IN, USA) at the indicated time points. Proteins were electrophoresed on 6–15% SDS polyacrylamide gels and were transferred to polyvinylidene difluoride membranes (Hybond-P; GE Healthcare, 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) at room temperature for 30 min. The primary antibodies used were: mouse anti-p53 monoclonal antibody (mAb) (Calbiochem, Darmstadt, Germany), mouse anti-p21^{WAF1} mAb (Calbiochem), mouse anti-MDM2 mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-BAX polyclonal antibody (pAb) (Santa Cruz Biotechnology), rabbit anti-poly (ADP-ribose) polymerase (PARP) pAb (Cell Signaling Technology, Beverly, MA, USA), mouse anti-Ad5 E1A mAb (BD PharMingen, Franklin Lakes, NJ, USA) and mouse anti-β-actin mAb (Sigma–Aldrich, St. Louis, MO, USA). The secondary antibodies used were: horseradish peroxidase-conjugated antibodies against rabbit IgG (GE Healthcare) or mouse IgG (GE Healthcare). Immunoreactive bands on the blots were visualised using enhanced chemiluminescence substrates (ECL Plus; GE Healthcare).

2.4. Cell viability assay

Cells were seeded on 96-well plates at a density of 1×10^3 cells/well 12 h before infection and were infected with OBP-301 or OBP-702 at MOIs of 0, 0.1, 1, 10 or 100 plaque-forming units (PFU)/cell. Cell viability was determined on days 2, 3 and 5 after virus infection using the Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN, USA), which is based on an XTT, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulphonic acid hydrate assay, according to the manufacturer's protocol. The 50% inhibiting dose (ID_{50}) value of OBP-301 and OBP-702 for each cell line was calculated using cell viability data obtained on day 5 after virus infection.

2.5. Flow cytometric analysis of active caspase-3 expression

Cells were incubated for 20 min on ice in Cytofix/Cytoperm solution (BD Biosciences, Franklin Lakes, NJ, USA), were labelled with phycoerythrin-conjugated rabbit anti-active caspase-3 mAb (BD Biosciences) for

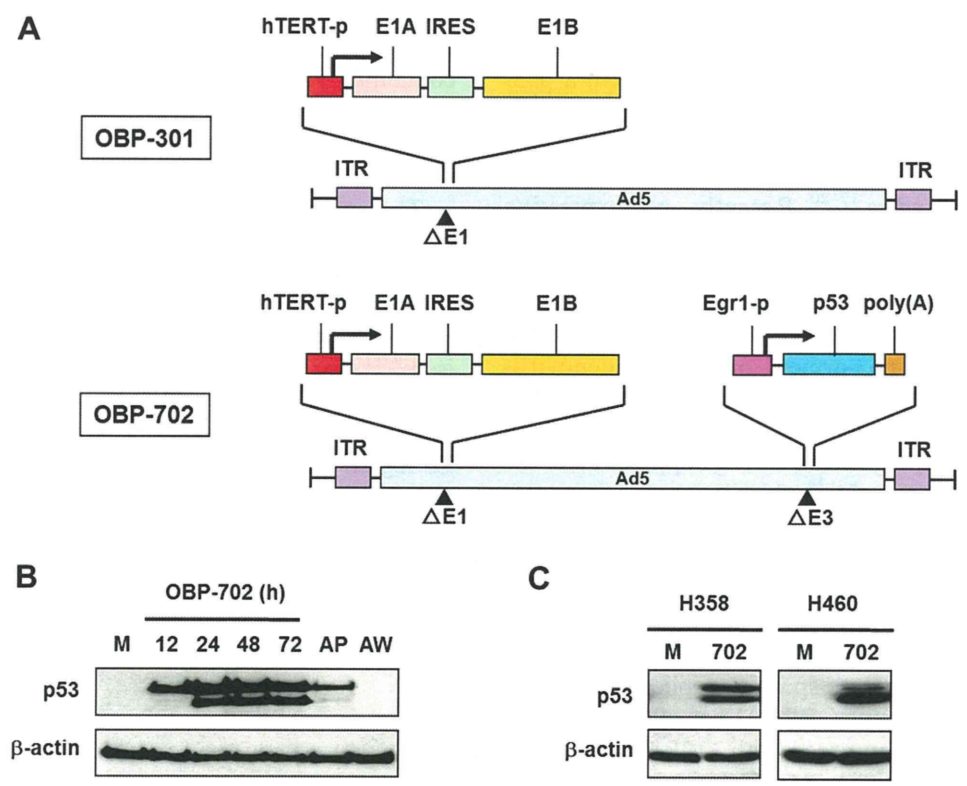

Fig. 1. p53 upregulation in human cancer cells infected with OBP-702. (A) Schematic diagrams of OBP-301 and OBP-702 structures. OBP-301 is a telomerase-specific replication-competent adenovirus, in which the *hTERT* promoter drives the expression of *E1A* and *E1B* genes that are linked with an IRES. OBP-702 is a p53-armed OBP-301, in which the *Egr-1* promoter drives expression of the *p53* gene that is inserted into the E3 region. (B) Expression of the p53 protein in p53-null H1299 cells infected with OBP-702 (10 MOI) at the indicated time points. A replication-deficient p53-expressing adenovirus Ad-p53 (AP) and a wild-type adenovirus Ad5 (AW) were also infected at an MOI of 10 for 24 h as a positive and negative control, respectively. Cell lysates were subjected to Western blot analysis with an anti-p53 antibody. β-Actin was assayed as a loading control. (C) Expression of the p53 protein in H358 and H460 cells infected with OBP-702 (702) at an MOI of 10 for 24 h. Mock-infected cells (M) were used as controls.

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

2.6. *In vivo subcutaneous H358 and T.Tn xenograft tumour models*

Animal experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Okayama University School of Medicine. The H358 and T.Tn cells (5×10^6 cells per site) were inoculated into the flanks of 5-week-old female athymic nude mice (Charles River Laboratories, Wilmington, MA, USA). When tumours reached approximately 5–6 mm in diameter, a 50 μl volume of solution containing OBP-301, OBP-702 or Ad-p53 at a dose of 1×10^8 PFU or phosphate buffered saline (PBS) was injected into the tumours for three cycles every 2 days. Tumour size was monitored by measuring tumour length and width using calipers. Each tumour volume was calculated using the following formula: tumour volume (mm^3) $= L \times W^2 \times 0.5$, where L is the length and W is the width. The survival rate of mice with H358 tumours or T.Tn tumours was assessed until 90 or 180 days, respectively, after first treatment.

2.7. *Statistical analysis*

Data are expressed as means ± standard deviation (SD). Student's t test was used to compare differences between groups. Log-rank test was also used to compare differences between groups in the survival rate of mice. Statistical significance was defined as a P value less than 0.05.

3. Results

3.1. *p53 induction in human cancer cells infected with OBP-702*

To examine the level of p53 expression induced by OBP-702 in human cancer cells, we first evaluated p53 expression of p53-null human lung cancer H1299 cells after OBP-702 infection using Western blot analysis. The p53 expression level was increased within 24 h after OBP-702 infection, and a high expression level was maintained for up to 72 h (Fig. 1B). OBP-702-induced p53 expression was higher than Ad-p53-induced p53 expression 24 h after infection. Detectable 40 kDa protein expression in OBP-702-infected H1299 cells may be due to higher p53 expression. In contrast, no p53 expression was induced by OBP-301 infection (data not shown). OBP-702 further induced p53 expression in other human lung cancer cells (H358 (p53-null) and H460 (wild-type p53)) and in human colon cancer cells (SW620 (mutant p53), LoVo cells (wild-type p53)) and human liver cancer cells (HepG2 (wild-type p53) and Huh7 (mutant p53)) (Fig. 1C and Supplementary Fig. 1A). These results indicate that OBP-702 efficiently induces exogenous p53 expression in human cancer cells independent of the status of endogenous p53.

3.2. *OBP-702 has enhanced antitumour activity against human cancer cells compared to OBP-301*

To compare the *in vitro* antitumour activity of OBP-702 and OBP-301, we used the two OBP-301-sensitive human cancer cells (H358 and H460) and the two OBP-301-resistant human cancer cells (T.Tn and HSC4) that were previously reported.[11] OBP-301-resistant cells showed lower the coxsackie and adenovirus receptor (CAR) expression compared to OBP-301-sensitive cells (data not shown). The cell viability of each cell line was assessed over 5 days after infection using the XTT assay. OBP-702 suppressed the viability of OBP-301-sensitive and OBP-301-resistant cells more efficiently than OBP-301, although at least 48 h are required for the sufficient viral replication (Fig. 2A). Furthermore, OBP-702 also showed increased antitumour activity against human colon and liver cancer cells compared to OBP-301 (Supplementary Fig. 1B). Calculation of the ID_{50} values indicated that all cell lines were more sensitive to OBP-702 than to OBP-301 (Supplementary Table S1). These results suggest that OBP-702 is more cytopathic for human cancer cells than OBP-301.

3.3. *Increased induction of apoptosis by OBP-702 compared to OBP-301 or Ad-p53*

We next investigated whether OBP-702 has a greater apoptotic effect than OBP-301 or Ad-p53. OBP-301-sensitive H358 cells and OBP-301-resistant T.Tn cells were each infected with OBP-702, OBP-301 or Ad-p53 at MOIs of 10 and 100 for 48 h, and apoptosis was analysed. Western blot analysis showed that OBP-702, but not OBP-301 or Ad-p53, induced the cleavage of PARP at 48 and 72 h after infection (Fig. 3A). Furthermore, flow cytometric analysis demonstrated that OBP-702 infection significantly increased the percentage of apoptotic H358 and T.Tn cells that expressed active caspase-3 compared to Ad-p53 infection (Fig. 3B and C). However, no apoptosis was induced after OBP-301 infection. These results suggest that OBP-702 has a stronger effect on apoptosis than Ad-p53 or OBP-301.

3.4. *Induction of apoptosis by OBP-702 through p53-dependent BAX upregulation and E1A-dependent p21 and MDM2 downregulation*

Overexpression of p53 is well known to induce apoptosis through induction of p53-downstream target genes.[14] To investigate the molecular mechanism of OBP-702-induced apoptotic cell death, the expression

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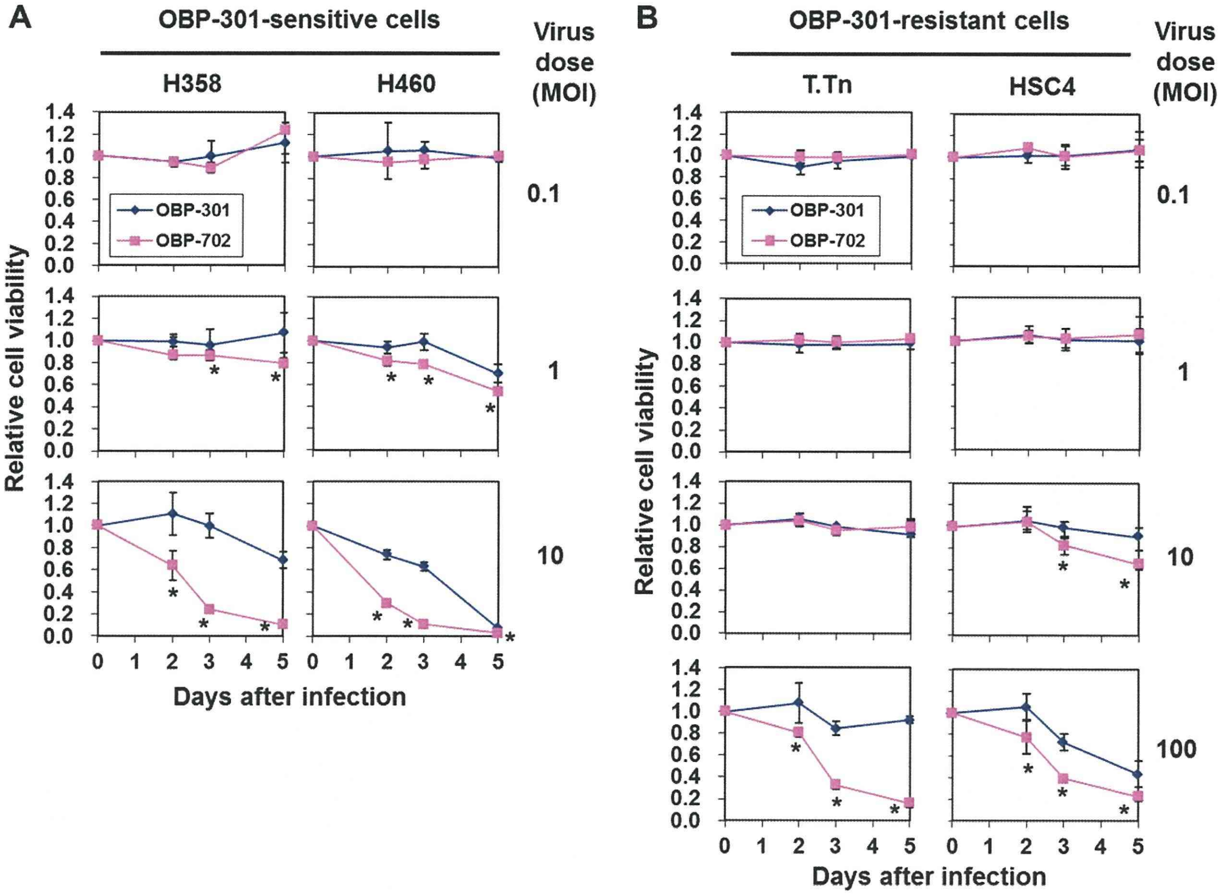

Fig. 2. OBP-702 has enhanced antitumour activity against human cancer cells compared to OBP-301. OBP-301-sensitive cells (H358 and H460) (A) and OBP-301-resistant cells (T.Tn and HSC4) (B) were infected with OBP-301 or OBP-702 at the indicated doses and cell viability was measured using the XTT assay on days 2, 3 and 5 after infection. Cell viability was calculated relative to that of the mock-treated group on each day, which was set at 1.0. Cell viability data are expressed as mean values ± SD ($n = 5$). Statistical significance was determined using Student's t test. $^{*}P < 0.05$. The data are representative of three separate experiments.

level of p53, and p53-downstream target proteins such as p21, BAX and MDM2, was evaluated by Western blot analysis. OBP-702 infection induced higher p53 expression than that induced by Ad-p53 between 24 and 72 h after infection (Fig. 4A). Ad-p53 infection upregulated the expression of p21, MDM2 and BAX proteins. In contrast, OBP-702 infection upregulated the BAX protein as well as Ad-p53, but expression of p21 and MDM2 was low despite strong p53 activation. PARP cleavage was observed 48 and 72 h after OBP-702 infection, consistent with suppression of p21 and MDM2 expression. Overexpression of the adenoviral E1A protein was observed in OBP-702-infected cells. These results suggest that OBP-702 upregulates p53 expression and subsequent BAX expression, but down-regulates p21 and MDM2 expression, resulting in the induction of apoptosis.

We recently reported that OBP-301 enhances Ad-p53-induced apoptosis through p53 overexpression and p21 suppression.[24] Furthermore, adenovirus-mediated E2F1 overexpression also enhanced Ad-p53-induced apoptosis through MDM2 downregulation.[26] Since adenoviral E1A is known to activate E2F1 expression,[27] we hypothesised that OBP-702-mediated E1A expression may enhance Ad-p53-induced apoptosis through suppression of p21 and MDM2 expression. To address this hypothesis, H358 cells were coinfected with E1A-deficient dl312 or E1A-expressing wild-type Ad5 after Ad-p53 infection. Ad-p53-induced p53 overexpression was enhanced in the Ad5-coinfected H358 cells, but not in the dl312-coinfected H358 cells (Fig. 4B). Consistent with p53 overexpression, BAX expression was also upregulated. However, despite the enhanced p53 expression, the expression of p21 and MDM2 proteins was lower in Ad5-coinfected cells than in dl312-coinfected cells. Furthermore, PARP cleavage was only detected in H358 cells 72 h after coinfection of Ad-p53 with Ad5. As expected, OBP-301 infection had no apparent effect of the expression of p53, and p53-downstream target proteins (Supplementary Fig. 2). These results suggest that adenoviral E1A suppresses the expression of p21 and MDM2 thereby enhancing apoptosis through p53-dependent BAX upregulation (Fig. 4C, Supplementary Fig. 3).

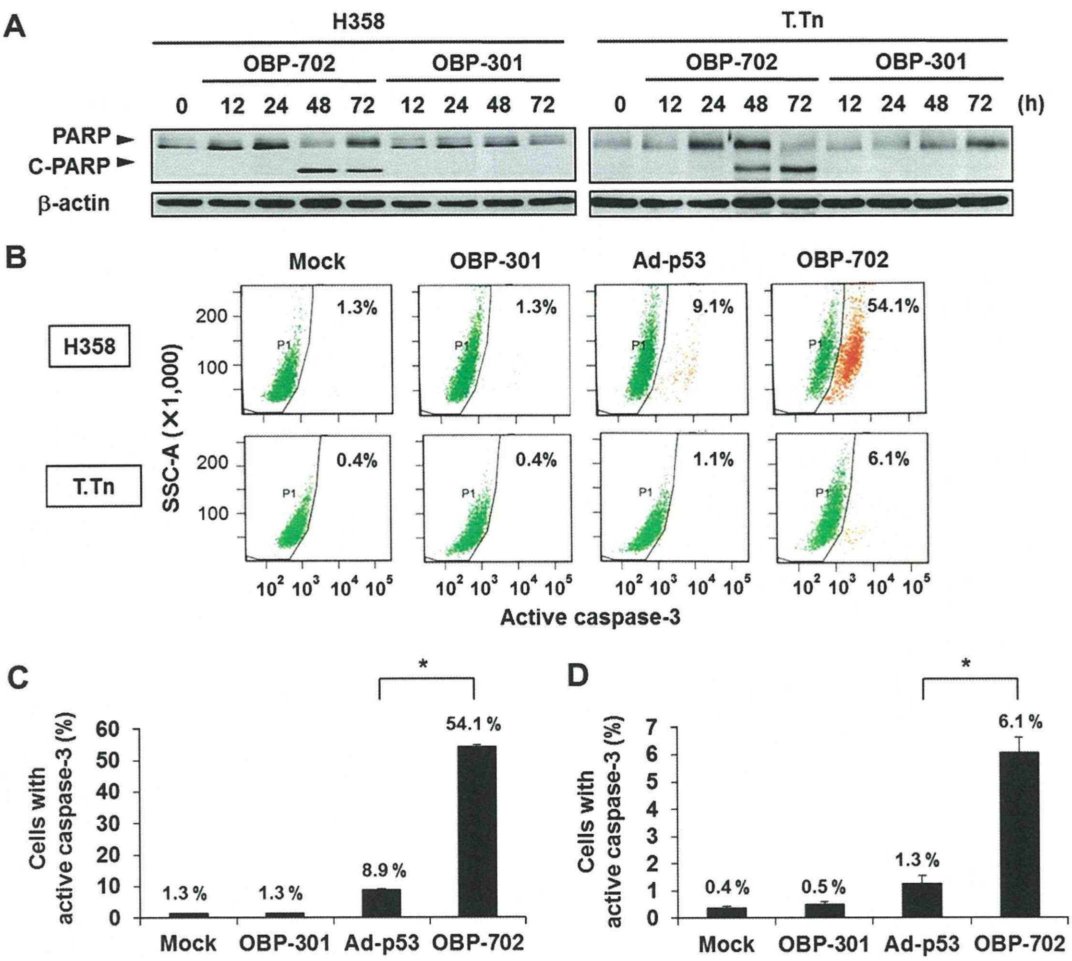

Fig. 3. OBP-702 induces increased apoptosis compared to OBP-301 or Ad-p53. (A) OBP-301-sensitive H358 cells and OBP-301-resistant T.Tn cells were infected with OBP-301 or OBP-702 at an MOI of 10 and 100, respectively, for 48 h. The level of cleaved PARP (C-PARP) and intact PARP in cell lysates was analysed using Western blotting. β-Actin was assayed as a loading control. (B–D), H358 and T.Tn cells were infected with OBP-702, OBP-301 or Ad-p53 at an MOI of 10 and 100, respectively, for 48 h. Mock-infected cells were used as controls. Caspase-3 activation was quantified using flow cytometric analysis. Representative flow cytometric data are shown (B). The mean percentage of H358 cells (C) and T.Tn cells (D) that express active caspase-3 was calculated based on three-independent experiments. Bars, SD. Statistical significance was determined using Student's t test. $^{*}P < 0.05$.

3.5. *Enhanced antitumour effect of OBP-702 in tumour xenograft animal models*

Finally, to assess the *in vivo* antitumour effect of OBP-702, we used subcutaneous H358 and T.Tn tumour xenograft models. OBP-702, OBP-301, Ad-p53 or PBS was intratumourally injected for three cycles every 2 days. OBP-702 administration significantly suppressed tumour growth compared to OBP-301, Ad-p53 or PBS in H358 and T.Tn tumour xenograft models (Fig. 5A). Furthermore, H358 tumour-bearing mice treated with OBP-702 significantly survived longer than those treated with OBP-301 or Ad-p53 (Fig. 5B). Although there was no significant difference in the survival rates between OBP-702-treated and OBP-301-treated mice with T.Tn tumours, OBP-702 treatment significantly increased the survival rate of T.Tn tumour-bearing mice compared to Ad-p53. These results suggest that OBP-702 eliminates tumour tissues more efficiently than OBP-301 or Ad-p53.

4. Discussion

Genetically engineered transgene-expressing armed oncolytic adenoviruses are expected to be a third-generation oncolytic virus for induction of a strong antitumour effect through induction of oncolytic and transgene-induced cell death.[6,13] Although the tumour suppressor *p53* gene is a potent therapeutic transgene for enhancement of an oncolytic adenovirus-mediated antitumour effect,[21–23] the molecular mechanisms by which p53 mediates enhancement of the antitumour effect remain unclear. In this study, we showed that the p53-expressing telomerase-specific oncolytic adenovirus OBP-702 exerted stronger *in vitro* and *in vivo* antitumour effects than OBP-301 or Ad-p53 (Figs. 2 and 5). This enhanced antitumour effect was due to p53-induced apoptosis, and adenoviral E1A enhanced this apoptosis via suppression of the expression of anti-apoptotic p21 and p53-inhibitory MDM2 (Figs. 3 and 4). Although

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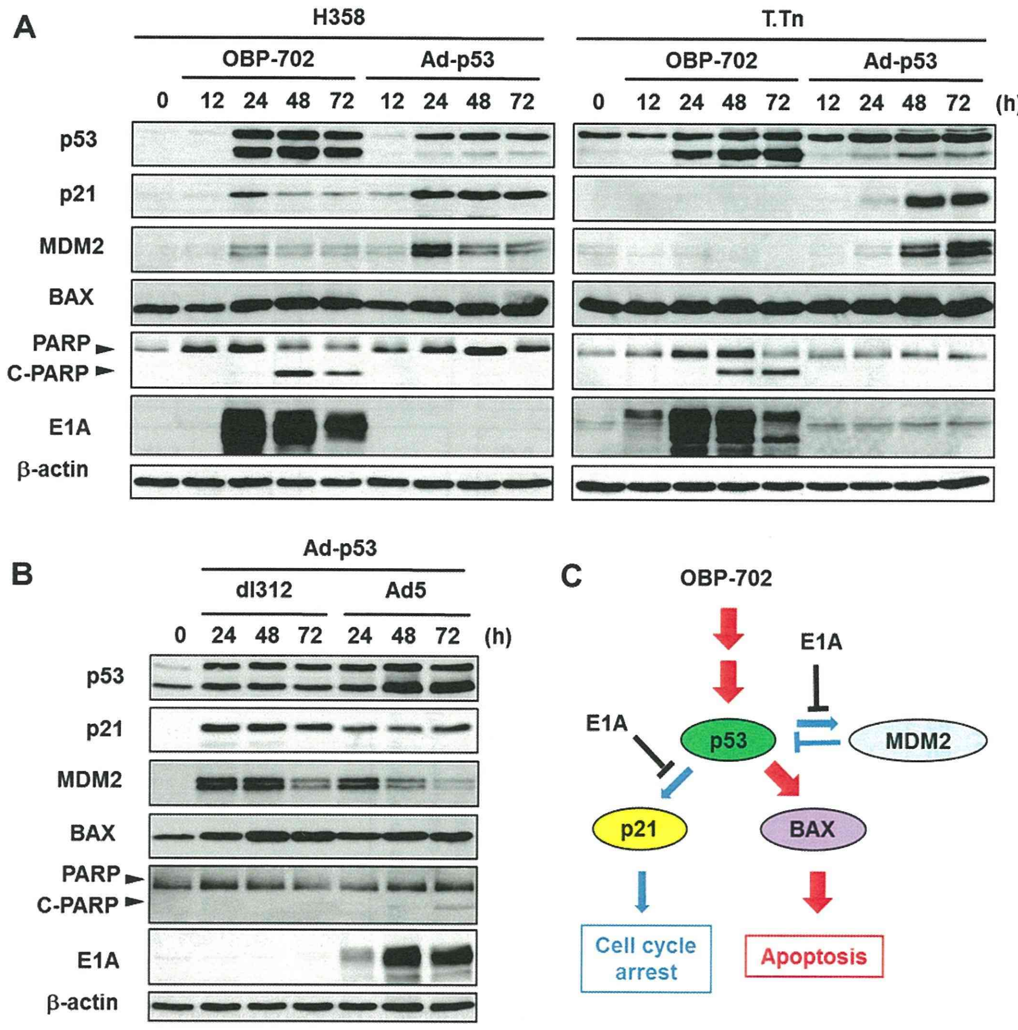

Fig. 4. OBP-702-mediated activation of p53, p53-target proteins and PARP in an E1A-dependent manner. (A) H358 and T.Tn cells were infected with OBP-702 or Ad-p53 at an MOI of 10 and 100, respectively, and infected cells were harvested at the indicated time points. The level of p53, p21, MDM2, BAX, PARP, cleaved PARP (C-PARP) and E1A proteins in cell lysates was analysed by Western blotting. β-Actin was assayed as a loading control. (B) H358 cells were infected with Ad-p53, following which they were coinfected with the E1A-deficient adenovirus (dl312) or an E1A-expressing wild-type adenovirus (Ad5) at the indicated time points. (C) Outline of OBP-702-mediated apoptosis induction through p53-dependent BAX upregulation and E1A-dependent downregulation of p21 and MDM2.

replication-competent adenovirus-mediated *p53* gene transduction has been suggested to exert an increased antitumour effect compared to replication-deficient Ad-p53 through replication-mediated p53 overexpression,[22] adenoviral E1A also enhanced p53-mediated apoptosis through suppression of expression of the p53-downstream targets p21 and MDM2 (Fig. 4). The adenoviral E1A protein has been previously shown to suppress p53-induced p21 and MDM2 expression.[28,29] E1A-mediated p21 and MDM2 suppression has also been shown to induce apoptosis in DNA-damaged cells that overexpress p53.[30,31] These reports support our findings that adenoviral E1A protein enhances p53-induced apoptosis through p21 and MDM2 suppression. It has recently been further shown that replication-deficient Ad-p53 enhances apoptosis through p21 suppression in combination with artificial microRNAs[32] or with OBP-301.[24] Thus, replication-competent oncolytic adenovirus-mediated *p53* gene transfer would strongly induce apoptosis not only through replication-dependent p53 overexpression, but also through E1A-dependent enhancement of p53-mediated apoptosis.

The molecular mechanism by which E1A suppresses p53-mediated upregulation of p21 and MDM2 remains unclear. Since adenoviral E1A has been shown to repress the expression of many target genes through activation of p300/CBP [cyclic adenosine monophosphate response element-binding protein (CREB)-binding protein] histone acetyltransferases that cause global histone modification,[33,34] p300/CBP activation may be involved in E1A-mediated p21 and MDM2 suppression. Indeed, E1A-mediated p21 and MDM2 suppression has been shown to be regulated in a p300/CBP dependent manner.[29,31] A recent report also suggested that an E1B-defective adenovirus activates p53 expression, but

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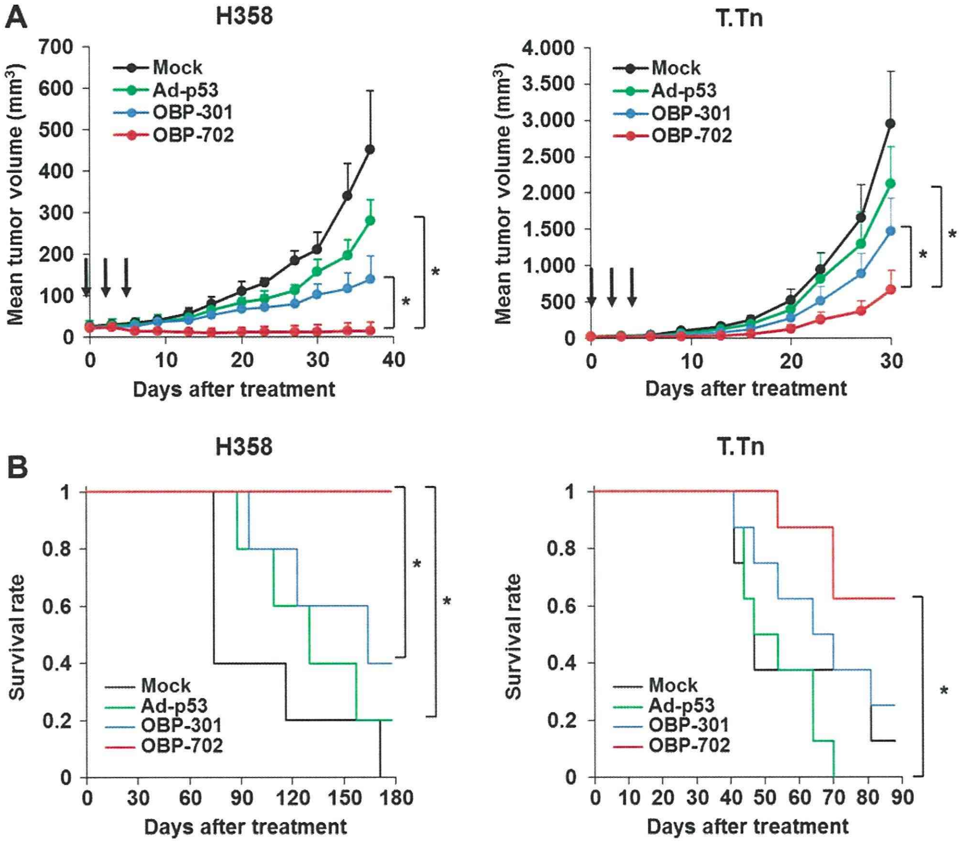

Fig. 5. Strong antitumour effect of OBP-702 on subcutaneous human tumours in xenograft models. (A) H358 or T.Tn cells (5×10^6 cells per site) were inoculated into the flank of 5-week-old female BALB/c *nu/nu* mice. When the tumours reached 3–5 mm in diameter, OBP-702 (10^8 PFU/tumour), OBP-301 (10^8 PFU/tumour), Ad-p53 (10^8 PFU/tumour) or PBS (Mock) was intratumourally injected on days 0, 2 and 4 (Black arrows). Tumour growth is expressed as the mean tumour volume ± SD in each group of H358 tumours ($n = 5$) or T.Tn tumours ($n = 8$). Statistical significance was determined using Student's *t* test. $^*P < 0.05$. The data are representative of three separate experiments. (B) Survival rate in each group of H358 tumours-bearing mice ($n = 5$) or T.Tn tumours-bearing mice ($n = 8$) was shown using the Kaplan–Meier method. Statistical significance was determined using log-rank test. $^*P < 0.05$.

suppresses p21 and MDM2 expression, through the binding of E1A with p300/CBP.[35] However, p300 disruption has also been shown to both increase p53 stability through MDM2 suppression, and to suppress p21 expression, resulting in apoptosis in UV-irradiated human cancer cells.[36] Therefore, the role of p300/CBP in adenoviral E1A-mediated p21 and MDM2 suppression may be cell type-specific.

It has recently been shown that siRNA-mediated p21 suppression enhances the antitumour effect of an oncolytic adenovirus,[37,38] suggesting that p21 suppression further induces oncolytic cell death. Oncolytic adenovirus-mediated cell death has been shown to be associated with autophagy-related cell death, which is distinct from apoptosis.[39,40] Autophagy has been shown to be positively regulated by p53,[14] but negatively regulated by p21.[41] These results suggest that p53 upregulation without p21 activation enhances autophagic cell death. Thus, oncolytic adenovirus-mediated p21 suppression may enhance not only p53-mediated apoptosis, but also autophagic cell death during the OBP-702-mediated antitumour effect.

Telomerase-specific replication-competent OBP-301 that possesses the *hTERT* gene promoter replicates, and induces an antitumour effect in, human cancer cells in a telomerase-dependent manner.[9–11] Previous reports have shown that Ad-p53-mediated p53 overexpression suppresses *hTERT* mRNA expression,[42,43] suggesting possible suppression of OBP-301 and OBP-702 replication by p53 overexpression. However, we previously reported that Ad-p53-mediated p53 overexpression did not suppress OBP-301 replication during combination therapy.[24] Shats et al. previously reported that knockdown of p21 eliminated the p53-dependent repression of *hTERT* mRNA expression.[44] Since OBP-702, or combination therapy of OBP-301 with Ad-p53, induces p53 overexpression together with E1A-mediated p21 downregulation, p53 overexpression may not suppress hTERT expression. Furthermore, we recently demonstrated that OBP-301 infection itself induces a 1.1- to 50-fold increase in *hTERT* mRNA expression in an E1A-dependent manner.[45] Thus, OBP-702-mediated p53 overexpression would induce apoptosis without affecting hTERT expression.

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An anti-tumour effect of Ad-p53-mediated gene therapy has been shown in various clinical studies.[17–20] We previously reported that Ad-p53 induces sensitivity to chemotherapeutic drugs, resulting in enhancement of the antitumour effect.[46,47] Since OBP-702-mediated p53 gene transfer has a stronger antitumour effect than Ad-p53 (Fig. 5), combination therapy of OBP-702 with chemotherapeutic agents may be a more effective antitumour therapy than monotherapy of OBP-702. The adenoviral E1A protein has been shown to enhance chemotherapy-induced apoptosis.[48,49] In particular, p21 suppression has been suggested to be involved in E1A-mediated chemosensitisation.[30] Indeed, artificial miRNA-mediated p21 suppression on Ad-p53-induced p53 overexpression enhanced tumour sensitivity to chemotherapeutic agents.[32] Thus, combination therapy of a p53-armed oncolytic adenovirus with chemotherapy may be a more efficient antitumour strategy for eradication of tumour cells through p53 and E1A-mediated chemosensitisation than monotherapy.

In conclusion, we have clearly demonstrated that the p53-expressing oncolytic adenovirus OBP-702 has a much stronger antitumour effect than OBP-301 or Ad-p53 through p53-mediated apoptosis that is enhanced by E1A-dependent p21 and MDM2 suppression. Oncolytic adenovirus-mediated *p53* gene transduction should therefore be a promising antitumour therapy for efficient elimination of tumour cells.

Conflict of interest statement

Yasuo Urata is an employee of Oncolys BioPharma Inc., the manufacturer of OBP-301 (Telomelysin). Other authors declare no potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.12.020.

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Genetically engineered oncolytic adenovirus induces autophagic cell death through an E2F1-*microRNA-7*-epidermal growth factor receptor axis

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Autophagy is known to have a cytoprotective role under various cellular stresses; however, it also results in robust cell death as an important safeguard mechanism that protects the organism against invading pathogens and unwanted cancer cells. Autophagy is regulated by cell signalling including microRNA (miRNA), a post-transcriptional regulator of gene expression. Here, we show that genetically engineered telomerase-specific oncolytic adenovirus induced *miR-7* expression, which is significantly associated with its cytopathic activity in human cancer cells. Virus-mediated *miR-7* upregulation depended on enhanced expression of the E2F1 protein. Ectopic expression of *miR-7* suppressed cell viability and induced autophagy by inhibiting epidermal growth factor receptor (EGFR) expression. Our results suggest that oncolytic adenovirus induces autophagic cell death through an E2F1-*miR-7*-EGFR pathway in human cancer cells, providing a novel insight into the molecular mechanism of an anticancer virotherapy.

Autophagy is well known to have a cytoprotective role and to contribute to the maintenance of cell survival under various cellular stresses, such as deprivation of nutrients,[1] hypoxia[2] and interruption of growth signaling.[3] The autophagic process has also been associated with the inhibition of tumor development. In fact, it has been reported that adenovirus infection induces autophagy-related cell death in infected cancer cells, leading to tumor suppression.[4–6] Furthermore, oncolytic adenoviruses induce autophagic cell death in human malignant glioma cells[7,8] and in brain tumor stem cells.[9] However, the molecular mechanism underlying virus infection-mediated autophagic cell death remains unclear.

MicroRNA (miRNA) is a small noncoding RNA consisting of 22 nucleotides, which post-transcriptionally suppresses the expression of many target genes by pairing with complementary nucleotide sequences in the 3′-untranslated regions of the target mRNA. A number of reports have indicated that miRNA can regulate diverse cell fates including cell proliferation,[10] the epithelial-mesenchymal transition,[11] apoptosis[12] and senescence[13] in cancer cells. Recently, Zhu *et al.* demonstrated inhibition of autophagy by *miR-30a*, which suppresses the autophagy-related *beclin 1* gene in human cancer cells,[14] suggesting the possible regulation of autophagy in cancer cells by miRNA. In addition, the Epstein-Barr virus[15] and the human cytomegalovirus[16] have been reported to modulate cellular miRNA expression in normal infected cells. The adenoviral E1A protein also downregulated *miR-520h* expression, resulting in an antitumor effect.[17]

These observations led us to examine whether genetically engineered telomerase-specific oncolytic adenovirus modulate cellular miRNA expression in human cancer cells. We previously developed an oncolytic adenovirus, OBP-301, which drives the expression of viral *E1A* and *E1B* genes linked with an internal ribosome entry site under the control of the

Key words: adenovirus, telomerase, microRNA, autophagy, EGFR
Abbreviations:: Ad5: wild-type adenovirus serotype 5; AVO: acidic vesicular organelle; CAR: coxsackie and adenovirus receptor; EGFR: epidermal growth factor receptor; GFP: green fluorescent protein; hTERT: human telomerase reverse transcriptase; LC3: microtubule-associated protein 1 light chain 3; 3-MA: 3-methyladenine; miRNA: microRNA; MOI: multiplicity of infection; PFU: plaque-forming units; RT-PCR: reverse transcription-polymerase chain reaction
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human telomerase reverse transcriptase (*hTERT*) promoter for virus replication and, therefore, induces oncolytic cell death in human cancer cells with high telomerase activity, but not in human normal cells without telomerase activity.[18] OBP-301 has an antitumor effect against a variety of human cancer cells in both *in vitro* and *in vivo* settings.[18,19] In this study, we investigated whether OBP-301- and wild-type adenovirus-mediated cytopathic activities are associated with autophagy induction in human cancer and normal cells. To address the molecular mechanism on the oncolytic adenovirus-induced autophagy, we assessed the global miRNA modulation in the infected cells and identified the miRNA-based autophagy induction system during adenovirus infection.

Material and Methods

Cell lines

The human nonsmall cell lung cancer cell lines H1299 and A549 were obtained from the American Type Culture Collection (Manassas, VA). The human esophageal cancer cell line T.Tn was purchased from Japanese Collection Research Bioresources (Osaka, Japan). The human normal lung fibroblast cell line NHLF was obtained from TaKaRa Biomedicals (Kyoto, Japan). The H1299 and T.Tn cells were maintained in RPMI 1640 medium, and A549 cells were maintained in Dulbecco's modified Eagle's medium containing a Nutrient Mixture (Ham's F-12). All media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. NHLF cells were cultured in the medium recommended by the manufacturer. The cells were routinely maintained at 37°C in a humidified atmosphere with 5% CO_2.

Recombinant adenovirus

Construction and characterization of the recombinant tumor-specific replication-selective adenovirus vector OBP-301 (Telomelysin) was previously reported.[18,19] Ad5 was the basal adenovirus for OBP-301 and was also used as another type of replication-competent adenovirus. Replication-deficient adenoviral vectors expressing E2F1 (Ad-E2F1) were used to induce E2F1 expression in infected cells, as previously reported.[20] OBP-301, Ad5 and Ad-E2F1 were purified using CsCl step gradient ultracentrifugation followed by CsCl linear gradient ultracentrifugation.

Infection of cells with OBP-301 or Ad5 and cell viability assay

Cells were seeded on 96-well plates at a density of 1×10^3 cells/well 12 hr before infection and were infected with OBP-301 or Ad5 at MOIs of 0, 1, 5, 10, 50 and 100 plaque-forming units/cell. Cell viability was determined on day 3 after infection using the Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol.

Transfection of cells with siRNA or miRNA and cell viability assay

Cells seeded at a density of 5×10^2 cells/well in 96-well plates were transfected with either p62 siRNA (Applied Biosystems, Foster City, CA) or with control siRNA (Applied Biosystems) at a concentration of 0, 1, 5 or 10 nM using HiPerfect transfection reagents (Qiagen, Valencia, CA). *miR-7* (Ambion, Austin, TX) or control miRNA (Ambion) was also transfected at the same concentrations. In contrast, EGFR siRNA (Applied Biosystems) or control siRNA (Applied Biosystems) was treated at a concentration of 0, 10 and 50 nM. Cells were pretreated with 3-methyladenine (3-MA) (200 nM) (Sigma–Aldrich, St. Louis, MO) for 2 hr before transfection to inhibit *miR-7*-mediated autophagy. Cell viability was determined on day 6 after transfection using the Cell Proliferation Kit II (Roche Molecular Biochemicals).

Western blot analysis

Cells were seeded in a 100-mm dish at a density of 1×10^5 cells/dish 12 hr before transfection and were transfected with either *miR-7* (Ambion) or with control miRNA (Ambion) at a concentration of 10 nM, or were infected with OBP-301 at the indicated MOIs. On day 3 after treatment, whole cell lysates were prepared in a lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl and 1% Triton X-100) containing a protease inhibitor cocktail (Complete Mini; Roche). Proteins were electrophoresed on 6–15% SDS polyacrylamide gels and were transferred to polyvinylidene difluoride membranes (Hybond-P; GE Health Care, Buckinghamshire, UK). Blots were blocked with 5% nonfat dry milk in TBS-T (Tris-buffered saline and 0.1% Tween-20, pH 7.4) at room temperature for 30 min. The primary antibodies used were: rabbit antimicrotubule-associated protein 1 light chain 3 (LC3) polyclonal antibody (pAb) (Medical & Biological Laboratories (MBL), Nagoya, Japan), rabbit anti-Atg5 pAb (Cosmo Bio, Tokyo, Japan), mouse anti-p62 monoclonal antibody (mAb) (MBL), mouse anti-Ad5 E1A mAb (BD PharMingen, Franklin Lakes, NJ), rabbit anti-E2F1 pAb (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-wild-type EGFR pAb (R&D Systems Inc., Minneapolis, MN) and mouse anti-β-actin mAb (Sigma-Aldrich). The secondary antibodies used were: horseradish peroxidase-conjugated antibodies against rabbit IgG (GE Healthcare), mouse IgG (GE Healthcare) or goat IgG (Chemicon International Inc., Temecula, CA). Immunoreactive bands on the blots were visualized using enhanced chemiluminescence substrates (ECL Plus; GE Healthcare).

Quantitative real-time reverse transcription-PCR analysis

Cells were seeded on six-well plates at a density of 3×10^4 cells/well 12 hr before infection and were infected with either OBP-301 or with Ad5 at the indicated MOIs. Total RNA was extracted from cells using a miRNeasy Mini kit (Qiagen).

Total RNA was extracted in dose-dependent experiments from cells infected at the indicated MOIs on day 3 after infection, and in time-course experiments from cells on days 0, 1, 2, 3 and 4 after infection. 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™ real-time PCR system. The expression of *miR-7* was 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.

miRNA microarray

The cells were seeded in 75T flasks at a density of 2.0×10^5 cells/flask 12 hr before infection and were infected with either OBP-301 or Ad5 using an MOI of 5. Total RNA, including miRNA, was extracted from the OBP-301-infected, Ad5-infected and mock-infected cells on day 3 after infection using a miRNeasy Mini kit (Qiagen) according to the manufacturer's protocol, and RNA concentrations were quantified using a NanoDrop spectrophotometer. The RNA samples were then used for microarray analysis, which was performed by Exiqon (Vedbaek, Denmark) (http://www.exiqon.com/). For this analysis, each RNA sample and a mixture of all samples were labeled with Hy3 or Hy5, respectively, and were hybridized with three dual-color miRNA microarray chips (miRCURY™ LNA Array version 10.0; Exiqon) in which 719 kinds of human miRNA probes were contained. Fifteen miRNAs showed more than a 50% difference in expression between the OBP-301- or Ad5-infected cells and the mock-infected cells (Supporting Information Fig. 4a). The expression levels of *miR-33a*, *miR-183*, *miR-483-3p* and *miR-7* were evaluated using real-time RT-PCR, as described above.

Infection of cells with E2F1-expressing adenoviral vectors and treatment with E2F1 siRNA

H1299 and A549 cells, seeded at a density of 3×10^4 cells/well in six-well plates, were infected with Ad-E2F1 at an MOI of 100 for 2 days. The same cell lines, seeded at the same density in six-well plates, were transfected with E2F1 siRNA (Applied Biosystems) or control siRNA (Applied Biosystems) at a concentration of 10 nM and, 24 hr later, were infected with 5 or 50 MOI (H1299 and A549 cells, respectively) of OBP-301 for 3 days. Total RNA and whole cell lysates were prepared from the infected cells, and the expression levels of *miR-7* and E2F1 were analyzed using real-time RT-PCR and western blotting, respectively.

Determination of autophagic cells using H1299-GFP-LC3 cells

H1299 cells stably transfected with GFP and LC3 fusion plasmid (GFP-LC3) were previously established.[9] After transfection with 50 nM *miR-7* (Ambion) or control miRNA (Ambion), GFP expression in the transfected cells was examined using a laser confocal microscope (Fluoview 300; Olympus, Tokyo, Japan). As a positive control, H1299-GFP-LC3 cells were serum-starved by culture in Hank's balanced salt solution for 4 hr before laser confocal microscopy (Olympus).

Flow cytometry

A549 cells, seeded at a density of 1×10^5 cells/dish in 100-mm dishes, were transfected with either 10 nM *miR-7* (Ambion) or control miRNA (Ambion) for 3 days. Following staining with Acridine Orange solution (1.0 μg/ml; Sigma–Aldrich) for 15 min, the cells were trypsinized and were analyzed using a flow cytometer (FACSArray; Becton Dickinson, San Jose, CA).

Statistical analysis

Determination of significant differences was assessed using Student's *t*-test. Correlations between the expression levels of *miR-7*, the cytopathic activity of OBP-301 and the expression level of EGFR were analyzed using Pearson's correlation coefficient. $p < 0.05$ was considered significant.

Results

The cytopathic effect of the oncolytic adenovirus OBP-301 is associated with induction of autophagy in human cancer cells

To investigate if the cytopathic effect of OBP-301 correlates with autophagy in human cancer cells, we used three human cancer cell lines (H1299, A549 and T.Tn), that showed different sensitivities to OBP-301.[19,21] The cytopathic effect of OBP-301 against each cell line was determined by assay of cell viability using the XTT assay (Fig. 1*a*). The H1299 and A549 cells showed high and moderate sensitivities, respectively, to OBP-301, but T.Tn cells were resistant. T.Tn cells showed lower expression level of coxsackie and adenovirus receptor (CAR) protein, but similar *hTERT* mRNA expression compared to H1299 and A549 cells (Supporting Information Figs. 1a and 2a). Consistent with CAR expression, T.Tn cells were less sensitive to adenovirus-mediated green fluorescent protein (GFP) induction compared to H1299 and A549 cells (Supporting Information Fig. 1b). In spite of high hTERT expression, the replication rate of OBP-301 was suppressed in T.Tn cells compared to H1299 and A549 cells (Supporting Information Fig. 2b). These results suggest that its resistance was due to impairment of virus infection and replication. Furthermore, as OBP-301 shows the tumor-specific cytopathic effect in a telomerase-dependent manner, the cell viability of human normal fibroblasts (NHLF), which show low CAR expression and no *hTERT* mRNA expression (Supporting Information Figs. 1a and 2a), was also determined after infection with OBP-301. As reported previously,[18] NHLF cells showed the resistance to OBP-301-mediated cytopathic effect (Fig. 1*a*). The cytopathic activity and replication rate of wild-type adenovirus serotype 5 (Ad5)

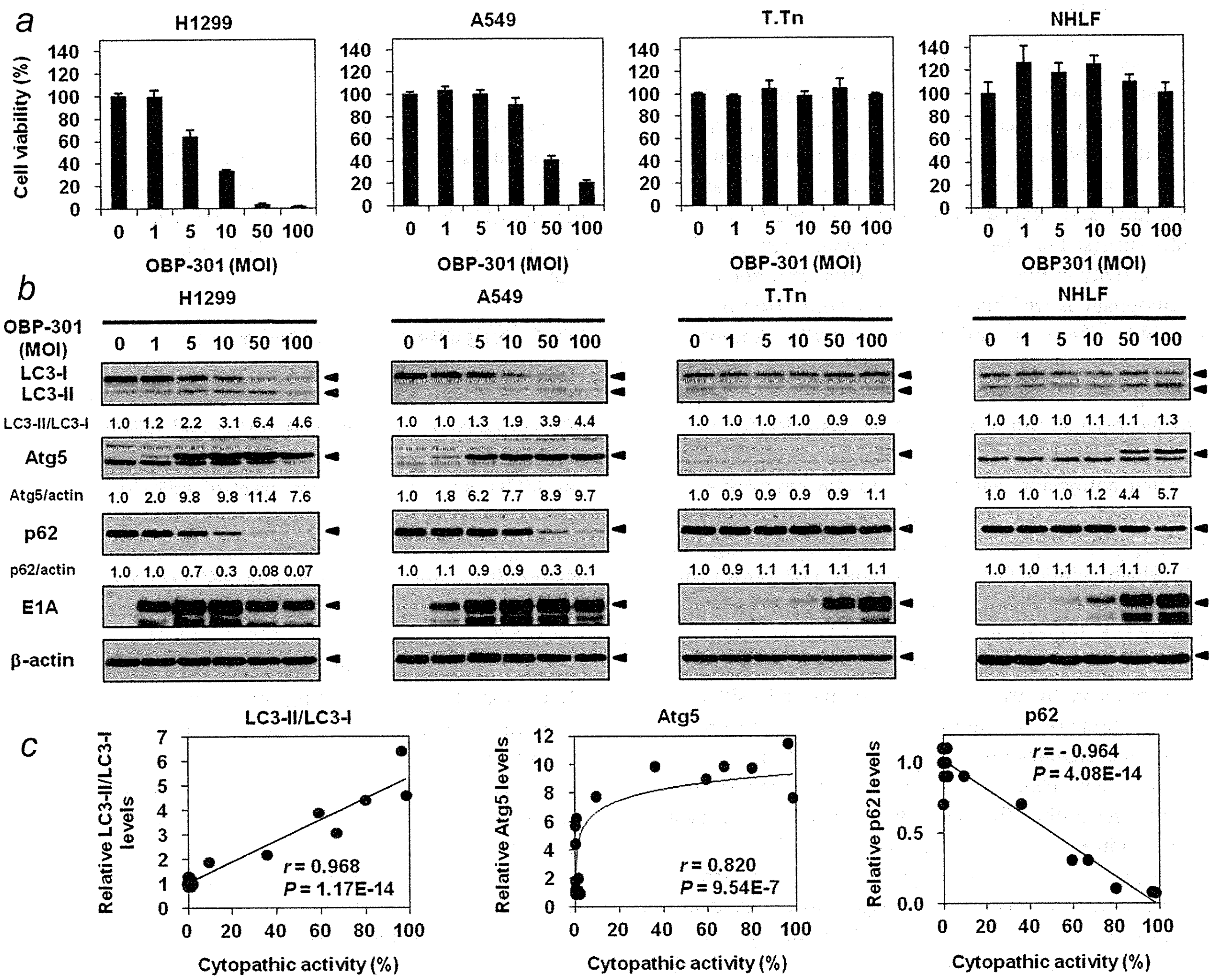

Figure 1. OBP-301-mediated induction of autophagic cell death in human cancer cells. (*a*) The cytopathic effect of OBP-301 in human cancer cells (H1299, A549 and T.Tn) and normal fibroblasts (NHLF). Cell viability was determined 72 hr after infection with OBP-301 at the indicated MOIs, using an XTT assay. Cell viability was calculated relative to that of mock infected cells, whose viability was set at 100%. (*b*) Expression of the autophagy marker proteins microtubule-associated protein 1 light chain 3 (LC3), Atg5, p62 and the viral E1A protein in H1299, A549, T.Tn and NHLF cells infected with OBP-301 at the indicated MOIs for 72 hr was assessed using Western blotting. β-actin was assayed as a loading control. The expression level of each protein was calculated relative to its expression in mock-infected cells, whose expression level was designated as 1. (*c*) There was a significant correlation between the expression levels of autophagy marker proteins (LC3-II, Atg5 and p62) and the cytopathic activity of OBP-301.

were also confirmed in all cell lines (Supporting Information Figs. 2 and 3). The data for the sensitivities to OBP-301 and Ad5 and expression levels of CAR and hTERT were summarized in Supporting Information Table 1.

Oncolytic adenovirus-mediated autophagy can be characterized by conversion of the microtubule-associated protein 1 light chain 3 (LC3)-I to the LC3-II form,[7,22,23] by upregulation of the autophagy-related protein Atg5[9,23] and by downregulation of the p62 protein.[23] Therefore, to analyze OBP-301 induction of autophagy, we determined the expression levels of LC3-I/II, Atg5 and p62 proteins in OBP-301-infected cells by Western blot analysis (Fig. 1*b*). OBP-301-sensitive H1299 and A549 cells exhibited conversion of LC3-I to LC3-II, Atg5 upregulation and p62 downregulation after OBP-301 infection using more than five multiplicity of infections (MOIs). However, the OBP-301-resistant T.Tn cells and NHLF cells showed no induction of autophagy. Adenoviral E1A expression was detected in all four cell lines after infection with OBP-301. To further evaluate the relationship between OBP-301-induced autophagy and cytopathic effect, OBP-301-sensitive cells were infected with OBP-301 at an MOI of 50, and the morphological and autophagic changes were analyzed at 0, 24, 48 and 72 hr after infection using conventional microscopy, Western blot analysis and electron microscopy (Supporting Information Figs. 3 and 4). Although no morphological and autophagic changes were observed at

24 hr, the detachment of round shaped cells and autophagic markers were induced at 48 hr and more increased at 72 hr after infection. At 72 hr after infection, autophagic vesicles were also confirmed in the OBP-301-infected H1299 and A549 cells under electron microscopy. Furthermore, we found the significant correlations between cytopathic effect of OBP-301 and autophagy-related markers, such as LC3-II/LC3-I ratio and expressions of Atg5 and p62 (Fig. 1*c*). These results suggest that there is a relationship between the cytopathic activity of OBP-301 and induction of autophagy.

Autophagy is well known to show both cytoprotective and cytopathic effects in human cancer cells. Autophagy has recently been shown to suppress tumorigenesis through p62 downregulation.[24] Furthermore, the accumulation of p62 proteins has been shown to be a critical factor for the survival of human cancer cells.[25] We, therefore, determined if OBP-301-mediated autophagy, which leads to downregulation of p62, is associated with cell survival or cell death. For this purpose, we analyzed the effect of siRNA-mediated downregulation of p62, which mimics OBP-301-mediated p62 downregulation, on the viability of noninfected H1299, A549 and T.Tn cells (Supporting Information Fig. 5). Using Western blotting analysis, we first confirmed complete suppression of p62 protein expression by transfection of 10 nM *p62* siRNA in all cell lines (Supporting Information Fig. 5a). Treatment with p62 siRNA significantly suppressed cell viability in all cell lines in a dose-dependent manner (Supporting Information Fig. 5b), suggesting that OBP-301-mediated downregulation of p62 induces cell death. In contrast to OBP-301-induced autophagic cell death, no apoptotic cell death, assessed by western blotting of caspase-3 cleavage, was observed in OBP-301-infected H1299 and A549 cells (Supporting Information Fig. 6). These results suggest that the cytopathic effect of OBP-301 is associated with autophagy-related cell death.

OBP-301 infection modulates miRNA expression in human cancer cells

To next investigate if OBP-301 induces autophagic cell death through modulation of miRNA expression in human cancer cells, OBP-301-sensitive H1299 cells were infected with OBP-301, and miRNA expression levels in the OBP-301-infected and mock-infected cells were analyzed using a miRNA microarray. Because wild-type Ad5 is the virus from which OBP-301 was generated, an Ad5-infected H1299 cell extract was also analyzed to clarify the candidate miRNAs modulated by infection with OBP-301 and/or Ad5. Fifteen miRNAs showed differences in expression that were higher than 50% in the OBP-301-treated and/or Ad5-treated cells compared to mock-treated cells (Supporting Information Fig. 7a). Of these 15 miRNAs, four miRNAs were downregulated and 11 miRNAs were upregulated. To further validate OBP-301-mediated modulation of miRNA expression, we further analyzed four miRNAs; two downregulated miRNAs (*miR-33a* and *miR-183*) and two upregulated miRNAs (*miR-483-3p* and *miR-7*), using same three RNA samples used for miRNA microarray by quantitative real-time RT-PCR (qRT-PCR) (Supporting Information Fig. 7b). Of these four miRNAs, the expression of *miR-7* was upregulated 2.94-fold and 1.91-fold in the OBP-301-treated and Ad5-treated cells, respectively, compared to mock-treated cells. This result for *miR-7* was consistent with the microarray data, whereas other three miRNAs showed different expression levels between microarray and qRT-PCR. Therefore, for further analysis, we focused on the role of *miR-7* in OBP-301-mediated oncolytic cell death.

miR-7 upregulation is associated with the cytopathic activity of OBP-301

To further confirm OBP-301-mediated *miR-7* upregulation, OBP-301-sensitive (H1299 and A549) and OBP-301-resistant (T.Tn and NHLF) cells were infected with OBP-301 at various MOIs, and the expression level of *miR-7* was examined using qRT-PCR. *miR-7* expression was dose-dependently upregulated in the OBP-301-infected H1299 and A549 cells, whereas T.Tn and NHLF cells showed no change in *miR-7* expression after OBP-301 infection (Fig. 2*a*). Time-dependent upregulation of *miR-7* expression was also observed in H1299 and A549 cells infected with OBP-301 at 5 and 50 MOIs, respectively (Supporting Information Fig. 8a). Furthermore, the level of *miR-7* upregulation after OBP-301 infection significantly correlated with the cytopathic activity of OBP-301 ($r = 0.954$, $p = 5.78E-13$) (Supporting Information Fig. 8b). Similar to OBP-301, Ad5 infection also dose-dependently upregulated *miR-7* expression, and this upregulation significantly correlated with the cytopathic activity of Ad5 ($r = 0.933$, $p = 8.94E-6$) (Supporting Information Fig. 9). These results suggest that *miR-7* upregulation is implicated in oncolytic adenovirus-mediated cell death.

E2F1 activation is involved in OBP-301-mediated miR-7 upregulation

Adenovirus infection has been shown to modulate many kinds of protein-coding genes through activation of the transcription factor, E2F1, induced by adenoviral E1A[26] and E4.[27,28] Furthermore, it has recently been shown that E2F1 regulates the expression of specific miRNAs in a transcription-dependent manner.[29] Therefore, we sought to assess the role of E2F1 in OBP-301-mediated *miR-7* upregulation. The Western blotting analysis revealed that OBP-301 infection at MOIs greater than five induced E2F1 protein expression in OBP-301-sensitive H1299 and A549 cells but not in OBP-301-resistant T.Tn cells (Fig. 2*b*). In contrast, NHLF cells showed slight increase in E2F1 expression after infection with high dose (more than 50 MOI) of OBP-301.The level of *miR-7* upregulation in these cells significantly correlated with the level of E2F1 expression ($r = 0.944$, $p = 4.48E-12$) (Supporting Information Fig. 8c). Furthermore, to investigate the E2F1-mediated *miR-7* upregulation, H1299 and A549 cells were infected with or without an E2F1-expressing replication-deficient adenoviral vector (Ad-E2F1) (100 MOI) for

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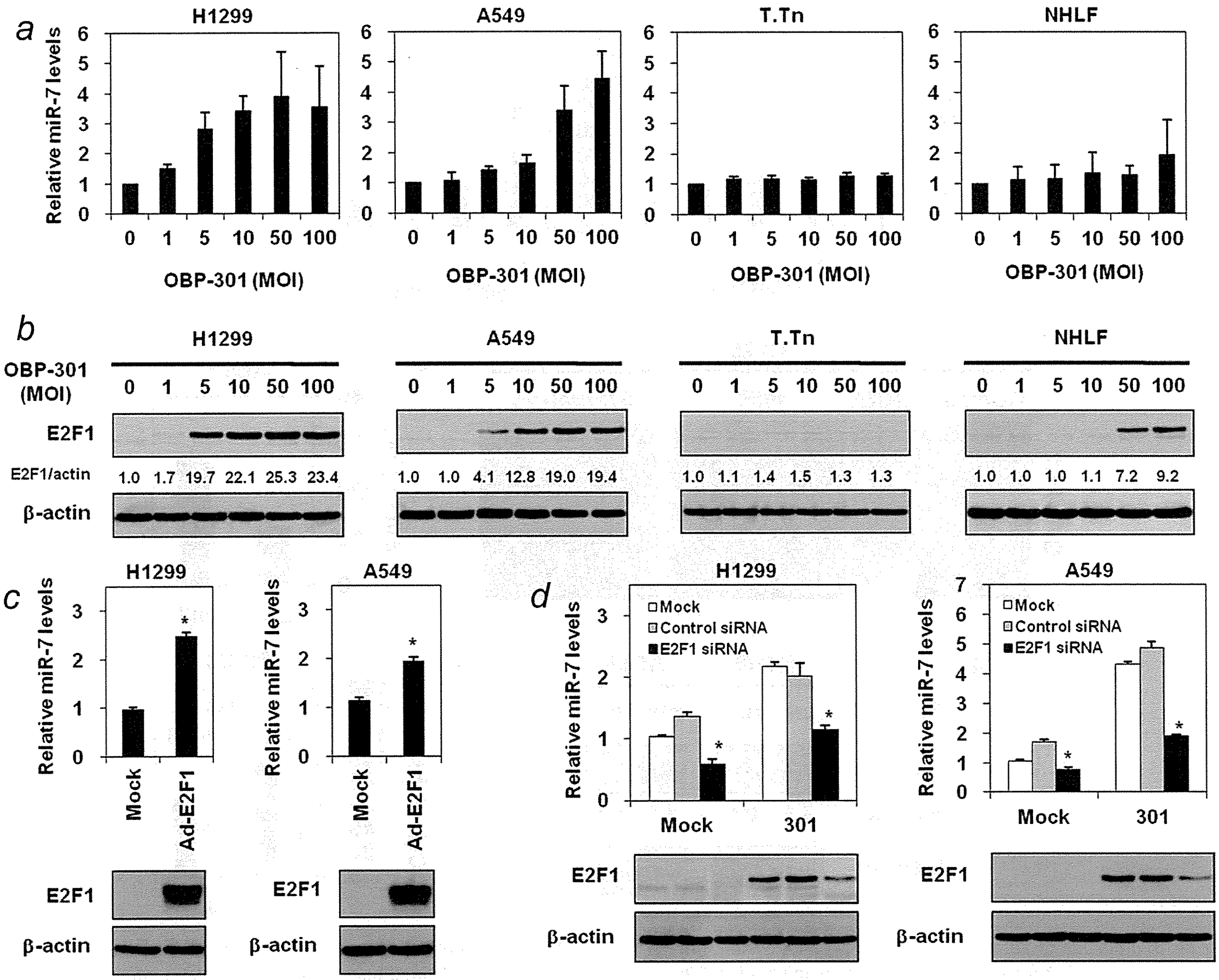

Figure 2. OBP-301-induced E2F1 expression upregulates *miR-7* expression in association with cytopathic activity in human cancer cells. (*a*) *miR-7* expression was assayed using qRT-PCR in H1299, A549, T.Tn and NHLF cells infected with OBP-301 at the indicated MOIs for 72 hr. The values of *miR-7* at 0 MOI and 0 hr were set at 1, and the relative levels of *miR-7* at the indicated MOIs and time points were plotted as fold induction. *miR-7* expression data are expressed as mean values ± SD (n = 3). (*b*) Western blot analysis of the expression of the E2F1 protein in H1299, A549, T.Tn and NHLF cells infected with OBP-301 at the indicated MOIs for 72 hr. E2F1 expression levels were calculated relative to actin expression in the same sample. E2F1/actin expression values at 0 MOI were set at 1.0. Relative levels of E2F1 expression were calculated as fold induction. (*c*) *miR-7* expression in H1299 and A549 cells infected with E2F1-expressing adenoviral vectors for 48 hr was assessed using qRT-PCR. The values of *miR-7* expression in the mock-infected cells were set at 1, and the relative levels of *miR-7* in the E2F1-overexpressing cells were plotted as fold induction. Expression of the E2F1 protein was confirmed by Western blot analysis using actin as a loading control. (*d*) *miR-7* and E2F1 expression in H1299 and A549 cells that were mock-pretreated or were pretreated with control siRNA or with E2F1 siRNA (10 nM) before mock-infection or to infection with OBP-301 at 5 and 50 MOIs, respectively. The values of *miR-7* expression in the mock-infected cells without pretreatment were set at 1, and relative levels of *miR-7* were plotted as fold induction. Expression of the E2F1 protein was confirmed by Western blot analysis. β-Actin was used as a loading control. For (*c*) and (*d*), *miR-7* expression data are expressed as mean values ± SD (n = 3). Statistical significance (*) was defined as $p < 0.05$.

48 hr. However, E1A-deleted control adenovirus was not used because other genes from E4 region might contribute to E2F1 activation.[27,28] Ectopic expression of E2F1 by infection with Ad-E2F1 significantly upregulated *miR-7* expression 2.48- and 1.96-fold in H1299 and A549 cells, respectively, compared to mock infection (Fig. 2*c*). Overexpression of the E2F1 protein by Ad-E2F1 infection was confirmed by Western blot analysis. Conversely, specific downregulation of E2F1 by pretransfection of E2F1 siRNA (10 nM) significantly suppressed the level of *miR-7* expression compared to mock or control siRNA treatment in both mock-infected and OBP-301-infected cells at 72 hr after OBP-301 infection following to siRNA treatment for 24 hr (Fig. 2*d*). Suppression of OBP-301-activation of E2F1 expression by pretreatment with E2F1

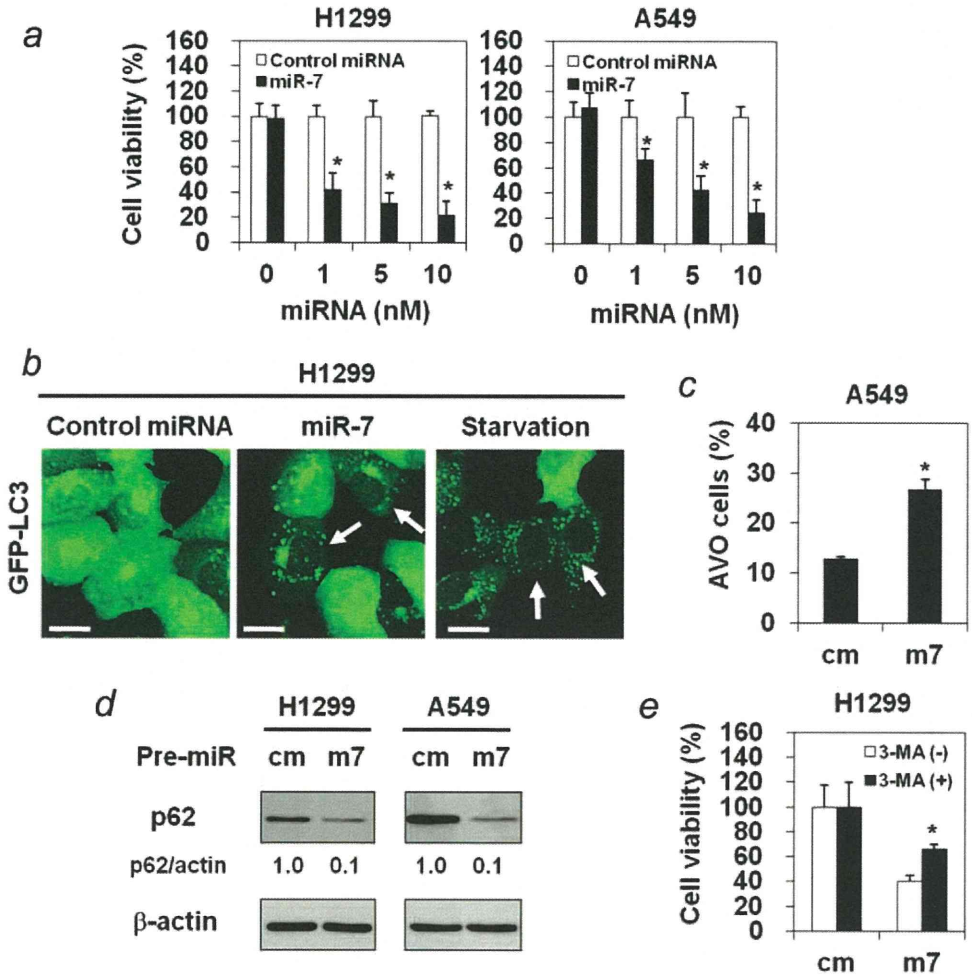

Figure 3. Induction of autophagy by *miR-7* overexpression. (*a*) The effect of transfection of the indicated concentrations of control miRNA (white bars) or *miR-7* (black bars) on the cell viability of H1299 and A549 cells was determined using XTT assay on day 6 after transfection. Cell viability of *miR-7*-transfected cells was calculated relative to that of control miRNA-transfected cells, which was set at 100%. (*b*) Immunofluorescence analysis of H1299 cells stably expressing the green fluorescent protein (GFP)-microtubule-associated protein 1 light chain 3 (GFP-LC3) fusion protein and transfected with 50 nM *miR-7* or control miRNA for 3 days. Serum-starved H1299-GFP-LC3 cells were used as a positive control. The white arrows indicate autophagic cells with punctate GFP expression in the cytoplasm. Scale bars: 50 μm. (*c*) Acidic vesicular organelles (AVO), present in A549 cells transfected with 5 nM *miR-7* (m7) or control miRNA (cm) for 3 days, were quantified by staining with acridine orange followed by flowcytometric analysis. The percentage of AVO cells, which are indicative of autophagy, are expressed as mean values ± SD ($n = 3$). (*d*) Expression of the p62 protein in H1299 and A549 cells transfected with 10 nM *miR-7* (m7) or control miRNA (cm) for 3 days was assayed by Western blotting. β-actin was assayed as a loading control. p62 expression levels were calculated relative to actin expression in the same sample. p62 expression levels were then calculated relative to the p62 expression levels of control miRNA-transfected cells, which were set at 1.0. (*e*) The viability of H1299 cells, transfected with 10 nM *miR-7* (m7) or with control miRNA (cm) following pretreatment with or without the autophagy inhibitor 3-methyladenine (3-MA), was assayed using the XTT assay. Cell viability data are expressed as mean values ± SD ($n = 5$). Statistical significance (*) was defined as $p < 0.05$.

siRNA was also confirmed by Western blot analysis. These results suggest that OBP-301-mediated *miR-7* upregulation depends mainly on activation of E2F1 expression.

miR-7 overexpression suppresses cell viability through induction of autophagy in human cancer cells

To determine if OBP-301-mediated *miR-7* overexpression is associated with cell death in human cancer cells, we introduced exogenous *miR-7*, or control miRNA, into OBP-301-sensitive H1299, A549 and T.Tn cells and investigated the effect of *miR-7* overexpression on cell viability (Fig. 3*a* and Supporting Information Fig. 10a). Ectopic expression of *miR-7* significantly suppressed cell viability in a dose-dependent manner compared to control miRNA in H1299 and A549 cells. However, T.Tn cells showed less sensitivity to *miR-7*-mediated suppression of cell viability compared to H1299 and A549 cells. In contrast, *miR-7*-overexpression did not induce apoptotic cell death (caspase-3 cleavage) in any of these cells (Supporting Information Fig. 11). These results indicate that *miR-7* overexpression suppresses cell viability through induction of nonapoptotic cell death in human cancer cells.

The observation of *miR-7*-mediated nonapoptotic cell death prompted us to investigate if *miR-7* overexpression induces autophagic cell death, because we had previously shown that autophagy may be involved in OBP-301-mediated oncolysis of H1299 cells[30] and observed the significant correlation between cytopathic activity of OBP-301 and autophagy induction (Fig. 1). To investigate *miR-7*-mediated induction of autophagy in cells, we used H1299 cells that were stably transfected with a GFP-LC3 fusion plasmid (GFP-LC3). *miR-7* overexpression in H1299-GFP-LC3 cells induced the appearance of autophagic cells with a punctate pattern of GFP-LC3 expression in the cytoplasm, similar to that observed in serum-starved cells (Fig. 3*b*). To quantify autophagy induced by *miR-7*, A549 cells that were transfected with *miR-7* or control miRNA were stained with acridine orange. The percentage of cells with stained acidic vesicular organelles (AVOs), which are indicative of autophagy, was then measured using flow cytometry. Transfection of A549 cells with *miR-7* significantly increased the percentage of AVO-positive cells compared to control miRNA (Fig. 3*c*). These results indicate that *miR-7* overexpression induces autophagy.

Because OBP-301 infection both upregulates *miR-7* and downregulates p62 in association with autophagy, we determined if *miR-7* overexpression might induce downregulation of p62 protein expression. *miR-7* transfection suppressed p62 expression compared to control miRNA in H1299 and A549 cells as shown by Western blotting (Fig. 3*d*). These results indicate that *miR-7* overexpression induces autophagy, resulting in p62 downregulation, in human cancer cells. To further examine if *miR-7* overexpression suppresses cell viability through induction of autophagy, the effect of the autophagy inhibitor, 3-MA, on *miR-7*-mediated suppression of cell viability was determined. Treatment of H1299 cells with 3-MA significantly attenuated *miR-7*-mediated suppression of cell viability (Fig. 3*e*), suggesting that *miR-7* does indeed mediate autophagic cell death.

EGFR downregulation by miR-7 overexpression is implicated in the OBP-301-mediated cytopathic effect

Recent evidence had shown that *miR-7* functions as a tumor suppressor by suppressing the expression of the epidermal growth factor receptor (EGFR),[31,32] which is strongly associated with tumor progression and poor prognosis in human cancers.[33] Furthermore, a recent report has shown that EGFR downregulation by siRNA induces autophagic cell death in human cancer cells.[34] We, therefore, next sought to determine if OBP-301 suppresses EGFR expression through *miR-7* upregulation. As shown in Figure 4*a*, OBP-301 infection suppressed EGFR expression in a dose-dependent manner in OBP-301-sensitive H1299 and A549 cells but not in OBP-301-resistant T.Tn and NHLF cells. The level of EGFR suppression was significantly associated with the level of *miR-7* upregulation ($r = -0.872$, $p = 2.64\text{E-}8$) (Fig. 4*b*) and with the cytopathic activity of OBP-301 ($r = -0.826$, $p = 6.73\text{E-}7$) (Fig. 4*c*), suggesting the involvement of *miR-7*-mediated EGFR suppression in the cytopathic effect of OBP-301. The ectopic expression of *miR-7* suppressed EGFR expression compared to control miRNA in H1299 and A549 cells (Fig. 4*d*). Furthermore, ectopic expression of E2F1 by infection with an Ad-E2F1 also downregulated EGFR expression compared to mock infection in H1299 and A549 cells (Fig. 4*e*). In contrast, treatment with EGFR siRNA significantly suppressed cell viability compared to control siRNA in H1299 and A549 cells (Fig. 4*f*). However, *miR-7*-resistant T.Tn cells showed about fivefold higher expression level of EGFR compared to H1299 and A549 cells (Supporting Information Fig. 10b). Even when T.Tn cells were transfected with *miR-7* at 10 nM, high expression levels of EGFR and p62 were maintained. The combined results suggest that OBP-301 infection induces *miR-7* expression through E2F1 activation and that E2F1-mediated *miR-7* upregulation suppresses EGFR expression, resulting in the induction of autophagy-related cell death (Fig. 4*g*).

Discussion

Tumor-specific replication-competent oncolytic virotherapy is emerging as a promising anticancer therapy for the induction of tumor-specific oncolytic cell death.[4] Although the possible involvement of autophagy in oncolytic adenovirus-mediated cell death has recently been suggested,[5–9] the molecular mechanism by which autophagic cell death is induced remains to be elucidated. In this study, we demonstrated that infection with the oncolytic adenovirus, OBP-301, upregulated *miR-7* expression and that this upregulation was associated with its cytopathic activity in human cancer cells. Furthermore, OBP-301-mediated E2F1 activation was involved in *miR-7* upregulation, which subsequently induced autophagy through suppression of EGFR expression in human cancer cells. Adenovirus infection is well known to induce the viral protein-mediated E2F1 activation and subsequent upregulation of many E2F1-target genes.[35] Recently, E2F1 has been shown to induce autophagy through upregulation of autophagy-related genes in a transcription-dependent manner.[36] In contrast, EGF is known to suppress autophagy through EGFR activation.[37] Furthermore, it has been shown that EGFR downregulation by EGFR siRNA causes autophagic cell death in human cancer cells.[34] Thus, oncolytic adenoviruses may activate E2F1 expression, resulting in the upregulation of autophagy-related genes and the downregulation of autophagy-suppressing genes via miRNA modulation. Subsequently, autophagy-related programmed cell death is induced.

OBP-301 induced higher levels of autophagy, replication rate and cytopathic activity than Ad5 in H1299 and A549 cells (Fig. 1 and Supporting Information Figs. 2 and 3).

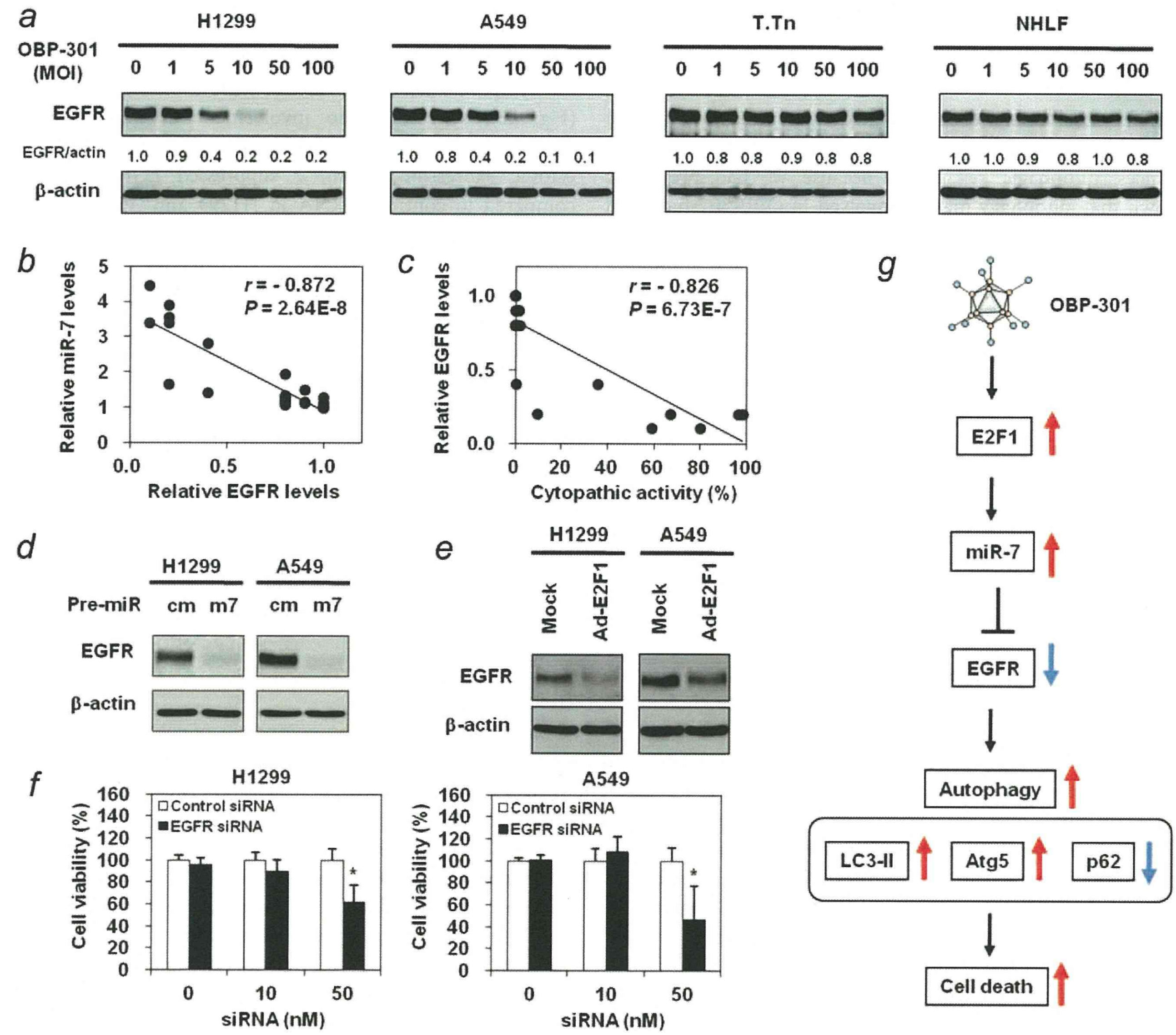

Figure 4. *miR-7*-mediated suppression of EGFR expression during OBP-301-mediated autophagic cell death. (*a*) Western blot analysis of EGFR protein expression in H1299, A549, T.Tn and NHLF cells infected with OBP-301 at the indicated MOIs for 72 h. EGFR expression levels were calculated relative to actin expression in the same sample. EGFR expression in OBP-301 infected cells was then calculated relative to that in mock-infected cells, whose value was set at 1. (*b*) There was a significant correlation between the expression level of EGFR and that of *miR-7*. (*c*) There was a significant correlation between the expression level of EGFR and the cytopathic activity of OBP-301. (*d*) Western blot analysis of the expression of the EGFR protein in H1299 and A549 cells transfected with 10 nM *miR-7* (m7) or with control miRNA (cm) for 72 hr. EGFR expression in the m7-transfected cell was calculated relative to that in the cm-transfected cells. β-actin was assayed as a loading control. (*e*) Western blot analysis of the expression of the EGFR protein in H1299 and A549 cells infected with E2F1-expressing adenoviral vectors for 48 hr. (*f*) Effect of EGFR siRNA transfection on the viability of H1299 and A549 cells. Cell viability was determined on day 6 after transfection using the XTT assay. Cell viability was calculated relative to that of mock-transfected cells, whose viability was set at 100%. Cell viability data are expressed as mean values ± SD ($n = 5$). Statistical significance (*) was defined as $p < 0.05$. (*g*) Outline of the E2F1-*miR-7*-EGFR axis during OBP-301-mediated autophagy.

Recent report has shown that adenovirus-mediated autophagy induction is associated with viral replication and oncolysis.[38] Autophagy inhibitor 3-MA has been suggested to inhibit the replication rate of Ad5 in A549 cells.[38] However, our collaborators have shown that pretreatment with 3-MA or Atg5 siRNA did not affect the replication and oncolysis of fiber-modified OBP-301 in human brain tumor cells.[22] Recently, we observed that OBP-301 infection upregulated *hTERT* mRNA expression and, subsequently, showed higher levels of replication rate and oncolysis than Ad5 in human sarcoma cells.[39] Therefore, the replication of OBP-301 may be less sensitive to autophagy inhibitor compared to Ad5 because of enhanced viral replication by *hTERT* promoter activation.

On the molecular mechanism of adenovirus-induced oncolysis, recent report has suggested that adenovirus-mediated autophagy induces caspase-8 activation in association with oncolysis in human leukemia cells and normal fibroblasts.[40] Recently, caspase-8 has been shown to be involved in not only apoptosis but also diverse cell fates including autophagy.[41] In this study, we observed that oncolytic adenovirus induces autophagic cell death, not apoptotic cell death, in human cancer cells (Fig. 1 and Supporting Information Fig. 6). These results suggest the functional role of caspase-8 in adenovirus-mediated autophagic cell death. Atg5-mediated autophagic cell death has recently been shown to be induced through interaction with Atg5 and Fas-associated protein with death domain (FADD),[42] which can also bind with caspase-8.[41] Thus, oncolytic adenovirus may contributes to autophagic cell death through activation of Atg5-FADD-caspase-8 network. Furthermore, although transfection with p62 siRNA suppressed p62 expression more strongly than OBP-301, the inhibitory effect of p62 siRNA was lower than OBP-301 in the cell viability of H1299, A549 and T.Tn cells (Fig. 1 and Supporting Information Fig. 5). These results suggest that OBP-301-mediated p62 downregulation not only suppresses oncogenic p62 function but also contributes to autophagy-related cell death.

We demonstrated that OBP-301-mediated activation of E2F1 expression upregulated *miR-7* expression in human cancer cells. E2F1 has recently been shown to regulate both oncogenic and tumor-suppressive miRNAs. The cluster of oncogenic miRNAs in the *miR-19-72* polycistron has been shown to be upregulated by E2F1.[43] In contrast, E2F1-inducible *miR-449a/b* has been shown to suppress cell proliferation and to induce apoptosis in human cancer cells.[44] Furthermore, Brosh *et al.* have suggested that 15 p53-repressed miRNAs, including the *miR-19-72* cluster and *miR-7,* are possibly regulated by E2F1,[45] which is consistent with our results that show E2F1-mediated *miR-7* upregulation. We previously reported that p53-inducible *miR-34a* suppresses E2F1 protein expression, resulting in downregulation of the E2F signaling pathway in human cancer cells.[13] These reports suggest possible cross-talk between E2F1, p53 and miRNAs. As adenovirus infection is well known to induce E2F1 expression,[35] but to suppress p53 expression,[46] the E2F1-inducible miRNA network may mainly assist the induction of autophagic cell death by oncolytic adenoviruses.

OBP-301-resistant T.Tn cells showed no induction of the E2F1-*miR-7*-EGFR axis, resulting in a lack of OBP-301-mediated autophagic cell death. Adenovirus infection is known to modulate E2F1 expression via two main viral factors, E1A and E4. E1A interacts with the phosphorylated retinoblastoma protein, resulting in the release of free E2F1.[26] In contrast, the adenoviral E4 19 kDa protein has been shown to enhance E2F1 protein levels through inhibition of proteasome-mediated E2F1 degradation.[27,28] Although the molecular basis for the lack of OBP-301-mediated E2F1 activation in T.Tn cells remains unclear, the cytopathic effect of an oncolytic adenovirus may mainly depend on E2F1 activation, leading to induction of autophagic cell death via modulation of E2F1-downstream target genes including miRNAs. On the role of another E2F family members during adenovirus infection, recent reports have suggested that adenovirus infection increases the E2F2 expression at the transcriptional level,[47] whereas the E2F4 expression is decreased.[48] Because it has been known that E2F2 is a transactivator as same as E2F1, but E2F4 functions as a transcriptional repressor, these E2F family members may function to induce the E2F-target gene network. Thus, further studies to address the role of E2F family members in OBP-301-mediated oncolytic cell death are warranted.

It has been recently shown that *miR-7* functions as a tumor suppressive miRNA by suppressing the expression of various EGFR signaling-related genes including that of *EGFR, insulin receptor substrate-2, Raf1* and *p21-activated kinase 1* in human cancer cells.[31,32,49] Consistent with these results, we observed that ectopic expression of *miR-7* suppressed cell proliferation and subsequently induced autophagic cell death through suppression of EGFR expression in human cancer cells. Regarding *miR-7*-mediated cell death, Webster *et al.* have suggested that nonapoptotic cell death is induced by *miR-7* transfection in human lung cancer A549 cells.[31] In contrast, Kefas *et al.* have shown that *miR-7* overexpression induces apoptotic cell death in human glioma cell lines.[32] These contradictory results suggest that *miR-7*-mediation of autophagic cell death may depend on the type of cancer cell in which it is expressed.

Overexpression or amplification of several types of EGFR gene isoforms is frequently observed in human cancers.[33] Recently, EGFR-targeting anticancer therapies, such as monoclonal antibodies and small molecule tyrosine kinase inhibitors, have been used to improve the clinical outcome of cancer patients. However, resistance to EGFR-targeting therapies is an issue that needs to be resolved. Furthermore, it has been recently reported that the EGFR regulates glucose transport that is required for the survival of cancer cells in an EGFR-kinase-independent manner.[34] This result suggests that not only inhibition of EGFR-kinase activity but also downregulation of the EGFR itself will be required for complete eradication of cancer cells. Recent report has further suggested that combination therapy of EGFR kinase inhibitor erlotinib with autophagy inducer rapamycin synergistically decreased the cell viability through increased autophagy in H1299 and A549 cells.[50] Our collaborators have also demonstrated that combination therapy of rapamycin with OBP-301 showed synergistic antitumor effect through activation of autophagy machinery in human brain tumor cells.[22] Taking the oncolytic adenovirus-mediated EGFR suppression and autophagy via *miR-7* induction into consideration, combination therapy of oncolytic adenoviruses with rapamycin may provide novel anticancer strategies that potentially have antitumor effects against cancer cells that are resistant to EGFR-targeting therapies.

In conclusion, we provide evidence, for the first time, that an oncolytic adenovirus induces autophagic cell death in human cancer cells through induction of *miR-7* upregulation via enhancement of E2F1 expression and through suppression of oncogenic EGFR expression. An understanding of oncolytic adenovirus-mediated modulation of the cellular miRNA network would provide novel insights into the antitumor mechanism of oncolytic virotherapy.

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