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Preferential Targeting of Disseminated Liver Tumors Using a Recombinant Adeno-Associated Viral Vector

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Abstract

A novel selectively targeting gene delivery approach has been developed for advanced hepatocellular carcinoma (HCC), a leading cause of cancer mortality whose prognosis remains poor. We combine the strong liver tropism of serotype-8 capsid-pseudotyped adeno-associated viral vectors (AAV8) with a liver-specific promoter (HLP) and microRNA-122a (*miR-122a*)-mediated posttranscriptional regulation. Systemic administration of our AAV8 construct resulted in preferential transduction of the liver and encouragingly of HCC at heterotopic sites, a finding that could be exploited to target disseminated disease. Tumor selectivity was enhanced by inclusion of *miR-122a*-binding sequences (ssAAV8-HLP-TK-122aT4) in the expression cassette, resulting in abrogation of transgene expression in normal murine liver but not in HCC. Systemic administration of our tumor-selective vector encoding herpes simplex virus-thymidine kinase (*TK*) suicide gene resulted in a sevenfold reduction in HCC growth in a syngeneic murine model without toxicity. In summary, we have developed a systemically deliverable gene transfer approach that enables high-level expression of therapeutic genes in HCC but not normal tissues, thus improving the prospects of safe and effective treatment for advanced HCC.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide with more than 500,000 new cases annually.[1] Surgical resection and liver transplantation can be curative, but only in a small number of patients where the disease is diagnosed early. Other treatment options such as chemo-embolization and radiofrequency ablation are only partially effective in controlling localized tumors, and are associated with a high rate of metastatic recurrence. Advanced HCCs are typically resistant to chemo- and radiotherapy, while targeted therapy with sorafenib has resulted in only modest survival benefits.[2] With overall 5-year survival rate for patients with advanced disease remaining disappointingly low at approximately 10%, there is a pressing need to develop novel therapeutic approaches.Gene therapy has emerged as a promising strategy for cancer treatment. Clinical successes have recently been reported in patients with advanced metastatic solid tumors as well as leukemia.[3–8] Among vectors available for gene transfer to the liver, there has been considerable interest in adeno-associated viral vectors (AAV) in part because of their excellent safety profile. These vectors when pseudotyped with serotype 8 capsid (AAV8) have a remarkable tropism for the liver, thus making them highly suited for gene therapy of HCC.[9,10] Unlike other viral vectors such as adenovirus and poxvirus, the prevalence of neutralizing antibodies to AAV8 in humans is low, enabling effective transduction of the liver following a simple systemic bolus infusion of AAV8, as illustrated in patients with severe hemophilia B.[11] Systemic administration of therapeutic vectors offers the possibility of targeting disseminated HCC, which is highly desirable for patients with advanced HCC.

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Safe and effective gene therapy mandates selective expression of the therapeutic genes in tumor cells specifically, to maximize efficacy and avoid injury to normal surrounding tissue. However, tumor-selective delivery and/or expression of the therapeutic gene have thus far been elusive. In this study a novel tumor-targeting gene therapy approach was developed by combining the strong liver tropism of AAV8 with transcription control of a small synthetic liver-specific promoter (*HLP*) and *miR-122a* posttranscriptional regulation of transgene expression. *MiR-122a* suppresses gene expression in the liver following binding to partial complementary sequences within target mRNA. *MiR-122a* is expressed at high levels in the liver but is significantly downregulated or undetectable in advanced HCC.[12–18] We, therefore, hypothesized that following systemic administration of AAV8, expression of a transgene containing *miR-122a*-binding sequences in the 3′-untranslated region will be suppressed in healthy hepatocytes but not advanced HCC (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/hum). To test this hypothesis, the delivery and expression of AAV8 transgenes containing *miR-122a*-binding sites was systematically evaluated in various models, including a syngeneic murine model of metastatic HCC.

Materials and Methods

Cell lines

HuH-7 and SK-Hep-1 cells were obtained from ATCC. BNL-1h cell line was kindly provided by Dr. Tao MH (Graduate Institute of Life Sciences). *MiR-122a* expression in the cell lines was assessed by mirVana miRNA Detection Kit (Life Technologies).

Vector design and production

Sequences complementary to *miR-122a* and *miR-223* were cloned downstream of the ORF for *luciferase* or *HSV-tk* (TK) genes under the control of the HLP promoter, and flanked by the AAV inverted terminal repeats (ITRs) (Figs. 1a and 2d). rAAV8 vector stocks were produced as described previously.[19]

In vitro *AAV functional studies*

An amount of 1×10^5 vector particles of rAAV8 vectors were used to transduce various cell lines. Luciferase activity was measured by the Luciferase Assay System (Promega). TK activity was assessed by adding 5 μg/ml of ganciclovir (GCV) to the media the day after cell transfection. Cells

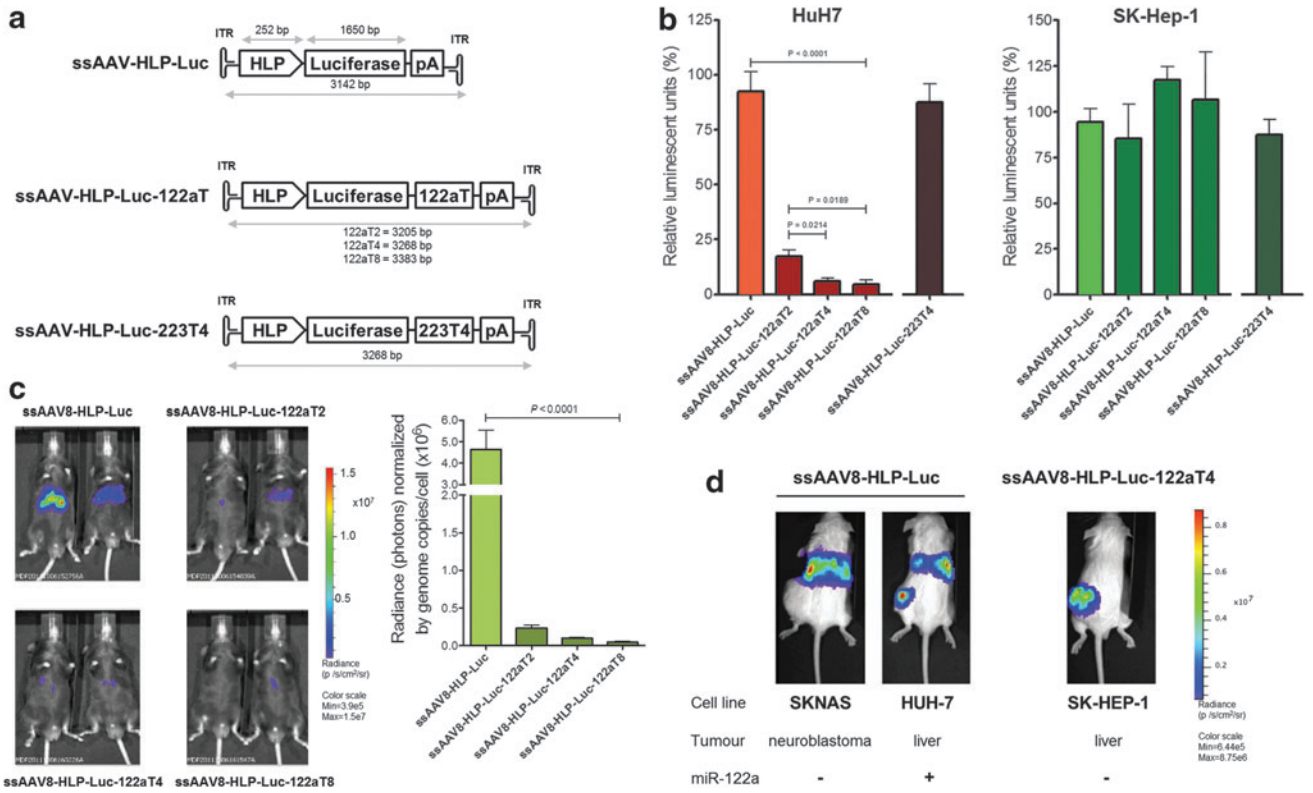

FIG. 1. Optimization of *miR-122a*-mediated silencing of rAAV8 transgene expression. **(a)** AAV8 expression cassette design. *Luciferase* was cloned downstream the HLP promoter with none, two, four, or eight repeated *miR-122a* (or four *miR-223*)-binding sequences in the 3′-UTR region. **(b)** Luciferase expression in HuH7 and SK-Hep-1 cells was transduced with these vectors (MOI = 1×10^5/cell; $n=3$). **(c)** Bioluminescence in C57Bl/6 mice 1 week after intravenous (iv) injection (1×10^{11} vg/mouse) (left). Genome copy number in liver samples (right). **(d)** HUH-7, SK-Hep-1, or SKNAS cell lines were injected in the flank of three cohorts ($n=6$/cohort) of NOD/SCID immunodeficient mice, followed 4 weeks later by tail vein injection of ssAAV8-HLP-Luc or ssAAV8-HLP-Luc-122aT4 vectors (1×10^{11} vg/mouse). Representative bioluminescence imaging of HUH-7 (left) and SKNAS (middle) mice injected with ssAAV8-HLP-Luc. Luciferase expression in mice bearing SK-Hep-1 xenograft injected with ssAAV8-HLP-Luc-122aT4 (right).

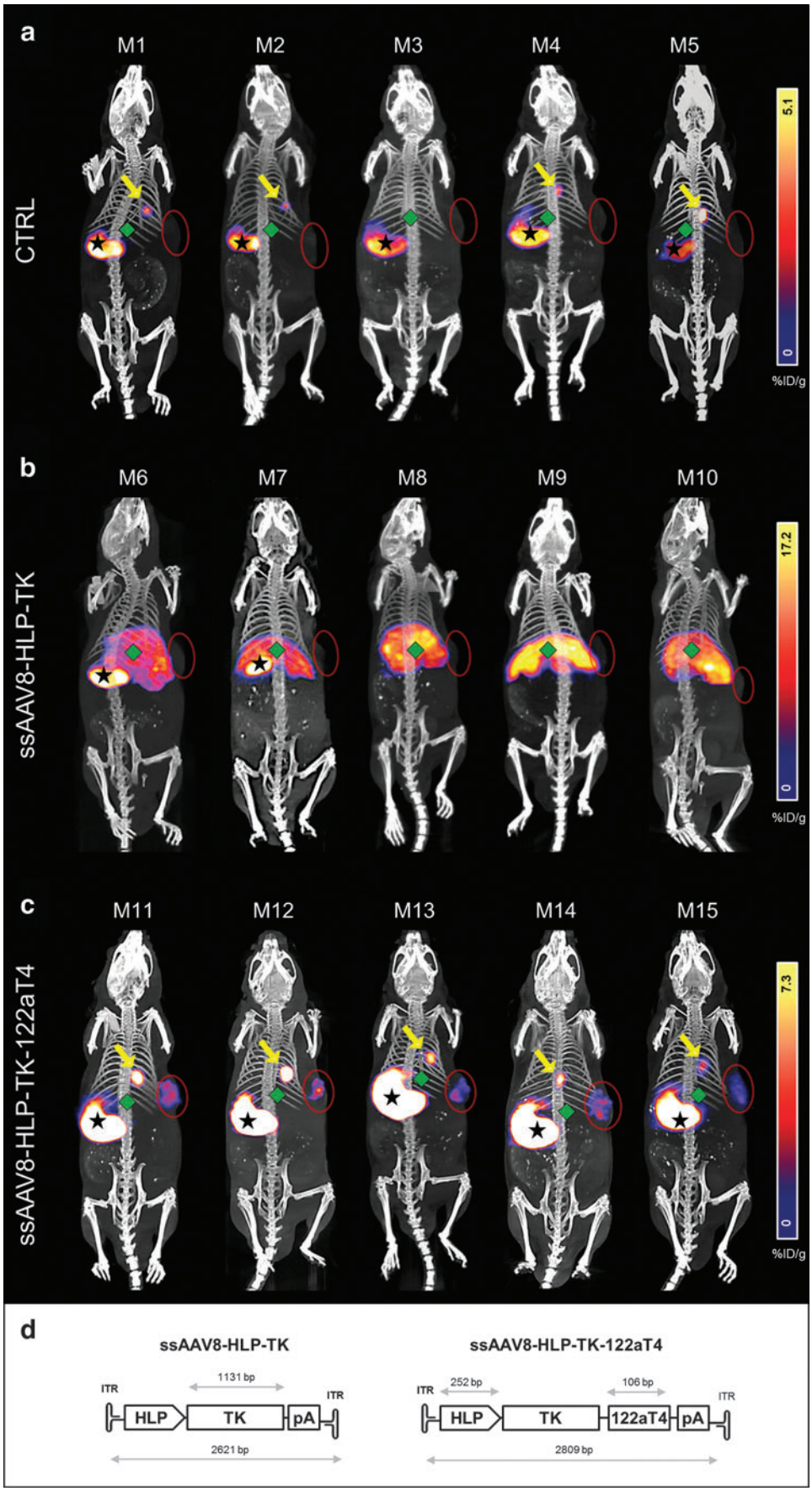

FIG. 2. SPECT/CT imaging showing cancer-specific expression of TK following iv administration of ssAAV8-HLP-TK-122aT4. NOD/SCID mice ($n = 5$/cohort) bearing human SK-Hep-1 xenografts (red circles) transduced with **(a)** no vector, **(b)** ssAAV8-HLP-TK vector, or **(c)** ssAAV8-HLP-TK-122aT4 vector. Four days after vector injection, SPECT-CT images were acquired **(a** and **b)** 30 min and **(c)** 3 hr after injection of [^{125}I]FIAU. Bars represent radiotracer uptake (%ID/g). Stars indicate stomach, diamonds indicate liver, and arrows indicate gallbladder. **(d)** *TK* gene cloned into our expression cassette to generate AAV-HLP-TK and AAV-HLP-TK-122aT4.

were counted by flow cytometry on day 5 using counting beads.

Western blot and IHC

Whole cell lysates were prepared in RIPA lysis buffer and subjected to immunoblotting using goat anti-HSVtk (vN-20, 1:200) or mouse anti-GAPDH (6C5, 1:1000) (Santa Cruz Biotechnology). Secondary antibodies were coupled to HRP and detected by ECL (Thermo Scientific). The same TK antibody was used also for IHC.

In vivo *AAV studies*

Animal work was performed under the authority of UK Home Office Project and Personal Licences regulations and was compliant with University College London ethical review committee guidelines. Mice were obtained from Charles River Laboratories Inc. AAV8 vectors encoding luciferase gene were injected by tail vein (1×10^{11} vg/mouse) suspended in 200 μl of X-vivo10. Luciferase expression was detected using D-Luciferin (Melford Laboratories), which was injected intraperitoneally (IP) at a dose of 200 μg/mouse, as substrate, using the IVIS Imaging System 100 Series (Perkin Elmer).

In vivo *HCC animal models*

Six- to eight-week-old male BALB/c mice received 1×10^{6} BNL-1h cells by intrasplenic injection under anesthesia. An amount of 1×10^{11} vg/mouse of ssAAV8-HLP-TK-122aT4 resuspended in 200 μl of X-vivo10 were injected into the tail vein on day 21 after tumor implantation, followed 5 days later by daily injection of GCV (25 mg/kg/mouse). An amount of 1×10^{7} SK-Hep-1 cells were injected in the flank of 6–8-week-old NOD/SCID immunocompromised mice to establish subcutaneous xenografts. An amount of 1×10^{11} vg/mouse of ssAAV8-HLP-TK-122aT4 vector (phosphate buffered saline [PBS] as control) were injected by tail vein followed by daily IP injection of GCV (25 mg/kg/day) for 12 days. Tumor volume was calculated using the ellipsoidal formula: (length$\times$width^2) 1/2. ALT and AST activity was measured using a Beckman Coulter AU680 analyzer (Beckman Coulter).

SPECT-CT imaging

FIAU was radiolabeled with [^{125}I] as previously described.[20] Six- to eight-week-old NOD/SCID mice received 1×10^{7} SK-Hep-1 cells by subcutaneous injection in the flank. Twenty-one days later, 1×10^{11} vg/mouse of vectors or PBS as control were administered via tail vein. SPECT-CT images were obtained 4 days later using a dedicated small animal scanner (Mediso). Anesthetized mice were then injected intravenously with 100–200 μl 20–30 MBq [^{125}I]FIAU. SPECT-CT images were collected 5, 30, and 180 min postinjection. Whole-body CT images were acquired and reconstructed using the Nucline software (Mediso). Images were fused using VivoQuant software (inviCRO), and then normalized to the administered activity to parameterize images in terms of %ID/g. Manually drawn three-dimensional volumes of interest were used to determine radiotracer accumulation in units of %ID/g.

Vector distribution

A qPCR assay SYBR Green (QuantiFast SYBR Green PCR Kit; Quiagen) was used to evaluate the biodistribution of rAAV8 vectors in genomic DNA using the following primers: HLP forward 5′-CAGGACGCTGTGGTTTCTG-3′ and reverse 5′-TGCCTGAAGCTGAGGAGAC-3′; GAPDH forward 5′-GGAGTCCACTGGCGTCTTCAC-3′ and reverse 5′-GAGGCATTGCTGATGATCTTGAGG-3′.

Statistical analysis

Data analysis was performed using unpaired and one-sample *t*-test (GraphPad Software Inc.). $p<0.05$ was considered statistically significant.

Results

Optimizing miR-122a*-mediated silencing of AAV transgene expression*

Three serotype 8-pseudotyped, single-stranded AAV vectors were developed containing our previously described liver-specific promoter (HLP) in preference to constitutively active promoters.[21] This is because our preliminary studies with AAV8 vectors showed significant transgene expression in nonhepatic tissues in addition to high level of expression in the liver with constructs containing constitutively active promoters, which increases the risk of off-target toxicity (Supplementary Fig. S2). Included in these HLP-driven vectors was the firefly luciferase reporter gene containing either two, four, or eight tandem repeats of *miR-122a*-binding sequences in the 3′-UTR (AAV-HLP-Luc-122aT2, AAV-HLP-Luc-122aT4, and AAV-HLP-Luc-122aT8, respectively) (Fig. 1a). The control vector (AAV-HLP-Luc) did not contain any *miR-122a*-binding sites. Since AAV transgene expression peaks at 3–5 days and primary human hepatocytes can only be maintained in culture for $\sim$3 days, the initial assessment of these vectors was performed in two cell lines with different levels of *miR-122a* expression.[22] The SK-Hep-1 is an immortalized, human endothelial cell line derived from the ascitic fluid of a patient with adenocarcinoma of the liver, which has been used in numerous studies of liver cancer.[23–25] These cells have undetectable levels of *miR-122a* and therefore serve as an *miR-122a*-negative cell line for the assessment of our AAV vectors. HuH7, a well-differentiated human HCC cell line, has some residual *miR-122a*, although approximately 10-fold lower than normal hepatocytes[26] and was therefore used as an *miR 122a*-positive cell line (Supplementary Fig. S3).

An amount of 1×10^{5} HuH7 or SK-Hep-1 cells were transduced with AAV-HLP-Luc-*miR-122a*T vectors and the AAV-HLP-Luc control vector at a multiplicity of infection (MOI) of 1×10^{5}. Incorporation of *miR-122a*-binding sites downstream of a luciferase gene had no effect on bioluminescence in the SK-Hep-1 (Fig. 1b). However, luminescence in HuH7 cells declined from 91$\pm$9% RLU (percentage of relative luminescence units, normalized to nontransduced [NT] cells) in cells transduced with the ssAAV8-HLP-Luc vector to 17$\pm$3%RLU in ssAAV8-HLP-Luc-122aT2 ($p=0.0012$) and 6$\pm$1%RLU in ssAAV8-HLP-Luc-122aT4 ($p=0.0006$). The difference in luminescence levels between HUH7 cells transduced with ssAAV8-HLP-Luc-122aT4 and ssAAV8-HLP-Luc-122aT8 was not significant ($p=0.60$).

Importantly, overall cell numbers at 72 hr after gene transfer were similar, suggesting that overexpression of *mir-122a*-binding site did not affect the cell proliferation or survival rate (Supplementary Fig. S4).

AAV-mediated luciferase expression was assessed *in vivo* 1 week after tail vein administration of 1×10^{11} vg/mouse into male C57Bl/6 mice. As described before, the liver was preferentially transduced after tail vein administration of ssAAV8-HLP-Luc vector because of the natural liver tropism of AAV8.[9,10,27] Two *miR-122a*-binding sites in the ssAAV8-HLP-Luc-122aT2 vector decreased luciferase expression by 35-fold. Luminescence was almost undetectable in the liver of mice transduced with ssAAV8-HLP-Luc-122aT4 and ssAAV8-HLP-Luc-122aT8 vectors (Fig. 1c). Analysis of the transgene copy number in liver by qPCR assay showed that the average copy number/cell in each cohort of mice was similar (0.70 ± 0.2) and did not account for the difference in transgene expression observed between different vectors.

These data indicated that inclusion of a minimum of four tandem repeats of *miR-122a*-binding sites in the 3′-UTR of the AAV expression cassette allows repression of transgene expression in *miR-122a*-positive HuH7 cells or murine hepatocytes but not in cells lacking *miR-122a*. That this was an *miR-122a*-specific effect was suggested by the fact that similar moderation of expression was not seen with an identical vector containing four *miR-223*-binding sites in place of *miR-122a* target sequences (Fig. 2b).

Preferential transduction of human HCC xenografts in ectopic sites following systemic administration of AAV8 vectors

Although AAV8 has a strong liver tropism, it was unclear if the serotype 8 would be equally efficient at transducing HCC cells in ectopic sites—an important issue in patients with metastases. Therefore, 1×10^{7} HuH-7 or SKNAS (*miR-122a*-negative human neuroblastoma cell line) were each injected in the subcutaneous space of 12 male NOD/SCID mice. Four weeks later, when the tumors in these animals were of equal size, mice bearing each of the tumors types were divided into equal groups that received a single tail vein injection of either ssAAV8-HLP-Luc or ssAAV8-HLP-Luc-122aT4 (1×10^{11} vg/mouse).

A week later, bioluminescence was observed in the liver of both cohorts of tumor-bearing mice following transduction with ssAAV8-HLP-Luc. Additionally, bioluminescence was detected in the HCC xenografts within the subcutaneous space but not the neuroblastoma xenografts (Fig. 1d, left). Quantitative PCR analysis of tissues isolated 6 weeks after gene transfer showed that the liver had the highest proviral copy number, with 6 ± 2 and 10 ± 3 copies/cell in HuH7 and SKNAS xenograft cohorts, respectively. The transgene copy number in HuH7 xenografts was 0.9 ± 0.2 copies/cell, as compared with 0.01 ± 0.003 copies/cell in SKNAS xenografts. Transgene copy number in kidneys of both cohorts was comparable at approximately 0.05 ± 0.004 copies/cells (Supplementary Fig. S5). These results suggest preferential transduction of HuH7 cells at ectopic sites by AAV8 vectors. In separate studies using *miR-122a*-negative SK-Hep-1 cells, luciferase expression was detected only in SK-Hep-1 xenografts following tail vein administration of ssAAV8-HLP-Luc-122aT4 vector with no signal from the normal liver (Fig. 1d, right). This is consistent with downregulation of AAV transgene expression in the *miR-122a*-positive normal murine liver cells but not in the *miR-122a*-negative SK-Hep-1 tumor cell line.

Efficacy of AAV8 vectors encoding the TK suicide gene against human HCC cells in vitro

The *luciferase* sequence was replaced with the *TK* suicide gene to generate AAV-HLP-TK and AAV-HLP-TK-122aT4 (Fig. 2d). An amount of 1×10^{5} HuH7 cells were transduced with ssAAV8-HLP-TK or ssAAV-HLP8-TK-122aT4 at an MOI of 10^{5}. Three days later, cells were exposed to varying concentrations of GCV (0.1–5 μg/ml). Cell survival was assessed after 72 hr of GCV treatment and expressed as a percentage of NT cells. GCV at a concentration of 5 μg/ml was not toxic to NT HuH7 cells. Dose-dependent killing of ssAAV8-HLP-TK-transduced HuH7 cells was observed with only $14\pm3\%$ cell survival following exposure to 5 μg/ml of GCV (Supplementary Fig. S6B). By comparison, the ssAAV8-HLP-TK-122aT4 vector-transduced HuH7 cells appeared to be less sensitive to killing by GCV, as illustrated by the survival of approximately $53\pm4\%$ of cells following 3 days of treatment with 5 μg/ml of the GCV. Western blot analysis demonstrated significantly lower TK expression in the ssAAV8-HLP-TK-122aT4-transduced HuH7 cells when compared with cells transduced with an identical vector lacking the *miR-122a*-binding sequences (Supplementary Fig. S6B). This is most likely because of a reduction in translation of the proviral mRNA following binding of *miR-122a* to its target sequences within the AAV expression cassette. In contrast, in *miR-122a*-negative cell lines, SK-Hep-1 and BNL-1h (a murine embryonic liver cell; Supplementary Fig. S3) showed no difference in GCV-mediated killing following transduction with either ssAAV8-HLP-TK or ssAAV-HLP8-TK-122aT4 (Supplementary Fig. S6C and D).

Radionucleotide imaging confirms selective gene transfer to HCC xenografts resulting in potent antitumor activity following systemic administration of ssAAV8-HLP-TK-122aT4

SK-Hep-1 cells were injected subcutaneously in the flank of 6–8-week-old male NOD/SCID mice, and 3 weeks later the animals were divided into 3 groups ($n=5$/cohort), each receiving 1×10^{11} vg/mouse of ssAAV8-HLP-TK, ssAAV8-HLP-TK-122aT4, or an equivalent volume of PBS (control group) via the tail vein. The radiolabeled TK substrate [^{125}I]FIAU was injected 4 days after vector administration followed by SPECT-CT imaging at regular intervals. As expected, there was nonspecific radiochemical uptake in the stomach and gallbladder in all groups of animals, including PBS controls. A high level of ^{125}I radioactivity was observed within 5 min of administration of substrate in the liver of animals transduced with ssAAV8-HLP-TK, consistent with high TK levels within hepatocytes (Fig. 2b and Supplementary Figs S7 and S8).

This radioactivity signal gradually declined over a period of 1 hr. In contrast, the ^{125}I radioactivity signal in the liver of mice transduced with ssAAV8-HLP-TK-122aT4 vector was low or undetectable at all time points and comparable to

levels observed in control animals that received PBS. This suggests that expression of TK is suppressed in normal murine hepatocytes by the interaction of high levels of endogenous *miR-122a* with the complementary binding sequences within the HLP-TK-122aT4 transgene (Fig. 2a and c, and Supplementary Figs S7 and S8). A relatively strong radioactive signal was observed in the SK-Hep-1 xenografts 3 hr after [^{125}I]FIAU injection in the cohort transduced with ssAAV8-HLP-TK-122aT4, suggesting that despite the lack of signal in the liver of these animals the *TK* transgene was expressed at significant levels in the *miR-122a*-negative tumor cells (Fig. 2c and Supplementary Figs S7 and S8). Signal in the SK-Hep-1 xenografts in the ssAAV8-HLP-TK mice remained negative at all time points presumably because of the rapid breakdown of [^{125}I]FIAU by the high levels of TK in the liver of these animals (Fig. 2b).

Consistent with the imaging data, tail vein administration of ssAAV8-HLP-TK-122aT4 followed by 12 days of GCV (25 mg/kg) treatment resulted in reduction of growth of SK-Hep1 tumors in the subcutaneous space (from 100 ± 10 pre-GCV to $129 \pm 49\,mm^3$ post-GCV) (Fig. 3a). TK expression in the xenografts was confirmed by immunohistochemistry (Fig. 3b), which showed diffuse staining, consistent with efficient transduction of >70% of tumor cells in the subcutaneous implant following systemic administration of ssAAV8-HLP-TK-122aT4. In mice transduced with ssAAV8-HLP-TK-122aT4 or ssAAV8-HLP-TK but treated with PBS as opposed to GCV, the tumor size increased approximately threefold from 138 ± 22 to $436 \pm 92\,mm^3$ and 103 ± 19 to $359 \pm 78\,mm^3$ over a 12-day period, respectively (Fig. 3a). GCV treatment in mice transduced with ssAAV8-HLP-TK resulted in death of all animals within 8 days associated with a >100-fold (2900 ± 1197 IU/I) increase in ALT levels over that observed in naïve untransduced mice treated with GCV alone (23 ± 3 IU/I). This is consistent with killing of hepatocytes expressing TK (Fig. 3b) as a result of conversion of GCV to phosphorylated GCV, which inhibits replication of DNA. ALT values in mice transduced with ssAAV8-HLP-TK-122aT4 vector were marginally higher at day 8 of GCV when compared with naïve untransduced controls at

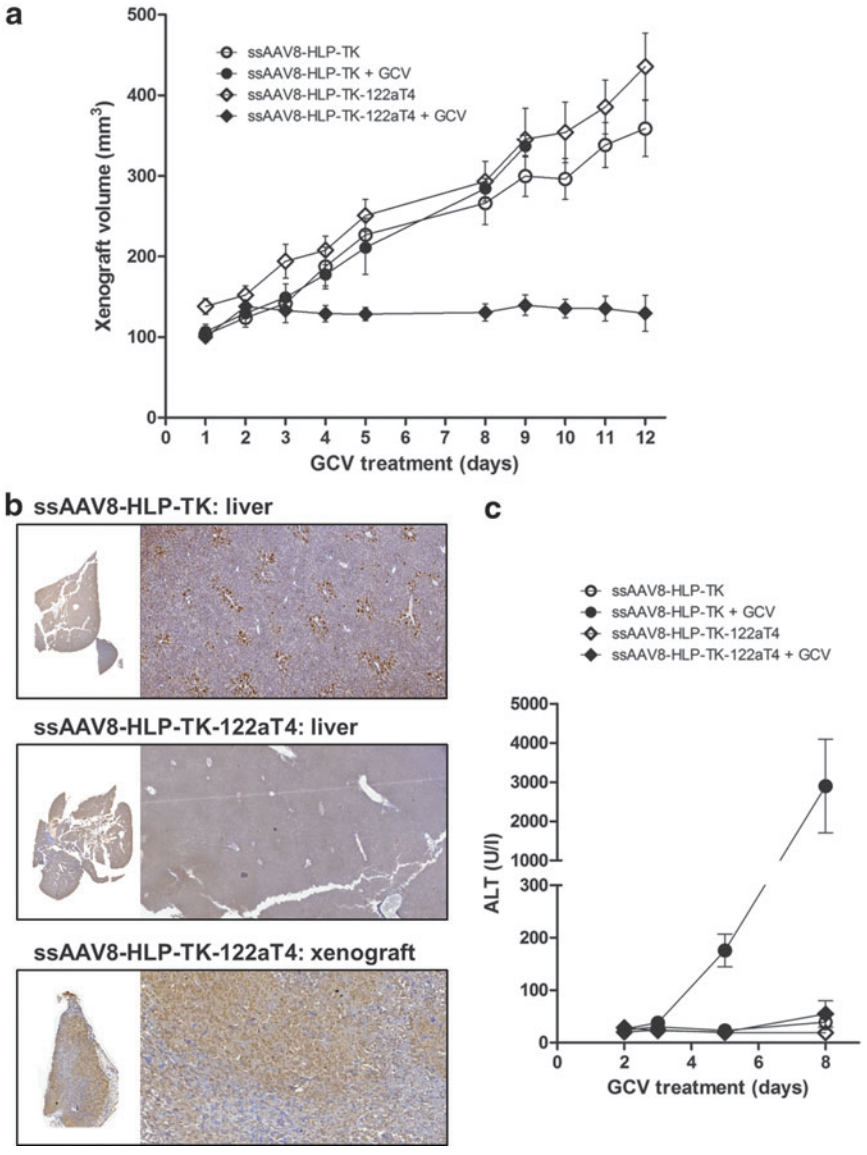

FIG. 3. Efficacy of ssAAV8-HLP-TK-122aT4/GCV in a xenografts model of human liver cancer. **(a)** SK-Hep-1 xenograft volumes in NOD/SCID mice after transduced with ssAAV8-HLP-TK and ssAAV8-HLP-TK-122aT4 vectors followed GCV (or PBS) treatment ($n = 6$/cohort). **(b)** Representative IHC for TK detection in the liver and xenograft. **(c)** ALT values. GCV, ganciclovir; PBS, phosphate buffered saline.

55 ± 25 IU/I, suggesting that liver toxicity was minimized by the inclusion of *miR-122a*-binding sequences within the transgene cassette (Fig. 3c and Supplementary Table S1).

Systemic administration of ssAAV8-HLP-TK-122aT4 is effective in a syngeneic metastatic murine HCC model

Tumor selectivity of the ssAAV8-HLP-TK-122aT4 vector was further assessed in a challenging syngeneic model of HCC in which BNL-1h *miR-122a*-negative murine cells, subclones of the BALB/c chemically transformed BNL-1ME A.7R.1 hepatic cell line, were injected into spleens of BALB/c mice to establish tumor in disseminated sites. Three weeks later the mice received a bolus tail vein injection of 1×10^{11} vg/mouse of ssAAV8-HLP-TK-122aT4. Five days after gene transfer, half of the animals received ($n = 6$) GCV IP at a dose of 25 mg/kg/day for 5 days. The remaining mice (control) received PBS. Five days after GCV treatment the mice were sacrificed and the number of hepatic and extra-hepatic lesions was counted in a blinded manner.

The combination of ssAAV8-HLP-TK-122aT4/GCV resulted in a significant reduction in the number of grossly visible lesions in the liver (Fig. 4) (mean = 0.8; range 1–4) compared with control mice (mean = 6; range 4–9). This difference was highly significant ($p = 0.004$). Similarly, there was a reduction in the number and size of tumor nodules in the spleen, as well as tumors outside of the hepato-splenic tissues, in ssAAV8-HLP-TK-122aT4/GCV-treated mice (Table 1). Consistent with these macroscopic findings, there was little or no evidence of tumor on histological evaluation of sections of liver derived from mice treated with GCV (Fig. 4).

Table 1. Total Number of Tumor Nodules in the Two Cohorts of ssAAV8-HLP-TK-122aT4 Mice After GCV Treatment (PBS as Control)

	Organs				
Treatment	*Liver*	*Spleen*	*Mesentery*	*Gut*	*Kidney*
PBS	36	21	9	1	1
GCV	5	4	1	0	1

GCV, ganciclovir; PBS, phosphate buffered saline.

BALB/c mice were implanted with the syngeneic BNL-1h cells. Three weeks later, animals received a tail vein injection of ssAAV8-HLP-TK-122aT4 (1×10^{11} vg/mouse). Five days later, animals received GCV (PBS as control) intraperitoneally (25 mg/kg/day) for 5 days. Mice were killed at day 31 of the study and the number metastases in the major organs was counted.

Discussion

In this study we aimed to extend our clinical success with AAV8 vectors in hemophilia B patients to develop a new selectively targeting gene therapy approach for advanced HCC, a leading cause of cancer deaths worldwide. Recent insights into the pathogenesis of HCC were exploited to develop a systemically deliverable gene transfer strategy that facilitated HCC-specific expression of transgenes, not only within primary tumor, but also in associated metastases. Specifically, our approach combined three aspects of vector design: a vector serotype with preferential liver tropism, a liver-specific promoter, and posttranscriptional regulatory mechanism inhibiting gene expression in healthy liver tissue. Bioluminescence and clinically translatable noninvasive molecular-genetic imaging using TK and radiolabeled TK substrate analog[28] showed that AAV8 is highly efficient at transducing HCC in the liver and heterotopic sites following systemic administration.

Our comparative studies demonstrated that HCC xenografts in the subcutaneous space had a higher transgene copy number than neighboring tissues, or other tumors such as neuroblastoma, following systemic administration of AAV8 vector. This ability to ''home'' to tumors in heterotopic sites after systemic administration of AAV8 raised the possibility of targeting disseminated tumor, thus offering an advantage over other cancer gene therapy approaches.[29] This aspect was exploited in murine models to show a reduction in tumor growth in *miR-122a*-negative human SK-Hep1 xenografts implanted into the subcutaneous space. Additionally, efficacy of our tumor-targeting approach was established in a syngeneic mouse model of metastatic HCC following tail

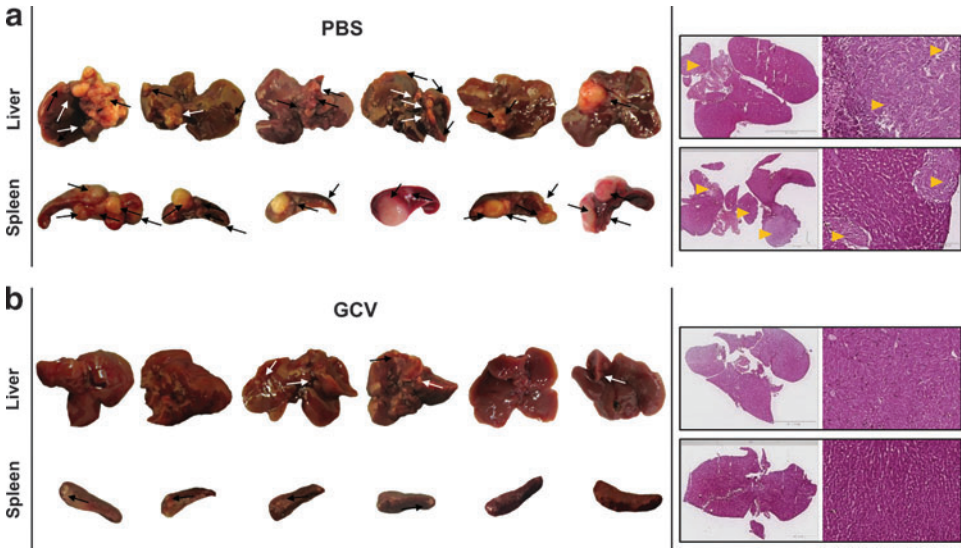

FIG. 4. Efficacy of ssAAV8-HLP-TK-122aT4/GCV in a metastatic syngeneic model of hepatocellular carcinoma (HCC). Balb/C mice were injected intrasplenically with BNL-1h cells followed 3 weeks later by a single iv injection of ssAAV8-HLP-TK-122aT4 and treated 5 days after GCV (PBS as control) ($n = 6$/cohort). Liver and spleen from **(a)** control and **(b)** GCV mice. Arrows indicate sites of tumors. Representative H&E staining of two liver showing healthy tissue and tumors (yellow arrows).

vein administration of ssAAV8-HLP-TK-122aT4 without toxicity. Thus, the hitherto unknown ability of AAV8 vectors to preferential transduce tissue of liver origin provides a unique opportunity to target disseminated disease in patients with advanced stage HCC. While the mechanism behind this remains unclear, we speculate that the upregulation of laminin receptor on HCC cell may play a role.[30]

A critical aspect of our strategy was the ability to minimize ''off-target toxicity'' of the therapeutic gene, which was achieved by exploiting the differential expression of *miR-122a*, which is abundantly expressed in normal hepatocytes but almost undetectable in HCCs. Specifically, incorporating a minimum of four tandem repeats of the *miR-122a*-binding sequence in the 3′ end of an AAV8 expression cassette significantly downregulated *TK* suicide gene expression in normal liver tissue, presumably secondary to proviral mRNA degradation following the binding of *miR-122a* with its complementary target sequence in the AAV expression cassette. This reduced hepatocellular toxicity without compromising antitumor potency of ssAAV8-HLP-TK-122aT4 in *miR-122a*-negative tumor cell lines. Several studies demonstrate absence or very low levels of *miR-122a* in biopsy samples from over 70% of patients with advanced HCC. These individuals generally have aggressive poorly differentiated HCC and have a particularly poor prognosis even with radical therapy involving sorafenib in combination with chemo- and/or radiotherapy.[12,31–33]

These patients need new treatment options and would, therefore, be highly suited to our HCC targeted gene therapy approach. Serum levels of circulating *miR-122a* have been proposed as a new marker for prediction of survival of patients with liver cirrhosis.[34] However, *miR-122a* is not downregulated in cirrhosis to the same extent as in HCC, thus enabling differential targeting of HCC tumor cells with our AAV expression cassette.[35] The potential benefits of AAV-based gene therapy for HCC will need to be balanced with the potential risk of AAV-mediated oncogenesis. This risk is relatively low as AAV proviral DNA integrates in the host genome at a much lower frequency than is the case with integrating vectors such as those based on oncoretroviruses.[36–41] This is consistent with the fact that wild-type AAV infection in humans, though common, is not associated with oncogenesis. However, an increased incidence of HCC has been reported in the mucopolysaccharidoses type VII mouse model following perinatal gene transfer of AAV.[42] Studies on other murine models have failed to recapitulate this finding; in addition, tumors have not been observed in larger animal models or over 500 patients who have received AAV vectors.[10,43] Therefore, the available data do not preclude the use of AAV vectors for gene therapy of HCC.

Differential expression of *miR-122a* in normal liver tissue and advanced HCC has been exploited previously for the purpose of constructing oncolytic viruses that replicate in HCC but not in normal hepatocytes, thus reducing off target toxicity.[44,45] Similarly, Xiao and colleagues demonstrated that the insertion of *miR-122a* target sequences into the 3′-UTR of AAV vectors encoding marker genes or secretable proteins resulted in reduction in transgene mRNA and protein levels by over 50-fold in the liver.[46] However, ours is the first study to systematically examine the use of miRNA-based posttranscriptional regulatory mechanisms in the context of AAV vectors to deliver therapeutic gene to HCC in animal models.

Collectively, the ability to administer vector systemically to target disseminated malignancy, together with HCC-restricted expression of the transgene, provides significant advantages over other gene therapy approaches involving adenovirus or oncolytic poxvirus that have been tested.[29,47,48] In some of these studies, the vector had to be administered intratumorally to minimize ''off target'' toxicity as well as overcome antiviral immunity arising from previous exposure to wild-type virus or immunization.[48] Consequently, the long-term efficacy was impaired, as only tumors easily accessed by intervention radiological techniques could be treated.

In summary, the synergistic effect of the three elements described, natural tropism of a viral vector, promoter specificity, and posttranslational regulation, provides a tumor-selective strategy for targeting advanced HCC. This approach provides a new opportunity for delivering a variety of therapeutic genes to HCC without concerns of significant toxicity to normal hepatocytes.

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Author Disclosure Statement

A.M.D. and A.C.N. are listed as inventors in a patent filing for HLP promoter. All other coauthors have no conflicts of interest to disclose.

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