

## ARTICLE

# Systemic gene transfer reveals distinctive muscle transduction profile of tyrosine mutant AAV-1, -6, and -9 in neonatal dogs

Chady H Hakim<sup>1</sup>, Yongping Yue<sup>1</sup>, Jin-Hong Shin<sup>1,5</sup>, Regina R Williams<sup>2</sup>, Keqing Zhang<sup>1</sup>, Bruce F Smith<sup>2,3</sup> and Dongsheng Duan<sup>1,4</sup>

The muscular dystrophies are a group of devastating genetic disorders that affect both skeletal and cardiac muscle. An effective gene therapy for these diseases requires bodywide muscle delivery. Tyrosine mutant adeno-associated virus (AAV) has been considered as a class of highly potent gene transfer vectors. Here, we tested the hypothesis that systemic delivery of tyrosine mutant AAV can result in bodywide muscle transduction in newborn dogs. Three tyrosine mutant AAV vectors (Y445F/Y731F AAV-1, Y445F AAV-6, and Y731F AAV-9) were evaluated. These vectors expressed the alkaline phosphatase reporter gene under transcriptional regulation of either the muscle-specific Spc5-12 promoter or the ubiquitous Rous sarcoma virus promoter. Robust skeletal and cardiac muscle transduction was achieved with Y445F/Y731F AAV-1. However, Y731F AAV-9 only transduced skeletal muscle. Surprisingly, Y445F AAV-6 resulted in minimal muscle transduction. Serological study suggests that the preexisting neutralization antibody may underlie the limited transduction of Y445F AAV-6. In summary, we have identified Y445F/Y731F AAV-1 as a potentially excellent systemic gene transfer vehicle to target both skeletal muscle and the heart in neonatal puppies. Our findings have important implications in exploring systemic neonatal gene therapy in canine models of muscular dystrophy.

*Molecular Therapy — Methods & Clinical Development* (2014) **1**, 14002; doi:10.1038/mtm.2014.2; published online 5 March 2014

## INTRODUCTION

The muscular dystrophies refer to a group of monogenic inherited muscle diseases characterized by progressive muscle wasting and weakness.<sup>1–3</sup> Although individually they are rare, collectively the muscular dystrophies affect a large population of patients worldwide. The first muscular dystrophy gene, dystrophin, was cloned by Kunkel *et al.* in 1987.<sup>4</sup> Mutations in the dystrophin gene result in Duchenne muscular dystrophy, Becker muscular dystrophy, and X-linked dilated cardiomyopathy. Since then, more than 40 disease-causing genes have been identified for various types of muscular dystrophy.<sup>5</sup> Breakthroughs in gene discovery have led to strong enthusiasm for curing these inherited muscle diseases by gene therapy.<sup>6–8</sup> However, unlike other single-gene disorders (such as hemophilia), afflicted muscle tissues are distributed all over the body in muscular dystrophy. Systemic gene therapy is essential in order to change the natural history of dystrophic muscle diseases.

Adeno-associated virus (AAV) is the first, and currently, only gene transfer vector capable of effective bodywide transduction.<sup>9</sup> AAV-mediated whole-body correction has been demonstrated in murine models of muscular dystrophies.<sup>10–13</sup> However, systemic AAV delivery remains a daunting challenge in large mammals.<sup>14</sup>

The AAV capsid is composed of 60 subunits of viral protein 1, 2, and 3 in a 1:1:13 ratio. Srivastava *et al.* recently developed a novel class of capsid-modified AAV vectors called tyrosine mutants.<sup>15</sup> In these vectors, one or multiple surface-exposed tyrosine (Y) residues of the

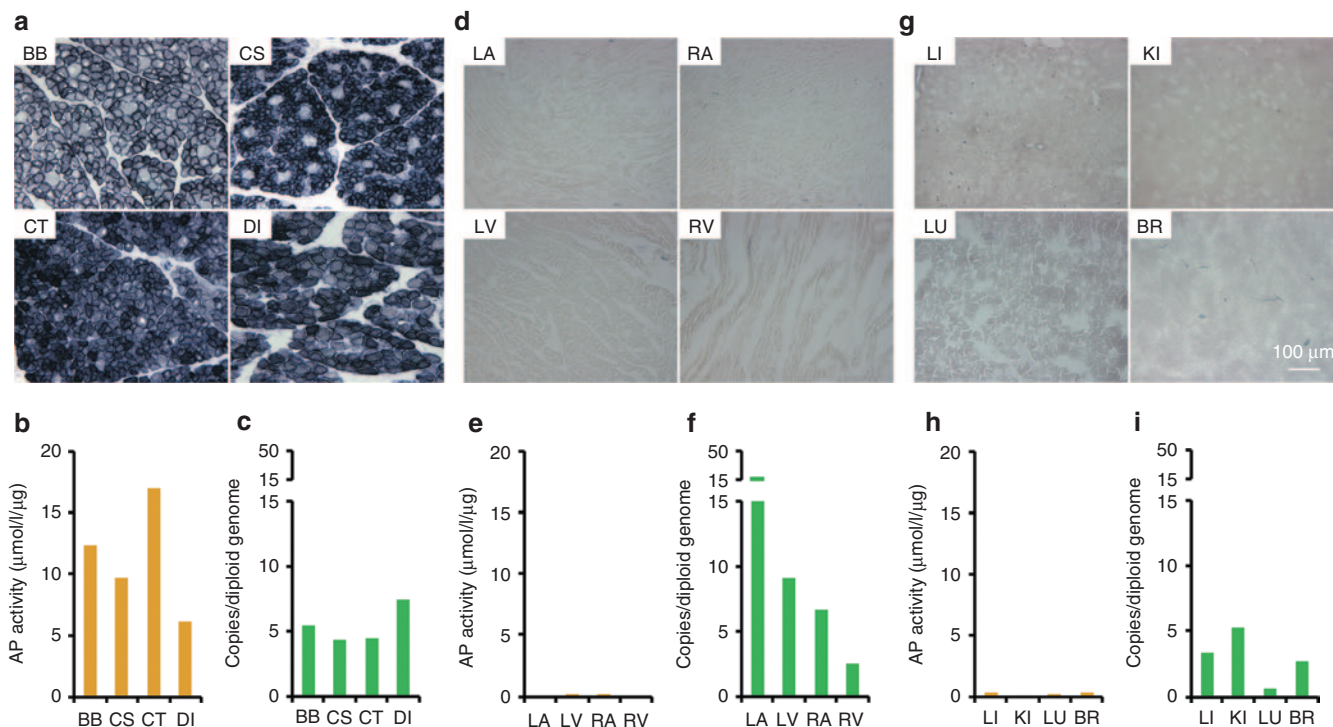
AAV capsid are changed to phenylalanine (F). Studies from several laboratories suggest that tyrosine-mutated AAV may have superior gene transfer efficiency.<sup>15–19</sup> In this study, we evaluated three tyrosine-mutated AAV vectors (Y445F/Y731F AAV-1, Y445F AAV-6, and Y731F AAV-9) for systemic gene delivery in neonatal dogs. These vectors expressed the heat-resistant human placental alkaline phosphatase (AP) gene under transcriptional regulation of either the muscle-specific Spc5-12 promoter or the ubiquitous Rous sarcoma virus (RSV) promoter. Robust skeletal and cardiac muscle transduction was achieved in the puppy that received Y445F/Y731F AAV-1. The puppy injected with Y731F AAV-9 only showed skeletal muscle transduction. Interestingly, minimal muscle expression was detected in three puppies that were treated with Y445F AAV-6. Serological study suggests that reduced Y445F AAV-6 transduction may relate to a high titer of the AAV-6 neutralization antibody (NAb) at birth.

## RESULTS

Y731F AAV-9 resulted in bodywide skeletal muscle transduction but no heart transduction

We have previously shown that systemic AAV-9 injection leads to widespread skeletal, but not cardiac, muscle transduction in neonatal dogs at the dose of  $\geq 2 \times 10^{11}$  vg/g.<sup>20</sup> To evaluate the systemic transduction profile of Y731F AAV-9, we delivered  $1.36 \times 10^{11}$  vg/g of the mutant AAV-9 SpC5-12.AP vector to a puppy (Monica #4) (Figure 1, Supplementary Figure S1a, and Table 1). Six weeks

<sup>1</sup>Department of Molecular Microbiology and Immunology, School of Medicine, The University of Missouri, Columbia, Missouri, USA; <sup>2</sup>Scott-Ritchey Research Center, College of Veterinary Medicine, Auburn University, Auburn, Alabama, USA; <sup>3</sup>Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, Alabama, USA; <sup>4</sup>Department of Neurology, School of Medicine, The University of Missouri, Columbia, Missouri, USA; <sup>5</sup>Current address: Department of Neurology, Pusan National University Yangsan Hospital, Yangsan, Republic of Korea. Correspondence: D Duan (duand@missouri.edu)  
Received 20 November 2013; accepted 5 January 2014



**Figure 1** Systemic Y731F AAV-9 injection in a newborn puppy (Monica #4,  $1.36 \times 10^{11}$  vg/g of SPC5-12.AP) resulted in bodywide skeletal muscle transduction. Representative photomicrographs of AP histochemical staining in (a) skeletal muscles, (d) heart muscles, and (g) internal organs. Scale bar = 100  $\mu\text{m}$  and it applies to all images. AP activity quantification results are shown for (b) skeletal muscles, (e) heart muscles, and (h) internal organs. The AAV genome copy number is shown for (c) skeletal muscles, (f) heart muscles, and (i) internal organs. AAV, adenoassociated virus; AP, alkaline phosphatase; BB, biceps brachii; BR, brain; CS, cranial sartorius; CT, cranial tibialis; DI, diaphragm; KI, kidney; LA, left atrium; LI, liver; LU, lung; LV, left ventricle; RA, right atrium; RV, right ventricle.

**Table 1** Summary of experimental animals

Dog name	Capsid	Promoter	Volume ( $\mu\text{l/g}$ )	AAV dose (vg/g)	Study duration (m)
Monica #4	Y731F AAV-9	Spc5-12	13.6	$1.36 \times 10^{11}$	1.5
Rover	Y445F AAV-6	RSV	7.5	$0.75 \times 10^{11}$	1.7
Monica #6	Y445F AAV-6	RSV	15.7	$1.57 \times 10^{11}$	1.5
Peter	Y445F AAV-6	Spc5-12	9.8	$1.47 \times 10^{11}$	9.2
Bing	Y445F/731F AAV-1	Spc5-12	12.2	$1.87 \times 10^{11}$	1.5

AAV, adenoassociated virus.

after injection, we performed whole-body necropsy. Uniform robust AP expression was observed in skeletal muscles throughout the body. Representative histochemical staining results from the biceps brachii, cranial sartorius, cranial tibialis, and the diaphragm are shown in Figure 1a and Supplementary Figure S1a. Enzymatic activity quantification revealed an average activity of  $11.32 \mu\text{mol/l}/\mu\text{g}$  (range:  $6.14$ – $17.04 \mu\text{mol/l}/\mu\text{g}$ ) (Figure 1b). Minimal AP expression was observed in the heart and internal organs (liver, kidney, lung, and brain) (Figure 1d, e, g and h). Although AP expression was substantially higher in skeletal muscle than that of other tissues, interestingly, the vector genome copy number in skeletal muscle ( $4.39$ – $7.42$  copies per diploid genome) did not differ much from that of the heart ( $2.58$ – $18.19$  copies per diploid genome), liver ( $3.39$  copies per diploid genome), kidney ( $5.22$  copies per diploid genome), and brain ( $2.75$  copies per diploid genome)

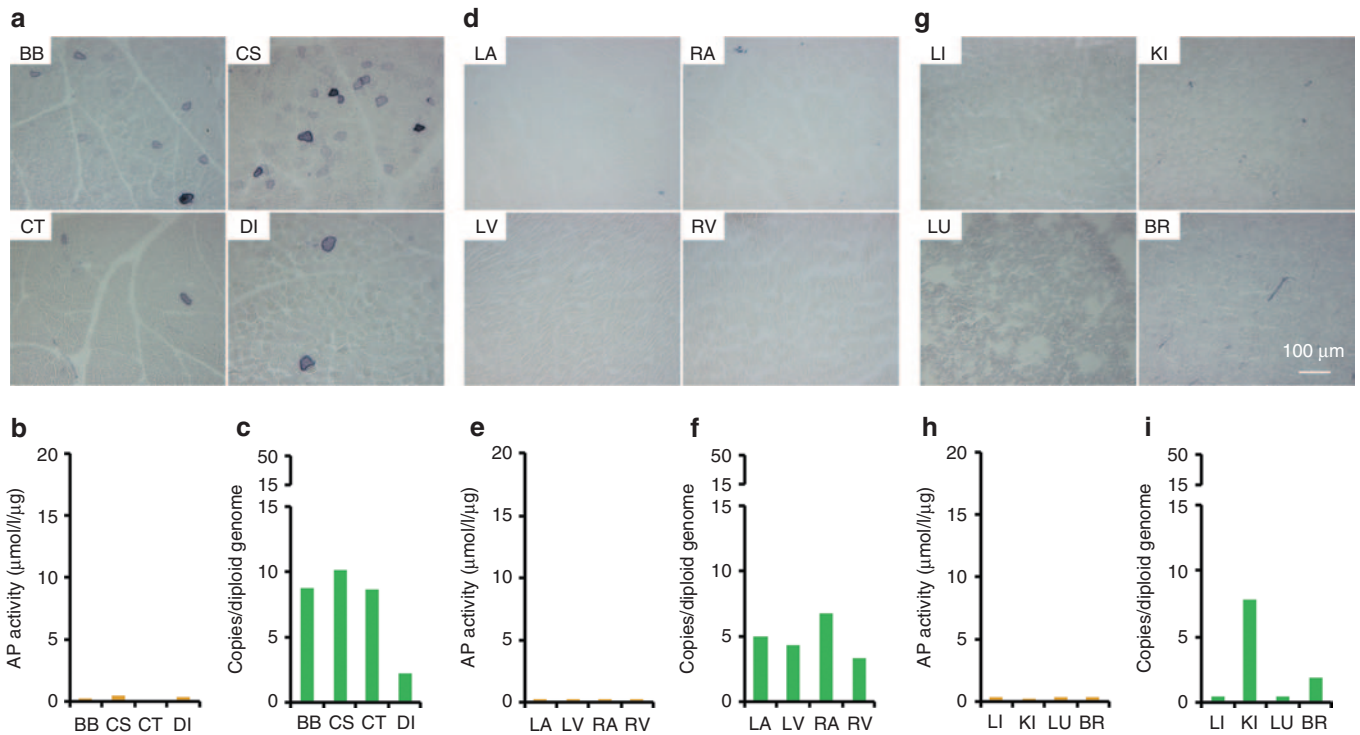
(Figure 1c,f,i). The only tissue that showed very low AAV copy number was the lung ( $0.57$  copies per diploid genome) (Figure 1i).

#### Y445F AAV-6 resulted in nominal muscle transduction

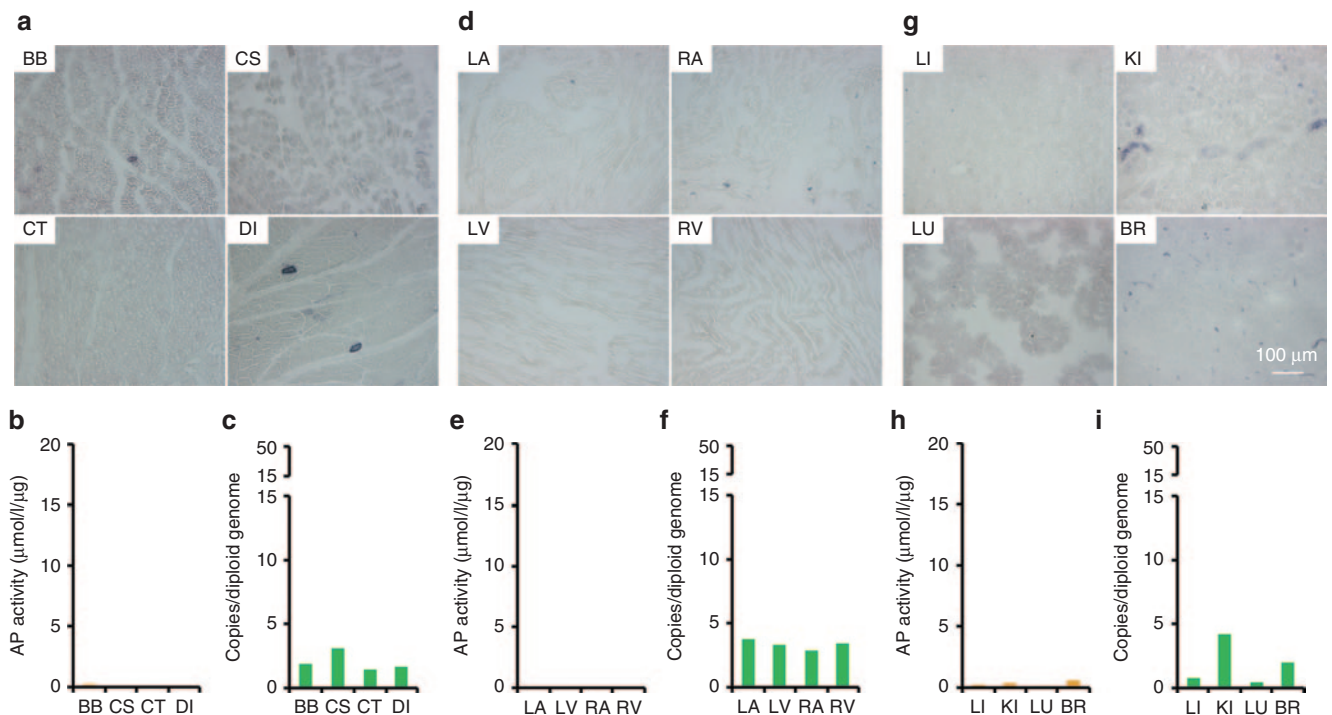
Tyrosine 445–mutated AAV-6 outperformed original AAV-6 following intramuscular injection in mice.<sup>16</sup> To test whether Y445F AAV-6 provides superior systemic transduction efficiency in neonatal dogs, we delivered Y445F AAV-6 to three newborn puppies via the jugular vein (Table 1). Two dogs (Rover and Monica #6) received the RSV.AP vector. The third dog (Peter) was injected with the SPC5-12.AP vector.

A necropsy was performed on Rover at 7 weeks after systemic injection of  $0.75 \times 10^{11}$  vg/g of RSV.AP Y445F AAV-6. Similar to the findings with Y731 AAV-9 injection (Monica #4) (Figure 1), there was nominal AP expression in the heart and internal organs (Figure 2 and Supplementary Figure S1a). Surprisingly, limited numbers of AP-positive cells and very low AP activity ( $0.18$ – $0.56 \mu\text{mol/l}/\mu\text{g}$ ) were observed in skeletal muscles (Figure 2). Nevertheless, the tissue AAV genome copy number ( $2.28$ – $10.12$ ,  $3.28$ – $6.72$ , and  $0.43$ – $7.87$  copies per diploid genome for skeletal muscle, heart, and internal organs, respectively) was quite comparable with those seen in Monica #4 (Y731F AAV-9 injection) (Figure 1).

One possible explanation for the lack of transduction in Rover could be the low vector dose (Table 1). To exclude this possibility, we doubled the dose and injected  $1.57 \times 10^{11}$  vg/g of RSV.AP Y445F AAV-6 to Monica #6 (Figure 3, Supplementary Figure S1b, and Table 1). Necropsy 6 weeks later revealed lower AP expression (AP activity,  $0.08$ – $0.24 \mu\text{mol/l}/\mu\text{g}$ ) and lower AAV genome copy number ( $1.50$ – $3.17$ ,  $2.91$ – $3.78$ , and  $0.46$ – $4.24$  copies per diploid genome for skeletal muscle, heart, and internal organs, respectively) (Figure 3).



**Figure 2** Transduction profile of Rover, a puppy that received  $0.75 \times 10^{11}$  vg/g of RSV.AP Y731F AAV-6 at birth. Representative photomicrographs of AP histochemical staining in (a) skeletal muscles, (d) heart muscles, and (g) internal organs. Scale bar = 100  $\mu\text{m}$  and it applies to all images. AP activity quantification results are shown for (b) skeletal muscles, (e) heart muscles, and (h) internal organs. The AAV genome copy number is shown for (c) skeletal muscles, (f) heart muscles, and (i) internal organs. AAV, adenoassociated virus; AP, alkaline phosphatase; BB, biceps brachii; BR, brain; CS, cranial sartorius; CT, cranial tibialis; DI, diaphragm; KI, kidney; LA, left atrium; LI, liver; LU, lung; LV, left ventricle; RV, right ventricle; RA, right atrium.



**Figure 3** Transduction profile of Monica #6, a puppy that received  $1.57 \times 10^{11}$  vg/g of RSV.AP Y731F AAV-6 at birth. Representative photomicrographs of AP histochemical staining in (a) skeletal muscles, (d) heart muscles, and (g) internal organs. Scale bar = 100  $\mu\text{m}$  and it applies to all images. AP activity quantification results are shown for (b) skeletal muscles, (e) heart muscles, and (h) internal organs. The AAV genome copy number is shown for (c) skeletal muscles, (f) heart muscles, and (i) internal organs. AAV, adenoassociated virus; AP, alkaline phosphatase; BB, biceps brachii; BR, brain; CS, cranial sartorius; CT, cranial tibialis; DI, diaphragm; KI, kidney; LA, left atrium; LI, liver; LU, lung; LV, left ventricle; RV, right ventricle; RA, right atrium.

As the vector used in the Y731F AAV-9 study contains the SPc5-12 promoter instead of the RSV promoter (Table 1), we tested Y445F AAV-6 in puppy Peter with  $1.47 \times 10^{11}$  vg/g of the SPc5-12.AP vector (Figure 4 and Supplementary Figure S1b). Peter was harvested at the age of 9.2 months. AP expression in Peter was quite low (Figure 4), consistent with the results of Rover and Monica #6 (Figures 2 and 3).

While AP expression in skeletal muscles of all three Y445F AAV-6-infected puppies was very low (Figures 2–4), fairly strong AP expression was seen in the tongues of Rover and Monica #6, although it was still much lower than that of other tyrosine mutants described in this article (Supplementary Figure S2). Compared with Rover and Monica #6, Peter had less expression in the tongue. However, it was still higher than that of the remaining skeletal muscles in this dog (Figure 4a and Supplementary Figure S2).

AAV-6 neutralization antibodies were present at birth in all experimental puppies

To understand the potential mechanism(s) underlying reduced transduction of Y445F AAV-6, we measured the titer of the preinjection NAb (Table 2).<sup>21</sup> All puppies showed low levels of AAV-9 NAb ( $\leq 1:5$ ) except for Peter (1:40). However, a consistently high preinjection NAb titer (1:80) was found in all four puppies described above (Table 2).

Y445F/Y731F double tyrosine mutant AAV-1 efficiently transduced both skeletal muscle and the heart

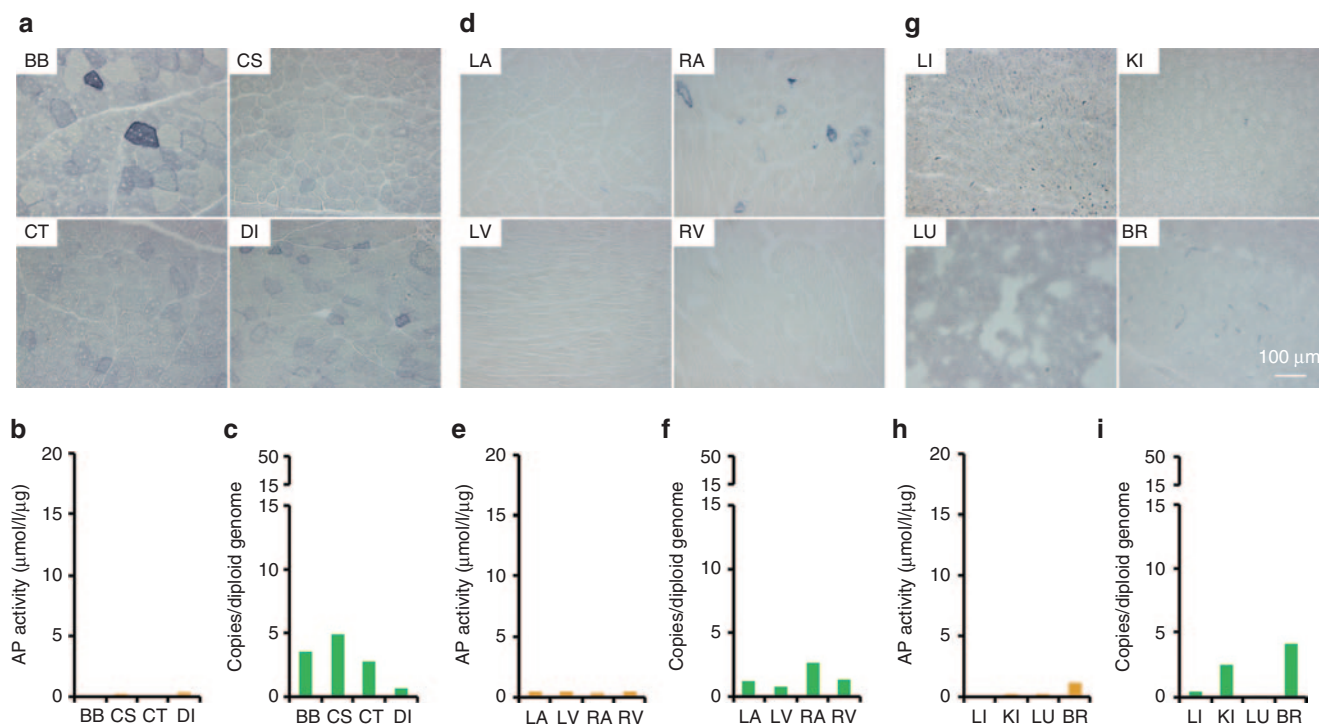
While screening for the AAV-6 neutralization antibody in newborn puppies, we found one puppy (Bing) with a very high AAV-6 NAb titer (1:640) (Table 2). As AAV-1 shares significant homology

with AAV-6,<sup>22</sup> we tested the Y445F/Y731F double tyrosine mutant AAV-1 SPc5-12.AP vector in Bing at the dose of  $1.87 \times 10^{11}$  vg/g (Figure 5 and Supplementary Figure S1c). The dog was euthanized 6 weeks after gene transfer (Table 1). Much to our surprise, there was robust AP expression in every muscle (Figure 5). The average AP activity level in skeletal muscle ( $12.99 \mu\text{mol/l}/\mu\text{g}$ ; range:  $9.87\text{--}18.75 \mu\text{mol/l}/\mu\text{g}$ ) reached that of Y731 AAV-9 (Monica #4) (Figures 1b and 5b). Importantly, high AP expression was observed in the heart (Figure 5d,e). The average level of AP activity from four heart chambers in Bing ( $3.79 \mu\text{mol/l}/\mu\text{g}$ ) was at least 20-fold higher than that of Monica #4 ( $0.12 \mu\text{mol/l}/\mu\text{g}$ ). Vector genome quantification also revealed an apparently higher copy number in skeletal muscle (average: 9.76, range:  $5.47\text{--}16.34$  copies per diploid genome) and the heart (average: 27.13, range:  $13.79\text{--}48.30$  copies per diploid genome) in Bing (Figure 5c,f).

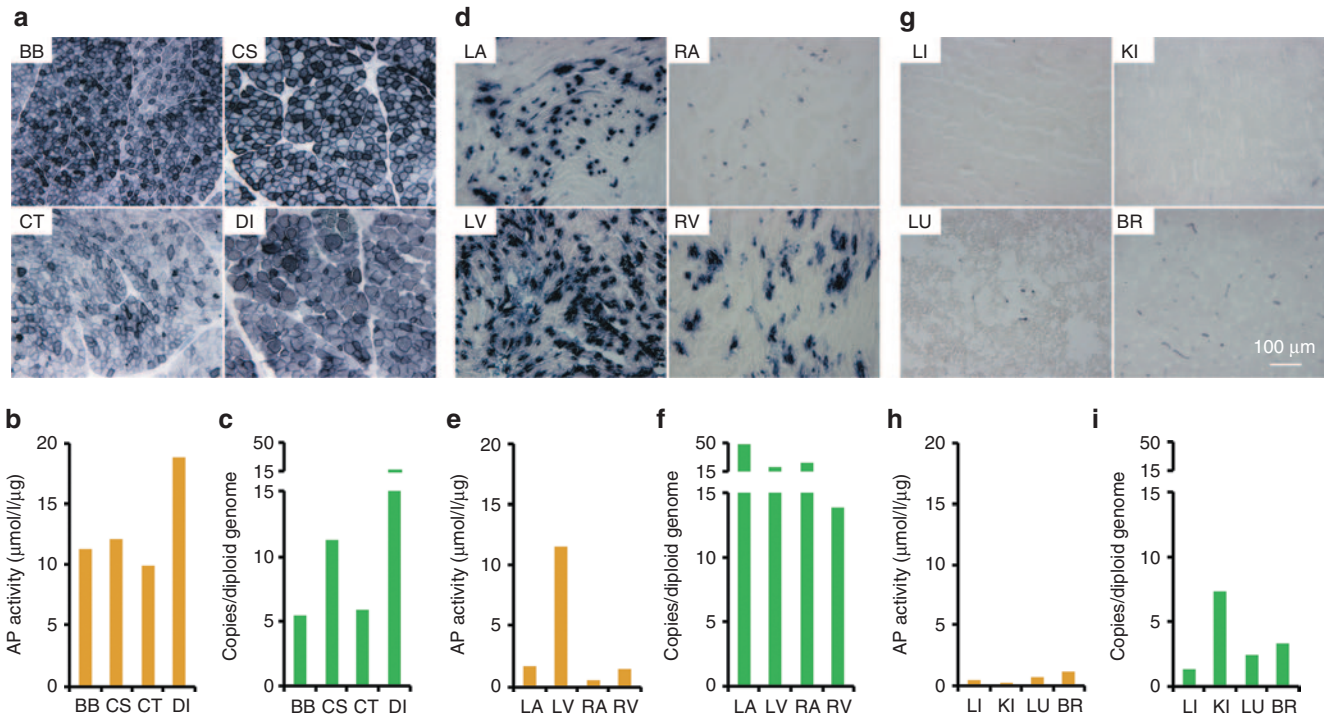
**Table 2** Summary of NAb analysis

Dog name	Capsid	Preinjection NAb	
		AAV-6	AAV-9
Monica #4	Y731F AAV-9	80	<5
Rover	Y445F AAV-6	80	5
Monica #6	Y445F AAV-6	80	<5
Peter	Y445F AAV-6	80	40
Bing	Y445F/731F AAV-1	640	<5

AAV, adenoassociated virus; Nab, neutralization antibody.



**Figure 4** Transduction profile of Peter, a puppy that received  $1.47 \times 10^{11}$  vg/g of SPc5-12.AP Y731F AAV-6 at birth and harvested at 9.2 months later. Representative photomicrographs of AP histochemical staining in (a) skeletal muscles, (d) heart muscles, and (g) internal organs. Scale bar = 100 μm and it applies to all images. AP activity quantification results are shown for (b) skeletal muscles, (e) heart muscles, and (h) internal organs. The AAV genome copy number is shown for (c) skeletal muscles, (f) heart muscles, and (i) internal organs. AAV, adenoassociated virus; AP, alkaline phosphatase; BB, biceps brachii; CS, cranial sartorius; CT, cranial tibialis; DI, diaphragm; LV, left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium; LI, liver; KI, kidney; LU, lung; and BR, brain.



**Figure 5** Neonatal intravascular Y445F/Y731F AAV-1 injection in a newborn puppy (Bing,  $1.87 \times 10^{11}$  vg/g of SPc5-12.AP) resulted in robust transduction in the heart and skeletal muscle. Representative photomicrographs of AP histochemical staining in (a) skeletal muscles, (d) heart muscles, and (g) internal organs. Scale bar = 100  $\mu$ m and it applies to all images. AP activity quantification results are shown for (b) skeletal muscles, (e) heart muscles, and (h) internal organs. The AAV genome copy number is shown for (c) skeletal muscles, (f) heart muscles, and (i) internal organs. AAV, adenoassociated virus; AP, alkaline phosphatase; BB, biceps brachii; CS, cranial sartorius; CT, cranial tibialis; DI, diaphragm; LV, left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium; LI, liver; KI, kidney; LU, lung; and BR, brain.

## DISCUSSION

In this study, we evaluated systemic gene transfer of Y445F/Y731F AAV-1, Y445F AAV-6, and Y731F AAV-9 in newborn dogs. Our results suggest that Y445F/Y731F AAV-1 may represent an excellent candidate vector to test bodywide neonatal gene therapy in canine models of muscular dystrophy in the future (Figure 5). Y731F AAV-9 has limited transduction in the heart but it can still be used for whole body skeletal muscle gene delivery (Figure 1). Y445F AAV-6, on the other hand, is not effective for systemic gene transfer to either skeletal or cardiac muscle in newborn puppies (Figures 2–4).

In the muscular dystrophies, usually both skeletal and cardiac muscles are damaged. An effective gene therapy will require robust transduction in skeletal muscle and the heart. Three AAV serotypes including AAV-6, -8, and -9 have been successfully used to test whole body (skeletal and cardiac muscles) gene therapy in neonatal/adult/aged rodent models of muscular dystrophy.<sup>10,12,23–27</sup> However, systemic AAV transfer in large mammals has lagged behind. So far, bodywide AAV delivery has only been tested in newborn puppies.<sup>20,28,29</sup> We reported the first case of intravascular AAV gene transfer in neonatal dogs in 2008.<sup>20</sup> In that study, we used AAV-9 because this serotype was considered as a “cardiotropic” vector. Despite efficient whole body skeletal muscle transduction, surprisingly, AAV-9 did not transduce the heart.<sup>20</sup> To overcome this hurdle, we initiated two different studies. In one study, we tested AAV-8.<sup>28</sup> We found that AAV-8 yielded robust skeletal muscles and myocardial transduction at a high dose ( $\geq 7.14 \times 10^{11}$  vg/g). At the same time, we decided to explore surface tyrosine-mutated AAV viruses because these mutants were more potent than their wild-type counterpart.<sup>15–19</sup> One of the puppies (Bing) in this cohort of

the study received  $1.87 \times 10^{11}$  vg/g of Y445F/Y731F AAV-1. We not only observed robust bodywide skeletal muscle transduction but also detected strong expression in the heart of this puppy (Figure 5). Our results suggest that Y445F/Y731F AAV-1 may represent another highly promising AAV serotype (besides AAV-8) to treat both skeletal muscle disease and cardiomyopathy in dystrophic dogs.

We have previously demonstrated that AAV-9 can efficiently transduce whole body skeletal muscle at a dose of  $\geq 2 \times 10^{11}$  vg/g.<sup>20</sup> However, when tested at a lower dose ( $1 \times 10^{11}$  vg/g), AAV-9 only resulted in partial skeletal muscle transduction. Specifically, some muscles (such as biceps brachii, extensor carpi radialis, and cranial sartorius) were highly transduced (>80% myofibers expressed transgene). Other muscles (such as cranial tibialis and brachial triceps) only had 20–70% myofibers transduced. Yet a third group of muscles (such as rectus femoris, biceps femoris, gastrocnemius, and the diaphragm) were barely transduced (<5% myofiber expressed transgene).<sup>20</sup> With Y731F AAV-9, every skeletal muscle was highly transduced (>90% of myofiber showed AP expression) including the ones that were moderately (such as cranial tibialis) or minimally (such as the diaphragm) transduced by low dose AAV-9 (Figure 1). Diaphragm transduction is especially important since diaphragm insufficiency-associated respiratory failure is the leading cause of death in muscular dystrophy. Although additional studies are needed to corroborate our findings, our results suggest that Y731F AAV-9 is at least as efficient as (if not more efficient) than unmodified AAV-9.

The SPc5-12 promoter is a synthetic muscle promoter.<sup>30</sup> It has been used to drive strong transgene expression in dog muscle by local gene transfer.<sup>31,32</sup> Results from two puppies (Monica #4 and Bing) suggest that this promoter can also be used for systemic muscle gene transfer in dogs (Figures 1 and 5).

Studies from several laboratories suggest that AAV-6 may not be ideal for intravascular delivery in dogs due to the high titer of pre-existing NAb and species-specific interaction with the galectin 3-binding protein in dog serum.<sup>21,33,34</sup> However, Arnett *et al.* proposed that AAV-6 is suitable for systemic gene delivery in dogs.<sup>35</sup> To further study this question, we delivered Y445F AAV-6 to three newborn puppies at the doses that were about 50% of (Rover; Figure 2) or equivalent to (Monica #6 and Peter; Figures 3 and 4) what we used for Y731F AAV-9 study (Monica #4) (Figure 1 and Table 1). These injections were conducted at two different locations (The University of Missouri and Auburn University). The vectors contain either the ubiquitous RSV promoter or muscle-specific SPc5-12 promoter. Necropsy was performed between the ages of 1.5 and 9.2 months (Table 1). We found nominal AP expression in skeletal muscles and the heart in all three Y445F AAV-6-infected dogs (Figures 2–4). Additional studies indicated that all three dogs had a high NAb titer (1:80) for AAV-6 before gene transfer (Table 2).<sup>21</sup> The correlation between the high AAV-6 NAb titer and the lack of transgene expression in Y445F AAV-6-infected dogs is suggestive of an immune-mediated inhibition mechanism.

We also detected a high level of AAV-6 NAb in Monica #4 (Table 2). However, systemic Y731F AAV-9 injection resulted in efficient skeletal muscle expression in this puppy. This piece of data suggests that AAV-6 NAb does not impact on AAV-9 transduction in neonatal puppies. AAV-6 is a naturally occurring hybrid of AAV-1 and AAV-2.<sup>22</sup> The capsid protein sequence of AAV-1 and -6 are 99% identical. Antibodies against the intact AAV-1 particles have been shown to cross-react with AAV-6 due to the binding of the same region on the surface.<sup>36</sup> We identified a newborn puppy (Bing) with a 1:640 NAb titer to AAV-6 (Table 2). Surprisingly, the high-level AAV-6 NAb did not compromise the transduction of Y445F/Y731F AAV-1 in Bing (Figure 5). This unexpected observation suggests that Y445F/Y731F AAV-1 may represent a NAb escape mutant.

There are several inherent limitations of the study. The first is the relatively small sample size. Although more puppies were used in this study (five dogs compared with three to four dogs used in previous studies),<sup>20,28,29,40</sup> additional confirmative studies may further corroborate our finding (especially for Y445F/Y731F AAV-1 and Y731F AAV-9). What is equally important is to extend our study to dystrophic puppies/therapeutic genes in the future. Results from those studies will reveal potential differences between normal and affected dogs and set the foundation for neonatal gene therapy in human patients. A second limitation is the lack of side-by-side comparison with unmodified vectors. Among three AAV serotypes used here, we have only studied unmodified AAV-9 in a previous report.<sup>20</sup> The data here seem to suggest a slightly better performance with Y731F AAV-9. The original goal of our study is to identify one serotype that can transduce both skeletal muscle and the heart (rather than to determine whether tyrosine mutants outperform wild-type capsids). The observation with Y731F AAV-9 suggests that future in-depth studies are warranted in order to better understand the significance/contribution of surface tyrosine mutation to AAV-9 gene transfer in neonatal dogs.

An intriguing finding is the lack of direct correlation between the viral genome copy number and the AP expression level. Monica #4, Rover, Monica #6, and Bing were harvested at the approximately same age (6–7 weeks). Monica #4 and Bing yielded 45-fold higher AP activity (mean  $\pm$  SEM,  $12.16 \pm 1.44$   $\mu\text{mol/l}/\mu\text{g}$ ; range: 6.14–18.75  $\mu\text{mol/l}/\mu\text{g}$ ) than Rover and Monica #6 (mean  $\pm$  SEM,  $0.27 \pm 0.06$   $\mu\text{mol/l}/\mu\text{g}$ ; range: 0.08–0.56  $\mu\text{mol/l}/\mu\text{g}$ ) in skeletal muscle ( $P < 0.00001$ ). Yet the AAV genome copy number in Monica #4 and

Bing (mean  $\pm$  SEM,  $7.61 \pm 1.48$  copies per diploid genome; range:  $4.39 \pm 16.34$  copies per diploid genome) was not different from that of Rover and Monica #6 (mean  $\pm$  SEM,  $4.77 \pm 1.31$  copies per diploid genome; range: 1.50–10.12 copies per diploid genome) ( $P = 0.174$ ). Currently, we do not have a solid explanation for this discrepancy. However, there are several possibilities. For example, in the case of Rover and Monica #6, AAV may have been trapped in the muscle tissue but not inside myocytes. Alternatively, the vector genome may be in a transcriptionally incompetent status or partially degraded. Future studies will clarify this issue. Nevertheless, our results caution the use of the vector genome copy number as the only parameter to evaluate AAV transduction.

Another provocative finding is the relatively high transduction efficiency of Y445F AAV-6 in the tongue. Since the tongue is not an immune privileged organ, we cannot explain this result by immune evasion. Whether preferential tongue transduction relates to the anatomic structure or other yet undefined factors requires additional investigations in the future. Nevertheless, this finding suggests that we should be cautious not to extrapolate systemic muscle transduction efficiency from a single muscle.

In summary, our results suggest that Y445F/Y731F AAV-1 may serve as an excellent vector system to target both the heart and skeletal muscle in neonatal dogs. On the other hand, caution is advised when using Y445F AAV-6 for skeletal muscle and/or heart transduction in newborn puppies.

## MATERIALS AND METHODS

### Animals

All animal experiments were approved by the Animal Care and Use Committees of the University of Missouri and the Auburn University and were in accordance with the National Institutes of Health guidelines. Five normal male puppies were used in this study (Table 1). All experimental dogs were generated by artificial insemination and were on a mixed genetic background of golden retriever, Labrador retriever, beagle, and Welsh corgi. The genotyping was determined by PCR as we previously described.<sup>37,38</sup>

### Recombinant tyrosine mutant AAV vector

The construction of the Y445F AAV-6 and Y731F AAV-9 capsid genes has been published before.<sup>16,17</sup> The Y445F/Y731F AAV-1 capsid gene was generated using a similar strategy.<sup>15</sup> Briefly, the coding sequences of the tyrosine residues at position 445 and 731 of the AAV-1 capsid gene were changed to that of phenylalanine by site-directed mutagenesis (position 445, from TAC to TTT; position 731, from TAC to TTC). The pcis.RSV.AP plasmid was used to generate the stock RSV.AP vector. This plasmid has been described previously.<sup>20</sup> The pcis.SPc5-12.AP plasmid (also called pCH1) was engineered by replacing the RSV promoter of pcis.RSV.AP with the synthetic SPc5-12 muscle promoter using standard molecular cloning methods.<sup>30</sup> Recombinant endotoxin-free tyrosine mutant AAV-1, -6, and -9 vectors were generated using the triple plasmids transfection method we described previously.<sup>39</sup> Viral titer was determined using the Fast SYBR Green Master Mix kit (Bio-Rad, Hercules, CA) by quantitative PCR (qPCR) in an ABI 7900 HT qPCR machine (Applied Biosystems, Foster City, CA). As RSV.AP and SPc5-12 vectors have different promoters, we opted to use primer sets located in the AP transgene for viral titer (vector genome particle) determination. The forward primer for viral titer quantification is 5' CCAGATGACTACAGCCAAGGTG. The reverse primer for viral titer quantification is 5' GGCTCAAAGACCCATGAGAT.

### Systemic AAV delivery

Systemic gene transfer was performed in awake newborn puppies within 48 hours of birth.<sup>20,28,40</sup> Briefly, the puppy was gently restrained and the neck hair was shaved. A catheter was placed in the jugular vein and secured with a surgical tape. AAV vector was delivered as a single bolus injection through the jugular vein. Viral dose was expressed as viral genome particles per gram body weight (vg/g). Three puppies (Bing, Peter and Rover) received

neonatal injection at the University of Missouri. Two puppies (Monica #4 and #6) received neonatal injection at Auburn University. Experimental details (dog name, AAV capsid, promoter, volume injected in each puppy, viral dose, and the study duration) are described in Table 1.

### Examination of AP reporter gene expression

At the end of the study, a full necropsy was performed using our published protocol.<sup>20,28</sup> Freshly harvested tissues were either directly frozen in liquid nitrogen or embedded in a liquid nitrogen isopentane cryobath in optimal temperature cutting media (Sakura Finetek, Torrance, CA). AP expression was evaluated by histochemical staining on tissue sections and enzymatic activity assay using tissue lysate.<sup>20,28,40</sup> Histochemical staining was performed on 8- $\mu$ m cryosections. Briefly, samples were fixed in 0.5% glutaraldehyde for 10 minutes and washed in 1 mmol/l MgCl<sub>2</sub>. Endogenous AP activity was inactivated by incubating the slides at 65 °C for 45 minutes. The slides were then washed with a prestaining buffer (100 mmol/l Tris-HCl, pH 9.5, 50 mmol/l MgCl<sub>2</sub>, 100 mmol/l NaCl) and stained in freshly prepared staining solution (165 mg/ml 5-bromo-4-chloro-3-indolylphosphate-p-toluidine, 330 mg/ml nitroblue tetrazolium chloride, 50 mmol/l levamisole) for 5–20 minutes. Enzymatic AP activity was quantified using the Stem TAGTM AP activity assay kit (Cell Biolabs, San Diego, CA) according to manufacturer's instruction. Prior to the assay, tissue lysate was incubated at 65 °C for 1 hour to inactivate endogenous AP activity.

### AAV genome copy determination

Genomic DNA was extracted from frozen tissue samples. The AAV genome copy was determined using SYBR green-based qPCR. A set of primers located within the AP gene (but different from what we used in viral titer determination) was used to quantify the viral genome copy number. The forward primer is 5' GACTGAGCCCATGACACCAA. The reverse primer is 5' CATCTGTCTCGACCCACTG.

### CONFLICT OF INTEREST

The authors declared no conflict of interest.

### ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (NIH) HL-91883 (to D.D.) and AR-49419 (to D.D.), Muscular Dystrophy Association (to D.D.) and Parent Project Muscular Dystrophy (to D.D.). We thank the Immunology Core at the Gene Therapy Program at the University of Pennsylvania for the testing of AAV neutralizing antibodies. We acknowledge the Gene Therapy Resource Program of the National Heart, Lung, and Blood Institute, NIH for providing the immunology test. We thank Arun Srivastava (University of Florida, Gainesville, FL) for providing tyrosine mutant AAV capsid packaging plasmids. We thank the Inovio Pharmaceuticals, Inc. (Blue Bell, PA) for providing the SPc5-12 promoter. We recognize the excellent technical assistance provided by Xiufang Pan, Marianne Hakim, Han Tran, Lalitha Kesetty, and Kasun Kodippili.

### REFERENCES

- Rahimov, F and Kunkel, LM (2013). The cell biology of disease: cellular and molecular mechanisms underlying muscular dystrophy. *J Cell Biol* **201**: 499–510.
- Flanigan, KM (2012). The muscular dystrophies. *Semin Neurol* **32**: 255–263.
- McNally, EM and Pytel, P (2007). Muscle diseases: the muscular dystrophies. *Annu Rev Pathol* **2**: 87–109.
- Kunkel, LM (2005). 2004 William Allan Award address. Cloning of the DMD gene. *Am J Hum Genet* **76**: 205–214.
- Davies, KE and Nowak, KJ (2006). Molecular mechanisms of muscular dystrophies: old and new players. *Nat Rev Mol Cell Biol* **7**: 762–773.
- Morgan, JE and Partridge, TA (1992). Cell transplantation and gene therapy in muscular dystrophy. *Bioessays* **14**: 641–645.
- Chamberlain, JS (1992). X-linked dystrophies: from gene localization to gene therapy. *Curr Opin Neurol Neurosurg* **5**: 610–614.
- Mendell, JR, Rodino-Klapac, L, Sahenk, Z, Malik, V, Kaspar, BK, Walker, CM *et al.* (2012). Gene therapy for muscular dystrophy: lessons learned and path forward. *Neurosci Lett* **527**: 90–99.

- Mingozzi, F and High, KA (2011). Therapeutic *in vivo* gene transfer for genetic disease using AAV: progress and challenges. *Nat Rev Genet* **12**: 341–355.
- Gregorevic, P, Allen, JM, Minami, E, Blankinship, MJ, Haraguchi, M, Meuse, L *et al.* (2006). rAAV6-microdystrophin preserves muscle function and extends lifespan in severely dystrophic mice. *Nat Med* **12**: 787–789.
- Odom, GL, Gregorevic, P, Allen, JM and Chamberlain, JS (2011). Gene therapy of mdx mice with large truncated dystrophins generated by recombination using rAAV6. *Mol Ther* **19**: 36–45.
- Zhang, Y, Yue, Y, Li, L, Hakim, CH, Zhang, K, Thomas, GD *et al.* (2013). Dual AAV therapy ameliorates exercise-induced muscle injury and functional ischemia in murine models of Duchenne muscular dystrophy. *Hum Mol Genet* **22**: 3720–3729.
- Wang, B, Li, J, Fu, FH 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.
- Duan, D (2011). Duchenne muscular dystrophy gene therapy: Lost in translation? *Res Rep Biol* **2011**: 31–42.
- Zhong, L, Li, B, Mah, CS, Govindasamy, L, Agbandje-McKenna, M, Cooper, M *et al.* (2008). Next generation of adeno-associated virus 2 vectors: point mutations in tyrosines lead to high-efficiency transduction at lower doses. *Proc Natl Acad Sci USA* **105**: 7827–7832.
- Qiao, C, Zhang, W, Yuan, Z, Shin, JH, Li, J, Jayandharan, GR *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.
- Petrs-Silva, H, Dinculescu, A, Li, Q, Min, SH, Chiodo, V, Pang, JJ *et al.* (2009). High-efficiency transduction of the mouse retina by tyrosine-mutant AAV serotype vectors. *Mol Ther* **17**: 463–471.
- Markusic, DM, Herzog, RW, Aslanidi, GV, Hoffman, BE, Li, B, Li, M *et al.* (2010). High-efficiency transduction and correction of murine hemophilia B using AAV2 vectors devoid of multiple surface-exposed tyrosines. *Mol Ther* **18**: 2048–2056.
- Dalkara, D, Byrne, LC, Lee, T, Hoffmann, NV, Schaffer, DV and Flannery, JG (2012). Enhanced gene delivery to the neonatal retina through systemic administration of tyrosine-mutated AAV9. *Gene Ther* **19**: 176–181.
- Yue, Y, Ghosh, A, Long, C, Bostick, B, Smith, BF, Kornegay, JN *et al.* (2008). A single intravenous injection of adeno-associated virus serotype-9 leads to whole body skeletal muscle transduction in dogs. *Mol Ther* **16**: 1944–1952.
- Shin, JH, Yue, Y, Smith, B and Duan, D (2012). Humoral immunity to AAV-6, 8, and 9 in normal and dystrophic dogs. *Hum Gene Ther* **23**: 287–294.
- Xiao, W, Chirmule, N, Berta, SC, McCullough, B, Gao, G and Wilson, JM (1999). Gene therapy vectors based on adeno-associated virus type 1. *J Virol* **73**: 3994–4003.
- Wang, Z, Zhu, T, Qiao, C, Zhou, L, Wang, B, Zhang, J *et al.* (2005). Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. *Nat Biotechnol* **23**: 321–328.
- Gregorevic, P, Blankinship, MJ, Allen, JM, Crawford, RW, Meuse, L, Miller, DG *et al.* (2004). Systemic delivery of genes to striated muscles using adeno-associated viral vectors. *Nat Med* **10**: 828–834.
- Bostick, B, Shin, JH, Yue, Y and Duan, D (2011). AAV-microdystrophin therapy improves cardiac performance in aged female mdx mice. *Mol Ther* **19**: 1826–1832.
- Bostick, B, Yue, Y, Lai, Y, Long, C, Li, D and Duan, D (2008). Adeno-associated virus serotype-9 microdystrophin gene therapy ameliorates electrocardiographic abnormalities in mdx mice. *Hum Gene Ther* **19**: 851–856.
- Bostick, B, Shin, JH, Yue, Y, Wasala, NB, Lai, Y and Duan, D (2012). AAV micro-dystrophin gene therapy alleviates stress-induced cardiac death but not myocardial fibrosis in >21-m-old mdx mice, an end-stage model of Duchenne muscular dystrophy cardiomyopathy. *J Mol Cell Cardiol* **53**: 217–222.
- Pan, X, Yue, Y, Zhang, K, Lostal, W, Shin, JH and Duan, D (2013). Long-term robust myocardial transduction of the dog heart from a peripheral vein by adeno-associated virus serotype-8. *Hum Gene Ther* **24**: 584–594.
- Kornegay, JN, Li, J, Bogan, JR, Bogan, DJ, Chen, C, Zheng, H *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, X, Eastman, EM, Schwartz, RJ and Draghia-Akli, R (1999). Synthetic muscle promoters: activities exceeding naturally occurring regulatory sequences. *Nat Biotechnol* **17**: 241–245.
- Brown, PA, Bodles-Brakhop, AM, Pope, MA and Draghia-Akli, R (2009). Gene therapy by electroporation for the treatment of chronic renal failure in companion animals. *BMC Biotechnol* **9**: 4.
- Koo, T, Okada, T, Athanasopoulos, T, Foster, H, Takeda, S and Dickson, G (2011). Long-term functional adeno-associated virus-microdystrophin expression in the dystrophic CXMDj dog. *J Gene Med* **13**: 497–506.
- Rapti, K, Louis-Jeune, V, Kohlbrenner, E, Ishikawa, K, Ladage, D, Zolotukhin, S *et al.* (2012). Neutralizing antibodies against AAV serotypes 1, 2, 6, and 9 in sera of commonly used animal models. *Mol Ther* **20**: 73–83.
- Denard, J, Beley, C, Kotin, R, Lai-Kuen, R, Blot, S, Leh, H *et al.* (2012). Human galectin 3 binding protein interacts with recombinant adeno-associated virus type 6. *J Virol* **86**: 6620–6631.

- 35 Arnett, AL, Garikipati, D, Wang, Z, Tapscott, S and Chamberlain, JS (2011). Immune Responses to rAAV6: The Influence of Canine Parvovirus Vaccination and Neonatal Administration of Viral Vector. *Front Microbiol* **2**: 220.
- 36 Gurda, BL, DiMattia, MA, Miller, EB, Bennett, A, McKenna, R, Weichert, WS *et al.* (2013). Capsid antibodies to different adeno-associated virus serotypes bind common regions. *J Virol* **87**: 9111–9124.
- 37 Fine, DM, Shin, JH, Yue, Y, Volkmann, D, Leach, SB, Smith, BF *et al.* (2011). Age-matched comparison reveals early electrocardiography and echocardiography changes in dystrophin-deficient dogs. *Neuromuscul Disord* **21**: 453–461.
- 38 Smith, BF, Yue, Y, Woods, PR, Kornegay, JN, Shin, JH, Williams, RR *et al.* (2011). An intronic LINE-1 element insertion in the dystrophin gene aborts dystrophin expression and results in Duchenne-like muscular dystrophy in the corgi breed. *Lab Invest* **91**: 216–231.
- 39 Shin, JH, Yue, Y and Duan, D (2012). Recombinant adeno-associated viral vector production and purification. *Methods Mol Biol* **798**: 267–284.
- 40 Yue, Y, Shin, JH and Duan, D (2011). Whole body skeletal muscle transduction in neonatal dogs with AAV-9. *Methods Mol Biol* **709**: 313–329.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivative Works 3.0 License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/3.0/>