INTRODUCTION
Duchenne muscular dystrophy (DMD) is both the most common and one of the most severe inherited myopathies affecting up to 1 in 3500 live male births.\(^1,2\) The genetically recessive myopathy results from mutations in the dystrophin gene, cytogenetically located on the X-chromosome at p21.1.\(^3\) Dystrophin is the largest known vertebrate gene in which an estimated one-third of DMD-causing mutations arise spontaneously. Mutations in the dystrophin gene, mostly due to out-of-frame deletions, lead to loss of functional dystrophin, which is a critical myocyte structural protein.\(^5\)

DMD has extensive systemic manifestations and substantial morbidity.\(^5\) Cardiomyopathy develops in nearly all patients by adulthood, at which point it confers a grave prognosis with a 5-year survival of 50%.\(^6,7\) Medical therapy may delay the progression of the cardiomyopathy but has not been shown to affect mortality.\(^8,9\)

One novel therapeutic approach aims to convert the out-of-frame mutation responsible for the disease into an in-frame mutation, thereby restoring the open reading frame and translation of a truncated, and presumably, quasi-functional protein. Binding an antisense oligonucleotide to specific cis-acting splicing signals in the primary transcript target sequence interferes with recognition of that splicing substrate resulting in deletion or skipping of one or more exons. These specific deletions, by design, restore the translational reading frame. The anti-sense sequence can be delivered by a recombinant adeno-associated virus (rAAV) that contains an expression cassette producing a small nuclear (sn) U7 RNA modified with a short anti-sense sequence complementary to the cis-acting splicing signals of the targeted exon(s) (U7smOPT). This approach was proven effective in tissue culture\(^10\) and in dystrophin null (mdx) strain mice.\(^11,12\) Translating this proof of concept to the clinic requires successful demonstration of this approach in a clinically relevant, large animal DMD model. The golden retriever muscular dystrophy (GRMD) dog is the best-established large animal DMD model. The golden retriever muscular dystrophy (GRMD) dog is the best-established large animal DMD model. The large mass of the GRMD dogs poses significant challenge in terms of viral quantities and delivery approaches needed in order to achieve effective gene therapy.

We have developed a large-scale rAAV production process yielding a vector with dual canine U7 smOPT exon-skipping expression cassettes (Figure 1)\(^,16\) and assessed the efficacy of various delivery methods into the myocardium of healthy and sick animals.
RESULTS

The rAAV6 was delivered to the left ventricular myocardium of the treated dogs either by intracoronary infusion \((n=3)\) or by transendocardial injections \((n=5)\). One homozygous female GRMD dog served as negative control and received sham injections. Both the delivery methods were well tolerated without any hemodynamic changes or significant arrhythmias. Initial experience with intracoronary delivery of rAAV6 of either green fluorescent protein \((n=2)\) or U7 small nuclear RNA \((snRNA)\) \((n=1)\) showed minimal transduction efficiency as evidenced by low rAAV DNA in the myocardium. Therefore, subsequent experiments were performed using transendocardial injections.

X-ray fused with magnetic resonance imaging (MRI) (XFM) successfully guided the transendocardial injections by overlaying the myocardial contours and structures over the fluoroscopy images viewed by the operator (Figure 2), thus enabling \(30 \pm 10\) target injections per animal. By updating injection locations in real-time, XFM provided a three-dimensional map for distributing the injections as uniformly as possible. Previously injected areas were avoided and untreated regions were identified and targeted enabling additional transendocardial injections at those locations (Figure 2). The extensive distribution of injections was demonstrated also by T2*-weighted MRI shortly after the injections based on the presence of the supraparamagnetic iron oxide (SPIO) particles in the injected myocardium (Figure 3). Healthy animals

\[\text{AAV U7smOPT E6E8}\]

| ITR | U7 | U7 | U7 | U7 | U7 | ITR |
|-----|----|----|----|----|----|-----|
| ESE 6 |    |    |    |    |    |     |
| ESE 8 |    |    |    |    |    |     |
| 1303 nt |    |    |    |    |    |     |

Figure 1. Diagram of the rAAVU7 smOPT E6E8 vector genome. The 1303-nt single-stranded rAAVU7smOPT E6E8 vector genome is represented schematically. The AAV type 2-derived ITRs are represented by rectangles with diagonal slashes. The two U7smOPT expression cassettes are identical except for the short anti-sense sequences complementary to the exon 6 and exon 8 exon splice enhancers (exonic enhancement element) of the canine dystrophin gene (vertical and horizontal slashes, respectively). Sequences homologous to the non-coding murine U7 snRNA are represented by rectangles labeled ‘U7’ and the positions of the smOPT modification are indicated.

| AAV U7smOPT E6E8 |
|------------------|
| ITR | U7 | U7 | U7 | U7 | U7 | ITR |
| ESE 6 |    |    |    |    |    |     |
| ESE 8 |    |    |    |    |    |     |
| 1303 nt |    |    |    |    |    |     |

Figure 2. Transendocardial injection locations on X-ray fused with MRI (XFM) images. X-ray fused with MRI (XFM) overlays heart contours and structures onto X-ray fluoroscopy. In multiple projections this provides three-dimensional orientation and guidance for transendocardial injections. (a) Short-axis and long-axis (b) views of the heart are demonstrated with the XFM contours overlaid on the fluoroscopy. The green contour represents the left ventricular epicardium, blue the left ventricular endocardium, yellow the right ventricular endocardium, blue lines indicate the mitral valve, and red lines indicate the locations of the aortic valve.
RT-PCR products were generated corresponding to transcripts with D6, 7, and 9, suggesting that the sequential order of splicing remains intact.

**Dystrophin western blot detection**

Dystrophin protein was detected in protein extracted from treated heart samples by western blot analysis (Figure 9). The amount of dystrophin expressed in the treated heart samples corresponds to a relatively low level compared with normal heart samples. Every normal cardiomyocyte expresses dystrophin, however, only transduced GRMD myocytes are capable of dystrophin expression. The amount of dystrophin protein represents an average value of both transduced and untransduced cells that were present in each randomly selected sample.

**Histology and immunohistochemistry**

Immunohistochemistry (IHC) using anti-dystrophin immune serum demonstrated extensive expression of dystrophin protein throughout the treated heart of GRMD dogs (Figure 10). Control GRMD dogs had no detectable dystrophin staining in myocardial samples. The highest levels of IHC fluorescence corresponded to the myocardial segments that had the highest amounts of vector.
Patients who develop Duchenne cardiomyopathy currently have a grave prognosis with no therapeutic options. We therefore evaluated a novel exon-skipping gene therapy approach for the treatment of Duchenne cardiomyopathy. This preclinical study was performed in GRMD canines which are the closest animal model before translation into clinical research. An injection catheter was introduced into the left ventricular cavity by means of percutaneous arterial access. This catheter was used retrograde across the aortic valve to deliver rAAV6-U7smOPT into the myocardium using MRI roadmaps co-registered with live X-ray fluoroscopy. This approach allowed wide transendocardial distribution of the injections, induced extensive exon skipping, and rescued dystrophin expression in the diseased cardiomyocytes.

As a monogenic recessive disease, DMD is an attractive target for gene-based therapy. However, several aspects of DMD, in particular, create challenges in developing effective gene therapy approaches. The large size of the dystrophin gene (~2.5 Mbp) and the resulting mRNA of the full-length protein (~12 knt) preclude standard 'gene' replacement approaches. An AAV-based approach to deliver mini- or micro- dystrophin cDNAs, which can produce a truncated but functional dystrophin protein, faces difficulties because the size of the cDNAs exceed the length of wild-type AAV genomes resulting in less efficient vector genome encapsidation and poor vector yields. Exon-skipping provides an alternative approach for restoring the dystrophin open reading frame. This process blocks transcript and exon-specific recognition signals consisting of intronic elements (pyrimidine tract, branch point, and so on), or exonic splice enhancer element, and canonical splice donators and acceptors. Thus, by targeting a specific splicing signal with the complementary element, one or more exons may be deleted or skipped, thereby restoring the translational reading frame. Initially developed using synthetic antisense oligonucleotides, exon skipping has been adapted as a component of snRNA, either the splicingosomal U1 snRNA or a modified U7 snRNA that interacts with the spliceosome, U7 smOPT.22 By introducing a short, complementary sequence into the 3'-end of the snRNA, the transcript can target a specific splicing signal in the dystrophin pre-mRNA. This approach is currently undergoing clinical evaluation. Although the cellular pathways for salvaging or degrading polynucleotides do not metabolize synthetic oligonucleotides, these oligonucleotides do not persist indefinitely either intracellularly or systemically. Thus, this approach requires repeated, systemic administrations. To overcome this limitation an alternative approach has been developed in which the snRNA expression cassette has been adapted using synthetic oligonucleotides. This approach effectively induced skipping in the mdx mouse, which due to a transition in exon 23, a stop codon prematurely terminates translation.

Although myocardial gene therapy demonstrated promising results in small animal models, translating these methods into large animal models and humans has been non-trivial. Several approaches to achieve effective transduction of the myocardium have been tested and reported with varying degrees of success in terms of histologic or phenotypic effects. Intracoronary infusion of the desired vector seems to be the most promising approach but has yielded varying results. To improve efficiency rates, several improvements have been proposed including reinfusion of the vector through the coronary veins or cardiac fluoroscopy. This approach allowed wide transendocardial distribution of the injections, induced extensive exon skipping, and rescued dystrophin expression in the diseased cardiomyocytes.
recirculation of the delivered vector. In the present study, we failed to achieve detectable cardiomyocyte transduction following intracoronary infusion of the vector with proximal flow occlusion. On the basis of initial experience of transendocardial delivery of viral vectors we have pursued a related approach. However, as the left ventricular cavity is a complex three-dimensional structure an appropriate approach to efficiently target all myocardial locations would require guiding by an imaging modality that would be able to depict three-dimensional structures. Bish et al. performed rAAV transendocardial injections with fluoroscopic guidance, this allowed two-dimensional guidance without the ability to identify any ventricular structures such as the papillary muscles or valves. While demonstrating efficient gene transfer in two areas (septum and left ventricular free wall), the robustness of the distribution with such an approach is unknown. To address this limitation we used an advanced multimodality imaging methodology that combines MRI-derived contours of regions of interest and overlaid these contours on top of the standard fluoroscopic views. These merged images accurately represented the relevant cardiac structures. This approach allowed accurate guidance and real-time assessment of injection locations. Indeed, in the present study, basal areas were less effectively targeted; however, this limitation is attributed to sub-optimal catheter shapes that are intended for use in humans.

In the present study, 3 months post treatment, the effects of vector-mediated exon-skipping were evident. The concentration of vector DNA in randomly selected samples ranged from 981 to 41 vg per cell and dystrophin expression was restored efficiently. As anticipated, there was good correlation between the amount of injections and the viral DNA in the tissue, however, because the DNA processed for PCR and RNA processed for RT-PCR were extracted from different specimens, the exon-skipped RNA and vector genome copy numbers results did not correlate.

Using large-scale rAAV production, we were able to evaluate two routes of administration for treating dystrophic dogs. This capability is essential for translating treatments from small animal proof-of-concept studies to demonstrating feasibility in a clinically relevant, large animal model of DMD.

The phenotypic effect of this exon-skipping gene therapy was modest, demonstrating stable left ventricular function without any significant improvement in 3-month follow-up. The treated GRMD dogs had advanced skeletal muscle disease, with dramatic muscle wasting and weakness, and some required percutaneous gastrostomy feeding due to an impaired swallowing mechanism and recurrent pulmonary aspiration of gastric contents. Despite having dramatic clinical evidence of skeletal muscle disease well before 1 year of age, GRMD dogs typically do not have clinical signs of cardiomyopathy until much later. Nonetheless, all GRMD dogs in this study had advanced cardiomyopathy with impaired LV function at the time of treatment. It is conceivable that even the most effective treatment would not be able to reverse LV dysfunction in this set of animals. Thus, the present results that demonstrate prevention of further deterioration in LV function might be viewed as encouraging.

The present study has several limitations. Our attempts to deliver the rAAV via the coronary approach failed. It is possible that supplementing our techniques with the previously reported methods might have increased the efficiency of gene transfer via the intracoronary approach. The rAAV6-U7smOPT vector
restored dystrophin expression in most of the tested samples. Distributing the injections uniformly throughout the LV myocardium and prolonging the dwell time for each injection is difficult without inducing cardioplegia. Thus, it is conceivable that subsequent to withdrawing the needle from the injection site, the vector refluxes along the needle track and does not diffuse interstitially. Despite the high doses of concentrated rAAV6-U7smOPT, it is likely that the heart contractions caused leakage of the vector back into the ventricular cavity, thereby limiting the amount of vector remaining in situ. Finally, because of the difficulty in breeding and availability of GRMD canines, the number of GRMD canines that were used in this study was relatively small. It is possible that larger number of animals and longer follow-up would have yielded more meaningful phenotypic data. No control dogs were used for comparison in the functional studies.

MATERIALS AND METHODS
Animals and study design
Animal procedures were approved by the institutional Animal Care and Use Committee and performed according to contemporary National Institutes of Health guidelines. A total of nine dogs were used, including...
four normal male beagles (11.9 ± 1.1 kg) and five 7–11 month old affected GRMD dogs (four male and one homozygous female) (12.1 ± 3.1 kg). Of these, three dogs (two normal dogs and one GRMD dog) received intracoronary rAAV infusion, five (two normal dogs (beagles) and three GRMD dogs, same sire different dams) received transendocardial rAAV delivery and one homozygous female GRMD dog served as control (a littermate of an experimental GRMD affected male dog). Animals were pretreated by oral amiodarone, atenolol and prophylactic cefazolin. Anesthesia was induced by midazolam, propofol and butorphanol and was maintained by sevoflurane inhalation and fentanyl infusion. Vascular femoral access was achieved with a 4-Fr micropuncture kit followed by insertion of 6- or 8-Fr and 5-Fr sheaths in the right femoral artery and vein respectively. Vascular hemostasis was achieved by manual pressure and application of D-stat Dry (Vascular Solutions, Minneapolis, MN, USA).

Bivalirudin (Angiomax, The Medicines Co, Parsippany, NJ, USA) was used as a procedural anticoagulant due to the binding affinity of AAV6 capsids with heparin that effectively neutralizes the biological activity of rAAV vectors. An initial bolus of 0.75 mg/kg was followed by continuous infusion of 1.75 mg/kg h⁻¹ maintained by sevoflurane inhalation and fentanyl infusion. Pressure and application of D-stat Dry (Vascular Solutions, Minneapolis, MN, USA). Treated animals were survived up to three months after the procedure. Immediately following euthanasia, hearts were explanted and were sliced systematically in a predefined mode through the short axis of the left ventricle into 17 segments at the basal segment (segments 1–6), mid-cavitary (segments 7–12), and apical (segment 17), according to standardized segmentation of the left ventricle. Random samples were taken from each segment and fresh frozen (-80°C) in cryopreservation medium (Tissue-Tek O.C.T. compound, Sakura, The Netherlands).

Adeno-associated virus vector
rAAV serotype 6 (rAAV6) was produced in Spodoptera frugiperda (SF9) insect cells using recombinant baculovirus expression vectors (BEVs) as previously described. Briefly, cells were cultivated in a 250 l single-use bioreactor (2001 working volume) in serum-free insect cell medium (SFX Insect Cell Medium, HyClone, Logan, UT, USA) at 28 °C with 30% dissolved O₂ (dO₂) (Integrity PadReactor, ATMI Lifesciences, Bloomington, MN, USA). Temperature and DO set points were maintained during cell growth and vector production with control loops responding to outputs from the appropriate probes (TruVu and TruLogic Control System, Finesse Solutions, San Jose, CA, USA). Late log-stage SF9 cells from shake-flasks were diluted into the bioreactor's minimum working volume (40 l) and expanded to the target volume by serial dilution to the final working volume (200 l). Two BEV vectors were required for rAAV production: one BEV for AAV-2 rep proteins and AAV6 cap proteins and the second BEV provided the ITR-bearing vector genome. Rather than using free extracellular BEVs in solution for rAAV production, a predetermined amount of concentrated, cryopreserved baculovirus-infected insect cells were added to the 200 l culture (baculovirus-infected insect cells: producer cell = 1:1000). The producer cell line was selected only until the baculovirus cycle arrest occurred at the target cell density of 5 × 10⁶ cells ml⁻¹. The downstream process for rAAV purification involved the following series of steps as previously described: Homogenization, nuclease digestion, clarification and microfiltration, immunaffinity column chromatography, tangential flow filtration for concentration and diafiltration and final filtration and sterile dispensing into single-use vials. Depending on the homogeneity of the vector, gel exclusion chromatography step was performed (Superdex 200, GE LifeSciences, Piscataway, NJ, USA).

The vector particles, consisting of AAV6 capsid proteins and a linear single-stranded DNA genome were characterized for composition and quantity. Following immunaffinity chromatography, the vector genome concentration was determined by two methods: PCR using vector-specific oligonucleotide primers, and by SYBR gold fluorescent dye binding to the vector-extracted DNA. Tangential flow filtration was used to achieve the final vector concentration and buffer exchange into phosphate-buffered saline (PBS; Pellicon 2, Millipore Corp, Billerica, MA, USA). The protein composition of the AAV6 capsid: VP1, VP2, and VP3, was analyzed by SDS-polyacrylamide gel electrophoresis and silver staining (Pierce Thermo Fisher Scientific, Rockford, IL, USA).

The vector genome has two cassettes, each consisting of a U7 promoter, a modified U7snRNA (U7snMOPT), and short sequence complementary to the exonic enhancement element of exons 6 and 8. The terminal palindromes were derived from AAV2. (Supplemental Table 1, Figure 1).

Intra-coronary delivery
Coronary injections were performed in anticoagulant-treated animals during balloon occlusion to delay washout. Engaging coronary arteries in these small animals required 5Fr short pediatric transfemoral diagnostic catheters (JL1.5–2.0, Merit Medical, South Jordan, UT, USA). The left anterior descending and circumflex coronary arteries were injected separately using over-the-wire coronary angioplasty balloons (2.0–3.0 mm diameter and 8.0–12.0 mm length as needed) and 0.014″ coronary guidewires positioned in the mid left anterior descending or proximal circumflex segments. Preconditioning of the left ventricular myocardium (to help the animal tolerate prolonged ischemia) was accomplished by two consecutive cycles of two minute ischemia by coronary balloon inflation followed by five minute reperfusion (balloon deflation). Next, the solution containing the AAV vector stock (1013 copies ml⁻¹) was delivered through the coronary balloon catheter lumen while inflating the balloon to occlude distal coronary artery flow (5 min). The same protocol was followed for the second vessel.

Transendocardial delivery
Transendocardial injections were performed under fluoroscopic guidance using X-ray fused with MRI (XFM). Injections were performed using a 7-Fr endomyocardial 27G injection needle catheter (Stiletto, Boston Scientific, Natic, MA, USA). Briefly, the Stiletto catheter was steered retrograde across the aortic valve into the left ventricle cavity by means of two coaxial guiding catheters (8-Fr and 6-Fr IMA and JR guiding catheters) (Vista Brite-Tip, Cordis Corp, Bridgewater, NJ, USA). The endocardium was injected repeatedly (0.2 ml per site x 20–40 sites for each procedure) with the intention to distribute the vector homogenously throughout the left ventricular myocardial mass. No attempts were made to inject into the right ventricle free wall.

The injection cocktail included virus and both MRI and X-ray contrast agents. Each 200 μl injection was comprised of 1.5 × 10¹⁴ vg rAAV stock (10¹⁴ vg ml⁻¹) and 50 μg of SPIO (50 mg ml⁻¹ stock, BioMag 547, Bangs Laboratories Inc., Fishers IN, USA), and 50 μl iopamidol, a fluoroscopy contrast agent (Isovue 300, Bracco Therapeutics, Princeton, NJ, USA). The control group received identical solution; however, the viral volume was replaced by saline. The delivery of this solution enabled correlation between real-time localization of the injection sites under XFM (iopamidol) with 3-dimensional, anatomic, early post-procedural analysis of the injections according to the SPIO particles void signal in T₂ weighted MR images.

We were careful to identify, record and verify each injection site in order to distribute the virus widely. Because the injectate contained iopamidol, the three-dimensional position of each injection was recorded under XFM to avoid overlap. The injectate also included SPIO MRI contrast, a MRI scan immediately after injections showed a complementary image of injections throughout the heart. Finally, these images were compared with histopathology.

X-ray fused with magnetic resonance imaging
XFM combines pre-acquired 3-dimensional MR image roadmaps of anatomic structures (the left ventricular myocardium in this experiment) and standard X-ray fluoroscopy images that provide high resolution images of the injection catheter and radiocontrast. This co-registration, or fusion enables 3-dimensional targeting of injections into soft tissue that is otherwise not visible using X-ray alone.

For XFM we combined MRI volumes of the heart and X-ray fluoroscopy as previously described. Briefly, a set of external fiducial multimodality markers (Beekey, Bristol, CT, USA) were placed on the animal’s chest and back. T₁-weighted gradient echo MR images and a set of X-ray fluoroscopy projections were used to register the two modalities. Relevant cardiac structures (for example, left and right ventricles, aorta) were segmented from MR images and projected onto live X-ray fluoroscopy. This co-registration, or fusion enabled 3-dimensional targeting of injections into soft tissue that is otherwise not visible using X-ray alone.

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incorporating parallel imaging with an acceleration factor of two, repetition time (TR)/echo time (TE) 3.2/1.4 ms; flip angle 80°; field of view 300 × 300 mm; matrix 192 × 192 pixels; slice thickness 6 mm; bandwidth 930 Hz per pixel. Argus Function software (Siemens Medical Solutions) was used to calculate LV ejection fraction (LVEF). T2*-weighted, FLASH sequence with TR/TE 6/3.5 ms; flip angle 15°; field of view 250 × 160 mm; matrix 192 × 125 pixels; slice thickness 6 mm; bandwidth 260 Hz per pixel was used to identify injection sites by means of the SPIO iron-induced signal voids. Images were analyzed using a commercial cardiac MRI workstation (Argus, Siemens).

DNA analysis
Total DNA from a random sample from each of the 17 predefined myocardium segments was extracted and purified using Gentra Puregene kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. PCR reactions were performed as described previously.43 Products were analyzed on ethidium bromide stained 2% agarose gels and sequenced. Six pg per geloid package was used to calculate the vector copy number per cell using 5 × 10^6 bp as the approximate size of the canine geloid package.

RNA analysis
Total RNA from each of the 17 predefined myocardium segments was extracted from randomly sampled specimens using a commercially available kit (RNeasy, Qiagen). The RNA was reverse transcribed to cDNA using Superscript I or II (RT) system (Invitrogen, Carlsbad, CA, USA). Canine dystrophin-specific exons 3 and exon 10 primers were used for the reaction at 55 °C for 30 min. The RT reaction was used for nested PCR reactions: First using a pair of external primers, and subsequently, with a pair of internal primers. The forward primers (F) for both external and internal primers were in exon 3 and the reverse primers (R) were in exon 10. External primers43: F 5'-GGAGAACGACGATAGA-G-3’; R 5’-TACCTCTTCCGACAT-CATT AG-3’. Internal primers: F 5’-GGAGACCGCTCCATAGCT-3’; R 5’-TACCTCTTCCGACATCATTAG-3’. The PCR reactions were analyzed using ethidium bromide stained agarose gel electrophoresis. The open reading frames of the three isolated PCR products were determined by DNA sequence analysis.

To increase the sensitivity of detecting the dystrophin mRNA, nested PCR using ‘outer’ and ‘inner’ set of primer pairs was used following reverse transcription of extracted cardiomyocyte RNA. Both sets of primer were specific to dystrophin exons 3 and 10, amplified the region targeted by the exon-skipping vector. The predicted sizes of the wild-type and GRMD (A7) RT-PCR products are 911 bp and 792 bp, respectively (Figure 3, ‘Normal Dog’ and ‘GRMD dog untreated’ lanes). The rAAV-U7sMOPT vector was designed to induce skipping of exons 6 and 8 generating a Δ6, 7, and 8 transcript with a predicted size of 437 bp. However, exon 9 deletion occurs cryptically producing a Δ6, 7, 8, and 9 dystrophin RT-PCR product that is 305 nt.

Dystrophin western blot analysis
Western blot analyses were performed on total protein extracted from previously frozen O.C.T. embedded heart segment sections (10 µm thickness) mounted on uncoated glass slides. Protein was extracted using a modified SDS-protein gel loading buffer as previously described consisting of 4% SDS, 125 mM Tris-HCl (pH 8.8), 40% glycerol, phenyl-methylsulfonyl fluoride (0.5 mM), and 100 µM dithiothreitol.46 Equivalent volumes of the protein extracts were fractionated using precast polyacrylamide gradient gel electrophoresis (NuPAGE Tris-Acetate SDS polyacrylamide gels, Invitrogen) and electrophoretically transferred to polyvinylidene fluoride membranes in transfer buffer (NuPAGE Transfer Buffer) containing 20% methanol (constant 30V, overnight at 4 °C). Protein loads were adjusted by digital analysis of common protein bands in Coomassie blue stained gels. Standard western blot conditions were used for membrane blocking, washing, staining, and chemiluminescence. Blocking buffers (BSs): PBST—PBS (50 mM Tris-HCl (pH 7.6) and 150 mM NaCl), 0.1% Tween-20, and 5% non-fat dry milk (w/v) (BB1) and BB2, PBST with 5% bovine serum albumin (w/v). Washing buffer: phosphate buffered saline and 0.1% Tween-20. Chemiluminescence reagent: SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). After blocking the membranes (30 min at ambient temperature), the primary antibodies were diluted and added to the BB solutions. The anti-dystrophin-peptide antibody (rabbit polyclonal) (ab15277, Abcam Inc., Cambridge, MA, USA) was diluted 1:200 in BB1, and incubated overnight at 4° on a rocking platform platform. Following incubation, the membranes were washed in the BB1, 3 × 10 min at room temperature, and the diluted secondary antibody, 1.500 goat anti-rabbit IgG – horse-radish peroxidase (HRP) (AS278, Sigma-Aldrich, St Louis, MO, USA), was added to the BB1 for 1 h at room temperature with agitation. The solution with the secondary antibody was removed and the membrane was washed in PBST (3 × 10 min). After the final wash, the luminol substrate was added (SuperSignal West Dura Extended Duration Substrate, Thermo Scientific) and the chemiluminescence signal was detected using a charged-coupled device camera and digital imaging processor (G:BOX Chemi, Syngene, Frederick, MD, USA). The data were analyzed using software (Gene Tools, Syngene) provided with the imaging system.

IHC and histology
Tissue specimens were sectioned using a cryostat microtome into 5 to 10 µm thick slices and mounted onto silane–treated glass slides and fixed with 10% formalin for 10 min at room temperature then washed (3 × 10 min) in PBS. For IHC, the formalin-fixed tissue sections were incubated in BB with either 5% goat serum or 5% bovine serum diluted in PBS, depending on the primary antisera source, for 30 min at ambient temperature. The BB was removed and primary antisera was applied. For dystrophin detection, a rabbit polyclonal antisera (ab15277, Abcam) was diluted (1:1000) into fresh 2% goat serum in PBS and applied to the section and incubated overnight at 4°C in a humidified chamber. The antibody solutions were removed and the samples were washed with PBS (3 × 10 min) at room temperature. Fluorescently labeled secondary antibodies were diluted in bovine serum - PBS and applied to the samples and incubated at room temperature for one hour in a dark plastic chamber to exclude light. The antibody solutions were then removed and washed with PBS. Coverslip mounting medium was applied (Vectorshield Mounting Medium with DAPI) (Vector Laboratories Inc, Burlingame, CA, USA) and a coverslip was placed over the tissue sample.

Data analysis
Statistical analysis used InStat (GraphPad Software Inc., La Jolla, CA, USA). Continuous parameters are reported as mean ± standard deviation, tested using two-tailed paired t-test. A P-value < 0.05 was considered significant. Correlation between number of injections and presence of viral DNA was assessed by Spearman’s rank correlation test.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)