In Vivo Reprogramming of hTERT by Trans-splicing Ribozyme to Target Tumor Cells

ARTICLE *in* MOLECULAR THERAPY · FEBRUARY 2008

Impact Factor: 6.23 · DOI: 10.1038/sj.mt.6300282 · Source: PubMed

10 AUTHORS, INCLUDING:

Seong-Wook Lee
Dankook University
92 PUBLICATIONS **1,559** CITATIONS

SEE PROFILE

In Vivo Reprogramming of hTERT by *Trans*-splicing Ribozyme to Target Tumor Cells

Seung-Hee Hong[1,*], Jin-Sook Jeong[2], Yoon-Jong Lee[1], Haeng-Im Jung[1], Kyoung-Sook Cho[1], Chang-Min Kim[1], Byung-Su Kwon[3], Bruce A Sullenger[4], Seong-Wook Lee[3] and In-Hoo Kim[1]

[1]*Research Institute & Hospital, National Cancer Center, Goyang, South Korea;* [2]*Department of Pathology and Medical Research Center for Cancer Molecular Therapy, Dong-A University College of Medicine, Busan, South Korea;* [3]*Department of Molecular Biology, Institute of Nanosensor and Biotechnology, Dankook University, Seoul, South Korea;* [4]*Department of Surgery, Center for Translational Research, Duke University Medical Center, Durham, North Carolina, USA*

We have developed and validated a new tumor-targeting gene therapy strategy based upon the targeting and replacement of human telomerase reverse transcriptase (hTERT) RNA, using a *trans*-splicing ribozyme. By constructing novel adenoviral vectors harboring the hTERT-targeting *trans*-splicing ribozymes with the downstream reporter gene (Ad-Ribo-LacZ) or suicide gene (Ad-Ribo-HSVtk) driven by the cytomegalovirus (CMV) promoter, we demonstrated that this viral system selectively marks tumor cells expressing hTERT or sensitizes tumor cells to prodrug treatments. We confirmed that Ad-Ribo-LacZ successfully and selectively delivered a ribozyme that performed a highly specific *trans*-splicing reaction into hTERT-expressing cancer cells, both *in vitro* and in a peritoneal carcinomatosis nude mouse model. We also determined that the hTERT-specific expression of the suicide gene in the Ad-Ribo-HSVtk, and treatment with the corresponding prodrug, reduced tumor progression with almost the same efficacy as the strong constitutive CMV promoter-driven adenovirus, both in cancer cell lines and in nude mouse HT-29 xenografts. These observations provide the basis for a novel approach to cancer gene therapy, and demonstrate that *trans*-splicing ribozymes can be employed as targeting anti-cancer agents which recognize cancer-specific transcripts and reprogram them, thereby combating cancerous cells.

Received 20 March 2007; accepted 16 July 2007; published online 14 August 2007. doi:10.1038/sj.mt.6300282

INTRODUCTION

Recent gene therapy for the treatment of cancer has focused on the development of novel strategies to enhance both efficacy and safety. Gene targeting may represent an attractive approach to that end, given that the objective of gene therapy is the eradication of cancer cells without harming normal cells.[1] In the effort to achieve this, three significant approaches have been attempted: transcriptional targeting, transduction, and exploitation of cancer-associated cellular pathways.[2] However, the ideal of precise and effective cancer cell-specific targeting remains a significant challenge.[3] In this study, we evaluated a new tumor-targeting gene therapy tool that utilizes a *trans*-splicing ribozyme, and we explored the efficacy of a recombinant adenovirus encoding for a ribozyme as a potential therapeutic agent against human cancers.

The self-splicing group I intron from *Tetrahymena thermophila* mediates the *trans*-splicing of an exon ligated at its 3′-end onto a targeted and separate 5′ exon RNA both *in vitro*[4] and in bacteria[5] and mammalian cells.[6] These *trans*-splicing ribozymes have been used for correcting defective transcripts that cause human genetic or malignant diseases.[7–11] Ribozyme-mediated *trans*-splicing has also been used in the induction of selective cytotoxicity in target cells.[12] Recently, we showed that the *trans*-splicing ribozyme specifically and selectively replaces viral transcripts with a new RNA that exerts anti-viral effects by recognizing the viral-specific RNA, and catalyzing the in-frame fusion of desired RNA sequences.[13] Moreover, we developed a ribozyme that reprogrammed human telomerase reverse transcriptase (hTERT) RNA to selectively induce transgene activity in cancer cells expressing hTERT, thereby demonstrating that *trans*-splicing ribozymes can serve as anti-cancer agents.[14] We selected hTERT-targeting *trans*-splicing ribozyme because it is expressed at high levels in most malignant tumors, but is not detected in normal postmitotic cells.[15,16] Thus far, however, there has been no proof of the efficacy of ribozyme-mediated *trans*-splicing in inducing therapeutic genes *in vivo*, in a tumor-specific manner.

In this context, we evaluated the feasibility of using an adenoviral vector encoding for an hTERT-targeting *trans*-splicing ribozyme as a tumor-targeting gene therapy tool. Targeting the 5′-untranslated region of hTERT messenger RNA, we assessed the efficacy and specificity of the *trans*-splicing reaction in $hTERT^+$ and $hTERT^-$ cells. We also attempted to determine whether reporter gene activity could be induced selectively in a peritoneal carcinomatosis mouse model by systemic delivery of the adenoviral vector, and whether the ribozyme would trigger selective

**Present address:* *Korea Human Resource Development Institute for Health and Welfare, Seoul, South Korea.*
***Correspondence:** Seong-Wook Lee, Department of Molecular Biology, Dankook University, San8, Hannam-Dong, Yongsan-Gu, Seoul 140-714, South Korea. E-mail: SWL0208@dankook.ac.kr or In-Hoo Kim, Molecular Imaging & Therapy Branch, Research Institute, National Cancer Center, 809 Madu-dong, Ilsan-gu, Goyang-si, Gyeonggi 410-769, South Korea. E-mail: ihkim@ncc.re.kr*

cytotoxicity in hTERT-expressing cells. We also analyzed the therapeutic effect of the adenovirus on mice implanted subcutaneously with hTERT-expressing human cancer cells.

RESULTS

Ad-Ribo-LacZ induces reporter gene expression selectively in hTERT$^+$ cell lines

By evaluating telomerase activity, we confirmed hTERT$^+$ in HT-29, SNU-407, HepG2, SNU-398, and SBC-5 cell lines, and hTERT$^-$ in an SKLU-1 cell line. Telomerase activity was highest in SBC-5 cells and relatively high in the other hTERT$^+$ cell lines when compared with the commercial positive control. However, no telomerase activity was detected in the SKLU-1 cells (**Figure 1a**). We then assessed whether new gene activity could be induced selectively in hTERT$^+$ cells by Rib21AS-3′exon-LacZ ribozyme, which *trans*-splices the β-galactosidase gene (*lacZ*) onto the hTERT transcript and allows for the monitoring of β-galactosidase expression. We observed high β-galactosidase levels in cells infected with Ad-LacZ, regardless of hTERT status. In contrast, Ad-Ribo-LacZ infection conferred elevated β-galactosidase activity only in the hTERT$^+$ cells (**Figure 1b**). Moreover, Ad-Ribo-LacZ was observed to induce β-galactosidase activity in hTERT$^+$ cells almost as efficiently (80–90%) as Ad-LacZ control virus did. The histological analysis of β-galactosidase activity verified the selectivity of Ad-Ribo-LacZ with regard to transgene expression. When infected with Ad-LacZ, all tested cell lines expressed β-galactosidase and were visualized by 5-bromo-4-chloro-3indoyl N-acetyl-β-D-galactosaminide (X-gal) staining. However, Ad-Ribo-LacZ was observed to induce β-galactosidase expression only in hTERT$^+$ cells, and not in the hTERT$^-$ SKLU-1 cells (**Figure 1c**). This indicates that Ad-Ribo-LacZ induces transgene expression both efficiently and selectively, in an hTERT-dependent manner.

In order to determine whether ribozyme-mediated transgene expression induces the *trans*-splicing reaction with hTERT RNA at the predicted site, we infected hTERT$^+$ HT-29 and hTERT$^-$ SKLU-1 cells with various multiplicity of infection (MOI) of Ad-Ribo-LacZ, and then analyzed β-galactosidase expression by reverse transcriptase-polymerase chain reaction (PCR). We detected *trans*-spliced molecules (TSMs) only in the hTERT$^+$ cells and not in the hTERT$^-$ cells, even though ribozymes (represented as *lacZ* RNA production) were present in the hTERT$^-$ cells (**Figure 1d**). Furthermore, the quantity of TSM produced correlated with the quantity of transfected Ad-Ribo-LacZ. Sequence analysis of the amplified fragment confirmed that the Ad-Ribo-LacZ correctly targeted hTERT's uridine, at position 21, and inserted *lacZ* RNA at its 3′exon (**Supplementary Figure S1**).

We also observed reduced telomerase activity in the hTERT$^+$ cells, as a consequence of the *trans*-splicing reaction of Ad-Ribo-LacZ. Since telomerase activity depends on hTERT RNA expression, endogenous telomerase activity should be reduced by Ad-Ribo-LacZ transfection. In order to test this, we assayed telomerase activity in the HT-29 cells following infection with Ad-Ribo-LacZ and Ad-LacZ. As expected, Ad-Ribo-LacZ infection reduced telomerase activity in a dose-dependent manner (10, 20, 23, and 33% reduction at MOI of 1, 10, 50, and 100, respectively). In contrast, Ad-LacZ infection did not effect a reduction in telomerase activity (**Figure 1e**). Collectively, these results clearly indicate that the Ad-Ribo-LacZ-mediated *trans*-splicing reaction is highly specific for its target sequence, and that the ribozyme in Ad-Ribo-LacZ (Rib21AS-3′exon-LacZ) effectively executes the cleavage/*trans*-ligation activity as designed.

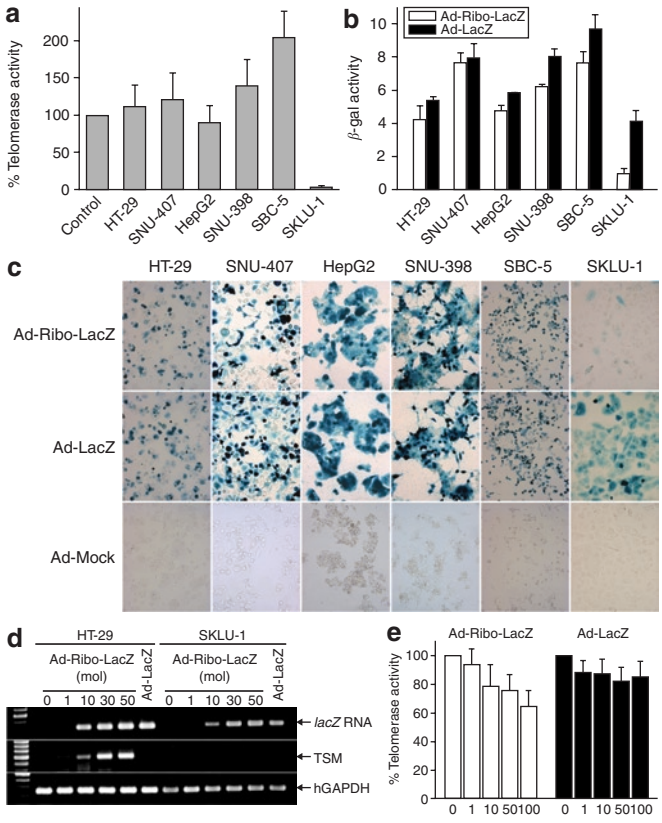

Figure 1 Human telomerase reverse transcriptase (hTERT) RNA-dependent reporter gene expression and telomerase activity reduction, caused by the hTERT-specific ribozyme Rib21AS-3′exon-LacZ. (**a**) Telomerase activity levels in the cell lines used in this experiment are shown here. Telomerase activity levels were compared with a control arbitrarily set at 100%. Values are the mean values ± SD for triplicate assays. (**b**) Assessment of β-galactosidase activity following the transduction of either Ad-Ribo-LacZ or Ad-LacZ into hTERT$^+$ cells and hTERT$^-$ cell SKLU-1. Forty-eight hours later, specific enzyme activities were determined as described in **Supplementary Materials and Methods**. The mean values of measurements performed in triplicate are shown, with the bars indicating SD. (**c**) Histochemical staining with X-gal in various cancer cells after transfection by Ad-Ribo-LacZ, Ad-LacZ, or Ad-Mock. After staining, cells were observed at ×100 magnification with the aid of a light microscope, and photographed. (**d**) Allele-specific reverse transcriptase-polymerase chain reaction of *trans*-spliced product from HT-29 cells transfected with Ad-Ribo-LacZ. The hTERT$^+$ HT-29 and hTERT$^-$ SKLU-1 cells were transfected with Ad-Ribo-LacZ at 1, 10, 30, or 50 multiplicity of infection (MOI), and cells treated with 50 MOI Ad-LacZ were used as a negative *trans*-splicing control. The production of ribozyme or transgene RNA is represented by the amplification of *lacZ* RNA. As an internal control, human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) RNA was amplified. (**e**) Dose-dependent reductions in the expression level of endogenous hTERT RNA by Ad-Ribo-LacZ. The relative telomerase activity levels in HT-29 cells transfected with Ad-Ribo-LacZ (open bars) and Ad-LacZ (closed bars) at 0, 1, 10, 50, and 100 MOI are shown. The activity in the non-transfected HT-29 cells was set at 100%. The ratios relative to positive control were plotted as the mean values ± SD for quaternary assays.

Transgene induction by Ad-Ribo-LacZ in hTERT-expressing tumor is selective *in vivo* as well

In view of the fact that Rib21AS-3′exon-LacZ selectively induced transgene expression in target RNA-expressing cells *in vitro*, we tested whether an adenovirus harboring the ribozyme also functions similarly *in vivo*. In a preliminary study, we systemically administered Ad-Ribo-LacZ or Ad-LacZ (1×10^9 plaque forming units) to female Balb/c mice by intravenous injection via tail veins, killed them 2 days later, and examined various tissues. Using PCR analysis with vector-specific primers, we detected high levels of Ad-Ribo-LacZ DNA in the liver, significantly lower levels in the lung, adipose tissue, and spleen, and barely detectable levels in other tissues (**Supplementary Figure S2a**). We further analyzed transgene expression by histochemical analysis (X-gal staining). Interestingly, mice injected with Ad-Ribo-LacZ evidenced little or no reporter gene activity, even in virus-laden tissues. We did, however, observe diffuse and extensive β-galactosidase expression in the liver and distinct cortical glomerular expression in the kidneys of Ad-LacZ-transfected mice (**Supplementary Figure S2b**).

Next, we tested whether the ribozyme-harboring adenovirus had the potential for tumor-targeted anti-cancer gene therapy in a colorectal adenocarcinoma–derived peritoneal carcinomatosis model. Ten days after peritoneal carcinomatosis induction, we randomly grouped mice and injected them with Ad-Ribo-LacZ (n = 5), Ad-LacZ (n = 5), or phosphate-buffered saline (PBS) (n = 3). We injected 1×10^9 plaque forming units of virus in 400 µl PBS intraperitoneally three times at 2-day intervals. Two days after the final virus injection, we killed the mice. Prior to analysis, we randomly selected one mouse from each virus-injected group and examined its abdominal organs macroscopically after X-gal staining for *lacZ* expression. We determined that Ad-Ribo-LacZ induced *lacZ* expression in tumor nodules, but not in other organs. In contrast, in the mice treated with Ad-LacZ, multifocal X-gal staining was observed in tumor nodules as well as on the exteriors of the liver and gastroenteric serosa (**Figure 2a**). However, the X-gal staining appeared heterogeneous in this case, suggesting that viruses tend to spread in a non-homogeneous manner after intraperitoneal administration.[17] We then analyzed the non-tumor-containing tissues of the mice remaining in each group for *lacZ* expression. In mice injected with Ad-LacZ, we observed β-galactosidase expression on the surfaces of the liver, stomach, and esophagus. In Ad-Ribo-LacZ-treated mice, we detected no β-galactosidase expression (**Figure 2b**). We also conducted PCR in order to verify the efficacy of transgene

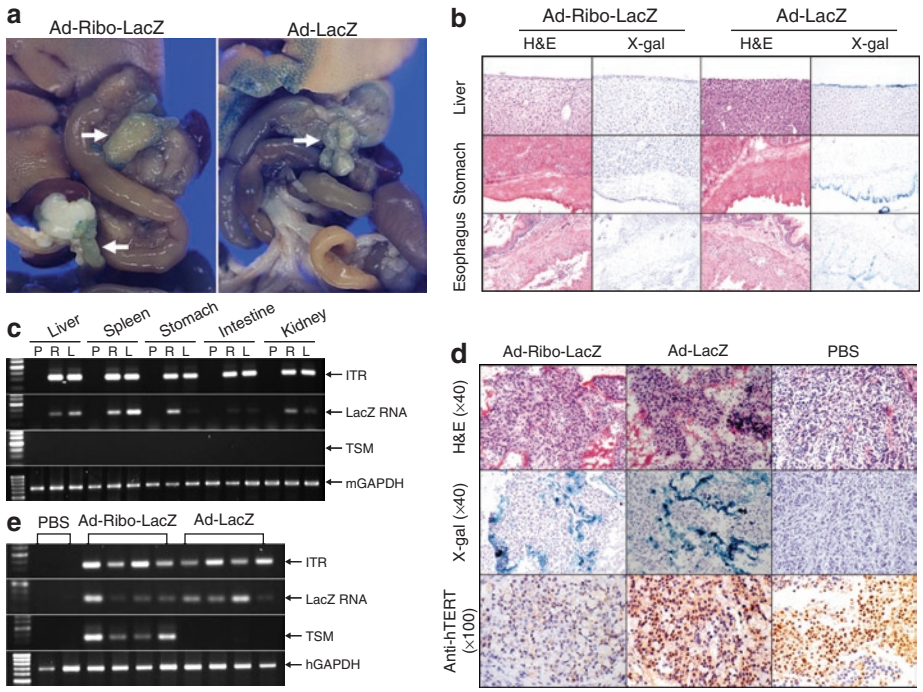

Figure 2 Selective induction of transgene expression by Ad-Ribo-LacZ in mouse cancer models. (**a**) β-galactosidase expression pattern detected by X-gal staining *in toto* in the peritoneal cavities of mice with established peritoneal carcinomatosis, intraperitoneally injected with Ad-Ribo-LacZ or Ad-LacZ. Arrows indicate tumor nodules. (**b**) Representative histochemical findings of transgene expression patterns in abdominal organs. Histologic findings (hematoxylin and eosin (H&E), ×40) and β-galactosidase expression (X-gal, ×40) are shown in non-tumor tissues from Ad-Ribo-LacZ or Ad-LacZ-injected peritoneal carcinomatosis model mice. (**c**) Tissue distribution and transgene expression patterns of injected viruses in non-tumor tissues of mice with established peritoneal carcinomatosis. The distribution of injected viruses (Lane R, Ad-Ribo-LacZ; Lane L, Ad-LacZ), transgene expression or ribozyme production, and the production of *trans*-spliced molecule detected in various tissues by polymerase chain reaction (PCR) for the inverted terminal repeats (ITR) region, reverse transcriptase-PCR (RT-PCR) for *lacZ* RNA (LacZ RNA), and *trans*-spliced molecule (TSM), respectively. Tissues from phosphate-buffered saline (PBS)-injected mice (Lane P) served as a negative control. The RT-PCR product of mouse glyceraldehyde-3-phosphate dehydrogenase (mGAPDH) served as an internal control. (**d**) Representative histochemical findings in developed tumor nodules. Histologic findings (H&E, ×40), β-galactosidase expression (X-gal, ×40), and human telomerase reverse transcriptase (hTERT) expression (Anti-hTERT, ×100) are shown in tumor nodules from Ad-Ribo-LacZ- or Ad-LacZ-injected mice with peritoneal carcinomatosis. (**e**) Presence and transgene expression patterns of injected viruses in tumor nodules; analysis similar to the one described in **c**. Each lane represents a tumor nodule from an individual mouse. The endogenous human GAPDH (hGAPDH) served as an internal control.

expression. We found that intraperitoneal viral introduction into mouse abdominal organs successfully delivered viruses, as determined by transgene expression. However, no TSM was detected in non-tumorous mouse organs, even in the Ad-Ribo-LacZ injected group, thereby indicating that the *trans*-splicing reaction specifically targeted hTERT only *in vivo* (**Figure 2c**). We observed no significant differences between individuals within groups.

We then analyzed at least three tumor nodules from each group to see whether differences existed in the transgene expression patterns. We observed β-galactosidase expression in the developed tumor nodules of mice injected with Ad-Ribo-LacZ or Ad-LacZ. Furthermore, the Ad-Ribo-LacZ-treated mice, when compared against the Ad-LacZ- or PBS-treated mice, had markedly lower hTERT expression in tumor nodules (**Figure 2d**). PCR analysis detected viral inverted terminal repeats and *lacZ* RNA in tumor nodules infected with Ad-Ribo-LacZ and Ad-LacZ, suggesting that the viruses efficiently dispersed within the tumor nodules. Using reverse transcriptase-PCR we determined whether the Rib21AS-3′exon-LacZ specifically targeted hTERT$^+$ tumor nodules. As in the *in vitro* targeting studies with the HT-29 cells, we detected correctly targeted TSMs only in tumor nodules transfected with Ad-Ribo-LacZ (**Figure 2e**). Moreover, rapid amplification of cDNA end reverse transcriptase-PCR analysis revealed that all of the *trans*-splicing products generated in the tumors of mice injected with Ad-Ribo-LacZ (20 clones) resulted only from reactions with the hTERT RNA (data not shown). These results demonstrate that adenovirus-mediated *trans*-splicing reactions by hTERT-specific ribozymes both effectively and specifically induce transgene expression *in vivo*.

Efficacy and specificity of suicide gene therapy using *trans*-splicing ribozyme

We constructed a recombinant adenovirus, Ad-Ribo-HSVtk, for therapeutic use. In order to determine whether Ad-Ribo-HSVtk induces herpes simplex virus thymidine kinase (*HSVtk*) gene expression in the presence of hTERT RNA, we exposed HT-29 and SKLU-1 cells to different MOI of Ad-Ribo-HSVtk, Ad-HSVtk, or Ad-Mock, and assayed for [^{3}H]fluoroganciclovir (PCV) cellular uptake, a radio-labeled HSVtk substrate. Rib21AS-3′exon-HSVtk mediated the uptake of [^{3}H]PCV in HT-29 cells approximately 20% less efficiently than Ad-HSVtk did. In contrast, SKLU-1 showed approximately 80% lower [^{3}H]PCV uptake following Ad-Ribo-HSVtk infection (**Figure 3a**). Additionally, in order to determine whether adenovirus-mediated transduction with Rib21AS-3′exon-HSVtk renders hTERT$^+$ cells sensitive to ganciclovir (GCV), we treated HT-29 and SKLU-1 cells with varying amounts of Ad-HSVtk, Ad-Ribo-HSVtk, or Ad-Mock, and evaluated cell survival after adding GCV (final concentration of 20 µmol/l). As expected from our results with Ad-Ribo-LacZ, Ad-Ribo-HSVtk caused a dose-dependent reduction in cell survival, to approximately the same degree as Ad-HSVtk did. However, Ad-Ribo-HSVtk had only a minimal effect on hTERT$^-$ SKLU-1 cells (**Figure 3b**). To assess whether the cytotoxicity was dependent on GCV concentration, we conducted the same experiment using a fixed dose of adenovirus and varying GCV concentrations (**Supplementary Figure S3**). Ad-Ribo-HSVtk killed

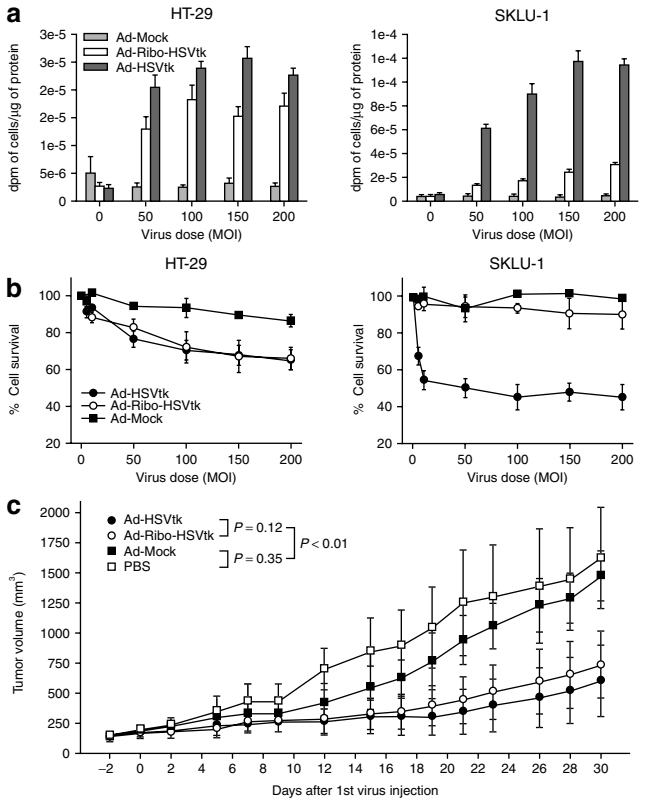

Figure 3 Therapeutic efficacy and specificity of Ad-Ribo-HSVtk *in vitro* and *in vivo*. (**a**) Thymidine kinase activity of Ad-Ribo-HSVtk, Ad-HSVtk, or Ad-Mock at various multiplictiy of infection (MOI). HT-29 and SKLU-1 cells were infected with adenoviruses and exposed with media containing [^{3}H]PCV. Radioactivity levels were then measured. For each of the uptake studies, at least two experiments were carried out, with three simultaneous samples in each experiment. Data were expressed as the net accumulation of probe in dpm cells/dpm medium/µg total protein ± SD. (**b**) Efficacy and specificity of the tumor-killing activity of Ad-Ribo-HSVtk *in vitro*. hTERT$^+$ HT-29 and hTERT$^-$ SKLU-1 were transfected with Ad-Ribo-HSVtk, Ad-HSVtk, or Ad-Mock at various MOI. After 5 days, cell viability was determined by MTS assay. Error bars indicate the mean values ± SD for triplicate assays. (**c**) Antitumor efficacy of Ad-Ribo-HSVtk *in vivo*. The tumor xenografts, approximately 140 mm^3 in size and composed of hTERT$^+$ HT-29 cells, were injected with Ad-Ribo-HSVtk/GCV (n = 12), Ad-HSVtk/GCV (n = 12), Ad-Mock/GCV (n = 9) or PBS/PBS (n = 9). Tumor size was measured on alternate days. Error bars indicate the mean values ± SD.

hTERT$^+$ HT-29 cells but not hTERT$^-$ SKLU-1 cells, whereas Ad-HSVtk was cytotoxic, independent of hTERT status. These *in vitro* results demonstrate that Ad-Ribo-HSVtk induces *HSVtk* in an hTERT RNA-dependent manner, and sensitizes hTERT$^+$ HT-29 cells to GCV.

We then assessed the therapeutic effects of Ad-Ribo-HSVtk *in vivo*, using tumor xenografts established with hTERT$^+$ HT29 cells in nude mice. After the tumors became palpable, we intratumorally injected viruses with either Ad-Ribo-HSVtk or Ad-HSVtk (treatment groups), and Ad-Mock or PBS (controls), and then administered GCV or PBS intraperitoneally every day for 10 days. Thirty days after the viral injections, the tumor volumes (in cubic millimeters) were 610.40 ± 296.54 for the Ad-HSVtk/GCV group; 744.24 ± 279.38 for the Ad-Ribo-HSVtk/GCV group; 1,482.10 ± 205.67 for the Ad-Mock/GCV group; and 1,628 ± 421.19 for the PBS/PBS group (**Figure 3c**). As is evident, tumor growth was

reduced significantly in treatment groups, as compared to controls (analysis of variance; $P < 0.0001$). Interestingly, we detected no statistically significant differences in the tumor growth inhibitory effects of Ad-HSVtk/GCV and Ad-Ribo-HSVtk/GCV (analysis of variance; $P = 0.12$). These results indicate that the hTERT-specific therapeutic adenovirus Ad-Ribo-HSVtk is as potent as Ad-HSVtk in suppressing tumor growth.

DISCUSSION

The ability of therapeutic genes to selectively eradicate rapidly proliferating cancer cells, without exerting toxic effects on normal cells, determines the success or failure of gene therapy in cancer.[2,3,18] Herein, we present a gene therapy strategy against cancer with specificity and efficacy based on the concept that the group I *trans*-splicing ribozyme, which targets hTERT transcripts expressed in cancer cells, would recognize its own substrates, switch them into "suicide" gene transcripts, and promote cell death. For the ribozyme delivery system, we selected adenovirus, which has been used in several *in vitro* and *in vivo* preclinical analyses.[19–22] We constructed novel adenoviral vectors harboring hTERT-targeting ribozymes with downstream *lacZ* (Ad-Ribo-LacZ) or *HSVtk* (Ad-Ribo-HSVtk) driven by a cytomegalovirus (CMV) early promoter. We analyzed the Ad-Ribo system for effective and specific delivery in animal models. By monitoring reporter gene activity, we verified that this viral system successfully delivers a ribozyme that performs a highly specific *trans*-splicing reaction with hTERT pre-messenger RNA, both *in vitro* and *in vivo*. We observed the reduction of hTERT and hTERT-dependent β-galactosidase expression in various cancer cell lines and mouse tumor models with $hTERT^+$ HT-29 cells. No β-galactosidase was expressed in non-tumor tissues from non-tumor bearing or mouse cancer models transfected with Ad-Ribo-LacZ. Moreover, *trans*-spliced products were not detected in non-tumor tissues in the Ad-Ribo-LacZ-treated mice. Notably, no mis-spliced products were generated in the tumor nodules of mice injected with the ribozyme-encoding adenovirus. These results indicate the *in vivo* target specificity of the *trans*-splicing ribozyme.

We also evaluated the efficacy of this adenoviral system. Telomerase activity was reduced in a viral dose-dependent manner when Ad-Ribo-LacZ was transfected into the HT-29 cells. Telomerase inhibition, however, reached a plateau at 100 MOI of virus administered *in vitro*, with approximately 30% reduction in telomerase activity. Interestingly, there were no significant differences in the antitumor effects of Ad-Ribo-HSVtk and Ad-HSVtk, thereby suggesting that 10–30% of delivered *tk* gene activity is sufficient for effective tumor killing. In addition, we determined that reporter gene activity in $hTERT^+$ cells infected with Ad-Ribo-LacZ, was only slightly (10–20%) lower than those infected with Ad-LacZ, and the effects were independent of internal telomerase activities. These results indicate that the efficacy of Ad-Ribo-LacZ is almost as high as Ad-LacZ in cells expressing target RNA. The efficacy of the adenoviral transfer, however, may influence the therapeutic effects of this system. As shown in the peritoneal carcinomatosis model, whole abdominal organs were not evenly transfected by Ad-LacZ, and *trans*-spliced products were detected only in 66% of tumor nodules transfected with Ad-Ribo-LacZ. This may reflect either the inefficacy of intraperitoneal delivery[17] or organ tropisms of the adenovirus. However, in view of the fact that the efficacy of viral delivery and promoter activity were indistinguishable between Ad-LacZ and Ad-Ribo-LacZ, the efficiency of *trans*-splicing ribozymes is unlikely to be a major limitation for the application of this technique to human anti-cancer therapy. Concerns regarding the utilization of adenoviral vectors for clinical tumor treatment include the existence of preexisting immunity against adenovirus in the majority of human populations, and the rapid immune response to the vector after initial injection.[23] The development of immune response limits the use of repeated inoculations of the adenoviral vector, as we employed in this study. Thus, strategies to circumvent the shortcomings of adenovirus, such as serotype switching,[24] may be required for an eventual successful approach to cancer therapy with ribozyme-encoding adenoviral vectors.

Most importantly, we showed that the hTERT-specific delivery of the *HSVtk* suicide gene and treatment with the corresponding prodrug (GCV) reduced tumor progression with almost the same efficacy as was observed with the strong constitutive CMV promoter-driven adenovirus, both in cancer cell lines and nude mouse HT-29 xenografts. These results indicate that ribozymes, in an adenovirus system, confer specificity without reducing viral transfection efficacy and transgene expression, even in an *in vivo* model. One frequently used approach in targeted gene therapy for cancer involves the use of adenovirus harboring a tissue/tumor-specific promoter that controls the expression of a therapeutic gene in a tissue/tumor-specific manner.[20,22] However, two drawbacks of this strategy are the loss of specificity caused by *cis*-acting sequences in the adenoviral genome, and expression levels lower than the current benchmark CMV promoter because of its weaker promoter activity.[2,25] These drawbacks might be overcome by using the new gene therapy strategy proposed here, which is based on the targeting and replacement of hTERT RNA through a *trans*-splicing ribozyme under the control of the CMV promoter. Detailed toxicological studies are, however, required before Ad-Ribo-HSVtk can be considered for application in humans. We selected hTERT RNA as a target, because telomerase activity is elevated in 85–90% of human cancers, and the continuous growth of advanced malignancies correlates with their telomerase reactivation.[26–29] In the murine model, normal cell damage is limited, because mice express murine-type TERT. However, human stem cells, germ cells in regenerating tissues, and normal human tissues have detectable telomerase activity.[30,31] Therefore, the systemic delivery of Ad-Ribo-HSVtk may generate toxic side effects. However, this toxicity can be minimized because stem cells proliferate only in an intermittent fashion, and their telomerase activities are negligible during quiescence.[32] Moreover, adenovirus tropisms potentially limit the infection of stem cells.[33]

In conclusion, we demonstrate that a *trans*-splicing ribozyme may represent a good candidate for cancer therapy, because of its dual function. As compared to the simple delivery of a traditional therapeutic genetic payload, one major advantage of this approach is that, by invading a tumor-specific RNA, the targeted expression of the therapeutic gene product is guaranteed, and the level of

expression of the target gene product is reduced simultaneously. Although considerable progress has been made in developing *trans*-splicing ribozyme for therapeutic applications, no evidence of the *in vivo* specificity and efficacy of the ribozymes was available. Importantly, in this study, we have shown that novel adenoviral vectors harboring group I *trans*-splicing ribozymes work both selectively and efficiently to target cancer in animal models. If it were possible to design a *trans*-splicing ribozyme targeting a tumor-specific oncogene or a drug-resistant gene, then therapeutic gene delivery with target gene inhibition could exert an additive, or perhaps a synergistic, anticancer effect. This study establishes the potential of group I ribozyme-mediated *trans*-splicing in the design of RNA-based cancer gene therapies.

MATERIALS AND METHODS

Generation of recombinant adenoviruses expressing trans-splicing ribozyme. hTERT-specific *trans*-splicing ribozymes were constructed as previously described.[14] In brief, Rib21AS ribozyme directed at U21 on hTERT RNA was generated to contain an extended internal guide sequence, such as an extension of the P1 helix, an additional 6-nucleotide-long P10 helix, and a 325-nucleotide-long antisense sequence complementary to the downstream region of the targeted hTERT RNA uridine (**Supplementary Figure S4**). We determined that the specificity and activity of the *trans*-splicing ribozyme in human cells were increased by modifications of the ribozyme.[14] Complementary DNA (cDNA) as a 3′ exon encoding for *HSVtk* or the bacterial *lacZ* gene was inserted between *Nru*I and *Xba*I downstream of the modified group I intron expression construct harboring the Rib21AS. Because the target sequence (U21) was present in the leader region, a new start codon (AUG) was inserted into the 5′-end of the 3′ exon with Kozak sequences.[34] The resultant ribozyme cDNA flanked by *lacZ* (Rib21AS-3′exon-LacZ) or *HSVtk* (Rib21AS-3′exon-HSVtk) was subcloned into a pAvCvSv shuttle vector containing the CMV promoter. The pAvCvSv shuttle vector harboring the expression cassette of Rib21AS-3′exon-LacZ or Rib21AS-3′exon-HSVtk was co-transfected into HEK293 cells with the adenoviral backbone vector pJM17, using calcium phosphate precipitation. Several clones were isolated by plaque assays, amplified in 293 cells and purified by double cesium chloride gradient ultracentrifugation as previously described.[35] We designated the adenovirus products harboring ribozymes with *HSVtk* and *lacZ* as Ad-Ribo-HSVtk and Ad-Ribo-LacZ, respectively. As a control, we employed adenoviruses with *HSVtk* (Ad-HSVtk) or *lacZ* (Ad-LacZ) driven by the CMV promoter and Ad-Mock, which harbors only the adenoviral backbone.

Cellular studies. Human colon tumor cell lines (HT-29 and SNU-407), hepatoma cell lines (HepG2 and SNU-398), lung carcinoma cell lines (SBC-5 and SKLU-1), and adenoviral E1-transformed human embryonic kidney cells (HEK293) were utilized. SNU-398 and SNU-407 were generously provided by Dr. Jae-Gahb Park and Ja-Lok Ku (Korean Cell Line Bank, Seoul, South Korea). HepG2, HT-29, SKLU-1, and HEK293 cells were purchased from American Type Culture Collection (Manassas, VA), and SBC-5 cells from the Japanese Collection of Research Bioresources (Osaka, Japan). All cells used in this experiment were maintained according to the standard procedures. Cell proliferation (MTS) assays were performed using standard protocols with some modifications. The cells were seeded at 5×10^3 cells/well (0.1 ml) in 96-well plates and incubated overnight at 37 °C. They were then exposed to varying MOI of adenoviruses and maintained for the required duration (5–7 days). For determining GCV sensitivity after the adenoviral transfer of thymidine kinase genes, GCV was applied following 24 hours of viral infection and maintained for 5 days. At the end of the experiments, 20 µl of CellTiter 96 Aqueous One solution reagent (Promega, Madison, WI) in 100 µl of Opti-MEM was added to each well, and incubated for 1–4 hours, on the basis of the rate of change of color. Cell viability was estimated by monitoring the absorbance at 490 nm. All experiments were performed at least twice in simultaneous sets of three per experiment. Enzyme assays for telomerase activity, β-galactosidase activity, and thymidine kinase activity were carried out as indicated in **Supplementary Materials and Methods**.

Experimental animals. Four- to five-week-old male Balb/cAnNCrj-nu/nu nude mice and female Balb/c mice (Charles River Laboratories, Wilmington, MA) were utilized in this study. The animals were kept under specific pathogen-free conditions and maintained in a Korean Food and Drug Administration animal facility in accordance with Association for Assessment and Accreditation of Laboratory Animal Care International Animal Care policy (Accredited Unit-Korea Food and Drug Administration: unit number-000996). They were acclimated to laboratory conditions for at least 1 week before use. The peritoneal carcinomatosis model of colon cancer was established by intraperitoneally injecting male nude mice with 5×10^6 HT-29 cells. Prior to cell injection, NK cells were depleted in order to facilitate the metastasis of HT-29 cells. In order to inhibit NK cell activity, 50 µl of anti-asialo GM1 (rabbit) antibody (Wako Pure Chemicals, Osaka, Japan) was intraperitoneally injected 2 days prior to this experiment. HT-29 cell viability was determined by the trypan blue exclusion test, and only a single cell suspension of >90% viability was used. The animals showed evidence of peritoneal carcinomatosis within 8–12 days, which was readily detectable by gross inspection and a binocular stereomicroscope. Subcutaneous tumors were induced by injecting 5×10^6 HT-29 cells (200 µl) into the flank regions of male nude mice. We measured tumor size every 2–3 days with calipers and calculated tumor volume using length (L) and width (W), as $LW^2/2$. When the tumors reached a size of 140 mm^3 (10 days after inoculation), we randomly assigned them to treatment groups, excluding those with tumors that were too large (>190 mm^3) or too small (<90 mm^3). Adenoviruses (5×10^7 plaque forming units/200 µl) or 200 µl of PBS were intratumorally injected three times at 3-day intervals. One day after the initial injection of adenovirus injection, GCV treatment was initiated in the treatment groups. GCV was intraperitoneally injected (50 mg/kg twice a day) for 10 days, whereas PBS was injected in the PBS-treated group.

Statistical analyses were performed using Statistical Analysis System software (SAS Institute, Cary, NC). Inter-group differences were assessed by analysis of variance. In the case of highly skewed distribution of measurements and small sample sizes, we employed nonparametric statistical tests (Kruskal–Wallis test for overall comparison and Wilcoxon rank-sum test for pair-wise comparison). All data are expressed as the mean values ± SD. P-values < 0.01 were considered statistically significant.

PCR analyses. The presence of adenoviruses was detected by PCR. The DNAs were isolated from various tissues and tumors with TRIzol reagent (Invitrogen, Carlsbad, CA), and the inverted terminal repeat region of adenovirus was amplified. The primers used were 5′-AGCC AATATGATAATGAGGGGGTG-3′ and 5′-TACGCGCTATGAGTAACA CAAA-3′. In order to determine the transgene expression levels or TSMs produced in the murine cells, tissues, and tumors, we conducted reverse transcriptase-PCR. Total RNA from adenovirus-transfected cells or tissues was isolated with TRIzol reagent according to the manufacturer's protocols. cDNAs were synthesized from the RNA using Omniscript reverse transcriptase (Qiagen, Valencia, CA) with the primer for the 3′-end of *lacZ* (5′-ACGCAACTCGCCGCACATCTGAA-3′). Transgene expression levels were determined by amplifying cDNAs with *lacZ* primers (5′-ATGGTCGTTTTACAACGTCGTGAC-3′ and 5′-GGGCT CGAGCGGATTGACCGTAATGGAA-3′). The TSM was detected by amplification with the 5′ primer specific for the *trans*-splicing junction (5′-GGGGAATTCAGCGCTGCGTCCTGCT-3′) and with a 3′ primer specific for the *lacZ* sequence.

β-galactosidase staining and immunohistochemistry. TERT-dependent β-galactosidase expression was qualitatively analyzed by X-gal staining. For X-gal staining, the cells were plated at 2×10^5 cells per well in 6-well plates. One day later, all of the cells except for the SKLU-1 cells were infected with adenoviruses at MOI of 30. The SKLU-1 cells were infected at MOI of 100. After 48 hours, the cells were stained with X-gal according to the manufacturer's instructions, using a β-galactosidase staining kit (Invitrogen, Carlsbad, CA). X-gal staining was performed on tissue sections 2 days after viral injection. The mice were killed, and the organs and tumors were removed, sectioned, and stored in liquid nitrogen or RNA solution. Eight micrometer frozen sections were fixed with 2% paraformaldehyde in a 100 mmol/l of PBS (pH 7.4) for 10 minutes at room temperature. The sections were washed twice in PBS, and stained overnight with X-gal solution at 37 °C. The X-gal solution was freshly prepared by mixing a solution containing 3 mmol/l potassium ferricyanide, 3 mmol/l potassium ferrocyanide, 1.3 mmol/l $MgCl_2$, 0.01% sodium deoxycholate, and 2% of a solution containing 40 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (GB Tech, St. Louis, MO) in 1 ml of dimethylformamide at a pH of 7.4. The sections were counterstained with hematoxylin and observed under a light microscope. X-gal staining *in toto* was also performed as described previously,[36] 2 hours after fixing the whole abdominal organs and tumor nodes in 2% formaldehyde.

Immunostaining was performed with the avidin–biotin–peroxidase complex technique, and detected with a DAKO EnVision Kit (Dako, Carpinteria, CA). Immunohistochemistry was conducted on formalin-fixed and paraffin-embedded specimens that were de-waxed and re-hydrated at graded alcohols. Endogenous peroxidase was blocked by the immersion of sections in 3% aqueous hydrogen peroxide for 10 minutes, and antigen retrieval was performed with 10 minute microwave treatment in 10 mmol/l of citrate buffer, pH 6.0. Diluted primary antibodies (1:100) for anti-hTERT (Santa Cruz Biotechnology, Santa Cruz, CA) were applied for 1 hour at room temperature. After incubation with the primary antibody, sections were incubated with the secondary antibody and the avidin–biotin–peroxidase complex. The slides were lightly counterstained with hematoxylin and eosin.

ACKNOWLEDGMENTS

The authors wish to thank Kazuhiro Oka (Baylor College of Medicine, Houston, TX) for providing the adenovirus vector and technical advice, and Kyu-Won Jung (National Cancer Center, South Korea) for the statistical analyses. This work was supported by a National Cancer Center Grant (0110240-3); by grants to S.W.L. from the Korea Research Foundation (KRF-2001-015-DP0572) and the Korea Science and Engineering Foundation (M10534000004-06N3400-00410); and a grant to J.S.J. from the Korea Science and Engineering Foundation through the Medical Research Center for Cancer Molecular Therapy at Dong-A University.

SUPPLEMENTARY MATERIAL

Figure S1. Sequence analysis of *trans*-spliced transcripts in Ad-Ribo-LacZ-transfected HT-29 cells.

Figure S2. Distribution of Ad-Ribo-LacZ in tissues of normal mice after systemic administration.

Figure S3. Anti-tumor effect of Ad-Ribo-HSVtk in a GCV dose-dependent manner.

Figure S4. Schematic diagram of the *trans*-splicing ribozyme.

Supplementary Materials and Methods.

REFERENCES

1. McCormick, F (2001). Cancer gene therapy: fringe or cutting edge? *Nat Rev Cancer* **1**: 130–141.
2. Wu, L, Johnson, M and Sato, M (2003). Transcriptionally targeted gene therapy to detect and treat cancer. *Trends Mol Med* **9**: 421–429.
3. Tarner, IH, Müller-Ladner, U and Fathman, CG (2004). Targeted gene therapy: frontiers in the development of 'smart drugs'. *Trends Biotechnol* **22**: 304–310.
4. Been, MD and Cech, TR (1986). One binding site determines sequence specificity of *Tetrahymena* pre-rRNA self-splicing, *trans*-splicing, and RNA enzyme activity. *Cell* **47**: 207–216.
5. Sullenger, BA and Cech, TR (1994). Ribozyme-mediated repair of defective mRNA by targeted, *trans*-splicing. *Nature* **371**: 619–622.
6. Jones, JT, Lee, SW and Sullenger, BA (1996). Tagging ribozyme reaction sites to follow *trans*-splicing in mammalian cells. *Nat Med* **2**: 643–648.
7. Lan, N, Howrey, RP, Lee, SW, Smith, CA and Sullenger, BA (1998). Ribozyme-mediated repair of sickle beta-globin mRNAs in erythrocyte precursors. *Science* **280**: 1593–1596.
8. Phylactou, LA, Darrah, C and Wood, MJ (1998). Ribozyme-mediated *trans*-splicing of a trinucleotide repeat. *Nat Genet* **18**: 378–381.
9. Rogers, CS, Vanoye, CG, Sullenger, BA and George, AL (2002). Functional repair of a mutant chloride channel using a *trans*-splicing ribozyme. *J Clin Invest* **110**: 1783–1789.
10. Watanabe, T and Sullenger, BA (2000). Induction of wild-type p53 activity in human cancer cells by ribozymes that repair mutant p53 transcripts. *Proc Natl Acad Sci USA* **97**: 8490–8494.
11. Shin, KS, Sullenger, BA and Lee, SW (2004). Ribozyme-mediated induction of apoptosis in human cancer cells by targeted repair of mutant p53 RNA. *Mol Ther* **10**: 365–372.
12. Ayre, BG, Köhler, U, Goodman, HM and Haseloff, J (1999). Design of highly specific cytotoxins by using *trans*-splicing ribozymes. *Proc Natl Acad Sci USA* **96**: 3507–3512.
13. Ryu, KJ, Kim, JH and Lee, SW (2003). Ribozyme-mediated selective induction of new gene activity in hepatitis C virus internal ribosome entry site-expressing cells by targeted *trans*-splicing. *Mol Ther* **7**: 386–395.
14. Kwon, BS, Jung, HS, Song, MS, Cho, KS, Kim, SC, Kimm, K *et al.* (2005). Specific regression of human cancer cells by ribozyme-mediated targeted replacement of tumor-specific transcript. *Mol Ther* **12**: 824–834.
15. Shay, JW and Bacchetti, S (1997). A survey of telomerase activity in human cancer. *Eur J Cancer* **33**: 787–791.
16. Shay, JW and Wright, WE (2002). Telomerase: a target for cancer therapeutics. *Cancer Cell* **2**: 257–265.
17. Lechanteur, C, Princen, F, Lo Bue, S, Detroz, B, Fillet, G, Gielen, J *et al.* (1997). HSV-1 thymidine kinase gene therapy for colorectal adenocarcinoma-derived peritoneal carcinomatosis. *Gene Ther* **4**: 1189–1194.
18. Dong, JY and Worathanadharm, J (2005). Gene therapy vector design strategies for the treatment of cancer. *Future Oncol* **1**: 361–373.
19. Kanerva, A and Hemminki, A (2004). Modified adenoviruses for cancer gene therapy. *Int J Cancer* **110**: 475–480.
20. Cascalló, M and Alemany, R (2004). Adenovirus-mediated gene transfer to tumor cells. *Methods Mol Biol* **246**: 121–138.
21. Ghosh, SS, Gopinath, P and Ramesh, A (2006). Adenoviral vectors: a promising tool for gene therapy. *Appl Biochem Biotechnol* **133**: 9–29.
22. St George, JA (2003). Gene therapy progress and prospects: adenoviral vectors. *Gene Ther* **10**: 1135–1141.
23. Bangari, DS and Mittal, SK (2006). Current strategies and future directions for eluding adenoviral vector immunity. *Curr Gene Ther* **6**: 215–226.
24. Vogels, R, Zuijdgeest, D, van Rijnsoever, R, Hartkoorn, E, Damen, I, de Béthune, MP *et al.* (2003). Replication-deficient human adenovirus type 35 vectors for gene transfer and vaccination: efficient human cell infection and bypass of preexisting adenovirus immunity. *J Virol* **77**: 8263–8271.
25. Martin-Duque, P, Jezzard, S, Kaftansis, L and Vassaux, G (2004). Direct comparison of the insulating properties of two genetic elements in an adenoviral vector containing two different expression cassettes. *Hum Gene Ther* **15**: 995–1002.
26. Kim, NW, Piatyszek, MA, Prowse, KR, Harley, CB, West, MD, Ho, PL *et al.* (1994). Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**: 2011–2015.
27. Masutomi, K and Hahn, WC (2003). Telomerase and tumorigenesis. *Cancer Lett* **194**: 163–172.
28. Smith, LL, Coller, HA and Roberts, JM (2003). Telomerase modulates expression of growth-controlling genes and enhances cell proliferation. *Nat Cell Biol* **5**: 474–479.
29. Meyerson, M, Counter CM, Eaton, EN, Ellisen, LW, Steiner, P, Caddle SD *et al.* (1997). hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell* **90**: 785–795.
30. Masutomi, K, Yu, EY, Khurts, S, Ben-Porath, I, Currier, JL, Metz, GB *et al.* (2003). Telomerase maintains telomere structure in normal human cells. *Cell* **114**: 241–253.
31. Yui, J, Chiu, CP and Lansdorp, PM (1998). Telomerase activity in candidate stem cells from fetal liver and adult bone marrow. *Blood* **91**: 3255–3262.
32. Forsyth, NR, Wright, WE and Shay, JW (2002). Telomerase and differentiation in multicellular organisms: turn it off, turn it on, and turn it off again. *Differentiation* **69**: 188–197.
33. Jaffe, HA, Danel, C, Longenecker, G, Metzger, M, Setoguchi, Y, Rosenfeld, MA *et al.* (1992). Adenovirus-mediated *in vivo* gene transfer and expression in normal rat liver. *Nat Genet* **1**: 372–378.
34. Kozak, M (1978). How do eucaryotic ribosomes select initiation regions in messenger RNA? *Cell* **15**: 1109–1123.
35. Kobayashi, K, Oka, K, Forte, T, Ishida, B, Teng, B, Ishimura-Oka, K *et al.* (1996). Reversal of hypercholesterolemia in low density lipoprotein receptor knockout mice by adenovirus-mediated gene transfer of the very low density lipoprotein receptor. *J Biol Chem* **271**: 6852–6860.
36. Hajri, A, Wack, S, Lehn, P, Vigneron, JP, Lehn, JM, Marescaux, J *et al.* (2004). Combined suicide gene therapy for pancreatic peritoneal carcinomatosis using BGTC liposomes. *Cancer Gene Ther* **11**: 16–27.