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Dear Dr Lian-Sheng Ma
President and Company Editor-in-Chief of "***World Journal of Gastroenterology***"

We are delighted to learn that the Senior Editor and reviewer#1 have recommended the publication. We appreciate the reviewer's time, effort, his/her constructive criticism and recommendation, and are pleased to respond to their itemized comments below. Please find the revised version of "**Non-transmissible Sendai virus vector encoding c-myc suppressor FBP-interacting repressor for cancer therapy** (WJG-6545R)" written by Kazuyuki Matsushita et al. We respond to all the criticisms and comments made by the reviewer 1, and modified the text, figures and figure legends. There are some changes in order to respond to the reviewer's comments written in red and deleted sentences with lines in blue. We believe that our revised version of manuscript is strengthened and clarified thanks to constructive criticisms made by the senior editor and the reviewer#1.

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

Yasuji Ueda was added for his experimental contribution.

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

ESPS manuscript NO:6545

The manuscript has been improved according to the suggestions of a reviewer.

A. Major points.

Response to 6545-edited file from the editorial office.

ABSTRACT

(before revision)

AIM: 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. However, detailed c-myc control mechanism through FIR remains largely unknown in cancers. For instance, 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. 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. In this study, the authors outlined a novel therapeutic strategy to target and suppress *c-myc* in human cancers by expressing human FIR using a fusion gene-deficient Sendai virus (SeV/dF/FIR) which is inherently non-transmissible.

(after revision)

AIM: To investigate a novel therapeutic strategy to target and suppress *c-myc* in human cancers by FUSE-Binding Protein-Interacting Repressor (FIR).

(before revision)

Results: 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 FIRΔexon2, led to c-Myc overexpression with phosphorylation at Ser62. HA-FIR suppressed CAT activity from 1.5kb *c-myc* promoter. HA-FIR also suppressed endogenous

c-Myc expression and induced apoptosis in HeLa and SW480 cells.

(after revision)

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 (FIRΔexon2), 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 FIRΔexon2, led to c-Myc overexpression with phosphorylation at Ser62. HA-FIR suppressed CAT activity from 1.5kb *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.

(before revision)

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

(after revision)

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.

Text

All the point-by-point corrections in the text by a professional English proofreader are listed in another file entitled "Matsushita K (WJG-6545R)_English proofreading".

Reviewer#1: Comments to the Author

(Inquiry)

1) There is no explanation about Fig. 1B in the text and the corresponding legend.

(Reply)

The explanation was added in the text as follow.

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 (Figure1B).

The explanation was added in the corresponding Figure 1B legend as follow.

(Fig 1B legend) HA-FIR transfected cells definitively associated with apoptosis revealed by TUNEL assay in HeLa, SW480 and LoVo cells.

(Inquiry)

2) In page 12, the authors describe that they examined SeV/dF/GFP transduction efficiency using nine human and five tumor cell lines, but they intend not to present the data. Because the data seems to be very informative in case of clinical application of the virus for various tumors, the authors should present the results (probably as Supplementary data).

(Reply)

We appreciate this reviewer's comments in a positive and productive way. We added supplementary figure 2. *SeV/dF/GFP* expressed in rat's cells. SeV/dF/GFP transduction efficiency was examined rat's non-tumorous hepatocytes, smooth muscle cells, lung vascular endothelial cells and neurons those were relatively difficult for gene-transfer by ordinal procedures (references 32-35). Prominently, SeV/dF/GFP transduction efficiency to those cells was sufficient and satisfactory (Figure S2). Accordingly, Yasuji Ueda who performed the

experiments was added as a coauthor.

(Inquiry)

3) In Fig. 3B legend, there is no description about H2O2 experiment that must be a positive control.

(Reply)

According to the reviewer's advice, the sentence that "5mM H_2O_2 was used as a positive control" was added in Fig. 3B legend.

(Inquiry)

4) In Fig. 4, although the authors state that they examined the effect of SeV/dF/FIR on the cell growth of SW480 cells, there is no data. It is required to demonstrate that SeV/dF/FIR actually reduce c-myc expression and induce apoptosis in tumors.

(Reply)

We agree the reviewer's comment. This is our misunderstanding. We revised as follows.

(before revision) page12 in the text.

"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)."

(after revision)

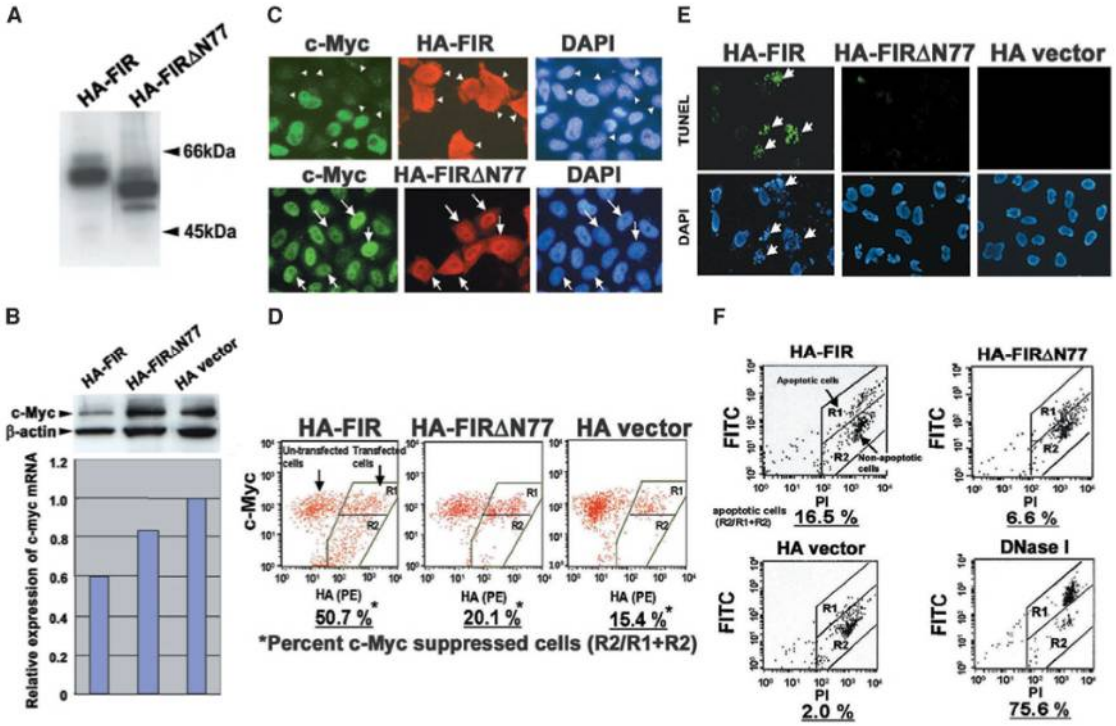
"Additionally, SeV/dF/FIR, but not SeV/dF/GFP, significantly suppressed cell growth in HeLa cells indicated by MTS assay (Figure 4A) and ~~SW480 cells~~ xenografted HeLa cells beneath the right thigh of mouse (Figure 4B) when analyzed by Dunnet's test for multiple comparisons."

Further, we have previously demonstrated and reported that SeV/dF/FIR actually reduce c-myc expression and induce apoptosis in tumors (reference 29, **Kitamura A,** Matsushita K, Takiguchi Y, Shimada H, Tomonaga T, Matsubara H, Inoue M, Hasegawa M, Sato Y, Levens D, Tatsumi K, Nomura F. Synergistic effect of non-transmissible Sendai virus vector encoding the c-myc suppressor FUSE-binding protein-interacting repressor plus cisplatin in treatment of malignant pleural mesothelioma. *Cancer Sci* 2011; **102**:1366-73 [PMID: 21435101 Doi: 10.1111/j.1349-7006.2011.01931.x]).

Thus we added the next sentence in the text (page 12).

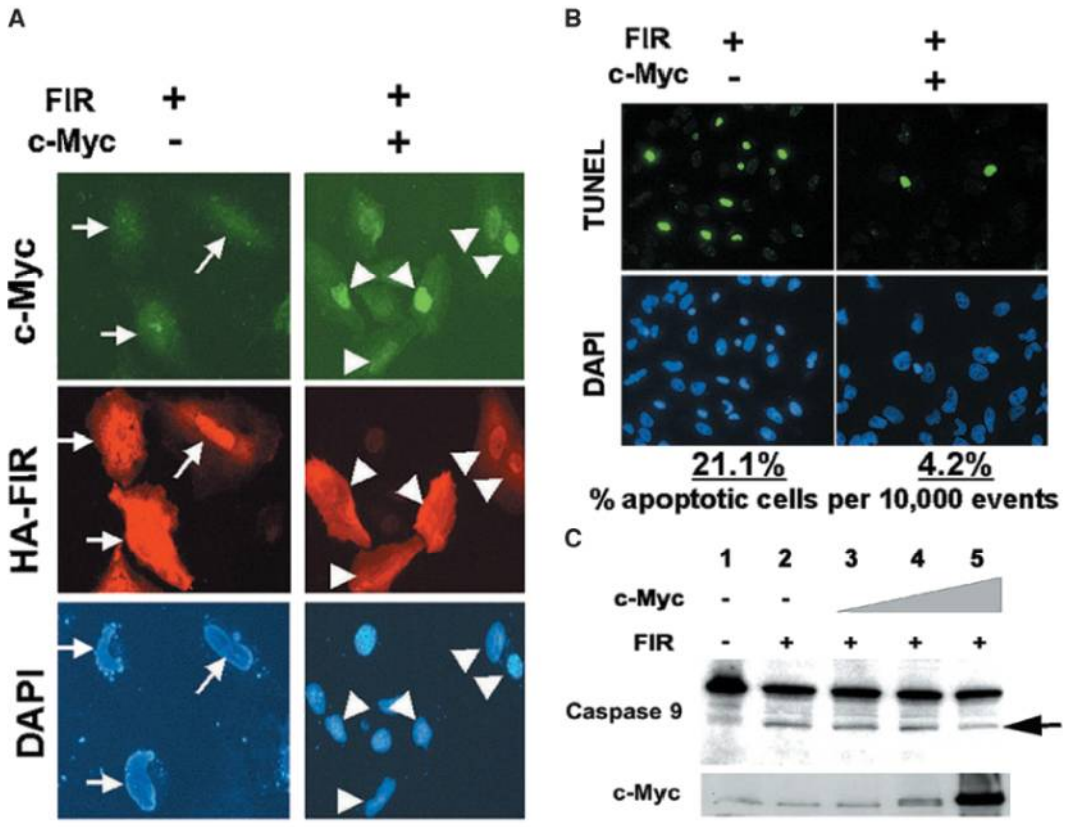
"We have previously demonstrated and reported that SeV/dF/FIR actually reduce c-myc expression and induce apoptosis in tumors[29]."

FIR suppresses activated but not basal *c-myc* transcription[26]. As preported previously, Spliceostain A (SSA) increased c-Myc expression in Fig. 4. As expected, SeV/dF/FIR suppressed SSA-activated *c-myc* expression in HeLa cells as shown in Fig. 4B. Further, FIR reduce endogenous c-myc expression and induce apoptosis in HeLa cells. Also SeV/dF/FIR actually reduce c-myc expression in Fig 5B. Further, the authors previously reported that exogenous FIR (HA-tagged FIR) actually reduce c-myc expression and induce apoptosis in tumors (reference 7, Matsushita K, Tomonaga T, Shimada H, Higashi M, Shioya A, Matsubara H, Harigaya K, Nomura F, Libutti D, Levens D, et al.: An essential role of alternative splicing of c-myc suppressor FIR in carcinogenesis. *Cancer Res* 2006; **66**: 1409-1417).



Amino terminus of FIR is necessary for both endogenous c-Myc suppression and apoptosis induction. (A) 100 fmol of HA-FIR or HA-FIRΔN77 was transfected into HeLa cells in 6-well plate. Twenty-four hours after transfection, both proteins and cDNAs were prepared from those cells. 5μg of proteins were loaded to 7.5% of polyacrylamide gel following western blot with anti-HA antibody. (B) Relative expression of *c-myc* mRNA and protein levels were detected by real-time PCR and western blot, respectively. β-actin was used for internal control. (C) Cells were immunostained with antibodies against c-Myc (left, green) or HA (middle, red). Arrowheads and arrows show the cells in which

HA-FIR and HA-FIRΔN77 were expressed, respectively. c-Myc expression was markedly reduced in most HA-FIR-expressing cells (arrowheads) when compared to HA-FIRΔN77-expressing cells (arrows). (D) c-Myc repression by HA-FIR or HA-FIRΔN77 transfection was quantified by flow cytometry. The transfected cells were identified as PE-positive cells shown along the x-axis. FITC-positive cells along the y-axis show c-Myc expression. The percentage of c-Myc suppressed cells in HA-FIR transfected cells is 50.7 %, vs. 20.1 % in HA-FIRΔN77 and 15.4 % in the HA vacant vector. (E) Examination of apoptotic cells by TUNEL assay. 150 fmoles of HA-FIR, HA-FIRΔN77, vacant vector plasmids were transfected to HeLa cells in 6-well plate and 48 hours later TUNEL assay was performed. Upper panels show apoptotic cells (arrows) after HA-FIR transfection to HeLa cells. Middle and right panels are HA-FIRΔN77 and control (HA vacant vector) transfected cells, respectively. (F) Quantification of apoptotic cells by two-color analysis. The cells were identified as PI-positive cells, shown along the x-axis, or FITC-positive, along the y-axis. Apoptotic cells are shown in the upper-gated areas in each panel as indicated in the figure. The percentage of apoptotic cells, per 10,000 events, in HA-FIR transfection is 16.5 %, vs. 6.6% in HA-FIRΔN77, 2.0 % in the HA vector or 75.6 % in DNase I treatent, respectively.



FIR-induced apoptosis is prevented by enforced expression of c-Myc. (A) 600ng of pcDNA3.1-FIR were transfected into semiconfluent HeLa cells on a 6-well plate with or without c-Myc expression plasmids (pcDNA3.1-*c-myc*). c-Myc expression was remarkably suppressed when FIR alone was

transfected (left, top and middle, arrows) whereas overall c-Myc expression was elevated when *c-myc* plasmids were cotransfected with FIR plasmids (right, top and middle, arrowheads). Staining with DAPI showed that nuclei were swollen and degraded in FIR transfected cells (left, bottom, arrows), vs. normal appearing in FIR and c-Myc co-expressing cells (right, bottom, arrowheads). **(B)** The number of apoptotic cells caused by FIR was drastically reduced when co-expressed with c-Myc. The percentage of apoptotic cells caused by FIR alone was 21.1%, but decreased to 4.2 % when FIR and c-Myc were co-expressed. **(C)** Proteins were extracted 48 hrs after transfection and western blot was performed. Caspase 9 cleavage by FIR was gradually diminished along with the increase of *c-myc* co-transfection (arrow). 1; no plasmids. 2; 600ng of pcDNA3.1-FIR alone. 3; 600ng of pcDNA3.1-FIR plus 1 ng of pcDNA3.1-*c-myc*. 4; 600ng of pcDNA3.1-FIR plus 10 ng of pcDNA3.1-*c-myc*. 5; 600ng of pcDNA3.1-FIR plus 60 ng of pcDNA3.1-*c-myc*.

What does the vertical axis of Figure 4A represent?
(Reply)
The vertical axis of Figure 4A represents the relative ratio of cell growth of SeV/dF/FIR as compared to SeV/dF/GFP.

The legend does not correspond to the panels. In the legend, +SD should be ± SD, and SeVΔF/FIR and SeVΔF/GFF should be SeV/dF/FIR and SeV/dF/GFP, respectively.
(Reply)
In Figure4 legend, +SD was corrected to ± SD, and SeVΔF/FIR and SeVΔF/GFF were changed to SeV/dF/FIR and SeV/dF/GFP, respectively.

(Inquiry)
5) In Fig. 5 legend, two days (72 hours) should be three days (72 hours).
(Reply)
In Fig. 5 legend, two days (72 hours) was corrected to three days (72 hours).

What does GL2 represent in Figure 5A?
(Reply)
Luciferase GL2 duplex as a negative control for siRNA targeting 5'-CGTACGCGGAATACTTCGA -3'.

Following information was added in the "Materials and methods".

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 hours at 37°C in a 5% CO_2 incubator.

In Figure S1, the authors describe that they observed complete regression of xenografted Yes-5 tumors after several injections of SeV/dF/FIR. Is this the only one case, or did they observe other similar cases? If this is due to some immunological effects as stated by the authors, other cases are supposed to be observed. If so, how much percentage?

(Reply)

In Figure S1, the observed complete regression of xenografted Yes-5 tumors after several injections of SeV/dF/FIR is only one case in our experiment. And no other similar case was observed. Thus it is unclear that this observation is due to some immunological effects as stated by the authors. Further investigation is required to reveal thorough mechanism.

(Inquiry)

6) The authors investigated endogenous FIR-interacting binding proteins (Table 1). This analysis is nice and provides valuable information about the FIR-binding proteins (although there is no verification). However, they state that they performed this analysis for avoiding unexpected side effects of SeV/dF/FIR in case of clinical use. It is unclear how the authors predict possible side effects and what conclusions they drew from these data. The authors should discuss this point.

(Reply)

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. So far no severe side effects were observed in animal model experiment[29], however, for clinical safety, SeV/dF/FIR is preferable for local tumor growth control rather than systemic cancer therapy. For example, enhancing the cell killing activity of carbon ion irradiation or x-ray irraditation.

(Inquiry)

7) In Figure S2, FIRΔN77 that lacks the repression domain still shows substantial repressive activity. How do the authors explain this activity?
Is there other repression domain in the molecule? Also, it is obscure how the data is essential for demonstrating the therapeutic efficacy of SeV/dF/FIR. The authors should discuss these points.

(Reply)

One reason why FIRΔN77 that lacks the repression domain still shows substantial repressive activity is explained as follows. First, because FIR forms dimer at dimerization domain, FIRΔN77 and authentic FIR makes heterodimer in case *c-myc* transcriptional repression. According to reviewer's comments, it is not necessary to explain the concept of therapeutic efficacy of SeV/dF/FIR. The authors deleted the original Figure S2 and added a revised novel Figure S2 instead, thanks.

B. Minor points.

Response to 6545-edited file from the editorial office.

1. The author contributions are as follows.

Matsushita K designed research; Matsushita K, Ueda Y and Inoue M performed research; Shimada H, Tomonaga T, Matsubara H and Nomura F contributed new reagents/analytic tools and scientific discussions; Matsushita K analyzed data; and Matsushita K wrote the paper.

2. Materials and methods.

(deleted)

Plasmids (page7)

~~and the FIR deleted of its first seventy-seven amino acids (HA-FIRΔ**exon2)**~~

~~A reporter plasmid that contained the *c-myc* promoter upstream of the chloramphenicol~~

~~acetyl transferase (CAT) gene was used for the CAT assay[19].~~

(added) page8.

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 hours at 37°C in a 5% CO_2 incubator.

(deleted) page11.

~~*Chloramphenicol acetyl transferase (CAT) assay*~~

~~HeLa cells were cultured in DMEM (Gibco-BRL, NY, USA) with 10% fetal calf serum. Cells were transfected by electroporation, harvested 48 hours after transfection, and assayed for CAT activity as previously described[19].~~

3. Results.

(deleted) page 14.

~~***HA-FIR suppresses CAT transcription from c-myc promoter as well as endogenous c-myc gene transcription***~~

~~To confirm the amino terminal requirement for FIR repression of the *c-myc* promoter, full-length FIR (1-542) or FIR mutant deleted of its amino terminal repression domain FIRΔN77 (78-542) were cotransfected with a CAT reporter plasmid driven by *c-myc* promoter and assayed for CAT activity. As expected, the first seventy-seven amino acids of FIR were required to repress activated-transcription. Although full-length FIR potently inhibited CAT expression in a dose dependent manner, deleting its amino terminus (FIRΔN77) enfeebled repressor activity (FigureS2).~~

4. ACKNOWLEDGEMENTS

The authors thank to Dr David Levens (NCI, NIH, USA) for ~~CAT assay and~~ scientific discussions,

5. REFERENCES

According to the suggestions from editorial office, we add PubMed citation numbers and DOI citation to the reference list and list all authors and revise throughout. The author provided the first page of the paper without PMID and DOI.
PMID (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed)
DOI (http://www.crossref.org/SimpleTextQuery/)

Reference 19 was deleted.

Reference 32 was added.

4. Figure legends was revised as follows.

(deleted FigureS2 legend)

~~FigureS2. Amino terminus of FIR is important for the suppression of transcription from the *c-myc* promoter. HA-FIR and HA-FIRΔN77 were co-transfected with 750ng of the CAT reporter plasmid driven by the *c-myc* promoter in HeLa cells and CAT activity was measured. No plasmid (lanes 1,2), 36 fmol of HA vector alone (lanes 3, 4), 18 (lanes 5, 6) and 36 (lanes 7, 8) fmol of HA-FIR, 18 (lanes 9, 10) and 36 (lanes 11, 12) fmol of HA-FIRΔN77 were transfected to HeLa cells, respectively. Experiments were performed duplicate. Lower panel shows histograms of the average of CAT activity in multiple experiments. The average of relative CAT activity of lanes 5 and 6 (18 fmol of HA-FIR), lanes 7 and 8 (36 fmol of HA-FIR), lanes 9 and 10 (18 fmol of HA-FIRΔN77), and lanes 11 and 12 (36 fmol of HA-FIRΔN77) were 0.25, 0.16, .052, and 0.43, respectively compared to lanes 3 and 4 (36 fmol of HA vector).~~

(revised FigureS2 legend)

FigureS2. SeV/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.

Sincerely,

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