Leukemia-related transcription factor TEL/ETV6 expands erythroid precursors and stimulates hemoglobin synthesis

Minenori Eguchi-Ishimae,[1,2] Mariko Eguchi,[1,2] Kazuhiro Maki,[1] Catherine Porcher,[3] Ritsuko Shimizu,[4] Masayuki Yamamoto[5] and Kinuko Mitani[1,6]

[1]Department of Hematology, Dokkyo Medical University School of Medicine, Tochigi 321-0293; [2]Department of Pediatrics, Ehime University, Ehime 791-0295, Japan; [3]MRC Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford OX3 9DS, United Kingdom; [4]Institute of Basic Medical Sciences and Center for TARA, University of Tsukuba, Ibaraki 305-8577; [5]Medical Biochemistry, Tohoku University School of Medicine, Miyagi 980-8575, Japan

(Received 24 July, 2008/Revised November 7, 2008; December 18, 2008/Accepted December 24, 2008/Online publication March 11, 2009)

TEL/ETV6 **located at chromosome 12p13 encodes a member of the E26 transformation-specific family of transcription factors.** ***TEL*** **is known to be rearranged in a variety of leukemias and solid tumors resulting in the formation of oncogenic chimeric protein. Tel is essential for maintaining hematopoietic stem cells in the bone marrow. To understand the role of TEL in erythropoiesis, we generated transgenic mice expressing human** ***TEL*** **under the control of** ***Gata1*** **promoter that is activated during the course of the erythroid-lineage differentiation (*GATA1-TEL* transgenic mice). Although *GATA1-TEL* transgenic mice appeared healthy up to 18 months of age, the level of hemoglobin was higher in transgenic mice compared to non-transgenic littermates. In addition, CD71$^+$/TER119$^+$ and c-kit$^+$/CD41$^+$ populations proliferated with a higher frequency in transgenic mice when bone marrow cells were cultured in the presence of erythropoietin and thrombopoietin, respectively. In transgenic mice, enhanced expression of *Alas-e* and *β-major globin* genes was observed in erythroid-committed cells. When embryonic stem cells expressing human *TEL* under the same *Gata1* promoter were differentiated into hematopoietic cells, immature erythroid precursor increased better compared to controls as judged from the numbers of burst-forming unit of erythrocytes. Our findings suggest some roles of TEL in expanding erythroid precursors and accumulating hemoglobin. (*Cancer Sci* 2009; 100: 689–697)**

TEL (also known as *ETV6*) gene is frequently involved in recurring chromosomal translocations as well as deletions in various hematopoietic malignancies, suggesting its role as a tumor suppressor gene.[1,2] *TEL* encodes a member of the ETS family of transcription factors and has the ETS DNA binding domain in its C-terminal side[3] and the Pointed domain with oligomerization capacity in its N-terminal side.[4] Between the Pointed and ETS domains is the central domain that ascribes TEL with transcriptional repression activity by recruiting repressor complexes including histone deacetylase and nuclear corepressors,[5–9] one of the characteristic properties of TEL among ETS transcription factors.

Tel plays some important roles in development and hematopoiesis. Complete ablation of the *Tel.* gene in mice results in embryonic lethal phenotype at day 10.5–11.5 post-coitus with impaired yolk sac angiogenesis.[10] At that time, primary erythropoiesis in the yolk sac is intact. Detailed analysis of hematopoietic differentiation and proliferation capacity of *Tel–/–* cells was carried out by means of generation of chimeric mice which consist of *Tel–/–* cells as well as wild-type cells to avoid embryonic lethality.[11] The observation that cells lacking both *Tel* alleles fail to contribute to hematopoiesis in the neonatal bone marrow, but do not in the yolk sac and fetal liver, indicating an active role of Tel on hematopoietic stem cells to recruit to the bone marrow microenvironment or to be maintained in the bone marrow niche to construct bone marrow hematopoiesis by producing their progeny. Conditional inactivation of *Tel* in adult mice results in complete loss of hematopoietic stem cells in the bone marrow[12] which is consistent with the finding obtained from the chimera analysis. These findings indicate the function of Tel is as a selective and essential regulator of stem cells. However, detailed functions of TEL in hematopoietic cell differentiation are still unknown. One approach to scrutinize the function of TEL is to see the outcome after enforced expression of *TEL* gene in a specific lineage and at a specific stage of hematopoietic differentiation, as expression of *TEL* gene is suggested to be ubiquitous and continuous during differentiation.[5,10,11] We have previously reported that upon induction of erythroid differentiation by chemical compounds, a murine erythroid leukemic cell line MEL differentiates to mature erythroid cells more effectively by overexpression of the *TEL* gene.[13] In addition, overexpressed *TEL* in an erythroid/megakaryocytic-committed human leukemic cell line UT7/GM promotes erythroid differentiation and inhibits megakaryocytic maturation.[14] All these data suggest that TEL might have some impact on terminal hematopoietic differentiation along the erythroid and megakaryocytic lineages.

GATA1, encoding a zinc-finger transcription factor, plays a central role in erythropoiesis by regulating transcription of genes such as *δ-aminolevulinic acid synthase-erythroid* (*ALAS-E*) and *α/β-globin* genes.[15–20] Gata1 is essential for primary erythropoiesis[21–23] and regulates maturation and apoptotic induction of definitive erythropoiesis.[24,25] Although *GATA1* is expressed in multipotential progenitor cells, albeit at a low level,[26,27] a drastic increase of *GATA1* expression is observed upon erythroid-lineage commitment, resulting in further progression of the erythroid differentiation pathway.[28,29] Regulation of *GATA1* expression at an appropriate stage is quite crucial for proper erythroid lineage development and its expression is controlled precisely through the erythroid-specific regulatory region of the gene.[30,31]

To understand the effects of *TEL* on erythropoiesis, we have established transgenic mice and embryonic stem (ES) cells that express human *TEL* specifically in the erythroid-committed cells under the control of the erythroid-specific *Gata1* promoter. Forced expression of *TEL* in the erythroid-committed cells resulted in higher hemoglobin (Hb) levels in the mice and promoted

[6]To whom correspondence should be addressed. E-mail: kinukom-tky@umin.ac.jp

doi: 10.1111/j.1349-7006.2009.01097.x

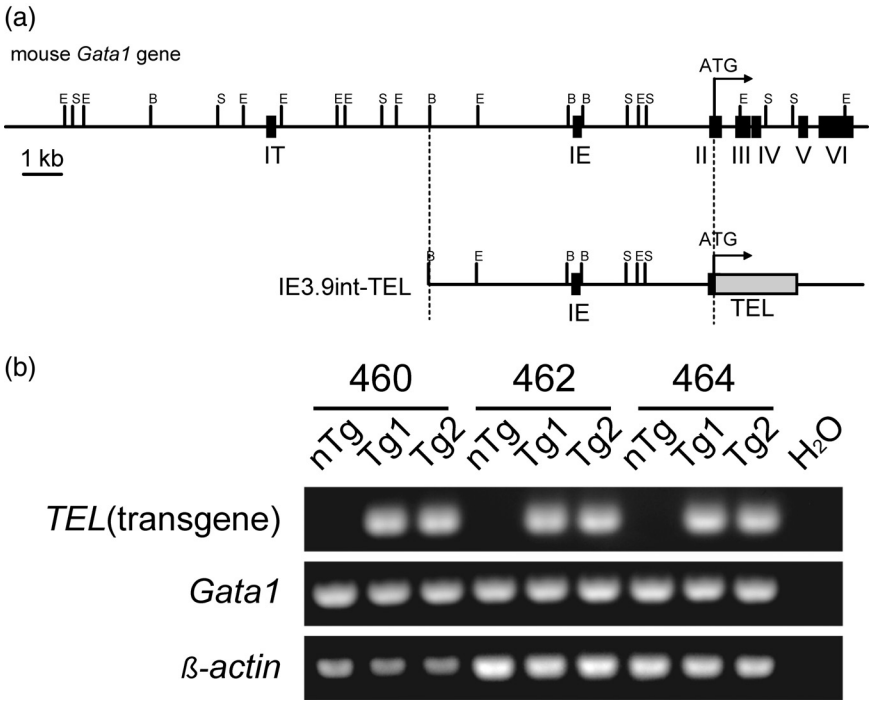


Fig. 1. Expression of TEL transgene in *GATA1-TEL* transgenic mice. (a) Schematic representation of the *Gata1* promotor region and the pIE3.9int-TEL construct used for the generation of *GATA1-TEL* transgenic mice. The pIE3.9int vector contains 3.9 kb *Gata1* promoter region upstream of exon IE.[30] The coding sequence of wild-type human *TEL* cDNA was connected to the first ATG codon in exon II of mouse *Gata1* gene. Abbreviations for the restriction enzyme sites are E, *Eco*RI; B, *Bam*HI; S, *Sac*I. (b) Expression of *Gata1*-driven *TEL* transgene was confirmed by reverse transcription polymerase chain reaction (RT-PCR) using bone marrow cells extracted from *GATA1-TEL* transgenic mice of lines 460, 462 and 464. Forward primers for the first and second PCR were both located on exon IE of mouse *Gata1* gene and reverse primers on exon II of human *TEL* gene. Tg, *Gata1-TEL* transgenic mouse; nTg, non-transgenic littermate.

expansion of erythroid progenitor following erythropoietin (EPO) stimulation *in vitro*. The erythroid-specific genes *Alas-e* and *β-major globin* were more highly expressed in $CD71^{high}$/$TER119^{+}$ erythroid precursor in the bone marrow of *GATA1-TEL* transgenic mice than non-transgenic mice. In ES cell culture experiments, when day 7 embryoid body (EB) was subjected to hematopoietic colony assay, higher numbers of BFU-E were formed in *GATA1-TEL* transgenic cells. These data indicate that TEL could regulate both proliferation and differentiation of erythroid cells.

Materials and methods

Transgenic vector and generation of GATA1-TEL transgenic mice. pIE3.9intLacZ vector which contains mouse *Gata1* promoter region 3.9 kb upstream of exon IE was described previously.[30] LacZ-coding region was removed from pIE3.9intLacZ and replaced by a coding sequence of wild-type human *TEL* gene downstream of native ATG codon of *Gata1* (pIE3.9int-TEL, Fig. 1a). *GATA1-TEL* transgenic mice were generated by microinjection of pIE3.9int-TEL vector to fertilized mouse oocytes isolated from superovulated BDF1 mice (Clea Japan Inc., Tokyo, Japan). Genomic DNA was prepared from tails of liveborn mice and genotyping was performed by polymerase chain reaction (PCR) using a combination of primers located on an intron sequence upstream of exon II of mouse *Gata1* (G1 HD-8369f) and human *TEL* cDNA (TEL-91r, TEL-117r). Sequences of primers are listed in Supporting Table S1. Peripheral blood counts were performed using particle counter PCE-170 (ERMA Inc, Tokyo, Japan). Serum EPO levels were evaluated using Quantikine Mouse/Rat Epo Immunoassay (R & D Systems, Minneapolis, MN, US).

Reverse transcriptase-mediated PCR (RT-PCR). Total RNA was prepared using RNeasy kit (Qiagen, Valencia, CA, US) with DNaseI treatment and then reverse transcribed with random hexamers using MMLV reverse transcriptase (Stratagene, La Jolla, CA, US). Reverse transcription products were amplified by PCR with specific primers using standard procedures. To examine the expression of *GATA1-TEL* transgene, forward primers for PCR amplification were designed on exon IE of *Gata1* (mGATA1-3f, mGATA1-28f) and reverse primers on human *TEL* sequence (TEL-91r, TEL-117r). Upon transcription from integrated pIE3.9int-TEL sequences, exon IE of mouse *Gata1* is connected to human *TEL* sequence replacing *Gata1*-coding sequence, which could be assessed specifically by RT-PCR with this combination of primers. Expression of endogenous *Gata1* was examined with mGATA1-3f and mGATA1-336r (located on exon III of *Gata1*). The details of primer sequences are shown in Table S1. The products were electrophoresed on 2% agarose gels and stained by ethidium bromide.

Real-time quantitative PCR. Quantitative PCR was performed with a SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, US) as indicated in the manufacturer's protocol using 10 ng cDNA template and 200 μM each primer per reaction. Reactions were run and analyzed on ABI7700 (Applied Biosystems). All reactions were performed in duplicate, and were analyzed using SDS software (Applied Biosystems). Primer sets to analyze expression levels of endogenous *Gata1*, transgenic *GATA1-TEL* and total (endogenous + exogenous) *TEL* transcripts were mGATA1–50f and mGATA1-303r, mGATA1-28f and GIHRD-TEL-r (*hTEL* in Supporting Table S2), TEL-1005f and TEL-1082r (for bone marrow cells, *hmTEL(3)* in Supporting Table S2), and TEL-829f and TEL-921r (for ES cells, *hmTEL(2)* in Supporting Table S2), respectively. Forward and reverse primers of both *hmTEL(3)* and *(2)* are located on exons V and VI of mouse and human *TEL* genes and can simultaneously amplify both mouse and human *TEL* transcripts because the sequences of this region are almost identical between these two species. *Hypoxanthine phosphoribosyl-transferase* (*Hprt*) was used as a control gene for normalization to account for variations in template input, as described previously.[32] The details of primer sequences are shown in Supporting Table S2.

Differentiation in liquid cultures. Bone marrow cells harvested from femurs of mice were dispersed into single cell suspensions and were cultured in the presence of recombinant murine EPO (3 U/mL) and stem cell factor (SCF) (50 ng/mL), or thrombopoietin (TPO, 20 ng/mL), interleukin (IL)-3 (10 ng/mL) and IL-6 (10 ng/mL). Cells were examined after 8 days by fluorescence activated cell sorting (FACS). Murine recombinant SCF, IL-3 and IL-6 were purchased from Peprotec (London, UK), and EPO from R & D Systems (Minneapolis, MN, US).

doi: 10.1111/j.1349-7006.2009.01097.x

Table 1. Peripheral blood count of GATA1-TEL transgenic mice and littermate controls

	TEL460		TEL462		TEL464	
	nTg	Tg	nTg	Tg	nTg	Tg
No. of mice	11	20	14	30	7	50
WBC ($\times 10^3/\mu L$)	9.8 ± 4.8	10.5 ± 3.5	11.1 ± 4.3	9.8 ± 3.4	8.6 ± 4.0	7.7 ± 3.0
RBC ($\times 10^6/\mu L$)	8.6 ± 0.5	9.1 ± 0.4*	8.7 ± 0.4	8.7 ± 0.4	8.7 ± 0.5	8.8 ± 0.4
Hb (g/dL)	16.5 ± 1.0	17.4 ± 1.0*	17.1 ± 0.8	17.8 ± 1.3*	16.4 ± 0.8	17.1 ± 3.9
Ht (%)	37.7 ± 3.5	38.9 ± 1.8	40.0 ± 3.1	41.0 ± 4.2	36.8 ± 1.9	37.7 ± 2.1
Plt ($\times 10^4/\mu L$)	164.4 ± 50.1	148.5 ± 32.6	128.1 ± 38.1	123.3 ± 56.0	122.0 ± 14.9	110.1 ± 25.9
MCV (fl)	43.5 ± 2.3	42.9 ± 1.2	46.1 ± 3.4	46.9 ± 3.9	42.5 ± 1.4	43.0 ± 1.4
MCH (pg)	19.1 ± 0.7	19.2 ± 0.8	19.8 ± 0.8	20.4 ± 0.9*	19.0 ± 0.7	19.5 ± 4.6*
MCHC (%)	44.1 ± 3.1	44.9 ± 1.7	42.9 ± 3.3	43.8 ± 4.4	44.7 ± 2.0	45.5 ± 10.5

*Significantly higher compared to littermate controls ($P < 0.05$).
Tg, *GATA1-TEL* transgenic mouse; nTg, non-transgenic littermate; Hb, hemoglobin; Ht, Hematocrit; Plt, platelet; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

Flow cytometry and cell sorting. Single cell suspensions were prepared from bone marrow or cultured cells and were then analyzed by flow cytometry using fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies against c-kit, TER119 (BD Biosciences Pharmingen, San Diego, CA, US) or CD41, CD71 (eBioscience, San Diego, CA, US). The stained cells were analyzed by FACSCalibur (Becton Dickinson, San Jose, CA, US) or sorted on FACSAria (Becton Dickinson).

ES cell growth and differentiation. Mouse ES cells (J1) were electroporated with pIE3.9int-TEL transgenic vector connected to *neomycin resistance* gene or mock pIE3.9int-neo vector, and selected with G418 (Sigma, St Louis, MO, US). ES cells were maintained on gelatinized plates in TX-WES cell culture medium (Thromb-X, Leuven, Belgium) with supplement of recombinant murine leukemia inhibitory factor (LIF, AMRAD, Melbourne, Australia). For the generation of EBs, ES cells were trypsinized and plated at various densities in differentiation cultures. Differentiation of EBs was carried out in 82-mm Petri-grade dishes in Iscove's modified Dulbecco's Medium (IMDM) supplemented with 15% fetal calf serum (FCS), 2 mM L-glutamine (Gibco/BRL, Gaithersburg, MD, US), 200 μg/mL transferrin, 0.5 mM ascorbic acid (Sigma), and 4.5×10^{-4} M 1-thioglycerol (Sigma). Cultures were maintained in a humidified chamber in a 5% CO_2/air mixture at 37°C.

Colony assays of EBs. To differentiate hematopoietic precursors, EBs were dissociated at day 7 and cells were plated in 1% methylcellulose containing 10% FCS, 5% protein-free hybridoma medium (PFHM-II; Gibco/BRL), 2 mM L-glutamine, 200 μg/mL transferrin and following cytokines for colony forming unit of granulocyte/erythrocyte/macrophage/megakaryocytic (CFU-GEMM) assay: SCF (100 ng/mL), TPO (5 ng/mL), EPO (2 U/mL), IL-11 (5 ng/mL), IL-3 (1 ng/mL), granulocyte/macrophage-colony stimulating-factor (GMCSF) (30 ng/mL), granulocyte-colony stimulating factor (G-CSF) (30 ng/mL), macrophage-colony stimulating factor (M-CSF) (5 ng/mL) and IL-6 (5 ng/mL), and for BFU-E assay: SCF (100 ng/mL), TPO (5 ng/mL) and EPO (2 U/mL). Murine recombinant GM-CSF, M-CSF, G-CSF and IL-11 were purchased from Peprotec. Cultures were maintained at 37°C with 5% CO_2. The numbers of colonies comprising more than 40 cells were scored after 7 days, and myeloid, erythroid and mixed colonies were defined based on their morphology.

***In vitro* differentiation of EB-derived c-kit$^+$/CD71$^+$ cells on OP9 layer.** EBs were dissociated at day 6 of differentiation and c-kit$^+$/CD71$^+$ cells were separated by FACSAria. Sorted c-kit$^+$/CD71$^+$ cells were plated onto OP9 stromal cell[33,34] layer supplemented with EPO (3 U/mL) and SCF (50 ng/mL) to promote erythroid differentiation and cultured for 8 days before FACS analysis. OP9 cells were maintained in α-modified minimum essential media (α-MEM, Gibco-BRL) supplemented with 20% FCS.

Statistical analysis. A two-tailed Student's *t*-test was used to determine the difference between non-transgenic and *GATA1-TEL* transgenic samples.

Results

Generation of *GATA1-TEL* transgenic mice. Three transgenic lines (460, 462 and 464) were established with pIE3.9int-TEL transgenic construct. Expression of transgene (i.e. human *TEL*) from integrated *Gata1-TEL* sequences was confirmed by RT-PCR with bone marrow cells of transgenic mice and their littermates. As expected, expression of the transgene was seen in the bone marrow of all of the transgenic mice examined, and the representative data are shown in Fig. 1(b). Expression of endogenous *Gata1* was also confirmed with the same bone marrow RNA samples. *GATA1-TEL* transgenic mice of all the lines appeared healthy up to 18 months of age without any symptoms.

Higher Hb concentration in *GATA1-TEL* transgenic mice. Blood counts were examined using peripheral bloods obtained from *GATA1-TEL* transgenic mice and their littermates (Table 1). As a result, Hb concentration was significantly higher in two transgenic lines (460 and 462), and red blood cell (RBC) count was also higher in one of the lines (460). Although not statistically significant, Hb concentration was also higher in the other transgenic line (464). There were no significant differences in white blood cell and platelet counts between *GATA1-TEL* transgenic mice and their littermate controls of any lines. Then, we evaluated serum EPO levels of *GATA1-TEL* transgenic mice and their litters of the three lines 460, 462 and 464. Mean EPO levels of transgenic and non-transgenic mice were 108 ± 21 pg/mL ($n = 10$) and 123 ± 30 pg/mL ($n = 10$) in line 460, 221 ± 65 pg/mL ($n = 8$) and 250 ± 105 pg/mL ($n = 11$) in line 462, and 134 ± 54 pg/mL ($n = 4$) and 142 ± 85 pg/mL ($n = 4$) in line 464. Although the differences were not statistically significant, there was a tendency that serum EPO levels were lower in the transgenic mice of all the lines, suggesting that serum EPO levels were negatively regulated by increased Hb in the transgenic mice.

CD71^{high}/TER119$^+$ cells expanded better from the bone marrow cells of transgenic mice than littermate controls. When populations of granulo-monocytic, erythroid, megakaryocytic and immature hematopoietic cells in the bone marrow were assessed by FACS analysis using antibodies against Gr-1/Mac-1, CD71/TER119, c-kit/CD41 and c-kit/CD34, no apparent difference between *GATA1-TEL* transgenic mice and their littermate controls was observed (data not shown). Colony forming cell (CFC) assay also revealed no significant difference between them (data not shown). However, when bone marrow cells were cultured in the

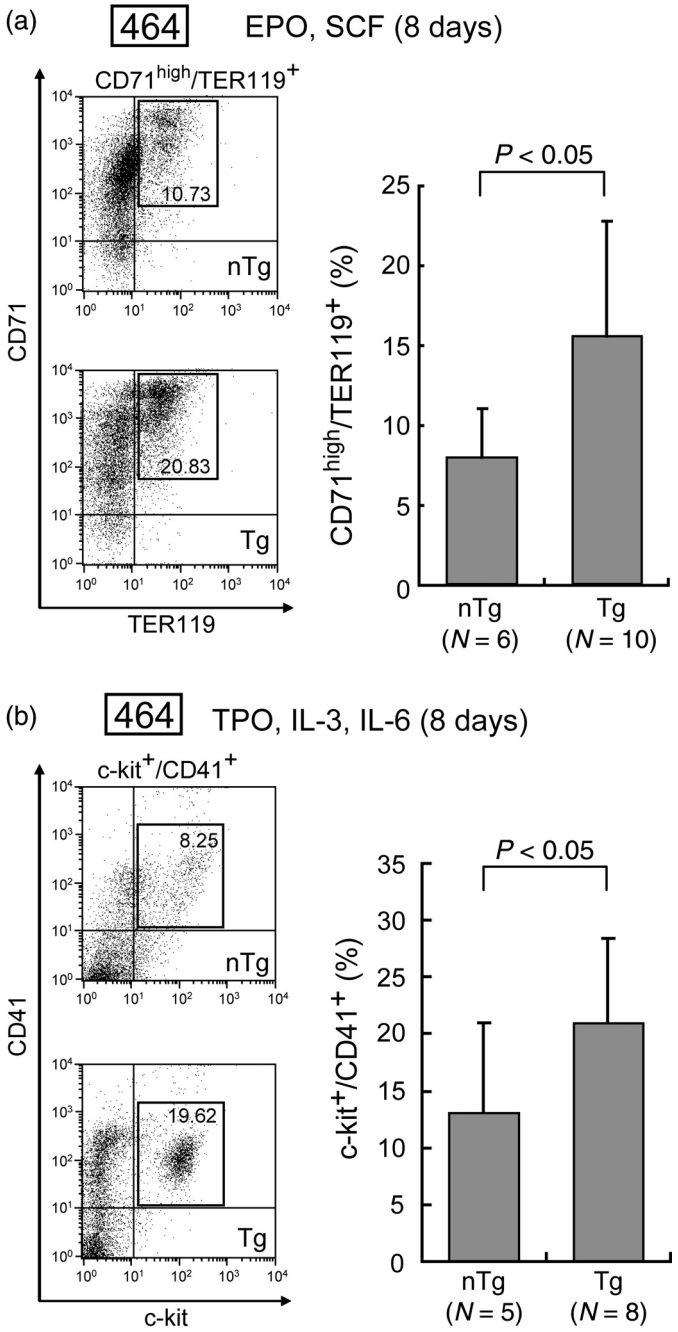


Fig. 2. Differentiation of bone marrow cells into erythroid and megakaryocytic precursors. Bone marrow cells were extracted and cultured in the presence of recombinant murine (a) erythropoietin (EPO) (3 U/mL) and Stem cell factor (SCF) (50 ng/mL), or (b) thrombopoietin (TPO) (20 ng/mL), interleukin (IL)-3 (10 ng/mL) and IL-6 (10 ng/mL). Cells were examined after 8 days of culture by fluorescence-activated cell sorter, which revealed that bone marrow cells obtained from *GATA1-TEL* transgenic mice showed higher populations of (a) CD71^{high}/TER119^{+} cells, or (b) c-kit^{+}/CD41^{+} cells compared to those from littermate controls. In the left panels, the representative data from non-transgenic (nTg) and transgenic (Tg) mice of line 464 are shown. In the right panel, indicated are average and standard deviation of five (a) or four (b) independent experiments using lines 460, 462 and 464. Numbers in parenthesis indicate numbers of mice analyzed in each group. Tg, *GATA1-TEL* transgenic mouse; nTg, non-transgenic littermate.

presence of EPO and SCF for 7 days, CD71^{high}/TER119^{+} population, corresponding to proerythroblast to basophilic erythroblast[35] expanded more efficiently from the bone marrow cells of *GATA1-TEL* transgenic mice compared to those of littermate controls (Fig. 2a). In addition, c-kit^{+}/CD41^{+} population was also obtained more abundantly in the transgenic mice following 7 days of culture with TPO, IL-3 and IL-6 (Fig. 2b).

The expression levels of *Alas-e* and *β-major globin* genes are higher in CD71high/TER119^{+} erythroblast of transgenic mice than littermate controls. Given that *GATA1-TEL* transgenic bone marrow cells gave rise to more CD71^{high}/TER119^{+} erythroblast upon stimulation with EPO, *Gata1*-driven *TEL* expression might alter proliferation and/or differentiation abilities of immature erythroid progenitors. To find out the molecular basis, bone marrow cells were separated into three populations according to the expression levels of CD71 and TER119 (Fig. 3A-a,b,c), and expression of genes related to erythroid proliferation/differentiation was examined by quantitative PCR. The most differentiated erythroid population in the panel is represented as CD71^{-}/TER119^{+} (Fig. 3A-c), whereas the CD71^{high}/TER119^{+} population (Fig. 3A-b) contains more immature but erythroid-committed progenitors, which are derived from the CD71^{int}/TER119^{-} population (Fig. 3A-a) consisting of not only erythroid-committed progenitors but also other lineages-committed progenitors such as myeloid cell and megakaryocyte. CD71^{int}/TER119^{-} population was positive for c-kit, and gave rise to both myeloid and erythroid colonies (data not shown). The proportions of these three populations were comparable between *GATA1-TEL* transgenic mice and their littermates (data not shown).

Expression of endogenous *Gata1* existed in the CD71^{int}/TER119^{-} population at a low level, and was then highly induced to a maximum level at the CD71^{high}/TER119^{+} stage in both transgenic and non-transgenic mice (Table 2 and Fig. 3B). Corresponding to this *Gata1* expression, total expression of endogenous + exogenous *TEL* gene was maintained at a relatively high level at the CD71^{high}/TER119^{+} stage in the *GATA1-TEL* transgenic bone marrow cells, showing a striking contrast to the control cells in which endogenous *Tel* gene was markedly down-regulated to the lowest level at this stage (Table 2). This suggested that exogenous *TEL* expression overlaid endogenous *Tel* expression at this stage in the transgenic mice. Then, expression levels of the genes that are involved in erythropoiesis were examined in these three populations (Table 2). As a result, higher expression of *Alas-e* and *β-major globin* genes was constantly observed in the CD71^{high}/TER119^{+} cells of *GATA1-TEL* transgenic mice than control mice (the former with a statistical significance but the latter without; Table 2 and Fig. 3C). In addition, expression of *erythroid Kruppel-like factor* (*Eklf*) was higher in *GATA1-TEL* transgenic mice at the stage of CD71^{int}/TER119^{-} population, but without a statistical significance. There was no difference in expression levels of *Fli1*, *stem cell leukemia* (*Scl*) and other hematopoietic transcription factor-encoding genes as well as *EPO receptor* (*Epor*) gene between transgenic mice and littermate controls.

Because *GATA1-TEL* transgenic bone marrow cells produced a more abundant population of megakaryocytic progenitors (c-kit^{+}/CD41^{+}), we also separated c-kit^{+}/CD41^{+} populations from bone marrow cells of *GATA1-TEL* transgenic mice and their littermates, and the expressions of endogenous *Gata1* and *endogenous + exogenous TEL* genes in this population were examined by quantitative PCR. *Gata1* mRNAs were abundantly expressed at comparable levels in both types of mice, and the expression of *endogenous + exogenous TEL* gene was higher in *GATA1-TEL* transgenic mice with a statistical significance, as expected (Supporting Fig. S1). Thus, exogenous *TEL* expression might support expansion of c-kit^{+}/CD41^{+} megakaryocytic progenitors *in vitro*.

Generation of *Gata1-TEL*-expressing ES cells. To analyze the effects of TEL in early erythropoiesis, ES cells in which human *TEL*

doi: 10.1111/j.1349-7006.2009.01097.x

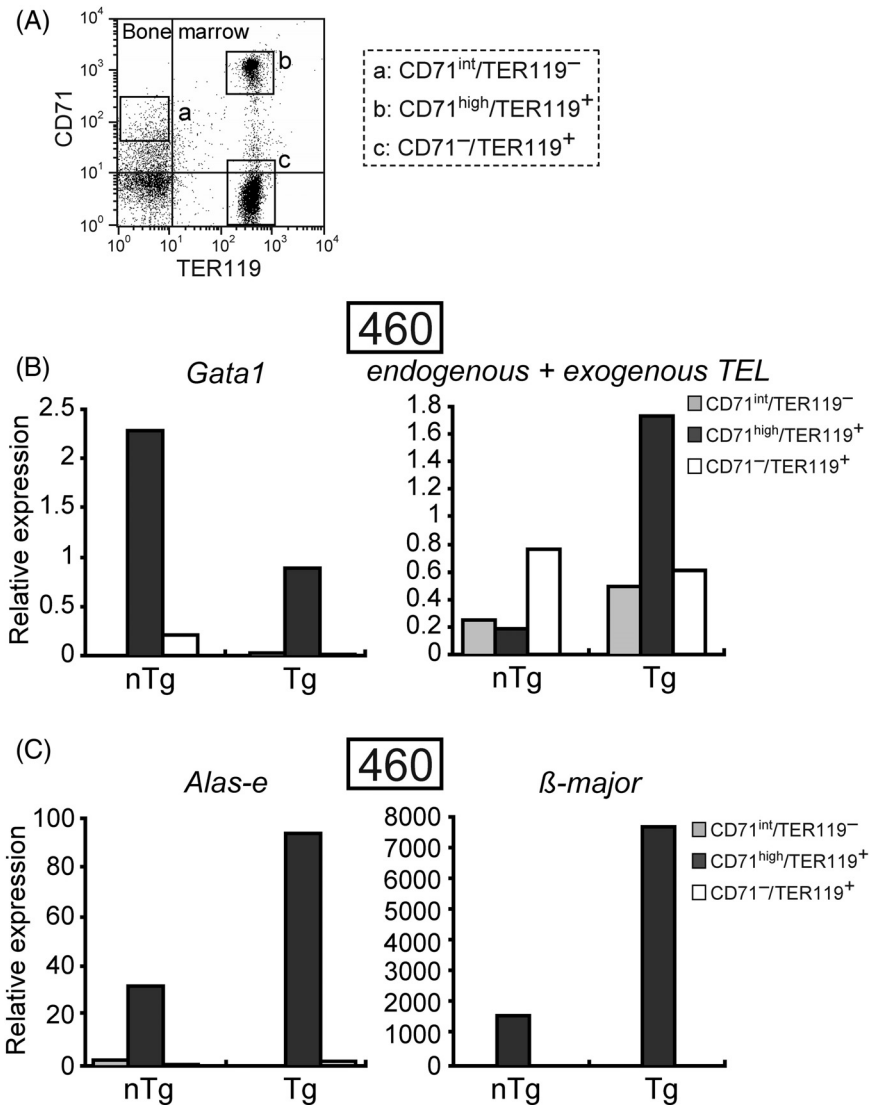
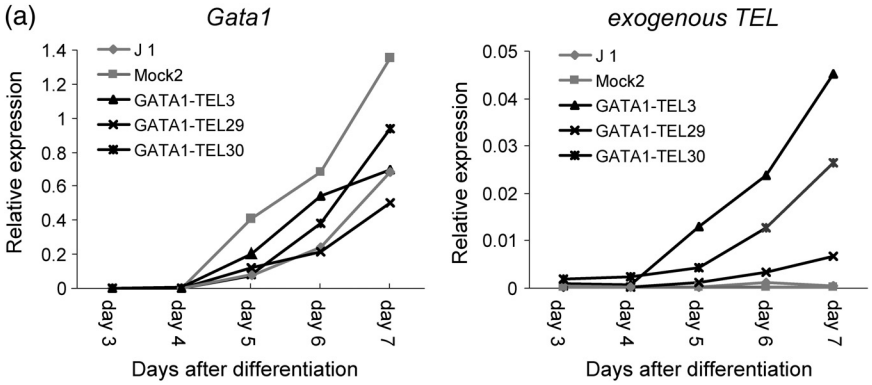


Fig. 3. Quantitative PCR of the genes involved in erythropoiesis. (A) To compare the expression of erythroid-related genes between *GATA1-TEL* transgenic mice and control littermates, bone marrow cells were sorted for CD71^{int}/TER119^{-} (a), CD71^{high}/TER119^{+} (b) and CD71^{-}/TER119^{+} (c), representing different stages of erythroid differentiation, and then subjected to quantitative PCR analysis. The result of FACS analysis shown in Fig. 3 A came from a non-transgenic litter mouse. There was no difference in the expression pattern of each population between non-transgenic and transgenic mice. (B) Representative results of quantitative PCR for endogenous *Gata1* and endogenous + exogenous *TEL* in each stage of erythroid differentiation from animals of line 460. The highest expression of *TEL* gene was obtained in CD71^{high}/TER119^{+} population in the *GATA1-TEL* transgenic mice, in concordance with the highest expression of endogenous *Gata1* among the three populations. (C) Representative results of quantitative PCR for *Alas-e* and *β-major globin* genes from animals of line 460. Tg, *GATA1-TEL* transgenic mouse; nTg, non-transgenic littermate.

Table 2. Quantitative analysis of transcripts expressed in different stages of erythropoiesis

	CD71^{int}/TER119^{-}		CD71^{high}/TER119^{+}		CD71^{-}/TER119^{+}	
	nTg	Tg	nTg	Tg	nTg	Tg
Gata1	0.05 ± 0.05	0.63 ± 0.62	3.40 ± 2.41	1.46 ± 0.78	0.54 ± 0.29	0.57 ± 0.71
hmTEL	0.76 ± 0.67	1.73 ± 1.48	0.18 ± 0.05	1.20 ± 0.65*	1.19 ± 0.73	0.93 ± 0.48
Gata2	0.13 ± 0.08	0.90 ± 1.10	0.04 ± 0.01	0.22 ± 0.32	0.44 ± 0.36	0.41 ± 0.45
Runx1	0.38 ± 0.29	0.93 ± 0.71	0.47 ± 0.15	0.57 ± 0.34	0.79 ± 0.26	0.91 ± 0.20
Scl	0.06 ± 0.02	0.12 ± 0.09	1.79 ± 0.60	1.56 ± 0.75	0.12 ± 0.18	0.19 ± 0.31
Fli1	1.13 ± 1.01	4.32 ± 7.39	0.19 ± 0.01	5.77 ± 10.16	2.20 ± 0.98	3.31 ± 3.51
Eklf	0.11 ± 0.07	0.64 ± 0.52	10.6 ± 3.26	6.52 ± 4.53	1.39 ± 1.80	0.56 ± 0.12
Epor	0.19 ± 0.11	0.11 ± 0.11	1.89 ± 0.73	2.14 ± 1.05	0.73 ± 1.06	0.05 ± 0.06
β-major globin	2.10 ± 2.38	18.0 ± 5.80	2804 ± 1970	6730 ± 4775†	298 ± 288	83.3 ± 78.9
Alas-e	1.92 ± 1.36	1.01 ± 0.67	46.3 ± 12.2	143 ± 36**	10.7 ± 11.6	3.62 ± 1.97

Every numerical value indicates fold difference relative to hypoxanthine-guanine phosphoribosyltransferase (HPRT) calculated by $2^{-(\Delta CT)}$.
(Δ cycle of threshold (CT), mean CT of indicated gene – mean CT of HPRT)
Average and standard deviation from two mice of line 460 and one mouse from 462 are shown.
*Significantly higher compared to control ($P < 0.01$).
**Significantly higher compared to control ($P < 0.003$).
†There is no significant difference though higher expression levels were observed in transgenic mice compared to controls in each experiment.
Tg, *GATA1-TEL* transgenic mouse; nTg, non-transgenic littermate.



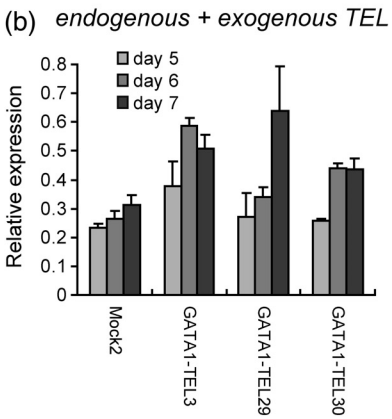


Fig. 4. Expression of *Gata1* and *TEL* during differentiation of embryonic stem (ES) cells. (a) Undifferentiated original J1, mock-transfected (Mock2) and *Gata1-TEL*-overexpressing (GATA1-TEL3, 29 and 30) ES cells were deprived of leukemia inhibitory factor to initiate differentiation and analyzed for the expression of endogenous *Gata1* gene and *TEL* transgene under the control of *Gata1* IE3.9int promoter. Expression of *TEL* transgene was observed from day 5 of differentiation in concordance with the expression of endogenous *Gata1* gene. (b) Total amount of *TEL* transcript (endogenous + exogenous) in differentiating embryoid body (EB) cells. Mouse and human *TEL* transcripts were simultaneously amplified as described in Materials and methods using primers TEL-829f and TEL-921r located on exons V and VI of mouse and human *TEL* gene. Average and standard deviation of two independent experiments are shown. After day 6 of differentiation, total amount of *TEL* was higher in *GATA1-TEL* EBs than in mock EBs.

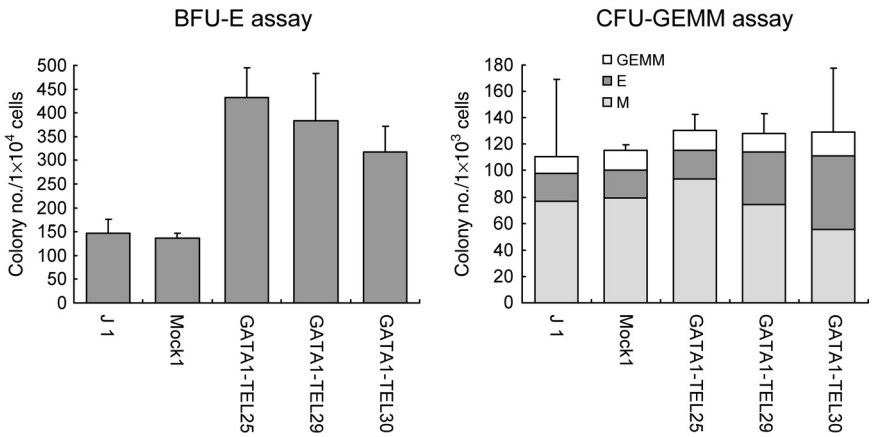


Fig. 5. Enhanced erythroid colony formation in *GATA1-TEL* embryoid body (EB) cells. Undifferentiated J1, mock-transfected (Mock1) and *Gata1-TEL*-overexpressing (GATA1-TEL25, 29 and 30) embryonic stem cells were deprived of leukemia inhibitory factor to form differentiated EBs. EBs at day 7 of differentiation were collected and subjected to BFU-E (supplemented with SCF, thrombopoietin [TPO] and erythropoietin [EPO]) and CFU-GEMM (supplemented with SCF, TPO, EPO, interleukin [IL]-11, IL-3, GM-CSF, G-CSF, M-CSF and IL-6) assays. Average and standard deviation of at least two independent experiments are shown. *Gata1-TEL*-expressing EB cells showed higher BFU-E activity than controls, while no difference was observed in CFU-GEMM activity. GEMM, mixed colony; E, erythroid colony; M, myeloid colony.

gene is induced under the control of IE3.9int *Gata1* promoter (*GATA1-TEL* ES) were established. The ES cells were maintained and differentiated into hematopoietic cells as described previously.[36] When expression of *Gata1* and *TEL* was examined during differentiation of ES cells by quantitative PCR analysis, endogenous *Gata1* transcript began to increase from day 5 of removal of LIF, which gradually increased afterwards (Fig. 4a), possibly due to an increment of erythroid-committed cells in the whole cell population. There was no statistical difference in the amount of endogenous *Gata1* mRNA between *GATA1-TEL* ES cells and control cells during days 5–7 of EB culture. Exogenous *TEL* gene expression from the integrated *GATA1-TEL* vector showed precisely a similar pattern to endogenous *Gata1* expression, starting to express around day 5 of differentiation and gradually increasing afterwards. In *GATA1-TEL* ES cells, total amount of endogenous + exogenous *TEL* transcript was higher compared to control ES cells after day 6 of differentiation (Fig. 4b).

Higher erythroid activity of *Gata1-TEL*-expressing ES cells. Day 7 EBs following removal of LIF were subjected to CFC assay. Erythroid colony-forming activity (BFU-E) of *GATA1-TEL* EBs was significantly higher than that of control EBs, while there was no difference observed in the activity of multipotential progenitors (CFU-GEMM) between them (Fig. 5). This result indicated that *GATA1-TEL* EB cells might have an increased number of erythroid-committed progenitors or be more prone to commit to the erythroid lineage at day 7 of differentiation.

Day 6 EB-derived c-kit$^+$/CD71$^+$ cells efficiently differentiated into CD71^{high}/TER119$^+$ erythroid precursors on OP9. Erythroid differentiation of *GATA1-TEL* and control ES cells was also assessed by coculture with OP9 as described previously with some modifications.[37] Day 7 EBs were replated onto OP9 stromal

doi: 10.1111/j.1349-7006.2009.01097.x

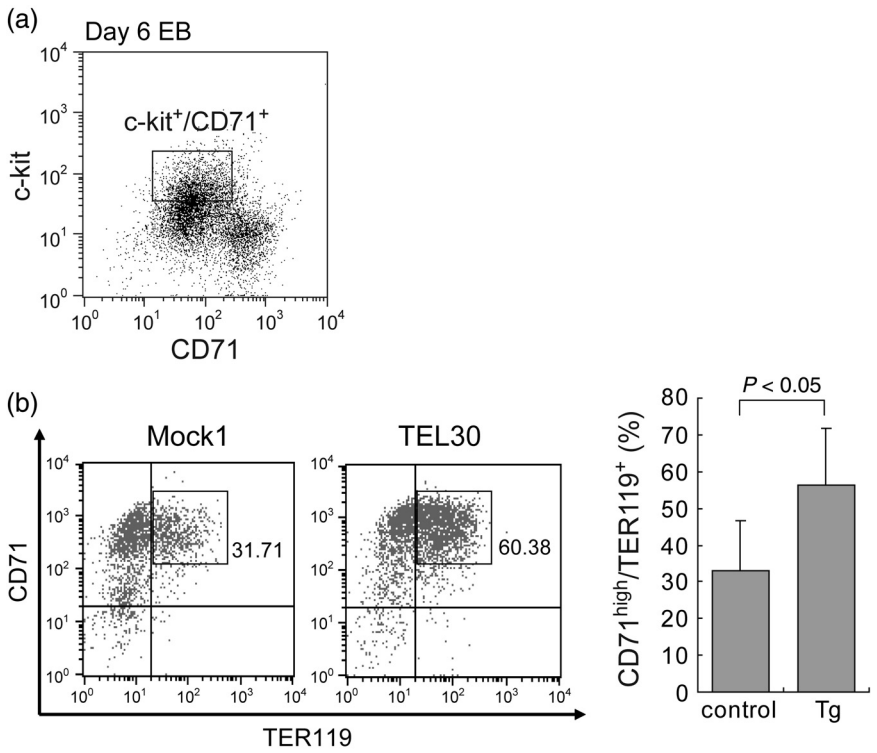


Fig. 6. *In vitro* erythroid differentiation of embryoid body (EB)-derived c-kit$^+$/CD71$^+$ cells. Undifferentiated embryonic stem cells were deprived of leukemia inhibitory factor to form differentiated EBs. After 6 days of differentiation, c-kit$^+$/CD71$^+$ cells (shown in panel a) were separated by fluorescence-activated cell sorter (FACS) and subjected to erythroid differentiation assay on OP9 stromal cell layer. The result of FACS analysis shown in (a) came from non-transgenic cells. There was no difference in the population of EB-derived c-kit$^+$/CD71$^+$ cells between non-transgenic and transgenic cells. (b) The CD71^{high}/TER119$^+$ fraction after 8 days of culture with erythropoietin and SCF on OP9 layer. In the left panel, the representative data of mock-transfected (Mock1) and GATA1-TEL30 are shown. In the right panel, average and standard deviation of control and transgenic (Tg) cells are shown. The results of control and Tg are derived from the combined data in at least two independent experiments of J1, Mock1 and 2, and GATA1-TEL3, 25 and 30, respectively. Day 6 transgenic EB-derived c-kit$^+$/CD71$^+$ cells produced higher numbers of CD71^{high}/TER119$^+$ cells compared to the controls.

layer and cultured for another 8 days supplemented with EPO and SCF. Erythroid differentiation was then assessed by FACS analysis. *GATA1-TEL* and control EB cells produced comparable amounts of the CD71^{high}/TER119$^+$ erythroid progenitor population (data not shown). This result indicated the possibility that usage of whole EB cells avoided detecting increased abilities of transgenic erythroid progenitors to expand, and/or that timing could be earlier when transgenic erythroid progenitors in EBs showed increased abilities.

Erythroid differentiation of ES cells is considered to begin around day 5 corresponding to the initiation of expression of a key transcription factor, Gata1. Generally, when immature EBs differentiate into the erythroid lineage, increment of CD71 expression as well as loss of c-kit expression is observed. To figure out the effect of *TEL* transgene in immature hematopoietic progenitors, day 6 EB-derived c-kit$^+$/CD71$^+$ cells, which are considered to have multipotential in hematopoietic differentiation, were separated by FACS and subjected to short-term culture on OP9 stroma with EPO and SCF (Fig. 6a). There was no difference in the amount of day 6 EB-derived c-kit$^+$/CD71$^+$ populations between non-transgenic and transgenic EBs (data not shown). After 8 days of coculture with OP9, almost all EB-derived cells were differentiated to the erythroid lineage, showing a high level of CD71 expression (Fig. 6b). The population of CD71^{high}/TER119$^+$ cells, which are erythroid-committed and equivalent to proerythroblast, was significantly higher in the *GATA1-TEL* EB-derived c-kit$^+$/CD71$^+$ cells compared to the controls.

Discussion

For the purpose of investigating TEL's functions in erythropoiesis, we in this study generated transgenic mice and ES cells expressing human *TEL* under the control of erythroid-specific *Gata1* promoter. Each system could have highlighted different aspects of TEL's roles in *Gata1*-expressing cells. We have divulged two roles of the transcription factor in erythropoiesis; one is the expansion of immature erythroid precursor and the other is the augmentation of Hb accumulation. Thus, we conclude that TEL affects proliferation and differentiation of erythroid-committed cells by distinctive mechanisms.

We precisely studied the expression levels of endogenous *Gata1*, and endogenous and exogenous (*Gata1* promoter-driven) *TEL* transcripts during the progression of erythroid differentiation by fractionating CD71^{int}/TER119$^-$, CD71^{high}/TER119$^+$ and CD71$^-$/TER119$^+$ populations in the bone marrow of non-transgenic and transgenic mice. In both types of mice, endogenous *Gata1* transcripts were induced with the highest level in the CD71^{high}/TER119$^+$ cells belonging to the stage of proerythroblast[38] and then markedly declined afterwards, which represents essential functions of Gata1 to activate transcription of *globin* and heme biosynthetic genes. On the other hand, expressional changes of endogenous *Tel.* transcripts in a physiological setting of erythroid differentiation have not as yet been described. The endogenous TEL expression in the non-transgenic mice was found to be low in the CD71^{high}/TER119$^+$ population, while relatively high in both the CD71^{int}/TER119$^-$ and CD71$^-$/TER119$^+$ populations (Table 2), for which we could not uncover a biological meaning at this moment. Considering that the exogenous expression in the transgenic mice was up-regulated at the CD71^{high}/TER119$^+$ proerythroblast stage, consistent with the highest expression of endogenous *Gata1* at this stage, we can conclude that our transgenic system successfully led to overexpression of exogenous *TEL* transcript in the *Gata1*-expressing cells. In addition, c-kit$^+$/CD41$^+$ megakaryocytic progenitors also highly expressed endogenous *Gata1* gene and the transgenic c-kit$^+$/CD41$^+$ cells showed high expression of exogenous *TEL* gene as well.

This transgenic event caused several differences between the transgenic and control mice. One is further up-regulation of *Alas-e* and *β-globin* genes in the CD71^{high}/TER119$^+$ population of *GATA1-TEL* transgenic mice. These data indicate that TEL directly or indirectly exaggerates the transcription of genes involved in Hb synthesis. In *GATA1-TEL* transgenic mice, the level of Hb concentration in the peripheral blood was higher, and with a statistical significance. We have previously reported by evaluating Hb accumulation with benzidine staining that overexpressed *TEL* stimulates erythroid differentiation in UT7/GM and MEL

cells.[13,14] In both the cell lines, expressional levels of *β-globin* and *ALAS-E* mRNAs were higher in the *TEL*-overexpressing cells. These previous data are consistent with those observed here in the CD71^{high}/TER119^{+} population of bone marrow cells in the *GATA1-TEL* transgenic mice. The transgenic mice expressing deletion mutants of TEL that lack the Pointed or the ETS domain did not show any alterations in Hb concentration (data not shown). Because these deletions abolish major molecular functions of TEL as a transcription factor, TEL appears to reinforce Hb synthesis through transcriptional regulation at the CD71^{high}/TER119^{+} stage. Considering that endogenous *Gata1* expression in the erythroid fraction of transgenic bone marrow cells was not increased compared to that of non-transgenic cells, it could not be plausible that TEL up-regulates the transcription of *Gata1* gene itself. Although we do not have any evidence that TEL and GATA1 physically associate with each other, functions of each molecule may cross-talk in the transcriptional regulation of *β-globin* and *ALAS-E* genes. ETS-binding consensus sequences are not found in the promoter region of *Alas-e* gene, suggesting that TEL works indirectly to stimulate transactivation of the gene. On the other hand, because *β-globin* gene contains an ETS-binding consensus sequence (GGAA/T) in its promoter region, TEL might directly activate the expression of *β-globin* gene, although TEL is currently known only as a transcriptional repressor. Notably, the expression of *Eklf* that activates the promoter of *β-globin* gene[39] was higher at the immature CD71^{int}/TER119^{-} stage in the transgenic mice, which may also partly have contributed to the up-regulation of *β-globin* gene in the CD71^{high}/TER119^{+} stage.

Enforced *TEL* expression in transgenic mice not only caused accelerated Hb accumulation but also expanded the immature progenitor at the earlier stage where the expression of endogenous *Gata1 has* not been fully activated yet. When cultured in the presence of EPO and TPO, transgenic bone marrow cells produced a more abundant population of CD71^{high}/TER119^{+} (erythroid-committed) and c-kit^{+}/CD41^{+} (megakaryocyte-committed) cells than control cells, respectively. This observation suggests a stimulatory function of TEL in propagating immature erythroid progeny and possibly erythrocyte/megakaryocyte common progenitors that can make a commitment to either of the erythroid or megakaryocytic lineage. Although the levels of *Epor* transcript in *TEL*-expressing CD71^{high}/TER119^{+} cells were comparable to those in controls, we could not deny the possibility that TEL affects intracellular EPO signals. The molecular mechanisms underpinning TEL's functions in expansion of immature erythroid precursor remain unknown. On the other hand, we at this moment cannot discuss the exact reason that exogenous *TEL* expression led to *in vitro* expansion of megakaryocytic progenitor in the presence of TPO, but did not cause an increased production of platelets in mice. However, considering that overexpressed TEL accelerates erythroid differentiation but inhibits megakaryocytic maturation in UT7/GM cells,[14] TEL may preferably drive the erythroid commitment in erythrocyte/megakaryocyte common progenitors also in mice and its overexpression may not result in higher production of platelets.

We also took advantage of *in vitro* differentiation of ES cells to clarify TEL's role in early hematopoiesis. The expressions of endogenous *Gata1* and exogenous *TEL* concomitantly commenced at day 5 of EB culture in differentiation media without LIF, and gradually increased together subsequently. We found that total levels of endogenous + exogenous *TEL* transcripts were higher at day 6 or 7 in the *GATA1-TEL* transgenic EB cells than in control cells. Interestingly, when assayed day 7 EB-derived cells on methylcellulose, numbers of BFU-E colonies derived from *GATA1-TEL* transgenic EB cells revealed a significant increase compared to those from control cells. In the liquid culture on OP9 cells in the presence of EPO and SCF, c-kit^{+}/CD71^{+} cells sorted from day 6 transgenic EB produced a more abundant population of erythroid-committed CD71^{high}/TER119^{+} cells than non-transgenic control cells. These observations also argue the function of TEL in expanding erythroid progenitor or accelerating definitive erythroid commitment.

In summary, we verify two compelling functions of TEL exerted at the different stages of erythroid differentiation. At the earliest stage of erythroid differentiation, TEL could proliferate erythrocyte/megakaryocyte common progenitors and/or favor growth of the erythroid lineage-committed cells. At the late stage of differentiation, TEL can accelerate terminal erythroid differentiation through stimulating Hb synthesis. Although TEL is not essential for erythropoiesis in the fetus and adult mice, TEL could be activated under the condition of hematopoietic stresses such as anemia and hypo-oxygenemia. We currently have observed no difference between non-transgenic and transgenic mice in recovery of Hb levels after bleeding experiments. Further analyses with different strategies to induce hematopoietic stress are required to address this issue. Finally, to clarify precise mechanisms for TEL to promote the propagation of erythroid progenitor, unknown downstream target genes of TEL that could be critical in the erythroid commitment and proliferation, are under investigation in our laboratory using comprehensive microarray systems.

Acknowledgments

This work was financially supported by the Grants-in-Aid from the Ministries in Japan of Education, Culture, Sports, Science and Technology (17016068), and Health, Labour and Welfare, and Japanese Society for the Promotion of Science (17659295, 17390283, 18591085, 19591135).

The authors thank Professor T. Nakano (Department of Pathology, Medical School and Graduate School of Frontier Biosciences, Osaka University) for kindly providing us with OP9 cells. We also thank Kirin Brewery Co Ltd. for kindly supplying TPO for this project and Ms. Yuki Shinozaki for technical support.

References

1 Mavrothalassitis G, Ghysdael J. Proteins of the ETS family with transcriptional repressor activity. *Oncogene* 2000; **19**: 6524–32.
2 Bohlander SK. ETV6: a versatile player in leukemogenesis. *Semin Cancer Biol* 2005; **15**: 162–74.
3 Golub TR, Barker GF, Lovett M, Gilliland DG. Fusion of PDGF receptor beta to a novel ets-like gene, Tel., in chronic myelomonocytic leukemia with t (5;12) chromosomal translocation. *Cell* 1994; **77**: 307–16.
4 Kim CA, Phillips ML, Kim W *et al*. Polymerization of the SAM domain of TEL in leukemogenesis and transcriptional repression. *Embo J* 2001; **20**: 4173–82.
5 Hiebert SW, Sun W, Davis JN *et al*. The t(12;21) translocation converts AML-1B from an activator to a repressor of transcription. *Mol Cell Biol* 1996; **16**: 1349–55.
6 Chakrabarti SR, Nucifora G. The leukemia-associated gene TEL encodes a transcription repressor which associates with SMRT and mSin3A. *Biochem Biophys Res Commun* 1999; **264**: 871–7.
7 Lopez RG, Carron C, Oury C, Gardellin P, Bernard O, Ghysdael J. TEL is a sequence-specific transcriptional repressor. *J Biol Chem* 1999; **274**: 30132–8.
8 Guidez F, Petrie K, Ford AM *et al*. Recruitment of the nuclear receptor corepressor N-CoR by the TEL moiety of the childhood leukemia-associated TEL-AML1 oncoprotein. *Blood* 2000; **96**: 2557–61.
9 Wang L, Hiebert SW. TEL contacts multiple co-repressors and specifically associates with histone deacetylase-3. *Oncogene* 2001; **20**: 3716–25.
10 Wang LC, Kuo F, Fujiwara Y, Gilliland DG, Golub TR, Orkin SH. Yolk sac angiogenic defect and intra-embryonic apoptosis in mice lacking the Ets-related factor TEL. *Embo J* 1997; **16**: 4374–83.
11 Wang LC, Swat W, Fujiwara Y *et al*. The TEL/ETV6 gene is required specifically for hematopoiesis in the bone marrow. *Genes Dev* 1998; **12**: 2392–402.
12 Hock H, Meade E, Medeiros S *et al*. Tel/Etv6 is an essential and selective regulator of adult hematopoietic stem cell survival. *Genes Dev* 2004; **18**: 2336–41.
13 Waga K, Nakamura Y, Maki K *et al*. Leukemia-related transcription factor TEL accelerates differentiation of Friend erythroleukemia cells. *Oncogene* 2003; **22**: 59–68.

doi: 10.1111/j.1349-7006.2009.01097.x

14 Takahashi W, Sasaki K, Kvomatsu N, Mitani K. TEL/ETV6 accelerates erythroid differentiation and inhibits megakaryocytic maturation in a human leukemia cell line UT-7/GM. *Cancer Sci* 2005; **96**: 340–8.
15 Yamamoto M, Takahashi S, Onodera K, Muraosa Y, Engel JD. Upstream and downstream of erythroid transcription factor GATA-1. *Genes Cells* 1997; **2**: 107–15.
16 Ohneda K, Yamamoto M. Roles of hematopoietic transcription factors GATA-1 and GATA-2 in the development of red blood cell lineage. *Acta Haematol* 2002; **108**: 237–45.
17 Crispino JD. GATA1 in normal and malignant hematopoiesis. *Semin Cell Dev Biol* 2005; **16**: 137–47.
18 Migliaccio AR, Rana RA, Vannucchi AM, Manzoli FA. Role of GATA-1 in normal and neoplastic hemopoiesis. *Ann N Y Acad Sci* 2005; **1044**: 142–58.
19 Pan X, Ohneda O, Ohneda K *et al*. Graded levels of GATA-1 expression modulate survival, proliferation, and differentiation of erythroid progenitors. *J Biol Chem* 2005; **280**: 22385–94.
20 Shimizu R, Yamamoto M. Gene expression regulation and domain function of hematopoietic GATA factors. *Semin Cell Dev Biol* 2005; **16**: 129–36.
21 Pevny L, Simon MC, Robertson E *et al*. Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* 1991; **349**: 257–60.
22 Fujiwara Y, Browne CP, Cunniff K, Goff SC, Orkin SH. Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1. *Proc Natl Acad Sci USA* 1996; **93**: 12355–8.
23 Takahashi S, Onodera K, Motohashi H *et al*. Arrest in primitive erythroid cell development caused by promoter-specific disruption of the GATA-1 gene. *J Biol Chem* 1997; **272**: 12611–15.
24 Weiss MJ, Keller G, Orkin SH. Novel insights into erythroid development revealed through in vitro differentiation of GATA-1 embryonic stem cells. *Genes Dev* 1994; **8**: 1184–97.
25 Suwabe N, Takahashi S, Nakano T, Yamamoto M. GATA-1 regulates growth and differentiation of definitive erythroid lineage cells during in vitro ES cell differentiation. *Blood* 1998; **92**: 4108–18.
26 Cheng T, Shen H, Giokas D, Gere J, Tenen DG, Scadden DT. Temporal mapping of gene expression levels during the differentiation of individual primary hematopoietic cells. *Proc Natl Acad Sci USA* 1996; **93**: 13158–63.
27 Shivdasani RA, Orkin SH. The transcriptional control of hematopoiesis. *Blood* 1996; **87**: 4025–39.
28 Nerlov C, Graf TPU. 1 induces myeloid lineage commitment in multipotent hematopoietic progenitors. *Genes Dev* 1998; **12**: 2403–12.
29 Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 2000; **404**: 193–7.
30 Onodera K, Takahashi S, Nishimura S *et al*. GATA-1 transcription is controlled by distinct regulatory mechanisms during primitive and definitive erythropoiesis. *Proc Natl Acad Sci USA* 1997; **94**: 4487–92.
31 Kobayashi M, Yamamoto M. Regulation of GATA1 gene expression. *J Biochem* 2007; **142**: 1–10.
32 Vandesompele J, De Preter K, Pattyn F *et al*. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; **3**: RESEARCH0034.
33 Nakano T, Kodama H, Honjo T. Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science* 1994; **265**: 1098–101.
34 Nakano T, Kodama H, Honjo T. *In vitro* development of primitive and definitive erythrocytes from different precursors. *Science* 1996; **272**: 722–4.
35 Zhang J, Socolovsky M, Gross AW, Lodish HF. Role of Ras signaling in erythroid differentiation of mouse fetal liver cells: functional analysis by a flow cytometry-based novel culture system. *Blood* 2003; **102**: 3938–46.
36 Robertson SM, Kennedy M, Shannon JM, Keller G. A transitional stage in the commitment of mesoderm to hematopoiesis requiring the transcription factor SCL/tal-1. *Development* 2000; **127**: 2447–59.
37 Kitajima K, Tanaka M, Zheng J, Sakai-Ogawa E, Nakano T. In vitro differentiation of mouse embryonic stem cells to hematopoietic cells on an OP9 stromal cell monolayer. *Meth Enzymol* 2003; **365**: 72–83.
38 Suzuki N, Suwabe N, Ohneda O *et al*. Identification and characterization of 2 types of erythroid progenitors that express GATA-1 at distinct levels. *Blood* 2003; **102**: 3575–83.
39 Bieker JJ. Probing the onset and regulation of erythroid cell-specific gene expression. *Mt Sinai J Med* 2005; **72**: 333–8.

Supporting Information

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

Fig. S1. Quantitative PCR of the *Gata1* and *TEL* genes expressed in megakaryocytic progenitors.

Table S1. Sequences of primers used for polymerase chain reaction (PCR) and reverse transcription (RT-PCR).

Table S2. Sequences of primers used for quantitative polymerase chain reaction.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.