derived from fetal origin gradually diminish after birth (asymptomatic phase). Additional spontaneous genetic lesions occur during the TMD stage of DS patients, and transform the hyperproliferative megakaryocytic progenitors into malignant leukemic cells, or alternatively patients achieve complete remission for life after the elimination of all affected cells. Mice harboring *Gata1* gene mutations never experience this leukemia, probably because they never find the second mutation(s) leading to leukemogenesis.

ation in the megakaryocytic progenitor population, but these mice were also reported to not develop megakaryocytic leukemia (Vannucchi *et al.* 2002). Although c-Kit^{+}CD71^{+} erythroid progenitors, which have a morphologically and immunophenotypically similar appearance to c-Kit^{+}CD71^{+} leukemic cells, have already accumulated in *Gata1*$^{G1.05/X}$ embryos and pups, these cells are not leukemogenic on transplantation into nude mice (Shimizu *et al.* 2004a). In the case of human DS-associated TMD and AMKL, it has been reported that approximately 20% of those TMD patients have a risk to develop DS-AMKL, whose leukemic blasts bear the same clonal mutations in the *GATA1* locus as do TMD blasts (Hitzler *et al.* 2003). The latent period between regressed TMD and first detection of AMKL leukemia may represent the time required to accumulate additional genetic hits that lead to leukemia. These summary observations suggest that additional genetic events must be required to transform the accumulated progenitor cells that result from the initial GATA1 deficiency. An intriguing observation is that the CD41^{+} megakaryocytic cells accumulated in the ΔNTR pups express erythroid marker genes, which shows a very good agreement with the observation that the blast cells of TMD and leukemic cells of AMKL also express similar erythroid genes (Ito *et al.* 1995; Bozner 2002). Based on these observations, we speculate that additional mutations that arise in the megakaryocytic progenitor population eventually yield the ΔNTR and ΔNTR^{null} mouse leukemias.

Recently, it was reported, from examining murine knock-in and conditional knockout mutants, that megakaryocytic progenitors in early stage mouse embryos were sensitive to *GATA1* gene mutations and acquire a growth advantage (Li *et al.* 2005). This report claimed that cells expressing an N-terminally truncated GATA1 protein are sensitized under the unusual condition of trisomy 21, so that proliferating megakaryocytic cells persist during the perinatal period and give rise to TMD, whereas this same population of cells are only transiently observed in a very narrow window and disappear by mid-gestation in normal mice that are disomic for chromosome 21 (Li *et al.* 2005). In contrast, we routinely observed abnormally high accumulation of megakaryocytic cells in the liver and circulating blood of perinatal ΔNTR

and ΔNTR^{null} mice. As these rescued mice do not have any mutations except for the genetically manipulated *Gata1* gene, attenuated expression of gene(s) on chromosome 21 seem not to be required for the development of TMD-like megakaryocytic progenitor accumulation.

One plausible explanation for this discrepancy is the difference in tissue culture conditions used in the two studies. Although Tpo is known to sustain CFU-Mk formation by cells derived from yolk sacs and E13.5 livers, additional cytokines are also required for CFU-Mk culture from adult bone marrow cells (Xu *et al.* 2001). In particular, IL-3 and IL-6 are known to support the development of early progenitors to mature megakaryocytes in combination with high concentrations of Tpo (Kaushansky 1995). Therefore, we tested elevated Tpo concentrations in combination with IL-3 and IL-6 for the detection of hyper-proliferating megakaryoblasts in late fetal liver cells, and these results revealed that proliferating megakaryocytic cells reside even in E18.5 liver cells. In contrast, large CFU-Mk colonies are formed when E12.5 or E15.5 liver cells from ΔNTR or ΔNTR^{null} embryos are grown in the presence of low concentrations of Tpo. However, in the latter culture condition, large CFU-Mk colonies are scarce when embryos developed further and were not observed in cultures developed from E18.5 liver cells. It was previously reported that bipotential megakaryocytic/primitive erythroid progenitors derived from hemangioblast precursors were predominant in yolk sacs and early fetal livers (Tober *et al.* 2007), so these may be the cells visualized under the latter culture condition.

In the present study, we exploited transgenic complementation/rescue to examine NT domain function in the pathogenesis of TMD. The advantage of such a transgenic rescue strategy over other approaches is that the quantity of exogenous GATA1 protein varies and can be precisely determined in independent transgenic lines, so that any GATA1 function that is impaired in some aspect of hematopoiesis can be compensated by expression of either wild-type or mutant GATA1 protein. A nice example of this strategy was recently provided in a mouse model of X-linked thrombocytopenia caused by an inherited mutation in *GATA1* that results in disruption of the critical GATA1-FOG1 interaction (Shimizu *et al.* 2004b). Although almost all knock-in mice that express a mutant GATA1 that cannot interact effectively with FOG1 died *in utero* (Chang *et al.* 2002), more abundant expression of the same mutant protein (as a transgene) enabled rescue of the embryonic lethal dyserythropoietic phenotype in the GATA1-deficient mice (Shimizu *et al.* 2004b).

Similarly, we found that, although GATA1-deficient mice are rescued from early embryonic lethality by ΔNT-G1 transgene expression at endogenous GATA1 levels, approximately 70% of the rescued mice died at a late embryonic stage *in utero* because of defective definitive erythropoiesis (Shimizu *et al.* 2001). In contrast, on abundant expression of the ΔNT-G1 transgene, both *Gata1* knockout and knockdown lines of mice are rescued and these rescued mice are an excellent platform for these reported leukemia studies. As all GATA1 transgenes employed were stably transmitted through multiple generations, in the rescued mice all erythroid and megakaryocytic cells express a mutant GATA1 protein in a consistent and reproducible manner.

In conclusion, we report the discovery that *Gata1* ΔNT mutation alone is sufficient to recapitulate abnormal proliferation and accumulation of megakaryocytic progenitors in embryonic and perinatal mouse livers *in vivo*. This transient megakaryoblastosis appears to be correlated to the changes in the sensitivity of megakaryocytic progenitors to the GATA1 ΔNT mutation during the hematopoietic environment switching from fetal liver to the spleen and bone marrow. These results suggest that human DS-TMD and -AMKL cases show more severe and pleiotropic abnormalities (Massey 2005) than those displayed by the ΔNT mutant mouse, perhaps as a consequence of combinatorial events, such as DS-related dysfunction of hematopoietic stem cells (Holmes *et al.* 2006) or altered immunological environments (Ugazio *et al.* 1990); nonetheless, we believe that this mouse model provides a good approximation of human TMD. Furthermore, additional mutation(s) to the *GATA1/Gata1* NT deletion appears to be necessary to transform those hyperproliferative megakaryocytic cells related to TMD into overt leukemic cells in both mouse and human. This model significantly clarifies our understanding of the distinct molecular mechanisms by which the GATA1 protein can contribute to leukemogenesis.

Experimental procedures

Animals

Experimental procedures were approved by the Institutional Animal Experiment Committee of the Tohoku University,

and experiments were carried out in accordance with the Regulation for Animal Experiments in Tohoku University. Survival time was measured from time of birth to the end of follow-up. Thirty to 50-μL of peripheral blood was collected from individual pups in EDTA. Hematopoietic indices of perinatal pups were determined using a hemocytometer (Nihon Koden).

Histological and immunohistochemical analyses

Fetal livers were fixed for 90 min in 4% paraformaldehyde solution in 0.1 M phosphate buffer and then dehydrated at 4 °C with 40, 80 and 100% methanol in PBS. After embedding in polyester wax, the tissues were sectioned and incubated with monoclonal anti-integrin αIIb antibody or N6 antibody (Santa Cruz) at 4 °C, and subsequently incubated with a biotinylated secondary antibody. Avidin–biotin complex immunocytochemistry (Vector Laboratory) was used to detect specific signals. For acetylcholine esterase (AchE) staining (Xu *et al.* 2001), fetal livers were embedded in Tissue-Tek OCT compound (Sakura Finetechnical) and quick-frozen.

Flow cytometry

Mononuclear cells were prepared by Histopaque-1083 density centrifugation, and incubated with monoclonal antibodies or their respective isotype controls. Dead cells were excluded by the addition of 2 μg/mL propidium iodide (PI). Flow cytometry was carried out using a FACSCalibur and the CellQuest Pro software (Becton Dickinson). For ploidy analysis, MNC were incubated fluorescein isothiocyanate (FITC)-conjugated anti-CD41 and fixed in 70% ethanol. Cells were washed and resuspended in CATCH medium (Levine & Fedorko 1976), subsequently incubated with 1-mg/mL RNase A. After 30 min incubation with 50 μg/mL PI, more than 1×10^4 $CD41^+$ cells were analyzed by FACSCalibur and ModFit software (Becton Dickinson).

Cell isolation

For cytospin analyses, MNC of peripheral blood were stained with FITC-conjugated anti-CD41 antibody, subsequently incubated with anti-FITC microbeads. Megakaryocytes were purified using a magnet-activated cell-sorting separation column. For semiquantitative and quantitative RT-PCR analyses, fetal liver cells were stained with FITC-conjugated anti-CD41 and phycoerythrin-conjugated anti-CD61 antibody and isolated by FACSAria.

Semiquantitative and quantitative RT-PCR

Total RNA was prepared by ISOGEN-LS (Nippon Gene). cDNA was synthesized using Superscript III (Life Technologies). Quantitative RT-PCR was carried out using qPCR MasterMix Plus for SYBR Green I (Eurogentec) with ABI PRISM 7300 (PE-Applied Biosystems). Expression levels were normalized to *Gapdh*. Primer sequences are available on request.

In vitro culture assay

To determine megakaryocyte proliferation, 3×10^5 cells were cultured in Methocult-M3231 medium (Stem Cell Technologies) with 50 ng/mL Tpo, 10 ng/mL IL-3 and 20 ng/mL IL-6 (cytokines were the generous gift of Kirin Brewery). After 6 days, cultured cells were stained for AchE activity. The colonies with a diameter of more than 0.1 mm were counted. Collagen-based megakaryocytic CFU (CFU-Mk), CFU-E and CFU-granulocyte/macrophage (CFU-GM) assays were carried out as previously described (Shimizu *et al.* 2004b).

Statistics

We carried out statistical analyses using StatView software. The unpaired Student's *t*-test was used to generate *P*-values for all datasets. Error bars represent standard deviation.

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Supporting Information/Supplementary material

The following Supporting Information can be found in the online version of the article:

Figure S1 Expression of GATA1 mutant proteins in transgenic mice.

Figure S2 Erythroid phenotype of rescued embryos.

Figure S3 Megakaryocytic phenotypes of rescued embryos.

Figure S4 Megakaryopoiesis in postnatal hematopoietic organs.

Additional Supporting Information may be found in the online version of this article

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