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RESEARCH ARTICLE

Comparison of high-capacity and first-generation adenoviral vector gene delivery to murine muscle in utero

R Bilbao[1,5], DP Reay[1,5], E Wu[1], H Zheng[1], V Biermann[2], S Kochanek[2,3] and PR Clemens[1,4]
[1]Department of Neurology, University of Pittsburgh, Pittsburgh, PA, USA; [2]Center for Molecular Medicine, University of Cologne, Cologne, Germany; [3]Division of Gene Therapy, University of Ulm, Ulm, Germany; and [4]Neurology Service, Department of Veterans Affairs Medical Center, Pittsburgh, PA, USA

In utero gene delivery could offer the advantage of treatment at an early stage for genetic disorders such as Duchenne muscular dystrophy (DMD) in which the inevitable process of muscle degeneration is already initiated at birth. Furthermore, treatment of fetal muscle with adenoviral (Ad) vectors is attractive because of a high density of Ad receptors, easy vector accessibility due to immaturity of the basal lamina and the possibility of treating stem cells. Previously, we demonstrated the efficient transduction of fetal muscle by high-capacity Ad (HC-Ad) vectors. In this study, we compared HC-Ad and first-generation Ad (FG-Ad) vectors for longevity of lacZ transgene expression, toxicity and induction of immunity after direct vector-mediated in utero gene delivery to fetal C57BL/6 mice muscle 16 days after conception (E-16). The total amount of β-galactosidase (βgal) expressed from the HC-Ad vector remained stable for the 5 months of the study, although the concentration of βgal decreased due to muscle growth. Higher survival rates that reflect lower levels of toxicity were observed in those mice transduced with an HC-Ad vector as compared to an FG-Ad vector. The toxicity induced by FG-Ad vector gene delivery was dependent on mouse strain and vector dose. Animals treated with either HC-Ad and FG-Ad vectors developed non-neutralizing antibodies against Ad capsid and antibodies against βgal, but these antibodies did not cause loss of vector genomes from transduced muscle. In a mouse model of DMD, dystrophin gene transfer to muscle in utero using an HC-Ad vector restored the dystrophin-associated glycoproteins. Our results demonstrate that long-term transgene expression can be achieved by HC-Ad vector-mediated gene delivery to fetal muscle, although strategies of vector integration may need to be considered to accommodate muscle growth.
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Introduction

Gene therapy offers the potential of effective treatments for hereditary genetic diseases. However, three major issues currently impede progress in gene therapy research. First, there is limited spread of genetic vectors to widespread tissues, such as skeletal muscle. Second, production of large quantities of viral vectors is difficult and costly. Third, vector capsid proteins or expression from therapeutic transgenes triggers immunity. For example, in murine models of Duchenne muscular dystrophy (DMD), a lethal degenerative muscle disease caused by the genetic absence of muscle dystrophin, there is evidence that dystrophin delivery to muscle postnatally can result in anti-dystrophin and anti-vector immune responses.[1] *In utero* gene delivery to the fetus offers an alternative to postnatal gene delivery that addresses each of these limitations. Specifically, fetal gene delivery can accomplish gene transfer when tissue mass is small, tissue barriers such as the basal lamina surrounding muscle fibers are not yet developed, stem cells may be relatively abundant and the immune system is immature.

There is a limited, but growing literature on studies of adenoviral (Ad) and adeno-associated viral (AAV) vector-mediated gene delivery *in utero*. Routes of delivery include intraplacental,[2,3] intra-amniotic,[4] intraperitoneal,[5,6] intrahepatic,[2,4,5] intravascular[7,8] and intramuscular.[4,8,9] To date, only intramuscular administration *in utero* has resulted in muscle transgene expression. Previous reports demonstrate excellent gene transfer to fetal murine skeletal muscle by direct intramuscular injection to the fetus using Ad[2,4,8,9] and AAV vectors.[2,4,8] Both Ad and AAV vectors for fetal gene delivery accomplished spread to multiple muscles in the vicinity of the intramuscular injection site.[2,9] Experiments employing fetal gene transfer for delivery of both secreted therapeutic proteins such as clotting factor IX[8] and human growth hormone[4] and cellular marker gene proteins such as β-galactosidase (βgal)[2,4,9] and luciferase[6] show expression of transgenes into the adult life of the animal.

Correspondence: Dr PR Clemens, Department of Neurology, University of Pittsburgh, Room S-520 Biomedical Science Tower, Pittsburgh, PA 15213, USA
[5]These authors contributed equally to this work
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Our understanding of the induction of immunity after fetal gene transfer is evolving. Important factors likely include antigenicity of the vector and protein expressed from the transgene, type of transgene (cellular *versus* secreted), gestational age of the fetal recipient at the time of gene delivery, and mouse strain. In one study of intramuscular gene delivery to fetal C57BL/6 mice, no antibodies against the Ad vector or transgene product expressed from either an Ad or AAV vector were detected.[8] In another study, however, *in utero* delivery of Ad and AAV vectors into B6/129 F1 mice resulted in the production of Ad and AAV vector neutralizing antibodies, respectively.[4] No study, regardless of vector or route of delivery, has demonstrated tolerance induced by *in utero* gene delivery such that repeated postnatal gene delivery was successful. The data to date suggest that antigens presented by gene delivery *in utero* may be largely ignored, but do not induce tolerance to either vector antigens or foreign transgene proteins.[4,5] One study, however, suggests that tolerance can be induced to factor IX.[10]

We have previously shown high transduction levels of βgal in fetal muscle 2 days after a single intramuscular *in utero* injection of an HC-Ad vector.[11] The application of fetal gene transfer as a treatment for DMD requires further preclinical studies to optimize gene delivery of a muscle protein. In this study, we compare gene transfer efficacy and gene expression longevity for HC-Ad and FG-Ad vectors that carry the same *lac*Z expression cassette. We compare the toxicity caused by these vectors and the humoral immune response induced by transgene and viral proteins. As a first step in the application of this technology to a muscle disease model, we test whether HC-Ad vector-mediated dystrophin delivery to dystrophin-deficient *mdx* mouse muscle can provide restoration of the dystrophin–glycoprotein complex (DGC).

Results

Efficacy of in utero gene transfer to skeletal muscle depended on dose and dilution volume of injected vector

To establish the optimal delivery conditions for intramuscular Ad vector injections into mouse fetuses 16 days after conception (E-16), we tested injectate volume, vector dose, needle type and injection flow rate in the C57BL/6 mouse strain using an FG-Ad vector carrying the *lac*Z gene driven by the human cytomegalovirus (CMV) promoter. To test the effect of injectate volume, 2×10^7 BFU (blue forming units) of FG-Ad vector was diluted in either 2 or 5 μl of lactated Ringer's buffer. At 2 days after *in utero* injections, we collected fetal hind limbs for quantification of βgal expression by ONPG assay. We found that βgal expression in muscle was significantly higher when the vector was diluted in 5 μl of buffer as compared to 2 μl (Figure 1a). Although gene delivery was intramuscular, low levels of gene expression were found in other organs. The larger delivery volume resulted in greater spreading of Ad vector to other organs including heart, liver, lung and the mother's liver. Muscle morphology in the region of the injection was normal with either a 2 or 5 μl injectate volume (data not shown). Second, we determined whether Ad vector transduction was dose dependent by injecting three

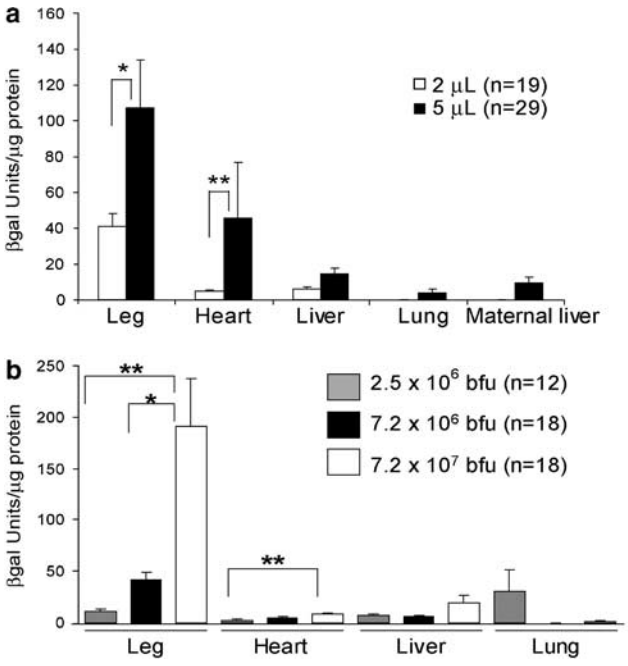

Figure 1 *Transduction efficiency by in utero intramuscular delivery is dependent on Ad vector dilution volume and dose. C57BL/6 fetal mice at E-16 gestation received an intramuscular injection in utero with (a) 2×10^7 BFU of FG-Ad vector per pup diluted to either 2 or 5 μl with lactated Ringer's buffer, or (b) different doses of FG-Ad vector per pup, 2.5×10^6, 7.2×10^6 or 7.2×10^7 BFU, diluted to 5 μl of lactated Ringer's buffer. After 2 days, fetal hind limbs, organs (heart, liver, lung) and mother's liver were collected. Levels of expression are shown as mean ± s.e. of units of βgal per μg of muscle protein. n = number of pups studied (*P < 0.05,**P < 0.001).*

different doses of FG-Ad vector (2.5×10^6, 7.2×10^6 and 7.2×10^7 BFU/fetus), each diluted to 5 μl with lactated Ringer's buffer. After 2 days, hind limbs and other organs were collected from the fetuses. As shown in Figure 1b, we found increasing levels of gene delivery to hind limb muscle with increasing doses of FG-Ad vector. Third, we compared the gene transfer efficiency using either a pulled glass needle or a 34G Hamilton needle and performing the injection at different flow rates using a syringe pump. In our experience, diluting the Ad vector in 5 μl of lactated Ringer's buffer and using a Hamilton needle with a flow rate of 10 μl/min provided optimal direct gene transfer to E-16 fetal muscle.

Muscle transduced with HC-Ad vector in utero maintained a stable total level of βgal expression up to 5 months

To evaluate the effect of the presence or absence of viral genes in FG-Ad or HC-Ad vectors, respectively, on transduction efficiency, we carried out *in utero* intramuscular injections of 2×10^7 BFU of FG-Ad and HC-Ad vectors to E-16 fetuses. Both FG-Ad and HC-Ad vectors used in this study had the same CMV-driven *lac*Z expression cassette and the same Ad serotype 5 capsid. In contrast to the FG-Ad vector, which retains most of the viral genome, but is rendered replication defective by deletions of the E1 and E3 regions, the HC-Ad vector lacks all viral genes.[12] We quantified transgene expression at 2 days, 1 month and 5 months after intramuscular gene delivery *in utero* (Figure 2 and Table 1).

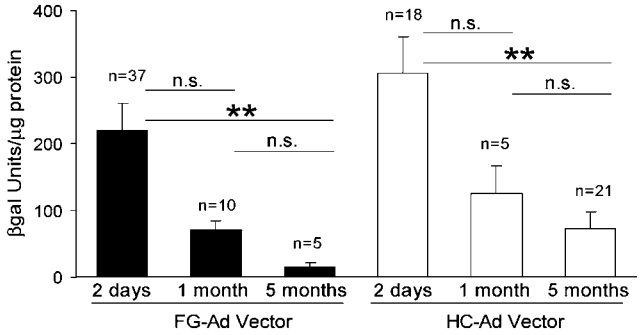

Figure 2 *Long-term transgene expression from FG-Ad and HC-Ad vectors delivered in utero. C57BL/6 E-16 fetuses received an intramuscular injection of FG-Ad or HC-Ad vector in the hind limb. Muscles were collected 2 days, 1 month and 5 months after in utero infection and analyzed for βgal expression by ONPG assay. Levels of expression are shown as mean ± s.e. of units of βgal per total μg of muscle protein. n = number of pups studied (**P < 0.001, n.s. = not significant). The background βgal expression (expressed as U/μg protein) in uninjected muscles from age-matched controls was 4.0, 8.6 and 7.9 for 2-day, 1-month and 5-month samples, respectively. These background levels were subtracted from the sample values to yield the βgal expression values shown.*

Table 1 Mean total βgal units in the hind limb as a function of time after *in utero* gene transfer

Time after gene transfer	*Total amount of hind limb muscle protein (mg)*	*FG-Ad vector, total βgal units*	*HC-Ad vector, total βgal units*
2 days	1.6	350 098	489 605
1 month	10.3	718 963	1 289 648
5 months	32.2	477 056	2 308 689

For *in utero* studies performed in mice, it is not possible to inject the fetuses close to the cervix without increasing the rate of pregnancy loss. We, therefore, routinely injected approximately 4–8 pups per each pregnant mouse avoiding the fetuses in close proximity to the cervix. The fetal mice killed 2 days after gene transfer were collected directly from the uterine cavity, and thus the injection status of each fetus was known. All injected fetuses were included in the study. After natural delivery, the only means to identify injected pups was to test all hind limb muscle for βgal expression. If there was any detectable βgal expression in any muscle, the animal was included in analysis. If there was no detectable βgal expression in any muscle, we assumed that the pup was not injected. In extensive experience with this experimental strategy, we find that the percentage of pups injected is approximately equivalent to the percentage of mice demonstrating vector transgene expression. Therefore, we feel that we are capturing all injected mice for study by this approach. However, we cannot completely exclude the possibility that a mouse scored as uninjected was in fact injected, but had a very low level of expression.

Quantitative analysis of βgal concentration in muscle protein (Figure 2) demonstrated a decline over time after a single *in utero* administration for both HC-Ad and FG-Ad vectors. However, this decline reflected, in part, the marked increase in total leg muscle protein from E-16 to 5 months of age. As shown in Table 1, the total amount of βgal protein in HC-Ad vector-transduced legs did not decline, and appeared to increase over the 5-month experiment. We also looked at the βgal expression by X-gal staining of entire limbs and muscle sections. As shown in Figure 3a and c, the intramuscular injection of HC-Ad vector into the hind limb of E-16 mice provided

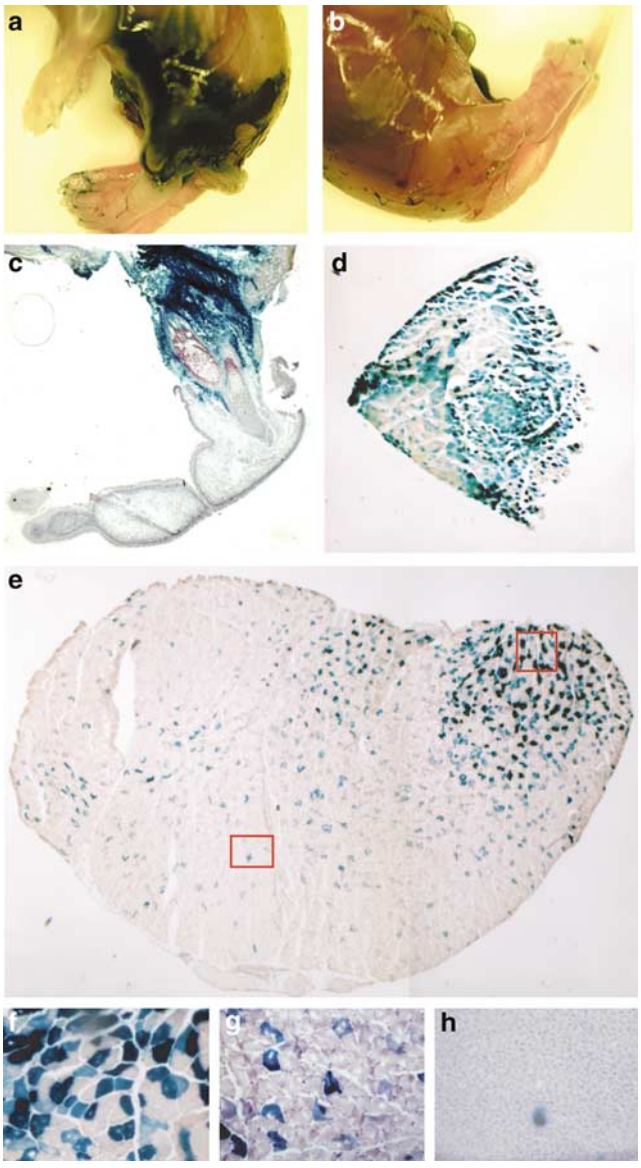

Figure 3 *Histochemical demonstration of βgal expression from the HC-Ad vector. βgal expression was detected by X-gal staining 2 days, 1 month and 5 months after in utero HC-Ad delivery. (a) The skinned whole embryo was stained by immersion in X-gal solution 2 days after gene delivery demonstrating βgal expression in muscles of the transduced hind limb. (b) The opposite hind limb that was not injected with HC-Ad vector was also stained by immersion in X-gal solution serving as a negative control. (c) X-gal-stained longitudinal section of transduced hind limb 2 days after gene transfer showing diffusion of vector within muscle tissue. (d) X-gal-stained transverse section of a transduced gastrocnemius muscle collected 1 month after gene transfer. (e) X-gal-stained transverse section of a transduced gastrocnemius muscle collected 5 months after gene transfer. (f, g) Higher magnification of boxed regions of the muscle section shown in (e) shows a mosaic pattern of expression with different levels of expression in different fibers. (h) Mother's liver 2 days after intramuscular injection of HC-Ad vector to fetuses she carried in utero showing a hepatocyte expressing βgal.*

a wide diffusion of the vector throughout the injected leg. At 1 month after HC-Ad vector delivery *in utero*, we found widespread βgal expression in several muscles. A region of a transduced gastrocnemius muscle is shown in Figure 3d. βgal expression persisted in transduced muscles even 5 months after HC-Ad vector delivery (Figure 3e). In a given cross-section, the level of βgal expression was variable, possibly reflecting the diffusion of βgal protein longitudinally within individual myofibers (Figure 3f and g). Inflammatory cells were not observed in transduced muscles (Figure 3d–g). X-gal staining (Figure 3h) and quantitative assay of mothers' liver 2 days after *in utero* injection revealed a variable level of βgal expression ranging from no expression to nearly 30 U/μg protein (mean 10 U/μg protein).

Viral DNA from both FG-Ad and HC-Ad vectors persisted for up to 5 months

To determine the stability of viral DNA over time, we used real-time polymerase chain reaction (PCR) to quantify the number of vector particles in all transduced leg muscles (Figure 4). The number of particles for both vectors remained stable up to 5 months, suggesting that the decrease in the total level of βgal expression for the FG-Ad vector (Table 1) may not have been caused by clearance of FG-Ad genomes.

In utero transfer of FG-Ad and HC-Ad vectors induced an antibody response to vector and transgene proteins

We examined whether the immune system responded to *in utero* delivery of FG-Ad or HC-Ad vectors by measuring antibodies against Ad capsid and βgal proteins. Assays for total antibodies against Ad capsid (Figure 5a) and βgal protein (Figure 5b) and neutralizing antibodies against Ad capsid were carried out on serum collected from 1- and 5-month-old mice that received intramuscular FG-Ad and HC-Ad vector delivery *in utero*. Five months after *in utero* injection, the majority of mice that received either FG-Ad or HC-Ad vector administration developed antibodies against Ad capsid proteins and approximately half of the mice developed antibodies against βgal. Neutralizing antibodies against Ad were not detected in either the FG-Ad or HC-Ad vector treatment group, indicating that the total anti-Ad antibodies observed were not neutralizing of Ad infection, and therefore would not preclude repeat vector administration. Antibodies against βgal and Ad capsid proteins were also induced in mothers carrying the treated pups (Figure 6). However, similar to our data in the transduced pups, anti-Ad antibodies detected in mothers' serum were not neutralizing antibodies.

In utero FG-Ad vector administration was associated with lower survival rates than HC-Ad vector administration

To determine the toxicity of FG-Ad and HC-Ad vector administration to E-16 fetal muscle, we calculated the percentage of surviving pups 10 days after birth, following vector delivery *in utero* and compared this to survival after injection of phosphate-buffered saline (PBS) alone (Table 2). Statistical analysis demonstrated a significant decrease in survival in those mice that were infected with 2×10^7 BFU of FG-Ad vector but not with 2×10^7 BFU of HC-Ad vector as compared to those of a

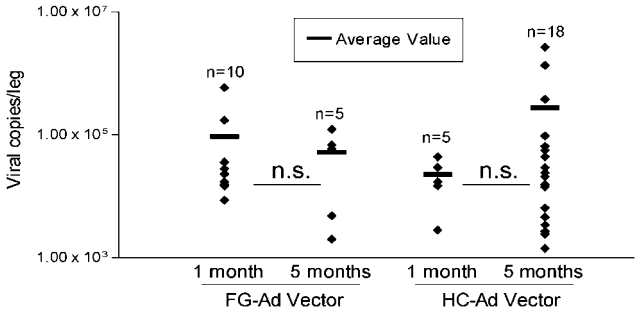

Figure 4 *Vector genome levels of FG-Ad and HC-Ad vectors were stable in muscle for 5 months after in utero gene transfer. Viral DNA was quantified in transduced muscles by real-time PCR 1 and 5 months after intramuscular FG-Ad and HC-Ad vector delivery in utero. Individual and mean values are shown. n = number of pups studied (n.s. = not significant).*

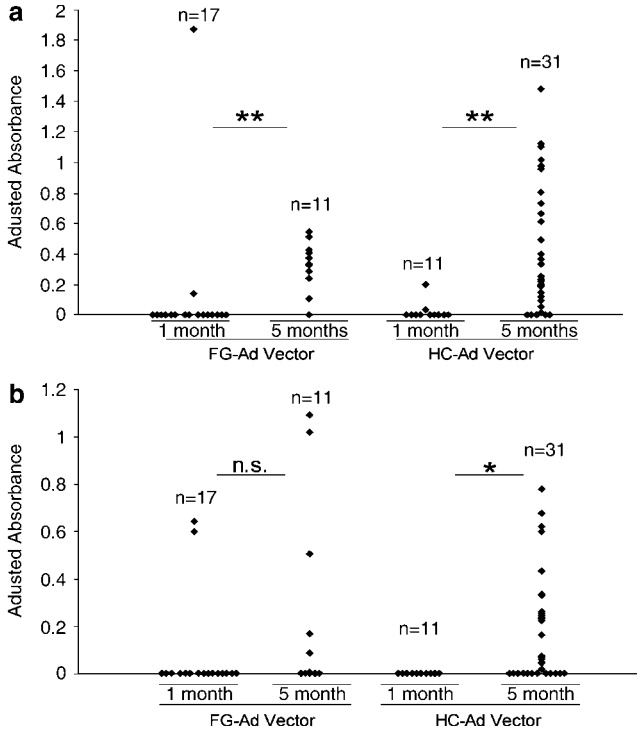

Figure 5 *In utero FG-Ad and HC-Ad vector gene delivery to muscle induced total antibodies against Ad capsid and βgal proteins. Detection in serum samples from injected animals, diluted 1:100, of total antibodies against (a) Ad capsid proteins and (b) βgal 1 and 5 months after intramuscular gene delivery of pups in utero with FG-Ad and HC-Ad vectors. n = number of pups studied (*P < 0.05, **P < 0.001, n.s. = not significant).*

PBS-injected control group. However, a lower dose of FG-Ad vector (1×10^7 or 5×10^6 BFU/pup) did not elicit significant toxicity (Table 2). We excluded wild-type adenovirus or endotoxin contamination of the FG-Ad vector preparation as possible explanations for FG-Ad vector toxicity by looking for cytopathic effect on A549 cells after FG-Ad vector infection *in vitro* and by testing for the presence of endotoxin in the vector preparation. We then tested whether the dose-dependent toxicity of FG-Ad vector administration *in utero* was also mouse strain dependent by injecting a range of doses of FG-Ad vector (2×10^7, 1×10^7 and 5×10^6 BFU/pup)

intramuscularly in E-16 fetal CD-1 mice. No significant toxicity of FG-Ad at any of the doses tested was observed in CD-1 mice at 10 days of age following *in utero* gene delivery.

HC-Ad vector dystrophin gene delivery to mdx mice in utero

An HC-Ad vector carrying the full-length murine dystrophin cDNA driven by the muscle creatine kinase (MCK) promoter (AdmDys)[13] was delivered intramuscularly to one hind limb of E-16 fetal *mdx* mice. At 9 weeks after vector delivery, muscles from both hind limbs were collected for dystrophin, α-sarcoglycan and β-sarcoglycan immunostaining. Figure 7 shows an example of dystrophin delivery *in utero* in which recombinant dystrophin expression from the HC-Ad vector delivered to muscle restored both α-sarcoglycan and β-sarcoglycan expression to the muscle membrane.

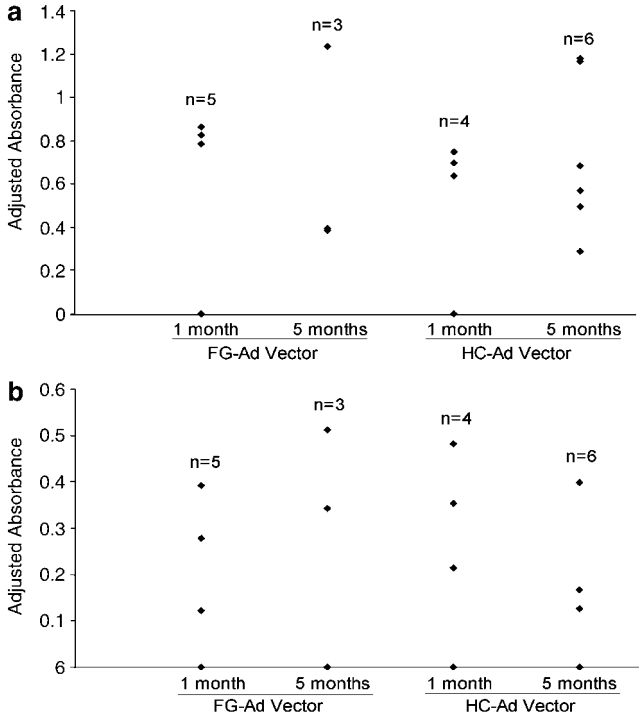

Figure 6 *Maternal antibodies were induced against Ad capsid and βgal proteins after gene delivery to pups in utero. Detection in maternal serum samples, diluted 1:100, of total antibodies against (a) Ad capsid proteins and (b) βgal 1 and 5 months after intramuscular gene delivery of pups in utero with FG-Ad and HC-Ad vectors. n = number of mothers studied.*

Discussion

In utero dystrophin gene delivery to muscle will require the use of vectors with large insert capacities such as the HC-Ad vector. We showed previously that the HC-Ad vector can efficiently transduce fetal muscle when studied soon after intramuscular delivery.[11] In this study, we demonstrated that HC-Ad vector gene delivery to muscle *in utero* resulted in stable total expression levels of βgal protein for at least 5 months. However, the dramatic increase in muscle mass that occurs between a late gestation fetus and an adult mouse resulted in a decreasing concentration of βgal expressed per μg of muscle protein. Furthermore, we showed that the HC-Ad vector can deliver the dystrophin cDNA to E-16 fetal *mdx* muscle by an *in utero* intramuscular injection resulting in restoration of the DGC to the muscle membrane.

To facilitate further studies in this preclinical murine model of *in utero* muscle gene transfer for DMD, we optimized the experimental conditions of vector dose, injectate volume and flow rate and needle type. Since most previous *in utero* gene delivery studies with Ad vectors have been performed using FG-Ad vectors, we sought to compare long-term expression, toxicity and immunity induced by FG-Ad and HC-Ad vectors. Using FG-Ad vectors, Mitchell *et al*[2] and Yang *et al*[9] detected a mosaic pattern of muscle fibers expressing βgal 6 and 16 weeks, respectively, after intramuscular vector delivery *in utero*. Our observations of the pattern of βgal expression 2 days, 1 month and 5 months after fetal intramuscular FG-Ad and HC-Ad vector delivery were similar.

Although the concentration of βgal in muscle protein declined over time after *in utero* gene transfer with either the FG-Ad or the HC-Ad vector, the total levels of βgal expression in muscle transduced with HC-Ad vector appeared to increase over 5 months, while with the FG-Ad vector total levels of βgal expression decreased between 1 and 5 months. To pursue this further, we quantified viral genomes in transduced muscle and observed persistence of viral DNA for at least 5 months for both FG-Ad and HC-Ad vectors suggesting that the loss of transgene expression from the FG-Ad vector *in utero* was not due to the elimination of transduced cells. We previously reported similar results comparing FG-Ad and HC-Ad vector administration to muscle during the neonatal period.[14] This phenomenon, observed with both *in utero* and neonatal Ad vector administration, suggests that retained viral genes in the FG-Ad vector diminish expression from the CMV-driven transgene carried by

Table 2 Toxicity of adenoviral vectors *in utero* in C57BL/6 and CD-1 mouse strains

	C57BL/6					*CD-1*			
	PBS	*FG-Ad vector, 2×10^7 BFU*	*FG-Ad vector, 1×10^7 BFU*	*FG-Ad vector, 5×10^6 BFU*	*HC-Ad vector, 2×10^7 BFU*	*PBS*	*FG-Ad vector, 2×10^7 BFU*	*FG-Ad vector, 1×10^7 BFU*	*FG-Ad vector, 5×10^6 BFU*
No. injected	48	89	21	20	81	30	32	35	33
No surviving	21	17	7	8	33	26	27	31	29
% survival	44	19	33	40	41	87	84	89	88
CHITEST value		0.000002	0.31	0.72	0.59		0.62	0.73	0.86

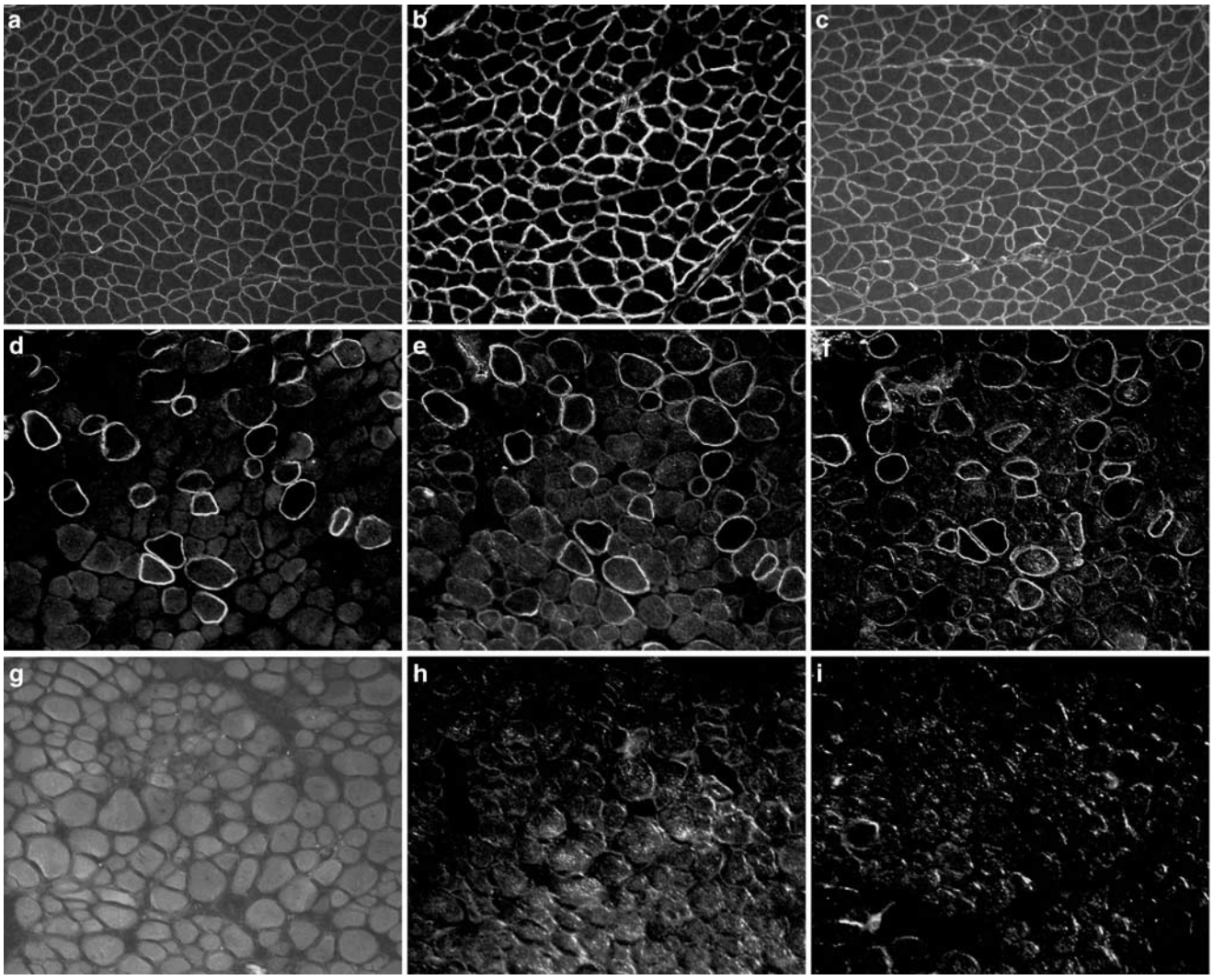

Figure 7 *Restoration of the dystrophin glycoprotein complex in the tibialis anterior muscle after in utero dystrophin gene transfer. Immunohistochemical detection of dystrophin, α-sarcoglycan and β-sarcoglycan expression in muscle 9 weeks after in utero intramuscular gene delivery to the hind limb of an E-16 mdx mouse. Serial sections demonstrate restoration of α-sarcoglycan and β-sarcoglycan in those fibers expressing recombinant dystrophin from HC-Ad vector, AdmDys (d,e,f). The opposite tibialis anterior muscle (g,h,i) and C57BL/6 normal muscle (a,b,c) provide negative and positive controls, respectively. Immunstaining for dystrophin (a,d,g), α-sarcoglycan (b,e,h) and β-sarcoglycan (c,f,i) is shown.*

the vector, either at a transcriptional or a translational level.

Survey of the literature on the immunity induced by muscle gene transfer using Ad vectors *in utero* does not reveal a clear consensus. This likely reflects experimental differences between studies, such as differences of vectors, mouse strain and fetal stage at gene delivery. We therefore report our determination of the humoral response induced to Ad capsid antigens and βgal protein in the C57BL/6 mouse strain with both FG-Ad and HC-Ad vector gene delivery to E-16 fetal muscle. The anti-Ad antibody response was low at 1 month for both the FG-Ad and HC-Ad vector-treated animals. However, there was a statistically significant increase in this response for both groups at 5 months. Most FG-Ad and HC-Ad vector-treated animals did not show an anti-βgal humoral response at 1 month, whereas approximately half of the vector-treated animals had anti-βgal antibodies present at 5 months. Despite this humoral immune response to both Ad proteins and βgal, the stable level of viral copy number in muscle indicates that this humoral response does not cause elimination of vector genomes from transduced muscle.

To further characterize the immune response, we studied whether the anti-Ad antibodies detected 5 months after *in utero* delivery were neutralizing antibodies. No neutralizing anti-Ad antibodies were observed. In accordance with our data, Jerebtsova *et al*[4] only detected neutralizing anti-Ad antibodies 14 days after birth following administration of FG-Ad vector *in utero* at a 1:4 dilution, accomplished by pooling plasma samples. This low level of neutralizing anti-Ad antibodies did not prevent a second vector administration postnatally. However, a third vector administration was not successful, indicating that immunological tolerance was not achieved.

Our previous report showed that the maternal liver is transduced with Ad vector at a low level by fetal intramuscular administration.[11] Here we show that the level of maternal gene transfer is higher with a larger

injectate volume. We found that the level of βgal expression in the maternal liver was quite variable from mouse to mouse. This likely reflects the degree to which the intramuscular injection introduces viral vector into the fetal circulation and then into the maternal circulation. Approximately 4–8 pups per mother are injected, which enhances the possibility of maternal transduction with viral vector. Furthermore, we observed both anti-Ad and anti-βgal antibodies in maternal serum. Similar to that observed in the transduced pups, anti-Ad antibodies found in maternal serum were not neutralizing. These results showing maternal gene transfer and antibody production demonstrate the need for further preclinical studies to address carefully the immune and other effects of *in utero* gene transfer for the mother. Potential routes for viral vector to reach the maternal liver in these experiments include vector that gains access to the maternal peritoneum by backflow from fetal limb injection and from the fetal circulation through the placenta to the maternal circulation.

In this study, we compared the toxicity caused by *in utero* intramuscular injection of FG-Ad and HC-Ad vectors by studying survival after gene transfer as compared to a PBS injection. Note that a mouse transgenic for Ad serotype 5 genome with E1 and E3 deletions demonstrated embryonic lethality with incomplete penetrance, suggesting that Ad genes are toxic to the developing mouse.[15] In our study, Ad vector doses above 10^7 BFU/embryo resulted in toxicity with the FG-Ad vector but not with the HC-Ad vector in the C57BL/6 strain. In contrast, the FG-Ad vector did not induce toxicity in CD-1 mice. In accordance with our data, Yang *et al*[9] reported an optimal survival rate of 83% before pup delivery that dropped to 55% at 4 weeks of age using a dose of 10^9 particles of FG-Ad vector per BALB/c embryo. Therefore, the toxicity induced by the FG-Ad vector is dependent on both vector dose and mouse strain. Our data confirm that the HC-Ad vector is less toxic for *in utero* gene delivery than the FG-Ad vector.

Finally, we explored the possibility of using an HC-Ad vector to deliver the 14 kb dystrophin cDNA driven by the muscle-specific MCK promoter to fetal *mdx* muscle. In contrast to adults and neonates, where the injected virus remains localized to the injection site in the muscle,[16] we could deliver dystrophin to two muscles of the hind limb after a single *in utero* injection. *In utero* dystrophin delivery using the HC-Ad vector resulted in restoration of the DGC to the muscle membrane.

In summary, our data demonstrate that the HC-Ad vector is less toxic than the FG-Ad vector for *in utero* gene transfer. Although gene expression from the HC-Ad vector was stable and vector genomes were not eliminated from muscle tissue transduced *in utero*, the tremendous growth of muscle coupled with the episomal nature of Ad vectors resulted in a decreasing concentration of βgal in muscle tissue. However, dystrophin is a more stable protein than βgal, and we show here that dystrophin gene delivery *in utero* restores proper expression of the dystrophin binding partners at the muscle membrane. It has been shown previously that dystrophin-expressing muscle fibers have a biochemical advantage for survival over dystrophin-deficient muscle fibers.[17] Therefore, future studies are warranted to explore the long-term therapeutic effects of dystrophin gene delivery *in utero* using HC-Ad vectors.

Materials and methods

Adenoviral vectors

The construction of the recombinant vectors used in these studies has been described in detail previously. The HC-Ad vector contains the short noncoding inverted terminal repeats (ITRs) of the viral genome and packaging signal, but no viral coding sequences.[18] The FG-Ad vector is a first-generation Ad vector with deletions of the E1 and E3 viral genes.[19] Both recombinant Ad vectors encode the *Escherichia coli lacZ* gene driven by the human CMV promoter. Each virus was titered by counting BFU after infection of 293 cells to limiting dilution as previously described.[11] The vector particle numbers were also quantified by real-time PCR to determine vector genome copies according to the method previously described.[11] The BFU/vector genome copies ratio was 1:11 for the FG-Ad vector and 1:37 for the HC-Ad vector.

AdmDys is an HC-Ad vector with a vector genome comprised of the Ad left ITR, HPRT stuffer DNA, MCK promoter, murine dystrophin cDNA/pA and Ad right ITR. The vector titer and the level of helper virus, AdLC8cluc,[20] were determined by real-time PCR. Helper virus level was less than 1% of the vector preparation.[13] The structure of the HC-Ad vectors was confirmed by restriction digest of vector DNA isolated from purified virions. There was no detectable recombination.

Adenoviral vector delivery to E-16 mice

Timed-pregnant (16 days gestation) C57BL/6 and CD-1 mice were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA). Fetal injections were performed under a dissection microscope using sterile techniques as described in detail previously.[11,21] The Ad vector was diluted in lactated Ringer's buffer. Intramuscular (2 or 5 μl of solution/fetus) injections were performed using a 33G needle (Hamilton). After surgery, the cages were placed on a warming mat for 24 h to allow complete recovery of the animals.

β-Galactosidase expression studies by ONPG and X-gal staining

For prenatal gene transfer analysis, the pregnant mice were killed by CO_2 inhalation 48 h after gene delivery and the E-18 pups were removed from the uteruses. To visualize βgal expression, embryos and mothers' livers were snap frozen and stored at −80°C. Cryostat sections were fixed in 0.5% glutaraldehyde in PBS. After three PBS washes, sections were incubated with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/ml X-gal and 1 mM $MgCl_2$) overnight at 37°C and counterstained with Harris' hematoxylin. Sections of uninjected muscle stained with X-gal showed no blue stain. To quantify βgal expression, hind limb muscles were collected from injected pups. Protein extracts from muscle were prepared by adding 75 μl of TEES (25 mM Tris-HCl pH 8.0, 2.5 mM EDTA pH 8.0, 2.5 mM EGTA pH 7.4, 5% SDS) to each sample. βgal activity and protein concentration were measured as described previously.[11,22] Concurrently measured background βgal activity levels in uninjected muscle extracts are subtracted from all sample values.

For postnatal gene transfer studies, pups were naturally delivered and nursed by their own mothers. At 1 and 5 months after delivery, mice were killed by CO_2 inhalation and hind limb muscles were collected individually for βgal expression studies. The visualization and quantification of βgal expression were performed as described above. Protein concentration was determined as described above.

To calculate the concentration of βgal per leg, we divided the total units of βgal by the total amount of muscle protein for each injected leg. To compare βgal production between 2-day-, 1- month and 5-month-old mice, we multiplied the mean concentration of βgal by the average amount of total muscle protein per leg for each vector at each time point.

Viral DNA quantification by real-time PCR

The diffusion of Ad vector through the leg muscles after *in utero* intramuscular injection is heterogeneous resulting in an unequal infection of each muscle. Therefore, viral DNA was only quantified in muscles that demonstrated βgal protein expression after confirming by real-time PCR of several muscles that muscles that do not express βgal did not contain detectable viral DNA.

Following protein extraction from Ad vector-injected muscles, total DNA was isolated from the remaining pellet using the TRIzol reagent (Life Technologies, Gaithersburg, MD, USA). Each sample of muscle DNA was amplified for *lac*Z and endogenous mouse apolipoprotein B (Apo-B) (a single-copy gene used as an internal control) genes. Conditions for real-time PCR were described previously.[11,21] The total number of viral copies in each injected leg was calculated by multiplying the sum of normalized number of viral copies per leg by the total number of nuclei per ng of protein at each age. We calculated by real-time PCR that the averages of nuclei per ng of protein in 1- and 5-month-old mice are 46.4 and 189.6, respectively.

Toxicity due to in utero Ad vector injection

Experimental mouse viability was determined as the percent of newborn mice surviving 10 days after delivery out of the total number of injected pups. To compare the expected results to observed results, we used the Microsoft Excel CHITEST function. The expected results for each Ad vector were calculated using the percent survival observed by PBS injection alone.[23] We considered a CHITEST value of <0.05 to be statistically significant.

To test for wild-type adenovirus contamination of HC-Ad or FG-Ad vector preparations, we infected A549 cells with 10^5 particles/cell.[24] After a 2-week incubation, all cells on the plate were positive for βgal when examined by X-gal staining but there was no evidence of cytopathic effect. To test for endotoxin in HC-Ad or FG-Ad vector preparations, PYROTELL (CAPE COD, Falmouth, MA, USA) was used following the directions from the manufacturer. The endotoxin levels in both viruses were below 0.03 endotoxin units (EU)/ml.

Antibody response

Detection of neutralizing antibodies to Ad vectors and total antibodies against Ad capsids and βgal was carried out on serum collected from 1- and 5-month-old experimental mice and mothers as described previously.[1,25] All serum samples were diluted 1:100 to detect total antibodies against Ad and βgal, after confirming that this dilution yielded results in the linear range of detection. For the neutralizing antibody assay, serum samples were diluted 1:2 prior to incubation with 6×10^4 infectious units of FG-Ad vector carrying the *lac*Z gene. Each serum and Ad mixture was incubated for 1 h at 37°C and added to confluent A549 cells for infection at 37°C, 5% CO_2 for 1 h. Following infection, cells were washed and complete media were added and the cells were allowed to incubate at 37°C, 5% CO_2 for 24 h. Cells were stained with X-gal as previously described and positive cells were counted. The neutralizing antibody titer was determined by the reciprocal of the serum dilution whereby a 50% reduction in positive cells was observed.

Detection of dystrophin, α-sarcoglycan and β-sarcoglycan expression

Timed-pregnant (16 days gestation) C57BL/10ScSnDmdmdx/J (*mdx*) mice (The Jackson Laboratory, Bar Harbor, ME, USA) were transduced with 5.2×10^7 viral genome copies/embryo by intramuscular injection. At 9 weeks after delivery, mice were killed and muscle was collected. Immunostaining for dystrophin α-sarcoglycan and β-sarcoglycan was carried out as described previously with modifications.[26] Muscle cryosections of 10 μm were blocked with the blocking solution of the MOM kit and the biotin blocking solution (Vector Laboratories, Burlingame, CA, USA), as suggested by the manufacturer. Sections were incubated with a mouse anti-dystrophin antibody (NCL-DYS2, Vector Laboratories) at a dilution of 1:20, a mouse anti-α-sarcoglycan antibody (NCL-L-a-SARC) at a dilution of 1:250 or a mouse anti-β-sarcoglycan antibody (NCL-L-b-SARC) at a dilution of 1:250 followed by a biotinylated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at a dilution of 1:250, and Streptavidin-Alexa (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at a dilution of 1:1000. All antibodies were diluted in antibody diluent from DAKO (Carpinteria, CA, USA). Positive and negative controls for dystrophin, α-sarcoglycan and β-sarcoglycan were muscles from C57BL/6 and *mdx* mice, respectively.

Statistical analysis

Nonparametric Kruskal–Wallis and Dunn's multiple comparison tests were conducted for statistical studies to compare the expression from different vector doses (Figure 1b) and to compare between different time points (Figure 2). All other statistical analyses were performed with the nonparametric Mann–Whitney test. All *P*-values were two-tailed and were considered statistically different (*) or very different (**) when the associated probability was less than 0.05 or 0.001, respectively.

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References

1 Gilchrist SC, Ontell MP, Kochanek S, Clemens PR. Immune response to full-length dystrophin delivered to Dmd muscle by a high-capacity adenoviral vector. *Mol Ther* 2002; **6**: 359–368.
2 Mitchell M *et al*. Long-term gene transfer to mouse fetuses with recombinant adenovirus and adeno-associated virus (AAV) vectors. *Gene Therapy* 2000; **7**: 1986–1992.
3 Turkay A, Saunders T, Kurachi K. Intrauterine gene transfer: gestational stage-specific gene delivery in mice. *Gene Therapy* 1999; **6**: 1685–1694.
4 Jerebtsova M, Batshaw ML, Ye X. Humoral immune response to recombinant adenovirus and adeno-associated virus after *in utero* administration of viral vectors in mice. *Pediatr Res* 2002; **52**: 95–104.
5 Lipshutz GS, Flebbe-Rehwaldt L, Gaensler KM. Reexpression following readministration of an adenoviral vector in adult mice after initial *in utero* adenoviral administration. *Mol Ther* 2000; **2**: 374–380.
6 Lipshutz GS *et al*. *In utero* delivery of adeno-associated viral vectors: intraperitoneal gene transfer produces long-term expression. *Mol Ther* 2001; **3**: 284–292.
7 Schachtner S, Buck C, Bergelson J, Baldwin H. Temporally regulated expression patterns following *in utero* adenovirus-mediated gene transfer. *Gene Therapy* 1999; **6**: 1249–1257.
8 Schneider H *et al*. Sustained delivery of therapeutic concentrations of human clotting factor IX – a comparison of adenoviral and AAV vectors administered *in utero*. *J Gene Med* 2002; **4**: 46–53.
9 Yang EY *et al*. Persistent postnatal transgene expression in both muscle and liver after fetal injection of recombinant adenovirus. *J Pediatr Surg* 1999; **34**: 766–772.
10 Waddington SN *et al*. *In utero* gene transfer of human factor IX to fetal mice can induce postnatal tolerance of the exogenous clotting factor. *Blood* 2003; **101**: 1359–1366.
11 Bilbao R *et al*. Fetal muscle gene transfer is not enhanced by an RGD capsid modification to high-capacity adenoviral vectors. *Gene Therapy* 2003; **10**: 1821–1829.
12 Kochanek S. High-capacity adenoviral vectors for gene transfer and somatic gene therapy. *Hum Gene Ther* 1999; **10**: 2451–2459.
13 Jiang Z *et al*. CTLA4Ig delivered by high-capacity adenoviral vector induces stable expression of dystrophin in *mdx* mouse muscle. *Gene Therapy* 2004; **11**: 1453–1461.
14 Chen HH *et al*. DNA from both high-capacity and first-generation adenoviral vectors remains intact in skeletal muscle. *Hum Gene Ther* 1999; **10**: 365–373.
15 Camargo FD *et al*. Germline incorporation of a replication-defective adenoviral vector in mice does not alter immune responses to adenoviral vectors. *Mol Ther* 2000; **2**: 496–504.
16 O'Hara AJ *et al*. The spread of transgene expression at the site of gene construct injection. *Muscle Nerve* 2001; **24**: 488–495.
17 Pegoraro E *et al*. Genetic and biochemical normalization in female carriers of Duchenne muscular dystrophy: evidence for failure of dystrophin production in dystrophin-competent myonuclei. *Neurology* 1995; **45**: 677–690.
18 Biermann V *et al*. Targeting of high-capacity adenoviral vectors. *Hum Gene Ther* 2001; **12**: 1757–1769.
19 Wills KN *et al*. Development and characterization of recombinant adenoviruses encoding human p53 for gene therapy of cancer. *Hum Gene Ther* 1994; **5**: 1079–1088.
20 Parks RJ *et al*. A helper-dependent adenoviurs vector system-removal of helper virus by cre-mediated excision of the viral packaging signal. *Proc Natl Acad Sci USA* 1996; **93**: 13565–13570.
21 Bilbao R *et al*. Binding of adenoviral fiber knob to the coxsackievirus-adenovirus receptor is crucial for transduction of fetal muscle. *Hum Gene Ther* 2003; **14**: 645–649.
22 Bouri K *et al*. Polylysine modification of adenoviral fiber protein enhances muscle cell transduction. *Hum Gene Ther* 1999; **10**: 1633–1640.
23 MacKenzie TC *et al*. Efficient transduction of liver and muscle after *in utero* injection of lentiviral vectors with different pseudotypes. *Mol Ther* 2002; **6**: 349–358.
24 Anderson RD *et al*. A simple method for the rapid generation of recombinant adenovirus vectors. *Gene Therapy* 2000; **7**: 1034–1038.
25 Jiang ZL *et al*. Local high-capacity adenovirus-mediated mCTLA4Ig and mCD40Ig expression prolongs recombinant gene expression in skeletal muscle. *Mol Ther* 2001; **3**: 892–900.
26 Lu QL, Partridge TA. A new blocking method for application of murine monoclonal antibody to mouse tissue sections. *J Histochem Cytochem* 1998; **46**: 977–984.