In vitro gene expression induced by Ad vectors containing miR-122a target sequences

In order to examine *in vitro* transgene expression levels induced by an Ad vector carrying the miR-122a-target sequences, human and mouse cultured cells expressing various levels of miR-122a were transduced using the Ad vectors. Quantitative reverse transcriptase-PCR analysis demonstrated that mouse primary hepatocytes and HuH-7 cells expressed large amounts of miR-122a. In contrast, low or negligible levels of miR-122a were found in SK HEP-1 cells and MS1 cells (**Figure 2a**). Ad-L-122aT mediated a 3- to 70-fold lower relative expression of *firefly* luciferase in mouse primary hepatocytes and HuH-7 cells than did Ad-L or Ad-L-controlT (**Figure 2b**). Superior suppression of *firefly* luciferase expression was observed in primary mouse hepatocytes than in HuH-7 cells, probably because of the higher levels of miR-122a expression in the former than in the latter. In contrast, both Ad-L and Ad-L-122aT exhibited similar levels of *firefly* luciferase expression in SK HEP-1 cells and in MS1 cells. These results suggest that the insertion of the miR-122a target sequences in the 3′-UTR region of the transgene expression cassette resulted in a reduction in transgene expression by Ad vectors in cells expressing miR-122a. The insertion of the control target sequences, which are reversed sequences of the miR-122a target sequences, did not affect *firefly* luciferase production in the mouse primary hepatocytes and MS1 cells. In contrast, Ad-L-controlT was shown to produce a significant reduction in *firefly* luciferase expression both in HuH-7 cells and SK-HEP1 cells. It is possible that certain miRNAs are associated with the reverse sequences of the miR-122a target sequences, and that these might reduce transgene expression.

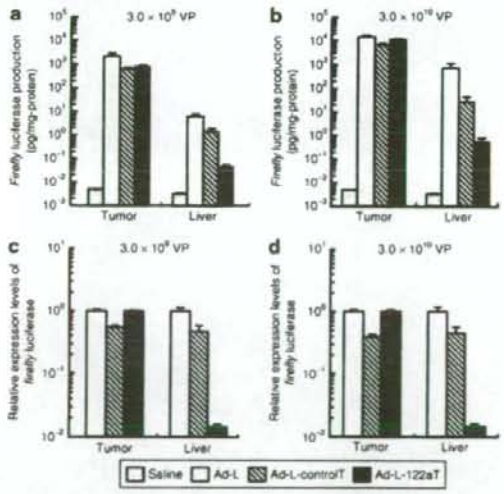

Figure 3 ***In vivo*** **transduction efficiency of adenovirus (Ad) vectors containing the miR-122a target sequences after intratumoral injection.** B16 tumor-bearing mice were intratumorally injected with Ad vectors, and the tumors and livers were recovered and subjected to luciferase expression analysis 48 hours after injection. *Firefly* luciferase production in the tumors and livers by Ad vectors at (**a**) 3.0×10^9 virus particles (VP) and (**b**) 3.0×10^{10} VP. Relative *firefly* luciferase expression levels in the tumors and livers at (**c**) 3.0×10^8 VP and (**d**) 3.0×10^{10} VP. *Firefly* luciferase production levels were normalized to *renilla* luciferase production levels. Data are presented as mean value ± SE ($n = 6$).

Insertion of the miR-122a target sequences resulted in a dramatic reduction in hepatic transgene expression from Ad vectors

In order to determine whether Ad-L-122aT exhibits a liver-specific reduction in *firefly* luciferase expression following *in vivo* application, the production levels of *firefly* and *renilla* luciferase in tumors and in the liver were determined after intratumoral injection of the Ad vectors at 3.0×10^9 virus particles (VP) or 3.0×10^{10} VP into B16 tumor-bearing mice. We confirmed that the B16 tumors expressed only scant amounts of miR-122a. In contrast, abundant expression of miR-122a was found in the liver, *i.e.*, more than 10^5-fold of the level in the B16 tumors (data not shown). Ad-L-122aT was shown to mediate similar or marginally lower absolute amounts of *firefly* luciferase production in B16 tumors at both doses, when compared with the amounts mediated by other Ad vectors (**Figure 3a** and **b**). In contrast, hepatic *firefly* luciferase production mediated by Ad-L-122aT was significantly reduced, *i.e.*, ~1/1,500 and 1/50 of those mediated by Ad-L and Ad-L-controlT, respectively, at 3.0×10^{10} VP. The relative expression levels of *firefly* luciferase were comparable at both doses (**Figure 3c** and **d**), although the absolute amount of *firefly* luciferase production in the liver by Ad-L-122aT at 3.0×10^{10} VP was approximately tenfold higher than that at 3.0×10^9 VP. The *firefly* luciferase production levels of Ad-L-controlT in the tumors and liver were slightly lower than those of Ad-L, as observed in HuH-7 cells and SK HEP-1 cells. Other organs, including spleen and heart, exhibited almost similar levels of *firefly* luciferase in response to each of the Ad vectors (data not shown). Similar results were observed in a study of Balb/c mice-bearing Colon26 tumors (data not shown). Quantitative PCR analysis of the Ad vector genome confirmed the absence of significant differences in the amounts of vector genomes of Ad-L, Ad-L-controlT, and Ad-L-122aT in the tumors and liver samples (**Figure 4**). These results indicate that the insertion of the

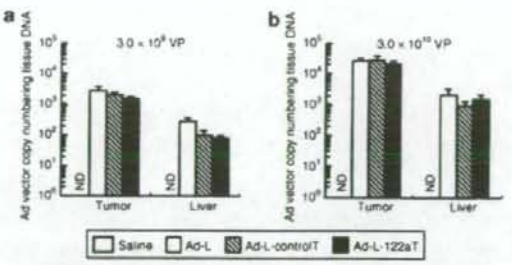

Figure 4 ***In vivo*** **biodistribution of adenovirus (Ad) vectors containing the miR-122a target sequences after intratumoral injection.** B16 tumor-bearing mice were intratumorally injected with Ad vectors, and tumors and livers were recovered and subjected to Ad vector copy number analysis by quantitative PCR 48 hours after the injection. Ad vector copy number in the tumor and liver induced by Ad vectors at (**a**) 3.0×10^9 virus particles (VP) and (**b**) 3.0×10^{10} VP. Data are presented as mean value ± SE ($n = 6$). ND, not detectable.

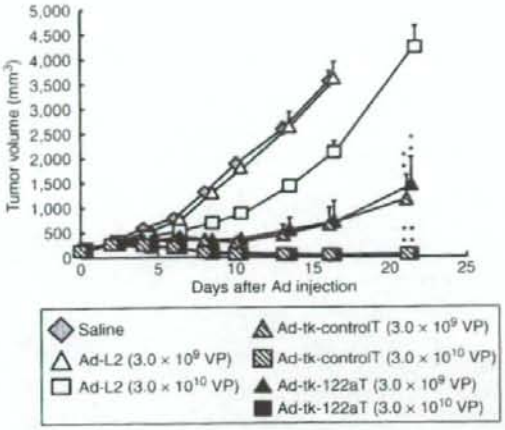

Figure 5 Antitumor activity of herpes simplex virus thymidine kinase–expressing adenovirus (Ad) vectors following intratumoral injection. Established B16 tumors were intratumorally injected on day 0 with saline, Ad-L2, Ad-tk-controlT, or Ad-tk-122aT. The mice were administered intraperitoneal injections of ganciclovir at 75 mg/kg once a day for 10 days. Tumor growth was monitored by calculating tumor size. Each point represents the mean value ± SE from 7–9 mice. Significant difference, $^{**}P < 0.005$ compared with Ad-L2.

miR-122a target sequences in Ad vectors significantly reduces transgene expression in the liver without altering transduction in the tumor, after intratumoral injection.

Antitumor effects of HSVtk-expressing Ad vectors

In order to evaluate the utility of Ad vectors containing the miR-122a target sequences in HSVtk/GCV suicide gene therapy to treat cancer, we constructed an Ad vector containing an miR-122a-regulated HSVtk expression cassette (Ad-tk-122aT) (**Figure 1e**), and examined the antitumor effects of HSVtk-expressing Ad vectors in B16 tumor–bearing mice. A single intratumoral injection of Ad-tk-122aT or Ad-tk-controlT resulted in a significant and vector dosage-dependent regression in the tumors (**Figure 5**). We did not find any significant differences between the antitumor activities of Ad-tk-122aT and Ad-tk-controlT. Ad-L2 at 3.0×10^{10} VP slightly suppressed tumor growth; however, the antitumor effects of Ad-L2 were less marked than those of Ad-tk-122aT and Ad-tk-controlT. These results indicate that the insertion of miR-122a target sequences does not exert any influence on the antitumor effects achieved by HSVtk-expressing Ad vectors after intratumoral injection.

Dramatically reduced hepatotoxicity with Ad-tk-122aT

In order to examine the hepatotoxicity associated with systemic leakage of HSVtk-expressing Ad vectors after intratumoral injection, histopathological examination of the liver was performed 6 days after Ad vector injection. Ad-L2 did not induce any apparent hepatotoxicity at either of the doses used, although infiltration of a few inflammatory cells was seen (**Figure 6b** and **c**, upper left). However, Ad-tk-controlT at 3.0×10^{9} VP provoked substantial

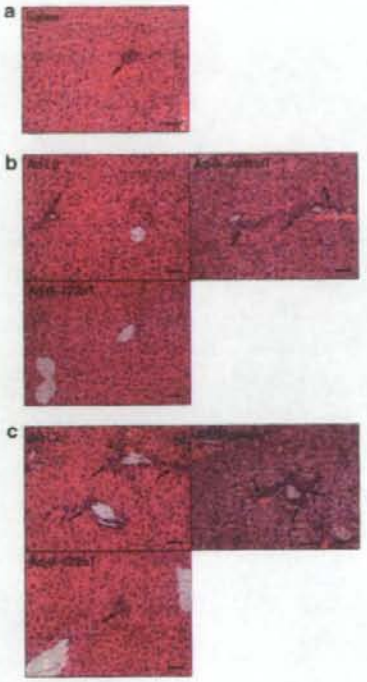

Figure 6 Histopathological analysis of the livers of mice that received intratumoral injections of saline, Ad-L2, Ad-tk-controlT, or Ad-tk-122aT. Representative sections of livers of mice injected intratumorally with (**a**) saline, (**b**) Ad-L2 (upper left), Ad-tk-controlT (upper right), or Ad-tk-122aT (lower left) at 3.0×10^{9} virus particles (VP), or (**c**) each of the vectors at 3.0×10^{10} VP. B16 tumor–bearing mice were treated as described in **Figure 5**. Six days after the injection, the livers were isolated, and histopathological analysis was performed using hematoxylin and eosin staining. The arrows point to inflammatory cell infiltration in the liver. The scale bar = 50 μm. Ad, adenovirus.

inflammatory cell infiltration in the liver (**Figure 6b**, upper right). Furthermore, Ad-tk-controlT at 3.0×10^{10} VP caused severe hepatotoxicity, including swelling of hepatocytes and inflammatory cell infiltration in all areas of the liver examined (**Figure 6c**, upper right). In contrast, low or negligible levels of inflammatory cell infiltration were observed in the livers of Ad-tk-122aT-injected groups at both doses, *i.e.*, 3.0×10^{9} VP and 3.0×10^{10} VP (**Figure 6b** and **c**, lower left). The levels of inflammatory cell infiltration and the extent of degeneration of hepatocytes were both comparable in the livers of Ad-L2-treated mice and in those of Ad-tk-122aT-treated mice. In this series, we also measured serum alanine aminotransferase (ALT), an enzymatic biomarker of hepatotoxicity, after intratumoral injection of Ad vectors (days 4 and 6). Ad-tk-122aT did not cause significant elevation in serum ALT levels at either of the doses used; however, on day 6, Ad-tk-controlT at 3.0×10^{9} VP induced a nonsignificantly higher level of serum ALT than did Ad-L2 and Ad-tk-122aT (**Figure 7a**). Furthermore, we identified a significant elevation in the serum ALT levels of mice treated with Ad-tk-controlT at 3.0×10^{10} VP

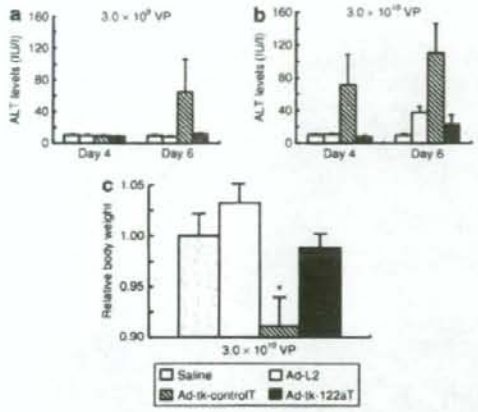

Figure 7 Elevation in serum alanine aminotransferase (ALT) levels and loss in body weight in mice after intratumoral injection of adenovirus (Ad) vectors. (a, b) Serum ALT levels after Ad vector injection. B16 tumor-bearing mice were treated with Ad vectors at 3.0×10^9 virus particles (VP) or 3.0×10^{10} VP. Blood samples were collected through retro-orbital bleeding 4 or 6 days after the injection. Data are presented as mean value ± SE ($n = 6$). **(c)** Relative body weights of mice 10 days after Ad vector injection. The body weights of mice treated with saline were normalized to 1.00. Data are presented as mean value ± SE ($n = 7$–9). *$P < 0.05$ in comparison with Ad-tk-122aT.

on day 6 as compared to those of mice treated with Ad-tk-122aT (**Figure 7b**). In addition, whereas mice that received Ad-tk-controlT at 3.0×10^{10} VP showed significant reduction in body weight, mice treated with Ad-tk-122aT at 3.0×10^{10} VP did not show any such loss in body weight (**Figure 7c**). Out of the seven mice that received Ad-tk-controlT at 3.0×10^{10} VP, two died after day 13, probably on account of HSVtk-induced hepatic damage; however no mice in any of the other groups died during the experiment. These data indicate that insertion of miR-122a target sequences dramatically reduces the hepatotoxicity caused by Ad vector–mediated HSVtk expression.

DISCUSSION

The potential of HSVtk/GCV suicide gene therapy has been extensively described.[1–5] Ad vectors are considered to be the most suitable for the delivery of the HSVtk gene into tumor cells; however, the problem of HSVtk/GCV-mediated toxicity in normal tissue, primarily in the liver, needs to be addressed. This study was undertaken to explore the efficacy of using a miRNA-regulated transgene expression system for circumventing unwanted transgene expression in the liver without disturbing transduction in the tumor, in the context of Ad vectors. Intratumoral injection of an Ad vector containing the liver-specific miR-122a-regulated HSVtk expression cassette yielded efficient antitumor effects without severe liver toxicity.

We incorporated four tandem copies of the miR-122a target sequences into the transgene expression cassette in order to suppress the hepatic expression of the gene introduced by Ad vectors. The copy numbers of miRNA target sequences are expected to play an important role in miRNA-regulated expression systems. Doench *et al.* reported that an increase in the copy number of miRNA target sequences resulted in greater suppression of transgene expression.[26] We also confirmed that when either two or four copies of the miR-122a target sequences were included in the transgene expression cassette, the greater number of copies (four in this case) was associated with greater suppression of transgene expression. Therefore four copies of the miR-122a target sequences were incorporated in the 3′-UTR region.

In order to address safety concerns relating to the clinical use of Ad vector–mediated HSVtk/GCV systems, the use of tumor-specific promoters has been extensively examined. Several studies have demonstrated that transgene expression levels driven by tumor-specific promoters in the tumor are often lower than those of ubiquitously active promoters, including the cytomegalovirus (CMV) promoter. Several types of tumor-specific promoters, including the telomerase reverse transcriptase promoter,[27] carcino-embryonal antigen promoter,[12] and thyroglobulin core promoter,[28] have been used for tumor-specific HSVtk expression by Ad vectors. These promoters render tumor cells sensitive to GCV in a tumor cell–specific pattern; however, the sensitivity of the transduced cells to GCV was lower than that of cells transduced with the CMV promoter, at least *in vitro*.[12,27,28] Efficient transduction in tumor cells is crucial in a HSVtk/GCV system, although the bystander effect can kill the neighboring untransduced cells. In addition, loss of the tumor specificity of promoters after incorporation into the Ad vector genome has been previously reported.[29–31] On the other hand, we clearly showed that a miR-122a-regulated expression system in the context of Ad vectors achieves both efficient transduction in the tumor and reduced transgene expression in the liver. These results indicate that the miRNA-regulated transgene expression system is suitable for the regulation of transgene expression by Ad vectors.

We utilized a liver-specific miRNA, miR-122a, to suppress transgene expression by Ad vectors in the liver, because injection of HSVtk-expressing Ad vectors provokes severe hepatotoxicity, given their highly hepatotropic nature. Insertion of the miR-122a target sequences significantly reduced transgene expression in the liver; however, no suppression of transgene expression by Ad-L-122aT was observed in the other organs (data not shown), because only negligible amounts of miR-122a are expressed in organs other than the liver.[23] In order to prevent transgene expression, not only in the liver but also in other organs, it is important to incorporate target sequences of miRNA that are ubiquitously expressed, but that are specifically downregulated in the targeted tumor. Recent studies using miRNA array systems have revealed that several miRNAs such as let-7,[32] miR-143,[33] and miR-145[33,34] were significantly downregulated in the target tumor. The use of target sequences to these miRNAs might further increase the safety profiles of HSVtk-expressing Ad vectors.

One major concern in using the miR-122a-regulated expression system is its potential influence on endogenous targets of miR-122a such as cationic amino acid transporter-1.[23] Cationic amino acid transporter-1 expression levels in the liver were not determined in this study; however, it is unlikely that recruitment of miR-122a to the target sequences inserted into the transgene

expression cassette leads to a loss of regulation of natural miR-122a targets, because expression levels of miR-122a in mouse hepatocytes would be much higher than those of *firefly* luciferase mRNA, which possesses the miR-122a target sequences in the 3′-UTR. The results of a study by Brown *et al.* also suggested that miRNA-regulated expression of natural targets is not perturbed by large amounts of miRNA substrate.[21] These results could also account the suppression efficiency (ratio of *firefly* luciferase production to *renilla* luciferase production) being maintained to the same extent at both doses, *i.e.*, 3.0×10^9 VP and 3.0×10^{10} VP. Sufficient levels of miR-122a would be expressed in the hepatocytes to suppress the transgene expression by Ad vectors at the doses used in this study.

As mentioned earlier, the efficiency of transgene suppression in the liver was comparable at 3.0×10^9 VP and 3.0×10^{10} VP; however, Ad-L-122aT at 3.0×10^{10} VP produced higher amounts of *firefly* luciferase in the liver than did 3.0×10^9 VP (**Figure 3**). These results suggest that an additional strategy, in addition to the miRNA-regulated expression system, will still be required to achieve further reduction in the absolute levels of transgene products in the liver. Alteration of virus tropism by capsid modification would be a promising approach in this context. Our group of researchers have previously demonstrated that fiber-mutant Ad vectors in the fiber HI loop containing an Arg-Gly-Asp peptide, which exhibits α_v-integrin tropism, achieved more efficient transduction in B16 tumors and reduction in hepatic transduction after intratumoral injection, than with the use of conventional Ad vectors.[13] The inclusion of an Arg-Gly-Asp peptide led to an increase in the affinity of Ad vectors for tumor cells and a reduction in the systemic leakage of Ad vectors. The combined use of fiber-mutant Ad vectors and a miRNA-regulated expression system would further enhance antitumor effects and reduce hepatotoxicity.

The Ad vector doses used in this study were higher than those in most clinical suicide gene therapies.[1,6,35] Given that the body weight of mice is ~20 g, the injected doses of 3.0×10^9–10^{10} VP/mice are equivalent to 1.5×10^{11}–10^{12} VP/kg. Serious hepatotoxicity was not frequently found in clinical trials using HSVtk-expressing Ad vectors. However, in order to obtain more efficient therapeutic effects, higher doses of Ad vectors would be required. An increase in the injected doses leads not only to higher antitumor effects but also to more severe hepatotoxicity. This study demonstrates that a miR-122a-regulated HSVtk expression system makes it possible to increase the injected dose of HSVtk-expressing Ad vectors without producing HSVtk-induced hepatic damage, thereby leading to greater antitumor efficacy.

In summary, the use of a miRNA-regulated transgene expression system dramatically reduced the level of hepatic side effects otherwise encountered in Ad vector–mediated suicide gene therapy for cancer. Moreover, this approach has the potential to enhance the safety and effectiveness of Ad vectors, not only in this particular context, but also as part of other therapeutic strategies.

MATERIALS AND METHODS

Mice and cells. Female C57BL/6 mice of ages 5–6 weeks, were obtained from Nippon SLC (Hamamatsu, Japan). B16 cells (a mouse melanoma cell line) were cultured in minimum essential medium supplemented with 10% fetal calf serum and antibiotics. HuH-7 cells (a human well-differentiated hepatocellular carcinoma cell line, RCB1366, obtained from the JCRB Cell Bank, Tokyo, Japan), 293 cells (a human embryonic kidney cell line), SK HEP-1 cells (a human hepatoma cell line), and MS1 cells (a mouse pancreatic islet endothelial cell line) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Mouse primary hepatocytes were isolated from C57BL/6 mice and cultured as previously described.[36]

Plasmids and Ad vectors. Ad vectors containing miRNA target sequences were constructed by means of an improved *in vitro* ligation method described previously.[37–39] A CMV promoter–driving *firefly* luciferase–expressing shuttle plasmid with four tandem copies of perfectly complementary sequences to miR-122a in the 3′-UTR of the *firefly* luciferase expression cassette, pCMVL1-122aT, was constructed as described: A *NotI/KpnI* fragment of pCMVL1[39] was ligated with oligonucleotides 1 (5′-GGCCACAAACACCATTGTCACACTCCACAGCACAAACACCATTGTCACACTCCA*TTAATTAA*GCGGTAC-3′) and 2 (5′-CGC*TTAATTAA*TGGAGTGTGACAATGGTGTTTGTGCTGTGGAGTGTGACAATGGTGTTTGT-3′) (perfectly complementary sequences to miR-122a and *PacI* recognition sequences are indicated by underlining and italics, respectively), resulting in pCMVL1-122aT-1. The *PacI/KpnI* fragment of pCMVL1-122aT-1 was then ligated with oligonucleotides 3 (5′-ACAAACACCATTGTCACACTCCAGGACACAAACACCATTGTCACACTCCAGTAC-3′) and 4 (5′-TGGAGTGTGACAATGGTGTTTGTGTCCTGGAGTGTGACAATGGTGTTTGTAT-3′), resulting in pCMVL1-122aT. pCMVL1-controlT, which contains four tandem copies of reverse sequences of perfectly complementary sequences to miR-122a as control sequences, was similarly constructed by introducing the following oligonucleotides into pCMVL1: 5′-GGCC**ACCTCACACTGTTACCACAAACA**CGACA**CCTCACACTGTTACCACAAACA***TTAATTAA***GCGGTAC-3′, 5′-CGC*TTAATTAA***TGTTTGTGGTAACAGTGTGAGG**TGTCG**TGTTTGTGGTAACAGTGTGAGGT**-3′, 5′-**ACCTCACACTGTTACCACAAACA**CAGG**ACCTCACACTGTTACCACAAACA**GTAC-3′, and 5′-**TGTTTGTGGTAACAGTGTGAGGT**ATC**TGTTTGTGGTAACAGTGTGAGGT**AT-3′ (reverse sequences of perfectly complementary sequences to miR-122a are indicated in boldface letters).

For the construction of the Ad vector plasmids, I-*CeuI*/PI-*SceI*-digested pCMVL1, pCMVL1-122aT, or pCMVL1-controlT was ligated with the I-*CeuI*/PI-*SceI*-digested Ad vector plasmid pAdHM20-RL3 containing a *renilla* luciferase expression cassette in the E3-deleted region, yielding pAdHM20-L, pAdHM20-L-122aT, or pAdHM20-L-controlT, respectively. pAdHM20-RL3 was prepared by ligation of *ClaI*-digested pAdHM20 (ref. 40) and *Csp45*I-digested pHM11-CMVRL. pHM11-CMVRL, containing a *renilla* luciferase expression cassette driven by the CMV promoter, was constructed using pHM11 (ref. 41) and pGL4.70 (Promega, Madison, WI). Plasmids for the HSVtk-expressing Ad vectors (pAdHM4-tk-122aT and pAdHM4-tk-controlT) were constructed as described earlier, using pAdHM4 (ref. 37) and pHM-CMVHSVtk. pHM-CMVHSVtk was constructed by replacing the *firefly* luciferase gene in pCMVL1 with the HSVtk gene in pHM3-CMVtk.[13] For generating Ad vector particles (Ad-L, Ad-L-122aT, Ad-L-controlT, Ad-tk-122aT, and Ad-tk-controlT), each vector plasmid was digested with *PacI* to release the recombinant viral genome, and the Ad vector particles were transfected into 293 cells plated on 60-mm dishes with SuperFect transfection reagent (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. A conventional Ad vector expressing *firefly* luciferase, Ad-L2, was prepared earlier.[17] All Ad vectors were propagated in 293 cells, purified by two rounds of cesium chloride-gradient ultracentrifugation, dialyzed, and stored at −80 °C. The VPs were determined using a spectrophotometric method,[42] and the biological titers were measured using the Adeno-X rapid titer kit (Clontech, Mountain View, CA). The ratio of particle-to-biological titer was between 9 and 21 for each Ad vector used in this study.

miRNA expression analysis. Total RNA, including miRNA, was extracted from the cultured cells using ISOGEN (Nippon Gene, Tokyo, Japan). Reverse transcription reactions were performed using the TaqMan MicroRNA reverse transcription kit (Applied Biosystems, Foster City, CA) in conjunction with the miR-122a-specific primers. In order to quantify miR-122a expression, real-time PCR was performed using the TaqMan miRNA assay kit for hsa-miR-122a and the ABI prism 7000 system (Applied Biosystems). Amplification of U6 served as an endogenous control to normalize the miR-122a expression data.

In vitro *gene expression analysis.* HuH-7, SK HEP-1, and MS1 cells were seeded at 6×10^4 cells/well on 24-well plates. Mouse primary hepatocytes were seeded at 1×10^5 cells/well on collagen-coated 12-well plates. On the following day, the cells were infected with Ad-L, Ad-L-122aT, or Ad-L-controlT at 1.5 hours at 300 VP/cell. Following a 48-hour incubation, *firefly* and *renilla* luciferase activity levels in the cells were measured using a Dual-Luciferase Assay Kit (Promega).

In vivo *gene expression analysis.* B16 cells (5×10^5 cells/50 μl) were intradermally inoculated into the abdomens of C57BL/6 mice. Six days later, when the tumor diameter exceeded 5–8 mm, 50 μl of Ad-L, Ad-L-122aT, or Ad-L-controlT was intratumorally injected at a dose of either 3.0×10^8 VP or 3.0×10^{10} VP. Forty-eight hours after the injection, the mice were killed and the tumors and organs were removed and homogenized as previously described.[43] The activity levels of *firefly* and *renilla* luciferase in the homogenates were measured using the Dual-Luciferase Assay Kit. The protein content was measured with a Bio-Rad assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard.

Tissue distribution of Ad vectors. The Ad DNA contents in the liver and tumor were quantified using a real-time PCR assay, as previously described.[44] Briefly, total DNA, including the Ad vector DNA, was isolated from each organ using an Automatic Nucleic Acid Isolation System (NA-2000; Kurabo, Osaka, Japan). After isolation, the total DNA concentrations were determined, and the Ad DNA contents were quantified using a TaqMan fluorogenic detection system (ABI Prism 7700 sequence detector; Perkin-Elmer Applied Biosystems, Foster City, CA).

In vivo *antitumor effects of HSVtk-expressing Ad vectors.* Established B16 tumors in C57BL/6 mice, as described earlier, were injected on day 0 with 50 μl of Ad-tk-122aT, Ad-tk-controlT, or Ad-L2 at a dose of either 3.0×10^8 VP or 3.0×10^{10} VP. The mice also received daily injections of GCV (75 mg/kg) intraperitoneally for 10 days. Tumor growth was monitored by calculating the tumor volume from caliper measurements. Tumor volumes were calculated according to the formula: (tumor volume; mm^3) = (major axis; mm) × (minor axis; $mm)^2 \times 0.5236$.

Toxicity studies. Four and six days after the Ad vector was injected into the tumors as described, blood samples were collected through retro-orbital bleeding. The samples were placed on ice for 2–3 hours and then centrifuged for 10 min to collect the serum. The serum ALT levels were determined using a transaminase-CII kit (Wako Pure Chemical Industries, Osaka, Japan). For the histopathological examination of liver sections, the mice were killed 6 days after the Ad vector injection, and their livers were collected. The extracted livers were washed, fixed in 10% formalin, and embedded in paraffin. After sectioning of the organs, the tissue samples were dewaxed in ethanol, rehydrated, and stained with hematoxylin and eosin. The liver sections were then examined under a microscope.

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