

Autologous Myoblast Transfer: A Combination of Myoblast Transplantation and Gene Therapy

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Abstract

Duchenne Muscular Dystrophy (DMD) is an inherited muscle disease that is characterized by a lack of dystrophin expression in the membrane cytoskeleton of muscle fibers. This lack of dystrophin is responsible for muscle fiber necrosis which leads to the muscle atrophy and progressive muscle weakness, characteristic of the DMD pathology. Myoblast transplantation and gene therapy based on viral vectors have been shown to be efficient in the delivery of genes to skeletal muscle but has been hindered by several limitations. Results from our laboratory and others have shown that adenovirus and herpes simplex virus efficiently infect neonatal muscles, however, within a few days of mouse development, the muscle is largely refractory to transduction with both viral vectors. Since myoblasts possess the ability to fuse with immature and mature myofibers, and viral vectors can efficiently transduce myoblasts, the *ex vivo* technique can be used as an alternative method to achieve viral gene delivery to mature muscle fibers. We have demonstrated that the myoblast-mediated gene transfer may be used to deliver viral vectors (Adenovirus and herpes simplex virus type 1) into mature muscle. In addition, we have shown that the *ex vivo* approach using mdx myoblasts engineered with adenoviral vectors, which carry the full-length dystrophin gene, is capable of restoring dystrophin to the injected mdx muscle. Our results suggest that the *ex vivo* approach may be an alternative way of achieving viral gene delivery to mature muscle and may contribute to the elimination of one of the major hurdles facing the application of gene therapy to skeletal muscle.

Key words: dystrophin, ex-vivo, adenovirus, gene therapy, myoblast transfer.

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Duchenne Muscular Dystrophy (DMD) is an X-linked recessive muscle disease characterized by a lack of dystrophin expression in the sarcolemma of the muscle fiber [6, 28, 68, 81]. Dystrophin is a 427 kDa protein which is associated with a large oligomeric complex of glycoproteins called dystrophin associated proteins (DAPs) which provide a linkage to the extracellular matrix [21, 41, 50, 51, 57]. The lack of dystrophin in dystrophic muscle and the consequent absence of the DAPs disrupt the linkage between the subsarcolemal cytoskeleton and the extracellular matrix [50, 51, 57], resulting in muscle fiber necrosis and progressive muscle weakness [10, 52, 74].

An inbred strain of mice (mdx) which has been broadly used as an animal model for this pathology, has a point mutation in the same gene (dystrophin) resulting in dystrophin deficiency in muscle fibers [12, 28, 66]. Since the lack of dystrophin in this animal model also results in a drastic

reduction in the dystrophin associated proteins, the mdx mice represent a good biochemical model for DMD [15].

The main goal of gene transfer for DMD is to restore dystrophin to as many muscle cells as required to alleviate the muscle weakness associated with this pathology. Myoblast transplantation and gene therapy using several vectors have been extensively investigated as approaches for the introduction of dystrophin into DMD muscle.

Myoblast transplantation (MT) consists of the implantation of myoblasts into dystrophin deficient muscle in order to create a reservoir of normal myoblasts which can consequently fuse with dystrophic muscle fibers and restore dystrophin [59]. The potential for myoblast transplantation for gene transfer is derived from a considerable number of animal experiments [4, 30, 33, 34, 42, 45, 48, 53, 58, 59, 71]. Since DMD is a rapidly progressive, fatal disease several research groups have begun clinical trials of

myoblast transplantation in DMD patients based on successful animal experiments [26, 31, 32, 36, 43, 49, 70]. Although the success of this approach in DMD patients has been found to be limited, some very important results were obtained from these clinical trials, such as: 1) the restoration of dystrophin positive muscle fibers into injected DMD muscles, 2) a transitory increase in the strength of the injected muscles, and 3) the induction of immunological responses from the implanted myoblasts which eventually produced immunorejection of the injected cells [26, 31, 32, 43, 49, 70].

Gene therapy using naked DNA (plasmids containing the gene of interest under the control of an appropriate promoter) is an approach which has been employed for the introduction of foreign genes into muscle. Direct injection of naked or complexed DNA (liposomes) carrying reporter genes and dystrophin into muscle has been found to be relatively inefficient [1]. However, even though humoral and cellular immunity have been detected in response to direct DNA injection [44], transgene expression following plasmid DNA inoculation has persisted over longer time periods [75].

Gene transfer via viral vectors is another approach for gene delivery to muscle, and much recent effort has been directed toward developing viral vectors (retrovirus, adenovirus, adeno-associated virus and herpes simplex virus type 1) that are capable of delivering genes to muscle cells. Since retroviral vectors require the presence of dividing cells for integration and expression, they have been employed to introduce a truncated form of the dystrophin gene (Becker) into myoblasts *in vitro*, but incapable of infecting non-dividing cells such as muscle fibers [16, 17]. Furthermore, their relatively low titer (10^5 - 10^6 pfu/ml) and the risk of their integration in the vicinity of proto-oncogenes, greatly limits the use of these vectors for gene therapy of DMD.

Adenovirus (AV) possesses advantages as a viral vector for skeletal muscle, such as: the ability to produce a high titer of recombinant virus (10^{10} - 10^{11} virus particle/ml), the capacity to infect a wide variety of cell types and their ability to infect quiescent, non-dividing cells. In fact, intramuscular inoculation of adenoviral vectors has been used extensively to infect and deliver foreign reporter genes to muscle cells *in vitro* and muscle fibers *in vivo* [2, 60, 61, 73]. Even though adenovirus appears to be a preferred vector for skeletal muscle cells, some limitations have been described concerning its use as a gene delivery vector to muscle. These limitations include differential transducibility with adenovirus throughout muscle maturation, immunological responses induced by AV transduction, ($> 10^7$ pfu/ml) and a restricted packaging capacity [19, 20, 67, 72, 79, 80]. However, the development of new mutant adenovirus deleted of all viral genes which expand the packaging capacity may open new opportunity for full-length dystrophin delivery to skeletal muscle [13, 46].

Adeno-associated virus (AAV) vectors represent another promising system for gene transfer to skeletal muscle.

AAV has been found capable of infecting newborn as well as mature myofibers with a high efficiency [14, 23, 76]. In addition, a long term persistence of reporter gene expression (up to 1.5 years) has been observed in muscle following AAV transduction [14, 23, 76]. The ability of the AAV genome to integrate with the host chromosome may contribute to the long term persistence of the reporter gene expression mediated by this vector [8, 24, 55, 63].

It has been observed that HSV-1 is also capable of infecting both myoblasts and differentiated myotubes (immature muscle fibers) in cell culture [37]. It has also been shown that HSV-1 can infect a significant number of muscle fibers in newborn mice and in some myofibers of adult animals. However, some limitations have been observed with the first generation of HSV-1 vectors, including differential transducibility throughout the maturation of muscle fibers, cytopathic effects, and immunological problems associated with the intramuscular inoculation of this viral vector [38, 39]. However, new engineered HSV-1 vectors which may be able to overcome some of these liabilities are currently under characterization in our laboratory [40]. The combination of myoblast transplantation and gene therapy in an *ex vivo* approach may help to overcome limitations facing the application of gene transfer to skeletal muscle such as, the inability of viral vectors to efficiently transduce mature skeletal muscle fibers.

This paper summarizes the current information and the most recent achievements pertaining to the use of autologous myoblast transfer to mediate viral gene transfer in mature muscle. We present data on *ex vivo* gene transfers using various viral vectors such as adenovirus and herpes simplex virus type 1 in mature muscle. The efficacy of viral gene delivery to mature myofibers will be compared between the *ex vivo* and the direct gene transfer approaches. In addition, we will discuss the data relating to the delivery of full-length dystrophin to dystrophic mdx muscle using this *ex vivo* approach and the potential of this approach as an avenue to alleviate the muscle weakness of the DMD patient.

Materials and Methods

A mdx myoblast cell line was used as a vehicle for the *ex vivo* gene transfer. This cell line was isolated from a transgenic animal carrying a thermolabile SV40 T-antigen under the control of an inducible promoter [54]. This permanent mdx cell line proliferates indefinitely under permissive conditions (33°C with gamma interferon) and undergoes normal differentiation at 37°-39°C without gamma interferon [54]. The adenoviral vector used for this project was a first generation adenovirus carrying the LacZ reporter gene under the control of the HCMV promoter. We have also used a mutant adenoviral vector (Ad-DYSpgal) deleted for all the viral genes and carrying the full-length dystrophin and the LacZ reporter genes (see below). The herpes simplex virus vector used for this experiment was a new mutant HSV-1 vector deleted for multiple immediate early genes (ICP4, ICP22 and ICP27)

carrying the LacZ gene under the control of the HCMV promoter [40]. The construction, propagation and purification of the adenovirus [35, 46] and herpes simplex virus [37-40] have previously been reported.

Myoblast transduction with adenovirus and herpes simplex virus type 1

The immortalized cell line was first grown in culture for 5 days in proliferating medium containing DMEM and 15% fetal bovine serum (Gibco-BRL; NY USA). 5×10^6 myoblasts were infected with the adenovirus using a multiplicity of infection of 25 (MOI = 25). At 24 hours post-infection, the myoblasts were detached using trypsin (0.25%) for approximately one minute, centrifuged for five minutes at 3500 rpm and the myoblast pellet was reconstituted with 25 μ l of Hank's balanced salt solution (HBSS, Gibco-BRL, NY USA). The harvested cells were transplanted using the technique described below. The same amount of immortalized myoblasts were infected with herpes simplex virus using an MOI of 5. The same procedure following the infection was carried out as described above for the adenovirus. As a control, some myoblast cultures, which were infected with adenovirus and HSV-1, were assayed for LacZ reporter gene expression to determine the level of viral infection *in vitro*. The transduced myoblast cultures were stained for the LacZ reporter gene using the staining technique described below.

Direct and ex vivo gene transfer

Forty-eight hours prior to transplantation, the gastrocnemius muscles of normal mice (C-57 BL10J+/+) were irradiated with gamma rays (30 Grays) to inactivate host satellite cells. The muscle was also injected with a myonecrotic agent, notexin (0.1 μ g), to trigger muscle regeneration twenty-four hours prior transplantation [27]. Then 25 μ l of the transduced myoblast solution (5×10^5 myoblasts) was injected intramuscularly into the exposed gastrocnemius muscle.

A similar amount of viral vectors used for transducing myoblast *in vitro* was directly injected into the contralateral muscle. The viral solution was also injected into the exposed gastrocnemius muscle of the contralateral side which was also irradiated and destroyed with notexin.

The injected mice were sacrificed two days post-injection, and the gastrocnemius muscles from the injected legs were removed and snap frozen in isopentane pre-cooled in liquid nitrogen. The muscles were then cryostat sectioned in entirety at 10 μ m thickness and assayed for LacZ reporter gene expression.

β -Galactosidase Staining

P-galactosidase staining was performed using a histochemical technique previously described by Sanes et al. [64]. The sectioned muscle was incubated overnight with X-Gal substrate at 37°C and then counterstained with hematoxylin and eosin following a protocol previously described [35]. The number of transduced myofibers in

each group were counted, statistically compared (ANOVA Test, Stat Viewtm 512⁺, Brain Power Inc., CA, USA) and the significance was shown with a P-value at < 0.05.

Adenoviral vector carrying full-length dystrophin

The mdx cell line was infected with an adenoviral vector, AdDYSgal, carrying the full-length dystrophin cDNA under the control of the muscle creatine kinase promoter (MCK) and the LacZ reporter gene under the control of the human cytomegalovirus (HCMV) promoter as previously described [46]. One million mdx myoblasts were infected with AdDYSgal using a multiplicity of infection of 25 (MOI = 25). To follow the early fate of the injected myoblasts, fluorescent latex microspheres (0.5 μ m, FLMs) were added to the cultured myoblasts at a dilution of 1:1000 for 24 hrs. [47, 65].

A total of 25 μ l of the transduced myoblast solution (1×10^6 myoblasts) was injected intramuscularly into the irradiated and notexin destroyed mdx muscle using a protocol similar to that described earlier. The injected mice were sacrificed five days after injection, and the gastrocnemius muscles from the injected and non-injected legs were removed and snap frozen in isopentane pre-cooled in liquid nitrogen. The mdx muscle was cryostat sectioned in its entirety at 10 μ m thickness and prepared for Lac Z and dystrophin staining (see below).

Muscle sections were fixed with cold acetone (100%) for one minute and rinsed with phosphate buffered saline (PBS) containing 0.5% Tween 20 (PBS-T). Following a blocking step with 5% horse serum in PBS for 30 minutes, the primary antibody (polyclonal rabbit anti-dystrophin, 6-10 antibody) was incubated in a 1:1000 dilution on the muscle sections overnight. After subsequent washings, the sections were incubated with a goat anti-rabbit antibody conjugated to biotin in a 1/500 dilution for 1 hour (Vector Laboratories; CA, USA). After removal of the secondary antibody, the sections were rinsed in PBS and finally incubated with streptavidin-fluorescein (FITC) 1:100 for 1 hour (Vector Laboratories; CA, USA). Muscle sections for dystrophin, P-galactosidase and fluorescent latex microspheres should be "stained" were viewed under light microscopy and epifluorescence (Nikon), digitized with a video camera (Optronics DEI-750) and frame grabber.

Results

A schematic representation of the *ex vivo* approach is presented in Figure #1. The *ex vivo* gene transfer approach is a form of somatic cell gene transfer that consisted of first establishing a primary myoblast cell culture which is then transduced with a viral vector carrying a reporter gene *in vitro*. These transduced myoblasts were then harvested and injected into the host animal's skeletal muscle. In our system, the myoblasts were not directly isolated from the host animal but rather, were isolated from an animal which is genetically indistinguishable (allograft). In a true autologous myoblast transfer, the primary myoblasts would be isolated from a muscle biopsy taken from the same host

Myoblast Mediated Ex-Vivo Gene Transfer

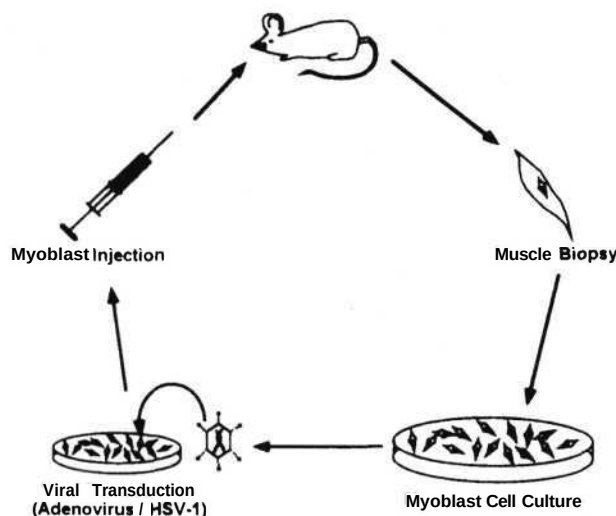


Figure 1. Schematic representation of the myoblast mediated ex vivo gene transfer approach.

animal. The myoblast mediated *ex vivo* gene transfer has been investigated in our experiment using three different viral vectors including, first generation adenovirus, third generation adenovirus carrying the full-length dystrophin and new mutant herpes simplex virus type I.

We have observed that adenovirus can be successfully delivered to mature muscle using the *ex vivo* gene transfer technique (see Figure #2B). Moreover, as previously reported, the adenoviral vector can also be delivered in mature muscle using the direct viral-vector injection approach (see Figure 2A). We have observed that the transduced myofibers obtained following the *ex vivo* approach had a small diameter and were located in the vicinity of the injected site, suggesting that many of these fibers were formed by the fusion of the injected myoblasts. The number of transduced myofibers found in the injected muscle following the *ex vivo* approach was significantly higher than that observed using the direct gene transfer of the same amount of virus $P < 0.05$ (E).

Similarly, we have observed that HSV-1 can be delivered to mature muscle by using the *ex vivo* approach (Figure #2D). In addition, the number of transduced myofibers using the same amount of HSV-1 particles were found to be significantly higher when using the *ex vivo* approach rather than the direct gene transfer approach (Figure #2C, D, and E). We have observed that the HSV-1 remains located outside the mature muscle fibers following the direct gene transfer approach (Figure #2G) in contrast to that observed with the *ex vivo* gene transfer of the same vector (Figure #2D). The transduced myofibers following the *ex vivo* approach based on HSV-1 vector were also located in the vicinity of the injected site and were also the small diameter (D).

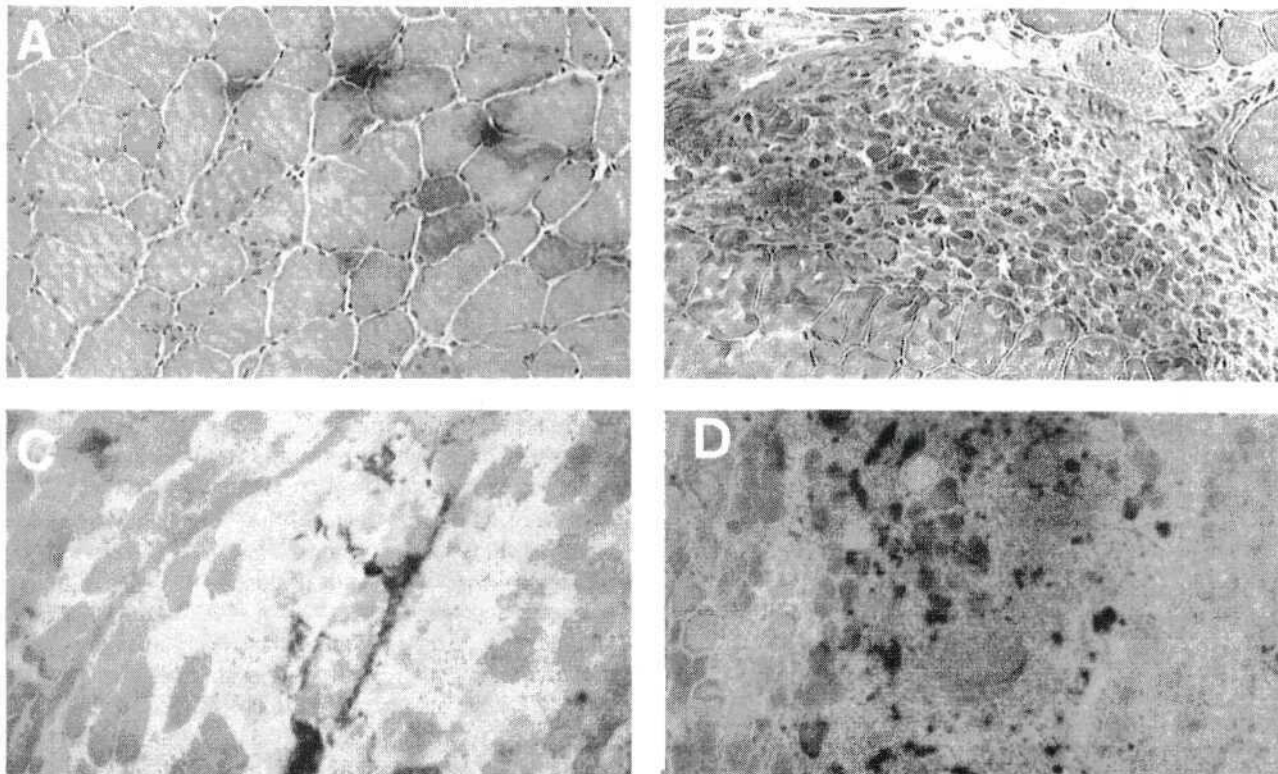
We have shown that myoblasts transduced with an adenoviral vector expressing the full-length dystrophin and the LacZ reporter gene were capable of fusing to form

myotubes *in vitro* expressing these proteins (data not shown). The transplantation of these transduced myoblasts into dystrophic mdx muscle, resulted also in the presence of myofibers expressing β -galactosidase and full-length dystrophin (see Figure #3 A, B). The dystrophin and LacZ positive myofibers were found in the vicinity of the injected site. The co-localization of dystrophin (green fluorescence) with FLM's (red fluorescence) suggests that these myofibers were formed by the fusion of the injected myoblasts (Figure #3B) and were not revertant fibers, which would be caused by a spontaneous mutation [29]. However, the dystrophin positive myofibers which are not FLMs positive, are probably revertant myofibers and not formed by the fusion of the injected myoblasts. Finally, the presence of FLMs inside the dystrophin negative myofibers may be due to many of the injected myoblasts dying and releasing FLMs which can either stay in the interstitial space or be phagocytized by host myofibers.

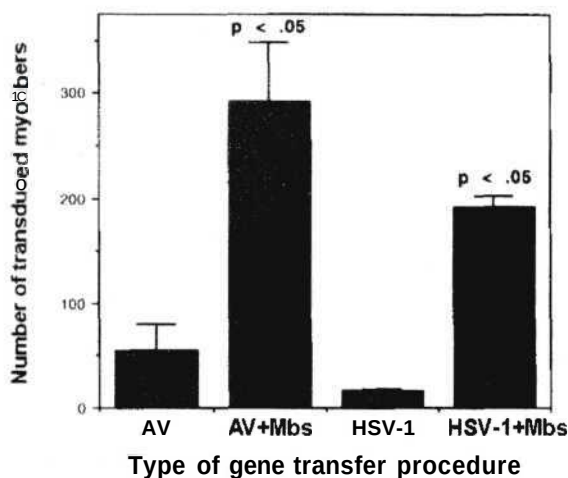
Discussion

One major impediment facing the successful implementation of gene therapy to muscle is the restricted infectivity of mature muscle fibers with viral vectors. This phenomenon has been observed using retrovirus, adenovirus and more recently with herpes simplex virus type 1 based vectors (HSV-1). Even though the mechanism underlying the rapid reduction of retrovirus-mediated gene delivery concomitant with the maturation of muscle fibers is probably related to the inability of the viral vector to transduce post-mitotic muscle fibers, the reason is still unclear for adenovirus and HSV-1. Our findings indicate that the poor level of transduction mediated by adenovirus and HSV-1 may be related through different mechanisms (paper in preparation). Since the depletion of myoblasts *in vitro* using Arac prevents the adenovirus infection of myotubes [3], it appears likely that adenoviral vectors may require myoblasts as a vehicle to transduce myofibers. In fact, supporting this, a higher level of transduction was observed with adenovirus in regenerating muscle (where many myoblasts were fused to regenerate muscle) than in age-matched control muscle [2]. If the adenoviral vector requires myoblasts to transduce myotubes, then it is likely that approaches which promote muscle regeneration by releasing myoblasts are potential alternatives for increasing the efficiency of direct gene transfer of adenovirus in mature myofibers.

Based on parallel studies of HSV-1 infections, we have reported that the failure of HSV-1 infection of mature myofibers is probably due to the maturation of the basal lamina, which physically blocks the virus' accessibility to mature myofibers [38, 39]. In support of this hypothesis, it has been shown that HSV-1 infects with a similar efficiency *in vitro* both myoblasts, and pure cultures of myotubes, where only a rudimentary basal lamina is found. This experiment suggests that the HSV-1 vector is capable of directly infecting myotubes without requiring myoblasts. Additionally, by using antibodies against HSV-



E Ex-vivo versus direct gene transfer



I and collagen IV, a major constituent of the basal lamina, it has been observed that HSV-1 cannot transduce mature myofibers due to the maturation of the basal lamina which acts as a physical barrier to HSV-1 transduction [38, 39]. In support of this hypothesis, it has been noted that an intermediate level of HSV-1 transduction is observed in adult myofibers of *dy/dy* mice which have a defective basal lamina due to merosin deficiency [5, 69, 77, 78]. Thus, HSV-1 transduction in mature myofibers will require methods which will permeabilize the basal lamina and allow HSV-1 penetration and transduction of mature myofibers.

Figure 2. Adenovirus and Herpes simplex virus type 1 mediated gene transfer to mature skeletal muscle. Direct adenoviral injection transduces mature skeletal muscle fibers with a lower efficiency (A) than the same amount of adenovirus in an ex vivo approach (B). Similar results are achieved with HSV-1 which shows a higher efficacy of transduction when HSV-1 is used to transduce the myoblasts which are injected into the mature muscle (D) than the direct injection of the same amount of virus (C). In fact, the number of transduced myofibers are significantly higher using this ex-vivo approach than the direct viral injection for both virus (E). (magnification: A, B = 20X; C, D = 10X).

Since myoblasts possess the ability to fuse with both immature and mature muscle fibers, and adenovirus and HSV-1 can efficiently transduce myoblasts, the ex vivo gene transfer approach may represent a plausible alternative for viral gene delivery to mature muscle. The myoblast mediated ex vivo gene transfer has been investigated by our laboratory and others, using different viral vectors including adenovirus, retrovirus and herpes simplex virus type 1.

Ex vivo gene transfer of LacZ reporter gene

The myoblast mediated ex vivo gene transfer of adenovirus carrying the β -galactosidase and luciferase reporter genes has resulted in an efficient gene transfer and expression of the reporter genes into the injected muscle [35]. The ex vivo approach allows an efficient gene transfer

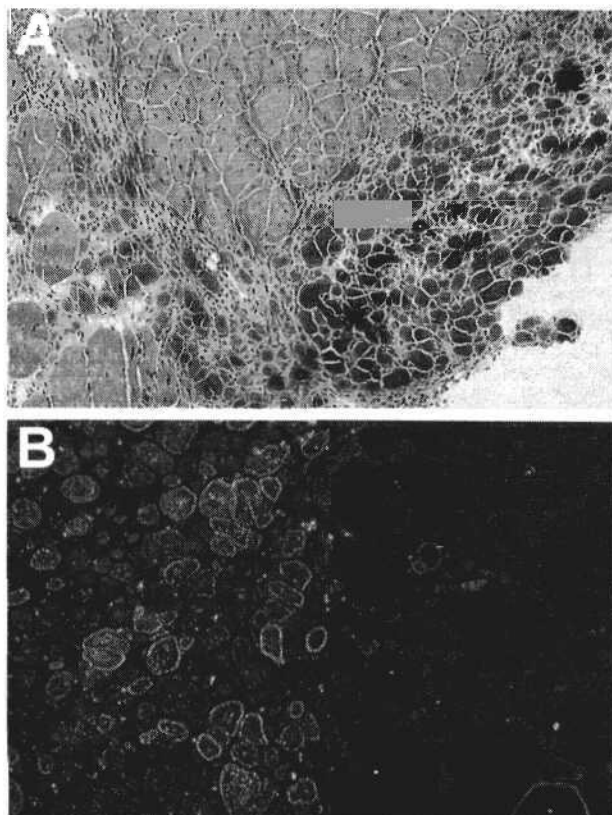


Figure 3. Myoblast mediated *ex vivo* gene delivery of full length dystrophin and β -galactosidase reporter gene to mature mdx skeletal muscle. A large number of transduced myofibers expressing LacZ reporter gene are found in the mdx injected muscle with the transduced myoblasts (A). In addition, dystrophin-positive myofibers are also detected at the same area in the mdx injected muscle with engineered myoblasts (B). The co-localization of the dystrophin-positive myofibers (green fluorescence) with the fluorescent latex microspheres (red fluorescence) suggests that the dystrophin-positive myofibers are the result of the fusion of the injected myoblasts and not revertant fibers due to a spontaneous mutation. (B). (magnification: A, B = 20X).

to skeletal muscle without apparent immunological problems associated with direct viral injection [35]. However, some problems related to myoblast transplantation still remain with this approach, since the vast majority of the injected myoblasts were lost rapidly, usually within 48 hours posttransplantation [7, 22, 35]. The cause of the accelerated death of the injected cells remains unclear, but some explanations have been offered including: 1) only a low proportion of these injected myoblasts are capable of supporting muscle regeneration and/or 2) an inflammatory response induced by the myoblast injection.

We have shown, as previously reported, that adenovirus can be successfully delivered to mature muscle using the *ex vivo* gene transfer approach [11, 35]. Moreover, a higher efficiency of gene transfer via adenovirus is observed using

the *ex vivo* gene transfer as compared to the direct viral vector injection in mature muscle (see Figure 2). This suggests that the *ex vivo* technique may be an efficient approach to efficiently deliver adenoviral transferred therapeutic genes to mature muscle.

We have recently observed that the myoblast mediated *ex vivo* gene transfer approach can also be used to deliver new mutant HSV-1 to mature skeletal muscle. In fact, HSV-1 transduced myoblasts are also capable of retaining their ability to proliferate and fuse forming myotubes *in vitro* expressing the β -galactosidase reporter gene [11]. The efficiency of gene transfer has also been found higher than the direct injection of the same viral solution.

Retrovirally infected myoblasts have also been found capable of preserving their ability to proliferate and fuse into myotubes *in vitro* and are capable of expressing the β -galactosidase reporter gene. The transplantation of the transduced myoblasts in muscle *in vivo* results in an efficient gene transfer to skeletal muscle [62]. In addition, a persistent transgene expression of up to 30 days has been achieved using the transplantation of myoblast mediated gene transfer of retroviral vectors in skeletal muscle *in vivo*. We have also observed that a higher efficiency of gene transfer to mature muscle occurs when the retroviral vector is delivered by the *ex vivo* approach rather than the direct gene transfer to mature muscle (data not shown).

Myoblast mediated *ex vivo* dystrophin delivery in mdx mice

We have developed a model which enables us to test the feasibility of an autologous myoblast mediated gene transfer to deliver full-length dystrophin in mature mdx muscle. In our study, primary mdx myoblasts were utilized as vehicles for *ex vivo* gene transfer to muscle. The myoblasts were transduced *in vitro* with an adenoviral vector expressing the full length dystrophin gene and LacZ reporter gene. We have shown that transduced myoblasts are capable of fusing and forming myotubes *in vitro* expressing β -galactosidase and full-length dystrophin. The transplantation of mdx transduced myoblasts has been found also capable of restoring full-length dystrophin in mature mdx muscle. The co-localization of dystrophin and FLM's suggests that myofibers are formed by the fusion injected myoblasts and are not revertant fibers which are caused by a spontaneous point mutation [29]. Moreover, we have also observed that we can successfully restore the 35 kDa and P-43 kDa sarcoglycans in the injected MDX muscle (data not shown) indicating that the restored dystrophin is capable of functioning normally [25].

The myoblast mediated *ex vivo* approach may possess attributes that make it useful as an effective gene transfer to skeletal muscle. A benefit in the use of myoblast mediated *ex vivo* gene transfer, is that these cells can not only serve as a vehicle for gene complementation, but also are able to create a reservoir of myogenic cells capable of regenerating damaged myofibers. Since the muscle weakness in DMD patients is not typically noticed until five

years of age, it appears that the dystrophic muscle of younger children is functionally rescued by the large population of satellite cells which continuously regenerates the chronically damaged myofibers. However, as the patient ages and the satellite cells undergo mitotic senescence [9, 56], progressive muscle weakness appears. The *ex vivo* approach may not only achieve the restoration of myoblasts capable of introducing dystrophin to dystrophic myofibers, but which are also capable of regenerating the muscles. In addition, this approach may enhance the total number of myoblasts required to alleviate the muscle weakness characteristic of this disease.

The current objectives in using the *ex vivo* approach to restore dystrophin to the muscle of the DMD patient involves the transplantation of a sufficient number of myoblasts that are capable of restoring dystrophin and thus eventually decrease the muscle weakness associated with this disease. Since it has been demonstrated that satellite cells isolated from DMD patients show a limited proliferative capacity, especially when taken from older patients [9], one potential hurdle facing the application of the *ex vivo* approach involves the cultivation of a sufficient number of myoblasts to alleviate muscle weakness characteristic of this disease. However, it is believed that it may be possible to isolate myoblasts from muscles which are less affected by the disease which will then serve as a better source of myogenic cells for the *ex vivo* approach [18].

In conclusion, the results show that the *ex vivo* gene transfer method may be used as an alternative approach to achieve a viral gene transfer to mature myofibers. This approach may eventually aid in the development of a gene therapy for the treatment of muscle disease such as Duchenne Muscular Dystrophy. We have shown that this technique provides a better gene transfer in mature muscle using adenovirus and HSV-1 as compared to the conventional direct gene transfer approach. Finally, it is clear that the limitations related to myoblast transfer such as the rapid loss of the injected myoblasts post-transplantation, as well as the low spreading of the injected cells post-implantation have to be clearly understood to eventually apply this technology to the treatment of DMD patients.

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References

- [1] Acsadi G, Dickson G, Love DR, Jani A, Walsh FS, Gurusinghe A, Wolff JA, Davies KE: Human dystrophin expression in MDX mice after intramuscular injection of DNA constructs. *Nature* 1991; 352: 815-818.
- [2] Acsadi G, Jani A, Massie B, Simoneau M, Holland P, Blaschuk K, Karpati G: A differential efficiency of adenovirus-mediated *in vivo* gene transfer into skeletal muscle cells at different maturity. *Hum Mol Gen* 1994a; 3: 579-584.
- [3] Acsadi G, Jani A, Huard J, Blaschuk K, Massie B, Holland P, Lochmuller H, Karpati G: Cultured human myoblasts and myotubes show markedly different transducibility by replication-defective adenovirus recombinant. *Gene Therapy* 1994b; 1: 338-340.
- [4] Alameddine HS, Dehaupas M, Fardeau M: Regeneration of skeletal muscle fibers from autologous satellite cells multiplied *in-vitro*. *Muscle and Nerve* 1989; 12: 544-555.
- [5] Hayashi, YK, Koga, R, Goto, JH, Lee, H, Ishii, T, Tsukahara, T, Takeda, S, Woo, M, Nonaka, I, Matsuzaki, T, Sugita, H: Laminin in animal models for Duchenne muscular dystrophy; defect of laminin M in skeletal and cardiac muscles and peripheral nerve of the homozygous dystrophic dy/dy mice. *Proc Jpn Acad Ser B* 1993; 69: 259-264.
- [6] Arahata K, Ishiura S, Ishiguro T, Tsukahara T, Suhara Y, Eguchi C, Ishihara T, Nonaka I, Ozawa E, Sugita H: Immunostaining of skeletal and cardiac muscle surface membrane with antibody against Duchenne Muscular Dystrophy peptide. *Nature* 1988; 333: 861-863.
- [7] Beauchamp JR, Morgan JE, Pagel CN, Partridge TA: Quantitative studies of the efficacy of myoblast transplantation. *Muscle & Nerve* 1994; 18: S261.
- [8] Berns KI, Linden RM: The cryptic life style of adeno-associated virus. *Bioessays* 1995; 17: 237-245.
- [9] Blau RM, Webster C, Pavlath GK: Defective myoblasts identified in Duchenne Muscular Dystrophy. *Proc Natl Acad Sci USA* 1983; 81: 4856-4860.
- [10] Bonilla E, Samitt AF, Miranda AP, Hays G, Salviati S, Dimauro S, Kunkel LM, Hoffman EP, Rowland LP: Duchenne Muscular Dystrophy: deficiency of

- dystrophin at the muscle cell surface. *Cell* 1988;54: 447-452.
- [11] Booth DK, Floyd SS, Day CS, Glorioso JC, Kovesdi I, Huard J: Myoblast mediated *ex vivo* gene transfer to mature muscle. *Tissue Engineering* 1997; vol. 3 #2: 125-133.
- [12] Bulfield G, Silier WG, Wright PAL, Moore KJ: X-chromosome-linked muscular dystrophy (MDX) in the mouse. *Proc Natl Acad Sci USA* 1984; 81: 1189-1192.
- [13] Clemens PR, Kochanek S, Sunada Y, Chan S, Chen SS, Campbell KP, Caskey CT: *In vivo* muscle gene transfer of full-length dystrophin with an adenoviral vector that lacks all viral genes. *Gene Therapy* 1996; 3: 965-972.
- [14] Clark KR, Sferra TJ, Johnson PR: Recombinant adeno-associated viral vectors mediate long-term transgene expression in muscle. *Hum Gene Ther* 1997; 8: 659-669.
- [15] Cox GA, Cole NM, Matsumura K, Phelps SF, Hauschka SD, Campbell KP, Faulkner JA, Chatnberlain JS: Over-expression of dystrophin in transgenic MDX mice eliminates dystrophic symptoms without toxicity. *Nature* 1993; 364: 725-729.
- [16] Dunkley MG, Davies KE, Walsh FS, Morris GE, Dickson G: Retroviral-mediated transfer of a dystrophin minigene into MDX mouse myoblasts *in vitro*. *Febs Letter* 1992; 296 #2: 128-134.
- [17] Dunkley MG, Wells DJ, Walsh FS, Dickson G: Direct retroviral-mediated transfer of dystrophin minigene into mdx mouse muscle *in vivo*. *Hum Mol Genet* vol. 2 96: 717-723.
- [18] Engel A, Yamamoto M, Fischbeck KH: Muscular Dystrophies; Dystrophinopathies. Myology, 2nd Edition. McGraw-Hill, Inc, 1994; 1133-1187.
- [19] Engelhardt JF, Litzky L, Wilson JM: Prolonged transgene expression in cotton rat lung with recombinant adenovirus defective in E2A. *Hu Gene Ther* 1994; (A), 5: 1217-1229.
- [20] Engelhardt JF, Ye X, Doranz B, Wilson JM: Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory responses in mouse liver. *Proc Natl Acad Sci USA (B)* 1994; 91: 6196-6200.
- [21] Ervasti JM, Campbell KP: Membrane organization of the dystrophin-glycoprotein complex. *Cell* 1991; 66: 1121-1131.
- [22] Fan Y, Maley M, Beilharz M, Grounds M: Rapid death of injected myoblasts in myoblast transfer therapy. *Muscle & Nerve* 1996, 19: 853-860.
- [23] Fisher KJ, Jooss K, Alston J, Yang Y, Haecker SE, High K, Pathak R, Raper SE, Wilson JM: Recombinant adeno-associated virus for muscle directed gene therapy. *Nature Medicine* 1997; 3 #3: 306-312.
- [24] Flotte TR, Carter BJ: Adeno-associated virus vectors for gene therapy. *Gene Therapy* 1995; 2: 357-362.
- [25] Floyd SS, Clemens PR, Ontell, Kochanek S, Day CS, Yang J, Hauschka SD, Balkir L, Morgan J, Feero GW, Epperly M, Huard J: *Ex vivo* gene transfer using adenovirus mediated full-length dystrophin delivery to dystrophic muscles. *Gene Therapy* In press, 1997.
- [26] Gussoni E, Pavlath PK, Lanctot AM, Sharma K, Miller RG, Steinman L, Blau RM: Normal dystrophin transcripts detected in DMD patients after myoblast transplantation. *Nature* 1992; 356: 435-438.
- [27] Harris JB, Johnson MA, Karlsson F: Pathological responses of rat skeletal muscle to a single subcutaneous injection of a toxin isolated from the venom of the Australian tiger snake, *Notechis scutatus scutatus*. *Clin Exp Pharmacol Physiol* 1975; 2: 383-404.
- [28] Hoffman EP, Brown J, Kunkel LM: Dystrophin: the protein product of the Duchenne Muscular Dystrophy locus. *Cell* 1987; 51: 919-928.
- [29] Hoffman EP, Morgan JE, Watkins SC, Slayter HS, Partridge TA: Somatic reversion/suppression of the mouse mdx phenotype *in vivo*. *J Neurol Sci* 1990; 99: 9-25.
- [30] Huard J, Labrecque C, Dansereau G, Robitaille L, Tremblay JP: Dystrophin expression in myotubes formed by the fusion of normal and dystrophic myoblasts. *Muscle and Nerve* 1991; 14: 178-182.
- [31] Huard J, Bouchard JP, Roy R, Malouin F, Dansereau G, Labrecque C, Albert N, Richards CL, Lemieux B, Tremblay JP: Human myoblast transplantation: preliminary results of four cases. *Muscle and Nerve* 1992a; 15: 550-560.
- [32] Huard J, Roy R, Bouchard JP, Malouin F, Richards CL, Tremblay JP: Human myoblast transplantation between immunohistocompatible donors and recipients produces immune reactions. *Transpl Proc* 1992b; 24 #6: 3049-3051.
- [33] Huard J, Roy R, Guerette B, Verreault S, Tremblay G, Lille S, Tremblay JP: Human myoblast transplantation in immunodeficient and immunosuppressed mice: Evidence of rejection. *Muscle and Nerve* 1994a; 17: 224-234.
- [34] Huard J, Verreault S, Roy R, Tremblay M, Tremblay JP: High efficiency of muscle regeneration following human myoblast clone transplantation in SCID mice. *J Clin Invest* 1994b; 93: 586-599.

- [35] Huard J, Acsadi G, Jani A, Massie B, Karpati G: Gene transfer into skeletal muscles by isogenic myoblasts. *Human Gene Therapy* 5: 949-958.
- [36] Huard J, Roy R, Bouchard JP, Labrecque C, Dansereau G, Lenlieux B, and Tremblay JP: Myoblast transplantation produced dystrophin-positive muscle fibers in a 16-year-old patient with Duchenne Muscular dystrophy. *Clin Sci* 1991; 81: 287-288.
- [37] Huard J, Goins B, Glorioso JC: Herpes simplex virus type 1 vector mediated gene transfer to muscle. *Gene Therapy* 1995; (B). 2: 1-9.
- [38] Huard J, Feero WG, Watkins SC, Hoffman EP, Rosenblatt DJ, Glorioso JC: The basal lamina is a physical barrier to herpes simplex virus-mediated gene delivery to mature muscle fibers. *J Virol* 1996; 70 #11: 8117-8123.
- [39] Huard J, Akkaraju G, Watkins SC, Pike-Cavalcoli M, Glorioso JC: LacZ gene transfer to skeletal muscle using a replication defective herpes simplex virus type 1 mutant vector. *Hum Gene Therapy* 1997; 8: 439-452.
- [40] Huard J, Krisky D, Oligino T, Marconi P, Day CS, Watkins SC, Glorioso JC: Gene transfer to muscle using herpes simplex virus-based vectors. *Neuromuscular Disorder*, In press, 1996.
- [41] Ibraghinov-Beskrovnya O, Ervasti JM, Leville CJ, Slaughter CA, Senett SW, Campbell KP: Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature* 1992; 355: 696-702.
- [42] Karpati G, Pouliot Y, Zubrzycka-Gaarn EE, Carpenter S, Ray PN, Worton RG, Holland P: Dystrophin is expressed in mdx skeletal muscle fibers after normal myoblast implantation. *Am J Pathol* 1989; 135: 27-32.
- [43] Karpati G, Ajdukovic D, Arnold D, Gledhill RB, Guttman R, Holland P, Koch PA: Myoblast transfer in Duchenne Muscular Dystrophy. *Ann Neurol* 1993; 34: 8-17.
- [44] Katsumi A, Emi N, Abe A, Hasegawa Y, Ito M, Saito H: Humoral and cellular immunity to an encoded protein induced by direct DNA injection. *Hum Gene Ther* 1994; 5: 1335-1339.
- [45] Kinoshita I, Vilquin JT, Guerette B, Asselin I, Roy R, Tremblay JP: Very efficient myoblast allotransplantation in mice under FK506 immunosuppression. *Muscle and Nerve* 1994; 17: 1407-1415.
- [46] Kochanek S, Clemens PR, Mitani K, Chen HH, Chan S, Caskey CT: A new adenoviral vector: Replacement of all viral coding sequences with 28 Kb of DNA independently expressing both full-length dystrophin and β -galactosidase. *Proc Natl Acad Sci USA* 1996; 93: 5731-5736.
- [47] Labrecque C, Huard J, Dansereau G, Robitaille L, Tremblay JP: A new technique to identify hybrid myotubes *in vitro* without culture fixation. *J Histochem Cytochem* 1991; 39#1: 139-143.
- [48] Law PK, Goodwin TG, Li HJ: Histoincompatible myoblast injection improves muscle structure and function of dystrophic mice. *Transplantation Proc* 1988a; 20: 1014-1019.
- [49] Law PK, Goodwin TG, Fang Q, Chen M, Li HJ, Florendo A, Kirby D, Bertorini T, Herrod H, Golden G: Pioneering development of myoblast transfer therapy, in Angelini C, Danieli GA, Fontanari D (eds): *Muscular Dystrophy Research. Excerpta Medica*. International Congress Series 934, New-York, Elsevier, 1991, pp 109-116.
- [50] Matsumura K, Ervasti JM, Ohlendieck K, Kahl SD, Campbell KP: Association of dystrophin-related protein with dystrophin-associated proteins in MDX mouse muscle. *Nature* 1992; 360: 588-591.
- [51] Matsumura K, Campbell KP: Dystrophin-glycoprotein complex: its role in the molecular pathogenesis of muscular dystrophies. *Muscle & Nerve* 1994; 17: 2-15.
- [52] Menke A, Jokusch H: Decreased osmotic stability of dystrophin less muscle cells from the MDX mice. *Nature* 1991; 349: 69-71.
- [53] Morgan JE, Hoffman EP, Partridge TA: Normal myogenic cells from newborn mice restore normal histology to degenerating muscle of the MDX mouse. *J Cell Biol* 1990; 111: 2437-2449.
- [54] Morgan JE, Beauchamps JR, Pagel CN, Peckham M, Atalio P, Jat PS, Noble NM, Farmer K, Partridge TA: Myogenic cell lines derived from transgenic mice carrying a thermolabile T antigen: A model system for the derivation of tissues-specific and mutation-specific cell lines. *Developmental Biology* 1994; 162: 486-498.
- [55] Muzyczka N: Use adeno-associated virus as a general transduction vector for mammalian cells. *Curr Top Microbiol Immunol* 1992; 158: 97-129.
- [56] Ontell W, Hughes D, Hauschka SD, Ontell M: Transient neonatal denervation alters the proliferative capacity of myosatellite cells in dystrophic (129ReJ^{dy/dy}) muscle. *J Neurobiol* 1992; Vol 23, No 4: 407-419.
- [57] Ozawa E, Yoshida M, Suzuki A, Mizuno Y, Hagiwara Y, Noguchi S: Dystrophin-associated proteins in muscular dystrophy. *Hum Mol Genet* 1995 4: 1711-1716.
- [58] Partridge TA, Morgan JE, Coulton GR, Hoffman EP, Kunkel LM: Conversion of MDX myofibers from dystrophin negative to positive by injection of normal myoblasts. *Nature* 1989; 337: 176-179.

- [59] Partridge TA: Myoblast transfer: A possible therapy for inherited myopathies. *Muscle and Nerve* 1991; 14: 197-212.
- [60] Quantin B, Perricaudet LD, Tajbakhsh S, Mandell JL: Adenovirus as an expression vector in muscle cells *in vivo*. *Proc Natl Acad Sci USA* 1992; 89: 2581-2584.
- [61] Ragot T, Vincent M, Chafey P, Vigne E, Gdgenkrantz H, Couton B, Cartaud J, Briand P, Kaplan JC, Perricaudet M, Kahn A: Efficient adenovirus mediated gene transfer of a human minidystrophin gene to skeletal muscle of MDX mice. *Nature* 1993; 361: 647-650.
- [62] Salvatori G, Ferrari G, Messogiorno A, Servidel S, Colette M, Tonalli P, Giarassi R, Cosso G, Mavillo F: Retroviral vector-mediated gene transfer into human primary myogenic cells lead to expression in muscle fibers *in vivo*. *Hum Gene Therapy* 1993; 4: 713-723.
- [63] Samulski RJ: Adeno-associated virus: integration at a specific chromosomal locus. *Curr Op Genet Dev* 1993; 3: 74-80.
- [64] Sanes JR, Rubenstein JLR, Nicolas JF: Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. *Embo J* 1986; 5: 5133-5142.
- [65] Satoh A, Huard J, Tremblay JP: Use of fluorescent latex microspheres (FLMS) to follow the fate of transplanted myoblasts. *J Histochem Cytochem* 1993; 41: 1579-1582.
- [66] Sincinsky P, Geng Y, Ryder-Cook A, Barnard E, Darlinson M, Barnard P: The molecular basis of muscular dystrophy in the MDX mouse: A point mutation. *Science* 1989; 244: 1578-1580.
- [67] Smith TAG, Mehaffey MG, Kayda DB, Saunders JM, Yei S, Trapnell BC, McClell A, Kaleko M: Adenovirus mediated expression of therapeutic plasma levels of human factor IX in mice. *Nature Genetics* 1993; 5: 397-402.
- [68] Sugita H, Arahata K, Ishiguro T, Sohara Y, Tsukahara T, Ishiura J, Eguchi C, Nonaka I, Ozawa E: Negative Immunostaining of Duchenne Muscular Dystrophy (DMD) and MDX muscle surface membrane with antibody against synthetic peptide fragment predicted from DMD cDNA. *Proc Jpn Acad* 1988; 64: 37-39.
- [69] Sumada U, Bemier SM, Kozak CA, Yamada Y, Campbell KP: Deficiency of merosin in dystrophin cy mice and genetic linkage of laminin M. chain gene to dy locus. *J Biol Chem* 1994; 269: 13729-13732.
- [70] Tremblay JP, Malouin F, Roy R, Huard J, Bouchard JP, Satoh A, Richards CL: Results of a blind clinical study of myoblast transplantations without immunosuppressive treatment in young boys with Duchenne Muscular Dystrophy. *Cell Transplantation* 1993; 2: 99-112.
- [71] Vilquin JT, Wagner E, Kinoshita I, Roy R, Tremblay JP: Successful histocompatible myoblast transplantation in dystrophin-deficient NMJ mouse despite the production of antibodies against dystrophin. *Jour Cell Biol* 1995a; 131 #4: 975-988.
- [72] Vilquin JT, Guerette B, Kinoshita I, Roy B, Goulet M, Gravel C, Roy R, Tremblay JP: FK506 immunosuppression to control the immune reactions triggered by first-generation adenovirus-mediated gene transfer. *Human Gene Therapy* 1995b; 6: 1391-1401.
- [73] Vincent M, Ragot T, Gilgenkrantz H, Couton D, Chafey P, Gregoire A, Briand P, Kaplan JC, Kahn A, Perricaudet M: Long-term correction of mouse dystrophic degeneration by adenovirus-mediated transfer of a mini dystrophin gene. *Nature Genet* 1993; 5: 130-134.
- [74] Watkins SC, Hoffman EP, Slayter HS, Kunkel LM: Immunoelectron microscopic localization of dystrophin in myofibers. *Nature* 1988; 333: 863-866.
- [75] Wolff JA, Ludtke JJ, Acsadi G, Williams P, Jani A: Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum Mol Genet* 1992; 1: 363-369.
- [76] Xiao X, Li J, Samulski RJ: Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *Journal of Virology* 1996; 70: 8098-8108.
- [77] Xu H, Christmas P, Wu XR, Wewer UM, Engvall E: Defective muscle basement membrane and lack of M-laminin in the dystrophic dy/dy mouse. *Proc Natl Acad Sci USA* 1994; 91: 5572-5576.
- [78] Xu H, Wu XR, Wewer UM, Engvall: Murine muscular dystrophy caused by a mutation in the laminin $\alpha 2$ (Lamal) gene. *Nature Genet* 1994; 8: 297-302.
- [79] Yang Y, Nunes FA, Berencsi K, Furth EE, Gonczol E, Wilson JM: Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc Natl Acad Sci USA* 1994; 91: 4407-4411.
- [80] Yang Y, Nunes FA, Berencsi K, Gonczol E, Engelhard JF, Wilson JM: Inactivation of E2A in recombinant adenoviruses improves the prospect for gene therapy in cystic fibrosis. *Nature Genetics* 1994; 7: 362-369.
- [81] Zubryzcka-Gaarn EE, Bulman DE, Karpati G, Burghes Belfall B, Klamut HJ, Talbot J, Hodges RS, Ray PN, Worton RG: The Duchenne Muscular Dystrophy gene is localized in the sarcolemma of human skeletal muscle. *Nature*. 1988; 333: 466-469.