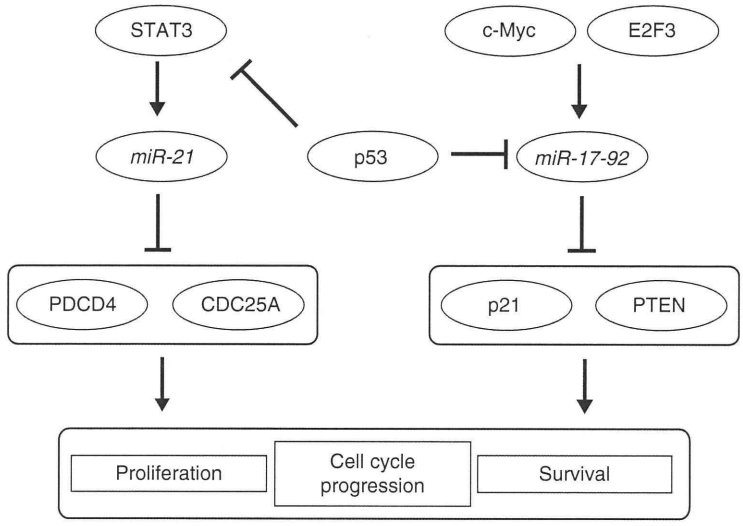

Figure 4. Oncogenic roles of *miR-21* and *miR-17-92* cluster in gastrointestinal cancer cells. STAT3-induced *miR-21* overexpression downregulates target genes (*PDCD4* and *CDC25A*), leading to proliferation, cell cycle progression and survival in human cancer cells. In contrast, c-Myc or E2F3 induction of the *miR-17-92* cluster downregulates target genes (*p21* and *PTEN*), leading to cell proliferation, cell cycle progression and survival in human cancer cells. p53 can suppress expression of *miR-21* and the *miR-17-92* cluster.

mechanism of the *miR-17-92* cluster (Figure 4), the *miR-17-92* cluster suppresses many target genes including *CDKN1A* (*p21*), phosphatase and tensin homologue (*PTEN*) and bcl-2 interacting mediator of cell death (*BIM*), thereby enhancing cell proliferation, cell cycle progression and cell survival [74].

4. miRNAs as novel biomarkers in gastrointestinal tumors

It has recently been predicted that it may be possible to detect aberrant miRNA expression in plasma, which could function as a novel biomarker for the early detection of various human cancers [75]. Indeed, the expression levels of oncogenic *miR-21* and the *miR-17-92* cluster in plasma were significantly higher in patients with gastrointestinal cancers compared with healthy controls [76-78]. Furthermore, overexpression of *miR-21* was detected in fecal miRNAs from patients with colorectal tumors including adenoma and adenocarcinoma [79]. These reports suggest that detection of oncogenic miRNAs that are highly expressed in the blood and stools of patients with gastrointestinal tumors is a promising screening system for early diagnosis of these tumors. Furthermore, the expression levels of *miR-17-3p* and *miR-92* among the *miR-17-92* cluster was significantly reduced after surgery in plasma of patients with colorectal cancers [78]. Thus, re-overexpression of oncogenic miRNAs in blood and stool may be also useful biomarker for the early detection of tumor recurrence after surgical resection in gastrointestinal cancer patients.

5. Potential role of miRNAs in cancer gene therapy for gastrointestinal tumors

5.1 Upregulation of tumor-suppressive *miR-34* expression

5.1.1 *Conventional therapy*

Conventional anticancer therapy, such as chemotherapy and radiation, induces *miR-34* expression in human cancer cells that have normal p53 and *miR-34* function (Figure 5) [12,24-27]. However, since more than 50% of human gastrointestinal tumors lack normal p53 function [28-33] and are therefore deficient in p53-induced *miR-34* expression, novel anticancer therapy that can induce *miR-34* expression in these tumors needs to be developed.

5.1.2 *p53-expressing adenovirus*

One possible method that might be effective for induction of *miR-34* in human cancer cells that have no functional p53 expression due to mutation or to chromosome 17p13 loss (Figure 2) is to infect the tumor cells with a p53-expressing adenovirus vector (Ad-p53) (Figure 5). Previous studies have shown that adenovirus-mediated overexpression of the p53 gene suppresses cell proliferation and tumor growth through induction of apoptotic cell death in human gastric cancer cells [80,81]. We previously reported that adenovirus-mediated wild-type p53 transfer efficiently suppressed cell proliferation, tumor growth and angiogenesis in human colon cancer cells [82,83]. Furthermore, induction of p53 overexpression by the Ad-p53 vector, in combination with aspirin treatment, enhances apoptotic cell

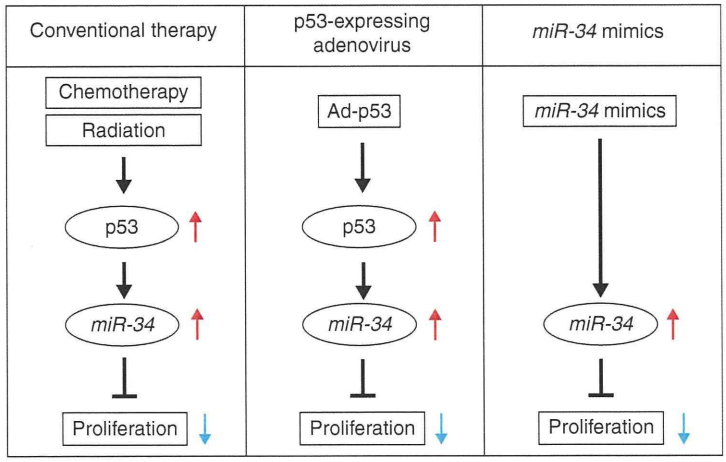

Figure 5. Suppression of aberrant cell proliferation through *miR-34* upregulation by conventional therapy, by a p53-expressing adenovirus or by *miR-34* mimics in human cancer cells. Left panel: conventional therapy, such as chemotherapy and radiation, upregulates *miR-34* expression through p53 activation in human cancer cells that have normal p53 and *miR-34* function. Middle panel: a p53-expressing adenovirus results in exogenous p53 expression, leading to *miR-34* expression, in human cancer cells that lack normal p53 function. Right panel: *miR-34* mimics result in *miR-34* expression in human cancer cells that lack normal *miR-34* function.

death through inhibition of NF-κB expression in human colon cancer cells [84]. These findings suggest that adenovirus-mediated p53 overexpression is a promising antitumor therapy for gastrointestinal tumors. Adenovirus-mediated p53 overexpression may induce *miR-34* expression, thereby contributing to the suppression of tumor growth in gastrointestinal tumors.

Several Phase I clinical trials have shown that treatment with recombinant, replication-deficient Ad-p53 was well tolerated in patients with NSCLC [85-87]. However, the low transduction rate of p53 gene transfer by replication-deficient Ad-p53 is major problem that needs to be overcome in order to improve the clinical outcome in patients with advanced cancers. We recently reported that combination therapy of Ad-p53 with a replication-competent oncolytic adenovirus enhances and sustains the expression level of p53, leading to enhanced apoptotic cell death of human cancer cells [88]. Furthermore, a conditionally replication-competent p53-expressing adenovirus also enhances and sustains p53 gene expression [89], which probably leads to strong *miR-34* expression in human cancer cells.

5.1.3 *miR-34 mimics*

In the case of human cancer cells that have no functional *miR-34* expression due to promoter methylation and/or loss of chromosome 1p36 or 11q23 (**Figure 2**), direct *miR-34* upregulation by *miR-34* mimics should be attempted (**Figure 5**). We previously reported that ectopic expression of *miR-34a* suppressed cell viability and induced subsequent senescence-like growth arrest in human colon cancer cells that expressed either wild-type or mutated *p53* genes [12]. Furthermore, *miR-34a* overexpression was recently reported to suppress tumor sphere formation of *p53*-mutated human gastric cancer cells [90]. Since tumor sphere formation is one of the characteristics of cancer stem cells [91], restoration of *miR-34a* expression may be a promising antitumor therapy against cancer stem cells in gastrointestinal tumors. Indeed, an antitumor effect of *miR-34a* overexpression has been recently shown in human cancer stem cells in the pancreas [92] and the brain [93]. Exploration of the antitumor effect of *miR-34a* mimics against cancer stem cells in gastrointestinal tumors is warranted.

5.2 Downregulation of oncogenic *miR-21* expression

Since a variety of human cancer cells including gastrointestinal tumors have been shown to overexpress *miR-21* [53-59], development of a cancer gene therapy that would suppress oncogenic *miR-21* overexpression would be a promising antitumor therapy against many human cancers. Several strategies, such as anti-inflammatory drugs, antisense oligonucleotides and miRNA sponges, have been suggested to efficiently suppress oncogenic miRNA expression in human cancer cells.

5.2.1 *Anti-inflammatory drugs*

The anti-inflammatory drug, Curcumin, has been recently shown to downregulate *miR-21* expression in human pancreatic cancer cells [94]. Since Curcumin can inhibit IL-6-mediated STAT3 activation [95], which induces *miR-21* expression in human colon cancer cells [63], Curcumin treatment may downregulate *miR-21* expression in human colon cancers.

5.2.2 *Antisense oligonucleotides*

Antisense oligonucleotides have been frequently used in *in vitro* experiments to directly suppress the expression of oncogenic miRNAs. A *miR-21* antisense oligonucleotide has been shown to suppress *miR-21* expression in human gastric cancer cells, resulting in suppression of cell proliferation and induction of apoptotic cell death [58]. In *in vivo* settings, a *miR-21* antisense oligonucleotide efficiently suppresses the tumor growth of human breast cancer cells [9] and human glioma cells [96]. These results suggest that the use of *miR-21* antisense oligonucleotides is a promising antitumor therapy against gastrointestinal tumors.

5.2.3 *miRNA sponges*

Overexpression of a miRNA sponge, which contains multiple binding sites for a specific miRNA, has been shown to downregulate the inhibitory effect of endogenous miRNA against many target genes [97]. Recently, it has been shown that a miRNA sponge for *miR-10b*, whose expression is significantly associated with breast cancer metastasis, can suppress *miR-10b* expression as efficiently as an antisense oligonucleotide and contributes to the suppression of lung metastasis in an orthotopic breast tumor animal model [98]. Thus, miRNA sponges may also be a promising antitumor therapy for the suppression of oncogenic *miR-21* overexpression in human gastrointestinal tumors.

5.3 Delivery of miRNA-based cancer gene therapy in gastrointestinal tumors

Establishment of delivery systems to induce efficient antitumor effect without normal tissue damage is an important issue for the miRNA-based cancer gene therapy. If gastrointestinal cancers are observed under gastroendoscopy or colonoscopy, the intratumoral injection of miRNA mimics, adenoviral vectors, antisense oligonucleotide and miRNA sponges can be performed. However, if the gastrointestinal tumors are with distal organ metastasis, systemic delivery of miRNA-based cancer gene therapy should be considered.

6. Conclusions

Diverse genetic alterations have been shown by many cancer researchers to play a role in the pathogenesis of gastrointestinal tumors and a 'multi-step colon carcinogenesis theory' has been established by Vogelstein's group [99] since the 1990s. However, since non-coding miRNAs have been shown to be deregulated in a variety of human cancers including gastrointestinal tumors [2,3], in order to understand the pathogenesis of gastrointestinal tumors it will be necessary to determine the molecular mechanism of the interaction between protein-coding genes and non-coding miRNA genes [100]. Thus, an understanding at the molecular level of miRNA-related cancer progression would provide a novel platform for the development of miRNA-based tumor diagnosis and cancer gene therapy for the treatment of patients with gastrointestinal tumors.

7. Expert opinion

Recent advances in molecular biology have revealed the aberrant expression of many miRNAs in a variety of human cancers including gastrointestinal tumors, suggesting a potential role of miRNAs in tumor initiation, progression and metastasis. Indeed a number of reports have indicated that miRNAs can regulate diverse cell fates in human normal and cancer cells. The miRNAs *miR-192/194/215* and *miR-7* have recently been shown to play functional roles during differentiation of human intestinal epithelial cells. In contrast, human gastrointestinal tumors show downregulation of tumor suppressive miRNAs (*miR-34* and *miR-143/145*) and upregulation of oncogenic miRNAs (*miR-21* and *miR-17-92*). Interestingly, the tumor suppressor p53 can induce both differentiation-related and tumor-suppressive miRNAs, whereas it can further suppress oncogenic miRNAs in gastrointestinal epithelium and tumors. These data suggest that restoration of *p53* expression is a promising cancer gene therapy against gastrointestinal tumors. However, the low transduction rate of *p53* gene transfer by a replication-deficient Ad-p53 is a major problem that needs to be overcome in order to improve the clinical outcome in patients with advanced cancers. In human cancers with *miR-34* dysfunction, restoration of *miR-34* rather than of p53 may be effective for induction of *miR-34* expression. Furthermore, suppression of oncogenic miRNA expression in combination with *miR-34* restoration may be a more effective therapy than restoration of p53. Thus, understanding of the molecular mechanism of miRNA-related cancer progression would provide a novel platform for the development of miRNA-based cancer gene therapy for the treatment of patients with gastrointestinal tumors. Furthermore, the development of an early detection system for oncogenic miRNAs that are highly expressed in blood and stool would improve the clinical outcome of patients with gastrointestinal tumors.

Declaration of interest

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EXPERT REVIEWS

Telomerase-specific oncolytic virotherapy for human gastrointestinal cancer

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Replication-selective tumor-specific viruses present a novel approach for treatment of neoplastic disease. These vectors are designed to induce virus-mediated lysis of tumor cells after selective viral propagation within the tumor. Human telomerase is highly active in more than 85% of primary cancers, regardless of their tissue origins, and its activity correlates closely with human telomerase reverse transcriptase (hTERT) expression. We constructed an attenuated adenovirus 5 vector (OBP-301), in which the hTERT promoter element drives the expression of *E1* genes. Since only tumor cells that express telomerase activity are able to activate this promoter, the hTERT proximal promoter allows for preferential expression of viral genes in tumor cells, leading to selective viral replication and oncolytic cell death. Lymphatic invasion is a major route for cancer cell dissemination, and adequate treatment of locoregional lymph nodes is required for curative treatment in patients with gastrointestinal tumors. In this article we show that intratumoral injection of OBP-301 mediates effective *in vivo* purging of metastatic tumor cells from regional lymph nodes, which may help optimize treatment of human gastrointestinal malignancies.

KEYWORDS: adenovirus • colorectal cancer • lymph node • metastasis • telomerase

Viruses are the simplest form of life, carry genetic material and are capable of entering host cells efficiently. Because of these properties, many viruses have been adapted as gene-transfer vectors [1–3]. Adenoviruses have been studied extensively and are well-characterized. Adenoviruses are large dsDNA viruses with tropism for many human tissues such as bronchial epithelia, hepatocytes and neurons. Furthermore, they are capable of transducing nonreplicating cells and can be grown to high titers *in vitro*, which allows for their potential clinical use. High titers of replication-defective adenoviruses can be produced and have been successfully used in eukaryotic gene expression [1,4,5]. Numerous studies using *in vitro* and animal models have tested a wide variety of adenoviral gene-therapy agents and have reported potential beneficial effects for different target diseases, including tolerability and safety [6–9].

Oncolytic viruses that can selectively replicate in tumor cells and lyse infected cells have been extensively investigated as novel anticancer agents [3,10,11]. These vectors are designed to induce virus-mediated lysis of tumor cells after selective viral propagation within the tumor cell while remaining innocuous to normal tissues [12]. Clinical trials of intratumoral injection of Onyx-015, which is an adenovirus with the *E1B* 55-kDa gene deleted and engineered to selectively replicate in and lyse p53-deficient cancer cells [13], alone or in combination with cisplatin/5-fluorouracil, have been conducted in patients with recurrent head and neck cancer [14,15]. However, subsequent studies have clarified that the capacity of Onyx-015 to replicate independently of the cell cycle does not correlate with the status of p53 [16], but is determined by late viral RNA export [17].

The optimal treatment of human cancer requires improvement of the therapeutic ratio to increase the cytotoxic efficacy on tumor cells and decrease that on normal cells. This may not be an easy task because the majority of normal cells surrounding tumors are sensitive to cytotoxic agents. Thus, to establish reliable therapeutic strategies for human cancer, it is important to seek genetic or epigenetic targets present only in cancer cells. One of the targeting strategies has involved the use of tissue-specific promoters to restrict gene expression or viral replication in specific tissues. A large number of different tissue-specific promoters have been used for virotherapy applications for targeting tumors derived

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from various tissues; however, tumor-specific, rather than tissue-specific, promoters would be more advantageous. For example, the promoter of human telomerase reverse transcriptase (hTERT) is highly active in most tumor cells but inactive in normal somatic cell types.

This article highlights some very promising advances in cancer therapeutic technologies using the hTERT promoter against human gastrointestinal cancer, especially for regional lymph node metastasis.

Lymph node metastasis in human gastrointestinal cancer

Lymph node status provides important information for both the diagnosis and treatment of human gastrointestinal cancer. Lymphatic invasion is a major route for cancer cell dissemination, and lymph node metastases represent an aggressive tumor behavior and are associated with a high rate of regional recurrence, which portends a poor outcome and may produce marked morbidity [18–20]. Therefore, adequate resection of the locoregional lymph nodes is required for curative treatment in patients with gastrointestinal malignancies such as esophageal, gastric and colorectal cancers [21,22]. Extended lymphadenectomy, however, may greatly impair quality of life, especially for patients with early-stage epithelial neoplasms of the GI tract [23]. These primary tumors can be removed by new endoluminal therapeutic techniques such as endoscopic submucosal dissection; however, patients with submucosal invasion, lymphovascular infiltration of cancer cells, or undifferentiated histology often become candidates for surgical organ resection with lymphadenectomy because there is a risk of regional lymph node metastasis, although the frequency is relatively low [24]. For example, resection of upper gastrointestinal organs such as gastrectomy and esophagectomy may result in weight loss and microgastria. Thus, a less invasive procedure to selectively treat lymph node metastasis would benefit these patients by allowing them to avoid prophylactic surgery.

Telomerase activity for transcriptional cancer targeting

One of the hallmarks of cancer is unregulated proliferation of a certain cell population, which eventually affects normal cellular function in the human body, and this almost universally correlates with the reactivation of telomerase. Tumor cells can maintain telomere length predominantly due to telomerase, and its activity is detected in approximately 85% of malignant tumors [25], whereas telomerase is absent in most normal somatic tissues [26], with a few exceptions, including peripheral blood leukocytes and certain stem cell populations [27,28]. The strong association between telomerase activity and malignant tissue suggests that telomerase can be a plausible target for the diagnosis and treatment of cancer [29].

The enzyme telomerase is a ribonucleoprotein complex responsible for the addition of TTAGGG repeats to the telomeric ends of chromosomes and contains three components: a RNA subunit (known as hTR, hTER or hTERC) [30], telomerase-associated protein (hTEP)1 [31], and a catalytic subunit (hTERT) [32,33]. Both hTR and hTERT are required for the reconstitution of telomerase activity *in vitro* [34] and, therefore, represent the minimal catalytic core of telomerase in humans [35]. Both hTR and hTERT transcripts are easily detectable in cancer cells but are either absent or exist in low levels in normal cells [36]. However, the hTR promoter is always stronger than hTERT, with presumably more background [37]. Thus, the hTERT promoter region can be substantially used as a fine-tuning molecular switch that works exclusively in tumor cells.

hTERT promoter-driven telomerase-specific oncolytic adenovirus

The use of modified adenoviruses that replicate and complete their lytic cycle preferentially in cancer cells is a promising strategy for the treatment of cancer. One approach to achieve tumor specificity of viral replication is based on the transcriptional control of genes that are critical for virus replication, such as *E1A* or *E4*. As described earlier, telomerase, especially its catalytic subunit hTERT, is expressed in the majority of human cancers and the hTERT promoter is preferentially activated in human cancer cells [25]. Thus, the broadly applicable hTERT promoter might be a suitable regulator of adenoviral replication. Indeed, it has been reported previously that transcriptional control of *E1A* expression via the hTERT promoter could restrict adenoviral replication to telomerase-positive tumor cells and efficiently lyse tumor cells [38–43]. Furthermore, Kuppuswamy *et al.* have recently developed a novel oncolytic adenovirus (VRX-011), in which the replication of the vector targets cancer cells by replacing the adenovirus *E4* promoter with the hTERT promoter [44]. VRX-011 is also able to overexpress the adenovirus death protein (also known as E3–11.6K), which is required for efficient cell lysis and the release of virions from cells at late stages of infection.

The adenovirus *E1B* gene is expressed early in viral infection and its gene product inhibits E1A-induced p53-dependent apoptosis, which in turn promotes the cytoplasmic accumulation of late viral mRNA, leading to a shutdown of host cell protein synthesis. In most vectors that replicate under the transcriptional control of the *E1A* gene including hTERT-specific oncolytic adenoviruses, the *E1B* gene, is driven by the endogenous adenovirus E1B promoter. However, Li *et al.* have demonstrated that transcriptional control of both *E1A* and *E1B* genes by the α-fetoprotein promoter with the use of the internal ribosome entry site significantly improved the specificity and therapeutic index in hepatocellular carcinoma cells [45]. Based on the aforementioned information, we developed telomelysin (OBP-301), in which the tumor-specific hTERT promoter regulates both the *E1A* and *E1B* genes (Figure 1). Telomelysin is expected to control viral replication more stringently, thereby providing better therapeutic effects in tumor cells, as well as attenuated toxicity in normal tissues [46].

In vitro & *in vivo* anti-tumor efficacy of telomelysin in human gastrointestinal cancer

The majority of human cancer cells acquire immortality and unregulated proliferation by the expression of hTERT [25], and therefore it has been hypothesized that hTERT-specific telomelysin possesses a broad-spectrum antineoplastic activity against a variety of human tumors [46,47]. Telomelysin induced selective

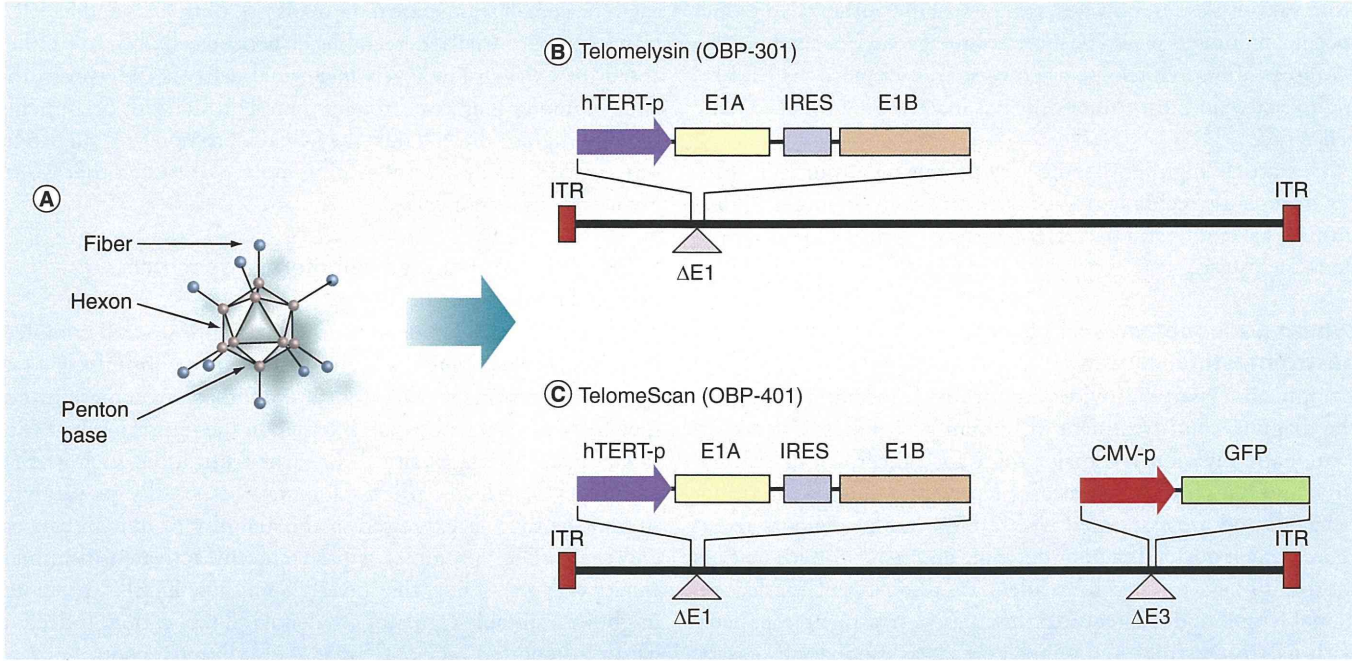

Figure 1. Structures of telomerase-specific oncolytic adenoviruses. (A) Schematic representation depicting the major structural components of the adenovirus (hexon, penton base and fiber). **(B)** Telomelysin (OBP-301), in which the promoter element drives the expression of *E1A* and *E1B* genes linked with an internal ribosome entry site. **(C)** TelomeScan (OBP-401) is a telomerase-specific replication-competent adenovirus variant, in which the *GFP* gene is inserted under a CMV-p into the E3 region for monitoring viral replication.
CMV-p: Cytomegalovirus promoter; GFP: Green fluorescent protein; hTERT-p: Human telomerase reverse transcriptase promoter; IRES: Internal ribosome entry site; ITR: Inverted terminal repeat.

E1A and E1B expression in cancer cells, which resulted in 5–6-log viral replication 3 days after infection; on the other hand, telomelysin replication was attenuated up to 2 logs in cultured normal cells [46,47].

In vitro cytotoxicity assays demonstrated that telomelysin could efficiently kill various types of human gastrointestinal cancer cell lines including esophageal cancer, gastric cancer and colorectal cancer in a dose-dependent manner [48]. These data clearly demonstrate that telomelysin exhibits desirable features for use as an oncolytic therapeutic agent, as the proportion of cancers potentially treatable by telomelysin is extremely high.

The *in vivo* anti-tumor effect of telomelysin was also investigated by using athymic mice carrying xenografts, because most murine tumor cells are known to express low levels of coxsackie and adenovirus receptor (CAR). Intratumoral injection of telomelysin into human colorectal tumor xenografts resulted in a significant inhibition of tumor growth and enhancement of survival [46,47]. Macroscopically, massive ulceration was noted on the tumor surface after injection of high-dose telomelysin, indicating that telomelysin induced intratumoral necrosis due to direct lysis of tumor cells by viral replication *in vivo* [49].

In vivo lymphatic spread of virus on regional lymph nodes

The therapeutic potential of viral agents against primary tumors as well as their systemic biodistribution targeting distant metastases has been intensively investigated [3,10,50]. However, few studies have examined the ability of the virus to traffic to the regional draining lymph nodes. Recently, Burton *et al.* showed that replication-deficient adenovirus could be successfully transported to the regional lymph nodes and noninvasively detect metastasis by expressing the prostate-specific reporter gene in an orthotopic prostate xenograft [51].

To verify that oncolytic adenoviruses traffic through the lymphatics to the regional lymph nodes, we used an orthotopic mouse model of human rectal cancer with spontaneous lymph node metastasis. We have demonstrated that intratumoral injection of the telomerase-specific, replication-selective, green fluorescent protein (GFP)-expressing adenovirus TelomeScan (OBP-401) (Figure 1) could efficiently visualize metastatic lymph nodes with GFP fluorescence signals in human cancer xenograft models [52,53]. These studies suggest the possible application of the adenovirus vectors as a lymphotropic agent for the treatment of lymph node metastasis.

In vitro purging of human colorectal cancer cells by telomelysin

In vitro purging experiments demonstrated that telomelysin infection could selectively eliminate human tumor cells in the presence of human or mouse lymphocytes [54]. We used TelomeScan to visualize viable human tumor cells after purging with telomelysin, as we have previously shown the high sensitivity and specificity of this molecular-imaging method [52,53]. It has been reported that the fiber-modified adenovirus serotype 5 (Ad5) and the

adenovirus vector based on another serotype such as Ad35 is able to efficiently transduce exogenous genes into hematopoietic cells, including stem cells. The unmodified Ad5, however, can rarely infect these cells because of the lack of CAR expression [55]. Indeed, Ad5-based telomelysin had no apparent effects on the viability of lymphocytes *in vitro*. These results suggest that normal lymphocytes in the regional lymph nodes could be strictly protected from telomelysin-induced oncolysis, because lymphocytes are not permissive for telomelysin infection and viral replication is also unlikely to occur in normal cells due to their low telomerase activity [27].

In vivo anti-tumor effect of telomelysin on lymph node metastasis

Mice bearing orthotopic human colorectal tumors received three courses of telomelysin intratumoral injections every 2 days, starting 2 weeks after tumor inoculation. Histopathological examination of the excised total lymph nodes showed that telomelysin treatment considerably reduced the metastatic rates. We also used a simple real-time *Alu* PCR assay to quantify the few metastatic human tumor cells in a background of large numbers of mouse host cells [54]. This human-specific amplification method enabled us to detect human tumor cells in a linear range of 10^3–10^8 cells/sample and monitor the time-dependent exponential growth of spontaneous lymph node metastasis from orthotopic colorectal tumor xenografts. In accordance with the histologically confirmed results, the *Alu* PCR assay indicated that intratumoral injection of telomelysin into the primary tumors significantly inhibited lymph node metastasis with high levels of viral replication.

We also used TelomeScan and a 3D optical detection system (IVIS® 200). After 2 weeks of orthotopic implantation of human colorectal tumor cells, telomelysin was administered intratumorally for five cycles. We then used the IVIS 200 imaging system to explore the abdominal cavity at laparotomy following a single injection of TelomeScan into the tumors. The number of GFP-positive lymph nodes and the GFP signal levels of individual lymph nodes were much higher in mock-treated control mice than in telomelysin-treated mice. Indeed, the sum of GFP fluorescence intensity in the abdominal cavity was significantly lower in mice treated with telomelysin, confirming the *in vivo* biological purging effect of telomelysin. The fact that two independent and highly sensitive approaches showed comparable results suggests a potent *in vivo* purging effect of oncolytic virotherapy on regional lymph nodes.

For effective treatment of metastatic tumors, intravenously infused chemotherapeutic drugs must be distributed in sufficient concentrations into the tumor sites; oncolytic viruses, however, are still able to replicate in the tumor, cause oncolysis and then release virus particles that could reach the distant metastatic lesions. Moreover, intratumoral injection can avoid hepatotoxicity that may be induced by systemic adenoviral administration. Therefore, intratumoral administration that causes the release of newly formed virus from infected tumor cells is theoretically suitable for oncolytic virus rather than systemic administration.

Preoperative intratumoral administration of telomelysin against lymph node metastasis

Currently, surgery and radiation are the most effective and clinically reliable local management strategies for human malignancies, including lymphatic metastases. Indeed, ionizing radiation targeting the lower half of the mouse body, including primary tumors and the para-aortic lymphatic area, significantly inhibited lymph node metastasis, although systemic toxicity such as weight loss was remarkable in irradiated mice compared with mice treated with telomelysin. In fact, total-body irradiation at a dose of 10 Gy has been reported to be lethal in mice because of acute radiation syndromes involving the hematopoietic system and GI tract [56]. We demonstrated that regional injection of telomelysin might be more simple and safe than radiotherapy as a treatment for metastatic lymph nodes [54].

We also assessed the effect of surgical resection of primary rectal tumors on lymph node metastasis. Unexpectedly, metastatic tumor cells in the lymph nodes considerably increased after surgical removal of primary rectal tumors, presumably due to the spread of tumor cells into the lymphatic circulation during the surgical procedure. Another possible explanation of this phenomenon includes a decrease in angiogenic inhibitors such as angiostatin and endostatin secreted from the primary tumor mass [57]. By contrast, intratumoral injection of telomelysin prior to surgical resection significantly inhibited lymph node metastasis. Telomelysin causes viral spread into the regional lymphatic area and selectively replicates in neoplastic lesions, resulting in eradication of lymph node metastasis. Tumor cells infected with telomelysin in the primary tumors are also unable to metastasize to the regional lymph nodes. Therefore, although the surgical procedure itself has the potential to promote regional metastasis, the preoperative treatment with telomelysin may prevent this undesirable event.

Clinical application of telomelysin

Preclinical models suggest that telomelysin could selectively kill a variety of human cancer cells *in vitro* and *in vivo* via intracellular viral replication regulated by hTERT transcriptional activity. Pharmacological and toxicological studies in mice and cotton rats have demonstrated that none of the animals treated with telomelysin showed signs of viral distress (e.g., ruffled fur, weight loss, lethargy or agitation) or histopathological changes in any organs at autopsy. These promising data led us to design a Phase I clinical trial of telomelysin as a monotherapy.

The protocol "A Phase I injection study of intratumoral injection with telomerase-specific replication-competent oncolytic adenovirus, telomelysin (OBP-301) for various solid tumors", sponsored by Oncolys BioPharma, Inc., is an open-label, Phase I, three-cohort dose-escalation study [58,59]. The trial commenced following approval from the US FDA in October 2006. The study has been completed to assess the safety, tolerability and feasibility of intratumoral injection of the agent in patients with advanced solid cancer. The doses of telomelysin were escalated from low to high virus particles in one log increment. In total, 16 patients with a variety of solid tumors such as melanoma, head

and neck cancer, breast cancer, lung cancer, and sarcomas were treated with a single-dose intratumoral injection of telomelysin and then monitored over 1 month.

All patients received telomelysin without dose-limiting toxicity. Common grade 1 and 2 toxicities included injection-site reactions (pain, induration) and systemic reactions (fever, chills). The data of pharmacokinetics and biodistribution of telomelysin may be of interest. Clinical trials of intratumoral and intravenous administration of CG7870, a replication-selective oncolytic adenovirus genetically engineered to replicate preferentially in prostate tissue, demonstrated a second peak of the virus genome in the plasma [60,61], suggesting active viral replication and shedding into the bloodstream. In fact, circulating viral DNA was transiently (<6 h after injection) detected in plasma in 13 out of 16 patients within 24 h of injection. This dose-dependent initial peak in circulating virus was followed by a rapid decline; however, three patients demonstrated evidence of prolonged viral replication through the detection of plasma viral DNA at days 7 and 14, suggesting telomelysin replication in primary tumors. One of these three patients had disappearance of the injected malignant lesion and locoregional uninjected satellite nodules, fulfilling a definition of complete response at day 28. Seven patients fulfilled the Response Evaluation Criteria In Solid Tumors (RECIST) definition for stable disease 56 days after treatment, although six patients showed a 6.6–43% reduction in tumor size. Thus, telomelysin is well-tolerated and warrants further clinical studies for solid cancer.

Expert commentary

There have been very impressive advances in our understanding of the molecular aspects of human gastrointestinal cancer and in the development of technologies for the genetic modification of viral genomes. Transcriptional targeting is a powerful tool for tumor selectivity in cancer therapy, and the hTERT-specific oncolytic adenovirus achieves a more strict targeting potential due to the amplified effect of viral replication. Several independent studies that used different regions of the hTERT promoter and different sites of adenoviral genome responsible for viral replication have shown that the hTERT promoter allows adenoviral replication as a molecular switch and induces selective cytopathic effects in a variety of human tumor cells [38–40,46–48]. Among these viral constructs, to the best of our knowledge telomelysin seems to be the first hTERT-dependent oncolytic adenovirus that has been used in a clinical trial based on preclinical pharmacological and toxicological studies. Thus, telomerase-specific targeted oncolytic adenovirus holds promise for the treatment of human cancer.

Nevertheless, many ethical and technical hurdles remain to be tackled and must be solved before virotherapy ever reaches routine clinical application. Safety considerations in the manufacture of the virus and clinical protocols are among the most important issues to be studied. Another important issue is to find ways to improve virus cell binding and entry. Although telomelysin showed a broad and profound anti-tumor effect in human cancer originating from various organs, one weakness of telomelysin is that virus infection efficiency depends upon CAR expression, which may not be highly expressed on the cell surface of some types of human cancer cells. Thus, tumors that have lost CAR expression may be refractory to infection with telomelysin. Since modification of fiber protein is an attractive strategy for overcoming the limitations imposed by CAR's dependence on telomelysin infection, we modified the telomelysin fiber to contain the Arg–Gly–Asp (RGD) peptide, which binds with high affinity to integrins ($\alpha v\beta 3$ and $\alpha v\beta 5$) on the cell surface, on the HI loop of the fiber protein. The resultant adenovirus, termed telomelysin–RGD or OBP-405, mediated not only CAR-dependent virus entry but also CAR-independent, RGD-integrin-dependent virus entry [47,62]. Telomelysin–RGD has an apparent oncolytic effect on human cancer cell lines with extremely low CAR expression. These data suggest that fiber-modified telomelysin–RGD exhibits a broad target range by increasing infection efficiency, although one needs to be cautious regarding increased toxicity since hematopoietic cell populations such as dendritic cells can be efficiently infected with RGD-modified adenovirus [63].

It has been shown that telomelysin delivered to the primary tumor site is able to spread into the regional draining lymphatics, selectively replicate in neoplastic foci, and then reduce the number of tumor cells in metastatic lymph nodes in an orthotopic human colorectal cancer xenograft model [54]. This virus-mediated molecular surgery for lymph node metastasis mimics the clinical scenario of lymphadenectomy; the technique, however, seems to be safer and less invasive. Moreover, we have demonstrated that preoperative delivery of telomelysin into primary tumors prevented the exacerbation of lymph node metastasis by surgical procedures. Telomelysin may offer advantages over other oncolytic viruses targeting lymphatic metastasis, as its safety profile as well as biodistribution pattern after intratumoral delivery have already been confirmed in a Phase I clinical trial for various types of solid tumors [58,59]. Our study provides evidence for the *in vivo* purging effect of telomelysin in regional lymph nodes that is sufficiently reliable to support this approach. Thus, Phase II studies of telomerase-specific virotherapy targeting lymph node metastasis in human cancer patients are warranted.

Five-year view

A possible future direction for telomelysin includes combination therapy with conventional therapies such as chemotherapy, radiotherapy, surgery, immunotherapy, and new modalities such as antiangiogenic therapy. Since the results of a Phase I clinical trial demonstrated that even partial elimination of the tumor induced by intratumoral injection of telomelysin could be clinically beneficial, the combination approaches may lead to the development of more advanced biological therapy for human cancer. The combination of systemic chemotherapy and local injection of telomelysin has previously been shown to be effective [64–66]. As a replication-deficient adenovirus could replicate in cancer cells and enhance the anticancer effect when cotransfected with telomelysin that could produce E1 protein, we demonstrated the synergistic effects of telomelysin combined with an E1-deleted replication-deficient adenoviral vector expressing human wild-type *p53* tumor-suppressor gene (Ad5CMV-p53; Advexin) [67,68]. Telomelysin is also synergistic with ionizing radiation against

human esophageal cancer cells, and we clarified the E1B 55 kDa-mediated mechanism used by telomelysin to inhibit DNA repair. Peri- or post-operative administration of telomelysin may be also valuable as adjuvant therapy in areas of microscopic residual disease at tumor margins to prevent recurrence or regrowth of tumors.

The field of targeted oncolytic virotherapy is progressing considerably and is rapidly gaining medical and scientific acceptance. Although many technical and conceptual problems remain to be solved, ongoing and future clinical studies will no doubt continue to provide important clues that may allow substantial progress in human gastrointestinal cancer therapy.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Key issues

- Adenoviruses are capable of transducing nonreplicating cells and can be grown to high titers *in vitro*, which allows for their potential use clinically as gene-transfer vectors.
- Adequate resection of the locoregional lymph nodes is required for curative treatment in patients with gastrointestinal malignancies; however, a less invasive method to selectively treat lymph node metastasis would benefit patients by allowing them to avoid a prophylactic surgery.
- Our team has developed telomelysin (OBP-301), in which the tumor-specific hTERT promoter controls viral replication stringently, thereby providing better therapeutic effects in tumor cells, as well as attenuated toxicity in normal tissues.
- Telomelysin delivered to the primary tumor site is able to spread into the regional draining lymphatics, selectively replicate in neoplastic foci, and then reduce the number of tumor cells in metastatic lymph nodes.
- Preoperative delivery of telomelysin into primary tumors prevents the exacerbation of lymph node metastases by surgical procedures.
- Phase II studies of telomerase-specific virotherapy targeting lymph node metastasis in human cancer patients are warranted.

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Clinical Cancer Research

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

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

Determination of miRNA expression levels in human normal and tumor cells

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

Infection with TRADs

Cells were seeded into 24-well plates at 5×10^4 cells/well. On the following day, cells were infected with TRADs at a multiplicity of infection (MOI) of 0.4 or 2 (for cancer cell lines), or of 10 (for normal cells), for 2 hours. Following incubation for 3 (for cancer cell lines) or 5 days (for normal cells), total DNA, including viral genomic DNA, was isolated from the cells using a DNeasy Blood & Tissue Kit (Qiagen). After isolation, the Ad genomic DNA contents were quantified using an ABI Prism 7000 system (Applied Biosystems) as previously described (25). The Ad genome copy numbers were normalized by the copy numbers of glyceroaldehyde-3-phosphate-dehydrogenase (GAPDH). Cell viability was also examined by crystal violet staining and Alamar blue assay at the indicated time points. To examine the miRNA-specific suppression of TRAD replication in normal human cells, 50 nmol/L of 2′-*O*-methylated antisense oligonucleotide complementary to miR-143 or miR-199a (Gene Design Inc.) was transfected into normal cells using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the cells were infected with TRADs and replication of TRADs was evaluated as described above.

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

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

Statistical analysis

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

Results

Replication of the conventional TRAD in normal human cells

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

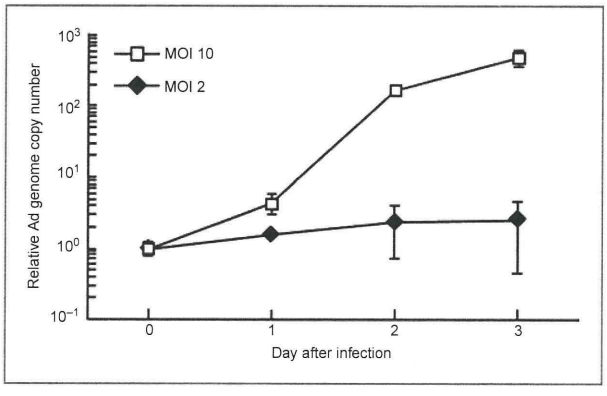

Figure 1. Replication of the conventional TRAD in WI38 cells. WI38 cells were infected with the conventional TRAD at an MOI of 2 or 10 for 2 hours. At the indicated time points, the copy numbers of the Ad genome and *GAPDH* gene were determined by real-time PCR. The ratio of the copy number of the Ad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

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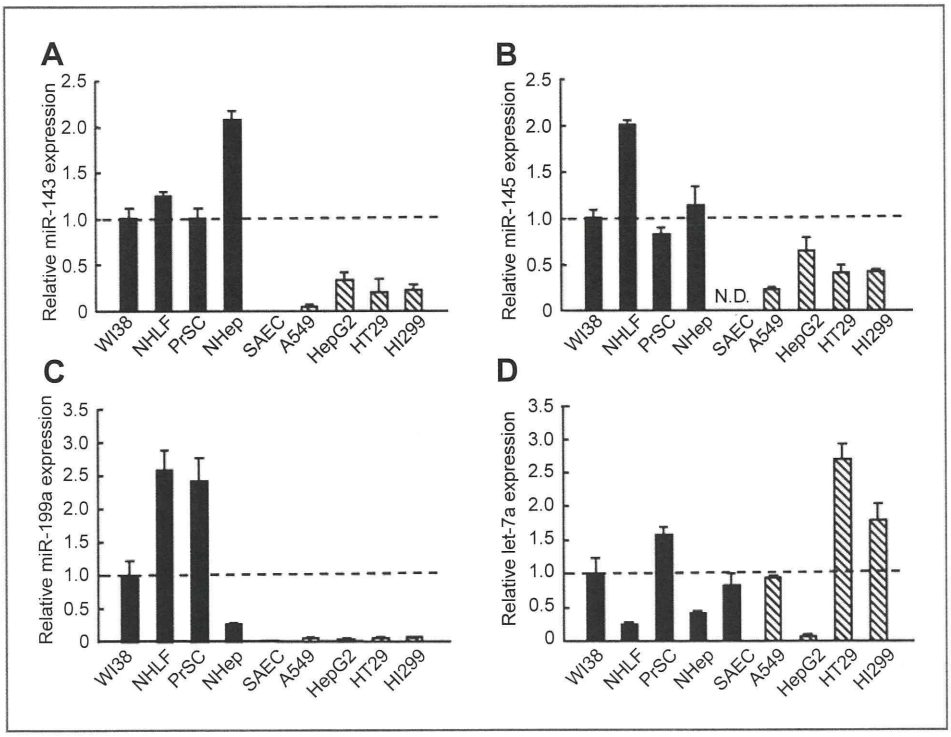

Figure 2. MiRNA expression levels in the human normal (solid bar) and tumor cells (hatched bar). MiRNA expression was determined by real-time RT-PCR. The ratio of miRNA to U6 expression levels was normalized by the data of WI38 cells. The data are shown as the means ± SD ($n = 3$). N.D., not detected.

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

MiRNA expression levels in human tumor and normal cells

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

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

Next, to develop TRADs carrying a miRNA-regulated *E1* gene expression cassette (TRAD-miRT), we incorporated 4 copies of the perfectly complementary sequences for miR-143, -145, -199a, or let-7a into the 3′-UTR of the *E1* gene expression cassette (Fig. 3A). In TRADs, the *E1A* gene was connected with the *E1B* gene via IRES. We found that the expression of both the first and second gene in the IRES-containing expression cassette was suppressed in an miRNA-dependent manner by insertion of the miR-122a complementary sequences into the region downstream of the second gene in miR-122a-expressing Huh-7 cells, not in HepG2 cells, which express a low level of miR-122a (Supplementary Fig. S1), although it remains controversial whether miRNA-mediated posttranscriptional regulation can occur in an IRES-containing expression cassette (28–30). All TRADs were efficiently grown in normal 293 cells, and the ratios of infectious titers to physical titers were comparable among all the TRADs, including the parental TRAD.

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

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

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

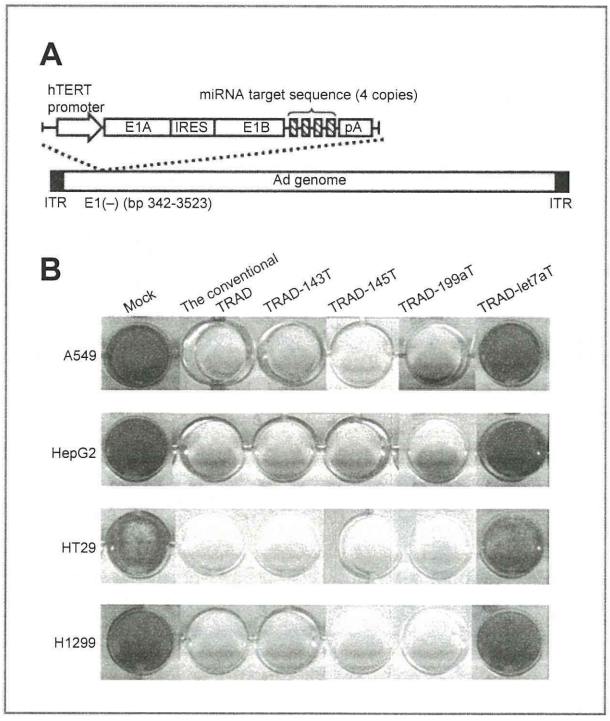

Figure 3. Replication and oncolytic activity of TRADs containing the miRNA complementary sequences in the tumor cells. A, a schematic diagram of a TRAD containing the miRNA-regulated *E1* gene expression system. ITR: inverted terminal repeat. B, crystal violet analysis of the cytopathic effects of TRADs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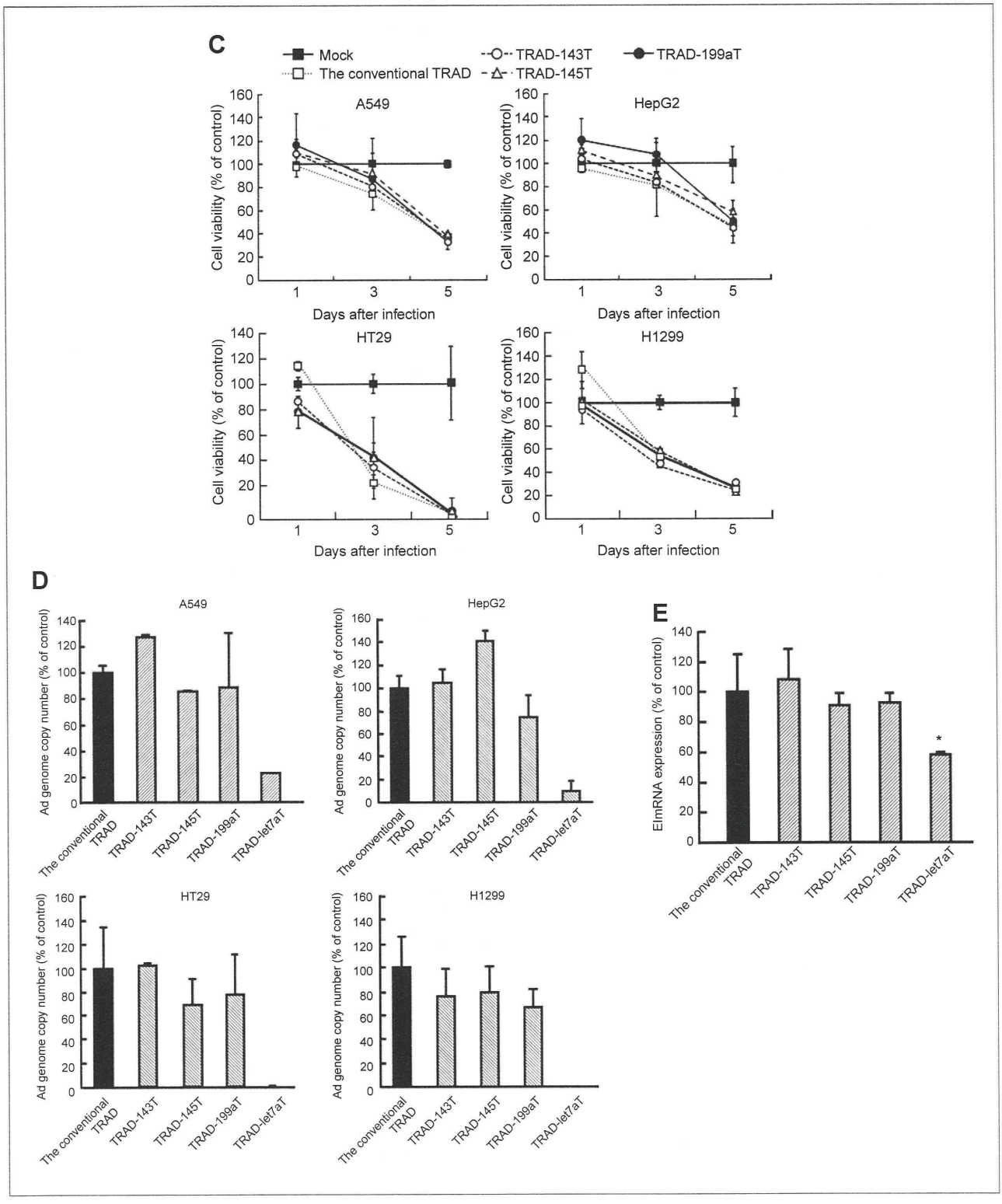

Figure 3. (*Continued*)