Dual programmed cell death pathways induced by p53 transactivation overcome resistance to oncolytic adenovirus in human osteosarcoma cells

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Abbreviations: hTERT, human telomerase reverse transcriptase; MOI, multiplicity of infection; PFU, plaque-forming units; ID_{50}, 50% inhibiting doses; GFP, green fluorescent protein; PARP, poly (ADP-ribose) polymerase; LC3, microtubule-associated protein 1 light chain 3; DRAM, damage-regulated autophagy modulator; RT-PCR, reverse transcription-polymerase chain reaction; Ct, threshold cycle; PBS, phosphate buffered saline; 3D-CT, three-dimensional computed tomography; miRNA, microRNA; EGFR, epidermal growth factor receptor.

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Abstract

Tumor suppressor p53 is a multifunctional transcription factor that regulates diverse cell fates, including apoptosis and autophagy in tumor biology. p53 overexpression enhances the antitumor activity of oncolytic adenoviruses; however, the molecular mechanism of this occurrence remains unclear. We previously developed a tumor-specific replication-competent oncolytic adenovirus, OBP-301, that kills human osteosarcoma cells, but some human osteosarcoma cells were OBP-301-resistant. In this study, we investigated the antitumor activity of a p53-expressing oncolytic adenovirus, OBP-702, and the molecular mechanism of the p53-mediated cell death pathway in OBP-301-resistant human osteosarcoma cells. The cytopathic activity of OBP-702 was examined in OBP-301-sensitive (U2OS and HOS) and OBP-301-resistant (SaOS-2 and MNNG/HOS) human osteosarcoma cells. The molecular mechanism in the OBP-702-mediated induction of two cell death pathways, apoptosis and autophagy, was investigated in OBP-301-resistant osteosarcoma cells. The antitumor effect of OBP-702 was further assessed using an orthotopic OBP-301-resistant MNNG/HOS osteosarcoma xenograft tumor model. OBP-702 suppressed the viability of OBP-301-sensitive and OBP-301-resistant osteosarcoma cells more efficiently than OBP-301 or a

replication-deficient p53-expressing adenovirus (Ad-p53). OBP-702 induced more

profound apoptosis and autophagy when compared with OBP-301 or Ad-p53.

E1A-mediated *miR-93*/*106b* upregulation induced p21 suppression, leading to

p53-mediated apoptosis and autophagy in OBP-702-infected cells. p53 overexpression

enhanced adenovirus-mediated autophagy through activation of damage-regulated

autophagy modulator (DRAM). Moreover, OBP-702 suppressed tumor growth in an

orthotopic OBP-301-resistant MNNG/HOS xenograft tumor model. These results

suggest that OBP-702-mediated p53 transactivation is a promising antitumor strategy to

induce dual apoptotic and autophagic cell death pathways via regulation of miRNA and

DRAM in human osteosarcoma cells.

Key words: oncolytic adenovirus; telomerase; p53; apoptosis; autophagy

Introduction

Osteosarcoma is one of the most common malignant tumors in young children (1, 2). Current treatment strategies, which consist of multi-agent chemotherapy and aggressive surgery, have significantly improved the cure rate and prognosis of patients with osteosarcoma. In fact, over the past 30 years, the 5-year survival rate has increased from 10% to 70% (3-5). Even in osteosarcoma patients with metastases at diagnosis, the 5-year survival rate has reached 20% to 30% in response to chemotherapy and surgical removal of primary and metastatic tumors (6). However, treatment outcomes for patients with osteosarcomas have further improved over the last few years. Therefore, the development of novel therapeutic strategies is required to improve the clinical outcomes in patients with osteosarcomas.

Tumor-specific replication-competent oncolytic viruses are being developed as novel anticancer therapy, in which the promoters of cancer-related genes are used to regulate virus replication in a tumor-dependent manner. More than 85% of all human cancers express high telomerase activity to maintain the length of the telomeres during cell division, while normal somatic cells seldom show this enhance telomerase activity (7, 8). Telomerase activity has also been detected in 44% to 81% of bone and soft tissue

sarcomas (9, 10). Telomerase activation is closely correlated with the expression of the

human telomerase reverse transcriptase (*hTERT)* gene (11). Based on these data, we

previously developed a telomerase-specific replication-competent oncolytic adenovirus

OBP-301 (Telomelysin), in which the *hTERT* gene promoter drives the expression of

the *E1A* and *E1B* genes (12). A phase I clinical trial of OBP-301, which was conducted

in the United States on patients with advanced solid tumors, indicated that OBP-301

was well tolerated by patients (13). Recently, we reported that OBP-301 efficiently

killed human bone and soft tissue sarcoma cells (14, 15). However, some osteosarcoma

cell lines were not sensitive to the antitumor effect of OBP-301. Therefore, to efficiently

eliminate tumor cells with OBP-301, its antitumor effects need to be enhanced.

Cancer gene therapy is defined as the treatment of malignant tumors via the

introduction of a therapeutic tumor suppressor gene or the abrogation of an oncogene.

The tumor-suppressor *p53* gene has an attractive tumor suppressor profile as a potent

therapeutic transgene for induction of cell cycle arrest, senescence, apoptosis and

autophagy (16). Dual cell death pathways, such as apoptosis and autophagy, induced by

p53 transactivation are mainly involved in the suppression of tumor initiation and

progression. However, among the p53-downstream target genes, p21, which is most

rapidly and strongly induced during the DNA damage response, mainly induces cell

cycle arrest through suppression of apoptotic and autophagic cell death pathways (17, 18). Thus, p21 suppression may be a more effective strategy for the induction of apoptotic and autophagic cell death pathways in tumor cells, particularly when the tumor-suppressor *p53* gene is overexpressed in tumor cells in response to cancer gene therapy.

A p53-expressing replication-deficient adenovirus (Ad-p53, Advexin) has previously been reported to induce an antitumor effect in the *in vitro* and *in vivo* settings (19, 20) as well as in some clinical studies (21-24). We recently reported that combination therapy with OBP-301 and Ad-p53 resulted in a more profound antitumor effect when compared to monotherapy with either OBP-301 or Ad-p53 (25). Moreover, we generated armed OBP-301 expressing the wild-type *p53* tumor suppressor gene (OBP-702) and showed that OBP-702 suppressed the viability of various types of epithelial malignant cells more efficiently than did OBP-301 (26). OBP-702 induced a more profound apoptotic cell death effect when compared to Ad-p53, likely via adenoviral E1A-mediated suppression of anti-apoptotic p21 in human epithelial malignant cells. However, it remained unclear whether OBP-702 efficiently induces an antitumor effect in human non-epithelial malignant cells, including osteosarcomas.

In the present study, we investigated the *in vitro* cytopathic efficacy of the

p53-expressing telomerase-specific replication-competent oncolytic adenovirus,

OBP-702, in human osteosarcoma cells, and we compared the induction level of

apoptotic and autophagic cell deaths in OBP-301-resistant human osteosarcoma cells

infected with OBP-301, OBP-702 and Ad-p53. The molecular mechanism by which

OBP-702 mediates induction of apoptosis and autophagy was also investigated. Finally,

the *in vivo* antitumor effect of OBP-702 was evaluated using an orthotopic

OBP-301-resistant human osteosarcoma xenograft tumor model.

Materials and Methods

Cell lines

The human osteosarcoma cell lines, HOS and SaOS-2, were kindly provided

by Dr. Satoru Kyo (Kanazawa University, Ishikawa, Japan). These cells were

propagated as monolayer cultures in Dulbecco's modified Eagle's medium. The human

osteosarcoma cell line, U2OS, was obtained from the American Type Culture Collection

(Manassas, VA, USA) and was grown in McCoy's 5a medium. The human

osteosarcoma cell line, MNNG/HOS, was purchased from DS Pharma Biomedical

(Osaka, Japan) and was maintained in Eagle's minimum essential medium containing

1% nonessential amino acids. All media were supplemented with 10% fetal bovine

serum, 100 U/ml penicillin and 100 mg/ml streptomycin. The normal human lung

fibroblast cell line, NHLF, was obtained from TaKaRa Biomedicals (Kyoto, Japan).

NHLF cells were propagated as monolayer culture in the medium recommended by the

manufacturer. Although cell lines were not authenticated by the authors, cells were

immediately expanded after receipt and stored in liquid N_2. Cells were not cultured for

more than 5 months following resuscitation. The cells were maintained at 37°C in a

humidified atmosphere with 5% CO_2.

Recombinant adenoviruses

The recombinant telomerase-specific replication-competent adenovirus

OBP-301 (Telomelysin), in which the promoter element of the *hTERT* gene drives the

expression of *E1A* and *E1B* genes, was previously constructed and characterized (12,

27). For OBP-301-mediated induction of exogenous *p53* gene expression, we recently

generated OBP-702, in which a human wild-type *p53* gene expression cassette was

inserted into the *E3* region (Supplementary Fig. S1) (26). Ad-p53 is a

replication-defective adenovirus serotype 5 vector with a *p53* gene expression cassette

at the *E1* region (19, 20). Recombinant viruses were purified by ultracentrifugation

using cesium chloride step gradients, their titers were determined by a plaque-forming

assay using 293 cells, and they were stored at -80°C.

Cell viability assay

Cells were seeded on 96-well plates at a density of 1×10^3 cells/well 24 h

before viral infection. All cell lines were infected with OBP-702 at multiplicity of

infections (MOI) of 0, 0.1, 1, 10, 50 or 100 plaque forming units (PFU)/cell. Cell

viability was determined on days 1, 2, 3 and 5 after virus infection using Cell

Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN, USA). The 50%

inhibiting dose (ID_{50}) value of OBP-702 for each cell line was calculated using cell

viability data obtained on day 5 after virus infection.

Time-lapse confocal laser microscopy

Green fluorescent protein (GFP)-expressing MNNG/HOS (MNNG/HOS-GFP)

cells were established by stable transfection with GFP expression plasmid using

Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA). MNNG/HOS-GFP and NHLF

cells were seeded in 35-mm glass-based dishes at a density of 1×10^5 cells/dish 24 h

before infection and were infected with OBP-702 at an MOI of 10 PFU/cell for 72 h.

Phase-contrast and fluorescence time-lapse recordings were obtained to concomitantly

analyze cell morphology and GFP expression using an inverted FV10i confocal laser

scanning microscopy (OLYMPUS; Tokyo, Japan).

Western blot analysis

SaOS-2 and MNNG/HOS cells, seeded in a 100-mm dish at a density of $1 \times$

10^5 cells/dish, were infected with OBP-301, OBP-702 or Ad-p53 at the indicated MOIs.

In contrast, SaOS-2 cells were transfected with 10 nM *miR-93* (Ambion, Austin, TX,

USA), *miR-106b* (Ambion) or control miRNA (Ambion) 24 h before Ad-p53 infection

and infected with Ad-p53 at an MOI of 100 for 48 h. Whole-cell lysates were prepared

in a lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100)

containing a protease inhibitor cocktail (Complete Mini; Roche, Indianapolis, IN, USA)

at the indicated time points. Proteins were electrophoresed on 6% to 15% sodium

dodecyl sulfate polyacrylamide gels and were transferred to polyvinylidene difluoride

membranes (Hybond-P; GE Health Care, Buckinghamshire, UK). Blots were blocked

with 5% non-fat dry milk in TBS-T (Tris-buffered saline and 0.1% Tween-20, pH 7.4).

The primary antibodies used were: rabbit anti-PARP polyclonal antibody (pAb) (Cell

Signaling Technology, Beverly, MA, USA), mouse anti-p53 monoclonal antibody

(mAb) (Calbiochem, Darmstadt, Germany), mouse anti-p21^WAF1 mAb (Calbiochem),

rabbit anti-E2F1 pAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse

anti-Ad5 E1A mAb (BD PharMingen, Franklin Lakes, NJ, USA), rabbit

anti-microtubule-associated protein 1 light chain 3 (LC3) pAb (Medical & Biological

Laboratories (MBL), Nagoya, Japan), mouse anti-p62 mAb (MBL), rabbit anti-

damage-regulated autophagy modulator (DRAM) pAb (Abgent, San Diego, CA, USA),

and mouse anti-β-actin mAb (Sigma-Aldrich, St. Louis, MO, USA).

Flow cytometric analysis

To analyze the active caspase-3 expression, cells were incubated for 20 min on

ice in Cytofix/Cytoperm solution (BD Biosciences, Franklin Lakes, NJ, USA), were

labeled with phycoerythrin-conjugated rabbit anti-active caspase-3 mAb (BD

Biosciences) for 30 min, and were then analyzed using FACS array (BD Biosciences).

To evaluate the sub-G1 population, which is a apoptosis indicator, in SaOS-2

cells after virus infection, SaOS-2 cells were seeded in a 100-mm dish at a density of 1

x 10^6 cells/dish 24 h before viral infection, and were infected with mock, OBP-301,

Ad-p53 or OBP-702 at an MOI of 10 PFUs/cell for 48 h. Cells were trypsinized and

resuspended in original supernatant to ensure that both attached and non-attached cells

were analyzed. Cells stained with propidium iodide were analyzed using FACS array

(BD Bioscience, Franklin Lakes, NJ, USA).

Quantitative real-time reverse transcription-PCR analysis

To evaluate the expressions of *miR-93* and *miR-106b* in tumor cells after

OBP-702 infection, SaOS-2 and MNNG/HOS cells were seeded on 6-well plates at a

density of 2×10^5 cells/well 24 h before viral infection and were infected with OBP-702

at MOIs of 0, 1, 5, 10, 50 or 100 PFU/cell. Three days after virus infection, total RNA

was extracted from the cells using a miRNeasy Mini kit (Qiagen, Valencia, CA, USA).

The concentration and quality of RNA were assessed using a Nanodrop

spectrophotometer. cDNA was synthesized from 10 ng of total RNA using the TaqMan

MicroRNA Reverse Transcription kit (Applied Biosystems), and quantitative real-time

RT-PCR was performed using the Applied Biosystems StepOnePlus^{TM} real-time PCR

System. The expressions of *miR-93* and *miR-106b* were defined from the threshold

cycle (Ct), and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method after

normalization with reference to the expression of U6 snRNA.

***In vivo* orthotopic MNNG/HOS xenograft tumor model**

Animal experimental protocols were approved by the Ethics Review

Committee for Animal Experimentation of Okayama University School of Medicine.

MNNG/HOS cells (5×10^6 cells per site) were inoculated into the tibias of female

athymic nude mice aged 6 to 7 weeks (CLEA Japan, Tokyo, Japan). Palpable tumors

developed within 21 days and were permitted to grow to approximately 5 to 6 mm in

diameter. At that stage, a 50-µl volume of solution containing OBP-702, OBP-301 or

Ad-p53 at a dose of 1×10^8 PFU or phosphate buffered saline (PBS) was injected into

the tumors for three cycles every 2 days. Tumor volume was monitored by computed

tomography (CT) imaging once a week after virus infection.

Three-dimensional computed tomography imaging

The tumor volume and formation of osteolytic lesions were evaluated using

three-dimensional computed tomography (3D-CT) imaging (ALOKA Latheta LCT-200;

Hitachi Aloka Medical, Tokyo, Japan). The tumor volume was calculated by INTAGE

Realia® software (Cybernet Systems, Tokyo, Japan).

Histopathologic analysis

Tumors were fixed in 10% neutralized formalin and embedded in paraffin

blocks. Sections were stained with hematoxylin/eosin (H&E) and analyzed by light

microscopy.

Statistical analysis

Data are expressed as means ± SD. Student's *t* test was used to compare

differences between groups. Statistical significance was defined as a *P* value less than

0.05.

Results

***In vitro* cytopathic efficacy of OBP-702 against human osteosarcoma cell lines**

To evaluate the *in vitro* cytopathic activity of OBP-702, we used the two

OBP-301-sensitive human osteosarcoma cells (HOS and U2OS) and the two

OBP-301-resistant human osteosarcoma cells (SaOS-2 and MNNG/HOS) that were

recently described (14). The cell viability of each cell was assessed over 5 days after

infection using the XTT assay. OBP-702 infection suppressed the viability of

OBP-301-sensitive and OBP-301-resistant cells in dose- and time-dependent manners

(Fig. 1A and 1B). When the ID_{50} values of OBP-702 in all four human osteosarcoma

cells were compared with those of OBP-301 calculated in a previous report (14), all cell

lines were more sensitive to OBP-702 than to OBP-301 (Table 1). The ID_{50} values of

OBP-702 were also lower than those of Ad-p53 (Supplementary Fig. S2). However,

OBP-702 did not exhibit any cytopathic effect in NHLF cells (Fig. 1B). When

GFP-expressing MNNG/HOS-GFP cells were co-cultured with human normal NHLF

cells, OBP-702 infection showed a cytopathic effect (confirmed by observation of

round-shaped morphological changes) in MNNG/HOS-GFP cells, but not in NHLF cells

(Fig. 1C). These results indicate that OBP-702 was more cytopathic than OBP-301 for

human osteosarcoma cells, but was not cytopathic for normal human cells.

Increased induction of apoptosis by OBP-702 when compared with OBP-301 or

Ad-p53

We next investigated whether OBP-702 induces more profound apoptosis when

compared with OBP-301 or Ad-p53. OBP-301-resistant SaOS-2 and MNNG/HOS cells

were infected with OBP-702, OBP-301 or Ad-p53, and apoptosis was assessed by

Western blot and flow cytometric analyzes. Western blot analysis showed that SaOS-2

cells exhibited the cleavage of PARP after infection with OBP-702 (more than 5 MOIs)

or Ad-p53 (more than 50 MOIs), whereas MNNG/HOS cells had the cleavage of PARP

after infection with OBP-702 (more than 5 MOIs), but not Ad-p53 (Fig. 2A). In contrast,

OBP-301 did not induce apoptosis (data not shown). Furthermore, flow cytometric

analysis demonstrated that OBP-702 infection (10 MOIs) significantly increased the

percentage of apoptotic cells with active caspase-3 when compared with Ad-p53 or

OBP-301 at same doses in SaOS-2 and MNNG/HOS cells (Fig. 2B and 2C). Cell cycle

analysis also showed that OBP-702 (10 MOIs) induced the highest percentages of

sub-G1 population in SaOS-2 cells when compared with Ad-p53 or OBP-301 at same

doses (Fig. 2D). These results suggest that OBP-702 induces increased apoptosis when

compared with Ad-p53 or OBP-301 in human osteosarcoma cells.

p53 induction in human osteosarcoma cells infected with OBP-702

To investigate the molecular mechanism of OBP-702-induced apoptosis in

human osteosarcoma cells, we evaluated p53 expression after OBP-702 infection in

SaOS-2 (p53 null) and MNNG/HOS (p53 mutant) cells in which endogenous p53

expression level was confirmed by Western blot analysis (Supplementary Fig. S3).

OBP-702 efficiently induced p53 expression in SaOS-2 and MNNG/HOS cells (Fig.

3A). The level of p53 expression was higher in OBP-702-treated cells than in

Ad-p53-treated cells (Fig. 3A). In spite of OBP-702-induced high p53 expression,

p53-downstream target p21 protein was induced only in Ad-p53-treated cells.

To investigate the effect of exogenous p53 overexpression in virus replication,

we next compared the replication abilities of OBP-702 and OBP-301 in p53-null

SaOS-2 cells by measuring the relative amounts of *E1A* copy numbers. The *E1A* copy

number of OBP-702 was similar to that of OBP-301 in SaOS-2 cells (Supplementary

Fig. S4). These results indicate that OBP-702 efficiently induces exogenous p53

expression without affecting p21 expression and virus replication in human

osteosarcoma cells.

OBP-702-mediated upregulation of *miR-93* and *miR-106b* suppresses p21

expression

Adenoviral E1A protein has been shown to activate E2F1 expression (28),

which is a multifunctional transcription factor that regulates diverse cell fates through

induction of many target genes, including small non-coding microRNAs (miRNAs) (29).

Recently, E2F1-inducible *miR-93* and *miR-106b* have been shown to suppress p21

expression in human cancer cells (30). Therefore, we sought to investigate whether

OBP-702 induces expressions of E2F1 and E2F1-regulated miRNAs (*miR-93* and

miR-106b). OBP-702 infection activated E2F1 expression along with E1A accumulation

in SaOS-2 and MNNG/HOS cells (Fig. 3B). The expression levels of *miR-93* and

miR-106b were increased in association with E2F1 activation in OBP-702-infected

SaOS-2 and MNNG/HOS cells (Fig. 3C). In contrast, E1A-deleted Ad-p53 infection did

not increase expressions of E2F1 and E2F1-regulated *miR-93* and *miR-106b* (data not

shown). Next, we assessed whether upregulation of *miR-93* and *miR-106b* efficiently

suppresses p21 expression induced by Ad-p53-mediated p53 overexpression. Ad-p53

infection at MOIs of 10 and 100 efficiently induced p21 expression at 48 h after

infection in SaOS-2 cells (Supplementary Fig. S5). When SaOS-2 cells were infected

with Ad-p53 at an MOI of 100 for 48 h, pre-transfection with *miR-93*, *miR-106b* or both

efficiently suppressed Ad-p53-induced p21 expression (Fig. 3D). Interestingly, both

miR-93 and *miR-106b* -transfected SaOS-2 cells showed the 1.5-fold increased

expression of cleaved PARP (C-PARP) in consistent with remarkable p21

downregulation when compared with those transfected with control miR. However, the

expression level of C-PARP was not increased in the *miR-93* or *miR-106b*-transfected

SaOS-2 cells, although transfection with *miR-93* or *miR-106b* moderately decreased p21

expression. These results suggest that OBP-702 suppresses p21 expression through

E1A-dependent upregulation of E2F1-inducible both *miR-93* and *miR-106b* and

contributes to induction of apoptosis.

Increased induction of autophagy by OBP-702 when compared with OBP-301

Recently, we demonstrated that oncolytic adenovirus OBP-301 mainly induces

programmed cell death in association with autophagy rather than apoptosis in human

tumor cells (31). Therefore, we next investigated whether OBP-702 induces more

profound autophagy than does OBP-301. Western blot analysis revealed that OBP-702

infection showed increased autophagy, which was confirmed by conversion of LC3-I to

LC3-II (increased ratio of LC3-II/LC3-I) and p62 downregulation, when compared with

OBP-301 in MNNG/HOS cells (Fig. 4A). Moreover, the expression level of the

p53-induced modulator of autophagy, DRAM (32), was decreased after OBP-301

infection, but its expression was maintained after OBP-702 infection (Fig. 4A). Since

p53-mediated p21 overexpression is known to inhibit both apoptosis and autophagy (17,

18), we further evaluated whether miR-mediated p21 suppression is involved in the

enhancement of p53-mediated autophagy induction. Ad-p53-induced autophagy was

enhanced by *miR-93* and *miR-106b* -mediated p21 suppression (Fig. 4B). These results

suggest that OBP-702 induces more profound autophagy than does OBP-301 and that

this effect occurs via p53-mediated DRAM activation and miR-mediated p21

suppression.

Enhanced antitumor effect of OBP-702 in an orthotopic xenograft tumor model

Finally, to assess the *in vivo* antitumor effect of OBP-702, we used an

orthotopic MNNG/HOS tumor xenograft model. OBP-702, OBP-301, Ad-p53 or PBS

was intratumorally injected for three cycles every 2 days. OBP-702 administration

significantly suppressed tumor growth when compared with OBP-301, Ad-p53 or PBS

in an orthotopic MNNG/HOS tumor model (Fig. 5A and B). 3D-CT examination

revealed that OBP-702-treated tumors had less bone destruction than did OBP-301- or

Ad-p53-treated tumors (Fig. 5C). On histopathologic analysis, there were large necrotic

areas in OBP-702-treated tumors, but not in OBP-301- or Ad-p53-treated tumors (Fig.

5D). Moreover, the expression of the cell proliferation marker, Ki67, was also decreased,

especially in OBP-702-treated tumor cells (Supplementary Fig. S6). These results

suggest that OBP-702 eliminates tumor tissues more efficiently when compared with

OBP-301 or Ad-p53.

Discussion

We previously reported that telomerase-specific replication-competent

oncolytic adenovirus OBP-301 has strong antitumor activity in a variety of human

epithelial and non-epithelial malignant cells (12, 14, 27). However, some human

osteosarcoma cells were resistant to the cytopathic activity of OBP-301 (14). In this

study, we demonstrated that a novel p53-expressing oncolytic adenovirus, OBP-702,

had increased *in vitro* and *in vivo* antitumor effects than did OBP-301 in human

osteosarcoma cells (Fig. 1 and 5). OBP-702 induced increased apoptosis in association

with p53 upregulation and p21 downregulation when compared with

replication-deficient Ad-p53 (Fig. 2 and 3A). E1A-dependent upregulation of *miR-93*

and *miR-106b* was involved in OBP-702-mediated suppression of p21 expression (Fig.

3). Moreover, p53-mediated DRAM activation with p21 suppression enhanced oncolytic

adenovirus-mediated autophagy induction (Fig. 4). Recent studies suggest that

transgene-expressing armed oncolytic adenoviruses are a promising antitumor strategy

for induction of oncolytic and transgene-induced cell death (33). Although p53

overexpression has been shown to enhance antitumor activity of oncolytic adenoviruses

(34), the molecular mechanisms by which p53 mediates enhancement of the antitumor

effect remain unclear. Recently, we reported that OBP-702 induces profound apoptosis

through p53-dependent BAX upregulation and E1A-dependent p21 and MDM2

downregulation in epithelial malignant cells (26). Thus, oncolytic adenovirus-mediated

p53 overexpression likely induces dual apoptotic and autophagic cell death pathways

through p53-dependent BAX/DRAM activation and E1A-dependent p21/MDM2

suppression with E2F1-inducible *miR-93/106b* upregulation (Fig. 6).

OBP-702 efficiently suppressed the cell viability of both OBP-301-sensitive

and OBP-301-resistant osteosarcoma cells (Fig. 1). We previously reported that

OBP-301-resistant SaOS-2 cells have no *hTERT* mRNA expression (Table 1),

suggesting that SaOS-2 cells maintain telomere length through alternative lengthening

of telomeres (ALT). As *hTERT* gene promoter is used for tumor-specific replication of

OBP-301, ALT-type human osteosarcoma cells like SaOS-2 cells may be resistant to

OBP-301. However, ALT-type SaOS-2 cells showed similar sensitivity to OBP-702 as

well as non-ALT-type MNNG/HOS cells (Fig. 1 and Table 1). These results suggest that

p53 overexpression overcomes resistance to OBP-301 in ALT-type SaOS-2 cells. Since

the replication rate of OBP-702 was almost similar that of OBP-301 in ALT-type

SaOS-2 cells (Supplementary Fig. S2), p53-induced cell death pathway would suppress

the cell viability of ALT-type human osteosarcoma cells.

OBP-702-mediated p53 overexpression induced two types of programmed cell

deaths (i.e., apoptosis and autophagy), thereby contributing to the enhancement of the

antitumor effect of OBP-301 in human osteosarcoma cells (Fig. 2 and 4). Since

p53-downstream target p21 functions as a suppressor of apoptosis and autophagy (17,

18), p21 suppression may be a critical factor to induce dual programmed cell death

pathways in response p53 overexpression. Suppression of p21 expression by genetic

deletion or artificial p21-target microRNA has been shown to enhance the

Ad-p53-induced apoptosis (18, 35). Inactivation of p21 by adenoviral E1A has been

shown to enhance apoptosis in chemotherapeutic drug-treated human colon cancer cells

that overexpress p53 (36). Genetic deletion of p21 has been also shown to induce

autophagy in mouse embryonic fibroblasts treated with C(2)-ceramide or

gamma-irradiation (17). In contrast, p21 overexpression inhibited the Ad-p53-mediated

apoptosis induction (18). Thus, E1A-mediated p21 downregulation would enhance

p53-induced apoptosis and autophagy in OBP-702-infected cells.

E1A-dependent E2F1 activation and subsequent upregulation of

E2F1-inducible miRNAs efficiently suppressed p21 expression, leading to the

enhancement of p53-induced apoptosis and autophagy, in OBP-702-infected

osteosarcoma cells (Fig. 2, 3 and 4). Recent studies suggest that the cross-talk between

p53 and E2F1 play a role in the regulation of diverse cell fates (37). For example,

co-expression of p53 and E2F1 contributes to induction of apoptosis (38, 39). We

previously demonstrated that E2F1 enhanced Ad-p53-mediated apoptosis through

p14ARF-dependent MDM2 downregulation (39) and that OBP-702 infection showed

E1A-dependent MDM2 downregulation in association with apoptosis (26). Recently,

E2F1 has been shown to suppress MDM2 expression by suppressing the promoter

activity (40) or by inducing upregulation of *miR-25/32*, which targets MDM2 (41).

Furthermore, E2F1-inducible *miR-93/106b* enhanced Ad-p53-induced apoptosis and

autophagy via p21 suppression (Fig. 3D and 4B). Therefore, the cooperation between

the MDM2-p53-p21 pathway and the E2F1-miRNA pathway may be involved in the

induction of apoptotic and autophagic cell death in response to OBP-702.

OBP-702-mediated p53 overexpression enhanced autophagy that was induced

by oncolytic adenovirus in human osteosarcoma cells. OBP-702 infection induced

increased expression of DRAM and decreased expression of p62 when compared with

OBP-301 (Fig. 4), suggesting that OBP-702-mediated p53 overexpression enhances

autophagy through DRAM activation. We recently reported that OBP-301 induces

autophagy through E1A-dependent activation of E2F1-*miR-7* pathway and subsequent

suppression of epidermal growth factor receptor (EGFR) (31). Restoration of p53

expression enhances the sensitivity to EGFR inhibitors in human cancer cells (42).

Moreover, EGFR downregulation by transfection of specific antisense oligonucleotide

promotes the differentiation status of human osteosarcoma U2OS cells (43). Thus,

OBP-702 may induce differentiation as well as cell death through autophagy activation

by DRAM upregulation and EGFR downregulation in human osteosarcoma cells.

The 3D-CT imaging system was a useful method to assess both tumor volume

and bone destruction status in MNNG/HOS tumors. OBP-702-treated tumors were

smaller and had less bone destruction when compared with PBS-, Ad-p53- or

OBP-301-treated tumors (Fig. 5A and 5C). Recent reports have suggested that

zoledronic acid suppresses tumor growth as well as osteolytic components in human

osteosarcoma xenograft tumor models (44, 45). These results suggest that combination

therapy with OBP-702 and zoledronic acid may be more effective and more protective

against bone destruction in human osteosarcomas. Further study using a 3D-CT imaging

system may provide important information regarding bone destruction status in

osteosarcomas treated with OBP-702 and zoledronic acid.

Adenovirus-mediated p53 gene therapy exerts an antitumor effect in human

osteosarcoma cells (46). However, the antitumor activity of replication-deficient Ad-p53

is limited in some human osteosarcoma cells (47). Ad-p53-mediated p53 overexpression

increases the sensitivity of human osteosarcoma cells to the chemotherapeutic drugs,

cisplatin and doxorubicin (48). A synergistic antitumor effect between doxorubicin and

roscovitine was also associated with autophagy induction in human osteosarcoma U2OS

cells (49). As OBP-702 induced more profound apoptosis and autophagy than did

OBP-301 or Ad-p53 (Fig. 2 and 4), combination therapy with OBP-702 and

chemotherapeutic agents may be more effective than monotherapy with OBP-702.

Moreover, a recent report has shown that p53-armed replication-competent oncolytic

adenovirus is a safe antitumor agent in rodents and nonhuman primates (50). However,

for clinical application of OBP-702, it must be necessary to establish the systemic

delivery method and confirm the host biological contributions in cancer patients.

Although there are some unsolved issues, the combination of p53-armed oncolytic

adenovirus and chemotherapy may provide us a promising antitumor strategy against

human osteosarcoma cells.

In conclusion, we clearly demonstrated that the p53-expressing oncolytic

adenovirus OBP-702 has a much stronger antitumor effect than does OBP-301.

Oncolytic adenovirus-mediated p53 gene transduction may induce dual apoptotic and

autophagic cell death pathways through p53-dependent activation of cell death inducers

and E1A-dependent suppression of cell death inhibitors, resulting in the enhancement of

antitumor effect.

Acknowledgements

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nonhuman primates. Toxicol Sci. 2008;106:242-50.

Table 1. Comparison of ID_{50} values of OBP-301 and OBP-702 in various human osteosarcoma cell lines

Cell lines	Sensitivity to OBP-301	Cell type*	Relative hTERT mRNA expression	ID_{50} value** (MOI)		Ratio[†]
				OBP-301	OBP-702	(OBP-702/OBP-301)
SaOS-2	Resistant	ALT	negative	98.1	5.5	0.06
MNNG/HOS	Resistant	non-ALT	1	97.3	6.7	0.07
U2OS	Sensitive	ALT	0.3	38.2	1.2	0.03
HOS	Sensitive	non-ALT	4.3	43.0	4.5	0.10

* Alternative lengthening of telomeres (ALT).

** The ID_{50} values of OBP-702 and OBP-301 were calculated from the data of XTT assay on day 5 after infection.

[†] The ratio was calculated from the division of the ID_{50} value of OBP-702 by the ID_{50} value of OBP-301.

Figure legends

Fig. 1. *In vitro* **cytopathic effect of OBP-702 in human osteosarcoma cell lines. A**,

OBP-301-sensitive (HOS and U2OS) and OBP-301-resistant (SaOS-2 and

MNNG/HOS) human osteosarcoma cells were infected with OBP-702 at the indicated

multiplicity of infection (MOI), and cell viability was quantified over 5 days using the

XTT assay. Cell viability was calculated relative to that of the mock-infected group on

each day, which was set at 1.0. Cell viability data are expressed as mean values ± SD (n

= 5). B, four human osteosarcoma cells and one normal fibroblast NHLF cells were

seeded 24 h before viral infection, and were infected with OBP-702 at the indicated

MOIs, and cell viability was examined on day 5 using the XTT assay. Cell viability was

calculated relative to that of the mock-infected group, which was set at 1.0. Cell

viability data are expressed as mean ± SD (n = 5). **C**, time-lapse images of cytopathic

effect of OBP-702 in co-culture of green fluorescent protein (GFP)-expressing

MNNG/HOS cells with human normal fibroblast NHLF cells. MNNG/HOS-GFP cells

co-incubated with NHLF cells were recorded for 72 h after OBP-702 infection at an

MOI of 10. Three images on the left are low-magnification, and one image on the right

is high-magnification of the area outlined by a white square. Left scale bars, 100 μm.

Right scale bar, 10 μm.

Fig. 2.   OBP-702 induces increased apoptosis when compared with OBP-301 or

Ad-p53. A, OBP-301-resistant SaOS-2 and MNNG/HOS cells were infected with

OBP-702 or Ad-p53 at the indicated MOIs for 72 h. Cell lysates were subjected to

Western blot analysis for the cleaved PARP (C-PARP) and PARP. β-actin was assayed

as a loading control. **B-D**, SaOS-2 and MNNG/HOS cells were infected with OBP-702,

OBP-301 or Ad-p53 at an MOI of 10 for 48 h. Mock-infected cells were used as control.

Caspase-3 activation was quantified using the flow cytometric analysis. Representative

flow cytometric data were shown (**B**). The mean percentage of SaOS-2 cells and

MNNG/HOS cells that express active caspase-3 was calculated based on

three-independent experiments (**C**). The cell cycle state was analyzed by flow cytometry

in SaOS-2 cells after staining with propidium iodide. Representative cell cycle data

were shown (**D**). The percentage of sub-G1 population was expressed in each graph.

Bars, SD. Statistical significance was determined using Student's *t* test. *, *P* < 0.05.

Fig. 3.   OBP-702 induces p53 overexpression with E1A-mediated p21 suppression

via *miR-93* and *miR-106b* activation. A, expression of the p53 and p21 proteins in

SaOS-2 and MNNG/HOS cells infected with OBP-702 or Ad-p53 at the indicated MOIs

for 72 h was assessed using Western blot analysis. **B**, expression of the E2F1 and viral

E1A proteins in SaOS-2 and MNNG/HOS cells infected with OBP-702 at the indicated

MOIs for 72 h was assessed using Western blot analysis. **C**, expression of *miR-93* and

miR-106b was assayed using qRT-PCR in SaOS-2 cells infected with OBP-702 at the

indicated MOIs for 72 h on three-independent experiments. The values of *miR-93* and

miR-106b at 0 MOI were set at 1, and the relative levels of *miR-93* and *miR-106b* at the

indicated MOIs were plotted as fold induction. Bars, SD. Statistical significance was

determined by Student's *t* test. *, $P < 0.05$.   **D**, SaOS-2 cells were transfected with 10

nM *miR-93*, *miR-106* or control miRNA 24 h before Ad-p53 infection at an MOI of 100.

At 48 h after Ad-p53 infection, the expression levels of p53, p21, PARP and cleaved

PARP (C-PARP) were examined by Western blot analysis. β-actin was assayed as a

loading control. By using ImageJ software, the expression level of C-PARP protein was

calculated relative to its expression in the control miR-treated cells, whose expression

level was designated as 1.0.

Fig. 4.   **OBP-702 induces increased autophagy when compared with OBP-301. A**,

MNNG/HOS cells were infected with OBP-702 or OBP-301 at the indicated MOIs for

72 h. Cell lysates were subjected to Western blot analysis for LC3, p62 and DRAM. **B**,

SaOS-2 cells were transfected with 10 nM *miR-93*, *miR-106* or control miRNA 24 h

before Ad-p53 infection. At 48 h after Ad-p53 infection at an MOI of 100, the

expression levels of LC3, p62 and DRAM were examined by Western blot analysis.

β-actin was assayed as a loading control. By using ImageJ software, the ratio of

LC3-II/LC3-I expressions was calculated relative to its expression in the mock-infected

cells, whose expression level was designated as 1.0.

Fig. 5. Antitumor effect of OBP-702 in an orthotopic MNNG/HOS osteosarcoma

xenograft model. A, athymic nude mice were inoculated intratibially with

MNNG/HOS cells (5×10^6 cells/site). Twenty-one days after inoculation (designated as

day 0), Ad-p53, OBP-301 or OBP-702 was injected into the tumor with 1×10^8 PFUs on

days 0, 2 and 4 (black arrows). PBS was used as a control. Three mice were used for

each group. Each tumor volume was assessed by computed tomography (CT)

examination. Tumor growth was expressed as mean tumor volume ± SD. Statistical

significance was determined by Student's *t* test. *, $P < 0.05$. **B**, macroscopic appearance

of MNNG/HOS tumors in nude mice on days 0 and 28 after treatment with PBS,

Ad-p53, OBP-301 or OBP-702. Tumor masses are outlined by a dotted line. **C**,

three-dimensional (3D)-CT images of MNNG/HOS tumors. The tumor volumes were

calculated by the image viewer (INTAGE Realia) based on 3D-CT images of tumors

after trimming. The white arrowheads indicate the osteolytic areas within tumor tissues

treated with PBS, Ad-p53 or OBP-301. Left side images are low-magnification, and

right side images are high-magnification of the area outlined by a white square. **D**,

histological analysis of the MNNG/HOS tumors. Tumor tissues were obtained on day

28 after first treatment with PBS, Ad-p53, OBP-301 or OBP-702. Paraffin-embedded

sections of MNNG/HOS tumors were stained with hematoxylin and eosin solutions.

There were large necrotic areas in MNNG/HOS tumors treated with OBP-702. Left side

images are low-magnification, and right side images are high-magnification of the area

outlined by a white square. Left scale bars, 500 μm. Right scale bars, 100 μm.

Fig. 6. Outline of OBP-702-mediated induction of dual programmed cell death

pathways. OBP-702 infection induces apoptosis and autophagy, leading to cell death,

through p53-dependent BAX/DRAM upregulation and E1A-dependent p21

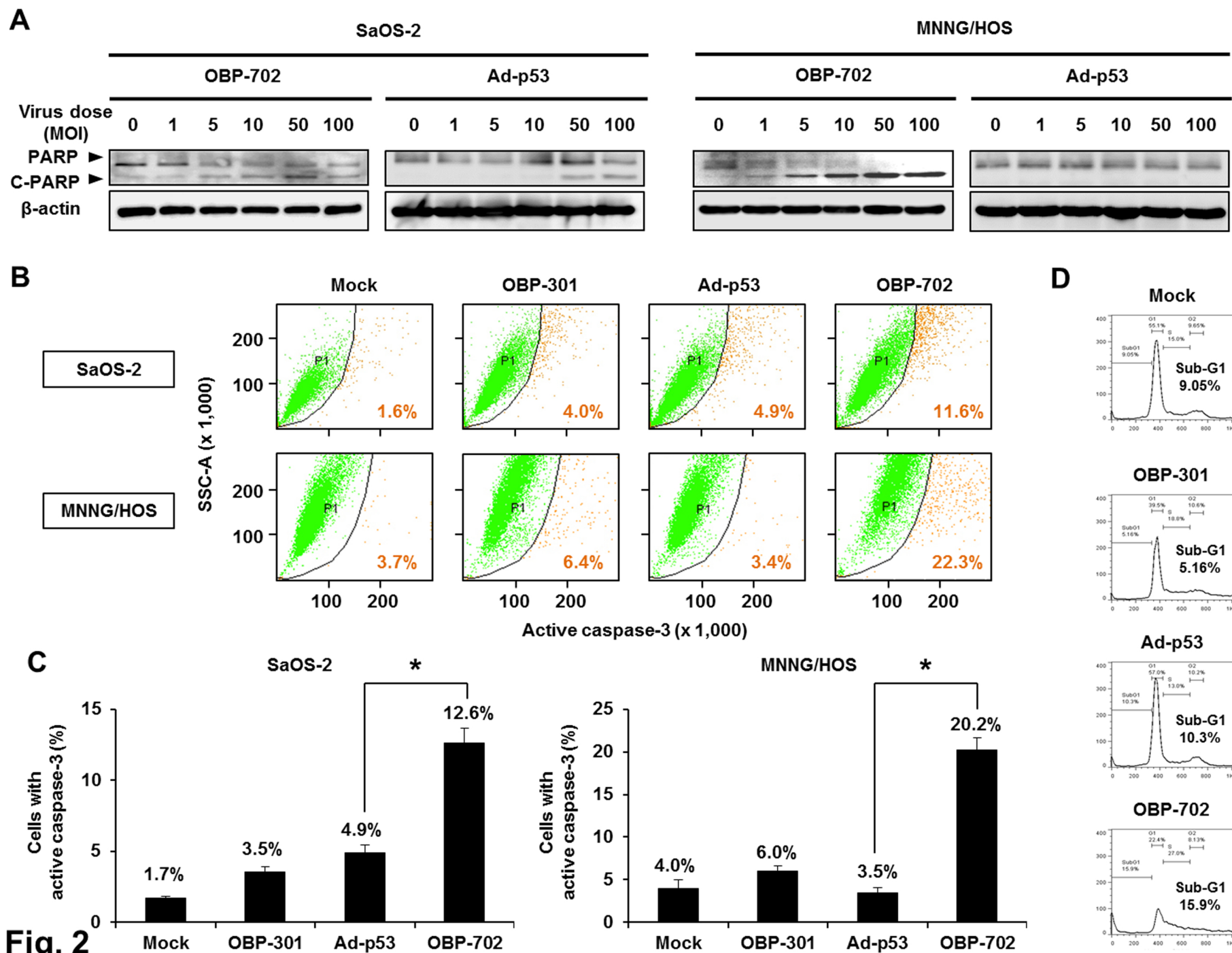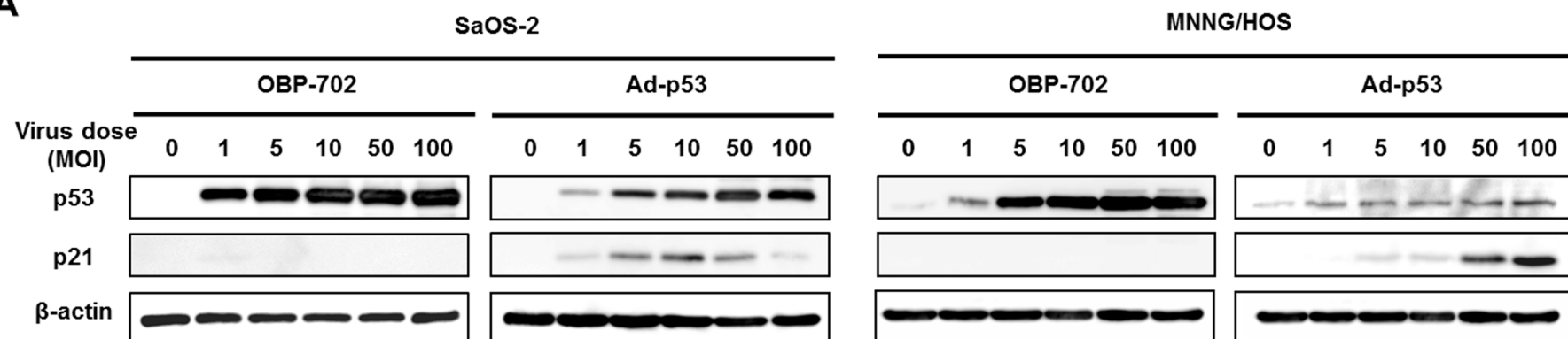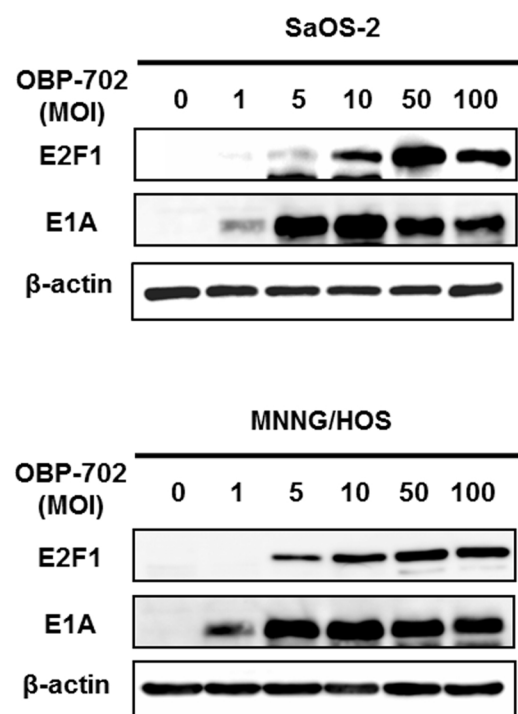downregulation via E2F1-inducible *miR-93/106b* activation.

Fig. 1

Fig. 2

Fig. 3

A

OBP-702

Virus dose (MOI)	0	1	5	10	50	100
LC3-I ►						
LC3-II ►						
LC3-II/LC3-I	1.0	1.2	1.4	1.7	2.1	2.3
p62						
DRAM						
β-actin						

OBP-301

Virus dose (MOI)	0	1	5	10	50	100
LC3-I ►						
LC3-II ►						
LC3-II/LC3-I	1.0	1.2	1.3	1.2	1.4	1.7
p62						
DRAM						
β-actin						

B

Ad-p53	–	+	+	+	+
Control miR	+	+	–	–	–
miR-93	–	–	+	–	+
miR-106b	–	–	–	+	+
LC3-I ►					
LC3-II ►					
LC3-II/LC3-I	1.0	2.1	3.1	2.8	3.1
p62					
DRAM					
β-actin					

Fig. 4

Fig. 5

Fig. 6