K137R Mutation on Adeno-Associated Viral Capsids Had Minimal Effect on Enhancing Gene Delivery *In Vivo*

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Abstract

The adeno-associated viral (AAV) vector has emerged as an attractive vector for gene therapy applications. Development of AAV vectors with enhanced gene transduction efficiency is important to ease the burden of AAV production and minimize potential immune responses. Rational mutations on AAV capsids have gained attention as a simple method of enhancing AAV transduction efficiency. A single-amino acid mutation, K137R, on AAV1 and AAV8 was recently reported to increase liver transgene expression by 5–10-fold. To determine whether the same mutation on other AAV serotypes would result in similar gene enhancement effects, K137R mutants were generated on AAV7, AAV8, and AAV9, and their effects were evaluated *in vivo*. Two reporter genes were utilized: the nuclear *LacZ* gene driven by the cytomegalovirus promoter and the *luciferase* gene driven by the CB promoter. Surprisingly, we found no difference in luciferase gene expression in the liver or other tissues using either the wild-type AAV8 capsid or AAV8-K137R. *LacZ* gene expression in the liver by AAV8-K137R was about onefold higher than that of wild-type AAV8. However, no difference was found in other tissues, such as skeletal muscle and cardiac muscle. In addition, no difference was found in transgene expression with either AAV7-K137R or AAV9-K137R mutants. Our results indicated that the K137R mutation on AAV7, AAV8, and AAV9 had minimal to no effect on transduction efficiency *in vivo*.

Introduction

ADENO-ASSOCIATED VIRAL (AAV) vectors have been successfully applied in phase I clinical trials in patients with Leber congenital amaurosis and hemophilia B. The benefits of using AAV include the lack of pathogenicity, the broad tissue tropism, and long-term transgene expression (Daya and Berns, 2008). A number of clinical trials utilizing AAV have been carried out worldwide (Manno *et al.*, 2003; Kaplitt *et al.*, 2007; Maguire *et al.*, 2008, 2009; Mueller and Flotte, 2008), and several challenges have been identified based upon their results (Grieger and Samulski, 2012). These challenges include tissue-specific tropism of AAV vectors, high quantity and high quality of recombinant AAV vectors, and immune response to AAV capsids and transgenes (Grieger and Samulski, 2012). To ease the burden of AAV production and minimize the immune response against AAV capsids, it is important to develop AAV vectors that provide enhanced gene expression at significantly low vector doses to achieve successful gene transfer in patients.

The AAV capsid influences transduction efficiency of the AAV vector at several steps, including vector binding to cell surface receptors, internalization, cytoplasmic trafficking to the nuclear membrane, vial uncoating, and second-strand synthesis. One of the most effective methods used to improve AAV transduction efficiency is using alternative AAV serotypes. Currently, at least 12 naturally occurring serotypes of AAV have been isolated (Gao *et al.*, 2002; Wu *et al.*, 2006; Grieger and Samulski, 2012). Different AAV serotypes exhibit distinct tissue tropism and interact with different cellular receptors (Zincarelli *et al.*, 2008). In addition to the utilization of isolated naturally occurring AAV serotypes, generation of novel AAV variants with capsid libraries (Schaffer and Maheshri, 2004; Muzyczka and Warrington, 2005; Yang *et al.*, 2009) and rational designs (Asokan *et al.*, 2010; Bowles *et al.*, 2012) are also important ways to expand AAV capsid versatility and improve AAV vector transduction efficiency.

One such strategy of rationally designed AAV was tyrosine-mutant AAVs (Zhong *et al.*, 2008a). This strategy was based

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upon previous observation that epidermal growth factor receptor protein tyrosine kinase negatively affects AAV2 vector transduction by phosphorylation of tyrosine in the viral capsid, leading to the capsid being targeted for proteasomal degradation and ultimately preventing nuclear localization of the vector (Zhong *et al.*, 2007). Zhong *et al.* (2008a) hypothesized that mutations of the surface-exposed tyrosine residues might allow the vectors to evade phosphorylation, subsequent ubiquitination, and prevent proteasome-mediated degradation. Seven surface-exposed residues were chosen for mutagenesis on the AAV2 vector. Y445F and Y731F showed the greatest impact on transduction efficiency with up to 29-fold gene expression increase observed in mouse hepatocytes after intravenous administration (Zhong *et al.*, 2008a). However, the transduction enhancement mediated by tyrosine mutation appeared to be AAV serotype specific or dependent on tissue type and route of administration (Qiao *et al.*, 2012). For example, the Y to F mutants of AAV8 and AAV9 have 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), but not in the skeletal or cardiac muscle (Qiao *et al.*, 2012).

In an effort to search for simple and efficient method to improve transduction efficiency of AAV vector, Gabriel *et al.* (2013) hypothesized that the mutation of surface-exposed amino acids other than tyrosine on AAV capsid may facilitate AAV intracellular trafficking. Specifically, eight serine (S), seven threonine (T), and nine lysine (K) residues on AAV2 capsid were mutated to alanine (A) and arginine (R) individually or in combination. Targeted modifications of those amino acids were thought to circumvent the host-cellular serine/threonine kinase phosphorylation and ubiquitination of the viral capsids. Indeed, delivering the mutated AAV2 vector *in vivo* by tail vein injection resulted in higher vector copy numbers (up to 4.9-fold) and transgene expression in hepatocytes (up to 14-fold) (Gabriel *et al.*, 2013). This strategy was also applied to the other serotypes by the same group such as AAV1, AAV5 (Sen *et al.*, 2013a), and AAV8 (Sen *et al.*, 2013b), and similar increases of gene transduction efficiency (up to 16-fold) were observed. In particular, the single–amino acid 137 K-R mutation on AAV1 (AAV1-K137R) and AAV8 (AAV8-K137R) resulted in consistently higher hepatocyte transgene expression *in vivo* with a 5–10-fold increase compared with the original capsids (Sen *et al.*, 2013a,b). Therefore, the single–amino acid 137K-R mutation on AAV capsids was proven to be a simple and efficient method to improve AAV transduction efficiency *in vivo*. We set out to study whether the current mutant, such as AAV8-K137R, will mediate similar gene expression enhancement effects in organs other than the liver. In addition, we also wanted to examine whether a mutation on other serotypes such as AAV9 will have comparable gene transfer enhancement effects, as AAV9 is a better option for muscle-directed gene therapy (Inagaki *et al.*, 2006; Kornegay *et al.*, 2010).

Materials and Methods

Generation of K137R AAV plasmids and AAV vector production

The original AAV9 plasmid was obtained from Dr. J. Wilson laboratory of the University of Pennsylvania (Philadelphia, PA) (Bish *et al.*, 2008). The AAV7 and AAV8 plasmids were commercially synthesized (Wang *et al.*, 2005). The K-R mutant packaging plasmids were generated via site-specific mutagenesis. The primers were as follows: AAV7-K137R(+): ACG GCT CCT GCA AAG AAG AGA C; AAV8-K137R(+): ACG GCT CCT GGA AAG AAG AGA C; AAV7 and AAV8-K137R(−): CCT AGC GCC TTC CTC AAC CAG AC; AAV9-K137R(+): ACG GCT CCT GGA AAG AAG AGG C; AAV9-K137R(−): CCT AGC CGC TTC CTC AAC CAG AC. Recombinant AAV vectors were generated by the triple-plasmid transfection method (Xiao *et al.*, 1998). Viral vectors were purified by polyethylene glycol precipitation followed by double CsCl gradient purification (Ayuso *et al.*, 2010). After three time changes of dialysis in virus dialysis buffer ($1\times$ phosphate-buffered saline, 25 mannitol, 6 mM MgCl$_2$) at room temperature for 6–8 hr or at 4°C for overnight, vector genome titers were determined by DNA dot blot and confirmed by quantitative polymerase chain reaction (PCR).

In vivo *gene transfer*

All animal experiments were approved by the Institutional Animal Care and Use Committee. The breeding pairs of ICR mice were purchased from Taconic (Hudson, NY), and C57BL6 and BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, ME). Four or five mice were included in each group.

Vector genome copy number 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 both endogenous control (*glucagon* gene) and AAV vector were developed as described elsewhere (Qiao *et al.*, 2012).

Luciferase imaging and luciferase activity assay

BALB/c mice at the age of 6 weeks received 1×10^{11} particles of AAV8/luc via retro-orbital injection. One week later, images were taken using a Xenogen IVIS Lumina imaging system (Caliper Life Sciences–PerkinElmer, Hopkinton, MA) after intraperitoneal injection of D-luciferin substrate (120 mg/kg; NanoLight Technology, Pinetop, AZ). Bioluminescence image was analyzed with Living Image software (PerkinElmer, Waltham, MA).

At week 2 after AAV/luc injection, mice were euthanized and different tissues were harvested for *in vitro* luciferase activity assay. Luciferase activity in tissues was determined using a luminometric assay (Promega, Madison, WI) and normalized to the total recovered protein determined by the Bradford assay.

Statistical analysis

Data were presented as means ± standard deviation. Comparison between two groups was analyzed with the Student's *t*-test; $p < 0.05$ was considered statistically significant.

Results

K137R mutants of AAV7, AAV8, and AAV9 in ICR mice with LacZ *reporter*

To investigate whether the simple amino acid mutation method would reproduce the impressive gene expression

enhancement for other serotypes, we created AAV7-K137R and AAV9-K137R, in addition to the AAV8-K137R packaging plasmid (see Materials and Methods for details) (Sen *et al.*, 2013b). The mutations were independently confirmed by DNA sequencing. The original and K137R mutant of AAV7, AAV8, and AAV9 vectors were produced containing the nuclear *LacZ* (*LacZnls*) gene driven by a ubiquitous cytomegalovirus (CMV) promoter. The *LacZ* gene was chosen as a reporter because it could be easily visualized in transduced tissue and conveniently quantified by β-galactosidase activity in the tissue (Qiao *et al.*, 2012). No difference was found in AAV yields among all six vectors generated, indicating that the point mutations did not compromise the vector packaging capacity (Supplementary Fig. S1; Supple-

mentary Data are available online at www.liebertpub.com/hgtb).

The AAV vectors (5×10^{11} vg/mouse) were delivered into 4-week-old ICR mice via tail vein injection ($n = 5$ for each group), and all mice were euthanized within 2 weeks postinjection. We chose to deliver AAV vectors in young adult mice, as this is an efficient way to achieve skeletal muscle and cardiac muscle gene transfer in addition to hepatic gene expression. First, different tissues were cryo-sectioned and subjected to *LacZ* staining. As shown in Fig. 1, only a moderate increase of *LacZ* expression in liver was observed by *LacZ* staining for the AAV8-K137R group as compared with the original AAV8 group (Fig. 1a). There was no apparent difference of *LacZ* expression in the liver for AAV9-K137R
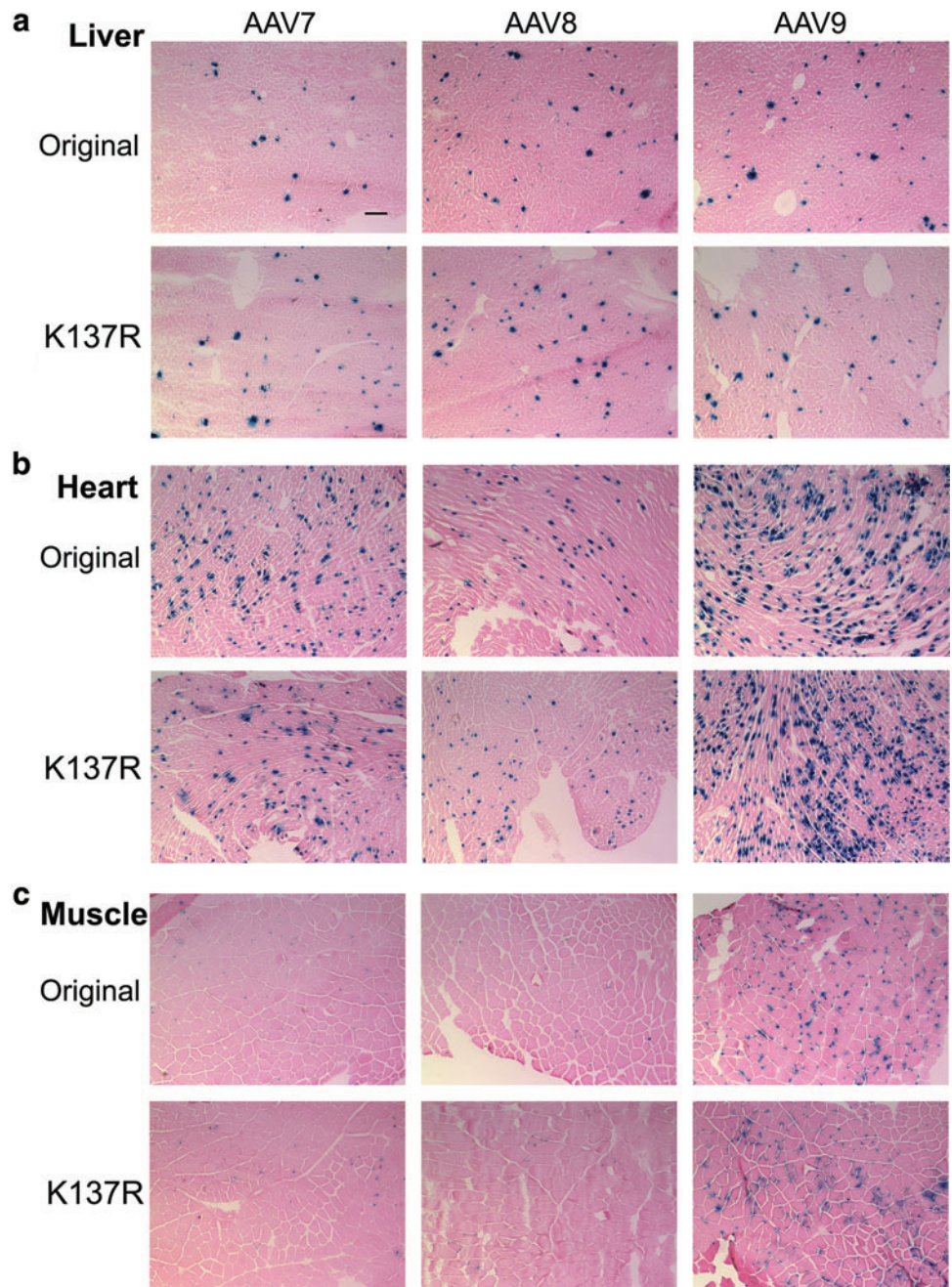

FIG. 1. Comparison of original and K137R mutants of AAV7-, AAV8-, and AAV9-cmv-*LacZnls* vectors in ICR mice via *LacZ* staining. About 5×10^{11} vg of AAV vectors were delivered into 1-month-old ICR male mice via tail vein injection, and the mice were euthanized 2 weeks postinjection ($n = 5$ for each group). **(a)** *LacZ* staining of liver tissue. The nucleus *LacZ* was stained blue (scale bar: 100 μm). **(b)** *LacZ* staining of heart. **(c)** *LacZ* staining of muscle. AAV, adeno-associated virus. Color images available online at www.liebertpub.com/hgtb

versus AAV9 group and AAV7-K137R versus AAV7 group (Fig. 1a). Aside from the liver, we focused on the muscle (TA muscle) and heart to see whether the K-R mutation could enhance gene expression in these tissues. As shown in Fig. 1, no significant difference in *LacZ* expression was found in either the heart or the muscle for any of the compared serotypes (AAV7, AAV8, and AAV9) (Fig. 1b and c). Next, *LacZ* activity from different tissues was measured and quantified. The quantitation data were relatively consistent with the *LacZ* staining data. A moderate difference (an increase by 1-fold) of *LacZ* activity was observed only in the liver for the AAV8-K137R group (2,404 ± 995 RU/μg protein) as compared with the original AAV8 (1,156 ± 454 RU/μg protein) group (Fig. 2a; $n = 5$, $p < 0.05$). The increase of *LacZ* activity in the liver was not apparent for AAV7-K137R versus AAV7 and for AAV9-K137R versus AAV9 (Fig. 2a). Similar to the *LacZ* staining results, there was no significant difference of *LacZ* activity in the cardiac muscle (Fig. 2a) or the skeletal muscle (Fig. 2a; $n = 5$, $p > 0.05$). Finally, total DNA was extracted from the liver, and quantitative PCR was performed to determine AAV vector DNA amounts. The higher AAV vector DNA copy numbers were expected in the mutant groups, since the K137R mutation on AAV capsid was supposed to decrease capsid ubiquitination and improve intracellular trafficking of AAV DNA (Sen *et al.*, 2013b). Indeed, the vector DNA of hepatocytes was doubled in the AAV8-K137R group (6.17 ± 2.7 copies/cell) as compared with the AAV8 group (3.12 ± 1.11 copies/cell) (Fig. 2b). However, there was no significant difference of vector DNA copy number of hepatocytes for AAV7-K137R (3.25 ± 0.38 copies/cell) versus AAV7 (2.22 ± 0.32 copies/cell) and for AAV9-K137R (4.54 ± 1.0 copies/cell) versus AAV9 (4.67 ± 0.92 copies/cell) (Fig. 2b).

K137R mutants of AAV7, AAV8, and AAV9 in BL6 mice with LacZ reporter

Different genetic background of mice may occasionally lead to different outcomes of AAV gene transfer. Therefore, we evaluated the K-R mutated vectors in inbred C57BL/6 mice, in addition to ICR mice. K-R mutant and corresponding original AAV vectors (2 × 10^{11} vg/mouse) were injected into 3-month-old C57BL/6 mice via tail vein injection. This time the less vector particles were utilized to rule out gene expression saturation issue, and the treated mice were euthanized earlier to avoid the concern of CMV promoter shutting off in the liver because of longer gene expression time (Qiao *et al.*, 2011). The treated mice were euthanized 1 week postinjection, and various tissues were cryo-preserved.

We analyzed only the liver tissue because very limited gene expression appeared in the muscle and heart with a low dose of the AAV vector. Overall, the data obtained from the C57BL/6 mice were in agreement with the ICR mice data. A slight increase of *LacZ* expression in the liver was observed in the AAV8-K137R group as compared with the original AAV8 group (Supplementary Fig. S2a). Also, *LacZ* activity displayed slight increases (by 1.7-fold differences) in expression in the liver for the AAV8-K137R group (742 ± 144 RU/μg protein) compared with the original AAV8 group (270 ± 219 RU/μg protein) (Supplementary Fig. S2a; $n = 4–5$, $p < 0.05$). The *LacZ* expression did not show dramatic increases in hepatocytes for either AAV7-K137R group or AAV9-K137R group (Supplementary Fig. S2a and b; $n = 5$, $p > 0.05$).

AAV8-K137R mutant with luciferase reporter

A previous study indicated that the K137R-mutated AAV8 vector resulted in a 10-fold increase of hepatic gene transfer compared with the wild-type AAV8 (Sen *et al.*, 2013b); however, our current data showed only moderate increase of hepatic gene transfer. These conflicted results prompted us to repeat the similar experiments in a different lab using a different reporter gene. Therefore, the AAV8-K137R packaging plasmid was re-created and used to package *luciferase* transgene, which was driven by the chicken β-actin promoter with CMV enhancer (CB), because the CB promoter has been recognized to render long-term efficient gene expression *in vivo* in many tissues, including the liver (Xu *et al.*, 2001; Wang *et al.*, 2003). The AAV8 and AAV8-K137R vectors (1 × 10^{11} particles) were delivered into the 6-week-old Balb/C mice via retro-orbital injection ($n = 4–5$ per group). The live mice were subjected to optimal image study to examine luciferase intensity at week 1 postinjection. As shown in Fig. 3a, the optimal image did not display significant differences in luciferase intensity between the AAV8 and AAV8-K137R group. The mice were euthanized at week 2 postinjection, and various tissues, including heart, spleen, liver, lung, kidney, and muscle (gastrocnemius), were carefully dissected and subjected to luciferase activity assay. Again, we failed to see any significant difference in luciferase expression between mutated AAV8-K137R and original AAV8 groups for all the tissues examined (Fig. 3b; $n = 4–5$, $p > 0.05$).

Discussion

AAV vector has been recognized as an attractive vector for gene therapy applications (Manno *et al.*, 2003; Kaplitt *et al.*,
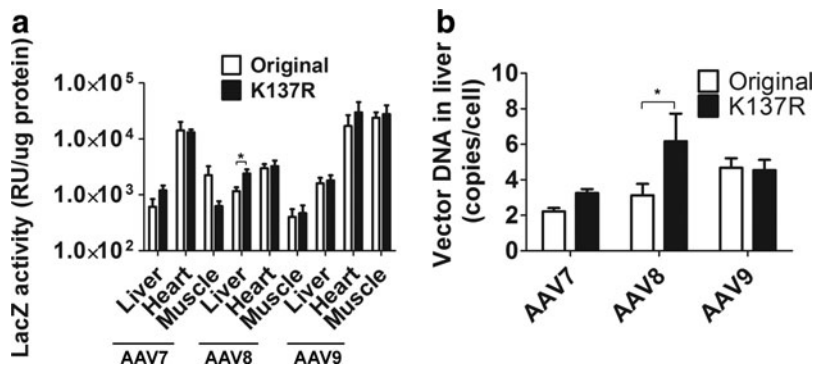

FIG. 2. Quantitation of *LacZ* activity and vector DNA copy numbers. **(a)** Quantitation of *LacZ* activity (*$p < 0.05$, and the Student's *t*-test was applied for the statistics). **(b)** AAV vector DNA copy numbers in liver tissue.
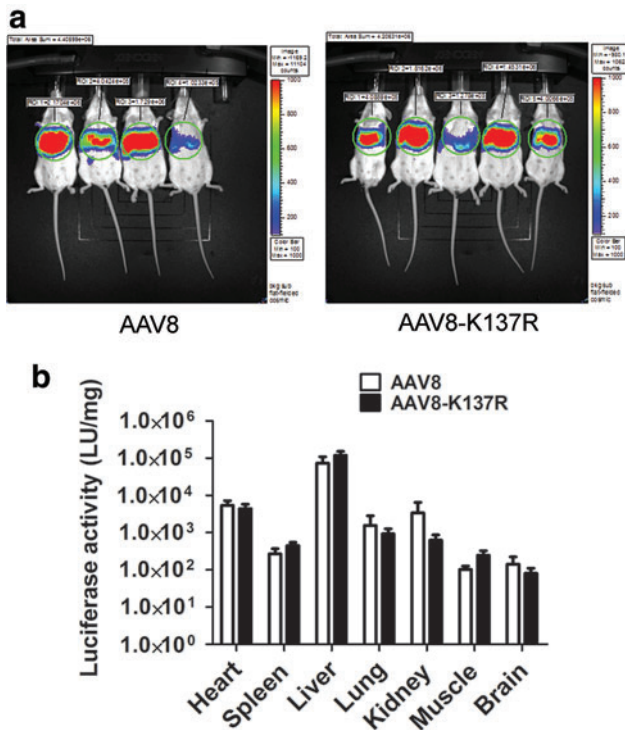
FIG. 3. Transgene luciferase expression after systemic administration of AAV/luc vector. About 1×10^{11} particles of the AAV8/luc or AAV8-K137R vector were administered into 6-week-old Balb/C mice via retro-orbital injection. At week 1 after AAV vector injection, the imaging was performed with 10 sec exposure after injection of D-luciferin substrate **(a)** At week 2, different tissues were collected and luciferase activity was measured; relative light units of luciferase levels are shown as normalized to protein levels determined using a Bradford assay. Error bars indicates standard deviation **(b)**. Color images available online at www.liebertpub.com/hgtb

2007; Hauswirth *et al.*, 2008; Maguire *et al.*, 2009; Nathwani *et al.*, 2011). Our lab has a long-standing interest in developing AAV vectors for muscle-directed gene therapy application, such as gene therapy for muscular dystrophies (Wang *et al.*, 2000; Qiao *et al.*, 2005; Zhu *et al.*, 2005). 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. Because high vector doses are often required in large animals (Kornegay *et al.*, 2010) and eventually in human patients (Mendell *et al.*, 2010), improvement in every step of 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. Mutation of the tyrosine residues on the capsid might allow the vectors to evade phosphorylation mediated by tyrosine kinase and subsequent ubiquitination. Therefore, more vector can emerge out of the trafficking pathway and enter the nucleus for transgene expression (Zhong *et al.*, 2008b). Mutation of serine/threonine to alanine or lysine (K) to arginine (R) on AAV capsid shares the same principle as tyrosine mutation. The rationale is to alter the enzymatic (kinase/ubiquitination ligase) targets on AAV capsid to circumvent capsid ubiquitination and increase the transduction efficiency of these vectors (Gabriel *et al.*, 2013). This strategy was originally attempted on AAV2 capsid, and the mutant AAV2 vector resulted in the increase of transgene expression up to 14-fold (Gabriel *et al.*, 2013). Later, AAV1, AAV5 (Sen *et al.*, 2013a), and AAV8 (Sen *et al.*, 2013b) were mutated, making it appear that most of the mutant vectors mediated much higher transgene expression than the original capsid. In particular, the AAV8-K137R mutant resulted in a significantly higher vector copy numbers in the liver (22-fold), lungs (9.7-fold), and muscle (8.4-fold) tissues when compared with the original AAV8 vectors (Sen *et al.*, 2013b). AAV1-K137R also demonstrated much higher (up to sixfold) luciferase expression. Since the lysine K137 residue was conserved across most of the AAV serotypes, we hypothesized that K137R mutation on other serotypes would result in the similar gene enhancement effects.

K137R mutants were generated on AAV7, AAV8, and AAV9 vectors. Two reporter genes were utilized: the nuclear *LacZ* gene driven by CMV promoter and *luciferase* gene driven by CB promoter. Several mouse strains, including Balb/C, ICR, and C57BL/6, were included. The experiments were even performed in two independent labs. Quite surprisingly, we did not observe the dramatic gene enhancement effects for any of the K137R mutants. Only moderate increase of transgene expression (up to 1.7-fold) in the liver was noticed for AAV8-K137R containing *LacZ* reporter. We did not observe any significant gene enhancement effects on other tissues such as skeletal muscle and cardiac muscle for AAV8-K137R. No significant increase of transgene expression was found in any of the tissues for AAV7-K137R and AAV9-K137R. The results were somewhat disappointing, but nonetheless informative and useful.

Some possible explanations for the conflicting results of our current study (an increase by 1-fold) and those of others (10-fold increase) (Sen *et al.*, 2013b) were deduced. The same mutant AAV8-K137R was used in both studies. However, they had a different reporter. In our study, both nuclear *LacZ* and luciferase reporters were used, whereas GFP reporter was used in the other report (Sen *et al.*, 2013b). The sensitivity of different reporters might contribute to various fold changes of transduction efficiency. Other factors, including incorrect vector titers, dilutions, and inconsistent vector potency from batch to batch, could also cause inconsistent results.

Nevertheless, tyrosine/serine/threonine mutation on AAV capsids represents a novel and distinct strategy to enhance AAV transduction efficiency by improving intracellular trafficking of AAV capsids. However, caution needs to be taken when applying this strategy, since it is both serotype specific and tissue specific. 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, both AAV8 and AAV9 are the most robust vectors for liver gene delivery. Also, AAV8 has been found to be effective in intracellular trafficking and uncoating (Nakai *et al.*, 2005). On the other hand, AAV2 is ineffective in the above-mentioned 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

K137R mutation on AAV7 and AAV9 did not increase transgene expression, we speculate that intracellular trafficking, in particular the escape of proteasomal degradation, may not be the rate-limiting step for these two vectors in the studied tissues.

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Author Disclosure Statement

No competing financial interests exist.

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