Mature erythrocyte membrane homeostasis is compromised by loss of the GATA1-FOG1 interaction

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GATA1 plays essential roles in erythroid gene expression. The N-terminal finger of GATA1 (GATA1-Nf) is important for association with FOG1. Substitution mutations in GATA1-Nf, such as GATA1^{V205M} that diminish the GATA1-FOG1 association, have been identified in human thrombocytopenia and anemia cases. A mouse model of human thrombocytopenia has been established using a transgenic complementation rescue approach; GATA1-deficient mice were successfully rescued from embryonic lethality by excess expression of GATA1^{V205G}, but res-cued adult mice suffered from severe thrombocytopenia. In this study, we examined GATA1-deficient mice rescued with GATA1^{V205G} at a comparable level to en-dogenous GATA1. Mice rescued with this level of GATA1^{V205G} rarely survive to adult-hood. Rescued newborns suffered from severe anemia and jaundice accompa-nied with anisocytosis and spherocyto-sis. Expression of *Slc4a1*, *Spna1*, and *Aqp1* genes (encoding the membrane pro-teins band-3, α-spectrin, and aquaporin-1, respectively) were strikingly diminished, whereas expression of other canonical GATA1-target genes, such as *Alas2*, were little affected. Lack of these membrane proteins provoked perturbation of mem-brane skeleton. Importantly, the red cells exhibited increased reactive oxygen spe-cies accumulation. These results thus demonstrate that the loss of the GATA1-FOG1 interaction causes a unique combi-nation of membrane protein deficiency and disturbs the function of GATA1 in maintaining erythroid homeostasis. (*Blood*. 2012;119(11):2615-2623)

Introduction

GATA1 is a founding member of the GATA family of transcription factors. Because GATA1, GATA2, and GATA3 are highly ex-pressed in hematopoietic lineages, these factors are collectively referred to as hematopoietic GATA factors.[1] The expression of hematopoietic GATA factor genes are strictly regulated through distinct molecular mechanisms, consequently each GATA molecule shows specificity in its expression profile.[1] In particular, GATA1 is expressed in lineage-committed cells, such as erythroid, megakaryo-cytic, eosinophilic, mast, and dendritic cells.[2,3] GATA1 plays key roles in regulating the differentiation and survival of these hemato-poietic lineage cells. Targeted knockout of the *Gata1* gene leads to in utero lethality of the mutant embryos because of defects in primitive and definitive hematopoiesis.[4,5] Conditional *Gata1* gene knockout gives rise to red cell aplasia and severe thrombocytope-nia.[6] In addition, GATA1 dysfunction is predictive of specific leukemias in mice and humans.[2]

GATA1 contains 3 functional domains, that is an N-terminal transactivation (NT) domain, an N-terminal zinc finger (Nf) domain, and a C-terminal zinc finger (cf) domain.[7] These 3 do-mains have been shown to be important for GATA1 function.[7] In transgenic complementation rescue analyses, GATA1 lacking ei-ther the Nf or cf domain poorly rescues GATA1-deficient mice from embryonic lethality, which indicates the importance of both Nf and cf domains in GATA1 function in vivo.[7] The Nf domain interacts with FOG1 (friend of GATA1), and also stabilizes the DNA binding of GATA1.[8,9] FOG1 was originally identified as a cofactor of GATA1, but it also retains the potential to associate with GATA2.[8,10] In the Nf domain of GATA1, specific amino acid residues responsible for the interaction with FOG1 are located on the opposite surface of the protein to those involved in DNA contact.[11,12]

Clinical observations further support the importance of the Nf domain. Patients harboring substitution mutations in the amino acid residues, which are important either for the FOG1 interaction, or the DNA interaction, suffer from hereditary thrombocytopenia and dyserythropoiesis.[13] A human case with the substitution mutation of valine 205 to methionine in GATA1 (GATA1^{V205M}), which impairs the association of GATA1 with FOG1, has been reported. Patients with this mutation rarely survive until birth without in utero red blood cell transfusion.[14] Similarly, mice harboring a substitution mutation at valine 205 to glycine (GATA1^{V205G}) were embryonic lethal because of insufficient eryth-ropoiesis.[15] These mutant mouse analyses have also shown that the GATA1-FOG1 interaction is essential for the terminal maturation of megakaryocytes, but it is less important in early megakaryopoi-esis as long as the GATA2-FOG1 interaction is conserved.[15,16] Instead of the Nf domain, the NT domain of GATA1 is required in early megakaryopoiesis.[17] Thus, although the function of GATA1 in erythropoiesis appears to be highly dependent on FOG1 and the importance of the GATA1-FOG1 interaction has been delineated in megakaryopoiesis, it still remains to be clarified how FOG1 is involved in GATA1 function during erythroid differentiation.

To address GATA1 function in vivo, we used a transgenic complementation rescue approach. In this approach, wild-type

(WT) or mutant GATA1 molecules are expressed in transgenic mice under the regulatory influence of the *GATA1* hematopoietic regulatory domain (G1HRD).[7] Transgenic expression of WT GATA1 under the regulatory influence of G1HRD nicely rescues GATA1-deficient mice from embryonic lethality, and the rescued mice show normal hematologic indices.[7] In contrast, transgenic expression of specific GATA1 mutants results in hematologic abnormalities. One important advantage of this approach is that transgenic expression of distinct levels of GATA1 is possible. A dyserythropoietic phenotype can be rescued to a certain extent by the expression of excess amounts of mutant GATA1. Consequently, rescued mice with an extended life span enable us to analyze later defects. Indeed, we previously demonstrated that GATA1-deficient pups rescued with transgenic expression of GATA1[V205G] at a level comparable with endogenous GATA1 rarely survived to adulthood, whereas excess amounts of GATA1[V205G] created a mouse model that provided a phenocopy of inherited thrombocytopenia in humans.[16]

In this study, we found that the GATA1-FOG1 interaction was especially important for the expression of genes involved in erythrocyte homeostasis. Transgenic expression of the GATA1[V205G] mutant with decreased ability to interact with FOG1 rescued mice from embryonic lethality. However, the mice were prone to die from acute hemolytic events triggered by delivery at birth because of membrane fragility. This phenotype could be partially avoided by more abundant expression of the GATA1[V205G] mutant. Consequently, mice occasionally escaped from neonatal death and survived until adulthood with abnormal red cell membrane function. In these rescued mice, expression levels of genes related to membrane integrity were decreased, whereas expression levels of genes for heme biosynthetic enzymes and globins were relatively preserved. Interestingly, we also noted increased accumulation of intracellular reactive oxygen species (ROS). The analysis of these mice provides a novel insight into the causes of the hemolytic anemia associated with hereditary hematopoietic disease.

Methods

Hematologic and scanning electron microscopy analyses

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. Adult mice were bled from the retro-orbital plexus. Peripheral blood was also collected from perinatal pups euthanized by decapitation, followed by 6-times dilution with saline. Hematopoietic indices were determined using a hemocytometer (Nihon Koden). Reticulocytes were counted after staining blood with new methylene blue using staining capillary tubes (VC-RO75 NM; Terumo). Red cell distribution width (RDW) was calculated by the following formula using the parameters provided by red-cell flow cytometry: RDW (%) = 100 × (SD of red cell volume)/(mean cell volume). For the analysis of drug-induced hemolysis, mice from the age of 6 to 12 months were treated with phenylhydrazine at a dose of 3.75-μg/Kg body weight for 2 consecutive days. A small amount of blood sample was collected from the tail vein into microhematocrit tube. Plasma concentrations of total and direct bilirubin were measured using Fuju Dry-Chem system 7000V according to the manufacturer's protocol (Fuji Film). Indirect-bilirubin was determined by subtracting the concentration of direct bilirubin from the total bilirubin concentration. Plasma concentration of erythropoietin was determined using Quantikine Mouse/Rat Erythropoietin ELISA kit (R&D Systems). For the scanning electron microscopy analysis, peripheral blood was fixed in 0.75% glutaraldehyde solution in 0.1M phosphate buffer (pH 7.4). Imaging of samples was conducted using JEOL 5600LV scanning electron microscope (JEOL).

Spleens were fixed in 3.7% formaldehyde solution in 0.02M phosphate buffer (pH 7.4). After embedding in paraffin, the tissues were sectioned and stained with H&E solution. Microscope images were captured by a Leica DMRBE (Leica) and recorded using a DP71 camera (Olympus) and DP Controller software (Olympus). Acquired images were processed with Photoshop Elements 4.0 software (Adobe).

Intracellular ROS accumulation

For the intracellular ROS measurement, erythrocytes were stained with 10μM 2′,7′-dichlorodihydrofluorescein diacetate (Invitrogen Life Technologies) for 30 minutes at 37°C with or without 50μM hydrogen peroxide. Stained cells were analyzed by flow cytometry using FACS-Calibur (Becton Dickinson).

Analysis of erythroid membrane proteins

Peripheral blood was collected in ethylenediaminetetraacetic acid (EDTA) and washed with PBS and then lysed with 1 mL of 1mM hypotonic phosphate buffer (pH 7.4). After centrifugation at 16 000*g* at 4°C, pellets were resuspended in Laemmli buffer and sonicated. Samples were denatured in the presence of 2-mercaptoethanol and separated by sodium dodecyl sulfate (SDS) 8% polyacrylamide gel electrophoresis (PAGE). To visualize the red cell membrane proteins, the gel was fixed and stained with 0.25% Coomassie brilliant blue-R250 dissolved in 5% acetic acid and 50% methanol solution, subsequently decolorized by 5% methanol and 7% acetic acid solution. For immunoblotting, proteins in SDS-PAGE gel were transferred on to Immobilon-P membrane (Millipore). The membrane was blocked for 1 hour in a solution containing 0.1% triton X-100, 4% nonfat milk, and 1% BSA. Blotted proteins were sequentially probed by primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Invitrogen-Life Technologies). Immunoreactive signals were detected by enhanced chemiluminescence plus Western blotting detection reagents (GE Healthcare). Antibodies against α-spectrin, β-spectrin, ankyrin, protein 4.1R, and protein 4.2 were as previously described.[18] Antibodies against band-3 (AE1; Millipore), aquaporin-1 (H-55), and actin (AB5; BD Pharmingen) were purchased.

Osmotic fragility test

Peripheral blood was collected in 0.1 mg/mL heparin-sodium solution and subsequently diluted with PBS to obtain a final hematocrit value of 10%. Red cells were suspended in a series of solutions with NaCl ranging from 0.2% to 0.6% as final concentration. After incubation for 30 minutes at room temperature, samples were centrifuged at 700*g* at 4°C and the supernatant were collected. Hemoglobin concentration in the supernatant was measured from the absorbance at 540 nm with a Spectra-MAX microplate reader (Molecular Devices).

Flow cytometry

Mononuclear cells were prepared from embryonic day 18.5 (E18.5) livers and neonatal spleens, and stained with FITC-conjugated anti-CD71, PE-conjugated anti-TER119, and allophycocyanin-conjugated anti–c-Kit antibodies (all purchased from BD Pharmingen), for 30 minutes on ice. FITC, PE, and allophycocyanin-conjugated rat IgG were used as isotype matched controls. Dead cells were excluded by the addition of 2 μg/mL propidium iodide.

Cell sorting

Mononuclear cells prepared from E18.5 livers were treated with Biotin-conjugated anti-TER119 antibody (BD Pharmingen) for 30 minutes on ice. After washing with cold PBS, cells were treated with streptavidin-conjugated Magnet Beads (QIAGEN) for 30 minutes on ice and then resuspended in PBS with 2% FBS. TER119-positive cells were collected by using a magnet stand.

Semi-quantitative and quantitative RT-PCR

Total mRNA from spleen or sorted cells was prepared by using Isogen (Nippon Gene). First strand cDNA was synthesized with Super Script II

reverse transcriptase (Invitrogen Life Technologies). Polymerase chain reaction (PCR) amplification was performed with Ex-Taq DNA polymerase (Takara Bio). The amount of cDNA was adjusted by dilution to give rise to the equivalent amount of *Hprt* expression. Primer sequences are described in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Quantitative reverse transcription (RT)–PCR was carried out using qPCR-Mastermix Plus for SYBR Green I (Eurogentec) with ABI7300 Real-Time PCR system (Applied Biosystems-Life Technologies). Expression levels were normalized to *Gapdh*. Primer sequences are described in supplemental Table 2.

Chromatin immunoprecipitation

Fetal liver cells were cross-linked with 0.4% formaldehyde and neutralized with glycine. After lysing the nuclei, samples were sonicated to produce DNA fragments of approximately 300 to 500 bps. Aliquots of each sample were kept as input DNA controls. The remaining samples were precleared using Dynabeads Protein-A (Invitrogen Life Technologies) in combination with affinity pure rabbit anti–rat IgG (Jackson ImmunoResearch Laboratories), subsequently immunoprecipitated with rat anti-GATA1 N6 antibody (Santa Cruz Biotechnology). Normal rat IgG (Santa Cruz Biotechnology) was used as a negative control for immunoprecipitation. After incubation for 1 hour at 55°C with 1% SDS and 0.1M sodium bicarbonate containing 200 μg/mL RNase, cross-links were reversed by incubation at 65°C with the same solution supplemented with 300 μg/mL proteinase K. PCR was performed using primers described in supplemental Table 3.

Statistical analysis

Statistical analyses were performed with a Student *t* test. Probability values of $P < .05$ were considered statistically significant.

Results

Defects in the GATA1-FOG1 interaction cause fatal dyserythropoiesis during the perinatal period

Expression of GATA1[V205G] mutant protein under the control of G1HRD at levels higher than the WT mouse level can rescue GATA1-deficient (*Gata1[G1.05]*/Y) mice efficiently from embryonic lethality.[16] In contrast, we rarely obtained rescued mice when GATA1[V205G] mutant protein was expressed at levels comparable with endogenous GATA1.[16] To delineate the importance of the GATA1-FOG1 interaction for embryonic erythropoiesis, we conducted in vivo complementary rescue analyses using 2 transgenic mouse lines expressing GATA1[V205G] at either levels comparable with (line A) or higher than (line B) the WT GATA1 level. For the sake of simplicity, we collectively refer to these rescued mice as V205GR, and the mice rescued with line-A and line-B transgenic mouse lines as V205GR-A and V205GR-B, respectively.

We first determined the genotype of embryos during the late gestation period. Numbers of live V205GR-A and V205GR-B embryos were comparable with those expected by Mendelian genetics (Figure 1A open bars), and they were easily distinguishable from WT littermates by their anemic appearance (supplemental Figure 1), indicating that transgenic expression of GATA1[V205G] at endogenous levels is sufficient to rescue *Gata1[G1.05]*/Y mice from embryonic lethality, but the expression appears insufficient for full-rescue of GATA1 function.

In contrast to the rescued pups at embryonic stage, frequencies of rescued pups dropped dramatically within 3 days after birth (Figure 1A filled bars). The expressions of mutant *Gata1* transcripts in V205GR-A and V205GR-B pups were comparable with or approximately 8-fold higher than WT *Gata1* mRNA, respectively in age-matched WT pups (Figure 1B). These results indicate
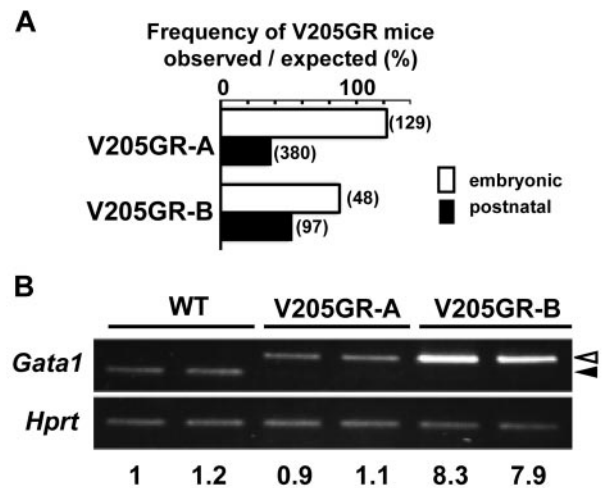

Figure 1. V205GR are prone to die from massive anemia during the perinatal period. (A) The frequency of live V205GR pups at embryonic (E18.5; open bars) or postnatal (within 3 days after birth; filled bars). Rescue efficiency was calculated as previously described.[16] Total number of pups in each transgenic rescue line is indicated in parentheses. (B) Transgene derived (open arrowhead) or endogenous (filled arrowhead) *Gata1* mRNA expression in GATA1[V205G] transgenic mouse lines. The mRNA levels were determined by semi-quantitative RT-PCR. *Hprt* mRNA is used as an internal control. *Gata1* expression level normalized to *Hprt* is shown under each lane. The most left WT sample is set to 1.

that a lethal event caused by the disturbed GATA1-FOG1 interaction occurs in V205GR neonates.

V205GR neonates show anemia with erythroid hyperplasia

To investigate the cause of the neonatal death, we measured indices of red cells from 5 V205GR-A and 3 V205GR-B neonates of 0 to 3 days old. Eight WT neonates were used as controls. Red blood cell count (RBC), hemoglobin concentration (Hb), and hematocrit value (Hct), were significantly reduced in V205GR-A and V205GR-B mice (Figure 2A), in addition to a marked reduction in platelet count (data not shown). Mean corpuscular volume (MCV) was also significantly reduced in the rescued pups, whereas mean cell hemoglobin content (MCH) was similar, which indicates cell dehydration. Increased mean cell hemoglobin concentration (MCHC) of red cells of rescued mice confirmed cell dehydration (Figure 2A).

The size of V205GR neonatal spleens was slightly smaller than that of their littermates (data not shown). Histologic examinations revealed that the splenic architecture was altered by expansion of the red pulp area in V205GR-A neonates. The demarcation lines between the red and white pulps were not well defined (Figure 2B). High magnification views showed that the number of nucleated cells was significantly elevated in the red pulp of V205GR-A neonates compared with that of WT littermates, although a considerable number of enucleated RBCs were also observed in the spleen of V205GR-A neonates (Figure 2C).

Flow cytometry analysis showed that the frequency of c-Kit⁻ TER119⁺ erythroid cells was slightly increased in V205GR-A neonatal spleens. Of note, whereas the frequency of erythroblasts in the c-Kit⁻ CD71⁺TER119⁺ fraction, which correspond to orthochromatophilic erythroblasts,[19] was preserved in V205GR-A pups, the TER119 expression in V205GR-A erythroblasts was decreased compared with those in WT littermates (mean intensity; 281 vs 2800; Figure 2D). This observation suggests that erythroid differentiation leading up to the stage of orthochromatophilic erythroblasts may not be disturbed markedly in V205GR-A pups, although they have a decreased assembly of glycophorin.[20,21]
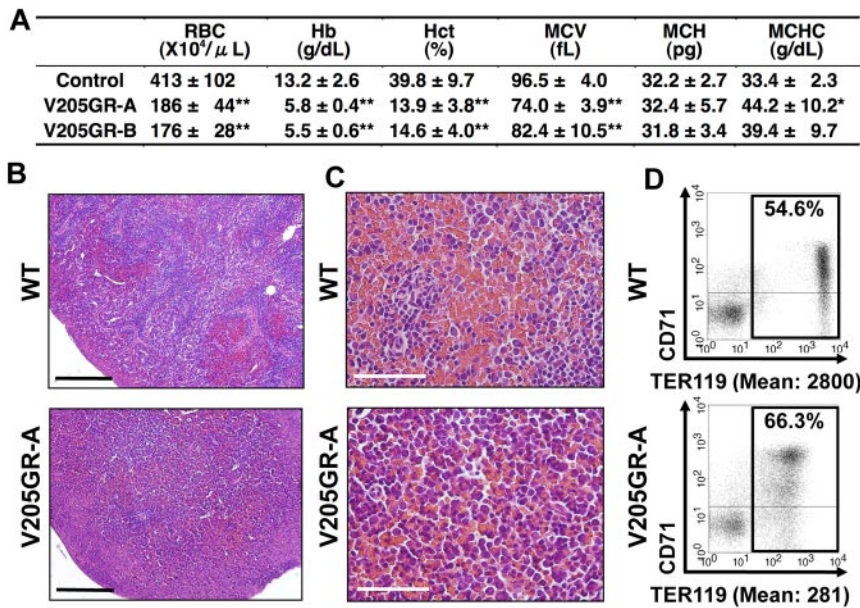
A

	RBC (X10^4/μL)	Hb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)
Control	413 ± 102	13.2 ± 2.6	39.8 ± 9.7	96.5 ± 4.0	32.2 ± 2.7	33.4 ± 2.3
V205GR-A	186 ± 44**	5.8 ± 0.4**	13.9 ± 3.8**	74.0 ± 3.9**	32.4 ± 5.7	44.2 ± 10.2*
V205GR-B	176 ± 28**	5.5 ± 0.6**	14.6 ± 4.0**	82.4 ± 10.5**	31.8 ± 3.4	39.4 ± 9.7

Figure 2. Erythroid phenotypes of V205GR neonates. (A) Erythroid parameters of V205GR neonates. Eight WT, 5 V205GR-A, and 3 V205GR-B neonates aged 0 to 3 days old were used. (*$P < .05$, **$P < .01$). (B-C) Histologic examination of 2-day-old neonatal spleens of V205GR-A (bottom panels) and WT littermate (top panels). Scale bars correspond to 0.5 mm in panel B and 100 μm in panel C, respectively. (D) The expression profile of CD71 and TER119 in the c-Kit$^-$ erythroid population. Mononuclear cells derived from 1-day-old neonatal spleens of V205GR-A (bottom panel) and WT littermates (top panel) were used. The frequencies and mean intensity values of TER119$^+$ cells were shown in the panels.

We next examined expressions of erythroid 5-aminolevulinate synthase (*Alas2*), erythroid Krüppel-like factor (*Eklf*), erythropoietin receptor (*EpoR*), and globin (*Hba-a1* and *Hbb-b1*) genes, which are known to be regulated by GATA1. Expressions of these genes were not changed, whereas *Gata2* gene expression was elevated in V205GR-A mouse spleens compared with those in WT mice (supplemental Figure 2). Importantly, expression of glycophorin A (*Gypa*) was preserved in the V205GR spleen. We hypothesize that

discrepancy between expression levels of glycophorin A on developing erythroblast and *Gypa* transcript in V205GR erythroblasts is probably the result of altered membrane trafficking.

V205GR neonates suffer from spherocytic hemolytic anemia

Through serial neonatal examinations, we noticed that V205GR-A neonates were easily distinguished by their yellowish skin from
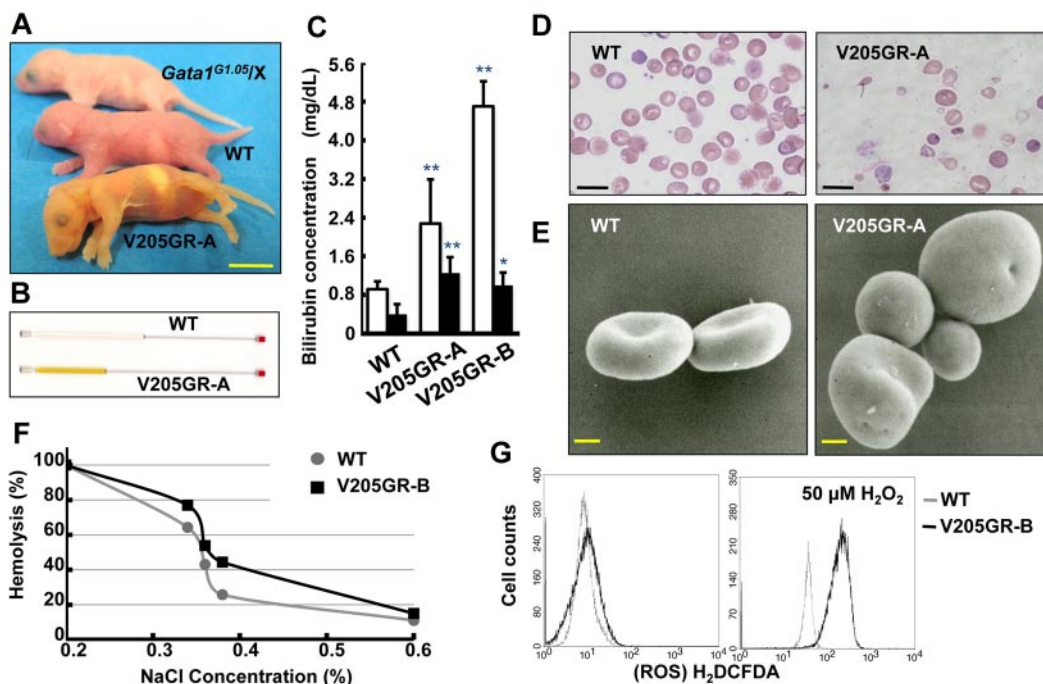

Figure 3. Abnormal appearance of V205GR characterized as spherocytic hemolytic anemia. (A) Macroscopic appearance of 3-day-old neonates. Note that V205GR-A neonate shows (lower pup) marked yellowish skin pallor compared with WT littermate (middle pup), whereas *Gata1$^{G1.05}$*/X (upper pup) shows significant anemia. (B) Plasma color derived from WT (top) and V205GR-A (bottom). (C) Bilirubin concentration in the neonatal plasmas. Ten WT, 4 V205GR-A, and 3 V205GR-B neonates at the age of 2 to 3 days old were used for this experiment (open bars, total-bilirubin; filled bars, indirect-bilirubin; *$P < .05$, **$P < .01$). (D) Abnormal red blood cells in the blood smears. Various sizes of red cell fragments were observed in V205GR-A at 2 days old (right panel). The left panel is the WT control. Scale bars correspond to 20 μm. (E) Scanning electron microscopic analysis of erythrocytes prepared from V205GR-A newborn (right panel) and WT control (left panel). Scale bars correspond to 1 μm. (F) Osmotic fragility curves of erythrocytes from V205GR-B (black line) or WT (gray line) pups. Percent hemolysis was calculated for each solution based on the absorbance value obtained from 0.2% concentration. (G) ROS levels in erythrocytes of WT (dotted line) and V205GR-B (solid line) pups without (left panel) or with (right panel) addition of exogenous H_2O_2 (50μM).

their littermates. A representative photo is shown in Figure 3A. This phenotype was strikingly different from that of E18.5 rescued embryos (supplemental Figure 1), which suggests that a dramatic hemolytic event was triggered by the delivery. Plasma color was also markedly yellowish with a high concentration of total and indirect-bilirubin (Figure 3B-C). In addition, many poikilocytes and tear-drop shaped cells were observed in blood smear samples (Figure 3D), although such deformed erythrocytes were not seen in the E18.5 rescued embryos (data not shown). In addition, aniso-cytic and spherocytic erythrocytes lacking central pallor were abundantly observed in both embryos and neonates (Figure 3D).

Scanning electron microscopic analysis revealed that erythro-cytes in V205GR-A mice varied in size with a typical spherocytic morphology, whereas those in WT had a discoid appearance (Figure 3E). Using peripheral blood from V205GR pups we performed osmotic fragility tests. Both V205GR-A and V205GR-B erythrocytes are fragile to hypo-osmotic stress (Figure 3F). We also found that the intracellular ROS concentration was significantly elevated in both erythrocytes (Figure 3G). These findings indicate that V205GR neonates are prone to die because of severe hemolytic crisis with spherocytosis.

Interaction of GATA1 and FOG1 is crucial for membrane protein gene expression

Hereditary spherocytosis (HS) is one of the most common inherited human red cell membrane disorders in humans. HS is characterized by a structural abnormality of the red cell membrane because of quantitative and/or qualitative defects in membrane proteins. To measure amounts of plasma membrane proteins, we performed an SDS-PAGE. We found a cluster of membrane proteins including α-spectrin, β-spectrin, and ankyrin almost disappeared in the V205GR-A mouse erythrocytes, whereas band-3, protein 4.1R, and 4.2 were decreased to a lesser extent (Figure 4A). The reduction of major membrane proteins in V205GR-A erythrocytes was con-firmed by immunoblotting analyses (Figure 4B). These results strongly argue that deficiency of multiple membrane structural proteins gave rise to the severe hemolysis in V205GR neonates.

Accumulating lines of evidence demonstrate that GATA1 is one of the key molecules involved in the regulation of membrane protein gene expression.[22-27] It has been reported that cis-regulatory loci in membrane protein genes are cooccupied by GATA1 and FOG1 in erythroid cells.[28] Because membrane proteins progres-sively accumulate during erythroid differentiation,[29] we hypoth-esized that accumulation of such proteins might be modified by an imbalance of erythroid differentiation status and/or ineffective erythropoiesis. Therefore, on examination of membrane protein expression in V205GR erythrocytes we sorted TER119^{+} cells from the E18.5 livers, as TER119 marks erythroblasts in the mature stages.

All membrane structure protein genes examined by RT-PCR were abundantly expressed in WT, whereas expressions of band-3 (*Slc4a1*) and α-spectrin (*Spna1*) genes were markedly reduced in V205GR-A embryos (Figure 5A). Expression of the aquaporin-1 (*Aqp1*) gene was also reduced (Figure 5A). In contrast, expressions of other erythroid-membrane proteins, such as β-spectrin (*Spnb1*), ankyrin 1 (*Ank1*), protein 4.1R (*Epb41*), protein 4.2 (*Epb42*), and glycophorin (*Gypa*) were preserved at levels comparable with endogenous expression. Reduced expressions of *Slc4a1*, *Spna1*, and *Aqp1* were further confirmed by quantitative PCR analyses in erythroblasts of V205GR-A and V205GR-B embryos and neonates (Figure 5B). Because band-3 and α-spectrin are core molecules for membrane complex formation,[30,31] we envisaged that translocation

of β-spectrin, ankyrin 1 and glycophorin A to the erythrocyte membrane was disturbed concomitant with the reduction of α-spectrin and band-3.

We next examined expression levels of globin genes and erythrocyte metabolic enzyme genes. The *Hba-a1*, *Hbb-b1*, and pyruvate kinase (*Pklr*) genes in the V205GR embryos were expressed at a normal level (Figure 5A). ϵy-*Globin* was not detectable in V205GR nor in WT littermates even after 32 amplifi-cation cycles, excluding the possibilities that globin chain imbal-ance or persistent expression of embryonic globin influenced the hemolytic event. On the other hand, expression of the glucose-6–phosphatase dehydrogenase (*G6pd*) gene varied in individuals; 2 of 6 V205GR-A embryos had a reduced expression level, whereas the expression in the remaining 4 was similar to those of WT littermates. *Slc4a1* gene expression was reduced in all 6 embryos (data not shown).

We previously reported that the Nf domain of GATA1 was important for gene regulation of heme biosynthesis enzymes in definitive erythroid progenitors.[7] To explore the contribution of GATA1-FOG1 interaction in heme biosynthesis, we also examined expressions of 8 enzymes of the heme biosynthesis pathway. The expressions of these genes were at normal levels except for the *Alas2* gene, which encodes the first and rate-limiting enzyme of the heme biosynthesis pathway in erythroid cells. We found that the *Alas2* gene expression decreased and magnitude of the decrease varied substantially in embryos (Figure 5A).

GATA1 binding to membrane protein genes

To verify that the *Slc4a1* and *Aqp1* genes are direct targets of GATA1 in vivo, we performed chromatin immunoprecipitation
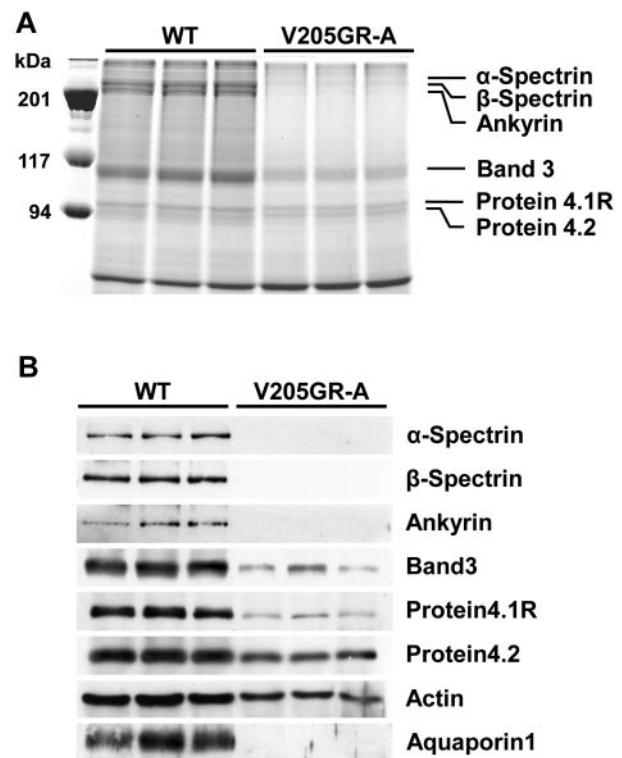

Figure 4. Expression of majority of membrane proteins was reduced in V205GR erythrocytes. Coomassie brilliant blue-stained SDS-PAGE profile (A) and relative immunoblotting expression levels of erythroid membrane proteins (B) in red blood cell ghosts. Three WT and 3 V205GR-A newborn pups were used for this experiment. Loading volume was adjusted by the original number of erythrocytes used for preparation of cell ghosts.
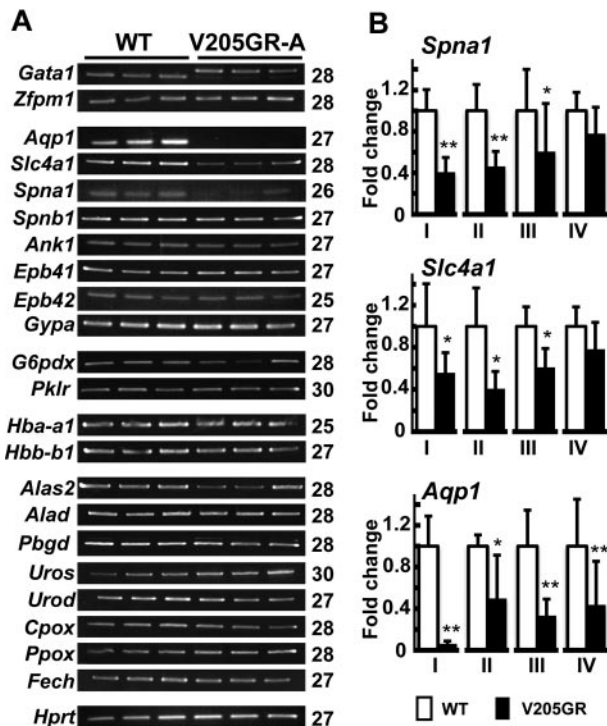
Figure 5. Expression of several genes coding for membrane proteins was selectively reduced in V205GR erythrocytes. (A) Expression of erythroid-related genes in V205GR-A TER119^{+} cells. Amplification cycles are shown in the right side of the figure. Note that the expressions of *Spna1*, *Slc4a1*, and *Aqp1* genes were specifically reduced in V205GR-A. (B) Expression of *Spna1*, *Slc4a1*, and *Aqp1* was analyzed by quantitative RT-PCR in erythroblasts TER119^{+} derived from E18.5 livers of V205GR-A (I), P2 spleens of V205GR-A (II), P3 spleens of V205GR-A (III), and P0 spleens of V205GR-B (IV). The values were represented as a mean of 3 independent age-matched and genotype-matched samples. The mean value of the transcripts in corresponding WT pups was set to one. ($^*P < .05$, $^{**}P < .01$)

(ChIP) assays using E18.5 liver cells. Previous transfection studies revealed that the *Slc4a1* gene harbors multiple active GATA-binding motifs in the upstream region of the erythroid-specific promoter,[28] although there is no report of active GATA motifs in the *Aqp1* gene. Because GATA motifs often reside in the intronic regions of membrane protein genes,[28] in this study we surveyed the sequence similarity of intronic regions between human and mouse genes. We found the presence of conserved GATA motifs in the first intron of the *Slc4a1* and *Aqp1* genes. In ChIP assays we identified clear accumulation of GATA1 occupancy in the first intron region of these genes (Figure 6). Consistent with these findings, GATA1 activated the expression of *Slc4a1* and *Aqp1* enhancer-based reporters, although the activity was abrogated by the incorporation of mutations to the GATA motifs (supplemental Figure 3). We could not detect accumulation of GATA1 occupancy in the upstream promoter region of the *Slc4a1* gene (data not shown). Similarly, although it has been reported that GATA1 activated the *Spna1* gene expression through GATA motifs in the promoter and the region 3′ to the promoter and GATA1 occupancy was identified in these regions,[24,25,28] we could not detect GATA1 occupancy in these loci in our ChIP assays (data not shown).

To examine whether FOG1 is required for stabilizing GATA1 binding in vivo, we performed ChIP analyses using E18.5 V205GR-A liver cells. FOG1 involvement in the DNA binding of GATA1 has been reported to differ between genes, that is, FOG1-deficiency compromised the GATA1 ability to stably associate with the β-*globin* and *Gata2* genes,[32,33] but not with the *c-Kit* gene.[34] Importantly, we found that GATA1 occupancies of the first

intron region of the *Slc4a1* and *Aqp1* genes were comparable with the E18.5 V205GR-A and WT littermate livers (Figure 6), which indicates that the FOG1 interaction is dispensable for binding of GATA1 to the first intron regions of these genes.

V205GR-B mice suffer from chronic hemolysis and exhibit hemolytic crisis on exposure to drugs

Although approximately one-half of the V205GR-B pups died during the first 5 days, V205GR-B pups that escaped from neonatal death matured to adulthood (data not shown). V205GR-A pups rarely survived beyond day 5 after birth. We therefore examined the hematologic indices of adult V205GR-B mice. As shown in Figure 7A, they showed mild anemia with reticulocytosis and high RDW. Scanning electron microscopy revealed spherocytic morphology of V205GR-B mouse erythrocytes (supplemental Figure 4). In adult V205GR-B mice erythropoietin concentration was significantly increased (Figure 7B), and the spleen was enlarged with erythroid hyperplasia (Figure 7C-E), which indicates that active erythropoiesis compensated for the anemia caused by chronic hemolysis. Although no marked decreases in the expression levels of various membrane proteins was noted, the levels of *Slc4a1, Spna1*, and *Aqp1* transcripts were significantly reduced in V205GR-B mice (supplemental Figure 5). V205GR-B mice developed a more severe hemolytic anemia after low dose exposure to phenylhydrazine (Figures 7F-G). The expression of *Alas2* and *Hbb-b1* genes were unaltered in the V205GR-B mice (supplemental Figure 5), which suggests that individual mice that were able to maintain near normal expression levels of erythroid genes, including *Alas2* and *Hbb-b1*, are spared from the neonatal death and grew up to adulthood with well-compensated hemolytic anemia.

To our surprise, although spherocytes are usually fragile in the hypo-osmotic condition, erythrocytes from adult V205GR-B mice are resistant to hypo-osmotic stress (supplemental Figure 6). Hemolysis occurred less frequently in V205GR-B erythrocytes
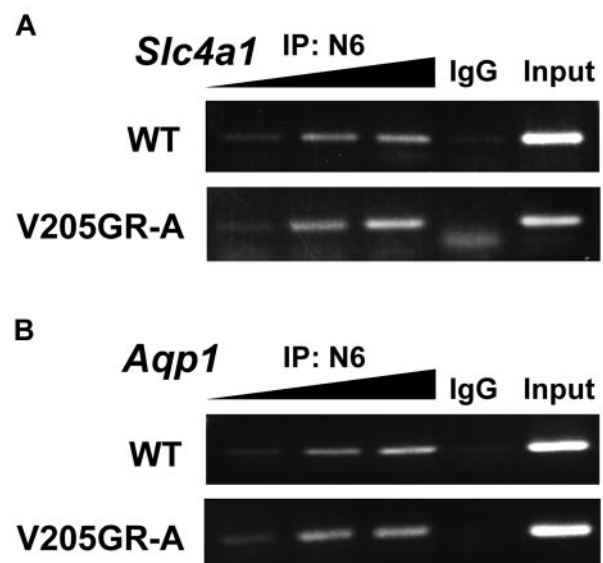

Figure 6. In vivo binding of GATA1 to the membrane protein genes. The in vivo association of GATA1 with *Slc4a1* (A) and *Aqp1* (B) genes was evaluated with ChIP assay in fetal livers of WT (top panel) and V205GR-A (bottom panel) mice. DNA fragments immunoprecipitated with GATA1-N6 antibody were diluted 1, 2, and 3 times, and rendered for 35 cycles of amplification. Samples of the input DNA were used in the PCR reactions to confirm that equal amounts of DNA were present in the samples. A control immunoprecipitation reaction with control IgG was also included.
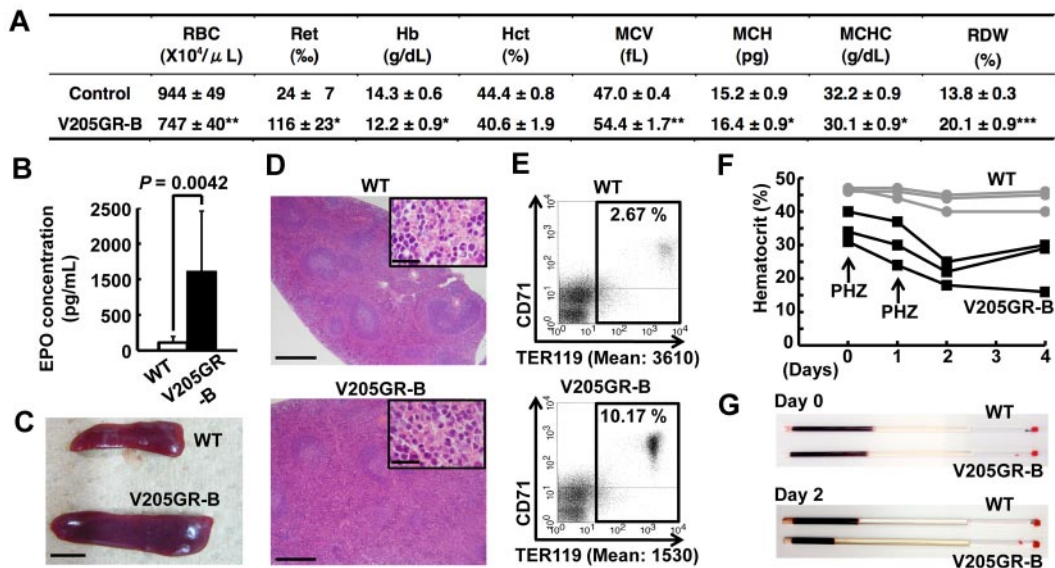
Figure 7. Erythroid phenotypes of V205GR adult mice. (A) Red cell parameters of adult V205GR mice. Three V205GR-B and 3 WT mice were used. (*P < .05, **P < .01, ***P < .005). (B) Plasma erythropoietin concentration was significantly elevated in V205GR-B mice. Nine WT (open bars) and 8 V205GR-B (filled bars) mice at the age of 4 to 12 months were used. (C) Spleen of a 6-month-old V205GR-B mouse was markedly enlarged. Scale bars correspond to 5.0 mm. (D) Histologic examination of spleens of V205GR-B (bottom panel) and WT littermate (top panel) shown in panel C. High magnitude image of red pulp area is shown in insets. Note that a large number of enucleated mature erythrocytes are observed in the enlarged red pulp area of V205GR-B spleen. Scale bars correspond to 0.5 mm and 20 μm. (E) Mature erythroid population (c-Kit⁻CD71⁺TER119⁺) was increased in the spleen of V205GR-B (bottom panel) compared with WT littermate (top panel). The frequencies and mean intensity values of TER119⁺ cells are shown in the panels. (F) Changes of hematocrit values taken from 3 V205GR-B (black lines) and 3 WT (gray lines) mice after exposure to a low dose of phenylhydrazine. (G) Yellowish plasma was observed in V205GR-B mice after phenylhydrazine treatment.

even after a 2-hour incubation in the hypotonic solution, which was enough for water diffusion to equilibrium (data not shown). In addition, we found that the intracellular ROS concentration was significantly elevated in erythrocytes from V205GR-B mice (supplemental Figure 6). We envisage that the defect in the FOG1 interaction influences expression of multiple GATA1 target genes, resulting in these complex phenotypes observed in the rescued mice.

Discussion

In this study, we found that transgenic expression of GATA1[V205G] at comparable levels with endogenous GATA1 appears insufficient to fully rescue GATA1-deficient mice, leading to premature death of newborns because of hemolytic anemia. This premature lethality can be abrogated by more abundant expression of the GATA1[V205G] mutant protein, but high-level expression of GATA1[V205G] is still insufficient for the maintenance of erythroid homeostasis, which indicates the presence of a dose-dependent compensatory feature[35] in the GATA1[V205G] mutant. A recent genome-wide ChIP study revealed that multiple erythroid transcription factors are coassociated in regions close to membrane protein genes and cooccupancy with GATA1 and FOG1 are frequently observed.[28] Importantly in this study however, we found that the GATA1[V205G] mutant protein lacking the FOG1 interaction activates *Ank1* and *Epb41* genes in vivo to levels comparable with those WT GATA1 activates, which indicates that the GATA1-FOG1 interaction is not essential for the expression of these genes even if GATA and FOG1 are colocalized in ChIP analyses. Our study thus demonstrates that there are 2 types of GATA1 target genes in erythroid cells. One type that heavily depends on the GATA1 association with FOG1 and the other that does not depend on the GATA1-FOG1 interaction.

HS is a common congenital hemolytic disorder caused by quantitative and/or qualitative defects in erythroid membrane proteins.[36] Phenotypic severities of the diseases are closely linked to the membrane protein level, mutation type, and/or genetic background.[30,37] Abnormality of band-3 and α-spectrin is the common cause of HS transmitted in an autosomal dominant fashion. In murine models, heterozygotes bearing either *Slc4a1* or *Spna1* amorphic allele experience mild and fully compensated hemolytic anemia.[38-40] In contrast, both V205GR-A and V205GR-B mutant mice suffered from severe hemolytic anemia during neonatal period, even though more than 50% levels of *Slc4a1* and *Spna1* transcripts were preserved. Furthermore, adult V205GR-B mice showed phenotype resembling the HS patients with well-compensated hemolytic anemia with relatively normal levels of expression of erythroid membrane proteins. A plausible explanation for this discrepancy between gene expression levels and phenotype is to assume that the reductions of multiple GATA1 target genes, which are participating in erythrocyte stability, synergistically generate the spherocytic phenotype in V205GR-B pups. Indeed, we previously observed that mice carrying excess amounts of a GATA1 mutant molecule lacking the Nf domain showed spherocytosis without the reduction of major membrane structure proteins, including band-3 and α-spectrin.[41] Therefore, the hemolytic event might be accelerated by the reduction of other membrane skeletal proteins and/or enzymes related to erythrocyte metabolism caused by the disturbed GATA1-FOG1 interaction.

We also found that the expression of the *Aqp1* gene is markedly disturbed in the V205GR mice. Aquaporin-1 is a water-channel abundantly expressed in erythrocytes as well as in kidney. Simple *Aqp1* gene knockout mice died as a result of water depletion with dehydration, but the mice showed no hematopoietic phenotype except for reduced water-permeability of erythrocytes.[42] A case of human *AQP1* polymorphism trait has been reported showing only a subtle phenotype of reduced red cell surface area and short life span of erythrocytes.[43] Recently, it was reported that membrane expression of aquaporin-1 was down-regulated during maturation of

reticulocytes into mature erythrocytes and this process is dependent on the osmotic condition of the environment.[44] We surmise that targeting-deficiency of aquaporin-1 may worsen the hemolysis event in V205GR pups, which may be triggered by the environmental changes during delivery.

The erythrocytes from V205GR neonates appear to be fragile against the hypo-osmotic stress, suggesting that various stresses render the erythrocytes at this stage to the serious hemolytic events. We also found that the intracellular ROS level is elevated in the V205GR erythrocytes. Erythrocytes have an extensive antioxidant defense system because of their fundamental function as oxygen carriers. Although expression of glucose-6–phosphatase dehydrogenase was not significantly changed, ROS detoxification mechanisms might be impaired in V205GR mice. The defect in GATA1-FOG1 interaction may give rise to an antioxidant imbalance in erythrocytes, leading to the destruction of membrane integrity. We surmise that decreased fragility against hypo-osmotic stress in adult V205GR mouse erythrocytes may in part be because of the membrane rigidity caused by membrane protein hyperoxidation, as observed in erythrocytes lacking AMP-activated protein kinase, which is important for maintaining redox homeostasis in erythrocytes.[45] We propose that the differences between the neonatal and adult V205GR mice in osmotic fragility test and other features are caused by the subtle changes in expression level of GATA1^{V205G} in individuals. V205GR mice with sufficient expression of GATA1^{V205G} to sustain the compensatory erythropoiesis and to overcome acute/chronic hemolytic conditions are selected to develop to adulthood.

In approximately 10% of human HS cases mutations have not been identified.[46] We envisage at least 2 possibilities as the cause of these unclassified HS cases. One possibility is to assume that mutations reside in regulatory regions of specific membrane protein genes. Indeed, a hypomorphic allele with a mutation in the mouse *Ank* gene promoter has been established and homozygous mutant mice showed an HS-like phenotype resembling the human case.[47] Together with the finding that the functional binding sites of transcription factors are frequently located in intronic regions,[28] mutation in such regulatory regions have a latent potential to cause HS. Another possibility is to assume that mutations reside in a molecule critical for regulation of membrane proteins. It has been reported that a single amino acid mutation in EKLF (erythroid Krüppel-like factor) causes spherocytosis in mice because of the impaired DNA binding of this mutant molecule to the dematin gene.[48] EKLF has also been shown to be important for membrane structural gene expression.[49] GATA1 dysfunction affects expression of multiple target genes simultaneously and consequently V205GR mice show complicated phenotypes and unexpected alterations in hematologic parameters. For example, the HS-like erythrocytes in V205GR mice are resistant to hypo-osmotic pressure. Based on these results, we propose that the *GATA1* gene may be one of the candidates responsible for human unclassified HS.

Authorship

Contribution: A.H. performed most experiments, and contributed to the project design and preparation of the paper; R.S. and M.Y. contributed to the project design, supervised the study, and wrote the paper; N.M. provided sophisticated expertise and wrote the paper; and all authors contributed to the integration and discussion of the results.

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