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Integrating vector and stem cell-based strategies for gene therapy of Duchenne muscular dystrophy

Review Article

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Summary

We review novel gene transfer strategies proposed to be suitable for the treatment Duchenne Muscular Dystrophy (DMD): use of hybrid adeno-retroviral vectors to stably replace dystrophin ultimately in patients lacking this gene and the potential intravenous application of stem cells and monocytes for targeted gene transfer. We discuss the limitations of current vector technology and demonstrate the need for continual evolution of vector design that is required before gene therapy of complex monogenic diseases such as DMD becomes a reality.

I. Introduction

DMD is a debilitating X-linked muscle wasting disease affecting 1 in 3500 newborn boys, about one-third of these cases arise from spontaneous mutations (Emery, 1993). The dystrophin gene is one of the largest known spanning some 2.5 Mb (hence the high rate of spontaneous mutations) and comprises 79 exons spliced into a 14 kb cDNA. The encoded protein has a molecular mass of 427 kDa and is located in the sarcolemmal membrane (**Figure 1**). The primary functions of dystrophin are the maintenance of myofibre integrity and the mediation of intracellular signal transduction (Brenment et al, 1995; Grady et al, 1999; Bredt, 1999). The N-terminus of dystrophin binds actin, anchoring the protein to the intracellular matrix, whereas the C-terminus binds to the Dystrophin Associated Glycoprotein (DAG) complex, thought to be involved in signalling. The DAG complex in turn spans the sarcolemmal membrane binding the basal lamina and forms the link to the extracellular matrix. The intervening region is made up of actin-like rod domains and hinge regions that serve as a buffer during muscle contraction. In the absence of dystrophin muscle fibres are disorganised, degenerate and after several cycles of regeneration are replaced by fatty tissue resulting in loss of contractile strength. There are a number mouse models available for this disease, but the most commonly used is the naturally occurring *mdx* mouse, which contains a point mutation on exon 23 encoding a stop codon resulting in the termination of gene expression (Rydercook et al, 1988). It is the scope of this review to discuss some of the evolving genetic therapeutic techniques that may be applicable to the treatment of this otherwise incurable disease.

II. Current viral-based gene therapy of DMD

Due to its large size it is difficult to construct viral vectors expressing the full-length dystrophin gene. However, there are truncated forms available, lacking some of the rod and hinge regions (England et al, 1990; Yuasa et al, 1998, Wang and Xiao, 2000). The most

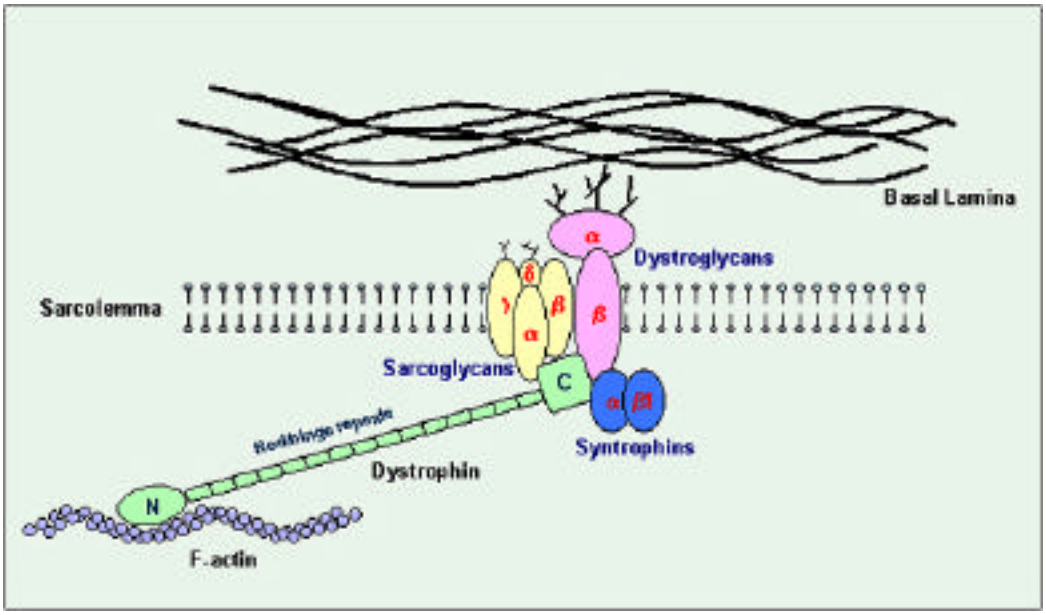

Figure 1. Schematic diagram of the dystrophin associated glycoprotein complex. Dystrophin confers structural integrity upon muscle fibres by linking the surrounding extracellular matrix to the cytoskeleton via the dystrophin associated glycoprotein complex. The long intervening region comprising of rod and hinge repeat regions absorbs stress induced during muscle contraction

widely used is mini-dystrophin (6.3 kb) isolated from a patient suffering from Becker Muscular Dystrophy (a mild form of DMD) (England et al, 1990). Overexpression of this mini-gene has been shown to restore expression of the DAG complex at the sarcolemma (Vincent et al, 1993; Rafael et al, 1994), reduce the extent muscle degeneration (Vincent et al, 1993; Decrouy et al, 1997), maintain myofibre integrity (Decrouy et al, 1997) and increase the force generating capacity of dystrophic muscle (Yang et al, 1998). In recent years shorter micro-dystrophin constructs ranging from 3.7-4.5kb have been developed and shown to provide a few of these therapeutic benefits (Yuasa et al, 1998; Wang and Xiao, 2000). A number of the current viral vectors incorporating full-length and truncated dystrophin genes in their design with potential application for the treatment of DMD are listed below.

(a) Adenovirus. Recombinant viral vectors based on the adenovirus are the most extensively used vectors for gene transfer in skeletal muscle (Quantin et al, 1992; Alameddine et al, 1994; Ascadi et al, 1996; Clemens et al, 1996; Petrof et al, 1996; Floyd et al, 1998; Yuasa et al, 1998; van Deutekom et al, 1999). However, first generation adenoviral-mediated mini-dystrophin expression in the muscle is short-term in nature, only lasting up to two months post-injection (Ascadi et al, 1996), although this can be extended by treatment with FK506 (Lochmuller et al, 1996). It is generally accepted that adenoviral-mediated expression elicits a powerful cytotoxic T-cell response resulting in the clearance of transduced fibres and a reduction in the force generating capacity of the muscle (Lochmuller et al, 1996, Petrof et al, 1996). Moreover, first and second generation vectors are limited in that they are only able to accommodate the 6.3kb mini-dystrophin construct with a simple promoter/enhancer element. However, their ease of use, ability to replicate to high titres in complementing cell lines and high infectivity of a number of cell types has led to the further development of this vector. Newer generations of adenoviral vectors have all viral genes deleted and comprise only the *cis* elements (ITRs) required for replication with the packaging signal and a stuffer fragment of DNA (Ascadi et al, 1996; Clemens et al, 1996; Chen et al, 1997; Balkir et al, 1998). These vectors have the capacity to express the full-length dystrophin gene driven by complex muscle specific promoters and a second reporter gene (Chen et al, 1997). Development of third generation vectors has led to a marked improvement in the duration of transgene expression *in vivo* due to their ability to evade the immune response (Chen et al, 1997). Although these recent developments in adenoviral vectorology have been promising this vector is not well suited to DMD because its genome is maintained episomally. As the vector is non-integrating it is likely the therapeutic gene would be lost during pathological turnover of muscle cells in dystrophic tissue, notwithstanding the stability of mature muscle fibres expressing dystrophin (Vincent et al, 1993).

(b) Adeno-Associated Virus. Vectors based on this virus hold great promise for gene therapy of a number of diseases where the defective gene is relatively small. In its wild-type form the virus is non-pathogenic and integrates at a specific site within chromosome 19 (Linden et al, 1996). However, *rep*-deleted recombinant AAV vectors do not integrate into this specific region (Ponnazhagan et al,

1997). In order to solve this problem hybrid AAV/Adenoviral (Lieber et al, 1999; Recchia et al, 1999; Ueno et al, 2000) and AAV/Herpes virus (Fraefel et al, 1997; Johnston et al, 1997; Costantini et al, 1999) vectors have been constructed to provide the *rep* gene *in trans*. Interestingly, the long-term gene expression obtainable from AAV is not associated with a deleterious immune response making them ideal vectors to express small heterologous secreted proteins, e.g. ApoE3 and clotting Factor IX, using skeletal muscle as an expression platform (Athanasopoulos et al, 2000). Although AAV vectors are efficient at infecting both mature and immature muscle (Pruchnic et al, 2000), their small size is a major drawback for the treatment of DMD. The maximum insert capacity is only 5.0 kb thus restricting the type of gene that can be inserted to synthetic micro-dystrophin constructs whose application to the treatment of DMD is expected to be limited (Yuasa et al, 1998).

(c) Retrovirus. During the life cycle of the retrovirus its genome integrates into the infected cell subsequent to cell division. As such retroviral vectors hold great promise for the treatment of DMD due to the capacity of degenerated muscle tissue to regenerate, a process mediated by muscle satellite stem cells (Fassati et al, 1995). However, injection of neat retroviral suspensions into dystrophic muscle is not an efficient means of delivery because the administration of low viral titres achievable during the vector preparation stage is insufficient to transduce the relatively low proportion of dividing myoblasts in dystrophic tissue (Fassati et al, 1995). This can be overcome by implanting retroviral producer cells into the target muscle resulting in efficient stable transduction of fibres provided muscle degeneration is induced (Fassati et al, 1996), and has even been shown to be an efficient means of introducing mini-dystrophin into dystrophic tissue for long-term expression (Dunckley et al, 1993; Fassati et al, 1997). However, there are major safety limitations in implanting such a cell line into patients considering the severe inflammatory response and formation of palpable tumours derived from producer cells observed in *mdx* mice treated in this manner (Fassati et al, 1996).

(d) Lentivirus. Lentiviral vectors have two distinct advantages over retroviral vectors; firstly, they are able to stably transduce non-dividing cells and secondly, they can be produced to much higher titres enabling them to be efficiently applied *in vivo* (Sakoda et al, 1999). A number of researchers have shown that lentiviral vector can mediate expression of transgene in skeletal, cardiac and smooth muscle cells *in vivo* for up to one year with minimal cytotoxicity (Kafri et al, 1997; Sakoda et al, 1999; Seppen et al, 2001). Despite these studies the application of lentiviral vectors to muscle-related disease has not occurred at the rate one would anticipate. This is likely a consequence of concerns over safety as most current vectors are based on Human Immunodeficiency Virus. This scenario is likely to change as lentiviral vector technology evolves into systems based on forms of the virus that are restricted to productive life cycles in other species. Indeed vectors based on Feline Immunodeficiency Virus have now been shown to efficiently transduce a number of human cell types (Johnson et al, 1999; Curren et al, 2000) and even hamster skeletal muscle (Johnson et al, 1999). If these studies can be extended into human skeletal muscle then there may be a significant future for lentiviral-mediated gene therapy of DMD.

(e) Herpesvirus. Vectors based on the herpes simplex virus type 1 (HSV-1) represent a potentially useful system for the treatment of DMD. The virus is able to accommodate up to 30 kb of heterologous DNA, making it an ideal vector to express full-length dystrophin. Indeed, replication defective HSV-1 vectors with single and triple mutations in the immediate early genes have been shown to efficiently deliver both mini- and full length dystrophin to muscle fibres *in vivo* (Akkaraju et al, 1999), although the long-term efficacy of HSV-1-mediated gene transfer is yet to be examined. Given the well-established link to cytotoxicity further disabled HSV-1 vectors may have to be developed in order to obtain prolonged expression.

Each of the vectors listed above have features that are advantageous to gene therapy but no one comprises all the elements required for an ideal gene transfer vector e.g. large insert capacity, capability to evade the immune response, ability to integrate safely into the host genome and with minimum toxicity to target cells. In order to address this issue a number of researchers have developed hybrid viral vectors. These include gene delivery systems based on Adenovirus/Retrovirus (Feng et al, 1997; Lin, 1998; Ramsey et al, 1998; Caplen et al, 1999; Duisit et al, 1999; Roberts et al, 2001), Adeno-Associated Virus/Herpes Simplex Virus (Fraefel et al, 1997; Johnston et al, 1997; Costantini et al, 1999), Adenovirus/Adeno-Associated Virus (Recchia et al, 1999; Lieber et al, 1999; Ueno et al, 2000), Semliki Forest Virus/Retrovirus (Li and Garoff, 2001), Epstein-Barr virus/Retrovirus (Tan et al, 1999), Herpes Simplex Virus/Retrovirus (Parrish et al, 1999), and Poxvirus/Retrovirus (Holzer et al, 1999). The hybrid adeno-retroviral vector system may be particularly applicable to the treatment of DMD. Both dividing and non-dividing muscle cells are efficiently infected with adenoviral vectors and the retrovirus confers an integrative capacity to transduced cells (Reynolds et al, 1999). Production of functional retroviral vector using this hybrid system is a two-step process; target cells are infected with adenoviruses expressing retrovirus structural genes and proviral sequences. Infected cells release functional retroviral vector, which then tranduces neighbouring cells, resulting in the stable integration of the therapeutic gene (**Figure 2**). Using adenovirus templates to produce retroviral vector in this manner offers an opportunity to produce retroviral vector *in situ*, thus reducing complement-mediated lysis and increasing the efficiency

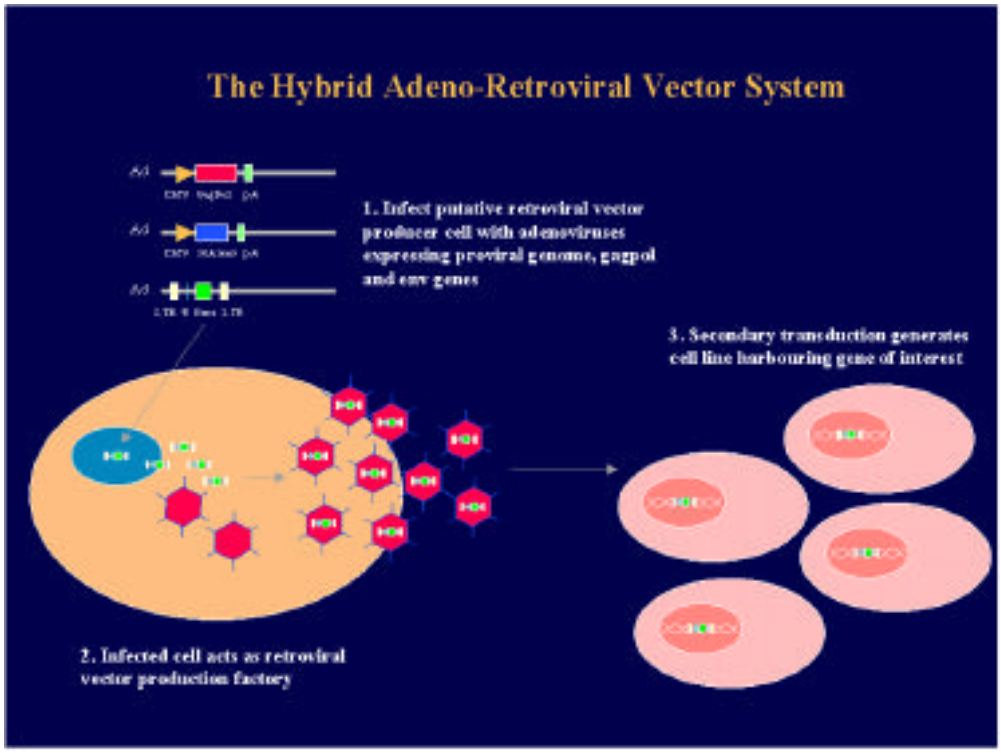

Figure 2. Hybrid adeno-retroviral vector system. Putative producer cells are infected with adenoviral vectors expressing the retroviral genome (AdLXIN), Gag-Pol (Adgagpol) and amphitrophic envelope protein (Ad10A1env). The infected cells assemble retroviral vector, which is released into the surrounding environment and transduces neighbouring cells. The proviral genome integrates during cell division creating a stable cell line expressing therapeutic transgene.

of retroviral vector transduction at the target site. Vectors constructed using this approach have already been used in two rodent models of cancer and shown to efficiently transduce rapidly dividing cancer cells (Feng et al, 1997; Caplen et al, 1998). One aspect of DMD pathology makes it an ideal target for gene therapy by *in situ* delivery of retroviral vector. Muscle fibres not expressing dystrophin degenerate and are subsequently replaced by proliferating myoblast stem cells during regeneration. If existing muscle fibres were allowed to act as a platform for retroviral production then regenerating myoblasts could be transduced by newly produced retroviral vector in the surrounding milieu.

III. Novel strategies for the treatment of DMDA. Muscle as a platform for retroviral vector production

Proliferating myoblasts and mature differentiated myotubes have been shown to act as efficient retroviral producer cells *in vitro* when using hybrid adeno-retroviral vectors (**Figure 3**; Roberts et al, 2001a). The most efficient hybrid adeno-retroviral vector system comprises three adenoviral vectors expressing retroviral components; Adgag-pol, Ad10A1env and AdLXIN (adenoviral vector encoding retroviral genome with gene of interest). The triple vector system has been shown to be a more efficient means of producing retroviral vector when compared to the two vector system, where *gag*, *pol* and *env* are expressed from one adenoviral vector (Lin, 1998, Roberts et al, 2001a). Myoblasts are able to generate retroviral vector titres of $5x10^4$ cfu/ml after 48 hours, which drops significantly after a couple of days. Interestingly, post-mitotic myotubes generate higher titres of retroviral vector (up to $3x10^5$ cfu/ml) and production from these mature muscle cells does not drop over time (Roberts et al, 2001a). This would suggest that post-mitotic cells are more efficient at sustained production of retroviral vectors compared to dividing cells. This postulation was confirmed when cell division was inhibited in immature cultures of myotubes proposed to contain a higher proportion of myoblasts as an increase in retroviral vector production was observed (Roberts et al, 2001a). It is likely that proliferating myoblasts sequester retroviral vector subsequent to its production resulting in the generation of lower levels of vector for harvesting. Moreover, as the adenovirus is episomal, during proliferation of the producer cell all the components required for retroviral vector production are lost, thus over

Adenoviral Vector Only **Hybrid Adeno-Retroviral**

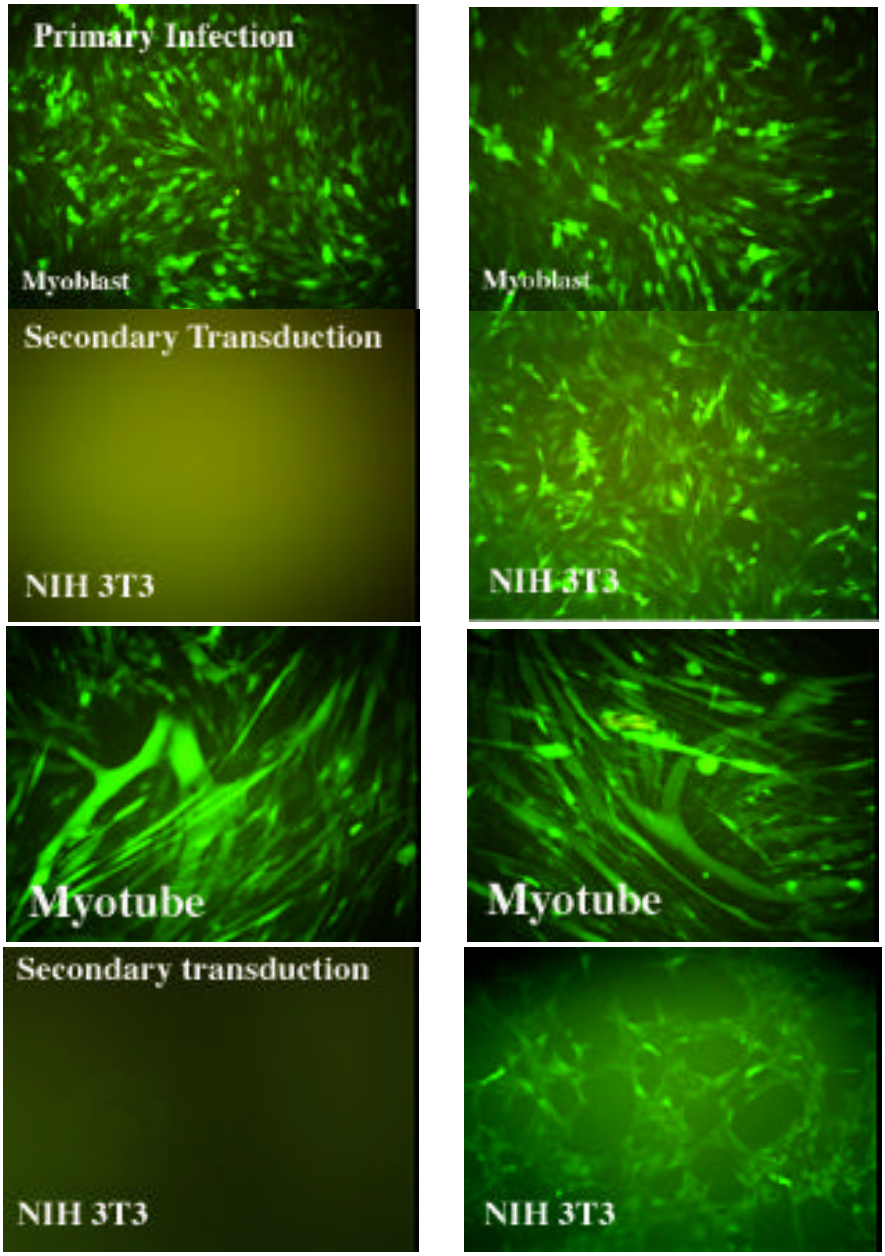

Figure 3. Cultures of myoblasts and myotubes produce retroviral vector. Proliferating immature myoblasts and post-mitotic differentiated myoblasts were infected with adenoviral vector alone or with the hybrid adeno-retroviral system expressing GFP. After two days medium was harvested from infected myocytes and used to transduce semi-confluent cultures of NIH 3T3 cells. One day subsequent to transduction geneticin was added and the transduced 3T3 cells were cultured for several weeks. Only NIH 3T3 cells treated with medium isolated from hybrid adeno-retroviral infected myoblasts and myotubes survived in the presence of geneticin, thus indicating the successful production of retroviral vector.

time fewer myoblasts will contain the elements required for retroviral vector production. These observations suggested that mature skeletal muscle might serve as an efficient long-term production platform for retroviral vector production given that the majority of cells in the muscle are post-mitotic myofibres.

The ability of muscle cells *in vitro* to produce relatively high titres of retroviral vector holds great promise for the gene therapy of DMD. Particularly since these results have recently been reproduced *in vivo*. In a recent study Tibialis Anterior (TA) muscle from *mdx* mice was injected with the adeno-retroviral vector system expressing the LacZ gene (Roberts et al, submitted). Retroviral vector production was allowed to occur for one week and primary muscle cultures were prepared from the infected tissue. After the primary myoblast cultures had

fused to form myotubes *LacZ* expression was analysed revealing that colonies of transduced myotubes only formed in cultures isolated from TA muscles infected with all the components required to make a retroviral vector (Roberts et al, submitted). Moreover, there was a ten-fold increase in the overall number of cells expressing β-galactosidase. We then examined the effect of retroviral production on the overall number of myofibres expressing *LacZ* in a TA muscle. After one week in normal *mdx* mice,retroviral vector production led to a two-fold increase in the number of myofibres expressing *LacZ* (Roberts et al, submitted). However, after four weeks the number of fibres expressing *LacZ* had fallen significantly suggesting an immune response to vector and transgene sequences had resulted in the destruction of transduced fibres (Roberts et al, submitted). Similar analysis in immunodeficient nude *mdx* mice revealed that by allowing the TA to act as a factory of retroviral vector production for four weeks there was a five-fold increase in the total number of *LacZ*-expressing myofibres (Roberts et al, submitted). These data suggest that provided muscle regeneration is induced, the hybrid adeno-retroviral vector system may be a good way to stably transduce skeletal muscle provided the issues of adenoviral-mediated immunogenicity and retroviral promoter shutdown are addressed.The hybrid adeno-retroviral vector system has also been used to slow the progression of muscular dystrophy in *mdx* mice. Neonates treated with hybrid adeno-retroviral vectors comprising a 3.7kb micro-dystrophin construct expressed the therapeutic transgene throughout most of the treated muscle (**Figure 4**). This efficient micro-dystrophin expression resulted in the restoration of components of the DAG complex (β-dystroglycan, α-sarcoglycan and β-sarcoglycan) that would otherwise be absent in dystrophic tissue (Roberts et al, submitted). Moreover, expression of micro-dystrophin decreased the total number of degenerating myofibres in the TA muscle of *mdx* mice. Taken with the data indicating restoration of the DAG complex, it is likely that expression of micro-dystrophin from hybrid adeno-retroviral vectors partially corrects the dystrophic phenotype. Moreover, the authors developed a novel nested PCR method to monitor retroviral integration (**Figure 5**). By using this procedure a specific product indicative of integration was detected only in animals injected with all the components required to produce retroviral vector, indicating that LTR duplication had occurred and the retroviral provirus had integrated into the muscle cell genome (Roberts et al, submitted).

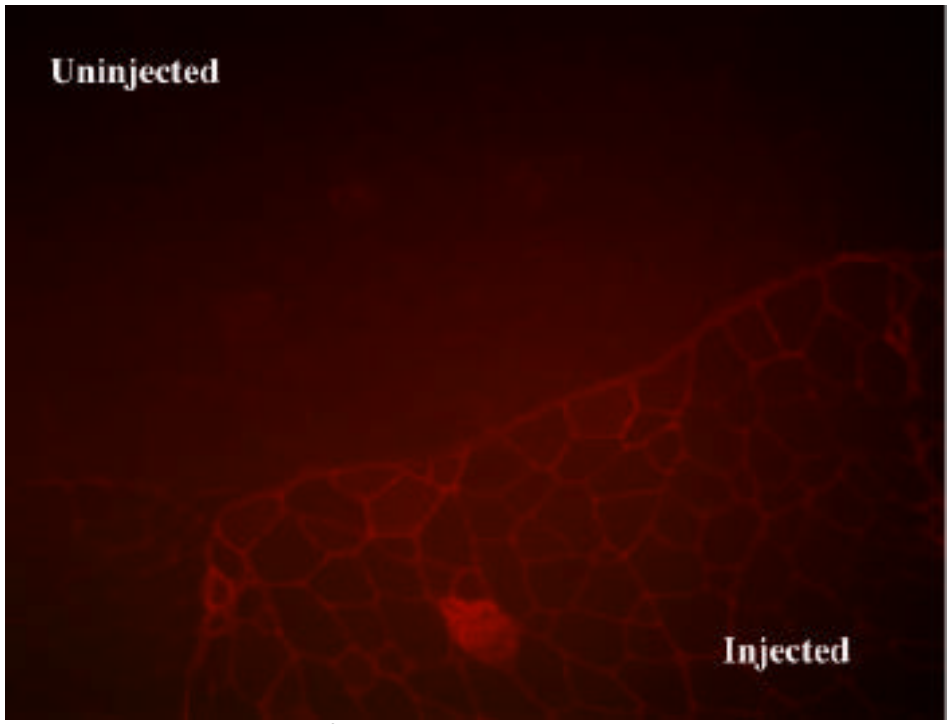

Figure 4. Adeno-retroviral-mediated expression of microdystrophin in muscle. Muscle injected with components required for the production of retroviral vector expressing micro-dystrophin (lower section) or uninjected (upper section) and stained with antibody against the C-terminus of dystrophin. Note the efficient expression of the micro-dystrophin construct localises to the sarcolemmal membrane conferring structural integrity to the muscle.

B. Strategies Based on Cell-Mediated Gene Transfer

As an alternative to viral vector-based strategies it has been proposed that intravenous transplantation of bone marrow stem cells from healthy individuals may serve to act as a stable source of dystrophin-expressing muscle satellite stem cells provided sufficient numbers of cells locate to the muscle (Gussoni et al, 1999). Intravenous transplantation of whole bone marrow, haematopoietic and muscle-derived stem cells from wild-type C57BL/10 mice can reconstitute lethally irradiated *mdx* recipients with all myeloid cell lineages. Moreover, up to 10% of muscle fibres from the TA muscle in recipient mice were found to express dystrophin derived from donors after three months (Gussoni et al, 1999). In a separate study a similar level of dystrophin expression (12%) in *mdx* mice intra-arterially transplanted with muscle-derived cells was only shown to occur subsequent to severe muscle damage in muscle groups near the injected artery (Torrente et al, 2001). Although stem cell-mediated therapy of DMD holds great promise a recent study has demonstrated the extremely low efficiency of this technique in the *mdx4cv* mouse model (Ferarri et al, 2001). The *mdx4cv* model has a stop codon mutation in exon 53 of the dystrophin gene preventing the formation of revertant dystrophin-expressing fibres that arise after exon skipping and allow for the expression of truncated functional forms of dystrophin. Less than one percent of muscle fibres were found to express dystrophin at any time over ten months in *mdx4cv* mice injected with whole bone marrow cells. The cumulative data from these studies would suggest that stem cell mediated recruitment of dystrophin-expressing myoblasts to dystophic muscle might only occur through revertant fibres. If this were indeed proven to be the case then the application of this type of therapy to the treatment of DMD will be extremely limited.

It has also been proposed that circulating monocytes may be able to deliver dystrophin constructs to the site of muscle degeneration provided they can be induced to produce retroviral vector (Parrish et al, 1996). During the degeneration of skeletal muscle large numbers of monocytes and macrophages that act to clear muscle cell debris infiltrate the damaged tissue (**Figure 6**). Using a hybrid HSV-1 amplicon/retroviral vector system Parrish and colleagues were able to convert a monocyte/macrophage cell line into retroviral producing cells releasing retroviral vector capable of transducing dividing myoblasts (Parrish et al, 1999). However, the overall efficiency of this technique was found to be extremely low as less than 0.1 % of myoblasts were transduced by retroviral vector produced from macrophages. This was likely a consequence of the toxicity that the HSV-1 vector conferred on the producer monocytes coupled with the low level of HSV-1-mediated monocyte infection (approximately 1% of monocytes were proposed to be producer cells). Given the high efficiency of adenoviral-mediated human monocyte/macrophage transduction (**Figure 7**), we proposed to use the hybrid adeno-retroviral vector system in a similar monocyte-mediated targeting approach. In preliminary studies we used monocyte/macrophages infected with hybrid adeno-retroviral vectors expressing green fluorescent protein

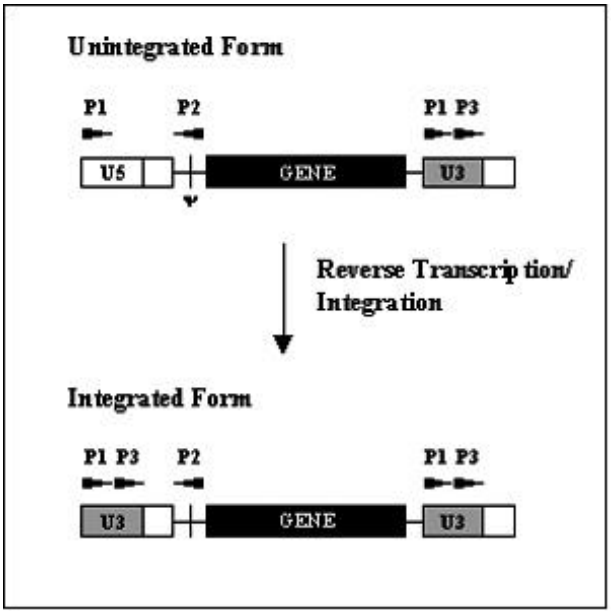

Figure 5. Novel PCR-based technique to detect integrated retroviral sequences in genomic DNA. During retroviral reverse transcription of genomic RNA and subsequent integration into the host genome the 3'-LTR U3 sequence duplicates to form the 5'-LTR U3 sequence of the proviral integrant. Forward primer 1 binds to both the 5' and 3' LTR and is used in conjunction with reverse primer 2, which binds to the retroviral packaging signal, to amplify target sequences. Nested PCR is then employed using identical reverse primer 2 with forward primer 3 that specifically binds the retroviral 3'LTR. Therefore, amplicon only accumulates subsequent to retroviral LTR duplication and is indicative reverse transcription and integration.

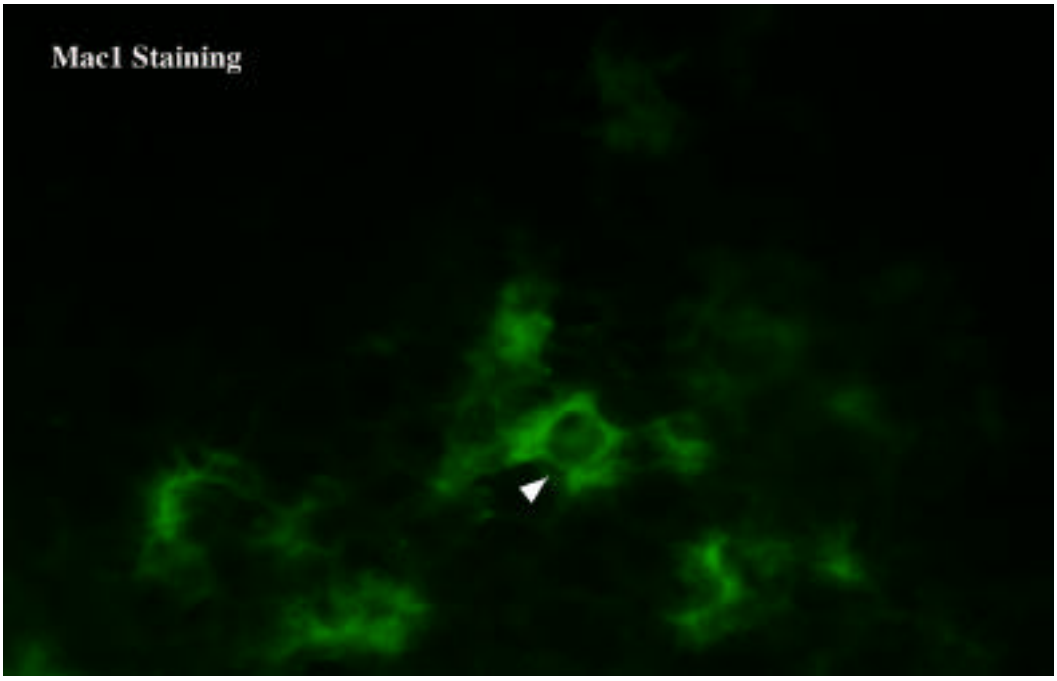

Figure 6. Monocyte localisation to dystrophic tissue. A 10 μm muscle section is stained with antibody recognising the Mac-1 cell surface marker present only on murine monocyte/macrophages. Note the accumulation of signal around a number of myofibres (indicated by arrowhead) that are likely to be degenerating.

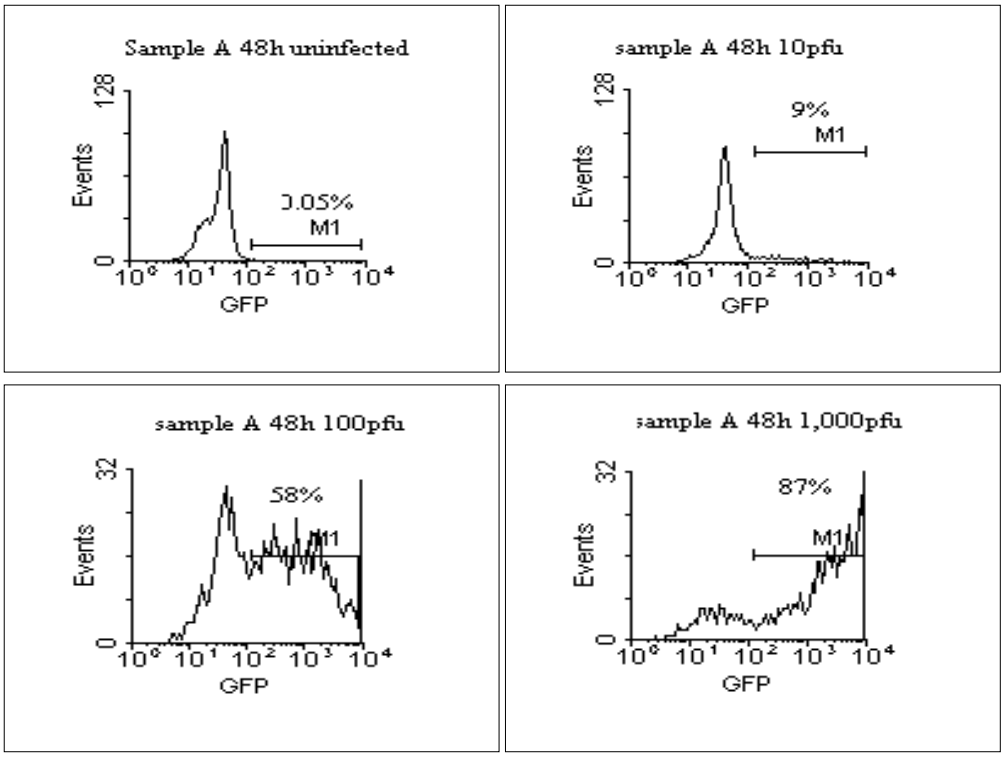

Figure 7. Adenoviral-mediated expression of transgene in human monocytes. FACS analysis of human monocyte infected with adenoviral vector expressing green fluorescent protein (GFP). Dose response indicates some 60 % of cells can be transduced by using a relatively low dose of vector (approximately 100 plaque forming units per monocyte).

Figure 8. Gene transfer to muscle cells using monocytes as delivery vehicles. Mouse monocytes were infected with adenoviral vector alone (**A**) or with adeno-retroviral vector capable of producing retrovirus expressing GFP (**B**). Infected monocytes were co-cultured with primary cultures from *mdx* muscle at the myoblast stage. After two weeks post-mitotic myotubes formed and were analysed for GFP expression by fluorescent microscopy. A four-fold increase in GFP expression amongst myotubes was observed in cultures infected with hybrid adeno-retroviral vector

(GFP) to transduce proliferating cultures of primary myoblasts from the *mdx* mouse (Roberts et al, 2000). We achieved a four-fold increase in GFP expression in myotubes co-cultured with macrophages producing retroviral vector over negative controls (**Figure 8**). However, retroviral vector production was also found to be inefficient in monocyte/macrophages when using the adeno-retroviral vector system, presumably because adenoviral infection of macrophages results in the release of cytokines (Kristofersson et al, 1997; Zhang et al, 2001), which may attenuate expression from the retroviral LTR (Kitamura, 1999). It will be necessary to optimise this method by employing retroviral elements with hybrid CMV/LTR promoters and adenoviral vectors with increased deletions and lower cytotoxicity to improve the efficiency of this approach before examining its feasibility *in vivo*.

IV. Conclusions

Considering that researchers are yet to discover the perfect gene transfer vector a number of groups have started to combine the favourable elements from different viral vectors to construct chimeras. One such hybrid viral system has particular application for the treatment of DMD. Using a hybrid adeno-retrovirus, differentiated myotubes have been shown to efficiently produce retroviral vector. Moreover, skeletal muscle acts as an efficient platform for retroviral vector production resulting in increased transduction of myofibres *in vivo*. These observations have direct applications for the treatment of DMD. Indeed, expression of micro-dystrophin mediated from a hybrid adeno-retroviral vector partially corrects the dystrophic phenotype of *mdx* mice. Furthermore, expression of transgene was shown to be stable as indicated by the detection of retroviral vector sequences in genomic DNA isolated from transduced muscle fibres. Due to the high rate of muscle turnover observed in DMD patients it is essential that an effective therapy should involve the targeted delivery of therapeutic transgene so that it is expressed for life. In order to achieve this goal gene transfer systems based on integrating viral vectors and muscle stem cells must be further developed. Indeed, future studies may reveal that successful gene therapy of DMD will only arise from a marriage between viral and cell-mediated gene transfer techniques.

References

Akkaraju GR, Huard J, Hoffman EP, Goins WF, Pruchnic R, Watkins SC, Cohen JB, and Glorioso JC (**1999**) Herpes simplex virus vector-mediated dystrophin gene transfer and expression in MDX mouse skeletal muscle. **J. Gene Med.** 1, 280-289.

Alameddine HS, Quantin B, Cartaud A, Dehaupas M, Mandel JL, and Fardeau M (**1994**) Expression of a recombinant dystrophin in mdx mice using adenovirus vector. **Neuromusc. Disord.** 4, 193-203.

Ascadi G, Jani A, Massie B, Simoneau M, Holland P, Blaschuk K, and Karpati G (**1994**) Cultured human myoblasts and myotubes show markedly different transducibility by replication-defective adenovirus recombinants. **Gene Ther.** 3, 579-584.

Ascadi G, Lochmuller H, Jani A, Huard J, Massie B, Prescott S, Simoneau M, Petrof BJ, and Karparti G (**1996**) Dystrophin expression in muscles of mdx mice after adenovirus-mediated in vivo gene transfer. **Hum. Gene Ther. 7,** 129-140.

Athanasopoulos T, Fabb SA, Dickson JG (**2000**) Gene therapy vectors based on adeno-associated virus: Characteristics and applications to acquired and inherited diseases (Review). **Int. J. Mol. Med.** 6, 363-375.

Bredt DS (**1999**) Knocking signalling out of the dystrophin complex. ***Nat. Cell Biol.*** 1, E89-E91.

Brenmen JE, Chao DS, Xia H, Aldape K, and Bredt DS (**1995**) Nitrogen-oxide synthase complexed with dystrophin and absent from skeletal-muscle sarcolemma in Duchenne Muscular-Dystrophy. **Cell** 82, 743-752.

Caplen NJ, Higginbotham JN, Scheel JR, Vahanian N, Yoshida Y, Hamada H, Blaese RM, and Ramsey WJ (**1999**) Adeno-retroviral chimeric viruses as in vivo transducing agents. **Gene Ther.** 6, 454-459.

Chen HH, Mack LM, Kelly R, Ontell M, Kochanek S, and Clemens PR (**1997**) Persistence in muscle of an adenoviral vector that lacks all viral genes. **Proc. Nat. Acad. Sci. USA.** 95, 1645-1650.

Clemens PR, Kochanek S, Sunada Y, Chan S, Chen HH, Campbell KP, and Caskey CT (**1996**) In vivo muscle gene transfer of full-length dystrophin with an adenoviral vector that lacks all viral genes. **Gene Ther.** 3, 965-72.

Costantini LC, Jacoby DR, Wang S, Fraefel C, Breakefield XO, and Isacson O (**1999**) Gene transfer to the nigrostriatal system by hybrid herpes simplex virus/adeno-associated virus amplicon vectors. **Hum. Gene Ther**. 10, 2527-2537.

Culver KW, Ram Z, Walbridge S, Ishii H, Oldfield EH, and Blease RM (**1992**) In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. **Science** 256, 1550-1552.

Curran MA, Kaiser SM, Achacoso PL, and Nolan GP (**2000**) Efficient transduction of nondividing cells by optimized feline immunodeficiency virus vectors. **Mol. Ther.** 1, 31-38.

Decrouy A, Renaud JM, Davis HL, Lunde JA, Dickson G, and Jasmin BJ (**1997**) Mini-dystrophin gene transfer in mdx4cv diaphragm muscle fibers increases sarcolemmal stability. **Gene Ther.** 4, 401-408.

Duisit G, Salvetti A, Moullier P, and Cosset FL (**1999**) Functional characterization of adenoviral/retroviral chimeric vectors and their use for efficient screening of retroviral producer cell lines. **Hum. Gene Ther.** 10, 189-200.

Dunckley MG, Wells DJ, Walsh FS, and Dickson G (**1993**) Direct retroviral-mediated transfer of a dystrophin minigene into mdx mouse muscle in vivo. **Hum. Mol. Genet.** 2, 717-723.

Emery AE (**1993**) Duchenne muscular dystrophy - Meryon's disease. **Neuromuscul. Disord.** 3, 263-6.

England SB, Nicholson LVB, Johnson MA, Forrest SM, Love DR, Zubrzyckagaarn EE, Bulman DE, Harris JB, Davies KE (**1990**) Very mild muscular-dystrophy associated with the deletion of 46 % of dystrophin. **Nature** 343, 180-182.

Fassati A, Wells DJ, Serpente PAS, Walsh FS, Brown SC, Strong PN, and Dickson G (**1997**) Genetic correction of dystrophin deficiency and skeletal muscle remodelling in adult mdx mouse via transplantation of retroviral producer cells. **J. Clin. Invest.** 100, 620-628.

Fassati A, Wells DJ, Walsh F, and Dickson G (**1996**) Transplantation of retroviral producer cells for in vivo gene transfer into mouse skeletal muscle. **Hum. Gene Ther.** 7, 595-602.

Fassati A, Wells DJ, Walsh FS, and Dickson G (**1995**) Efficiency of in vivo gene transfer using murine retroviral vectors is strain dependent in mice. **Hum. Gene Ther.** 6, 1177-1183.

Feng M, Jackson WH, Goldman CK, Rancourt C, Wang M, Dusing SK, Siegal G, and Curiel DT (**1997**) Stable in vivo gene transduction via a novel adenoviral/retroviral chimeric vector. **Nature Biotech.** 15, 866-870.

Ferrari G, Stornaiuolo A, and Mavilio F (**2001**) Failure to correct murine muscular dystrophy. **Nature** 411, 1014-1015.

Floyd SS, Clemens PR, Ontell MR, Kochanek S, Day CS, Yang J, Haushka SD, Balkir R, Morgan J, Moreland MS, Feero GW, Epperly M, and Huard J (**1998**) Ex vivo gene transfer using adenovirus-mediated full-length dystrophin delivery to dystrophic muscles. **Gene Ther**. 5, 19-30.

Fraefel C, Jacoby DR, Lage C, Hilderbrand H, Chou JY, Alt FW, Breakefeild XO, and Majzoub JA (**1997**) Gene transfer into hepatocytes mediated by helper virus-free HSV/AAV hybrid vectors. **Mol. Med.** 3, 813-825.

Grady RM, Grange RW, Lau KS, Maimone MM, Michol MC, Stull JT, and Sanes JR (**1999**) Role for alpha-dystrobrevin in the pathogenesis of dystrophin-dependent muscular dystrophies. **Nat. Cell Biol.** 1, 215-220.

Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, Kunkel LM, and Mulligan RC (**1997**) Dystrophin expression in the mdx mouse restored by stem cell transplantation. **Nature** 401, 390-394.

Holzer GW, Mayrhofer JA, Gritschenberger W, Domer F, and Falkner FG (**1999**) Poxviral/retroviral chimeric vectors allow cytoplasmic production of transducing defective retroviral particles. **Virology** 253, 107-114.

Johnston JC, Gasmi M, Lim LE, Elder JH, Yee JK, Jolly DJ, Campbell KP, Davidson BL, and Sauter SL (**1999**) Minimum requirements for efficient transduction of dividing and nondividing cells by feline immunodeficiency virus vectors. **J. Virol.** 73, 4991-5000.

Johnston KM, Jacoby D, Pechan PA, Fraefel C, Borghesani P, Schuback D, Dunn RJ, Smith FI, and Breakefield XO (**1997**) HSV/AAV hybrid amplicon vectors extend transgene expression in human glioma cells. **Hum. Gene Ther.** 8, 359-370.

Kafri T, Blomer U, Peterson DA, Gage FH, and Verma IM (**1997**) Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. **Nat. Genet.** 17, 314-317.

Kafri T, Morgan D, Krahl T, Sarvetnick N, Sherman L, and Verma I (**1998**) Cellular immune response to adenoviral vector infected cells does not require de novo viral gene expression: Implications for gene therapy. **Proc. Nat. Acad. Sci. USA.** 95, 11377-11382.

Kitamura M (**1999**) Bystander macrophages silence transgene expression driven by the retroviral long terminal repeat. **Biochem. Biophys. Res. Commun.** 257, 74-78.

Kristoffersen AK, Sindre H, Mandi Y, Rollag H, and Degre M (**1997**) Effect of adenovirus 2 on cellular gene activation in blood-derived monocytes and macrophages. **APMIS.** 105, 402-409.

Li KJ and Garoff H (**1998**) Packaging of intron containing genes into retrovirus vectors by alpha virus vectors. **Proc. Nat. Acad. Sci. U S A.** 95, 3650-3654.

Lieber A, Steinwaerder DS, Carlson CA, and Kay MA (**1999**) Integrating adenovirus-adeno-associated virus hybrid vectors devoid of all viral genes. **J. Virol.** 73, 9314-9324.

Lin X (**1998**) Construction of new retroviral producer cells from adenoviral and retroviral vectors. **Gene Ther.** 5, 1251-1258.

Linden RM, Ward P, Giraud C, Winocour E, and Berns KI (**1996**) Site-specific integration by adeno-associated virus. **Proc. Nat. Acad. Sci. USA.** 93, 11288-11294.

Lochmuller H, Petrof BJ, Pari G, Larochelle N, Dodelet V, Wang Q, Allen C, Prescott S, Massie B, Nalbabtoglu J, and Karpati G (**1996**) Transient immunosuppression by FK506 permits a sustained high-level dystrophin expression after adenovirus-mediated dystrophin minigene transfer to skeletal muscles of adult dystrophic (mdx) mice. **Gene Ther.** 3, 706-716.

Parrish E, Peltekian E, Dickson G, Epstein AL, and Garcia L (**1999**) Cell engineering for muscle gene therapy: Extemporaneous production of retroviral vector packaging macrophages using defective herpes simplex virus type 1

vectors harbouring gag, pol, env genes. **Cytotechnology** 30, 173-180.

Parrish EP, Cifuentes-Diaz C, Li ZL, Vicart P, Paulin D, Dreyfus PA, Peschanski M, Harris AJ, and Garcia L (**1996**) Targeting widespread sites of damage in dystrophic muscle: engrafted macrophages as potential shuttles. **Gene Ther.** 3, 13-20.

Petrof BJ, Lochmuller H, Massie B, Yang L, Macmillan C, Zhao JE, Nalbantoglu J, and Karpati G (**1996**) Impairment of force generation after adenovirus-mediated gene transfer to muscle is alleviated by adenoviral gene inactivation and host CD8+ T cell deficiency. **Hum. Gene Ther.** 7, 1813-1826.

Ponnazhagan S, Erikson D, Kearns WG, Zhou SZ, Nahreini P, Wang XS, and Srivastava A (**1997**) Lack of site-specific integration of the recombinant adeno-associated virus 2 genomes in human cells. **Hum. Gene Ther.** 8, 275-284.

Pruchnic R, Cao BH, Peterson ZQQ, Xiao X, Li J, Samulski RJ, Epperly M, and Huard J (**2000**) The use of adeno-associated virus to circumvent the maturation-dependent viral transduction of muscle fibers. **Hum. Gene Ther.** 11, 521-536.

Quantin B, Perricaudet LD, Tajbakhsh S, and Mandel JL (**1992**) Adenovirus as an expression vector in muscle cells in vivo. **Proc. Nat. Acad. Sci. USA.** 89, 2581-2584.

Rafael JA, Sunada Y, Cole NM, Campbell KP, Faulkner JA, and Chamberlain JS (**1994**) Prevention of dystrophic pathology in mdx mice by a truncated dystrophin isoform. **Hum. Mol. Genet.** 3, 1725-1733.

Ramsey WJ, Caplen NJ, Li Q, Higginbotham JN, Shah M, and Blaese RM (**1998**) Adenovirus vectors as transcomplementing templates for the production of replication defective retroviral vectors. **Biochem. Biophys. Res. Commun.** 246, 912-919.

Recchia A, Parks RJ, Lamartina S, Toniatti C, Pieroni L, Palombo F, Ciliberto G, Graham FL, Cortese R, La Monica N, and Colloca S (**1999**) Site-specific integration mediated by a hybrid adenovirus/adeno-associated virus vector. **Proc. Nat. Acad. Sci. USA.** 96, 2615-2620.

Reynolds PN, Feng M, and Curiel DT (**1999**) Chimeric viral vectors – the best of both worlds? **Mol. Med. Today** 5, 25-31.

Roberts M, Patterson S, and Dickson G (**2000**) Novel strategies for the treatment of DMD: evaluating the feasibility of using macrophages as retrovirus-producing cells. **Mol. Ther.** 1, S331.

Roberts ML, Athanasopoulos T, Pohlschmidt M, Duisit G, Cosset FL, and Dickson G (**2001**) Post-mitotic, differentiated myotubes efficiently produce retroviral vector from adeno-retrovirus templates. **Gene Ther.** 8, 1580-1586.

Rydercook AS, Sicinski P, Thomas K, Davies KE, Worton RG, Barnard EA, Darlison MG, and Barnard PJ (**1988**) Localization of the mdx mutation within the mouse dystrophin gene**. EMBO. J.** 7, 3017-3021.

Sakoda T, Kasahara N, Hamamori Y, and Kedes L (**1999**) A high-titer lentiviral production system mediates efficient transduction of differentiated cells including beating cardiac myocytes. **J. Mol. Cell. Cardiol.** 31, 2037-2047.

Seppen J, Barry SC, Harder B, and Osborne WR (**2001**) Lentivirus administration to rat muscle provides efficient sustained expression of erythropoietin. **Blood** 98, 594-596.

Tan BT, Wu L, and Berk AJ (**1999**) An adenovirus-Epstein-Barr virus hybrid vector that stably transforms cultured cells with high efficiency. **J. Virol.** 73, 7582-7589.

Torrente Y, Tremblay JP, Pisati F, Belicchi M, Rossi B, Sironi M, Fortunato F, El Fahime M, D'Angelo MG, Caron NJ, Constantin G, Paulin D, Scarlato G, and Bresolin N (**2001**) Intraarterial injection of muscle-derived CD34(+)Sca-1(+) stem cells restores dystrophin in mdx mice. **J. Cell Biol.** 152, 335-348.

Ueno T, Matsumura H, Tanaka K, Iwasaki T, Ueno I, Fujinaga K, Asada K, and Kato I (**2000**) Site-specific integration of a transgene mediated by a hybrid adenovirus/adeno-associated virus vector using the Cre/loxP-expression switching system. **Biochem. Biophys. Res. Commun.** 273, 473-478.

Van Deutekom JCT, Cao B, Pruchnic R, Wickham TJ, Kovesdi I., and Huard J (**1999**) Extended tropism of an adenoviral vector does not circumvent the maturation-dependent transducibility of mouse skeletal muscle. **J. Gene Med.** 1, 393-399.

Vincent N, Ragot T, Gilgenkrantz H, Couton D, Chafey P, Gregoire A, Briand P, Kaplan JC, Kahn A, and Perricaudet M (**1993**) Long-term correction of mouse dystrophic degeneration by adenovirus-mediated transfer of a minidystrophin gene. **Nature Genet.** 5, 130-134.

Wang B, Li J, and Xiao X (**2000**) Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy in mdx mouse model. **Proc. Nat. Acad. Sci. USA.** 97, 13714-13719.

Yang L, Lochmuller H, Luo J, Massie B, Nalbantoglu J, Karpati G, and Petrof BJ (**1998**) Adenovirus-mediated dystrophin minigene transfer improves muscle strength in adult dystrophic (MDX) mice. **Gene Ther.** 5, 369-379.

Yuasa K, Miyagoe Y, Yamamoto K, Nabeshima Y, Dickson G, and Takeda S (**1998**) Effective restoration of dystrophin-associated proteins in vivo by adenovirus-mediated transfer of truncated dystrophin cDNAs. **FEBS. Letts.** 425, 329-336.

Zhang Y, Chirmule N, Gao GP, Qian R, Croyle M, Joshi B, Tazelaar J, and Wilson JM (**2001**) Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. **Mol. Ther.** 3, 697-707.

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