

by ELISA (Fig. 6A). In addition, *in vivo* VEGF levels in the peri-infarct tissue were significantly higher in the CD34⁺ cell-treated than saline-treated group (Fig. 6B, left), although *in vivo* levels of bFGF differed little between the two groups (Fig. 6B, right).

DISCUSSION

Although gained with small numbers of cynomolgus monkeys, our data suggest that the direct transplantation of bone marrow CD34⁺ cells, even without coronary bypass grafts or percutaneous coronary intervention, results in improved regional blood flow and cardiac function after myocardial infarction in nonhuman primates. Furthermore, we have tried to see the contribution of transplanted CD34⁺ cells to the

repair of ischemic myocardium. To this end, we genetically marked CD34⁺ cells with GFP using an SIV-based lentiviral vector before transplantation. Lentiviral vectors can transduce nondividing cells unlike oncoretroviral vectors, and thus the *ex vivo* culture period with multiple cytokines to allow cell cycling can be reduced to 1 day or less [20, 22, 23]. This is the great advantage of using lentiviral vectors over oncoretroviral vectors for transduction of multipotent stem cells, given that extended *ex vivo* culture of stem cells may result in loss of multilineage differentiation and engraftment abilities [24]. Human immunodeficiency virus (HIV)-1-based lentiviral vectors can efficiently transduce human cells, but not Old World monkey cells [25]. According to a recent report, a species-specific cytoplasmic component confers the innate

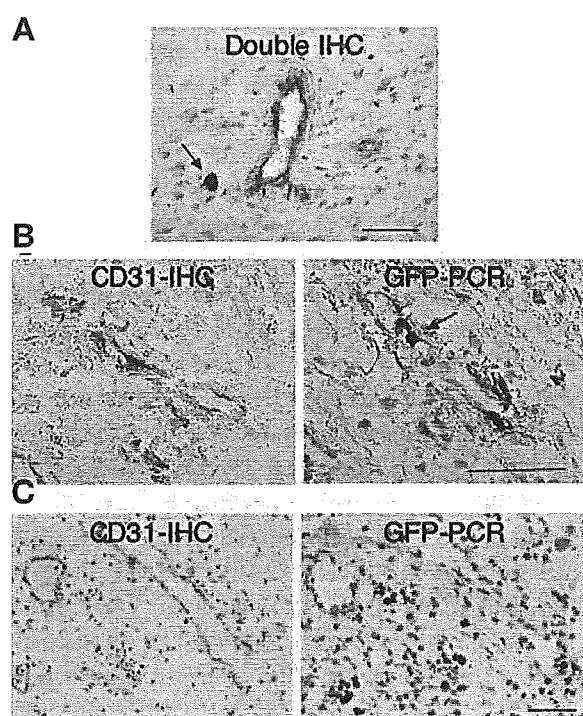


Figure 5. *In vivo* fate of transplanted cells. Cardiac sections were prepared at 2 weeks after transplantation. (A): Double immunohistochemistry (IHC) with anti-CD31 and anti-green fluorescent protein (GFP) in the peri-infarct region of the CD34⁺ cell-treated myocardium. Some cells (arrow) were positive for both CD31 (stained in brown) and GFP (stained in black), but such cells were rare. (B, C): Serial sections from the peri-infarct region of the CD34⁺ cell-treated myocardium. One section (left) was stained with anti-CD31 (stained in brown), and the other (right) was assessed by *in situ* polymerase chain reaction (PCR) for proviral GFP sequences (stained in black). (B): Some CD31-positive endothelial cells contained the GFP-provirus (arrow, right panel), but such cells were rare. (C): Transplanted cell progeny (cells positive for GFP-provirus in the right panel) were not incorporated in vessels (cells positive for CD31 in the left panel). Bar = 50 μm.

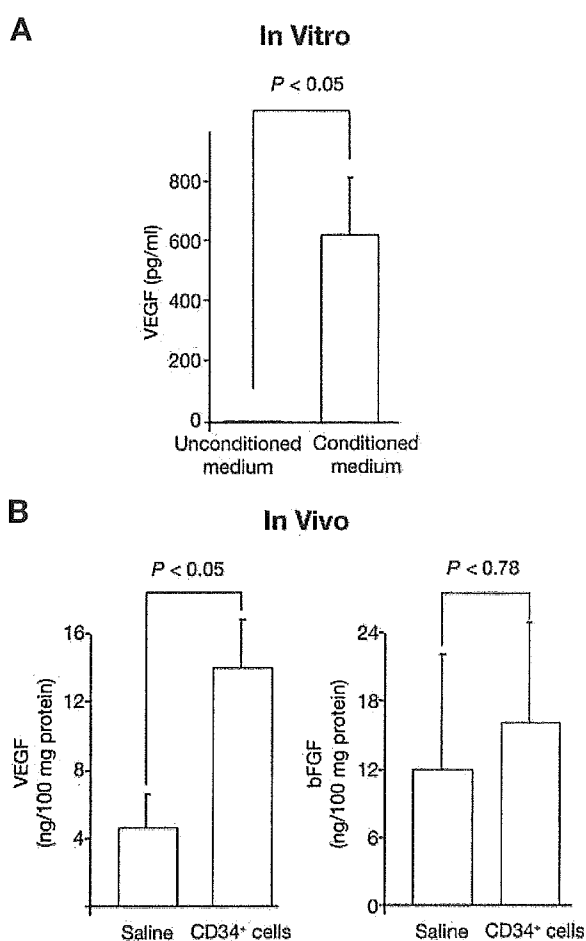


Figure 6. VEGF is implicated in the neoangiogenesis. (A): Unconditioned and conditioned media of *in vitro* CD34⁺ cell cultures for endothelial differentiation were examined for VEGF by ELISA. The average \pm standard error of six culture dishes is shown. (B): Lysates (three samples per monkey) from the peri-infarct region of the CD34⁺ cell-treated (monkey, $n = 3$) and saline-treated (monkey, $n = 3$) myocardium were prepared and examined for VEGF and basic fibroblast growth factor (bFGF) by ELISA. Data are shown as the average \pm standard error. Abbreviation: VEGF, vascular endothelial growth factor.

postentry restriction to HIV-1 infection in simian cells [26]. Unlike HIV-1-based lentiviral vectors, SIV-based ones can efficiently transduce simian hematopoietic stem/progenitor cells [21]. In this study, we also used an SIV-based lentiviral vector and achieved the efficient gene transfer into simian CD34⁺ cells.

As a result of this marking study, we found only a few GFP-positive cells incorporated into the vascular structure in the ischemic myocardium at 2 weeks after transplantation. GFP-positive cardiomyocytes were not detectable. The existence of GFP-positive endothelial cells can be explained by fusion events [27, 28]. However, if that is the case, GFP-positive cardiomyocytes should have also been detected, given that cardiomyocytes are even easier targets of fusion than endothelial cells [11, 29]. Whether fusion occurred or not, only a few transplanted cells gave rise to nonhematopoietic cells in our primate model.

There are several possible explanations for the very low prevalence of transplanted cell-derived endothelial cells or cardiomyocytes in the ischemic myocardium. First, 2 weeks was too short or the number of transplanted cells was too small to see the nonhematopoietic differentiation. However, the cardiac function and regional blood flow were ameliorated by this time point and with this number of transplanted cells. Thus, if transplanted cell-derived, nonhematopoietic differentiation was a reason for the improvement, transplanted cells at this number should have given rise to such cells by this time point. In fact, Orlic et al. [8] observed transplanted cell-derived endothelial cells and cardiomyocytes within 11 days after transplant in mice. In addition, we observed the endothelial differentiation from CD34⁺ cells within 7 days *in vitro* (Fig. 1). However, we cannot formally rule out a possibility that inflammatory responses after generation of infarction might have negative effects on engraftment of transplanted cells. Second, the SIV vector failed to transduce stem or progenitor cells that might be responsible for nonhematopoietic differentiation. Even if the transduction was successful, the cytokine treatment during the transduction or GFP expression in the cells spoiled the differentiation abilities. However, we have shown that the SIV vector successfully transduced cells that were capable of differentiating into GFP-expressing endothelial cells (Fig. 1). We have not examined the differentiation ability to cardiomyocytes, because the method to differentiate CD34⁺ cells to cardiomyocytes *in vitro* has not been well established. Thus, we cannot formally rule out the possibility that the *ex vivo* culture spoiled the ability to differentiate to cardiomyocytes or reduced the ability to differentiate to endothelial cells. Third, cells expressing xenogeneic GFP were rejected via immune responses.

However, 2 weeks is too short to allow immune elimination of GFP-expressing cells in monkeys [30, 31]. Fourth, the GFP expression was shut down because of transcriptional silencing *in vivo*, resulting in negative immunostaining with anti-GFP. To examine this possibility, we tried to detect the provirus (vector integrated into genome) in the cardiac tissue by *in situ* PCR and found again that only a few CD31-positive endothelial cells contained the GFP-provirus (Fig. 5B), thus arguing against transcriptional silencing-based negative immunostaining with anti-GFP. Taken together, we concluded that most transplanted cell progeny were not incorporated into the repaired, nonhematopoietic tissues.

Our results are in agreement with recent reports that transplanted hematopoietic cells are unable to transdifferentiate into nonhematopoietic cells in ischemic myocardium in mice [9–11]. Our studies confirm and extend these findings in a couple of ways. First, we show that the cardiac function can be indeed significantly improved after injection of hematopoietic cells in a nonhuman primate model, although the above studies used murine myocardial infarction models and did not address the potential beneficial effects of hematopoietic cell injection. Second, the improvement is unlikely to be the result of generation of transplanted cell-derived endothelial cells or cardiomyocytes. Finally, we have found that cultured CD34⁺ cells secrete VEGF and that the CD34⁺ cell-treated myocardium contains a significantly higher level of VEGF than the saline-treated myocardium. This observation raises a possibility that some angiogenic cytokines secreted from transplanted cells (paracrine effects) potentiate angiogenic activity of endogenous cells. VEGF would be a candidate. Despite this, the delivery of a single agent (VEGF) failed in clinical trials for cardiac ischemia [32]. *In situ* multiple cytokine production and coordinated action may be essential for clinical benefits [33, 34]. It will be important to explore and identify cytokines responsible for the paracrine effect. If transplanted cells serve as cytokine factories rather than stem cells in ischemic tissues, it is not surprising that not only stem cells but other types of cells may also work [35]. The concept of stem cell therapeutics for ischemic diseases needs additional consideration.

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TEF, an antiapoptotic bZIP transcription factor related to the oncogenic E2A-HLF chimera, inhibits cell growth by down-regulating expression of the common β chain of cytokine receptors

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Gain and/or loss of function mediated by chimeric transcription factors generated by nonrandom translocations in leukemia is a key to understanding oncogenesis. E2A–hepatic leukemia factor (HLF), a chimeric basic region/leucine zipper (bZIP) transcription factor expressed in t(17;19)–positive leukemia cells, contributes to leukemogenesis through its potential to inhibit apoptosis. To identify physiologic counterparts of this chimera, we investigated the function of other bZIP factors that bind to the same DNA sequence recognized by E2A-HLF. Here, we show

that thyrotroph embryonic factor (TEF), which shares a high level of sequence identity with HLF and recognizes the same DNA sequence, is expressed in a small fraction of each subset of hematolymphoid progenitors. When TEF was introduced into FL5.12 interleukin 3 (IL-3)–dependent cells, TEF protected the cells from apoptosis due to IL-3 deprivation. Unexpectedly, TEF also almost completely down-regulated expression of the common β (β c) chain of cytokine receptors. Consequently, TEF-expressing cells accumulated in G₀/G₁ phase without un-

dergoing apoptosis. These findings suggest that TEF is one of the apoptotic regulators in hematopoietic progenitors and controls hematopoietic-cell proliferation by regulating the expression of the β c chain. In contrast, E2A-HLF promoted cell survival more efficiently than TEF but did not down-regulate β c chain expression, suggesting that E2A-HLF retains ideal properties for driving leukemic transformation. (Blood. 2005;105:4437-4444)

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Introduction

Since key systems that regulate cell survival have been conserved through evolution, relatively simple organisms such as *Caenorhabditis elegans* often provide insight into the complex mechanisms that control cell fate in mammals.¹ The genetic approach demonstrated that 2 factors, cell death specification protein 1 (CES-1) and CES-2, regulate the programmed cell death of serotonergic neurosecretory motor (NSM) neurons in *C. elegans* in a cell type–specific fashion.² Further studies revealed that CES-2 is a transcription factor that belongs to the basic region/leucine zipper (bZIP) superfamily (Figure 1).³ CES-2 negatively regulates CES-1, a member of the snail family of zinc finger transcription factors,⁴ and CES-1 appears to determine the fate of NSM sisters by negatively regulating the expression of *eglI*, a BH3-only proapoptotic member of the B cell lymphoma (Bcl-2) superfamily.⁵⁻⁷

Transcription factors that bind to the DNA sequence elements recognized by CES-2 might play important roles in cell type–specific cell death in mammals. The E2A–hepatic leukemia factor (HLF) fusion transcription factor is a good example. This chimera is derived from childhood acute pro-B-cell leukemia harboring a t(17;19) chromosomal translocation.⁸⁻¹⁰ It contains the transactivation domains of E2A and the bZIP DNA-binding and dimerization domain of HLF (Figure 1). E2A-HLF promotes the anchorage-independent growth of murine fibroblasts as a homodimer that

depends on both the transactivation domain of E2A and the bZIP domain of HLF.^{11,12} E2A-HLF induces the expression of annexin II,¹³ annexin V, and sushi-repeat protein upregulated in leukemia (SRPUL),¹⁴ which have been postulated to play paraneoplastic roles in coagulopathies and bone invasion, as well as groucho-related genes, and E2A-HLF also suppresses Runt-related 1 (RUNX1).¹⁵ In addition, we and others established transgenic mice expressing *E2AHLF* that develop T-lineage lymphoid malignancies.^{16,17} Of note, we demonstrated that E2A-HLF binds avidly to the sequence recognized by CES-2 and protects cells from apoptosis due to growth factor deprivation without promoting cell proliferation.¹⁸⁻²⁰ The close homology between the bZIP domains of HLF and CES-2 suggests that E2A-HLF subverts a cell-death pathway through a mechanism similar to that used by CES-2 in the worm. Indeed, we have identified SLUG, a zinc finger transcription factor closely related to CES-1, as one of the downstream targets of E2A-HLF in human leukemias associated with the 17;19 translocation and found that SLUG has antiapoptotic potential.^{21,22}

These findings suggest that a cell-death pathway in mammalian hematopoietic cells is normally regulated by bZIP factors that exhibit a DNA-binding specificity similar to that of CES-2 or E2A-HLF. HLF is obviously a good candidate for the regulator of apoptosis in hematopoiesis; however, HLF is not expressed in

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hematopoietic progenitors and, moreover, enforced expression of HLF in cytokine-dependent cells failed to inhibit apoptosis.^{18,19} We then identified a bZIP factor, E4 promoter binding protein (E4BP4)/nuclear factor regulated by IL-3 (NFIL3) (Figure 1), as a physiologic counterpart of E2A-HLF. E4BP4 has nearly identical DNA-binding specificity to CES-2 and its expression is tightly regulated by interleukin (IL-3) in IL-3-dependent cell lines such as FL5.12 and Baf-3. Moreover, enforced expression of E4BP4 delays apoptosis of IL-3-deprived cells.^{23,24} These data suggested that subversion of the roles of antiapoptotic bZIP factors normally regulated by cytokines is 1 critical aspect of leukemogenesis induced by E2A-HLF.

Other bZIP factors may also play important roles in the regulation of hematopoietic cell survival. HLF is a member of the proline- and acidic amino acid-rich (PAR) bZIP family, which also includes thymotroph embryonic factor (TEF)²⁵ and albumin promoter D-box-binding protein (DBP)²⁶ (Figure 1). TEF was originally cloned as a factor expressed in the developing anterior pituitary gland that could *trans*-activate the *TSH β* promoter,²⁵ whereas DBP was cloned as a liver-enriched transcriptional activator that binds to the D element of the albumin promoter.²⁶ It has been well documented that the PAR proteins as well as E4BP4 are implicated in circadian control.²⁷⁻³³ PAR proteins may also be involved in the regulation of cell death because all 3 PAR proteins exhibit nearly identical DNA-binding specificity to that of E4BP4 and CES-2.^{25,26,28,29,34-37} Here we demonstrate that TEF promotes the survival of FL5.12 cells due to IL-3 starvation. Unexpectedly, we also found that TEF potently represses the expression of the β subunits of the IL-3 receptor, blocking proliferation and leading to arrest of the cell cycle in the G₀/G₁ phase, even in the presence of IL-3.

Materials and methods

Constructs of eukaryotic expression vectors

Expression plasmids containing the cDNAs of wild-type *TEF* (pMT-TEF), a mutated *TEF* (pMT-TEF basic mutant [BX]), and *E2AHLF* (pMT-E2A-



Figure 1. Schematic representation of the CES-2, TEF, DBP, HLF, E2A-HLF, and E4BP4/NFIL3 proteins. The CES-2 protein in *C. elegans* contains a basic region/leucine zipper (bZIP) domain in the carboxyl-terminal region and acts as a transrepressor. TEF, DBP, and HLF contain a proline- and acidic amino acid-rich (PAR) domain as well as a bZIP domain in the carboxyl-terminal region, which shows high level of sequence identity to that of CES-2. TEF, DBP, and HLF each also contain a *trans*-activation domain (TAD) and can act as transcriptional activators. The E2A-HLF fusion protein, which is expressed in t(17;19)-positive leukemia cells, retains the 2 transactivation domains in the amino terminal of E2A (AD1 and AD2) but not its basic region/helix-loop-helix domain, which is replaced by the bZIP domain of HLF. E2A-HLF has a joining region (■ with arrowhead) generated at the breakpoint but not the basic region extension (BRE; indicated with □) nor the PAR domain of HLF, which contribute to sequence-specific DNA binding. E4BP4/NFIL3 contains a bZIP domain in its amino-terminal region and has nearly identical DNA-binding specificity as that of CES-2, TEF, DBP, HLF, and E2A-HLF. E4BP4 acts as either a transactivator or transrepressor depending on complexes formed by its TAD or transrepression domain (TRD) in its carboxyl-terminal region.

HLF) were constructed with the pMT-CB6⁺ eukaryotic expression vector (a gift from F. Rauscher III, Wistar Institute, Philadelphia, PA), which contains the inserted cDNA under the control of a sheep metallothionein promoter as well as the neomycin resistance gene driven by the simian virus 40 early promoter. The cDNA encoding a basic mutant of TEF (TEF/BX) that contained substitutions for 6 of the amino acid residues in the basic region critical for DNA binding (TRRKKNVAAK mutated to TSPKSYNVPPK; single-letter code) was made by polymerase chain reaction (PCR) mutagenesis.

Cell culture and cell survival assay

FL5.12 cells, which are murine IL-3-dependent cells, were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 0.5% 10T1/2 cell-conditioned medium as a source of IL-3. Transfectants were generated by electroporation using 2×10^7 cells and 80 μ g of DNA with a gene pulser (Bio-Rad, Hercules, CA) set at 300 mV and 960 μ F. Cells were then cultured in 24-well dishes and selected in the presence of the neomycin analog G418 (0.6 mg/mL) for 2 weeks. The induction of protein expression with Zn in G418-resistant cells was confirmed by immunoblot analysis. Cell growth and cell survival experiments were performed using 3 independent pools of cells, and the results of representative data of multiple experiments are shown. For cell survival assays, cells growing exponentially in IL-3-containing medium were precultured for 16 hours in the presence or absence of 100 μ M ZnSO₄. Cells were washed with IL-3-free medium twice and were adjusted to 5×10^5 cells per milliliter. Viable cell counts were determined by trypan blue dye exclusion.

Immunoblot analysis

Cells were solubilized in Nonidet P-40 lysis buffer (150 mM NaCl, 1.0% Nonidet P-40, 50 mM Tris [tris(hydroxymethyl)aminomethane; pH 8.0]), and total cellular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After wet electrotransfer onto polyvinylidene difluoride membranes, proteins were detected with the following primary antibodies: anti-HLF c-terminal (C),³⁵ anti-p44/42 mitogen-activated protein kinase (MAPK; New England BioLabs, Beverly, MA), anti-p70S6K (New England BioLabs), anti-Akt (New England BioLabs), antiphospho-signal transducers and activators of transcription 5 (anti-phospho-STAT5; Tyr694; New England BioLabs), anti-phospho-p44/42MAPK (Thr202/Tyr204; New England BioLabs), anti-phospho-p70S6K (Thr421/Ser424; New England BioLabs), and anti-phospho-Akt (Ser473; New England BioLabs) rabbit serum, or anti- β -tubulin (PharMingen, San Diego, CA), antiphosphotyrosine (Upstate Biotechnology, Lake Placid, NY), and anti-STAT5 (Transduction Laboratories, Lexington, KY) mouse monoclonal antibodies. Then, the blots were stained by horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG) and IgM secondary antibodies (MBL, Nagoya, Japan), respectively, and subjected to enhanced chemiluminescence detection (Amersham Life Science, Arlington Heights, IL).

Flow cytometric analysis

Cells (2×10^5) were washed with phosphate-buffered saline (PBS) and incubated with rat monoclonal antibodies specific for the mouse IL-3 receptor α chain (5B11)³⁸ or the mouse IL-3 receptor β chain (AIC2A; β_{IL3} ; 9D3)³⁹ on ice for 30 minutes. Cells were washed with PBS and stained with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (Jackson Immuno Research Laboratories, West Grove, PA). For detection of the common β chain of the IL-3 receptor (AIC2B; β_C), cells were incubated with an anti-mouse β_C chain hamster monoclonal antibody (MBL), followed by incubation with an FITC-conjugated goat anti-hamster IgG secondary antibody (Jackson Immuno Research Laboratories). Cells were analyzed by flow cytometry (FACS Calibur; Becton Dickinson, San Jose, CA).

Northern blot analysis

Total cellular RNA was isolated by the guanidinium-cesium chloride method. RNA samples (approximately 20 μ g per lane) were separated by

electrophoresis in 1% agarose gel containing 2.2 M formaldehyde, transferred to nylon membranes, and hybridized with the appropriate probes according to the standard procedure. For Northern blot analysis of multiple human hematolymphoid tissues, the blot was purchased from Clontech (Palo Alto, CA). Unique cDNA probes in the 3' untranslated region of the PAR genes,⁴⁰ a 1.3-kilobase (kb) *Xho*I fragment of mouse IL-3 receptor α -chain cDNA, and a 0.8-kb *Xho*I/*Bam*HI fragment of mouse IL-3 receptor β_{IL3} chain cDNA were used as probes.

Analysis of gene expression in subsets of hematopoietic stem and progenitor cells

Cells (5×10^3) with surface marker expression patterns typical of hematopoietic stem cells (HSCs) and various types of myeloid and lymphoid progenitors were sorted from mouse bone marrow by fluorescence-activated cell sorting (FACS), as previously described.⁴¹ Total RNA was prepared with TRIZOL reagent and subjected to cDNA synthesis with the SuperScript first-strand cDNA synthesis system (GIBCO, Carlsbad, CA). PCR was performed for 40 cycles of 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 45 seconds with the following primers: 5'-ACCATCTTCTCTACTGCCATCTTTCAG and 5'-GTACTTGGTCTCGTACTGGACACGATG for first-round PCR; 5'-GTGATCTGGTTCTCCTTCAG for nested PCR amplification of *Tef*; and 5'-CACAGGACTAGAACACCTGC and 5'-GCTGGTAAAAAGGACCTCT for PCR amplification of *Hprt*.

Electrophoretic mobility shift assay

Binding reactions in electrophoretic mobility shift assay (EMSA) were performed with a ³²P-end-labeled DNA oligonucleotide probe (2×10^4 counts per minute [cpm]) in 10 μ L of binding buffer (12% glycerol, 12 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.9], 4 mM Tris [pH 7.9], 133 mM KCl, 300 mg of bovine serum albumin per milliliter) and 5 μ L of nuclear proteins extracted from FL5.12 cells by standard procedures, as previously described.^{35,42} The oligonucleotide probes used were wild-type HLF consensus sequence (CS) (5'-GCTA-CATATTACGTAATAAGCGTT-3'); wild-type β -casein for STAT5 (5'-AGATTTCTAGGAATTCATCC-3'); and mutant β -casein (5'-AGATTTAGTTAATTCATCC-3'). As a carrier DNA, 1.5 μ g of sheared calf thymus DNA or poly-d(I/d)(C) was added to the reaction mixture testing for binding to HLF-CS or β -casein probe, respectively. The entire mixture was incubated at 30°C for 15 minutes. In the competition inhibition experiments, an approximately 100-fold molar excess of the unlabeled oligonucleotide was added to the reaction mixture. One microliter of polyvalent HLF(C) antiserum or preimmune rabbit serum was added to the nuclear lysates and incubated at 4°C for 30 minutes prior to the DNA-binding reaction. Nondenaturing polyacrylamide gels containing 4% acrylamide and 2.5% glycerol were prerun at 4°C in a high-ionic-strength Tris-glycine buffer for 30 minutes, loaded with the samples containing protein-DNA complexes, run at 35 mA for approximately 90 minutes, dried under vacuum, and analyzed by autoradiography.

Results

Expression of *TEF* in hematolymphoid tissues

The 3 members of the PAR family of proteins are differentially expressed in tissues and organs.^{8,10,40} In contrast to HLF, which shows tissue-specific expression, TEF and DBP are expressed ubiquitously, although their expression in normal hematopoietic and lymphoid tissues has not been clarified. First, we performed Northern blot analysis of human hematolymphoid tissues using a specific cDNA probe. As shown in Figure 2A, *TEF* was expressed in the spleen, lymph node, thymus, and fetal liver but was nearly undetectable in peripheral blood leukocytes and bone marrow. *DBP* was widely expressed in hematolymphoid tissues, whereas *HLF* was expressed in the fetal liver alone. Second, subsets of mouse hematolymphoid progenitors were sorted from the bone marrow

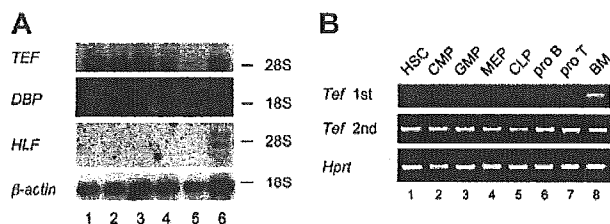


Figure 2. Expression of *TEF* during hematopoiesis. (A) Northern blot analysis of human hematolymphoid tissues. The blot was hybridized with human *TEF*, *DBP*, *HLF*, and β -actin cDNA probes. Lane 1, spleen; lane 2, lymph node; lane 3, thymus; lane 4, peripheral blood leukocyte; lane 5, bone marrow (BM); and lane 6, fetal liver. The mobility of 28S and 18S rRNA was indicated. (B) Patterns of *Tef* expression in murine myeloid and lymphoid progenitors in different stages of development. cDNA was synthesized from total RNA extracted from 5000 cells of each hematopoietic progenitor subset that had been sorted from bone marrows by FACS (lanes 1-7) as well as unpurified bone marrow mononuclear cells (lane 8). The cDNA was subjected to PCR with primers specific for the murine *Tef* (top panel) and *Hprt* (bottom panel) genes. The PCR product of amplification of *Tef* was subjected to nested PCR (middle panel). HSC indicates hematopoietic stem cells; CMP, common myeloid progenitor; GMP, granulocyte-monocyte progenitor; MEP, megakaryocyte-erythrocyte progenitor; and CLP, common lymphoid progenitor.

and were subjected to reverse transcriptase-PCR (RT-PCR) for *Tef* (Figure 2B).²² Although *Tef* was detectable in unpurified bone marrow mononuclear cells, the RT-PCR product was virtually undetectable in each subset of progenitors (Figure 2B top panel). Upon performing nested PCR using each PCR product as a template, *Tef* was detected in each subset (Figure 2B middle panel). These results suggested that *Tef* is expressed in hematolymphoid progenitors but its expression is low or restricted to a small fraction of each subset of progenitors.

Establishment of FL5.12 cells conditionally expressing TEF

To analyze the function of TEF in hematopoietic cells, we established FL5.12 cells that inducibly expressed TEF by the addition of Zn using an expression vector (pMT-CB6⁺) under the control of a metallothionein promoter. Immunoblot analysis using the anti-HLF(C) antibody that effectively recognizes HLF, DBP, and TEF proteins³⁴ revealed the induction of TEF protein in cells transfected with pMT-TEF when cultured in medium containing 100 μ M Zn (Figure 3A lane 4). No endogenous or leaky expression of TEF protein was detected (Figure 3A lanes 1-3). Time course analysis revealed that protein expression of TEF was detectable within 4 hours after the addition of Zn (Figure 3B lane 2) and that it reached a plateau after 16 hours (Figure 3B lanes 4-5). The expression level of TEF was related to the concentration of Zn. Upon the addition of 75 μ M Zn, the TEF expression level was approximately 20% of that induced by 100 μ M Zn (Figure 3C lanes 4-5), whereas no significant induction was obtained by the addition of less than 50 μ M Zn (Figure 3C lanes 2-3). We also established FL5.12 cells that conditionally expressed TEF/BX (Figure 3A lanes 5-6), which is a mutant form of TEF (see "Constructions of eukaryotic expression vectors"), or E2A-HLF (Figure 3A lanes 7 and 8). EMSA using the HLF-CS probe that contains the consensus binding sequence of the PAR proteins¹⁷ detected specific protein-DNA complexes in nuclear extracts from cells expressing either TEF (Figure 3D lanes 3-4) or E2A-HLF (Figure 3D lanes 7-8) but not in nuclear extracts from cells expressing TEF/BX (Figure 3D lanes 5-6). The lack of DNA-binding ability of TEF/BX was not due to aberrant subcellular localization because immunofluorescence studies showed that TEF/BX was localized in the nucleus (data not shown).

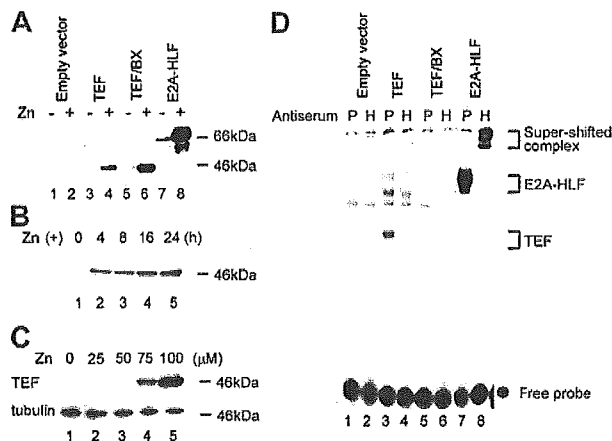


Figure 3. Establishment of FL5.12 cells conditionally expressing TEF, TEF/BX, or E2A-HLF. (A) Immunoblot analysis of FL5.12 cells using HLF(C) antiserum. Representative pools of FL5.12 cells transfected with the empty vector (lanes 1-2), pMT-TEF (lanes 3-4), pMT-TEF/BX (lanes 5-6), or pMT-E2A-HLF (lanes 7-8) were cultured in the presence (even lanes) or absence (odd lanes) of 100 μ M ZnSO₄ for 16 hours. (B) Time course analysis of TEF expression in pMT-TEF-transfected FL5.12 cells. FL5.12 cells transfected with pMT-TEF were cultured in IL-3-containing medium in the presence of 100 μ M Zn for the indicated periods of time and subjected to immunoblot analysis using the HLF(C) antiserum. (C) TEF protein expression in pMT-TEF-transfected FL5.12 cells cultured in the presence of different concentrations of Zn. FL5.12 cells transfected with pMT-TEF were cultured in the presence of Zn at the indicated concentrations for 16 hours and subjected to immunoblot analysis using the HLF(C) antiserum. The expression of tubulin was also analyzed as control. (D) Antibody-perturbed electrophoretic mobility shift analysis (EMSA) with the HLF-CS probe. FL5.12 cells transfected with the empty vector (lanes 1-2), pMT-TEF (lanes 3-4), pMT-TEF/BX (lanes 5-6), or pMT-E2A-HLF (lanes 7-8) were cultured with 100 μ M of Zn for 16 hours. Nuclear extracts from these cells were incubated with either preimmune (P; odd lanes) or anti-HLF(C) (H; even lanes) antiserum. Brackets show the mobility of DNA-protein complexes containing the indicated proteins and the ● indicates unbound, labeled oligonucleotide probes.

TEF but not E2A-HLF induced G₀/G₁ arrest

When TEF expression was induced by the addition of Zn in proliferating pMT-TEF-transfected FL5.12 cells cultured in IL-3-containing medium, cell growth was gradually disturbed and finally arrested within 36 hours (Figure 4A). In the dye exclusion assay, the viability of TEF-transfected cells was over 90% at 60 hours after the addition of Zn (data not shown). Growth arrest was observed when the Zn concentration was higher than 75 μ M (Figure 4B). Cells transfected with pMT-TEF/BX or the empty vector proliferated exponentially in culture medium containing 100 μ M Zn (Figure 4A), indicating that the growth inhibition depends on the sequence-specific DNA-binding activity of TEF and not on the cytotoxic effects of Zn. Cells transfected with pMT-E2A-HLF grew more slowly than cells transfected with the empty vector but did not undergo growth arrest (Figure 4A). Growth arrest observed in pMT-TEF-transfected FL5.12 cells was reversible after the withdrawal of Zn (Figure 4C). Cells preincubated in IL-3-containing medium in the presence of 100 μ M Zn for 12 hours were washed with medium and subsequently cultured in IL-3-containing medium either in the presence or absence of 100 μ M Zn. The expression of TEF was gradually decreased and became undetectable 72 hours after withdrawal of Zn (Figure 4D lanes 3, 5, 7), whereas TEF levels were maintained in the cells that were continued in culture in the presence of Zn (Figure 4D lanes 2, 4, 6). Interestingly, the growth arrest of cells cultured in the absence of Zn was reversed and cells began cycling approximately 72 hours after withdrawal of Zn, whereas the growth arrest and cell viability were maintained in the cells cultured in the presence of Zn (Figure 4C). Moreover, the growth of cells rescued by the withdrawal of Zn

could be arrested again upon the subsequent readdition of Zn (Figure 4C).

To determine the cause of growth arrest, cell cycle analysis was performed using flow cytometry (Table 1). Upon the addition of Zn, TEF-expressing cells in the G₀/G₁ phase gradually accumulated and nearly 80% of the cells were in the resting phase after 48 hours. On the other hand, it was unlikely that cell death contributed to this growth arrest because the population in the sub-G₀/G₁ phase always comprised less than 10% of TEF-expressing cells (data not shown). In addition, the G₀/G₁-arrested pMT-TEF-transfected FL5.12 cells were capable of re-entering the cell cycle when Zn was withdrawn from the culture medium (Figure 4E). By contrast, in cells transfected with the empty vector, regardless of the presence or absence of Zn, more than 50% of the cells were in the S phase whereas nearly 40% of the cells were in the G₀/G₁ phase. Consistent with the slow growth rate, the number of E2A-HLF-expressing cells in the S phase slightly decreased after the addition of Zn.

TEF and E2A-HLF promoted cell survival

Next, we analyzed cell viability in the absence of IL-3 (Figure 4F). Cells were precultured in the presence or absence of Zn in IL-3-containing medium for 16 hours, washed with IL-3-free medium twice, and cultured in IL-3-free medium with or without Zn. Cells expressing E2A-HLF survived more than 72 hours in IL-3-free medium, as previously reported.^{18,19} TEF also promoted cell survival in IL-3-free medium for 48 hours, but the number of viable cells gradually decreased after 72 hours even though TEF expression was maintained (data not shown). Cells expressing TEF/BX rapidly underwent apoptosis in a manner similar to that of the control cells transfected with the empty vector. These data indicated that TEF protects IL-3-dependent FL5.12 cells from apoptosis in the absence of the cytokine and that this activity depends on its DNA-binding activity.

TEF inactivated IL-3 signaling pathways

To clarify the mechanism of G₀/G₁ arrest induced by TEF, we analyzed the tyrosine phosphorylation of cellular proteins. TEF-transfected cells were preincubated in IL-3-containing medium with Zn for 18 hours, then incubated in IL-3-deficient medium with Zn for 12 hours, and then restimulated by IL-3. As previously reported by others,⁴³⁻⁴⁵ rapid tyrosine phosphorylation of a wide variety of proteins was observed in cells transfected with the empty vector (Figure 5A lanes 1-4) but not in cells expressing TEF (Figure 5A lanes 5-8), suggesting that TEF inactivated signals from the IL-3 receptor. To verify the observation made in Figure 5A more precisely, we analyzed the STAT5 pathway, MAPK pathway, and phosphatidylinositol 3-kinase (PI3K) pathway by monitoring the phosphorylation status of not only STAT5⁴⁶ and p44/42 MAPK⁴⁷ but also p70S6K⁴⁸ and Akt,⁴⁹ which are downstream proteins in the PI3K pathway⁴⁷ (Figure 5B). Each FL5.12 transfectant was cultured in IL-3-free medium in the presence or absence of Zn for 12 hours following 18 hours' preincubation in IL-3-containing medium with or without Zn, respectively; subsequently exposed to IL-3 for 5 minutes; and processed for immunoblot analysis. In the absence of Zn, IL-3 stimulation of cells transfected with pMT-TEF (Figure 5B lanes 3-4) as well as cells transfected with pMT-TEF/BX (Figure 5B lanes 5-6), pMT-E2A-HLF (Figure 5B lanes 7-8), or the empty vector (Figure 5B lanes 1-2) rapidly induced the phosphorylation of STAT5. In the presence of Zn, phosphorylation of STAT5 was completely abrogated in cells

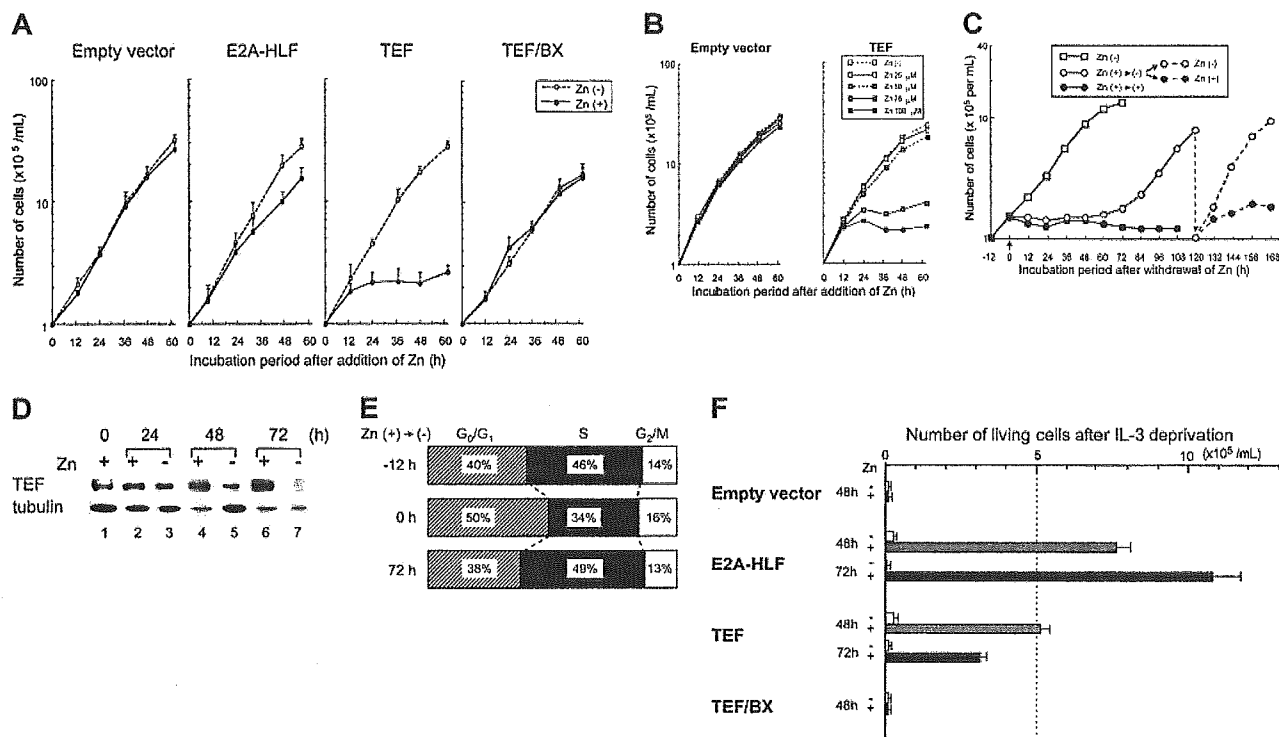


Figure 4. Growth arrest and cell survival of FL5.12 cells transfected with the TEF expression vector. (A) Growth curves of FL5.12 cells transfected with the empty vector, pMT-TEF, pMT-TEF/BX, or pMT-E2A-HLF. Cells were adjusted to 1×10^5 cells per mL and cultured in IL-3-containing medium in the presence (●) or absence (○) of 100 μ M Zn. Representative data of multiple experiments using 3 independent pools are shown. Bars indicate standard error. (B) Growth of FL5.12 cells transfected with the empty vector or pMT-TEF in IL-3-containing medium in the presence of 0, 25, 50, 75, or 100 μ M of Zn. (C) Growth curves of pMT-TEF-transfected FL5.12 cells after withdrawal of Zn. □ indicates the growth of cells cultured in IL-3-containing medium in the absence of Zn. Cells were preincubated in the presence of 100 μ M Zn for 12 hours, washed with medium, adjusted to 1.5×10^5 cells per mL, and then cultured in IL-3-containing medium either in the presence (●) or absence (○) of 100 μ M Zn. One hundred twenty hours after withdrawal of Zn, cells cultured in the absence of Zn were adjusted to 1×10^5 cells per mL and cultured in the presence (●-●) or absence (○-○) of 100 μ M Zn. The means of triplicate samples are indicated. (D) Time course analysis of TEF expression in pMT-TEF-transfected FL5.12 cells after withdrawal of Zn. Cells were preincubated in the presence of 100 μ M Zn for 12 hours, washed with medium (lane 1), then cultured in IL-3-containing medium in the presence (lanes 2, 4, and 6) or absence (lanes 3, 5, and 7) of 100 μ M Zn for the indicated periods of time and subjected to immunoblot analysis using the HLF(C) antiserum. The expression of tubulin was also analyzed as control. (E) Cell cycle analysis of pMT-TEF-transfected FL5.12 cells. Cells cultured in IL-3-containing medium (top; - 12 hours) were preincubated with 100 μ M of Zn for 12 hours (middle; 0 hour), washed with medium, and subsequently cultured in IL-3-containing medium in the absence of Zn for 72 hours (bottom; 72 hours). ▨ indicates G₀/G₁, ■, S; and □, G₂/M. (F) Number of viable cells after IL-3 deprivation of FL5.12 cells. FL5.12 cells transfected with the empty vector, pMT-TEF, pMT-TEF/BX, or pMT-E2A-HLF were precultured in IL-3-containing medium in the presence or absence of 100 μ M of Zn for 16 hours. Cells were then washed with IL-3-free medium, adjusted to 5×10^5 cells per mL, and cultured without IL-3 either in the presence (▨ and ■) or absence (□) of Zn, respectively. Upon deprivation of IL-3 for 48 or 72 hours, the numbers of viable cells as determined by trypan-blue dye exclusion are indicated. The mean data of 3 independent experiments are shown. Bars indicate standard error.

transfected with the TEF expression vector (Figure 5B lanes 11-12), whereas it was rapidly induced in the other transfectants (Figure 5B lanes 9-10 and 13-16). The same results were observed with regard to the phosphorylation of p44/42 MAPK, p70S6K, and Akt. These results indicated that TEF, acting through its DNA-binding activity, blocked IL-3-signaling pathways whereas E2A-HLF did not. This was supported by the results of EMSA (Figure 5C), which demonstrated rapid induction of protein-DNA complexes containing STAT5 following restoration of IL-3⁴² in nuclear extracts from cells transfected with the empty vector (Figure 5C

lanes 1-6) but not in cells transfected with pMT-TEF (Figure 5C lanes 7-10).

TEF induces down-regulation of the IL-3 receptor β chains

The inactivation of IL-3 signaling observed in Figure 5 could be explained by down-regulation of the IL-3 receptor by TEF. In mice, the high-affinity IL-3 receptor is a heterodimer of the IL-3-specific α chain and either of the β_{IL3} chain or the β_C chain, the latter of which is shared with the IL-5 and granulocyte-macrophage

Table 1. Cell-cycle analysis of FL5.12 cells in the presence of IL-3

Zn ⁺ , h*	Empty vector, %			TEF, %			E2A-HLF, %		
	G ₀ /G ₁	S	G ₂ /M	G ₀ /G ₁	S	G ₂ /M	G ₀ /G ₁	S	G ₂ /M
0	37	56	7	32	54	14	30	63	7
8	ND	ND	ND	39	43	17	ND	ND	ND
16	ND	ND	ND	54	27	18	ND	ND	ND
24	41	52	7	61	19	20	47	40	13
48	39	53	8	79	9	12	35	50	14

Data of representative pools of each transfectant are indicated. ND indicates not determined. *Hours of incubation with Zn.

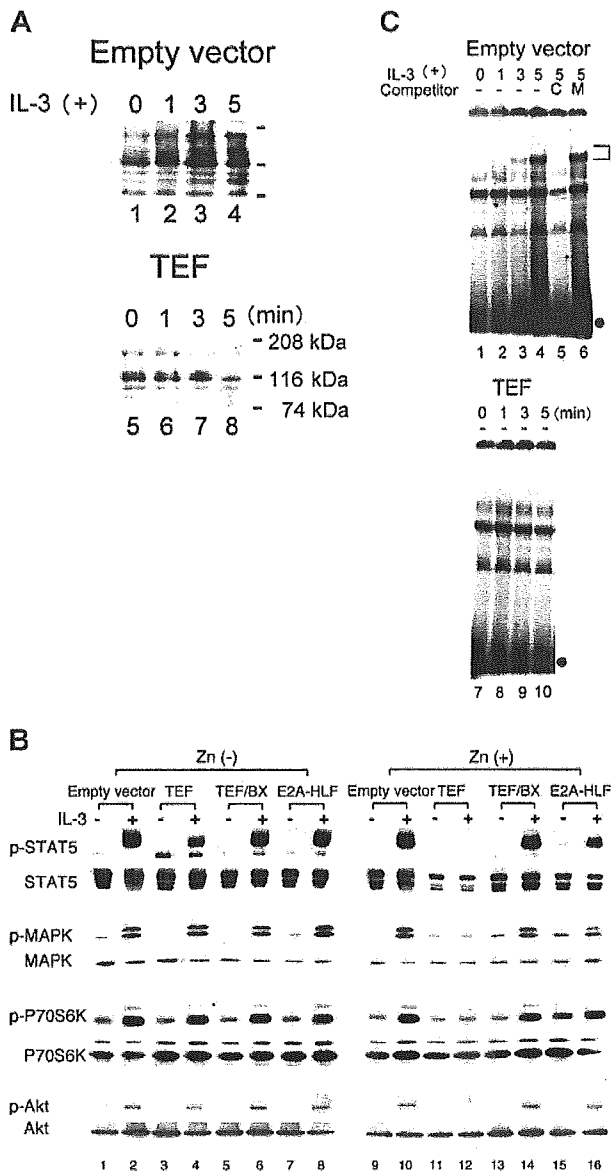


Figure 5. Phosphorylation of cellular proteins by IL-3 restoration. FL5.12 cells transfected with the empty vector, pMT-TEF, pMT-TEF/BX, or pMT-E2A-HLF were cultured in IL-3-containing medium in the presence or absence of Zn for 16 hours, and then the cells were transferred to IL-3-deficient medium in the presence or absence of Zn for 12 hours. Thereafter, cells were restimulated with IL-3 for the indicated periods of time. (A) Immunoblot analysis of FL5.12 cells that had been transfected with the empty vector (lanes 1-4) or pMT-TEF (lanes 5-8) and then cultured in IL-3-containing medium in the presence of Zn using an antiphosphotyrosine monoclonal antibody. (B) Immunoblot analysis for the phosphorylated (p) and nonphosphorylated forms of STAT5, p44/42 MAPK, p70S6K, and Akt in transfectants that had been restored with IL-3 for 5 minutes in the absence (lanes 1-8) or the presence (lanes 9-16) of Zn. The blots were probed with antiphospho-STAT5, antiphospho-p44/42 MAPK, antiphospho-p70 S6K, and antiphospho-Akt as well as anti-STAT5, anti-p44/42 MAPK, anti-p70S6K, and anti-Akt antibodies. (C) Activation of STAT5 DNA-binding by IL-3 restoration. EMSA was performed using nuclear lysates from FL5.12 cells that had been transfected with either the empty vector (lanes 1-6) or pMT-TEF (lanes 7-10) with a β -casein sequence as a probe. In the competition inhibition, an approximately 100-fold molar excess of the unlabeled β -casein sequence oligonucleotide (C; lane 5) or β -casein sequence oligonucleotide with mismatches (M; lane 6) was added in the reaction mixture. A bracket indicates the mobility of specific DNA-protein complexes and ● indicates unbound, labeled oligonucleotide probes.

colony-stimulating factor (GM-CSF) receptors.⁵⁰ Northern blot analysis was performed using an α -chain cDNA probe and a β_{IL3} chain cDNA probe; the latter recognizes both the β_{IL3} and β_C transcripts because of their extremely high levels of sequence identity (Figure 6A).⁵¹ Upon the addition of Zn to TEF-transfected

cells, the mRNA level of the β chains rapidly decreased and was almost undetectable within 16 hours. In contrast, the addition of Zn to cells transfected with either the empty vector or pMT-TEF did not change the expression level of α -chain mRNA.

Next, we analyzed the cell surface expression of the β_{IL3} , β_C , and α chains of the IL-3 receptor by flow cytometry using specific antibodies for each chain (Figure 6B). Consistent with the results of Northern blot analysis, within 24 hours after the addition of Zn, nearly complete down-regulation of the expression of both the β_{IL3} and β_C chains was observed in cells transfected with pMT-TEF but not in cells transfected with the empty vector, pMT-E2A-HLF, or pMT-TEF/BX. These data indicated that TEF down-regulates expression of the β chains in a DNA-binding-dependent fashion. Moreover, we found up-regulation of the expression of the α chain in TEF-expressing cells but not in cells transfected with the empty vector, pMT-TEF/BX, or pMT-E2A-HLF. Since Northern blot

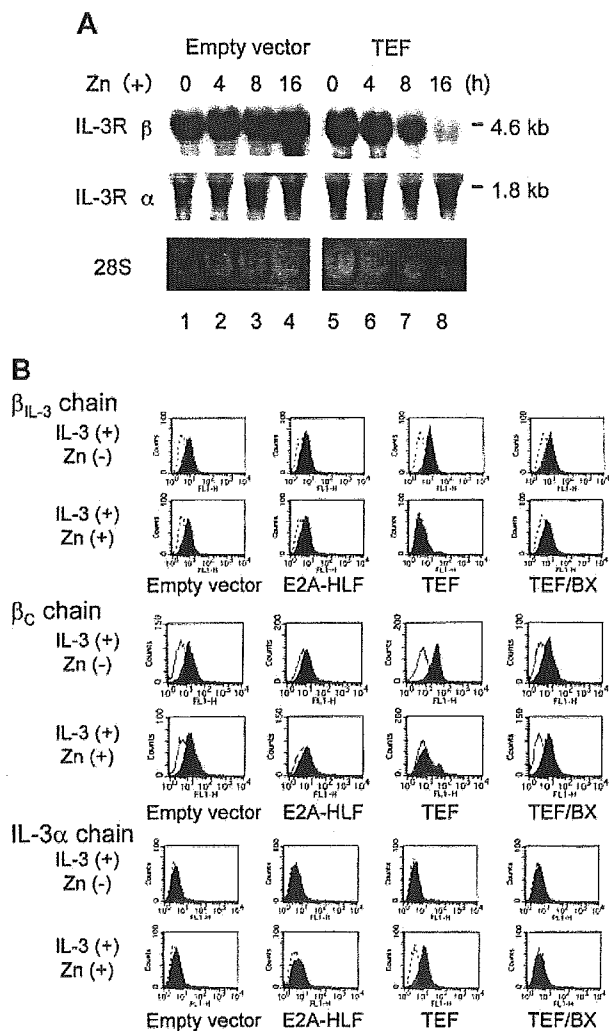


Figure 6. Northern blot analysis and flow cytometric analysis of FL5.12 cells. (A) Northern blot analysis for the IL-3 receptor. Total RNA was extracted from FL5.12 cells that had been transfected with the empty vector (lanes 1-4) or pMT-TEF (lanes 5-8) and cultured in the presence of IL-3 with Zn for the indicated periods of time. The blot was hybridized with mouse cDNA probes specific for IL-3 receptor β chains and α chain. The 28S rRNA visualized with ethidium bromide staining is shown in the bottom panel. (B) Flow cytometric analysis for surface expression of the IL-3 receptor. FL5.12 cells transfected with the empty vector, pMT-E2A-HLF, pMT-TEF, or pMT-TEF/BX were cultured in IL-3-containing medium in the presence or absence of Zn for 24 hours. Cells were analyzed with the specific antibodies for mouse β_{IL3} , β_C , or α chains. Dotted or solid lines indicate the histograms of control staining, and filled curves indicate those of specific antibodies.

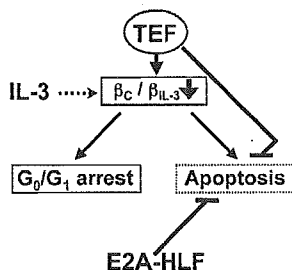


Figure 7. Hypothetical roles of TEF and E2A-HLF. TEF down-regulates the expression of the common β (β_c) chain and inactivates IL-3 signaling pathways, which are critical for both cell proliferation and cell survival. In addition, TEF promotes cell survival in the absence of survival signalings downstream of IL-3. Consequently, cells in the G_0/G_1 phase accumulated without undergoing apoptosis. In contrast, E2A-HLF blocks apoptosis without regulating the expression of cytokine receptors.

analysis revealed that the level of mRNA expression of the α chain did not change (Figure 6A), up-regulation of the cell surface α -chain expression by TEF was due to posttranscriptional mechanism(s), probably inhibition of internalization caused by down-regulation of the expression of β chains.⁵² These observations indicated that TEF disrupts formation of the IL-3 receptor by down-regulating the cell surface expression of the β chains, thus rendering FL5.12 cells insensitive to IL-3.

Discussion

In this study, using a Zn-inducible system, TEF protein expression was induced in pMT-TEF-transfected FL5.12 cells within 4 hours after the addition of Zn (Figure 3B). The mRNA expression levels of the β subunits of the IL-3 receptor were reduced and became barely detectable within 16 hours (Figure 6A), and surface expression of the β chains was almost completely down-regulated within 24 hours (Figure 6B). Subsequently, the IL-3 signaling pathways were lost (Figure 5) and cells underwent cell cycle arrest in G_0/G_1 phase within 36 hours (Figure 4A; Table 1). However, the cells did not undergo apoptosis (Figure 4A) because TEF protected FL5.12 cells from apoptosis induced by IL-3 deprivation (Figure 4F). Thus, TEF induced FL5.12 cells to remain in a resting state, either in the presence or absence of IL-3 (Figure 7). These unique functions of TEF depended on its DNA-binding potential because a TEF mutant with amino acid substitutions in its basic region (TEF-BX) completely lost these activities (Figures 4-6). However, there is no potential binding site for TEF in the promoter regions of the 2 β -chain genes in the mouse^{51,53} and TEF is known as a transcriptional activator.^{29,37} Thus, TEF likely down-regulates transcription of the β -chain genes indirectly.

The β_c chain is common to the receptors of a series of major cytokines including IL-5 and GM-CSF as well as IL-3 and is expressed by the majority of hematopoietic progenitors (H.I. and A.K., unpublished observation, May 2004; Militi et al⁵⁴), which expand in response to multiple growth factors during normal hematopoiesis. Therefore, it is reasonable that the *Tef* expression level in each hemolymphoid progenitor was low (Figure 2B). TEF expression may be restricted to a small fraction of progenitors

that are in the resting phase and do not express the β_c chain. TEF might play an important role in preventing the exhaustion of hematopoietic progenitors by rendering a portion of the progenitors to be insensitive to growth factors. Establishment of Tef-deficient mice and extensive analysis of their hematopoiesis would elucidate the biologic significance of this unique transcription factor that regulates both cell survival and expression of cytokine receptors in hematopoietic progenitors.

This study also provides insights into functional differences between oncogenic chimeras and their normal counterparts. Similar to TEF, the E2A-HLF chimera inhibited apoptosis caused by IL-3 deprivation (Figure 4F; Inaba et al,¹⁸ Inukai et al,¹⁹ Altura et al²⁰). However, unlike TEF, E2A-HLF did not down-regulate the expression of β subunits of the IL-3 receptor. As a result, cells expressing E2A-HLF proliferated in the presence of IL-3 (Figure 4A) and survived in the absence of IL-3 (Figure 4F). These findings suggest that the chimeric transcription factor retains the ability to drive leukemic transformation with maximum efficiency by disrupting multiple transcriptional networks. Since TEF and HLF share a high degree of sequence identity in their bZIP domains, the difference in the ability to regulate the levels of growth factor receptors between TEF and E2A-HLF is most likely caused by differences in their *trans*-activation domains (Figure 1). Alternatively, subtle changes in their DNA-binding specificity might contribute to the loss of activity of E2A-HLF to regulate cytokine receptors. The DNA-binding specificity of the PAR bZIP transcription factors is reported to be influenced by the sequences flanking the basic region, such as the basic region extension (BRE), fork region, and PAR domains.³⁶ Because the BRE and PAR domains of HLF are not included in the E2A-HLF chimera (Figure 1), the DNA-binding potential of E2A-HLF to suboptimal sites is substantially impaired.³⁴

The antiapoptotic potential of TEF that depends on its DNA-binding activity supports a model that the consensus binding sequence recognized by the PAR factors as well as E2A-HLF, E4BP4, and CES-2 could serve as a regulatory switch for programmed cell death.^{18-20,23,24} However, the detailed downstream pathways in mammalian systems have not yet been elucidated. In *C. elegans*, CES-2 appears to down-regulate CES-1 expression.^{5,6} Although we previously identified the *SLUG* gene, a mammalian homologue of CES-1, as one of the downstream responders of E2A-HLF in human leukemia cells with t(17;19) translocation,²³ neither TEF nor E2A-HLF was able to induce its expression in FL5.12 cells (data not shown). Thus, there must be other critical downstream pathway(s) for the antiapoptotic potential of TEF and E2A-HLF. Identification of downstream pathway(s) of TEF for its antiapoptotic potential and β_{IL3} and β_c chain gene regulation will be critical for clarifying transcriptional regulation driving normal hematopoiesis as well as leukemogenesis by E2A-HLF.

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RESEARCH ARTICLE

Efficient and stable Sendai virus-mediated gene transfer into primate embryonic stem cells with pluripotency preserved

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Efficient gene transfer and regulated transgene expression in primate embryonic stem (ES) cells are highly desirable for future applications of the cells. In the present study, we have examined using the nonintegrating Sendai virus (SeV) vector to introduce the green fluorescent protein (GFP) gene into non-human primate cynomolgus ES cells. The GFP gene was vigorously and stably expressed in the cynomolgus ES cells for a year. The cells were able to form fluorescent teratomas when transplanted into immunodeficient mice. They were also

able to differentiate into fluorescent embryoid bodies, neurons, and mature blood cells. In addition, the GFP expression levels were reduced dose-dependently by the addition of an anti-RNA virus drug, ribavirin, to the culture. Thus, SeV vector will be a useful tool for efficient gene transfer into primate ES cells and the method of using antiviral drugs should allow further investigation for regulated SeV-mediated gene expression. Gene Therapy (2005) 12, 203–210. doi:10.1038/sj.gt.3302409
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Introduction

Since human embryonic stem (ES) cell lines have the ability to both proliferate indefinitely and differentiate into multiple tissue cells,^{1,2} they are expected to have clinical applications as well as to serve as models for basic research and drug development. Although efficient and stable gene transfer into primate ES cells would be useful for such purposes, it has been difficult and only lentiviral vectors have been successful in achieving it.^{3–5} We have previously developed Sendai virus (SeV) vectors that replicate in the form of negative-sense single-stranded RNA in the cytoplasm of infected cells and do not go through a DNA phase.⁶ SeV vectors can efficiently introduce foreign genes without toxicity into airway epithelial cells,⁷ vascular tissue,⁸ skeletal muscle,⁹ synovial cells,¹⁰ retinal tissue,¹¹ and hematopoietic progenitor cells.¹² Here we report that the SeV-mediated gene transfer into primate ES cells is very efficient and stable even after the terminal differentiation of the cells. In addition, we show that SeV-mediated transgene expression levels can be reduced by the addition of a ribonucleoside analog, ribavirin, to the culture. Ribavirin is a mutagen and inhibitor of viral RNA polymerase.^{13,14} It shows antiviral activity against a variety of RNA viruses and is used to treat infections of hepatitis C virus in combination with interferon- α .^{15,16} and of lassa

fever virus.¹⁷ The method of using antiviral drugs might offer a novel approach for regulated SeV-mediated gene expression in primate ES cells.

Results

SeV-mediated gene transfer into ES cells

In this study, we have used an SeV vector, which is capable of self-replication but incapable of transmitting to other cells.⁶ The vector does not encode the fusion (F) protein (Figure 1a), which is essential for viral entry into cells. It can be propagated only in a packaging cell line expressing the F protein. The green fluorescent protein (GFP) gene was introduced after the leader sequence of the vector genome. Cynomolgus ES cells¹⁸ were exposed to the SeV vector for 24 h. Flow cytometric analysis at 2 days after infection showed that 15, 38, and 61% of cells fluoresced at 2, 10, and 50 transducing units (TU) per cell, respectively (Figure 1b). The gene transfer efficiency of about 60% is comparable to or even better than that for lentiviral vectors.³ We confirmed that the undifferentiated cell fractions remained unchanged after the infection with SeV vector, as assessed by the expression of undifferentiated markers, alkaline phosphatase and SSEA-4 (data not shown). The GFP expression after infection was stable at least for a month. On the other hand, the GFP gene transfer to cynomolgus ES cells with adenovirus- and adeno-associated virus (AAV)-based vectors resulted in much lower expression levels (<20% by flow cytometry) and the levels declined to zero within a week after infection (Figure 1c).

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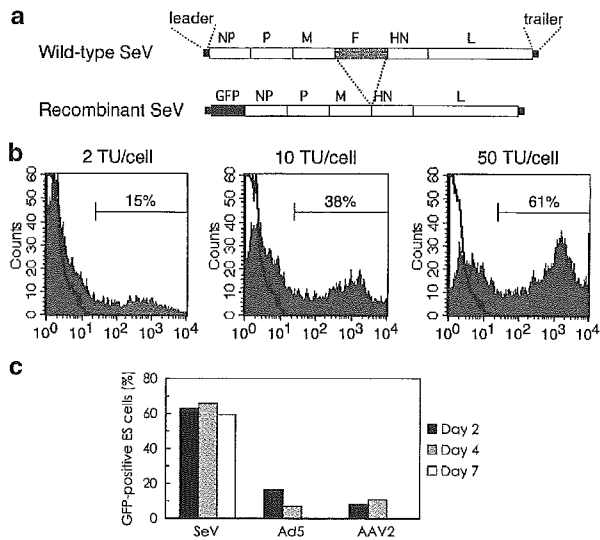


Figure 1 High-level transgene expression in cynomolgus ES cells after infection with SeV vector. (a) Schematic diagrams of the wild-type SeV genome and recombinant F-defective SeV carrying the GFP gene (SeV vector in this study). The SeV genome is 15 384 nucleotides long and its genes (NP, P, M, F, HN, and L) are in order from 3' to 5' in the negative-strand RNA. In the SeV vector, the entire fusion (F) gene was removed and the GFP gene was introduced at a unique NotI site between the leader sequence and NP gene. (b) The GFP expression by the SeV vector in cynomolgus ES cells. Cynomolgus ES cells were infected with the SeV vector at 2, 10, and 50 TU/cell. The flow cytometric profiles at day-2 postinfection are shown in gray. The white areas indicate uninfected ES cells. The fractions of GFP-positive cells are indicated. (c) The GFP expression levels in cynomolgus ES cells infected with the SeV (50 TU/cell), adenovirus serotype 5 (Ad5, 3.4×10^2 g.c./cell), and AAV serotype 2 (AAV2, 2.4×10^4 g.c./cell) vectors. The fractions of GFP-positive cells were examined by flow cytometry at 2, 4, and 7 days postinfection.

We plucked fluorescent ES cell colonies under a fluorescent microscope once at 1 month after infection and propagated them. After this selection procedure, approximately 90% of the ES cells expressed GFP (Figure 2a and b) and the high-level expression was stable for a year as assessed by flow cytometry (Figure 2c, upper). The mean fluorescence intensity per cell was also stable (Figure 2c, lower), indicating that the replicating vector genome was almost equally delivered to each cell of all progeny. The self-replication of the SeV vector in infected cells was confirmed by RNA-PCR that amplified the viral RNA genomic sequence (Figure 3a). The GFP cDNA sequence, however, could not be detected by DNA-PCR in the infected cells (Figure 3b), indicating that no DNA phase was involved in the GFP expression.

Pluripotency of infected ES cells

The SeV-infected, fluorescent cynomolgus ES cells were able to form fluorescent tumors when transplanted into immunodeficient mice (Figure 4a–c). The fluorescence was observed uniformly by fluorescent microscopy (Figure 4d and e). The tumors consisted of all three embryonic germ layer cells (Figure 4f–i). Thus, the SeV-infected ES cells were capable of forming teratomas and the SeV infection did not spoil the pluripo-

tency of ES cells. The infected, fluorescent cynomolgus ES cells were also able to generate fluorescent embryoid bodies (Figure 5a and b), MAP-2-positive neurons (Figure 5c), clonogenic hematopoietic colonies (Figure 5d and e), and mature functional (NBT test-positive) neutrophils (Figure 5f and g), all of which fluoresced. In addition, the GFP expression levels were not decreased during the teratoma formation or differentiation, indicating that no 'silencing' of the transgene occurred.

Drug-inducible reduction of transgene expression

Next, we examined whether ribavirin inhibits the replication and transcription of the SeV vector resulting in a reduction of transgene expression. We first used a rhesus monkey kidney cell line (LLC-MK2) to test the effect of ribavirin on the replication and transcription of the SeV vector. LLC-MK2 is a standard control cell line for SeV infection. Ribavirin was added at various concentrations 2 days after the infection. The formation of viral particles quantified by the hemagglutination assay decreased drastically upon the addition of ribavirin (Figure 6a). The decrease was dependent on the dose of ribavirin. The GFP expression was also depressed dose-dependently (Figure 6b). Thus, ribavirin dose-dependently inhibits the replication and transcription of the SeV vector in LLC-MK2 cells. The toxicity associated with ribavirin was not observed in LLC-MK2 cells.

We then examined the effect of ribavirin on SeV-infected, fluorescent cynomolgus ES cells. The addition of ribavirin also resulted in a dose-dependent reduction of GFP expression in the cells (Figure 6c). Although the GFP expression was almost completely inhibited after a 3-day exposure with 4 mM of ribavirin, the cells could not be propagated thereafter. Ribavirin at high concentrations (>1 mM) hampered the proliferation of cynomolgus ES cells. With lower concentrations (0.5–0.75 mM) of ribavirin, the GFP expression level decreased by half. After the discontinuation of ribavirin treatment, the cells could be propagated and nearly regained the original level of GFP expression. The undifferentiated cell fractions were unchanged after the discontinuation as assessed by alkaline phosphatase and SSEA-4 staining (Figure 6d).

Discussion

There are several advantages in using SeV vectors over other vectors. (i) SeV vectors can infect nondividing, quiescent cells as well as dividing cells unlike oncoretroviral vectors.^{7–11} Thus, they can be used to infect cells that are terminally differentiated as well as at various stages of differentiation, whether they are dividing or not. (ii) SeV vector-mediated gene transfer does not require a DNA phase. Thus, there is no concern about the unwanted integration of foreign sequences into the host genome unlike with oncoretroviral or lentiviral vectors. (iii) Transgene expression is stable even in dividing cells since the SeV vector replicates by itself in the cytoplasm of host cells. On the other hand, gene transfer using nonreplicating adenoviral and AAV vectors resulted in decreased levels of transgene expression in dividing cells over time, since the non-replicating transgene was

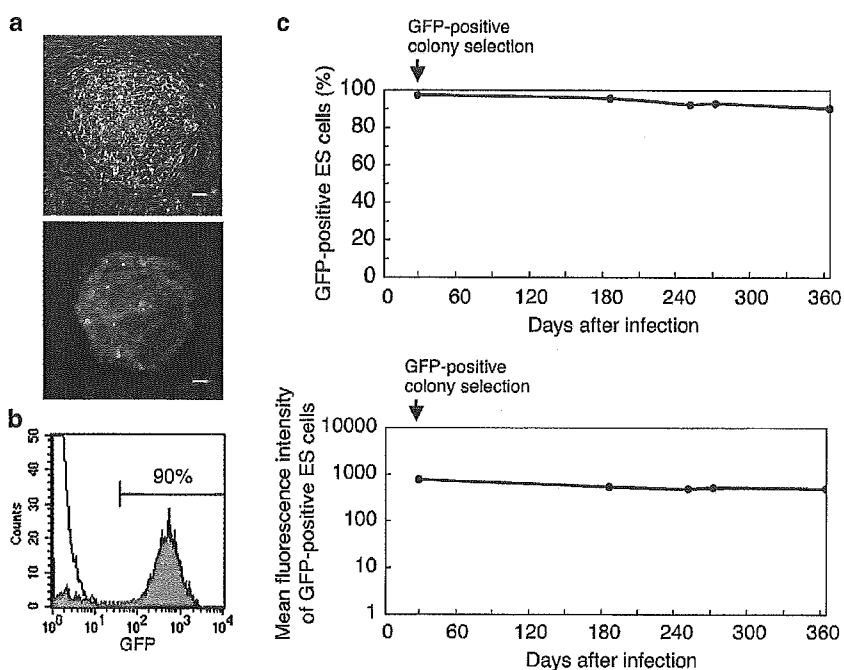


Figure 2 Stable SeV-mediated transgene expression in cynomolgus ES cells. Fluorescent ES cell colonies were plucked under a fluorescent microscope once at 1 month after infection and the cells were further propagated. (a) Phase-contrast (upper) and fluorescence (lower) images of a cynomolgus ES cell colony at day 370 after infection. Bar = 100 μ m. (b) Flow cytometric analysis of SeV-infected cynomolgus ES cells at day 370 after infection (shown in green). The percentage of GFP-positive cells is indicated. Uninfected, parental cynomolgus ES cells are indicated by another line (white area). (c) The percentage of GFP-positive cells (upper) and mean fluorescence intensity per GFP-positive cell (lower) after infection with the SeV vector at 10 TU/cell are shown as a function of time (days).

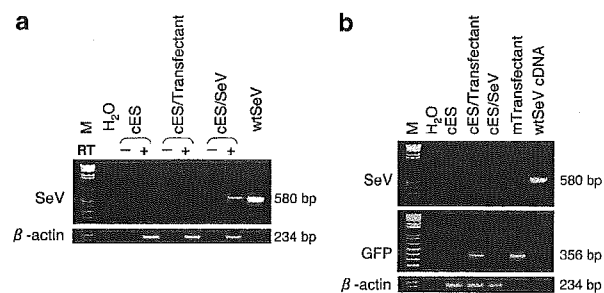


Figure 3 DNA-independent replication and transcription of SeV vector. Total cellular RNA and DNA were extracted from cynomolgus ES cells at day 284 after infection with the SeV vector. RNA-PCR (a) and DNA-PCR (b) for the SeV RNA genome or GFP sequence were conducted. The cynomolgus β -actin sequence was used as an internal control. In the RNA-PCR (a), negative results obtained without reverse transcriptase (designated RT-) confirmed that the amplified products were not derived from cellular DNA. M, 100-kb DNA ladder; cES, naive cynomolgus ES cells; cES/Transfectant, cynomolgus ES cells stably expressing the GFP gene after transfection; cES/SeV, cynomolgus ES cells infected with the SeV vector; wtSeV, wild-type SeV genome; mTransfectant, a GFP-positive mouse cell line after transfection.

diluted out. (iv) The SeV vector is much less unlikely to generate wild-type virus *in vitro* or *in vivo* than oncoretroviral and lentiviral vectors, since homologous recombination between RNA genomes is very rare indeed in negative-strand RNA viruses.¹⁹ (v) The SeV genome is not subject to cellular epigenetic modifications

such as methylation, and thus it is unlikely that methylation-based silencing of transgene expression occurs.

No cytotoxic or differentiating effect on ES cells associated with the SeV infection was observed in our study. However, the wild-type SeV contains immunogenic surface proteins, hemagglutinin-neuraminidase (HN) and F proteins, which potentially induce antibody responses.^{20,21} For future clinical applications, it would be desired that as many viral genes as possible are deleted from the vector backbone to permit reapplication, improve the safety, and lessen the possible toxicity of SeV vectors. To this end, we have developed a series of attenuated SeV vectors that are F gene-deleted,⁶ F gene-deleted with preferable mutations,²² M gene-deleted,²³ or have deletions of both F and M genes.²⁴ The modified vectors would be safer for *in vivo* use.

Ribavirin at high concentrations seems toxic to ES cells; presumably, it directly hampers viability and proliferation potential of ES cells. However, we cannot tell whether the observed toxicity is simply due to its toxicity to ES cells, as feeder cells are more highly sensitive to ribavirin than ES cells. In fact, while feeder cells died at 1 mM of ribavirin, cocultured ES cells were alive at this concentration for some time. Cynomolgus ES cells lose pluripotency and proliferation potential without feeder cells. Thus, the observed toxicity to ES cells may also be a secondary event following the injury of feeder cells. Whether the cytotoxicity is primary or secondary, it will be necessary to find modified compounds of less cytotoxicity.

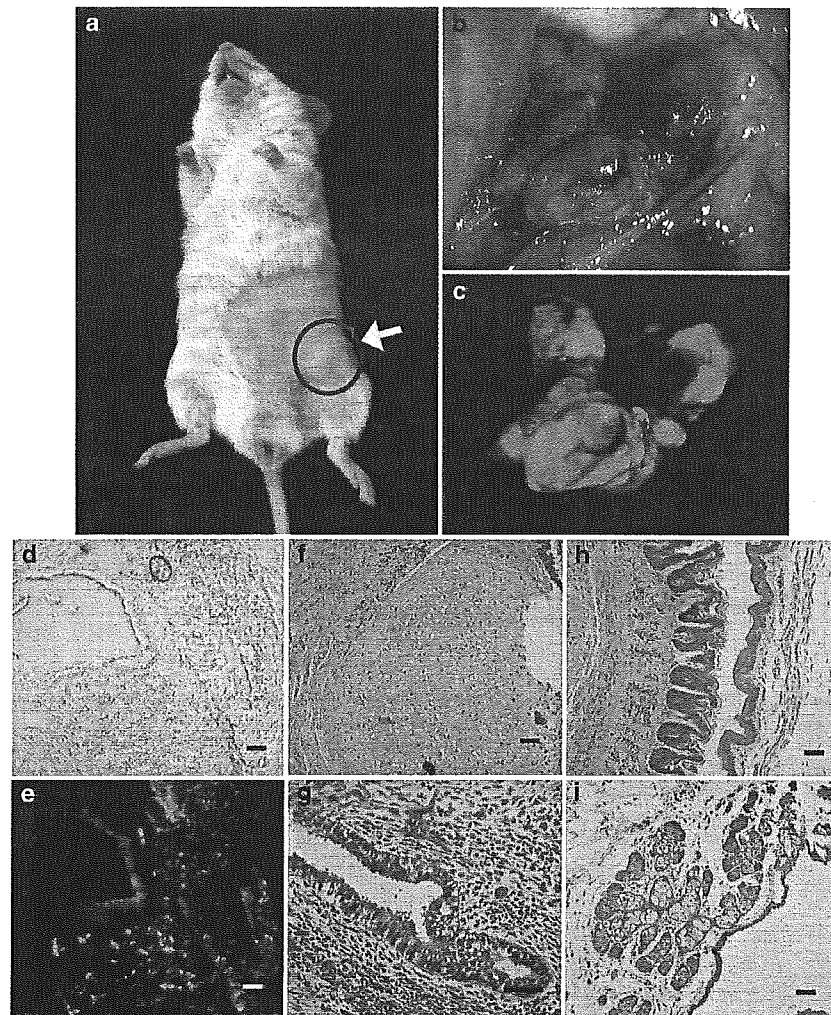


Figure 4 Pluripotency of SeV-infected cynomolgus ES cells. Tumors formed in NOD-SCID mice after inoculation of the SeV-infected cynomolgus ES cells (a). The tumor was fluorescing ((b), bright field; (c), dark field). Fluorescence was observed uniformly in the tumor under a fluorescent microscope ((d), bright field; (e), dark field). The tumor contained all three embryonic germ layer cells; cartilage (f), ciliated columnar epithelium (g), skin (h), and sebaceous gland (i) (stained with hematoxylin and eosin). Bar = 100 μ m.

Materials and methods

Cell culture

Cynomolgus ES cells (CMK6) were maintained on a feeder layer of mitomycin C (Kyowa, Tokyo, Japan)-treated mouse (BALB/c) embryonic fibroblasts as described previously.¹⁸ The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 15% ES cell-qualified fetal calf serum (FCS; Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma, St Louis, MO, USA), 2 mM glutamine (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin, Irvine Scientific, Santa Ana, CA, USA). The ES cell colonies were routinely passaged every 3–4 days after dissociation with a combined approach of 0.25% trypsin (Invitrogen) digestion and mechanical cutting. Alkaline phosphatase staining was conducted with an Alkaline Phosphatase Chromogen Kit

(Biomedex, Foster City, CA, USA). Embryoid bodies were produced by culturing ES cell aggregates in Petri dishes. LLC-MK2 cells (1×10^6) were grown in six-well plates and cultured in Eagle's minimal essential medium (Invitrogen) supplemented with 10% FCS.

Vectors

The F-defective SeV vector carrying the GFP gene was constructed as previously described.⁶ The vector titer was 1.8×10^9 TU/ml determined by counting fluorescent cells after the infection of LLC-MK2 cells. Gene transfer was conducted by adding various concentrations of the SeV vector solution to culture media. After 24 h of incubation, the cells were washed twice with phosphate-buffered saline (PBS) and fresh medium was added. In some experiments, ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide; Sigma) was added at various concentrations to the culture media after infection. The

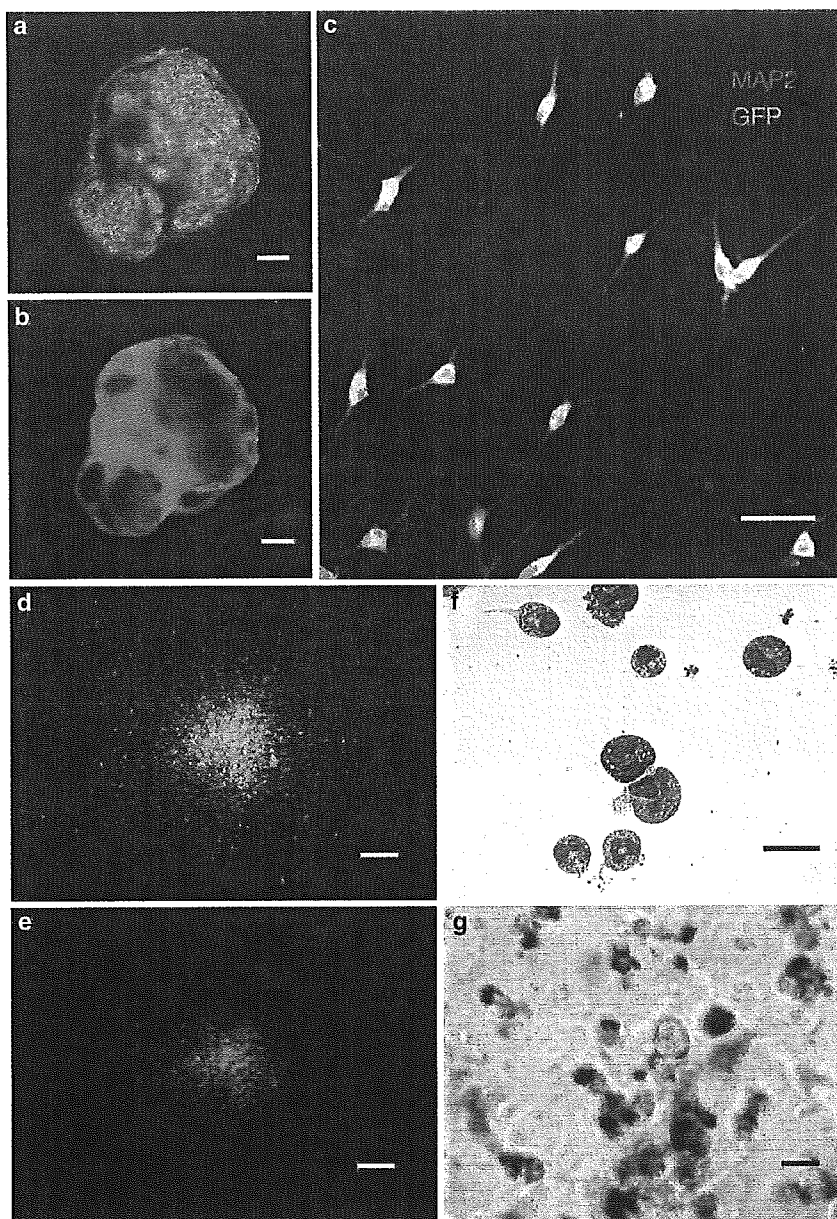


Figure 5 Stable transgene expression during differentiation. A day-20 cystic embryoid body was observed under a fluorescent phase-contrast microscope, confirming that the embryoid body was fluorescing ((a), bright field; (b), dark field). After infection with the SeV vector, fluorescent cynomolgus ES cells differentiated into neural cells. Double immunostaining with anti-GFP (green) and anti-MAP-2 (red) confirmed that differentiated neural cells expressed GFP (c). Yellow cells indicate GFP-expressing neurons. SeV-infected, fluorescent cynomolgus ES cells also differentiated into fluorescent hematopoietic cells. A clonogenic hematopoietic colony was fluorescing ((d) bright field; (e), dark field). A cytospin specimen of hematopoietic colony cells (Wright-Giemsa staining) showed that the cells were mature granulocytes (f). The infected ES cell-derived, fluorescent neutrophils were positive for NBT (stained in black (g)). Bar = 100 μ m (a, b, g); 50 μ m (c, f); 500 μ m (d, e).

viral particles in infected cells were quantified by a hemagglutination assay as described previously.²⁵

An adenovirus serotype 5-based vector carrying the GFP gene was constructed as reported.²⁶ It contained the cytomegalovirus (CMV) promoter, simian virus (SV)-40 intron, and SV-40 polyadenylation signal. An AAV serotype 2-based vector expressing the GFP gene under the control of the chicken β -actin promoter with the CMV immediate-early enhancer (a gift from Dr J Miyazaki)

was prepared as described previously.²⁷ Gene transfer experiments were performed using 3.4×10^2 genome copies (g.c.)/cell of the adenoviral vector or 2.4×10^4 g.c./cell of the AAV vector. The period of exposure was 48 h.

Flow cytometry

GFP and SSEA-4 expression was analyzed on a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA) using the

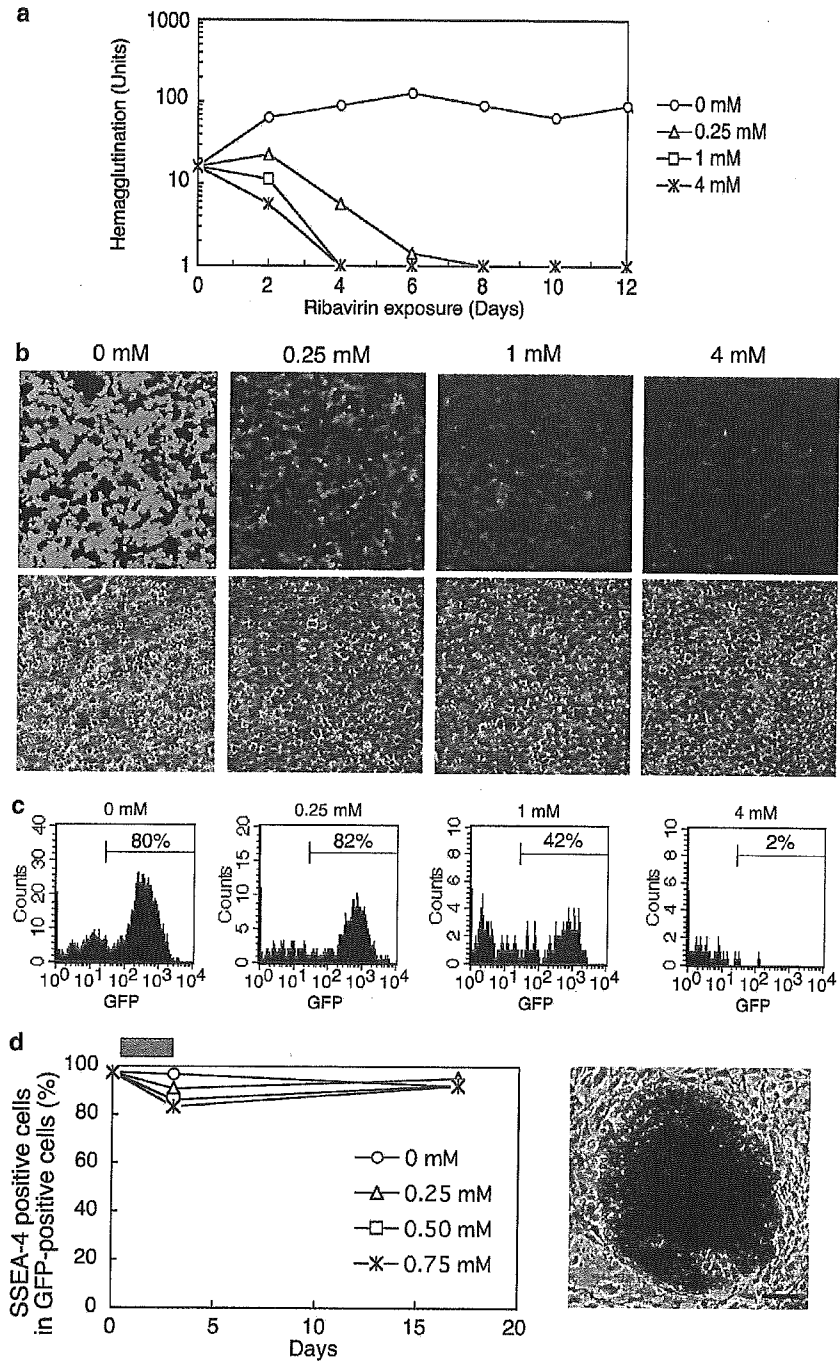


Figure 6 Ribavirin-regulated transgene expression. (a) A rhesus kidney cell line (LLC-MK2) was infected with the SeV vector at 3 TU/cell. Ribavirin was started at various concentrations on day 2 after the infection. The formation of viral particles in the infected LLC-MK2 cells was examined by the hemagglutination assay. (b) The ribavirin-treated LLC-MK2 cells were observed under a fluorescent microscope after an 8-day exposure of ribavirin (upper, dark field; lower, bright field). (c) Ribavirin was added at various concentrations to the SeV-infected, fluorescent cynomolgus ES cells. The GFP expression was assessed by flow cytometry after a 3-day exposure of ribavirin. (d) The fractions of SSEA-4-positive ES cells were assessed by flow cytometry with anti-SSEA-4 before and after a 3-day exposure of ribavirin and are shown as a function of time (days) in the left panel. A gray bar indicates ribavirin treatment. ES cells were stained for alkaline phosphatase (in red) at day 21 after a 3-day exposure of 0.75 mM ribavirin and are shown in the right panel. Bar = 100 μ m.

CellQuest software (Becton Dickinson). For SSEA-4 staining, cells were incubated with a primary antibody, anti-SSEA-4 (MC-813-70; Chemicon, Temecula, CA, USA), and then a secondary antibody, PE-conjugated

F(ab')₂ fragment of rabbit anti-mouse immunoglobulins (DakoCytomation, Glostrup, Denmark). Cocultured BALB/c feeder cells could be distinguished from cynomolgus ES cells by using PE-conjugated anti-mouse

H-2d (SF1-1.1; PharMingen, San Diego, CA, USA), which does not react to cynomolgus cells but does react to BALB/c cells.

Teratoma formation

Cynomolgus ES cells (approximately 10^6 cells per site) were injected subcutaneously into the hind leg of 6- to 8-week-old nonobese diabetic/severe combined immunodeficient mice (Jackson Laboratory, Bar Harbor, ME, USA). The resulting tumors (usually 9–12 weeks after the injection) were dissected and fixed in 4% paraformaldehyde. For histological analysis, samples from the tumors were embedded in paraffin and stained with hematoxylin and eosin. To observe GFP fluorescence, samples were embedded in OTC compound (Sakura, Zoeterwoude, Netherlands), frozen, sectioned, and examined under a fluorescence microscope.

Hematopoietic differentiation

The mouse bone marrow stromal cell line OP9 was maintained in α -modified minimum essential medium (Invitrogen) supplemented with 20% FCS as described previously.²⁸ For induction of hematopoietic differentiation, ES cells were seeded onto a mitomycin C-treated confluent OP9 cell layer in six-well plates. Medium to support the differentiation was described elsewhere.²⁹ Cells at day 18 were placed in Methocult GF+ media (StemCell Technologies, Vancouver, Canada) at 1×10^4 and 1×10^5 cells per plate and clonogenic hematopoietic colonies were produced. After 14 days, individual colonies were removed and spun onto glass slides. Cells were stained with the Wright–Giemsa method. The nitro blue tetrazolium (NBT, Sigma) reduction test was performed on the cells as a granulocyte functional assay according to a previously described method.³⁰

Neural differentiation

The induction of neural differentiation was carried out as described previously.³¹ Day-4 embryoid bodies were plated onto tissue culture dishes and nestin-positive cells were selected in DMEM/F12 medium supplemented with 5 μ g/ml of insulin (Sigma), 50 μ g/ml of transferrin (Sigma), 30 nM selenium chloride (Sigma), and 5 μ g/ml of fibronectin (Sigma) for 5 days. Cells were then trypsinized and plated in polyornithine-coated dishes (15 μ g/ml) and expanded in N2 medium³² supplemented with 1 μ g/ml of laminin (Sigma) and 10 μ g/ml of basic fibroblast growth factor (bFGF; Roche, Basel, Switzerland) for 6 days. Differentiation was induced by removal of bFGF. To confirm the neural differentiation, cells were stained with anti-human MAP-2. Briefly, cells were fixed in 4% paraformaldehyde in PBS and incubated with anti-human MAP-2 (HM-2; Sigma; diluted 1:4000) and then by Alexa Fluor 594-labeled antibody (diluted 1:500; Molecular Probe, Eugene, OR, USA). The samples were examined under a fluorescence microscope.

DNA-PCR

DNA-PCR for the SeV genome and GFP sequences was carried out as follows. DNA was extracted using the QIAamp DNA mini kits (Qiagen, Hilden, Germany) and 250 ng was used for each PCR with ExTaq (Takara, Shiga, Japan). Amplification conditions were 30 cycles of 94°C for 1 min, a variable annealing temperature (noted

below) for 1 min, and 72°C for 1 min. The amplified products were run on 2% agarose gel and visualized by ethidium bromide staining. Primer sequences, annealing temperatures and product sizes were as follows: the SeV vector genome sequence: 5'-AGA GAA CAA GAC TAA GGC TAC C-3' and 5'-ACC TTG ACA ATC CTG ATG TGG-3' (55°C, 580 bp); the GFP sequence: 5'-CGT CCA GGA GCG CAC CAT CTT C-3' and 5'-GGT CTT TGC TCA GGG CGG ACT-3' (60°C, 356 bp). the cynomolgus β -actin sequence: 5'-CAT TGT CAT GGA CTC TGG CGA CGG-3' and 5'-CAT CTC CTG CTC GAA GTC TAG GGC-3' (60°C, 234 bp).

RNA-PCR

RNA-PCR for the SeV RNA genomic sequence was carried out as follows. Total RNA was extracted using RNA STAT-60 (Tel-Test, Friendswood, TX, USA). Reverse transcription was conducted by using Taqman reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). The product (250 ng) after the reverse transcription was used for the subsequent PCR as described above.

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