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A TGFβ Signaling Inhibitor, SB431542, Inhibits Reovirus-mediated Lysis of Human Hepatocellular Carcinoma Cells in a TGFβ-independent Manner

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Abstract. *Background/Aim: Oncolytic reovirus, which is a non-enveloped virus possessing a 10-segmented double-stranded RNA genome, has been anticipated as a novel class of antitumor agent. Hepatocellular carcinoma (HCC) is considered to be a target suitable for reovirus-mediated virotherapy. Transforming growth factor (TGF)-β plays an important role in the pathogenesis of HCC. TGF-β-signaling inhibitors have proceeded to clinical trials as potential antitumor agents for HCC. On the other hand, TGF-β is involved in induction of expression of cathepsins B and L, which are important for reovirus infection. It remains to be examined whether TGF-β signaling inhibitors affect reovirus-mediated lysis of HCC cells. The aim of this study was to evaluate the effects of TGF-β-signaling inhibitors on tumor cell lysis efficiency of reovirus in human HCC cells. Materials and Methods: Reovirus was added to four types of human HCC cell lines pretreated with one of three TGF-β type I receptor inhibitors: SB431542, A-83-01, or galunisertib (LY2157299). Cell viability, virus genome copy numbers, and virus protein expression were evaluated following reovirus infection. Results: SB431542 significantly inhibited reovirus-mediated killing of human HCC cell lines, while A-83-01 and galunisertib did not inhibit. Conclusion: These data indicate that SB431542 inhibited reovirus-mediated lysis of human HCC cells in a TGF-β signaling-independent manner.*

Oncolytic viruses, which specifically infect tumor cells and induce tumor cell death, have attracted much attention as a new class of antitumor agent. More than 10 oncolytic viruses have been developed so far. Several types of oncolytic viruses have proceeded to clinical trials against various types of tumors and showed promising results. Among the various types of oncolytic viruses, mammalian orthoreovirus type 3 Dearing (hereafter reovirus), which is a non-enveloped virus possessing 10-segmented double-stranded RNA genome, is the most promising oncolytic virus due to its unique properties (1, 2). Reovirus not only exhibits efficient oncolytic activities on a variety of tumor cell lines, but also efficiently induces antitumor immunity. In addition, reovirus can be intravenously administered to patients because of its superior safety profile. Combination therapy using reovirus and antitumor agents have shown promising therapeutic effects on various types of tumors.

Hepatocellular carcinoma (HCC), which is derived from well-differentiated hepatocytes, is the most common primary liver cancer and the third leading cause of cancer-related deaths in the world (3, 4). HCC mainly occurs in patients with chronic liver diseases and cirrhosis; it is difficult to treat due to its rapid development and confers a poor prognosis for survival. Therefore, it is important to develop effective therapeutics for the treatment of HCC. The transforming growth factor (TGF)-β signaling pathway is considered to be a promising therapeutic target in HCC because this pathway

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is involved in the pathogenesis of HCC and is often activated in HCC (5, 6). TGF-β signaling inhibitors have proceeded to clinical trials in HCC (7).

HCC is an ideal target for reovirus-mediated virotherapy because RAS, which is a key factor for reovirus-mediated oncolysis (8), is often activated in human HCC (9, 10). In addition, reovirus efficiently accumulates in the liver following systemic administration (11). Two lysosome proteases, cathepsins B and L, playing a crucial role in infection with reovirus, are often up-regulated in various types of tumor, including HCC (12, 13). These cathepsins degrade the outer capsid protein of reovirus, yielding infectious subviral particles (ISVPs). ISVPs can disrupt the endosomal/lysosomal membrane and invade the cytosol, resulting in expression of viral proteins and progeny virus production. Tumor cells showing high activity levels of cathepsins B and L are susceptible to reovirus-mediated oncolysis (14, 15).

Expression levels of cathepsins B and L have been demonstrated to be elevated by TGF-β (16, 17), which led us to hypothesize that TGF-β signaling inhibitors might suppress the expression of cathepsins B and L, resulting in inhibition of reovirus infection in human HCC cells. However, it remained to be examined whether TGF-β signaling inhibitors actually consequently inhibit reovirus-mediated killing of human HCC cells.

In this study, we examined the effects of TGF-β signaling inhibitors on reovirus-mediated killing of human HCC cells.

Materials and Methods

Cell lines and reagents. Cells of the human hepatocellular carcinoma cell lines HepG2 (JCRB1054; JCRB Cell Bank, NIBIO, Osaka, Japan), Hep3B, HLE (JCRB0404), PLC/PRF/5 (JCRB0406), and the human non-HCC cell lines HEK293 (a human embryonic kidney cell line), U87 (a human glioblastoma cell line) and Mewo (a human skin melanoma cell line) were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and antibiotics at 37˚C in an atmosphere with 5% CO_2. L929 cells were maintained in Joklik's modified Eagle's minimal essential medium with 5% fetal bovine serum, 1% L-glutamine, and antibiotics at 37˚C in an atmosphere with 5% CO_2. The TGF-β type I receptor inhibitor SB431542 was purchased from Tocris Bioscience (Bristol, UK). A-83-01 and galunisertib, which are inhibitors of TGF-β type I receptor, were obtained from Merck (Darmstadt, Germany) and Focus Biomolecules (Plymouth Meeting, PA, USA), respectively. An siRNA against receptor-interacting serine/threonine kinase 2 (RIPK2), which is inhibited by SB431542 *via* an off-target effect (18), was purchased from Thermo Scientific (Waltham, MA, USA).

Reovirus. Reovirus (kindly provided by Dr. T Etoh and A Nishizono, Oita University, Oita, Japan) was grown in L929 cells and purified by CsCl ultracentrifugation, followed by overnight dialysis, as previously described (14). Biological titers of reovirus were determined by a plaque-forming assay using L929 cells. ISVPs were freshly prepared by treating purified virions with chymotrypsin immediately prior to infection for 15 min at 37˚C as previously described (19). All digestions were performed in a buffer containing 200 μg/ml chymotrypsin, 150 mM NaCl, 15 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.6).

Reovirus-mediated tumor cell lysis. For evaluation of the cell lysis activity of reovirus, all cell lines were seeded at 0.5×10^4 cells per well in a 96-well plate. On the following day, TGF-β signaling inhibitors were added to the cells at the following concentrations, SB431542: 2 μM and 10 μM; galunisertib: 2 μM and 10 μM; A-83-01: 0.2 μM and 1 μM. Following a 24-h incubation, cells were infected with reovirus and ISVPs at a multiplicity of infection (MOI) of 20. Cell viability was measured at 72 h post-infection using a cell counting kit-8 (Dojindo Laboratories, Kumamoto, Japan).

For the determination of viability of human HCC cells with RIPK2 knockdown, following reovirus infection, HepG2 and Hep3B cells were transfected with RIPK2 siRNA at a final concentration of 20 μM using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA). Reovirus was added to *RIPK2*-knockdown cells at an MOI of 20 at 48 h after siRNA transfection. Cell viability was assessed at 72 h post-infection as described above.

Real-time reverse transcriptase-polymerase chain reaction analysis of the viral genome. HepG2, Hep3B, HLE, and PLC/PRF/5 cells were seeded at 5×10^4 cells per well in a 24-well plate. On the following day, TGF-β signaling inhibitors were added to the cells at the following concentrations, SB431542: 10 μM; galunisertib: 10 μM; A-83-01: 1 μM. Following a 24-h incubation, cells were infected with reovirus at an MOI of 20. Total RNA, including viral genomic RNA, was isolated from the cells following 72-h infection using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Real-time reverse transcriptase-polymerase chain reaction analysis of the reovirus genome was performed as previously described (14).

Western blotting. For assessment of TGF-β production in the cells, HepG2, Hep3B, HLE, and PLC/PRF/5 cells were lysed in RIPA buffer, followed by western blotting analysis using anti-human TGF-β1 antibody (3C11 at 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a primary antibody. In order to evaluate the phosphorylation levels of SMAD family member 2 (SMAD2), HepG2 cells were seeded at 1×10^5 cells/well in a 12-well plate. On the following day, cells were incubated with TGF-β signaling inhibitors (10 μM SB431542; 10 μM galunisertib; 1 μM A-83-01) for 24 h. Recombinant human TGF-β (Peprotech, Rocky Hill, NJ, USA) was added to the cells at a final concentration of 10 ng/ml. Following a 1-h incubation, cell lysates were prepared using RIPA buffer. Western blotting was performed using phospho-SMAD2 antibody (138D4, 1:1000) (Cell Signaling Technology, Danvers, MA, USA). For assessment of sigma 3 protein production by reovirus, HepG2, Hep3B, HLE, and PLC/PRF/5 cells were seeded at 2×10^4 cells per well in a 12-well plate. On the following day, the cells were incubated with TGF-β signaling inhibitors as above for 24 h. Cells were then infected with reovirus at an MOI of 20 for 72 h, following by western blotting analysis of sigma 3 expression using anti-reovirus sigma 3 monoclonal antibody (1:200) (Developmental Studies Hybridoma Bank, Iowa City, IA, USA).

Expression analysis of junction adhesion molecule-A on the cell surface. HepG2, Hep3B, HLE, and PLC/PRF/5 cells were recovered following a 24-h incubation in the presence of SB431542 at 10 μM. Cells were then labeled with mouse monoclonal antibody to

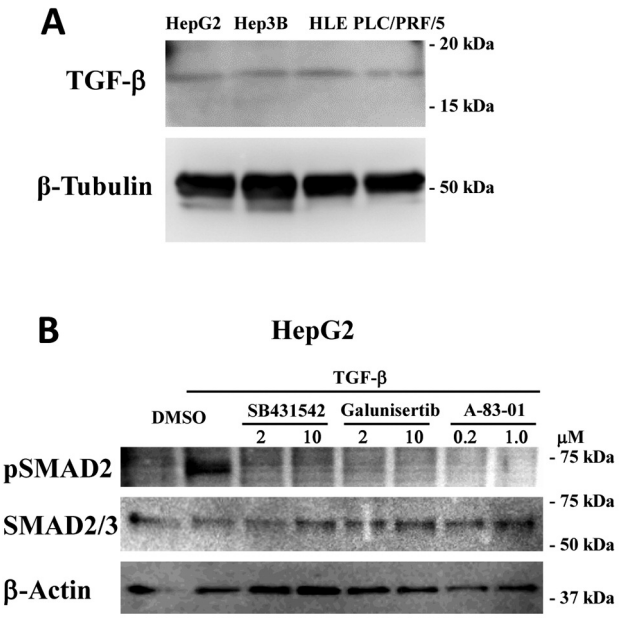

Figure 1. *Inhibition of phosphorylation of SMAD family members SMAD2/3 by transforming growth factor (TGF)-β signaling inhibitors in human hepatocellular carcinoma (HCC) cells. A: TGF-β1 expression in human HCC cells. B: Inhibition of phosphorylation of SMAD2 by TGF-β signaling inhibitors. Cells pretreated with TGF-β signaling inhibitors were incubated with recombinant TGF-β2 (10 ng/ml) for 1 h, followed by western blotting analysis. DMSO: Dimethyl sulfoxide.*

junction adhesion molecule-A (JAM-A) (F11; Hycult Biotech, Uden, the Netherlands) or purified mouse IgG1, isotype control (BD Pharmingen, Franklin Lakes, NJ, USA), then incubated with phycoerythrin-conjugated goat anti-mouse IgG secondary antibody (BD Pharmingen). Flow cytometric analysis was performed using a MACS Quant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany). The data were analyzed using FlowJo flow cytometry data analysis software (TreeStar, San Carlos, CA, USA).

Cathepsin activity in cells. The activity levels of cathepsins B and L in HepG2 and Hep3B cells treated with TGF-β signaling inhibitors (SB431542: 2 μM and 10 μM; galunisertib: 2 μM and 10 μM; A-83-01: 0.2 μM and 1 μM) for 24 h were measured using an Innozyme Cathepsin B Activity Assay Kit (Calbiochem, San Diego, CA, USA) and Innozyme Cathepsin L Activity Assay Kit (Calbiochem), respectively, according to the manufacturer's instructions.

Results

TGF-β expression in human HCC cells. In order to examine the expression levels of TGF-β in human HCC cell lines, western blotting analysis was performed. The results showed that all four human HCC cell lines produced detectable levels of TGF-β1 (Figure 1A). Next, in order to examine whether TGF-β signaling inhibitors at the concentrations used in this study significantly inhibited TGF-β signaling, the phosphorylation status of SMAD2 was examined. SB431542, A-83-01, and galunisertib inhibit TGF-β receptor 1 (ALK5), the type 1 receptor serine/threonine kinase activin A receptor type 1B (ALK4) and activin A receptor type 1C (ALK7), respectively (19-21). All three TGF-β signaling inhibitors significantly inhibited phosphorylation of SMAD2 in HepG2 cells (Figure 1B). There were no apparent differences in the SMAD2/3 levels induced by the TGF-β signaling inhibitors. These data indicate that the TGF-β signaling inhibitors significantly inhibited TGF-β signaling at the concentrations used in this study.

Reovirus-mediated lysis of human HCC cells in the presence of TGF-β signaling inhibitors. In order to examine whether treatment with TGF-β signaling inhibitors suppressed reovirus-mediated lysis of human HCC cells, reovirus was added to human HCC cell lines pretreated with TGF-β signaling inhibitors. Neither A-83-01 nor galunisertib promoted or inhibited reovirus-mediated killing of any of the human HCC cell lines tested (Figure 2A). On the other hand, SB431542 at 10 μM significantly restored the viability of all human HCC cell lines following reovirus infection. None of the TGF-β signaling inhibitors inhibited the reovirus-mediated lysis of human non-HCC cell lines, HEK293, U87 and Mewo (Figure 2B). These results indicate that SB431542 inhibited reovirus-mediated lysis of human HCC cells in a TGF-β signaling-independent manner.

Inhibition of reovirus infection by SB431542. Next, in order to examine whether SB431542 inhibited reovirus infection of human HCC cells, reovirus genome copy numbers and virus protein production in the presence of TGF-β signaling inhibitors were examined. SB431542 mediated a more than 30% reduction in the reovirus genome copy numbers in all human HCC cell lines following a 72-h infection (Figure 3A). Sigma 3 protein levels were significantly reduced by SB431542 in HepG2, Hep3B, and HLE cells, although no apparent reduction was observed in SB431542-treated PLC/PRF/5 cells (Figure 3B). Neither A-83-01 nor galunisertib significantly reduced the virus genome copy numbers or sigma 3 protein levels in these cell lines. These data indicate that SB431542 inhibited reovirus infection in human HCC cells.

Mechanism of SB431542-mediated inhibition of reovirus infection. In order to reveal the mechanism of SB431542-mediated inhibition of reovirus infection in human HCC cells, we examined the expression levels of JAM-A, which is a primary receptor for reovirus (22), on human HCC cells following treatment with SB431542. No apparent differences in JAM-A expression levels were observed between SB431542-treated and dimethyl sulfoxide-treated cells (Figure 4A). Next, we examined the expression and activity levels of cathepsins B and L following treatment with TGF-

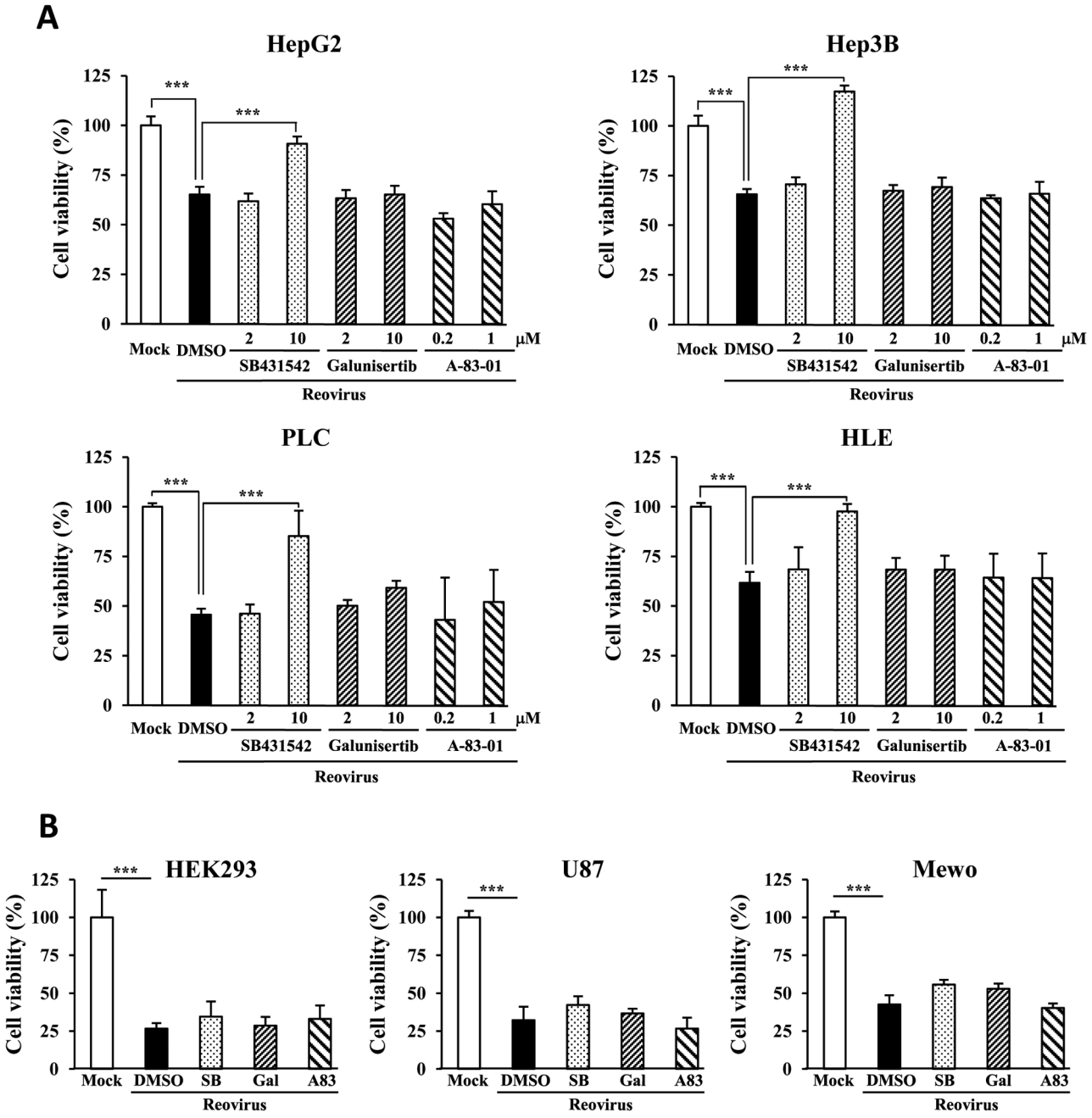

Figure 2. *Reovirus-mediated lysis of human hepatocellular carcinoma cells (A) and non-hepatocellular carcinoma cells HEK293, U87, and Mewo cells (B) in the presence of transforming growth factor-β signaling inhibitors. Cells were preincubated with TGF-β signaling inhibitors for 24 h, followed by infection with reovirus at a multiplicity of infection of 20. Cell viabilities were determined after a 72-h infection. SB: 10 μM SB431542; Gal: 10 μM galunisertib; A83: 1 μM A-83-01. DMSO: Dimethyl sulfoxide. The data are expressed as means±SD (n=3-4). ***Significantly different at p<0.001.*

β signaling inhibitors. No statistically significant reduction in the expression or activity levels of cathepsin B was observed in HepG2 or Hep3B cells treated with SB431542 (Figure 4B and D). SB431542 slightly reduced the mRNA level of cathepsin L in HepG2 cells (Figure 4C) but the activity level in HepG2 or Hep3B cells was not significantly affected by SB431542 (Figure 4E). Moreover, SB431542 significantly restored cell viability following treatment with ISVPs (Figure 4F). ISVPs mediated efficient tumor cell lysis even when cathepsins B and L were inhibited (15). The SB431542-

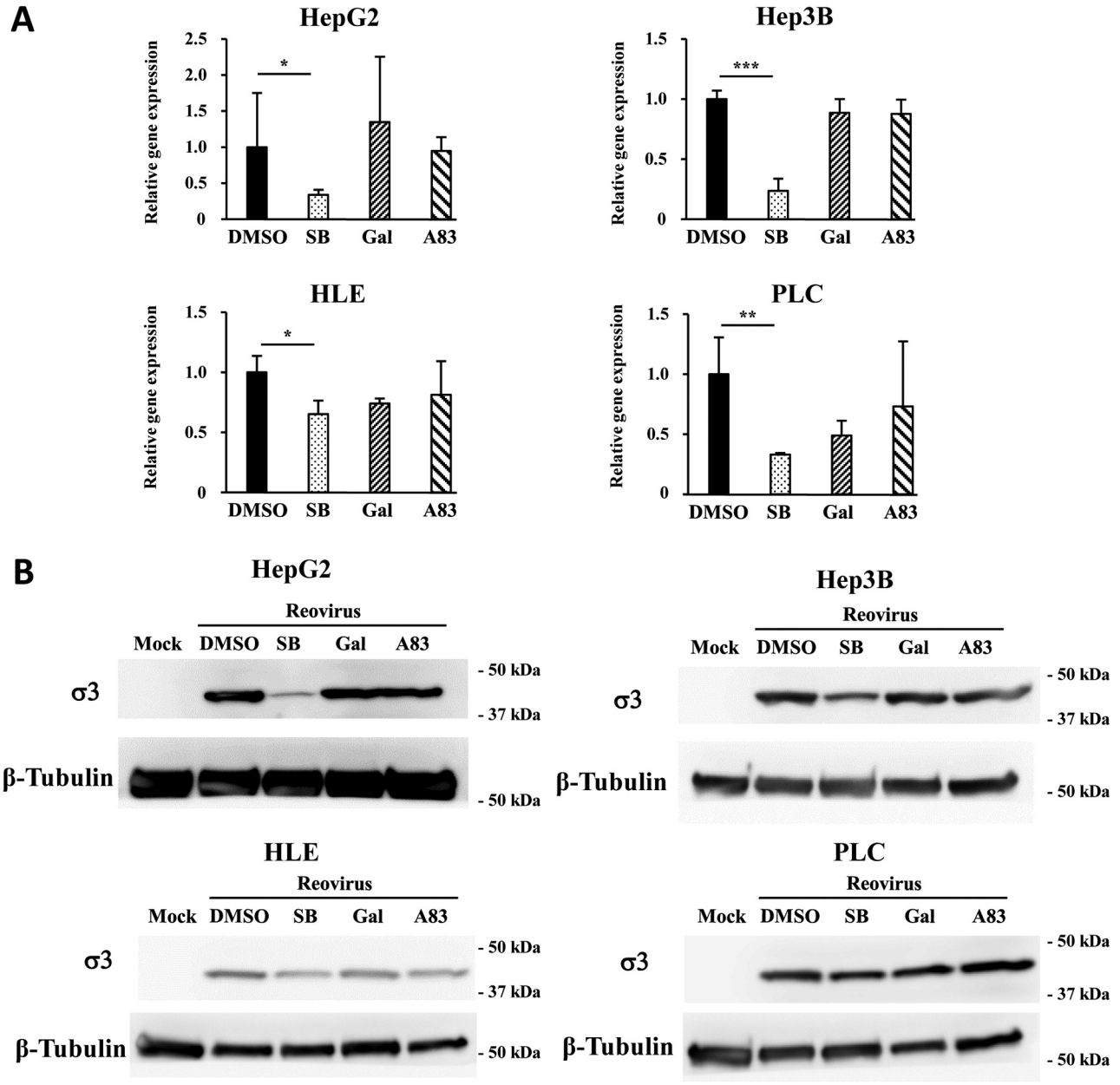

Figure 3. *Reovirus genome copy number (A) and virus protein level (B) in human hepatocellular carcinoma cells pretreated with transforming growth factor (TGF)-β signaling inhibitors following a 72-h infection with reovirus. Cells were pretreated with TGF-β signaling inhibitors SB431542 (SB), galunisertib (Gal), A-83-01 (A83) for 24 h, followed by reovirus infection at a multiplicity of infection of 20. Total RNA and cell lysates were recovered 72 h after reovirus infection, followed by real-time reverse transcriptase-polymerase chain reaction analysis (relative to glyceraldehyde 3-phosphate dehydrogenase) and western blotting analysis. DMSO: Dimethyl sulfoxide. The data are expressed as means±SD (n=4). Significantly different at *p<0.05, **p<0.01 and ***p<0.001.*

mediated viability of ISVP-treated cells was not largely different from that in the cells treated with reovirus or SB431542. These data indicate that SB431542 did not significantly reduce the expression or activity levels of cathepsins B or L. These data indicate that SB431542 did not significantly alter the expression levels of JAM-A, or the activity levels of cathepsins B or L in human HCC cells.

RIPK2 was not involved in SB431542-mediated inhibition of reovirus infection. In order to examine other cellular factors

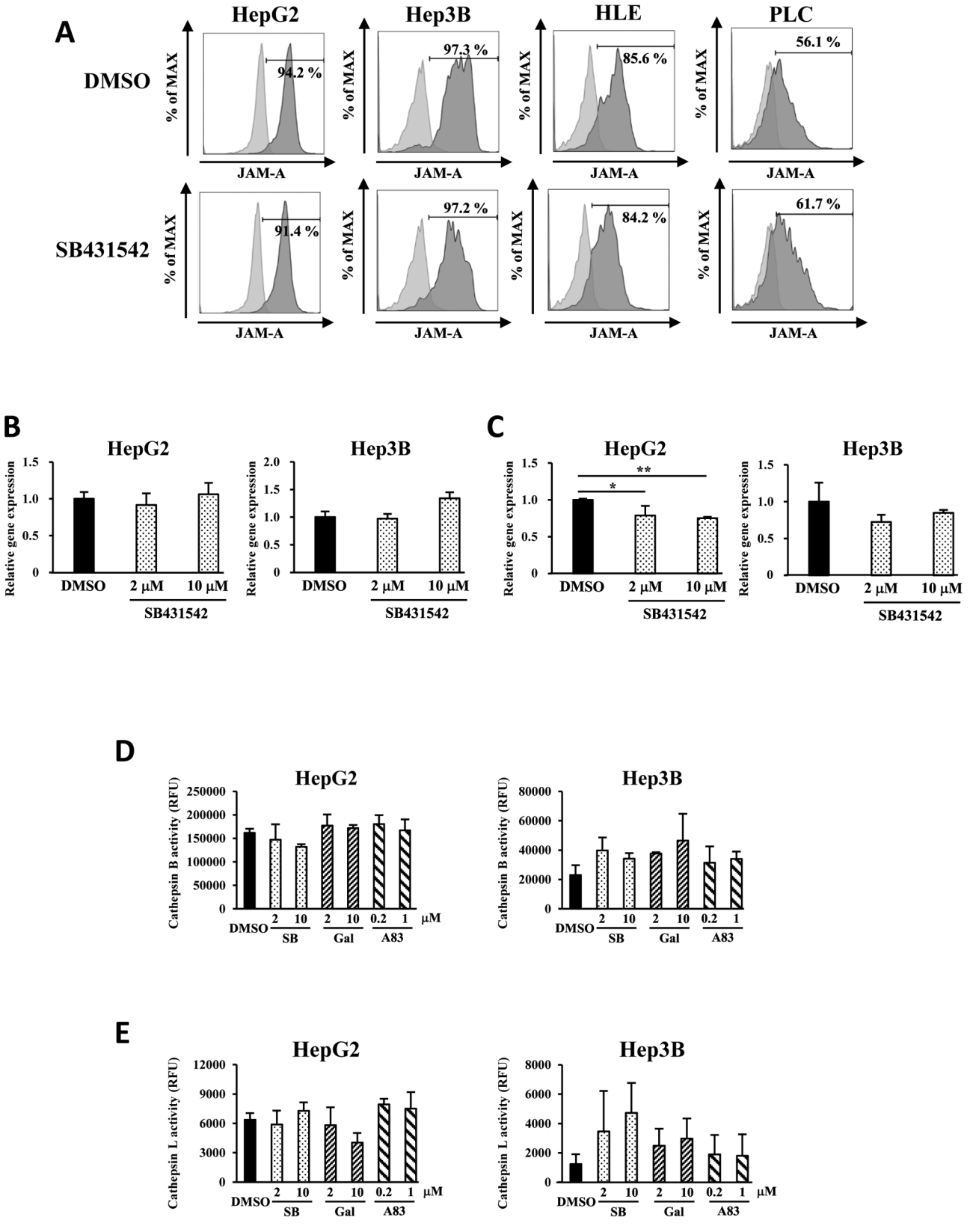

Figure 4. *Continued*

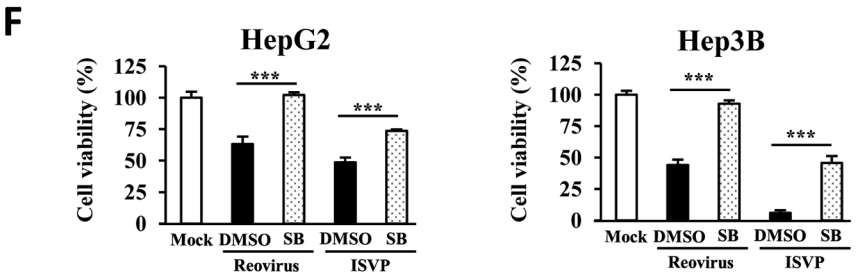

Figure 4. *Effects of SB431542, an inhibitor of transforming growth factor-β type I receptor, on HepG2 and Hep3B human hepatocellular carcinoma cells. A: Expression of junction adhesion molecule-A (JAM-A), a primary receptor of reovirus, on the cell surface following SB431542 treatment. Cells were incubated with SB431542 at 10 μM for 24 h then mRNA levels of cathepsins B (B) and L (C) (relative to glyceraldehyde 3-phosphate dehydrogenase) were analyzed. Levels of enzymatic activity of cathepsins B (D) and L (E) were determined in HepG2 and Hep3B cells pretreated with TGF-β signaling inhibitors SB431542 (SB), galunisertib (Gal), A-83-01 (A83) for 24 h. F: Infectious subviral particles (ISVP) were added to cells pretreated with SB431542. Cell viability was determined at 72 h following treatment with ISVPs. DMSO: Dimethyl sulfoxide; RFU: relative fluorescence units. The data are expressed as means±SD (n=4). Significantly different at *p<0.05, **p<0.01 and ***p<0.001.*

involved in SB431542-mediated inhibition of reovirus infection in human HCC cells, *RIPK2* was knocked down by an siRNA against *RIPK2*. A previous study reported that SB431542 inhibited RIPK2 *via* an off-target effect (18). In our experiment, an siRNA against RIPK2 mediated more than 95% reduction in *RIPK2* mRNA level following transfection (Figure 5A). Reovirus led to similar levels of tumor lysis in HepG2 and Hep3B cells transfected with siRNA against *RIPK2* and a control siRNA (Figure 5B). These data indicate that RIPK2 was not largely involved in the SB431542-mediated inhibition of reovirus infection in human HCC cells and that SB431542 inhibited reovirus-mediated lysis of human HCC cells *via* an unknown mechanism except for inhibition of TGF-β signaling.

Discussion

TGF-β is involved in various cellular activities, including tumor malignancy, maintenance of stemness of stem cells, and epithelial and mesenchymal transition. Numerous TGF-β signaling inhibitors have been developed, and have been widely used in basic studies. Among the TGF-β signaling inhibitors developed so far, SB431542 is most widely used. SB431542 is often used for differentiation of stem cells, activation of immune cells, and inhibition of myofibroblast differentiation *via* inhibition of TGF-β signaling (23-25). This study demonstrated that SB431542, but not A-83-01 or galunisertib, significantly inhibited the reovirus-mediated killing of human HCC cells. The concentrations of SB431542 used in this study have often been used in cultured cells for inhibition of TGF-β signaling (23, 26, 27). SB431542 is a specific inhibitor of ALK5. In addition to ALK5, ALK4 and ALK7 are inhibited by SB431542 (20). A-83-01 and galunisertib also inhibit not only ALK5 but also ALK4 and ALK7 (20, 21). SB431542, A-83-01, and galunisertib interact with the ATP-binding site in ALK5 (28, 29). Our findings indicate that SB431542 inhibited the reovirus-mediated killing of human HCC cells in a TGF-β signaling-independent manner.

SB431542 did not inhibit reovirus-mediated lysis of HEK293, U87, or Mewo cells, suggesting that SB431542-mediated inhibition of reovirus infection occurs only in human HCC cells. The reason for this is unclear, although presumably human HCC cell-specific molecules which are involved in reovirus infection would be inhibited by SB431542.

Reovirus is a highly promising oncolytic virus due to its superior properties. Although reovirus monotherapy exhibited efficient antitumor effects in animal models (30, 31), combination therapies of reovirus and various types of antitumor agents have been carried out and have exhibited promising results in clinical trials (32, 33). When antitumor agents are used in combination with reovirus, we should be vigilant to ensure that the antitumor agents do not inhibit the infection of tumor cells with reovirus. As described above, TGF-β was demonstrated to enhance the expression of cathepsins B and L (16, 17), which are lysosomal proteinases crucial for reovirus infection. These findings led us to hypothesize that TGF-β signaling inhibitors would inhibit reovirus-mediated killing of tumor cells. Contrary to our hypothesis, this study demonstrated that inhibition of TGF-β signaling did not suppress reovirus-mediated killing of tumor cells, at least under the *in vitro* culture conditions used here. The expressions of cathepsins B and L are regulated by several transcriptional factors other than SMAD2/3 (34, 35). Thus, signaling pathways other than TGF-β/SMAD signaling appear to have mediated the expression of cathepsins B and L in the HCC cell lines used in this study.

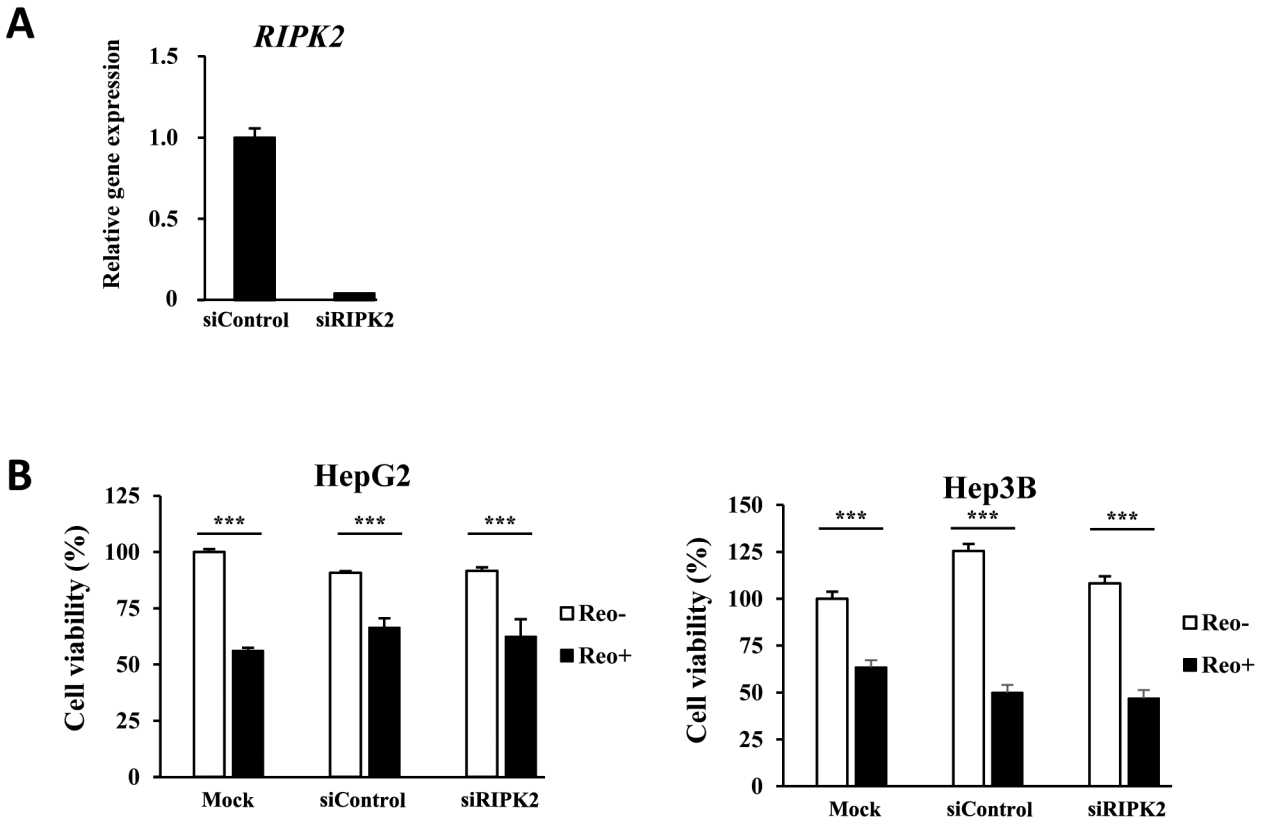

Figure 5. *Receptor-interacting serine/threonine-protein kinase 2 (RIPK2) is not involved in SB431542-mediated inhibition of reovirus infection in human hepatocellular carcinoma cells. A: Knockdown efficiency of an siRNA against RIPK2 in HepG2 and Hep3B cells. B: Cell viability of hepatocellular carcinoma cells with RIPK2 knockdown following reovirus infection. Cells were transfected with an siRNA against RIPK2 at 20 μM for 48 h, followed by infection with reovirus at a multiplicity of infection of 20. Cell viabilities were determined at 72 h after reovirus infection. The data are expressed as means±SD (n=4). ***Significantly different at p<0.001.*

It remains to be evaluated whether TGF-β signaling inhibitors inhibit or promote the *in vivo* antitumor effects of reovirus. The expression and activity levels of cathepsins B and L in tumor cells different under *in vitro* and *in vivo* conditions. Alain *et al*. demonstrated that reovirus efficiently infected tumor cells resistant to reovirus-mediated *in vitro* oncolysis when tumor cells were subcutaneously transplanted because of elevation in the activity of cathepsins B and L in the subcutaneous tumors, compared with *in vitro*-cultured tumor cells (15). TGF-β signaling might be more largely involved in the expression and activity of cathepsins B and L in tumor cells under *in vivo* than under *in vitro* culture conditions. On the other hand, TGF-β is involved in the suppression of antitumor immunity (36). TGF-β inhibition results in further up-regulation of reovirus-induced activation of antitumor immunity. Previous studies demonstrated efficient activation of antitumor immunity by the combinatorial use of TGF-β inhibitors and oncolytic viruses (37, 38).

In summary, this study demonstrated that SB431542 inhibited reovirus-mediated lysis of human HCC cells *via* an unknown mechanism in a TGF-β signaling-independent manner. The other TGF-β inhibitors, A-83-01 and galunisertib, did not inhibit reovirus-mediated lysis of human HCC cells. This study suggests that the off-target effects of small-molecule drugs should be borne in mind when they are used in combination therapies with oncolytic viruses.

Conflicts of Interest

The Authors declare no competing interests.

Authors' Contributions

II and NS designed and performed the experiments, analyzed the data, and wrote the article. TK performed the experiments. HM supervised the project and reviewed the article. FS conceived and designed the project, performed the experiments, analyzed data, and wrote the article.

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