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

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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.

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.

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 Cell 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'-ATTGCGGCCGCCAAGGTTCAATGGC-GACGGCGACCATAGC-3' and 5'-ATTGCGGCCGC-GATGAACTTTCACCCTAAGTTTTTCTTACTACG-GTCACGCAGAGAGGTCACTGTTATCAAAACGC-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™ AQueous 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-di-methylthiozol-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). 5×10^6 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 1×10^{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
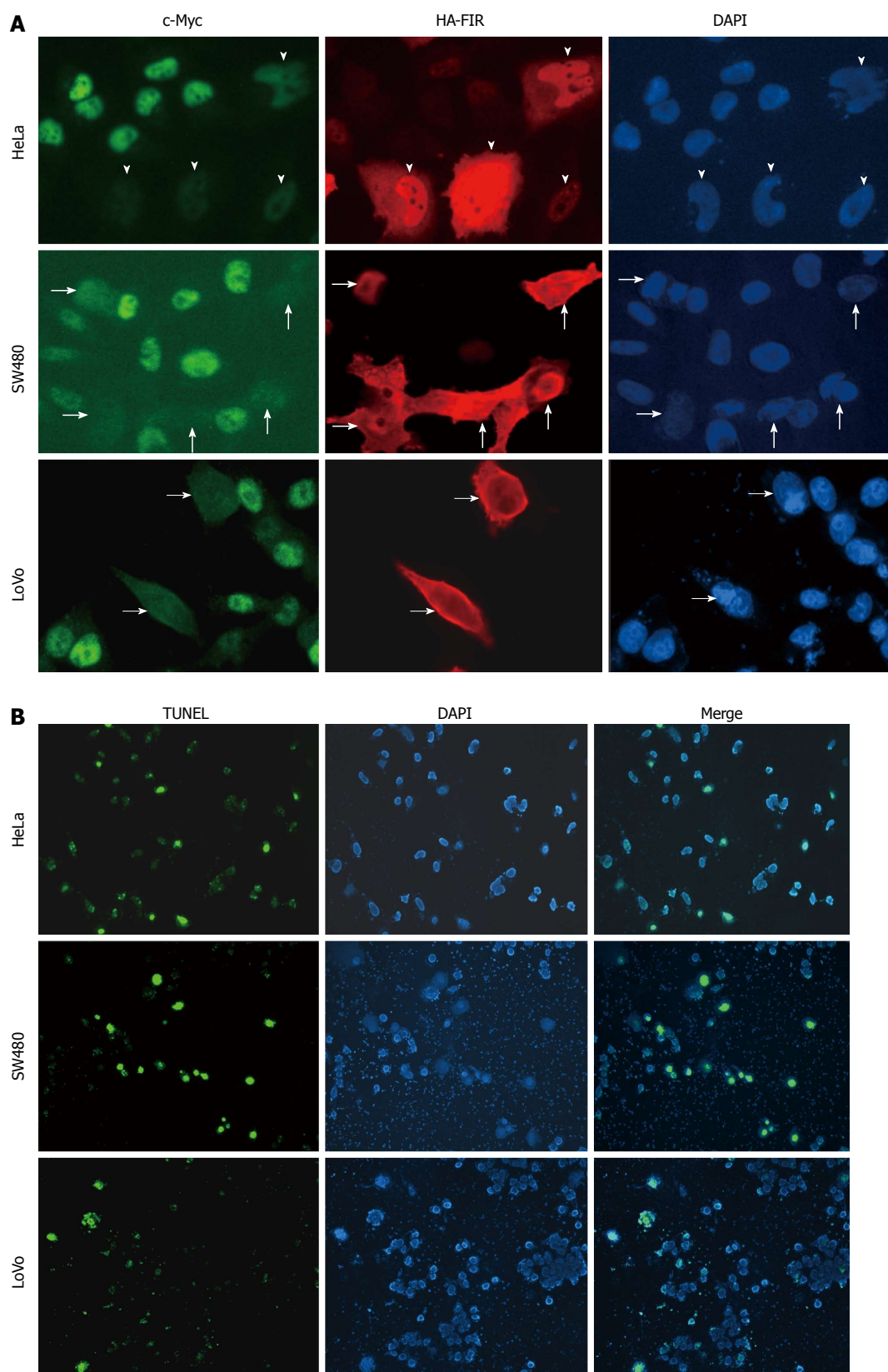
Figure 1 Far up stream element-binding protein-interacting repressor suppresses endogenous c-Myc in SW480 and LoVo cells as well as HeLa cells. A: 100 fmol of HA-FIR was transfected into the colon cancer cell lines, SW480 and LoVo cells, as well as HeLa cells (cervical squamous cell carcinoma cells) 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.
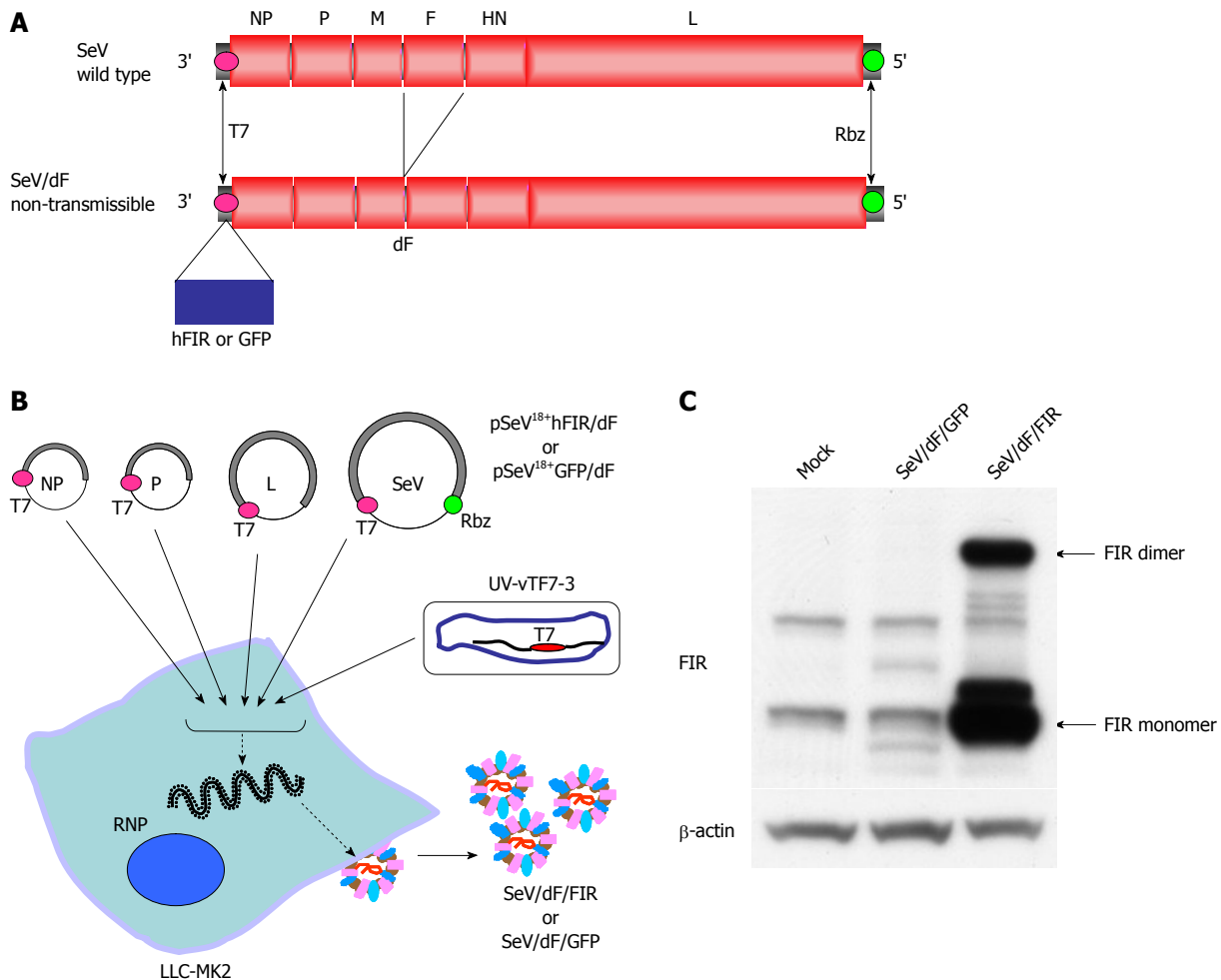
Figure 2 Structures and procedures for generating fusion gene-deficient Sendai virus/dF/far up stream element-binding protein-interacting repressor (SeV/dF/FIR) or Sendai virus/dF/green fluorescent protein vectors (SeV/dF/GFP) from Sendai virus genome RNA. A: Schematic genome structures of wild-type (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

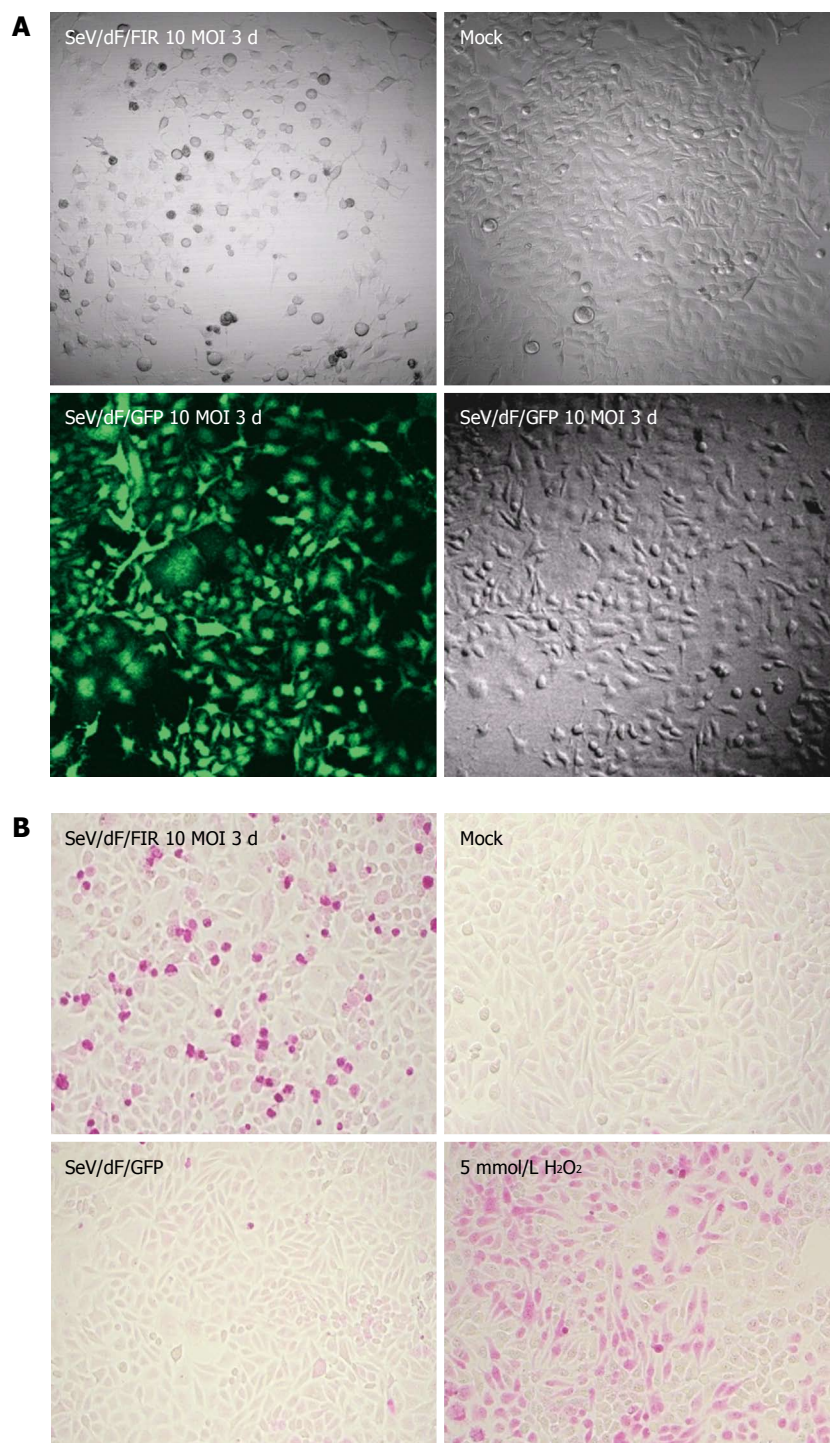Overexpression of c-Myc has been known to promote

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-
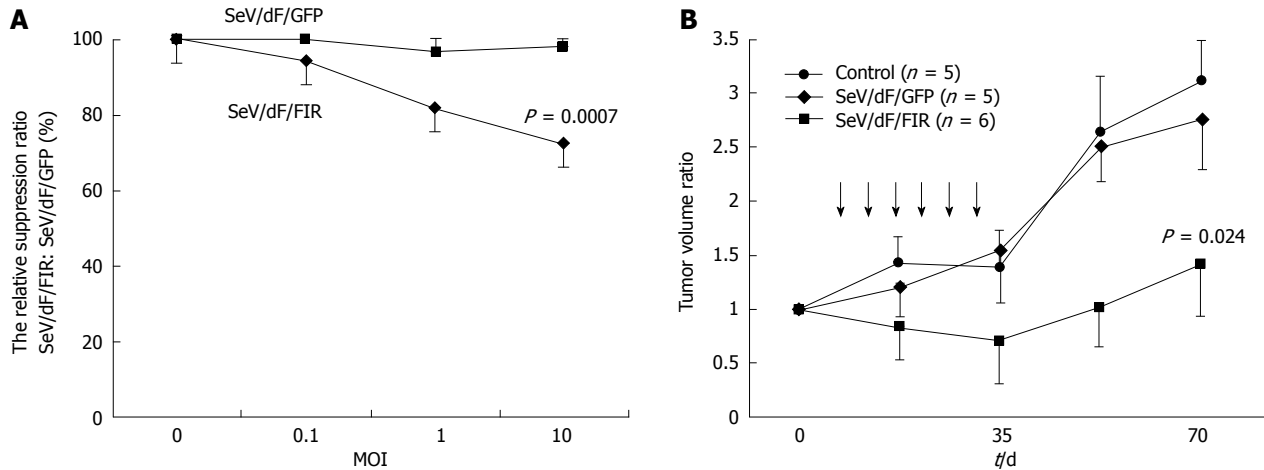
Figure 4 Sendai virus/dF/far up stream element-binding protein-interacting repressor decreased HeLa cell growth *in vitro* and in a xenograft animal model. HeLa cells (A) and SW480 cells (B) were infected with 0, 0.1, 1, and 10 MOI of SeV/dF/FIR or SeV/dF/GFP vectors and cell growth was measured by MTS 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 5×10^6 HeLa cells were xenografted into the right thigh of Balbc/nu/nu mice, the tumor size was approximately 7-8 mm. 3.0×10^7 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.
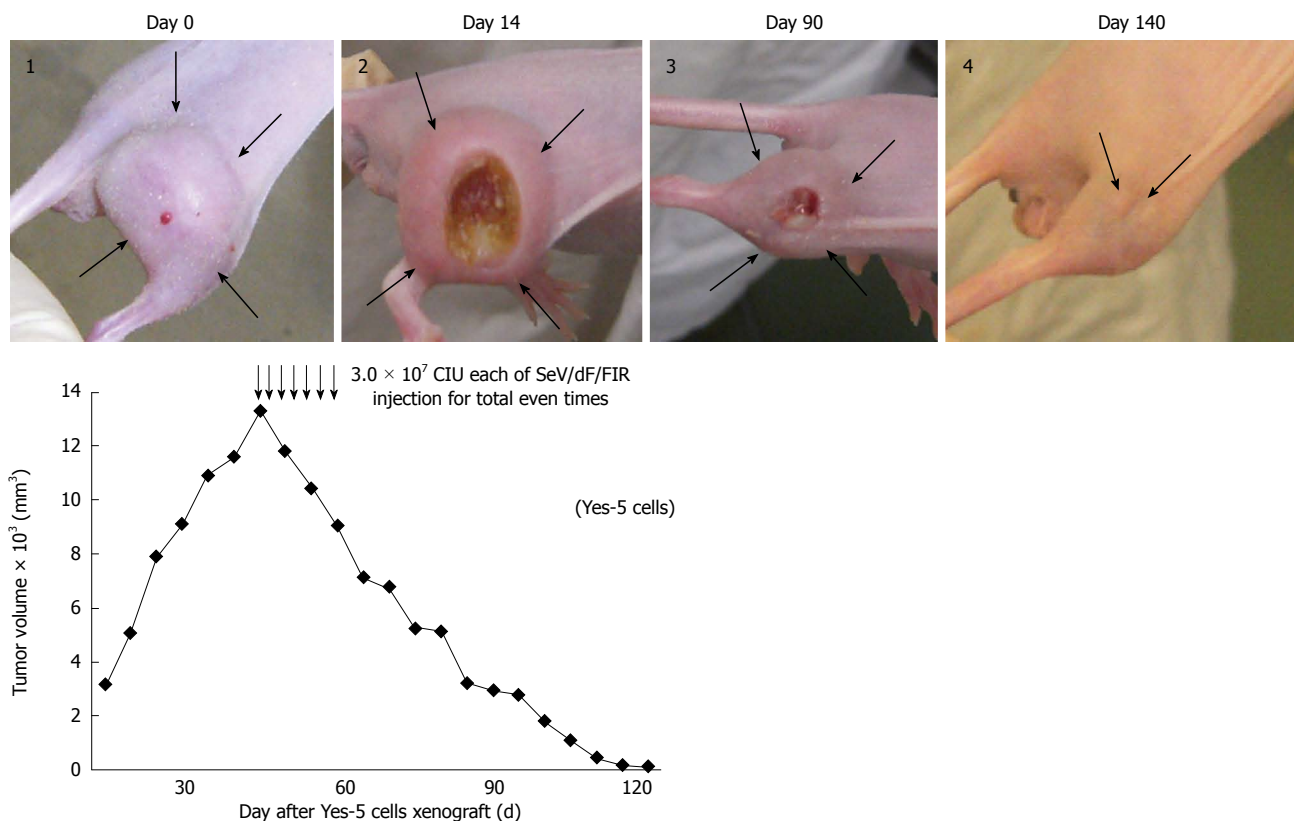

Figure 5 Sendai virus/dF/Far up stream element-binding protein-interacting repressor vector showed anti-tumor activity in a mouse xenograft model. 10^6 Yes-5 cells were xenografted into the right thigh of Balbc/nu/nu mice, and the tumor size was approximately 15 mm in diameter at Day 0. 3.0×10^7 CIU 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 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 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, Cytoplasmic 1-like or poly (A) binding protein, cytoplasmic 5
PABPC1 | PABPC4	Poly (A) binding protein, cytoplasmic 1 Or poly A binding protein, cytoplasmic 4
PABPC1L2B | PABPC4	Poly (A) binding protein, cytoplasmic 1-like 2B 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

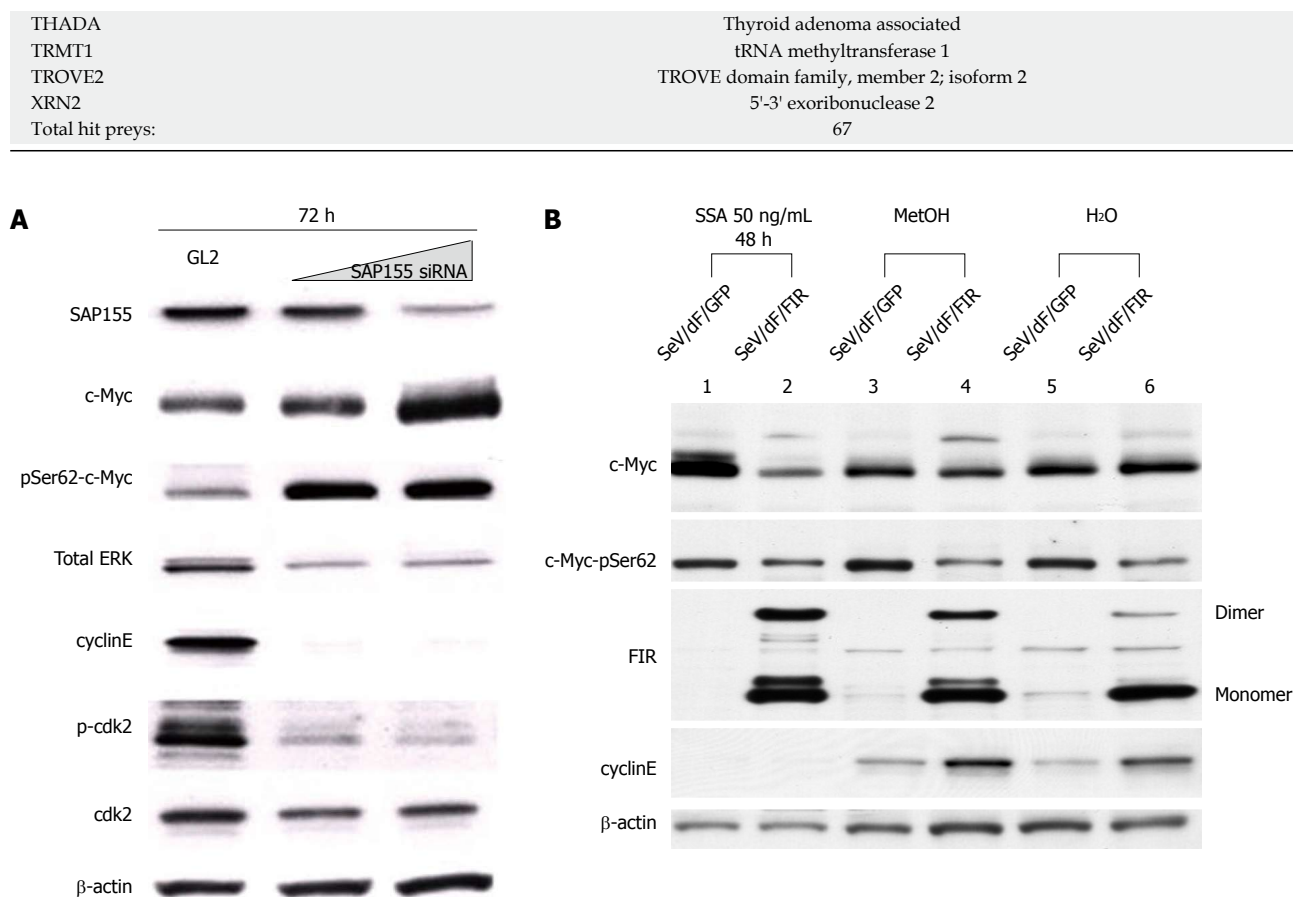| THADA	Thyroid adenoma associated
TRMT1	tRNA methyltransferase 1
TROVE2	TROVE domain family, member 2; isoform 2
XRN2	5'-3' exoribonuclease 2
Total hit preys:	67

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$_2$O). 10 MOI of SeV/dF/FIR apparently suppressed activated c-Myc expression, whereas SeV/dF/FIR did not influence basal expression (MetOH or H$_2$O). 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 ad-

vantage 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
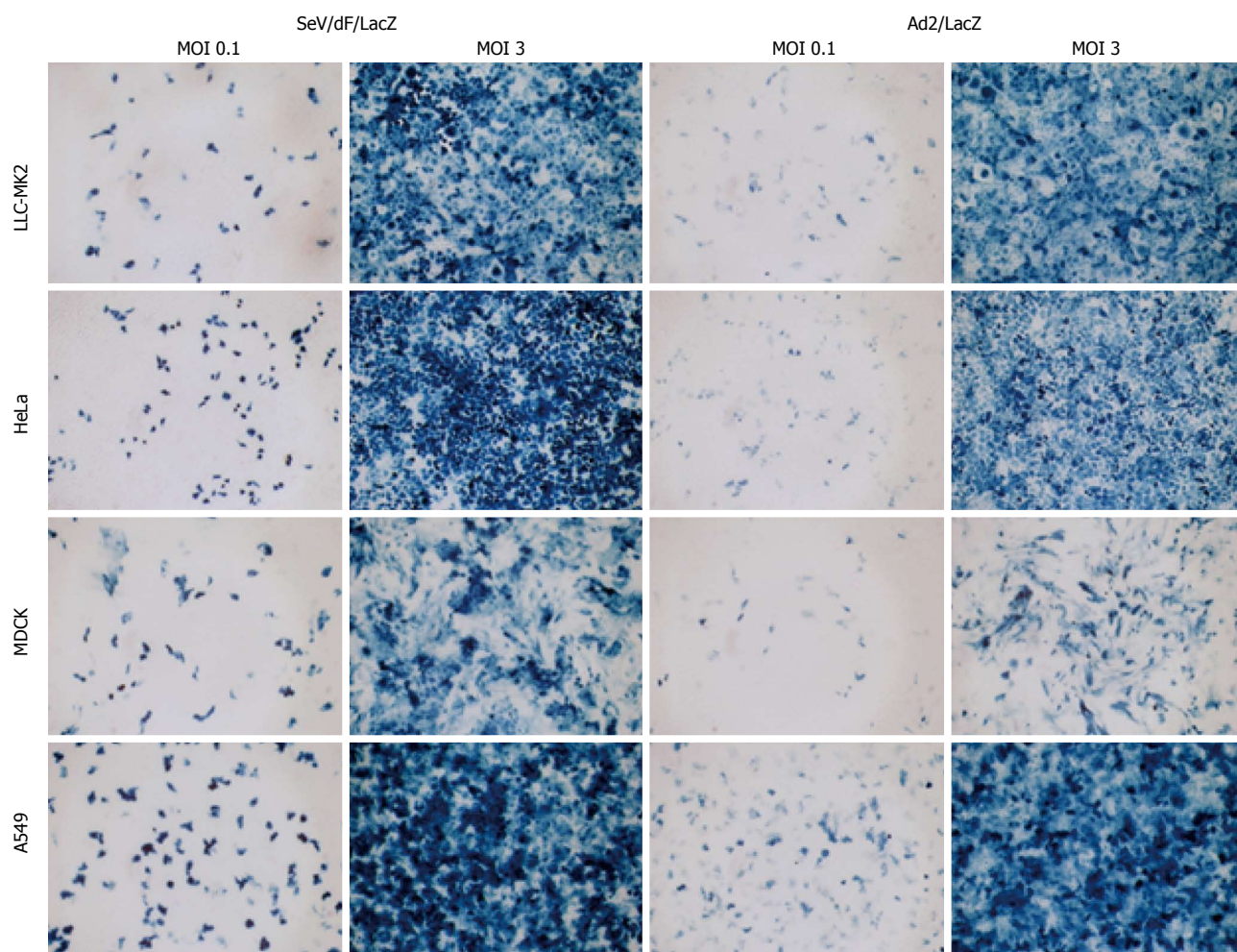
Figure 7 Sendai virus/dF/LacZ transduction efficiency was examined in some human and animal cell lines. Confluent culture of LLC-MK2 (macaque kidney fibroblasts), HeLa (human adenocarcinoma cells), MDCK (canine kidney cells), and A549 (human lung carcinoma cells) were infected with LacZ expressing SeV 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, al-

though further clinical and basic research are required to explain the precise mechanism of tumor suppression by FIR expressing vectors.

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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