### Registration of *FIR* heterozygous knockout mice [FIR$^{fl/+}$/Cre(+)]

FIR heterozygous knockout mice were established, registered, and made available at the National Institute of Biomedical Innovation (http://animal.nibio.go.jp/j_FIR.html) and the experimental animal division of the RIKEN Bioresource Center, Japan (RBRC No. RBRC05542; http://www2.brc.riken.jp/lab/animal/search.php). Briefly, two loxP sites were inserted upstream of *FIR* exon 3 and downstream of exon 5, respectively, and a PGK-neo cassette and a loxP site was inserted downstream of exon 5. *FIR*-deficient mice could be generated by crossing with tissue-specific Cre mice to give *Mus musculus* C57BL/6-FIR<tm1>/CU.

### TP53-null mice; *TP53*$^{-/-}$

The p53-null mice (*TP53*$^{-/-}$) were purchased from RIKEN BRC (Bio-Resource Center, Tsukuba, Japan; BRC_No 01361, strain name C57BL-p53+/-).

### CAG-Cre-transgenic mice

These mice were a gift from Dr T. Miki [47].

### Measuring the bodyweight and survival curves of mice

The bodyweight of all mice was measured twice a week after the age of 7 weeks. The nose-to-anus length was also measured (Figure S2).

### Immunocytochemistry

Cancer cells were prepared for immunocytochemistry as described previously [8].

### Flow cytometry and cell sorting

The antibodies used for immunostaining and flow cytometry are listed in Table S6. Flow cytometry and cell sorting were performed as described previously [48, 49]. Briefly, linage surface marker antibodies for thymocytes, splenocytes, peripheral blood cells, and bone marrow were Gr-1 (Ly-G6, bone-marrow derived cells), Mac-1 (CD11b, granulocyte, macrophage, etc), B220, CD4 (helper/induced T cell marker), CD8α (cytotoxic T cell marker), CD45.2 (Leukocyte common antigen). Dead cells were eliminated by staining with propidium iodide (1 μg/mL; Sigma-Aldrich). After cell surface staining, intracellular staining was performed using a FITC-conjugated anti-c-Myc antibody. Intraprep™ (Beckman Coulter) was used for fixation and permeabilization. All flow cytometric analyses and cell sorting were performed on FACSAria II or FACSCanto II (BD Biosciences) and analyzed using FACSDiva software (BD Biosciences) and Flowjo (Tree Star, Ashland, OR, USA).

### siRNA

*c-myc* and *TP53* siRNA duplexes were purchased from Sigma-Aldrich (Tokyo, Japan). The transient transfection of siRNAs was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The transfected cells were cultured for 48–72h at 37°C in a $CO_2$ incubator. The target sequences for the siRNAs are listed in Supplementary Table 4.

### Bleomycin treatment

The DNA-damaging agent bleomycin was purchased from Sigma-Aldrich (sulfate powder from *Streptomyces verticillus*; Tokyo, Japan; Lot no.BCBG6499V; PCode, 101203713), dissolved in distilled $H_2O$ at a concentration of 5 mg/mL, and stored at −20°C. HLF cells were seeded in 6-well plates and incubated at 37°C/5% $CO_2$ until confluent (approximately 24 h). Immediately before drug treatment, the media were removed and replaced with fresh culture media. Cells were treated with 30 μg/mL bleomycin alone or co-treated with adenovirus vectors.

### Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using TRIzol LS solution (Invitrogen) and reverse-transcribed using the ThermoScript RT-PCR system (Invitrogen) with oligo-dT primers. qRT-PCR was performed using an ABI Prism 7300 Thermal Cycler (Applied Biosystems) with FastStart Universal Probe Master (Roche) and Universal Probe Library (Roche). Primers and probes for mouse were listed (Table S5B).

### FIR and FIRΔexon2 adenovirus vectors

FIR and FIRΔexon2 adenovirus vectors were prepared as described previously [18].

### Protein extraction and western blotting

Culture media were removed and the cells were washed twice with cold (4°C) PBS, lysed with 1:20 β-mercaptoethanol and 2x sample buffer, and incubated at 100°C for 5 min. Whole cell lysates were assayed for

protein content (Bio-Rad, Hercules, CA, USA), and 10 μg protein samples were separated using SDS-PAGE on 7.5% and 10%–20% XV PANTERA gels. They were then transferred to polyvinylidene fluoride membranes using a tank transfer apparatus, and the membranes were blocked with 0.5% skimmed milk in PBS overnight at 4°C. Membranes were incubated with primary antibodies for 1 h at room temperature, followed by three 10-min washes with PBS/0.01% Tween 20. Membranes were then incubated with secondary antibodies, followed by three 15-min washes with PBS/0.01% Tween 20. Details of the antibodies used in this study are listed in Table S6. Antigens on the membranes were detected using enhanced chemiluminescence detection reagents (GE Healthcare UK Ltd., Buckinghamshire, UK).

### RNA-sequencing

Total RNA was extracted form sorted $CD4^{+}CD8^{+}$ and $CD4^{low+}CD8^{+}$ thymic lymphoma cells from *FIR*$^{+/-}$*TP53*$^{-/-}$ and *FIR*$^{+/+}$*TP53*$^{-/-}$ mice using TRIzol. The RNA quality was analyzed using a 2100 Bioanalyzer system (Agilent, Santa Clara, CA) to confirm that their RINs (RNA integrity numbers) were > 7.0. RNA-seq was performed to analyze genome-wide gene expression levels. Specifically, RNA-seq libraries were prepared using a TruSeqStranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA) followed by sequencing using a HiSeq1500 genome sequencer (Illumina), according to the manufacturer's protocol. The gene expression levels in *FIR*$^{+/-}$*TP53*$^{-/-}$ mice were compared with those in *FIR*$^{+/+}$*TP53*$^{-/-}$ mice, and the top 100 upregulated genes (Table S2C) were analyzed using KEGG (Kyoto Encyclopedia of Genes and Genomes) software (http://www.genome.jp/kegg/). Signaling pathways with a FDR (false discovery rate) < 1.0 were selected as significantly activated pathways.

### Statistical analysis

The expression of SAP155 and FIR was compared in the lungs, intestine, heart, kidney, and liver of FIR heterozygous knockout adult mice and 14-day-old fetal mice (E14) using Student's *t*-tests and the Wilcoxon test. The WBC, RBC, and platelet counts, organs' weight curve, and the ratio of *FIRΔexon2*/*FIR* mRNA were analyzed statistically using Student's *t*-tests. Overall survival curves were generated using the Kaplan–Meier method and analyzed statistically using log-rank tests. Statistical analyses were performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA).

### Accession number and genetic information of FIR genome

Ensemble NM_014281.

### Conflicts of interest

We have no potential conflicts of interest to disclose.

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ORIGINAL ARTICLE

# Non-transmissible Sendai virus vector encoding *c-myc* suppressor FBP-interacting repressor for cancer therapy

Kazuyuki Matsushita, Hideaki Shimada, Yasuji Ueda, Makoto Inoue, Mamoru Hasegawa, Takeshi Tomonaga, Hisahiro Matsubara, Fumio Nomura

Kazuyuki Matsushita, Fumio Nomura, Department of Molecular Diagnosis, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan
Hideaki Shimada, Department of Surgery, School of Medicine, Toho University, Tokyo 143-8541, Japan
Yasuji Ueda, Makoto Inoue, Mamoru Hasegawa, DNAVEC Corporation, 6 Ohkubo, Tsukuba City, Ibaraki 300-2611, Japan
Takeshi Tomonaga, Proteome Research Center, Proteome Research Project, National Institute of Biomedical Innovation, Osaka 567-0085, Japan
Hisahiro Matsubara, Department of Frontier Surgery, Chiba University, Graduate School of Medicine, Chiba 260-8670, Japan
Author contributions: Matsushita K designed the research; Matsushita K, Ueda Y and Inoue M performed the research; Shimada H, Tomonaga T, Hasegawa M, Matsubara H and Nomura F contributed new reagents/analytic tools and scientific discussions; Matsushita K analyzed the data and wrote the paper.
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Correspondence to: Kazuyuki Matsushita, MD, PhD, Associate Professor, Department of Molecular Diagnosis, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-Ku, Chiba 260-8670, Japan. kmatsu@faculty.chiba-u.jp
Telephone: +81-43-2227171 Fax: +81-43-2262169
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## Abstract

**AIM:** To investigate a novel therapeutic strategy to target and suppress *c-myc* in human cancers using far up stream element (FUSE)-binding protein-interacting repressor (FIR).

**METHODS:** Endogenous c-Myc suppression and apoptosis induction by a transient FIR-expressing vector was examined *in vivo via* a HA-tagged FIR (HA-FIR) expression vector. A fusion gene-deficient, non-transmissible, Sendai virus (SeV) vector encoding FIR cDNA, SeV/dF/FIR, was prepared. SeV/dF/FIR was examined for its gene transduction efficiency, viral dose dependency of antitumor effect and apoptosis induction in HeLa (cervical squamous cell carcinoma) cells and SW480 (colon adenocarcinoma) cells. Antitumor efficacy in a mouse xenograft model was also examined. The molecular mechanism of the anti-tumor effect and c-Myc suppression by SeV/dF/FIR was examined using Spliceostatin A (SSA), a SAP155 inhibitor, or SAP155 siRNA which induce c-Myc by increasing FIRΔexon2 in HeLa cells.

**RESULTS:** FIR was found to repress *c-myc* transcription and in turn the overexpression of FIR drove apoptosis through *c-myc* suppression. Thus, FIR expressing vectors are potentially applicable for cancer therapy. FIR is alternatively spliced by SAP155 in cancer cells lacking the transcriptional repression domain within exon 2 (FIRΔexon2), counteracting FIR for c-Myc protein expression. Furthermore, FIR forms a complex with SAP155 and inhibits mutual well-established functions. Thus, both the valuable effects and side effects of exogenous FIR stimuli should be tested for future clinical application. SeV/dF/FIR, a cytoplasmic RNA virus, was successfully prepared and showed highly efficient gene transduction in *in vivo* experiments. Furthermore, in nude mouse tumor xenograft models, SeV/dF/FIR displayed high antitumor efficiency against human cancer cells. SeV/dF/FIR suppressed SSA-activated c-Myc. SAP155 siRNA, potentially produces FIR Δexon2, and led to c-Myc overexpression with phosphorylation at Ser62. HA-FIR suppressed endogenous c-Myc expression and induced apoptosis in HeLa and SW480 cells. A *c-myc* transcriptional suppressor FIR expressing SeV/dF/FIR showed high gene transduction efficiency with significant antitumor effects and apoptosis induction in HeLa and SW480 cells.

**CONCLUSION:** SeV/dF/FIR showed strong tumor growth

suppression with no significant side effects in an animal xenograft model, thus SeV/dF/FIR is potentially applicable for future clinical cancer treatment.



**Key words:** Cancer gene therapy; *c-myc* suppressor; Far up stream element-binding protein-interacting repressor; Sendai virus vector

**Core tip:** The authors performed *in vivo* experiments and included an animal model to examine the Sendai virus/dF/Far Up Stream Element-Binding Protein-Interacting Repressor for cancer gene therapy to minimize side effects for clinical use.

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## INTRODUCTION

c-Myc plays an essential role in cell proliferation and tumorigenesis. *c-myc* activation was also shown to be required for skin epidermal and pancreatic beta-cell tumor maintenance in c-MYC-ER$^{TAM}$ transgenic mice[1]. High *c-myc* expression level in colorectal cancer tissues was associated with poor long-term survival of colorectal cancer patients[2]. The far up stream element (FUSE) is a sequence required for correct expression of the human *c-myc* gene[3]. The FUSE is located at 1.5 kb upstream of *c-myc* promoter P1, and binds the FUSE binding protein (FBP), a transcription factor which stimulates *c-myc* expression in a FUSE-dependent manner[4]. Yeast two-hybrid analysis revealed that FBP binds to a protein that has transcriptional inhibitory activity termed the FBP interacting repressor (FIR). FIR interacts with the central DNA binding domain of FBP[5]. Recently, FIR was found to engage the TFIIH/p89/XPB helicase and repress *c-myc* transcription by delaying promoter escape[5,6]. Furthermore, exogenous FIR expression represses endogenous *c-myc* transcription, and drives apoptosis due to the decrease in c-Myc[7]. Although these observations indicate that cancer therapies targeting *c-myc* suppression by FIR may be a useful strategy, the mechanism of the antitumor effect of FIR should be determined in detail prior to clinical testing. For example, first, FIR is alternatively spliced in colorectal cancer lacking the transcriptional repression domain within exon 2 (FIRΔexon2)[7]. Second, FIR and FIRΔexon2 form a homo- or hetero-dimer, which complexes with SAP155, a subunit of the essential splicing factor 3b (SF3b) subcomplex in the spliceosome, and is required for correct P27Kip1 (P27) pre-mRNA splicing, after which P27 arrests cells in G1[8]. Third, SAP155 is required for correct FIR pre-mRNA splicing and thus the FIR/FIRΔexon2/SAP155 interaction bridged *c-myc* and p27 expression[9]. Accordingly, SAP155-mediated alternative splicing of FIR serves as a molecular switch for *c-myc* expression[9]. Finally, spliceostatin A (SSA), a natural SF3b inhibitor, markedly inhibited P27 expression by disrupting its pre-mRNA splicing and reducing cdk2/cyclinE expression[10]. Taken together, these findings suggest that exogenous FIR stimuli potentially affect the FIR/FIRΔexon2/SAP155 interaction which is pivotal for the cell cycle, cancer development and differentiation.

In this study, a fusion gene-deficient human FIR-expressing Sendai virus vector (SeV/dF/FIR) was prepared for future cancer therapy for the following reasons; Sendai virus (SeV), a member of the Paramyxoviridae family, has envelopes and a nonsegmented negative-strand RNA genome. The SeV genome contains six major genes in tandem on a single negative-strand RNA. Three proteins, the nucleoprotein (NP), phosphoprotein (P) and large protein (L; the catalytic subunit of the polymerase) form a ribonucleoprotein complex (RNP) with the SeV RNA. Matrix proteins (M) contribute to the assembly of viral particles, hemagglutinin-neuraminidase (HN) and fusion proteins (F) engage in the attachment of viral particles and infiltration of RNPs into infected cells. Importantly, SeV does not transform cells by integrating its genome into the cellular genome[11]. Therefore, SeV can mediate gene transfer and expression to a cytoplasmic location using cellular tubulin[12], thereby avoiding possible malignant transformation due to the genetic alteration of host cells. These are the safety advantages of SeV. Recently, a novel SeV vector was established where an enhanced green fluorescent protein (EGFP) reporter gene was inserted at the 3'-end of fusion gene-deficient SeV genomic RNA (SeV/dF/EGFP)[13]. This SeV/dF/EGFP is incapable of self-replication, but capable of infecting various cells, including human smooth muscle cells, hepatocytes, and endothelial cells, thus the SeV/dF/EGFP has a broad spectrum of gene transfer activity[9,10]. In this study, SeV/dF/FIR was prepared following the method for SeV/dF/EGFP[12,13]. The validity of SeV/dF/FIR for cancer therapy was examined in animal xenograft models as SeV/dF vectors have been shown to be applicable for clinical use[14-18]. The clinical use of SeV/dF/FIR for cancer therapy is also discussed.

## MATERIALS AND METHODS

### Plasmids

Full-length FIR cDNA (HA-FIR) was cloned into the pCGNM2 vector plasmid to introduce the hemagglutinin (HA)-tag at the amino termini[7]. Full-length FIR cDNA was cloned into the p3xFLAG-CMV-14 vector (Sigma, MO, United States) to introduce the Flag-tag at the amino termini for the selection of FIR-Flag in 293T cells (performed by Dr. T.N.). Plasmids were prepared by

CsCl ultra-centrifugation or the Endofree® Plasmid Maxi Kit (Qiagen, MD, United States) and the DNA sequences were verified.

### Tumor cell lines

HeLa cells (human cervical squamous cell carcinoma cells), LoVo and SW480 cells (human colon cancer cell lines) and LLC-MK2 a rhesus monkey kidney cell line were purchased from the American Type Culture Collection (Manassas, VA, United States). Yes-5, a human esophageal squamous cell carcinoma cell line was established by Dr Takuo Murakami (Yamaguchi University, Yamaguchi, Japan). All cell lines were cultured at 37 ℃ in a humidified atmosphere containing 5% $CO_2$. All tumor cell lines, except LLC-MK2 cells [which were maintained in DMEM; Dulbecco's Modified Eagle's Medium (Gibco BRL, NY, United States)] were cultured in tissue flasks or Petri dishes containing RPMI-1640 (Gibco, NY, United States) supplemented with 10% heat-inactivated FBS and penicillin (100 units/mL), streptomycin (0.1 mg/mL), and 2 mmol/L glutamine.

### Immunocytochemistry, protein extraction and immunoblotting

Immunocytochemistry was performed as described previously[7]. Protein extraction and immunoblotting are described elsewhere[8,9].

### siRNA against FIR or SAP155

SAP155 siRNA duplexes were purchased from Sigma Aldrich. The target sequences for SAP155 siRNA oligonucleotides were listed previously[8]. Luciferase GL2 duplex was used as a negative control for siRNA targeting 5'-CGTACGCGGAATACTTCGA-3'. Transient transfection of siRNA was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The transfected cells were cultured for 72 h at 37 ℃ in a 5% $CO_2$ incubator.

### Apoptosis detection

Apoptotic cells were detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay according to the manufacturer's instructions (Apoptosis Detection System, Fluorescein, Promega, WI, United States) as described previously[7]. Apoptosis detection by APOPercentage apoptosis assay™ (Funakoshi Co., Ltd., Tokyo, Japan) was performed according to the manufacturer's instructions[9].

### Construction of SeV vector

Human FIR cDNA was amplified with a pair of NotI site-tagged primers containing SeV-specific transcriptional regulatory signal sequences, (End and Start, italicized below) 5'-ATTGCGGCCGCCAAGGTTCAATGGCGACGGCGACCATAGC-3' and 5'-ATTGCGGCCGCGATGAACTTTCACCCTAAGTTTTTCTTACTACGGTCACGCAGAGAGGTCACTGTTATCAAAACGC-3'. The amplified fragment was introduced into the NotI site of the parental SeV vector cDNA, $pSeV^{18+}b(+)/dF$[15], to generate $pSeV^{18+}$hFIR/dF. $pSeV^{18+}$hFIR/dF was transfected to LLC-MK2 cells which were preliminarily infected with psoralen- and long-wave UV-treated vaccinia virus vTF7-3, expressing T7 polymerase. The cells were then washed twice with DMEM, and cultured for 24 h in DMEM containing cytosine β-D-arabinofuranoside (AraC; 40 μg/mL) and trypsin (7.5 μg/mL). LLC-MK2/F7/A cells expressing the F protein were suspended in DMEM containing AraC and trypsin, and layered onto the transfected cells, and cultured at 37 ℃ for an additional 48 h. The recovered vector in the culture supernatants was propagated using the LLC-MK2/F7/A cells. A GFP expression vector (SeV/dF/GFP) was prepared as previously described[8]. The viral vectors were further amplified by several rounds of propagation. The virus titers of the recovered vectors were determined by their infectivity and expressed using cell-infectious units (CIU). These vectors were frozen at -80 ℃ until use.

### SeV/dF/GFP-mediated green fluorescent protein transduction efficiency

One million LLC-MK2 cells and HeLa cells were seeded in six-well plates and transduced with SeV/dF/GFP when monolayers reached 60%-80% confluence. As the standard inoculation procedure for vaccination, the monolayers were washed twice with PBS and overlaid with serum-free medium containing SeV/dF/GFP at a multiplicity of infection (MOI) of 0, 1, 10, 50, 100, or 300. After incubation at 37 ℃ for 90 min, non-adsorbed virus was removed, medium containing 10% FBS was added, and the cells were incubated for more than 48 h at 37 ℃. The transduction studies were carried out in triplicate for each MOI. Microscopy was used to detect transduced cells by GFP fluorescence. At 72 h after transduction, the GFP-transduced cells were analyzed for GFP expression using a FACS Calibrator (BD Pharmingen, Franklin Lakes, NJ, United States).

### MTS assay to assess cell viability

The inhibitory effects of viruses on the proliferation of cultured cells were examined using the CellTiter96™ $AQ_{ueous}$ One Solution Proliferation Assay (Promega, Madison, WI, United States). In brief, five thousand cells were plated in each well on day 0. On day 1, 24 h later, HeLa cells were infected with SeV/dF/FIR or SeV/dF/GFP as the control at 0.1 to 10 MOI, and cultured for 2 d. On day 3, cell viability was quantified by measuring the absorbance at 570 nm after incubation with the tetrazolium compound [3-(4,5-dimethylthiozol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), and an electron coupling agent, phenazine ethosulfate (PES)] (Promega, Madison, WI, United States) for 4 h. The absorbance at 570 nm was measured by a Mutiabel Counter™, ARVOSX WAIIAC™ (Perkin Elmer, MA, United States). The results are shown as percentages of the uninfected control cells.

### Mice

Six- to eight-week male immuno-competent Balbc/nu/

nu mice were purchased from Clea Japan (Tokyo, Japan) and housed in the Animal Maintenance Facility at Chiba University under specific pathogen-free conditions. All animal experiments were approved by the Committee of the Ethics on Animal Experiments in the Faculty of Medicine, Chiba University and carried out following the Guidelines for Animal Experiments in the Faculty of Medicine, Chiba University, Chiba, Japan and The Law and Notification of the Government. Mouse experiments were carried out at least twice to confirm the results.

### Tumor xenograft experiments

The *in vivo* inhibition of tumorigenicity of HeLa cells (human cervical squamous cell carcinoma cells) was examined by SeV/dF/FIR or SeV/dF/GFP injection (as the control). $5 \times 10^6$ cells/50 μL PBS of HeLa cells were injected under the skin in the right thigh of nude mice (6-wk old males). Tumor growth was observed and the long and short diameter measured for tumor volume calculation. Thirty days after inoculation, tumors grew up to 5-8 mm in diameter in 18 of 18 mice (100%). Tumor size was calculated using the formula, $(a \times b^2)/2$, where a and b represent the larger and smaller diameters, respectively, and was monitored every 3 d.

### FIR-binding protein identification

The methods for the direct nanoflow liquid chromatography-tandem mass spectrometry system with FIR-FLAG transiently transfected 293T nuclear extracts have been described previously[19-23].

## RESULTS

### HA-FIR suppresses endogenous c-Myc expression with apoptosis induction in vivo

To examine endogenous *c-myc* gene suppression by FIR, HA-FIR expression plasmids were transfected into HeLa cells, and c-Myc expression was visualized by immunostaining with anti-c-Myc antibodies (Figure 1A; upper panels: HA-FIR is red; c-Myc is green). c-Myc levels were greatly diminished in HA-FIR expressing cells (arrows), demonstrating that FIR represses endogenous c-Myc expression in SW480 (Figure 1A; middle panels) and LoVo cells (Figure 1A; lower panels). After HA-FIR expression plasmids were transfected into HeLa, SW480 or LoVo cells, apoptotic cells were visualized by TUNEL assay. HA-FIR transfected cells were definitively associated with apoptosis (Figure 1B).

### FIR protein expression by fusion gene-deficient SeV/dF/FIR

SeV/dF/FIR and SeV/dF/GFP were prepared as described in Materials and Methods (Figure 2A, B). SeV/dF/FIR vectors were infected into LLC-MK2 or HeLa cells. FIR protein expression level was examined by western blot with anti-FIR antibody (6B4) (Figure 2C). At least $1 \times 10^{10}$ CIU of fusion gene-deficient SeV/dF/FIR virus particles were prepared at amplification for use in the experiments.

### Transduction efficiency of the SeV/dF/GFP vector in various human tumor cell lines

Nine human and five murine tumor cell lines plus non-tumor cells, propagated *in vitro* were collected, transduced by SeV/dF/GFP, and examined for gene transduction efficiency. Flow cytometric analyses showed dose-dependent GFP expression, and optimal expression was obtained at a MOI of 10-100; > 90% GFP-positive tumor cell lines were detected at a MOI over 10 (Figure 3A and data not shown). Furthermore, SeV/dF/FIR, but not SeV/dF/GFP significantly suppressed HeLa cell (human cervical squamous carcinoma) growth as shown by the APOPercentage assay (Figure 3B), indicating SeV/dF/FIR suppresses tumor cell growth by apoptosis *in vivo*.

### SeV/dF/FIR vector showed anti-tumor activity in the mouse xenograft model

SeV/dF/FIR, but not SeV/dF/GFP, significantly suppressed cell growth in HeLa cells (Figure 4A) and SW480 cells when analyzed by Dunnet's test for multiple comparisons (Figure 4B). Of note, xenograft tumors 2 cm in diameter disappeared completely following SeV/dF/FIR treatment, indicating that SeV/dF/FIR has immunological effects (Figure 5)[24,25].

### FIR was co-immunoprecipitated with SAP155

If SeV/dF/FIR is to be tested clinically, endogenous FIR-interacting proteins should be identified to avoid unexpected side effects. For this purpose, the FIR-FLAG tag vector was transiently expressed in 293T cells and co-immunoprecipitated with anti-FLAG conjugated beads to detect FIR-binding proteins[19-23] (Table 1). As reported previously, FBP (Far upstream element-binding protein)[26,27], SAP155[28], and SRp54 (splicing factor, arginine/serine rich-12)[28] were identified as candidate FIR-binding proteins. To date, no significant side effects have been observed following SeV/dF/FIR treatment including our study[29].

### SeV/dF/FIR suppressed SSA-activated c-Myc

We previously reported that the adenovirus vector encoding FIRΔexon2 (Ad-FIRΔexon2) activates not only *c-myc* transcription, but also c-Myc protein expression in HeLa cells[8]. However, the extent of c-Myc protein activation by Ad-FIRΔexon2, evaluated by western blot analysis, could not be explained solely by *c-myc* transcription activation[8]. Therefore, we hypothesized that c-Myc protein should be modified by Ad-FIRΔexon2 to be more stable. Ad-FIRΔexon2 expression leads to increased levels of c-Myc phosphorylated at Ser62 (data not shown), indicating that stable c-Myc protein accumulates in cells[30,31]. As reported previously, SAP155 siRNA inhibited FIR pre-mRNA splicing and generated FIRΔexon2[8,9]. In fact, SAP155 siRNA increased levels of c-Myc phosphorylated at Ser62 and Ad-FIRΔexon2 (Figure 6A). In other words, Ad-FIRΔexon2, which lacks the transcriptional repressor

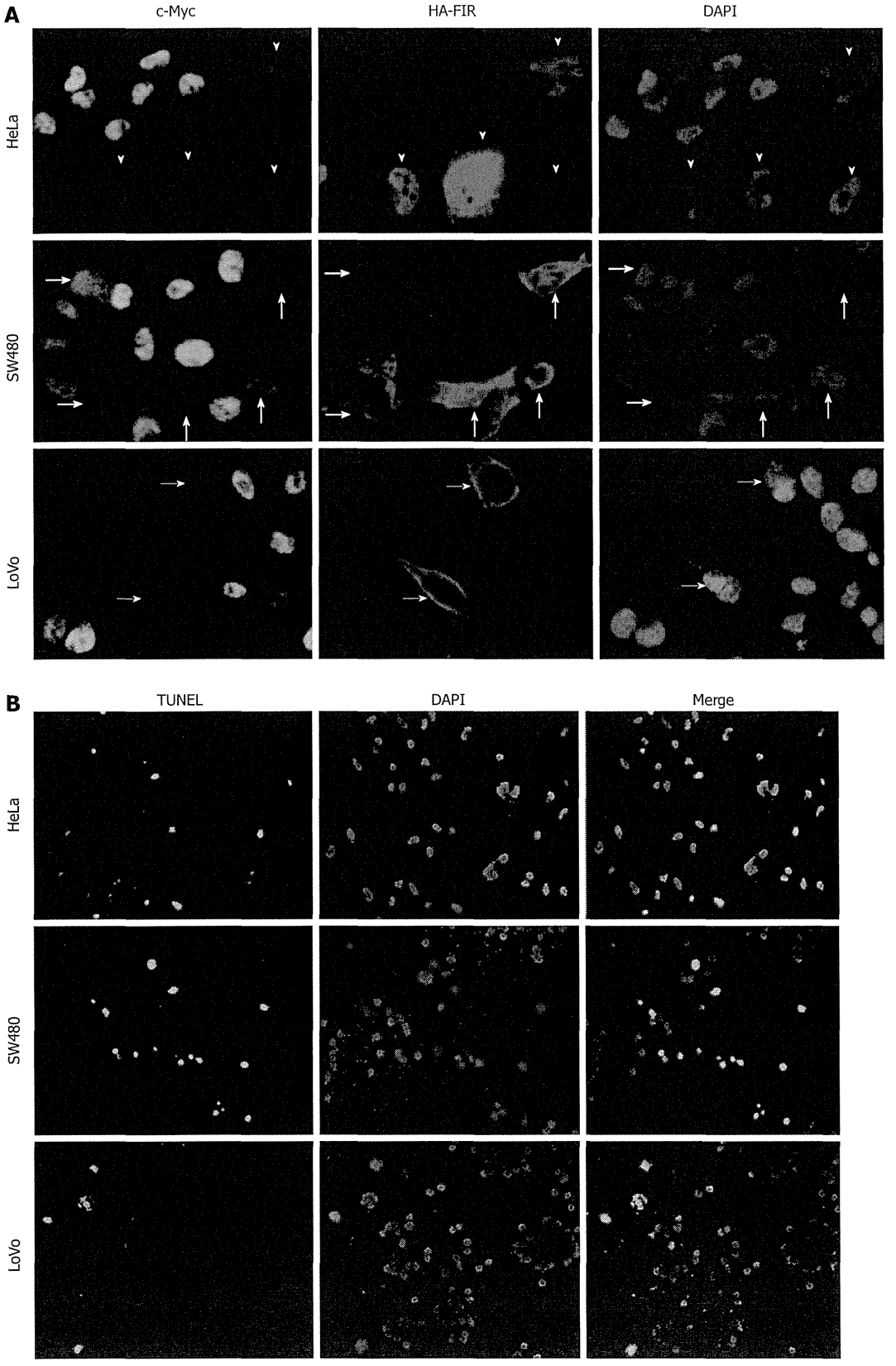


**Figure 1 Far up stream element-binding protein-interacting repressor suppresses endogenous c-Myc in SW480 and LoVo cells as well as HeLa cells.** A: 100 fmol of HA-FIR was transfected into the colon cancer cell lines, SW480 and LoVo cells, as well as HeLa cells (cervical squamous cell carcinoma cells) in a 6-well plate. After 48 h transfection, cells were fixed and immunostained against c-Myc (left, green) or HA (middle, red) antibodies. Arrowheads (HeLa), thick arrows (SW480) and thin arrows (LoVo) show the cells in which HA-FIR plasmids were expressed. c-Myc expression (left, green) was markedly reduced in most HA-FIR-expressing cells (middle, red) (indicated by arrowheads and arrows); B: HA-FIR transfected cells were definitively associated with apoptosis as revealed by TUNEL assay in HeLa, SW480 and LoVo cells. Nuclear DNA was stained with DAPI (right, blue). FIR: FBP Interacting Repressor; FBP: FUSE-Binding protein; FUSE: Far Upstream Element.

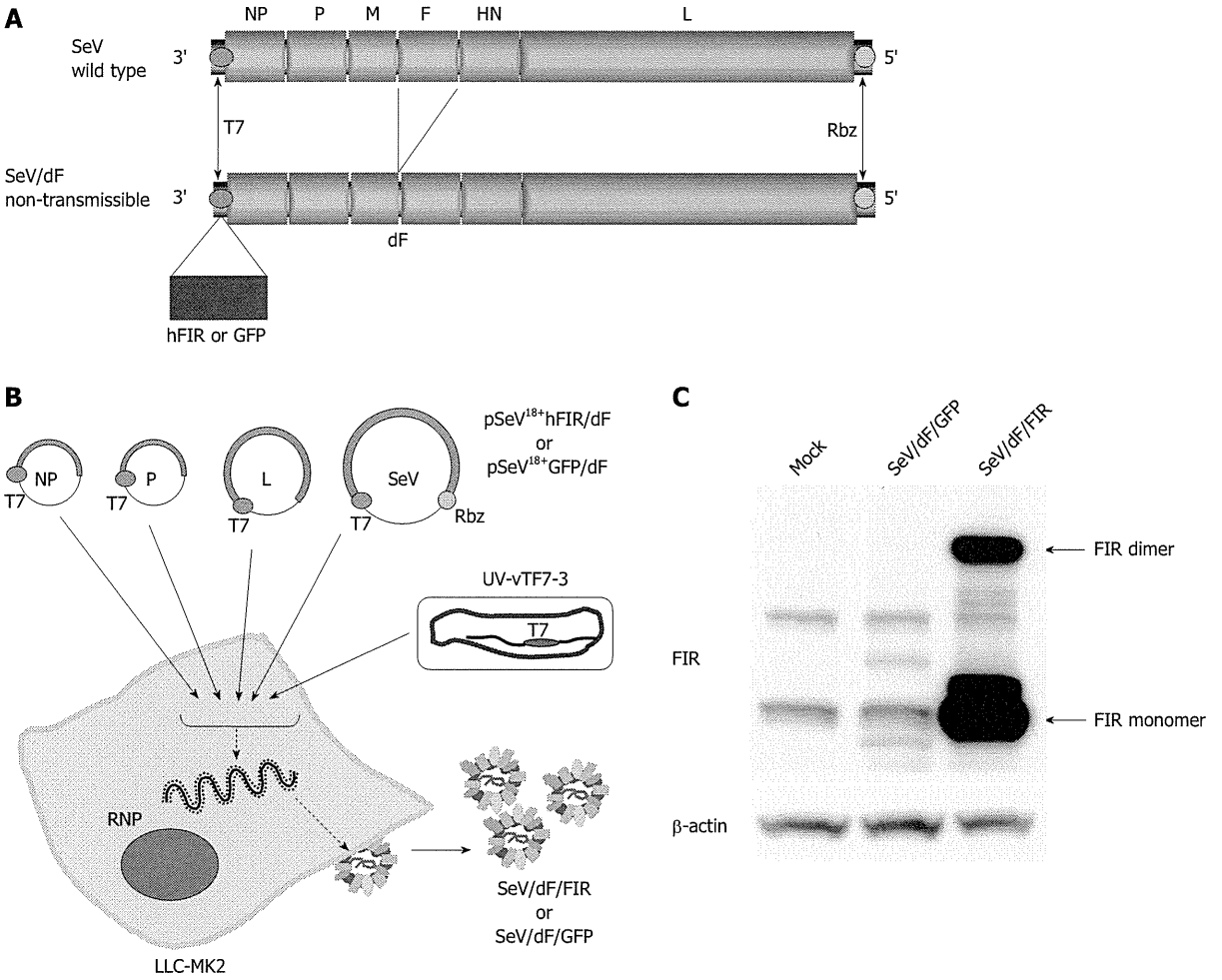


**Figure 2 Structures and procedures for generating fusion gene-deficient Sendai virus/dF/far up stream element-binding protein-interacting repressor (SeV/dF/FIR) or Sendai virus/dF/green fluorescent protein vectors (SeV/dF/GFP) from Sendai virus genome RNA.** A: Schematic genome structures of wild-type (SeV) and fusion gene-deficient (SeV/dF; non-transmissible) vector carrying the human FIR (*hFIR*) gene or jellyfish green fluorescent protein (GFP). The open reading frame or the *FIR* or *GFP* gene was inserted with the SeV-specific transcriptional regulatory signal sequences. T7; T7 promoter, Rbz; hepatitis delta virus ribozyme sequence; B: Schematic representation of the procedure for generating the fusion gene-deficient SeV/dF/FIR or SeV/dF/GFP. SeV/dF/FIR or SeV/dF/GFP virus particles were propagated using fusion protein-expressing packaging cells (LLC-MK2/F7/A) after preparation in LLC-MK2 cells using the four plasmids driven by a recombinant vaccinia virus expressing T7 RNA polymerase which had been inactivated with psoralen and long-wave UV light (UV-vTF7-3); C: SeV/dF/FIR virus vectors were infected into HeLa cells and whole cell proteins were extracted for western blot analysis. SeV/dF/FIR expresses FIR proteins. FIR: FBP Interacting Repressor; FBP: FUSE-Binding protein; FUSE: Far Upstream Element; SeV: Sendai virus; NP: Nucleoprotein; P: Phosphoprotein; L: The catalytic subunit of the polymerase large protein forms a ribonucleoprotein complex (RNP) vector and was transfected separately with the SeV RNA. See Materials and Methods.

domain, directly or indirectly activated *c-myc* expression not only through transcription, but also through protein level, suggesting that FIRΔexon2 acts in opposition to the repressor function by FIR[8].

In this study, the effect of SeV/dF/FIR was examined to determine whether it suppresses the increase in c-Myc after SSA treatment. SeV/dF/FIR suppressed SSA-induced c-Myc activation (Figure 6B, compare lane 2 with lane 1), but not basal c-Myc expression (Figure 6B, compare lanes 4 to 3 and 6 to 5, respectively). These results were consistent with previous reports that FIR suppresses activated, but not basal, *c-myc* transcription[6]. These observations suggest that the increase in *c-myc* by either SAP155 siRNA or SSA treatment is due to reduced FIR activity, or an increase in the ratio of FIRΔexon2/FIR in HeLa cells. Taken together, these results suggest that SeV/dF/FIR is potentially clinically applicable for cancer therapy as it counteracts SSA-activated c-Myc (Figure 6B, compare lane 2 with lane 1) as well as endogenous c-Myc (Figure 1A).

### *SeV/dF vector transduction*

*F* gene-deficient SeV vectors (SeV/dF) can transduce cells in a wide range of tissues such as respiratory, nervous, muscular, epithelial and immune tissues[11,32-35]. Transduction efficiency to cell lines from various tissues was examined and compared to the adenovirus vector expressing LacZ (Ad5/LacZ) at the same MOI. The transduction efficiency of SeV/dF to those cells was even higher than that of adenovirus vector (Figure 7).

## DISCUSSION

Overexpression of c-Myc has been known to promote

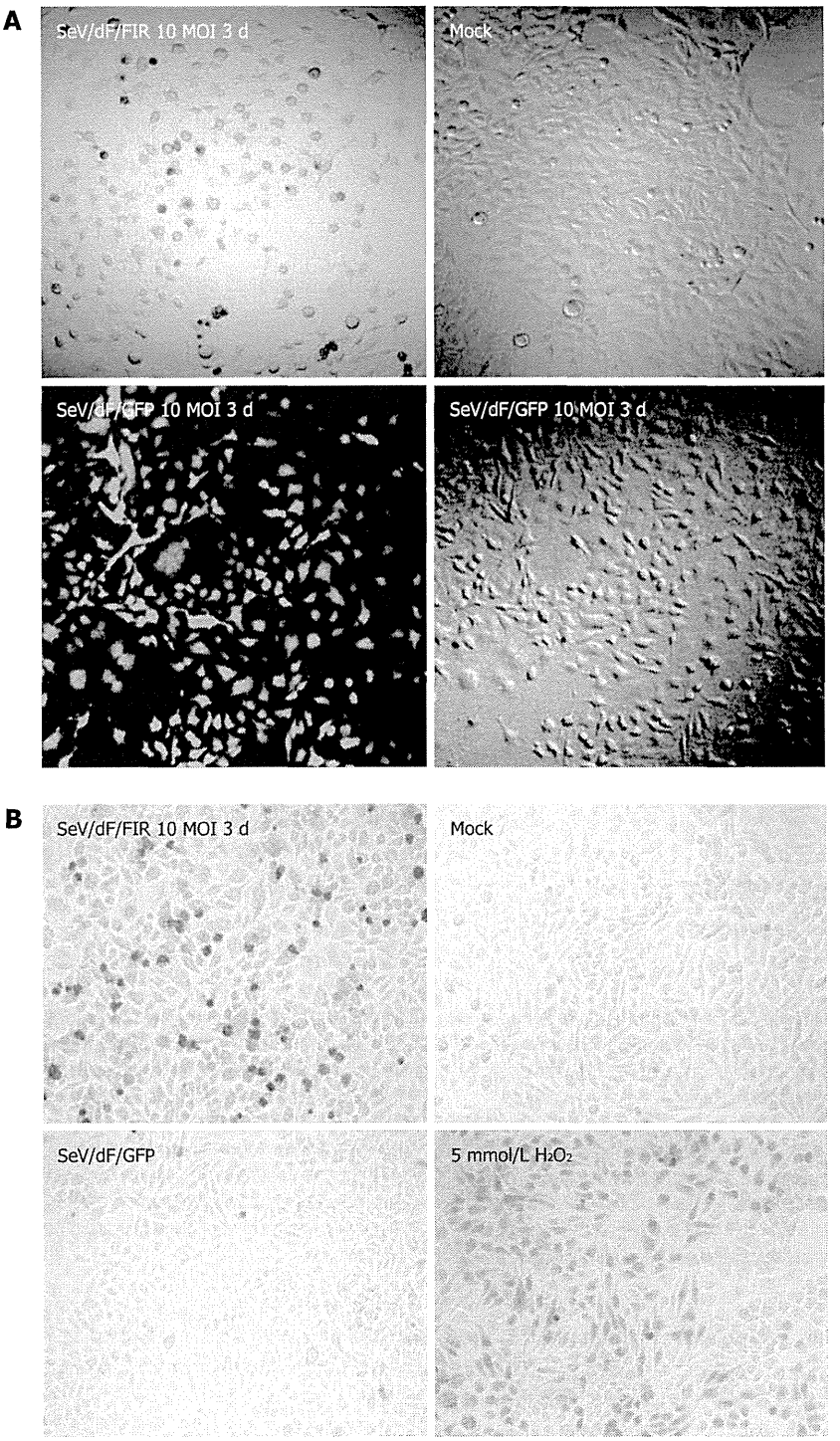


**Figure 3 High efficiency of Sendai virus/dF/green fluorescent protein vectors to HeLa cells and Sendai virus/dF/green fluorescent protein vectors indicates significant cell growth inhibition with apoptosis.** A: HeLa cells were infected with a 10 MOI of SeV/dF/FIR virus vector and cultured in DMEM for 3 d (72 h). The same amount of 10 MOI SeV/dF/GFP was also used to infect the control. As shown in the left lower panel, SeV/dF/GFP infected almost 100% cells (green). Under these conditions, SeV/dF/FIR significantly inhibited cell growth as shown in the left upper panel. Mock and 10 MOI SeV/dF/GFP showed no cell growth inhibition as seen in the right upper and lower panels; B: HeLa cells infected by 10 MOI SeV/dF/FIR for 3 d showed significant cell damage revealed by the APO Percentage apoptosis assay™, compared to mock or the same conditions of SeV/dF/GFP virus infected HeLa cells. 5 mmol/L $H_2O_2$ was used as a positive control. FIR: FBP Interacting Repressor; FBP: FUSE-Binding protein; FUSE: Far Upstream Element; SeV: Sendai virus; GFP: Green fluorescent protein; MOI: Multiplicity of infection.

cell growth, proliferation and immortalization, whereas a reduction in c-Myc induces apoptosis. The recent genetic construction of the mouse in which the expression of *c-myc* can be switched on or off *in vivo* has emphasized the significance of c-Myc expression in tumorigenesis. Ectopic *c-myc* expression in hematopoietic cells using the tetracycline regulatory system caused malignant T cell lymphomas and acute myeloid leukemia; subsequent inactivation of the transgene caused regression of established tumors[36]. These observations have provided encourage-

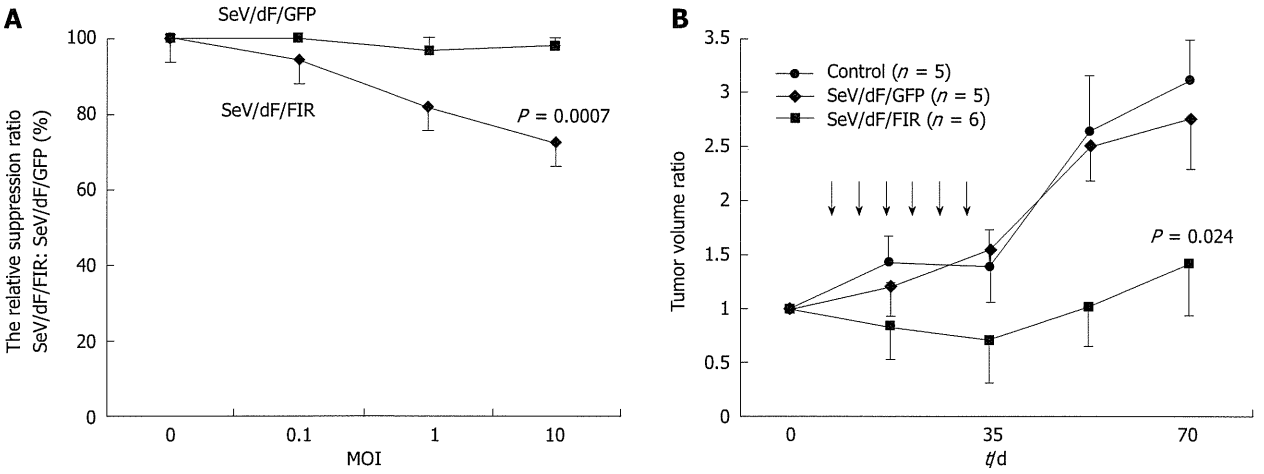


**Figure 4 Sendai virus/dF/far up stream element-binding protein-interacting repressor decreased HeLa cell growth *in vitro* and in a xenograft animal model.** HeLa cells (A) and SW480 cells (B) were infected with 0, 0.1, 1, and 10 MOI of SeV/dF/FIR or SeV/dF/GFP vectors and cell growth was measured by MTS assay (see Materials and Methods). Results are shown as the percentage of cell number at day 0. Points, mean of three separate experiments; bars, SD. Statistical significance was analyzed by Dunnett's test for multiple comparisons (SeV/dF/FIR *vs* SeV/dF/GFP, $P < 0.007$). Two weeks after $5 \times 10^6$ HeLa cells were xenografted into the right thigh of Balbc/nu/nu mice, the tumor size was approximately 7-8 mm. $3.0 \times 10^7$ CIU of SeV/dF/FIR or SeV/dF/GFP vector were injected directly around the tumor, and the tumor growth was observed and measured every three days as described in Materials and Methods. Results are shown as the ratio of tumor volume compared to the size at day 0. The tumor volume at day 0 of SeV/dF/FIR ($n = 6$), SeV/dF/GFP ($n = 5$), and control ($n = 5$) were 1173.1 + 259.2, 836.0 + 259.2, and 972.2 + 327.0 (average ± SD) $mm^3$, respectively. The average tumor volume at day 0 was estimated as 1 in each experiment. Arrows indicate the injection of SeV/dF/FIR or SeV/dF/GFP vectors. FIR: FBP Interacting Repressor; FBP: FUSE-Binding protein; FUSE: Far Upstream Element; SeV: Sendai virus; GFP: Green fluorescent protein; MOI: Multiplicity of infection.

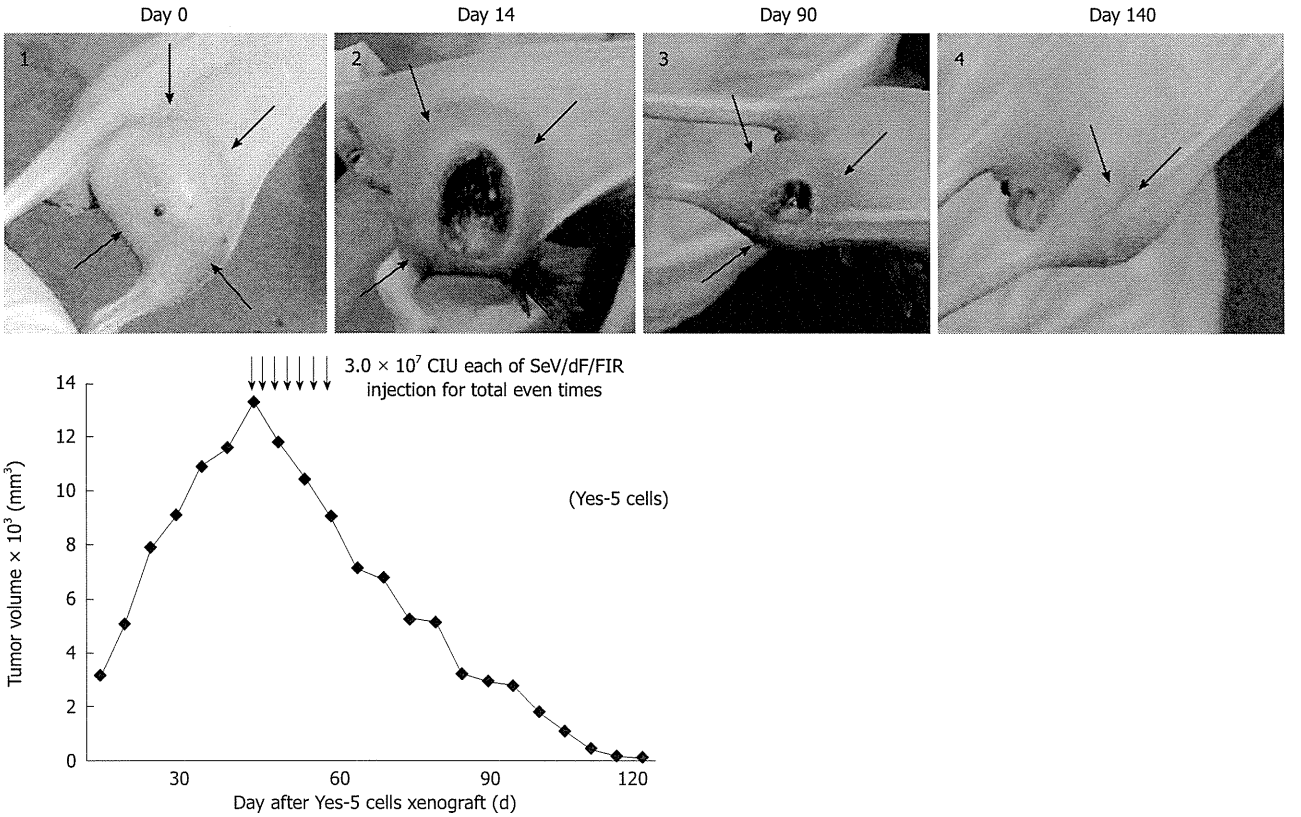


**Figure 5 Sendai virus/dF/Far up stream element-binding protein-interacting repressor vector showed anti-tumor activity in a mouse xenograft model.** $10^6$ Yes-5 cells were xenografted into the right thigh of Balbc/nu/nu mice, and the tumor size was approximately 15 mm in diameter at Day 0. $3.0 \times 10^7$ CIU of SeV/dF/FIR vectors were injected directly around the tumor every two days, seven times in total. Tumor growth was observed and measured every two days as described in Materials and Methods. Ulcer formation was observed in the center of the tumor (day 14 after SeV/dF/FIR injection). Tumor size was significantly diminished with ulcer formation (day 90) and disappeared completely during surveillance (day 140). Thick arrows in the images indicate the tumor margin. Thin arrows indicate the injection of SeV/dF/FIR vectors into the tumor. FIR: FBP Interacting Repressor; FBP: FUSE-Binding protein; FUSE: Far Upstream Element; SeV: Sendai virus; CIU: Cell-infectious units.

**Table 1 Co-immunoprecipitaed proteins with far up stream element-binding protein-interacting repressor in 293T cells**

| Hit preys | |
|---|---|
| CDKN2AIP | CDKN2A interacting protein |
| CDYL | Chromodomain protein, Y chromosome-like |
| DAZAP1 | DAZ associated protein 1 |
| DDX17 | DEAD box polypeptide 17 |
| DDX5 | DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 |
| ELAVL1 | ELAV-like 1 |
| FAM120A | Oxidative stress-associated Src activator |
| FUBP1 \| FUBP3 \| KHSRP | Far upstream element-binding protein family |
| FUBP1 \| KHSRP | Far upstream element-binding protein family |
| HNRNPA1 | Heterogeneous nuclear ribonucleoprotein A1 |
| HNRNPA1 \| HNRNPA1L2 | Heterogeneous nuclear ribonucleoprotein; A1 or A1-like |
| HNRNPA1 \| HNRNPA1L2 \| LOC402562 | Nuclear ribonucleoprotein A1 family |
| HNRNPA2B1 | Heterogeneous nuclear ribonucleoprotein A2/B1 |
| HNRNPA3 | Heterogeneous nuclear ribonucleoprotein A3 |
| HNRNPAB | Heterogeneous nuclear ribonucleoprotein A/B; isoform a |
| HNRNPAB \| HNRNPD | Heterogeneous nuclear ribonucleoprotein; A/B or D |
| HNRNPD | Heterogeneous nuclear ribonucleoprotein D; isoform c |
| HNRNPH1 | Heterogeneous nuclear ribonucleoprotein H1 |
| HNRNPK | Heterogeneous nuclear ribonucleoprotein K |
| HNRNPL | Heterogeneous nuclear ribonucleoprotein L |
| HNRNPM | Heterogeneous nuclear ribonucleoprotein M; isoform a |
| HNRNPR | Heterogeneous nuclear ribonucleoprotein R |
| HNRNPU | Heterogeneous nuclear ribonucleoprotein U; isoform a |
| HNRNPUL1 | Heterogeneous nuclear ribonucleoprotein U-like 1 |
| HNRPDL | Heterogeneous nuclear ribonucleoprotein D-like |
| IFIT5 | Interferon-induced protein with tetratricopeptide repeats 5 |
| IGF2BP1 | Insulin-like growth factor 2 mRNA binding protein 1 |
| IGF2BP1 \| IGF2BP3 | Insulin-like growth factor 2 mRNA binding protein; 1 or 3 |
| IGF2BP2 | Insulin-like growth factor 2 mRNA binding protein 2 |
| IGF2BP3 | Insulin-like growth factor 2 mRNA binding protein 3 |
| ILF2 | Interleukin enhancer binding factor 2 |
| KHDRBS1 | KH domain containing, RNA binding, signal transduction associated 1 |
| KHDRBS1 \| KHDRBS2 \| KHDRBS3 | KH; domain containing, RNA binding, signal transduction associated 1<br>Or domain-containing, RNA-binding, signal transduction- |
| KHSRP | KH-type splicing regulatory protein |
| KIAA1967 | p30 DBC protein |
| LARP1 | La related protein |
| LRPPRC | Leucine-rich PPR motif-containing protein |
| LSM12 | LSM12 homolog |
| MAGOH \| MAGOHB | Mago-nashi homolog; or B |
| MATR3 | Matrin 3 |
| MOV10 | Mov10, Moloney leukemia virus 10, homolog |
| MSI2 | Musashi 2; isoform a |
| PABP3 \| PABPC1 \| PABPC3 | Poly (A) binding protein, cytoplasmic 1<br>Or poly (A) binding protein, cytoplasmic 3 |
| PABPC1 | Poly (A) binding protein, cytoplasmic 1 |
| PABPC1 \| PABPC1L \| PABPC5 | Poly (A) binding protein, cytoplasmic 1 or poly(A) -binding protein,<br>Cytoplasmic 1-like or poly (A) binding protein, cytoplasmic 5 |
| PABPC1 \| PABPC4 | Poly (A) binding protein, cytoplasmic 1<br>Or poly A binding protein, cytoplasmic 4 |
| PABPC1L2B \| PABPC4 | Poly (A) binding protein, cytoplasmic 1-like 2B<br>Or poly A binding protein, cytoplasmic 4 |
| PABPC4 | Poly A binding protein, cytoplasmic 4 |
| PABPC4 \| PABPC4L | Poly A binding protein, cytoplasmic 4 |
| PABPN1 | Poly (A) binding protein, nuclear 1 |
| PCBP1 \| PCBP2 \| PCBP3 \| PCBP4 | Poly (rC) binding protein; 1 or 2 or 3 or 4 |
| PCBP2 \| PCBP3 | Poly (rC) binding protein; 2 or 3 |
| PTBP1 | Polypyrimidine tract-binding protein 1; isoform d |
| SF3B1 | Splicing factor 3b, subunit 1 |
| SF3B14 | Splicing factor 3B, 14 kDa subunit |
| SF3B3 | Splicing factor 3b, subunit 3 |
| SF3B4 | Splicing factor 3b, subunit 4 |
| SFPQ | Splicing factor proline/glutamine rich (polypyrimidine tract binding |
| SFRS11 | Splicing factor, arginine/serine-rich 11 (SRp54) |
| SFRS12 | Splicing factor, arginine/serine-rich 12 |
| SNRPD1 | Small nuclear ribonucleoprotein D1 polypeptide 16 kDa |
| SSB | Autoantigen La |
| SYNCRIP | Synaptotagmin binding, cytoplasmic RNA interacting protein |

| | |
|---|---|
| THADA | Thyroid adenoma associated |
| TRMT1 | tRNA methyltransferase 1 |
| TROVE2 | TROVE domain family, member 2; isoform 2 |
| XRN2 | 5'-3' exoribonuclease 2 |
| Total hit preys: | 67 |

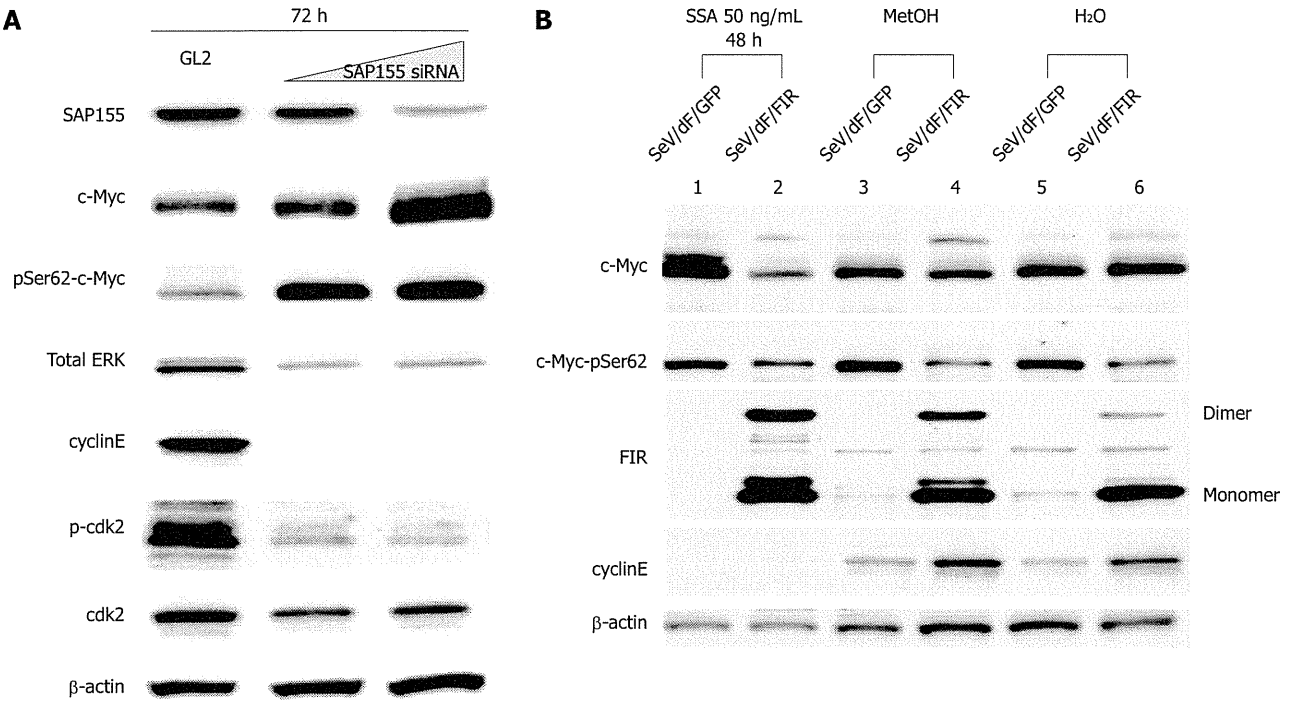


**Figure 6 SAP155 siRNA induces c-Myc activation with ErK phosphorylation, but suppresses phosphorylated-cdk2/cyclinE expression. HeLa cells were treated with SAP155 siRNA for three days (72 h).** A: SAP155 siRNA, as well as SSA treatment, increased not only c-Myc expression level, but also c-Myc phosphorylation at both Ser62, but suppressed phosphorylated-cdk2 and cyclinE in a dose-dependent manner. Thus, SAP155 siRNA activates c-Myc potentially *via* inhibiting endogenous FIR pre-mRNA splicing; B: FIR Sendai virus (SeV/dF/FIR) reversed the cytotoxicity of SSA by suppressing activated endogenous c-Myc. HeLa cells were treated with 50 ng/mL SSA for 48 h with control (MetOH and $H_2O$). 10 MOI of SeV/dF/FIR apparently suppressed activated c-Myc expression, whereas SeV/dF/FIR did not influence basal expression (MetOH or $H_2O$). FIR: FBP Interacting Repressor; FBP: FUSE-Binding protein; FUSE: Far Upstream Element; SeV: Sendai virus; GFP: Green fluorescent protein; MOI: Multiplicity of infection; SSA: Spliceostatin.

ment for the future development of cancer therapies based on targeting individual oncogenes such as *c-myc*. We have previously reported that FIR strongly represses endogenous *c-myc* transcription, and induces apoptosis[7] and is thus applicable for cancer treatment. In this study, first, we demonstrated that *c-myc* suppressor FBP-interacting repressor (FIR) strongly repressed endogenous *c-myc* transcription and induced apoptosis in SW480, LoVo (human colon cancer cell lines) as well as HeLa cells (human cervical squamous cancer cell line). Second, SeV/dF/FIR showed strong anti-tumor effects in both cultured cells and xenograft tumor growth in an animal model. These results provide new insight into a new therapeutic target for tumor treatment.

What type of suitable vector should be selected and how should FIR expressing vectors be conveyed to cancers? Sendai virus is an RNA virus and exists only in the cytoplasm, hence it is relatively safe as it does not affect chromosomes. In addition, SeV does not transform cells by integrating its genome into the cellular genome, thereby avoiding possible malignant transformation due to the genetic alteration of host cells; this is a safety advantage of SeV. For this reason, we chose SeV and prepared a fusion gene-deficient SeV/dF/FIR vector. The fusion gene-deficient SeV vector cannot transmit to F protein-non-expressing cells as F protein is essential for viral infection. The fusion gene-deficient SeV vector in this study does not require helper virus for reproduction, but is self-replicable in infected cells. Thus, the fusion gene-deficient SeV vector has several advantages over expressing vectors as a gene delivery system for human disease including cancer treatment. First, the fusion gene-deficient SeV vector is not pathogenic in humans. Second, the virus replicates only in the cytoplasm, therefore does not affect chromosome DNA in host cells. Third, SeV vector shows highly efficient gene transfer to a wide spectrum of cells, even to smooth muscle cells, nerve cells, or endothelial cells which are generally difficult to infect. Fourth, the SeV vector shows highly efficient gene transfer to a wide spectrum of cells, even to smooth muscle cells and does not generate wild-type virus in packaging cells. Recently, a gene-deficient SeV (SeV/dF) vector alone demonstrated tumor suppression by activating dendritic cells (DCs)[24], or if granulocyte

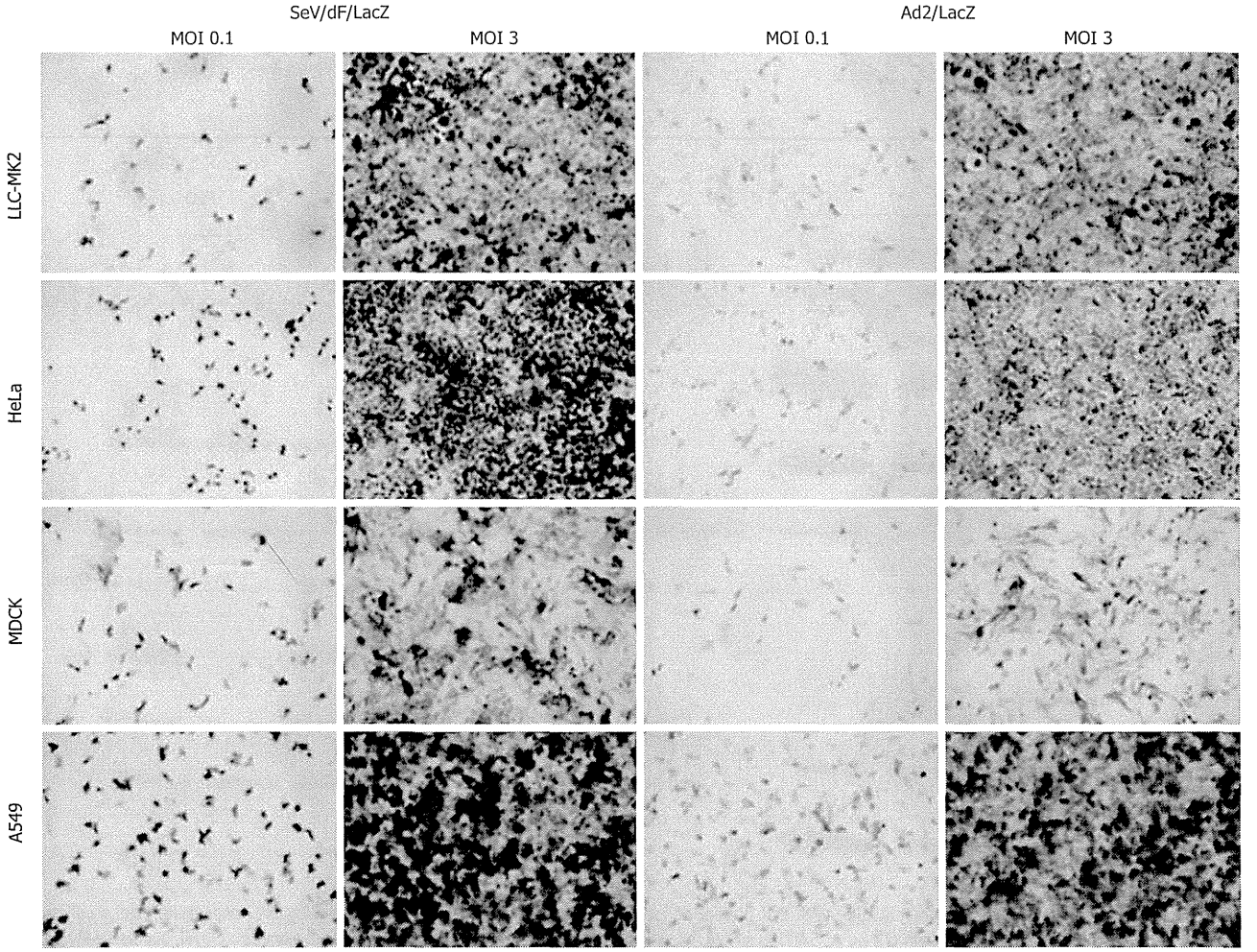


**Figure 7 Sendai virus/dF/LacZ transduction efficiency was examined in some human and animal cell lines.** Confluent culture of LLC-MK2 (macaque kidney fibroblasts), HeLa (human adenocarcinoma cells), MDCK (canine kidney cells), and A549 (human lung carcinoma cells) were infected with LacZ expressing SeV vector (SeV/dF/LacZ) at a MOI of 0.1 or 3.0. LacZ expressing Adenovirus vector (Ad5/LacZ) was used as a control. Two days after infection, the cells were stained with X Gal. SeV: Sendai virus.

macrophage colony-stimulating factor was encoded, it produced autologous tumor vaccines[25]. Therefore, the SeV/dF/FIR vector in this study may suppress tumor growth by a dual function through c-Myc suppression of tumor cells and DC activation. Furthermore, SeV/dF/FIR showed a synergistic effect with cisplatin in the treatment of malignant pleural mesothelioma[29]. FIR-binding proteins are basically classified into four categories (Table 1); (1) RNA binding proteins and splicing factors; (2) transcription factors and chromatin remodeling proteins; (3) actin-binding proteins; and (4) signal transduction and protein kinase families. This suggests that FIR potentially engages in some different intracellular events, such as RNA transport, DNA damage repair and pre-mRNA splicing. Accordingly, the side effects of SeV/dF/FIR need to be considered before clinical use, such as pre-mRNA splicing disturbance[8,9], DNA damage repair[37] or intracellular protein transport interference. For clinical safety, SeV/dF/FIR is preferable for local tumor growth control rather than systemic cancer therapy.

Taken together, these findings show that SeV/dF/FIR is a promising approach for cancer gene therapy, although further clinical and basic research are required to explain the precise mechanism of tumor suppression by FIR expressing vectors.

## ACKNOWLEDGMENTS

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## COMMENTS

### *Background*

Far Up Stream Element-Binding Protein-Interacting Repressor (FIR) is a *c-myc* transcriptional repressor. Thus, FIR expressing vectors are applicable for cancer therapy. In this study, the authors examined a novel therapeutic strategy to suppress *c-myc* in human cancers using a fusion gene-deficient Sendai virus (SeV/dF/FIR) which is inherently non-transmissible.

### Research frontiers

As c-myc transcriptional control is largely unknown, modulation of c-myc regulation by SeV/dF/FIR for cancer therapy should be monitored, strictly and skeptically, from several aspects. This study revealed that SeV/dF/FIR is effective for cancer gene therapy without significant side effects in a xenograft model.

### Innovations and breakthroughs

SeV/dF/FIR showed high gene transduction efficiency with significant antitumor effects and apoptosis induction in HeLa and SW480 cells. In the xenograft model, SeV/dF/FIR showed strong suppression of tumor growth with no significant side effects.

### Applications

SeV/dF/FIR is potentially applicable for future clinical cancer treatment as SeV/dF/FIR suppresses endogenous c-Myc as well as Spliceostatin A (SSA)-activated c-Myc.

### Terminology

FUSE: Far Upstream Element which is required for correct c-myc transcription. FBP: FUSE-Binding protein which has strong transcriptional activity. FIR: FBP interacting repressor which is a critical transcriptional repressor of c-myc gene. SeV: Sendai virus, a member of the Paramyxoviridae family, has envelopes and a nonsegmented negative-strand RNA genome. The SeV genome contains six major genes in tandem on a single negative-strand RNA. DC: Dendritic cell. The gene-deficient SeV (SeV/dF) vector alone demonstrates tumor suppression by activating dendritic cells (DCs).

### Peer review

The authors performed the enthusiastic experiments *in vivo* and animal model to examine the SeV/dF/FIR for cancer gene therapy to minimize the side effect for the clinical use.

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# The translation elongation factor eEF2 is a novel tumor-associated antigen overexpressed in various types of cancers

YUSUKE OJI[1], NAOYA TATSUMI[1], MARI FUKUDA[2], SHIN-ICHI NAKATSUKA[12], SAYAKA AOYAGI[2], ERIKA HIRATA[2], ISAMU NANCHI[2], FUMIHIRO FUJIKI[3], HIROKO NAKAJIMA[3], YUMIKO YAMAMOTO[2], SYOHEI SHIBATA[2], MICHIYO NAKAMURA[2], KANA HASEGAWA[1], SAYAKA TAKAGI[2], IKUYO FUKUDA[2], TOMOKO HOSHIKAWA[2], YUI MURAKAMI[2], MASAHIDE MORI[13], MASAYOSHI INOUE[4], TETSUJI NAKA[5], TAKESHI TOMONAGA[15], YOSHIFUMI SHIMIZU[16], MASASHI NAKAGAWA[17], JUNICHI HASEGAWA[18], RIICHIRO NEZU[18], HIDENORI INOHARA[6], SHUICHI IZUMOTO[7], NORIO NONOMURA[8], TOSHIKI YOSHIMINE[7], MEINOSHIN OKUMURA[4], EIICHI MORII[9], HAJIME MAEDA[14], SUMIYUKI NISHIDA[10], NAOKI HOSEN[11], AKIHIRO TSUBOI[10], YOSHIHIRO OKA[5] and HARUO SUGIYAMA[2]

Departments of [1]Cancer Stem Cell Biology, [2]Functional Diagnostic Science, [3]Cancer Immunology, [4]Surgery, [5]Respiratory Medicine and Allergy, Rheumatic Diseases, [6]Otolaryngology and Sensory Organ Surgery, [7]Neurosurgery, [8]Urology, [9]Pathology, [10]Cancer Immunotherapy, and [11]Biomedical Informatics, Osaka University Graduate School of Medicine, Osaka; [12]Department of Pathology, Kansai Rosai Hospital, Hyogo; Departments of [13]Thoracic Oncology, and [14]General Thoracic Surgery, Toneyama National Hospital; [15]Laboratory of Proteome Research, National Institute of Biomedical Innovation, Osaka; [16]Department of Internal Medicine, Takarazuka City Hospital, Hyogo; [17]Department of Internal Medicine, Nissay Hospital; [18]Department of Surgery, Osaka Rosai Hospital, Osaka, Japan

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**Abstract.** Recent studies have shown that cancer immunotherapy could be a promising therapeutic approach for the treatment of cancer. In the present study, to identify novel tumor-associated antigens (TAAs), the proteins expressed in a panel of cancer cells were serologically screened by immunoblot analysis and the eukaryotic elongation factor 2 (eEF2) was identified as an antigen that was recognized by IgG autoantibody in sera from a group of patients with head and neck squamous cell carcinoma (HNSCC) or colon cancer. Enzyme-linked immunosorbent assay showed that serum eEF2 IgG Ab levels were significantly higher in colorectal and gastric cancer patients compared to healthy individuals. Immunohistochemistry experiments showed that the eEF2 protein was overexpressed in the majority of lung, esophageal, pancreatic, breast and prostate cancers, HNSCC, glioblastoma multiforme and non-Hodgkin's lymphoma (NHL). Knockdown of eEF2 by short hairpin RNA (shRNA) significantly inhibited the growth in four eEF2-expressing cell lines, PC14 lung cancer, PCI6 pancreatic cancer, HT1080 fibrosarcoma and A172 glioblastoma cells, but not in eEF2-undetectable MCF7 cells. Furthermore, eEF2-derived 9-mer peptides, EF786 (eEF2 786-794 aa) and EF292 (eEF2 292-300 aa), elicited cytotoxic T lymphocyte (CTL) responses in peripheral blood mononuclear cells (PBMCs) from an HLA-A*24:02- and an HLA-A*02:01-positive healthy donor, respectively, in an HLA-A-restricted manner. These results indicated that the *eEF2* gene is overexpressed in the majority of several types of cancers and plays an oncogenic role in cancer cell growth. Moreover, the *eEF2* gene product is immunogenic and a promising target molecule of cancer immunotherapy for several types of cancers.

## Introduction

Cancer immunotherapy consists of therapeutic approaches to elicit effective antitumor immunity through active or passive immunization. Recent studies have shown that cancer immunotherapy have potential to provide anticancer activity as a single agent or in combination with conventional surgery, radiation and chemotherapy as reviewed (1-4). These findings indicate that cancer immunotherapy should be a promising therapeutic option for the cancer treatment.

Strategies of cancer immunotherapy include antitumor monoclonal antibodies, cancer vaccines, adoptive transfer

*Correspondence to:* Professor Yusuke Oji, Department of Cancer Stem Cell Biology, Osaka University Graduate School of Medicine, 1-7 Yamada-oka Suita, Osaka 565-0871, Japan
E-mail: oji@sahs.med.osaka-u.ac.jp

*Key words:* eukaryotic elongation factor 2, tumor associated antigen, cytotoxic T lymphocyte, autoantibody, cancer immunotherapy

of *ex vivo* activated T and natural killer cells, and administration of antibodies or recombinant proteins that either costimulate immune cells or block immune inhibitory pathways (5). Among these strategies, cancer vaccines are approaches to specifically activate host T cells against tumor antigens. The target antigens of cancer vaccine should be: i) highly immunogenic; ii) expressed in a significant proportion of cancer patients; iii) not expressed (or expressed in limited populations) in normal tissues; and iv) required for cancer cell growth and/or survival. Although large number of tumor-associated antigens (TAAs) have been identified using recently developed new technologies such as SEREX and protein microarrays (6,7), there are limited number of antigens that fit all of these criteria in current cancer vaccines.

High level protein biosynthesis is one of the characteristics of cancer cell metabolism (8). Translation is regulated at the initiation and elongation step and deregulated in cancer through a variety of mechanisms (9). Eukaryotic elongation factor 2 (*eEF2*) is a gene that plays an essential role in the polypeptide chain elongation step. Cells control translation levels at elongation step through regulation of eEF2 activity under multiple biological conditions such as cell cycle progression (10) and genotoxic stress (11,12), or in response to endogenous carbon monoxide that exerts antiproliferative effects (13). Previously, we showed that eEF2 was overexpressed in the majority of gastric and colorectal cancers and promoted progression of $G_2$/M of the cell cycle in association with activation of Akt and a $G_2$/M regulator, cdc2 proteins, resulting in the enhancement of *in vitro* and *in vivo* cancer cell growth (14). However, the role for eEF2 in the tumorigenesis remains largely unknown and it is undetermined whether eEF2 can be a target molecule of molecule-targeted cancer therapy.

In the present study, we identified eEF2 as an antigen eliciting humoral immune responses in a group of patients with HNSCC or colorectal cancer by immunoblot analysis and showed that eEF2 was overexpressed in the majority of various types of cancers such as lung, esophageal, pancreatic, breast and prostate cancers, HNSCC, glioblastoma multiforme and NHL. Knockdown of eEF2 by shRNA significantly inhibited growth of cancer cells. Furthermore, eEF2-derived 9-mer peptides, EF786 (eEF2 786-794 aa) and EF292 (eEF2 292-300 aa), elicited cytotoxic T lymphocyte (CTL) responses in PBMCs from an HLA-A*24:02- and an HLA-A*02:01-positive healthy donors, respectively, in an HLA-A-restricted manner.

## Materials and methods

*Cell lines.* Lung cancer cell lines PC14 and LU99B, pancreatic cancer cell line PCI6, glioblastoma cell line A172, fibrosarcoma cell line HT1080, gastric cancer cell lines MKN28 and AZ-521, and breast cancer cell line MCF7 were cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum (FBS). Leukemia cell line K562, colon cancer cell line SW480, parent T2 and T2 cells with forced expression of either HLA-A24:02 (T2-2402) (15) or HLA-A02:01 (T2-0201) (16) were cultured in RPMI-1640 medium supplemented with 10% FBS. Leukemia cell line TF-1 was cultured in RPMI-1640 medium supplemented with 10% FBS containing 2 ng/ml human recombinant GM-CSF (Peprotech, Rocky Hill, NJ, USA).

*Sera samples.* Sera were obtained from 79 colorectal and 80 gastric cancer patients, 10 patients with head and neck squamous cell carcinoma (HNSCC) and 40 healthy individuals with informed consent at Osaka University Hospital and Osaka Rosai Hospital and stored at -80°C until use.

*Tissue samples.* Tumor tissues were obtained from 31 lung adenocarcinoma, 20 small-cell lung cancer, 15 esophageal squamous cell carcinoma, 21 HNSCC, 28 pancreatic cancer, 8 breast cancer, 16 glioblastoma, 4 prostate cancer and 50 NHL (40 diffuse large B-cell lymphoma and 10 follicular lymphoma) patients. All samples were obtained with informed consent at Osaka University Hospital, Toneyama National Hospital, NHO Osaka Minami Medical Center, and Higashiosaka City General Hospital.

*Western blot analysis.* Proteins were separated by SDS-PAGE and transferred to Immobilon polyvinylidene difluoride membrane. After blocking of non-specific binding, the membranes were incubated with the first antibodies, followed by incubation with the corresponding secondary antibodies conjugated with alkaline phosphatase, and visualized using BCIP/NBT kit (Nacalai Tesque, Kyoto, Japan). Polyclonal anti-EF2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-GAPDH (Chemicon International, Temecula, CA, USA) were used as the first antibodies.

*Density gradient isoelectric focusing.* Density gradient isoelectric focusing was performed by the method reported previously (17) with minor modifications. In brief, K562 cells ($5x10^7$ cells) were lysed in 2 ml of 0.1% Triton X-100/PBS. After centrifugation, the supernatant was collected as cytoplasmic fraction. Proteins of the cytoplasmic fraction were precipitated with acetone and the pellet was solved in 1 ml of $dH_2O$ containing 4% CHAPS and 7 M urea. Isoelectric focusing was carried out using an LKB column (NA-1720, Nihon-Eido Co., Tokyo, Japan) according to the manufacturer's instructions. On completion of the run, effluent fractions (3 ml each) were collected and twice dialyzed to 200 volume of de-ionized water for 18 h, and then the proteins were precipitated with acetone and stored at -80°C until use.

*MALDI-TOF mass spectrometry.* The bands on the silver stained gels were excised with surgical blazor. After dehydration with acetonitrile, the gel slice was dried with Speed Vac. The dried gels were digested with Trypsin (Promega, Madison, WI, USA) at 37°C for 24 h and the tryptic peptides were analyzed. All peptide mass fingerprinting (PMF) spectra were obtained by Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using an ultraflex spectrometer (Bruker Daltonics, Bremen, Germany). PMF data were then searched with MS-FIT software against NCBInr database.

*Immunohistochemistry.* Formalin-fixed tissue sections were cut from each paraffin-block. After dewaxing and rehydration, the sections were antigen retrieved using Pascal (Dako Cytometry, Glostrup, Denmark) and reacted with the first antibody at 4°C overnight and then reacted with Dako Envision kit/HRP (Dako Cytometry) at room temperature for 30 min.

After treatment with 3% $H_2O_2$ solution to reduce endogenous peroxidase activity, immunoreactive eEF2 protein was visualized using diaminobenzidine (DAB). The sections were then counterstained with hematoxylin. The intensity of stain in tumor cells was scored as positive (increased staining in carcinoma cells compared to that in normal cells) or negative (less or negative staining in carcinoma cells) by a pathologist. eEF2-H118 antibody (Santa Cruz Biotechnology) that recognized 741-858aa of eEF2 protein and Sigma-Aldrich #SAB4500695 antibody that recognized the N terminus of eEF2 protein were used as first antibodies. Non-immune rabbit immunoglobulin (Dako Cytometry) was used as negative control for non-specific staining.

*Sequencing.* The *eEF2* gene overexpressed in tumors was RT-PCR amplified and directly sequenced in both directions by the method previously described (14).

*Transient expression of shRNA targeting eEF2.* Two different shRNA vectors targeting eEF2 mRNA (shEF-1918 and shEF-2804 targeting 1918-1947 and 2804-2833 nt of eEF2 sequence, respectively) were prepared as described previously (14). shRNA targeting luciferase (shLuc) was used as a control. shRNA vectors were transiently expressed as described previously (14).

*Enzyme-linked immunosorbent assay (ELISA).* ELISA was established to measure serum eEF2 IgG Ab levels by a method previously reported (18) with modifications. ELISA 96-well plates were coated with recombinant GST-tagged eEF2 fragmented protein (Ref Seq NM_001961, 411-858 aa) (2 $\mu$g/well). Plates were blocked with TBS containing 0.05% Tween-20 and 1% gelatin. Sera were diluted at 1:100 in TBS containing 0.05% Tween-20 (0.05% TBST) and pre-absorbed by immobilized GST protein at 4°C overnight. Then, 100 $\mu$l of the diluted sera was added to each well for overnight incubation at 4°C. After washing, captured eEF2 IgG Ab was detected using ALP-conjugated goat anti-human IgG Ab (Santa Cruz Biotechnology) and BCIP/NBT kit. Then, absorbance at 550 nm was measured using a microplate reader. All sera were examined in duplicate. The titers of eEF2 IgG Ab were calculated by interpolation from the standard line which was constructed for each assay from the results of simultaneous measurements of serial dilutions of rabbit polyclonal eEF2 H-118 Ab using the corresponding second Ab (data not shown). eEF2 Ab titer that produces the absorbance at 550 nm equal to that produced by 1.0 $\mu$g/ml of eEF2 H-118 Ab in the ELISA system was defined as 1.0 EF2-reacting-unit (ERU).

*Synthetic peptides.* The primary amino acid sequence of human eEF2 was analyzed for consensus motifs for 9-mer peptides capable of binding to HLA-A*24:02 or 02:01 using ProPred-I computer algorithm (Table I). Then, the top 4 candidate peptides for HLA-A*02:01 and 24:02 each were synthesized at immunological grade (Sigma Genosys, Hokkaido, Japan). Synthesized peptide was solved in $dH_2O$ (2 mg/ml) and stored at -20°C until use.

*MHC stabilization assay.* Binding of the synthetic peptides to HLA-A*24:02 or 02:01 molecules was evaluated by MHC stabilization assay using antigen processing mutant T2-2402 or T2-0201 cells as described previously (19). Expression of HLA-A24 or HLA-A02 molecules was measured with a FACSort flow cytometer (BD Biosciences, San Jose, CA, USA) and the mean fluorescence intensity (MFI) was recorded.

*In vitro generation of eEF2 peptide-specific $CD8^+$ T cells.* PBMCs were obtained from an HLA-A*24:02-positive and an HLA-A*02:01-positive healthy donors by density gradient centrifugation. $CD4^+CD25^+$ Treg cells were depleted from PBMCs by using CD25 MicroBeads (Miltenyi Biotech, Auburn, CA, USA). For generation of autologous dendritic cells (DCs), $CD14^+$ monocytes were isolated from the donor PBMCs using BD IMag CD14 isolation kit (BD Bioscience) and cultured in X-VIVO15 (Bio Whittaker, Walkersville, MD, USA) supplemented with 1% human AB serum (Nabi, Miami, FL, USA) containing IL-4 (1,000 U/ml) and GM-CSF (800 U/ml). After 24 h, IL-1β (10 ng/ml), IL-6 (1,000 U/ml), TNF-α (10 ng/ml), and PGE-2 (1 $\mu$g/ml) were added to the culture for DC maturation and the cells were cultured for 48 h. DCs were pulsed with EF2 peptide at the concentration of 10 $\mu$g/ml in X-VIVO15 supplemented with 1% human AB serum at 37°C for 2 h, irradiated at 30 Gy, and washed 3 times with RPMI-1640 medium. Then, Treg-depleted PBMCs ($2x10^6$ cells) were stimulated by co-culture with the EF2 peptide-pulsed DCs at the DC: PBMC ratio of 1:10 in X-VIVO15 supplemented with 5% human AB serum. After 24 h of co-culture, IL-2 (20 U/ml) was added to the culture. The cultured cells were repeatedly stimulated with the EF2 peptide-pulsed, irradiated autologous PBMCs at 10-day intervals. After several times of re-stimulation, the cultured cells were maintained as the established T cell lines in X-VIVO15 supplemented with 5% human AB serum, IL-7 (10 IU/ml) and IL-15 (10 IU/ml) and used for cytotoxic assays.

*$^{51}Cr$ release cytotoxicity assay.* Effector cells were prepared from the established T cell lines using Human CD8 T Lymphocyte Enrichment Set-DM (BD Bioscience). Target cells (listed in Table III) were labeled with 100 $\mu$Ci of $^{51}Cr$ (Perkin-Elmer, Waltham, MA, USA) at 37°C for 1.5 h and the target cells ($1x10^4$ cells) were added to wells containing varying numbers of effector cells in 96-well plates. After 4 h of incubation at 37°C, 100 $\mu$l of supernatants were collected from each well and measured for radioactivity. The percentage of specific lysis was calculated as follows: percentage of specific lysis = (cpm of experimental release - cpm of spontaneous release)x100/(cpm of maximal release - cpm of spontaneous release). Radioactivity of the supernatant of the target cells that were cultured without effector cells and the radioactivity of target cells that were completely lysed by the treatment with 1% Triton X-100 was used for spontaneous and maximal release, respectively. The characteristics of target cells in cytotoxicity assay are listed in Table III.

*Statistics.* The statistical significance in a difference between arithmetical means of test groups was assessed by unpaired t-test or Kruskal-Wallis test. After Kruskal-Wallis test, Scheffe's F-test was used as a post hoc test.