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Single Tyrosine Mutation in AAV8 and AAV9 Capsids Is Insufficient to Enhance Gene Delivery to Skeletal Muscle and Heart

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Abstract

Site-directed mutations of tyrosine (Y) to phenylalanine (F) on the surface of adeno-associated viral (AAV) capsids have been reported as a simple method to greatly enhance gene transfer *in vitro* and *in vivo*. To determine whether the Y-to-F mutation could also enhance AAV8 and AAV9 gene transfer in skeletal muscle and heart to facilitate muscular dystrophy gene therapy, we investigated four capsid mutants of AAV8 (Y447F or Y733F) and AAV9 (Y446F or Y731F). The mutants and their wild-type control AAV8 and AAV9 capsids were used to package reporter genes (luciferase or β-galactosidase) resulting in similar vector yields. To evaluate gene delivery efficiencies, especially in muscle and heart, the vectors were compared side by side in a series of experiments *in vivo* in two different strains of mice, the outbred ICR and the inbred C57BL/6. Because AAV8 and AAV9 are among the most effective in systemic gene delivery, we first examined the mutant and wild-type vectors in neonatal mice by intraperitoneal injection, or in adult mice by intravenous injection. To our surprise, no statistically significant differences in transgene expression were observed between the mutant and wild-type vectors, regardless of the reporter genes, vector doses, and the ages and strains of mice used. In addition, quantitative analyses of vector DNA copy number in various tissues from mice treated with mutant and wild-type vectors also showed similar results. Finally, direct intramuscular injection of the above-described vectors with the luciferase gene into the hind limb muscles revealed the same levels of gene expression between mutant and wild-type vectors. Our results thus demonstrate that a single mutation of Y447F or Y733F on capsids of AAV8, and of Y446F or Y731F on AAV9, is insufficient to enhance gene delivery to the skeletal muscle and heart.

Introduction

Adeno-associated viral (AAV) vectors have been increasingly used as a vector of choice for *in vivo* gene delivery and gene therapy for many genetic diseases, such as hemophilia B (Manno *et al.*, 2003; Nathwani *et al.*, 2011), Leber's congenital amaurosis (Hauswirth *et al.*, 2008; Maguire *et al.*, 2008, 2009), Parkinson's disease (Kaplitt *et al.*, 2007), and hereditary muscular dystrophies (Wang *et al.*, 2000; Harper *et al.*, 2002; Mendell *et al.*, 2009, 2010). The lack of pathogenicity of the wild-type virus, the broad tissue tropism, as well as the persistence of transgene expression have bolstered the potential of AAVs as a class of safe and effective delivery vehicles for gene therapy applications (Daya and Berns, 2008). AAV2 was the first AAV serotype that was made into vectors for gene transfer in preclinical and clinical applications (Samulski *et al.*, 1982; Xiao *et al.*, 1999; Flotte *et al.*, 2003; Brantly *et al.*, 2009). Despite some clinical success (Maguire *et al.*, 2008, 2009), AAV2 is limited by the relatively low efficiency of gene transfer, slower onset of gene expression, and higher prevalence of preexisting immunity in human populations (Gao *et al.*, 2005). To overcome such drawbacks, many investigators have successfully explored either naturally occurring (Gao *et al.*, 2002) or genetically engineered and modified (Schaffer and Maheshri, 2004; Muzyczka and Warrington, 2005; Li *et al.*, 2008; Yang *et al.*, 2009) novel AAV capsids, which are the major determinants of tropism and transduction efficiency. To date more than 11 AAV serotypes (Wu *et al.*, 2006) and more than 100 naturally occurring primate AAV variants have been reported (Gao *et al.*, 2005). The efficiency of AAV transduction depends on the efficiency at each step of AAV infection: receptor binding, cell entry, intracellular trafficking, uncoating, second-stranded synthesis, vector genome stabilization, and so on (Daya and Berns,

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2008). Among them, intracellular trafficking (Hauck *et al.*, 2004) and second-strand synthesis (Ferrari *et al.*, 1996) have been identified as the rate-limiting steps (Ding *et al.*, 2005; Keiser *et al.*, 2010). Some serotypes of AAV are naturally more effective than others in this process to escape the endosomes and evade proteasomes in order to finally reach inside the cell nucleus.

One report described a new concept and simple method to enhance AAV particle intracellular trafficking by ablating the surface-exposed tyrosine residues, which have a hydroxyl group as a substrate for phosphorylation and subsequent ubiquitination (Zhong *et al.*, 2008b). It was hypothesized that mutations of the tyrosine to phenylalanine, which lacks the hydroxyl group, should allow the vectors to evade phosphorylation and subsequent ubiquitination and proteasome-mediated degradation. Indeed, mutations of the surface-exposed tyrosine in AAV2 vectors have been reported to dramatically enhance transduction efficiency both *in vitro* and *in vivo* (Zhong *et al.*, 2008a,b; Kauss *et al.*, 2011; Li *et al.*, 2011; Markusic *et al.*, 2011; Petrs-Silva *et al.*, 2011). Additional studies have shown variable degrees of enhancement of gene transfer and expression in other AAV serotypes (Petrs-Silva *et al.*, 2009; Qiao *et al.*, 2010; Dalkara *et al.*, 2011; Pang *et al.*, 2011).

Our laboratory has long been interested in gene therapy for muscular dystrophy and cardiomyopathy. Thus far, AAV8 and AAV9 have been identified as the most efficient vectors in crossing the blood vessel barrier to achieve systemic gene transfer in both skeletal and cardiac muscle (Wang *et al.*, 2005; Inagaki *et al.*, 2006; Kornegay *et al.*, 2010). In this study, we wished to investigate whether tyrosine mutation could enhance the gene transfer efficiencies of AAV8 and AAV9 to the muscle and heart of mice after systemic or intramuscular administration of the vectors. The improved AAV8 and AAV9 vectors should allow for improved transduction efficiency at a lower vector dose requirement and better safety profile. To this end, we have carried out a series of experiments in mice to compare the transduction efficiency of two AAV8 mutants (Y447F or Y733F) and two AAV9 mutants (Y446F or Y731F) with the original wild-type AAV8 and AAV9 counterpart vectors. To our surprise, we did not observe any statistically significant enhancement of transgene expression by mutation of tyrosine to phenylalanine (Y to F) at amino acid residue 447 or 733 on AAV8 and at amino acid residue 446 or 731 on AAV9 capsids. Potential reasons for the negative findings and a dramatic discrepancy between a previous report using identical AAV9 mutants in mice are discussed (Zhang *et al.*, 2009).

Materials and Methods

Recombinant AAV vectors

The AAV8 and AAV9 packaging plasmids containing the original (also termed *wild-type*) capsid gene were obtained from the J. Wilson laboratory of the University of Pennsylvania (Philadelphia, PA) (Bish *et al.*, 2008). The mutant AAV8 and AAV9 packaging plasmids containing the tyrosine (Y)-to-phenylalanine (F) mutations were provided by A. Srivastava of the University of Florida (Gainesville, FL) (Zhong *et al.*, 2008b). The AAV reporter vector plasmids pAAV-CMV-Luc and pAAV-CMV-LacZ (with nuclear localization signal, *nls*) have been described elsewhere (Qiao *et al.*, 2011). Recombinant AAV vectors were generated by the triple-plasmid transfection method (Xiao *et al.*, 1998). Viral vector stocks were purified by polyethylene glycol (PEG) precipitation followed by double CsCl gradient purification (Ayuso *et al.*, 2010). After three changes of dialysis in virus dialysis buffer (1× phosphate-buffered saline [PBS], 2% mannitol, 6 m*M* $MgCl_2$) at room temperature for 6 to 8 hr or at 4°C overnight, vector genome copy titers were determined by DNA dot blot and confirmed by quantitative PCR.

In vivo *gene transfer*

All animal experiments were approved by the institutional animal care and use committee. For the luciferase intramuscular injection and neonatal delivery studies, 6- to 8-week-old male ICR mice were purchased from Taconic (Hudson, NY). An amount equivalent to 3×10^{10} VG/injection was injected directly into the tibialis anterior (TA) and gastrocnemius (GAS) muscles. For neonatal delivery, different doses of AAV vector (1×10^{10} VG/pup for the low-dose group, and 1×10^{11} VG/pup for the high-dose group) were introduced into 3-day-old ICR pups via intraperitoneal injection. For systemic delivery, 3×10^{11} VG total was delivered via tail vein injection into 6- to 8-week-old C57BL/6 (BL6) mice, which were purchase from Jackson Laboratory (Bar Harbor, ME). All mice were killed 3 to 6 weeks posttreatment. There were three mice in each group for all previously mentioned studies.

Luciferase activity assay

Tissues (25–100 mg) were lysed and homogenized in luciferase lysis buffer (0.05% Triton X-100, 0.1 *M* Tris-HCl [pH 7.8], 2 m*M* EDTA) in the presence of proteinase inhibitors (cat. no. p2714; Sigma-Aldrich, St. Louis, MO). The homogenized lysate were extensively vortexed, and spun down at 4°C for 2 min. The supernatant were used for luciferase activity analysis. The analysis was performed according to a previously described protocol (Yu *et al.*, 2009), and a luciferase assay kit (cat. no. E1501) was purchased from Promega (Madison, WI).

Genome copy determination

Total DNA was extracted with a kit (DNeasy blood and tissue kit, cat. no. 69506; Qiagen, Valencia, CA). Vector copy number was determined with a 7300 real-time PCR system (Applied Biosystems, Foster City, CA). TaqMan assays for endogenous control of the glucagon gene were developed to normalize vector copy numbers. The sequences for mouse glucagon primers and probes were as follows: Glucagon-real-F (mouse), AAG GGA CCT TTA CCA GTG ATG TG; Glucagon-real-R (mouse), ACT TAC TCT CGC CTT CCT CGG; TaqMan mouse glucagon probe, FAM-cag caa agg aat tca-MGB. For all the AAV vectors, primers and probes were designed on the common bovine growth hormone (BGH) poly(A) sequence. Their sequences were as follows: BGH-F, AGC CTC GAC TGT GCC TTCTA; BGH-R, ATG CGA TGC AAT TTC CTCAT; BGH probe, FAM-TGC CAG CCA TCT GTTG. The copy number of delivered vector in a specific tissue per diploid cell was calculated as (vector copy number/endogenous control)×2.

X-Gal staining for expression of LacZ and LacZ activity analysis

For 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) staining, the cryo-thin-section slides were air-dried and fixed in X-Gal fixative buffer containing 2% formaldehyde and 0.2% glutaraldehyde in PBS. After washing with PBS, the slides were subjected to X-Gal staining buffer at 37°C overnight. The X-Gal staining buffer consisted of 0.1 *M* phosphate buffer (pH 7.3) supplemented with 2 m*M* $MgCl_2$, 5 m*M* potassium ferrocyanide (cat. no. P-9287; Sigma-Aldrich) and 5 m*M* potassium ferricyanide (cat. no. P-8131; Sigma-Aldrich). Before use, X-Gal was added at a final concentration of 1 mg/ml (Qiao *et al.*, 2002).

To quantitate LacZ expression, we used a Galacto-Light Plus kit (cat. no. T1007; Applied Biosystems). The manufacturer's protocol was strictly followed. The relative units of LacZ activity were normalized by total protein concentration, which was assessed with a Pierce BCA protein assay kit (cat. no. 23227; Pierce Biotechnology, Rockford, IL).

Statistical analysis

Data are presented as means ± standard deviation. Comparison among groups was performed by one-way analysis of variance (ANOVA) followed by Dunnett post hoc test (compare all columns vs. control group). $p < 0.05$ was considered statistically significant.

Results

Systemic gene delivery in neonatal ICR mice by intraperitoneal injection

Different genetic backgrounds of mice may occasionally lead to different outcomes of AAV gene transfer. We therefore evaluated the Y to F-mutated AAV8 and AAV9 vectors in outbred ICR mice as well as in inbred C57BL/6 mice. We started with neonatal and adult ICR mice, using two reporter genes: firefly luciferase (Luc) and β-galactosidase (LacZ). The Luc reporter provides a broad liner range for quantitative analysis, whereas the LacZ reporter can be used to visualize the individual cells in tissues and also to quantitate β-galactosidase activities. Both reporter genes were controlled by the strong ubiquitous cytomegalovirus (CMV) promoter. The mutant AAV8 (Y447F or Y733F) and AAV9 (Y446F or Y731F) packaging plasmids were generously provided by A. Srivastava (Zhong *et al.*, 2008b). The mutations were independently confirmed by DNA sequencing. There was no difference in viral vector yields among all four Y-to-F mutants and their wild-type AAV8 and AAV9 controls, suggesting that the point mutations did not compromise the vector packaging capacity.

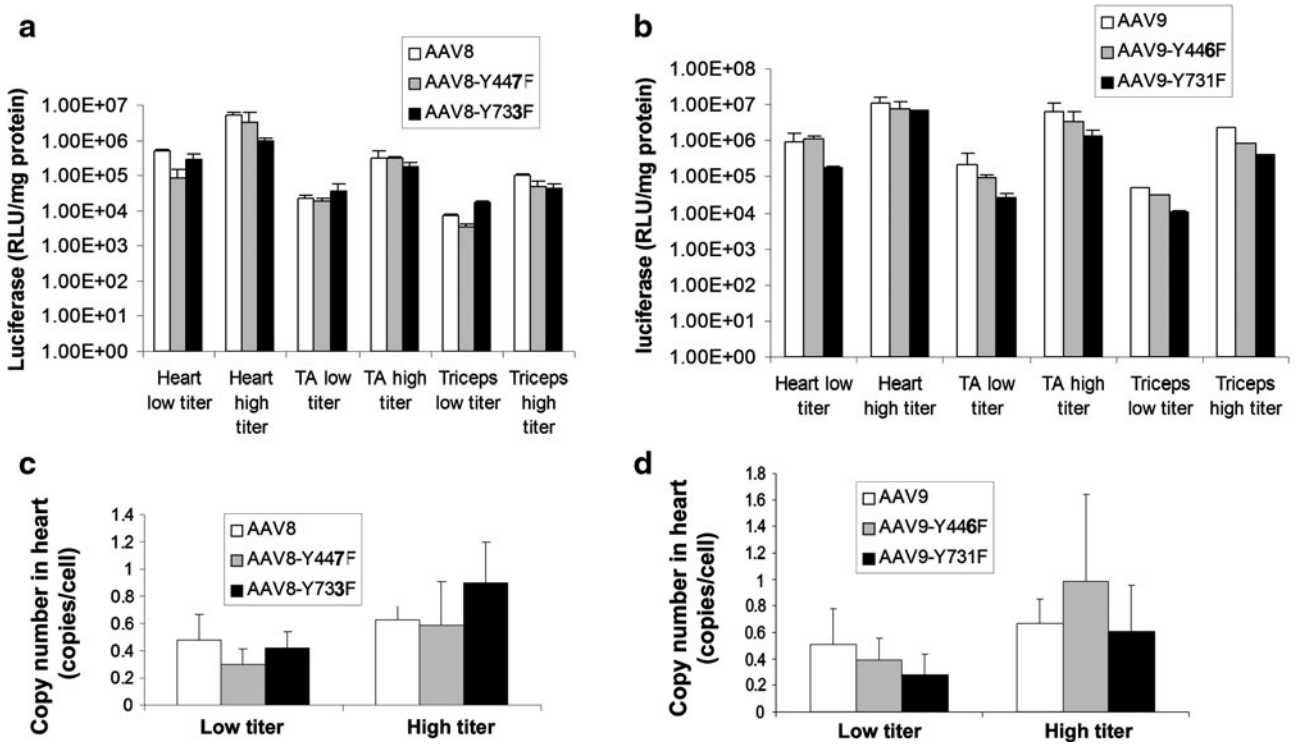

FIG. 1. Comparison of Y-to-F mutants and wild-type AAV8 and AAV9 vectors encoding luciferase by systemic delivery in neonatal ICR mice. The mutants and wild-type Luc vectors controlled by the CMV promoter were injected intraperitoneally into 3-day-old ICR mice (low dose, 1×10^{10} VG/mouse; high dose, 1×10^{11} VG/mouse). The mice were killed 6 weeks later for analyses of luciferase activities in samples from the heart, tibialis anterior (TA) and triceps muscles, and vector DNA copy numbers in the heart. **(a)** Luciferase activities of mice treated with mutant and wild-type AAV8-Luc vectors ($p > 0.05$ by one-way ANOVA, $n = 3$). RLU, relative light units. **(b)** Luciferase activities of mice treated with mutant and wild-type AAV9-Luc vectors ($p > 0.05$ by one-way ANOVA, $n = 3$). **(c)** DNA copy numbers in heart tissues from mice treated with mutant and wild-type AAV8-Luc vectors ($p > 0.05$, one-way ANOVA followed by Dunnett post hoc test, $n = 3$). **(d)** DNA copy numbers in heart tissues from mice treated with mutant and wild-type AAV9-Luc vectors ($p > 0.05$ by one-way ANOVA, $n = 3$).

We first evaluated the four mutants and wild-type AAV8 and AAV9 vectors in 3-day-old neonatal ICR mice by intraperitoneal injection, which is the most convenient approach to achieve systemic delivery to the skeletal muscle and heart with minimal vector dose requirement yet robust gene transfer efficiency (Wang *et al.*, 2005, 2009). Two vector doses were used (low dose, 1×10^{10} VG/mouse; high dose, 1×10^{11} VG/mouse) for all six AAV vectors (AAV8-Luc, AAV8-Y447F-Luc, AAV8-Y733F-Luc, AAV9-Luc, AAV9-Y446F-Luc, and AAV9-Y731F-Luc). Six weeks postinjection, the mice were killed and various muscle tissues and major organs were carefully dissected and immediately snap-frozen. Half of the muscle tissue samples were used for the luciferase activity assay and the other half for DNA extraction and quantitative analysis of vector copy numbers. To our surprise, the luciferase activity assay revealed no significant enhancement for any of the Y-to-F mutants when compared with their parental wild-type AAV8 and AAV9 controls (Fig. 1a and b). Subsequently, quantitative real-time PCR analyses were performed to evaluate vector DNA copy numbers in heart tissue, because both AAV8 and AAV9, especially AAV9, are effective in cardiac gene transfer by systemic delivery. Again, no significant and consistent differences in vector DNA copy numbers between mutant and wild-type vectors were observed (Fig. 1c and d) ($p>0.05$, one-way ANOVA followed by Dunnett post hoc test). The vector copy numbers are in general agreement with transgene expression levels, suggesting consistent vector binding and intracellular trafficking among the mutant and wild-type vectors.

In addition to luciferase, we also used the LacZ reporter to evaluate the mutant AAV8 and AAV9 vectors in neonatal ICR mice. Six AAV vectors (AAV8-LacZ, AAV8-Y447F-LacZ, AAV8-Y733F-LacZ, AAV9-LacZ, AAV9-Y446F-LacZ, and AAV9-Y731F-LacZ) were injected intraperitoneally into 3-day-old ICR pups at a dose of 5×10^{10} VG/mouse. The mice were killed 6 weeks after treatment. No significant enhancement of LacZ expression by mutant AAV8 and AAV9 vectors over their wild-type controls was observed in , heart, liver, or muscles (Fig. 2a and b). These results are in agreement with the findings in the luciferase experiments.

Systemic gene delivery in adult ICR mice by intravenous injection

We next investigated systemic delivery in adult ICR mice to further evaluate the performance of the mutant AAV8 and AAV9 vectors. The same six AAV-LacZ vectors used in the neonatal experiments were administered in 6- to 8-week-old ICR adult mice by tail vein injection at a vector dose of 3×10^{11} VG/mouse. Three weeks postinjection, the mice were killed and tissues were collected and analyzed. X-Gal staining of cryo-thin sections of the heart, liver, and muscles did not reveal any significant difference in LacZ expression between the mutant and wild-type vectors (Fig. 3a). Consistently, quantitative analysis of LacZ enzyme activities in the tissue homogenates revealed no statistically significant differences either between those vectors ($p>0.05$, one-way ANOVA) (Fig. 3b).

Systemic gene delivery in adult C57BL/6 mice by intravenous injection

In an earlier report C57BL/6 mice were used to examine the same Y-to-F mutants of AAV9 capsids used in our studies. However, highly dramatic enhancement of gene delivery by AAV9-Y731F was reported (Zhang *et al.*, 2009). For consistency we also used C57BL/6 mice in our study to rule out the possibility that different mouse strains gave rise to different outcomes. Similar to the experiments done in adult ICR mice, the same six AAV-CMV-Luc vectors of both mutant and wild-type AAV8 and AAV9 were injected into 6- to 8-week-old C57BL/6 mice via the tail vein, at a vector dose of 3×10^{11} VG/mouse. In addition, AAV-CMV-LacZ vectors of mutant and wild-type AAV9 were also injected into 6- to 8-week-old C57BL/6 mice via the tail vein, but in two dose groups (1×10^{11} and 3×10^{11} VG/mouse). The mice were killed 3 weeks postinjection. Once again, we did not observe any significant difference in either luciferase expression ($p>0.05$, one-way ANOVA; Fig. 4) or LacZ expression ($p>0.05$, one-way ANOVA; Fig. 5a and b) between mutant and wild-type AAV8 and AAV9 vectors. In addition, quantitative real-time PCR analysis of the LacZ vector DNA isolated from tissues did not reveal statistically significant difference in DNA copy numbers between mutant and wild-type vectors ($p>0.05$, one-way ANOVA; Fig. 5c and d). These results strongly demonstrated that the strains of mice did not make appreciable differences in the *in vivo* examination of the mutant and wild-type AAV8 and AAV9 vectors.

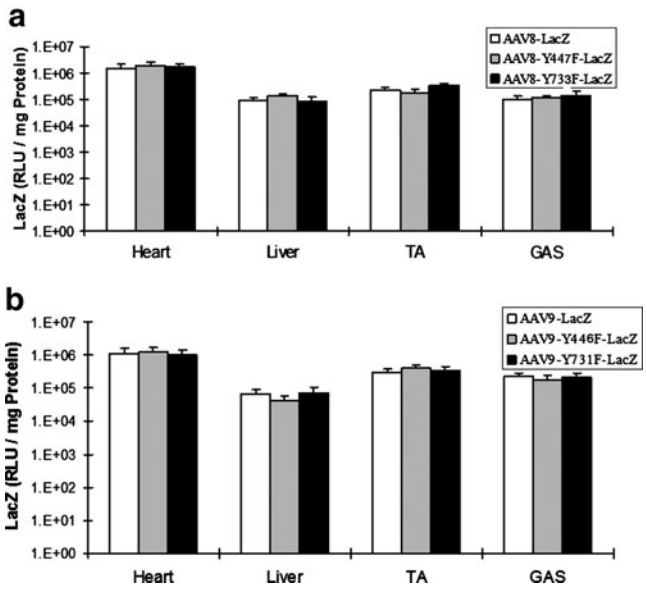

FIG. 2. Comparison of Y-to-F mutants and wild-type AAV8 and AAV9 vectors encoding LacZ reporter by systemic delivery into neonatal ICR mice. Mutants and wild-type LacZ-vectors controlled by the CMV promoter were injected intraperitoneally into 3-day-old ICR mice at a dose of 5×10^{10} VG/mouse. The mice were killed 6 weeks later for analysis of β-galactosidase activities in samples from heart, liver, and tibialis anterior (TA) and gastrocnemius (GAS) muscles ($p>0.05$ by one-way ANOVA, $n=3$).

Local intramuscular injection in ICR mice

Previously, we have observed that two tyrosine mutants, AAV6-Y445F and AAV6-Y731F, achieved enhanced gene transfer and expression of luciferase reporter gene by a few fold over their parental wild-type AAV6 vectors after

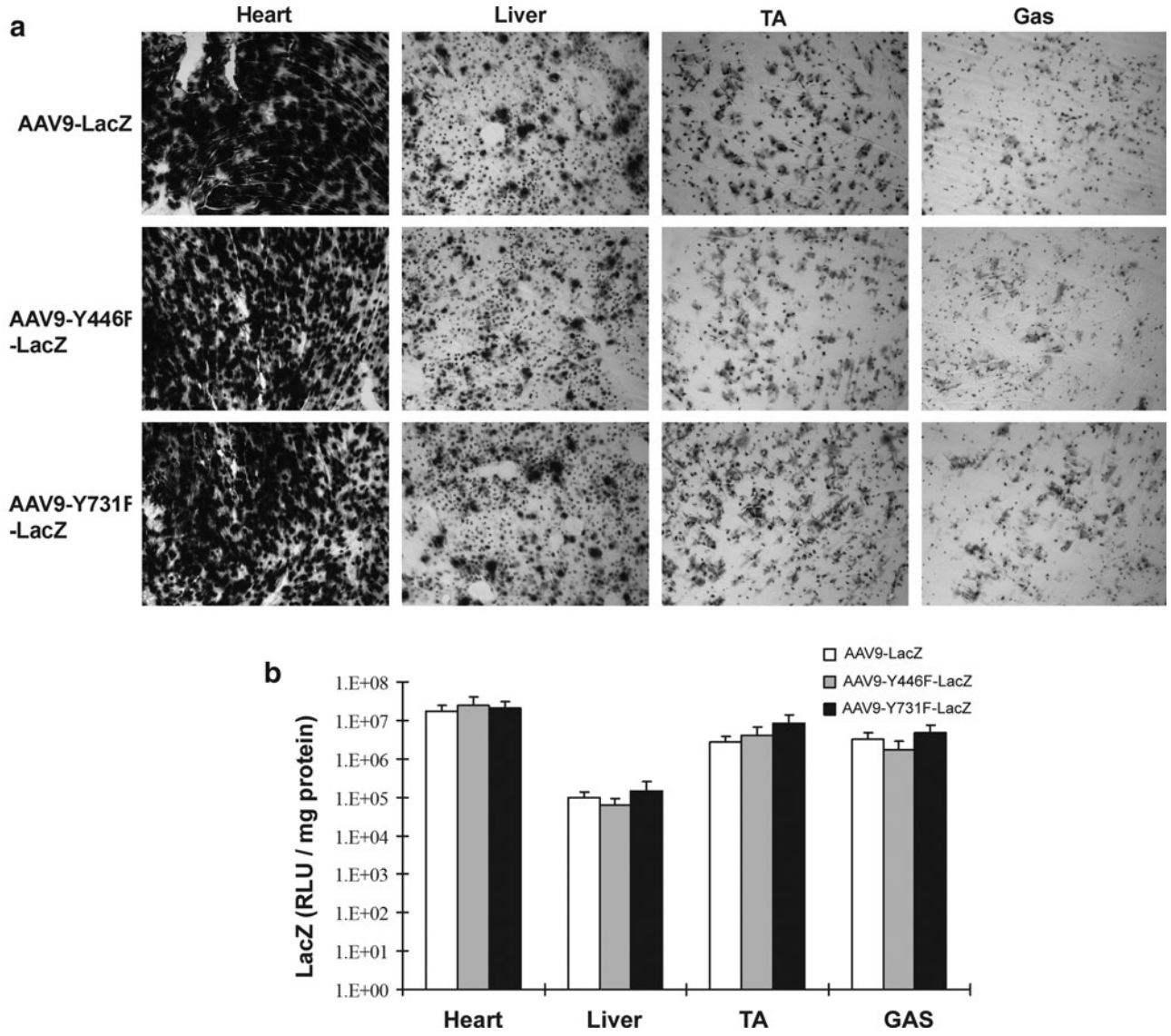

FIG. 3. Comparison of Y-to-F mutants and wild-type AAV9 vectors encoding LacZ reporter by systemic delivery in adult ICR mice. The mutants and wild-type LacZ vectors controlled by the CMV promoter were injected via the tail vein into 6- to 8-week-old ICR mice at a dose of 3×10^{11} VG/mouse. The mice were killed 3 weeks postinjection. **(a)** Photographs of X-Gal staining of representative tissues. Note that the LacZ vectors contained a nucleus localization signal (*nls*) and show nuclear staining. TA, tibialis anterior; Gas, gastrocnemius. **(b)** Quantitation of LacZ expression by β-galactosidase activity assay ($p>0.05$ by one-way ANOVA, $n=3$).

intramuscular injection in the ICR mice (Qiao *et al.*, 2010). The enhancement was more appreciable at low vector doses. To determine whether similar enhancement could be achieved with the mutant AAV8 and AAV9 vectors, we carried out local intramuscular injection that delivered viral particles directly to the muscle tissues without the involvement of a blood vessel barrier. Low vector doses were used to avoid dose saturation. For each mouse, two muscles of the left hind leg, the tibialis anterior (TA) and gastrocnemius (GAS), were injected with 3×10^{10} VG/site in a 50-μl vector volume. Three weeks postinjection, the muscles were collected and subjected to luciferase activity assay. As shown in Fig. 6, we could not observe meaningful enhancement of transgene expression in any mouse in the Y-to-F mutant group. It appeared to have a trend of enhanced gene expression in AAV9-Y446F mutant vector-treated TA muscles, but the difference did not reach statistical significance (AAV9 comparison, $p=0.109$; AAV8 comparison, $p=0.6888$ by one-way ANOVA). These results suggest that the surface-exposed tyrosine mutation in AAV8 and AAV9 capsids could not significantly enhance their gene transfer efficiency in skeletal muscle tissues in mice.

Discussion

Muscular dystrophy gene therapy requires therapeutic gene transfer to most of the striated muscle cells in a body-wide manner to achieve desirable efficacies in patients. Muscle is the largest organ in the body and its demand for gene therapy vector is also the highest. For systemic gene

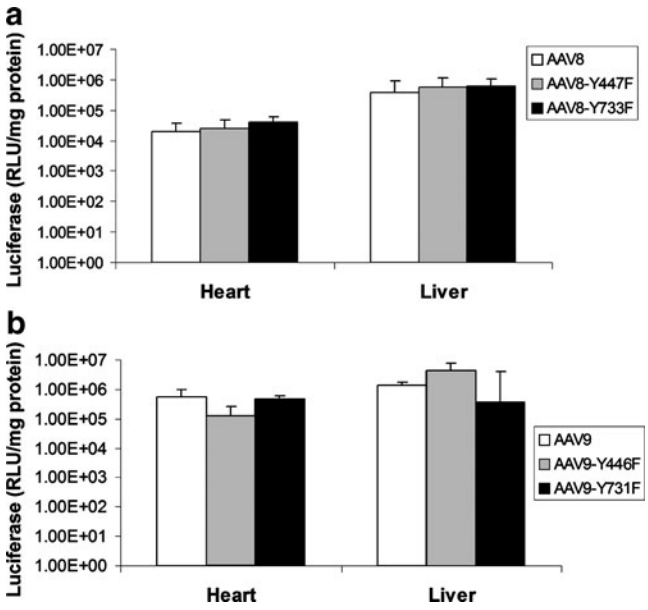

FIG. 4. Comparison of Y-to-F mutants and wild-type AAV8 and AAV9 vectors encoding luciferase in adult C57BL/6 mice. The mutant and wild-type Luc vectors controlled by the CMV promoter were injected via the tail vein into 6- to 8-week-old C57BL/6 mice at a dose of 3×10^{11} VG/mouse. The mice were killed 3 weeks postinjection ($p>0.05$ for all comparisons by one-way ANOVA, $n=3$). **(a)** Luciferase expression of wild-type and mutant AAV8 vectors. **(b)** Luciferase expression of wild-type and mutant AAV9 vectors.

delivery, AAV viral particles must cross the tight endothelial barrier before reaching the surface receptors on muscle cells. This places the bar even higher for AAV vectors to overcome. Because high vector doses are often required for muscle disease gene therapy in large animals (Kornegay *et al.*, 2010) and eventually in human patients, improvement in every step of the gene transfer process counts. The tyrosine-to-phenylalanine mutation was intended to overcome one of the rate-limiting steps in AAV infection, that is, intracellular trafficking. The design and principle behind the strategy is to reduce ubiquitination sites on the viral particles to minimize degradation, so that more of them can survive and emerge out of the trafficking pathway and enter the nucleus for transgene expression. AAV2 was the first serotype examined by this new method. Ablation of surface-exposed tyrosines dramatically enhanced gene transfer and expression, reported as more than 10-fold and up to 30-fold in some *in vitro* and *in vivo* experiments involving tissues such as the eye, liver, and hematopoietic cells (Zhong *et al.*, 2008a,b; Kauss *et al.*, 2011; Li *et al.*, 2011; Markusic *et al.*, 2011; Petrs-Silva *et al.*, 2011). The Y-to-F mutants of other serotypes, such as AAV8 and AAV9, have also been reported to variably increase gene expression in the eye and brain (Petrs-Silva *et al.*, 2009; Dalkara *et al.*, 2011; Pang *et al.*, 2011). AAV6 mutants also significantly enhanced gene expression in skeletal muscle by direct intramuscular injection, but not by systemic routes in either neonatal or adult mice. Mutant AAV6-Y445F was more effective than AAV6-Y731F. The enhancement effects varied in different muscle groups. For example, tibialis anterior muscle was more effectively treated than gastrocnemius muscle (Qiao *et al.*, 2010). Because AAV8 and AAV9

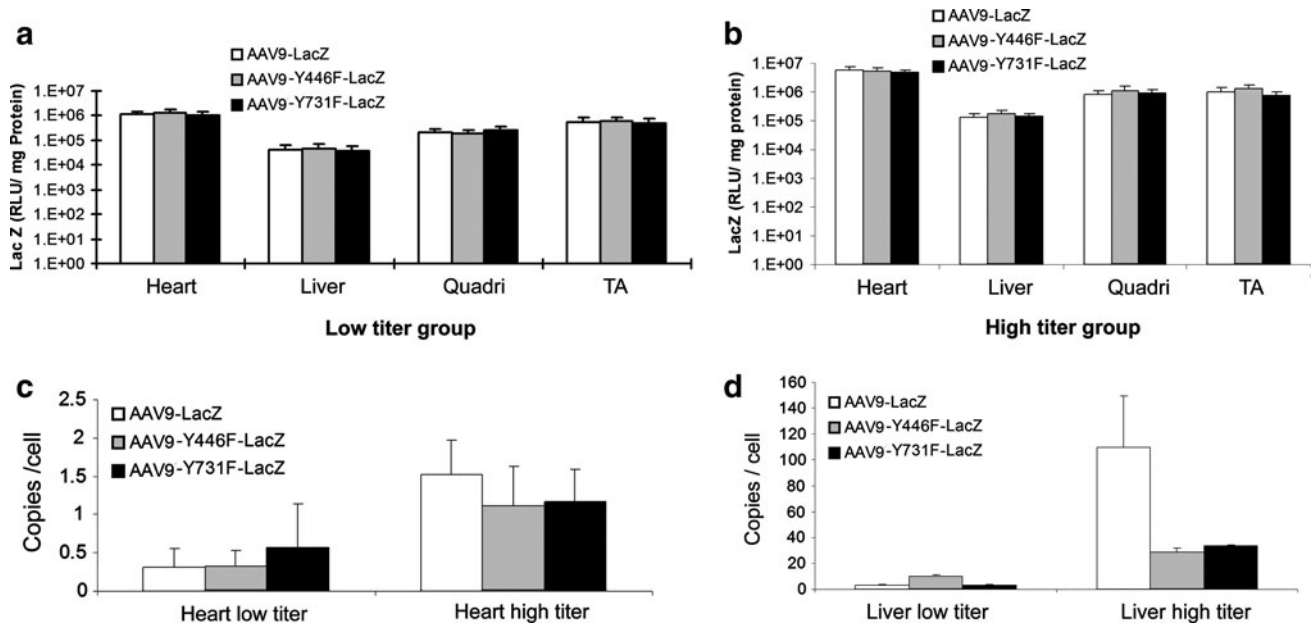

FIG. 5. Comparison of Y-to-F mutants and wild-type AAV9 vectors encoding LacZ in adult C57BL/6 mice. The mutant and wild-type LacZ vectors encoding nuclear LacZ were injected via the tail vein into 6- to 8-week-old C57BL/6 mice at two vector doses (low titer, 1×10^{11} VG/mouse; high titer, 3×10^{11} VG/mouse). The mice were killed 3 weeks postinjection ($p>0.05$ for all comparisons between mutant and wild-type vectors by one-way ANOVA, $n=3$). **(a)** Quantitation of LacZ expression by β-galactosidase activity assay in the low-titer group. **(b)** Quantitation of LacZ expression in the high-titer group. **(c)** Vector DNA copy numbers in heart tissue. **(d)** Vector DNA copy numbers in liver tissue.

mutant vectors are among the most effective vectors for muscle and heart gene transfer by systemic administration, we tested four mutants of AAV8 and AAV9, two for each, to determine whether the Y-to-F mutations could also greatly enhance systemic gene delivery to the muscle and heart. As described in Results, however, we did not find any statistically significant improvement in multiple experiments with two reporter genes at different doses, two strains of mice, and two different age groups (neonate and adult) during vector administration. In addition, we did not observe any enhancement effect in cultured U87 cells *in vitro* using the tyrosine mutant AAV8 and AAV9 vectors with both LacZ and Luc reporter genes (Supplementary Fig. S1; supplementary data are available online at www.liebertonline.com/hgtb). The negative findings are somewhat disappointing, but nonetheless informative and useful.

There could be a number of potential reasons and plausible explanations for the negative findings. First of all, the basis of the Y-to-F mutation is to enhance AAV particle intracellular trafficking. If a certain serotype of AAV in a given tissue/cell type is already efficient in this process, one would not expect significant improvement after all. For example, AAV8 is one of the most robust vectors for liver gene delivery. AAV8 is also found effective in intracellular trafficking and uncoating. On the other hand, AAV2 is ineffective in the above-cited processes in the liver and therefore poor in hepatic gene transfer, despite the fact that its receptor binding and entry into liver cells are nearly as effective as those of AAV8 (Thomas *et al.*, 2004). Given our observation that the Y-to-F mutations did not enhance AAV8 and AAV9 in liver, muscle, and heart, we suspect that intracellular trafficking, in particular the escape of proteasomal degradation, may not be the rate-limiting step for these two vectors in the above-cited tissues. Other steps, instead, such as cell surface receptor distribution in different cell types and tissues, may be more critical. In fact, the discovery of the AAV9 primary receptor, terminal N-linked galactose, strongly supports this scenario (Bell *et al.*, 2011; Shen *et al.*, 2011). These authors showed that treatment of tissues, for example, the airway, with recombinant sialidase to expose more terminal N-linked galactose residues dramatically enhanced AAV9 gene transfer to airway cells. On the other hand, the primary receptor for AAV2 is heparan sulfate proteoglycan (Summerford and Samulski, 1998), which is universally distributed in nearly all cell types but with variable abundance. It was observed that AAV2 preferentially infected skeletal muscle slow-twitch myofibers, which display more abundant heparin molecules (Pruchnic *et al.*, 2000). It remains elusive why AAV6 Y-to-F mutants were more effective in tibialis anterior muscle than in gastrocnemius muscle by intramuscular injection, and why they showed no effects by systemic administration (Qiao *et al.*, 2010).

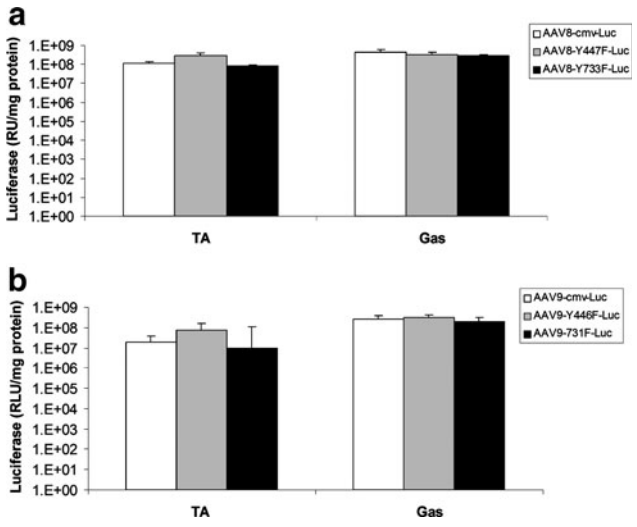

FIG. 6. Direct intramuscular injection of mutant and wild-type AAV8 and AAV9 vectors encoding luciferase into adult ICR mice. For each ICR mouse, two injection sites positioned in the middle belly of the tibialis anterior (TA) and gastrocnemius (Gas) muscles received AAV vector at a dose of 3×10^{10} VG in a 50-μl volume per site. Luciferase activities in the injected muscles were measured 3 weeks later and no significant difference was found ($p>0.05$ for all comparisons by one-way ANOVA, $n=3$). **(a)** Luciferase expression mediated by wild-type and mutant AAV8 vectors. **(b)** Luciferase expression mediated by wild-type and mutant AAV9 vectors.

Another possibility is that simultaneous ablations of multiple tyrosine residues are required in order to achieve significant enhancement of gene transfer by AAV8 and AAV9 vectors in muscle and heart. Although a single Y-to-F mutation enhanced AAV2 gene transfer by more than 1 log, triple mutation enhanced gene transfer efficiencies even more (Markusic *et al.*, 2011) *in intro* and also in the eye, liver, and muscle (Jayandharan *et al.*, 2011). One report showed that a double mutation of Y446F and Y731F enhanced AAV9 gene transfer in the retina and brain after systemic injection into neonatal mice, but without mentioning other tissues (Dalkara *et al.*, 2011). The results suggested that ubiquitination and viral particle degradation could be a rate-limiting step for AAV9-mediated gene transfer in the retina. However, it remains to be investigated whether it holds true in muscle, heart, and liver.

Finally, our negative finding of AAV8 and AAV9 mutants in mouse skeletal muscle and heart is also in stark contrast to the results of one report (Zhang *et al.*, 2009), despite the fact that both laboratories used the same mutants provided by the Srivastava laboratory. However, Zhang and colleagues observed multiple fold enhancement by the mutants, especially the AAV9-Y731F mutant, which rendered more than 10-fold enhancement over the wild-type AAV9 vector. Histochemical staining of the reporter alkaline phosphatase gene expression in thin sections of muscle and heart displayed even more dramatic enhancement. To reconcile the large discrepancy, we have searched for possible factors that may have contributed to the different results. One possible factor is the difference in mouse strains. In our experiments we initially used outbred ICR mice instead of C57BL/6 mice. However, when we repeated our experiments in adult C57BL/6 mice, no enhancement by the mutant AAV8 and AAV9 vectors was observed either (Figs. 4 and 5), ruling out mouse strain as a contributing factor. A second possible factor is the difference in reporter genes, that is, luciferase and LacZ versus human placenta alkaline phosphatase. We obtained essentially consistent results using both luciferase and LacZ reporter genes. The luciferase assay is well known for its low background whereas our LacZ has a nucleus localization signal, which displays nuclear staining that was

clearly shown in the tissue thin-section X-Gal staining (Fig. 3a). In skeletal muscle cells with their large cytoplasm, incomplete translation of LacZ to the nucleus could be seen, which was not due to background staining. By contrast, endogenous alkaline phosphatase activities are high in mouse tissues, which require heat inactivation to bleach the high background. If the process somehow failed, high background staining would occur. Other factors including incorrect vector titers, dilutions, and inconsistent vector potency from batch to batch would also contribute to inconsistent results. Although the precise reason(s) remain to be further investigated, we believe that it is informative to publish a negative finding so that other researchers will take it into consideration when designing and choosing vectors, in order to minimize unnecessary duplicated efforts and expense.

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Author Disclosure Statement

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References

Ayuso, E., Mingozzi, F., Montane, J., *et al.* (2010). High AAV vector purity results in serotype- and tissue-independent enhancement of transduction efficiency. Gene Ther. 17, 503–510.

Bell, C.L., Vandenberghe, L.H., Bell, P., *et al.* (2011). The AAV9 receptor and its modification to improve *in vivo* lung gene transfer in mice. J. Clin. Invest. 121, 2427–2435.

Bish, L.T., Morine, K., Sleeper, M.M., *et al.* (2008). Adeno-associated virus (AAV) serotype 9 provides global cardiac gene transfer superior to AAV1, AAV6, AAV7, and AAV8 in the mouse and rat. Hum. Gene Ther. 19, 1359–1368.

Brantly, M.L., Chulay, J.D., Wang, L., *et al.* (2009). Sustained transgene expression despite T lymphocyte responses in a clinical trial of rAAV1-AAT gene therapy. Proc. Natl. Acad. Sci. U.S.A. 106, 16363–16368.

Dalkara, D., Byrne, L.C., Lee, T., *et al.* (2011). Enhanced gene delivery to the neonatal retina through systemic administration of tyrosine-mutated AAV9. Gene Ther. (in press).

Daya, S., and Berns, K.I. (2008). Gene therapy using adeno-associated virus vectors. Clin. Microbiol. Rev. 21, 583–593.

Ding, W., Zhang, L., Yan, Z., and Engelhardt, J.F. (2005). Intracellular trafficking of adeno-associated viral vectors. Gene Ther. 12, 873–880.

Ferrari, F.K., Samulski, T., Shenk, T., and Samulski, R.J. (1996). Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. J. Virol. 70, 3227–3234.

Flotte, T.R., Zeitlin, P.L., Reynolds, T.C., *et al.* (2003). Phase I trial of intranasal and endobronchial administration of a recombinant adeno-associated virus serotype 2 (rAAV2)-CFTR vector in adult cystic fibrosis patients: A two-part clinical study. Hum. Gene Ther. 14, 1079–1088.

Gao, G., Vandenberghe, L.H., and Wilson, J.M. (2005). New recombinant serotypes of AAV vectors. Curr. Gene Ther. 5, 285–297.

Gao, G.P., Alvira, M.R., Wang, L., *et al.* (2002). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. Proc. Natl. Acad. Sci. U.S.A. 99, 11854–11859.

Harper, S.Q., Hauser, M.A., Dellorusso, C., *et al.* (2002). Modular flexibility of dystrophin: Implications for gene therapy of Duchenne muscular dystrophy. Nat. Med. 8, 253–261.

Hauck, B., Zhao, W., High, K., and Xiao, W. (2004). Intracellular viral processing, not single-stranded DNA accumulation, is crucial for recombinant adeno-associated virus transduction. J. Virol. 78, 13678–13686.

Hauswirth, W.W., Aleman, T.S., Kaushal, S., *et al.* (2008). Treatment of Leber congenital amaurosis due to *RPE65* mutations by ocular subretinal injection of adeno-associated virus gene vector: Short-term results of a phase I trial. Hum. Gene Ther. 19, 979–990.

Inagaki, K., Fuess, S., Storm, T.A., *et al.* (2006). Robust systemic transduction with AAV9 vectors in mice: Efficient global cardiac gene transfer superior to that of AAV8. Mol. Ther. 14, 45–53.

Jayandharan, G.R., Aslanidi, G., Martino, A.T., *et al.* (2011). Activation of the NF-κB pathway by adeno-associated virus (AAV) vectors and its implications in immune response and gene therapy. Proc. Natl. Acad. Sci. U.S.A. 108, 3743–3748.

Kaplitt, M.G., Feigin, A., Tang, C., *et al.* (2007). Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne *GAD* gene for Parkinson's disease: An open label, phase I trial. Lancet 369, 2097–2105.

Kauss, M.A., Smith, L.J., Zhong, L., *et al.* (2011). Enhanced long-term transduction and multilineage engraftment of human hematopoietic stem cells transduced with tyrosine-modified recombinant adeno-associated virus serotype 2. Hum. Gene Ther. 21, 1129–1136.

Keiser, N.W., Yan, Z., Zhang, Y., *et al.* (2010). Unique characteristics of AAV1, 2, and 5 viral entry, intracellular trafficking, and nuclear import define transduction efficiency in HeLa cells. Hum. Gene Ther. 22, 1433–1444.

Kornegay, J.N., Li, J., Bogan, J.R., *et al.* (2010). Widespread muscle expression of an AAV9 human mini-dystrophin vector after intravenous injection in neonatal dystrophin-deficient dogs. Mol. Ther. 18, 1501–1508.

Li, M., Jayandharan, G.R., Li, B., *et al.* (2011). High-efficiency transduction of fibroblasts and mesenchymal stem cells by tyrosine-mutant AAV2 vectors for their potential use in cellular therapy. Hum. Gene Ther. 21, 1527–1543.

Li, W., Asokan, A., Wu, Z., *et al.* (2008). Engineering and selection of shuffled AAV genomes: A new strategy for producing targeted biological nanoparticles. Mol. Ther. 16, 1252–1260.

Maguire, A.M., Simonelli, F., Pierce, E.A., *et al.* (2008). Safety and efficacy of gene transfer for Leber's congenital amaurosis. N. Engl. J. Med. 358, 2240–2248.

Maguire, A.M., High, K.A., Auricchio, A., *et al.* (2009). Age-dependent effects of *RPE65* gene therapy for Leber's congenital amaurosis: A phase 1 dose-escalation trial. Lancet 374, 1597–1605.

Manno, C.S., Chew, A.J., Hutchison, S., *et al.* (2003). AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. Blood 101, 2963–2972.

Markusic, D.M., Herzog, R.W., Aslanidi, G.V., *et al.* (2011). High-efficiency transduction and correction of murine hemophilia B using AAV2 vectors devoid of multiple surface-exposed tyrosines. Mol. Ther. 18, 2048–2056.

Mendell, J.R., Rodino-Klapac, L.R., Rosales-Quintero, X., *et al.* (2009). Limb-girdle muscular dystrophy type 2D gene therapy restores α-sarcoglycan and associated proteins. Ann. Neurol. 66, 290–297.

Mendell, J.R., Campbell, K., Rodino-Klapac, L., *et al.* (2010). Dystrophin immunity in Duchenne's muscular dystrophy. N. Engl. J. Med. 363, 1429–1437.

Muzyczka, N., and Warrington, K.H., Jr. (2005). Custom adeno-associated virus capsids: The next generation of recombinant vectors with novel tropism. Hum. Gene Ther. 16, 408–416.

Nathwani, A.C., Tuddenham, E.G., Rangarajan, S., *et al.* (2011). Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. N. Engl. J. Med. 365, 2357–2365.

Pang, J.J., Dai, X., Boye, S.E., *et al.* (2011). Long-term retinal function and structure rescue using capsid mutant AAV8 vector in the rd10 mouse, a model of recessive retinitis pigmentosa. Mol. Ther. 19, 234–242.

Petrs-Silva, H., Dinculescu, A., Li, Q., *et al.* (2009). High-efficiency transduction of the mouse retina by tyrosine-mutant AAV serotype vectors. Mol. Ther. 17, 463–471.

Petrs-Silva, H., Dinculescu, A., Li, Q., *et al.* (2011). Novel properties of tyrosine-mutant AAV2 vectors in the mouse retina. Mol. Ther. 19, 293–301.

Pruchnic, R., Cao, B., Peterson, Z.Q., *et al.* (2000). The use of adeno-associated virus to circumvent the maturation-dependent viral transduction of muscle fibers. Hum. Gene Ther. 11, 521–536.

Qiao, C., Wang, B., Zhu, X., *et al.* (2002). A novel gene expression control system and its use in stable, high-titer 293 cell-based adeno-associated virus packaging cell lines. J. Virol. 76, 13015–13027.

Qiao, C., Zhang, W., Yuan, Z., *et al.* (2010). Adeno-associated virus serotype 6 capsid tyrosine-to-phenylalanine mutations improve gene transfer to skeletal muscle. Hum. Gene Ther. 21, 1343–1348.

Qiao, C., Yuan, Z., Li, J., *et al.* (2011). Liver-specific microRNA-122 target sequences incorporated in AAV vectors efficiently inhibits transgene expression in the liver. Gene Ther. 18, 403–410.

Samulskim, R.J., Berns, K.I., Tan, M., and Muzyczka, N. (1982). Cloning of adeno-associated virus into pBR322: Rescue of intact virus from the recombinant plasmid in human cells. Proc. Natl. Acad. Sci. U.S.A. 79, 2077–2081.

Schaffer, D.V., and Maheshri, N. (2004). Directed evolution of AAV mutants for enhanced gene delivery. Conf. Proc. IEEE Eng. Med. Biol. Soc. 5, 3520–3523.

Shen, S., Bryant, K.D., Brown, S.M., *et al.* (2011). Terminal N-linked galactose is the primary receptor for adeno-associated virus 9. J. Biol. Chem. 286, 13532–13540.

Summerford, C., and Samulski, R.J. (1998). Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. J. Virol. 72, 1438–1445.

Thomas, C.E., Storm, T.A., Huang, Z., and Kay, M.A. (2004). Rapid uncoating of vector genomes is the key to efficient liver transduction with pseudotyped adeno-associated virus vectors. J. Virol. 78, 3110–3122.

Wang, B., Li, J., and Xiao, X. (2000). Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy in *mdx* mouse model. Proc. Natl. Acad. Sci. U.S.A. 97, 13714–13719.

Wang, B., Li, J., Fu, F.H., and Xiao, X. (2009). Systemic human minidystrophin gene transfer improves functions and life span of dystrophin and dystrophin/utrophin-deficient mice. J. Orthop. Res. 27, 421–426.

Wang, Z., Zhu, T., Qiao, C., *et al.* (2005). Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. Nat. Biotechnol. 23, 321–328.

Wu, Z., Asokan, A., and Samulski, R.J. (2006). Adeno-associated virus serotypes: Vector toolkit for human gene therapy. Mol. Ther. 14, 316–327.

Xiao, W., Chirmule, N., Berta, S.C., *et al.* (1999). Gene therapy vectors based on adeno-associated virus type 1. J. Virol. 73, 3994–4003.

Xiao, X., Li, J., and Samulski, R.J. (1998). Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. J. Virol. 72, 2224–2232.

Yang, L., Jiang, J., Drouin, L.M., *et al.* (2009). A myocardium tropic adeno-associated virus (AAV) evolved by DNA shuffling and *in vivo* selection. Proc. Natl. Acad. Sci. U.S.A. 106, 3946–3951.

Yu, C.Y., Yuan, Z., Cao, Z., *et al.* (2009). A muscle-targeting peptide displayed on AAV2 improves muscle tropism on systemic delivery. Gene Ther. 16, 953–962.

Zhang, W., Yue, Y., Ghosh, A., *et al.* (2009). Novel tyrosine mutant vectors expand the utilities of AAV-mediated muscle gene therapy. Poster 453, presented at the annual meeting of the American Society of Gene and Cell Therapy, May 29, 2009, San Diego, CA.

Zhong, L., Li, B., Jayandharan, G., *et al.* (2008a). Tyrosine-phosphorylation of AAV2 vectors and its consequences on viral intracellular trafficking and transgene expression. Virology 381, 194–202.

Zhong, L., Li, B., Mah, C.S., *et al.* (2008b). Next generation of adeno-associated virus 2 vectors: point mutations in tyrosines lead to high-efficiency transduction at lower doses. Proc. Natl. Acad. Sci. U.S.A. 105, 7827–7832.

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