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Systemic delivery of AAV8 *in utero* results in gene expression in diaphragm and limb muscle: Treatment implications for muscle disorders

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Abstract

One of the major challenges in the treatment of primary muscle disorders, which often affect many muscle groups, is achieving efficient, widespread transgene expression in muscle. *In utero* gene transfer can potentially address this problem by accomplishing gene delivery when the tissue mass is small and the immune system is immature. Previous studies with systemic *in utero* adeno-associated viral (AAV) vector serotype 1 gene delivery to embryonic day 16 (E-16) pups resulted in high levels of transduction in diaphragm and intercostal muscles, but no detectable transgene expression in limb muscles. Recently newer AAV serotypes such as AAV8 have demonstrated widespread and high transgene expression in skeletal muscles and diaphragm by systemic delivery in adult and neonatal mice. We tested AAV8 vector gene delivery by intraperitoneal administration in E-16 mice *in utero*. Using an AAV8 vector carrying a *lac*Z reporter gene, we observed high level transduction of diaphragm and intercostal muscles and more moderate transduction of multiple limb muscles and heart. Our current studies demonstrate the potential of AAV8 to achieve widespread muscle transduction *in utero* and suggest its therapeutic potential for primary muscle disorders.

Introduction

Several genetically-determined muscle disorders are caused by loss-of-function mutations and present at birth or in early childhood. Viral vectors delivered *in utero* offer a potential means of gene therapy for these disorders that primarily affect striated muscle.

Adeno-associated viral (AAV) vectors provide efficient gene delivery to striated muscle tissues.[1] Recently identified AAV serotypes have varied tissue tropism and transduction efficiency. Among the currently known serotypes, AAV8 demonstrates superior potential to deliver transgenes to muscle tissues.[2–9] Using systemic delivery of AAV8 vectors, reporter gene and therapeutic gene transfer studies in postnatal mice and hamsters demonstrated efficient transduction of skeletal muscle and heart in neonates and adults.[6,7] Similarly Nakai et al. demonstrated the ability of AAV8 vector to deliver a transgene to skeletal muscle by

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systemic gene delivery in adult mice.[6] Other studies also showed AAV8 vector effectively transduced nonhuman primate skeletal muscles.[5]

In disease models, AAV8 vectors have demonstrated potential for therapeutic benefit. In response to AAV8 vector-mediated myostatin-inhibitor gene transfer to *mdx* mice, skeletal muscle mass increased.[4] AAV8 vector-mediated α-glucosidase gene transfer decreased glycogen storage in skeletal muscle of a mouse model of Pompe disease.[3,9]

Furthermore, the AAV8 vector appeared to be less immunogenic[10] and demonstrated more rapid uncoating post-internalization[11] compared to the AAV2 vector. In addition, the AAV8 vector also demonstrated good potential to deliver transgenes in various animal models such as mouse, rat and non-human primates.[7,12–14] The AAV8 vector also showed high level transduction in various tissues such as pancreatic cells[15], neuronal cells[16,17], liver cells[18], and smooth muscle cells.[17]

Although AAV8 vectors have been extensively studied in various animal models and in different age groups, their transduction potential *in utero* remains unknown. Preclinical studies have shown that *in utero* gene delivery results in good expression of transgene extending into adulthood. Intramuscular injection of AAV1[19] and AAV2[20] vectors into fetal muscle demonstrated good gene expression. However, systemic delivery of AAV1 vector *in utero* did not transduce limb muscle tissue to any significant degree.[19] This study therefore aims to determine the distribution of AAV8 vector particles to different tissues and levels of expression after *in utero* systemic delivery.

Materials and methods

Production of AAV8lacZ vector

AAV8 vector carrying a *lac*Z expression cassette driven by the human cytomegalovirus (HCMV) promoter (AAV8*lac*Z) vector stocks were generated by the triple-plasmid transfection method.[21] Briefly, the three plasmids are the AAV-CMV-lacZ vector plasmid, the mini-adeno helper plasmid, and the AAV8 packaging plasmid containing the AAV2 Rep gene and AAV8 Cap gene, which has an ATG to ACG start codon mutation to increase the vector yield.[22] AAV8*lac*Z viral particles were purified by double CsCl gradient centrifugation and dialyzed three times against PBS containing 5% Sorbitol. The titer of vector genomes was determined by a standard DNA dot-blot assay.

Mice and in utero AAV8lacZ vector administration

Timed pregnant CD1 mice (Harlan Sprangue Dawley Inc, Maryland) at embryonic day 16 (E-16) were used for these studies according to the protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee. The pregnant mice were anesthetized with an (intraperioneal) IP injection of 5mg/kg of xylazine and 50mg/kg of ketamine. After shaving the abdominal wall and cleaning the skin with betadine, a single vertical incision was made through the abdominal wall and peritoneal membrane to expose the uterus. Fetal injections were performed under sterile conditions and high magnification. AAV8*lac*Z was injected intraperitoneally. A fluorescent marker (fluorescent beads or fluorescent dextran) was also injected as a means to identify treated mice after birth. A total volume of 8–10 μl of AAV8*lac*Z solution/fetus was injected using a 33 G needle (Hamilton, USA) attached to a PB600 syringe dispenser (Hamilton, USA). A dose of 6.4×10^{11} vector genomes were injected per fetus. For this study, we injected 14 pups from 9 pregnant CD1 mothers to achieve 8 positive pups. A total of 8 treated and 8 untreated littermate pups were studied.

Between 2 and 5 days following injection, the naturally delivered pups were examined using a fluorescence microscope to identify those pups that were injected by the presence of the

fluorescent marker. At 9 weeks following injection, different tissues from the mother, the injected pups and uninjected littermates were analyzed for biodistribution of β-galactosidase (β-gal) expression and vector content.

Whole muscle staining

Tissues such as forelimb muscle, hindlimb muscle, heart, diaphragm, intercostal muscle and abdominal muscles were isolated from mice, washed in 1x PBS for 5 minutes and stained for β-gal expression. Whole muscle tissues were fixed with 0.5% glutaraldehyde and immersed in 5-bromo-4-chloro-3-indole-β-D-galactopyranoside (X-gal) staining solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/ml X-gal and 1 mM $MgCl_2$) for 1– 2 hours at 37°C. Stained tissues were imaged using a Fujipix 5400 camera.

Cryosections

The tissues that were analyzed were tibialis anterior, quadriceps, gastrocnemius, forelimb muscle, abdominal muscle, intercostal muscle, diaphragm, heart, liver, lung, spleen, and kidney. Tissue samples were snap-frozen in 2-methylbutane cooled with dry ice. Half of the sample was used for preparing cryo-sections using a cryostat (HM 550, Richard-Allan Scientific) and the other half was used to extract protein and DNA. The cryosections taken on glass slides were stained for β-gal expression. Sections were fixed with 0.5% glutaraldehyde, stained in X-gal staining solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/ml X-gal and 1 mM $MgCl_2$) for 2– 3 hours at 37°C, mounted and viewed with a Zeiss Axiophot microscope.

Immunohistochemistry

Tibialis anterior muscle sections were pretreated with a blocking solution (Mouse-on Mouse [M.O.M.]; Vector, Burlingame, CA) in order to prevent nonspecific antibody binding. Sections were then incubated with polyclonal rabbit anti-β-galactosidase (5′ Prime 3′ Prime, Inc., USA) diluted at 1:2000 and monoclonal anti-myosin (skeletal, slow) (Sigma, USA) diluted at 1:2000 for 2 hrs. After a 20 min wash in 1X phosphate buffered saline (PBS), the sections were incubated with a Cy3 AffiniPure Donkey Anti-Rabbit IgG (Jackson ImmunoResearch Inc., USA) diluted 1:300 with M.O.M. and an Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen, USA) diluted 1:100 with M.O.M. for 30 min at room temperature. Cell nuclei were stained with Hoechst. Muscle sections were visualized using fluorescence microscopy.

Ortho-nitro-phenyl galactopyranoside (ONPG) β-gal expression assay

To quantitate β-gal expression, the ONPG assay was performed on extracted protein from tissue samples.[23] Briefly, snap frozen tissue samples were treated with TEES buffer (25 mM Tris-HCl pH 8.0, 2.5 mM ethylenediaminetetraacetic acid [EDTA] pH 8.0, 2.5 mM ethyleneglycoltetraacetic acid [EGTA] pH 7.4, 5% sodium dodecyl sulfate [SDS]) on ice. Samples were then centrifuged at 14,000 rpm for 30 min and protein extracts were collected and stored at −80°C until analysis. Subsequently, the protein extracts were used for ONPG assay and bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). For the ONPG assay, serial dilutions of the protein extract were incubated at 37°C for 30 min in a 96-well plate with a buffer containing the enzyme substrate for the reaction. The optical density of the reaction was then read at wavelength 420 nm. The quantified samples were presented as units of β-gal activity/ng of protein.

Real time PCR assay

Total DNA was isolated from the muscle tissue by ethanol precipitation (Wizard genomic DNA purification kit, Promega, USA). Viral genomes were quantified in DNA from tissue samples using real-time PCR as previously described.[23] Briefly, a 50 μl PCR volume contained 10 μl of DNA, 200 nM of each primer, 200 nM probe and 25 μl of TaqMan Universal Master Mix

(PE Applied Biosystem, Foster City, CA, USA) containing 8% glycerol, 1X TaqMan buffer A, 5 mM $MgCl_2$, 400μM dUTP, 200μM dATP, dCTP, dGTP (each), AmpliTaq Gold (0.025 U/μl) and AmpErase UNG (0.01 U/μl). The vector genomes were calculated by amplifying *lac*Z and normalized to endogenous mouse apolipoprotein B (Apo-B) (a single copy gene used as an internal control) used to calculate the amount of DNA (and thus the number of nuclei) in each sample. The primers and probes for the *lac*Z24 and Apo B[25] genes have been previously described. The amount of DNA for each sample was calculated from the number of nuclei in each sample using the approximation that a murine diploid nucleus contains 6 pg of DNA. All real-time PCR assays were performed in MicroAmp optical 96-well reaction plates (PE Applied Biosystem, USA). Amplification conditions of 2 min at 50°C and 10 min at 95°C for the first cycle, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min were used. The results were presented as copies of vector particles per 1000 nuclei.

Results

Tissue biodistribution of *lac*Z expression after intraperitoneal administration of AAV8*lac*Z *in utero*

To determine the biodistribution and gene transfer efficiency of systemically delivered AAV8 *in utero,* 6.4×10^{11} vector genomes of AAV8*lac*Z were injected IP per fetus on E-16. At 9 weeks of age, tissues were collected from injected pups, uninjected littermates and mothers carrying the treated pups. To assess the results of gene delivery, all tissues were analyzed for the expression of β-gal by X-gal staining. By whole tissue staining, diaphragm, intercostal muscles and abdominal muscles from the injected pups had the highest levels of β-gal expression (Figure 1). Moderate levels of expression were seen in hindlimb muscles, forelimb muscles and heart. Minimal to undetectable levels of expression were observed in liver, lung, spleen and kidney (data not shown).

Histological analysis of *lac*Z expression after intraperitoneal administration of AAV8*lac*Z *in utero*

Cryosections of diaphragm, quadriceps, gastrocnemius, tibialis anterior, forelimb muscle and heart collected from injected pups at 9 weeks of age were stained for β-gal expression. Diaphragm exhibited β-gal expression in nearly all muscle fibers (Figure 2b). A mosaic pattern of muscle fibers expressing β-gal was observed in forelimb, tibialis anterior, quadriceps and gastrocnemius muscles (Figure 2). A mosaic pattern of β-gal expressing cells was observed in cross-sections of heart with most transgene-expressing cells in proximity to the ventricles.

Quantification of β-gal protein expression in various tissues after intraperitoneal administration of AAV8*lac*Z *in utero*

In order to quantify the expression of protein we performed the ONPG assay on protein extracts from all tissues. Diaphragm and intercostal muscles exhibited high levels of expression of 7.73 ± 1.63 and 6.48 ± 0.94 units of β-gal per ng of protein respectively (Figure 3a). Quadriceps, tibialis anterior, gastrocnemius and forelimb muscle, and heart had moderate levels of expression of 2.2 ± 0.46, 1.40 ± 0.28, 1.91 ± 0.41, 2.47 ± 0.43, and 1.39 ± 0.29 units of β-gal per ng of protein respectively (Figure 3b). Liver, lung, kidney and spleen showed very low levels of expression of 0.51 ± 0.21, 0.84 ± 0.13, 0.22 ± 0.11, and 0.14 ± 0.08 units of β-gal per ng of protein respectively (Figure 3b). There were no detectable levels of β-gal expression in diaphragm, intercostal muscles, quadriceps, gastrocnemius, tibialis anterior, forelimb, heart, liver, lung, spleen and kidney tissues collected from the mothers that carried the treated mice *in utero* (data not shown).

Quantification of AAV vector genomes in various tissues after intraperitoneal administration of AAV8*lacZ in utero*

To determine viral vector gene transfer efficiency, we quantified the number of viral particles in individual tissues by real time PCR. The highest levels of viral vector particles were observed in diaphragm and intercostal muscles. Diaphragm and intercostal muscles had 294.68 ± 51.13 and 251.76 ± 83.28 viral particles per 1000 nuclei respectively (Figure 3a). Quadriceps, tibialis anterior, gastrocnemius, forelimb muscle, and heart had 37.20 ± 8.92, 15.52 ± 3.03, 23.24 ± 5.31, 62.72 ±19.40, and 13.80 ± 4.13 viral particles per 1000 nuclei respectively (Figure 3b). We also quantified the number of viral particles in non-muscle tissues. Liver, lung, kidney, and spleen had 3.79 ± 1.28, 3.76 ± 1.28, 3.76 ± 1.35, and 1.32 ± 0.49 viral particles per 1000 nuclei respectively (Figure 3b). There were no detectable levels of vector genomes in diaphragm, intercostal muscles, quadriceps, gastrocnemius, tibialis anterior, forelimb, heart, liver, lung, spleen and kidney tissues collected from the mothers that carried the treated mice *in utero* (data not shown).

Systemic administration of AAV8*lacZ in utero* demonstrates preferential transduction of fast-twitch muscle fibers

To better understand the distribution of muscle fibers transduced by AAV8*lac*Z *in utero*, we correlated transduction efficiency with muscle fiber type. Myofibers can be classified as fast-twitch or slow-twitch based on the isoform of myosin heavy chain (MyHC) expressed.[26,27] Previous studies have shown that AAV2 preferentially transduced slow-twitch fibers and AAV6 transduced both fiber types in mice.[28–30] Similarly another study showed AAV9 preferentially transduced fast-twitch fibers in mice;[31] however AAV9 did not show any fiber type transduction preference in dogs.[32] To determine if the transduction by AAV8*lac*Z *in utero* correlated with fiber type we performed double immunostaining with antibodies raised against β-gal and slow-twitch MyHC. Interestingly, we observed that the majority of β-gal-expressing muscle fibers did not express slow-twitch MyHC. However, rare slow-twitch fibers were transduced (Figure 4).

Discussion

In this study, we systemically delivered AAV8 vector to E-16 mice *in utero* and analyzed the distribution of transgene expression and vector genomes in treated mice at 9 weeks of age. While considerable AAV8 gene therapy studies have been done in adult and neonatal mice, the biodistribution of AAV8 vector after *in utero* gene delivery has not been reported. We here provide the first report that widespread gene expression in various muscles, including forelimb, diaphragm, intercostal, heart, tibialis anterior, quadriceps, and gastrocnemius muscles after AAV8 *lac*Z *in utero* injection. In addition to demonstrating *lac*Z gene expression by X-gal staining, we quantified gene expression by ONPG assay, and quantified the number of gene copies by real time PCR. We observed a good correlation between the level of transgene protein expression and vector genome copy numbers in individual tissues. In particular, we observed highest levels of expression in diaphragm and intercostal muscles followed by other skeletal muscles. Interestingly, we noted minimal vector transduction in non-muscle tissues such as the liver, lung, spleen and kidney. Importantly, we observed no detectable levels of β-gal expression (by ONPG) or vector genomes (by Taqman real time PCR) in tissues collected from the mothers that carried the experimental mice *in utero*. Maternal tissues studied included diaphragm, intercostal muscles, quadriceps, gastrocnemius, tibialis anterior, forelimb, heart, liver, lung, spleen and kidney

Previous studies have shown the potential of AAV8 vectors to deliver transgenes to muscle tissue of postnatal mice.[2–9] With systemic administration, AAV8 vector exhibited better transduction in neonates than adults in mice suggesting systemic barriers to gene delivery that

develop with maturation of adult tissues.[7] This suggested that *in utero* gene delivery of AAV8 would offer even more efficient gene delivery with potential benefit for the treatment of genetic diseases in particular. Consistent with previous postnatal AAV8 vector studies we observed high gene transduction in skeletal muscles with *in utero* AAV8 vector gene transfer. In this respect, we noted a widespread and mosaic pattern of gene expression throughout various muscle groups both in the upper and lower limbs. Importantly, a high level of gene transduction was observed in diaphragm and intercostal muscles when gene delivery of AAV8 vector was accomplished *in utero*.

Some parallels can be drawn between gene delivery *in utero* and to neonatal mice by comparing our results with a prior AAV8 systemic delivery study in neonatal mice published by Wang et al.[7] In this study 1-day-old mouse pups treated with IP administration of AAV8 showed high transduction efficiency in various muscle tissues for up to 2 months. The dose and period of analysis of the neonatal study were comparable to our *in utero* study. Similar to the neonatal study, we observed expression in various muscle tissues, heart and diaphragm. Furthermore, similar to the neonatal study, our study also showed minimal to undetectable expression in non-muscle tissues such as liver, lung, kidney and spleen.

Previous studies of viral vectors injected *in utero* have exhibited promising, but varying abilities to deliver transgenes to different tissues.[19,20,33,34] Prior *in utero* viral vector-mediated gene delivery studies have been done with AAV1 and AAV2,[19,20,35,36] AAV5,[36] adenoviral vectors,[33,36,37] and lentiviral vectors.[38] While some of the previous *in utero* studies using viral vectors such as AAV1,[19] AAV2[19] and AAV5[36] demonstrated minimal skeletal muscle expression, our study using AAV8 *in utero* systemic gene delivery demonstrated significant levels of widespread gene delivery to skeletal muscles including forelimbs and hindlimbs. Furthermore, the high level of gene transduction in respiratory muscles was accomplished with a relatively low dose of AAV8 vector.

Different patterns of association of gene transduction to muscle fiber type have been observed for different AAV serotypes. While studies have shown AAV6 transduces both fast- and slow-twitch fibers in young adult mice (5–6 week old C57BL/6) in extensor digitorum longus (EDL) and soleus muscles[29] but with a trend towards preferential transduction of fast-twitch fibers treated in 3-day-old *mdx* hind limb muscle,28 it has also been shown that AAV2 preferentially transduces slow-twitch fibers in hindlimb muscle of both newborn and adult mice.30 Moreover, studies with AAV9 demonstrated that both fiber types were transduced in gastocnemius muscle of newborn dogs and that AAV9 transduction was independent of laminin receptor (LamR) expression,32 a known receptor of AAV9.39 In contrast, studies in soleus and TA muscles of newborn and young adult (7 week old) C57BL/10 mice demonstrated that AAV9 preferentially transduced fast-twitch fibers.31 Hence to further understand the transduction profile of AAV8 *in utero* we studied the fiber type transduction in TA muscle. We observed preferential transduction of fast-twitch fibers. However, we also noted transduction of rare slow-twitch fibers. Further studies will be required to understand the reasons for fiber type preference in transduction that appears to depend upon vector serotype and host factors such as species and age.

Studies have shown that many viral vectors injected systemically into adult mice preferentially transduce the liver.[2,7] However, our *in utero* gene delivery data, consistent with other *in utero* studies,[19,33,34] demonstrated minimal transduction of viral vectors into liver. It is interesting to note that even when delivered by the intrahepatic route, *in utero* injection of AAV2 provided minimal liver transduction (<1%) at days 21 and 70 post treatment in mice.[20] Similarly intramniotoic injection of AAV2 to transduce rabbit fetuses demonstrated no transduction into liver tissue.34 These and other studies19،33 demonstrated very low transduction of the liver by *in utero* gene transfer. This possibly is due to high cell turn over

and unique properties in the fetal liver compared to the adult liver.[40–42] From the standpoint of safety this is significant since liver transduction often leads to toxicity and has been an important limitation of clinical viral vector gene transfer studies.[43] Furthermore, liver transduction is generally not desired as a component of muscle gene transfer strategies.

Studies have shown that IP injections of vectors such as AAV1 and AAV2 have varying abilities to transduce the murine heart *in utero*.[19,44] Lipshutz et al. showed that while the gene expression of AAV2 in the peritoneum of CD1 mice continued to persist up to 18 months post *in utero* transuterine IP injection, the expression in heart declined.[44] Similarly, it has been shown that while the diaphragm showed high transgene expression for up to 4 weeks after intraperitoneal *in utero* treatment of AAV1, the heart demonstrated minimal expression in C57BL/6 mice.[19] Hence in order to understand the transduction ability of AAV8 vector in heart after *in utero* gene delivery, we performed X-gal staining and quantitative assessment of β-gal expression. We observed that although the transgene expression levels in heart did not reach as high as most limb muscles we did observe β-gal-expressing cells in close proximity to the ventricles with levels approaching that of the limb muscle tissues.

Given that many muscular dystrophy patients require ventilatory assistance and many die due to respiratory failure, the high amount of AAV8 vector transduction observed in respiratory muscles offers the potential to provide functional benefit to these patients. In addition to disorders due to deficiencies of muscle membrane proteins such as Duchenne muscular dystrophy, AAV8 also has potential as a gene delivery vector in other genetic muscle diseases where patients die due to respiratory failure such as Pompe disease.[45] Patients suffering from infantile-onset Pompe disease start showing symptoms at a median age of 1.6 months and die at a median age of 6–8 months.[45] Thus the significance of effective vector transduction into respiratory muscles prenatally could potentially help improve the clinical condition of these patients.

In this report we demonstrate the potential of systemic delivery of AAV8 vector *in utero* to achieve widespread muscle transduction with especially high levels in respiratory muscles. The findings suggest the therapeutic potential for delivering genes systemically to muscle cells of disease models by *in utero* gene delivery of AAV8 vectors.

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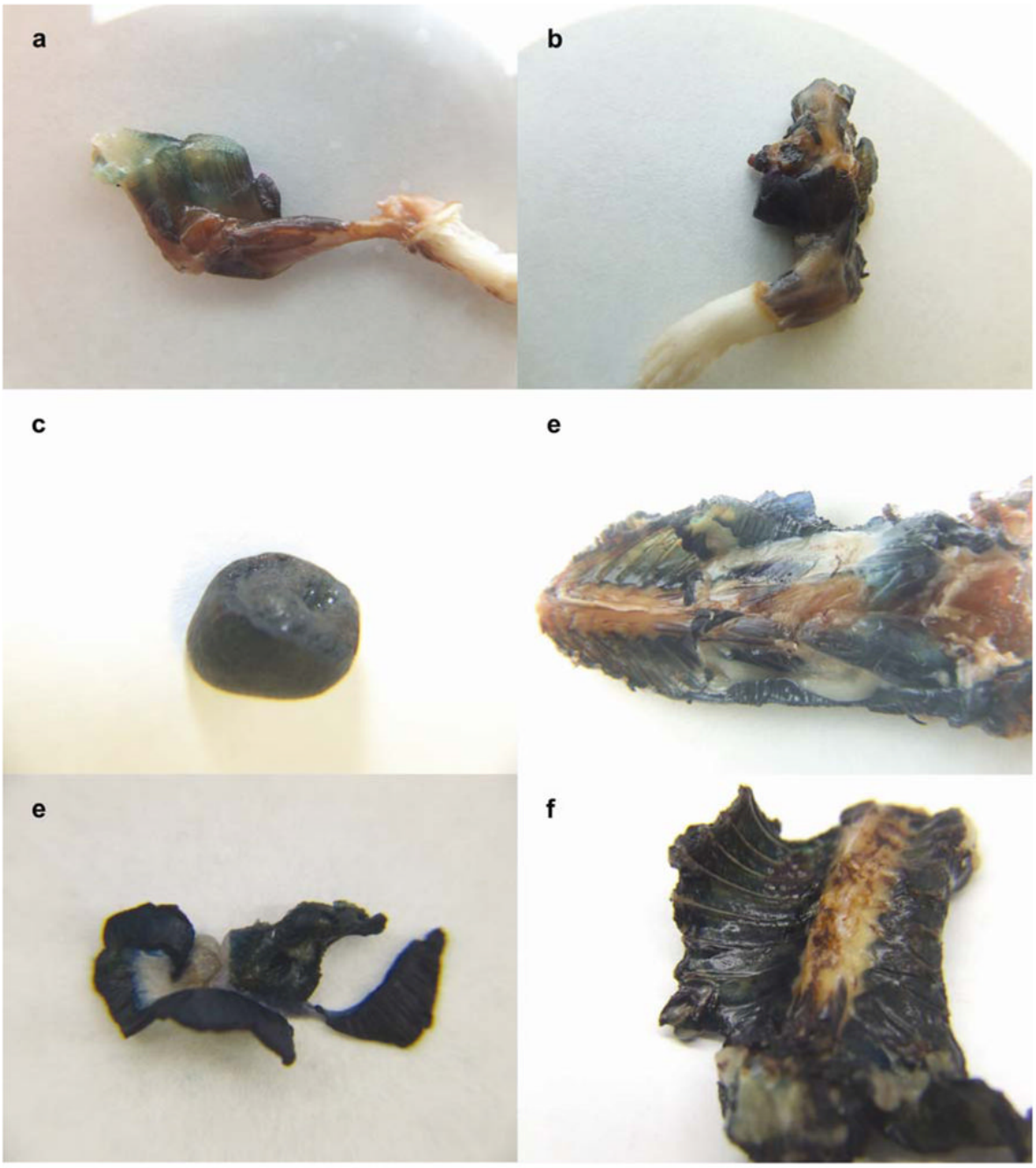

Figure 1. β-galactosidase (β-gal) expression in skeletal and cardiac muscle tissues

Tissues were collected 9 weeks after an intraperitoneal injection of 6.4×10^{11} vector genomes of AAV8*lac*Z into E-16 pups of pregnant CD1 mice and stained for β-gal expression using X-gal. X-gal staining was observed in hindlimb (**a**), forelimb (**b**), heart (**c**), abdominal muscles (**d**), diaphragm (**e**), and intercostal muscles (**f**).

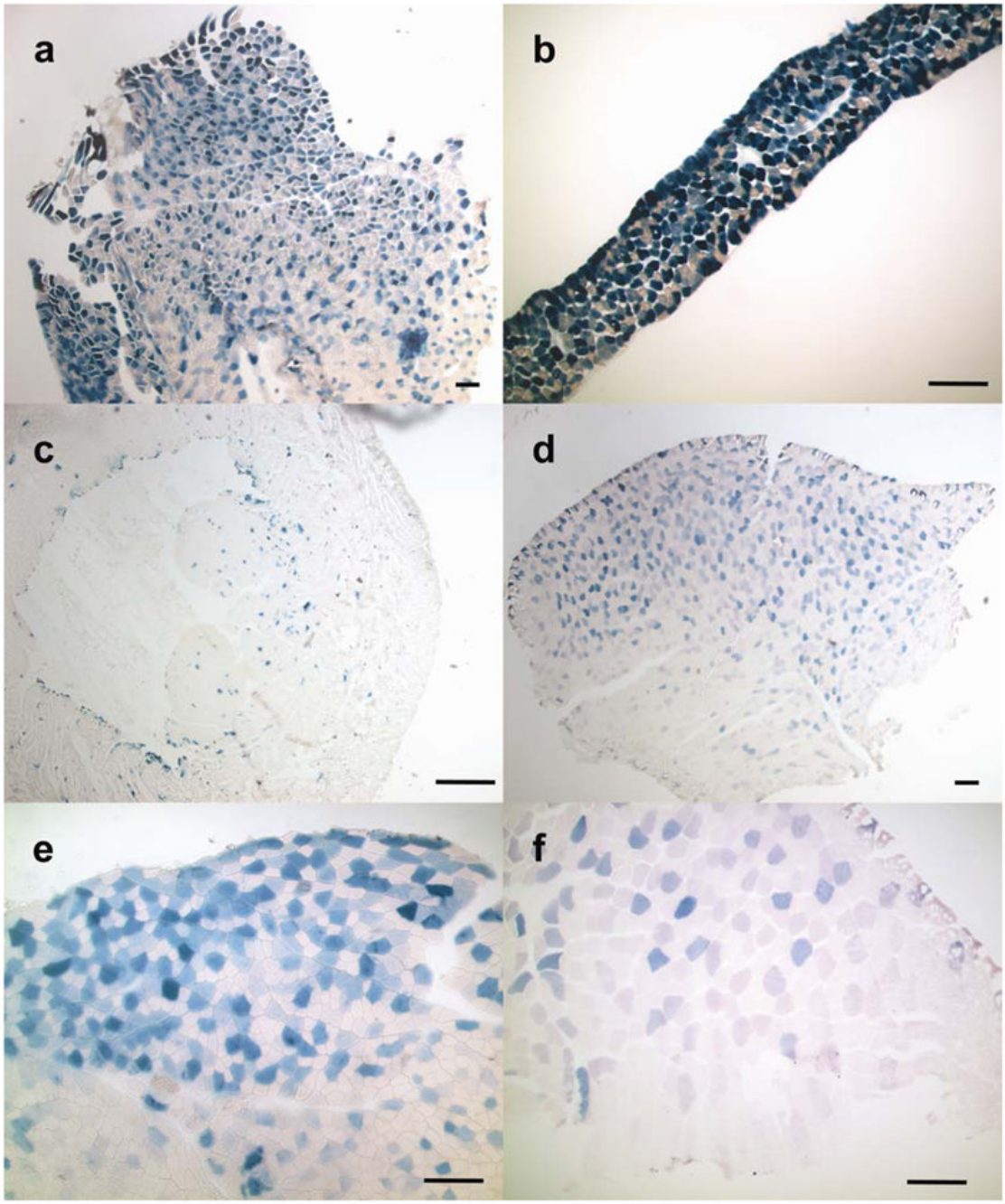

Figure 2. β-galactosidase (β-gal) expression in cryosections of muscle and heart

Tissues were sectioned and stained for β-gal 9 weeks following an intraperitoneal injection of 6.4×10^{11} vector genomes of AAV8*lac*Z into E-16 pups of pregnant CD1 mice. β-gal expression was observed in cryo-sections of forelimb (**a**), diaphragm (**b**), heart (**c**), tibialis anterior (**d**), quadriceps (**e**), and gastrocnemius (**f**) muscles. Scale bar = 200 μm.

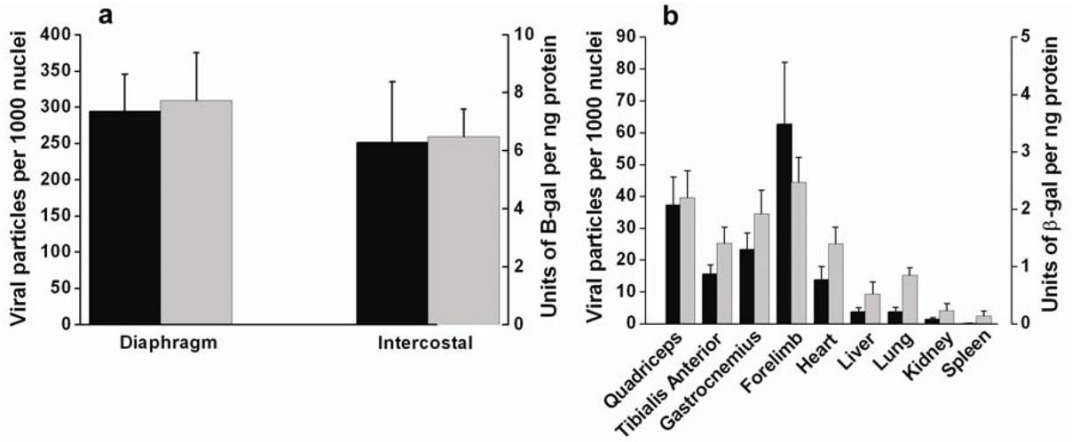

Figure 3. Biodistribution of vector particles of AAV8*lac*Z and expression of β-galactosidase (β-gal)

Tissues were collected 9 weeks after an intraperitoneal injection of 6.4×10^{11} vector genomes of AAV8*lac*Z into E-16 pups of pregnant CD1 mice. Viral particles and β-gal expression were quantified by real-time PCR and ONPG assay, respectively, in diaphragm and intercostal muscles (**a**) and quadriceps, tibialis anterior, gastrocnemius, forelimb muscle, heart, liver, lung, kidney and spleen (**b**). Quantification of viral particles is expressed as mean viral particles per 1000 nuclei. Quantification of β-gal expression is shown as units of β-gal per ng of protein. Error bars represent standard error; number of mice analyzed (n) = 8. Viral particles per 1000 nuclei ■; Units of β-gal per ng protein ■.

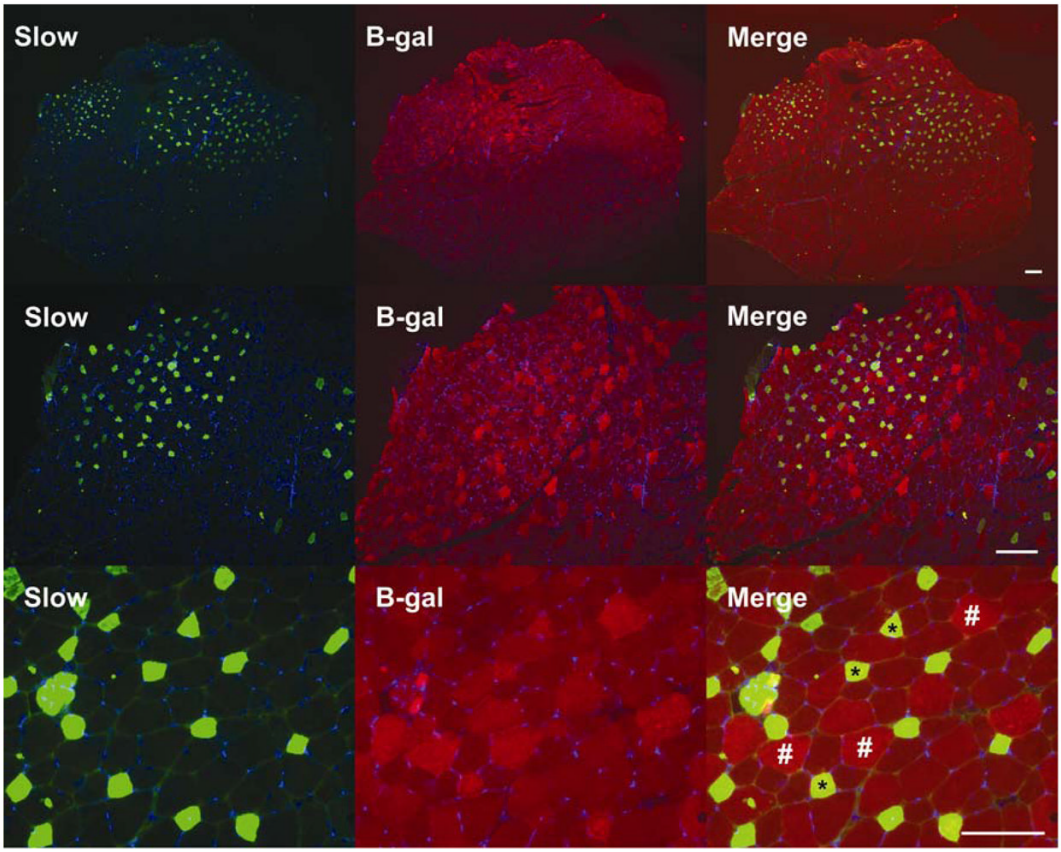

Figure 4. Expression of β-galactosidase (β-gal) in slow- and fast-twitch muscle fibers

Section of tibialis anterior muscle from a 9-week-old CD1 mouse treated *in utero* at E-16 with intraperitoneal administration of 6.4×10^{11} vector genomes of AAV8*lac*Z was immunostained for β-gal (red, β-gal) and slow-twitch myosin heavy chain (green, Slow). The merge image shows that most transduced fibers were fast-twitch fibers (examples labeled with #). Rare slow-twitch fibers were also transduced by AAV8*lac*Z (examples labeled with *). Nuclei are stained with Hoechst. Scale bar = 200 μm.