Gene Therapy (2015) **22,** 155–162

www.nature.com/gt

ORIGINAL ARTICLE

Tumor-specific suicide gene therapy for hepatocellular carcinoma by transcriptionally targeted retroviral replicating vectors

Y-H Lai, C-C Lin, S-H Chen and C-K Tai

Replicating virus vectors are attractive tools for anticancer gene therapy, but the potential for adverse events due to uncontrolled spread of the vectors has been a major concern. To design a tumor-specific retroviral replicating vector (RRV), we replaced the U3 region of the RRV ACE-GFP with a regulatory sequence consisting of the hepatitis B virus enhancer II (EII) and human α-fetoprotein (AFP) core promoter to produce ACE-GFP-EIIAFP, a hepatocellular carcinoma (HCC)-targeting RRV. Similar to ACE-GFP, ACE-GFP-EIIAFP exhibited robust green fluorescent protein (GFP) expression in HCC cells and, most importantly, it exhibited HCC-specific replication and did not replicate in non-HCC tumor cells or normal liver cells. We sequenced the promoter region of ACE-GFP-EIIAFP collected from serial infection cycles to examine the genomic stability of the vector during its replicative spread, and found that the vector could retain the hybrid promoter in the genome for at least six infection cycles. *In vitro* studies revealed that ACE-CD-EIIAFP and ACE-PNP-EIIAFP, which express the yeast cytosine deaminase and *Escherichia coli* purine nucleoside phosphorylase, respectively, exert a highly potent cytotoxic effect on HCC cells in the presence of their respective prodrugs. *In vivo*, ACE-CD-EIIAFP-mediated suicide gene therapy efficiently suppressed HCC tumor growth and no detectable RRV signal was observed in extratumoral tissues. These results suggest that the tumor-specific, suicide-gene-encoding RRV may fulfill the promise of retroviral gene therapy for cancer.

Gene Therapy (2015) **22,** 155–162; doi:10.1038/gt.2014.98; published online 30 October 2014

INTRODUCTION

Vectors derived from murine leukemia virus (MLV) have been widely employed for delivering genes to cancer cells *in vitro* and *in vivo*. MLV-based retrovirus vectors possess several inherent advantages in cancer gene therapy, including low immunogenicity,[1–3] transduction only to actively dividing cells[4–6] and stable expression of transgenes by integration into the target cell DNA. Conventional retrovirus vectors are replication defective because the viral genes *gag*, *pol* and *env* are removed to develop a safer gene delivery carrier; however, it has become clear that defective retrovirus vectors are generally incapable of efficient gene delivery when they are administered *in vivo*.[7–13]

To improve the gene delivery efficiency of retrovirus vectors, we have constructed a series of retroviral replicating vectors (RRVs) derived from MLV.[14–17] We have previously demonstrated that MLV-based RRVs containing transgenes inserted at the *env*-3′ untranslated region border can stably propagate over multiple infection cycles and transmit the inserted transgenes throughout a solid tumor *in vivo*, without detectable spread to extratumoral tissues.[18–20] Nonetheless, it would be advantageous to incorporate additional mechanisms so that RRVs selectively target tumor cells and thereby minimize the risk to normal tissues. A practicable approach for tissue-specific targeting of RRVs might be to replace the transcriptional control elements within the long terminal repeat (LTR) with tissue-specific promoters.[21–23] It was found that introducing prostate-specific probasin promoters to the LTR of RRVs resulted in both the replication and transgene expressions of RRVs being transcriptionally targeted to prostate cells.[24] In the present study, we investigated whether such a targeting strategy could be applied to target RRV replication to hepatocellular carcinoma (HCC) cells.

Transcriptional targeting of replication-defective retrovirus vectors to HCC cells can be achieved by introducing a human α-fetoprotein (AFP) promoter into the vector.[25,26] In the present study, we characterized two RRVs targeted to HCC by replacing the U3 region of LTR with a sequence consisting of the AFP core promoter fused to the AFP enhancer domain B or the enhancer II (EII) of the human hepatitis B virus (HBV). The 192-bp AFP enhancer domain B contains one hepatocyte nuclear factor (HNF)-1, one HNF-3 and two CCAAT/enhancer binding protein binding sites, and it has been shown to play a major role in the tissue-specific enhancement of the AFP promoter in HCC.[27] The 155-bp HBV EII consisting of one HNF-3, one HNF-4, two CCAAT/enhancer binding protein sites and some binding regions for other hepatic transcription factors has also been found to greatly enhance liver-selective transcriptional activity.[28] Our results show that the RRV with the U3 region replaced with a hybrid sequence of HBV EII and AFP core promoter expresses a high level of HCC-specific transcriptional activity. Furthermore, the growth of HCC infected with the targeted RRV carrying the yeast cytosine deaminase (CD)[29] or the *Escherichia coli* purine nucleoside phosphorylase (PNP)[30,31] suicide gene was suppressed in the presence of their respective prodrugs: 5-fluorocytosine (5-FC) and fludarabine phosphate (F-araAMP). Our results demonstrate that highly efficient and tumor-specific cell killing can be achieved both

Department of Life Science and Institutes of Molecular Biology and Biomedical Science, National Chung Cheng University, Min-Hsiung, Chia-Yi, Taiwan. Correspondence: Dr C-K Tai, Department of Life Science, National Chung Cheng University, 168 University Road, Min-Hsiung, Chia-Yi 621, Taiwan.
E-mail: biockt@ccu.edu.tw
Received 6 April 2014; revised 12 September 2014; accepted 17 September 2014; published online 30 October 2014

in vitro and *in vivo*, and furthermore that progressive and stable suicide gene transduction by transcriptionally targeted RRVs represents a unique property for achieving long-term therapeutic benefits.

RESULTS

Transcriptional activity of hybrid MLV LTRs in human cell lines

We constructed two hybrid MLV LTRs incorporating sequences from the AFP promoter. The hybrid LTRs were generated by replacing the MLV U3 with a sequence consisting of the AFP enhancer domain B and the AFP core promoter or a chimeric sequence consisting of the HBV EII and the AFP core promoter. The resulting hybrid LTRs were designated EAFP and EIIAFP, respectively. The EAFP, EIIAFP and wild-type MLV LTRs were inserted into the promoterless luciferase reporter plasmid pGL4-basic to generate pGL4-EAFP, pGL4-EIIAFP and pGL4-LTR, respectively (Figure 1a).

HEK293, U-87, MCF-7, Huh-7 and HepG2 cells were transfected with pGL4-EAFP and pGL4-EIIAFP to determine the transcriptional activity of the hybrid LTRs. As a positive control, pGL4-LTR containing the wild-type MLV LTR was used in parallel transfections. In non-HCC cells, including HEK293, U-87 and MCF-7, the wild-type LTR exhibited a high level of transcriptional activity, in contrast with the very low luciferase activities driven by EAFP and EIIAFP (Figure 1b). In the HCC cells Huh-7 and HepG2, both EAFP and EIIAFP were much more potent than wild-type LTR, increasing luciferase expression to levels at least fivefold higher than that for wild-type LTR. Together, these results show that the wild-type LTR possesses a universal transcriptional ability, and that hybrid LTRs exhibit HCC-specific transcriptional activity.

Evaluation of AFP protein levels in human cell lines

To evaluate whether AFP was expressed in an HCC-specific manner, we collected and quantified the supernatants harvested from cultured HepG2, Huh-7, THLE-2, U-87 and HEK293 cells. The AFP levels of the two HCC cell lines were found to be >700-fold higher than that of the three non-HCC cell lines (Table 1). Notably, the human liver epithelial cell line THLE-2 exhibited an extremely low level of AFP. Such differential expression of AFP between THLE-2 and HCC cells was strongly suggestive of the potential utility of hybrid LTRs in achieving HCC-specific transcription.

Replication of targeted vectors in HCC and non-HCC cells

To evaluate the ability of the hybrid LTRs to support HCC-specific transcription and replication of RRVs, we replaced the 3′ LTR of the RRV plasmid pACE-GFP with each of the two hybrid LTRs, producing two plasmids: pACE-GFP-EAFP and ACE-GFP-EIIAFP (Figure 2a). Various cell lines were infected with the virus vectors ACE-GFP, ACE-GFP-EAFP or ACE-GFP-EIIAFP at a multiplicity of infection of 0.05, and the replication kinetics of each vectors were measured by flow-cytometry analysis for green fluorescent protein (GFP) expression at each passage. As expected, the ACE-GFP vector could efficiently replicate in all cell lines tested, including HEK293, U-87, Huh-7, HepG2, MCF-7, T-47D and THLE-2, and

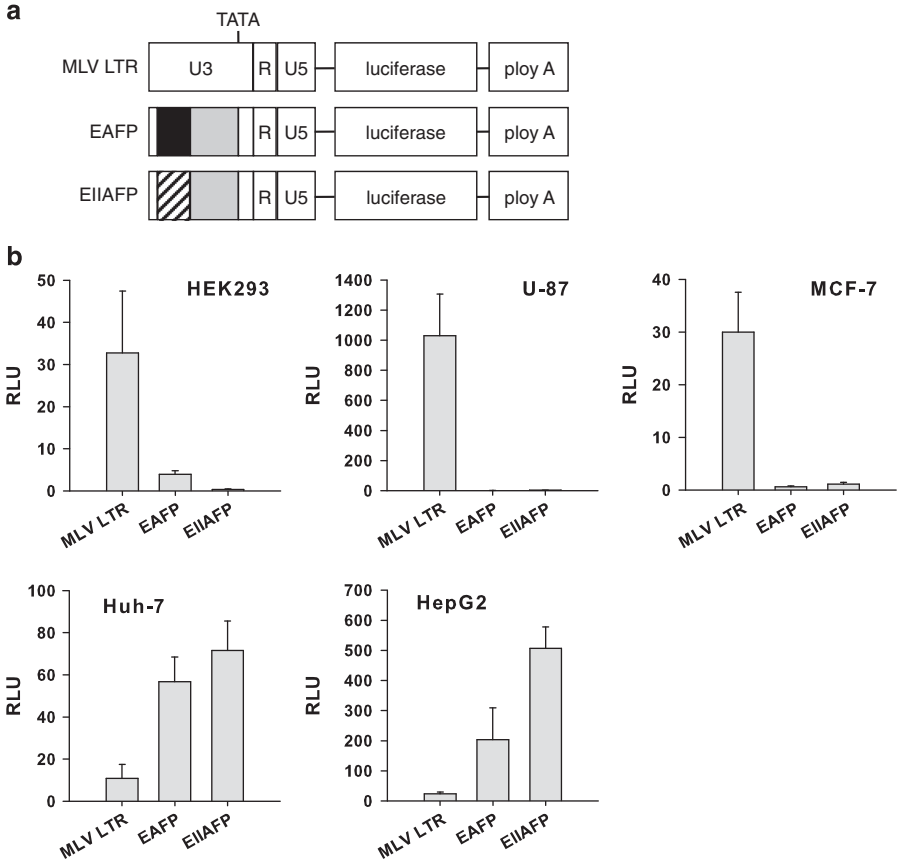

Figure 1. Transcriptional activity of hybrid LTRs in human cell lines. (**a**) Luciferase reporter constructs containing the wild-type MLV LTR and hybrid LTRs, EAFP and EIIAFP. TATA: MLV TATA box. Black box: AFP enhancer domain B. Gray box: AFP core promoter. Hatched box: HBV EII. (**b**) Each of the luciferase reporter plasmids was transfected into HEK293, U-87, MCF-7, Huh-7 and HepG2 cells. Luciferase activity in the transfected cell extracts is indicated by relative light units (RLUs). The activity measured for the control plasmid (pGL4-basic) in each cell line was assigned a value of 1. Results are the means obtained from three independent experiments. Error bars indicate s.d.

achieved complete transduction to each cell line (Figure 2b and Supplementary Figure 1). Huh-7 and HepG2 cells infected with ACE-GFP-EAFP or ACE-GFP-EIIAFP exhibited progressively increased transduction levels; however, there was no evidence of replication of either vector in non-HCC cells. In particular, ACE-GFP-EIIAFP replicated markedly faster than ACE-GFP-EAFP and slightly slower than ACE-GFP in HepG2 cells, indicating that the higher level of transcriptional activity of EIIAFP relative to that of EAFP enabled a more rapid spread of the vector.

Genetic stability and promoter specificity of ACE-GFP-EIIAFP during prolonged replication

We subjected ACE-GFP-EIIAFP to six serial infection cycles in HepG2 cultures and isolated genomic DNA from each cycle in order to investigate the genetic stability of the vector after long-term propagation. PCR was used to amplify the IRES-GFP cassette and hybrid LTR EIIAFP from the integrated ACE-GFP-EIIAFP provirus, and the resulting products were resolved on agarose gels. The full-length IRES-GFP and hybrid LTR sequences were the predominant species amplified in the HepG2 culture of each infection cycle, indicating that the vector genome is highly stable after long-term propagation *in vitro* (Figures 3a and b).

To examine the genetic integrity of the hybrid LTR following serial infection cycles, the hybrid LTR of integrated ACE-GFP-EIIAFP provirus from the sixth infection cycle was amplified by PCR, cloned and sequenced. Sequence analyses of the hybrid LTR obtained from six randomly chosen clones revealed that few point mutations occurred in the AFP core promoter, whereas barely any mutations were located in the six clones in any known transcription factor binding sites (Figure 3c). This indicated that the genetic integrity of the hybrid LTR was stably maintained during long-term propagation of the vector. The maintenance of the HCC specificity of serially passaged ACE-GFP-EIIAFP was further confirmed by the absence of replicative spread of the vector in THLE-2, HEK293 and MCF-7 cells exposed to viruses from each of the six infection cycles (data not shown).

Table 1. AFP protein levels in human cell lines

Cell	*AFP concentration (ng ml^{-1})*
HepG2	4899.6 ± 56.8
Huh-7	2511.4 ± 16.1
THLE-2	3.44 ± 1.05
U-87	3.23 ± 1.77
HEK293	0.91 ± 0.46

Supernatants harvested from 1×10^6 of HepG2, Huh-7, THLE-2, U-87 and HEK293 were subjected to enzyme-linked immunosorbent assay (ELISA) assay for quantification of secreted human α-fetoprotein (AFP). The values of AFP concentrations are presented as mean ± s.d.

Tissue-specific cytotoxicity achieved by EIIAFP-targeted RRVs

To convert the RRV into a therapeutic reagent, the CD and PNP coding sequences were cloned from pACE-CD[20] and pACE-PNP[16] and used to replace the GFP gene in pACE-GFP-EIIAFP, producing pACE-CD-EIIAFP and pACE-PNP-EIIAFP, respectively. We have previously confirmed the expression levels of CD and PNP using western blot analysis, indicating efficient expression of the CD and PNP gene products in cells after RRV transduction.[16,32]

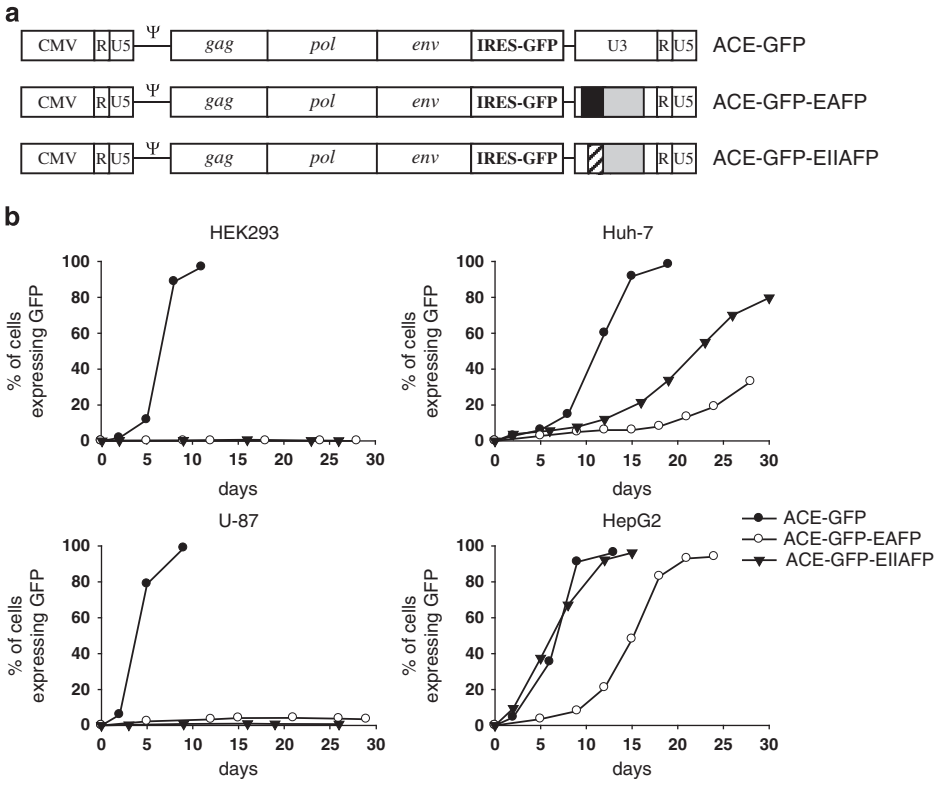

Figure 2. Replication of RRVs in HCC and non-HCC cells. (**a**) Structure of RRVs containing the wild-type MLV LTR and hybrid LTRs. Each vector contains an IRES-GFP positioned immediately downstream of the amphotropic *env* gene. CMV, cytomegalovirus immediate-early promoter; Ψ, packaging signal. (**b**) HEK293, U-87, Huh-7 and HepG2 cells were inoculated with RRVs at a multiplicity of infection of 0.05. At various time points after infection, the cells were analyzed by flow cytometry to determine the percentage of cells expressing GFP. The x axis indicates days after virus inoculation and y axis indicates % of cells expressing GFP.

To determine the functional dose range for the prodrugs used, HepG2 cells were incubated with 5-FC or F-araAMP at various concentrations, and the remaining viable cells were examined 4 days later. The half-maximal inhibitory concentration values of 5-FC and F-araAMP were determined as ~4.0 mM and ~3.5 μM, respectively (data not shown). In HepG2 cells, transduction by ACE-CD or ACE-CD-EIIAFP without adding the 5-FC prodrug had no significant effect on cell viability. Exposure to 0.5 mM 5-FC produced potent cell killing in both the ACE-CD- and ACE-CD-EIIAFP-transduced HepG2 cells (Figure 4a). Similar results were obtained when HepG2 cells transduced by ACE-PNP or ACE-PNP-EIIAFP were exposed to 0.5 μM F-araAMP prodrug (Figure 4b). For non-HCC cells such as U-87, the viability of ACE-PNP-transduced cells was also decreased by exposure to F-araAMP. In contrast, the viability of ACE-PNP-EIIAFP-transduced U-87 cells was not significantly affected by the presence of F-araAMP (Figure 4c). These results demonstrate that tissue-specific cytotoxicity can be achieved by the transduction of cells with EIIAFP-targeted RRVs followed by prodrug administration.

Replicative spread of RRVs *in vivo*

To examine the replication efficiency of HCC-targeting RRV *in vivo*, we injected a single low dose of ACE-GFP-EIIAFP (1×10^4 transducing units (TUs)) into a pre-established subcutaneous HepG2 tumor model and observed the spread of the vector in tumors by quantifying the GFP expression of resected tumors at

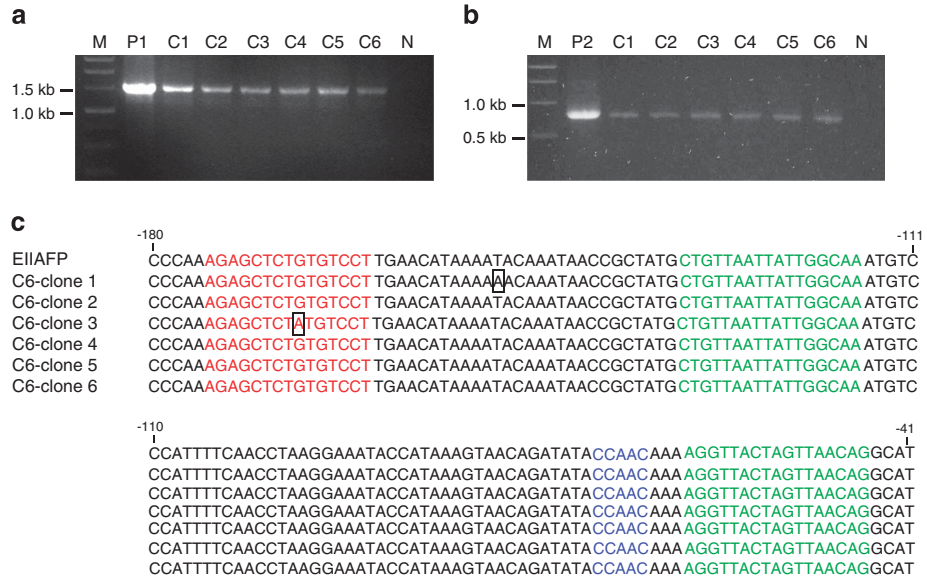

Figure 3. Genetic stability of ACE-GFP-EIIAFP during prolonged replication. HepG2 cells were infected with ACE-GFP-EIIAFP and passaged until a maximum percentage of GFP-positive cells was reached. The viral supernatant was collected from HepG2 culture at 7 days post infection, diluted 25-fold and used to initiate a new infection. This was repeated for six serial infection cycles. (**a**) PCR analysis of IRES-GFP insert region of serially passaged viruses. Genomic DNA of HepG2 at the end of each cycle was analyzed by PCR using primers in the MLV sequence flanking the IRES-GFP insert. The expected size of the full-length PCR product is ~1.5 kb. C1–C6, genomic DNA from HepG2 at infection cycles 1–6; M, DNA marker; N, genomic DNA from uninfected HepG2; P1, pACE-GFP-EIIAFP plasmid DNA. (**b**) PCR analysis of EIIAFP hybrid LTR of serially passaged viruses. Genomic DNA of HepG2 was analyzed by PCR using primers flanking the 5′ LTR. The expected size of the full-length PCR product is ~0.75 kb. P2, pZE-GFP, an RRV plasmid DNA containing the wild-type 5′ LTR of MLV as a positive control. (**c**) Sequence analysis of EIIAFP core promoter region of serially passaged viruses. PCR products of HepG2 at the sixth infection cycle were cloned and sequenced. Nucleotide substitutions in EIIAFP core promoter region are boxed. Nucleotide sequence in red indicates glucocorticoid-responsive element, nucleotide sequences in green indicate HNF-1 binding sites, and nucleotide sequence in blue indicates CAAT signal.

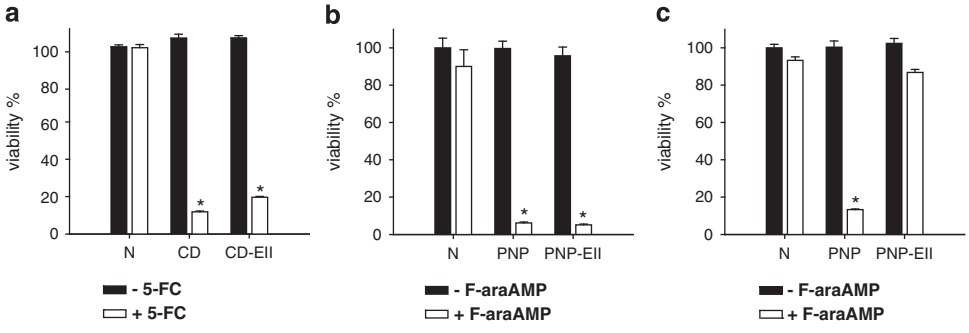

Figure 4. *In vitro* cytotoxicity achieved by RRVs. (**a**) RRV-transduced HepG2 cells were exposed to 0.5 mM 5-FC. (**b**) RRV-transduced HepG2 cells were exposed to 0.5 μM F-araAMP. (**c**) RRV-transduced U-87 cells were exposed to 0.5 μM F-araAMP. The viability of cells was determined in a quadruplicate repeat with MTS assay 4 days after prodrug treatment. CD, ACE-CD-transduced cells; CD-EII, ACE-CD-EIIAFP-transduced cells; −F-araAMP, without F-araAMP incubation; +F-araAMP, with F-araAMP incubation; −5-FC, without 5-FC incubation; +5-FC, with 5-FC incubation; N, uninfected cells; PNP, ACE-PNP-transduced cells; PNP-EII, ACE-PNP-EIIAFP-transduced cells. Error bars indicate s.d. *$P < 0.001$.

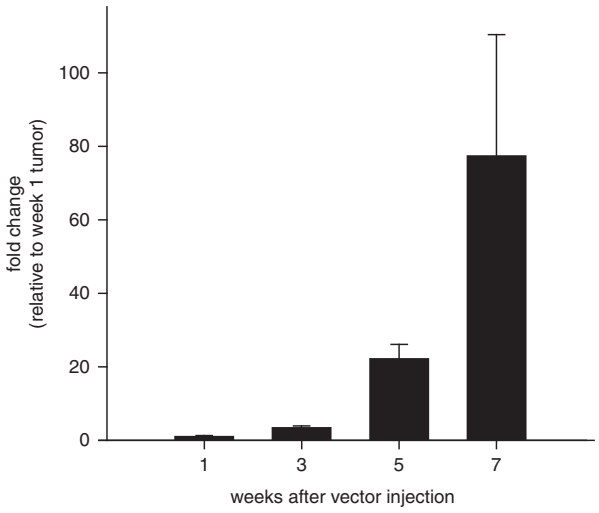

Figure 5. Replicative spread of ACE-GFP-EIIAFP in solid tumors. The ACE-GFP-EIIAFP viral vector (1×10^4 TUs) was injected into a pre-established subcutaneous HepG2 tumor model in nude mice. At 1, 3, 5 and 7 weeks after vector injection, tumors were removed from subsets of the mice and were analyzed for GFP fluorescence by flow cytometry. The GFP fluorescence measured for tumors removed 1 week after vector injection was assigned a value of 1. Error bars indicate s.e.

various time points. As expected, the percentage of GFP-positive cells in the tumors was low (~0.1%) at 1 week after vector injection, but thereafter increased rapidly over time, so that ~7.7% of the cells in the tumors were GFP positive at 7 weeks, demonstrating that ACE-GFP-EIIAFP is capable of efficient replication in HepG2 solid tumors (Figure 5). In addition, we examined the distribution of ACE-GFP-EIIAFP in the animals by performing a quantitative real-time PCR analysis of genomic DNA extracted from tumors and extratumoral organs using primers specific for the 4070A amphotropic envelope. As expected, the vector could be readily detected in the transduced HepG2 tumors by PCR amplification of the envelope sequence. However, no RRV signal was detectable in genomic DNA from liver, spleen and bone marrow in the same animals (data not shown). The HCC specificity of ACE-GFP-EIIAFP was also confirmed by the lack of replicative spread of the vector in subcutaneous U-87 tumors *in vivo* (data not shown).

We also compared the spreading efficiency of ACE-GFP and ACE-GFP-EIIAFP *in vivo* by preparing a cell mixture in which HepG2 cells infected with either vector were mixed with uninfected HepG2 cells at a ratio of 1% of the cell population, and then subcutaneously implanting this mixture into nude mice. In flow-cytometry analysis of resected tumors, the percentages of GFP-positive cells were determined to be 57.1 ± 18.3% (ACE-GFP) and 43.1 ± 7.1% (ACE-GFP-EIIAFP) at 7 weeks after tumor implantations, indicating that the replication of ACE-GFP-EIIAFP in the tumors was efficient, although slightly attenuated.

In vivo suicide gene therapy

To determine whether a low ratio of tumor cells expressing RRVs could achieve tumor suppression, we applied the prodrug 5-FC to mice bearing subcutaneous HepG2 tumors in which 1% of the cells initially expressed ACE-CD-EIIAFP. The growth of the subcutaneous tumors was significantly inhibited immediately after 5-FC administration, indicating the therapeutic effect achieved by RRV-mediated replicative spread of the CD transgene within the tumors (Figure 6a). To determine whether delivery of the CD gene mediated by direct inoculation of RRVs could

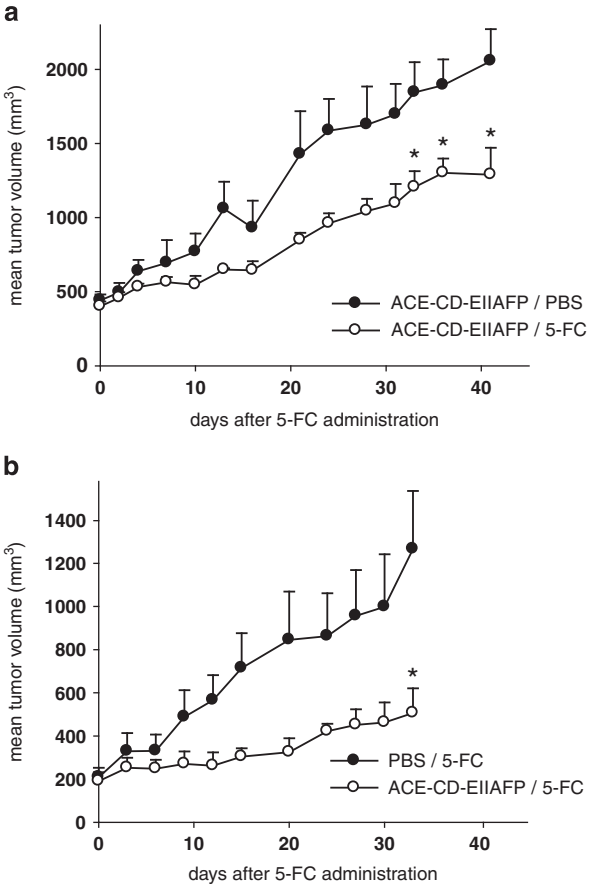

Figure 6. Therapeutic effect achieved by ACE-CD-EIIAFP *in vivo*. (**a**) Growth of ACE-CD-EIIAFP-transduced tumor in response to 5-FC treatments. Nude mice bearing subcutaneous HepG2 xenografts in which 1% of the cells initially expressed ACE-CD-EIIAFP were treated with 5-FC or phosphate-buffered saline (PBS), once every other day. Statistically significant differences in tumor size between the 5-FC-treated group and the PBS control group were observed. $^*P < 0.05$. (**b**) Growth of tumor in response to ACE-CD-EIIAFP inoculation plus 5-FC treatments. ACE-CD-EIIAFP (5×10^5 TUs) or PBS vehicle was injected into pre-established subcutaneous HepG2 tumors in nude mice. When mean tumor volume reached ~200 mm^3, the mice received intraperitoneal injections of 5-FC, once every other day, and tumor size was monitored. Statistically significant difference in tumor size between the ACE-CD-EIIAFP/5-FC-treated group and the PBS/5-FC control group was observed. Error bars indicate s.e. $^*P < 0.05$.

suppress tumor growth, we injected a single total dose of 5×10^5 TUs of ACE-CD-EIIAFP or phosphate-buffered saline into a pre-established subcutaneous HepG2 tumor model. When the mean tumor volume reached ~200 mm^3, the mice received intraperitoneal injections of 5-FC every other day, and tumor size was monitored. Treatment with ACE-CD-EIIAFP plus 5-FC resulted in a significant suppression of tumor growth, indicating the potential utility of this strategy for HCC therapy (Figure 6b).

DISCUSSION

Conventional replication-defective retrovirus vectors have largely failed to achieve significant transduction efficiencies and hence therapeutic benefits for cancer.[8] In contrast, RRVs have been considered to be a more efficient tool for delivering therapeutic genes to tumors. However, the potential for adverse events due to uncontrolled spread of the vectors has been a major concern.[33] This study evaluated the possibility of targeting replication of an

RRV to HCC by replacing transcriptional control elements within the U3 region of the vector with a regulatory sequence consisting of the HBV EII and AFP core promoter. Our results indicate that this approach can be used to generate RRVs that replicate efficiently and selectively in HCC tissues. Others have recently attempted the tissue-specific replication of RRVs. Duerner *et al.*[34] replaced the wild-type envelope of RRV with a protease-activatable envelope to allow viral replication in matrix metalloproteinase-rich tissue only. Lin *et al.*[35] incorporated a hematopoietic lineage-specific microRNA target sequence in RRV to restrict viral replication in hematopoietic lineage-derived cells and lymphoid tissues. However, neither of these studies evaluated the therapeutic application of the vectors for specific types of cancer.

Our results demonstrate that a strong enhancer/promoter is required to support efficient virus vector replication. Despite the level of transcriptional activity in HepG2 cells being much higher for EAFP than for wild-type MLV LTR, the replication kinetics of the corresponding ACE-GFP-EAFP vector were slow. The inclusion of the more effective EIIAFP in ACE-GFP-EIIAFP resulted in greatly improved replicative efficiency. In addition, the stability of the targeting sequences during virus vector replication would be a major concern when using a transcriptionally targeted RRV. Our results indicate that the EIIAFP sequence is faithfully preserved over multiple infection cycles, although a few mutations did occur in the AFP core promoter. In addition, the specificity of ACE-GFP-EIIAFP to HCC cells was found to be maintained after multiple infection cycles, further confirming the high stability of EIIAFP.

Transcriptionally targeted RRVs have been described previously,[24,36] but this is the first comprehensive report of a therapeutic application for such vectors, displaying highly efficient tumor-cell-selective replication and effective suicide gene transduction and cell killing of HCC cells both *in vitro* and *in vivo*. *In vitro* cytotoxicity studies have demonstrated highly efficient cell killing of HepG2 by EIIAFP-targeted RRVs ACE-CD-EIIAFP and ACE-PNP-EIIAFP, indicating that the gene-transfer efficiency of the vectors was as high as that of the untargeted vectors ACE-CD and ACE-PNP. For non-HCC cells such as U-87 glioma cell, the viability of ACE-PNP-EIIAFP-transduced cells was not significantly affected by the presence of F-araAMP (Figure 4c), demonstrating that tissue-specific cytotoxicity can be achieved by transducing the cells with ACE-PNP-EIIAFP followed by prodrug administration. In addition, U-87 transduced with ACE-CD-EIIAFP should also be included in the experiments to demonstrate the tissue-specific cytotoxicity of ACE-CD-EIIAFP following 5-FC treatment. However, in Figure 2b we have shown that EIIAFP-targeted RRVs (ACE-GFP-EIIAFP) could not replicate in U-87. We therefore believe that switching of the PNP suicide gene to CD should not affect the tissue-specific targeting property of EIIAFP-targeted RRVs.

The additional advantage of this therapeutic strategy is that the expression of the CD and PNP suicide genes in HCC cells can achieve the direct intracellular generation of active antineoplastic agents by converting 5-FC and F-araAMP to 5-fluorouracil and 2-fluoroadenine, respectively.[37,38] Both of these agents are freely diffusible across cell membranes, allowing them to spread from RRV-transduced to untransduced cells, thereby exerting more potent bystander effects on neighboring tumor cells.[31,39]

Our current *in vivo* results are consistent with and extend the findings of our previous study,[40] demonstrating that a single dose of cell-free vector supernatant followed by the administration of 5-FC was able to achieve profound inhibition of pre-established HCC xenografts. Certainly we agree that the PNP/F-araAMP suicide gene therapy system is more strongly cytotoxic to HepG2 cells *in vitro* (Figures 4a and b); however, the F-araAMP prodrug was found to be toxic in its own right after dephosphorylation to F-araA by plasma enzymes *in vivo*.[41,42] In addition, F-araAMP itself is a chemotherapy drug used in the treatment of hematological malignancies.[43,44] The CD/5-FC suicide gene therapy system was therefore chosen to reduce the unwanted cytotoxic effect of the prodrug itself, thus providing better experimental control for *in vivo* studies. Our *in vivo* investigation of vector spread in the present study as well as our experience from previous studies indicated that these animals were treated with 5-FC whereas their tumors were only partially transduced with ACE-CD-EIIAFP; therefore, growth would have presumably resumed in any untransduced tumor that did not experience bystander effects. This suggests that optimally effective doses of the RRV were not delivered, and that a higher or repeat dose of the RRV might help to achieve even more potent antitumor effects.

Notably, after intratumoral injection of ACE-GFP-EIIAFP *in vivo*, vector replication was restricted to the tumor tissue itself as demonstrated by real-time PCR analyses, indicating that introducing EIIAFP results in a vector with a high degree of tumor selectivity. The presented results indicate that the use of transcriptionally targeted RRVs may represent a promising therapeutic strategy for HCC because of their ability to efficiently and selectively transduce tumor cells *in vivo*, achieving stable integration and persistent transgene expression concomitant with progressive virus replication within the tumor.

MATERIALS AND METHODS

Cell lines

The human embryonic kidney cell lines HEK293 and 293T,[45] human HCC cell lines HepG2 and Huh-7, human mammary carcinoma cell line MCF-7 and human glioma cell line U-87 were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The human mammary carcinoma cell line T-47D was grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 10 μg ml^{-1} insulin. The immortalized human liver epithelial cell line THLE-2 was grown in Bronchial Epithelial Cell Basal Medium (Cambrex, East Rutherford, NJ, USA) supplemented with 10% fetal bovine serum, 0.5 μg ml^{-1} hydrocortisone, 15 ng ml^{-1} human epidermal growth factor and 5 μg ml^{-1} insulin.

AFP enzyme-linked immunosorbent assay

The supernatants of cultured cells were collected after brief centrifugation and the numbers of cells in the same dish were calculated at the day of harvest. The concentrations of secreted AFP in the supernatants were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Johnson and Johnson, New Brunswick, NJ, USA).

Construction of plasmids

To construct plasmids pACE-GFP-EAFP and pACE-GFP-EIIAFP, we replaced the 3′ LTR of pACE-GFP from the *Nhe*I site to the *Sac*I site just upstream of the MLV TATA box with a 417-bp sequence consisting of the AFP enhancer domain B and the AFP core promoter or a 359-bp chimeric sequence consisting of the HBV EII and the AFP core promoter, respectively. The AFP core promoter and enhancer B were amplified from human genomic DNA by PCR and HBV EII was amplified from the plasmid HBV/pGem3Z that contains a HBV genome (courtesy of Dr HL Wu, Hepatitis Research Center, National Taiwan University Hospital, Taipei, Taiwan). Luciferase reporter constructs were generated by using pGL4-basic (Promega, Madison, WI, USA). The sequence of the wild-type MLV LTR and both hybrid LTRs was amplified from pACE-GFP, pACE-GFP-EAFP and pACE-GFP-EIIAFP by using a 5′ primer containing an *Acc*65I site and a 3′ primer containing an *Xho*I site. These PCR products were then introduced into pGL4-basic at the *Acc*65I and *Xho*I sites to generate pGL4-LTR, pGL4-EAFP and pGL4-EIIAFP. To construct plasmids pACE-CD-EIIAFP and pACE-PNP-EIIAFP, we replaced the GFP gene in pACE-GFP-EIIAFP between the IRES and 3′ untranslated region with the CD and PNP coding sequences from pACE-CD and pACE-PNP, respectively.

Luciferase assay

Cultured cells at ~70% confluence in 6-well plates were transfected with 2 μg of pGL4 plasmids by using Lipofectamine Plus (Invitrogen, Carlsbad, CA, USA). Each culture was co-transfected with 2 μg of pGFPemd-CMV (PerkinElmer Life Sciences, Boston, MA, USA) to control for transfection efficiency. At 48 h post transfection, cells in each well were subjected to

Luciferase Assay System (Promega) for luciferase activity and flow-cytometry analysis for GFP expression.

Virus vector production and infection

Virus vectors were produced by transient transfection of the vector plasmids into 293T cells by using Lipofectamine Plus. At 2 days post transfection, the virus-containing supernatant was collected, passed through 0.45 μm syringe filters and frozen at −80 °C for later use. Alternatively, the filtered virus-containing supernatant was concentrated by using Retro-X Concentrator (Clontech, Mountain View, CA, USA). For titer determination, viral supernatant was added to ~20% confluent HepG2 cells in the presence of 4 μg ml^{-1} polybrene (Sigma, St Louis, MO, USA). Viral supernatant was then removed 24 h post infection, and cells were incubated in regular media with 50 μM AZT (Sigma) for 48 h and were analyzed for GFP expression using a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). Viral titer was represented as TUs. For *in vitro* infection experiments, cultured cells seeded on six-well plates were infected with virus vectors ACE-GFP, ACE-GFP-EAFP or ACE-GFP-EIIAFP at a multiplicity of infection of 0.05 in the presence of 4 μg ml^{-1} polybrene. At various time points post infection, the cells were analyzed for GFP expression using a FACScan.

Multiple cycle infections with ACE-GFP-EIIAFP

HepG2 cells at ~20% confluence were infected with ACE-GFP-EIIAFP at a multiplicity of infection of 0.05 and subsequently passaged and subjected to flow-cytometry analysis for GFP every 3–4 days until a maximum percentage of GFP-positive cells was reached. Serial infections with ACE-GFP-EIIAFP were carried out by transferring diluted viral supernatant from HepG2 culture at day 7 post infection to fresh HepG2 cultures to initiate a new infection cycle. This was repeated for six serial infection cycles. Genomic DNA from each infection cycle was isolated by Genomic DNA Mini Kit (Geneaid, Taipei, Taiwan).

PCR analysis and sequencing

The IRES-GFP cassette of the integrated ACE-GFP-EIIAFP provirus was amplified by PCR by using Taq DNA Polymerase Master Mix Red (Ampliqon, Odense M, Denmark), an upstream primer that binds just upstream of IRES (5′-TTACCACCTTAATCTCCACCATCA-3′), and a downstream primer that binds just downstream of GFP (5′-CCCCCCTTTTTCTGGAGACTAA-3′). The PCR products were resolved on agarose gels and visualized by ethidium bromide staining. In addition, the promoter region in the 5′ LTR of the provirus was amplified by PCR by using an upstream primer that binds the 18 5′-terminal nucleotides of the 5′ LTR U3 region (5′-AAAGA CCCCACCCGTAGG-3′) and a downstream primer that binds the MLV packaging signal (5′-GGTGTTCCGAACTCGTCAG-3′). The PCR products were separated by gel electrophoresis, purified with a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA), cloned into the yT&A cloning vector (Yeastern Biotech, Taipei, Taiwan) and sequenced.

In vitro cytotoxicity experiments

Uninfected, ACE-CD-transduced or ACE-CD-EIIAFP-transduced HepG2 cells were seeded onto replicate 96-well plates (2×10^3 cells per well). After overnight culture, the cells were exposed to 0.5 mM 5-FC (Sigma) or to control medium without 5-FC, and cell viability was determined 4 days after 5-FC treatment with MTS assay using the CellTiter Aqueous One Solution Cell Proliferation Assay kit (Promega). Similarly, the viabilities of ACE-PNP- or ACE-PNP-EIIAFP-transduced HepG2 and U-87 were measured with MTS assay 4 days after treatment with 0.5 μM F-araAMP (Berlex Laboratories, Richmond, CA, USA).

Viral replication assay *in vivo* in subcutaneous tumor models

2×10^7 HepG2 cells mixed with Matrigel (Becton Dickinson) were subcutaneously injected into the right dorsal flank of BALB/c nude mice (National Laboratory Animal Center, Taipei, Taiwan). When the tumor volumes reached ~50 mm^3, 1×10^4 TUs of ACE-GFP-EIIAFP vector was injected into the center of each tumor. At various time points (1, 3, 5 and 7 weeks) post virus injection, the tumors were excised and digested with collagenase (Invitrogen). The dissociated cells were filtered through a 100-μm cell strainer, pelleted by centrifugation, resuspended in culture medium containing 50 μM AZT and plated onto culture dishes. After overnight culture, the cells were trypsinized and immediately subjected to flow cytometry for GFP expression analysis. In a separate experiment, BALB/c nude mice were subcutaneously inoculated with cell suspensions (2×10^7) consisting of ACE-GFP-EIIAFP-transduced or ACE-GFP-transduced HepG2 mixed with uninfected HepG2 at a ratio of 1% of the cell population. At 7 weeks after tumor implantation, tumor cells were harvested as previously described and subjected to flow cytometry for GFP expression analysis.

Real-time PCR

To detect any integrated RRV sequences in genomes, real-time PCR was performed using an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). The primers were designed to target the 4070A amphotropic envelope gene (forward: 5′-ACCCTCAA CCTCCCCTACAAG-3′; reverse: 5′-GTTAAGCGCCTGATAGGCTC-3′). Apolipoprotein B gene for precise amounts of input genomic DNA was also quantified as an internal control (forward primer: 5′-CACG TGGGCTCCAGCATT-3′; reverse primer: 5′-TCACCAGTCATTTCTGCCTTTG-3′). Real-time PCR was done in 25 μl of reaction mixture containing genomic DNA extracted from tumors and extratumoral organs, 12.5 μl of 2× SYBR green real-time PCR master mix (Toyobo, Osaka, Japan) and 300 nM of each primer. Each sample was analyzed in triplicate, using 15 ng of genomic DNA in each reaction. Products were amplified by 45 cycles of successive incubation at 95 °C for 15 s and at 60 °C for 1 min. A standard curve for RRV copy number was generated by amplification of serially diluted RRV plasmid at specific copy numbers mixed into genomic DNA from spleen cells of uninfected mice.

In vivo HCC therapy by HCC-targeting RRV

Ten nude mice were subcutaneously inoculated with cell suspensions (2×10^7) consisting of ACE-CD-EIIAFP-transduced HepG2 mixed with uninfected HepG2 at a ratio of 1% of the cell population. When the tumors were ~400 mm^3 in size, five mice received intraperitoneal injections of 500 mg kg^{-1} 5-FC, once every other day whereas the remaining five mice received phosphate-buffered saline injections. The tumor volumes were measured every 3–4 days. In a second experiment, nude mice were inoculated with 2×10^7 HepG2 cells into the right dorsal flank. When the tumor volumes reached ~50–60 mm^3, 5×10^5 TUs of ACE-CD-EIIAFP or 50 μl of phosphate-buffered saline was injected into the center of each tumor. At 2 weeks after intratumoral injections of the vector or phosphate-buffered saline, all mice received intraperitoneal injections of 500 mg kg^{-1} 5-FC, once every other day.

Statistical analysis

Student's *t*-tests were performed for statistical analysis of cell viability, luciferase activity, GFP fluorescence and tumor volume.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Dr Noriyuki Kasahara for many helpful suggestions and discussion. We thank Chi Tsung Li for assistance with ELISA to detect AFP. This work was supported by a grant from the National Science Council of Taiwan (NSC99-2320-B-194-004-MY3).

REFERENCES

1 Ram Z, Culver KW, Walbridge S, Frank JA, Blaese RM, Oldfield EH. Toxicity studies of retroviral-mediated gene transfer for the treatment of brain tumors. *J Neurosurg* 1993; **79**: 400–407.
2 Hein A, Czub S, Xiao LX, Schwender S, Dorries R, Czub M. Effects of adoptive immune transfers on murine leukemia virus-infection of rats. *Virology* 1995; **211**: 408–417.
3 Traister RS, Lynch WP. Reexamination of amphotropic murine leukemia virus neurovirulence: neural stem cell-mediated microglial infection fails to induce acute neurodegeneration. *Virology* 2002; **293**: 262–272.
4 Culver KW, Ram Z, Wallbridge S, Ishii H, Oldfield EH, Blaese RM. In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science* 1992; **256**: 1550–1552.
5 Miller DG, Adam MA, Miller AD. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol Cell Biol* 1990; **10**: 4239–4242.

6 Varmus HE, Padgett T, Heasley S, Simon G, Bishop JM. Cellular functions are required for the synthesis and integration of avian sarcoma virus-specific DNA. *Cell* 1977; **11**: 307–319.
7 Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. *Nat Rev Genet* 2003; **4**: 346–358.
8 Rainov NG. A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. *Hum Gene Ther* 2000; **11**: 2389–2401.
9 Harsh GR, Deisboeck TS, Louis DN, Hilton J, Colvin M, Silver JS *et al.* Thymidine kinase activation of ganciclovir in recurrent malignant gliomas: a gene-marking and neuropathological study. *J Neurosurg* 2000; **92**: 804–811.
10 Kruse CA, Roper MD, Kleinschmidt-DeMasters BK, Banuelos SJ, Smiley WR, Robbins JM *et al.* Purified herpes simplex thymidine kinase Retrovector particles. I. In vitro characterization, in situ transduction efficiency, and histopathological analyses of gene therapy-treated brain tumors. *Cancer Gene Ther* 1997; **4**: 118–128.
11 Puumalainen AM, Vapalahti M, Agrawal RS, Kossila M, Laukkanen J, Lehtolainen P *et al.* Beta-galactosidase gene transfer to human malignant glioma in vivo using replication-deficient retroviruses and adenoviruses. *Hum Gene Ther* 1998; **9**: 1769–1774.
12 Ram Z, Culver KW, Oshiro EM, Viola JJ, DeVroom HL, Otto E *et al.* Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. *Nat Med* 1997; **3**: 1354–1361.
13 Smiley WR, Laubert B, Howard BD, Ibanez C, Fong TC, Summers WS *et al.* Establishment of parameters for optimal transduction efficiency and antitumor effects with purified high-titer HSV-TK retroviral vector in established solid tumors. *Hum Gene Ther* 1997; **8**: 965–977.
14 Logg CR, Logg A, Tai CK, Cannon PM, Kasahara N. Genomic stability of murine leukemia viruses containing insertions at the Env-3' untranslated region boundary. *J Virol* 2001; **75**: 6989–6998.
15 Tai CK, Logg CR, Park JM, Anderson WF, Press MF, Kasahara N. Antibody-mediated targeting of replication-competent retroviral vectors. *Hum Gene Ther* 2003; **14**: 789–802.
16 Tai CK, Wang W, Lai YH, Logg CR, Parker WB, Li YF *et al.* Enhanced efficiency of prodrug activation therapy by tumor-selective replicating retrovirus vectors armed with the Escherichia coli purine nucleoside phosphorylase gene. *Cancer Gene Ther* 2010; **17**: 614–623.
17 Lu YC, Luo YP, Wang YW, Tai CK. Highly efficient gene transfer to solid tumors in vivo by tumor-selective replicating retrovirus vectors. *Int J Mol Med* 2010; **25**: 769–775.
18 Logg CR, Tai CK, Logg A, Anderson WF, Kasahara N. A uniquely stable replication-competent retrovirus vector achieves efficient gene delivery in vitro and in solid tumors. *Hum Gene Ther* 2001; **12**: 921–932.
19 Wang WJ, Tai CK, Kasahara N, Chen TC. Highly efficient and tumor-restricted gene transfer to malignant gliomas by replication-competent retroviral vectors. *Hum Gene Ther* 2003; **14**: 117–127.
20 Tai CK, Wang WJ, Chen TC, Kasahara N. Single-shot, multicycle suicide gene therapy by replication-competent retrovirus vectors achieves long-term survival benefit in experimental glioma. *Mol Ther* 2005; **12**: 842–851.
21 Diaz RM, Eisen T, Hart IR, Vile RG. Exchange of viral promoter/enhancer elements with heterologous regulatory sequences generates targeted hybrid long terminal repeat vectors for gene therapy of melanoma. *J Virol* 1998; **72**: 789–795.
22 Ferrari G, Salvatori G, Rossi C, Cossu G, Mavilio F. A retroviral vector containing a muscle-specific enhancer drives gene expression only in differentiated muscle fibers. *Hum Gene Ther* 1995; **6**: 733–742.
23 Moore KA, Scarpa M, Kooyer S, Utter A, Caskey CT, Belmont JW. Evaluation of lymphoid-specific enhancer addition or substitution in a basic retrovirus vector. *Hum Gene Ther* 1991; **2**: 307–315.
24 Logg CR, Logg A, Matusik RJ, Bochner BH, Kasahara N. Tissue-specific transcriptional targeting of a replication-competent retroviral vector. *J Virol* 2002; **76**: 12783–12791.
25 Ido A, Nakata K, Kato Y, Nakao K, Murata K, Fujita M *et al.* Gene therapy for hepatoma cells using a retrovirus vector carrying herpes simplex virus thymidine kinase gene under the control of human alpha-fetoprotein gene promoter. *Cancer Res* 1995; **55**: 3105–3109.
26 Mawatari F, Tsuruta S, Ido A, Ueki T, Nakao K, Kato Y *et al.* Retrovirus-mediated gene therapy for hepatocellular carcinoma: selective and enhanced suicide gene expression regulated by human alpha-fetoprotein enhancer directly linked to its promoter. *Cancer Gene Ther* 1998; **5**: 301–306.
27 Nakabayashi H, Koyama Y, Suzuki H, Li HM, Sakai M, Miura Y *et al.* Functional mapping of tissue-specific elements of the human alpha-fetoprotein gene enhancer. *Biochem Biophys Res Commun* 2004; **318**: 773–785.
28 Kramer MG, Barajas M, Razquin N, Berraondo P, Rodrigo M, Wu C *et al.* In vitro and in vivo comparative study of chimeric liver-specific promoters. *Mol Ther* 2003; **7**: 375–385.
29 Kievit E, Bershad E, Ng E, Sethna P, Dev I, Lawrence TS *et al.* Superiority of yeast over bacterial cytosine deaminase for enzyme/prodrug gene therapy in colon cancer xenografts. *Cancer Res* 1999; **59**: 1417–1421.
30 Hong JS, Waud WR, Levasseur DN, Townes TM, Wen H, McPherson SA *et al.* Excellent in vivo bystander activity of fludarabine phosphate against human glioma xenografts that express the escherichia coli purine nucleoside phosphorylase gene. *Cancer Res* 2004; **64**: 6610–6615.
31 Sorscher EJ, Peng S, Bebok Z, Allan PW, Bennett Jr LL, Parker WB. Tumor cell bystander killing in colonic carcinoma utilizing the Escherichia coli DeoD gene to generate toxic purines. *Gene Therapy* 1994; **1**: 233–238.
32 Hiraoka K, Kimura T, Logg CR, Tai CK, Haga K, Lawson GW *et al.* Therapeutic efficacy of replication-competent retrovirus vector-mediated suicide gene therapy in a multifocal colorectal cancer metastasis model. *Cancer Res* 2007; **67**: 5345–5353.
33 Tai CK, Kasahara N. Replication-competent retrovirus vectors for cancer gene therapy. *Front Biosci* 2008; **13**: 3083–3095.
34 Duerner LJ, Schwantes A, Schneider IC, Cichutek K, Buchholz CJ. Cell entry targeting restricts biodistribution of replication-competent retroviruses to tumour tissue. *Gene Therapy* 2008; **15**: 1500–1510.
35 Lin AH, Timberlake N, Logg CR, Liu Y, Kamijima S, Diago O *et al.* MicroRNA 142-3p attenuates spread of replicating retroviral vector in hematopoietic lineage-derived cells while maintaining an antiviral immune response. *Hum Gene Ther* 2014; **25**: 759–771.
36 Metzl C, Mischek D, Salmons B, Gunzburg WH, Renner M, Portsmouth D. Tissue- and tumor-specific targeting of murine leukemia virus-based replication-competent retroviral vectors. *J Virol* 2006; **80**: 7070–7078.
37 Nakamura H, Mullen JT, Chandrasekhar S, Pawlik TM, Yoon SS, Tanabe KK. Multimodality therapy with a replication-conditional herpes simplex virus 1 mutant that expresses yeast cytosine deaminase for intratumoral conversion of 5-fluorocytosine to 5-fluorouracil. *Cancer Res* 2001; **61**: 5447–5452.
38 Parker WB, Allan PW, Shaddix SC, Rose LM, Speegle HF, Gillespie GY *et al.* Metabolism and metabolic actions of 6-methylpurine and 2-fluoroadenine in human cells. *Biochem Pharmacol* 1998; **55**: 1673–1681.
39 Huber BE, Austin EA, Richards CA, Davis ST, Good SS. Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. *Proc Natl Acad Sci USA* 1994; **91**: 8302–8306.
40 Lu YC, Chen YJ, Yu YR, Lai YH, Cheng JC, Li YF *et al.* Replicating retroviral vectors for oncolytic virotherapy of experimental hepatocellular carcinoma. *Oncol Rep* 2012; **28**: 21–26.
41 Hersh MR, Kuhn JG, Phillips JL, Clark G, Ludden TM, Von Hoff DD. Pharmacokinetic study of fludarabine phosphate (NSC 312887). *Cancer Chemother Pharmacol* 1986; **17**: 277–280.
42 Malspeis L, Grever MR, Staubus AE, Young D. Pharmacokinetics of 2-F-ara-A (9-beta-D-arabinofuranosyl-2-fluoroadenine) in cancer patients during the phase I clinical investigation of fludarabine phosphate. *Semin Oncol* 1990; **17**(5 Suppl 8): 18–32.
43 Danhauser L, Plunkett W, Keating M, Cabanillas F. 9-beta-D-arabinofuranosyl-2-fluoroadenine 5'-monophosphate pharmacokinetics in plasma and tumor cells of patients with relapsed leukemia and lymphoma. *Cancer Chemother Pharmacol* 1986; **18**: 145–152.
44 Danhauser L, Plunkett W, Liliemark J, Gandhi V, Iacoboni S, Keating M. Comparison between the plasma and intracellular pharmacology of 1-beta-D-arabinofuranosylcytosine and 9-beta-D-arabinofuranosyl-2-fluoroadenine 5'-monophosphate in patients with relapsed leukemia. *Leukemia* 1987; **1**: 638–643.
45 DuBridge RB, Tang P, Hsia HC, Leong PM, Miller JH, Calos MP. Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. *Mol Cell Biol* 1987; **7**: 379–387.

Supplementary Information accompanies this paper on Gene Therapy website (http://www.nature.com/gt)