**Name of journal:** **World Journal of Gastroenterology**

**ESPS Manuscript NO: 6545**

**Columns: ORIGINAL ARTICLES**

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

MatsushitaK *et al*. FIR-encoding Sendai virus vector for cancer gene therapy

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**Supported by** in part by the 21st Century COE (Center Of Excellence) Programs to Dr. Takenori Ochiai and by a Grant-in-Aid 18591453 to K.M from the Ministry of Education, Science, Sports and Culture of Japan

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**Received:** October 22, 2013 **Revised:** December 14, 2013

**Accepted:** January 19, 2014

**Published online:**

**Abstract**

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

**METHODS:** Endogenous c-Myc suppression and apoptosis induction by transient FIR-expressing vector was examined *in vivo* *via* HA-tagged FIR (HA-FIR) expression vector. Fusion gene-deficient, non-transmissible, Sendai virus (SeV) vector encoding FIR cDNA, SeV/dF/FIR, was prepared. SeV/dF/FIR was examined 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) cells. Antitumor efficacy in mice xenograft model was also examined. Molecular mechanism of anti-tumor effect and c-Myc suppression by SeV/dF/FIR was examined using Spliceostatin A (SSA), a SAP155 inhibitor, or SAP155 siRNA that induce c-Myc by increasing FIRexon2 in HeLa cells. Transcriptional activity by FIR from1.5 kb *c-myc* promoter was examined through chloramphenicol acetyl transferase (CAT) assay *in vitro*.

RESULTS: FUSE-binding protein-interacting repressor (FIR) has been found to repress *c-myc* transcription and in turn the overexpression of FIR drives 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 (FIRexon2), counteracting FIR for c-Myc protein expression. Further FIR forms complex with SAP155 and inhibits mutual well-established functions. Thus both valuable and side effects of exogenous FIR stimuli should be tested for future clinical application. SeV/dF/FIR, cytoplasmic RNA virus, successfully prepared and indicated the high efficiency gene transduction *in vivo* experiment. Further, in nude mice tumor xenograft models, SeV/dF/FIR displays high antitumor efficiency against human cancer cells. SeV/dF/FIR suppressed SSA-activated c-Myc. SAP155 siRNA, potentially produces FIRexon2, led to c-Myc overexpression with phosphorylation at Ser62. HA-FIR suppressed CAT activity from 1.5 kb *c-myc* promoter. HA-FIR also 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 effect and apoptosis induction in HeLa and SW480 cells.

**CONCLUSION:** SeV/dF/FIR showed strong tumor growth suppression with no significant side effects in animal xenograft model, thus SeV/dF/FIR is potentially applicable for future clinical cancer treatment.

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

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

Matsushita K, Shimada H, Ueda Y, Inoue M, Hasegawa M, Tomonaga T, Matsubara H, Nomura F. Non-transmissible Sendai virus vector encoding *c-myc* suppressor FBP-interacting repressor for cancer therapy. World J Gastroenterol 2014;

**Available from:**

**DOI:**

**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-ERTAM 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 proper 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 stimulating *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]. Further, exogenous FIR expression represses endogenous *c-myc* transcription, drives apoptosis by the decrease of c-Myc[7]. Although these observations indicate that cancer therapies targeting *c-myc* suppression by FIR will be hopeful strategy, FIR’s mechanisms of antitumor effect should be revealed in detail when it’s going to be tested clinically. For example, first, FIR is alternatively spliced in colorectal cancer lacking the transcriptional repression domain within exon 2 (FIRexon2)[7]. Second, FIR and FIRexon2 form a homo- or hetero-dimer, which makes a complex with SAP155, a subunit of the essential splicing factor 3b (SF3b) subcomplex in the spliceosome, and is required for proper P27Kip1 (P27) pre-mRNA splicing, after which P27 arrests cells in G1[8]. Third, SAP155 is required for proper FIR pre-mRNA splicing and thus FIR/FIRexon2/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 reduced cdk2/cyclinE expression[10]. Together, exogenous FIR stimuli potentially effects FIR/FIRexon2/SAP155 interaction that is pivotal for cell cycle, cancer development and differentiation.

In this study, fusion gene-deficient human FIR-expressing Sendai virus vector (SeV/dF/FIR) was prepared for future cancer therapy due to the following reasons; Sendai virus (SeV), a member of the family Paramyxoviridae, 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 protein (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]. In other words, 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 advantage of SeV. Recently, a novel SeV vector was established that an enhanced green fluorescent protein (EGFP) reporter gene was inserted at 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 has been also prepared following SeV/dF/EGFP[12,13]. The validity of SeV/dF/FIR is examined for cancer therapy in animal xenografts model because SeV/dF vectors have been revealed applicable to clinical use[14-18]. Clinical assignment of SeV/dF/FIR for cancer therapies is also discussed.

**MATERIALS AND METHODS**

*Plasmids*

Full-length FIR cDNA (HA-FIR) was cloned into the pCGNM2 vector plasmid, respectively, to introduce the hemagglutinin (HA)-tag at their amino termini[7]. Full-length FIR cDNA was cloned into the p3xFLAG-CMV-14 vector (Sigma, MO, USA) to introduce the Flag-tag at their amino termini for the selection of FIR-Flag in 293T cells (performed by Dr. T.N.). Plasmids were prepared by CsCl ultra-centrifugation or Endofree® Plasmid Maxi Kit (Qiagen, MD, USA) and the DNA sequences were verified.

***Tumor cell lines***

HeLa cells: a human cervical squamous cell carcinoma, LoVo and SW480: human colon cancer cell lines, 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 °C in a humidified atmosphere containing 5% CO2. All tumor-cell lines except for 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 are listed previously**[**8**]**. Luciferase GL2 duplex 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 °C in a 5% CO2 incubator.

***Apoptosis detection***

Apoptotic cells were detected by [Terminal deoxynucleotidyl transferase](http://en.wikipedia.org/wiki/Terminal_deoxynucleotidyl_transferase) dUTP nick end labeling (TUNEL) assay according to the manufacturer’s instructions (Apoptosis Detection System, Fluorescein. Promega, WI, United States) and described previously[7]. Apoptosis detection by APOPercentage apoptosis assayTM (Funakoshi Co., Ltd., Tokyo, Japan) was performed according to manufacturer’s instructions[9].

***Construction of SeV vector***

The 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’-ATTGCGGCCGCGATGAA*CTTTCACCCT*AAG*TTTTTCTTA*CTACGGTCACGCAGAGAGGTCACTGTTATCAAAACGC-3’. The amplified fragment was introduced into the NotI site of the parental SeV vector cDNA, pSeV18+b(+)/dF[15], to generate pSeV18+hFIR/dF. pSeV18+hFIR/dF was transfected to LLC-MK2 cells that 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 °C 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 severalrounds of propagation. The virus titers of the recovered vectors were determined by their infectivity and expressed using cell-infectious units (CIU). These vectors were keptfrozen at –80 °C until use.

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

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

*MTS assay for assessing cell viability*

The inhibitory effects of viruses on the proliferation of cultured cells were examined by the CellTiter96TMAQueousOne Solution Proliferation Assay (Promega, Madison, WI, United States). In brief, five thousand cells were plated in each well the day 0. The day 1, 24 h later, HeLa cells were infected with SeV/dF/FIR or SeV/dF/GFP as control at 0.1 to 10 MOI, and cultured for 2 d. The day 3, the 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 Mutiabel CounterTM, ARVOSX WAIIACTM (Perkin Elmer, MA, United States). The results are shown as percentages of control results of 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 xenografts experiments***

The *in vivo* inhibition of tumorigenicity of HeLa cells (human cevical squamous cell carcinoma) was examined by SeV/dF/FIR or SeV/dF/GFP injection (as control). 5 x 106 cells/50 μl PBS of HeLa cells were injected beneath the skin of right thigh of nude mice (balbc/nu/nu, 6-wk birth, males). Tumor growth was observed and measured the long and short diameter for tumor volume calculation. Thirty days after inoculation, tumor grew up to 5-8 mm in diameter in 18 of 18 mice (100%). Tumor size was calculated using for the formula, (a x b2)/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 the endogenous *c-myc* gene suppression by FIR, HA-FIR expression plasmids were transfected into HeLa cells, and c-Myc expression was visualized by immnostaining 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) as well. 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 or SeV/dF/GFP was prepared as described in materials and methods (Figure 2A, B). SeV/dF/FIR vectors were infected to LLC-MK2 or HeLa cells. FIR protein expression level was examined by western blot with anti-FIR antibody (6B4) (Figure 2C). At least 1x10E10 CIU of fusion gene-deficient SeV/dF/FIR virus particles were prepared at amplification for the use of experiments.

***Transduction efficiency of the SeV/dF/GFP vector to various human tumor-cell lines***

Nine human and five mouse tumor cell lines, including 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 MOI of 10–100; > 90% GFP positive tumor-cell lines were detected at MOI over 10 (Figure 3A and data not shown). Further, SeV/dF/FIR but not SeV/dF/GFP drastically suppressed HeLa cell (human cervical squamous carcinoma cells) growth as shown by Apopercentage assay (Figure 3B), indicating SeV/dF/FIR suppresses tumor cell growth with apoptosis *in vivo*.

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

Additionally, 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, xenografted tumor in size of 2cm in diameter disappeared completely by SeV/dF/FIR, indicating SeV/dF/FIR has some immunological effects (Figure 5)[24,25] .

*FIR was co-immunoprecipitated with SAP155*

If SeV/dF/FIR is going to be tested clinically, endogenous FIR-interacting proteins should be identifed to avoid unexpected side effects. For this purpose, 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 rotein)[26,27], SAP155[28], and SRp54 (splicing factor, arginine/serine rich-12)[28] were identified as candidates FIR-binding proteins. So far, no significant side effect was observed in SeV/dF/FIR treatment including our study[29].

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

We previously reported that adenovirus vector encoding FIRexon2 (Ad–FIRexon2) 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–FIRexon2, evaluated by western blot analysis, was too distinctive to be explained solely by *c-myc* transcription activation[8]. Therefore, we hypothesized that c-Myc protein should be modified by Ad–FIRexon2 to be more stable. Actually, Ad–FIRexon2 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 generates FIRexon2[8,9]. In fact, SAP155 siRNA increased levels of c-Myc phosphorylated at Ser62 as Ad–FIRexon2 (Figure 6A). In other words, Ad–FIRexon2, which lacks the transcriptional repressor domain, directly or indirectly activated *c-myc* expression not only through transcription but also through protein level, suggesting that FIRexon2 acts opposite to the repressor function by FIR[8].

In this study, the effect of SeV/dF/FIR was examined whether it suppresses the increase of 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, not basal, *c-myc* transcription[6]. These observations suggest that the increase of *c-myc* by either SAP155 siRNA or SSA treatment is due to the reduced FIR activity, or the increase of the ratio of FIRexon2/FIR in HeLa cells. Together, SeV/dF/FIR is potentially clinically applicable for cancer therapy because 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 vecors (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 adenovirus vector expressing LacZ (Ad5/LacZ) at the same MOI. Prominently, SeV/dF/ transduction efficiency to those cells was even higher than that of Adeno virus vector (Figure 7).

# DISCUSSION

Overexpression of c-Myc has been known to promote cell growth, proliferation and immortalization, wheras the reduction of c-Myc induces apoptosis. The recent genetic construction of mouse in which the expression of *c-myc* can be switched on or off *in vivo* has emphasized the significance of c-Myc expression for tumorigenesis. Ectopic *c-myc* expression in hematopoietic cells using the tetracycline regulatory system caused malignant T cell lymphomas and acute myeloid leukemia; the subsequent inactivation of the transgene caused regression of established tumors[36]. These observations have provided encouragement for the future development of cancer therapies based on targeting individual oncogenes such as *c-myc*. We have been reported that FIR strongly represses endogenous *c-myc* transcription, and induces apoptosis[7] and 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 induce apoptosis in SW480, LoVo (human colon cancer cell lines) as well as HeLa cells (human cervical squamous cancer cell line). Second, SeV/dF/FIR has shown strong anti-tumor effects to both cultured cells and xenografted tumor growth in animal model. These results indicate new insight for a new therapeutic target for tumor treatment.

So what kind of suitable vector should we select and how to convey FIR expressing vector to cancers? Sendai virus is RNA virus and exists only in the cytoplasm, hence relatively safe due to not affecting chromosome. Besides, 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 have chosen Sendai virus (SeV) and prepared fusion gene-deficient SeV/dF/FIR vector. The fusion gene-deficient SeV vector cannot transmit to F protein-non-expressing cells because F protein is indispensable for viral infection. The fusion gene-deficient SeV vector in this study does not require helper virus for its 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 has no pathogenicity to humans. Second, replicates only in the cytoplasm, therefore does not affect chromosome DNA of the host cells. Third, SeV vector shows high efficiency gene transfer to a wide spectrum of the cells even to smooth muscle cells, nerve cells, or endothelial cells generally difficult to be infected. Forth, SeV vector shows high efficiency gene transfer to a wide spectrum of the cells even to smooth does not generate wild-type virus in a packaging cells. Recently, gene-deficient SeV (SeV/dF) vector alone demonstrates tumor suppression by activating dendritic cells (DCs)[24] or if granulocyte macrophage colony-stimulating factor is encoded, it produces autologous tumor vaccines[25]. Therefore, SeV/dF/FIR vector in this study may suppress tumor growth by dual function through c-Myc suppression of tumor cells and DC activation. Further, SeV/dF/FIR showed synergistic effect with cisplatin in treatment of malignant pleural mesothelioma[29]. FIR-binding proteins indicated are basically classified into four categories (Table 1); those are (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. These results suggest 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, DNA damage repair or intracellular protein transport interference. For clinical safety, SeV/dF/FIR is preferable for local tumor growth control rather than systemic cancer therapy.

Together, SeV/dF/FIR is a promising approach for cancer gene therapy, although further enthusiastic clinical as well as basic research are required to explain the precise mechanism of tumor suppression by FIR expressing vectors.

ACKNOWLEDGEMENTS

The authors thank to Dr. David Levens (NCI, NIH, United States) for scientific discussions, Dr. Toru Natsume (Biomedicinal Information Research Center, National Institute of Advanced Industrial Science and Technology, Tokyo, Japan) for FIR-binding proteins analysis, and Dr. Minoru Yoshida (Chemical Genetics Laboratory, RIKEN Advanced Science Institute, Saitama, Japan) for a kind gift of Spliceostatin A (SSA).

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 studied a novel therapeutic strategy to suppress *c-myc* in human cancers by a fusion gene-deficient Sendai virus (SeV/dF/FIR) which is inherently non-transmissible.

*Research frontier*

Because c-myc transcriptional control remains 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 xenografted animal model.

*Innovations and breakthroughs*

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

*Applications*

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

*Terminology*

FUSE: Far Upstream Element that is required for proper c-myc transcription. FBP: FUSE-Binding protein that has strong transcriptional activity. FIR: FBP interacting repressor that is a critical transcriptional repressor of c-myc gene. SeV: Sendai virus**,** a member of the family Paramyxoviridae, 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. 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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**P-Reviewer:** Takenaga K **S-Editor:** Ma YJ **L-Editor:** **E-Editor:**

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 SW480 and LoVo cells, colon cancer cell lines, as well as HeLa cells, cervical squamous cell carcinoma, in 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 definitively associated with apoptosis 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 or Sendai virus/dF/green fluorescent protein vectors from Sendai virus genome RNA.** A:Schematic genome structures of wild type (SeV) and fusion gene-deficient (SeV/dF; non-transmissible) vector carring 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 by 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 form a ribonucleoprotein complex (RNP) vectors were transfected separately with the SeV RNA. See materials and methods

**Figure 3 High efficiency of Sendai virus/dF/green fluorescent protein vectors to HeLa cells and** **Sendai virus/dF/far up stream element-binding protein-interacting repressor indicates significant cell growth inhibition with apoptosis.** A: HeLa cells were infected 10 MOI of SeV/dF/FIR virus vetors and cultures in DMEM for 3 d (72 h). Same amount of 10 MOISeV/dF/GFP were also infected as control. As shown in left lower panel, SeV/dF/GFP infected almost 100% cells (green). At this condition, SeV/dF/FIR has indicated drastically inhibited cell growth as shown in left upper panel. Mock and 10 MOISeV/dF/GFP show no cell growth inhibition as shown in right upper and lower panels;B: HeLa cells infected by 10 MOISeV/dF/FIR for 3 d showed significant cell damage revealed by APO Percentage apoptosis assayTM, compared to mock or same conditions of SeV/dF/GFP virus infected HeLa cells. 5 mmol/L H2O2 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.

**Figure 4 Sendai virus/dF/far up stream element-binding protein-interacting repressor decreased HeLa cells growth *in vitro* and xenografted 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 versus SeV/dF/GFP, *P* < 0.007). Two weeks after 5 x 10E6 HeLa cells xenografting to right thigh of balbc/nu/nu mice, the tumor size reached to around 7-8 mm. 3.0 x 10E7 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 as compared to the size of of 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, 972.2 + 327.0 (average ± SD) mm3, respectively. These 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 mouse xenograft model.** 10E6 Yes-5 cells were xenografted to right thigh of balbc/nu/nu mice, the tumor size reached to around 15 mm in diameter at Day 0. 3.0 x 10E7 CIU of SeV/dF/FIR vectors were injected directly around the tumor every two days, seven times total. The tumor growth was observed and measured every two days as described in materials and methods. Ulcer formation was observed in the center of tumor (Day 14 after SeV/dF/FIR injection). Tumor size was drastically diminished with ulcer formation (Day 90) and disappeared completely during surveillance (Day 140). Thick arrows in the pictures indicate the margin of tumor. Thin arrows indicate the injection of SeV/dF/FIR vectors to the tumor. FIR: FBP Interacting Repressor; FBP: FUSE-Binding protein; FUSE: Far Upstream Element; SeV: Sendai virus; CIU: cell-infectious units.

**Figure 6 SAP155 siRNA induce 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 phosphorylasion at both Ser62, but suppressed phosphorulated-cdk2 and cyclinE in a dose dependent manner. Thus SAP155 siRNA activates c-Myc potentially *via* endogenous FIR IRdogenous *via* denFIR pre-mRNA splicing; B: FIR Sendai virus (SeV/dF/FIR) rescued 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 H2O). 10 MOI (multiplicity of infection) of SeV/dF/FIR apparently suppressed activated c-Myc expression by, whereas SeV/dF/FIR influenced less to basal expression (MetOH or H2O). 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 A.

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