Systemic *Trans*-Splicing Adeno-Associated Viral Delivery Efficiently Transduces the Heart of Adult *mdx* Mouse, a Model for Duchenne Muscular Dystrophy

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Abstract

Trans-splicing adeno-associated viral (tsAAV) vectors hold great promise for delivering large therapeutic genes. One potential application is in the treatment of Duchenne muscular dystrophy (DMD). In this case, it is necessary to transduce whole body muscle. We demonstrated body-wide AAV-9 tsAAV transduction in normal neonatal mice. However, it was not clear whether such an approach would work in diseased mice. In this study we delivered the AAV-9 alkaline phosphatase (AP) tsAAV vector (3×10^{12} vector genome particles per vector per mouse, tail vein injection) to 2-month-old *mdx* mice, the most widely used DMD model. Four months later, we observed widespread AP expression in the heart. It reached the same level as we have seen in normal neonatal puppy. Interestingly, myocardial transduction correlated with β -myosin heavy chain expression but not with LamR, the putative AAV-9 receptor. AP expression was also detected in various skeletal muscles but at levels much lower than in normal newborn mice. Despite the existing inflammatory milieu, we did not see any appreciable increase in CD4⁺ and CD8⁺ T cells and macrophages in striated muscles after systemic tsAAV infection. In summary, our results have paved the way for tsAAV-mediated gene therapy for Duchenne cardiomyopathy.

Introduction

A DENO-ASSOCIATED VIRUS (AAV) has been considered a promising viral vector for Duchenne muscular dystrophy (DMD) gene therapy (Athanasopoulos *et al.*, 2004; Blankinship *et al.*, 2006). DMD is a lethal, degenerative muscle disease caused by mutations in the dystrophin gene (Kunkel, 2005). The 12-kb dystrophin coding sequence has been a great challenge for the AAV vector, which has a 5-kb maximal packaging capacity. To overcome the size hurdle, investigators have developed synthetic micro- and minidystrophin genes. The microgenes carry only about 30% of the coding information and they are usually less than 4 kb. The minigenes are larger than 6 kb and contain about 50 to 60% of the coding sequence. Although a microgene can fit into a single AAV virion, it is less effective in ameliorating muscle disease (Harper *et al.*, 2002; Lai *et al.*, 2009). From the therapeutic standpoint, the minigene would be preferable to the microgene.

A series of dual-AAV vector systems have the capacity to carry the minigene. These include the overlapping, *trans*-splicing (ts) and hybrid vectors (Duan, 2006b; Ghosh and Duan, 2007; Ghosh *et al.*, 2008). Among these, the rationally

designed tsAAV vectors are particularly promising (Lai *et al.*, 2005, 2006, 2008). After local injection into dystrophin-deficient *mdx* mice, the transduction efficiency of the tsAAV minidystrophin vectors reached that of a single AAV microdystrophin vector (Lai *et al.*, 2005; Liu *et al.*, 2005). Because all body muscles are affected in DMD, the next critical step will be to achieve body-wide transduction with the tsAAV vectors. As a proof of principle, we first tested systemic AAV-9 tsAAV transduction in normal newborn mice (Ghosh *et al.*, 2007). Six weeks after intravenous delivery, we observed robust whole body muscle transduction. In skeletal and cardiac muscle, transduction efficiency reached approximately 80 and 50%, respectively (Ghosh *et al.*, 2007). These levels would meet the need for DMD gene therapy (Chamberlain, 2002; Duan, 2006a).

Despite the encouraging results, it remains to be determined whether systemic tsAAV delivery is achievable in a disease model such as adult *mdx* mice. Compared with normal neonatal mice, there are several challenges. First, AAV-9 has not yet been tested in dystrophic muscles. Disease-associated cellular and/or biochemical changes may alter AAV-9 transduction and/or tsAAV reconstitution in *mdx* muscle, especially in the context of systemic delivery.

Second, there is a 20-fold body weight difference between a newborn puppy and an adult mouse. Scaling up could represent an important barrier for systemic tsAAV transduction. Third, in our previous neonatal study, we observed transduction for only 6 weeks. A longer observation period would allow us to better evaluate the relative persistence of systemic tsAAV transduction.

To address these clinically relevant issues, we administered AAV-9 alkaline phosphatase tsAAV vectors (tsAAV-AP) to 2-month-old female *mdx* mice by a single bolus tail vein injection and evaluated transgene expression 4 months later. The highest AP expression was observed in the heart, which reached the 50% transduction threshold required for dystrophic cardiomyopathy gene therapy. Surprisingly, limited skeletal muscle transduction was observed. Nevertheless, we did not see a strong cellular immune reaction in either the heart or skeletal muscle in tsAAV-infected *mdx* mice. These results raise the hope of exploring tsAAV-mediated minidystrophin therapy for Duchenne cardiomyopathy. Further optimization of the technology may pave the way for future body-wide treatment with minidystrophin tsAAV vectors.

Materials and Methods

Animals

All animal experiments were approved by the animal care and use committee at the University of Missouri (Columbia, MO) and were in accordance with National Institutes of Health (NIH, Bethesda, MD) guidelines. The original dystrophin-deficient *mdx* mice (C57BL/10ScSn-*Dmd*^{mdx}/J) were purchased from Jackson Laboratory (Bar Harbor, ME). Experimental mice (2-month-old female) were obtained from a local breeding colony. All mice were housed in specific pathogen-free animal care facilities and kept under a 12-hr light (25 lx)/12-hr dark cycle with free access to food and water.

Recombinant AP tsAAV vector production

The *cis* plasmids used for AAV packaging (including pcisAV.AP.Donor and pcisAV.AP.Acceptor) have been described previously (Ghosh *et al.*, 2007). The reconstituted expression cassette expresses the human placental AP gene under the transcriptional regulation of the Rous sarcoma virus promoter and the simian virus 40 (SV40) polyadenylation signal. The AAV-9 packaging plasmid (pRep2/Cap9) was a gift from J. Wilson (University of Pennsylvania, Philadelphia, PA) (Gao *et al.*, 2004). Recombinant AAV-9 vectors were generated by a triple-plasmid transfection protocol described previously (Bostick *et al.*, 2007; Ghosh *et al.*, 2007; Yue *et al.*,

2008). Viral stocks were purified by three rounds of isopycnic CsCl₂ ultracentrifugation followed by three changes of HEPES buffer at 4°C for 48 hr. Viral titer and quality control were performed according to our previously published protocol (Bostick *et al.*, 2007; Ghosh *et al.*, 2007; Yue *et al.*, 2008). The average viral titer was 1×10^{10} vector genome (VG) particles/ μ l for both the donor and acceptor vectors.

Systemic tsAAV delivery

The study was performed in 2-month-old female *mdx* mice. The average body weight was 23.8 ± 0.5 g ($n = 10$). We first determined the maximal tolerable fluid volume for tail vein injection. Up to 600 μ l of saline was well received in this age group of female *mdx* mice without noticeable adverse response (such as respiratory stress and death). Systemic delivery was then performed in conscious mice by a single bolus injection through the tail vein. Before injection, AV.AP.Donor and AV.AP.Acceptor vectors were thoroughly mixed by repeated pipetting and gentle shaking at 4°C overnight. A total of 600 μ l of tsAAV-AP vectors (including 300 μ l of AV.AP.Donor and 300 μ l of AV.AP.Acceptor) was delivered to each mouse (25 μ l/g body weight). This yielded a dose of 3×10^{12} VG particles/vector/mouse or 1.26×10^{11} VG particles/vector/g body weight. After injection, mice were returned to the cage for recovery. All tsAAV-injected mice survived the procedure.

Reporter gene expression evaluation

Mice were euthanized 4 months after systemic tsAAV-AP infection. Various skeletal muscles (including the tibialis anterior, extensor digitorum longus, quadriceps, gastrocnemius, soleus muscles, diaphragm, and tongue) as well as several internal organs (the heart, lung, and liver) were harvested for AP staining/activity assays. *In situ* expression was determined by histochemical staining of 8- μ m cryosections as described previously (Duan *et al.*, 2000). AP activity in whole muscle lysate was measured with a StemTAG AP activity assay kit (Cell Biolabs, San Diego, CA) according to the manufacturer's instructions (Ghosh *et al.*, 2007). In these assays, endogenous heat-labile AP activity was first inactivated by incubating samples at 65°C for 60 min.

Other morphology examinations

Immunofluorescence staining for dystrophin and utrophin was performed essentially as described previously (Yue *et al.*, 2003; Lai *et al.*, 2005). Dystrophin was detected with a mouse monoclonal antibody against the dystrophin C-terminal domain (Dys-2, diluted 1:100; clone Dy8/6C5, IgG1; Novocastra, Newcastle, UK). Utrophin was examined with a mouse

FIG. 1. Systemic delivery of AAV-9 alkaline phosphatase (AP) tsAAV vectors resulted in sporadic skeletal muscle transduction in adult *mdx* mice. **(A)** Representative photomicrographs of AP (left) and dystrophin (right) staining in serial sections of selected skeletal muscles (gastrocnemius, quadriceps, tongue, and diaphragm). *Middle:* High-magnification images of the boxed areas at left. Asterisks mark the same myofiber in the respective serial sections. Arrow in the quadriceps panels indicate dystrophin-positive revertant fibers. Arrow in the diaphragm panels denote necrotic myofibers that were filled up with immunoglobulin. The scale bars in the second row apply to the top row. **(B)** Whole muscle lysate AP activity quantification from selected skeletal muscles. TA, tibialis anterior muscle; EDL, extensor digitorum longus muscle; Gastro, gastrocnemius muscle; Sol, soleus muscle. Solid columns, results from the current study ($n = 3$ for each group). Open columns, results from systemic tsAAV transduction in normal neonatal mice ($n = 5$) (Ghosh *et al.*, 2007). *Significantly higher than in the other groups; #significantly higher than in age- and sex-matched uninfected *mdx* skeletal muscle.

monoclonal antibody against the utrophin N-terminal domain (VP-U579, diluted 1:20; clone DRP3/20C5, IgG1; Vector Laboratories, Burlingame, CA). Slow and fast skeletal muscle fiber and cardiac β -MHC were determined by immunofluorescence staining according to a previously published protocol (Lai *et al.*, 2005). Slow twitch fiber (type I) was revealed with a monoclonal antibody against MHC-I (M8421, diluted 1:2000; clone NOQ7.5.4D, IgG1; Sigma-Aldrich, St. Louis, MO). Fast twitch fiber (type II) was revealed with a monoclonal antibody against MHC-II (VP-M665, diluted 1:20; clone WB-MHCf, IgG1; Vector Laboratories). Cardiac β -MHC was determined with the M8421 monoclonal antibody (Sigma-Aldrich) at a dilution of 1:4000. Embryonic MHC was detected with a monoclonal antibody against embryonic MHC (F1.652, diluted 1:250; IgG1; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). Capillaries were revealed by means of an alkaline phosphatase-metaniol yellow staining protocol described previously (Yue *et al.*, 2008). The AAV-9 receptor (laminin receptor) was detected by immunofluorescence staining with a rabbit polyclonal antibody (diluted 1:200; Abcam, Cambridge, MA) (Yue *et al.*, 2008). A VECTASTAIN ABC kit (Vector Laboratories) was used for immunohistochemical detection of CD4⁺ and CD8⁺ T cells and macrophages. Macrophages were recognized with a rat anti-mouse F4/18 antibody (#RM2920, diluted 1:200; clone CI:A3-1, IgG2b; Caltag Laboratories, Burlingame, CA). CD4⁺ T cells were revealed with a rat anti-mouse CD4 (L3T4) antibody (cat. no. 553727, diluted 1:800; clone GK1.5, IgG2b; BD Biosciences, San Jose, CA). CD8⁺ T cells were revealed with a rat anti-mouse CD8 (Ly-2) anti-

body (cat. no. 553027, diluted 1:800; clone 53-6.7, IgG2b; BD Biosciences).

Statistical analysis

Data are presented as means $\pm$ standard error of mean (SEM). Statistical analysis was performed with SPSS software (SPSS, Chicago, IL). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc analysis. Differences were considered significant when $p < 0.05$.

Results

Systemic tsAAV transduction in adult *mdx* skeletal muscle

To evaluate intravascular tsAAV transduction in dystrophic muscle, we delivered tsAAV-AP vector at 6×10^{12} vector genome (VG) particles/mouse to 2-month-old *mdx* mice via tail vein injection (3×10^{12} VG particles/mouse of each vector, AV.AP.Donor and AV.AP.Acceptor). AP expression was examined 4 months after gene delivery. We observed minimal to moderate transduction in various skeletal muscles. It ranged from about 5 to 15% on tissue cross-sections (Fig. 1A). In whole muscle lysate, the AP activity ranged from 6 to 22 $\mu\text{M}/\mu\text{g}$ protein (Fig. 1B). This represents approximately one tenth to one fourth of what we have seen in tsAAV-AP-infected normal neonatal mouse skeletal muscle (Fig. 1B) (Ghosh *et al.*, 2007).

To explore the potential mechanisms underlying the dramatic skeletal muscle transduction difference between adult

FIG. 2. AAV-9 AP tsAAV preferentially transduces fast twitch myofiber fibers in *mdx* skeletal muscle. Representative photomicrographs of serial sections stained for AP, fast twitch fiber and slow twitch fiber. *Top row:* Soleus muscle. *Bottom row:* Lateral head of the gastrocnemius muscle. Asterisk marks the same myofiber in the respective serial sections. Arrow, a few AP-positive slow fibers in the gastrocnemius muscle.

FIG. 3. Quantitative evaluation of tsAAV transduction in various types of myofibers in *mdx* mice. (A) Fiber type quantification in tsAAV-transduced myofibers. A total of 835 AP-positive myofibers were quantified from 4 independent samples (about 200 fibers per muscle sample). *Significantly higher than the other two groups; #significantly higher than that of the embryonic myofiber group. em, embryonic myofiber; pos, positive. (B–F) Representative photomicrographs of serial muscle sections stained for general histology by hematoxylin & eosin (B), AP (C), embryonic myosin heavy chain (D), type II myosin heavy chain (fast twitch myofiber) (E), and type I myosin heavy chain (slow twitch myofiber) (F). In (E), embryonic myofibers were also stained with the type II myosin heavy chain antibody. Arrows, AP-positive fast twitch fibers; arrowheads, AP-positive slow twitch fibers.

mdx mice and newborn normal mice, we examined whether the absence of dystrophin limited systemic tsAAV transduction. In *mdx* skeletal muscle, there exists about 1 to 2% dystrophin-positive myofibers (Lu *et al.*, 2000). These are called revertant fibers. Interestingly, we did not see a transduction preference for the revertant fibers (Fig. 1A, arrow in quadriceps panels).

We next examined fiber type preference. Although we observed a few AP-positive slow twitch myofibers (Fig. 2, arrows at bottom; Fig. 3B–F, arrowheads), the vast majority of AP-positive cells were fast twitch fibers (Fig. 3A). It is interesting to note that the only known AAV-9 receptor, LamR, was enriched in slow twitch myofibers (see Supplementary Fig. 1 at www.liebertonline.com/hum) (Akache *et al.*, 2006; Yue *et al.*, 2008). Besides slow and fast twitch myofibers, *mdx* skeletal muscle also contains a significant amount of embryonic/regenerating myofibers. Interestingly, these fibers were rarely transduced by the tsAAV vectors (Fig. 3).

Additional study on capillarity density suggests that systemic tsAAV transduction was not limited by capillary distribution (see Supplementary Fig. 2 at www.liebertonline.com/hum). The muscles (or the regions) that contain substantially more capillaries (presumably better blood perfusion) did not show high numbers of AP-positive cells (Supplementary Fig. 2).

Robust tsAAV transduction in the *mdx* heart

In contrast to skeletal muscle, we observed widespread transduction in the *mdx* heart (Fig. 4A). The highest AP expression was observed in the left ventricle, which reached about 50% of the cardiomyocytes, a level sufficient for treating Duchenne cardiomyopathy (Fig. 4B) (Yue *et al.*, 2004; Duan, 2006a; Bostick *et al.*, 2008). Overall, AP activity in the *mdx* heart reached that of the normal newborn mouse heart

(Fig. 4C) (Ghosh *et al.*, 2007). To understand the mosaic transduction pattern in the *mdx* heart, we performed AP staining and β -myosin heavy chain (MHC) immunofluorescence staining (Fig. 5A). Interestingly, there appeared to be a correlation between AP and β -MHC expression. More AP-positive cardiomyocytes were seen in areas that showed high levels of β -MHC expression (Fig. 5A). Next, we examined AAV-9 receptor LamR expression in the *mdx* heart. In contrast to skeletal muscle, LamR was highly expressed at the intercalated disks rather than inside cardiomyocytes (Fig. 5B). This was completely different from the intracellular expression pattern seen in AP-positive cardiomyocytes (Fig. 5B).

Systemic tsAAV delivery did not aggravate macrophage and T cell infiltration in *mdx* muscle

The inflammatory milieu in dystrophic muscle represents a great challenge for muscular dystrophic gene therapy. To determine whether systemic AAV-9 tsAAV-AP delivery provoked additional immune cell infiltration, we performed immunohistochemical staining with macrophage- and T cell-specific markers. In the absence of tsAAV-AP infection, macrophages were the predominant inflammatory cells in striated muscles (Fig. 6). There were also a few CD4⁺ and CD8⁺ T cells (Fig. 6). In tsAAV-AP-infected mice, we did not see an appreciable increase in immune cell infiltration in either heart or skeletal muscle (Fig. 6).

tsAAV transduction in other tissues

Besides striated muscles, we also detected a few AP-positive cells in the lung and liver (see Supplementary Fig. 3 at www.liebertonline.com/hum). In the lung, the transduced cells were mainly alveolar cells (Supplementary Fig. 3). Surprisingly, we detected an extraordinary amount of AAV genome in the liver (see Supplementary Fig. 4 at

FIG. 4. Systemic AAV-9 tsAAV delivery results in efficient myocardial transduction in the *mdx* heart. (A) A representative AP staining photomicrograph of a whole heart cross-section. (B) Representative photomicrographs of serial heart sections stained for AP, dystrophin, and utrophin. (C) Whole heart lysate AP activity quantification. Open bar, results from systemic tsAAV transduction in normal neonatal mice ($n = 3$); solid bars, results from the current study ($n = 3$ for each group). *Whole heart AP activity in uninfected *mdx* mice is significantly lower than in tsAAV-infected mice.

www.liebertonline.com/hum). For reasons yet unknown, we observed extremely high-level endogenous AP expression in the kidney of uninfected *mdx* mice (data not shown). This high background prevented us from reaching a conclusion about tsAAV transduction in the *mdx* kidney.

Discussion

The ability to deliver a larger therapeutic expression cassette is the primary motivation in developing the tsAAV vectors. Theoretically, the tsAAV system offers an opportunity to capitalize on the therapeutic benefit of the mini-

dystrophin gene for DMD treatment (Harper *et al.*, 2002; Duan, 2006b; Lai *et al.*, 2009). To achieve the long-term goal of tsAAV-mediated DMD gene therapy, we generated a pair of rationally designed minidystrophin tsAAV vectors (Lai *et al.*, 2005, 2008). Local injection into *mdx* mice resulted in robust transduction comparable to that of a single AAV vector (Lai *et al.*, 2005). However, the benefit of such an approach will be limited unless we can demonstrate efficient whole body muscle transduction in a dystrophic animal model. To build on our previous success in normal neonatal mice, in this work we tested the hypothesis that systemic tsAAV transduction is achievable in adult *mdx* mice.

FIG. 5. tsAAV-AP transduction correlates with β -MHC expression, but is independent of LamR expression in the *mdx* heart. (A) Representative photomicrographs of serial heart sections stained for AP and β -MHC. Top and bottom rows represent samples from two independent *mdx* hearts. L, area of low AP and β -MHC expression; H, area of high AP and β -MHC expression. (B) Representative photomicrographs of serial heart sections stained for AP and LamR. Asterisk marks the same cardiomyocyte in the serial sections. Arrow denotes LamR expression in an intercalated disk. Color images available online at www.liebertonline.com/hum.

After a single tail vein injection of the tsAAV-AP vectors, we observed variable, but in general low-level, transduction in various skeletal muscles (Fig. 1). However, robust tsAAV transduction was detected in the *mdx* heart (Fig. 4). Importantly, we did not see aggravated immune cell intrusion in tsAAV-transduced muscles (Fig. 6). Collectively, these results are encouraging but somewhat unexpected.

In our study with the same set of AAV-9 tsAAV-AP vectors, we delivered 3.5×10^{11} VG particles/vector/mouse to normal newborn mice (Ghosh *et al.*, 2007). This equals 2.3×10^{11} VG particles/vector/g body weight. In the current study, we used a dose of 3×10^{12} VG particles/vector/mouse

or 1.26×10^{11} VG particles/vector/g body weight. On the basis of the viral titer of 1×10^{10} VG particles/ μ l and a tolerable volume of 600 μ l/mouse, this is the maximal dose we can deliver to a 2-month-old *mdx* mouse. Although the total viral dose was about 10-fold higher in adult *mdx* mice, the dose per gram body weight was reduced by about 50%. The reduction in per-unit dose seems to correlate with the reduced transduction in skeletal muscle. However, it cannot explain our findings in the heart.

Age and disease status are two major differences between the current and previous studies. Although age-dependent differences have been shown in the aorta, retina, liver, and

FIG. 6. Systemic AAV-9 tsAAV infection does not elicit additional immune cell infiltration. **(A)** Representative photomicrographs of serial skeletal muscle sections stained for AP, macrophages, and CD4⁺ and CD8⁺ T cells. *Left column:* Results from an age- and sex-matched uninfected *mdx* mouse. *Right column:* Results from a tsAAV-infected *mdx* mouse. *Insets:* High-magnification images of the boxed regions in the respective low-power photomicrographs. They show rarely occurring CD4⁺ and CD8⁺ T cells. **(B)** Representative photomicrographs of serial heart sections stained for AP, macrophages, and CD4⁺ and CD8⁺ T cells. *Left column:* Results from an age- and sex-matched uninfected *mdx* mouse. *Right column:* Results from a tsAAV-infected *mdx* mouse. Arrows, rarely occurring CD4⁺ and CD8⁺ T cells. Color images available online at www.liebertonline.com/hum.

kidney after systemic AAV-9 transduction, age seems to have a minimal impact on striated muscle transduction (Bostick *et al.*, 2007). The primary cellular defect in DMD is the loss of sarcolemmal dystrophin. To determine whether dystrophin deficiency per se affects systemic tsAAV transduction, we examined AP expression in dystrophin-positive revertant myofibers (Fig. 1). We did not see preferential transduction in these myofibers (Fig. 1). This result suggests that the absence of dystrophin is not the limiting factor in systemic tsAAV transduction in *mdx* mice. Efficient cardiac transduction in the *mdx* heart further strengthened this conclusion (Fig. 4).

We also failed to see a correlation between systemic tsAAV transduction and regenerating (embryonic) or necrotic myofibers (Figs. 1 and 3). Similar to normal mouse muscle, fast twitch myofibers were also favored in *mdx* muscle (Figs. 2 and 3) (Bostick *et al.*, 2007). Additional studies on the LamR AAV-9 receptor and capillary density suggest that these factors were also unlikely to be responsible for the poor skeletal muscle transduction (Supplementary Figs. 1 and 2). We currently believe that the low transduction efficiency in *mdx* skeletal muscle may likely reflect the viral dose used in the study. In support of this notion, it has been shown that at the dose of 1.8×10^{12} VG particles/mouse, a tail vein-injected AAV-9 LacZ vector completely transduced both the heart and skeletal muscle in normal mice. However,

at the dose of 1×10^{11} VG particle/mouse, efficient transduction was observed only in the heart and there was barely any transduction in skeletal muscle (Inagaki *et al.*, 2006).

It has been established that mosaic expression in less than 20% myofiber cannot halt skeletal muscle disease in DMD (Phelps *et al.*, 1995). Our findings reveal an unexpected challenge in AAV-9 tsAAV-mediated minidystrophin gene therapy. Considering the fact that we have already used the maximal dose that an adult *mdx* mouse can tolerate, novel strategies must be developed to further enhance skeletal muscle transduction efficiency. In this regard, several strategies (such as tyrosine mutant modification and *in vivo* AAV capsid evolution) hold great promise in generating more potent AAV vectors (Zhong *et al.*, 2008; Yang *et al.*, 2009).

Several interesting observations were seen in the heart. We have shown that AAV-9 preferentially transduces the fast twitch fibers (type II MHC) in skeletal muscle. The slow twitch fibers (type I MHC) were minimally transduced (Fig. 3) (Bostick *et al.*, 2007). Cardiac β -MHC and skeletal muscle type I MHC (slow twitch fibers) share the same contractile properties. Surprisingly, β -MHC-positive cardiomyocytes were preferentially transduced in the *mdx* heart (Fig. 5A). A better understanding of this paradoxical phenomenon awaits future studies.

AAV-9 has been shown to efficiently transduce adult mouse liver after systemic gene delivery in normal mice

(Inagaki *et al.*, 2006; Bostick *et al.*, 2007). Surprisingly, we detected only minimal liver transduction in adult *mdx* mice despite the abundant amount of viral genome in the liver (Supplementary Figs. 3 and 4). Future studies are needed to clarify the underlying mechanism(s) of the aberrant tsAAV transduction pattern in *mdx* mouse liver.

The most striking finding of our study is the robust transduction of tsAAV vector in adult *mdx* heart. A single AAV-9 vector has been shown to preferentially transduce the heart in normal mice (Inagaki *et al.*, 2006; Pacak *et al.*, 2006; Bostick *et al.*, 2007). The tsAAV-9 vector has also been shown to result in robust cardiac transduction in normal neonatal mice (Ghosh *et al.*, 2007). The outstanding myocardial transduction and the apparent lack of provocative cellular immune response seen in adult *mdx* mice suggest that AAV-9 tsAAV may hold great promise for treating Duchenne cardiomyopathy. This will be particularly relevant to the gene therapy of X-linked dilated cardiomyopathy in which dystrophin is selectively lost in the heart but not in skeletal muscle.

Acknowledgments

This work was supported by grants from the National Institutes of Health (AR-49419; D.D.) and the Muscular Dystrophy Association (D.D.).

Author Disclosure Statement

No competing financial interests exist.

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Received for publication March 30, 2009;

accepted after revision July 23, 2009.

Published online: October 1, 2009.