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**ARTICLE**

# Strategies to generate high-titer, high-potency recombinant AAV3 serotype vectors

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Although recombinant adeno-associated virus serotype 3 (AAV3) vectors were largely ignored previously, owing to their poor transduction efficiency in most cells and tissues examined, our initial observation of the selective tropism of AAV3 serotype vectors for human liver cancer cell lines and primary human hepatocytes has led to renewed interest in this serotype. AAV3 vectors and their variants have recently proven to be extremely efficient in targeting human and nonhuman primate hepatocytes *in vitro* as well as *in vivo*. In the present studies, we wished to evaluate the relative contributions of the *cis*-acting inverted terminal repeats (ITRs) from AAV3 (ITR3), as well as the *trans*-acting Rep proteins from AAV3 (Rep3) in the AAV3 vector production and transduction. To this end, we utilized two helper plasmids: pAAVr2c3, which carries *rep2* and *cap3* genes, and pAAVr3c3, which carries *rep3* and *cap3* genes. The combined use of AAV3 ITRs, AAV3 Rep proteins, and AAV3 capsids led to the production of recombinant vectors, AAV3-Rep3/ITR3, with up to approximately two to fourfold higher titers than AAV3-Rep2/ITR2 vectors produced using AAV2 ITRs, AAV2 Rep proteins, and AAV3 capsids. We also observed that the transduction efficiency of Rep3/ITR3 AAV3 vectors was approximately fourfold higher than that of Rep2/ITR2 AAV3 vectors in human hepatocellular carcinoma cell lines *in vitro*. The transduction efficiency of Rep3/ITR3 vectors was increased by ~10-fold, when AAV3 capsids containing mutations in two surface-exposed residues (serine 663 and threonine 492) were used to generate a S663V+T492V double-mutant AAV3 vector. The Rep3/ITR3 AAV3 vectors also transduced human liver tumors *in vivo* approximately twofold more efficiently than those generated with Rep2/ITR2. Our data suggest that the transduction efficiency of AAV3 vectors can be significantly improved both using homologous Rep proteins and ITRs as well as by capsid optimization. Thus, the combined use of homologous Rep proteins, ITRs, and capsids should also lead to more efficacious other AAV serotype vectors for their optimal use in human gene therapy.

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## INTRODUCTION

Successful long-term gene transfer to liver is highly desirable for the treatment of not only various liver diseases, but also other metabolic disorders and plasma protein deficiencies. In the late 1990s, it was observed that the adeno-associated virus (AAV) serotype 2 (AAV2) vectors, when directly injected into mice via the tail-vein, accumulated predominantly in hepatocytes.[1,2] Since then, liver remains an extensively studied target tissue for AAV vector-mediated gene transfer. Although other serotypes, such as AAV8 and AAV5, have since been used for liver-directed gene delivery, one particular serotype, AAV3, has largely been ignored, owing to its poor transduction efficiency in most cell lines *in vitro* and mouse tissues *in vivo*. However, we observed that AAV3 vectors efficiently transduce human liver cancer cell lines and primary human hepatocytes in culture.[3,4] Subsequently, additional mechanistic studies revealed that AAV3 utilizes the human hepatocyte growth factor receptor as a cellular coreceptor to mediate gene delivery in human liver cells.[5] To further enhance the transduction efficiency of AAV3 vectors without interfering with its selective tropism, mutagenesis of the specific surface-exposed tyrosine, serine, threonine, and lysine residues, previously reported to circumvent capsid phosphorylation, ubiquitination, and subsequent proteasome-mediated degradation, and eventually to achieve enhanced viral intracellular trafficking and transgene expression by AAV vectors,[6–8] was also applied to AAV3 capsid. For example, a serine (S) residue at position of 663, and a threonine (T) residue at position of 492 on AAV3 capsid were mutagenized to generate a S663V+T492V double mutant, which was efficient in targeting both human liver tumors and primary human hepatocytes in chimeric mouse models *in vivo*.[9–11] The S663V+T492V-AAV3 vector also efficiently transduced the liver in nonhuman primates *in vivo* following intravenous delivery.[11] In addition, Lisowski *et al*.[12] also used a similar chimeric mouse model, whose liver was partially xenografted with human hepatocytes, to perform directed evolution from a shuffled AAV library. An engineered capsid, termed AAVLK-03, whose major VP3 protein has only one amino acid difference with the AAV3 VP3 protein, was

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obtained and shown to demonstrate selective tropism for human hepatocytes. Although there is some debate as to which serotype and AAV3 variant among AAV3, AAV3LK-03, and AAV8 is the optimal vector for targeting human hepatocytes,[13] all of these published studies have led to renewed interest in AAV3-based vectors.[14,15]

In addition to the viral capsid, modifications in the viral genome, especially the viral inverted terminal repeats (ITRs), have also provided an additional avenue to achieve enhanced transgene expression.[16] To date, the most successful modification on AAV viral genome is the development of self-complementary AAV vectors, which can bypass the requirement for viral second-strand DNA synthesis, and which has shown remarkable efficacy in the treatment of hemophilia B patients with self-complementary AAV8 vectors.[17] All AAV vectors, including all of the AAV3 vectors described above, contain transgene cassettes flanked by ITRs from AAV serotype 2 (ITR2). Previous studies involving the use of ITRs from AAV serotypes other than AAV2 in generating recombinant AAV vectors have yielded conflicting results. For example, it was reported that the combination of ITR8 with AAV8 capsids (AAV8/8) resulted in vectors that led to at least twofold increase in transgene expression in mouse liver, compared with AAV8 capsids pseudotyped with ITR2 (AAV2/8) vectors,[18] whereas ITRs from AAV serotypes 1–6 were reported to be interchangeable in terms of packaging efficiency into AAV8 capsids (AAV1/8–AAV6/8), but played no role in transgene expression in murine hepatocytes *in vivo*.[19] Moreover, in a recently proposed model of AAV encapsidation, the Rep proteins, together with the ITRs, were suggested to be functionally involved in packaging of the viral genome into viral capsid.[20,21] Thus, in the present studies, we wished to evaluate the relative contributions of the *cis*-acting ITRs from AAV3 (ITR3), as well as the *trans*-acting Rep proteins from AAV3 (Rep3) in the AAV3 vector production and transduction. Our data document that the combined use of Rep3 and ITR3 leads to generation of recombinant AAV3 vectors with increased titers as well as higher transduction efficiency in human liver cancer cell lines *in vitro* as well as in human liver tumors in a murine xenograft model *in vivo*.

## RESULTS

### AAV3 ITRs have no effect on recombinant plasmid-mediated transgene expression

Two sets of AAV3 plasmids were constructed, as illustrated in Figure 1a. In the first set, humanized recombinant green fluorescent protein (hrGFP) gene, driven by a cytomegalovirus promoter, was flanked by either ITR2 or ITR3. The size of viral genome was ~2.8 kb. In the second set, enhanced green fluorescent protein (EGFP) gene, also driven by a cytomegalovirus promoter, was flanked by either ITR2 or ITR3. To mimic the size of the wild-type (WT) AAV genome, which is 4.75 kb, a neomycin resistant gene (*neo*$^R$) expression cassette was inserted in between the polyadenylation signal and the ITR2/3. All plasmids were sequenced prior to their use in experiments. The accuracy of the DNA concentrations was confirmed both by spectrophotometry (data not shown) and agarose gel electrophoresis following restriction endonuclease digestion (Figure 1b). The results of transfection assays in Huh7 cells, as shown in Figure 1c,d, indicate that ITR2 and ITR3 are interchangeable in terms of plasmid-mediated transgene expression with both sets of transgene cassettes.

### Cap3 proteins are expressed at equivalent levels from either Rep2- or Rep3-containing helper plasmids

Two Cap3-expressing AAV3 helper plasmids were used. In the first plasmid, the *cap2* gene in a pAAV2 plasmid, which contains the WT AAV2 genome but without the ITRs, was replaced by the *cap3* gene (Figure 2a, top). The second plasmid contains the WT AAV3 genome, including the promoter region, the *rep3* gene and the *cap3* gene, but without the ITRs (Figure 2a, bottom). Both plasmids were transfected into HEK293 cells, together with the pHelper plasmid, which contains the essential adenoviral genes (*E2a*, *E4orf6*, and *VA*). Total protein was extracted 48 hours posttransfection from cells cotransfected with both Rep/Cap and pHelper plasmids to determine the levels of Cap3 protein expression. A representative Western blot, shown in Figure 2b, documented that both plasmids mediated similar levels of Cap3 proteins in the HEK293 cells. The conventional 1:1:10 ratios of VP1:VP2:VP3 proteins were also observed.

### Combination of homologous Rep proteins and ITRs leads to higher level of viral genome replication and encapsidation

We hypothesized that the combined use of Rep3 and ITR3 may be beneficial in the production of AAV3 vectors. To this end, both sets of the ITR-containing AAV3 plasmids were transfected into the HEK293 cells in the presence of either Rep2 (Figure 3a) or Rep3 (Figure 3b). The pHelper plasmid was also transfected in each group to support the AAV life cycle in these cells. Low-molecular-mass DNA was isolated 72 hours posttransfection, followed by *Dpn*I digestion for 4 hours. The DNA samples were then subjected to qPCR assays for the quantification of viral genome copy number. It is evident that in the presence of Rep2, both ITR2-containing plasmids resulted in a higher level of viral genome copy numbers. A higher viral genome copy number of ITR2/3-hrGFP was also observed compared with ITR2/3-EGFP-Neo, regardless of the origin of ITRs, which was most likely due to the subgenomic length of the viral DNA. Interestingly, in the presence of Rep3, whereas the same origin of ITR had no significant effect on genome replication of shorter viral DNA, it clearly produced a higher number of viral genome copy number when the genome size was similar to the WT AAV DNA. Thus, in all subsequent experiments, the set of longer viral genomes, pITR2/3-EGFP-Neo, was used to examine the potential benefit of Rep3 and ITR3.

Our hypothesis was further corroborated by quantifying the encapsidated vector genome copy numbers. From the triple-transfected HEK293 cells, crude lysates were prepared 72 hours posttransfection, as described in Materials and Methods. Samples were treated with both benzonase and universal nuclease for 4 hours, followed by extraction of viral encapsidated genomic DNA. The negative controls of this assay included HEK293 cells that were triple transfected with pITR2/3-EGFP-Neo, pHelper, and a plasmid containing the AAV2 *rep* gene but no *cap* genes.[22] In the negative control cells, viral genome replication occurs, but no viral capsid proteins are expressed. The results of viral encapsidation assays revealed that the encapsidated viral genomes in the negative control group were below the detection limitation by qPCR assays (data not shown). Importantly, the use of Rep3 and ITR3 to produce AAV3 vectors yielded approximately fourfold higher titers, compared with the group in which Rep2 and ITR2 were used (Figure 3c). To validate these observations, we performed viral encapsidation assays using S663V+T492V-AAV3 capsids. The results also showed approximately twofold increase in vector titers when Rep3 and ITR3 were used (Figure 3d).

### AAV3 vectors produced by homologous Rep proteins and ITRs lead to higher transduction efficiency

We further hypothesized that the use of the same origin of Rep and ITR may lead to improved transduction efficiency of AAV3

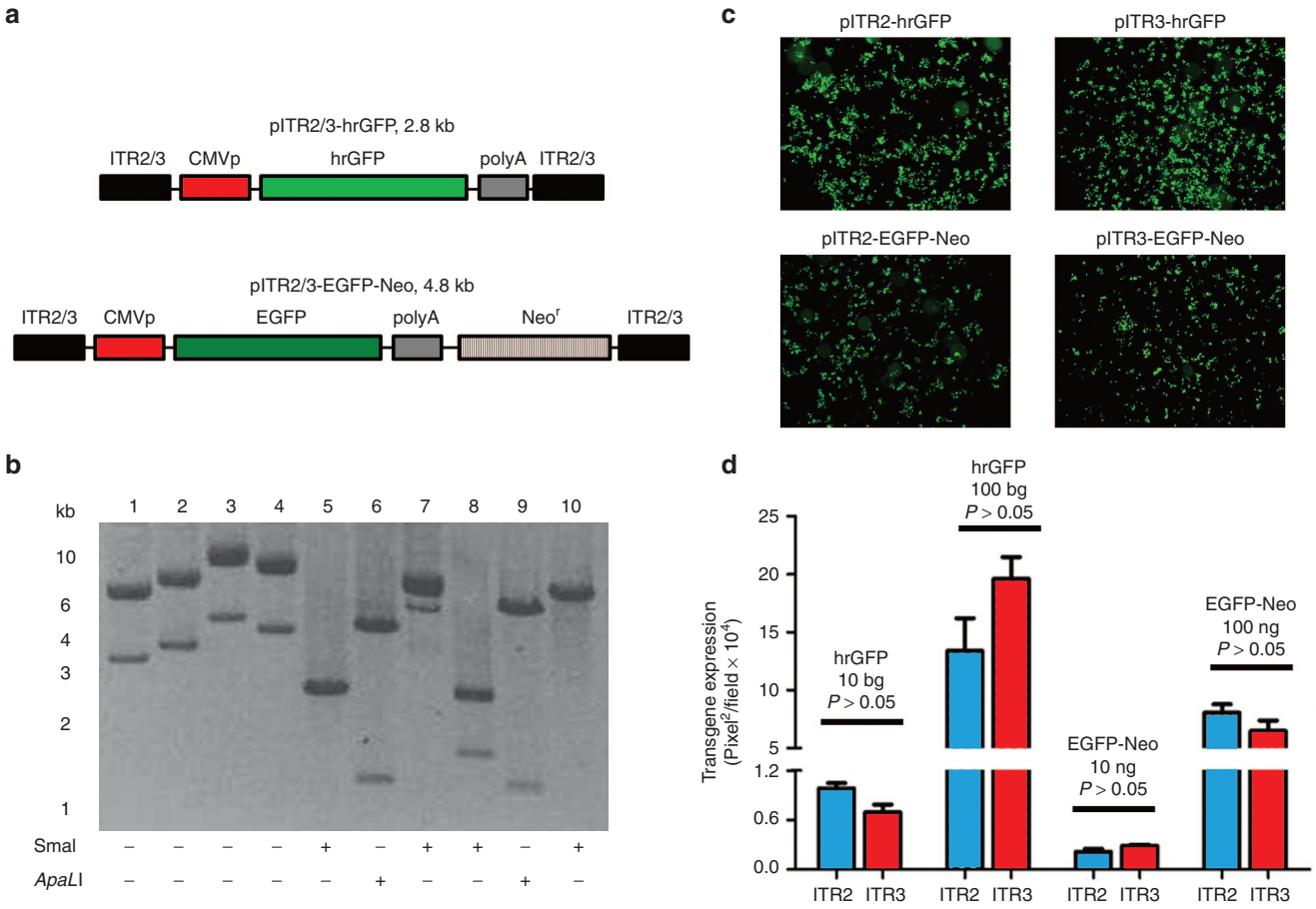


**Figure 1** Characterization of ITR2- and ITR3-containing AAV plasmids. (**a**) Schematic structures of recombinant AAV genomes. CMVp, cytomegalovirus promoter; EGFP, enhanced green fluorescent protein; hrGFP, humanized recombinant green fluorescent protein; ITR, inverted terminal repeat; *neo*$^R$, neomycin resistant gene; polyA, polyadenylation signal. (**b**) Quality and quantity of the plasmid DNA used in the present studies. Plasmids pITR2-hrGFP (lanes 1 and 5), pITR3-hrGFP (lanes 2, 6, and 7), pITR2-EGFP-Neo (lanes 3 and 8), and pITR3-EGFP-Neo (lanes 4, 9, and 10) were digested with the indicated restriction endonuclease enzymes for 1 hour and subjected to 1% agarose gel electrophoresis. (**c**, **d**) Analysis of GFP expression following transfection of Huh7 cells. Both representative (**c**) and quantitative (**d**) results are shown.

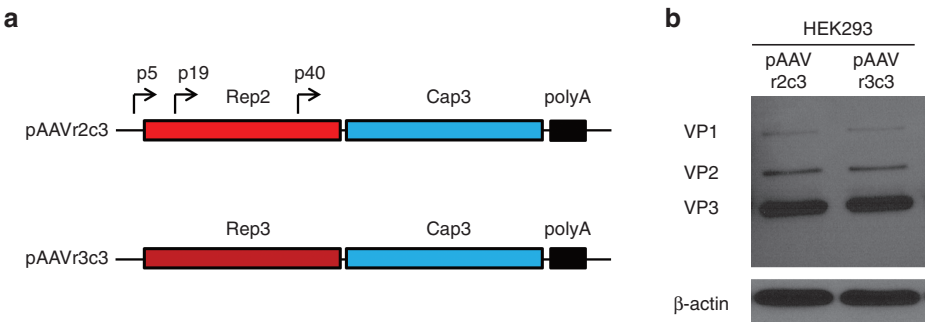


**Figure 2** Characterization of Rep- and Cap-containing AAV helper plasmids. (**a**) Schematic structures of AAV helper genomes. Arrows indicate the positions of known promoters of AAV2. (**b**) Western blot analysis of the expression level of AAV3 capsid proteins from different AAV helper plasmids. Total proteins were isolated from HEK293 cells that were transfected with the indicated plasmids, together with pHelper. β-actin was used as a loading control. AAV, adeno-associated virus; AAV2, AAV serotype 2; AAV3, AAV serotype 3.

vectors. To this end, purified WT-AAV3 and S663V+T492V-AAV3 vectors containing the EGFP-Neo transgene cassette were produced either in the presence of Rep2/ITR2 or Rep3/ITR3. Two human hepatocellular carcinoma (HCC) cell lines, Huh7 and LH86, were transduced with these vectors under identical conditions, and transgene expression was determined 72 hours post-transduction. Consistent with our previously published reports,[10] the S663V+T492V-AAV3 vectors led to >10-fold increase in the transduction efficiency. Interestingly, the data, as shown in Figure 4a,b, also indicated that the WT-AAV3 vectors, which were generated with ITR3, Rep3, and Cap3, transduced both human HCC cell lines approximately twofold more efficiently than those generated with ITR2, Rep2, and Cap3. Similar results were also obtained when S663V+T492V-AAV3 vectors, generated with Rep2/ITR2 versus Rep3/ITR3, were used to infect human HCC cell lines (Figure 4c,d).

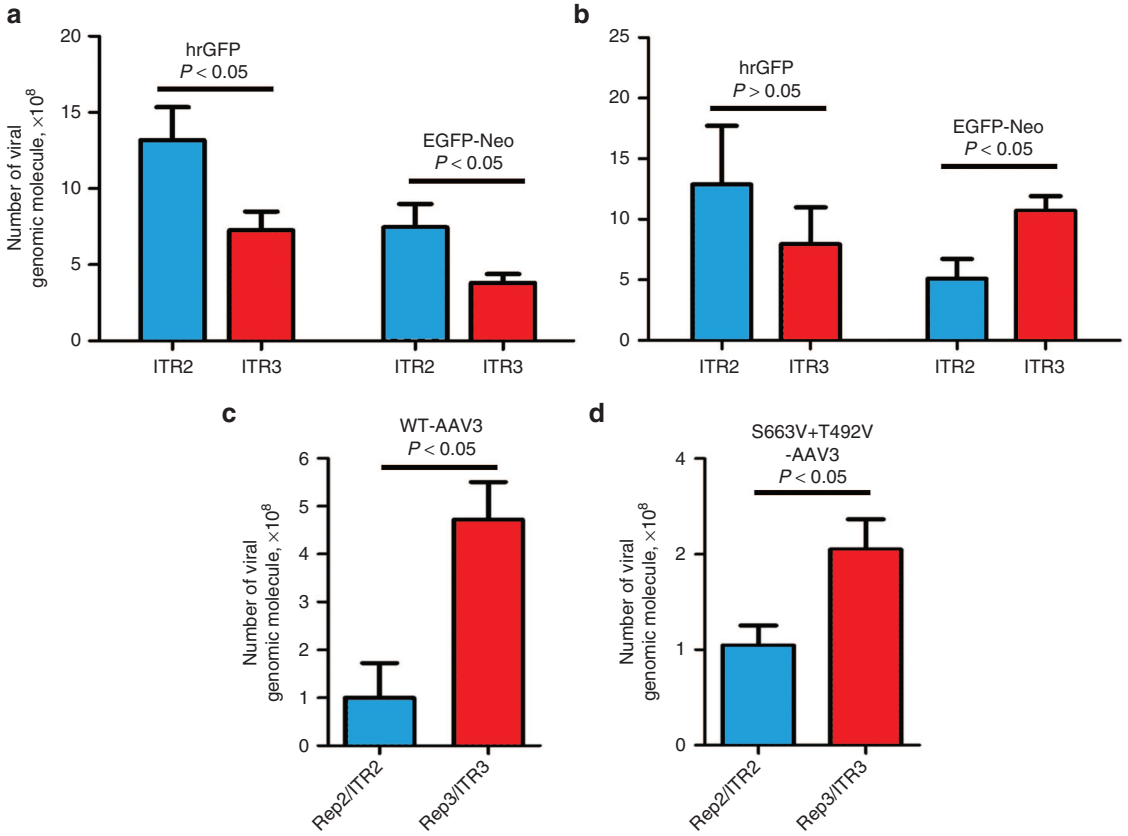


**Figure 3** Effect of ITRs on viral genome replication and encapsidation. (**a**, **b**) qPCR assays showing the genome copy numbers following rescue and replication of AAV genomes from recombinant plasmids. HEK293 cells were transfected with the indicated plasmids (ITR2/3-hrGFP or ITR2/3-EGFP-Neo), together with pHelper, in the presence of either (**a**) pAAVr2c3 or (**b**) pAAVr3c3. Low-molecular-mass DNA was isolated 72 hours post-transfection, followed by *Dpn*I digestion for 4 hours. (**c**, **d**) qPCR assay showing the viral genome copy numbers following encapsidation. HEK293 cells were transfected with (**c**) ITR2-EGFP-Neo and AAVr2c3, and with ITR3-EGFP-Neo and AAVr3c3; or with (**d**) ITR2-EGFP-Neo and AAVr2c3-S663V+T492V, and with ITR3-EGFP-Neo and AAVr3c3-S663V+T492V. pHelper plasmid was also included in each group. Viral encapsidation assays were performed 72 hours posttransfection. The number of AAV3 genome was determined by comparison with double-stranded plasmid pITR2/3-EGFP-Neo standards. Data were normalized by the group of Rep2/ITR2. EGFP, enhanced green fluorescent protein; ITR, inverted terminal repeat.

To further corroborate the results of *in vitro* experiments, we generated Huh7 tumor-bearing immune-deficient mouse models, as described previously.[23] When the tumor grew to 0.5 cm in diameter, AAV3-EGFP-Neo vectors, produced either with Rep2/ITR2 (Figure 5a, lanes 2–5) or with Rep3/ITR3 (Figure 5a, lanes 6–9), were injected intratumorally with $1\times10^{11}$ vgs/tumor. Forty-eight hours postvector administration, tumors were obtained, and transgene expression was evaluated by Western blot assays. A tumor without vector injection (Figure 5a, lane 1) was used as a negative control. These results, consistent with those of the *in vitro* experiments, indicated that the AAV3 vectors generated with Rep3/ITR3 transduced human liver tumors *in vivo* approximately twofold more efficiently than their counterpart produced with Rep2/ITR2 (Figure 5b). Taken together, our data suggest that the combined use of Rep3 and ITR3 leads to generation of recombinant AAV3 vectors at higher titers as well as higher transduction efficiency in human liver cancer cell lines *in vitro* and in human liver tumors in a murine xenograft model *in vivo*.

## DISCUSSION

Our long-term efforts have led to the identification of AAV3 as the most efficient serotype in transducing human hepatocytes.[3–5,9,10] However, in our recently published studies with a murine model with chimeric humanized liver, less than 30% of human hepatocytes were transduced by AAV3 vectors at a dose of $1\times10^{11}$ vgs/mouse, and the average transduction efficiency was determined to be ~36% of the liver in nonhuman primates at a dose of $1\times10^{13}$ vgs/kg in these animals.[11] Although our previous studies have revealed that site-directed mutagenesis of specific surface-exposed amino acids on AAV3 capsids can significantly increase the transduction efficiency,[9,10] and indeed, a fivefold increase in transduction with S663V+T492V-AAV3 vectors was observed in nonhuman primate livers,[11] it is obvious that additional strategies are warranted to further augment the transduction efficiency of AAV3 vectors. We reasoned that one such strategy might be the use of homologous AAV3 Rep proteins and AAV3 ITRs to generate AAV3 vectors, instead of the AAV2 Rep proteins and AAV2 ITRs, since AAV3 Rep proteins would be expected to mediate more efficient encapsidation of AAV3 ITR-containing vector genomes into AAV3 capsids. This hypothesis was tested experimentally in the present studies.

To date, extensive progress has been made by manipulating the viral capsids for the purpose of improving the AAV gene transfer ability.[24] On the other hand, few studies have focused on altering the only AAV *cis*-DNA element, the ITR. This is due to the critical role that ITRs play in the AAV life cycle, and modifications in the ITRs might be detrimental. Thus, we elected to substitute the entire ITR2 with ITR3, and explored the potential benefits of the combined usage of the homologous Rep proteins and ITRs. Our data indicated

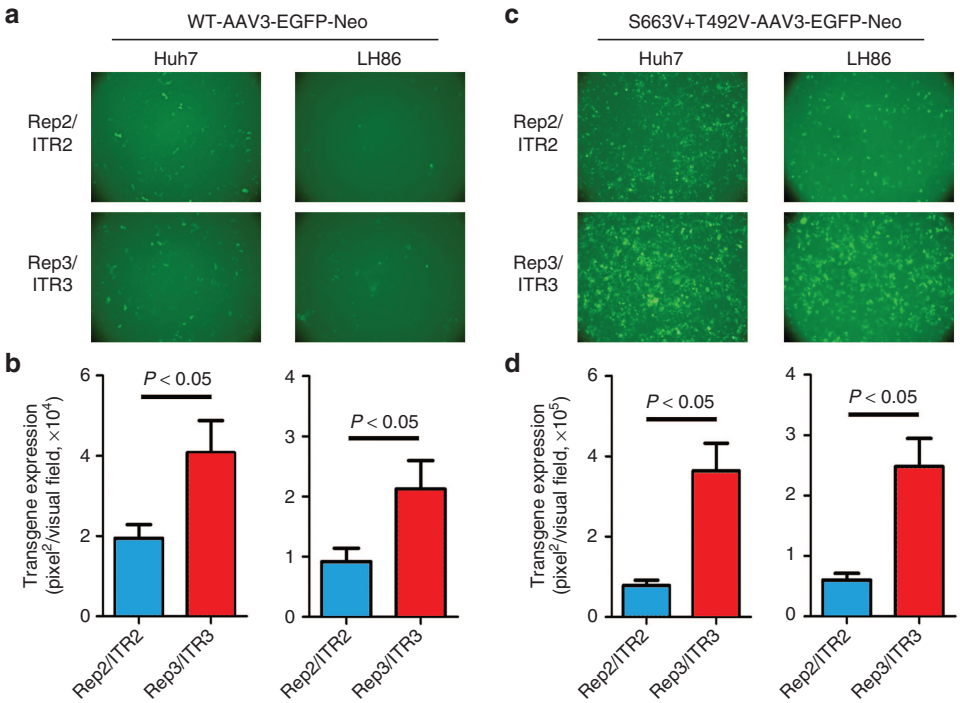


**Figure 4** Transduction efficiency of AAV3 vectors *in vitro*. (**a**, **b**) WT-AAV3 vectors and (**c**, **d**) AAV3-S663V+T492V vectors were produced by triple transfection with either the combination of Rep2/ITR2, or the combination of Rep3/ITR3. Human hepatocellular carcinoma cell lines, Huh7 and LH86, were transduced with the indicated viral vectors at an MOI of 5,000 vgs/cell. Transgene expression was determined by fluorescence microscopy 72 hours posttransduction. Both representative (**a**, **c**) and quantitative (**b**, **d**) results are shown. AAV, adeno-associated virus; ITR, inverted terminal repeat.

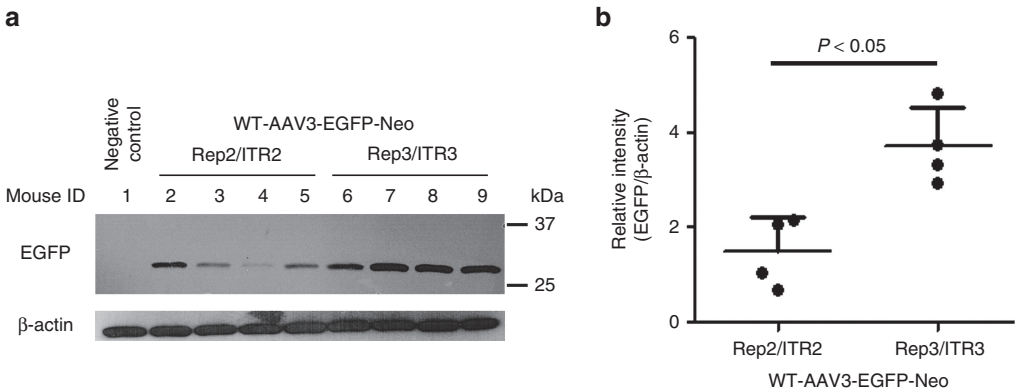


**Figure 5** Transduction efficiency of AAV3 vectors *in vivo*. Immune-deficient NSG mice ($n = 4$) were injected subcutaneously with $5 \times 10^6$ human Huh7 liver cancer cells on the ventral side of the neck between shoulder blades. When tumors grew to 0.5 cm in diameter, each tumor was injected with $1 \times 10^{11}$ vgs/tumor of AAV3-EGFP-Neo vectors, generated with either Rep2/ITR2 (lanes 2–5), or with Rep3/ITR3 (lanes 6–9) combinations. (**a**) EGFP expression in each tumor was determined by Western blotting 48 hours postvector administration. A tumor without vector injection (lane 1) was used as a negative control, and β-actin was used as a loading control. (**b**) Quantitation of EGFP expression in each tumor, normalized with β-actin expression. AAV, adeno-associated virus; EGFP, enhanced green fluorescence protein; ITR, inverted terminal repeat.

that the effect of Rep3 and ITR3 in viral genome rescue and replication is genome length-dependent. This combination resulted in an increased vector genome copy number when the length of viral genome was close to that of the WT AAV genome (4.75 kb). On the other hand, the combination of Rep2/ITR2 was beneficial when the genome length was shorter (2.8 kb). Our data are consistent with a previous report that in the presence of Rep2, when the genome length is less than 4.0 kb, the ITR2 and ITR3 are interchangeable in terms of viral genome replication.[19] However, it is important to note that the qPCR method, which was used in our studies, is quantitatively more sensitive than Southern or DNA dot blot assays. In addition to genome replication, the combined use of Rep3 and ITR3 significantly increased both titers and potency of AAV3 vectors. This is particularly remarkable considering that Rep2/ITR2 resulted in higher viral genome replication (Figure 3a), yet led to lower packaging efficiency in the AAV3 capsid (Figure 3c). Although the precise mechanism of AAV genome encapsidation still remains a puzzle,[21] it is clear that both Rep proteins and ITRs play critical roles together. It is also possible that the Rep3 protein is relatively less toxic to the packaging cells, which may facilitate higher vector titer/retention per cell basis. Based on the sequence alignments of both Rep and ITRs of AAV2 and AAV3 (Figure 6), it is evident that subtle differences exist. Additionally, it would be informative to determine the empty-to-full particle ratio of AAV3 vectors, which may also be

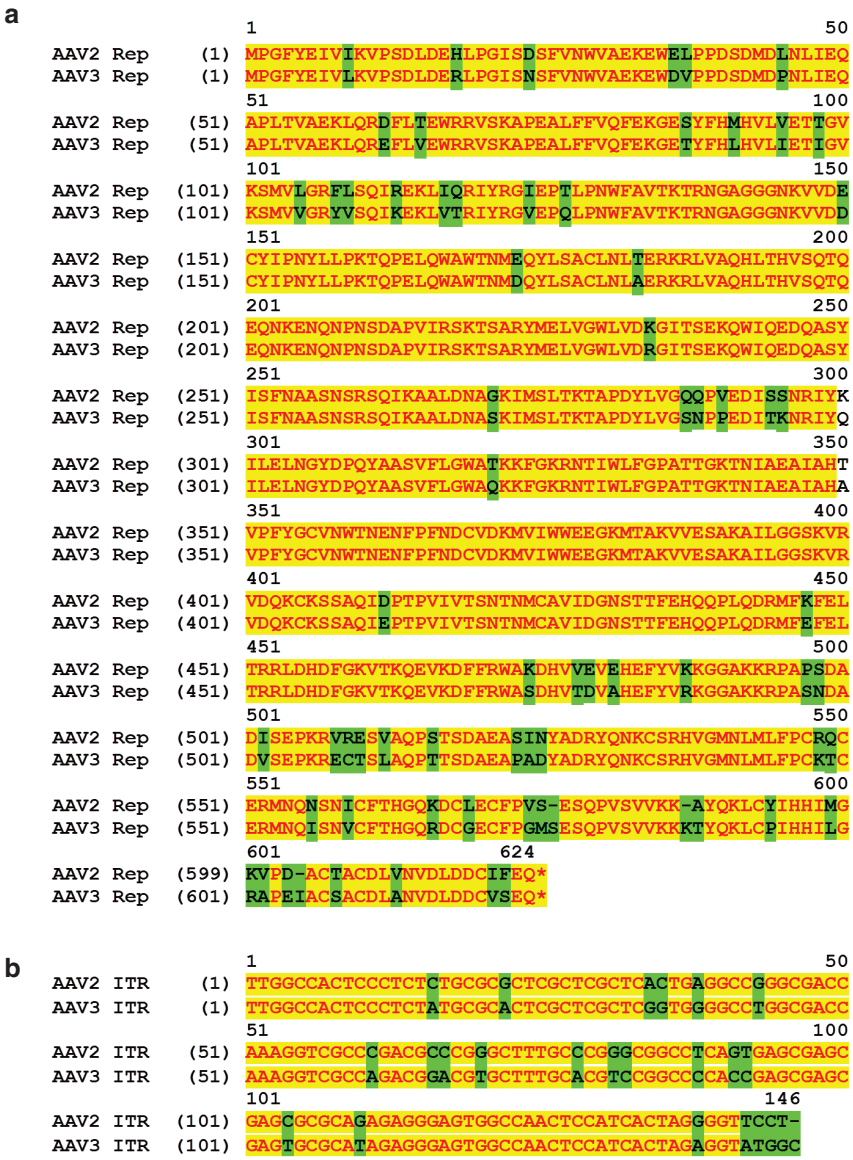


**Figure 6** Alignments of Rep and ITRs of AAV2 and AAV3. (**a**) Amino acid sequence identities and differences in AAV2 and AAV3 Rep proteins are highlighted in yellow and green, respectively. The stars at the end of the Rep sequences represent the stop codon. (**b**) Nucleotide sequence identities and differences in AAV2 and AAV3 ITRs are highlighted in yellow and green, respectively. These sequences were retrieved from NC_001401 and NC_001729 from the NCBI database. Alignments were performed using the Vector NTI® Software (Thermo Fisher Scientific). AAV, adeno-associated virus; ITR, inverted terminal repeat.

influenced by the homologous Rep and ITRs. Thus, further studies are warranted to examine whether these differences account for the improved vector titers and potency.

The most interesting finding from our studies is that the AAV3 vectors produced with Rep3 and ITR3 possess superior transduction efficiency compared with their counterparts produced with Rep2 and ITR2. Although further studies are warranted, it is clear that additional factors, such as the secondary structure of the ITR, or the viral second-strand synthesis, may significantly influence the ITR3-mediated increase in AAV3 transduction efficiency since the ITR3-containing plasmids showed no superior transfection efficiency compared with the ITR2-containing plasmids (Figure 1c,d). Alternatively, the use of nonhomologous Rep proteins, ITRs, and Cap proteins may result in encapsidation of subgenomic length DNA strands or defective viral genomes, instead of a full-length genome, as we[25] and others[26] have reported previously. It should be noted that in our viral transduction assays, a transgene cassette similar to the WT AAV genome length was used. A systemic comparison of various genome lengths, as we have previously reported,[27] is currently underway to fully elucidate the underlying molecular mechanisms of encapsidation.

During the past two decades, the roles of the D-sequence, a 20-nucleotide sequence in the ITR2, in AAV2 genome replication, encapsidation and gene expression, have become clear,[28–32] and has led to the development of D-sequence-deleted single-stranded AAV vectors[16] as well as D-sequence-deleted single-stranded DNA mini-vectors[33] with improved transduction efficiency. Manipulation of the D-sequence and terminal resolution site has also resulted in self-complementary AAV8 vectors that have been used successfully in a hemophilia B clinical trial.[17] However, since AAV2 and AAV3 ITRs have D-sequences that are different Figure 6b, especially the distal 10 nucleotides, it is of interest

to explore the role of D-sequence in the AAV3 genome rescue, replication, and encapsidation.

A recent report documented that of 193 patients with HCC, 11 contained an integrated genome sequence of the WT AAV2, and concluded that AAV2 is associated with oncogenic insertional mutagenesis in human HCC.[34] Although we[35] and others[36] have questioned this conclusion, additional studies are warranted to elucidate what role, if any, do vectors containing AAV3 ITRs play in HCC. Regardless, novel tools and findings reported here with AAV3 vectors are not only likely to be applicable to other AAV serotypes, but also promise to further enhance our understanding of the roles of Rep proteins and ITRs in AAV biology and vectorology.

## MATERIALS AND METHODS

### Cells and plasmids

Human embryonic kidney (HEK293) and HCC (Huh7 and LH86) cells were described previously.[7,37] All cells were maintained in complete Dulbecco's Modified Eagle Medium (Mediatech, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 1% penicillin and streptomycin (P/S, Lonza, Walkersville, MD). Cells were grown as adherent culture in a humidified atmosphere at 37 °C in 5% $CO_2$ and were subcultured after treatment with trypsin–versene mixture (Lonza, Walkersville, MD) for 2–5 minutes at room temperature (RT), washed and resuspended in complete Dulbecco's Modified Eagle Medium. Recombinant AAV2 and pHelper plasmids have been described previously.[38] A recombinant plasmid containing the WT AAV3 genome was a kind gift from Dr. Shin-ichi Muramatsu, Jichi Medical University, Japan.

### Plasmid transfection assays

Plasmid transfections were performed in 96-well plates using lipofecatimine LTX (Life Technologies, Grand Island, NY). Reporter gene expression was detected by fluorescence microscopy[39] 72 hours posttransfection. Western blot assays were performed 48 hours posttransfection.

### Western blot assays

Western blot assays were performed as previously described.[40,41] Briefly, cells were harvested and disrupted in a radio-immunoprecipitation assay lysis buffer. Following normalization for protein concentration, samples were boiled for 10 minutes with loading buffer, separated using 12% SDS-PAGE electrophoresis, electro transferred to nitrocellulose membranes (Bio-Rad), and probed with anti-AAV capsid antibody at 4 °C overnight. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000 dilution, GE Healthcare, Piscataway, NJ), and detected with an enhanced chemiluminescence substrate (MEMD Millipore, Billerica, MA).

### AAV DNA rescue and replication assays

Equivalent amounts of each of the ITR-containing AAV3 plasmid and Rep- and Cap- containing AAV helper plasmid were transfected in HEK293 cells together with plasmid pHelper. At 72 hours posttransfection, low-molecular-mass DNA samples were isolated by the procedure described by Hirt,[42] with modification and digested extensively with *Dpn*I at 100 U/ml for 4 hours in a buffer containing 50 mmol/l potassium acetate, 20 mmol/l Tris-acetate, 10 mmol/l magnesium acetate, and 1 mmol/l DTT. *Dpn*I-treated samples were analyzed by qPCR assays using primes specific for the CMV promoter.

### AAV DNA encapsidation assays

Equivalent amounts of each of the ITR-containing AAV3 plasmid and Rep- and Cap- containing AAV helper plasmid were transfected in HEK293 cells together with pHelper in six-well plates. Cells were harvested 72 hours posttransfection, subjected to three rounds of freeze-thaw and then digested with 50 U/ml Benzonase (Sigma-Aldrich, St. Louis, MO) and 50 U/ml Pierce Universal Nuclease (Thermo Fisher Scientific, Grand Island, NY) at 37 °C for 1 hour. Cell lysates were then centrifuged at 4,000 rpm for 30 minutes. Equivalent amounts of supernatants were deproteinized to release the AAV3 genome by incubation at 65 °C for 30 minutes in NaOH at a final concentration of 100 mmol/l. The viral DNA was purified by DNA Clean & Concentrator-25 Kit (ZYMO Research, Irvine, CA) and subjected to qPCR assays.[43]

### Recombinant AAV vector production

Highly purified stocks of AAV3 vectors were produced by triple plasmid transfection.[44] Briefly, HEK293 cells were cotransfected with three plasmids by polyethylenimine transfection protocol. Medium was replaced 6 hours posttransfection. Cells were harvested 72 hours posttransfection, subjected to three rounds of freeze-thaw and then digested with 50 U/ml Benzonase (Sigma-Aldrich, St. Louis, MO) and 50 U/ml Pierce Universal Nuclease (Thermo Fisher Scientific, Grand Island, NY) at 37 °C for 1 hour. Viral vectors were purified by iodixanol (Sigma, St. Louis, MO) gradient ultracentrifugation followed by ion exchange chromatography using HiTrap Q HP (GE Healthcare, Piscataway, NJ), washed with phosphate-buffered saline (PBS) and concentrated by centrifugation using centrifugal spin concentrators with 150 K molecular-weight cutoff. Viral vectors were finally resuspended in 500 µl PBS.

### Recombinant AAV vectors transduction *in vitro*

*In vitro* transduction assays were performed as described previously.[45] Cells were seeded in 96-well plates at 10,000 cells per well in complete Dulbecco's Modified Eagle Medium. AAV infections were performed in complete Dulbecco's Modified Eagle Medium medium for 2 hours, followed by extensive washes with PBS to remove the vector inoculum. Transgene expression was analyzed by fluorescence microscopy 72 hours posttransduction.

### Animal handling

All animal experiments were approved by the Institutional Animal Care and Use Committee and were performed according to the guidelines for animal care specified by the Animal Care Services at the University of Florida (Gainesville, FL). Six- to 10-week-old nonobese diabetic/severe-combined immune-deficient, interleukin 2-gamma-deficient (NSG) mice were purchased from Jackson Laboratory and maintained by the Animal Care Services at the University of Florida College of Medicine (Gainesville, FL). Mice were injected subcutaneously with 5 million human liver cancer cells on the ventral side of the neck between shoulder blades.[22] Animals were kept in sterile cages until the end of the experiments.

### Statistical analysis

Results are presented as mean ± SD. Differences between groups were identified using a grouped-unpaired two-tailed distribution of Student's *t*-test. $P$ values < 0.05 were considered statistically significant.

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