Figure 5. *In vitro* and *in vivo* antitumor effects of OBP-405 on OBP-301–resistant human sarcoma cell lines. A, expression of the integrins αvβ3 (left) and αvβ5 (right) on OUMS-27 and NMFH-1 cells. The cells were incubated with a monoclonal anti-αvβ3 integrin (LM609) or an anti-αvβ5 integrin (P1F6), followed by flow cytometric detection using an FITC-labeled secondary antibody. The gray histogram represents integrin antibody staining. The number at the top right-hand corner of each graph is the MFI. B, cytopathic effect of OBP-405 on OUMS-27 and NMFH-1 cells. The cells were infected with OBP-405 at the indicated MOI values, and cell survival over 5 days was quantified using an XTT assay. C, antitumor effect of OBP-405 in a subcutaneous OUMS-27 xenograft tumor model. Athymic nude mice were inoculated subcutaneously with OUMS-27 cells (5×10^6 cells/site). Fourteen days after inoculation (designated as day 0), OBP-301 (▲) or OBP-405 (■) was injected into the tumor, with 1×10^8 PFUs on days 0, 2, and 4. PBS (○) was used as a control. Ten 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).

suppression of tumor growth, whereas intervals of 1 day between injections were not effective. These results suggest that an interval of more than 2 days between injections is necessary to efficiently suppress tumor growth by repeated injections of OBP-301.

Antitumor effect of OBP-405 on OBP-301–resistant sarcoma cell lines

OUMS-27 and NMFH-1 cells are resistant to OBP-301 because they lack CAR expression (Fig. 1A and Supplementary Fig. S2). We previously developed a fiber-modified OBP-301, termed OBP-405, which can enter not only CAR-positive cancer cells but also CAR-negative cancer cells through binding to the cell surface integrins αvβ3 and αvβ5 (31). We therefore sought to evaluate the antitumor effect of OBP-405 on the OBP-301–resistant OUMS-27 and NMFH-1 cells. We first examined the expression levels of

the integrins αvβ3 and αvβ5 on the surface of these cells by flow cytometry (Fig. 5A). OUMS-27 and NMFH-1 cells expressed both integrin molecules. We next examined the effect of OBP-405 on OUMS-27 and NMFH-1 cell viability by using the XTT assay (Fig. 5B). OBP-405 efficiently suppressed cell viability of both of these cell lines in a dose- and time-dependent manner. We further assessed whether OBP-405 has an *in vivo* antitumor effect by assaying the effect of 3 intratumoral injections of OBP-301 or OBP-405, with 10^8 PFUs or of control PBS, into subcutaneous OUMS-27 tumor xenografts. As shown in Figure 5C, administration of OBP-405 resulted in significant suppression of tumor growth compared with OBP-301- or PBS-treated tumors 28 days after treatment. These results suggest that fiber-modified OBP-405 is a potential antitumor reagent that is effective against CAR-negative human sarcoma cells.
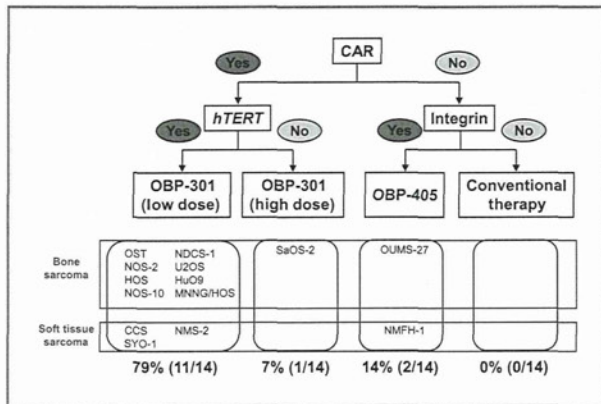
59

Figure 6. Outline of a therapeutic strategy for the use of telomerase-specific replication-selective oncolytic adenoviruses for human bone and soft tissue sarcoma cells. Assessment of CAR expression in tumor cells could serve as an indicator for OBP-301 or OBP-405 treatment. Of the 14 human sarcoma cell lines shown, the 12 CAR-expressing sarcoma cells (86%) should be treated with OBP-301 and the 2 sarcoma cells (14%) that lack CAR expression should be treated with OBP-405. The tumor expression level of hTERT mRNA would be useful in deciding the dose of OBP-301 to be used for treatment. The expression level of integrins on the tumor surface should be confirmed prior to OBP-405 treatment.

Discussion

Telomerase-specific replication-selective oncolytic adenoviruses are emerging as promising antitumor reagents for induction of tumor-specific cell death. We previously reported that OBP-301 has a strong antitumor effect on a variety of human epithelial malignant cells that have high telomerase activity (12, 13). However, nonepithelial malignant cells often show low telomerase activity and instead maintain telomere length through an ALT mechanism (20, 21). The effect of OBP-301 on human bone and soft tissue sarcoma cells has not been extensively examined. In this study, we showed that OBP-301 induced cell death in 12 of 14 human bone and soft tissue sarcoma cell lines (Fig. 1) and that the cytopathic activity of OBP-301 significantly correlated with tumor CAR expression (Fig. 2A). Furthermore, 2 ALT-type sarcoma cells showed low hTERT mRNA expression (Fig. 2B) but a similar sensitivity to OBP-301 compared with non–ALT-type cells because of hTERT mRNA upregulation by OBP-301 infection (Fig. 3). In contrast, 2 OBP-301–resistant sarcoma cells that lack CAR expression were highly sensitive to OBP-405, which can infect cells by binding to surface integrin molecules (Fig. 5). On the basis of these results, and with future clinical application in mind, we established a therapeutic strategy for the use of telomerase-specific oncolytic adenoviruses to treat patients with bone and soft tissue sarcomas (Fig. 6). This strategy involves assessment of the expression levels of CAR, hTERT, and integrins on human sarcoma cells, which would then allow easy selection of the most effective protocol for the treatment of patients by using oncolytic adenoviruses. Furthermore, as OBP-301 and OBP-405 show

the profound antitumor effect in the combination of various chemotherapeutic agents (43, 44), further evaluation for the strategy using OBP-301 and OBP-405 in combination with chemotherapy should be warranted.

The cytopathic activity of OBP-301 significantly correlated with CAR expression, but not with telomerase activity, of human sarcoma cells (Fig. 2). These results suggest that the cytopathic activity of OBP-301 depends primarily on infection efficiency rather than virus replication. Primary epithelial and nonepithelial malignant tumors frequently express CAR (23–30). However, CAR expression can often be downregulated by tumor progression (45, 46) or under hypoxic conditions (47), possibly leading to a low infection efficiency and resistance to OBP-301. Thus, for future clinical application of OBP-301, it may be necessary to overcome the resistance to OBP-301 that arises during tumor progression. A histone deacetylase (HDAC) inhibitor has been previously shown to enhance CAR expression on human cancer cells (48–50). Therefore, for the treatment of OBP-301–resistant sarcomas, it may be necessary to either upregulate CAR expression on tumor cells in combination with an HDAC inhibitor or use OBP-405 to kill tumor cells in an integrin-dependent manner (31).

ALT-type sarcoma cells that express a low level of hTERT mRNA showed sensitivity to OBP-301 that was similar to that of non–ALT-type sarcoma cells (Figs. 1 and 2). We further showed that OBP-301 infection upregulates hTERT gene expression and subsequently activates virus replication and cytopathic activity in ALT-type sarcoma cells (Fig. 3). These results suggest that the hTERT gene promoter is a useful tool for enhancement of the oncolytic adenoviruses not only because it induces tumor-specific virus replication but also because it enhances virus replication after infection. Indeed, the ALT-type sarcoma SaOS-2 cells that lack hTERT gene expression were relatively less sensitive to OBP-301 than the other ALT-type sarcoma U2OS cells that express low levels of hTERT mRNA (Figs. 1 and 2). We further observed that hTERT mRNA expression was not upregulated after OBP-301 infection of SaOS-2 cells (data not shown). These results suggest that if hTERT gene expression cannot be detected in tumor cells, then ALT-type sarcoma cells should be treated with high doses of OBP-301, or with OBP-405, to enhance OBP-301 infection efficiency (Fig. 6).

It is also worth noting in terms of future clinical application that an interval of more than 2 days between injections is necessary in order for repeated injections of OBP-301 to induce a strong antitumor effect in an SYO-1 animal xenograft model (Supplementary Fig. S5). We first expected that continuous injection of OBP-301 at intervals of 1 day, when tumors are of a minimum size, might be more effective in inducing an antitumor effect than injection at intervals of 2 days or 1 week. Surprisingly, continuous injection of OBP-301 at intervals of 1 day, for 3 days, could not induce an antitumor effect. There are 2 possible explanations for these results. The

60

first possibility is that 3 days of continuous injections may not provide enough time for OBP-301 to replicate and reach the minimal dose required for induction of an antitumor effect within tumor tissues. The second possibility is that OBP-301 may be less effective against more slowly proliferating tumor cells than it is against rapidly proliferating tumor cells because its replication rate would be lower in the more slowly proliferating cells. Although it remains unclear why continuous injection of OBP-301 was less effective, it is clear that repeated infection with OBP-301 at intervals of more than 2 days would be sufficient to exert an antitumor effect against human sarcoma tissues.

In conclusion, we have clearly shown that OBP-301 has strong *in vitro* and *in vivo* antitumor effects against human bone and soft tissue sarcoma cells. Telomerase-specific replication-selective oncolytic virotherapy would provide a new platform for the treatment of patients with bone and soft tissue sarcomas.

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62

Cancer Therapy: Preclinical

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

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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

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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

64

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/AflIII* 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/AflIII* 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-CeuI* after digestion with *NheI* followed by *Klenow* treatment, and then ligated with the *I-CeuI/PmeI* 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-SceI*-digested pSh-AIB-143T was ligated with the *I-CeuI/PI-SceI*-digested pAdHM3 (21), resulting in pAdHM3-AIB-143T. To generate TRADs, pAdHM3-AIB-143T was digested with *PacI* 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
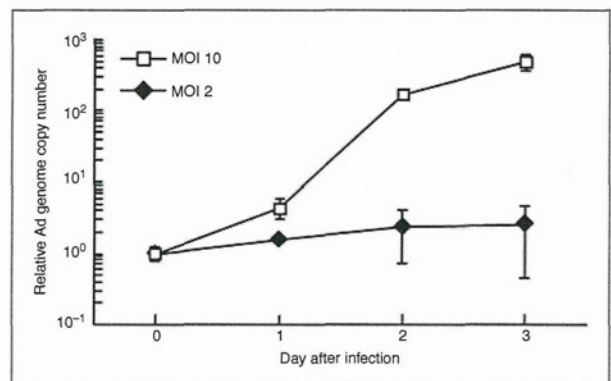

Figure 1. Replication of the conventional TRAD in WI38 cells. WI38 cells were infected with the conventional TRAD at an MOI of 2 or 10 for 2 hours. At the indicated time points, the copy numbers of the Ad genome and *GAPDH* gene were determined by real-time PCR. The ratio of the copy number of the Ad genome to that of GAPDH was normalized by the data on day 0. The data are shown as the means ± SD ($n = 3$).
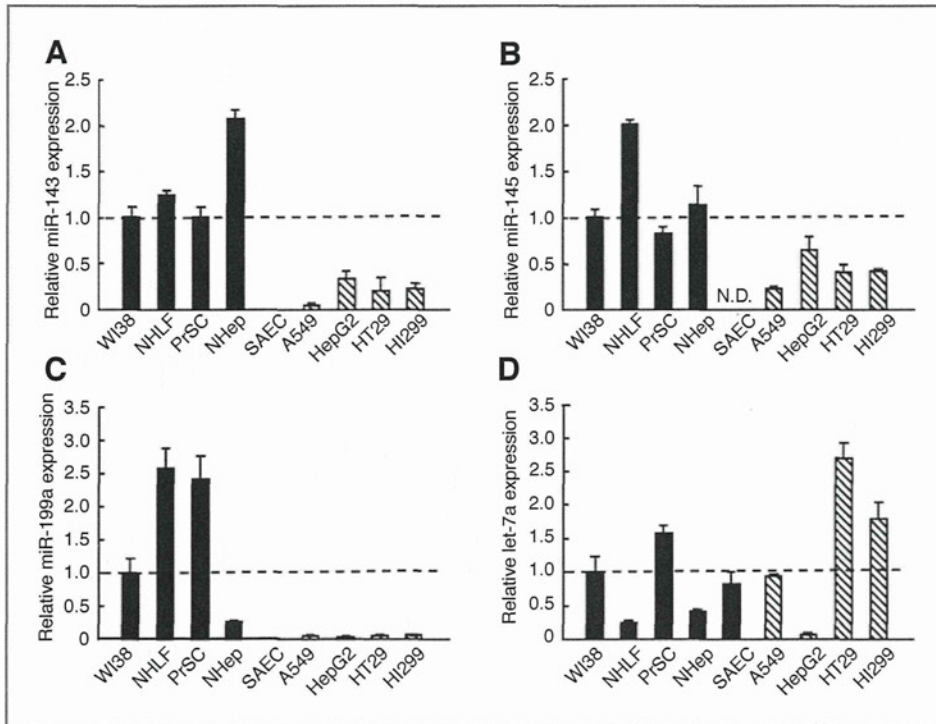
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Figure 2. MiRNA expression levels in the human normal (solid bar) and tumor cells (hatched bar). MiRNA expression was determined by real-time RT-PCR. The ratio of miRNA to U6 expression levels was normalized by the data of WI38 cells. The data are 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).

66

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 inhib-
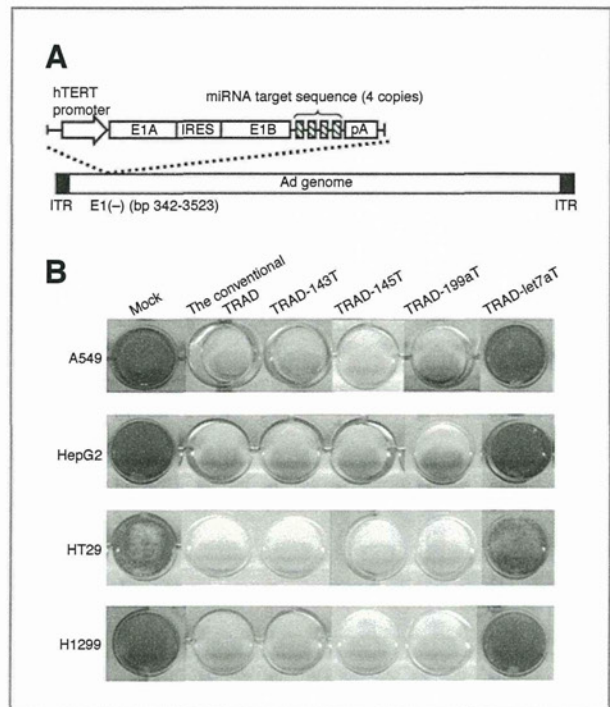

Figure 3. Replication and oncolytic activity of TRADs containing the miRNA complementary sequences in the tumor cells. A, a schematic diagram of a TRAD containing the miRNA-regulated *E1* gene expression system. ITR: inverted terminal repeat. B, crystal violet analysis of the cytopathic effects of TRADs 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). *, $P < 0.05$; **, $P < 0.005$.

ited 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.

67

Figure 3. (*Continued*)

68