Runx1 is involved in primitive erythropoiesis in the mouse

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Running Title

Runx1 in primitive erythropoiesis.

Word count

Abstract: 126 words, Text words: 2231words

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Abstract

Targeted disruption of the *Runx1/ AML1* gene in mice has demonstrated that it is required for the emergence of definitive hematopoietic cells, but that it is not essential for the formation of primitive erythrocytes. These findings led to the conclusion that Runx1 is a stage-specific transcription factor acting only during definitive hematopoiesis. However, the zebrafish and *Xenopus* homologues of Runx1 have been shown to play roles in primitive hematopoiesis, suggesting that mouse Runx1 might also be involved in the development of primitive lineages. In this study, we show that primitive erythrocytes in *Runx1*$^{-/-}$ mice display abnormal morphology and reduced expression of Ter119, Erythroid Kruppel-like factor (EKLF, KLF1), and GATA-1. These results suggest that mouse Runx1 plays a role in the development of both primitive and definitive hematopoietic cells.

Introduction

Recent studies have revealed that the process of hematopoiesis is largely conserved in vertebrates. During development, similar sequential waves of primitive and definitive hematopoiesis are observed in mammals, fish, and amphibians.[1-3] In the mouse embryo, primitive erythrocytes first appear in the yolk sac blood island at embryonic day (E) 7 and lead to the production of nucleated erythrocytes.[4,5] These erythrocytes (erythroblasts) start to circulate at 4 to 6 somite pairs (E8.25)[6] and are necessary for the survival of the embryo before establishment of definitive erythropoiesis, which is initiated in the fetal liver around E12 and is characterized by the production of enucleated erythrocytes.[4] The equivalent site of blood island in zebrafish embryo is known as the intermediate cell mass (ICM), which is located in the posterior part of embryo and provides the primitive erythrocytes.[3]

Runx1/AML1 encodes the DNA-binding subunit of the Runt domain transcription factor,[7] which plays crucial roles in processes of organ and tumor development, such as hematopoiesis, neural development, and leukemogenesis.[8,9] Runx1-deficient mice die around E12.5 due to severe hemorrhaging in the central nervous system (CNS) and a complete lack of definitive hematopoietic cells, including the aortic hematopoietic clusters, which are thought to comprise hematopoietic stem cells.[10-14] The specification of definitive hematopoietic stem cells by Runx1 has also been observed in the zebrafish embryo. Depletion of zebrafish Runx1 by antisense

morpholino oligonucleotides leads to a severe defect in definitive hematopoietic development in the dorsal aorta.[15,16]

Although Runx1 has been implicated in primitive hematopoiesis in zebrafish and *Xenopus* embryos,[15,17] its function during primitive erythropoiesis in the mouse has not been described despite its reported expression in this lineage.[13,18] In this study, we examined primitive erythrocytes in *Runx1*$^{-/-}$ mice and observed aberrant morphology and reduced expression of Ter119, Erythroid Kruppel-like factor (EKLF, KLF1), and GATA-1, in these cells.

Materials & Methods

Mice

Runx1-deficient mice (C57BL/6) have been previously described.[12] For visualizing GATA-1 expression in primitive erythrocytes, *IE3.9-LacZ* transgenic mice carrying a 3.9 kb *GATA-1* promoter-*LacZ* reporter gene (C57BL/6 x DBA/2 mixed background)[19] were crossed with Runx1-deficient mice. For the transgenic complementary rescue experiment, *G1-HRD-Runx1* transgenic mice carrying a 8.0 kb *GATA-1* hematopoietic regulatory domain –*Runx1* transgene (C57BL/6 x DBA/2 mixed background)[20] were crossed with Runx1-deficient mice.

Benzidine- and LacZ-staining

Embryos were fixed at 4°C for 2 hours in 1% formaldehyde, 0.2% glutaraldehyde, and 0.02% Nonidet P-40 in PBS. For LacZ-staining, embryos were incubated at 37°C overnight in 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 1 mg/ml X-Gal in PBS. For Benzidine-staining, embryos were incubated for 15 min in 14% Benzidine base (3% Benzidine in 90% glacial acetic acid/10% H_20) and 14% H_2O_2 in H_2O.

Scanning Electron Microscopy

Peripheral blood cells from E12.5 embryos were fixed with 1.6% glutaraldehyde, postfixed in 1% osmium tetraoxide. After dehydration with a graded series of alcohol

solutions, the samples were freeze-dried, coated with a layer of gold and observed under a scanning electron microscope (JEOL, JSM-6320F).

In vitro hematopoietic progenitor assay

A colony assay for the development of primitive erythroid lineages was performed as described.[21] Briefly, E8.5 whole embryos from each genotypes were treated with trypsin (Invitrogen) for 5min at 37℃, and washed with PBS containing 5% FBS. The cells were plated in 1% methylcellulose/IMDM supplemented with 10% Plasma derived serum (PDS), 5% Protein free hybridoma medium (PFHM) (Invitrogen), 12.5 mg/ml ascorbic acid (Wako), 2 mM L-glutamine, 10 mg/ml transferring (Boehringer Manheim), 39 ng/ml MTG (Sigma), 5 ng/ml stem cell factor (R&D systems), 50 ng/ml erythropoietin. After 3 days of culture, primitive erythroid colonies were counted.

Cell preparation and staining

Embryos were treated with Dispase II (Roche) and cell dissociation buffer containing EDTA/EGTA (Invitrogen) to make single cell suspensions. Non-specific binding was blocked by incubation in 5 % mouse serum for 15 min, followed by incubation with Oregon Green-conjugated anti-Ter119 antibody (a generous gift from Dr. S.-I. Nishikawa) for 30 min. β-galactosidase activity was detected using FluoReporter lacZ

Flow Cytometry Kit (Invitrogen). Briefly, dissociated cells were resuspended in 20 μl of PBS with 5% FCS prior to loading with 20 μl of 2 mM FDG (fluorescein di-β-D-galactopyranoside) and followed by incubation at 37°C for 75 sec. The uptake was stopped by the addition of 500 μl ice cold PBS with 5% FCS. Cells were analyzed with FACScan or FACS Vantage (Becton Dickinson). Mean fluorescence intensity (MFI) values were calculated using CellQuest software (Becton Dickinson).

Real-time RT-PCR

RNA was purified from E11.5 peripheral blood cells using the RNeasy Kit (Qiagen) and reverse transcribed by SuperScriptIII (Invitrogen). Real-time polymerase chain reactions (PCR) employing Taqman probes for *GATA-1* and *GAPDH* or with SYBR green for *EKLF* and *SCL* were performed in an ABI PRISM 7700 (Applied Biosystems). The forward and reverse primers used for PCR were as follows: EKLF, 5'-AGACTGTCTTACCCTCCATCAG-3' and 5'-GGTCCTCCGATTTCAGACTCAC-3'; SCL, 5'-CACTAGGCAGTGGGTTCTTTG-3' and 5'-GGTGTGAGGACCATCAGAAATCT-3'. The Primers and VIC-labeled probe for *GATA-1* were as follows: 5'-CAGAACCGGCCTCTCATCC-3', 5'-TAGTGCATTGGGTGCCTGC-3', and VIC-labeled 5'-CCC AAG AAG CGA ATG ATT GTC AGC AAA-3'.[22] cDNA quantities were normalized to the amount of *GAPDH* cDNA, which was quantified using Rodent *GAPDH* Control Reagents (VIC

probe, Applied Biosystems).

Gene array analysis

Total RNA from E10.5 wild type and *Runx1*$^{-/-}$ yolk sacs was extracted using RNeasy kit, and three same genotype samples were mixed equally. The quality and quantity of the isolated RNA was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies). As previously described,[23] Cy3- or Cy5-labeled cRNA was generated by the use of the Low RNA fluorescent Linear Amplification Kit (Agilent Technologies), and hybridized to **a** 20K Mouse Development Microarray (Agilent Technologies). The scanning was performed with an Agilent Technologies Microarray Scanner (Agilent Technologies), and the analysis was carried out using Excel software (Microsoft).

Results

Abnormal morphology of *Runx1*$^{-/-}$ primitive erythrocytes

To study the role of Runx1 in primitive erythropoiesis, we used a loss-of-function strategy. As reported previously, E10.5 *Runx1*$^{-/-}$ embryos were not anemic and were indistinguishable from wild type embryos.[10-12] *Runx1*$^{-/-}$ embryos began to hemorrhage in the CNS around E11.5, and died around E12.5. Firstly, to examine whether any morphological defects exist in Runx1-deficient primitive erythrocytes, we isolated peripheral blood from live E12.5 *Runx1*$^{-/-}$ embryos. Cytospin preparation of *Runx1*$^{-/-}$ peripheral blood revealed that 27% of primitive erythrocytes displayed a deformed shape of the erythrocytes, in contrast to the smooth, round shape of erythrocytes from *Runx1*$^{+/-}$ mice (Figure 1A-C). Consistently, scanning electron microscopic analysis revealed a rough surface and characteristic hole-like structures on *Runx1*$^{-/-}$ primitive erythrocytes (9 out of 20 erythrocytes, Figure 1G-I), not on *Runx1*$^{+/-}$ primitive erythrocytes (0 out of 16 erythrocytes, Figure 1D-F). As reported previously,[18] the number of primitive erythroid colonies derived from *Runx1*$^{-/-}$ embryos was similar to that derived from control *Runx1*$^{+/-}$ embryos, indicating that formation of primitive erythroid progenitors is normal in *Runx1*$^{-/-}$ embryos at E8.5 (Figure 2A). Benzidine staining of whole yolk sac revealed a normal number of hemoglobinized erythrocytes in *Runx1*$^{-/-}$ embryos at E8.5 (Figure 2B) and E10.5 (data not shown). Taken together, these results suggest that Runx1 is required for the

terminal maturation and / or maintenance of primitive erythrocytes.

Reduced expression of Ter119 in *Runx1*$^{-/-}$ primitive erythrocytes

To further characterize *Runx1*$^{-/-}$ primitive erythrocytes, we analyzed the expression of the erythroid surface marker, Ter119, by fluorescence activated cell sorting (FACS). Compared with *Runx1*$^{+/-}$ embryos, *Runx1*$^{-/-}$ embryos show a reduction of Ter119 expression at E9.5 and E10.5 (Figure 2C and D), indicating that impairment of *Runx1*$^{-/-}$ primitive erythrocytes already occurred at E9.5, at the molecular level.

Microarray analysis of RNA prepared from the *Runx1*$^{-/-}$ yolk sac

We next compared the gene expression profile of wild-type and *Runx1*$^{-/-}$ embryos using microarray analysis to identify potential genes that function downstream of Runx1 in primitive erythropoiesis. For this experiment, we used the E10.5 yolk sac, since this organ at this stage is filled with primitive erythrocytes. We found that the expression of 43 genes was reduced in the *Runx1*$^{-/-}$ yolk sac ($P < 0.01$, Table 1). These included at least two genes encoding factors known to be involved in erythropoiesis, *EKLF* and α-hemoglobin stabilizing protein (*AHSP*).[24-26]

Reduced expression of EKLF and GATA-1 in *Runx1*$^{-/-}$ embryos

The expression of *EKLF* and *AHSP* is regulated by GATA-1,[26,27] and knock-down of

GATA-1 in embryonic stem cells leads to reduced expression of Ter119,[28] leading us to speculate that *GATA-1* expression might be affected by Runx1 depletion. GATA-1 is a critical regulator of both definitive and primitive erythrocyte differentiation.[29] EKLF is also an important transcription factor involved in adult erythropoiesis and recently has been shown to be involved in the maintenance of both normal morphology and Ter119 expression in primitive erythrocytes.[24,25] To quantify the expression of the erythroid transcription factors, *GATA-1* and *EKLF,* in *Runx1*$^{-/-}$ embryos, we performed quantitative RT-PCR analysis. We also examined the expression of another erythroid transcription factor, SCL / Tal-1. Whereas the expression of *SCL* in E11.5 *Runx1*$^{-/-}$ peripheral blood cells was comparable to that observed in *Runx1*$^{+/-}$ and wild type blood cells, the level of both *GATA-1* and *EKLF* mRNAs was reduced by approximately 60% in *Runx1*$^{-/-}$ mice (Figure 3A).

To confirm and better visualize the reduced expression of GATA-1 in *Runx1*$^{-/-}$ primitive erythrocytes, we took advantage of mice transgenic for a *GATA-1* promoter-*LacZ* hybrid gene construct (Figure 3B). We previously reported that a *GATA-1* promoter element, 3.9kb upstream from the GATA-1 proximal promoter (IE) exon, is active in primitive but not definitive erythrocytes.[19] Thus, we introduced the *GATA-1* promoter-*LacZ* (*IE3.9-LacZ*) transgene into Runx1 mutant mice as a surrogate to measure GATA-1 expression in *Runx1*$^{-/-}$ primitive erythrocytes. Consistent with the RT-PCR analysis, expression of β-galactosidase from the

transgene was reduced in E10.5 *Runx1*$^{-/-}$ mice compared to *Runx1*$^{+/-}$ mice (Figure 3C and D). Similar results were obtained in E8.5 (Figure 3C) and E11.5 embryos (data not shown). Reduced expression of β-galactosidase in *Runx1*$^{-/-}$ mice was confirmed by FACS analysis (Figure 3E and F), suggesting that *GATA-1* expression is dependent upon *Runx1* in primitive erythrocytes.

Transgenic rescue of *Runx1*$^{-/-}$ primitive erythrocytes

To determine whether erythroid-specific reintroduction of Runx1 rescues the abnormal phenotype of *Runx1*$^{-/-}$ primitive erythrocytes, transgenic mice expressing Runx1 under the control of the *GATA-1* hematopoietic regulatory domain (*G1-HRD*, also called *IE3.9int*, Figure 4A)[19,20] were crossed into the *Runx1*$^{-/-}$ genetic background. The expression of GATA-1 was observed in the early erythroid progenitors, and *G1-HRD* significantly recapitulates the endogenous expression profile of GATA-1 in the erythroid lineage.[19] Therefore, although *GATA-1* expression was reduced by approximately 60% in *Runx1*$^{-/-}$ genetic background (Figure 3A), we assumed that this regulatory domain is suitable for our erythroid-specific rescue experiment. As expected, normal erythrocyte morphology and Ter119 expression were restored in *Runx1*$^{-/-}$ mice carrying the *G1-HRD-Runx1* transgene (Figure 4B-D). Thus, we conclude that the defect in primitive erythrocytes in *Runx1*$^{-/-}$ mice is cell autonomous.

Discussion

In this study, we demonstrated that *Runx1*$^{-/-}$ primitive erythrocytes exhibit some abnormal features. Impairment of primitive erythrocytes has also been observed in mice carrying a knocked-in *CBFβ-MYH11* gene.[30] CBFβ is a heterodimeric binding partner of Runx, and the fusion protein generated from the *CBFβ-MYH11* gene functions as a dominant negative inhibitor of Runx1 activity.[31] In addition, although data were not shown, Wang and coworkers had noticed subtle abnormalities in the morphology and staining intensity of primitive erythrocytes from *Runx1*$^{-/-}$ embryos. [11] These results further support our contention that Runx1 plays a role in primitive erythropoiesis in the mouse embryo.

Although the morphology of *Runx1*$^{-/-}$ primitive erythrocytes is not normal, we believe that these erythrocytes are nonetheless able to deliver oxygen to body tissues based on following reasons. First, benzidine staining experiments demonstrated that erythrocytes in *Runx1*$^{-/-}$ embryos exhibit nearly normal levels of hemoglobinization (Figure 2B). Second, mutant mice lacking *GATA-1* (*Alas*), which exhibit no functional primitive erythrocytes, die around E10.5,[32,33] indicating that intact primitive erythrocytes are essential for the survival of the embryo after E11. Unlike these latter mice, Runx1-deficient mice die instead around E12.5.

We showed here that expression of GATA-1 is at least partially dependent upon Runx1 expression in primitive erythrocytes (Figure 3). It is unknown, however,

whether Runx1 directly regulates *GATA-1* transcription. An interaction or association between Runx and GATA has been observed in other biological systems. Human RUNX1 and GATA-1 physically interact and synergistically activate the megakaryocyte gene *in vitro*.[34] In the Drosophila blood system, coexpression of the GATA factor, Serpent, and the Runt-related factor, Lozenge, enhances crystal cell overproduction.[35] Interestingly, mutations in *GATA-1* were found in the vast majority of Down's syndrome (constitutional trisomy 21) patients with acute megakaryoblastic leukemia (DS-AMKL), and an extra copy of *RUNX1* on chromosome 21 has been suspected to be responsible for the increased risk of DS-AMKL.[36] These observations suggest that Runx and GATA factors work cooperatively and may modulate one another's activity in a gene regulatory network.

We have uncovered a previously unrecognized role for Runx1 in mouse primitive erythropoiesis. Runx1 is also expressed during immature stages of definitive erythrocytes.[37] In addition, impairment of definitive erythroid differentiation has been observed in an AML patient with a t(8;21) translocation, which results in the production of an AML1-ETO fusion protein.[38] These observations suggest that Runx1 may play a role in definitive erythropoiesis. Although there is a report that there is no apparent reduction of the hemoglobin level in *Runx1*-deficient peripheral blood cells,[39] it is worth examining more extensively whether conditional loss of *Runx1* in erythroid lineage influences adult

erythropoiesis.

Acknowledgements

We would like to thank Dr. S.-I. Nishikawa for reagents and Dr. O. Nakajima for protocol.

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Table 1. Genes downregulated in *Runx1*$^{-/-}$ yolk sac

Systematic Name	Gene	Symbol	Wild-type signal	Runx1 KO signal	Fold Change
H3044F08-3	Mitogen inducible mig	MGC36836	3362.7	767.1	0.2281
L0240C12-3	Complement component 1, q subcomponent, alpha polypeptide	C1qa	3672.5	1220.3	0.3323
L0075B01-3	Mus domesticus strain MilP mitocondrion genome, complete sequence	-	30184.8	14344.7	0.4752
H3054H09-3	H3054H09-3	-	7727.4	4002.2	0.5179
M97200.1	Erythroid Kruppel-like factor	EKLF	4482.6	2440.6	0.5445
C0284A02-3	Hydroxysteroid dehydrogenase-1, delta<5>-3-beta	Hsd3b1	3796.8	2093.6	0.5514
L0258G12-3	Muscleblind-like	Mbnl	5640.8	3188.8	0.5653
H3126G01-5	H3126G01-5	-	82880.5	47827.7	0.5771
H3060E04-3	Protein kinase C type EC2.7.1-	6030436C20Rik	3320.5	1954.3	0.5886
H3114C08-3	α hemoglobin stabilizing protein	AHSP	8460.8	5144.7	0.6081
H3087E02-3	riken cDNA 4933436C10 gene	4933436C10Rik	9907.1	6159.5	0.6217
H3059D10-3	Cornichon homolog	Cnih	11741.2	7310.0	0.6226
J0460E10-3	J0460E10-3	-	5814.1	3646.0	0.6271
H3048G11-3	Biliverdin reductase B	Blvrb	10690.0	6713.2	0.6280
H3065D10-3	RAS-related protein-1a	Rap1a	19757.3	12435.3	0.6294
L0222E01-3	Rhesus blood group CE and D	Rhced	10397.4	6590.9	0.6339
H3056A02-3	H3056A02-3	-	4661.2	2981.7	0.6397
H3157F03-3	riken cDNA 2810465O16 gene	2810465O16Rik	5012.1	3214.5	0.6414
C0321H12-3	Cell growth regulator	1110038G02Rik	8733.7	5608.6	0.6422
H3076H08-3	H3076H08-3	-	20849.6	13503.4	0.6477
C0166A10-3	Carbonic anhydrase 2	Car2	27887.6	18109.0	0.6494

Genes downregulated less than 0.65-fold in Runx1-deficient yolk sacs were listed. Probe informations and gene annotations of Agilent Mouse Development Microarrays are available at the NIA mouse cDNA project home page (http://lgsun.grc.nia.nih.gov/cDNA/cDNA.html).

Figure legends

Figure 1. *Runx1*$^{-/-}$ primitive erythrocytes show defect in morphology. (A, B) May-Grunwald Giemsa stained cytospin preparation of peripheral blood cells at E12.5. While normal erythrocytes (arrowhead) can be seen in *Runx1*$^{-/-}$ embryo, 27% of them show apparent deformed shape (arrows). (C) Quantification of abnormal primitive erythrocytes in *Runx1*$^{+/-}$ (n=3) and *Runx1*$^{-/-}$ (n=4) embryos at E12.5. Asterisk indicates significance, P=0.002, Student *t* test. (D-I) Scanning electron microscopy of primitive erythrocytes at E12.5. Shown are representative pictures of primitive erythrocytes from *Runx1*$^{+/-}$ (n=3) and *Runx1*$^{-/-}$ (n=3) embryos. Characteristic large holes (white arrowheads) were observed in *Runx1*$^{-/-}$ erythrocytes (9/20), not in *Runx1*$^{+/-}$ erythrocytes (0/16). This difference is statistically significant (P=0.002 by Fisher's exact probability test). Original magnifications are x 400 for A and B, x 8000 for D, E, F and G, x 8500 for H, and x 9000 for I.

Figure 2. Analysis of primitive erythroid proginitors and differentiated cells in *Runx1*$^{-/-}$ embryo. (A) Primitive erythroid colonies from E8.5 *Runx1*$^{+/-}$ yolk sacs (n=3) and *Runx1*$^{-/-}$ yolk sacs (n=3). (B, C) Benzidine-staining of E8.5 embryos. Hemoglobinized cells were observed inside blood vessels of yolk sac. Similar staining levels were obtained for wild type and *Runx1*$^{-/-}$ embryos. Original magnifications are x 16.0. (D) Flow cytometric analysis of E9.5 yolk sac using

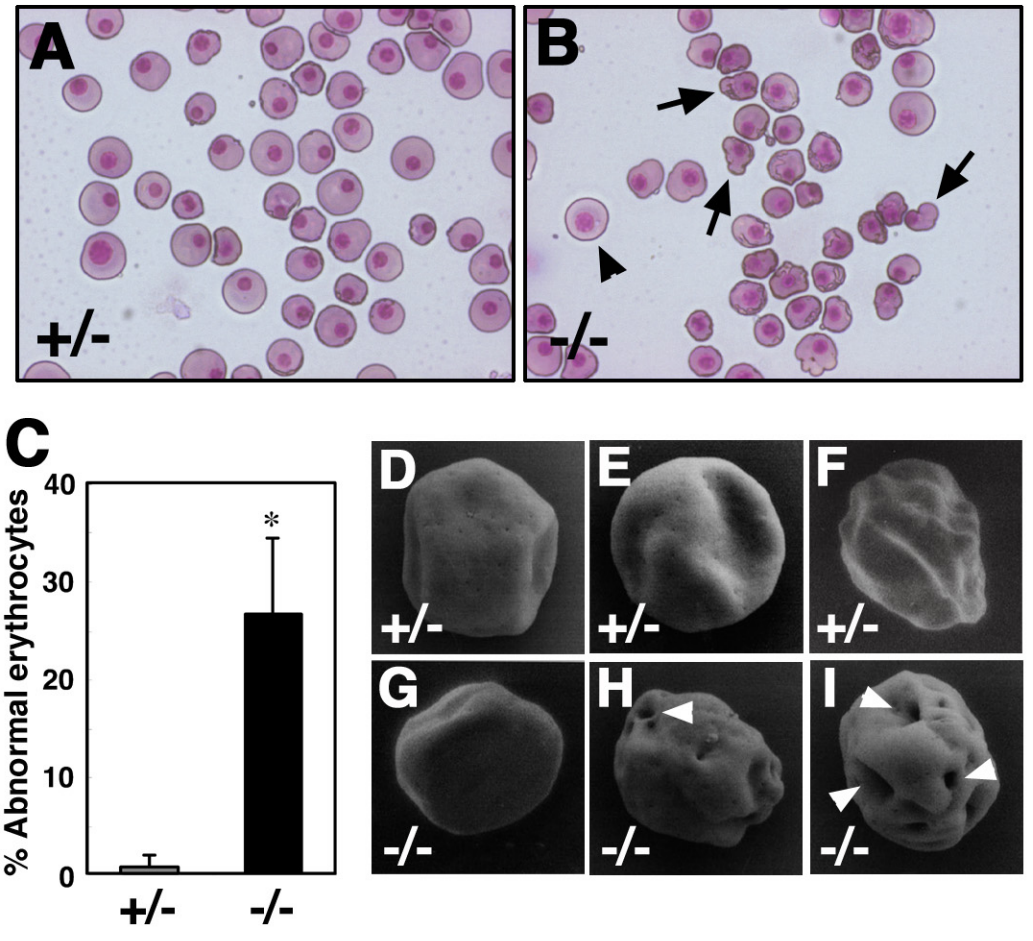
anti-Ter119 antibody. Black line, gray bold line, or red bold line represent Ter119 expression in *Runx1*$^{+/+}$, *Runx1*$^{+/-}$, and *Runx1*$^{-/-}$, respectively. (E) Relative MFI values of Ter119 for *Runx1*$^{+/+}$ (normalized to MFI=1), *Runx1*$^{+/-}$, and *Runx1*$^{-/-}$ embryos at E9.5 and E10.5. Bar graphs represents mean ratios ± standard deviations derived from *Runx1*$^{+/-}$ (n=3 E9.5, n=4 E10.5) and *Runx1*$^{-/-}$ (n=2 E9.5, n=4 E10.5) embryos. Significant differences in *Runx1*$^{-/-}$ mice from *Runx1*$^{+/-}$ mice were observed; ***P*=0.017, **P*=0.039 by Student *t* test.

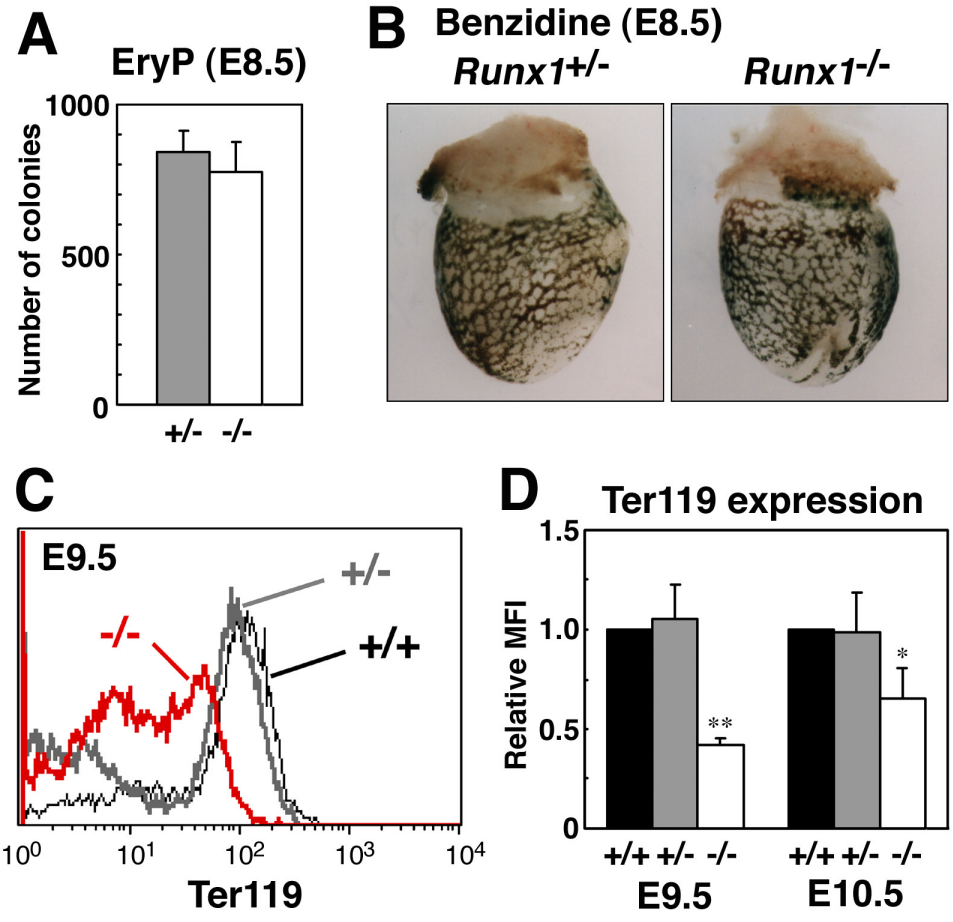
Figure 3. Reduced expression of GATA-1 and EKLF in *Runx1*$^{-/-}$ primitive erythrocytes. (A) Relative intensity levels were measured by real-time PCR and normalized to *GAPDH*. The Student *t* test was applied for statistical analysis. Significant differences in *Runx1*$^{-/-}$ mice from wild type were observed; ***P*=0.009, **P*=0.013. (B) Structure of the *GATA-1* promoter-*LacZ* (*IE3.9-LacZ*) transgene. IE indicates exon IE. (C) Representative pictures of *Runx1*$^{+/-}$::*IE3.9-LacZ* (n=7 E8.5, n=12 E10.5) and *Runx1*$^{-/-}$::*IE3.9-LacZ* (n=8 E8.5, n=4 E10.5) embryos. Expression of β-galactosidase was reduced in *Runx1*$^{-/-}$ embryo (C, right panels) compared to *Runx1*$^{+/-}$ embryo (C, left panels). (D) Cytospin preparation of blood cells from embryos shown in panel C (E10.5). Intensity of β-galactosidase staining was reduced in *Runx1*$^{-/-}$ cells (arrowheads) compared to *Runx1*$^{+/-}$ cells (arrows). (E) FACS analysis of GATA-1 (β-galactosidase) expression in E10.5 embryos. FDG was used as a

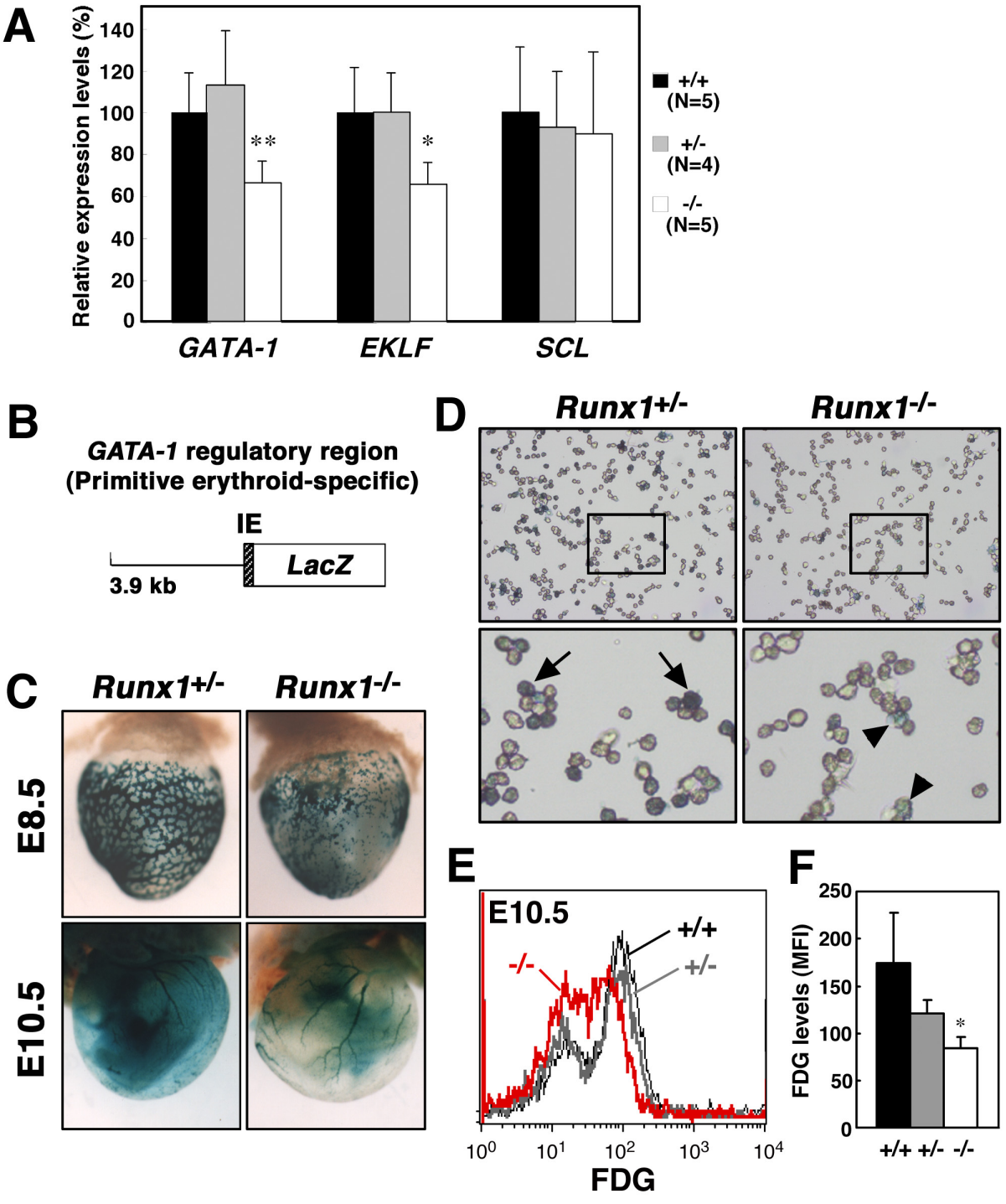
fluorescent substrate for β-galactosidase. Black line, gray bold line, or red bold line represent β-galactosidase expression in *Runx1*$^{+/+}$, *Runx1*$^{+/-}$, and *Runx1*$^{-/-}$, respectively. (F) MFI values of FDG for *Runx1*$^{+/+}$ (n=2) *Runx1*$^{+/-}$ (n=5) and *Runx1*$^{-/-}$ (n=2) embryos. Significant difference in *Runx1*$^{-/-}$ mice from *Runx1*$^{+/-}$ mice was observed; **P*=0.032 by Student *t* test. Original magnifications are x 16.0 for C (upper panels), x 7.2 for C (lower panels), and x 400 for D.

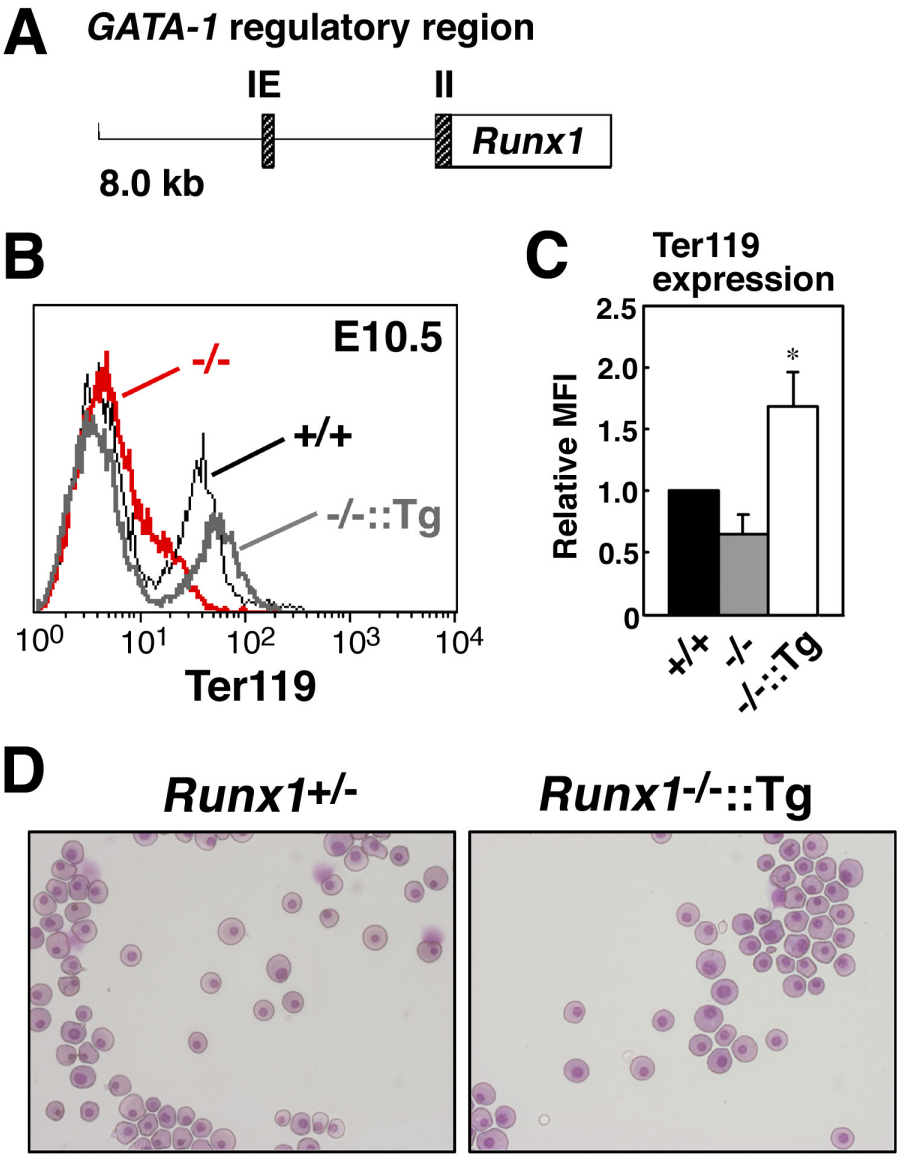
Figure 4. Rescue of defects in *Runx1*$^{-/-}$ primitive erythrocytes by transgenic introduction of Runx1. (A) Structure of the *G1-HRD-Runx1* (*IE3.9int-Runx1*) transgene. IE and II indicate exon IE and II, respectively. (B) Rescue of Ter119 expression on E10.5 *Runx1*$^{-/-}$ primitive erythrocytes by introducing *G1-HRD-Runx1* transgene. Black line, gray bold line, or red bold line represent Ter119 expression in *Runx1*$^{+/+}$, *Runx1*$^{-/-}$::*G1-HRD-Runx1* and *Runx1*$^{-/-}$, respectively. Note that overexpression of Runx1 in *Runx1*$^{-/-}$ erythrocytes induced slightly higher Ter119 expression. (C) Relative MFI values of Ter119 for *Runx1*$^{+/+}$ (normalized to MFI=1), *Runx1*$^{-/-}$, and *Runx1*$^{-/-}$::*G1-HRD-Runx1* embryos at E10.5. Bar graphs represents mean ratios ± standard deviations derived from *Runx1*$^{-/-}$ (n=4) and *Runx1*$^{-/-}$::*G1-HRD-Runx1* (n=4) embryos. Significant difference in *Runx1*$^{-/-}$::*G1-HRD-Runx1* mice from *Runx1*$^{-/-}$ mice was observed; **P*<0.001 by Student *t* test. (D) Rescue of abnormal morphology observed in *Runx1*$^{-/-}$ primitive erythrocytes. May-Grunwald Giemsa stained cytospin

preparation of peripheral blood cells from E12.5 embryos. Deformed shape observed in *Runx1*$^{-/-}$ erythrocytes (see Figure 1B) was rarely seen in *Runx1*$^{-/-}$::*G1-HRD-Runx1* erythrocytes.


A
+/-
B
-/-
C
% Abnormal erythrocytes
40
30
20
10
0
*
+/-
-/-
D
+/-
E
+/-
F
+/-
G
-/-
H
-/-
I
-/-


A
EryP (E8.5)
Number of colonies
1000
500
0
+/-
-/-
B
Benzidine (E8.5)
Runx1+/-
Runx1-/-
C
E9.5
+/-
-/-
+/+
10^0
10^1
10^2
10^3
10^4
Ter119
D
Ter119 expression
Relative MFI
1.5
1.0
0.5
0
**
*
+/+ +/- -/-
+/+ +/- -/-
E9.5
E10.5


A
Relative expression levels (%)
140
120
100
80
60
40
20
0
**
*
GATA-1
EKLF
SCL
+/+ (N=5)
+/- (N=4)
-/- (N=5)
B
GATA-1 regulatory region
(Primitive erythroid-specific)
IE
LacZ
3.9 kb
C
Runx1+/-
Runx1-/-
E8.5
E10.5
D
Runx1+/-
Runx1-/-
E
E10.5
+/+
+/-
-/-
10^0
10^1
10^2
10^3
10^4
FDG
F
FDG levels (MFI)
250
200
150
100
50
0
*
+/+ +/- -/-


A
GATA-1 regulatory region
IE
II
Runx1
8.0 kb
B
E10.5
-/-
+/+
-/-::Tg
10^0
10^1
10^2
10^3
10^4
Ter119
C
Ter119 expression
Relative MFI
2.5
2.0
1.5
1.0
0.5
0
*
+/+
-/-
-/-::Tg
D
Runx1+/-
Runx1-/-::Tg