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Development of an Optimized Promoter System for Exosomal and Naked AAV Vector-Based Suicide Gene Therapy in Hepatocellular Carcinoma

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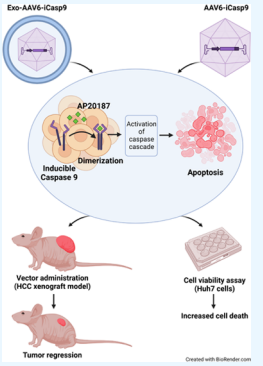
ABSTRACT: Suicide gene therapy is a promising strategy for the potential treatment of hepatocellular carcinoma (HCC). However, the lack of high transduction efficiency and targeted vectors in delivering the suicide genes to only the HCC cells is a major impediment. In the present study, we utilized an adeno-associated virus serotype 6 (AAV6) and its exosomal counterpart (exo-AAV) comprising of an inducible Caspase 9 (*iCasp9*) gene under the control of different promoter systems for targeting HCC cells. We employed a ubiquitous cytomegalovirus immediate early enhancer/chicken β actin promoter (CAG), a liver-specific promoter (LP1), and a baculoviral IAP repeat-containing protein 5 (BIRC5) promoter for liver and cancer cell-specific expression of *iCasp9*, respectively. We further evaluated these vectors in Huh7 cells for their ability to kill the target cells. BIRC5 and LP1 promoter-driven iCasp9 vectors demonstrated superior cytotoxicity when compared to CAG promoter-driven iCasp9 vectors. Further validation in a murine model of HCC demonstrated that the LP1-iCasp9 or Birc5-iCasp9-based AAV6 vectors contributed to tumor regression (∼2 fold) as effectively as the AAV6-CAG-iCasp9 vectors (∼1.9 fold). Similarly, exo-AAV6 vectors showed ∼2.1 to 2.8 fold superior *in vivo* tumor regression when compared to mock-treated animals. Our study has developed two novel promoters (LP1 or BIRC5) whose efficacy is comparable to a strong ubiquitous promoter in both AAV and exo-AAV systems. This expands the toolkit of AAV vectors for safe and effective treatment of HCC.



INTRODUCTION

Hepatocellular carcinoma (HCC) ranks as the third most frequent cause of mortality in cancer patients.[1] Surgery, chemotherapy, and radiotherapy are still the standard of care for HCC treatment.[2−4] However, aggressive characteristics of the disease, such as resistance to chemo- or radiotherapy,[5,6] and a high incidence of metastasis in HCC[7,8] lead to a higher recurrence rate even after tumor removal.[9,10] Thus, ideal therapeutic strategies are still being explored. Gene therapy has rapidly evolved as a viable strategy for the potential treatment of cancer by employing various vectors.[11,12] Adeno-associated virus (AAV) has emerged as the vector of choice for gene transfer among these vectors due to its beneficial characteristics, including wide tissue tropism, the capacity to infect both dividing and nondividing cells, low immune response, and long-term transgene expression in target cells.[13,14] However, some challenges, such as the need for a high vector dose for effective therapy and high production costs of the vectors, remain. Therefore, strategies that can increase the yield or simplify the production process are warranted. In this context, the exosome-associated AAV vectors (exo-AAVs) are reported to be therapeutically very efficient during ocular gene transfer[15] and in HCC.[16] They are also known to be resistant to neutralizing antibodies in comparison to conventionally purified AAV vectors[17,18] and thus could have significant therapeutic potential.

Several studies have utilized AAV vectors containing suicide genes for cancer therapy.[12,19−21] A nonimmunogenic *iCasp9* system has also been explored for suicide gene therapy of cancers, including HCC.[22] This analogue of Caspase 9 is derived from the mammalian Caspase 9 fused to a human FK506 binding protein (FKBP), allowing conditional dimerization to AP20187, a small, bioinert molecule.[22] While we have previously found that the AAV-iCasp9 system is effective in murine models of HCC[16,19,22] however, concerns related to off-target effects that could result from using a ubiquitous promoter such as cytomegalovirus (CMV) immediate early enhancer and chicken β actin promoter (CAG) driving the suicide gene remain. Thus, the development and directed expression of the *iCasp9* suicide gene, specifically within the HCC cells, is desirable for its clinical translation. We utilized the AAV6 serotype to improve phenotypic rescue in HCC as it

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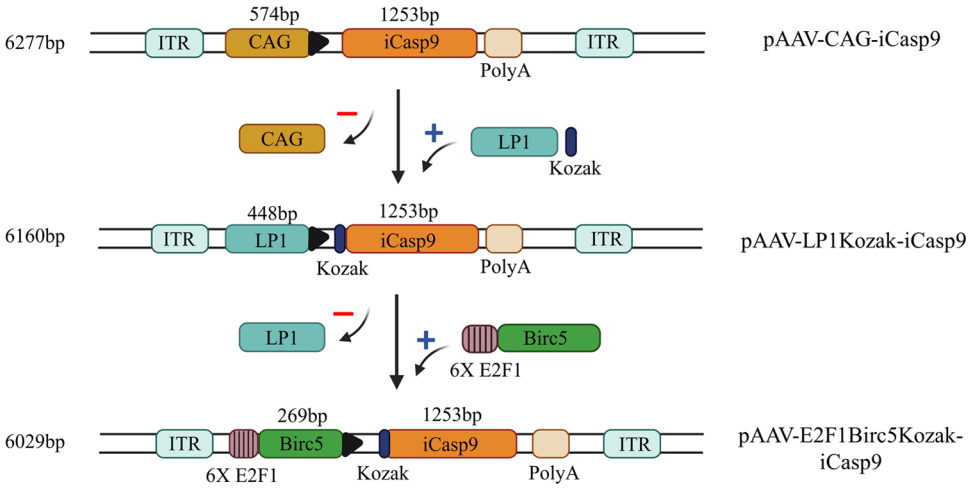

Figure 1. Schematic representation of the cloning strategy for the development of targeted vectors. The CAG promoter from the donor plasmid (*pAAV-CAG-iCasp9*[22]) was replaced with an LP1 promoter. A consensus Kozak sequence (GCCGCCACC) was added before the iCasp9 transgene to generate the pAAV-LP1Kozak-iCasp9 construct. For the second plasmid, the LP1 promoter was replaced by six repeats of the E2F1 binding site (6X E2F1) and a BIRC5 promoter. Cloning resulted in two plasmids, namely, *pAAV-LP1Kozak-iCasp9* and *pAAV-E2F1Birc5Kozak-iCasp9*.

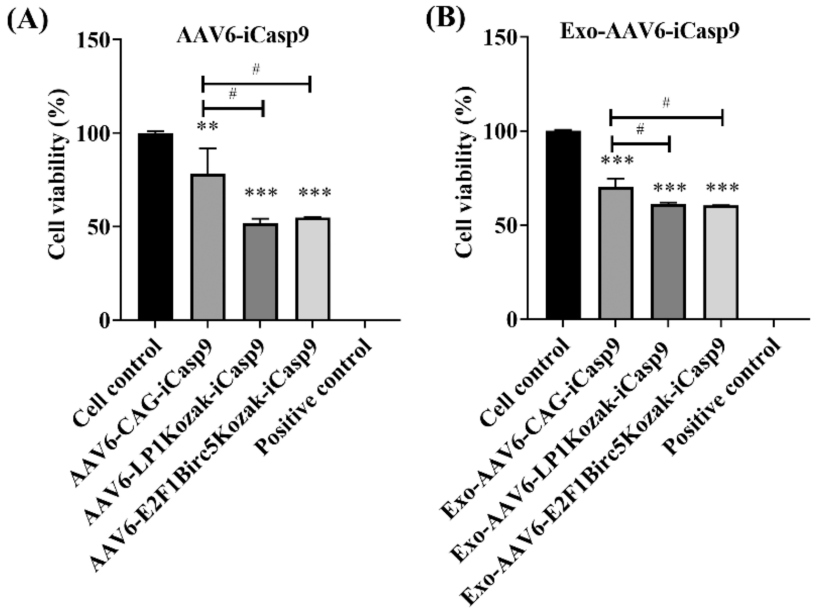

Figure 2. Cytotoxic effect of targeted AAV-iCasp9 and exo-AAV6-iCasp9 vectors ***in vitro***. The viability of Huh7 cells 72 h after transduction was determined using an ATP assay. Data are represented in comparison to mock-treated cells. Cell viability graph for (A) AAV-iCasp9 vectors and (B) exo-AAV6-iCasp9 vectors. Cell control: mock-treated; positive control: 0.1% Triton X-100. Data are presented as mean ± SD (for each condition, $n = 3$ replicates, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ in comparison to cell control, $\#p \leq 0.05$ in comparison to AAV6-CAG-iCasp9 or Exo-AAV6-CAG-iCasp9).

has been reported to target liver cells efficiently, including Huh7 cells.[23,24]

One of the most practical strategies to direct and limit the expression of the suicide gene product to a specific tissue, such as the liver, is to engineer the regulatory elements in the transgene-containing vectors. Previous studies have highlighted the potential of the LP1 promoter (consisting of core liver-specific elements from the human apolipoprotein hepatic control region) for liver-directed human factor IX expression.[25,26] This LP1 promoter has so far not been tested for delivering a suicide gene in the context of HCC. Similarly, baculoviral IAP repeat-containing 5 (*BIRC5*), also known as "Survivin", is one of the major drivers of the development and progression of multiple cancers, including HCC.[27,28] The *BIRC5* gene promoter is highly tumor-specific,[29,30] thus presenting the possibility of its inclusion as a promoter driving the suicide gene. Importantly, the functional promoter region of the *BIRC5* gene spans 1456 bp,[31] but a shorter fragment of 269 bp core promoter is reported to have necessary promoter activity and specificity to drive target gene expression in cancer cells and not in healthy noncancerous cells.[32] The inclusion of other cancer-specific regulatory elements in the transgene-containing vectors, such as cancer-specific transcription factors, may also be beneficial. One such element is E2F1, a transcription activator that recognizes and interacts with DNA through dimerization proteins via the E2 recognition

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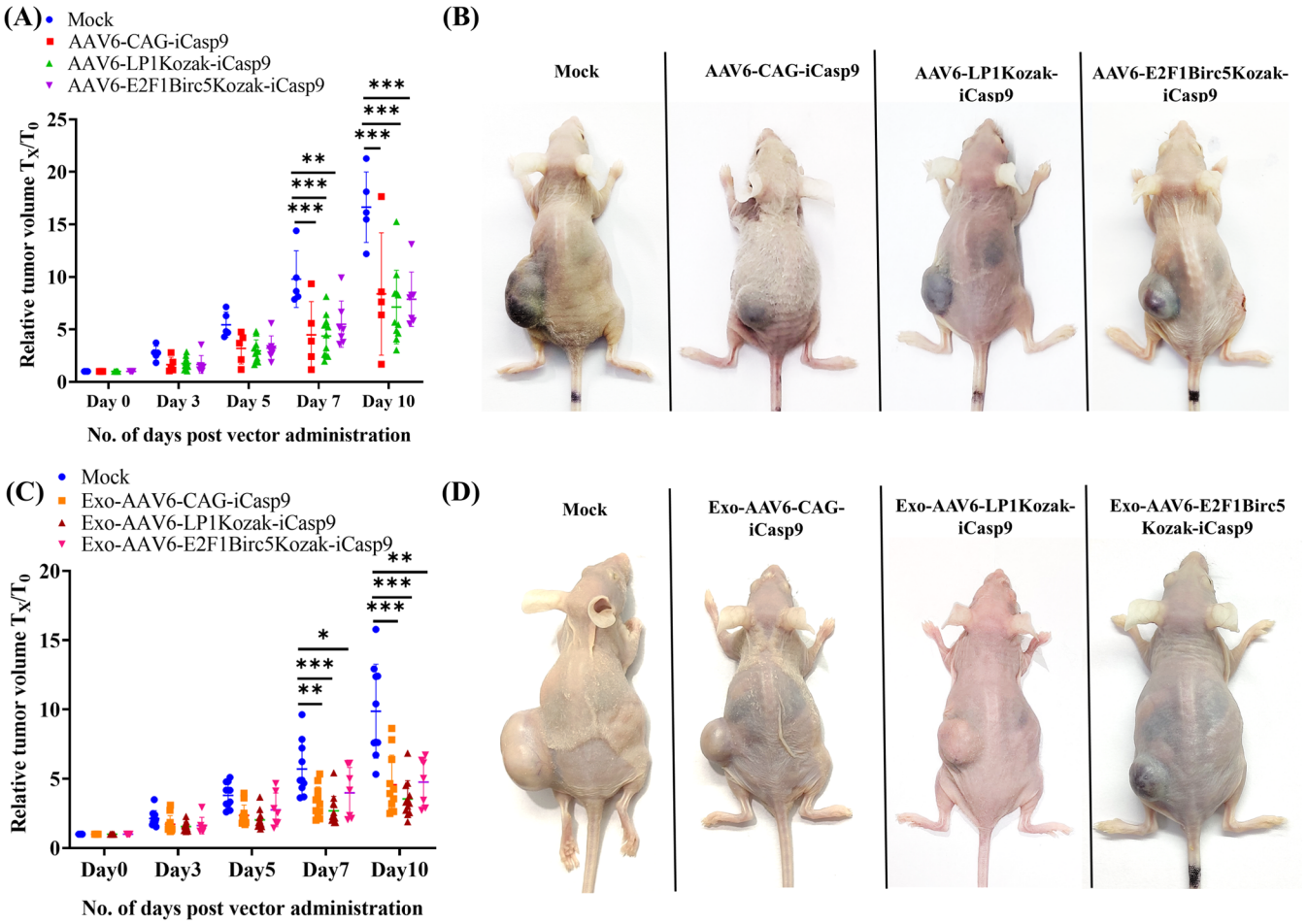

Figure 3. Therapeutic effect of AAV6-iCasp9 vectors and their exosome counterparts in an HCC xenograft model. Graphical representation of tumor regression *in vivo* after suicide gene therapy with naked AAV6-iCasp9 vectors (A) and Exo-AAV6-iCasp9 vectors (C). Representative images of test animals from different experimental groups 10 days post vector administration with AAV6-iCasp9 vectors (B) and Exo-AAV6-iCasp9 vectors (D). The treated groups AAV6-CAG-iCasp9 (n = 5), AAV6-LP1Kozak-iCasp9 (n = 11), and AAV6-E2F1Birc5Kozak-iCasp9 (n = 7) demonstrated significant tumor regression in comparison to mock-injected animals (n = 5). A similar pattern of tumor regression was also found in animals that received Exo-AAV6-CAG-iCasp9 (n = 11), Exo-AAV6-LP1Kozak-iCasp9 (n = 12), and Exo-AAV6-E2F1Birc5Kozak-iCasp9 (n = 7) vectors when compared to mock-injected animals (n = 10). Two-way ANOVA was performed for statistical analysis. Animals that died or had necrotic tumors were excluded from the analysis. Data are presented as mean ± SD, $^*p \leq 0.05$; $^{**}p \leq 0.01$; and $^{***}p \leq 0.001$ in comparison to mock.

site, 5′-TTTC[CG]CGC-3′.[33,34] E2F1 is also a cancer biomarker and has been shown to be associated with a number of cancers, including HCC.[35,36] Hence, together with a cancer-specific gene (*BIRC5*) promoter, it can help in fine-tuning the suicide gene expression within the cancer cells.

In the present study, we evaluated these targeted promoter systems for their efficacy, with the aim of increasing the therapeutic index and limiting the off-target toxicity of the naked AAV and exo-AAV vectors both *in vitro* and *in vivo*.

RESULTS

Development of Liver-Specific LP1 and Cancer-Specific BIRC5 Promoter-Containing iCasp9 Vectors. For the development of promoter-optimized AAV-iCasp9 vectors, a hybrid CAG promoter composed of the CMV immediate early enhancer and chicken β actin promoter from the *pAAV-CAG-iCasp9* (donor plasmid) was used, and the promoter was replaced with either an LP1 (along with Kozak) or BIRC5 promoter (along with E2F1 binding site). The developed vectors were validated by Sanger sequencing (GenScript, New Jersey). A schematic of the linear plasmid map is presented in Figure 1. The size of the plasmid obtained after cloning was ∼6.1 and ∼6.0 kb for *pAAV-LP1Kozak-iCasp9* and *pAAV-E2F1Birc5Kozak-iCasp9*, respectively.

AAV6-iCasp9 Vectors and Exo-AAV6-iCasp9 Exhibit Efficient Cytotoxicity In Vitro. We further corroborated the novel constructs in human hepatocellular carcinoma cell line (Huh7) after packaging AAV6-CAG-iCasp9, AAV6-LP1Kozak-iCasp9, and AAV6-E2F1Birc5Kozak-iCasp9. Our data demonstrates that all three vectors caused significant cell death in Huh7 cells. Cells infected with AAV6-CAG-iCasp9 vectors showed 78% cell viability, while those infected with AAV6-E2F1Birc5Kozak-iCasp9 (54%) and AAV6-LP1Kozak-iCasp9 (51%) vectors had reduced cell viability as well (Figure 2A). The survival of Huh7 cells between the AAV6-E2F1Birc5Kozak-iCasp9 and AAV6-LP1Kozak-iCasp9 vector-infected groups was comparable. When treated with Exo-AAV6-CAG-iCasp9, Exo-AAV6-LP1Kozak-iCasp9, and Exo-AAV6-E2F1Birc5Kozak-iCasp9 vectors, the Huh7 cell viability decreased to 70.31%, 61.17%, and 60.46%, respectively (Figure 2B). In both AAV6-iCasp9 and exo-AAV6-iCasp9 versions, AAV6-LP1Kozak-iCasp9 and AAV6-E2F1Birc5Kozak-iCasp9

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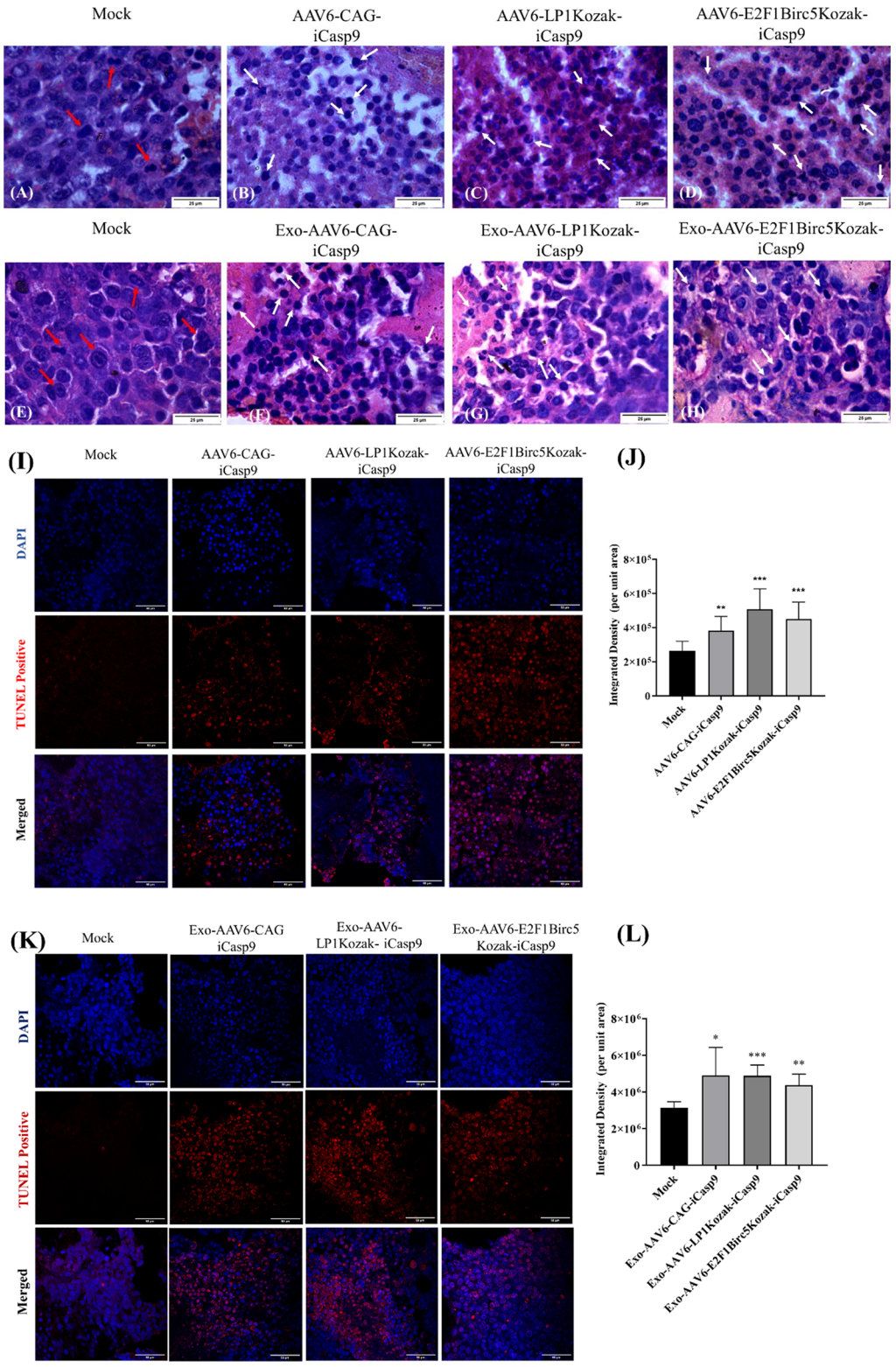

Figure 4. Morphological and molecular characterization of apoptotic cells in tumor tissue. Hematoxylin and eosin staining was performed on 10 μm sections of a paraffin-embedded tumor tissue, and microscopic examination was conducted using a Leica DMi8 (Leica microsystems, Wetzlar, Germany). (A–H) 100× images, Scale bar: 25 μm. Red arrows indicate mitotic and proliferating cells and white arrows indicate pyknotic, dense, or fragmented nuclei. TUNEL staining of tumor sections was performed, and confocal imaging was done (I, K) using an LSM780NLO Carl Zeiss GmbH (Carl Zeiss AG, Jena, Germany), 63× images, Scale bar: 50 μm. Quantification of TUNEL-positive cells in tumor sections using ImageJ software (J, L) is shown. Data are presented as mean ± SD, *$p \leq 0.05$; **$p \leq 0.01$; and ***$p \leq 0.001$ in comparison to mock.

exhibited a significant ($p \leq 0.05$) decrease in cell survival when compared to AAV6-CAG-iCasp9 vector-infected cells.

Tumor Growth Is Substantially Inhibited with Targeted Suicide Gene Vectors In Vivo. To confirm the therapeutic utility of AAV6-iCasp9 vectors and their exosomal

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versions, we tested them in a xenotransplantation model of HCC, as described earlier.[22] Briefly, athymic nude mice were transplanted with ∼5 million Huh7 cells in a mixture of 25% Matrigel to develop ectopic tumors of 100−150 mm^3 in size. These animals were either mock (PBS) administered or administered with 1×10^{10} vg of AAV6 vectors carrying inducible Caspase 9 intratumorally. Our data provided in Figure 3 show a significant regression in the tumor volume 7 days after treatment in all 3 treated groups: AAV6-CAG-iCasp9 (∼[approximately] 2.19 fold; $p \leq 0.001$), AAV6-LP1Kozak-iCasp9 (∼2.24 fold; $p \leq 0.001$), and AAV6-E2F1Birc5Kozak-iCasp9 (∼1.78 fold; $p \leq 0.01$) vectors when compared to animals that were mock-treated (Figure 3A,B). At day 10, a similar level of phenotypic rescue was observed in the treated groups AAV6-CAG-iCasp9 (∼1.99 fold; $p \leq 0.001$), AAV6-LP1Kozak-iCasp9 (∼2.33 fold; $p \leq 0.001$), and AAV6-E2F1Birc5Kozak-iCasp9 (∼2.11 fold; $p \leq 0.001$) vectors when compared to mock-treated animals. Similar data were obtained with Exo-AAV6-CAG-iCasp9 (∼1.96 fold; $p \leq 0.01$ at day 7 and ∼2.30 fold; $p \leq 0.001$ at day 10), Exo-AAV6-LP1Kozak-iCasp9 (∼2.27 fold; $p \leq 0.001$ at day 7 and ∼2.86 fold; $p \leq 0.001$ at day 10), and Exo-AAV6-E2F1Birc5Kozak-iCasp9 (∼1.60 fold; $p \leq 0.05$ at day 7 and ∼2.13 fold; $p \leq 0.01$ at day 10) vectors when compared to animals that were mock-treated (Figure 3C,D). The *iCasp9* transgene in combination with AP20187 resulted in tumor regression, as we have recently reported in another study that the vector alone lacks the ability to bring about cell death.[37] These data highlight that the LP1 or E2F1-Birc5 promoter is similar in efficacy in comparison to that of the robust CAG promoter system.

Detection of Apoptosis in Tumor Tissues after Suicide Gene Transfer. Treated animals were humanely euthanized, and tumors were isolated 10 days after gene therapy. Hematoxylin and eosin staining of tumor tissue sections was performed for morphological evaluation. The mock-treated tissue samples exhibited a higher number of mitotically active and large proliferating cells (Figure 4A,E). Histological analysis of the vector-treated groups depicted apoptotic cells, which presented with well-defined features, including cell shrinkage, condensation, and deep eosinophilia of the cytoplasm along with a pyknotic, round to irregular nucleus, as described earlier.[38] The AAV6-iCasp9- and exo-AAV6-iCasp9-treated groups demonstrated a high number of apoptotic cells with pyknotic nuclei (Figure 4B−D,F−H).

To determine the impact of *iCasp9*-based gene therapy at the molecular level, a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed. The imaging data from the tumor tissue from all three treated groups AAV6-CAG-iCasp9, AAV6-LP1Kozak-iCasp9, and AAV6-E2F1Birc5Kozak-iCasp9 and respective exosome counterparts showed a higher number of TUNEL-positive cells (red). This signifies massive cell death in the treated groups, as compared to the control group (Figure 4I,K). We further quantified the fluorescence signal from the TUNEL-positive cells, which revealed that all three treated groups AAV6-CAG-iCasp9, AAV6-LP1Kozak-iCasp9, and AAV6-E2F1Birc5Kozak-iCasp9 and the corresponding exo-AAVs had significantly higher integrated density per unit area of TUNEL positivity when compared to the mock group (Figure 4J,L).

DISCUSSION

The preferred course of treatment for HCC patients with early stage illness and poor liver function is surgical resection. However, despite recent advancements, hepatectomy remains linked to an increased rate of morbidity and mortality.[39,40] To preserve liver function, it is crucial to develop a strategy that exclusively targets HCC without harming normal cells. In our previous study, we utilized an AAV serotype 2-based vector to test the potency of *iCasp9* to induce cytotoxicity, where the transgene was under the influence of a strong ubiquitous promoter (CAG).[22] In the present study, we reasoned that the use of a liver-specific (LP1) or cancer-specific promoter (BIRC5), with additional molecular features such as Kozak and cancer-specific transcription factors, could be a better approach than using a ubiquitous promoter to limit off-target toxicity.

AAV6 vectors have been used in cancer therapies to deliver transgenes for therapeutic effect. In an earlier study, we utilized AAV6 to deliver a CAG promoter-driven *iCasp9* transgene for the therapeutic rescue of HCC and observed significant tumor regression in a mouse xenograft model.[22] By employing LP1 and BIRC5, we made an effort to make iCasp9 expression more targetable. We additionally incorporated a consensus Kozak motif for improved protein production into the vectors. Most eukaryotic cells use the Kozak sequence as a translation initiation site, and it has been observed that mutations in this area can alter the translation status and cause disease.[41] A Kozak consensus sequence has therefore been included in our study to boost translation of the coding region. This Kozak sequence has already been identified as a consensus sequence in 699 vertebrate mRNAs for initiation of translation.[42]

Specific targeting of liver/cancer cells is crucial in the treatment of HCC. Although the LP1 promoter has not been explored in this context previously, it has been used to drive coagulation factor IX expression to the liver[25,26] in the case of hemophilia B gene therapy. BIRC5 has been widely studied, and a recent study demonstrated that the herpes simplex virus thymidine kinase (HSV-TK) suicide gene system, when controlled via a survivin promoter, selectively inhibited HCC cell proliferation in a xenograft mice model by enhancing apoptosis, thereby increasing survival.[43] We also incorporated an E2F1 transcription factor binding site to elevate *iCasp9* expression. E2F1 is one of the members of the E2F family that often operates as a transcriptional activator for genes involved in cell cycle progression and nucleotide synthesis. The E2F1 promoter within an adenoviral vector has been used to demonstrate that a transgene driven by this promoter can cause tumor-selective gene expression and further eradicate established gliomas *in vivo*.[44] Taking a cue from these studies, we demonstrated the therapeutic effect of the *iCasp9* system when delivered with tissue/cancer-specific promoters using naked AAV6 vectors and their exosomal versions. The addition of the exosomal AAV fraction, which is otherwise discarded during routine recombinant vector packaging, is likely to reduce the cost associated with suicide gene therapy. Our results also demonstrate that the efficacy of the tissue-specific (LP1) and cancer-specific (BIRC5) promoter is similar to the highly robust CAG promoter system either in AAV or exo-AAV systems. Several studies have shown superior transgene expression with a strong CAG promoter system over liver-directed or cancer-specific promoters.[45,46] Although tissue-specific promoters are generally considered weak in nature when compared to ubiquitous promoters,[47] we observed that

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the phenotypic outcome with the LP1 or BIRC5 promoters employed here was similar to a strong CAG ubiquitous promoter *in vivo*. Similar observations have been made in the context of neuron-specific promoters that resulted in comparable green fluorescent protein (GFP) expression in the brain to the CAG promoter.[48] In our previous reports, we have utilized higher doses of vector where AAV2-iCasp9 and exo-AAV6-iCasp9 vectors were administered at a dose of 5×10^{10} vgs/animal[22] and 2×10^{10} vgs/animal,[16] respectively. Hence, in the current study, we tested a dose of 1×10^{10} vgs/animal to determine if phenotypic rescue is maintained at a lower dose. The observation of similar but specific cytotoxicity with the LP1 and E2F1/BIRC5 vectors, even at a 5-fold lower dose of vectors administered (1×10^{10} vgs vs 5×10^{10} vgs/animal) compared to the previous study,[22] augurs well for their potential use in humans.

Our study is proof-of-concept for the potential of targeted AAV6 vectors, whose outcomes we believe can be improved further. From our earlier studies,[16,22] we have innovated to generate different promoter systems (LP1 and BIRC5) in AAV6 serotype that demonstrate their potential for HCC treatment. However, in the absence of complete tumor ablation, further clinical use will be contingent upon their evaluation in combination with Sorafenib, the current standard of care, or novel chemotherapeutic agents as a chemogene therapy strategy to achieve maximum remission.

MATERIALS AND METHODS

Design and Synthesis of Modified Inducible Caspase 9 Constructs. The *iCasp9* transgene was cloned into the AAV backbone along with LP1 (liver-specific) and BIRC5 (cancer-specific) promoters. The plasmid *pAAV-CAG-iCasp9* was used as a donor plasmid, and the LP1[25] promoter was added in place of the CAG promoter along with a Kozak sequence (GCCGCCACC)[49] upstream of the *iCasp9* transgene to yield *pAAV-LP1Kozak-iCasp9*. The LP1 promoter was replaced with the BIRC5[32] promoter to generate the *pAAV-Birc5Kozak-iCasp9* vectors. To improve cancer cell targeting, six tandem repeats of the E2F1 factor binding site, 5′-TTTC[CG]CGC-3′,[33] were incorporated upstream of the BIRC5 promoter to yield *pAAV-E2F1Birc5Kozak-iCasp9*. The cloning and synthesis of these vectors were performed using a PCR based method named CloneEZ, and Sanger sequencing was performed for confirmation of cloning (Genscript).

Cell Lines and Reagents. The AAV packaging cell line (AAV293) was procured from Stratagene (San Diego, CA). The human hepatocellular carcinoma (Huh7) cell line was a kind gift from S. Das (Indian Institute of Science, Bengaluru). Ciprofloxacin was purchased from HiMedia Laboratories, Mumbai, India, and piperacillin from MP Biomedicals, Irvin, CA. Iscove's modified Dulbecco's medium (IMDM) and fetal bovine serum (FBS) were procured from Gibco (Life Technologies, Carlsbad, CA). Cells were cultured at 37 °C with 5% CO_2 and maintained in IMDM with 10% FBS, 10 μg/mL ciprofloxacin and piperacillin each, and sodium bicarbonate (Sigma-Aldrich, St Louis, MO).

Viral Vector Packaging and Quantification. The packaging cell line AAV293 was expanded to 40 Petri dishes (150 mm^3). At a 70−80% confluence, cells were transfected using three plasmids: AAV-rep/cap (*pAAV6WT*), an adenoviral helper plasmid (pHelper) with three separate transgenes *pAAV-CAG-iCasp9*, *pAAV-LP1Kozak-iCasp9*, or *pAAV-E2F1Birc5Kozak-iCasp9* in the presence of 0.1% polyethylenimine (PEI; Polysciences Inc., Taipei, Taiwan) as a transfecting reagent. AAV genome titers were estimated using a quantitative real-time polymerase chain reaction (qPCR).[50] Titers were generated from two replicate analyses and calculated as average vector genomes per milliliter (vgs/mL).

Exosome Isolation. Exosomes were harvested from the supernatant media collected 68−72 h post-transfection of AAV293 cells and purified using density gradient centrifugation, as described previously.[16] The supernatant media were centrifuged at 3000 rpm for 15 min for cell sedimentation, followed by 20,000*g* for 1 h to remove cell debris; further, centrifugation of the same media at 100,000*g* for 1.5 h yielded a pellet, which was resuspended in 200 μL of phosphate-buffered saline (PBS). Titers were estimated using qPCR and calculated as average vgs/mL.

Cell Cytotoxicity Assay. The viability was tested in Huh7, seeded at a cell density of 1.5×10^4 cells/well in a 48-well plate. Cells were transduced at a multiplicity of infection (MOI) of 10,000 with AAV6-CAG-iCasp9, AAV6-LP1Kozak-iCasp9, and AAV6-E2F1Birc5Kozak-iCasp9 and their respective Exo-AAV vectors. The dimerizer drug AP20187 (50 nM; Ariad Pharmaceuticals, Cambridge, MA) was added 24 h post-transduction, and cells were incubated for 48 h at 37 °C.[22] Percentage cell viability was measured using an ATP assay kit as per the manufacturer's protocol (CellTiter-Glo, Promega, Madison, WI).

Suicide Gene Therapy in a Xenograft Mouse Model of HCC. Athymic nude mice (8 to 10 weeks old) were used for the study. The animal experiments were approved by the Institutional Animal Ethics Committee (IIT Kanpur, India). The xenograft model was developed by injection of ∼5 million Huh7 cells[51] along with 25% ECM (extracellular matrix) gel (Sigma-Aldrich, St Louis, MO), subcutaneously. Animals were randomly divided into 4 groups: mock (n = 5), AAV6-CAG-iCasp9 (n = 5), AAV6-LP1Kozak-iCasp9 (n = 11), and AAV6-E2F1Birc5Kozak-iCasp9 (n = 7) after they developed tumors (100−150 mm^3).[52] With exosomal AAV vectors, the treatment groups were mock (n = 10), Exo-AAV6-CAG-iCasp9 (n = 11), Exo-AAV6-LP1Kozak-iCasp9 (n = 12), and Exo-AAV6-E2F1Birc5Kozak-iCasp9 (n = 7). Animals in the mock group received an equal volume of PBS. A dose of 1×10^{10} vgs of the vector was administered intratumorally, and dimerizer drug AP20187 (1 mg/kg body weight) was injected intraperitoneally, thrice at 48 h intervals. After 10 days of vector administration, the animals were humanely euthanized, and the tumor was collected. The tumor volume was calculated using the formula $V = (L \times W^2)/2$, where L and W are the longest and smallest diameters (mm), respectively. The relative tumor volume (RTV) in the treated and control mice was calculated as reported earlier, RTV = V_t/V_0, where V_t is the volume on each day and V_0 is the tumor volume at the beginning of the treatment.[53]

Histological Analysis. Tumors from the test groups were harvested and fixed in 10% buffered formalin overnight. After paraffin embedding, 10 μm sections were prepared. Hematoxylin and eosin staining was performed, and apoptotic bodies in the tumor tissue were identified using Leica DMi8 (Leica microsystems, Wetzlar, Germany).

In Situ TUNEL Assay. The tumor tissue was fixed in 10% buffered formalin. The samples were embedded in paraffin and sectioned into 10 μm slices using a microtome (Leica, Wetzlar, Germany). The TUNEL assay was performed to identify apoptotic cells according to standard protocols (Roche, Basel,

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Switzerland). Sections were also counterstained with DAPI (Thermo Fischer, Waltham, MA) and mounted using FluorSave (Sigma-Aldrich). Images were acquired by confocal imaging using an LSM780NLO Carl Zeiss GmbH (Carl Zeiss AG, Jena, Germany). A minimum of three tissue sections per tumor (2 tumors per group) were analyzed for quantification of TUNEL-positive cells, which was performed using ImageJ software.

Statistical Analysis. Statistical analysis was performed using either unpaired two-tailed Student's *t* test or by one-way/two-way analysis of variance test (ANOVA), as applicable. A p value of ≤ 0.05 was considered statistically significant. All analyses were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA).

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Author Contributions

V.S. conducted the experiments. S.P. and N.K. contributed to *in vivo* administrations. G.R.J. conceived, designed, and supervised the study. V.S. and G.R.J. wrote the manuscript.

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Notes

The authors declare the following competing financial interest(s): The authors declare that IIT Kanpur has filed patent applications on AAV vectors.

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