厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

骨髄異形成症候群のCD34 陽性細胞に発現する蛋白の同定

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研究要旨
プロテオミクス解析の手法を用いて骨髄異形成症候群(myelodysplastic syndromes:MDS)の幹細胞/前駆細胞レベルの細胞（CD34 陽性細胞）に存在する蛋白の網羅的な同定を試みた。その結果、不応性貧血（RA）の CD34 陽性細胞おいて、正常 CD34 陽性細胞と比較して有意に発現量が変化している蛋白スポットが認められた。それらの中には複数症例で共通なものが存在した。また、同一症例における CD34 陽性細胞由来蛋白の経時的検討の結果、RA から RA with excess blasts(RAEB)、あるいは RA から overt leukemia への移行に伴い、有意に発現量が変化している蛋白スポットが認められた。これらの蛋白は MDS の進行に関連している可能性があると思われる。これらのスポットについて質量分析を行い、RA に高発現する蛋白および RAEB に高発現する蛋白を同定した。これらの蛋白は本症の各病型の病因、病態に深く関わっていると考えられ、現在引き続き蛋白の同定および解析を進めている。

A．研究目的

本研究はMDS の各病型に特異的な、あるいは、MDS の進行に伴う特異的な MDS 血球の分子生物学的変化を CD34 陽性細胞のプロテオーム解析により、明らかにすることを目的とした。MDS はヘテロな疾患群であるので、MDS の進行に伴う特異的な分子生物学的変化を明らかにするためには、個々の症例における MDS 血球の経時的変化を解析する必要があると考えられる。すなわち、例えば不応性貧血（RA）から白血病（overt leukemia）に移行した症例で RA および overt leukemia 移行時の CD34 陽性細胞のプロテオーム解析を行うことにより、病型の進行に関わる蛋白を同定することができると考えられる。
このような MDS の進行に伴う蛋白の変化を CD34 陽性細胞レベルで解析することは極めて難しいと考えられてきた。その理由は2つある。
1つは、MDS の進行を認めた患者の進行前、進行後のサンプルを収集するためには、それなりの年月を要することである。他の1つは、RA の病型でプロテオーム解析に必要な量の CD34 陽性細胞を収集することは極めて難しいと考えられるからである。しかし本研究では、すでにMDS の進行を認めた患者の進行前および後の CD34 陽性細胞の収集が行われており、研究の遂行が可能であった。また、近年、Saturation dye と呼ばれる蛍光色素を用いて二次元ディファレンス電気泳動（2D-DIGE）を行うことにより、極めて微量（1 ゲル当たり5μg程度）の蛋白でも二次元電気泳動による蛋白解析が可能となっている。本研究ではこの方法を用いて、MDS 由来 CD34 陽性細胞のプロテオーム解析が可能であるかを明らかにすることも目的とした。

B．研究方法

MDS 患者の末梢血あるいは骨髄液から比重遠心法により単核球を分離し、イムノビーズ法を用いて CD34 陽性細胞を純化した。また、正常 CD34 陽性細胞についても同様な方法で純化した。その細胞から蛋白を抽出後、Saturation dye(GE Healthcare)を用いて蛋白を蛍光標識し、2D-DIGE を行った。1 次元目（等電点電気泳動）はIPG strip (PH 3-10 linear 18cm) を使用し、Multiphor II unit 装置(GE Healthcare)で泳動を行った。2 次元目は Ettan DALTsix Large Electrophoresis System（GE Healthcare）を用いて泳動を行った。泳動後、Image analyzer FLA-5000 (Fujifilm)を用いて蛍光スキャンを行い、蛋白スポットのデータを保存した。その後、ゲル上の蛋白スポットの蛋白発現量について画像解析ソフト(PDQuest: BIO-RAD)を用いて解析した。発現量に有意な変化を認めた蛋白スポットについて、ゲル内消化後、MALDI-TOF(Autoflex II TOF/TOF:BRUKER DALTONICS) を用いて質量分析を行い、当該蛋白を同定した。
(倫理面への配慮)
患者に対して本研究について説明した後，文書にて同意を得た上で検体の提供を受けた。

C．研究結果

ゲル上の蛋白スポットの蛋白発現量を解析した結果、RA のCD34 陽性細胞において、正常 CD34 陽性細胞と比較して有意に発現量が変化している蛋白スポットが認められた。それらの中には複数症例で共通なものが 20 スポット存在した。また、同一症例における CD34 陽性細胞由来蛋白の経時的検討の結果、RA から RA with excess blasts(RAEB)、あるいは RA から overt leukemia への移行に伴って、有意に発現量が変化している蛋白スポットが認められた。これらの有意差を認めたスポットについて質量分析を行ったところ、RA に高発現する蛋白を８個(NCBI データーベース, GI: 28872730, GI: 14249382

など)、RAEB に高発現する蛋白を 2 個 (GI: 5803187 など) 同定した。これらの蛋白は細胞内酵素、細胞骨格関連蛋白、リン脂質関連結合蛋白、プロテアソーム関連蛋白、遺伝子制御蛋白などであった。現在引き続き蛋白の同定および解析を進めている。

D．考察

MDS の中でも RA などでは収集できる CD34 陽性細胞数が極めて少ないため、二次元電気泳動による解析が極めて難しいと考えられるが、本研究の結果、Saturation dye を用いることにより、RA などの CD34 陽性細胞でもプロテオーム解析が可能であることが明らかになった。この手法を用いることにより、MDS の各病型の CD34 陽性細胞に特異的に存在する蛋白を網羅的に同定することができると考えられる。また、病型の進行に関わる特異的蛋白の同定も可能であると考えられる。
本研究により、正常 CD34 陽性細胞と比較して RA および RAEB において有意に発現量が変化している蛋白、MDS の進行に伴い有意に発現量が変化している蛋白の存在が明らかになった。また、質量分析により同定された蛋白は細胞内酵素、細胞骨格関連蛋白、リン脂質関連結合蛋白、プロテアソーム関連蛋白、遺伝子制御蛋白であった。それらはいずれも細胞の増殖、分化、機能に重要なものであることから、本症の病因、病態に深く関与している蛋白である可能性があると考えられる。これらの蛋白の中から、本症に対する新規分子標的治療の標的蛋白を見出すことができる可能性があると考える。

E．結論

MDS 由来 CD34 陽性細胞より抽出した蛋白のプロテオーム解析の結果、本症の各病型特異的に発現が変化している蛋白が存在した。それらの蛋白は MDS の病因・病態に関与しているものと考えられた。

F．健康危険情報

なし

G．研究発表

なし

H．知的財産権の出願・登録状況

1. 特許取得
2. 実用新案登録
3. その他
   いずれも予定なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

MDS の新規原因遺伝子 *CBL* 変異の同定と機能に関する研究

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研究要旨
高密度 SNP アレイを用いた 222 例の骨髄異形成症候群（MDS）のゲノムコピー数異常・アレル不均衡の解析により、全症例の約 30%に片親性二倍体(aUPD)が検出された。MDS において最も多くの症例で観察された 11 番染色体長腕(11q)aUPD の標的遺伝子として *CBL* 遺伝子変異を同定した。変異型 CBL は E3 ユビキチンリガーゼ活性が顕著に低下し、造血サイトカイン刺激後の受容体型チロシンキナーゼの活性化を遷延させることが、MDS/骨髄増殖性腫瘍（MPN）の発症に大きく関わっていると考えられた。また、治療上の観点からは恐らくチロシンキナーゼ活性の抑制が有用である可能性が示唆された。

A. 研究目的

骨髄異形成症候群(MDS)は高齢者に好発するが、現在高齢者に適した根治的治療がなく、また今後急速な少子高齢化による患者数の増加が危惧される。MDS では形態異常を伴う血球産生の異常から血球減少が共通に認められる一方、一部には急性骨髄性白血病(AML)への移行が認められるなど、多様な病態が含まれており、病態の理解と治療成績の向上の観点からは、MDS に含まれる多様な病態を明らかにした上で、個々の病態に即した分子標的療法を含む治療戦略を構築することが重要である。我々は、これまで行ってきた高密度 SNP アレイを用いた MDS の網羅的なゲノム解析において最も多くの症例で観察された 11 番染色体長腕 aUPD の標的遺伝子の同定と機能解析を行った。

B. 研究方法

解析に用いた MDS の SNP アレイデータは、昨年度までに解析を行った 171 例に 51 例を追加し、計 222 例より得たものである。GeneChip100K ないし 500K アレイによる解析をしたのち、我々が独自に開発したゲノムコピー数解析ツール CNAG/AsCNAR を用いてアレル特異的なゲノムコピー数を算出し、アレル不均衡の網羅的な解析を行った。本解析により同定された aUPD の集積領域から、新規標的遺伝子の同定を試みた。

（倫理面への配慮）

検討に用いた検体は、当該患者からインフォームドコンセントを得たのちに連結可能匿名化を施した。当院の倫理委員会の承認済みである。

C. 研究結果

MDS222 例のゲノム解析において、70 例(31.5％)の症例で aUPD が観察され、いくつかの染色体領域に集積をした。11q 領域の aUPD は正常核型の MDS/ MPN を中心に最も多くの症例(17 例)で観察された。我々は 11q-aUPD の最小領域から *CBL* 遺伝子のホモ変異を同定し、同変異は 11q-UPD と強く関連しており、UPD の標的となっていることを明らかにした。変異 *CBL* は NIH3T3 細胞を強く形質転換させがん遺伝子として機能していたが、その一方、正常 *CBL* は生体内ではがん抑制遺伝子として作用していることが、*CBL* ノックアウトマウスの解析から明らかとなった。*CBL* 変異は、E3 ユビキチンリガーゼ活性上重要なドメイン内に認められ、変異型 CBL ではユビキチンリガーゼ活性が顕著に低下しており、造血サイトカインなどの刺激後の受容体型チロシンキナーゼの活性化を遷延させた。変異型 *CBL* 導入細胞はサイトカイン刺激に高感受性となった。またこの効果は、*CBL* ノックアウト細胞ではより顕著であり、正常 *CBL* の導入により著明に減弱した。

D. 考察

MDS は、heterogeneity の大きな疾患群であるが、SNP アレイを用いた網羅的なゲノム解析を行うことにより、aUPD を含めたゲノム異常のパターンから、いくつかの亜型に分類可能であり、11q-aUPD に特徴づけられる亜型の標的遺伝子として *CBL* 変異を見出した。機能解析の結果、*CBL* 変異は MDS/MPN の発症に大きく関わり、aUPD により正常アレルが失われることが病態上も重要であると考えられた。また、治療上の観点からは恐らくチロシンキナーゼ活性の抑制が有用である可能性が示唆された。

E. 結論

SNP アレイによる網羅的かつ高感度なゲノム解析により、染色体分析では見出されない異常から、*CBL* 遺伝子変異を見出した。変異 CBL の機能解析により MDS/MPN の病態解明が進み、今後病態に即した治療法の開発が進むことが期待される。

F. 健康危険情報

なし

G．研究発表
論文発表

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H．知的財産権の出願・登録状況

1. 特許取得
2. 実用新案登録
3. その他

いずれも予定なし

III.　研究成果の刊行に関する一覧

研究成果の刊行に関する一覧表（論文）

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
Eguchi-Ishimae M, Eguchi M, Maki K, Porcher C, Shimizu R, Yamamoto M, Mitani K.	Leukemia-related transcription factor TEL/ETV6 expands erythroid precursors and stimulates hemoglobin synthesis.	Cancer Sci	100	689-697	2009
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研究成果の刊行に関する一覧表（論文）

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
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IV.　研究成果の刊行物・別刷

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

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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.[3,10,11] 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

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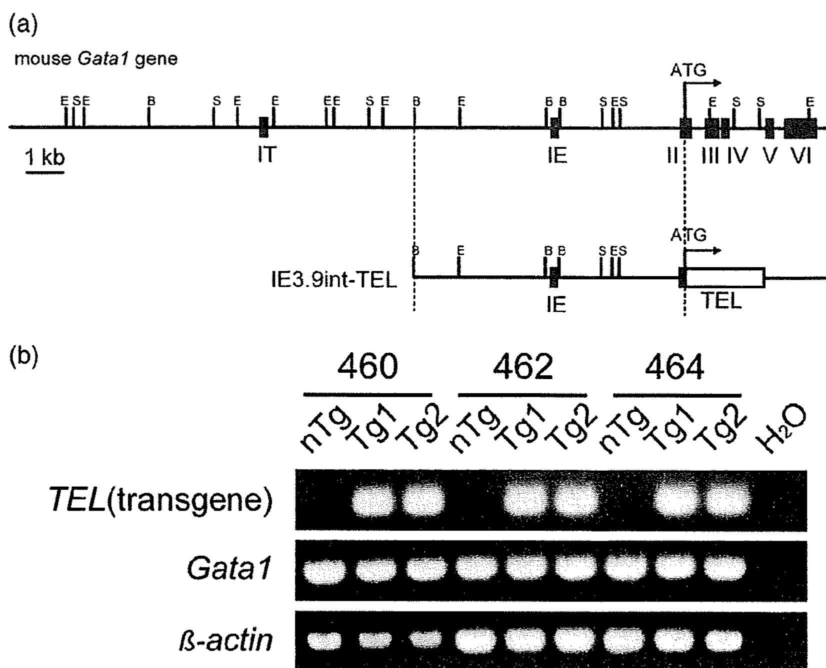

Fig. 1. Expression of TEL transgene in *GATA1-TEL* transgenic mice. (a) Schematic representation of the *Gata1* promotor region and the pIE3.9int-TEL construct used for the generation of *GATA1-TEL* transgenic mice. The pIE3.9int vector contains 3.9 kb *Gata1* promoter region upstream of exon IE.[30] 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).

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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 (× 10³/µL)	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 (× 10⁶/µL)	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 (× 10⁴/µL)	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

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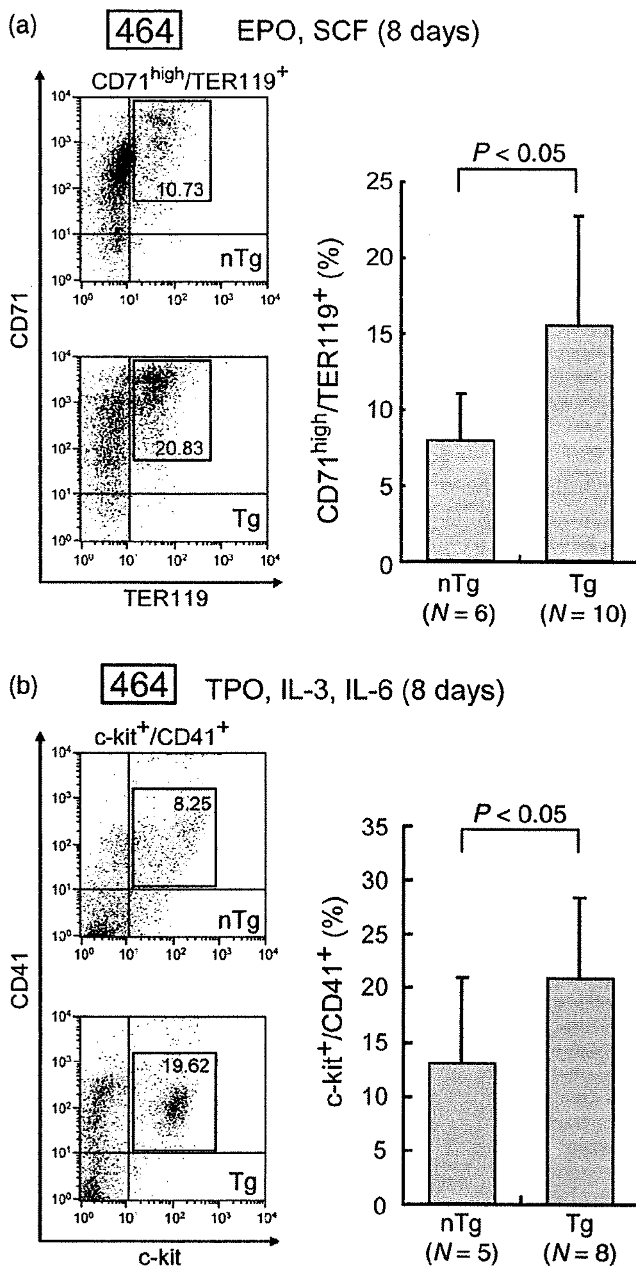

Fig. 2. Differentiation of bone marrow cells into erythroid and megakaryocytic precursors. Bone marrow cells were extracted and cultured in the presence of recombinant murine (a) erythropoietin (EPO) (3 U/mL) and Stem cell factor (SCF) (50 ng/mL), or (b) thrombopoietin (TPO) (20 ng/mL), interleukin (IL)-3 (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*

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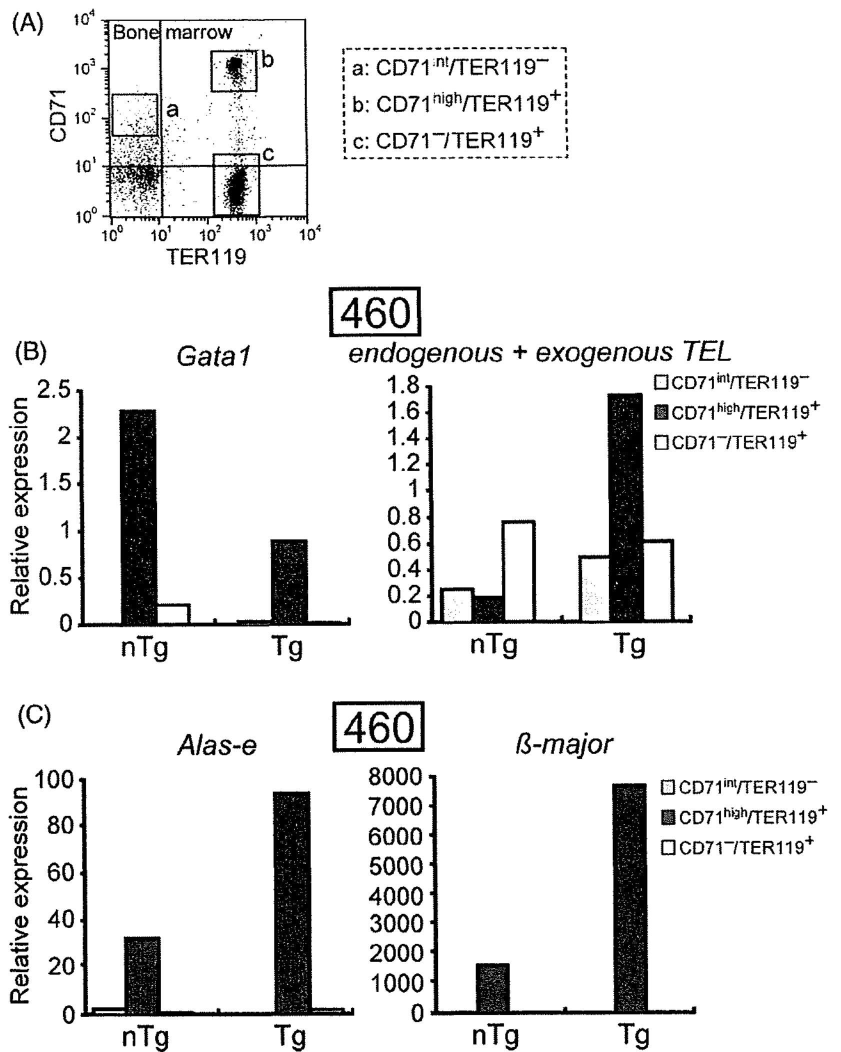

Fig. 3. Quantitative PCR of the genes involved in erythropoiesis. (A) To compare the expression of erythroid-related genes between *GATA1-TEL* transgenic mice and control littermates, bone marrow cells were sorted for CD71^{int}/TER119^{-} (a), CD71^{high}/TER119^{+} (b) and CD71^{-}/TER119^{+} (c), 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.

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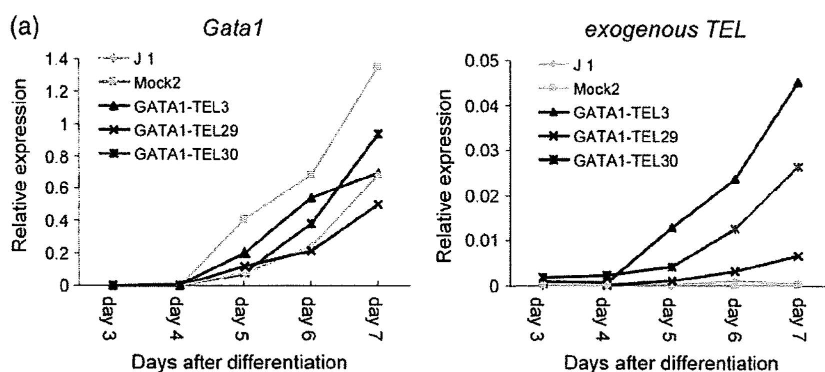

Fig. 4. Expression of *Gata1* and *TEL* during differentiation of embryonic stem (ES) cells. (a) Undifferentiated original J1, mock-transfected (Mock2) and *Gata1-TEL*-overexpressing (GATA1-TEL3, 29 and 30) ES cells were deprived of leukemia inhibitory factor to initiate differentiation and analyzed 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.

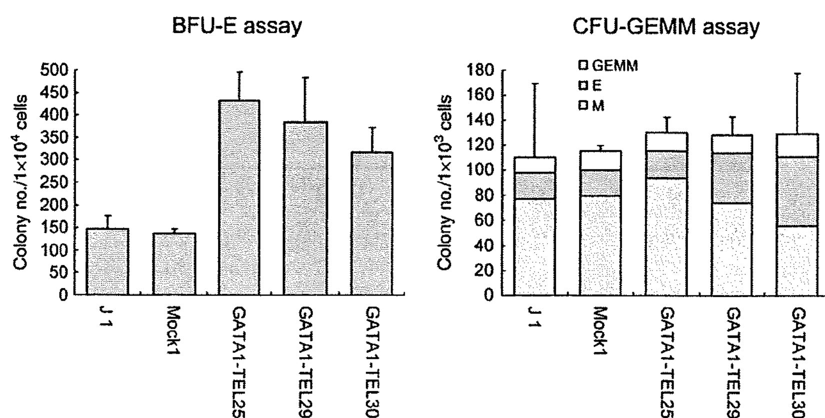

Fig. 5. Enhanced erythroid colony formation in *GATA1-TEL* embryoid body (EB) cells. Undifferentiated J1, mock-transfected (Mock1) and *Gata1-TEL*-overexpressing (GATA1-TEL25, 29 and 30) embryonic stem cells were deprived of leukemia inhibitory factor to form differentiated EBs. EBs at day 7 of differentiation 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

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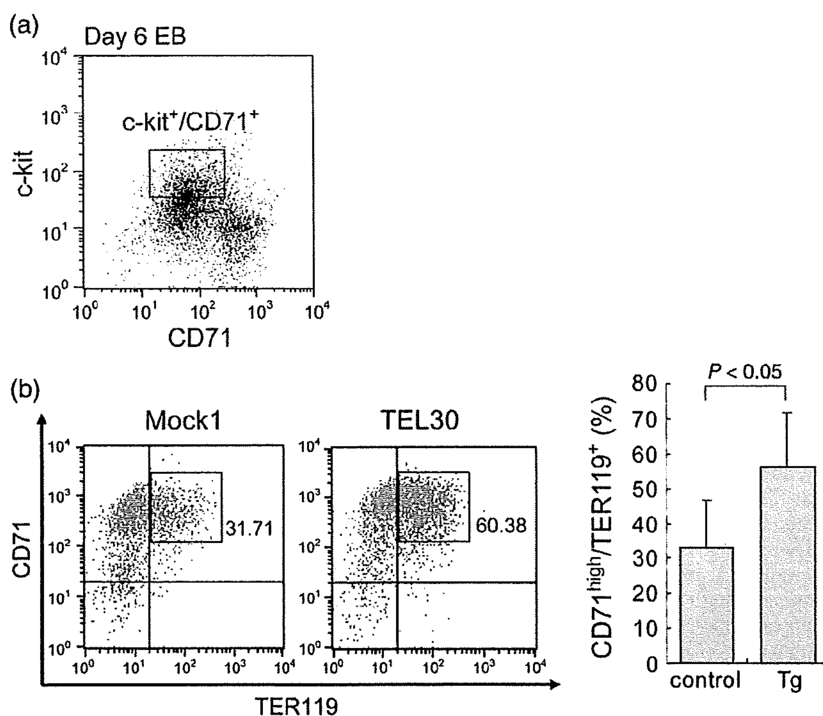

Fig. 6. *In vitro* erythroid differentiation of embryoid body (EB)-derived c-kit+/CD71+ cells. Undifferentiated embryonic stem cells were deprived of leukemia inhibitory factor to form differentiated EBs. After 6 days of differentiation, c-kit+/CD71+ cells (shown in panel a) were separated by fluorescence-activated 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 CD71high/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 CD71high/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 CD71high/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 CD71high/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 CD71int/TER119−, CD71high/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 CD71high/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 CD71high/TER119+ population, while relatively high in both the CD71int/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 CD71high/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 CD71high/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

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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.

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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.

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LETTER TO THE EDITOR

Enhanced expression of the *EVI1* gene in NUP98/HOXA-expressing leukemia cells

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The chromosomal translocation t(7;11)(p15;p15) is mainly observed in Asian patients with acute myelogenous leukemia (AML), particularly in the M2 subtype according to the FAB classification, myelodysplastic syndrome, and blastic crisis of chronic myelogenous leukemia [1–3]. This is the first identified chromosomal translocation involving 11p15, and the presence of this chromosomal abnormality is associated with poor prognosis in AML. The t(7;11)(p15;p15) translocation causes the *NUP98* gene on 11p15 to fuse to three different members of the *HOXA* family gene on 7p15, leading to the production of three different fusion genes, *NUP98/HOXA9*, *NUP98/HOXA11* and *NUP98/HOXA13*. The *NUP98* gene encodes a nucleoporin protein, which comprises nuclear pore complexes that facilitate mRNA export from the nucleus. The *HOXA* family genes encode HOX family transcription factors, which play important roles in survival of hematopoietic stem cells and the development of body segmentation. The fusion of the *NUP98* gene and the *HOXA* family genes generate chimeric NUP98/HOXA proteins, which universally have the FG-repeat domain derived from the NUP98 molecule and the DNA-binding plus PBX-heterodimerizing domains derived from the HOXA molecules.

The generation of the NUP98/HOXA fusion proteins is believed to have critical roles in the development of leukemia with t(7;11)(p15;p15) translocation. To date, two mechanisms have been proposed for the leukemogenesis by the NUP98/HOXA fusion proteins; disruption of the formation of functional nuclear pore complexes, and dysregulated functions of the HOXA family transcription factors. For the latter mechanism, it has been shown that NUP98/HOXA fusion proteins up-regulate the HOXA family responsive genes such as *HOXA7*, *HOXA9* and *MEIS1* [4], of which the up-regulation of *MEIS1* is particularly critical for NUP98/HOXA to cause full-blown leukemia [5, 6]. To further investigate the molecular mechanism of the NUP98/HOXA chimeric proteins in leukemia development, we analyzed gene expressions in human leukemic samples that expressed the *NUP98/HOXA* fusion transcripts.

After having obtained written informed consent from patients, bone marrow cells were collected following protocols approved by institutional review board. Patient samples analyzed in this study included four cases that had the t(7;11)(p15;p15) translocation (Case 1, therapy-related AML; Cases 2 and 3, M2; Case 4, M4), and six control cases that did not have the t(7;11)(p15;p15) translocation (Case 5, M1; Case 6, M2; Case 7, M5a; Case 8, M0; Cases 9 and 10, M1). Total RNA was extracted from the bone marrow cells of these patients, and gene expressions were analyzed using reverse-transcript PCR (RT-PCR) method. Cases 1 and 2 expressed *NUP98/HOXA9* transcript, and Case 3 expressed *NUP98/HOXA11* transcript, as verified in

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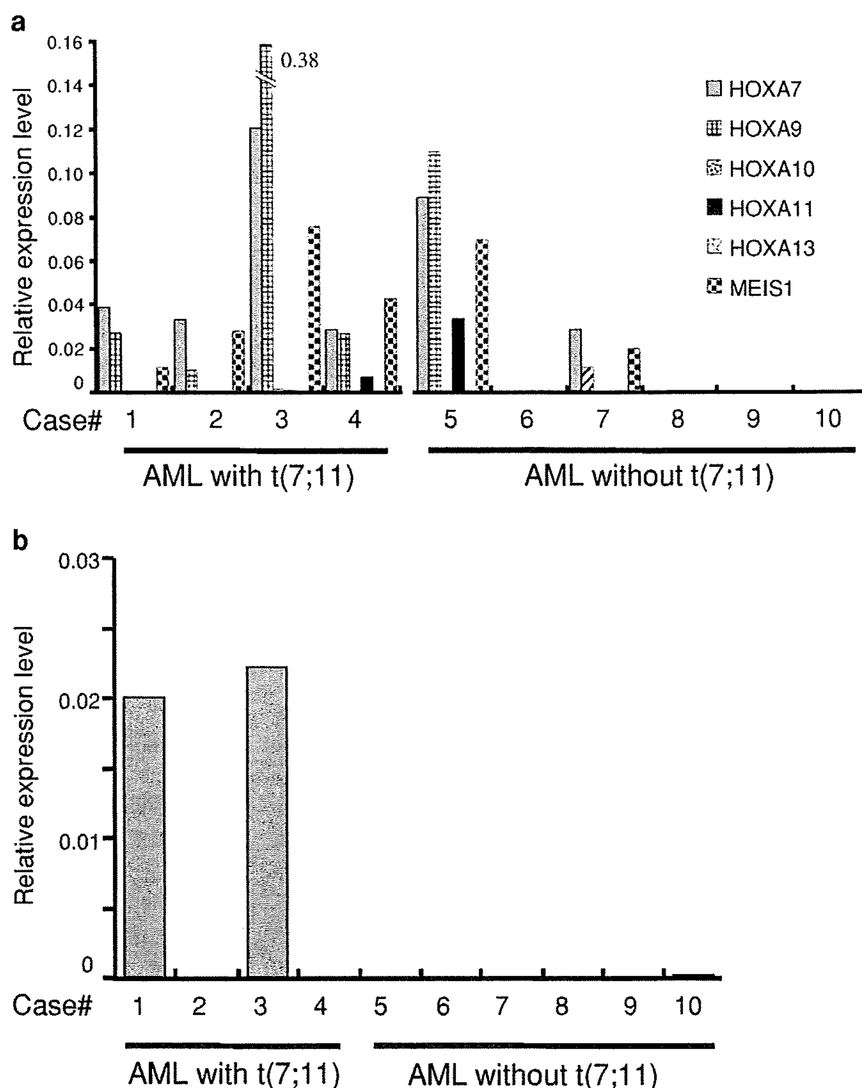

Fig. 1 **a** The expression levels of the *HOXA7*, *HOXA9*, *HOXA10*, *HOXA11*, *HOXA13*, and *MEIS1* genes in bone marrow cells from the patients were analyzed in quantitative RT-PCR analysis. The expression levels of the genes were normalized with that of the *β2-microglobulin* gene. Cases 1–4 had the t(7;11)(p15;p15) abnormality, and Cases 1–3 expressed NUP98/HOXA-fusion transcripts. Cases 5–10 did not have the t(7;11)(p15;p15) abnormality. **b** The expression levels of the *EVI1* gene were analyzed in the same bone marrow samples. The expression levels were also normalized with that of the *β2-microglobulin* gene

RT-PCR (data not shown). Case 4 had the t(7;11)(p15;p15) translocation, but did not express NUP98/HOXA chimeric transcripts including *NUP98/HOXA9*, *NUP98/HOXA10*, *NUP98/HOXA11* or *NUP98/HOXA13*. Analysis of the fusion transcript in Cases 1, 2 and 3 showed the following fusion patterns; in *NUP98/HOXA9*-expressing samples (Cases 1 and 2), the exon 12 of the *NUP98* gene was fused to the exon 1B of the *HOXA9* gene, and in *NUP98/HOXA11*-expressing sample (Case 3), the exon 12 of the *NUP98* gene was fused to the exon 2 of the *HOXA11* gene.

Then we analyzed the expressions of five *HOXA* family genes, *HOXA7, HOXA9, HOXA10, HOXA11* and *HOXA13*, and that of the *MEIS1* gene with quantitative RT-PCR analysis using sets of primers shown in supplemental Table 1. Of the six genes, three genes, *HOXA7*, *HOXA9* and *MEIS1*, are reported to be up-regulated by the NUP98/HOXA chimeric proteins. Consistently, our analysis confirmed that these genes were significantly up-regulated in the samples expressing *NUP98/HOXA* chimeric transcripts (Cases 1–3) (Fig. 1a). Cases 4 and 5 also expressed *HOXA7*, *HOXA9* and *MEIS1*. FISH analysis for Case 5 showed no rearrangement in the *MLL* gene (data not shown), suggesting the absence of MLL-fusion proteins that are known to induce the *HOXA* family gene expression. Therefore, the expression of *HOXA7*, *HOXA9* and *MEIS1* in Cases 4 and 5 is due to mechanisms other than the generation of NUP98/HOXA chimeric proteins or the MLL fusion proteins.

We next evaluated the expression of the *EVI1* gene, since the expression of *EVI1* is highly associated with poor prognosis in myeloid malignancies [7]. Strikingly, the expression of *EVI1* was up-regulated in two of the three samples expressing *NUP98/HOXA* chimeric transcripts (Fig. 1b). No expression of *EVI1* was detected in Case 4,

which had t(7;11)(p15;p15) but did not express *NUP98/HOXA* chimeric transcripts. Also, the six control samples (Cases 5–10) did not express *EVI1*. These data suggest that there is a potential link between the expression of *NUP98/HOXA* chimeric mRNA and the over-expression of the *EVI1* gene.

We also measured the expression levels of a particular isoform of *EVI1* mRNA, *MDS1/EVI1*, since our PCR primers detect both EVI1 and MDS1/EVI1 isoforms [8]. It is important to distinguish these two transcripts because EVI1 and MDS1/EVI1 have opposing effects; EVI1 is highly oncogenic while MDS1/EVI1 has an anti-oncogenic effect over EVI1 [9]. Consistently, EVI1 is over-expressed in human leukemia with chromosome 3q abnormalities, while MDS1/EVI1 is mostly detected in normal hematopoietic cells [9]. We used PCR primers that distinguish *EVI1* and *MDS1/EVI1* transcripts [10]. Our analysis revealed that *MDS1/EVI1* transcript was not expressed in the three *NUP98/HOXA*-expressing samples (data not shown), indicating that only the oncogenic form of *EVI1* mRNA was expressed in the two *EVI1*-expressing samples.

To test if NUP98/HOXA chimeric proteins stimulate the expression of the *EVI1* gene through its 5′ regulatory region, we constructed a reporter plasmid that has the 6.5 kb upstream region of the human *EVI1* gene fused to the *luciferase* gene (pGL3-EVI1-Luc). We then transfected the EVI1 reporter plasmid along with expression plasmids of wild-type-HOXA9, NUP98/HOXA9 or NUP98/HOXA11 into 293T cells and evaluated luciferase activity. Cotransfection of NUP98/HOXA9 or NUP98/HOXA11 expression plasmid showed significant increase in the luciferase activity, while that of wild-type-HOXA9 plasmid caused almost no effect (Fig. 2). This result indicates that both NUP98/HOXA9 and NUP98/HOXA11 fusion proteins have capacity to enhance the promoter activity of the *EVI1* gene, while HOXA9 does not have such capacity.

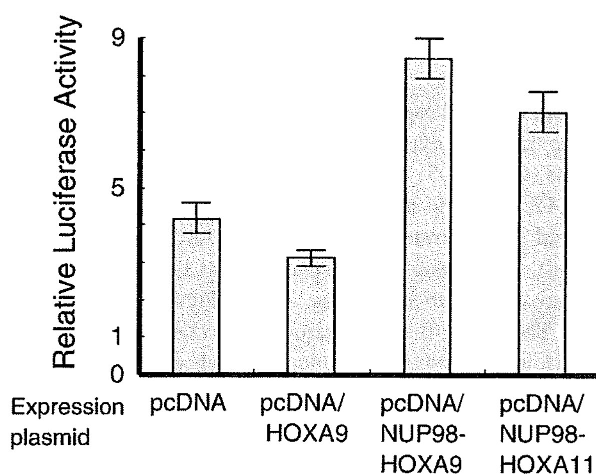

Fig. 2 The pGL3-EVI1-Luc (firefly) plasmid (320 ng) and pCMV-Luc (*Renilla*) (10 ng) were co-transfected into 293T cells with expression plasmids (80 ng) indicated. Cells were harvested 48 h post-transfection, and the firefly luciferase activity was normalized with control *Renilla* luciferase activity

We show that the *EVI1* gene is up-regulated in two out of three samples positive for *NUP98/HOXA* chimeric transcripts. This is the first study suggesting potential association between the presence of *NUP98/HOXA* chimeric transcripts and the over-expression of the *EVI1* gene. The over-expression of EVI1 is known to disrupt multiple signaling pathways, and is associated with various types of hematological malignancies [7]. Thus, this study suggests that the over-expression of *EVI1* causes the leukemic transformation of cells expressing *NUP98/HOXA* fusion proteins. Therefore, this study proposes another mechanism of leukemia development caused by t(7;11)(p15;p15) translocation, in addition to the two mechanisms introduced earlier.

Several studies have explored potential target genes down-stream of NUP98/HOX fusion proteins. Takeda et al. [11] have identified *EVI1* as one of the target genes induced by retrovirally transduced *NUP98/HOXA9* in human CD34-positive primary cells. Similarly, Palmqvist et al. [12] have identified *EVI1* among genes induced by two different NUP98-fusion proteins, NUP98/HOXA10 and NUP98/HOXD13, in murine bone marrow cells. In contrast, *EVI1* is not found in genes induced by NUP98/HOXA9 in a study by Jankovic et al. [13]. Despite the discrepancies, recurrent appearance of EVI1 in the two former studies suggests that EVI1 can potentially be induced by NUP98/HOX fusion proteins. The identification of *EVI1* transcript in our NUP98/HOXA-expressing samples provides clinical evidence that the induction of *EVI1* by NUP98/HOXA do occur in real leukemic cells.

Our reporter assay suggests that both NUP98/HOXA9 and NUP98/HOXA11 enhance the activity of the *EVI1* promoter. However, we could not find the specific consensus binding elements for HOXA9 or HOXA11 in the 6.5 kb promoter region. Thus, the activation of the *EVI1* promoter by NUP98/HOXA9 or NUP98/HOXA11 could be an indirect effect. Further study is needed to clarify the mechanism of the over-expression of the *EVI1* gene caused by the generation of NUP98/HOXA fusion proteins.

References

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