reported that 12% of advanced human colon cancers had a truncating mutation at codon 531 of the *SRC* gene, determining the importance of this mutation in the generation of colorectal cancers remained elusive according to the negative results in subsequent reports (Daigo et al., 1999; Wang et al., 2000; Laghi et al., 2001). In primary hematopoietic malignancies, no studies have demonstrated structural abnormalities of the SRC family of kinases.

In this study, we performed molecular analysis of a t(6;12)(q21;p13) observed as the sole chromosomal abnormality in a case of acute myelogenous leukemia (AML) and identified a SRC-like tyrosine kinase gene, *FRK* (Fyn-related kinase or *Rak*), on 6q21 (Cance et al., 1994; Lee et al., 1994) that is fused with *ETV6* (also called *TEL*), a gene frequently involved in chromosomal translocations in a variety of human leukemias (Golub et al., 1997). We found that the resultant chimeric protein, ETV6/FRK, is a transforming oncoprotein with elevated kinase activity. We also demonstrated that ETV6/FRK inhibits ETV6-mediated transcriptional repression in a dominant-negative manner, indicating that ETV6/FRK is a unique oncoprotein with dual functions. This is the first report showing the involvement of a SRC-like kinase gene (*FRK*) in primary human cancers.

MATERIALS AND METHODS

Case History

The patient was a 69-year-old Japanese woman with AML-M4, carrying the translocation t(6;12)(q21;p13) as the sole chromosomal abnormality in 8 of 20 examined bone marrow metaphase cells. After obtaining informed consent, a sample of her bone marrow was taken for use in this study. The patient did not respond to chemotherapy and died 5 months later.

Fluorescence In Situ Hybridization Analysis

Fluorescence in situ hybridization (FISH) analysis was performed as previously described (Pinkel et al., 1986) with a panel of biotin- and digoxigenin-labeled cosmid probes that contained different exons of *ETV6*, kindly provided by Dr. Peter Marynen (University of Leuven, Leuven, Belgium). The order and the relative locations of cosmids are depicted in Figure 1A.

3′-Rapid Amplification of cDNA End

To do the 3′-rapid amplification of cDNA end (RACE), total RNA was isolated from the leukemic sample as described previously (Ogawa et al., 1996). First-strand cDNA was synthesized from 2.5 μg of total RNA using the primer R2N6 as described previously by Peeters et al. (1997). The first polymerase chain reaction (PCR) was performed with primers T4F1 and R2N6R1 (Peeters et al., 1997). Then, a diluted product of the first PCR, along with primers T4F2 and R2N6R2, was used for the second, nested PCR (Peeters et al., 1997). The nucleotide sequences of the primers used in this study and the conditions for PCR are listed in Table 1. The PCR products were subcloned into the pCR® 2.1-TOPO® vector using a TOPO TA Cloning® kit (Invitrogen, Tokyo, Japan) and subjected to DNA sequencing by use of a 3100 Applied Biosystems automated sequencer (Applied Biosystems, Chiba, Japan).

Reverse Transcriptase-PCR

For the reverse transcriptase-PCR (RT-PCR), 5 μg of the total RNA was transcribed to cDNA with 2 units of Moloney murine leukemia virus reverse-transcriptase (MMLV-RT, Stratagene, La Jolla, CA) using a random hexamer. One-tenth of the synthesized cDNA was directed to PCR analysis. Primers T4F2 and FRK1198R were used to confirm the *ETV6/FRK* transcripts. The primers for detecting the reciprocal *FRK/ETV6* transcripts were FRK451F and TEL723R. For amplification of the wild-type *ETV6* and *FRK* transcripts, primers T4F2 and TEL723R and primers FRK808F and FRK1198R, respectively, were used. All the sequences of the RT-PCR products were verified by direct sequencing.

Plasmid Construction

Full-length *ETV6* cDNA tagged with a FLAG sequence at the 5′ end, a gift from Dr. Kinuko Mitani (Dokkyo University School of Medicine, Tochigi, Japan), was subcloned into the expression plasmid pME18S-neo (Invitrogen, San Diego, CA). A FLAG-tagged full-length *FRK* cDNA was isolated by RT-PCR from total RNA obtained from human placenta using primers *Eco*RI-FLAG-FRK and FRK-*Not*I-2058R and was cloned into pME18S-neo. The pME18S-neo-FLAG-ETV6/FRK vector was generated by replacement of the *Cla*I-*Not*I fragment of the pME18S-neo-FLAG-ETV6 vector with the *Cla*I-*Not*I fragment of *ETV6/FRK*, which was obtained by RT-PCR from the patient's bone marrow using primers TEL-*Cla*I-F and FRK-*Not*I-2058R, with subsequent digestion with *Cla*I and *Not*I. To construct a kinase-inactive mutant of ETV6/FRK, designated ETV6/FRK(K262R), a point mutation corresponding

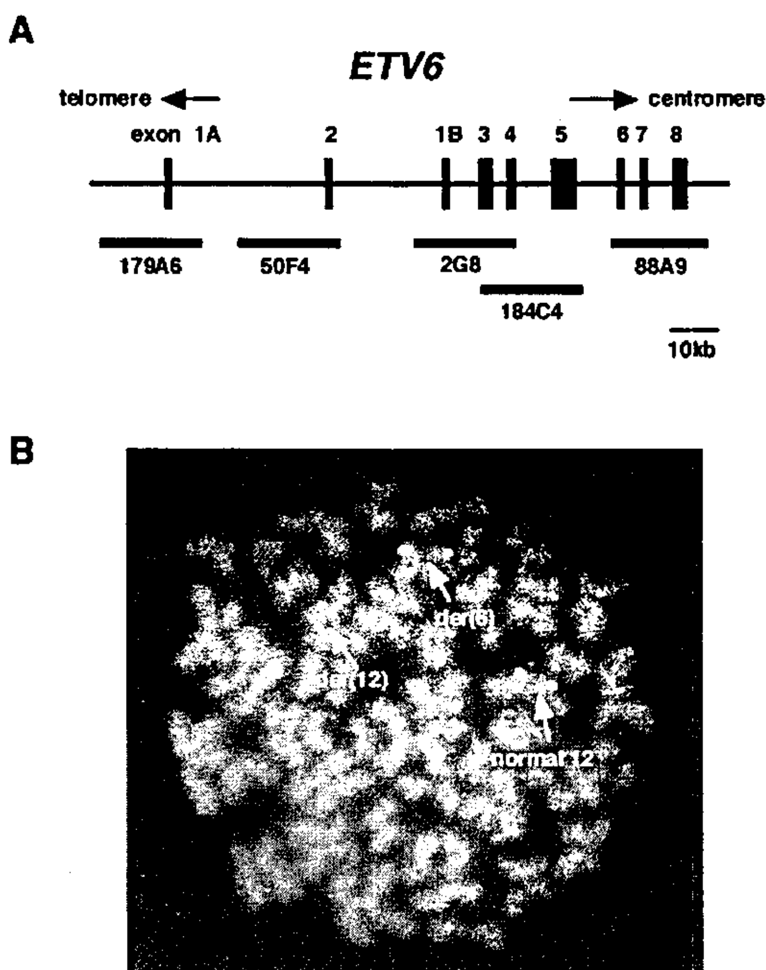

Figure 1. Analysis of breakpoint on chromosome 12. (A) A genomic map of *ETV6* and location of the cosmid probes used for FISH analysis. (B) FISH analysis of the patient's leukemic cells. The signals of the 2G8 probe (red) containing *ETV6* exons 1B, 3, and 4 are hybridized on the der(6) and on the normal 12p, whereas those of the 184C4 probe (green) containing *ETV6* exons 3–5 are found on the der(6), the der(12), and the normal 12p.

to a kinase-inactivating mutation in the ATP-binding site lysine residue (Lys262) of FRK was introduced into *ETV6/FRK* cDNA. A mutated fragment generated by PCR using the mutagenic primer FRK-K262R-*Bam*HI and the primer TEL-*Eco*RI-FLAG was spliced together with a C-terminal partial fragment of *FRK* into pME18S-neo. A FLAG-tagged full-length *FRK/ETV6* cDNA was constructed into the pME18S-neo vector by assembling partial fragments from *ETV6* and *FRK* and a fragment spanning the *FRK/ETV6* junction generated by RT-PCR using primers FRK451F and TEL723R. All the constructs were sequenced to confirm the fidelity of the sequence and conservation of the reading frame at the site of fusion.

Cell Lines, Transfection, and Cell Transformation Studies

For transient expression studies, 4×10^4 HeLa cells were seeded in each 60-mm dish and transfected with expression plasmid or plasmids 24 hr later by a lipofection method using Effectine™ Transfection Reagent (Qiagen, Hilden, Germany). Cells were incubated for 48 hr and harvested for analysis. NIH3T3 cells were transfected with expression plasmids, also using Effectine™, and selected in 400 μg/ml of G418 for 2 weeks. Ba/F3 clones stably expressing ETV6/FRK or other proteins were obtained by electroporation of each expression plasmid into Ba/F3 cells as previously described (Carroll et al., 1996) and subsequent isolation of individual G418-resistant subclones by limiting dilution. Expression of the transfected genes was evaluated by immunoblotting as previously described (Maki et al., 1999) using anti-FLAG-M2 monoclonal antibody (Sigma-Aldrich, St. Louis, MO). The soft-agar colony assay was performed as previously described (Kurokawa et al., 1996). After 21 days, all macroscopic colonies larger than 0.25 mm in diameter were counted. For growth curves, 2×10^4 G418-resistant Ba/F3 cells were washed 3 times with PBS and plated in IL-3-free medium on day 0, and viable cells were counted each day by trypan blue exclusion.

TABLE 1. Primers Used For 3′-RACE and (RT)-PCR Amplifications

Name	Sequence
R2N6	5′-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGC(N)6-3′
T4F1	5′-CATATTCTGAAGCAGAGGAAA-3′
R2N6R1	5′-CCAGTGAGCAGAGTGACG-3′
T4F2	5′-ACACAGCCGGAGGTCATACT-3′
R2N6R2	5′-GAGGACTCGAGCTCAAGC-3′
FRK1198R	5′-CTTCCCATACTTCGCAAAC-3′
FRK451F	5′-AGCAACATCTGTCAGAGGCT-3′
TEL723R	5′-GTAGGACTCCTGGTGGTTGTT-3′
FRK808F	5′-ATCGGAAGATCAGATGCAGAG-3′
*Eco*RI-FLAG-FRK	5′-GCGAATTCGTTGTGATGGGGGACTACAAGGACGAC GATGACAAGTCCGGGAGCAACATCTGTCAGAGGCT-3′
FRK-*Not*I-2058R	5′-ATTGCGGCCGCACTGATTGTGCAGTTGGTTGA-3′
TEL-*Cla*I-F	5′-CTTTCGCTATCGATCTCCTCA-3′
TEL-*Eco*RI-FLAG	5′-GCGAATTCGTTGTGATGGGGGACTACAAGGACGAC GATGACAAGTCCGGGTCTGAGACTCCTGCTCAGTG-3′
FRK-K262R-*Bam*HI	5′-TTGGATCCATTGAACCTGGTTTTAATGTTCTCACTG-3′

Thermal cycling profile was: 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min, with a final extension at 72°C for 10 min.

Immunoprecipitation, Immunoblotting, and Immune Complex Kinase Assay

Lysates were prepared by washing cells (1×10^6–1×10^7) with phosphate-buffered saline and then adding lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.0% NP-40, 1 mM EDTA, and 1 mM Na3VO4] containing 5 mM phenylmethyl-sulfonylfluoride and 1 μg/ml of aprotinin. After 10 min on ice, the samples were centrifuged at 12,000 g to remove insoluble particles. For immunoprecipitation, 1 mg of total cell lysate was incubated with anti-FLAG-M2 antibody for 1 hr at 4°C, after which 50 μl of Protein G-Sepharose beads (Amersham Biosciences, Uppsala, Sweden) was added. After rotating for 1 hr at 4°C, immunoprecipitates were washed 3 times and boiled in loading buffer for 5 min. Protein samples were separated on 6.5%–15% gradient SDS-polyacrylamide gels and transferred onto PVDF membranes (Millipore, Bedford, MA). Immunoblotting was performed as previously described (Maki et al., 1999) using either anti-FLAG-M2 antibody or antiphosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology Incorporated, Lake Placid, NY) as a primary antibody.

For the immune complex kinase assay, immunoprecipitates were washed 3 times and suspended in kinase buffer [40 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 5 mM $MnCl_2$]. For determination of kinase activity, 2.5 μg of either histone H2B or histone H4 (Roche Diagnostics K. K., Tokyo, Japan) was added to each reaction. Kinase reactions were initiated by the addition of 10 μCi of [γ-^{32}P] ATP (3,000 Ci/mmol; Amersham Biosciences Corp., Piscataway, NJ) and incubated at 30°C for 15 min. Reactions were stopped by the addition of loading buffer and analyzed by SDS-PAGE and exposure to a film.

Luciferase Assay

For the luciferase assay, 4×10^4 HeLa cells were transfected with 1 μg of the reporter plasmid (EBS)3tkLuc (Waga et al., 2003), a kind gift of Dr. Kinuko Mitani, along with the indicated amounts of the expression vectors. The total amount of DNA in weight was adjusted to be equal by adding pME18S-neo plasmid. Luciferase activities were determined as described previously (Maki et al., 1999). All transfection experiments were performed in duplicate at least 3 times.

RESULTS

Identification of the Breakpoint on Chromosome 12

We performed FISH experiments using several probes from the *ETV6* locus, on 12p13 (Fig. 1A). The signals from the cosmids containing exons 1–4 (179A6, 50F4, and 2G8) were found on the der(6) (Fig. 1B), whereas the signals from the cosmid containing exons 3–5 (184C4) were split to the der(6) and the der(12) (Fig. 1B), suggesting that the breakpoint on 12p13 was localized to *ETV6* exons 4–5. The signals on the normal 12p were always observed with all the indicated cosmid probes of the *ETV6* locus, suggesting that the non-

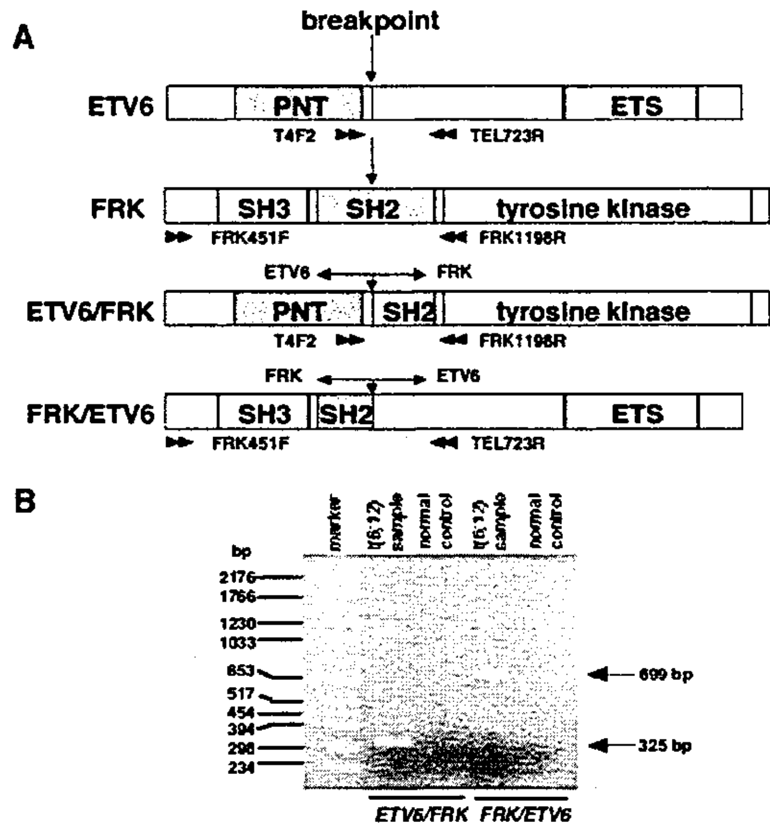

Figure 2. Identification of *ETV6/FRK* and *FRK/ETV6* fusion transcripts. (A) Schematic representation of wild-type *ETV6*, *FRK*, and the fusion transcripts. The breakpoints are indicated by vertical arrows. Horizontal arrows indicate the positions of RT-PCR primers (described in the Materials and Methods section). (B) Detection of *ETV6/FRK* as well as *FRK/ETV6* fusion transcripts by RT-PCR in the patient's leukemic sample. (C) Expression of *ETV6* and *FRK* in the patient's leukemic sample by RT-PCR.

translocated allele of *ETV6* was grossly intact with no large deletions.

Identification of the Fusion Partner of *ETV6*

To identify the unknown fusion partner of *ETV6*, 3′-RACE-PCR was performed. After two rounds of PCR, 3′-RACE-PCR products were successfully obtained. Sequencing analysis of the PCR products showed that exon 4 of *ETV6* was fused to exon 3 of *FRK* on 6q21, creating an *ETV6/FRK* fusion gene. The *FRK* gene encodes a SRC-like nonreceptor tyrosine kinase, consisting of the N-terminal SH3 and SH2 domains, the C-terminal kinase domain, and a short regulatory tail (Fig. 2A). The *ETV6/FRK* fusion gene produced a chimeric protein in which the entire pointed (PNT) oligomerization domain (also called helix–loop–helix domain) of ETV6 and the kinase domain of FRK were fused in-frame (Fig. 2A).

Detection of the *ETV6/FRK* and *FRK/ETV6* Fusion Transcripts

RT-PCR analysis was performed to confirm the fusion transcripts of the *ETV6* and *FRK* genes. Both reciprocal fusion transcripts, *ETV6/FRK* and *FRK/ETV6*, were specifically amplified from the leukemic sample but not from control bone marrow (Fig. 2B). Expression of wild-type *ETV6* and *FRK* also was detected in the leukemic sample (Fig. 2C). There were no mutations in the entire coding sequences of *ETV6*, *FRK*, *ETV6/FRK*, and *FRK/ETV6* (data not shown).

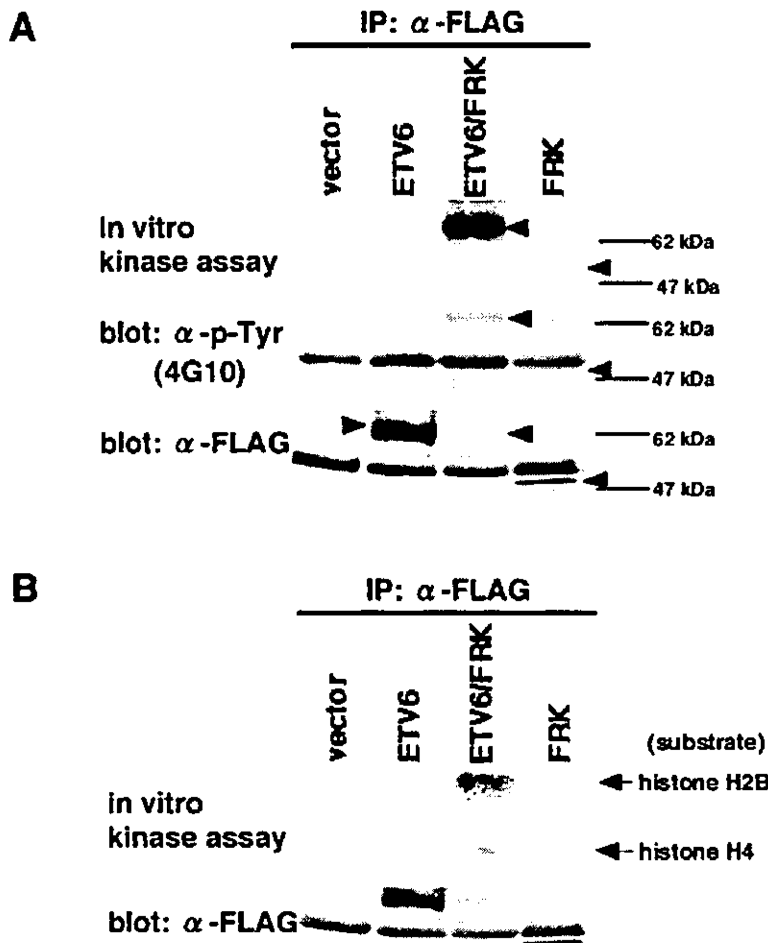

Figure 3. The ETV6/FRK tyrosine kinase is constitutively activated in HeLa cells. (A) Lysates of HeLa cells transfected with the indicated expression vectors were immunoprecipitated with an anti-FLAG-M2 monoclonal antibody and then analyzed by immune complex kinase assay (top) or immunoblotting with an antiphosphotyrosine antibody 4G10 (middle). The total amount of each protein was also assessed by immunoblotting with anti-FLAG-M2 antibody (bottom). Arrowheads show the proteins expressed or phosphorylated at an expected size. (B) Results of kinase assay performed with histones H2B (top) and H4 (middle).

Constitutive Activation of the ETV6/FRK Tyrosine Kinase

Because the ETV6/FRK fusion protein retained the kinase domain but lacked the SH3 domain and most of the SH2 domain, we examined its kinase activity. First, we compared the autophosphorylation status of ETV6/FRK and wild-type FRK. Either the ETV6/FRK fusion protein, wild-type FRK, or wild-type ETV6 FLAG-tagged at the N-terminus was introduced into HeLa cells, immunoprecipitated with an anti-FLAG-M2 monoclonal antibody, and then analyzed by the kinase assay or immunoblotting with an antiphosphotyrosine antibody 4G10 (Fig. 3A, top and middle). To compare expression levels, the same amounts of immunoprecipitate were also subjected to anti-FLAG blot (Fig. 3A, bottom). A high level of tyrosine phosphorylation occurred only in the ETV6/FRK protein (Fig. 3A, top and middle). A basal level of autophosphorylation also was detectable in the wild-type FRK (Fig. 3A, top), a finding in agreement with the previous data (Cance et al., 1994). However, the level of autophosphorylation was significantly lower than that of ETV6/FRK (Fig. 3A, top and middle). Next, we compared the ability of ETV6/FRK and wild-type FRK to phosphorylate exogenous substrates. When histone H2B or H4 was added to the kinase reaction, they were found to be phosphorylated to a greater extent in ETV6/FRK-expressing cells than in FRK-expressing cells (Fig. 3B), suggesting that the ETV6/FRK protein had elevated tyrosine kinase activity.

Cell Transformation by ETV6/FRK in a Kinase-Dependent Manner

To assay the transforming activities of ETV6/FRK, we stably expressed the cDNA-encoding ETV6/FRK or other proteins into the fibroblast cell line NIH3T3. We established 3 NIH3T3 clones expressing ETV6/FRK, 2 clones expressing FRK/ETV6, 2 clones expressing FRK, 2 clones expressing ETV6, and 2 clones expressing ETV6/FRK(K262R) (Fig. 4A), the kinase-inactive

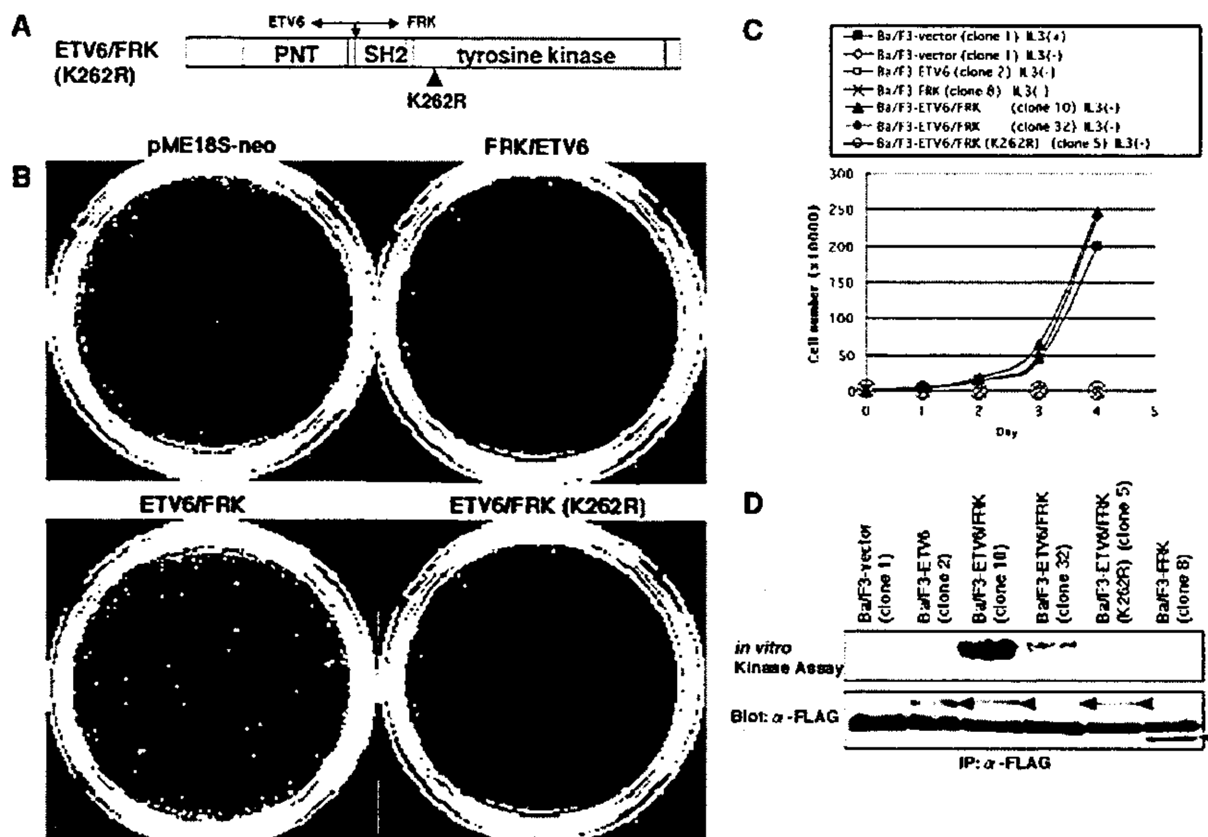

Figure 4. ETV6/FRK transforms NIH3T3 cells and Ba/F3 cells in a kinase-dependent manner. (A) Schematic representation of the kinase-inactive ETV6/FRK(K562R) mutant with a lysine-to-arginine mutation at the ATP binding site. (B) Soft-agar assay demonstrating macroscopic colony formation in ETV6/FRK-expressing NIH3T3 cells. (C) 2×10^4 Ba/F3 cells stably transfected with the indicated expression vectors were washed free of IL-3 and plated on day 0 in growth medium without IL-3. Viable cells were counted each day. Data of the representative clone(s) for each protein are presented. (D) Cell lysates of the indicated Ba/F3 clones were immunoprecipitated with an anti-FLAG-M2 antibody and then subjected to kinase assay (top) and immunoblotting with anti-FLAG-M2 antibody (bottom). Arrowheads show the proteins expressed at an expected size.

mutant of ETV6/FRK, confirmed by immunoblotting analysis (data not shown). The soft-agar assay was performed on each clone. Comparable results were obtained for the clones expressing the same proteins, and the representative data are presented. Only the NIH3T3 cells expressing intact ETV6/FRK were able to produce macroscopic colonies, whereas the NIH3T3 cells transfected with the empty vector or cells expressing the kinase-inactive mutant ETV6/FRK(K262R), the reciprocal FRK/ETV6 fusion protein, wild-type FRK, or wild-type ETV6 failed to grow colonies (Fig. 4B, Table 2). These results suggest that ETV6/FRK but not FRK/ETV6 contributes to neoplastic transformation in a kinase-dependent manner.

Next, we also examined the ability of ETV6/FRK to transform the murine hematopoietic cell line Ba/F3, which is strictly dependent on IL-3 for survival and proliferation. Following stable transduction by electroporation, we obtained 6 Ba/F3 clones expressing ETV6/FRK, 2 clones expressing FRK, 2 clones expressing ETV6, and 3 clones expressing ETV6/FRK(K262R), confirmed by immunoblotting analysis (data not shown). To assay the ability to confer independent proliferation of IL-3, each Ba/F3 clone was switched to growth medium without IL-3. Comparable results were obtained for the clones expressing the same proteins, and the representative data are presented. The Ba/F3 clones expressing ETV6/FRK showed sustained proliferation in the absence of IL-3 (Fig. 4C). In contrast, Ba/F3 cells transfected with the empty vector or cells expressing kinase-inactive mutant ETV6/FRK(K262R), wild-type FRK, and wild-type ETV6 were all unable to proliferate in the absence of IL-3 (Fig. 4C). Although the ETV6/FRK proteins expressed in the stable clones were constitutively autophosphorylated, the ETV6/FRK(K262R) mutants were not (Fig. 4D). These observations indicate that ETV6/FRK is a dominant oncoprotein and that constitutive activa-

TABLE 2. Transformation of NIH3T3 Cells By ETV6/FRK

Transfected DNA	No. of colonies[a]
pME18S-neo (vector)	0
pME18S-neo-ETV6	0
pME18S-neo-FRK	0
pME18S-neo-ETV6/FRK	15
pME18S-neo-ETV6/FRK(K262R)	0
pME18S-neo-FRK/ETV6	0

NIH3T3 cells were transfected with the indicated constructs, and stable transfectants were selected in G418. Cells were plated in soft agar. Macroscopic colonies were counted at day 21.
[a]Average of four experiments.

tion of the ETV6/FRK tyrosine kinase is necessary for ETV6/FRK-induced transformation.

Inhibition of ETV6-Mediated Transcription Repression by ETV6/FRK

Because ETV6 is an ETS transcription factor that acts as a transcriptional repressor (Lopez et al., 1999), we also investigated the transcriptional regulatory property of ETV6/FRK and its ability to modulate the function of wild-type ETV6. We transfected a previously described (EBS)3tkLuc reporter, in which the luciferase gene is placed under the control of an ETS responsive promoter (Waga et al., 2003), along with either wild-type ETV6, ETV6/FRK, or FRK/ETV6 into HeLa cells and evaluated luciferase activity. The results showed, in agreement with the previous finding (Waga et al., 2003), that there was decreased luciferase activity after cotransfection of (EBS)3tkLuc with the wild-type ETV6 expression plasmid (Fig. 5A). In contrast, no repression was observed when ETV6/FRK or FRK/ETV6 was expressed with the (EBS)3tkLuc reporter (Fig. 5A).

Because the oncoprotein ETV6/FRK lacks the ETS DNA binding site but still retains the PNT oligomerization domain, it is possible that it might affect ETV6-mediated transcriptional repression by heterodimerizing with ETV6. Notably, coexpression of ETV6/FRK abolished the transcriptional repression by ETV6 in a dose-dependent manner (Fig. 5B), suggesting that ETV6/FRK has a dominant-negative effect on ETV6-mediated transcriptional repression. In contrast, coexpression of the reciprocal FRK/ETV6 protein did not affect ETV6-mediated transcriptional repression (Fig. 5B). In control experiments, dose-dependent expression of the ETV6, ETV6/FRK, or FRK/ETV6 protein was confirmed by immunoblotting analysis (data not shown).

DISCUSSION

The t(6;12)(q21;p13) is a rare but recurrent reciprocal chromosome translocation in human leukemia (Hayashi et al., 1990; Katz et al., 1991; Raimondi et al., 1997). In this article, we report our finding that it generated novel fusion genes *ETV6/FRK* and *FRK/ETV6* in a case of AML. FRK belongs to a family of SRC kinases, as at the amino acid level, it has the highest homology, 50%, with FYN (Cance et al., 1994; Lee et al., 1994). Although several tyrosine kinase (TK) genes have been identified as fusion partners of *ETV6* (Golub et al., 1994; Papadopoulous et al., 1995; Lacronique et al., 1997; Peeters et al., 1997; Cazzaniga et al., 1999; Eguchi et al., 1999; Iijima et al., 2000; Kuno et al., 2001), this is the first report of a SRC-family tyrosine kinase gene being fused with *ETV6* and structurally altered in human cancers. In the resultant ETV6/FRK fusion protein, the entire PNT oligomerization domain of ETV6 and the kinase domain of FRK are fused in frame. We demonstrated that this ETV6/FRK fusion protein constitutively underwent autophosphorylation on its tyrosine residues. ETV6/FRK had elevated kinase activity compared to that in wild-type FRK. ETV6/FRK showed transforming activities in two cell lines, Ba/F3 and NIH3T3, indicating that ETV6/FRK is a dominant transforming oncoprotein. The kinase-inactive mutant ETV6/FRK(K262R) transformed neither of these two cell lines, indicating that the kinase activity of ETV6/FRK was essential for transformation. The reciprocal fusion protein FRK/ETV6, whose mRNA also was transcribed in the patient sample, did not have transforming activity. These data strongly suggest that the elevated kinase activity of the ETV6/FRK fusion protein directly contributes to the pathogenesis of leukemia with a t(6;12)(q21;p13).

Although activated variants of the SRC family kinases show transforming activities (Parker et al., 1984; Cartwright et al., 1987), the *SRC* and its family of genes rarely have been reported as being mutated or structurally altered in primary human tumors. Irby et al. (1999) reported that 12% of advanced human colon cancers in the United States had a truncating mutation at codon 531 of the *SRC* gene and that the mutation elevated kinase activity and promoted the potential for malignancy. However, three subsequent large-scale studies on advanced colorectal cancers in Japanese, northern European, Chinese, and Italian patients failed to detect the mutation (Daigo et al., 1999; Wang et al., 2000; Laghi et al., 2001), making the

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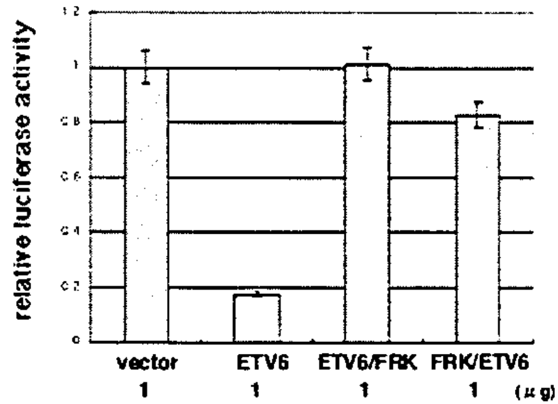

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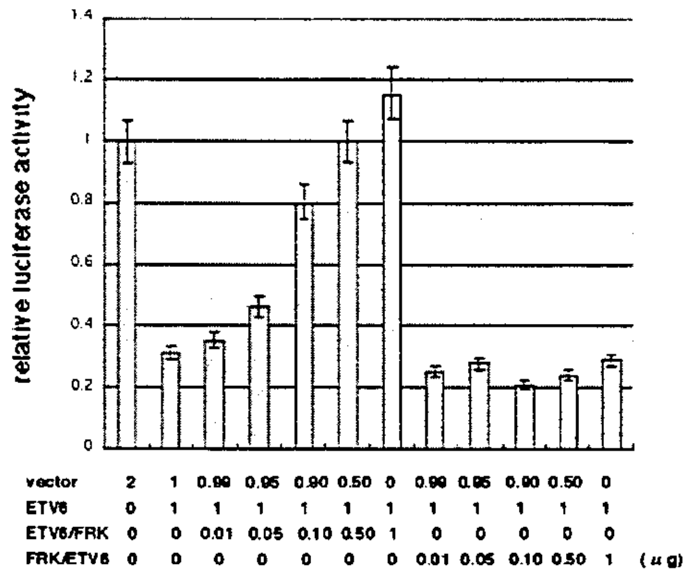

Figure 5. ETV6/FRK is a dominant-negative regulator of ETV6-mediated transcriptional repression in HeLa cells. (A) HeLa cells were transfected with 1 μg of (EBS)3tkLuc reporter plasmid along with 1 μg of the indicated expression vector. Bars show relative luciferase activities to the level when a control plasmid pME18S-neo was cotransfected with the corresponding reporter plasmid, and they present average results of duplicate experiments. (B) HeLa cells were transfected with 1 μg of (EBS)3tkLuc reporter plasmid along with 1 μg of pME-18S-neo-FLAG-ETV6 expression vector together with indicated amounts of pME18S-neo-FLAG-ETV6/FRK or pME18S-neo-FLAG-FRK/ETV6 expression vector. The results are presented as relative luciferase activities.

importance of this mutation controversial. In hematopoietic malignancies, two human T-cell acute lymphoblastic leukemia cell lines have been shown to have rearrangement of *LCK*, a SRC-family kinase gene (Tycko et al., 1991; Wright et al., 1994). In these two cell lines, HSB-2 and SUP-T12, the upstream promoter of the *LCK* gene was juxtaposed to the *TCRB* locus without any accompanying large structural abnormality of the LCK protein. *LCK* mRNA was elevated in the two cell lines (Tycko et al., 1991), and the HSB-2 cell line was later shown to carry several activating point mutations in the *LCK* gene (Wright et al., 1994), indicating that overexpression and/or activation of the LCK kinase would lead to cell transformation. On the other hand, the involvement of SRC family members in primary leukemia has not been reported previously. In this study, we showed that the structural abnormality of an SRC-like kinase gene, *FRK*, through translocation with *ETV6* can directly contribute to leukemogenesis through activation of the altered tyrosine kinase. In addition to the analysis of the current case with a t(6;12), we also performed a mutation analysis of the *FRK* gene in 20 hematopoietic cell lines but failed to detect activating mutations or structural abnormalities (data not shown). Thus, it is currently unclear whether FRK could be activated through other mechanisms such as activating mutations or translocations with other partner gene(s), although more intensive analyses may be required.

Two mechanisms could contribute to the constitutive activation of the ETV6/FRK kinase. First, in the ETV6/FRK fusion protein, the SH3 and SH2 domains of FRK are lost or disrupted, respec-

tively. Both the SH2 and SH3 domains are required to maintain the SRC family kinases in an inactive state: the SH2 domain binds to the C-terminal tyrosine residue in a phosphorylation-dependent manner, and the SH3 domain interacts with a short polyproline type II helix located between the SH2 domain and the kinase domain (Schindler et al., 1999; Xu et al., 1999; Young et al., 2001). These intramolecular interactions are believed to lock the molecule in a closed, inactive state, resulting in repression of kinase activity. In this regard, disruption of this closed conformation would activate the SRC family kinases and lead to cell transformation. In fact, some deletions or mutations in either the SH2 or the SH3 domain of SRC have been shown to activate its catalytic and/or transforming activities (Hirai and Varmus, 1990). Thus, the disruption of the SH3 and SH2 domains in ETV6/FRK may contribute to deregulation of kinase activity. Secondly, in the ETV6/FRK fusion protein, the entire PNT domain of ETV6 is fused to the kinase domain of FRK. As is the case with other ETV6/TK fusion proteins (Carroll et al., 1996; Golub et al., 1996; Jousset et al., 1997), the PNT domain would force dimerization of the ETV6/FRK protein and lead to constitutive tyrosine autophosphorylation and activation of the ETV6/FRK kinase.

The downstream signaling pathway mediated by ETV6/FRK still remains to be elucidated. The wild type FRK is expressed primarily in epithelial tissues (Cance et al., 1994), but also weakly in various hematopoietic cell line (data not shown). However, its functions or downstream signaling pathways remain largely unknown, especially in hematopoietic systems. The only known candidate endogenous downstream component of FRK is the SH2-domain adaptor protein SHB. According to recent reports, GTK, a rodent homologue of FRK, induces neurite outgrowth in PC12 cells and insulin stimulated signaling pathways in pancreatic insulin-producing cells via SHB (Anneren et al., 2000; Anneren and Welsh, 2002). In the present study, however, immunoblotting analysis failed to detect expression of the SHB protein in ETV6/FRK-expressing cells (data not shown). Thus, involvement of SHB in transformation by ETV6/FRK remains unclear. We also tested the phosphorylation status of several signaling molecules, including signal transducer and activator of transcription (STAT1, STAT3, STAT5, STAT6, extracellular signal-regulated kinase 1/2 (ERK1/2), P38 mitogen-activated protein kinase (P38 MAPK), phosphatidylinositol 3-kinase (PI3K), and phospholipase C (PLC)-gamma, in ETV6/FRK-expressing cells. However, we failed to detect any aberrant phosphorylation of these molecules in ETV6/FRK-expressing cells in comparison to FRK-expressing cells (data not shown). Future identification of the target substrate of ETV6/FRK might provide a novel insight into the mechanism of ETV6/FRK-induced transformation as well as of wild-type FRK-mediated signal transduction.

Finally, we demonstrated that ETV6/FRK had a dominant-negative effect over ETV6-mediated transcriptional repression. Because ETV6/FRK retains the PNT oligomerization domain of ETV6, ETV6/FRK may interfere with the transcriptional repression activity of ETV6 by heterodimerizing with wild-type ETV6. Our results indicate that ETV6/FRK is a novel oncoprotein with dual functions: deregulated tyrosine kinase activity and a dominant-negative modulation of transcriptional repression by ETV6. Because wild-type ETV6 appears to have tumor-suppressive activity (Romparey et al., 2000), its suppression by ETV6/FRK also could contribute to oncogenesis. It may be possible that ETV6/FRK can contribute to oncogenesis through two independent mechanisms: activation of the ETV6/FRK tyrosine kinase, which would lead to aberrant stimulation of the downstream signaling pathway, and inhibition of the tumor-suppressive functions of ETV6. This model suggests potential strategies for reversion of transformation by ETV6/FRK. Because the kinase-inactive mutant of ETV6/FRK is nontransforming, a specific inhibitor of the SRC family kinases may inhibit transformation by ETV6/FRK. Alternatively, overexpression of wild-type ETV6 also would interfere with the ability of ETV6/FRK to transform cells. Further experiments will explore these possibilities.

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Extra Views

Runx1/AML-1 Ranks as a Master Regulator of Adult Hematopoiesis

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ABSTRACT

Runx1 (AML-1) is a critical gene involved in human leukemogenesis, originally identified at the 21q22 breakpoint of the leukemic translocations of t(8;21)(q21;q22), and is thought to be involved in as much as 25% of human leukemia. It encodes a transcription factor that has close homology to a *Drosophila* protein, runt, and is found to play essential roles in regulation of hematopoietic systems. Really a gene disruption experiment unequivocally shows that Runx1 is absolutely required for the establishment of definitive or adult-type hematopoiesis. Moreover, accumulated evidence from a number of in vitro studies and findings in patients with familial platelet disorder with predisposition to acute myelogenous leukemia (FPD/AML) strongly suggests that it also commits to the control of hematopoietic system in adult life, although the in depth analysis of its roles in adult hematopoiesis has been largely hampered by premature lethality of *Runx1*-null animals. Recently we have developed conditional knockout mice in which *Runx1* is disrupted specifically in hematopoietic compartments after birth and dissected its roles in adult hematopoiesis. Notably, in these mice, maturation of megakaryocytes and development of both T and B lymphocytes were severely impaired, whereas hematopoietic progenitors were maintained or even expanded with apparently normal myeloid and erythroid differentiation in the periphery and bone marrow. Our findings clearly demonstrated differential requirement of *Runx1* in stem cell development and in its maintenance together with multi-modal functions of this transcription factor that are critically required for maturation of megakaryocytes and lymphocyte development, also providing a novel insight into how deeply and meticulously Runx1 is involved in regulation of mammalian hematopoiesis.

Mammalian hematopoietic development is believed to arise from two distinct cellular origins. In mice, the primitive erythroid cells that appears around day 7.5 postcoitus (E7.5) in the yolk sac is the first hematopoiesis thus far detected, known as primitive hematopoiesis, and is exclusively composed of large and nucleated erythrocytes.[1] On the other hand, the second wave of hematopoiesis, or definitive hematopoiesis, is heralded later in the ventral region of the aorta-gonad-mesonephros (AGM) region in E10.5 and consists of enucleated erythrocytes, myeloid cells and lymphoid progenitors, which ensures following expansion of hematopoietic stem cells (HSCs) and blood production in the fetal liver around E12.5.[2] These are finely regulated processes in which a bunch of genes are expressed in a well-coordinated manner and the growing lines of evidence suggest that these regulations are mediated by a number of hematopoietic transcription factors.

Runx1, also known as *AML-1*, *CBFA2* or *PEBP2αB* is a transcription factor first isolated from t(8;21)(q21;q22) and later in t(3;21)(q26;q22), t(12;21)(p13;q22), and t(16;21)(q24;q21) translocations found in human leukemia in which the aberrant fusion genes, *AML-1/ETO* and *AML-1/Evi1*, *TEL/AML-1*, and *AML-1/MTG16*, were generated, respectively.[3] It has high homology to the *Drosophila* segmentation gene, *runt*, and also has two mammalian homologues, Runx2 (AML-3) and Runx3 (AML-2), collectively called the *Runx* family transcription factors. It is shown that Runx1 dimerizes with the common β-subunit, CBFβ, to bind to its target sequences known as PEBP2 sequences and regulates a variety of hematopoietic lineage-specific genes, including interleukin-3, granulocyte-macrophage colony stimulating factor, macrophage colony stimulating factor receptor, neutrophil elastase, granzyme B, myeloperoxidase, neutrophil defensin, and subunits of the T-cell and B-cell antigen receptor, in cooperation with other transcription factors.[3] Runx1 is absolutely required for mouse embryogenesis and hematopoiesis, since *Runx1*-null embryos die at midgestation by E12.5 with massive hemorrhage in the central nervous system and complete effacement of definitive hematopoiesis in the fetal liver, though primitive erythropoiesis is preserved.[4,5] In fact it has been demonstrated using

Runx1-LacZ knock-in embryos that Runx1 is essential for development of HSCs from the endothelial cells in the embryonic AGM region,[6] where definitive hematopoiesis specifically arises from the ventral endothelial linings that express Runx1.

On the other hand, genetic analysis of Runx1 functions in adult life is largely limited because of the embryonic lethality of the homozygously gene-targeted mice, and our knowledge about its in vivo functions in adult hematopoiesis has mostly come from the analysis of murine models of *Runx1*-involving translocations, although there exist a large body of in vitro studies that argue its postnatal functions. Among these, the most intensively studied is *AML-1-ETO* generated in t(8;21)(q21;q22) translocation. Since this aberrant Runx1 (AML-1) fusion protein seems to have a dominant-negative effect on Runx1 functions and its knock-in mice recapitulate a *Runx1*-null phenotype, several mice models of t(8;21)(q21;q22) have been developed using inducible or retrovirus-mediated expression of the fusion protein in bone marrow in order to clarify its leukemogenic functions in vivo.[7-9] The common features of these mouse models are expanded hematopoietic progenitor pools and increased self-renewal capacity of stem cells with varying degrees of abnormalities in differentiation. While these observations provide important clues to the understanding of leukemogenic mechanism through AML-1-ETO and also of physiological Runx1 functions, it cannot be determined to what extent we are able to ascribe these phenotypes to loss of Runx1 functions.

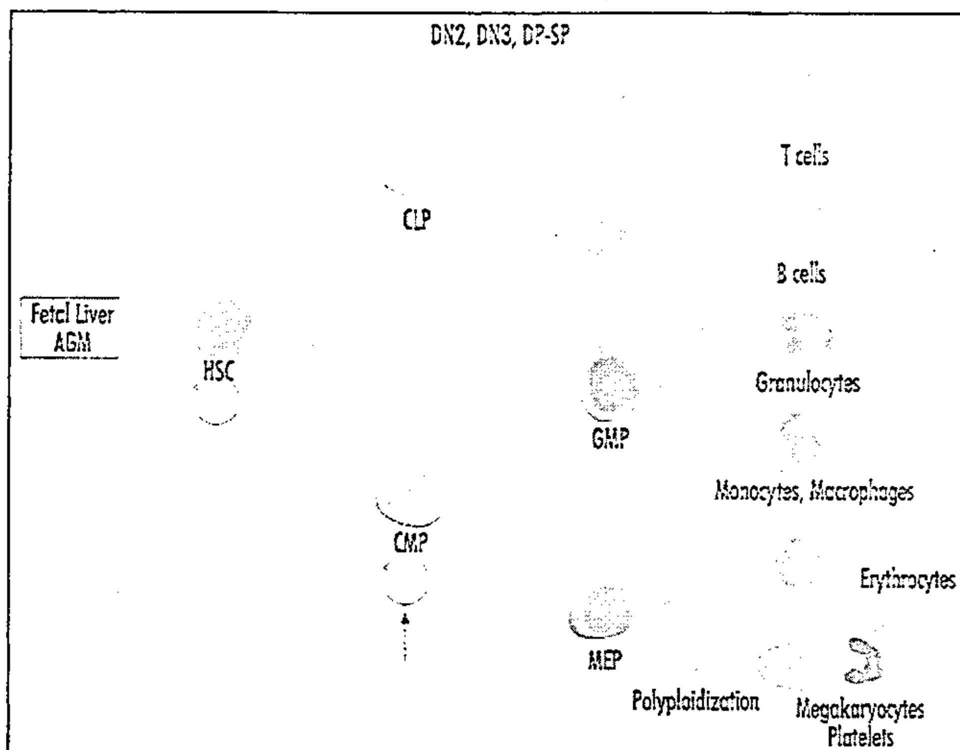

Figure 1. Function of Runx1 in adult hematopoietic maintenance. Red arrows: Runx1 is required. Runx1 is required for development of definitive hematopoiesis at the embryonic stage, several steps in T cell development, early B cell development, and polyploidization of the megakaryocytes, but not for maintenance of early hematopoietic progenitors. Runx1 also negatively regulates the number of myeloid progenitors (broken arrow).

In the article recently published, we analyzed the in vivo role of Runx1 in adult hematopoiesis using the conditional gene targeting system.[10] We generated mice in which exon 5 of the *Runx1* locus was flanked by two *loxP* sites and bred them with mice expressing an interferon-inducible Cre recombinase. With this approach, *Runx1* could be successfully disrupted in the hematopoietic compartments in the adult animals. Although the absence of *Runx1* during developmental stages results in total loss of definitive hematopoiesis and HSC generation, in our conditional knockout mice hematopoietic progenitors were maintained with normal myeloid as well as erythroid development despite complete lack of Runx1, demonstrating that Runx1 is not absolutely required for the maintenance of established adult HSCs per se. It was further supported by transplantation experiments of *Runx1*-null bone marrow cells, in which *Runx1*-null hematopoietic progenitors could fully repopulate recipients' bone marrow for at least three months. On the other hand, however, there exist severe defects about *Runx1*-null hematopoietic progenitors in producing platelets and mature lymphocytes.

A number of transcription factors, including c-Myb, GATA-1, GATA-2, SCL, and LMO-2, also participate in the regulation of the committed hematopoietic progenitors and are indispensable either for the development of embryonic hematopoiesis or for the expansion of HSCs, but their precise roles in maintenance of adult HSCs were largely unknown. However, of particular note is a recent report that SCL, a transcription factor, indispensable for the development of primitive erythropoiesis at the embryonic stage, is not essential for the maintenance of adult hematopoietic, although it is still required for erythroid and megakaryocytic differentiation of the committed progenitor cells.[11] In addition, Kunisato et al[12] demonstrated that SCL does not affect long term repopulating capacity of HSCs but direct their commitment to myeloid lineage using retrovirus-mediated gene transfer of a dominant-negative SCL mutant into HSCs. These findings on *SCL*-null mice are comparable to those on our *Runx1*-null animals, where Runx1 is required specifically for embryonic development of definitive hematopoiesis and regulation of lymphoid differentiation and megakaryocytic maturation, but is also dispensable for the maintenance of adult HSCs. Both examples may represent the functional multi-modality of hematopoietic transcription factors that participated in exquisite regulation of development and maintenance of hematopoietic systems. In our *Runx1*-null mice, there was the increased number of hematopoietic progenitor cells as well as the augmented replating capacity of these progenitors from the *Runx1*-knockout bone marrow. Because *Runx1* is mutated in FPD/AML[13] as well as some sporadic MDS cases,[14-16] it is intriguing in view of the leukemogenic role of Runx1 deficiency that the expansion of hematopoietic progenitors and increased self-renewal capacity have also been observed in the mouse models of t(8;21) (q21;q22)-carrying leukemias, indicating that the size of progenitor cell pool seems to be negatively regulated by Runx1 function, which may be overridden by AML-1-ETO. Although it is not clear whether the increased progenitor pool reflects differentiation block of downstream cell lineages or unregulated cell cycling or apoptosis of the progenitors, it may well be possible that the expanded progenitor pool also

contribute to leukemia development by, for example, increasing the chance of additional mutations. It should also be noted that neither *Runx1*-null mice nor murine models of t(8;21)(q21;q22) develop spontaneous leukemia, indicating additional mutations are required for development of full-blown leukemia.

As already mentioned, the *Runx1*-null mice show greatly reduced platelet counts with severely impaired maturation of megakaryocytes as evident from their smaller cell size, hypoploidy, and abnormalities in ultrastructure. Although the precise mechanism of this phenotype is still unclear, it is worth noting that the similar megakaryocytic maturation arrest at the polyploidization step is also observed in *Fli-1* knockout mice and *GATA-1* knockdown mice.[17-19] Since Runx1 and GATA-1 are known to physically interact with each other in megakaryocytes and promote megakaryocyte-specific gene expression,[20] it may be speculated that Runx1 regulates megakaryocytic maturation in cooperation with GATA-1 through affecting megakaryocyte-specific gene expression.

Mature lymphocyte production is also severely defective in both T and B lineages and competitively transplanted *Runx1*-null HSCs could not contribute to recipients' T or B populations in spite of the normal number of common lymphoid progenitors. *Runx1* is expressed in the developing thymocytes and in B cells, and is known to regulate the expression of T cell and B cell-specific genes. A previous study using T-cell specific *Runx1* knockout mice revealed the essential role of Runx1 in T cell development, in which Runx1 was shown to be required for development of double negative ($CD4^-CD8^-$) thymocytes in transition from $CD44^-CD25^+$ (DN3) to $CD44^-CD25^-$ (DN4) phenotypes.[21] However, our bone marrow reconstitution experiment using Runx1-null hematopoietic stem cells also demonstrated that Runx1 expression is absolutely required for more early stages of T cell development in transition from $CD44^+CD25^+$ (DN2) to DN3 double negative thymocytes. On the other hand, no direct evidence has been reported about the exact biological role of Runx1 in B cell development. Runx1-null bone marrow cells could not repopulate the peripheral T and B lymphocyte population of sublethally irradiated recipients while CLP fraction of the conditional knockout animals is not decreased. Therefore, our data revealed the previously unknown role of Runx1 in B cell development. The precise regulation of Runx1 in B cell development should be analyzed by further investigation, and B-cell lineage-specific targeting of the Runx1 gene is under way to clarify the physiological role of Runx1 in the committed B cells.

In conclusion, from the analysis of conditional Runx1-knockout mice, it became clear that Runx1 is not required for the maintenance of HSC functions in adult mice, but is indispensable for maturation and differentiation of various blood components including T and B lymphocytes, as well as megakaryocytes (Fig. 1). Our data not only recapitulate the human diseases FPD/AML and MDS related to Runx1, but also demonstrate that Runx1 is still a multi-role regulator in maintenance of the lineage-committed cells in adult hematopoiesis, although for HSCs, it is essential only once at the embryonic developmental stage.

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