

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

Hiroshi Tazawa^{1,2}, Shuya Yano², Ryosuke Yoshida², Yasumoto Yamasaki², Tsuyoshi Sasaki³, Yuuri Hashimoto², Shinji Kuroda², Masaaki Ouchi⁴, Teppei Onishi², Futoshi Uno², Shunsuke Kagawa², Yasuo Urata⁴ and Toshiyoshi Fujiwara²

¹ Center for Gene and Cell Therapy, Okayama University Hospital, Okayama, Japan

² Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

³ Department of Orthopaedic Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

⁴ Oncolys BioPharma Inc., Tokyo, Japan

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 hypoxia² 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 ade-

novirus 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 apoptosis¹² and senescence¹³ 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 virus¹⁵ and the human cytomegalovirus¹⁶ 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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Correspondence to: Toshiyoshi Fujiwara, Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan, Tel.: +81-86-235-7257, Fax: +81-86-221-8775, E-mail: toshi_f@md.okayama-u.ac.jp

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% CO₂.

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 $2^{-\Delta\Delta C_t}$ 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 exam-

ined 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. 1a). 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 (NHFL), 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 NHFL cells showed the resistance to OBP-301-mediated cytopathic effect (Fig. 1a). The cytopathic activity and replication rate of wild-type adenovirus serotype 5 (Ad5)

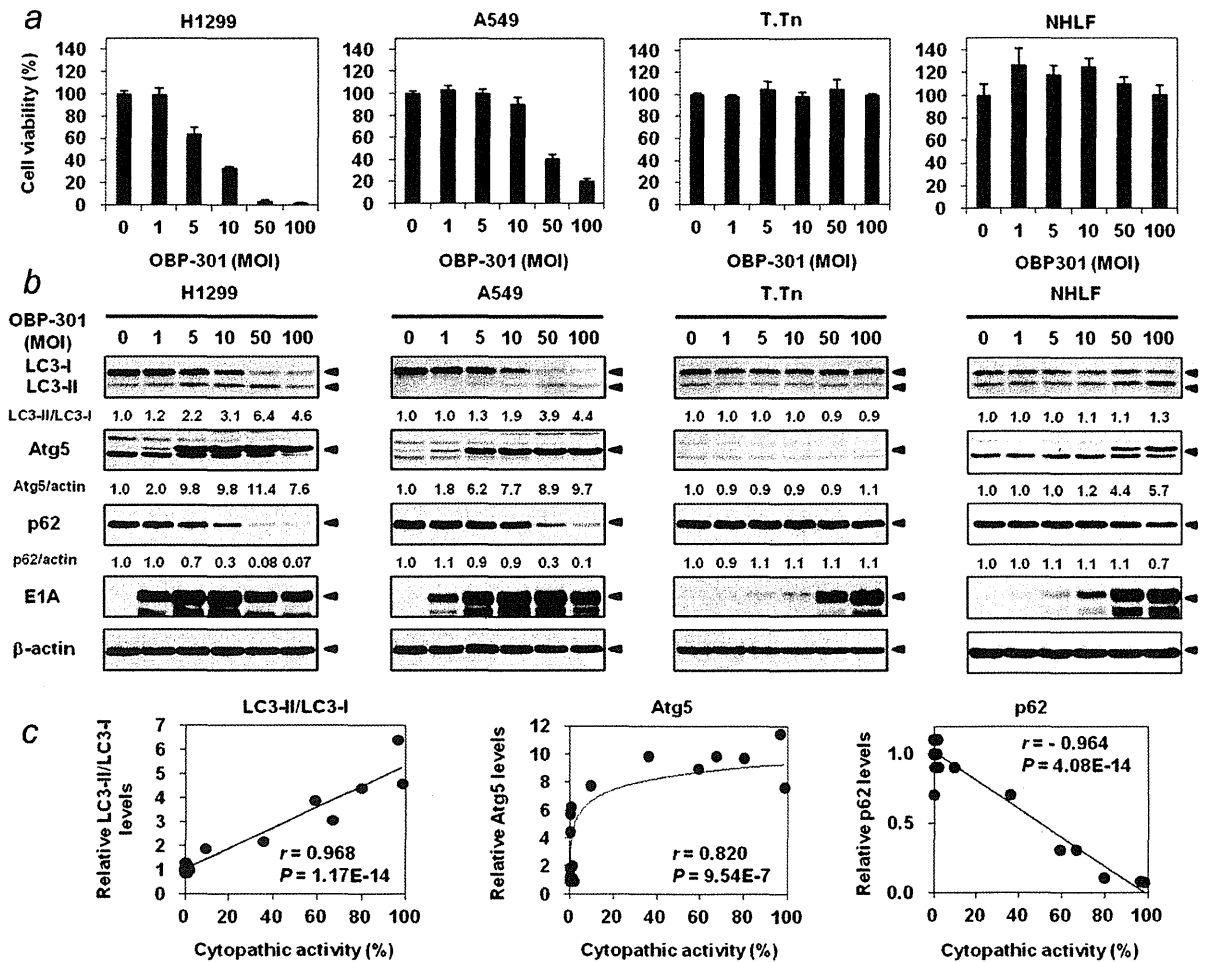


Figure 1. OBP-301-mediated induction of autophagic cell death in human cancer cells. (a) The cytopathic effect of OBP-301 in human cancer cells (H1299, A549 and T.Tn) and normal fibroblasts (NHLF). Cell viability was determined 72 hr after infection with OBP-301 at the indicated MOIs, using an XTT assay. 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. 1b). 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. 1c). 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. 2a). 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 E1A²⁶ 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. 2b). 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

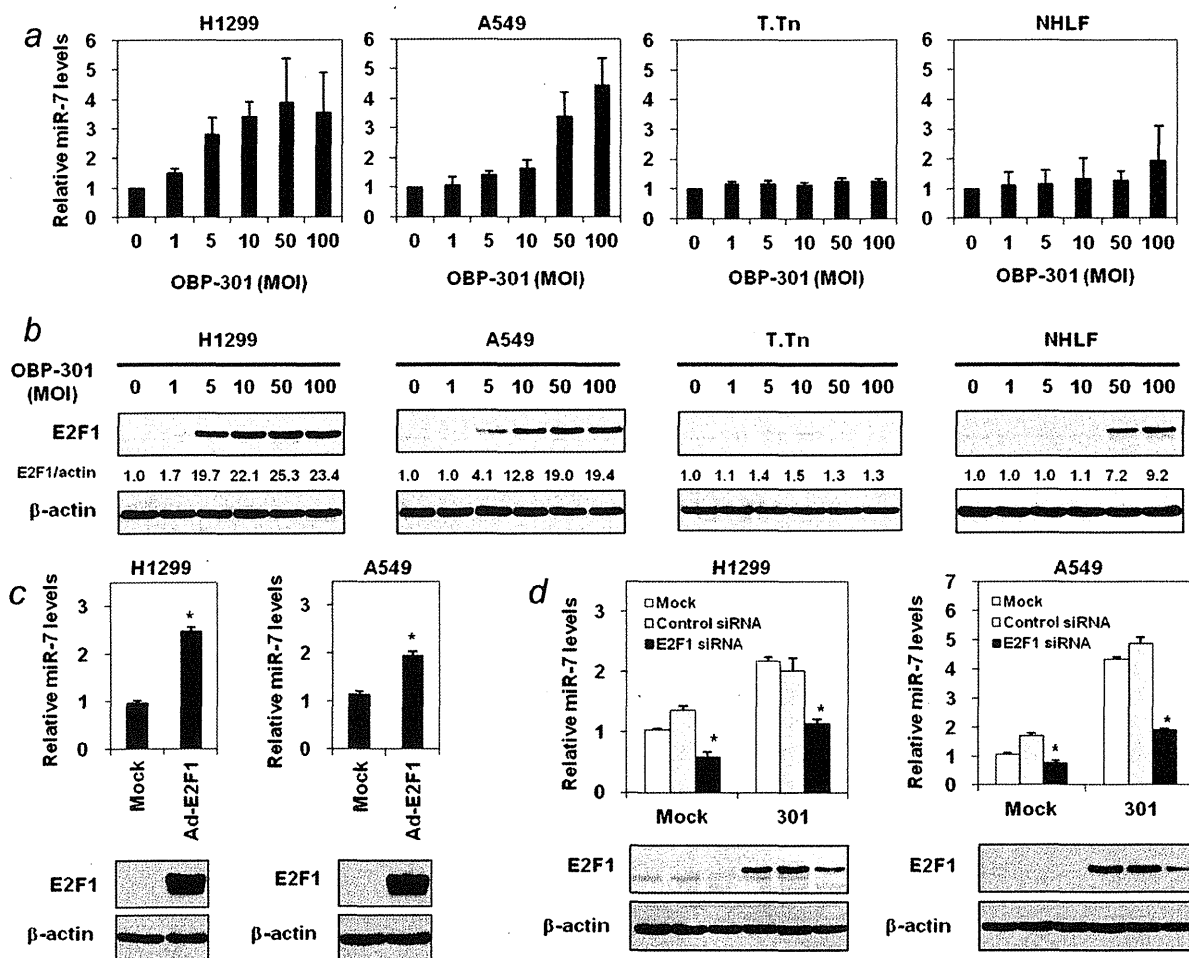


Figure 2. OBP-301-induced E2F1 expression upregulates *miR-7* expression in association with cytopathic activity in human cancer cells. (a) *miR-7* expression was assayed using qRT-PCR in H1299, A549, T.Tn and NHLF cells infected with OBP-301 at the indicated MOIs for 72 hr. The values of *miR-7* at 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 \pm 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 \pm 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. 2c). Overexpression of the E2F1 protein by Ad-E2F1 infection was confirmed by West-

ern 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. 2d). Suppression of OBP-301-activation of E2F1 expression by pretreatment with E2F1

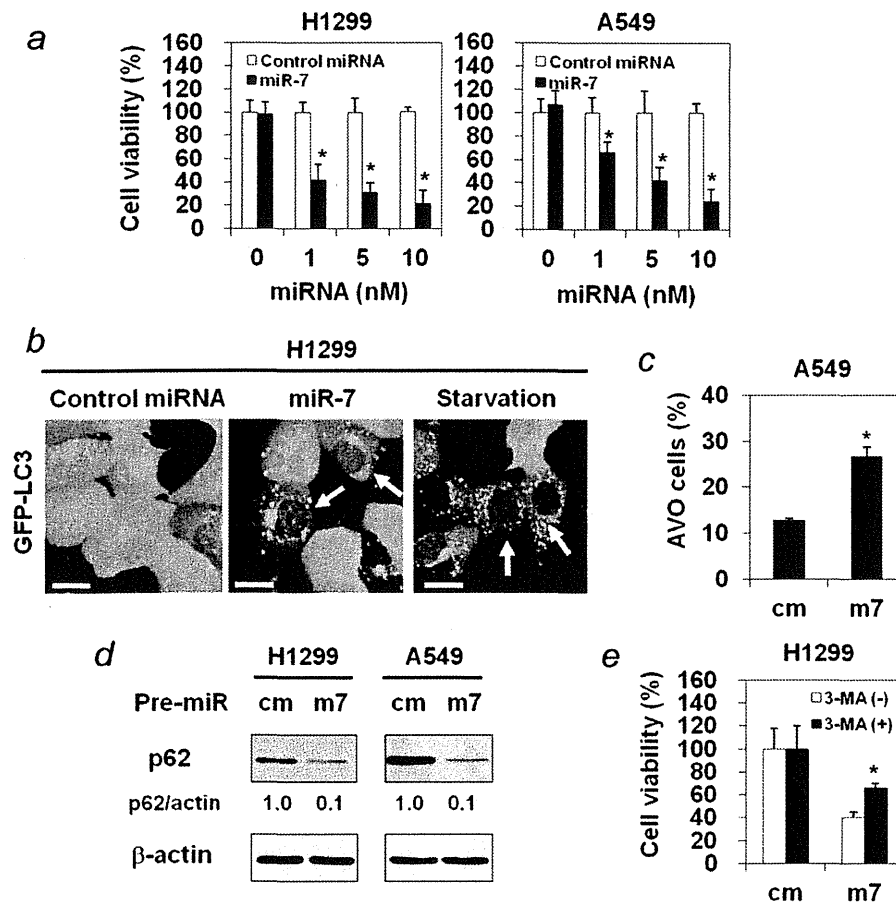


Figure 3. Induction of autophagy by *miR-7* overexpression. (a) The effect of transfection of the indicated concentrations of control miRNA (white bars) or *miR-7* (black bars) on the cell viability of H1299 and A549 cells was determined using XTT assay on day 6 after transfection. Cell viability of *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 μ m. (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 \pm 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 \pm 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. 3a 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 cells³⁰ 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. 3b). 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. 3c). 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. 3d). 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. 3e), 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 4a, 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 ($r = -0.872$, $p = 2.64E-8$) (Fig. 4b) and with the cytopathic activity of OBP-301 ($r = -0.826$, $p = 6.73E-7$) (Fig. 4c), 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. 4d). 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. 4e). In contrast, treatment with EGFR siRNA significantly suppressed cell viability compared to control siRNA in H1299 and A549 cells (Fig. 4f). 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. 4g).

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).

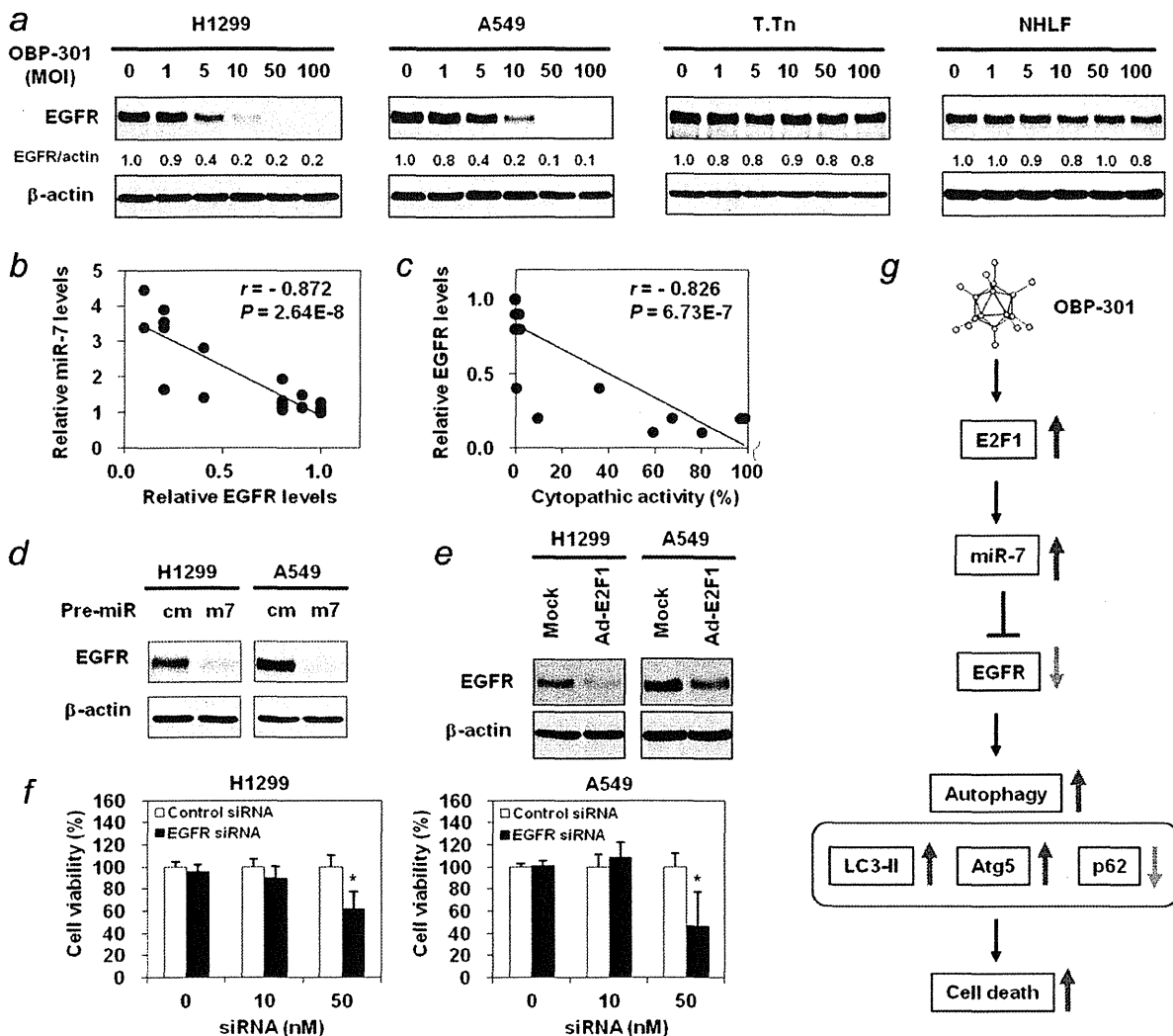


Figure 4. miR-7-mediated suppression of EGFR expression during OBP-301-mediated autophagic cell death. (a) Western blot analysis of EGFR protein expression in H1299, A549, T.Tn and NHLF cells infected with OBP-301 at the indicated MOIs for 72 h. EGFR expression levels were calculated relative to actin 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 contribute 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 onco-

lytic 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 anti-tumor mechanism of oncolytic virotherapy.

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Mechanism of resistance to trastuzumab and molecular sensitization via ADCC activation by exogenous expression of HER2-extracellular domain in human cancer cells

Ryosuke Yoshida · Hiroshi Tazawa · Yuuri Hashimoto · Shuya Yano · Teppei Onishi · Tsuyoshi Sasaki · Yasuhiro Shirakawa · Hiroyuki Kishimoto · Futoshi Uno · Masahiko Nishizaki · Shunsuke Kagawa · Toshiyoshi Fujiwara

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Abstract Trastuzumab, a humanized antibody targeting HER2, exhibits remarkable therapeutic efficacy against HER2-positive breast and gastric cancers; however, acquired resistance presents a formidable obstacle to long-term tumor responses in the majority of patients. Here, we show the mechanism of resistance to trastuzumab in HER2-positive human cancer cells and explore the molecular sensitization by exogenous expression of HER2-extracellular domain (ECD) in HER2-negative or trastuzumab-resistant human cancer cells. We found that long-term exposure to trastuzumab induced resistance in HER2-positive cancer cells; HER2 expression was downregulated, and antibody-dependent cellular cytotoxicity (ADCC) activity was impaired. We next examined the hypothesis that trastuzumab-resistant cells could be re-sensitized by the transfer of non-functional HER2-ECD. Exogenous HER2-ECD expression induced by the stable transfection of a plasmid

vector or infection with a replication-deficient adenovirus vector had no apparent effect on the signaling pathway, but strongly enhanced ADCC activity in low HER2-expressing or trastuzumab-resistant human cancer cells. Our data indicate that restoration of HER2-ECD expression sensitizes HER2-negative or HER2-downregulated human cancer cells to trastuzumab-mediated ADCC, an outcome that has important implications for the treatment of human cancers.

Keywords HER2 · Extracellular domain · Trastuzumab · ADCC · Adenovirus

Abbreviations

HER2	human epidermal growth factor receptor 2
ADCC	antibody-dependent cellular cytotoxicity
NK	natural killer
ECD	extracellular domain
MOI	multiplicity of infection
IGF-1R	insulin-like growth factor-1 receptor
Ad5	adenovirus type 5
tzb	trastuzumab

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R. Yoshida · Y. Hashimoto · S. Yano · T. Onishi · Y. Shirakawa · H. Kishimoto · F. Uno · M. Nishizaki · S. Kagawa · T. Fujiwara (✉)

Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan
e-mail: toshi_f@md.okayama-u.ac.jp

H. Tazawa · T. Fujiwara
Center for Gene and Cell Therapy, Okayama University Hospital, Okayama 700-8558, Japan

T. Sasaki
Department of Orthopaedic Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan

Introduction

Human epidermal growth factor receptor 2 (HER2) is a member of a receptor family associated with tumor cell proliferation, adhesion, migration, and differentiation [1]. Trastuzumab, a humanized monoclonal antibody that targets HER2, inhibits the HER2-mediated signaling pathway [2, 3] and also induces antibody-dependent cellular cytotoxicity (ADCC) [4–7]. The randomized clinical trial that led to the approval of trastuzumab for clinical use was conducted in combination with standard cytotoxic chemotherapy [8]. Subsequent trials have confirmed the utility of

trastuzumab in HER2-overexpressing breast cancer in various clinical scenarios [9]. Recently, the efficacy and safety of adding trastuzumab to chemotherapy in HER2-positive advanced gastric cancer was evaluated, and the combination therapy was found to be significantly superior to chemotherapy alone [10]. However, HER2 is overexpressed only in approximately 20 % of primary breast and gastric cancers [8, 11–13]. Moreover, even if the HER2 status is positive, the majority of patients that initially respond to trastuzumab eventually develop resistance [9, 14, 15]. Thus, more effective treatments against HER2-overexpressing cancer require a deeper understanding of the mechanisms of resistance to trastuzumab.

Several mechanisms for trastuzumab resistance have been proposed, including the truncation of the HER2 receptor into a constitutively activated form (p95HER2) [16, 17], increased cellular signaling through alternative receptor tyrosine kinases [18–20], and altered intracellular signaling involving the loss of PTEN [21–23], which increases Akt activity. However, the biochemical nature of the resistance mechanism is confusing and controversial. Although the activation of ADCC is an important antitumor mechanism of trastuzumab, few studies have examined the role of ADCC in trastuzumab resistance. ADCC relies on the binding of antigen–antibody complexes to Fc γ receptors expressed on immune cells, and it is mainly attributable to the activation of natural killer (NK) cells. In fact, ADCC and overall NK cell activity were found to correlate with responses to trastuzumab [24]. Tumor cells potentially avoid ADCC attack from therapeutic antibodies by various mechanisms, such as insufficient recruitment of effector cells into tumors and the reduction or elimination of antigen expression on tumor cells [25].

Several strategies have been proposed to re-sensitize resistant tumor cells to therapeutic antibodies. In particular, the modification of heterogeneous or decreased antigen expression in resistant tumor cells might overcome resistance by enhancing ADCC activity. HER2 contains an extracellular ligand-binding domain, a short hydrophobic transmembrane region, and a cytoplasmic tyrosine kinase domain, which is crucial for downstream signaling [26]. Therefore, we hypothesize that truncated HER2 without an intracellular domain could be used as a non-signaling target for ADCC.

In the present study, we analyzed the HER2 surface expression and ADCC susceptibility of HER2-positive human cancer cells following repeated exposure to trastuzumab. We found that the surviving cells had reduced HER2 expression and were consequently less susceptible to ADCC. Moreover, we explored the effect of exogenous overexpression of the extracellular domain (ECD) of HER2, which lacks an intracellular signaling fragment, in HER2-negative and trastuzumab-resistant human cancer cells.

Materials and methods

Cell lines and cell cultures

Three human mammary gland adenocarcinoma cell lines, SKBR3, BT474, and MCF7, were obtained from American Type Culture Collection. SKBR3 was cultured in McCoy's 5A medium. BT474 was cultured in Leibovitz's medium. MCF7 was cultured in DMEM supplemented with 2 mmol/ml L-glutamine. The human gastric adenocarcinoma cell lines MKN1 and MKN28 were obtained from Human Science Research Resources Bank and cultured in RPMI1640. Penicillin (100 units/ml), streptomycin (100 μ g/ml), and 10 % fetal bovine serum were added to the medium for each cell line.

Construction of plasmids and establishment of stable cell lines

Complementary DNAs of human full-length HER2 (HER2-wt) and truncated HER2 containing extracellular and transmembrane regions (HER2-ECD) were kindly provided by Dr. Mien-Chie Hung (M. D. Anderson Cancer Center). HER2-wt and HER2-ECD cDNAs were subcloned into the multi-cloning sites of the pcDNA3 vector. MCF7 breast cancer cells were transfected with the vectors expressing HER2-wt or HER2-ECD. For selection of stably transfected cells, cells were maintained in medium containing 0.2 mg/ml geneticin (G418), and single colonies were isolated.

Recombinant adenovirus

Replication-deficient adenoviral vector expressing the extracellular and transmembrane domains of HER2 (Ad-HER2-ECD) was constructed. Briefly, the HER2-ECD expression cassette that contains the human cytomegalovirus promoter, HER2-ECD cDNA, and the SV40 early polyadenylation signal was inserted between the XbaI and ClaI sites of pXCJL1. The HER2-ECD shuttle vector and the recombinant plasmid pJM17 were cotransfected into 293 cells (Ad5-transformed human embryonic kidney cell line). The culture supernatant of 293 cells showing the complete cytopathic effect was collected and used for subsequent infections. This virus was purified by ultracentrifugation in cesium chloride step gradients, and its titer was determined by a plaque-forming assay using 293 cells. Replication-deficient E1A-deleted adenovirus (dl312) was used as control adenovirus. The viruses were stored at -80°C before use.

Establishment of trastuzumab-acquired auto-resistance in HER2-positive cancer cells

To establish the trastuzumab-resistant cells, human breast cancer cell lines SKBR3 and BT474 expressing HER2 were

exposed to increasing concentrations of the anti-HER2 monoclonal antibody trastuzumab (Chugai Pharmaceutical Co.) for more than 3 months. Briefly, HER2-positive cancer cells were initially exposed to 50 mg/ml trastuzumab for 1 month followed by 100 mg/ml trastuzumab for 2 months. Trastuzumab was administered twice a week. Trastuzumab-resistant cells established by continuous exposure to trastuzumab were maintained in medium with 100 mg/ml trastuzumab. The trastuzumab-resistant cancer cells were cultured in medium without trastuzumab for 5 days before each experiment.

Western blotting analysis

Primary antibodies against HER2-ECD (Thermo Scientific), β -actin (Sigma Chemical, Co.); PTEN (Santa Cruz), HER2-intracellular domain, IGF1-R, pAkt, pmTOR, pHER3, Akt, and mTOR (Cell Signaling Technology) and peroxidase-linked secondary antibodies (Amersham) were used. Proteins were electrophoretically transferred to Hybond-polyvinylidene difluoride transfer membranes (GE Healthcare Life Science) and incubated with primary antibody, followed by peroxidase-linked secondary antibody according to the manufacturer's protocol. The Amersham ECL chemiluminescence system (GE Healthcare Life Science) was used to detect the peroxidase activity of the bound antibody. In experiments with replication-deficient adenoviral vector, cells were infected with Ad-HER2-ECD or dl312 at a multiplicity of infection (MOI) of 20 for 36 h.

Flow cytometric analysis

In experiments to measure the affinity to trastuzumab, tumor cells were pretreated with 100 μ g/ml of trastuzumab for 60 min at 37 °C. Tumor cells were fixed with 4 % paraformaldehyde in PBS for 10 min and then washed with PBS containing 0.5 or 1.5 % BSA. The cells were labeled with APC-conjugated rabbit monoclonal anti-HER2-ECD antibody (R&D Systems Inc.) or APC-conjugated AffiniPure F(ab')₂ fragment goat monoclonal anti-human IgG + IgM (H + L) antibody (Jackson ImmunoResearch Laboratories, Inc.) at room temperature for 45 min and analyzed by FACSaria instrument (BD Biosciences). The cell population was gated on forward scatter and side scatter. The intensity of staining was determined by the BD-FACS Software. In experiments with replication-deficient adenoviral vector, cells were infected with Ad-HER2-ECD at an MOI of 20 for 36 h.

Cell viability assay

Parental or trastuzumab-resistant human breast cancer cells were seeded on 96-well plates at a density of 1×10^3 cells/

well for 24 h. Then, trastuzumab was added to every well at the indicated concentration for 5 days. Cell viability was determined 5 days after trastuzumab treatment by using the Cell Proliferation Kit II (Roche Molecular Biochemicals) with the sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay, according to the manufacturer's protocol.

Cell proliferation assays

Cells were trypsinized and re-plated in 24-well plates at a density of 1×10^4 cells/well. Parental MCF7 cells, mock vector-treated MCF7 cells, MCF7-HER2-wt cells, and MCF7-HER2-ECD cells were analyzed. Cells were incubated for 12 h to allow for attachment, after which the zero time point was determined. In experiments with adenoviral vector, trastuzumab-resistant SKBR3 or BT-47 cells with downregulated HER2 expression or low HER2-expressing breast and gastric cancer cells were infected with replication-deficient adenovirus (20 MOI) 1 day after the zero time point.

Antibody-dependent cellular cytotoxicity (ADCC) assay

Peripheral blood mononuclear cells (PBMCs) were separated from peripheral blood obtained from healthy donors by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. After centrifugation, PBMCs were washed three times with PBS and suspended with medium containing 10 % FBS. Target cells were labeled with 50 μ Ci (1.85 MBq) of Na⁵¹Cr (Parkin Elmer, Waltham, MA, USA) for 60 min. Then, target cells (1×10^4 /well) and effector cells at various effector/target ratios were co-incubated in 200 μ l of X-VIVO medium in a 96-well U-bottomed plate for 4 h at 37 °C with trastuzumab (2 μ g/well; Chugai Pharmaceutical co.) or control antibody, rituximab (2 μ g/well; Chugai Pharmaceutical Co.). After 4 h of incubation, the radioactivity of the supernatant (100 μ l) was measured with a γ -counter. The percentage of specific lysis = $100 \times (\text{experimental count per minute (cpm)} - \text{spontaneous cpm}) / (\text{maximum cpm} - \text{spontaneous cpm})$. In experiments with replication-deficient adenovirus, target cells were infected with Ad-HER2-ECD or dl312 at an MOI of 20 for 36 h (MCF7 and MDA-MB-231 cells) or 24 h (trastuzumab-resistant SKBR3 or BT474, MKN1, and MKN28 cells) before the ADCC assay was performed.

Statistical analysis

A comparison of continuous variables between two groups for in vitro assays was performed with the two-sided Student's *t* test. At least three independent experiments were

performed. The differences between groups were considered to be statistically significant when the p values were <0.05 . Means and 95 % confidence intervals are reported, unless otherwise indicated. All data were analyzed with the statistical software SPSS 15.0 (SPSS, Inc, Chicago, IL, USA).

Results

Continuous exposure to trastuzumab downregulates cell-surface HER2 expression and impairs trastuzumab-mediated ADCC in HER2-positive human cancer cells

To elucidate the molecular mechanism of developing resistance to trastuzumab, we continuously treated HER2-positive SKBR3 and BT474 breast cancer cells with trastuzumab. Exposure to trastuzumab for 1 month reduced extracellular HER2 levels in both cell lines, and this downregulation was maintained for at least 2 months. HER2 downregulation did not recover following 5 days of incubation in the absence of trastuzumab (Fig. 1a). Intracellular HER2 expression was also reduced by long-term exposure to trastuzumab, although p95HER2 could not be detected (Supplemental Fig. 1). Flow cytometric analysis demonstrated that trastuzumab-exposed SKBR3 and BT474 cells showed decreased HER2 expression as compared with parental lines, leading to reduced affinity to trastuzumab (Fig. 1b).

SKBR3 and BT474 cells exposed to trastuzumab for 3 months were apparently more resistant to trastuzumab-mediated growth suppression *in vitro* (Fig. 1c). Western blotting analysis for assessment of the HER2-related signaling pathway demonstrated that phosphorylated Akt and mTOR expression were downregulated in these resistant cell lines. In contrast, insulin-like growth factor-1 receptor (IGF-1R) expression was notably enhanced following trastuzumab exposure in SKBR3 cells and constitutively high in BT474 cells without trastuzumab treatment (Fig. 1d). These results suggest that the development of resistance to trastuzumab at least partially depends on upregulation of an alternative signaling pathway downstream of other receptor tyrosine kinases such as IGF-1R. We further examined trastuzumab-mediated ADCC against parental and trastuzumab-resistant SKBR3 and BT474 cells by using PBMCs from healthy volunteer donors. Although apparent ADCC activity was observed in parental SKBR3 and BT474 cells, long-term exposure to trastuzumab significantly reduced this activity in both cell lines (Fig. 1e). Thus, impaired ADCC activity might be another possible mechanism contributing to acquired trastuzumab resistance.

Effects of exogenous HER2-ECD expression on *in vitro* growth and signaling pathways in human cancer cells

To investigate the effects of exogenous expression of HER2 protein on trastuzumab-mediated antitumor activity, we transfected human full-length HER2 (HER2-wt) and truncated HER2 cDNA containing extracellular and transmembrane regions (HER2-ECD) into low HER2-expressing MCF7 human breast cancer cells. HER2 overexpression contributes to breast cancer carcinogenesis, and studies have indicated that transfection of HER2-wt into mammary epithelial cells induces oncogenic transformation [27]. Indeed, stable HER2-wt-expressing MCF7 cells showed accelerated cell growth compared to parental MCF7 cells, whereas the growth pattern of HER2-ECD-transfected MCF7 cells was similar to that of parental MCF7 cells (Fig. 2a). Furthermore, transfection of HER2-wt, but not HER2-ECD, led to an increase in phosphorylated Akt and mTOR expression in the presence of HER3 ligand, HRG- β ; these results suggest that exogenous HER2-ECD expression did not trigger the signaling pathways of HER2/HER3, which is the most potent combination of receptors in human breast cancer cells [28, 29] (Fig. 2b).

We next explored whether exogenous expression of HER2-wt or HER2-ECD was altered by long-term exposure to trastuzumab. The expression of 185-kDa full-length HER2 protein was slightly reduced in the presence of trastuzumab, whereas 3-month treatment with trastuzumab resulted in a slight increase in 100-kDa HER2-ECD expression (Fig. 2c). However, flow cytometric analysis demonstrated that neither cell-surface HER2 expression nor trastuzumab binding affinity changed following long-term trastuzumab exposure (Fig. 2d). Furthermore, the ADCC activity of trastuzumab against MCF7 cells transfected with HER2-wt or HER2-ECD was maintained even after a 3-month exposure to trastuzumab (Fig. 2e). These results indicate that exogenous overexpression of HER2-wt or HER2-ECD could overcome trastuzumab-mediated downregulation of endogenous HER2 expression.

Exogenous HER2-ECD expression enhances trastuzumab-mediated ADCC activity in low HER2-expressing human cancer cells

We conducted a standard 4-h ^{51}Cr release assay with PBMCs from healthy volunteer donors to test the hypothesis that trastuzumab-mediated ADCC activity could be enhanced by exogenous overexpression of HER-ECD. With 5, 10, or 20 $\mu\text{g/ml}$ of trastuzumab, ADCC activity was significantly increased in both HER2-ECD- and HER2-wt-expressing MCF7 cells as compared to MCF7 cells transfected with control pcDNA3 vector. A low concentration of trastuzumab (1 $\mu\text{g/ml}$) failed to enhance ADCC (Fig. 3a).

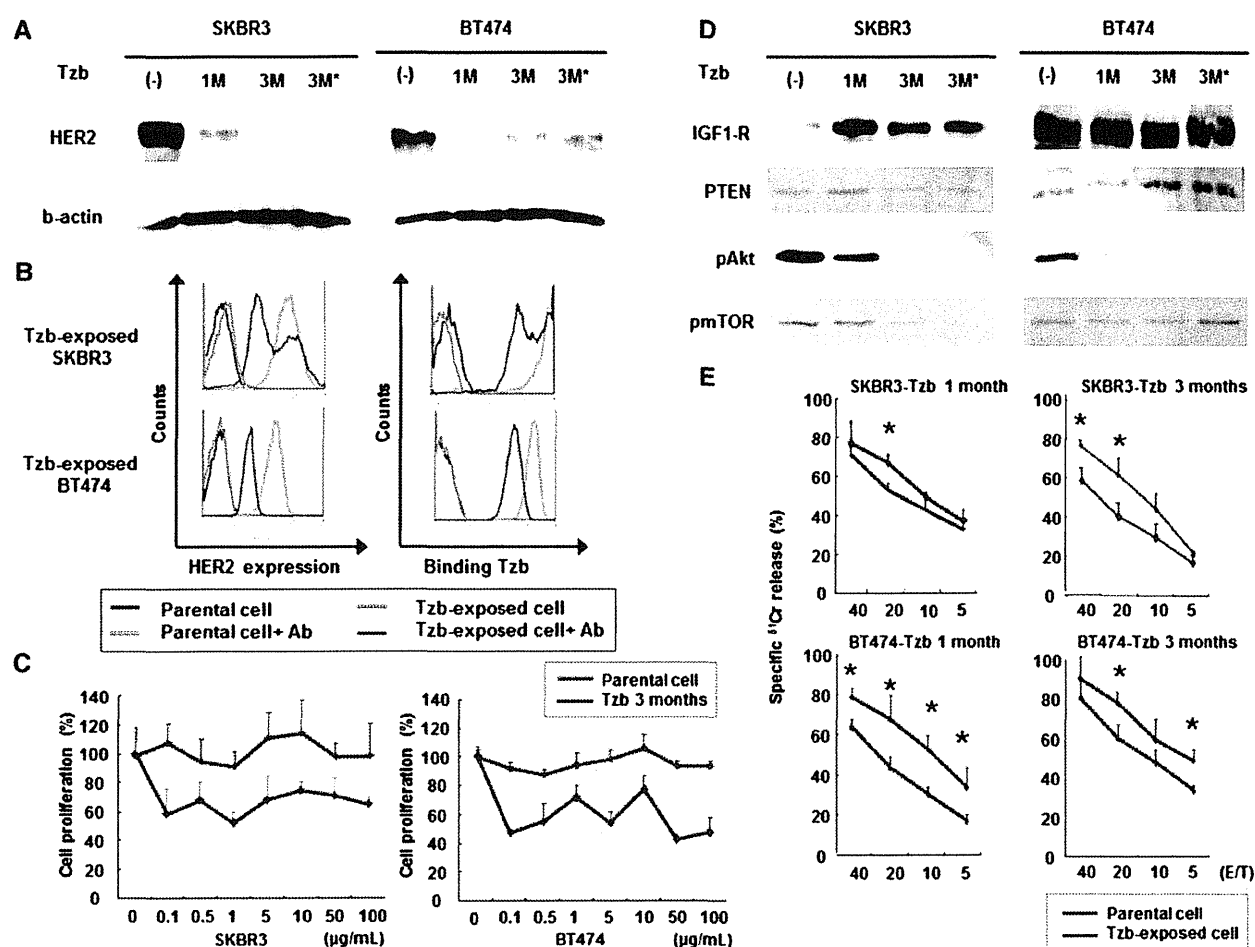


Fig. 1 Effects of continuous exposure to trastuzumab in HER2-over-expressing breast cancer cells. **a** Western blot analysis of HER2 expression. Human breast cancer SKBR3 and BT474 cells were initially incubated with 50 mg/ml trastuzumab (Tzb) for 1 month followed by 100 mg/ml trastuzumab for 2 months. *Cells were cultured in the absence of trastuzumab for 5 days before analysis. Equivalent amounts of protein from whole cell lysates were loaded into each lane. Blots were probed with anti-HER2-ECD antibody and visualized by using an ECL detection system. Equal loading of samples was confirmed by stripping each blot and reprobng with anti- β -actin antibody. **b** Flow cytometric analysis of HER2 expression and trastuzumab binding. Parental or trastuzumab-exposed cells were stained with APC-conjugated anti-HER2-ECD antibody to measure cell-surface

HER2 expression or treated with trastuzumab followed by incubation with APC-conjugated anti-human antibody to measure the amount of bound trastuzumab. **c** Parental or trastuzumab-exposed cells were further treated with the indicated doses of trastuzumab for 5 days, and cell viability was assessed by XTT assay. **d** Western blot analysis for assessment of HER2-related signaling pathway. Blots were probed with anti-IGF1-R, anti-PTEN, anti-phosphorylated Akt, or anti-phosphorylated mTOR antibody. **e** ADCC activity of trastuzumab-exposed SKBR3 or BT474 cells. Parental or trastuzumab-exposed cells were incubated with PBMCs from healthy donors in the presence of 10 μ g/ml of trastuzumab, and the cytotoxic activity was assessed by a 4-h standard ⁵¹Cr-release assay. Data represent the mean \pm SD of 3 wells at four different effector-to-target (E/T) ratios. * $p < 0.05$

Furthermore, effector cells showed significantly increased ADCC against HER2-ECD- or HER2-wt-expressing MCF7 cells as compared to pcDNA3-transfected cells at the effector/target cell ratios of 5:1, 10:1, 20:1, and 40:1 (Fig. 3b). These results indicate that exogenous overexpression of HER2-ECD may be an appropriate strategy to sensitize human cancer cells with low or reduced expression of HER2 to trastuzumab. Thus, we next examined the most efficient tool for gene transfer.

Efficient HER2-ECD overexpression in human cancer cell lines by a recombinant replication-deficient adenovirus vector

Modified adenovirus type 5 (Ad5) vectors have been used as a platform to deliver genes of interest into various types of human cells. We constructed a replication-deficient adenoviral vector containing a gene that encodes the extracellular domain of HER2 plus the transmembrane domain

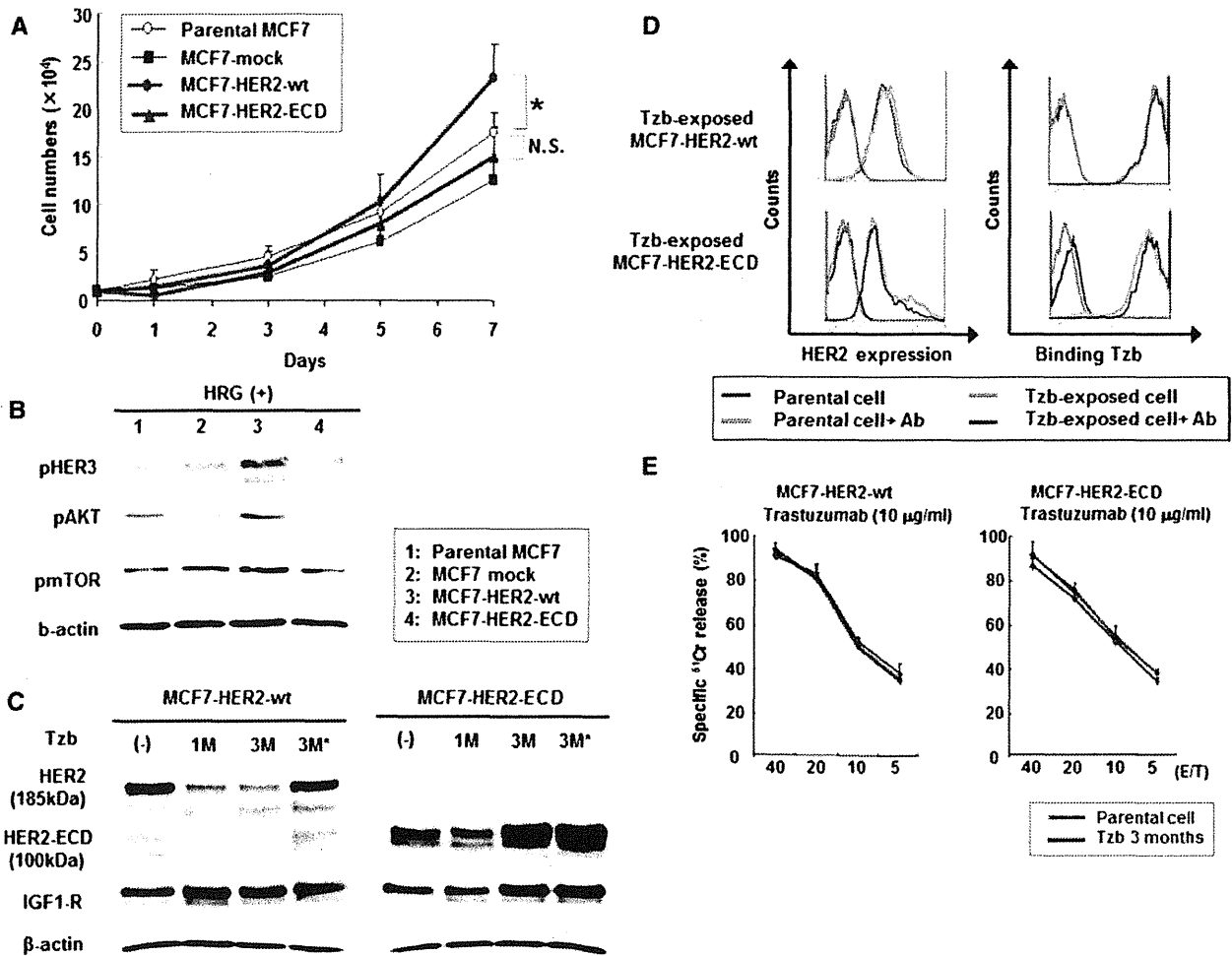


Fig. 2 Effects of exogenous HER2-ECD expression in low HER2-expressing MCF7 cells. **a** MCF7 human breast cancer cells were transfected with a vector expressing human full-length HER2 (HER2-wt) or truncated HER2 containing extracellular and transmembrane regions (HER2-ECD), or empty vector (mock). The cell growth was assessed for parental cells and stable clones. **p* < 0.05. **b** Western blot analysis of phosphorylated HER3, Akt, and mTOR. Cells were stimulated with HER3 ligand, heregulin- β . **c** Western blot analysis of MCF7 cells expressing HER2-wt (185 kDa) or HER2-ECD (100 kDa) after continuous exposure to trastuzumab for 1 month or 3 months. Cells were pre-

pared as described in the legend for Fig. 1a. **d** Flow cytometric analysis of HER2 expression and the amount of bound trastuzumab on parental or trastuzumab-exposed MCF7 cells expressing HER2-wt or HER2-ECD. Cells were stained and subjected to the analysis as described in the legend to Fig. 1b. **e** ADCC activity of parental or trastuzumab-exposed MCF7 cells expressing HER2-wt or HER2-ECD. The cytotoxic activity of PBMCs was assessed in the presence of 10 μ g/ml of trastuzumab by a 4-h standard 51 Cr-release assay. Data represent the mean \pm SD of 3 wells at four different E/T ratios

(Ad-HER2-ECD). To assess the efficient exogenous HER2-ECD overexpression by Ad-HER2-ECD infection, we used trastuzumab-resistant and low HER2-expressing human breast and gastric cancer cell lines. Various HER2 tests have demonstrated that the levels of HER2 expression in both MKN1 and MKN28 cells are low and that MKN7 cells overexpress HER2 antigen (Table 1). Indeed, the degree of HER2 expression correlated well with trastuzumab-mediated ADCC activity (Supplemental Fig. 2).

As expected, Ad-HER2-ECD infection at an MOI of 20 for 36 h resulted in a marked increase in the expression of 100-kDa HER2-ECD protein in trastuzumab-resistant

breast cancer cells (SKBR3 and BT474), low HER2-expressing breast cancer cells (MCF7 and MDA-MB-231), and low HER2-expressing gastric cancer cells (MKN1 and MKN28) as compared to mock- or control d1312-infected cells (Fig. 4a). Western blot analysis also demonstrated that Ad-HER2-ECD had no apparent effects on the HER2 signaling pathway such as Akt and mTOR expression as well as phosphorylated mTOR expression, although Ad-HER2-ECD and control d1312 induced phosphorylated Akt. Flow cytometric analysis confirmed the cell-surface expression of HER2-ECD in Ad-HER2-ECD-infected cells, which in turn leads to increased trastuzumab binding (Fig. 4b).

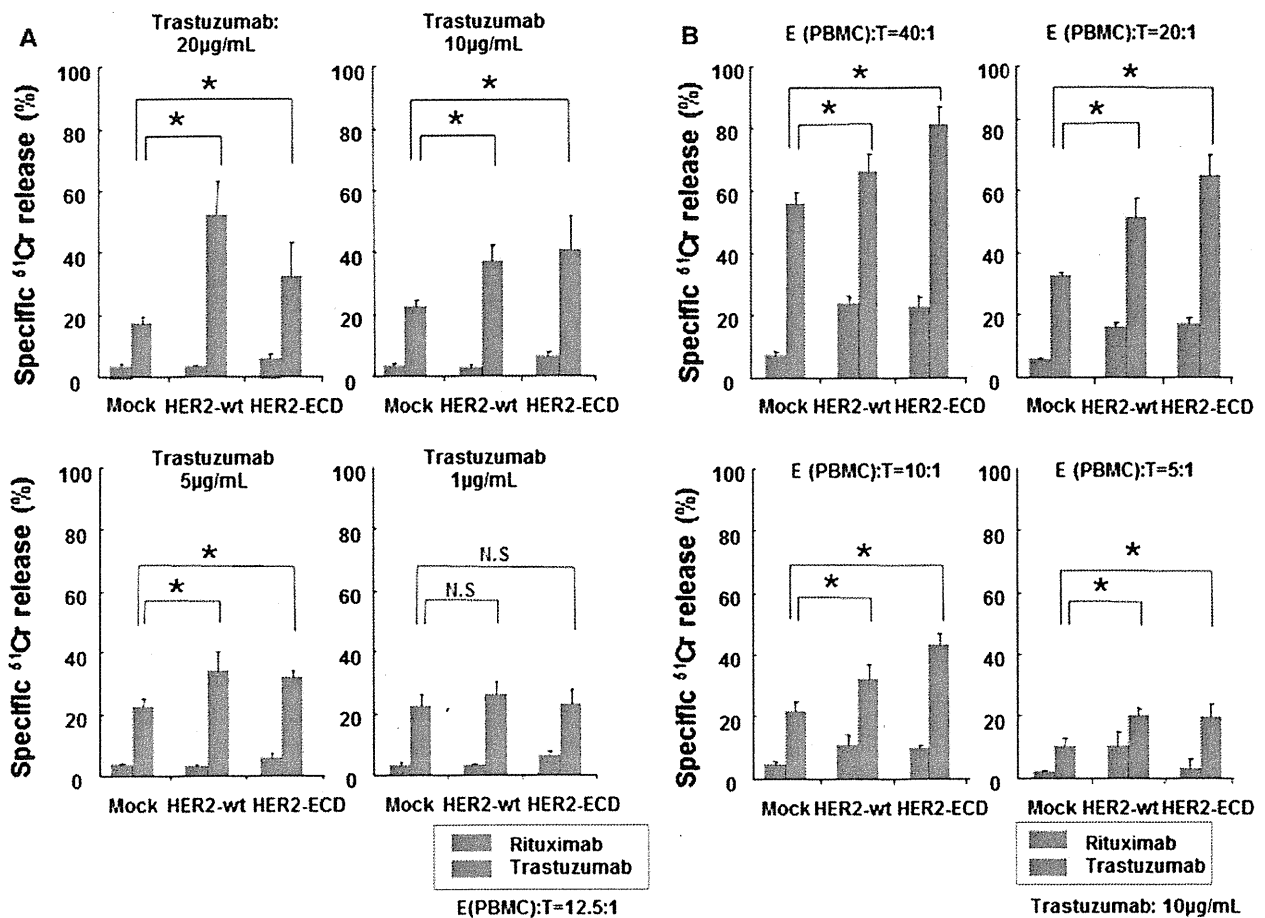


Fig. 3 Trastuzumab-mediated ADCC activity on HER2-ECD-expressing MCF7 cells. **a** The cytotoxic activity against MCF7 human breast cancer cells transfected with vector expressing HER2-wt or HER2-ECD or empty vector (mock) was assessed by a 4-h standard ⁵¹Cr-release assay in the presence of the indicated doses of trastuzumab or control rituximab. Data represent the mean ± SD of 3 wells at an E/

T ratio of 12.5:1. **p* < 0.05. **b** A 4-h ⁵¹Cr-release assay was also performed against MCF7 cells expressing HER2-wt or HER2-ECD, or mock-treated MCF7 cells in the presence of 10 µg/ml of trastuzumab or control rituximab. Data represent the mean ± SD of 3 wells at four different E/T ratios. **p* < 0.05

Table 1 HER2 expression status of gastric cancer cell lines

Cell lines	HER2 status		
	FACS (MFI)	HercepTest	Western blotting
MKN1	13	–	Weak
MKN7	106	3+	Strong
MKN28	30	–	Negative
MKN45	23	1+	Weak
NUGC3	9	–	Negative
KATO-III	30	2+	Medium

HER2-expressing status of six different gastric cancer cell lines measured by flow cytometry, immunocytochemistry (HercepTest), and Western blotting analysis

MFI mean fluorescence intensity, FACS fluorescence-activated cell sorting

Direct antitumor effects of Ad-HER2-ECD on trastuzumab-resistant or low HER2-expressing human cancer cells

Next, we assessed the cell growth pattern of trastuzumab-resistant SKBR3 and BT474 human breast cancer cells and low HER2-expressing MKN1 and MKN28 human gastric cancer cells following Ad-HER2-ECD infection. MCF7 cells that were stably transfected with the HER2-ECD plasmid showed a growth pattern similar to that of parental or control vector-transfected MCF7 cells (Fig. 2a). However, adenovirus-mediated overexpression of HER2-ECD unexpectedly induced a significant suppression of in vitro growth in all cell lines as compared to uninfected cells or cells infected with control dl312 (Fig. 5). These results suggest that Ad-HER2-ECD had a slight but significant direct

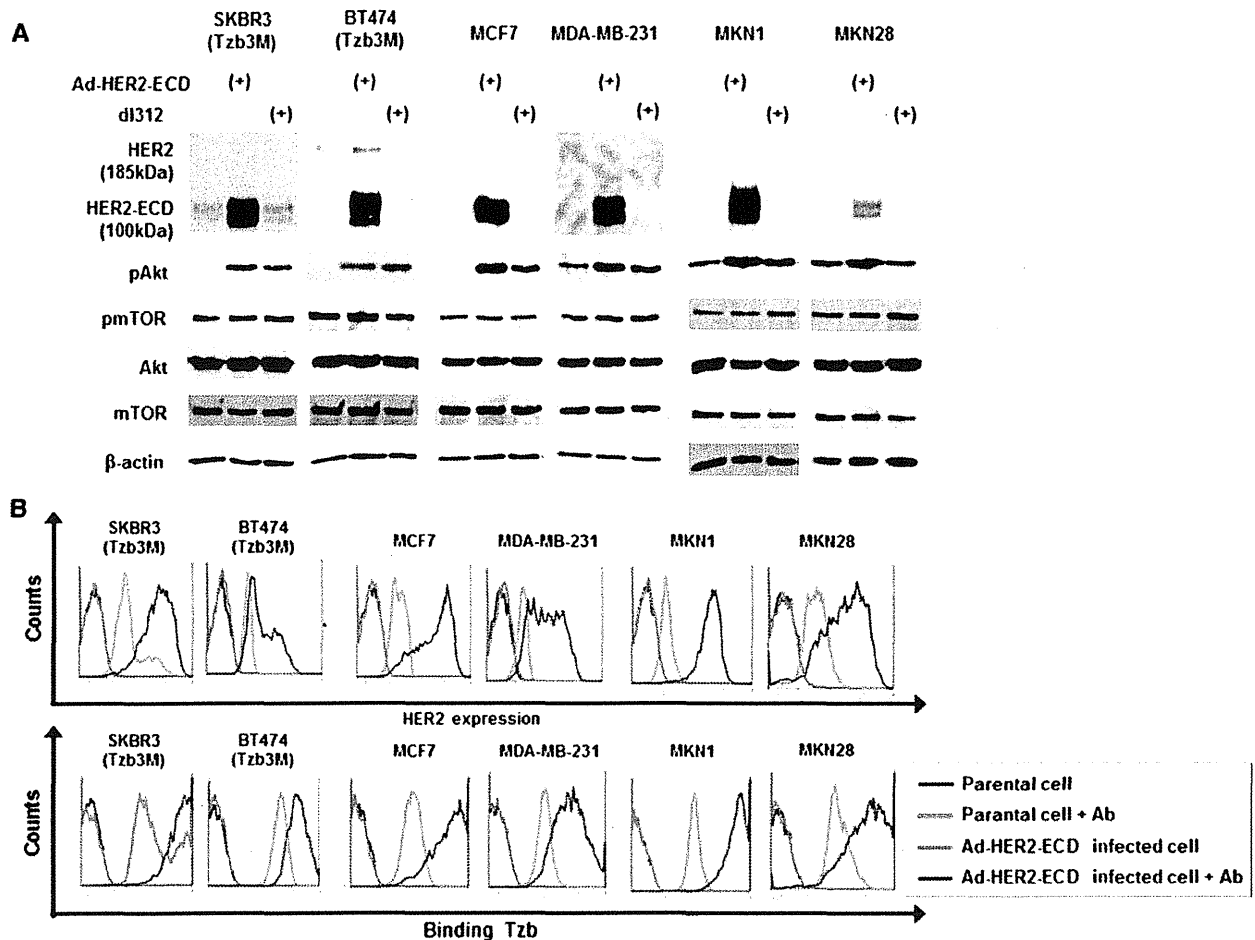


Fig. 4 Efficient HER2-ECD overexpression in human cancer cell lines by a recombinant replication-deficient adenovirus vector. **a** Western blot analysis of HER2-wt (185 kDa), HER2-ECD (100 kDa), and representative HER2-related signaling proteins in various types of human cancer cells. Trastuzumab-resistant breast cancer cells (SKBR3 and BT474), low HER2-expressing breast cancer cells (MCF7 and MDA-MB-231), and low HER2-expressing gastric cancer

cells (MKN1 and MKN28) were infected with replication-deficient adenoviral vector expressing exogenous HER2-ECD (Ad-HER2-ECD) or replication-deficient control adenovirus (dl312) at an MOI of 20 for 36 h. **b** Flow cytometric analysis of HER2 expression and the amount of bound trastuzumab in cells 36 h after Ad-HER2-ECD infection at an MOI of 20

antitumor effect on trastuzumab-resistant and low HER2-expressing human cancer cell lines in vitro.

Adenovirus-mediated HER2-ECD overexpression sensitizes trastuzumab-resistant or low HER2-expressing human cancer cells to trastuzumab-mediated ADCC

Finally, we examined whether Ad-HER2-ECD infection could overcome acquired resistance to trastuzumab-mediated ADCC in SKBR3 and BT474 human breast cancer cells. Enhancement of ADCC activity by Ad-HER2-ECD infection was also assessed in low HER2-expressing human breast and gastric cancer cell lines. Following Ad-HER2-ECD infection, trastuzumab-resistant (Fig. 6a) as well as low HER2-expressing cells (Fig. 6b, c) were more efficiently killed by ADCC, and a significant difference was

detected at all effector/target ratios in all cell lines, except trastuzumab-resistant SKBR3 cells, as compared to mock- or control dl312-infected cells. Thus, Ad-HER2-ECD-mediated exogenous expression of HER2-ECD could sensitize trastuzumab-resistant HER2-downregulated cells or low HER2-expressing cells to trastuzumab through ADCC activation in vitro.

Discussion

The nature of acquired resistance to trastuzumab is an area of active research in both the laboratory and the clinic. In the present study, we exposed HER2-positive breast cancer cells to trastuzumab continuously in vitro to induce resistance against this antibody and investigate the mechanisms