TEL2, an ETS Factor Expressed in Human Leukemia, Regulates Monocytic Differentiation of U937 Cells and Blocks the Inhibitory Effect of TEL1 on Ras-Induced Cellular Transformation

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ABSTRACT

TEL2 is a member of the ETS family of transcription factors, which is highly similar to TEL1/ETV6. It binds to DNA via the ETS domain and interacts with itself or TEL1 via the pointed domain. The expression of TEL2 in normal and leukemic hematopoietic cells suggests a role in hematopoietic development. In this article, we describe the role of TEL2 in hematopoietic differentiation and cellular transformation. Quantitative reverse transcription-PCR showed that the expression of TEL2 mRNA was down-regulated during monocytic differentiation of U937 and HL60 induced by 1,25-(OH)2 vitamin D3 and 12-O-tetradecanoylphorbol 13-acetate, respectively. Overexpression of TEL2 in U937 cells inhibited differentiation induced by vitamin D3. In contrast, overexpression of a TEL2 mutant lacking either the pointed domain or a functional ETS domain induced both differentiation of U937 cells and inhibited their growth in vitro and in vivo. In addition, these mutants blocked TEL2-mediated transcriptional repression of a synthetic promoter containing TEL2 binding sites. These data suggest that dominant-negative inhibition of TEL2 might cause differentiation. Quantitative reverse transcription-PCR demonstrated that TEL2 is expressed at higher level in some primary human leukemia samples than in normal bone marrow. Furthermore, overexpression of TEL2 in NIH3T3-UCLA cells blocked the inhibitory effect of TEL1 on Ras-induced cellular transformation. These results suggest that TEL2 may play an important role in hematopoiesis and oncogenesis.

INTRODUCTION

TEL1 (also referred as TEL/ETV6) and TEL2 (also referred as TELB/ETV7) are members of the ETS (E26-transformation specific) family of transcription factors (1, 2). The ETS family in mammals includes almost 30 different members, and all of them share a highly conserved 85 amino acid region, the ETS DNA binding domain (3, 4). This domain recognizes a purine-rich GGAA/T core motif in regulatory elements of various target genes including regulators of hematopoiesis (5, 6). Many studies indicate that members of the ETS family are involved in both normal and malignant hematopoiesis (7, 8). In particular, chromosomal translocations involving these genes provide direct evidence that the ETS family of genes is linked to human hematopoiesis/oncogenesis (9, 10).

The TEL1 gene on the human chromosome 12p13 was originally cloned from the break point of t(5;12)(q33;p13) in a case of chronic myelomonocytic leukemia (1). The TEL1 protein acts as a transcriptional repressor by recruiting several corepressors such as mSIN3A, N-COR, and SMRT (11–13). It physically interacts with other proteins of the ETS family such as TEL2 and FLI1 as well as with itself through its pointed (PNT) domain (2, 14, 15). Gene targeting studies in mice revealed that TEL1 is indispensable for normal yolk sac angiogenesis and homing of hematopoietic progenitors from fetal liver to bone marrow (16).

To date, >30 different chromosomal rearrangements involving TEL1 have been reported, and >10 different fusion partners have been cloned (17). These fusion genes encode in-frame fusion proteins that contribute to cellular transformation. The most frequent abnormality involving TEL1 in leukemia is the TEL-AML1 fusion caused by t(12;21)(p13;q22) in pediatric pre-B acute lymphoblastic leukemia (ALL) (18, 19). Interestingly, the second allele of the TEL1 gene is often deleted in these cases, suggesting that TEL1 is also a tumor suppressor (20, 21). In addition, overexpression of TEL1 induces a G1 arrest in fibroblasts and suppresses their transformation induced by mutated H-Ras protein (22). These data suggest that loss of TEL1 is likely to participate in malignant transformation of the cells.

TEL2 was originally cloned as a novel ETS family member closely related to TEL1 (2, 23, 24). The TEL2 gene on human chromosome 6p21 encodes a 38-kDa protein that localizes in the nucleus. Similar to TEL1, TEL2 contains a PNT domain and an ETS domain at its NH2- and COOH-terminal region, respectively. The amino acid sequence of the ETS domain of TEL2 is 85% identical to that of TEL1, whereas the PNT domain is 62% identical between these two molecules. Their similarity is much lower in the regions outside these domains. Gu et al. (24) discovered six different isoforms of TEL2 produced by alternative splicing. The longest isoform (TEL2b, referred as TEL2 in this article) contains both the PNT domain and the ETS domain, whereas the other isoforms lack either or both of these domains with exception of TEL2e, which has a different COOH-terminal end from TEL2. TEL2 can bind the DNA sequences recognized by TEL1 in vitro (2, 23, 24). In addition, TEL2 can associate with itself and with TEL1 through the PNT domain (2, 23) and depends on the presence of the ETS and PNT domain to act as a transcriptional repressor (23, 24). In contrast to the ubiquitous expression of TEL1, TEL2 mRNA is predominantly present in human bone marrow and spleen, suggesting a possible role in normal hematopoiesis (2, 24). Furthermore, TEL2 is also expressed in a variety of human tumors including leukemias (2, 23). Although direct evidence that links TEL2 to leukemogenesis (for example, a gene rearrangement) has not been found yet, its expression pattern and its similarity to TEL1 prompted us to speculate that dysregulated expression of TEL2 might be involved in oncogenesis.

In this article, we described a role of TEL2 in hematopoietic differentiation and cellular transformation. Overexpression of TEL2 in U937, a human myeloid leukemia cell line, blocked its monocytic differentiation induced by 1,25-(OH)2 vitamin D3 (vit-D3). In contrast, overexpression of TEL2 mutants lacking either the PNT domain or a functional ETS domain induced monocytic differentiation of U937 cells, suggesting that dominant-negative inhibition of TEL2 function induces differentiation. A quantitative reverse transcription-PCR (RT-PCR) analysis showed that TEL2 expression in some leukemia patient samples is higher than that in normal bone marrow. In addition, overexpression of TEL2 blocked the inhibitory effect of TEL1 on the transformation of NIH3T3 cells induced by mutant...
H-Ras. Taken together, these findings suggest that TEL2 may play key roles in hematopoiesis and oncogenesis.

MATERIALS AND METHODS

Cell Culture. The human leukemia cell lines U937 (25), HL60 (26), and Reh (27) were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS) in a 37°C incubator with 5% CO2. U937 cells (28) and their derivatives, stably expressing tetVP-16 from pUHD/TetVP16Puro (29) under the control of the TetVP-16-responsive promoter (30), were maintained as described previously (28). NH3T3-UCLA (31) and Phoenix-ECO (American Type Culture Collection, Manassas, VA), a packaging cell line for ectropic retrovirus vectors, were cultured as described (31).

Normal and Patient Samples. Normal human bone marrow cells were purchased from Cambrex (East Rutherford, NJ). Bone marrow samples from patients with newly diagnosed ALL were obtained after informed consent approved by the Institutional Review Board of the hospital. Bone marrow samples from patients with newly diagnosed acute myelogenous leukemia (AML) were kindly provided by Dr. Yoshiyuki Kosaka (Kobe University School of Medicine, Kobe, Japan).

RNA Isolation and cDNA Synthesis. RNA was isolated by using Trizol reagent (Invitrogen, Carlsbad, CA) or RNaseasy mini columns (Qiagen, Valencia, CA). cDNA was made from 2 μg of total RNA using an oligo-dT primer (Amersham Pharmacia Biotech, Piscataway, NJ) and Superscript II reverse transcriptase (Invitrogen) in 50-μl reaction solution according to the manufacturer’s direction.

Quantitative RT-PCR. For quantification of TEL2 mRNA levels (quantitative RT-PCR), we performed TaqMan PCR using an ABI PRISM 7900HT sequence detection system (Perkin-Elmer, Foster City, CA). Five μl (1/10 volume) of cDNA solution was diluted in 50 μl (final volume) of TaqMan PCR reaction solution according to the manufacturer’s direction with the following primers and a probe: forward primer (5′-AATGGGCTTCGCGA-GACTC-3′), reverse primer (5′-CAGGGCCAGGACATCTCTC-3′), and TaqMan probe (5′-AAATCACAAGACCGGGTGACATGACCTA-3′). For quantification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal control, the TaqMan human GAPDH control kit (Perkin-Elmer) was used. PCR reaction was performed according to the manufacturer’s recommendation. The data collection and analysis were performed with the SDS version 2.0 software (Perkin-Elmer). To generate standards for relative quantification, dilutions (triplicates of 2 × 10-1, 5 × 10-2, 1 × 10-2, 2 × 10-3, 5 × 10-4, and 1 × 10-4, these numbers were designated as dilution factors) of a cDNA solution made from 2 μg of total RNA of Reh cells (abundantly expressing TEL2) were PCR amplified. The value of the threshold cycle (Ct; the point during cycling when the PCR product is first obtained) was calculated for each PCR reaction and the dilution factors were used to determine the standard curves for TEL2 and GAPDH mRNA (Fig. 1A). The slope of these standard curves nearly matched the theoretical value of −3.33 (data not shown). The value representing TEL2 expression was obtained from the Ct value using the standard curve and divided by that of GAPDH for normalization. Relative expression levels of TEL2 were determined by comparing the normalized value (TEL2/GAPDH) in each sample to that in the reference sample included in the same reaction.

RT-PCR Detection of TEL2 Isomorphs. Detection of TEL2 isomorphs (TEL2b, TEL2c, and TEL2d) was carried out by RT-PCR as described (24). All of the isomorphs were amplified with the same primers, 5′-CGGCTCACA-GACAGAAGACGGG-3′ and 5′-CTGGAGACAGCTCATAACGGAC-3′. The PCR fragments of TEL2 (377 bp), TEL2c (241 bp), and TEL2 (212 bp) were sequenced and used as probes for Southern blot analysis. Images were obtained by Storm860 and ImageQuant version 5.1 (Molecular Dynamics, Sunnyvale, CA).

Vector Constructs. Full-length TEL2 cDNA (2) was cloned into the EcoRI sites of pUHD10s (29) and the bicistronic retroviral vector MSCV-IRES-GFP (the EcoRI site is 5′ of the IRES sequence; ref. 32), resulting in pUHD10s/TEL2 and MSCV-TEL2-GFP, respectively. TEL2 cDNA was also cloned into the expression vector pSCTOP (pSCTOP/TEL2; ref. 33). TEL1 cDNA (22) was cloned into the EcoRI site of MSCV-IRES-YFP (MSCV-TEL1-YFP). The pSRasm-5h-Ras/LSys12-Tk-C/CD8 retroviral vector was a gift from Dr. Martine Roussel (St. Jude Children’s Research Hospital, Memphis, TN). TEL2ΔPNT lacking the PNT domain (position 151 to 349, the first A of the open reading frame is 1) was generated by overlapping PCR using the following primers: 5′-CGAATACTCATAGCAGAGGAATGTG-3′, 5′-ACACACGCGGCTCGGTGACTGACGAGATGCGCTTC-3′, 5′-CGAGCCTCTGTGTTGTGGGGCCCTTTTTGAGGATCTCAG-3′, and 5′-CGAATTCTCTCAGAGGAGATTTTCTG-3′. TEL2ΔPNT mutant cDNA containing substitutions of arginines for leucines at codons 281 and 284 in its IRES binding domain was generated by overlapping PCR and cloned 5′ of the chloramphenicol acetyltransferase (CAT) reporter gene of pBLcat6 (ref. 34; pbTS/OCT-CAT). pPOU/VPI6 was constructed by inserting a COOH-terminal fragment of VP16 cDNA (264 bp) in-frame 3′ of the entire POU domain of Oct 6 driven by the cytomegalovirus promoter (35). The integrity of all of the cDNAs inserted was verified by sequencing.

Inducible Gene Expression in U937T Cells. Tet-off U937T cells inducibly expressing TEL2, TEL2ΔPNT, or TEL2ΔBM were generated as described previously (28, 36). Independent clones were cultured in the presence or absence of tetracycline and examined for tetracycline-regulated expression of wild-type or mutant TEL2 proteins by Western blot analysis with a TEL2 antibody (2).

Western Blot Analysis. Western blot analysis of TEL1 and TEL2 was performed as described previously (2, 37). Anti-H-Ras and anti-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Infect Immunofluorescence. Indirect immunofluorescence staining of TEL2 was carried out as described previously (2). Bound TEL2 antibodies were visualized with Alexa-488 (BD Pharmingen, San Diego, CA) or Cy3 (Amersham Pharmacia Biotech) -conjugated anti-rabbit IgG. Nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). Images were obtained using a BX-50 fluorescence microscope (Olympus, Tokyo, Japan) with a Spot camera (Diagnostic Instruments, Sterling Heights, MI).

Differentiation of the Cells and Surface Markers Analysis. Monocytic differentiation of U937 and HL60 cells was induced with 1 × 10-8 mol/L vit-D3 (Sigma) and 50 ng/ml 12-O-tetradecanoylphorbol 13-acetate (TPA; Sigma), respectively. In control experiments, the equal amount of ethanol was added, which was the solvent of vit-D3 or TPA. U937T cells inducibly expressing TEL2 were cultured with or without tetracycline for 24 h, after which vit-D3 was added. After an additional 72 hours the cells were subjected to flow cytometric analysis. Expression of cell surface markers for monocytic differentiation was examined by flow cytometer (FACS Caliber, BD Immunocytometry Systems) as described previously (28).

Cell Growth, Cell Cycle, and Apoptosis Analysis. U937T cells (1 × 105) expressing normal and mutant TEL2 were cultured in the presence (1 μg/ml) or absence of tetracycline. After assessment of the number of viable cells by trypan blue dye exclusion at 2,4, and 6 days after initiation of the cultures, the cell cycle distribution was examined by flow cytometric analysis of nuclei stained with propidium iodide (PI) (22). The percentage of apoptotic cells was analyzed on a flow cytometer after incubation with annexin V conjugated with fluorescence isothiocyanate (Roche Applied Science) and PI (38).

In vivo Tumor Formation Assay. In vivo growth of U937 cells expressing TEL2 or TEL2 mutants in NOD-SCID mice was tested as described (39). Three weeks after injection, mice were anesthetized and weighed. Preparatory assay. NIH3T3-UCLA cells (2 × 105 cells in 6-cm-diameter dish) were transfected using Fugene 6 with a combination of following plasmids: pBS/OCT-CAT, pPO/UVP16, rat β-actin promoter-driven secreted alkaline phosphatase (ALP) expression construct (35), pSCTOP/TEL2, pcDNA3/TEL2ΔPNT, pcDNA3/TEL2ΔBM, and pcSCTOP (as a carrier). After 48 h, cell lysates were tested for CAT activity using a CAT enzyme assay system (Promega). We monitored transfection efficiency by measuring the ALP activity in the medium (40). For normalization, the CAT activity was divided by the ALP activity in each transfection.

Retroviral Transduction. Ecotropic retroviruses were made by transfecting Phoenix-eco cells with retroviral vector plasmids (37). Retroviral trans-
Fig. 1. Expression of TEL2 mRNA is down-regulated during monocytic differentiation, and TEL2 overexpression inhibits differentiation of U937 cells induced by 1,25-(OH)2 vitamin D3 (vit-D3). A, standard curves of the quantitative RT-PCR analysis of TEL2 and GAPDH (internal control). Dilutions of cDNA generated from Reh cell RNA were subjected to TaqMan PCR. The value of the threshold cycle (Ct) obtained by TaqMan PCR and the dilution factor (DF) for each sample were plotted. B, the relative expression level of TEL2 mRNA in U937 and HL60 cells during differentiation. Monocytic differentiation was induced in U937 cells and HL60 cells by addition of vit-D3, and TPA, respectively. In the control culture, the same volume of ethanol (solvent for reagents) was added. The value of TEL2/GAPDH determined by TaqMan PCR was compared between samples with or without induction of differentiation to determine the relative expression level. The value for the sample without induction was set at 1. Data shown are the mean from triplicate data obtained in a representative experiment; bars, ±SE. The same experiment was repeated three times with similar results. C, TEL2 isoforms in U937 and HL60 cells. The expression of TEL2 RNA isoforms [TEL2 (TEL2b), TEL2c, and TEL2d] was analyzed by RT-PCR followed by Southern blot analysis. PCR was performed with a single primer set that amplifies all these isoforms. The sizes of the PCR products are as follows: 377 bp (TEL2), 247 bp (TEL2c), and 212 bp (TEL2d). The relative expression level of GAPDH determined by TaqMan PCR is indicated (mean, n = 3, the value of the sample without induction was set at 1). D, tetracycline-regulated expression of TEL2 in U937 cells. Cells cultured with or without tetracycline for 48 and 72 hours were harvested, and expression of the TEL2 protein was examined by Western blot analysis with TEL2 antibody. Detection of actin served as a loading control. E, subcellular localization of TEL2. Cytospin slides were prepared from cells cultured with or without tetracycline for 8, 24, and 48 hours and were analyzed by immunofluorescence with TEL2 antibody followed by anti-rabbit IgG conjugated with Alexa-488. Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI). F, flow cytometric analysis of the differentiation markers in U937 cells overexpressing TEL2. Cells were cultured with or without tetracycline for 24 hours and then cultured for an additional 72 hours in the presence or absence of vit-D3. In the culture without vit-D3, the same volume of ethanol was added as a control. Expression of CD11b and CD14 on the cell surface was analyzed by flow cytometry. The percentage of positive cells (bars, ±SE, n = 9) is shown. G, growth of U937 cells overexpressing TEL2. Cells were cultured with (○) or without (□) tetracycline, and the number of viable cells was determined by the trypan blue dye exclusion method at days 2, 4, and 6. Bars, ±SE (n = 9) is plotted.

RESULTS

TEL2 Expression Is Differentially Regulated during Myeloid Differentiation of Human Hematopoietic Cell Lines. TEL2 mRNA expression occurs in various cell types including hematopoietic cells (2, 24). To examine whether TEL2 expression is differentially regulated during hematopoietic differentiation, we induced monocytic differentiation of U937 and HL60 cells by adding 1 × 10-14 mol/L vit-D3 or 50 ng/ml TPA to the cultures, respectively. After 72 hours, TEL2 and GAPDH mRNA expression levels were determined by quantitative RT-PCR. Ct values obtained by TaqMan PCR and relative expression levels of mRNA showed a clear correlation (Fig. 1A). The relative expression level of TEL2 in U937 cells cultured with vit-D3 was 4-fold lower than in cells cultured without vit-D3 (Fig. 1B). A similar decrease in TEL2 expression was observed in HL60 cells cultured with TPA (Fig. 1B). This down-regulation suggested a role of TEL2 in myeloid development. Additional RT-PCR analysis also demonstrated expression of TEL2c in U937 and HL60 cells, a TEL2 isoform lacking the PNT domain (Fig. 1C). The intensity of the TEL2 band was lower than that of the TEL2c band in U937 cells, which does not necessarily mean that expression of TEL2 is lower than that of TEL2c, because the smaller TEL2c fragment amplifies more effectively than the TEL2 fragment. Both cell types down-regulated the expression of both isoforms during monocytic differentiation (Fig. 1C). An additional isoform without PNT domain, TEL2d, was also detected at a very low amount in HL60 cells during differentiation (Fig. 1C). This does not necessarily mean up-regulation of TEL2d transcription; however, it is possible that a relative difference in abundance could be caused by differences in half-life of the mRNAs.
encoding the different isoforms. These data suggest that induction of differentiation down-regulates all of the isoforms of TEL2.

**Overexpression of TEL2 Blocks Monocytic Differentiation of U937 Cells.** To elucidate the role of TEL2 in myeloid differentiation of U937 cells, we established a U937T cell line expressing TEL2 in a tetracycline-inducible manner (Tet-off). Using Western blot analysis we selected the clone with the strongest inducible TEL2 expression, designated U937TT/TEL2 (Fig. 1D). Using indirect immunofluorescence with TEL2 antibody we did not detect nuclear TEL2 in uninduced U937/T/TEL2 cells or in cells induced for 8 hours (Fig. 1E). However, 16 hours later, TEL2 was detected (Fig. 1E). Using Western blotting TEL2 could be detected after 48 hours of induction, reaching a maximum after 72 hours (Fig. 1D). The endogenous TEL2 protein level was too low to be detected by Western blotting. On the basis of these findings, we assessed whether forced TEL2 expression in U937TT/TEL2 cells would block differentiation. In the absence of tetracycline for 92 hours and without vit-D3 there was no induction of the monocytic differentiation marker CD11b (Fig. 1F), indicating that forced expression of TEL2 did not induce differentiation of U937 cells. Similar to parental U937 cells, addition of vit-D3 to uninduced U937TT/TEL2 cells resulted in CD11b expression in >90% of cells. In contrast, the percentage of CD11b-positive cells was significantly lower in the cells cultured without tetracycline in the presence of vit-D3 (P < 0.05, Fig. 1F), and expression of a second monocytic differentiation marker, CD14, was also much lower (P < 0.05, Fig. 1F). Moreover, tetracycline withdrawal did not inhibit the growth of U937TT/TEL2 cells (Fig. 1G), suggesting that overexpression of TEL2 blocks vit-D3-induced monocytic differentiation of U937 cells.

**Overexpression of TEL2ΔPNT and DNA Binding Mutants Induces Monocytic Differentiation of U937 Cells.** We next analyzed the functional importance of the TEL2 PNT and ETS domains in the blocking of differentiation of U937T cells. We generated U937TT cell lines expressing a TEL2 mutant lacking the entire PNT domain (Fig. 1H). Unlike TEL2DBM, this mutant protein on cell cycle was approximately constant during the following 24 hours (Fig. 2C). Overexpression of TEL2ΔPNT (U937TT/TEL2ΔPNT) or TEL2ΔPNT-Hi cells (Fig. 1H) showed that at 72 hours after induction, TEL2ΔPNT was localized in the nucleus (Fig. 2D), again showing that the inhibitory effect of TEL2ΔPNT protein on cell cycle was modest when expressed at the same level as TEL2DBM. Consistent with this, 64% of the TEL2ΔPNT-expressing U937TT cells were in G0/G1 phase 6 days after induction versus 56% of the control cells (Fig. 2E), again showing that the inhibitory effect of TEL2ΔPNT was less prominent than that of TEL2DBM.

**Dose-Dependent Effect of TEL2ΔPNT on Differentiation, Growth, and Survival of U937T Cells.** The Western blot in Fig. 3A shows that one of the U937T clones, U937TT/TEL2ΔPNT-Hi, expressed TEL2ΔPNT at an even higher level than U937TT/TEL2ΔPNT cells. Therefore, we repeated the differentiation and cell cycle experiments with this new clone and found that the percentage of CD18-positive cells increased from 30% in U937TT/TEL2ΔPNT cells (Fig. 2B) to 95% of U937TT/TEL2ΔPNT-Hi cells at 72 hours after induction (Fig. 3B). Although the induction of CD18 by TEL2ΔPNT-Hi equalled that of TEL2DBM in U937TT/TEL2DBM cells, the comparative induction of CD11b expression was less effective (Fig. 3B). As a result of the increased differentiation in TEL2ΔPNT-Hi cultures, the cell number after 6 days without tetracycline was much lower than that in cultures with tetracycline (Fig. 3C) or of that in cultures of U937TT/TEL2ΔPNT cells (Fig. 2D). This inhibitory effect was also reflected in the cell cycle analysis of U937TT/TEL2ΔPNT-Hi cells. Four days without tetracycline rendered 55% of the cells in G0/G1 phase versus 46% of cells in cultures with tetracycline (Fig. 3D). Inhibition became more prominent after 6 days with 30% of cells in S phase with tetracycline versus 10% without tetracycline but also with 32.4% of the cells in the sub-G0/G1 fraction (Fig. 3D) suggesting that a significant number of cells were apoptotic (41).

Annexin V-fluorescence isothiocyanate staining (38) indeed confirmed increased numbers of apoptotic (annexin V+/PI+) but not necrotic cells already after 4 days without tetracycline (Fig. 3E). This became more pronounced at 6 days after induction (Fig. 3E). Thus, the effect of the TEL2ΔPNT protein on differentiation, growth, and survival of U937T cells depends on its expression level.

**Overexpression of TEL2 Mutants Inhibits the Tumorigenicity of U937 Cells In vivo.** We next tested whether overexpression of TEL2DBM and TEL2ΔPNT affected the tumorigenicity of U937 cells after s.c. injection into NOD/SCID mice. Tumor weights of U937TT/TEL2DBM and U937TT/TEL2ΔPNT cells 21 days after injection were similar, whereas the weights of tumors produced by U937TT/TEL2ΔPNT, U937TT/TEL2ΔPNT-Hi, and U937TT/TEL2ΔPNT-Hi cells were significantly reduced (Fig. 4). In particular, the U937TT/TEL2ΔPNT-Hi tumors were much smaller, thus confirming that TEL2DBM and TEL2ΔPNT overexpressing cells are growth-inhibited.
TEL2 Mutants Block TEL2-Mediated Transcriptional Repression. The effects of TEL2DBM and TEL2ΔPNT on differentiation of U937 cells were opposite to those of TEL2 and, therefore, we examined whether this was caused by interference with the transcriptional function of TEL2 by these mutants. In the presence of POU-VP16, TEL2 repressed the transactivation of a POU-VP16-responsive promoter containing TEL binding sites, confirming the reported transcription repressor activity of TEL2 (23, 24). Cotransfection of pcDNA3/TEL2DBM with pSCTOP/TEL2 at a molar ratio of 0.3 significantly blocked the repression by TEL2, which became complete at a ratio of 1.6 (Fig. 5). Cotransfection of pcDNA3/TEL2ΔPNT also inhibited the TEL2 repressor activity (Fig. 5), but the effect only became significant at a molar ratio of pcDNA3/TEL2ΔPNT to pSCTOP/TEL2 of 5.5. Thus, although both TEL2 mutants inhibited TEL2 repressor activity, TEL2DBM was more effective than TEL2/H9004PNT.

TEL2 Expression Is Up-Regulated in Some Cases of Human Leukemia. The data above strongly suggested that TEL2 is involved in differentiation of human hematopoietic cells. Therefore, we examined expression of TEL2 in normal and leukemia patient bone marrow cells using quantitative RT-PCR analysis. Characteristics of the leukemia patients are summarized in Table 1. On the basis of the data that TEL2 mRNA was detected in primary samples of B-lineage ALL with t(12;21)(p13;q22) (23) and myeloid leukemia cell lines (24), we tested TEL2 mRNA expression in 23 B-lineage ALL [10 with t(12;21)(p13;q22)], 6 AML patient samples, and 6 normal bone marrow samples. In agreement with previous data (23, 24), TEL2 mRNA was detected by TaqMan PCR in all of the normal and leukemic samples with Ct values within the linear range of the standard curve. The relative expression levels of TEL2 in both ALL and AML were highly variable (Fig. 6A). If the expression level of TEL2 mRNA in a patient sample was higher than the upper limit value of the 95% confidential interval of the mean TEL2 expression level in normal bone marrow cells (n = 6), we considered TEL2 to be overexpressed. We found TEL2 overexpression in 1 FAB-M2 and 1 FAB-M5 of the 6 AML samples and in 1 t(12;21)(p13;q22) case and 1 pseudodiploid case among 23 B-lineage ALL samples (Fig. 6A). The mean relative

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Fig. 2. Overexpression of TEL2 mutants lacking either the PNT domain (TEL2ΔPNT) or a functional ETS domain (TEL2DBM) induces differentiation of U937 cells. A, tetracycline-regulated expression of TEL2ΔPNT and TEL2DBM in U937 cells. Cells cultured with or without tetracycline for 48 and 72 hours were harvested, and the expression of TEL2 mutants was examined by Western blot analysis with TEL2 antibody. Actin detection served as a loading control. B, subcellular localization of TEL2 mutants. Cytospin slides were prepared from the cells cultured with or without tetracycline for 72 hours. The slides were analyzed by immunofluorescence with TEL2 antibody followed by anti-rabbit IgG conjugated with Alexa-488 and observed by fluorescence microscopy. Nuclei were stained with DAPI. C, flow cytometric analysis of differentiation markers in U937 cells overexpressing TEL2ΔPNT or TEL2DBM. Cells were cultured with or without tetracycline for 48 or 72 hours. The percentage of cells positive for CD11b and CD18 on the cell surface was analyzed by flow cytometry. Mean (bars, ±SE) percentage of positive cells from triplicate data were plotted. D, growth of U937 cells overexpressing TEL2ΔPNT or TEL2DBM. Cells were cultured with or without tetracycline, and the number of viable cells was determined by the trypan blue dye exclusion method at days 2, 4, and 6. Mean (n = 9) was plotted; bars, ±SE. E, flow cytometric analysis of the cell cycle of U937 cells overexpressing TEL2ΔPNT or TEL2DBM. Cells were cultured with or without tetracycline for 4 or 6 days. Nuclei of the cells were stained with propidium iodide, and the DNA content was analyzed by flow cytometry. The percentage of cells in G0/G1, S, and G2-M phase of the cell cycle (mean; bars, ±SE, n = 3) is indicated at the side of each panel. For C and E, the result of a representative experiment is shown. Triplicate experiments yielded identical results.
expression level of TEL2 mRNA in AML was significantly higher than that in B-lineage ALL ($P < 0.05$), whereas it was comparable to that in normal bone marrow (Fig. 6A). Thus, the expression of TEL2 was elevated in at least some cases of human leukemia. When the expression of TEL2 isoforms was analyzed by RT-PCR in the AML samples (M2 and M5) with a high level of TEL2 expression, the presence of TEL2c and TEL2d was also detected (Fig. 6B), similar to what has been reported in normal bone marrow cells (24).

**TEL2 Relieves the Inhibitory Effect of TEL1 on Ras-Induced Cellular Transformation.** We and others reported that TEL1 inhibits Ras-induced transformation of NIH3T3 fibroblasts (22, 42). To examine whether the activity of TEL2 in cellular transformation is similar or different from TEL1, we transduced NIH3T3-UCLA cells with a combination of the following retroviral expression vectors: pSRArMV-Ha-Ras/Lys12-Tk-CD8, MSCV-TEL2-YFP, MSCV-TEL2-GFP, and MSCV-ires-GFP (mock control). After transduction, cells were FACS-sorted for expression of the different markers (CD8, GFP, and YFP), producing the following cell lines: UCLA-Ras/Mock, -Ras/TEL1, -Ras/TEL2, and -Ras/TEL1/TEL2. Western blot analysis demonstrated that H-Ras/Lys12 was expressed at an equal level in the different cell lines, and also the amount of TEL1 in UCLA-Ras/TEL1 and -Ras/TEL1/TEL2 cells was similar (Fig. 7A). TEL2 was clearly detected in UCLA-Ras/TEL1/TEL2 cells, whereas in murine cells, such as NIH3T3-UCLA cells, no TEL2 is detected, because mice do not possess an endogenous TEL2 gene (Fig. 7A). Immunofluorescence with TEL2 antibody showed that TEL2 localized in the nucleus of UCLA-Ras/TEL2 and UCLA-Ras/TEL1/TEL2 cells (Fig. 7B). In soft agar assays TEL2, unlike TEL1, did not inhibit Ras-induced transformation of NIH3T3-UCLA cells (Fig. 7, C and D), and UCLA-Ras/TEL1/TEL2 double-transduced cells gave rise to a significantly higher number of soft agar colonies than UCLA-Ras/TEL1 cells (Fig. 7, C and D; $P < 0.05$). This suggested that TEL2 relieves the inhibitory effect of TEL1 on Ras-induced transformation of NIH3T3 cells.

We next determined whether the PNT domain and/or ETS domain are necessary for this effect and generated UCLA-Ras/TEL1/TEL2ΔPNT and -Ras/TEL1/TEL2ΔDBM cells using MSCV-TEL2ΔPNT-GFP and MSCV-TEL2ΔDBM-GFP retroviral vectors. The expression levels of these TEL2 mutants were comparable with that of TEL2 in UCLA-Ras/TEL1/TEL2 cells (Fig. 7A). In addition, the expression levels of H-Ras/Lys12 and TEL1 were equal in UCLA-Ras/TEL1/TEL2, -Ras/TEL1/TEL2ΔPNT, and -Ras/TEL1/TEL2ΔDBM cells (Fig. 7A). Consistent with the previous experiment, TEL2ΔPNT was localized in the nucleus, whereas the TEL2ΔDBM was mainly present in the cytoplasm (Fig. 7B).

In soft agar assays both UCLA-Ras/TEL1/TEL2ΔPNT and UCLA-Ras/TEL1/TEL2ΔDBM cells gave the same number of colonies as UCLA-Ras/TEL1 cells alone (Fig. 7, C and D). This indicated that both the PNT and the ETS domains of TEL2 are important for relieving the inhibition of Ras-induced transformation by TEL1. It also suggested that TEL2ΔPNT could not compete for TEL1 targets, suggesting that TEL1 and TEL2 bind different target genes.
DISCUSSION

Our group and others reported previously the cloning of TEL2, a novel member of the ETS family of transcription factors highly related to TEL1/ETV6 (2, 23, 24). TEL2 is expressed in both normal and malignant hematopoietic cells and can associate with itself and TEL1. Despite these findings suggesting involvement of TEL2 in hematopoiesis and oncogenesis, no definitive data for such a role have been reported.

In this article, we provide evidence for a role of TEL2 in hematopoietic differentiation and cellular transformation. First, we showed that TEL2 expression is down-regulated during differentiation of human hematopoietic cells, which is reminiscent of the expression pattern of a number of other transcription factors that control hematopoiesis (43), including some members of the ETS family. For example, TEL1 is up-regulated during early erythroid differentiation of the murine erythroleukemia cell line MEL and then down-regulated at a late stage of differentiation (44). PU.1, a key regulator of myeloid development, is up-regulated during myeloid differentiation of a murine multipotent hematopoietic cell line EML (45), whereas it is down-regulated during the erythroid differentiation of MEL cells (46). We now report that TEL2 expression is down-regulated during monocytic differentiation of the human hematopoietic cell lines U937 and HL-60 suggesting a role for TEL2 in myeloid differentiation. However, the expression pattern of TEL2 during myeloid differentiation is different from that of PU.1. The latter accelerates lineage commitment and terminal differentiation of myeloid cells (47), whereas TEL2 expression might keep cells undifferentiated. During differentiation, TEL2c, the TEL2 isoform lacking the PNT domain, is down-regulated to the same extent as TEL2. Therefore, it is unlikely that an increase in the TEL2c/TEL2 ratio would cause the dominant-negative inhibition of TEL2 and subsequent differentiation.

On the basis of our expression analysis, we examined the effect of overexpression of TEL2 on the differentiation of U937 cells. Overexpression of TEL2 significantly blocked VitD3-induced monocytic differentiation, whereas it did not affect their growth, a result consistent with the expression pattern of endogenous TEL2 during differentiation. Recent studies have shown that overexpression of other ETS factors also affects differentiation of hematopoietic cells. In contrast to TEL2, overexpression of PU.1 in 32Dcl3, a murine myeloid progenitor line, enhances its granulocytic differentiation induced by granulocyte-colony stimulating factor (48). The difference in expression pattern and the effect of overexpression between these two ETS factors suggests a unique role of TEL2 in myeloid development.

Regarding erythroid development, overexpression of PU.1 blocks differentiation of human erythroleukemia cells. For example, TEL1 expression is up-regulated during early erythroid differentiation of the murine erythroleukemia cell line MEL and then down-regulated at a late stage of differentiation (44). PU.1 expression is up-regulated during myeloid differentiation of a murine multipotent hematopoietic cell line EML (45), whereas it is down-regulated during the erythroid differentiation of MEL cells (46). We now report that TEL2 expression is down-regulated during monocytic differentiation of the human hematopoietic cell lines U937 and HL-60 suggesting a role for TEL2 in myeloid differentiation. However, the expression pattern of TEL2 during myeloid differentiation is different from that of PU.1. The latter accelerates lineage commitment and terminal differentiation of myeloid cells (47), whereas TEL2 expression might keep cells undifferentiated. During differentiation, TEL2c, the TEL2 isoform lacking the PNT domain, is down-regulated to the same extent as TEL2. Therefore, it is unlikely that an increase in the TEL2c/TEL2 ratio would cause the dominant-negative inhibition of TEL2 and subsequent differentiation.

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Regarding erythroid development, overexpression of PU.1 blocks differentiation of human erythroleukemia cells.

Table 1 Characteristics of patients

<table>
<thead>
<tr>
<th>Cytogenetics</th>
<th>FAB</th>
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<tbody>
<tr>
<td>t(8;21)(q22;q22)/AML-ETO*</td>
<td>M2</td>
<td>2</td>
</tr>
<tr>
<td>t(15;17)(q22;q11)/PML-RARα*</td>
<td>M3</td>
<td>2</td>
</tr>
<tr>
<td>t(10;11)/MLL rearrangement†</td>
<td>M5</td>
<td>1</td>
</tr>
<tr>
<td>t(1;19)(p13;q34)</td>
<td>M7</td>
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* Detected by RT-PCR.
† Determined by fluorescence in situ hybridization.
‡ Detected by Southern blot analysis.
differentiation of MEL cells (49), whereas overexpression of TEL1 accelerates it (44). In contrast, we have no evidence that TEL2 is involved in erythroid differentiation, because overexpression of TEL2 in the human chronic myeloid leukemia cell line K562 did not affect its erythroid differentiation induced by sodium butyrate.1 In addition, overexpression of TEL1 blocks megakaryocytic differentiation of K562 cells induced by overexpression of Fli-1 (50). TEL1 can interact with FLI-1 through the PNT domain (15), suggesting that the heterodimerization of these ETS factors may interfere with differentiation of the cells. TEL2 can also interact with TEL1 through the PNT domain but not with FLI-1 (2, 23). It was conceivable that overexpressed TEL2 in U937 cells might bind endogenous TEL1 and inhibit its function in differentiation. However, unlike TEL2, overexpression of TEL1 in U937 cells did not affect their differentiation when cultured with or without vit-D3.1 Therefore, it is unlikely that inhibition of TEL1 function by overexpressed TEL2 causes the block in differentiation of U937 cells.

In our opinion, the data showing that TEL2 blocks differentiation of U937 cells is supported by the observation that overexpression of TEL2 in the human chronic myeloid leukemia cell line K562 did not affect its erythroid differentiation induced by sodium butyrate. In addition, overexpression of TEL1 blocks megakaryocytic differentiation of K562 cells induced by overexpression of Fli-1 (50). TEL1 can interact with FLI-1 through the PNT domain (15), suggesting that the heterodimerization of these ETS factors may interfere with differentiation of the cells. TEL2 can also interact with TEL1 through the PNT domain but not with FLI-1 (2, 23). It was conceivable that overexpressed TEL2 in U937 cells might bind endogenous TEL1 and inhibit its function in differentiation. However, unlike TEL2, overexpression of TEL1 in U937 cells did not affect their differentiation when cultured with or without vit-D3.1 Therefore, it is unlikely that inhibition of TEL1 function by overexpressed TEL2 causes the block in differentiation of U937 cells.

In our opinion, the data showing that TEL2 blocks differentiation of U937 cells is supported by the observation that overexpression of TEL2 PNT domain (TEL2/H9004PNT) or ETS domain (TEL2DBM) mutants induces monocytic differentiation. It has been shown previously that TEL1 lacking the PNT domain is able to bind to DNA sequences recognized by wild-type TEL1 (44). This mutant exerted a dominant-negative effect on TEL1-mediated transcriptional repression and blocked TEL1-induced erythroid differentiation of MEL cells (44).

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1 H. Kawagoe and G. C. Grosveld, unpublished observations.
Similar to TEL1, TEL2 protein lacking the PNT domain no longer functions as a transcriptional repressor but still binds to the target DNA (24). Similarly, the TEL1 mutant lacking the ETS domain acts as a dominant-negative inhibitor of TEL1 (42). Consistent with the previous findings, both TEL2 mutants inhibited TEL2-mediated transcriptional repression of a reporter construct. These observations suggest that TEL2ΔPNT and TEL2DBM inhibit endogenous TEL2 function in a dominant-negative manner and thereby induce the differentiation of U937 cells. However, we cannot rule out the possibility that TEL2ΔPNT interferes with the function of other ETS factors than TEL2 in U937 cells because of its relatively low (in vitro) DNA binding specificity. Similarly, it is still possible that TEL2DBM interacts with other ETS factors containing a PNT domain, although it does not bind to FLI-1 (23). We tried to rule out these uncertainties by TEL2 knockdown experiments, but despite attempts with three different short hairpin RNA vectors we were unable to knockdown expression of TEL2. Thus, additional studies will be needed to settle this issue. In addition, both mutants retarded the growth of U937 cells in vitro and in vivo. Particularly, TEL2DBM caused a marked G1 arrest in cells and suppressed their tumorigenicity in NOD/SCID mice. We believe this is caused by a differentiation-induced cell cycle exit rather than a primary effect of TEL2 on cell growth given that differentiation preceded the growth inhibition of these cells.

TEL2DBM accelerated differentiation and retarded growth of U937 cells more effectively than TEL2ΔPNT when overexpressed at a similar level. In agreement with this, TEL2DBM also blocked the TEL2-mediated transcriptional repression of a reporter more effectively than TEL2ΔPNT in a dose-dependent manner. A much higher amount of TEL2ΔPNT than TEL2DBM was required to induce differentiation of U937 cells to a similar extent. Such high levels of TEL2ΔPNT expression caused apoptotic cell death in U937 cells and strongly reduced their tumorigenicity in vivo. On the basis of the observation that the TEL2DBM protein is in the cytoplasm, it is reasonable to speculate that it binds to endogenous TEL2 through the PNT domain thereby preventing TEL2 from entering the nucleus. On the other hand, TEL2ΔPNT might compete with TEL2 by binding to the same target DNA. The former mechanism might inhibit the function of TEL2 more effectively.

The data supporting a role of TEL2 in human hematopoietic differentiation led us to investigate the expression of TEL2 in primary human leukemia samples. Dysregulated expression of some ETS factors is seen in leukemia. For instance, Fli-1 and PU.1 are activated in mouse erythroleukemia cells by the proviral integration of the Friend murine leukemia virus and spleen focus forming virus, respectively (8). Although TEL2 mRNA was detected in all of the normal and leukemic samples, elevated expression of TEL2 was only seen in some of the ALL (2 of 23) and AML (2 of 6) samples. In the AML samples, TEL2c and TEL2d were also detected. The expression level of these TEL2 isoforms lacking the PNT domain may not be high enough to induce differentiation of the leukemic cells. Although overexpression of TEL2 is not likely to be a common event in primary leukemias, we hypothesize that elevated TEL2 expression may contribute to leukemogenesis in at least some cases of human leukemia.

TEL1 not only plays a role in hematopoesis but also inhibits cellular transformation induced by mutant H-Ras in murine fibroblasts (22). Expression analysis by using the Cancer Profiling Array (BD Clontech) showed that the expression of TEL2 was significantly higher in lung and rectal cancers than in the respective normal tissues. Taken together with the data of TEL2 expression in leukemia samples, these findings led us to study the role of TEL2 in cellular transformation. Unlike TEL1, TEL2 did not inhibit Ras-induced transformation. When TEL2 is coexpressed with TEL1, it blocked the inhibitory effect of TEL1 on Ras-induced transformation. These results suggest that TEL2 plays a role in cellular transformation. Interestingly, TEL2DBM did not block the effect of TEL1. As discussed above, the overexpression data in U937 cells strongly suggest that TEL2DBM binds to TEL2 via the PNT domain and inhibits its function in a dominant-negative manner. A possible explanation for the difference in effect of TEL2DBM on TEL1 and TEL2 is that the TEL2 homodimer (including TEL2-TEL2DBM and TEL2DBM-TEL2DBM) might form preferentially over the TEL1-TEL2 heterodimer (including TEL1-TEL2DBM). Additionally, the effect of TEL2 depends on the PNT domain. Our data suggest that both homodimerization and DNA binding are necessary for TEL2 to exert its effect on cellular transformation.

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REFERENCES

TEL2 REGULATES DIFFERENTIATION AND TRANSFORMATION


TEL2, an ETS Factor Expressed in Human Leukemia, Regulates Monocytic Differentiation of U937 Cells and Blocks the Inhibitory Effect of TEL1 on Ras-Induced Cellular Transformation

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