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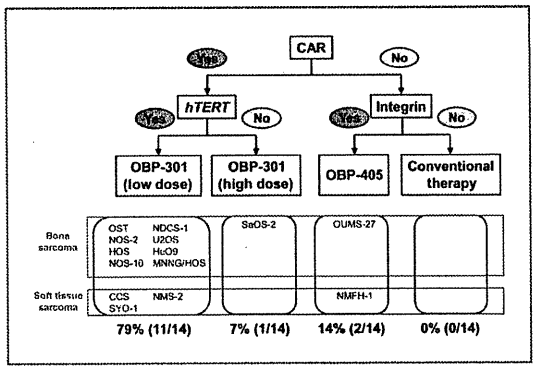

Figure 6. Outline of a therapeutic strategy for the use of telomerase-specific replication-selective oncolytic adenoviruses for human bone and soft tissue sarcoma cells. Assessment of CAR expression in tumor cells could serve as an indicator for OBP-301 or OBP-405 treatment. Of the 14 human sarcoma cell 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

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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.

Disclosure of Potential Conflict of Interest

Y. Urata is an employee of Oncolys BioPharma, Inc., the manufacturer of OBP-301(Telomelysin). The other authors disclosed no potential conflicts of interest.

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Cancer Therapy: Preclinical

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

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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

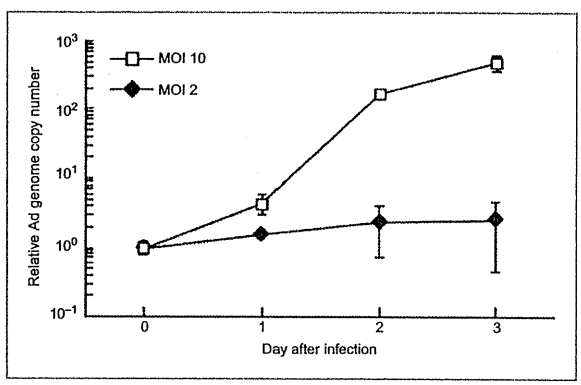

Figure 1. Replication of the conventional TRAD in WI38 cells. WI38 cells were infected with the conventional TRAD at an MOI of 2 or 10 for 2 hours. At the indicated time points, the copy numbers of the Ad genome and *GAPDH* gene were determined by real-time PCR. The ratio of the copy number of the Ad 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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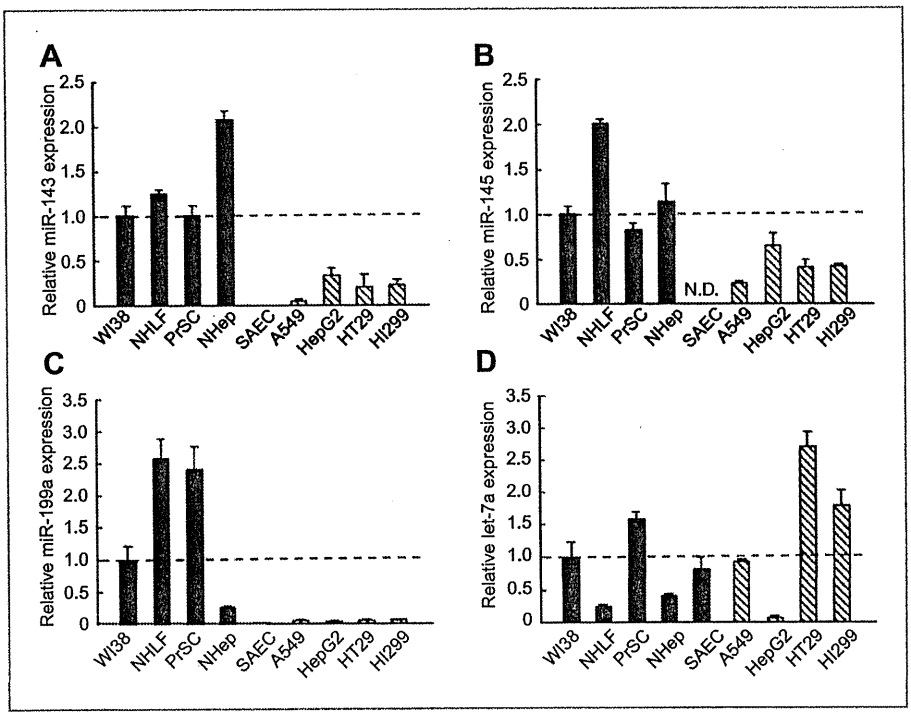

Figure 2. MiRNA expression levels in the human normal (solid bar) and tumor cells (hatched bar). MiRNA expression was determined by real-time RT-PCR. The ratio of miRNA to U6 expression levels was normalized by the data of WI38 cells. The data are shown as the means ± SD ($n = 3$). N.D., not detected.

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).

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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.

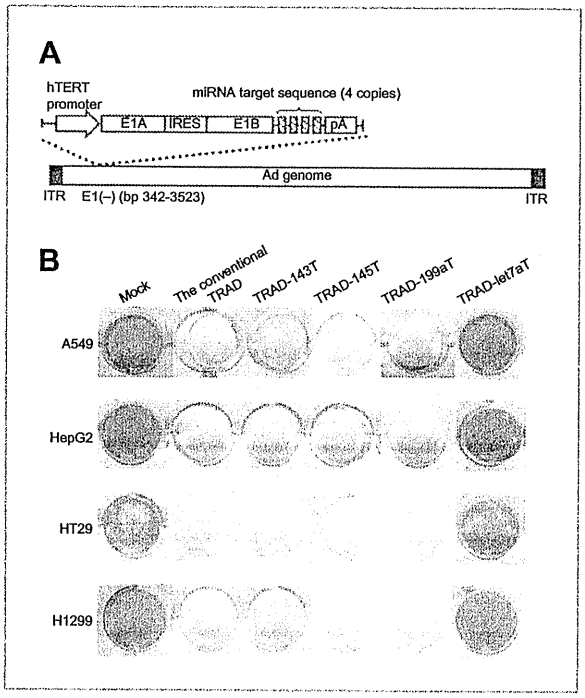

Figure 3. Replication and oncolytic activity of TRADs containing the miRNA complementary sequences in the tumor cells. A, a schematic diagram of a TRAD containing the miRNA-regulated *E1* gene expression system. ITR: inverted terminal repeat. B, crystal violet analysis of the cytopathic effects of TRADs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

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.

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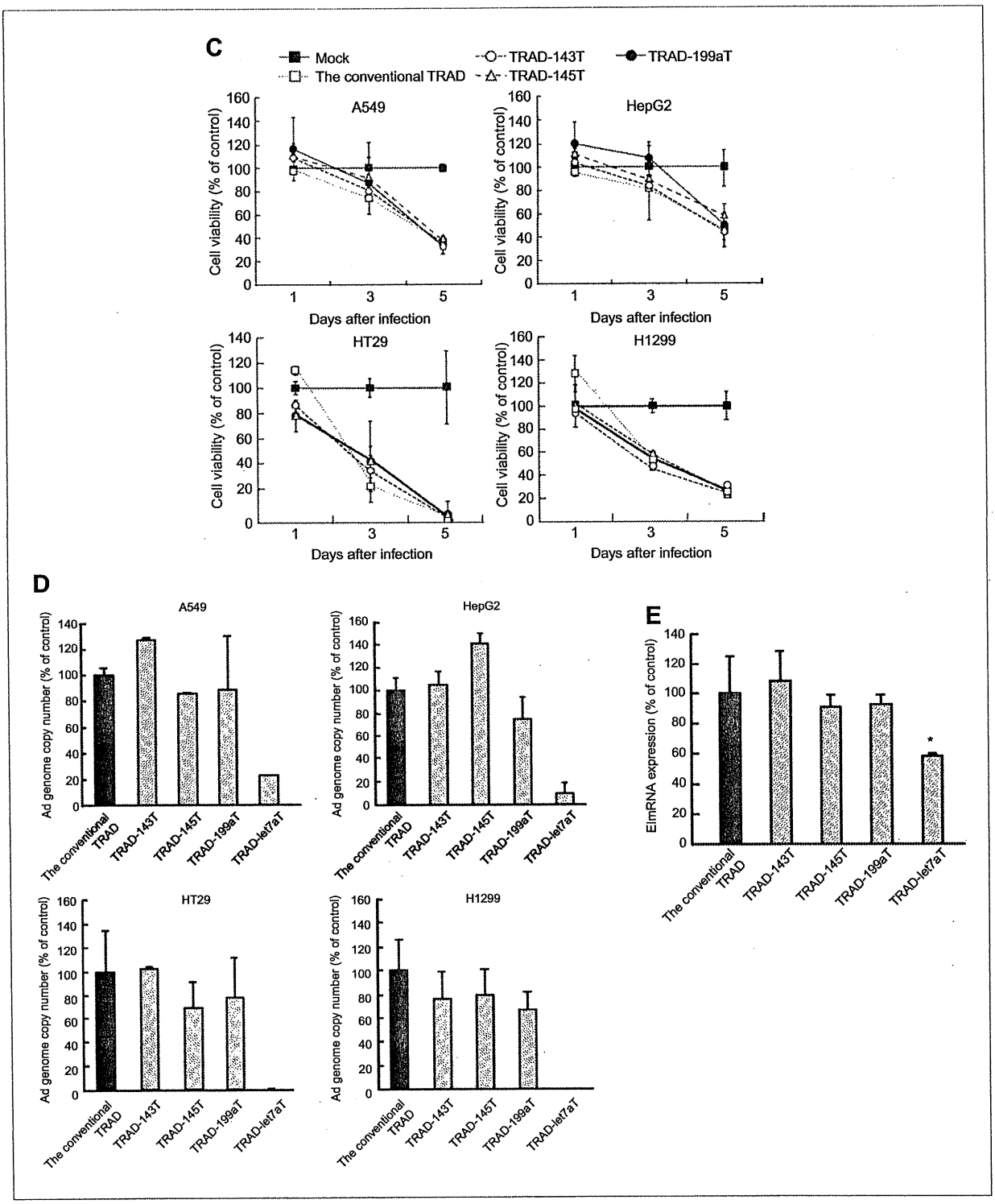

Figure 3. (*Continued*)

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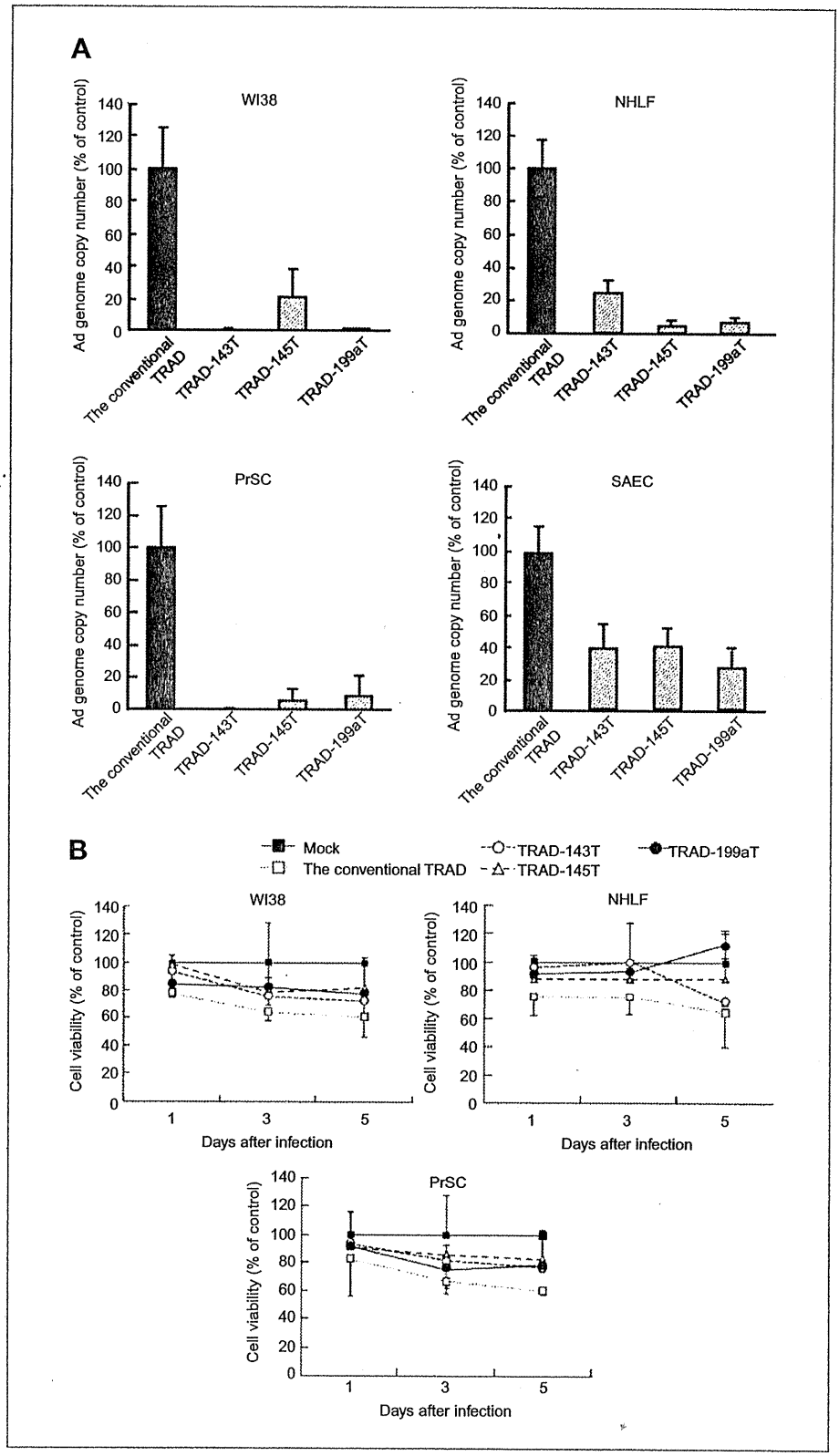

Figure 4. Reduced replication of TRADs in normal human cells by insertion of the miRNA complementary sequences. A, the viral genome copy numbers of TRADs in normal cells. The cells were infected with the TRADs at an MOI of 10 for 2 hours. Five days after infection, the viral genome copy numbers were 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 ± SD ($n = 3$–4). *, $P < 0.05$; **, $P < 0.005$.

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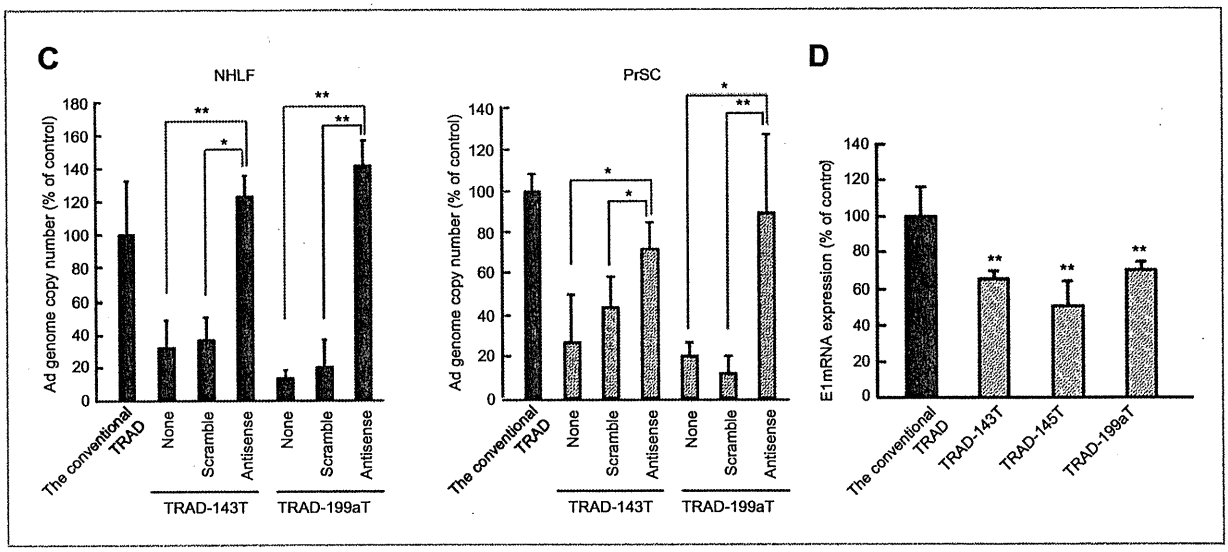

Figure 4. (*Continued*)

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 wascarried 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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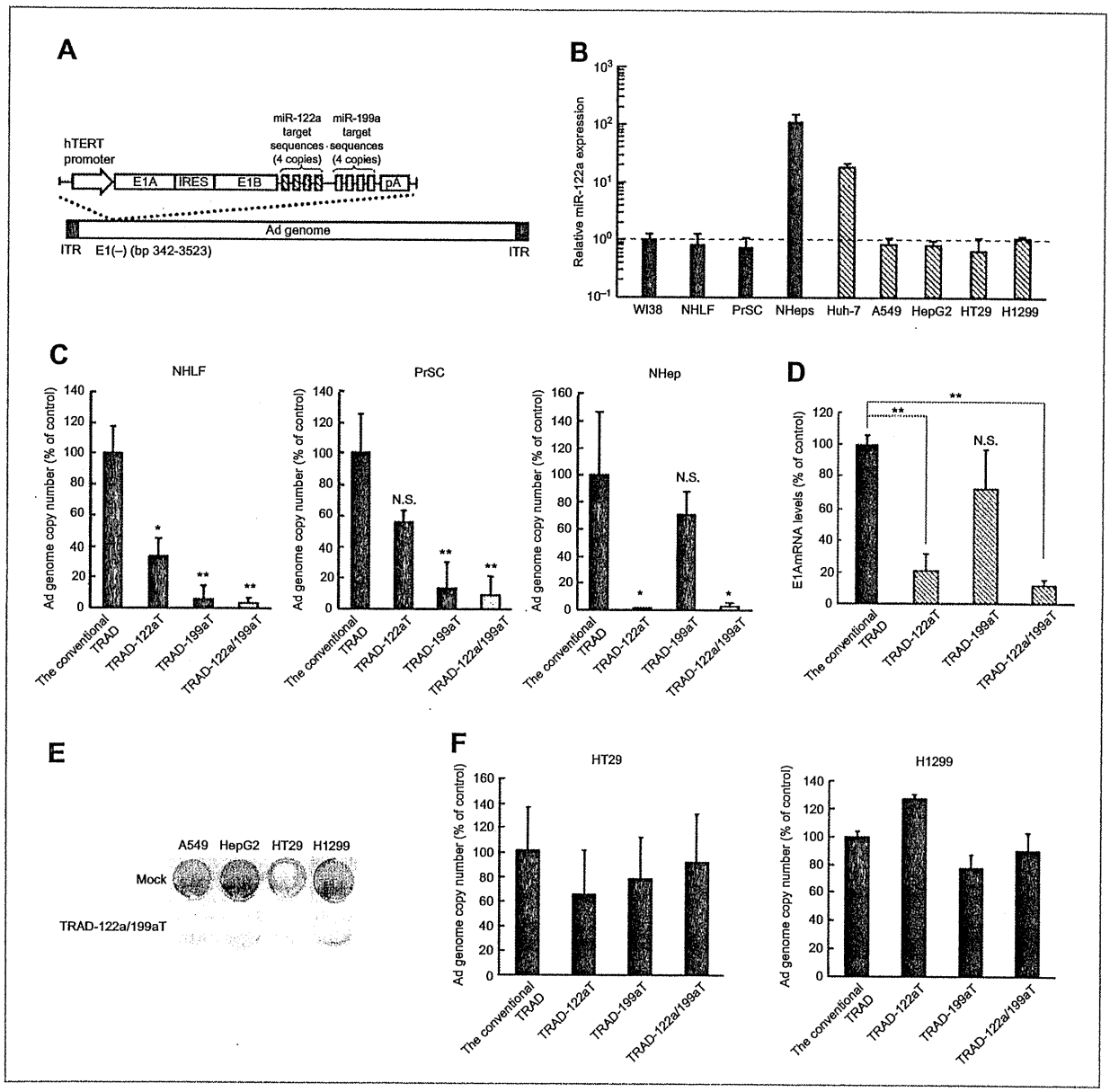

Figure 5. Tumor cell lysis activity and enhanced safety profile of TRAD-122a/199aT. A, a schematic diagram of TRAD-122a/199aT. B, miR-122a expression levels in the normal and tumor cells. C, the viral genome copy numbers of TRAD-122a/199aT in normal human cells. D, the E1A mRNA levels in NHep. E, crystal 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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Inhibition of mTOR by temsirolimus contributes to prolonged survival of mice with pleural dissemination of non-small-cell lung cancer cells

Toshiaki Ohara,[1] Munenori Takaoka,[1] Shinichi Toyooka,[2] Yasuko Tomono,[3] Toshio Nishikawa,[1] Yasuhiro Shirakawa,[1] Tomoki Yamatsuji,[1] Noriaki Tanaka,[1] Toshiyoshi Fujiwara[1] and Yoshio Naomoto[1,4,5]

[1]Departments of Gastroenterological Surgery, Transplant, and Surgical Oncology, [2]Cancer and Thoracic Surgery, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama; [3]Shigei Medical Research Institute, Okayama, Japan

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Temsirolimus (CCI-779), a recently synthesized analogue of rapamycin, specifically inhibits mTOR and has been approved for clinical use in renal cell carcinoma. Recent reports have indicated the growth inhibitory effect of temsirolimus in some cancers including non-small-cell lung carcinoma (NSCLC). In this study, we aimed to explore the potential therapeutic use of temsirolimus as a treatment for NSCLC. Using cultured NSCLC cells (A549, H1299, and H358), we determined the effect of temsirolimus on cell proliferation and its antitumor effects on subcutaneous tumors, as well as its contribution to the survival of mice having pleural dissemination of cancer cells, mimicking advanced NSCLC. Temsirolimus suppressed proliferation of NSCLC cells in a dose-dependent manner, with an IC_{50} of <1 nM. Western blot analysis revealed that temsirolimus treatment specifically inhibited the phosphorylation of mTOR and its downstream effectors in 1 h, accompanied by an increased cell population in the G_0/G_1 phase, but according to flow cytometry, the cell population did not increase in the sub-G_0 phase. When NSCLC subcutaneous tumor-bearing mice were treated with temsirolimus, tumor volume was significantly reduced (tumor volume on day 35: vehicle *vs* temsirolimus = 1239 *vs* 698 cm^3; $P < 0.05$). Furthermore, prolonged survival was observed in pleural disseminated tumor-bearing mice with temsirolimus treatment (median survival: vehicle *vs* temsirolimus = 53.5 *vs* 72.5 days; $P < 0.05$). These results suggest that temsirolimus could be useful for NSCLC treatment, due to its antiproliferative effect, and could be a potential treatment for advanced NSCLC, giving prolonged survival. (*Cancer Sci* 2011; 102: 1344–1349)

Lung cancer is one of the most aggressive malignancies with poor prognosis. It is estimated that more than 160 000 and 65 000 lung cancer patients in the USA and Japan, respectively, die each year.[1,2] A wide variety of new chemotherapy medicines have been developed and introduced in clinical practice, but the mortality rate has not been improved.[1] Recently, the strategy of drug development has focused on targeting particular molecules that are supposed to be critical for cancer progression. Several molecules in the growth factor receptor pathway are specifically targeted because those molecules are well recognized as being aberrantly regulated in cancers. For example, epidermal growth factor receptor (EGFR) and its downstream molecules are often upregulated due to gene amplification or mutation;[3,4] therefore, targeting EGFR is a major therapeutic strategy for non-small-cell lung carcinoma (NSCLC).[5] Gefitinib is a well-known small molecule inhibitor that selectively suppresses EGFR tyrosine kinase activity[6] and has been applied in the treatment of NSCLC.[7] Several studies have shown that gefitinib treatment has a drastic antitumor effect in a subset of NSCLC which had acquired certain types of EGFR mutation.[8,9] Since the appearance of gefitinib, several selective EGFR inhibitors have been developed. However, these drugs only revealed a minimal effectiveness due to the aberrant regulation of molecules located downstream from the receptor tyrosine kinase pathways including Ras-Raf-MAPK and phosphatidylinositol 3′-kinase (PI3K)-Akt.[10,11] Among them, mammalian target of rapamycin (mTOR) is one of the major effectors regulated by the PI3K-Akt signaling pathway and plays a central role in this stimulated growth.[12,13] Moreover, there is an upregulation of mTOR activity in many types of cancers including NSCLC.[14,15] Therefore, several compounds that selectively inhibit mTOR activity have been developed for clinical use.[16,17] Temsirolimus (CCI-779), an analogue of rapamycin, was recently synthesized to specifically inhibit mTOR and has provided prolonged survival of patients with renal cell carcinoma. It was also reported that temsirolimus showed a certain antitumor effect on other types of cancers including breast cancer,[18] glioblastoma,[19] neuroendocrine carcinomas,[20] and mantle cell lymphoma.[21] Moreover, temsirolimus has antitumor effects in other diseases such as lymphangioleiomyomatosis.[22] Based on these observations, we questioned whether temsirolimus treatment could be a potential therapeutic option for NSCLC. In this study, we evaluated the antiproliferative and antitumor effects of temsirolimus in NSCLC *in vitro* and *in vivo*, with an assessment of its survival advantage in an animal model of advanced NSCLC.

Materials and Methods

Cell lines and cultures. Three cancer cell lines that were established from human NSCLC (A549, H1299, and H358) were used in this study. A549 was cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) and H1299 and H358 were cultured in RPMI-1640 medium (Sigma-Aldrich) at 37°C in humidified air with 5% CO_2 These media were supplemented with 10% FCS (Hyclone, Logan, UT, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma-Aldrich).

Reagents. Temsirolimus, commercialized as Tricel by Wyeth K.K. (Madison, NJ, USA), was purchased from OZ International (Tokyo, Japan). The temsirolimus was diluted to the final concentration with culture media before an *in vitro* experiment. When temsirolimus was used *in vivo*, it was dissolved and diluted to a final concentration of 10 mg/kg with 0.9% sodium chloride.

Trypan blue exclusion assay. Cancer cells (5.0×10^3 per well) were plated directly in 24-well dishes with culture medium.

[4]To whom correspondence should be addressed.
E-mail: ynaomoto@med.kawasaki-m.ac.jp
[5]Present address: Department of General Surgery, Kawasaki Medical School, Kawasaki Hospital, Kita-ku, Okayama, Japan.

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After the cells entered into an exponential growth phase, they were treated with different concentrations of temsirolimus (0, 0.1, 1, 10, 100, or 1000 nM) for 48 h, stained with Trypan blue, and the number of viable cells was counted using a hemacytometer.

Apoptosis assay. Cells in apoptosis were determined by TUNEL assay using a MEBSTAIN Apoptosis kit II (MBL International, Woburn, MA, USA) according to the manufacturer's protocol. Briefly, cells (1.0×10^4 per well) were seeded on Lab-Tek 8-well permanox chamber slides (Becton Dickinson, Franklin Lakes, NJ, USA) and were treated with 10 nM/L of temsirolimus or with an equivalent volume of diluted DMSO (final concentration, 0.005%) as a control for 48 h. The TUNEL-positive cells were counted with a fluorescence microscope.

Cell cycle analysis by flow cytometry. For cell cycle analysis, cancer cells were plated in six well tissue culture plates and treated with different concentrations of temsirolimus (0, 1, 10, or 100 nM/L). After a 24-h treatment, the cells were harvested and stained with 20 mg/mL propidium iodide. The DNA content was analyzed with a fluorescence-activated cell sorter (FACScan; Becton Dickinson) using CellQuest software (BD Biosciences, San Jose, CA, USA).

Western blot analysis. Whole cell lysates and nuclear protein were extracted using M-PER buffer (Thermo Fisher Scientific, Rockford, IL, USA) and NE-PER buffer (Thermo Fisher Scientific) supplemented with protease inhibitors and phosphatase inhibitors. The protein concentration of the collected supernatants was determined and equal amounts of protein were electrophoresed under a reducing condition in gradient polyacrylamide gels (ATTO, Tokyo, Japan) and were then transferred onto PVDF filter membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies at 4°C overnight, followed by incubation with secondary antibodies at room temperature for 1 h. An Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ, USA) was used for signal detection. The antibodies used for Western blotting were phospho-mTOR (Ser2448), mTOR, phospho-p70 S6 kinase (Thr389), p70 S6 kinase, phospho-S6 ribosomal protein (Ser235/236), and hydroxy-HIF-1α (Pro564) (D43B5). All of them were obtained from Cell Signaling Technology (Beverly, MA, USA). β-Actin was obtained from Sigma-Aldrich. Horseradish peroxidase-conjugated rabbit anti-mouse IgG was obtained from Dako Cytomation (Glostrup, Denmark). Goat anti-rabbit IgG was obtained from American Qualex Antibodies (La Mirada, CA, USA).

Animal experiments. The protocol for the animal experiments was approved by the Ethics Review Committee for Animal Experiments of Okayama University (Okayama, Japan). Mice used in this study were purchased from Clea (Tokyo, Japan). A549 s.c. xenografts were produced on the backs of 6-week-old male BALB/c nu/nu mice by injecting 3×10^6 cells mixed with Matrigel (BD Biosciences) at a 1:1 ratio. After 7 days, the tumor-bearing animals were randomized into two groups that consisted of seven mice each: (i) temsirolimus (10 mg/kg given i.v. once/week for 5 weeks); and (ii) saline alone as a vehicle (given i.v. once/week for 5 weeks). Tumor volume was measured weekly (length × width × height). To create the A549 pleural dissemination model, 4×10^6 cancer cells were intrathoracically injected into the pleural cavity of 6-week-old male BALB/c nu/nu mice. After 7 days, the animals were randomized into two groups that consisted of eight mice each; (i) temsirolimus (10 mg/kg given i.p. once/week for 5 weeks); and (ii) saline alone as a vehicle. The drug was given once a week and lasted until the mice expired. Each animal experiment was repeated three times and the representative data is shown. The dose and schedule of temsirolimus treatment (10 mg/kg/week) in these animal experiments was decided based upon previous reports where the researchers used a range of 8–20 mg/kg/week.[23–25]

Immunohistochemistry. Surgically resected pleural membrane tissues from mice with disseminated pleural tumors from A549 cells were used for immunohistochemical study following procedures described previously.[26] Deparaffinized tissue sections were immersed in methanol containing 3% hydrogen peroxide to block endogenous peroxide activity. An autoclave pretreatment in citrate buffer was done for antigen retrieval. After incubation with a blocking buffer the sections were treated with an anti-phospho-mTOR rabbit mAb (Cell Signaling Technology) for 6 h at room temperature followed by immunobridging with Avidin DH-biotinylated HRP complex (Nichirei, Tokyo, Japan). Signal detection was done for 2–5 min using 3,3′-diaminobenzidine tetrahydrochloride dissolved to 50 mM/L Tris–HCl (pH 7.5) containing 0.001% hydrogen peroxide. The sections were counterstained with Mayer-hematoxylin. Monoclonal anti-human mouse Ki-67 antibody (MIB-1; Dako Cytomation) was used to calculate the Ki-67 labeling index by counting the number of positively stained cells per 1000 cancer cell nuclei for each section.

Statistical analysis. Student's *t*-test was used to compare data between two groups. Data represent the mean ± SD. Overall survival was calculated using the Kaplan–Meier method and compared by the log–rank test. $P < 0.05$ was considered statistically significant.

Results

Inhibition of mTOR by temsirolimus suppresses cell growth of NSCLC cells. First, we examined how effective temsirolimus was at inhibiting the proliferation of cultured NSCLC cells using a Trypan blue exclusion assay. As shown in Figure 1, temsirolimus suppressed the cell proliferation of A549, H1299, and H358 cells in a dose-dependent manner. The IC_{50} values were measured to examine the suppression of cell proliferation by temsirolimus in NSCLC cell lines. The IC_{50} for A549 cells was 0.76 nM and those for H1299 and H358 were 0.75 nM and 0.64 nM, respectively (Fig. 1). These data indicated that temsirolimus effectively inhibited the viability of NSCLC cells at a low concentration of <1 nM.

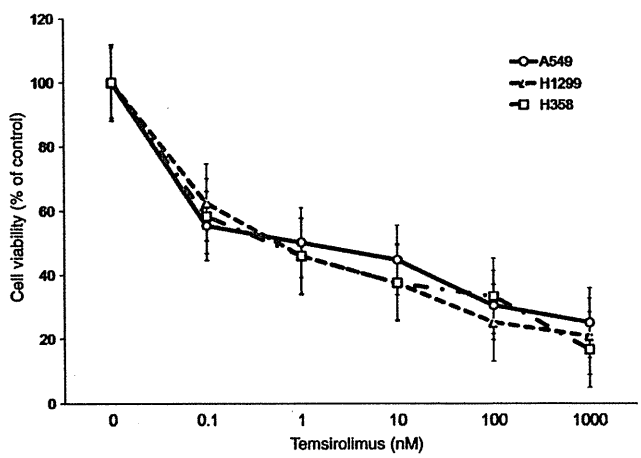

Fig. 1. Temsirolimus suppresses cell proliferation of non-small-cell lung carcinoma cells in a dose-dependent manner. Cultured cells were treated with the indicated concentrations of temsirolimus for 48 h and the number of viable cells was counted by the Trypan blue exclusion method. The IC_{50} values of A549, H1299, and H358 cells were 0.76, 0.75, and 0.64 nM, respectively.

Temsirolimus inhibited mTOR pathway in a dose- and time-dependent manner. Next, in order to evaluate the effect of temsirolimus on regulating activation in the mTOR pathway, we examined the phosphorylation of mTOR and its downstream effectors by Western blot analysis. As expected, the treatment with temsirolimus suppressed the activations of mTOR, p70 ribosomal S6 kinase, and S6 in a dose-dependent fashion in A549 (Fig. 2A). This inhibitory effect occurred after 1 h, and lasted at least 4 h (Fig. 2B). Similar results were obtained in another NSCLC cell line, H1299 (Fig. 2C,D). Furthermore, we assessed the expression status of cell cycle markers including $p21^{cip1}$, $p27^{kip1}$, and cyclinD1, whose expression is often modified by the inactivation of p70 S6 kinase and S6.[27] Interestingly, $p21^{cip1}$ was apparently induced by temsirolimus treatment, but we did not observe any change in cyclinD1 or $p27^{kip1}$ (Fig. S1). Because the upregulation of $p21^{cip1}$ is known to contribute to cell cycle arrest, these data suggest that the inhibition of the mTOR pathway using temsirolimus is a promising strategy to diminish the proliferation of NSCLC cells.

Temsirolimus treatment leads to G_1 cell cycle arrest but not cell death. Our next question was whether temsirolimus treatment is lethal to NSCLC cells. In order to answer this question, we carried out flow cytometry to analyze the cell cycle distribution in A549 and H1299 cell lines under temsirolimus treatment. Interestingly, temsirolimus treatment increased the cell population in the G_0/G_1 phase, but not in the sub-G_0 phase, which accounted for dead cells (Fig. 3). Furthermore, when we examined the amount of apoptosis by TUNEL assay, we did not observe a significant number of apoptotic cells (data not shown). Taken together, these results suggested that temsirolimus suppressed NSCLC cell proliferation by its cytostatic effect, not by cytotoxicity.

Temsirolimus reduces s.c. tumor growth of NSCLC cells. Next, we investigated the effect of temsirolimus on *in vivo* tumor growth. A549 s.c. xenografts were made. When 10 mg/kg temsirolimus was given weekly i.v. to the mice bearing the s.c. tumor, a significant delay of s.c. tumor growth was observed on day 35 (tumor volume: vehicle *vs* temsirolimus = 1239 *vs* 698 cm^3; $P < 0.05$) (Fig. 4). None of the mice died of drug-induced toxicity and no other significant adverse events were observed. Moreover, during the observation period (up to 35 days after cell inoculation), there was no significant change in body weight in either group (data not shown).

Temsirolimus treatment prolonged survival of mice with disseminated pleural tumors of NSCLC cells. As shown above, we found that temsirolimus had a cytostatic effect on NSCLC cells and showed a delay of s.c. tumor growth. Based on these results, we predicted that a major advantage of temsirolimus treatment would be an improvement in the survival of patients bearing NSCLC tumors, similar to renal cell carcinoma.[28] To investigate the effect of temsirolimus on survival, we made a pleural dissemination animal model by injecting A549 cells into the intrapleural cavity, to mimic an advanced clinical stage of NSCLC. A weekly i.p. injection of 10 mg/kg temsirolimus significantly prolonged the survival period of these pleural disseminated tumor-bearing mice (median survival: vehicle *vs* temsirolimus = 53.5 *vs* 72.5 days; $P < 0.05$) (Fig. 5A,B).

Macroscopic observation by opening the thoracic cavity of the mice showed that temsirolimus treatment obviously reduced the number and the volume of pleural disseminated tumors on day 21 after the inoculation of A549 cells in the thoracic cavity (Fig. 5C,D), although tumors were recognized in a bilateral thoracic cavity regardless of temsirolimus treatment. This result led us to speculate that temsirolimus reduced the growth of pleural disseminated tumors, leading to the prolonged survival of the tumor-bearing mice. Furthermore, immunohistochemical analysis revealed that phosphorylation of mTOR was strongly suppressed in the tumor tissues of the temsirolimus-treated mice (Fig. 5E,F).

The immunohistochemical analysis for Ki-67 using disseminated pleural tumor tissues revealed a significant decrease in the number of proliferating cells (determined by calculating the

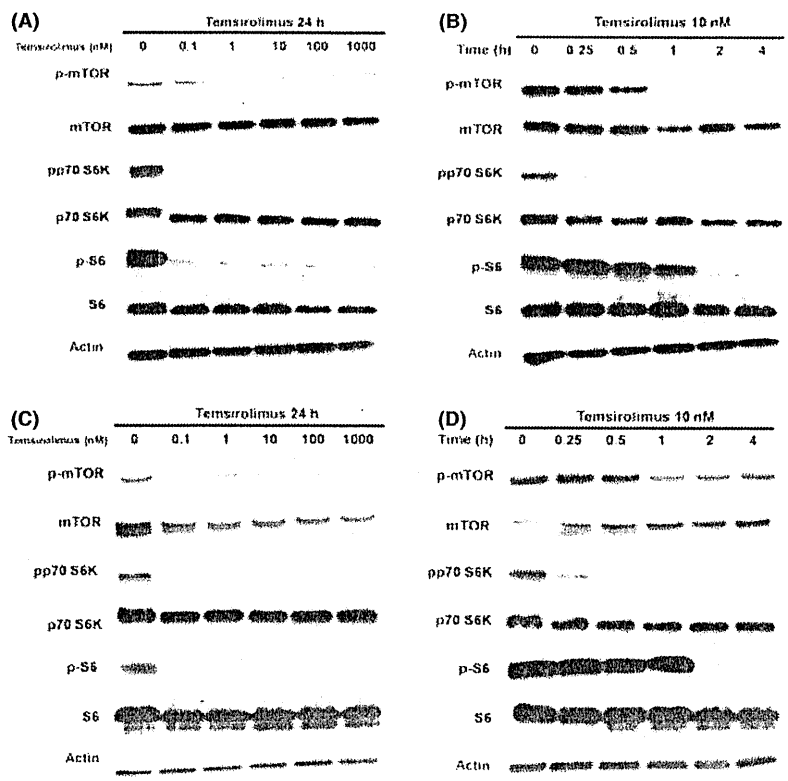

Fig. 2. Temsirolimus suppresses the activation of mTOR and its downstream effectors. Whole cell lysates of A549 (A,B) and H1299 (C,D) non-small-cell lung carcinoma cells that were treated with the indicated concentrations of temsirolimus were used for Western blot to determine the inhibitory effects on mTOR and its downstream effectors in dose-dependent (A,C) and time-course (B,D) studies. p-mTOR, phospho-mTOR; pp70 S6K, phospho-p70 S6 kinase; p-S6, phospho-S6 ribosomal protein.

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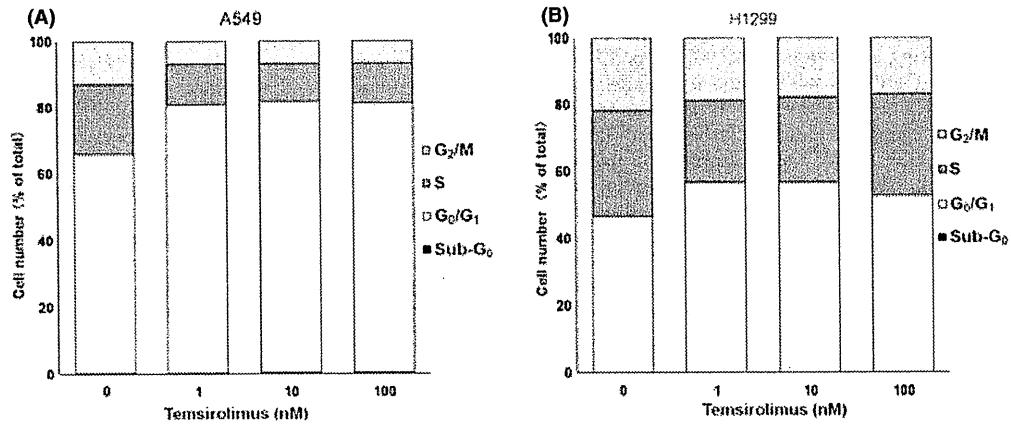

Fig. 3. Temsirolimus induces cell cycle arrest rather than cell death. A549 (A) and H1299 (B) non-small-cell lung carcinoma cells were treated with 10 nM temsirolimus for 24 h and the cell cycle distribution was analyzed by flow cytometry.

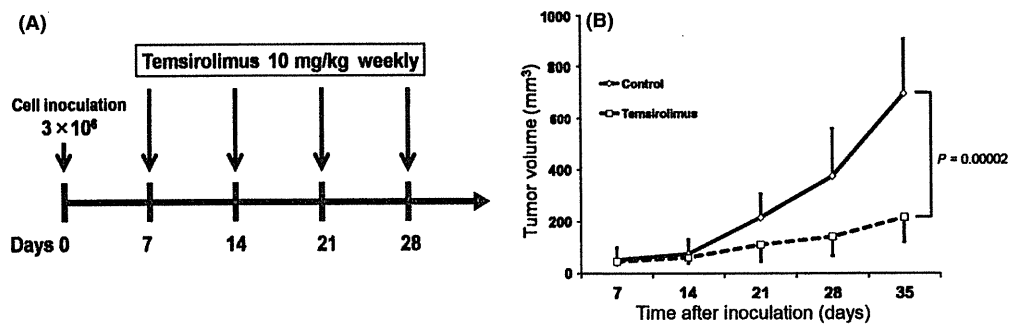

Fig. 4. Temsirolimus reduces the growth of s.c. tumors of A549 non-small-cell lung carcinoma cells. A549 cells were inoculated s.c. in the dorsum of nude mice (day 0) and i.v. injections of either temsirolimus (10 mg/kg) or saline as a vehicle were started from day 7 and continued once a week (A). Tumor volume was measured as a cube (length × width × height) and was tracked for up to 5 weeks (B). The representative data were taken from three independent experiments.

Ki-labeling index, defined in Materials and Methods) in the tissues treated with temsirolimus (temsirolimus, 0.106 ± 0.019; control, 0.191 ± 0.044; $P < 0.05$) (Fig. S2A). However, temsirolimus treatment did not increase the incidence of apoptosis in the tumor tissues, as checked by immunohistochemistry for cleaved caspase-3 (temsirolimus, 0.004 ± 0.002; control, 0.004 ± 0.002; $P > 0.05$) (Fig. S2B). These results were similar to our *in vitro* data, supporting our conclusion that the primary effect of temsirolimus is antiproliferative rather than cytotoxic. Thus, the advantage of *in vivo* temsirolimus treatment was to provide prolonged survival in advanced NSCLC tumor-bearing mice by suppressing tumor growth.

Inhibition of mTOR by temsirolimus suppresses the action of hypoxia inducible factor 1α (HIF-1α). Finally, we assessed the inhibition of mTOR by temsirolimus in NSCLC cells and tumors. Because recent reports have shown that the action of HIF-1α, a major transcriptional activator for angiogenesis and oncogenes, is regulated by the mTOR pathway,[29] and is therefore inhibited by temsirolimus *in vitro* and *in vivo*,[25,30] we also determined the effect of temsirolimus on the expression status of HIF-1α in the nuclei, where activated HIF-1α normally translocates.[31] Temsirolimus treatment suppressed the translocation of HIF-1α to the nucleus in all of NSCLC cells (Fig. S3A). As HIF-1α is known to play a critical role in cell proliferation and angiogenesis,[32] this inhibition of HIF-1α action by temsirolimus should at least partially contribute to its antiproliferative effect.

Regarding the antiangiogenic effect of temsirolimus by negatively regulating HIF-1α, we additionally determined the expression of vascular endothelial cell growth factor (VEGF), a known transcriptional target of HIF-1α. In cultured NSCLC cells, the amount of VEGF protein secreted in the culture medium was suppressed by temsirolimus treatment in a dose-dependent manner (Fig. S3B,C). Similarly, the production of VEGF mRNA expression, especially the 572-bp form of VEGF, was decreased in the pleural disseminated tumors of the mice that had temsirolimus treatment (Fig. S3D). The inhibition of HIF-1α/VEGF-mediated angiogenesis might also contribute to slowing tumor growth by temsirolimus treatment.

Discussion

Temsirolimus, an analogue of rapamycin, is a new molecular targeted agent and was first approved for the treatment of renal cell carcinoma. In terms of NSCLC, it was reported that inhibiting mTOR with rapamycin revealed a growth inhibitory effect in some NSCLC cell lines.[33] Temsirolimus was developed as an improved derivative of rapamycin,[34] and our data indicated its effectiveness by showing its potent inhibitory effect on cell proliferation of cultured NSCLC cells at a low concentration (as low as 1 nM). Concerning the antiproliferative effect of temsirolimus, our results reproduced the results of a previous report using rapamycin, which induced cell cycle arrest at the G_1 checkpoint and inhibited cell proliferation of murine NSCLC without inducing apoptosis.[23] In this study, temsirolimus suppressed the phosphorylations of p70 S6 kinase and S6 (Fig. 2). As the action of p70 S6 kinase and S6 is critical for

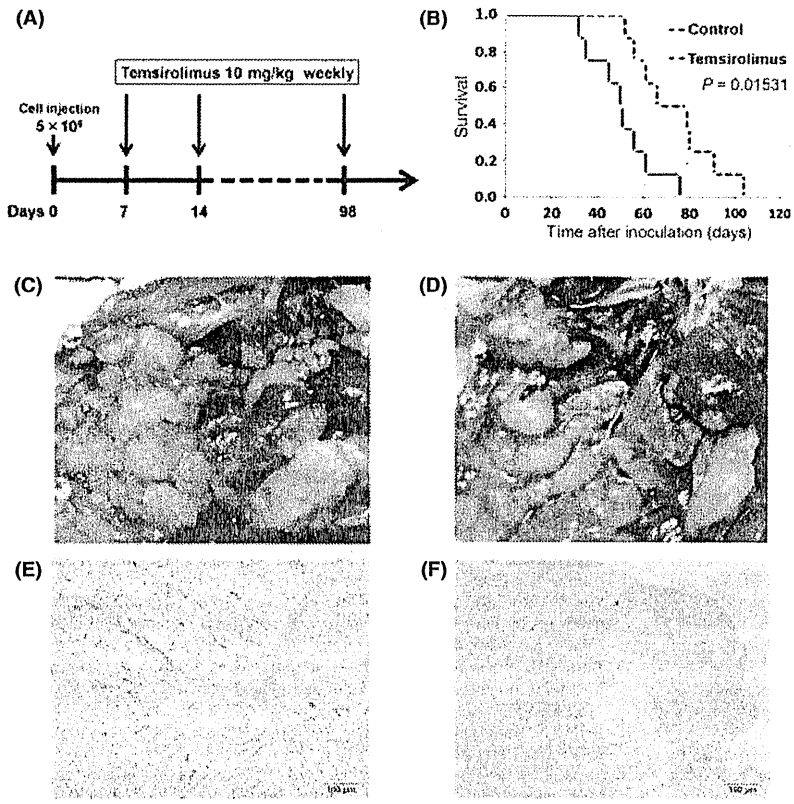

Fig. 5. Temsirolimus prolongs the survival of pleural disseminated tumor-bearing mice. A549 non-small-cell lung carcinoma cells were injected into the thoracic cavity of mice (day 0) and i.p. injections of either temsirolimus (10 mg/kg) or saline as a vehicle were started from day 7 and continued once a week (A). Cell survival periods were tracked to draw a survival curve by the Kaplan–Meier method (B). Representative images of macroscopic observation in the thoracic cavity on day 21 are shown (C, vehicle only; D, temsirolimus). Immunohistochemical examination of resected disseminated tumor tissues from the control mice (E) and temsirolimus treated mice (F) was carried out to assess the expression status of phosphorylated mTOR (day 21). Each photograph was taken at high magnification (×200). The experiment was repeated three times and the representative data are shown.

cell cycle progression,[27,35] the cytostatic effect of temsirolimus can be at least partially explained by the importance of p70 S6 kinase to cell cycle progression. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and Akt are also interesting molecules related to cell proliferation signals. A recent study using rapamycin[33] showed that the inhibition of mTOR by temsirolimus appeared to regain Akt activity (Fig. S1). According to a previous report,[36] PTEN was lost in H1299 cells by its promoter methylation, whereas it remained intact in A549 cells. Regardless of their PTEN expression, our data indicated the similar potent antiproliferative effects of temsirolimus on those cell lines (Fig. 1).

Using an animal model of pleural dissemination, a condition for human lung cancer patients with one of the worst survival rates, we observed that temsirolimus reduced the growth of both s.c. tumors and pleural disseminated tumors of NSCLC cells, and that the treatment significantly prolonged the survival of mice bearing disseminated pleural tumors (Fig. 5). It is noteworthy that the dose and schedule of temsirolimus treatment in this study followed those currently in clinical use for renal cell carcinoma, with no apparent adverse effects in the mice. Because this regimen has also been tolerated in several clinical studies for other cancers,[18–21] temsirolimus treatment might safely provide prolonged survival for advanced NSCLC patients, possibly due to its cytostatic effect.

One immunohistochemical study showed that there were differences in mTOR signaling activation depending on histological type.[14] According to that study, adenocarcinoma had more frequent ctivation of phosphorylated mTOR than squamous cell carcinoma. However, it was unclear what histological type of NSCLC temsirolimus treatment would be effective in clinical use. mTOR is frequently activated in adenocarcinoma, but the outcome differs depending on the mTOR expression.[37] In a future study, it would be intriguing to establish a more effective combination therapy with temsirolimus,[38] because mTOR activity can be modified by other effectors, such as growth factors[39] and nutrition.[40,41]

In conclusion, our data suggests that temsirolimus, with a cytostatic effect on cell proliferation, may be useful for NSCLC treatment in general and could give prolonged survival to advanced NSCLC cases with pleural dissemination specifically.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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