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RESEARCH ARTICLE

Highly efficient EIAV-mediated in utero gene transfer and expression in the major muscle groups affected by Duchenne muscular dystrophy

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Gene therapy for Duchenne muscular dystrophy has so far not been successful because of the difficulty in achieving efficient and permanent gene transfer to the large number of affected muscles and the development of immune reactions against vector and transgenic protein. In addition, the prenatal onset of disease complicates postnatal gene therapy. We have therefore proposed a fetal approach to overcome these barriers. We have applied β-galactosidase expressing equine infectious anaemia virus (EIAV) lentiviruses pseudotyped with VSV-G by single or combined injection via different routes to the MF1 mouse fetus on day 15 of gestation and describe substantial gene delivery to the musculature. Highly efficient gene transfer to skeletal muscles, including the diaphragm and intercostal muscles, as well as to cardiac myocytes was observed and gene expression persisted for at least 15 months after administration of this integrating vector. These findings support the concept of in utero gene delivery for therapeutic and long-term prevention/correction of muscular dystrophies and pave the way for a future application in the clinic.
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Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive disease occurring in one in 3500 males.[1] Characteristically, skeletal muscle degeneration after repeated rounds of necrosis is followed by the onset of fibrosis and eventually leads to muscle weakness and death.[1] Although improved nursing care and positive pressure ventilation to aid breathing allows some patients to reach the third decade of life, DMD sufferers are usually wheelchair bound near the first decade and respiratory or cardiac failure is the common cause of death.[2] The early onset of this disease, which begins to be visible histologically by the 18th–20th week of gestation[3] and presents clinically between 2 and 4 years of age, complicates postnatal gene therapy. In addition, efficient gene delivery to several affected muscles groups is technically difficult. However, widespread gene transfer to a large percentage of existing and rapidly expanding muscle cells *in utero* may overcome these difficulties. Based on the observation that skewed X-inactivation repressing up to 90% of dystrophin-expression from myonuclei does not result in disease manifestation and that prevention of pathology can be achieved in transgenic dystrophin-deficient *mdx* mice expressing recombinant dystrophin cDNA from 0.2 times endogenous levels it has been suggested that as few as 20% of genetically modified muscle cells may be sufficient to correct the DMD phenotype.[4,5]

Postnatal gene delivery is also complicated by the risk of cellular immune responses against the transgenic proteins as demonstrated in the mdx mouse model by loss of transgenic dystrophin-expressing fibres following dystrophin gene transfer.[6,7] In contrast, *in utero* gene transfer may avoid the development of immune reactions to the vector or transgene product. Long-term persistence of luciferase expression in liver and heart without the generation of antibodies to the vector or the transgenic reporter gene product has been demonstrated after *in utero* administration of a luciferase gene carrying adeno-associated virus (AAV),[8] and we have recently shown that postnatal tolerance to human factor IX can be achieved by application of an adenovirus vector expressing this protein *in utero*.[9]

Lentivirus vectors are well suited for potential long-term therapeutic expression of dystrophin since they are able to integrate into the genome of nondividing muscle cells[10] and are relatively nonimmunogenic.[11] These viruses can also be pseudotyped with envelopes, which may increase the tissue specificity of gene transfer for muscle cells. This has been demonstrated recently for direct intramuscular injection *in utero* comparing VSV-G,

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Ebola or Mokola pseudotyped HIV-1-based vectors carrying the β-galactosidase reporter gene.[12] In a similar study in neonatal mice, Kobinger *et al*[13] extended the number of pseudotypes investigated to include rabies, murine leukaemia virus (MuLV) and lymphocytic choriomeningitis virus (LCMV) envelope proteins. Not surprisingly, however, the intramuscular route of injection restricted expression primarily to the site of the injected muscles while other muscle groups necessary for the treatment of DMD were poorly transduced or nontransduced.

We have previously shown that the EIAV lentivirus, which is nonpathogenic to humans, can be used to efficiently transduce several tissues of the MF-1 fetal mouse when delivered systemically via the fetal yolk-sac vessels.[14] In this study, we have extended our previous *in utero* investigations to achieve widespread gene transfer and expression in several muscle groups important to the treatment of DMD. Using intraperitoneal, intrathoracic, supracostal, intramuscular and systemic routes of injection, we show that the EIAV vector can efficiently transduce muscle cells of the diaphragm and abdominal cavity, the intercostal muscles and the skeletal musculature of all injected limbs as well as the heart muscle.

Results

With the aim to achieve widespread and high-level gene transfer and expression in the most important muscle groups related to DMD, we applied β-galactosidase-expressing EIAV lentiviruses pseudotyped with VSV-G by single or combined injections via different routes to the MF1 mouse fetus on day 15 of gestation. Gene expression was investigated at either early (less than 7 days) mid (between 1 week and 5 months) or late time points. Initially, the fetuses were injected separately either systemically (i.v.), intramuscularly (i.m.), intraperitoneally (i.p.), intrathoracically or supracostally to determine transduction efficiency and fetal survival after each route of application. For the i.v., i.m. and i.p. routes of administration a minimum of 10 fetuses were injected per group in at least three dams to account for variation between litters. Fetal survival after the surgical procedure and injection of vector was between 68 and 91% (Table 1) compared with 95% for PBS-injected controls. The most important variable in determining fetal survival was vector toxicity and significant differences were noted between different batches of EIAV vector preparations. Insufficient animals ($n=2$) were injected via the intrathoracic and supracostal routes to determine accurately the percentage fetal survival, however, all of the treated animals were born and survived until they were killed. We then combined different routes simultaneously in two animals. Tissues were examined macroscopically and histologically following X-gal staining.

Table 1 Fetal survival after injection of EIAV vector via different routes

Route of injection	*Vector dose TU per fetus*	*Dams injected*	*Fetuses injected*	*Percentage survival*
i.v.	2×10^7	3	11	91
i.p.	1×10^7	3	10	70
i.m.	5×10^6	5	23	68

Systemic EIAV vector injection via fetal yolk-sac vessels

Situated in the plane of the yolk-sac membrane the yolk-sac vessels are accessible through the wall of the exposed uterus after midline laparotomy. Using colloidal carbon as a marker, we have previously demonstrated that injection through the yolk-sac vessel enables all tissues to be reached from the fetal circulation.[14] Injection of 2×10^7 transducing units (TU)/fetus i.v. resulted in widespread gene delivery. Highest gene expression was observed in the liver, as we have observed previously,[14] but expression of β-galactosidase was also observed in the heart and skeletal musculature after 7 and 14 days, which was maintained for up to 15 months. Levels of expression in the heart were variable but in all animals injected i.v. with VSV-G pseudotyped EIAV cardiac myocytes were transduced (Figure 1).

Targeting the peripheral skeletal musculature

A dose of 5×10^6 TU/fetus was injected directly into the fetal skeletal muscle of the hind limb. The small size of the mouse fetus (approximately 1 cm in length at day 15 of gestation) precludes injection of a particular muscle group, for example, the tibialis anterior as is often used for gene transfer experiments in adult animals. A substantial amount of vector spread with intense β-galactosidase expression was observed in the surrounding musculature following a single injection, which persisted for at least 5 months. In these animals, multiple muscle groups in the hind limb showed high levels of expression after a single *in utero* injection. Fetal injection of the hindlimb most commonly transduced the tibialis anterior and gastrocnemius of the lower leg and the gluteus maximus, biceps femoris and quadriceps of the upper leg. Representative examples of macroscopic staining of the hind limbs are shown in Figure 2. Histologically, longitudinal and transverse sections of the skeletal muscle confirmed strong gene expression along the length of the muscle fibres (Figure 2). In order to determine the percentage of myofibres transduced and extent of gene transfer throughout the muscle transverse frozen sections of the quadriceps of the injected leg were stained for β-galactosidase expression. The percentage myofibres transduced was between 0.5 and 23% (average 9.25 ± 3.4, $n=6$). Positive fibres were observed throughout the muscle however in most cases, expression of the transgene was highest near the surface of the muscle as observed macroscopically. In order to investigate reproducibility of vector administration and subsequent gene expression quantitative ELISA was performed on muscle homogenates prepared from the whole of the injected leg. It became necessary to analyse such a large volume of tissue in order to account for variation in the site of gene expression due to difficulties in injecting a particular muscle group *in utero* which hampers subsequent postnatal analysis of transduced tissues. However, using this method levels of expression were reproducible between animals, ranging from 420 to 912 pg β-galactosidase per mg of tissue (average 731.67 ± 156.64, $n=3$).

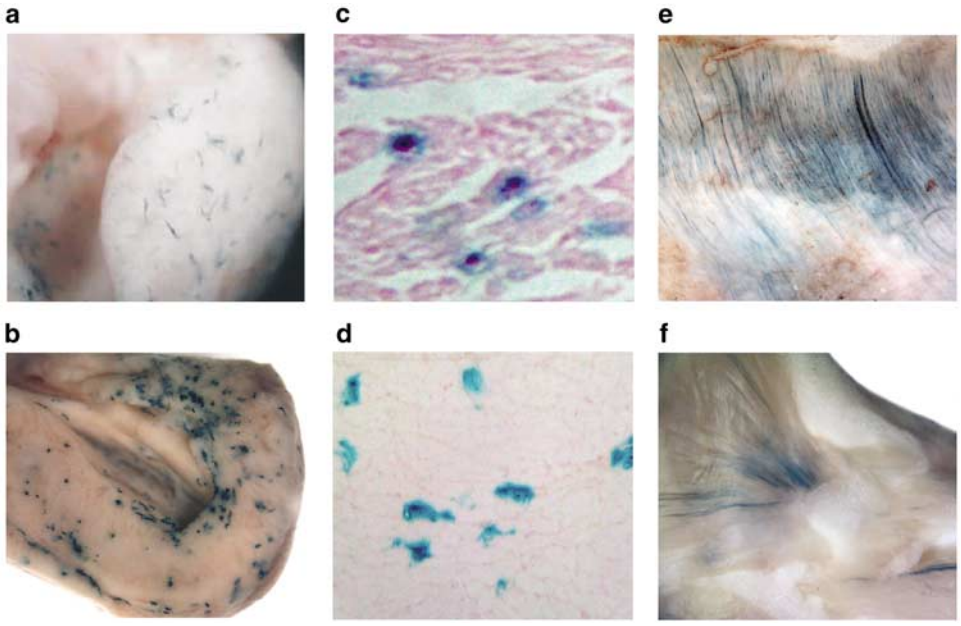

Figure 1 *β-Galactosidase expression in heart and skeletal following intravenous injection. X-gal staining after 14 days (SMART2 Z, cytoplasmic staining) (a) persisting up to 5 months (b). Histological sections demonstrating transduced cardiac myocytes of the heart 14 days (c) and 5 months (d) after SMART2 Z administration. Muscle fibres of the abdominal wall (f) and hindlimb (g) are also transduced after i.v. administration of SMART3 NZ, nuclear localizing vector which persists for at least 15 months.*

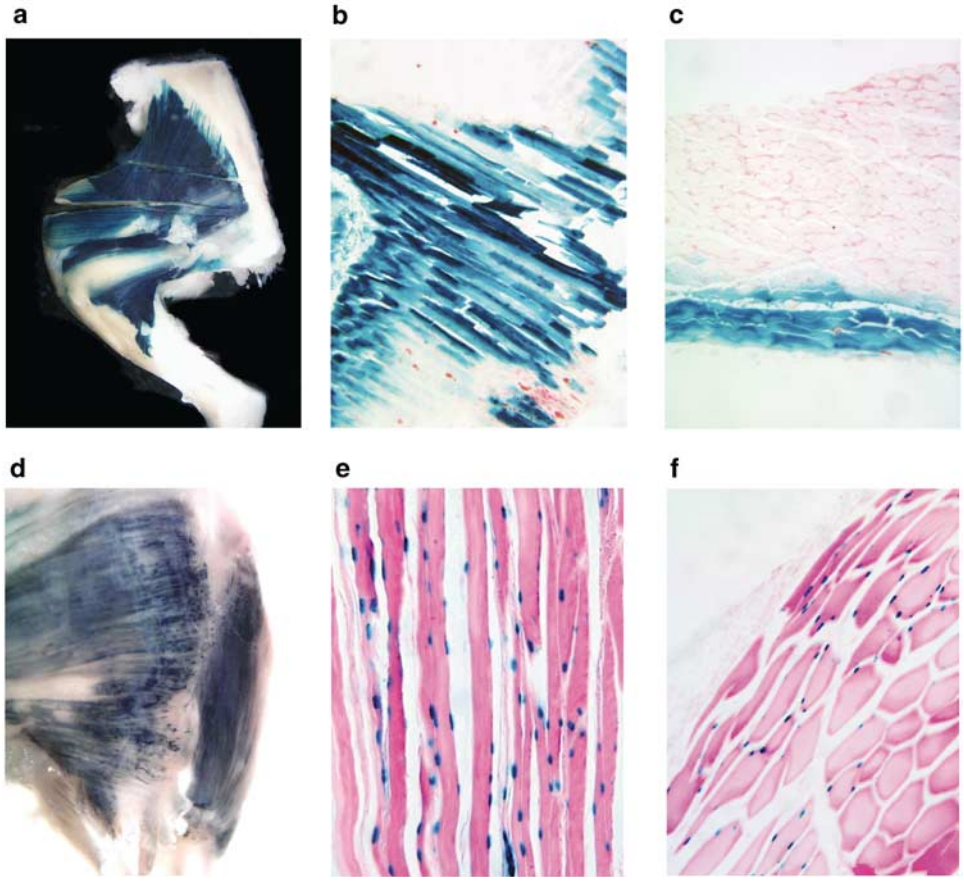

Figure 2 *Widespread expression in hind-limb muscle after a single intramuscular injection. β-Galactosidase expression in muscle 13 days after SMART2 Z (a–c) and 5 months after SMART3 NZ vector delivery (d–f). Longitudinal (b and e) and transverse (c and f) sections of the muscle prepared after X-gal staining.*

Targeting the respiratory musculature

Intrathoracic and supracostal EIAV vector injection. To increase transduction of the critical respiratory musculature, we first applied a vector dose of 1×10^7 SMART2 Z T.U/fetus. by intrathoracic injection. The needle was inserted through the chest wall and vector injected into the thoracic cavity. This resulted in extensive macroscopically detectable gene expression in approximately 80% of the myofibres of the innermost intercostal musculature as shown in a transverse section of the rib cage (Figure 3a and b). In addition, there was significant staining of the peripheral muscle fibres of the diaphragm but not of the external intercostal muscles. We therefore applied supracostal injection to another animal by inserting the needle under the skin over the surface of the ribs and injecting 1×10^7 TU/fetus. Extensive gene expression in the external intercostal muscles was achieved which was maintained for at least 1 month (Figure 3c). No gene expression was observed in the diaphragm following this route of administration and vector spread was limited to the external intercostal muscles.

Intraperitoneal EIAV vector injection. To reach the posterior muscle fibres of the diaphragm and muscles of the abdomen 1×10^7 TU of SMART2 Z particles were injected into the fetal intraperitoneal cavity. This resulted in comprehensive gene transfer to the diaphragm (Figure 3e). Histologically, a section cut in the longitudinal plane of the muscle showed gene expression in nearly 100% of the myofibres of the external surface of the diaphragm (Figure 3f). A transverse section showed gene transfer was limited to the posterior surface with no gene expression observed in myofibres of the anterior side (Figure 3g). In addition to gene transfer to the diaphragm extensive staining was also observed in the abdominal musculature following this route of administration and strong gene expression was still observed after 6 weeks in muscle fibres (Figure 3d). Transduction was limited to the muscle and there was no gene transfer to the liver or other organs within the peritoneal cavity after i.p. administration of VSV-G pseudotyped EIAV.

Multiple injections of EIAV vector

By combining the intrathoracic and i.p. routes, the musculature of the diaphragm was comprehensively transduced both anteriorly and posteriorly (Figure 4). The innermost intercostals and abdominal muscles were also stained to a similar degree when the injection routes were used individually. A combination of the i.v. and i.p. injection routes and i.m. injection of three limbs and a single flank resulted in widespread gene expression in all injected muscles and also to the diaphragm and heart (Figure 4). This combination of injection routes with a total dose of 5×10^7 TU did not result in any observable ill health or mortality to the animal prior to killing 6 weeks following fetal injection and all tissues also appeared histologically normal.

Immune responses to vector and transgene

One of the potential advantages of administration of gene therapy vectors to the fetus is avoidance of an immune response against the vector and transgenic proteins. In order to investigate this further, we looked for antibodies to the viral envelope protein VSV-G and the transgene β-galactosidase in the i.v. and i.m. injected groups of mice. Despite strong expression of β-galactosidase in the liver, muscle and other tissues in these fetally treated mice, which persisted for at least 5 months, no antibodies to either the transgene or the viral envelope protein VSV-G could be detected. In addition, tissue sections were scored for macrophages, polymorphonuclear neutrophils, CD4-and CD8-positive cells after immunohistochemical staining of injected muscles expressing β-galactosidase as an indication of a possible cellular cytotoxic immune response to the vector or transgene (Table 2, Figure 5). For each of the parameters there was no significant difference in the number of infiltrating inflammatory cells in the muscles of mice injected fetally with EIAV vector. In contrast, those injected with adenovirus at 4 weeks old had statistically significant elevated numbers of macrophages, polymorphonuclear neutrophils, CD4- and CD8-positive cells.

Discussion

The treatment of DMD by gene therapy is challenging for several reasons. The muscle degeneration characteristic of the DMD phenotype occurs in all muscle groups of the body at an early age, which makes postnatal treatment of this disease by multiple local injections very complicated and traumatic. *In utero* gene application on the other hand offers the possibility to reach a broader local population of fetal muscle cells by injection at several sites. The results reported here, which show extensive marker gene expression persisting up to 15 months in multiple muscle groups after EIAV vector application, strongly support this hypothesis. The extent of vector spread in adult animals injected *in utero* compares favourably with the large number of injections, and hence increased viral dose, that would be required in order to transduce such a large muscle mass in the adult and which has impeded efforts towards gene therapy of DMD. In addition, we have shown that the crucially important intercostal and diaphragm muscles can be efficiently transduced by vector delivery into the fetal rib cage, combined with supracostal and intraperitoneal injection in the same fetus. Intraperitoneal injection also provided gene transfer to the muscles of the abdominal cavity. Most importantly such combinations of delivery routes in the same animal did not affect fetal survival or tissue histology despite the increase in EIAV vector dose. Fetal survival after surgical intervention and administration of VSV-G pseudotyped EIAV vector is largely dependent upon vector toxicity, which was noted to vary between virus batches. However, new methods to purify and concentrate lentiviral vectors have been developed thus reducing contaminating protein debris, including VSV-G protein, which is toxic to target cells[15] resulting in increased fetal survival.

In contrast to the low level of muscle gene transfer by a VSV-G pseudotyped HIV-1 vector after direct injection of the hind limb described by MacKenzie *et al*[12] and Kobinger *et al*[13] we found that VSV-G pseudotyped EIAV resulted in high level and extensive gene transfer to these muscles. This may be due to differences between the EIAV and HIV vectors. Two viral vectors were

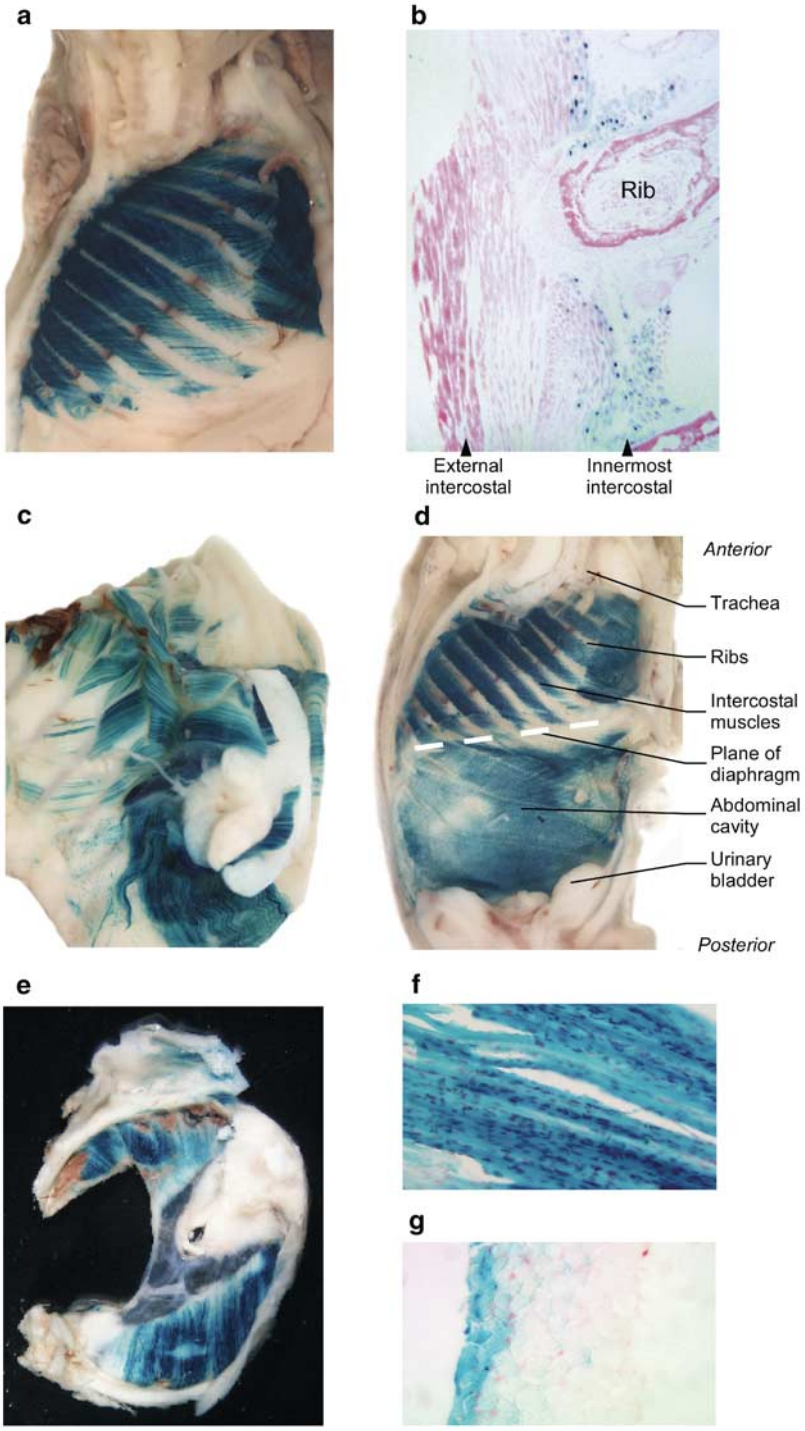

Figure 3 *Gene delivery to musculature after intrathoracic, supracostal and intraperitoneal injections. X-gal staining in the muscle fibres of the innermost (a and b) and external (c) intercostal muscles 5 days (intrathoracic injection) and 1 month (supracostal injection) after SMART 2Z injection, respectively. β-Galactosidase expression in muscle fibres of the abdominal wall and innermost intercostal muscles 4 days after combined intrathoracic and intraperitoneal injections of SMART3 NZ (d). Transduction of the diaphragm (e) 13 days after intraperitoneal injection of SMART2 Z. Longitudinal (f) and transverse (g) histological sections of the diaphragm.*

investigated in the course of this study, SMART2 Z and the nuclear localizing SMART3 NZ. SMART3 NZ, which has further deletions of viral sequence thus giving it an improved safety profile, was at least as effective as the earlier vector SMART2 Z at gene transfer to skeletal muscle. Intramuscular injection of vector resulted in consistently reproducible gene transfer to multiple muscle groups of the hindlimb, which was confirmed by macroscopic X-gal staining and measured concentrations of β-galactosidase protein by ELISA. Although reproducible, expression was greatest at the surface of the muscle. The studies of MacKenzie *et al*[12] and Kobinger *et al*[13] may also indicate that even higher levels of transduction could be achieved when applying the

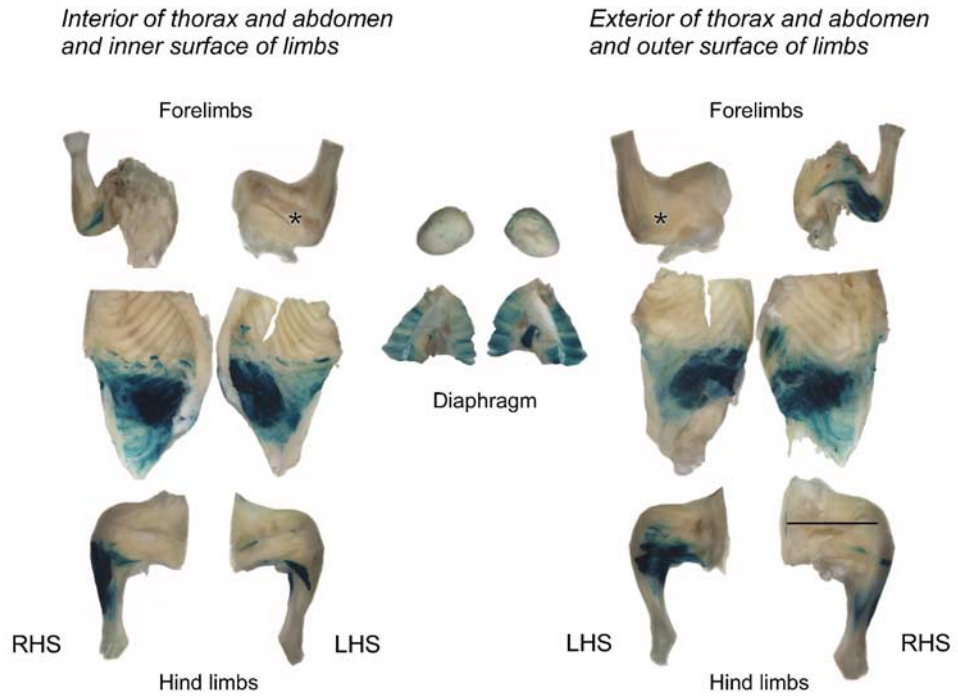

Figure 4 *Extensive staining of all muscle groups after multiple EIAV injections. β-Galactosidase expression in the injected muscles (i.m.), abdominal walls and diaphragm (i.p.) and heart and uninjected limb (*) (i.v.) 6 weeks after injection of SMART2 Z. LHS = left-hand side, RHS = right-hand side. The scale bar represents 2 cm.*

Table 2 Cellular immune response to intramuscular injection of vector

	Uninjected	*EIAV injected*	*Adeno injected*
Macrophages	15.6±2.3	19.8±3.0	75.5±15.8*
PMN	1.2±0.2	1.5±0.3	47.2±11.8*
CD4+	0.1±0.1	0.1±0.1	4.7±1.1*
CD8+	0±0	0.1±0.1	2.3±0.8*

EIAV-treated animals were injected at day 15 of gestation and immunohistochemical analysis of inflammatory cell infiltrates was performed when the mice were 4 weeks old. Uninjected muscle sections were prepared from age-matched controls. Animals (4-week old) injected with adenovirus (Adeno) served as positive controls. Muscle sections were prepared 3 days after injection.
PMN = polymorphonuclear neutrophils.
*$P < 0.05$ compared with uninjected muscle using *t*-test with Bonferroni correction.

Ebola or Mokola pseudotyped EIAV, and we are presently investigating these viral envelope proteins for gene transfer to the musculature.

The extent and intensity of β-galactosidase expression in the muscle was unchanged over time when comparing that observed at 1 week and 5 months, in agreement with our previously reported findings in the skeletal muscle and heart over a period of 1 year after i.v. administration of VSV-G pseudotyped SMART2 Z.[14] Maintenance of gene expression is most likely due to integration of the vector into the host genome and negligible degeneration of myofibres in MF-1 mice. These data are in agreement with recently published literature. O'Rourke *et al*[16] reported maintenance of EGFP expression over 3 months after i.m. injection of VSV-G pseudotyped EIAV to adult mice. Similarly, gene expression has been shown to be maintained for up to 14 months after i.m. injection of VSV-G pseudotyped HIV vectors to fetal and adult mice and rats.[10,12,13,16]

A lentiviral vector was chosen for this study because of its ability to integrate into target cells, allowing for the possibility of lifetime correction with a single administration of vector. Vector spread after i.v. injection of VSV-G pseudotyped EIAV has previously been shown by PCR analysis of fetally treated adult tissues 1 year postadministration. All tissues examined were, in varying degrees, positive for EIAV vector presence with the exception of the pancreas, white blood cells and notably the sperm.[14] Importantly for an integrating vector, despite continued expression of the transgene in fetally treated male and female adult mice their progeny show no expression of the transgene. Lentiviral vectors have previously been reported to transduce haematopoietic stem cells[17,18] and in our studies using VSV-G pseudotyped EIAV, cells of the bone marrow have been shown to be positive for vector presence by PCR analysis. However, vector is not detected in the white blood cells and further studies are ongoing to determine the exact cell types transduced.

Immune responses against vector and transgenic protein can result in loss of the transgenic protein in muscle. However, in the present study expression was maintained for at least 5 months and no antibodies to either β-galactosidase or the viral envelope protein VSV-G were detected. In addition to the absence of a humoral response there was no evidence of a cellular inflammatory response after fetal injection of the virus. The absence of an immune response is clearly not due to a failure to transduce sufficient cells and supports the concept that delivery of genes to the fetus may avoid transgene elimination. Further studies are in progress to ascertain whether tolerance has been achieved, as has been demonstrated after *in utero* administration of an

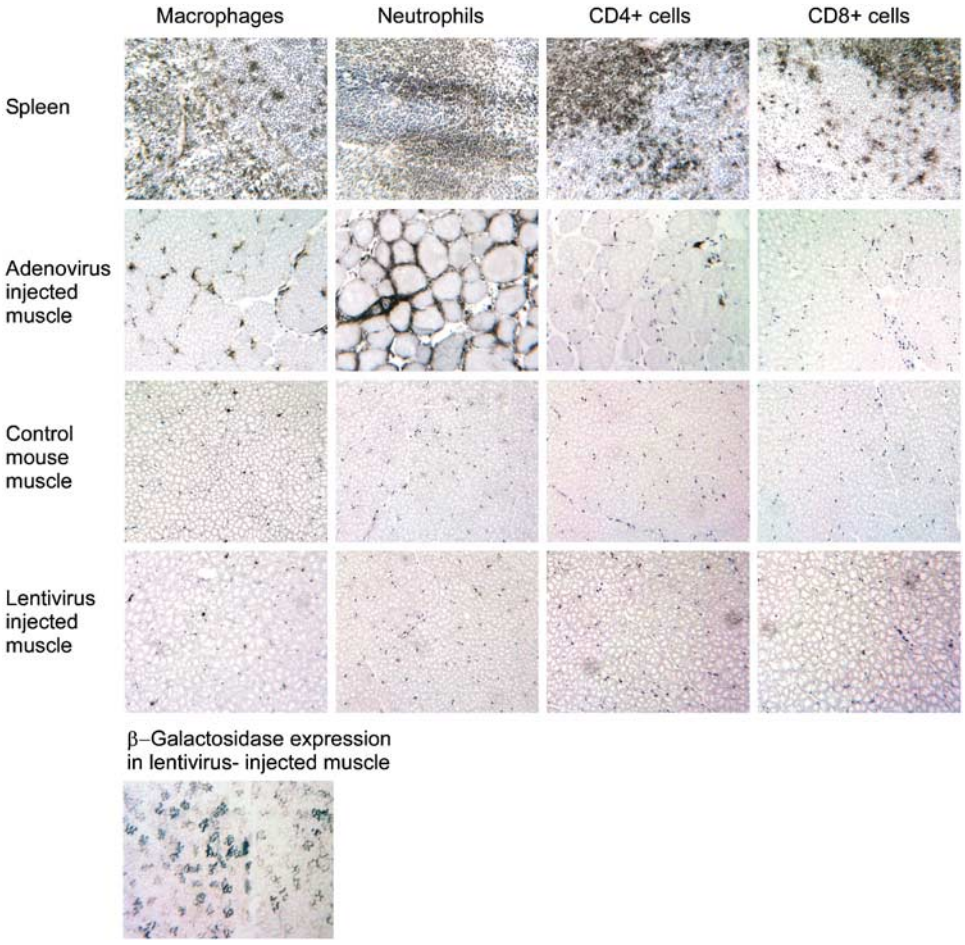

Figure 5 *Immunohistochemical analysis of cellular inflammatory response in muscle. Immuno staining for macrophages, polymorphonuclear neutrophils, CD4- and CD8-positive cells. EIAV-treated animals were injected at day 15 of gestation and immunohistochemical analysis of inflammatory cell infiltrates was performed when the mice were 4 weeks old. Uninjected muscle sections and spleen sections were prepared from age-matched controls. Animals (4-week old) injected with adenovirus (Adeno) served as positive controls. Muscle sections were prepared 3 days after injection.*

adenovirus vector encoding factor IX,[14] by readministration of vector to fetally treated adults.

Expression of dystrophin or minidystrophin in fetal myofibres after *in utero* gene delivery would prevent the repeated rounds of necrosis and associated inflammation observed in dystrophic muscle where dystrophin expression is totally lacking and avoid, or at least limit, the early onset of myodegeneration. Our EIAV vectors have a cloning capacity of up to 8.0 kb, and we are currently cloning mini- and microdystrophin constructs with expression driven by ubiquitous or muscle-specific promoters. These will be pseudotyped with alternative envelopes, including Ebola and Mokola, to investigate targeting of muscle progenitor cells.

In summary, we have shown here that the simultaneous *in utero* application of EIAV lentiviruses, by various routes, provides widespread reporter gene delivery to all the necessary muscle groups for gene therapy of DMD. We are therefore planning further studies to use EIAV vectors carrying minimal dystrophin gene constructs for future rescue experiments in the dystrophin-deficient mdx mouse model of DMD and demonstrate proof of principle for long-term prevention/correction of this debilitating disease.

Materials and methods

Production of high titre equine infectious anaemia virus (EIAV)

Human embryonic kidney 293 T cells were used to produce EIAV particles.[19,20] Cells were grown in DMEM supplemented with 10% fetal calf serum and transfected with plasmid vectors to transiently produce infectious virus particles as previously described.[21] Virus titration was performed on D17 canine cells and human HT1080 cells by seeding cells onto 12-well culture dishes at 8×10^4 cells/well followed by the addition of serially diluted virus. Virus was left on cells for 24 h before removal. Cells were washed twice in PBS then refed with fresh medium and incubated for a further 24 h. Virus titre was determined by X-gal staining as approximately 1×10^9 transduction units/ml in every case.

Virus construction

In the course of these experiments, two EIAV constructs were investigated. SMART2 Z is an EIAV vector expressing the β-galactosidase gene driven by the CMV promoter. It also contains the central polypurine tract

from EIAV (cppt)[22] and the WPRE element from the woodchuck hepatitis virus.[23] The construction of an EIAV construct expressing nuclear localizing β-galactosidase incorporating the SV40 nuclear localization signal (SMART3 NZ) was obtained by PCR amplification from the plasmid vector pNGVL1-ntbta-gal. Primer pairs 5′AGCGCAAAGCTTCGGACCGAGGTACCATGGATA AAGTTTTCCGGAATTCCGCAA3′ and 5′GCCGACCT GCGGCCGCCTCGAGATTATTATTTTTGACACCAGA CCAACTGGT3′ were used to insert *Hind*III and *Not*I restriction sites at each end of the 3.2 kb PCR products followed by restriction and ligation into *Hind*III and *Not*I restriction digested and dephosphorylated pSmart3G.

Assays to detect the presence of replication-competent lentiviruses in the viral preparations

To detect the presence of replication-competent EIAV virus, 293 T cells were seeded into 12-well plates at 1×10^5 cells/well. These cells were transduced 24 h later in duplicate with three viral vector preparations. The cells were passaged 1 in 5 every 3–4 days. Supernatant and cellular DNA were isolated at various time points. The supernatant samples were assayed for product-enhanced reverse transcriptase (PERT) activity[24] and the cellular DNA was analysed for integrated copies of EIAV genome.[25] No replication-competent EIAV virus was detected for virus preparations.

Administration of vector

Pregnant female MF1 mice (B&K Universal Ltd, UK) at 15–16 days gestation were used in this study. Under isofluorane anaesthesia, the uterus was exposed through a full-depth midline laparotomy. Each route of vector administration was accessed by transuterine injection using a 34-gauge needle (Hamilton). Systemic, peritoneal, thoracic and muscular delivery was achieved by administration of 20, 20, 10 and 5 μl of vector solution in PBS, respectively, into the yolk-sac vessel, peritoneal cavity or thoracic cavity or directly into the hind leg, respectively. Up to four fetuses were injected per dam. The laparotomy was closed in two stages using interrupted stitches of 6-0 silk suture and the mouse permitted to recover in a warm cage.

Tissue harvest and histology

For prenatal analysis, dams were killed by cervical dislocation and fetuses decapitated. For postnatal end point analysis, mice were anaesthetized with isofluorane and exsanguinated by cardiac puncture. Fetuses and mice less than a month old were placed in 100% ethanol for 2 h, injected with ethanol and halved sagittally. Carcasses from older mice were dismembered before fixation. To analyse the tissue expression of the β-galactosidase transgene tissue samples were placed in X-gal solution (1 mg/ml, pH 7.4) and incubated overnight at room temperature. Tissues were then fixed in 10% formalin, embedded in paraffin wax and 5 μm sections counterstained with eosin.

Detection of anti-VSV-G and anti-β-galactosidase antibodies

Antibodies against VSV-G and β-galactosidase were detected by ELISA. 96-well plates were coated at 4°C overnight with 1:150 000 dilution of β-galactosidase protein (10 ng) or 1:5000 dilution of VSV-G protein (10 ng) before washing with 0.2% Tween 20 in PBS. Serum samples diluted 1:100 or 1:500 in 4% BSA in PBS pH 8.25 were added to the wells and left for 2 h at room temperature. After washing horseradish peroxidase-conjugated rabbit anti-mouse antibody (DAKO, UK) diluted 1:1000 in 4% BSA in PBS pH 8.1 was added for 1 h at room temperature. This antibody detects immunoglobulin G1 (IgG1), IgG2a, IgG2b, IgG3, IgA and IgM. After washing, TMB+ substrate-chromogen solution (DAKO, UK) was added for exactly 3 min before stopping the reaction by addition of 3 M H_2SO_4. Absorbances were measured at 450 nm. In both assays, sample values were expressed as a ratio of sample absorbance *versus* absorbance of hyperimmune serum that was generated by repeated intramuscular injection of VSV-G and β-galactosidase protein. A standard curve constructed from dilutions of hyperimmune serum (1:8000 to 1:1024 000 for β-galactosidase, and 1:5000 to 1:640 000) was used to establish the range of sensitivity.

Detection of cell infiltrates

Frozen sections of muscle tissue were examined for the presence of macrophages and neutrophils (anti-CD68, MCA1957GA and antiallotypic marker, MCA771GA, Serotec Ltd. Oxford, UK), CD4- and CD8-positive cells (anti-L3T4, 550278 and anti-Ly-2, 550281, BD Biosciences, Oxford, UK). Visualization followed standard avidin–biotin peroxidase and diaminobenzidine treatment.

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