Supplementary Materials

Sasaki et al.

Preclinical Evaluation of Telomerase-specific Oncolytic Virotherapy for Human Bone and Soft Tissue Sarcomas

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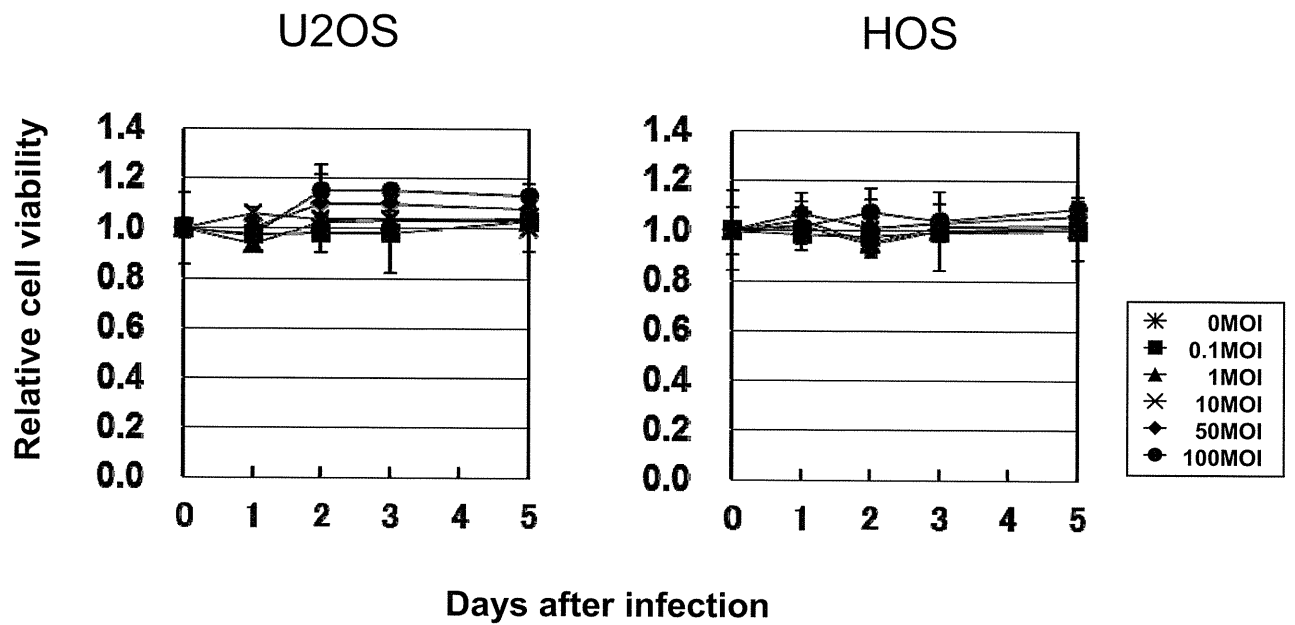
Supplementary Table 1
Upregulation of *hTERT* mRNA expression afterOBP-301 infection on human bone and soft tissue sarcoma cell lines

Supplementary Methods

Orthotopic subcutaneous SYO-1 tumor xenograft model

Animal experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Okayama University School of Medicine. Female athymic nude mice aged 6 to 7 weeks (Charles River Laboratories) were inoculated in the flank with 1×10^7 SYO-1 cells per site. Palpable tumors developed within 14 days and were permitted to grow to approximately 5-6 mm in diameter. At that stage, a 50 μl volume of solution containing OBP-301 or PBS was injected into the tumors. Tumor size was monitored by measuring the tumor length and width using calipers, and volumes were calculated using the formula: (L x W^2) x 0.5, where L is the length and W is the width of each tumor.

U2OS

HOS

Relative cell viability

Days after infection

* 0MOI
■ 0.1MOI
▲ 1MOI
✳ 10MOI
◆ 50MOI
● 100MOI

Supplementary Fig. S1.

Cytopathic effect of dl312 on human osteosarcoma U2OS and HOS cell limes. Cells were infected with dl312 at the indicated multiplicity of infection (MOI), and cell survival was quantified over 5 days using the XTT assay. Data are means ± SD.
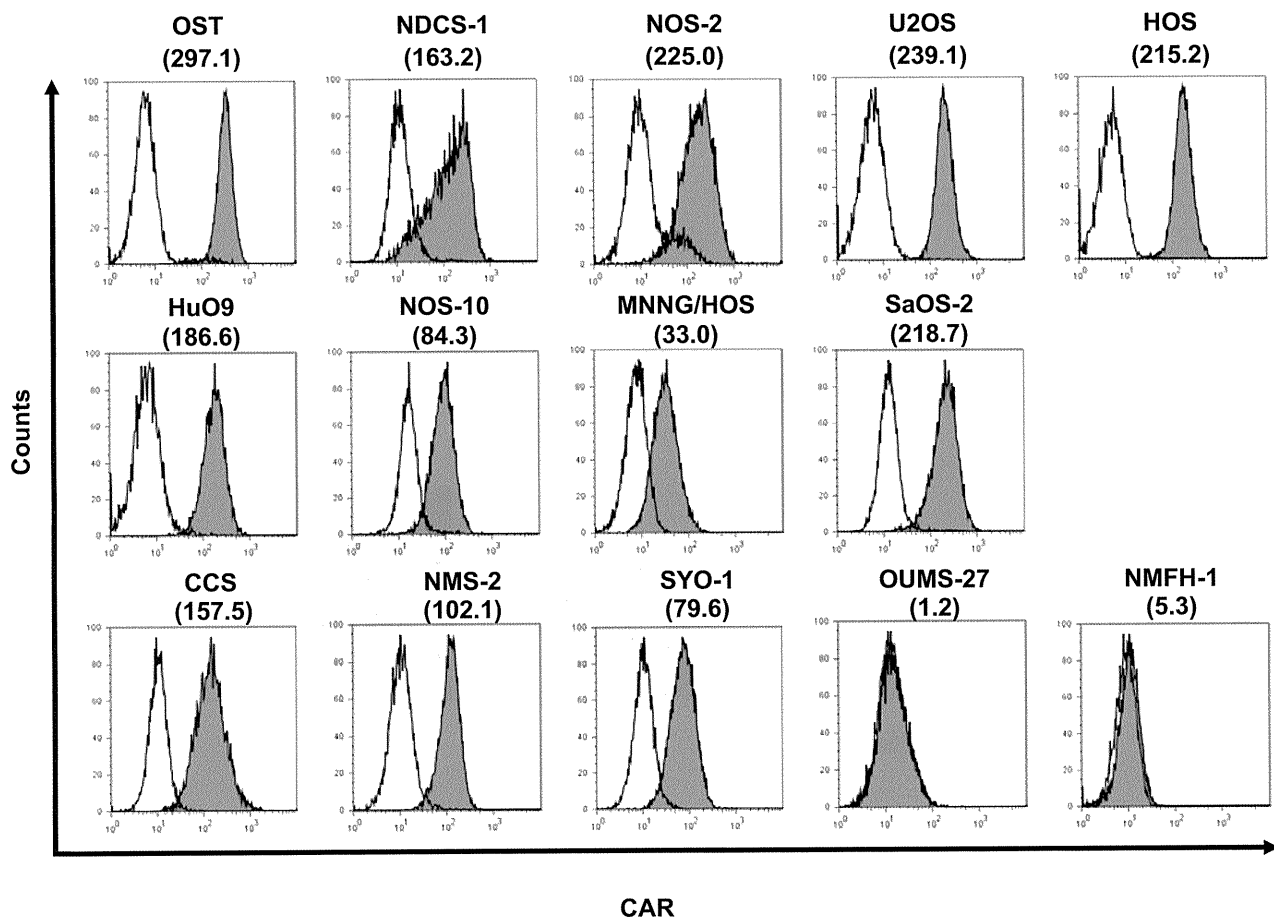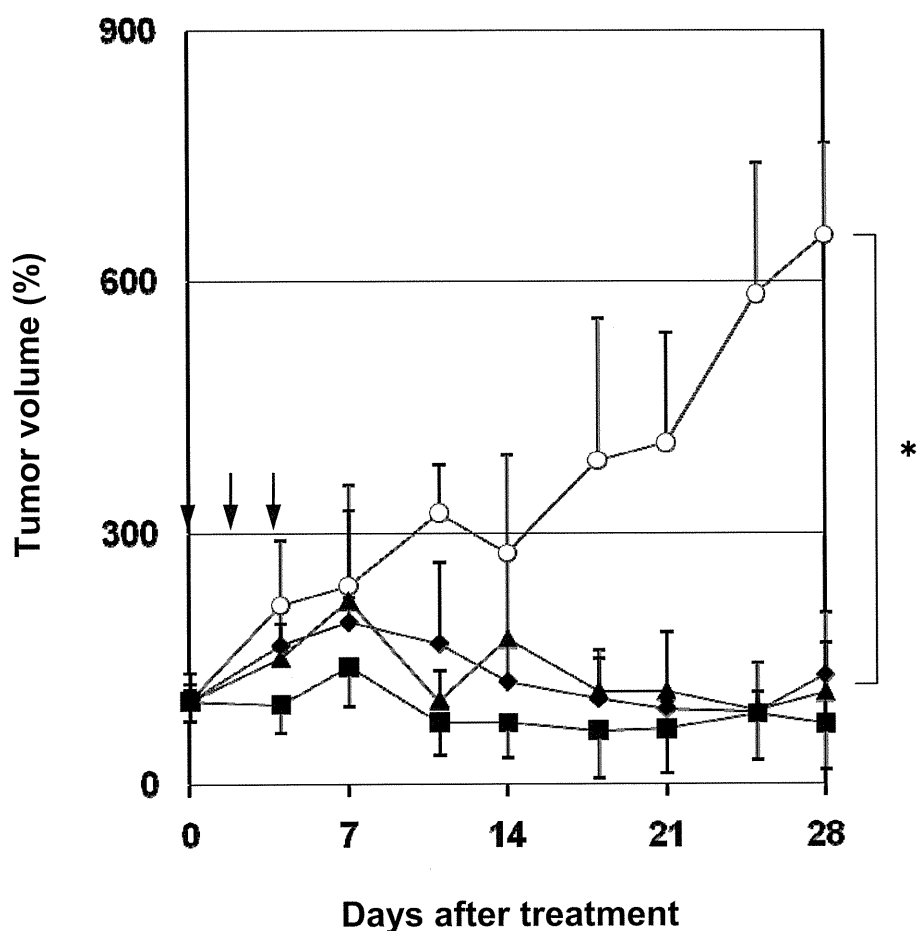
Supplementary Fig. S2

CAR expression levels on human bone and soft tissue sarcoma cell lines.
CAR expression levels on human bone and soft tissue sarcoma cell lines were
analyzed using flow cytometry. The cells were incubated with a monoclonal
anti-CAR (RmcB) antibody, followed by detection using a FITC-labeled
secondary antibody. The mean fluorescence intensity (MFI), which is a
measure of CAR expression, was calculated for each cell line and is indicated
in parentheses.
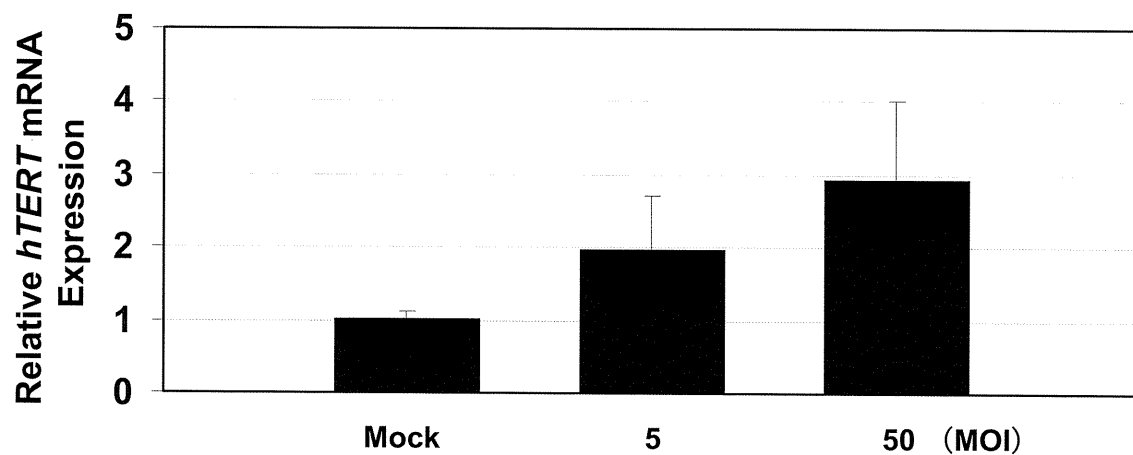
Supplementary Fig. S3

Expression of *hTERT* mRNA after infection with OBP-301 on OST human osteosarcoma cells. OST cells were infected with OBP-301 at an indicated MOIs and analyzed for *hTERT* mRNA expression 2 days after infection. OBP-301 infection at 5 and 50 MOI increased the *hTERT* mRNA expression 1.9- and 2.9- fold, respectively, relative to mock-infection in OST cells. Data are means ± SD.
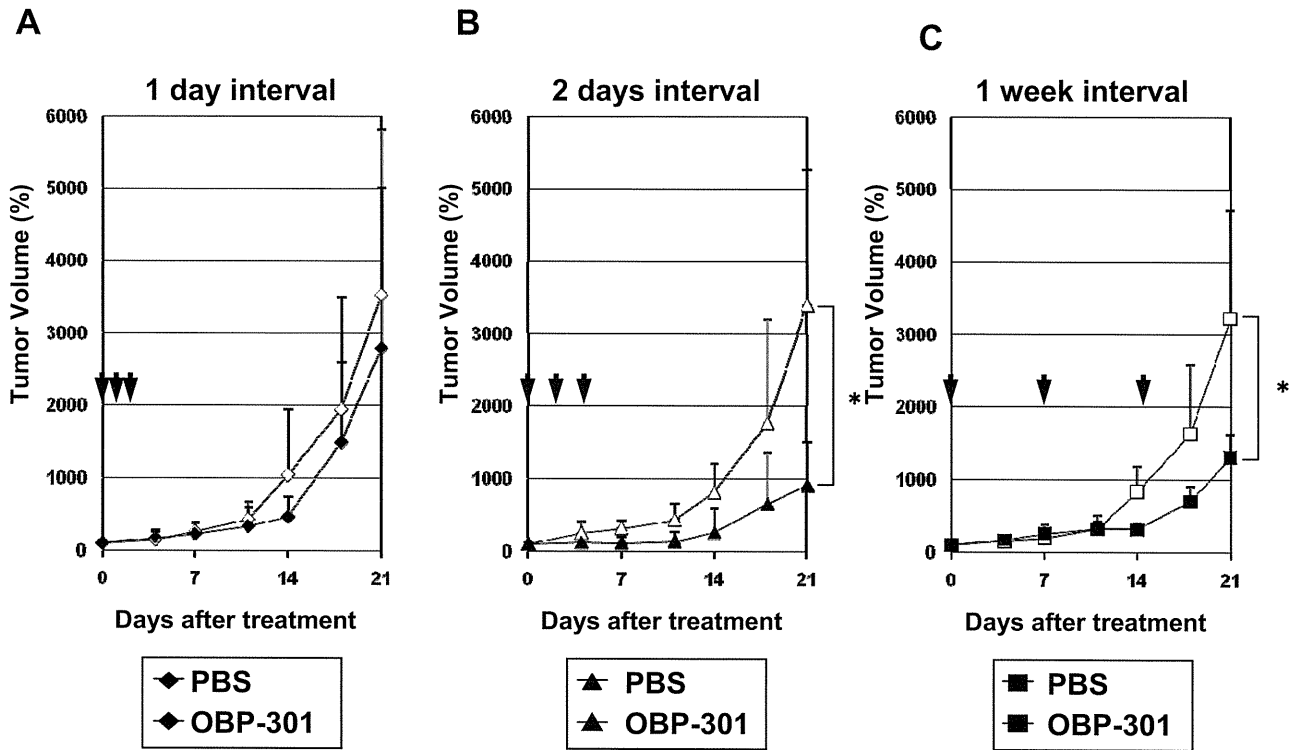
Supplementary Fig. S4

Antitumor effect of OBP-301 in an orthotopic OST bone sarcoma xenograft model. Athymic nude mice were inoculated subcutaneously with OST cells (5×10^6 cells/site). Fourteen days after inoculation (designated as day 0), OBP-301 was injected into the tumor using an MOI of 1×10^7 (◆), 1×10^8 (▲) or 1×10^9 (■) PFU on days 0, 2 and 4. PBS (O) was used as a control. Six mice were used for each group. Tumor growth was expressed as the mean tumor volume ± SD. Statistical significance (*) was determined as $P < 0.05$ (Student's t test).

Supplementary Fig. S5

Antitumor effect of repeated intratumoral injection of OBP-301 at different intervals in an orthotopic subcutaneous SYO-1 soft tissue sarcoma xenograft model. Athymic nude mice were inoculated subcutaneously with SYO-1 cells (1×10^7 cells/site). Fourteen days after inoculation, OBP-301 was injected three times into the tumor using an MOI of 1×10^9 PFU and using 1 day- (A), 2 day- (B) or 1 week- (C) intervals between injections. PBS was used as a control. Six mice were used for each group. Tumor growth was expressed as the mean tumor volume $\pm$ SD. Statistical significance (*) was determined as $P < 0.05$ (Student's t test).

Supplementary Table 1 Upregulation of hTERT mRNA expression after OBP-301 infection on human bone and soft tissue sarcoma cell lines

| Cell line | Tumor origin* | ID50 | Relative hTERT mRNA express | | Ratio[†] |
			Pre-infetcion	Post-infection	
MNNG/HOS	BS	97.3	1.0	7.1	7.1
NOS-10	BS	48.2	3.7	50.8	13.7
HuO9	BS	48.2	0.02	1.0	50.0
HOS	BS	44.6	4.3	7.1	1.7
U2OS	BS	38.0	0.3	1.3	4.3
NOS-2	BS	30.8	1.1	5.0	4.5
NDCS-1	BS	29.3	16.4	31.0	1.9
OST	BS	18.1	7.5	4.2	0.6
SYO-1	STS	38.2	35.2	99.4	2.8
NMS-2	STS	29.2	7.1	7.8	1.1
CCS	STS	17.6	13.1	22.8	1.7

*BS-Bone sarcoma; STS- Soft tissue sarcoma
†Ratio = Post-infection/Pre-infection

Clinical Cancer Research

Enhanced Safety Profiles of the Telomerase-Specific Replication-Competent Adenovirus by Incorporation of Normal Cell-Specific microRNA-Targeted Sequences

Kumiko Sugio[1,3], Fuminori Sakurai[1,3], Kazufumi Katayama[1], Katsuhisa Tashiro[3], Hayato Matsui[1,3], Kenji Kawabata[2,3], Atsushi Kawase[4], Masahiro Iwaki[4], Takao Hayakawa[5], Toshiyoshi Fujiwara[6], and Hiroyuki Mizuguchi[1,3]

Abstract

Purpose: Oncolytic adenoviruses (Ad) have been actively pursued as potential agents for cancer treatment. Among the various types of oncolytic Ads, the telomerase-specific replication-competent Ad (TRAD), which possesses an *E1* gene expression cassette driven by the human telomerase reverse transcriptase promoter, has shown promising results in human clinical trials; however, the *E1* gene is also slightly expressed in normal cells, leading to replication of TRAD and cellular toxicity in normal cells.

Experimental Design: To overcome this problem, we utilized a microRNA (miRNA)-regulated gene expression system. Four copies of complementary sequences for miR-143, -145, -199a, or let-7a, which have been reported to be exclusively downregulated in tumor cells, were incorporated into the 3′-untranslated region of the *E1* gene expression cassette.

Results: Among the TRAD variants (herein called TRADs) constructed, TRADs containing the sequences complementary to miR-143, -145, or -199a showed efficient oncolytic activity comparable to the parental TRAD in the tumor cells. On the other hand, replication of the TRADs containing the miRNA complementary sequences was at most 1,000-fold suppressed in the normal cells, including primary normal cells. In addition, to suppress the replication of the TRADs in hepatocytes as well as other normal cells, we constructed a TRAD containing 2 distinct complementary sequences for miR-199a and liver-specific miR-122a (TRAD-122a/199aT). TRAD-122a/199aT exhibited more than 10-fold reduction in viral replication in all the normal cells examined, including primary hepatocytes.

Conclusions: This study showed that oncolytic Ads containing the sequences complementary to normal cell-specific miRNAs showed significantly improved safety profiles without altering tumor cell lysis activity. *Clin Cancer Res; 17(9); 2807–18. ©2011 AACR.*

Introduction

Oncolytic adenoviruses (Ad) are genetically engineered Ads which can kill tumor cells by tumor cell-specific replication (1, 2). Several clinical trials using oncolytic Ads have been carried out, and promising results have been reported (3–5). Various types of oncolytic Ads have been developed, and can be mainly classified into 2 groups. One type of oncolytic Ads show tumor-selective replication via deletion of certain genes, such as the *E1B-55K* gene, which are dispensable for the replication of Ads in tumor cells. The other type of oncolytic Ads possess an *E1* gene expression cassette driven by tumor-specific promoters. Various types of tumor-specific promoters are used in oncolytic Ads, including the α-fetoprotein promoter (6), prostate-specific antigen promoter (7), osteocalcin promoters (8), and cyclooxygenase-2 promoter (9).

Among these oncolytic Ads possessing tumor-specific promoters, the telomerase-specific replication-competent

Authors' Affiliations: Departments of [1]Biochemistry and Molecular Biology and [2]Biomedical Innovation, Graduate School of Pharmaceutical Sciences, Osaka University; [3]Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation; [4]Department of Pharmacy, School of Pharmacy, [5]Pharmaceutical Research and Technology Institute, Kinki University, Osaka; and [6]Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Fuminori Sakurai, Department of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan. Phone: 81-6-6879-8188; Fax: 81-6-6879-8186; E-mail: sakurai@phs.osaka-u.ac.jp or Hiroyuki Mizuguchi, Department of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka, 565-0871, Japan. Phone: 81-6-6879-8185, Fax: 81-6-6879-8186; E-mail: mizuguch@phs.osaka-u.ac.jp

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Translational Relevance

Oncolytic adenoviruses (Ad) are promising anticancer agents and have been used in human clinical trials. However, though a lesser extent than in tumor cells, some oncolytic Ads also replicate in normal human cells, resulting in unexpected toxicity. In this study, we included a microRNA (miRNA)-regulated posttranscriptional detargeting system into a telomerase-specific replication-competent Ad (TRAD), which has been used in clinical trials. Complementary sequences for miR-143, -145, and -199a, which have been shown to be exclusively downregulated in tumor cells, were inserted into the E1 gene expression cassette. The TRAD containing these miRNA complementary sequences exhibited significantly reduced replication in normal cells (up to 1,000-fold reductions), including human primary cells, and comparable tumor cell lysis activity to the conventional TRAD. These results indicate that an miRNA-regulated posttranscriptional detargeting system offers a potential strategy to reduce the replication of TRAD in normal cells without altering tumor cell lysis activity, and makes it possible to increase the injected doses, leading to enhanced antitumor effects.

Ad (TRAD; also known as Telomelysin), which has an *E1* gene expression cassette driven by the human telomerase reverse transcriptase (hTERT) promoter, is one of the most promising oncolytic Ads (10, 11). A variety of tumor cells express telomerase and most normal cells do not, leading to tumor-selective efficient replication of TRAD. A phase I clinical trial using TRAD has already been carried out, and antitumor effects were shown in several patients (3). Combined therapy using anticancer agents and TRAD also has been shown to provide enhanced antitumor effects compared with either treatment alone (12). Another advantage of TRAD is that TRAD exerts antitumor effects on distant, uninjected tumors following intratumoral administration. TRAD efficiently replicates in the injected tumors and is disseminated from the injected tumors into the systemic circulation, resulting in infection of distant tumors with TRAD (11, 13). This property of TRAD has made it possible to visualize lymph mode metastasis by inclusion of the green fluorescence protein (GFP)-expression cassette into TRAD (14). However, these properties have led to the concern that TRAD also infects normal cells throughout the body after their dissemination from the injected tumors. Some oncolytic Ads, including TRAD, replicate to some extent in normal human cells although tumor-specific promoters are used probably because low levels of E1A expression can sufficiently support the replication of Ads (15). Previous studies have shown a more than 100-fold increase in Ad genome copy numbers in human primary fibroblasts 3 days after infection with TRAD (10, 11). Replication of TRAD in normal human cells might lead to unexpected cellular toxicity, therefore, in addition to a tumor-specific promoter, a system which can prevent

the replication of TRAD in normal human cells should be incorporated into TRAD.

To achieve this goal, we utilized a microRNA (miRNA)-regulated gene expression system. MiRNAs are small noncoding RNAs of approximately 22-nt in length, and are endogenously expressed. MiRNAs bind to imperfectly complementary sequences in the 3′-untranslated region (UTR) of the target mRNA leading to the suppression of gene expression via posttranscriptional regulation. More than 800 miRNAs have been identified and have been shown to be expressed in tissue- and cell-type-specific patterns. Furthermore, recent studies have shown that several miRNAs, including miR-143, -145, and let-7, are specifically downregulated in tumor cells, compared with normal cells (16–20). Thus we hypothesized that incorporation of the complementary sequences for miRNAs selectively downregulated in tumor cells into the *E1* expression cassette would prevent the replication of TRADs in normal human cells without altering the antitumor effects.

In the present study, miR-143, -145, -199a, and let-7a were selected as the miRNAs exclusively downregulated in tumor cells. Four copies of sequences perfectly complementary to these miRNAs were inserted into the 3′-UTR of the *E1* gene expression cassette in TRADs. TRADs containing the target sequences for miR-143, -145, or -199a exhibited not only efficient oncolytic activities comparable to the parental TRAD, but also significantly reduced levels of replication (up to 1,000-fold reductions) in normal cells, including human primary cells. Furthermore, insertion of sequences complementary to liver-specific miR-122a into the *E1* gene expression cassette, in addition to the miR-199a target sequences, resulted in a decrease in virus replication in primary hepatocytes as well as other primary cells.

Materials and Methods

Cells

A549 (a human non–small cell lung cancer cell line), HepG2 (a human hepatocellular carcinoma cell line), and 293 cells (a transformed embryonic kidney cell line) were cultured in Dulbecco's modified Eagle's Medium containing 10% fetal bovine serum (FBS) and antibiotics. HT29 (a human colorectal cancer cell line) and WI38 cells (a normal human lung diploid fibroblast) were cultured in Minimum Essential Medium containing 10% FBS and antibiotics. H1299 cells (a human non–small cell lung cancer cell line) were cultured in RPMI1640 containing 10% FBS and antibiotics. These cell lines were obtained from the cell banks, including the Japanese Collection of Research Bioresources (JCRB) cell bank. The normal human lung fibroblasts (NHLF), normal human prostate stromal cells (PrSC), normal human small airway epithelial cells (SAEC), and normal human hepatocytes (Nhep; Lonza) were cultured in the medium recommended by the manufacturer.

Construction of TRADs

All TRADs were prepared by means of an improved *in vitro* ligation method described previously (21–23). hTERT

promoter-driving *E1* gene-expressing shuttle plasmids having multiple tandem copies of sequences perfectly complementary to miRNAs in the 3′-UTR of the *E1* gene expression cassette were constructed as described below. A *KpnI/Afl*III fragment of pHMCMV5 (22) was ligated with oligonucleotides miR-143T-S1 and miR-143T-AS1, which contain miR-143 complementary sequences, resulting in pHMCMV5-143T-1. The sequences of the oligonucleotides are shown in Supplementary Table S1. Next, a *PacI/Afl*III fragment of pHMCMV5-143T-1 was ligated with oligonucleotides miR-143T-S2 and miR-143T-AS2. The resulting plasmid, pHMCMV5-143T, was digested with *I-Ceu*I after digestion with *Nhe*I followed by *Klenow* treatment, and then ligated with the *I-CeuI/Pme*I fragment of pSh-hAIB (10), in which the *E1A* and *E1B* genes linked with an internal ribosomal entry site (IRES) are located downstream of the hTERT promoter, creating pSh-AIB-143T. For the construction of vector plasmids for TRADs, *I-CeuI/PI-Sce*I-digested pSh-AIB-143T was ligated with the *I-CeuI/PI-Sce*I-digested pAdHM3 (21), resulting in pAdHM3-AIB-143T. To generate TRADs, pAdHM3-AIB-143T was digested with *Pac*I and was transfected into 293 cells using Superfect transfection reagent (Qiagen). All TRADs were propagated in 293 cells, purified by 2 rounds of cesium chloride gradient ultracentrifugation, dialyzed, and stored at −80°C. TRADs containing other miRNA complementary sequences were similarly constructed using the corresponding oligonucleotides (Supplementary Table S1). The parental TRAD was similarly prepared using pSh-AIB and pAdHM3. The virus particles (VP) and biological titers were determined by a spectrophotometrical method (24) and by using an Adeno-X rapid titer kit (Clontech), respectively. The ratio of particle-to-biological titer was between 6 and 9 for each TRAD used in this study.

Determination of miRNA expression levels in human normal and tumor cells

Total RNA, including miRNAs, was isolated from cells using Isogen (Nippon Gene). After quantification of the RNA concentration, miRNA levels were determined using a TaqMan MiRNA reverse transcription kit, TaqMan miRNA assay kit, and ABI Prism 7000 system (Applied Biosystems). Amplification of U6 served as an endogenous control to normalize the miRNA expression data.

Infection with TRADs

Cells were seeded into 24-well plates at 5×10^4 cells/well. On the following day, cells were infected with TRADs at a multiplicity of infection (MOI) of 0.4 or 2 (for cancer cell lines), or of 10 (for normal cells), for 2 hours. Following incubation for 3 (for cancer cell lines) or 5 days (for normal cells), total DNA, including viral genomic DNA, was isolated from the cells using a DNeasy Blood & Tissue Kit (Qiagen). After isolation, the Ad genomic DNA contents were quantified using an ABI Prism 7000 system (Applied Biosystems) as previously described (25). The Ad genome copy numbers were normalized by the copy numbers of

glyceroaldehyde-3-phosphate-dehydrogenase (GAPDH). Cell viability was also examined by crystal violet staining and Alamar blue assay at the indicated time points. To examine the miRNA-specific suppression of TRAD replication in normal human cells, 50 nmol/L of 2′-*O*-methylated antisense oligonucleotide complementary to miR-143 or miR-199a (Gene Design Inc.) was transfected into normal cells using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the cells were infected with TRADs and replication of TRADs was evaluated as described above.

Real-time reverse transcriptase PCR analysis for *E1A* gene expression

Cells were seeded as described above and were infected with TRADs at an MOI of 2 (for cancer cells) or 10 (for normal cells) for 1.5 hours. After a 24 hour-incubation, total RNA was isolated, and reverse transcription reaction was carried out using a SuperScript II First-Strand Synthesis System (Invitrogen). E1A mRNA levels were determined with the E1A-specific primers and probe using an ABI prism 7000 system (26). The E1A mRNA levels were normalized by the GAPDH mRNA levels.

Statistical analysis

Statistical significance ($P < 0.05$) was determined using Student's *t* test. Data are presented as means ± SD.

Results

Replication of the conventional TRAD in normal human cells

First, to examine replication of the conventional TRAD in normal human cells, WI38 cells, which are human embryonic lung fibroblasts, were infected with the conventional TRAD at an MOI of 2 or 10 (Fig. 1). The conventional TRAD did not highly replicate in WI38 cells at an MOI of 2; however, an almost 500-fold increase in the Ad genome
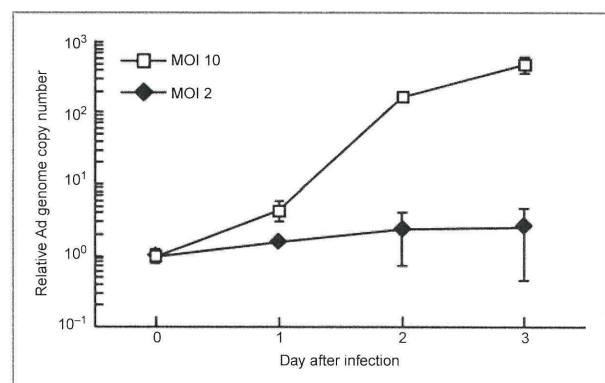

Figure 1. Replication of the conventional TRAD in WI38 cells. WI38 cells were infected with the conventional TRAD at an MOI of 2 or 10 for 2 hours. At the indicated time points, the copy numbers of the Ad genome and *GAPDH* gene were determined by real-time PCR. The ratio of the copy number of the Ad genome to that of GAPDH was normalized by the data on day 0. The data are shown as the means ± SD ($n = 3$).
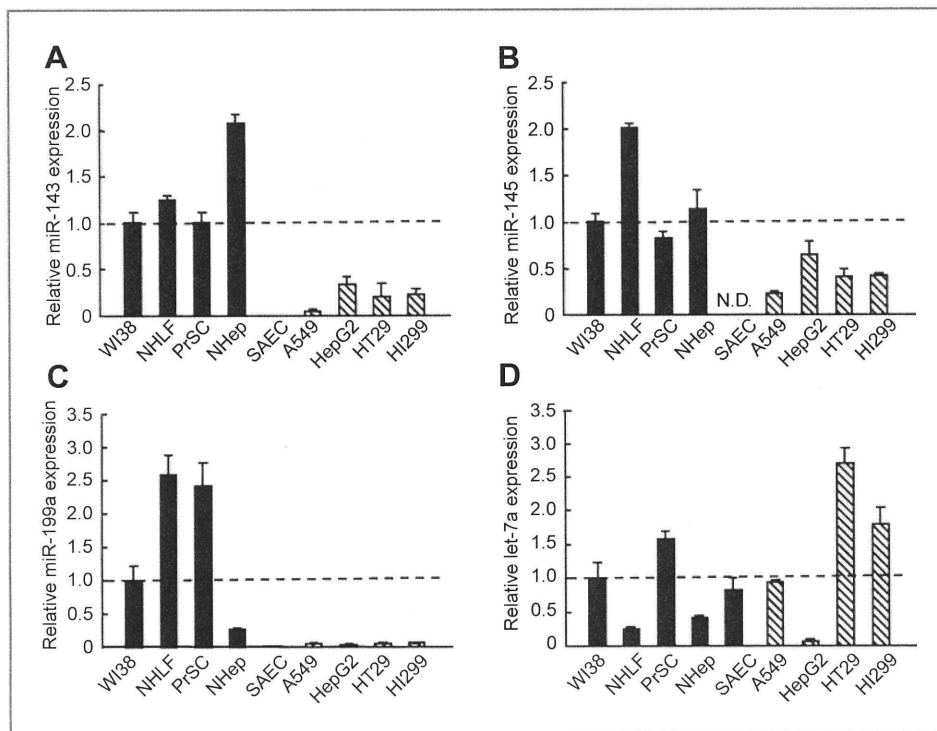
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Figure 2. MiRNA expression levels in the human normal (solid bar) and tumor cells (hatched bar). MiRNA expression was determined by real-time RT-PCR. The ratio of miRNA to U6 expression levels was normalized by the data of WI38 cells. The data are shown as the means ± SD ($n = 3$). N.D., not detected.

was found 3 days after infection at an MOI of 10. These data indicate that the conventional TRAD replicates in normal human cells at a high MOI, even though tumor-specific hTERT promoters are used for the E1 gene expression.

MiRNA expression levels in human tumor and normal cells

To examine the expression levels of miR-143, -145, -199a, and let-7a in the human normal and tumor cells, reverse transcriptase PCR (RT-PCR) analysis was carried out. Several studies have shown that these miRNAs are downregulated in various types of tumor cells isolated from cancer patients, compared with the corresponding normal tissues (16–18, 27). The expression levels of miR-143, -145, and -199a in the tumor cells were approximately 2- to 100-fold lower than those in the normal cells, although SAECs expression levels of miR-143, -145, and -199a were comparable or lower than those in the tumor cells (Fig. 2). In particular, a large reduction was found for miR-199a expression in all tumor cells, compared with the normal cells. On the other hand, the expression levels of let-7a in HT29 and H1299 cells were higher than those in the normal cells, although HepG2 cells expressed lower levels of let-7a than the normal cells. The absolute amounts of let-7a were more than 10-fold higher than those of the other miRNAs in all tumor and normal cells, except for NHLF, NHep, and HepG2 cells (data not shown).

Development of TRADs carrying an miRNA-regulated E1 gene expression system

Next, to develop TRADs carrying a miRNA-regulated E1 gene expression cassette (TRAD-miRT), we incorporated 4

copies of the perfectly complementary sequences for miR-143, -145, -199a, or let-7a into the 3′-UTR of the E1 gene expression cassette (Fig. 3A). In TRADs, the E1A gene was connected with the E1B gene via IRES. We found that the expression of both the first and second gene in the IRES-containing expression cassette was suppressed in an miRNA-dependent manner by insertion of the miR-122a complementary sequences into the region downstream of the second gene in miR-122a-expressing Huh-7 cells, not in HepG2 cells, which express a low level of miR-122a (Supplementary Fig. S1), although it remains controversial whether miRNA-mediated posttranscriptional regulation can occur in an IRES-containing expression cassette (28–30). All TRADs were efficiently grown in normal 293 cells, and the ratios of infectious titers to physical titers were comparable among all the TRADs, including the parental TRAD.

Tumor cell lysis activity and replication of TRAD-miRT in tumor cells

To examine whether or not the inclusion of the sequences complementary to the miRNAs downregulated in tumor cells would inhibit the tumor cell lysis activity of TRADs, the viability of tumor cells was evaluated after infection with the TRADs. Almost all tumor cells were lysed by TRAD-143T, -145T, and -199aT at 3 days after infection, although cell lysis by TRAD-let7aT was largely inhibited (Fig. 3B). Furthermore, time-course studies of cell viability showed that TRAD-143T, -145T, and -199aT exhibited cytopathic efficacies comparable to that of the parental TRAD in the tumor cells at an MOI of 0.4 (Fig. 3C). Similar results were obtained at an MOI of 2 (data not shown).

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We next examined the replication ability of the TRADs in the tumor cells by determining the viral genome copy numbers. TRAD-143T, -145T, and -199aT efficiently replicated in the tumor cells, and the viral genome copy numbers of TRAD-143T, -145T, and -199aT in the tumor cells were more than 500-fold higher than those in the normal cells (data not shown). In addition, TRAD-143T, -145T, and -199aT exhibited viral genome copy numbers similar to that of the conventional TRAD in all tumor cells (Fig. 3D). All TRADs except for TRAD-let7aT also expressed similar levels of E1A mRNA (Fig. 3E). In contrast, insertion of let-7a complementary sequences largely inhibited the replication in all tumor cells. The E1A mRNA level was also reduced by 42% in H1299 cells infected with TRAD-let7aT. Inefficient replication of TRAD-let7aT in the tumor cells corresponded to the low cytopathic effects described above. These results indicate that TRADs containing the complementary sequences for miR-143, -145, or -199a exhibit efficient E1 gene expression in the tumor cells and tumor cell lysis activity comparable to those of the conventional TRAD.

Reduced replication of TRAD-miRT in normal cells

To examine whether replication of TRADs in normal cells is suppressed by incorporation of the sequences complementary to the miRNAs downregulated in tumor cells, normal human cells were infected with the TRADs. The virus genome copy numbers of TRAD-143T, -145T, and -199aT were 5- to 1,000-fold reduced, compared with the conventional TRAD at 5 days following infection in WI38 cells (Fig. 4A). An approximately 3- to 300-fold reduction in the genome copy numbers of TRAD-143T, -145T, and -199aT was also observed in NHLF and PrSC. The replication of TRADs was also suppressed in SAEC by the insertion of the miRNA complementary sequences, although the expression levels of miR-143, -145, and -199a in SAEC were much lower than those in the other normal cells (Fig. 2). The suppressive effects of insertion of the miRNA target sequences were different among the cells; however, overall, the insertion of miR-199a complementary sequences mediated similar or higher suppressive effects on the replication of TRADs in all the normal cells examined, compared with insertion of the sequences complementary to miR-143 and -145. Replication of TRAD-199aT was inhibited by more than 10-fold in all the normal cells except for SAEC. We also examined the viabilities of the normal cells after infection with the TRADs. No apparent differences in cell viabilities were found among the TRADs by crystal violet staining (data not shown); however, Alamar blue assay showed that the average values of the normal cell viabilities were higher after infection with TRAD-miRT than after infection with the conventional TRAD (Fig. 4B). These results suggest that the suppression of TRAD replication by insertion of the miRNA complementary sequences results in the improvement of the TRAD safety profile in normal cells.

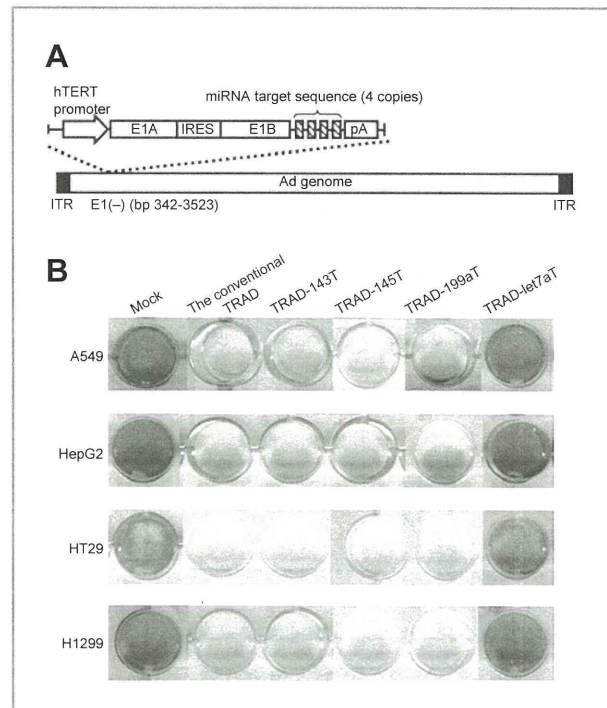Next, to evaluate whether the reduction in replication of TRAD-miRT was miRNA-dependent, miRNAs were inhibited by a 2′-O-methylated antisense oligonucleotide. NHLF and PrSC cells were transfected with the 2′-O-methylated antisense oligonucleotide against miR-143 or -199a, and then the cells were infected with the TRADs, 24 hour after transfection. In the cells transfected with the 2′-O-methylated antisense oligonucleotide against miR-143 or -199a, the reduction in the replication of TRAD-miRT was significantly restored, but the scramble 2′-O-methylated oligonucleotide did not significantly affect the replication of TRAD-miRT (Fig. 4C). These results indicate that the reduction in the replication of TRAD-miRT in the normal cells was miRNA-dependent.

Figure 3. Replication and oncolytic activity of TRADs containing the miRNA complementary sequences in the tumor cells. A, a schematic diagram of a TRAD containing the miRNA-regulated E1 gene expression system. ITR: inverted terminal repeat. B, crystal violet analysis of the cytopathic effects of TRADs in the tumor cells. The cells were infected with the TRADs at an MOI of 2 for 2 hours. Three days after infection, the cells were stained with crystal violet. The results are representative of at least 2 independent experiments. C, time-course study of the tumor cell lysis activity of TRADs by Alamar blue assay. The cells were infected with the TRADs at an MOI of 0.4 for 2 hours. At the indicated time points, the viability of the cells was analyzed by Alamar blue assay. The data were normalized by the data of the mock-infected group. D, the viral genome copy numbers of TRADs in the tumor cells. The cells were infected with the TRADs at an MOI of 2 for 2 hours. Three days after infection, the viral genome copy numbers were quantified by real-time PCR. The data was normalized by the data of the conventional TRAD group. E, the E1A mRNA levels in H1299 cells 24 hour after infection with the TRADs. The cells were infected with the TRADs at an MOI of 2 for 1.5 hours. Twenty-four hours after infection, the E1A mRNA levels were determined by real-time RT-PCR. The data was normalized by the data of the conventional TRAD group. All the data are shown as the means ± SD (n = 3–6). *, $P < 0.05$; **, $P < 0.005$.
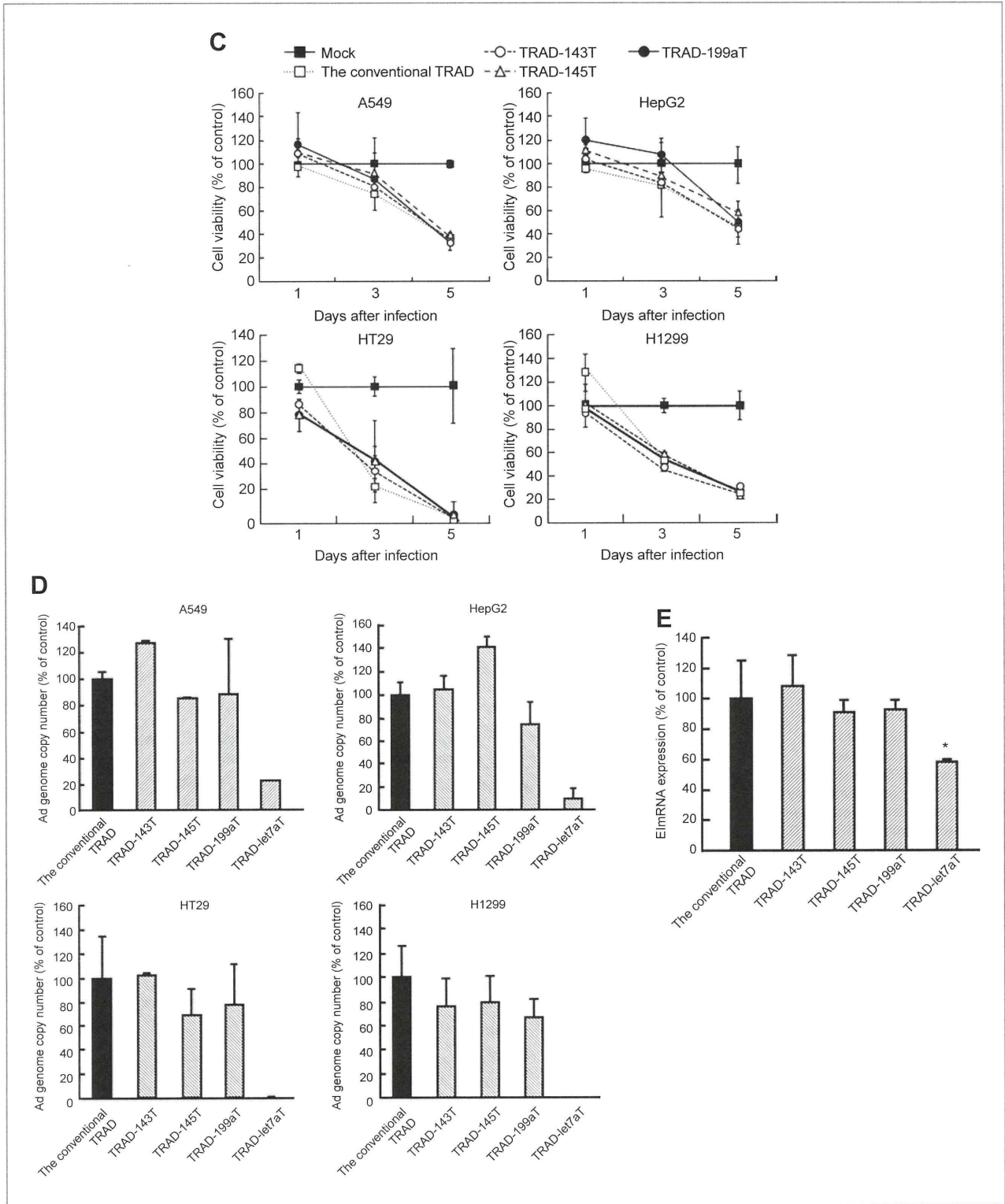
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Figure 3. (*Continued*)

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Figure 4. Reduced replication of TRADs in normal human cells by insertion of the miRNA complementary sequences. A, the viral genome copy numbers of TRADs in normal cells. The cells were infected with the TRADs at an MOI of 10 for 2 hours. Five days after infection, the viral genome copy numbers were determined by real-time PCR. B, time-course study of the normal human cell viabilities after infection with TRADs by Alamar blue assay. The cells were infected with the TRADs at an MOI of 10 for 2 hours. At the indicated time points, the viability of the cells was analyzed by Alamar blue assay. The data were normalized by the data of the mock-infected group. C, restoration of TRAD replication in human normal cells by 2'-O-methylated antisense oligonucleotides. The cells were transfected with 50 nmol/L of 2'-O-methylated antisense oligonucleotides for miR-143 or -199a. Twenty-four hours after transfection, the cells were infected with the TRADs at an MOI of 10, and the viral genome copy numbers were determined 5 days after infection with the TRADs. D, the E1A mRNA levels in normal human cells. The cells were infected with the TRADs at an MOI of 10 for 1.5 hours. Twenty-four hours after infection, the E1 mRNA levels were determined by real-time RT-PCR. The data was normalized by the data of the conventional TRAD group. All the data are shown as the means $\pm$ SD ($n = 3$–4). *, $P < 0.05$; **, $P < 0.005$.
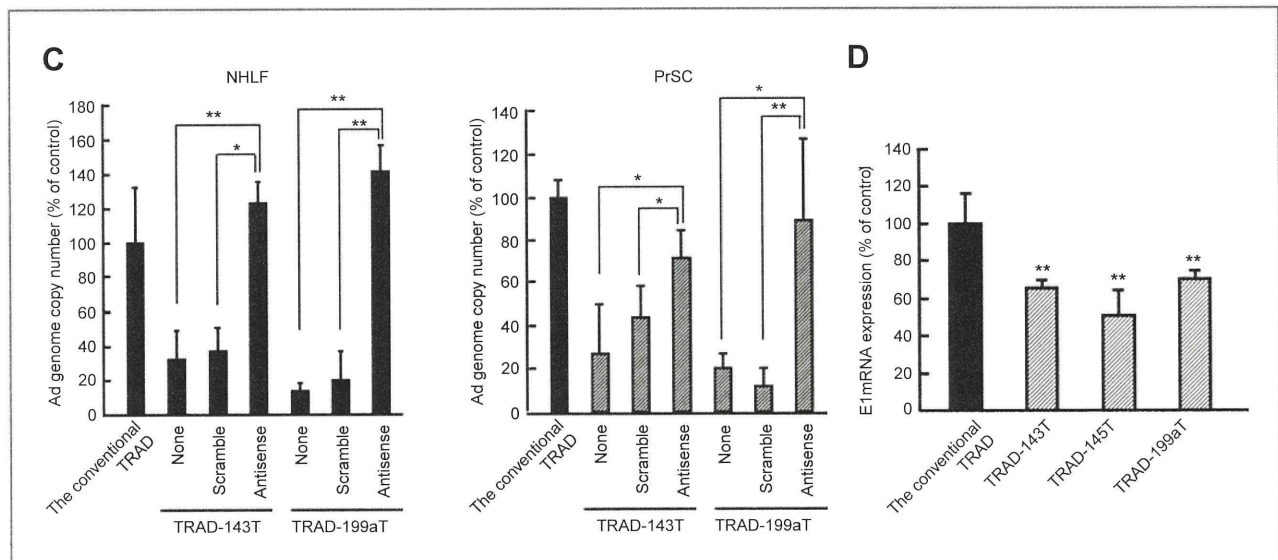
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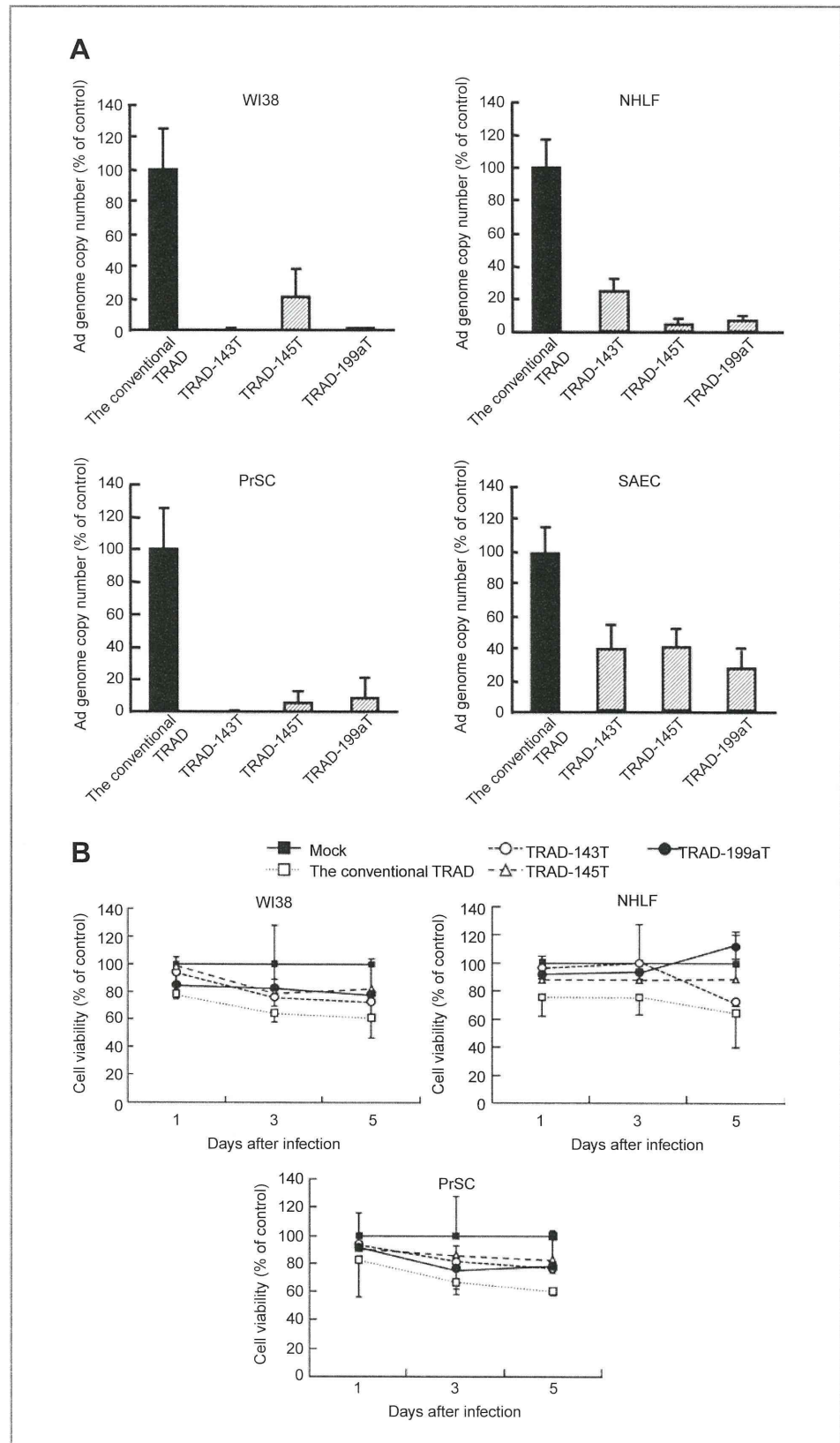
Figure 4. (*Continued*)

E1A expression by TRAD-miRT in normal cells

To determine whether incorporation of the miRNA complementary sequences into the *E1* gene expression cassette decreases the *E1* mRNA levels in normal human cells, real-time RT-PCR analysis for the E1A mRNA levels was carried out. The E1A mRNA levels were reduced by more than 30% for TRAD-143T, -145T, and -199aT, compared with the parent TRAD, in NHLF (Fig. 4D). The reduction in the E1A mRNA levels corresponded to the suppression in replication of TRAD-miRT, indicating that miRNA-mediated reduction in the *E1* gene expression resulted in a reduced replication of TRAD-miRT.

Development of TRADs containing the complementary sequences for liver-specific miRNA

To prevent the replication of TRADs in liver hepatocytes as well as other normal cells, we incorporated not only miR-199a complementary sequences but also sequences complementary to liver-specific miR-122a into the *E1* gene expression cassette, resulting in TRAD-122a/199aT (Fig. 5A). It is well known that Ads have high hepatic tropism, leading to efficient liver accumulation even after local administration. MiR-122a was expressed approximately 100- and 20-fold more abundantly in NHep and Huh-7 cells, respectively, than in the other normal human cells and tumor cells (Fig. 5B); conversely, the other normal cells expressed more than 10-fold lower levels of miR-122a than miR-143, -145, and -199a (data not shown). Incorporation of miR-122a complementary sequences alone significantly reduced the virus genome copy number of TRAD-122aT in NHLF and NHep; however, no statistically significant decrease in the genome copy number of TRAD-122aT was found in PrSC (Fig. 5C). On the other hand, insertion of miR-199a target sequences alone was less efficient than insertion of miR-122a target sequences in NHep, probably due to the lower expression of miR-199a

than miR-122a in NHep. By contrast, insertion of both miR-122a and miR-199a target sequences into the *E1* gene expression cassette efficiently reduced the replication of TRAD-122a/199aT by 10- to 50-fold in all normal cells examined. Significantly reduced replication of TRAD-122a and TRAD-122a/199aT was also found in Huh-7 cells, which are a hepatoma cell line highly expressing miR-122a and are often used as a model of hepatocytes (Supplementary Fig. S2). The incorporation of the miR-145 complementary sequences was also effective for suppressing the TRAD replication in NHep (Supplementary Fig. S3). The E1A mRNA levels were reduced for TRAD-122aT and -122a/199aT in NHep (Fig. 5D). In addition, TRAD-122a/199aT efficiently replicated in the tumor cells, resulting in efficient tumor cell lysis (Fig. 5E and F). These results indicate that replication of the TRADs in various types of normal human cells, including liver hepatocytes, is significantly reduced by insertion of the multiple target sequences to both miR-122a and -199a, without influencing the tumor cell lysis activity.

Discussion

The aim of this study was to prevent the replication of TRADs in normal human cells by incorporation of sequences complementary to miRNAs that are selectively downregulated in tumor cells, without altering the tumor cell lysis activity. Currently, there is no appropriate animal model which fully supports the *in vivo* replication of Ads and evaluation of the *in vivo* toxicity caused by oncolytic Ads, and thus it is important to be cautious in regard to oncolytic Ad-induced toxicity. To prevent the *E1* gene expression and replication of oncolytic Ads in normal cells as much as possible, an miRNA-mediated posttranscriptional detargeting system was included in TRADs, in
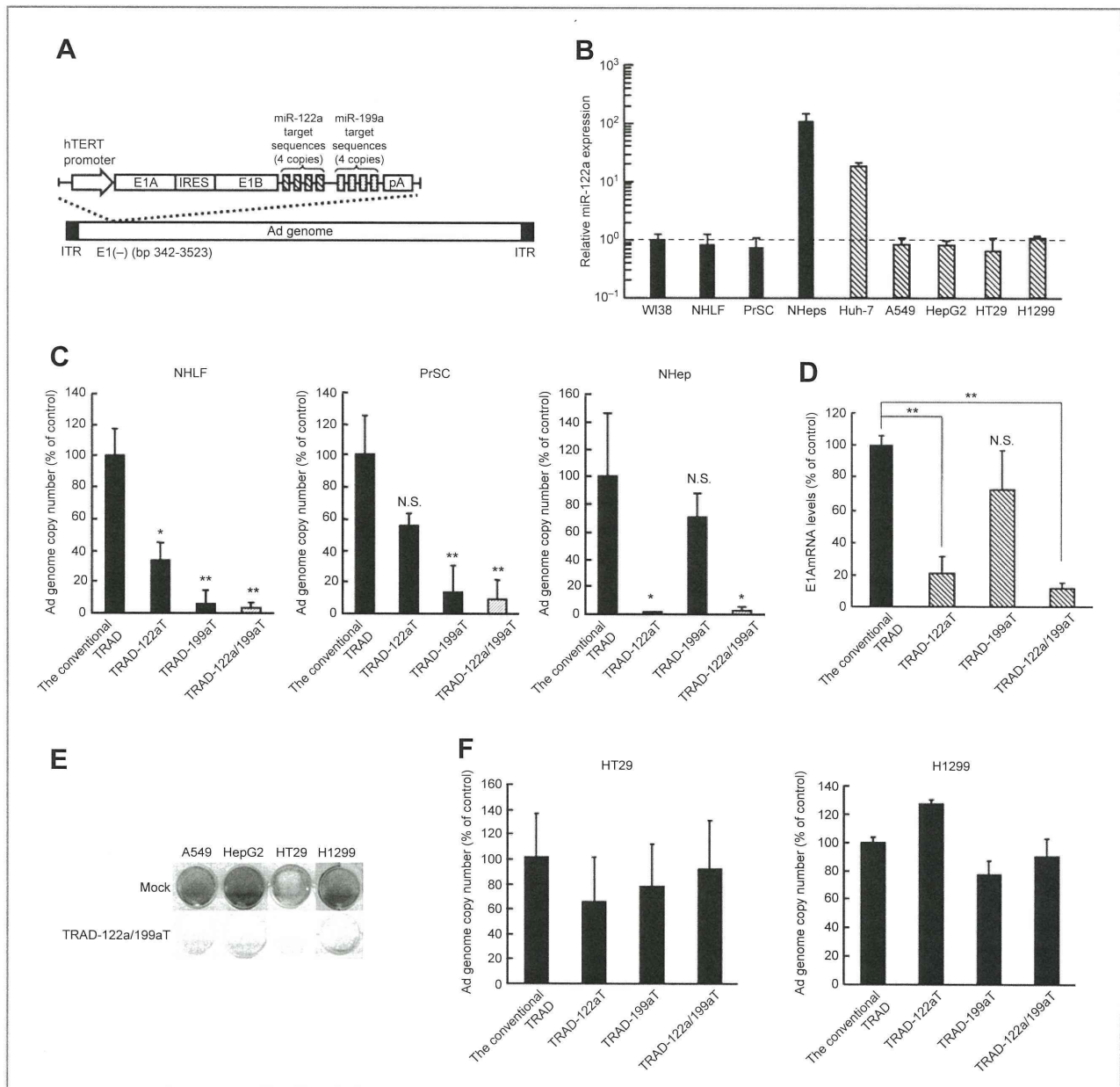
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Figure 5. Tumor cell lysis activity and enhanced safety profile of TRAD-122a/199aT. A, a schematic diagram of TRAD-122a/199aT. B, miR-122a expression levels in the normal and tumor cells. C, the viral genome copy numbers of TRAD-122a/199aT in normal human cells. D, the E1A mRNA levels in NHep. E, crystal violet analysis for the cytopathic effects of TRAD-122a/199aT. The results are representative of 2 independent experiments. F, the viral genome copy numbers of TRAD-122a/199aT in tumor cells. The tumor and normal cells were infected with the TRADs at an MOI of 2 (tumor cells) or 10 (normal cells) for 2 hours. The cells were stained with crystal violet 3 days after infection. The viral genome copy numbers were determined 3 (tumor cells) or 5 days (normal cells) after infection. For determination of the E1A mRNA levels, total RNA was isolated from NHep 24 hour after infection with the TRADs at an MOI of 10, and the E1A mRNA levels were determined by real-time RT-PCR. The data was normalized by the data of the conventional TRAD group. All the data are shown as the means ± SD ($n = 3–6$). N.S.: not significantly different. *, $P < 0.05$; **, $P < 0.005$.

addition to the transcriptional targeting system via tumor-specific promoters.

As described above, TRAD replicates in the injected tumors and is disseminated from the injected tumors into the systemic circulation, leading to infection of distant, uninjected tumors (11, 13, 14). This property of TRAD had led to a concern that TRAD could infect normal cells over

the whole body, including the hepatocytes, after dissemination from the injected tumors. It is crucial that such unexpected infection of normal cells by TRAD is prevented. Previous studies have shown that insertion of sequences complementary to liver-specific miR-122a reduced the replication of oncolytic Ads in Huh-7 cells, which are a model cell for hepatocytes (31–33). It is especially crucial

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to prevent the replication of TRAD in the liver, because Ad vectors have strong hepatotropism. However, TRAD also might infect normal cells other than hepatocytes, indicating that replication of oncolytic Ads in normal cells other than hepatocytes should also be suppressed. To prevent the replication of TRADs in other normal cells, we incorporated the sequences complementary to miR-143, -145, -199a, or let-7a, which are downregulated in the tumors and widely expressed in normal cells. The expression levels of these miRNAs in the tumor cells were lower than those in the normal cells in this study, and insertion of sequences complementary to miR-143, -145, or -199a significantly reduced the E1A mRNA levels and the replication of TRADs in the normal cells.

Overall, among the miRNA complementary sequences, the miR-199a complementary sequences appeared to be the most efficient at suppressing the replication of TRADs across all the normal cells except for hepatocytes; however, insertion of miR-199a target sequences alone failed to significantly reduce the replication of TRADs in the hepatocytes. To simultaneously prevent the replication of TRADs in various types of normal cells, including hepatocytes, we incorporated sequences complementary to miR-122a, which is abundantly expressed in hepatocytes, in addition to miR-199a target sequences. Brown and colleagues reported that a desired transgene expression pattern was achieved, depending on the miRNA expression profile, by incorporation of target sequences for 2 distinct miRNAs (34). TRAD-122aT/199aT exhibited more than 10-fold reduction in the replication in all the normal cells except for SAEC, although insertion of target sequences for miR-122a or miR-199a alone failed to suppress the replication of TRADs in either of the normal cells. Furthermore, TRAD-122aT/199aT and the parental TRAD mediated similar cytopathic efficacies in the tumor cells. These results indicate that replication of TRADs in not only hepatocytes but also other normal cells is simultaneously reduced by insertion of both miR-122a complementary sequences and sequences complementary to miRNAs highly expressed in normal cells, without altering the tumor cell lysis activity.

TRADs containing miR-122a complementary sequences are also considered to be promising for the treatment of liver cancer because miR-122a is significantly downregulated in liver cancer cells (35–37) leading to efficient replication and lytic activity of TRADs containing miR-122a complementary sequences in liver cancer cells. This study has shown that TRAD-122aT/199aT caused efficient cell lysis in a hepatocellular carcinoma cell line, HepG2 cells, while the replication of TRADs containing the miR-122a complementary sequences in normal hepatocytes, which highly express miR-122a, was significantly inhibited.

The expression levels of miRNAs are a crucial factor to suppress the gene expression by miRNAs. Brown and colleagues showed that miRNAs should be expressed at a concentration above the threshold (>100 copies/pg small RNA) to induce miRNA-regulated suppression of transgene expression (34). We were not able to precisely show the expression levels of miRNAs as the ratio of copies/pg small

RNA in this study; however, comparing the miRNA levels in this study with those reported by Brown and colleagues (34), we consider that the expression levels of miR-143, -145, and -199a in the normal cells were higher than 100 copies/pg small RNA, leading to efficient suppression of the replication of TRADs.

Several studies have shown that let-7, including let-7a, is significantly downregulated in tumor cells (16, 19, 20). Edge and colleagues reported that insertion of let-7a complementary sequences into the matrix protein expression cassette of the vesicular somatitis virus (VSV) suppressed the replication of VSV in human primary fibroblast MG38 cells; on the other hand, VSV carrying let-7a target sequences efficiently replicated in A549 cells (38). However, our data showed that cancer cell lines other than HepG2 cells expressed similar or higher levels of let-7a than the normal cells. In addition, the expression levels of let-7a were more than 10-fold higher than those of the other miRNAs in the tumor cells. Abundant let-7a expression leads to a reduction in the replication of TRAD-let7aT in tumor cells. Furthermore, the members of the let-7 family, including let-7b and let-7c, have the same seed sequence, suggesting that let-7 family members other than let-7a would also contribute to the significant suppression of replication of TRAD-let7aT. These results suggest that not only expression profiles of miRNAs but also absolute amounts of miRNA expression in the cells are of great importance for miRNA-regulated gene expression.

Our data showed that the E1A mRNA levels were reduced by approximately 30% to 50% for TRAD-143T, -145T, and -199aT, compared with the conventional TRAD 24 hour after infection with the normal cells. These reduction levels in the E1A mRNA were much smaller than those in the Ad genome copy numbers at 5 days after infection; however, these reductions in the E1A mRNA levels would lead to large differences in the Ad genome copy numbers after several virus replication cycles. More than 5-fold reductions in the E1A mRNA were found for TRAD-143T, -145T, and -199aT, compared with the parental TRAD, 5 days after infection with the normal cells (data not shown).

A phase I clinical trial of the parental TRAD was conducted, and serious adverse events were not observed (3). In this study, efficient replication of the conventional TRAD in WI38 cells was found at an MOI of 10; however, the conventional TRAD did not exhibit a high level of replication at an MOI of 2. It might be unlikely that such a high titer (MOI 10) of oncolytic Ad would infect organs distal from the injection points in clinical trials; however, normal cells around the injection points might be infected with a high titer of oncolytic Ad. In addition, even though no apparent replication of TRADs is observed in normal cells after infection of TRADs, the expression of Ad proteins, including E1A and E4 proteins, affects the cellular functions via various mechanisms (39–41). This study indicates that inclusion of an miRNA-regulated *E1* gene expression system in oncolytic Ads enhances the safety of oncolytic Ads and makes it possible to increase the injection doses, leading to superior therapeutic effects.

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In summary, we developed TRADs in which the *E1* gene expression is controlled by miRNAs more highly expressed in normal cells than tumor cells. The TRADs containing the sequences complementary to miR-143, -145, or -199a exhibited reduced replication in the normal cells without altering the tumor cell lysis activity. Furthermore, incorporation of both miR-199a and miR-122a target sequences significantly suppressed the replication in all human primary cells examined, including hepatocytes. TRAD-miRT has enhanced both the safety profiles and comparable tumor cell lysis activity to the parental TRAD, suggesting that TRAD-miRT offers great potential for the treatment of tumors.

Disclosure of Potential Conflicts of Interest

Toshiyoshi Fujiwara and Hiroyuki Mizuguchi are consultants to Oncolys BioPharma, Inc. No other potential conflicts of interest were disclosed.

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