Persistent Gene Transfer to Skeletal Muscle Mediated by Stably Transfected Early Myogenic Progenitor Cells

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Abstract

Duchenne muscular dystrophy is a progressive muscle weakness characterized by a lack of dystrophin expression in the sarcolemma of muscle fibers. Both myoblast transplantation and gene therapy based on direct and *ex vivo* gene transfer techniques have been investigated as ways to deliver dystrophin in dystrophic muscle. Although the myoblast-mediated *ex vivo* gene transfer approach has been found capable of improving viral gene transfer to skeletal muscle, the poor survival rate of the injected cells as well as the immune response against the virally transduced cells has significantly hindered the success of this technique. For this paper, we investigated the use of non-viral vectors through the *ex vivo* approach based on early myogenic progenitor cells, which have been found highly capable of surviving post-implantation, to improve both the cell survival rate and the long-term persistence of gene transfer to skeletal muscle. We transfected a population of early myogenic progenitor cells derived from mdx mice with a plasmid encoding β-galactosidase, mini-dystrophin and the neomycin resistance gene. The selected muscle cells were capable of expressing β-galactosidase and differentiating into myotubes expressing dystrophin *in vitro*. More importantly, the transplantation of the transfected cells can be used to deliver β-galactosidase and dystrophin in skeletal muscle of adult mdx mice. The infiltration of CD4+ and CD8+ activated lymphocytes at the injected site suggests that the persistence of the transfected myofibers is limited by the immune response. On the other hand, the persistent transgene expression observed with the same approach in transgenic mdx mice that express β-galactosidase (mdx/β-gal) suggests that immune response is triggered by the β-galactosidase reporter gene.

Key words: Duchenne muscular dystrophy, dystrophin, early myogenic progenitor cells, immune responses, mdx mouse, myoblast-mediated *ex vivo* gene transfer, plasmid DNA, β-galactosidase, transgenic mdx/β-gal.

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Duchenne muscular dystrophy (DMD) is an inherited muscle disease characterized by the absence of dystrophin in the membrane-associated cytoskeleton of muscle fibers [7, 29, 70, 82]. Dystrophin is associated with a large oligomeric complex of glycoproteins called dystrophin-associated proteins (DAPs), which provide linkage to the extracellular matrix [21, 39, 50, 51, 56]. The lack of dystrophin in dystrophic muscle and consequent absence of all DAPs disrupt the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix [50, 51, 56], resulting in muscle fiber necrosis and progressive muscle weakness [9, 53, 76]. The mdx mouse also lacks dystrophin and DAPs in skeletal muscle fibers and can therefore be used as an animal model for DMD [11, 29, 68].

Transplantation of normal myoblasts into dystrophin-deficient muscle has the potential of creating a reservoir of normal myoblasts capable of fusing with dystrophic muscle fibers and restoring dystrophin [58]. Animal experi-

ments [5, 37, 42, 55, 57] have shown that the transplantation of normal myoblasts results in dystrophin expression at the muscle fiber plasma membrane and subsequently improves the structure and function of dystrophic mouse muscle. However, long-term dystrophin expression after myoblast transplantation has been primarily hindered by immune-rejection. Prolonged efficiency of myoblast transplantation was observed only in immunodeficient nude and SCID mice [36, 38] and mice adequately immunosuppressed using FK-506 [45]. While initial clinical trials of myoblast transfer to DMD patients were unsuccessful, the studies demonstrated the transient restoration of dystrophin-positive muscle fibers in the injected DMD patients [26, 32, 33, 41, 52, 71, 74]. The failure of myoblast transfer in these clinical trials, like that observed in the animal models, was at least partially attributed to immune-rejection. Indeed, serum antibodies against the injected cell antigens, such as dystrophin and dystrophin-associated proteins, were detected in DMD patients after transplantation [26]. Other limitations, including the poor survival rate and spreading of the injected myoblasts within the injected muscle, have also contributed to the poor success of myoblast transplantation [5, 8, 22, 25, 26, 30, 32, 33, 36-38, 41, 42, 45, 52, 55, 57, 71, 74].

Gene therapy based on the direct injection of non-viral and viral vectors has been found capable of delivering genes to skeletal muscle. This technique has also been hindered by various hurdles. It has been reported that direct injection of naked or complex DNA (liposomes) encoding for either reporter genes or dystrophin can achieve gene transfer to skeletal muscle with a low toxicity and immunogenicity, but displays a relatively low efficiency of myofiber transfection [1, 43, 77]. Retroviral vectors can introduce truncated dystrophin to dystrophic myoblasts *in vitro* but cannot efficiently transduce differentiated muscle cells, such as myotubes and myofibers, because the retrovirus requires dividing cells for integration and expression [18, 19]. While adenovirus (AV) appears to be a relatively good vector for skeletal muscle cells, there are potential limitations on its use as a gene delivery vector to muscle: the differential transducibility of myofibers throughout muscle maturation, the immune-rejection induced by first-generation adenoviral vectors, and the high titer of viral recombinants often required for successful muscle transduction [2-4, 10, 15, 20, 24, 62, 63, 69, 72, 73, 75, 79]. Recent studies with mutant AV vectors in which all viral genes were removed suggest that these vectors may be able to circumvent the immunological problems against viral antigens [14, 28, 46, 47]. Finally, replication-defective herpes simplex virus type 1 (HSV-1) is a vector that can efficiently infect and express a foreign reporter gene in muscle cells *in vitro* and in newborn myofibers *in vivo*. Limitations for the use of the first-generation HSV-1 vector for muscle include inefficient transduction of mature myofibers, cytotoxicity, and immune-rejection [31, 34, 35]. More recently, recombinant adeno-associated viral vectors (rAAV) have been used as a gene delivery vehicle for skeletal muscle cells. Although a high efficiency of gene transfer occurs in mature muscle and a long-term transgene expression of up to 18 months has been observed in mouse skeletal muscle [44, 65, 78], the use of this viral vector is limited in its application for DMD by its restricted gene insert capacity (<5 Kb). The recent development of truncated dystrophin, which can effectively restore the dystrophin-associated protein and therefore protect the muscle fiber from degeneration, may offer the possibility to use the rAAV for an efficient delivery in the dystrophic muscle [80].

Another approach to gene complementation in skeletal muscle has been investigated: *ex vivo* gene transfer, which consists of a combination of myoblast transplantation and gene therapy. It has been shown that isogenic myoblasts transduced with adenovirus [30], retrovirus [66], and HSV-1 [10, 72, 73] *in vitro* can deliver reporter genes to muscle. The efficiency of gene transfer of viral vectors (same number of viral particles) has been found to be superior using the *ex vivo* versus the direct gene transfer approach [10]. Furthermore, we recently evaluated the feasibility of myoblast-mediated *ex vivo* gene transfer of full-length dystrophin in mdx mice [24]. We transduced myoblasts that were incapable of producing dystrophin with an adenoviral vector lacking all the viral genes but expressing full-length dystrophin and β-galactosidase [46]. We then evaluated the ability of these transduced myoblasts to restore the dystrophin protein in mdx muscle. Both the adenovirally transduced primary mdx myoblasts and an immortalized mdx cell line could express β-galactosidase and full-length dystrophin *in vitro*. These transduced myoblasts were subsequently injected into dystrophic mdx muscle, where the injected cells restored dystrophin as well as dystrophin-associated proteins [24]. The *ex vivo* procedure remained limited by a cellular immune response that provided only a transient transgene expression. In fact, 25 days after myoblast-mediated gene transfer, we observed massive infiltration of CD4+ and CD8+ activated lymphocytes in muscles injected with either the immortalized myoblast cell line or primary myoblasts. This demonstrates that the lack of persistence of the transgene expression using the *ex vivo* approach is, at least in part, attributed to immune-rejection [24].

For this paper, we investigated the use of the *ex vivo* approach based on early myogenic progenitor cells that display a high cell survival rate and non-viral vectors to overcome the limitations facing myoblast transplantation. Plasmid DNA encoding for the neomycin resistance gene, the β-galactosidase marker gene and mini-dystrophin was engineered and used to establish a stable transfection in highly purified mdx myogenic cells that were transplanted into dystrophic mdx mouse muscle. The level of gene transfer, the persistence of transfected myofibers (dystro-

phin/LacZ), and the presence of immune response against the transduced myofibers in both mdx and transgenic mdx/β-gal mice were monitored for up to 3 months post-injection.

Materials and Methods

The strategy of myoblast-mediated ex vivo gene transfer of non-viral vectors to skeletal muscle

Fig. 1 shows a schematic representation of the strategy used for myoblast-mediated *ex vivo* gene transfer of non-viral vectors. First, muscle-derived cells were isolated from the tibialis anterior of mdx muscle and highly purified by the preplate technique [59, 64]. These highly purified muscle-derived cells display both early myogenic and stem cell characteristics [49]. In fact, these cells express high levels of myogenic markers such as desmin [17], m-cadherin (a satellite cell marker, [40]), myogenin [4], MyoD [54], BCL-2 (a marker limited to cells in the early stage of myogenesis, [54]), and various markers of stem cells, including CD34 [6, 13, 23, 67], FLK-1 [81], and Sca-1 [27]. We therefore called these muscle-derived cells early myogenic progenitor cells [49].

Construction of plasmid for mini-dystrophin, LacZ, and neomycin resistance gene expression

The early myogenic progenitor cells were subsequently transfected with a plasmid encoding for the β-galactosidase reporter gene under the control of the human cytomegalovirus promoter (HCMV), the mini-dystrophin gene under the control of the chicken β-actin/cytomegalovirus (CAG) promoter, and the neomycin resistance gene under the control of the phospho-glycerate kinase (PGK) promoter (see Fig. 1). The construct used for transfection was generated in pBR322 as follows: A SmaI/SalI fragment containing the neomycin resistance gene under the PGK promoter from pPGK-NEO was cloned into the SmaI/SalI cut pIEPLacZ containing the LacZ gene under the HCMV promoter to generate pNEO-LacZ. A XhoI/SalI fragment containing the gene encoding a short version of dystrophin [80] from DysM3 (obtained from Dr. Takeda) was inserted into the SalI site in pNEO-LacZ to generate the plasmid of mini-dystrophin, LacZ, and neomycin resistance genes. The plasmid (Fig. 1) was linearized by SalI digestion before transfection.

Transfection and screening

The cells were transfected with 10 μg of the linear plasmid of mini-dystrophin, LacZ, and neomycin resistance genes and 40 μg of lipofectamine reagent according to the standard protocol (Gibco BRL, MD, USA). At 72 hours post-transfection, the transfected cells were selected under 3 mg/mL of geneticin (G418; Gibco BRL, MD, USA) for the additional 10 days until colonies appeared. Colonies were then picked up and grown until confluence. The clones carrying the gene encoding the mini-dystrophin,

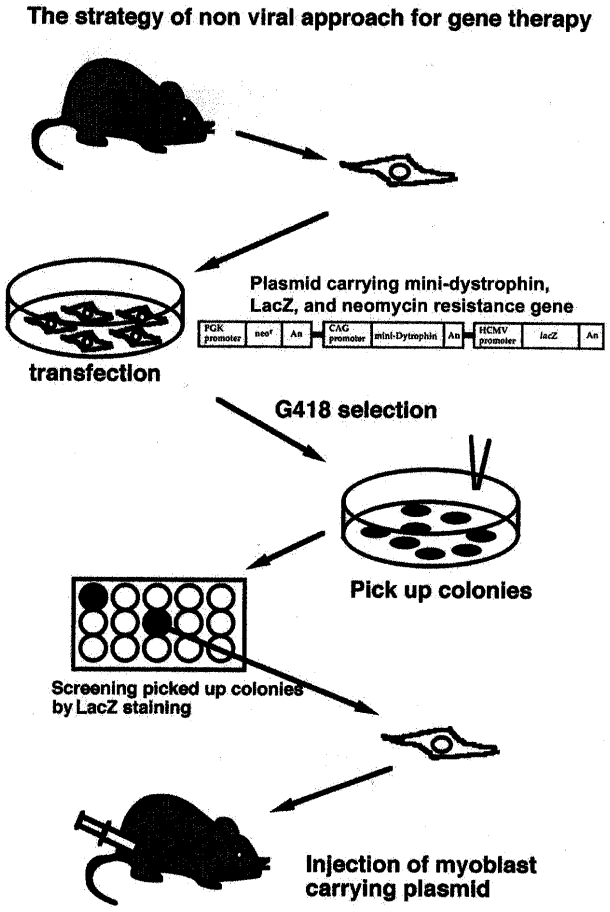


Figure 1. Schematic representation of the strategy used for muscle-derived cells' mediated ex vivo *gene transfer of non-viral vectors. A muscle biopsy was obtained, and the muscle-derived cells were isolated and purified by the preplate technique [67, 68]. The cells were subsequently transfected with the plasmid, selected under G418, and expanded to obtain large quantities of transfected myoblasts. The β-galactosidase reporter gene was under the control of the HCMV promoter. The mini-dystrophin gene was under the control of the CAG promoter. The neomycin resistance gene was under the control of the PGK promoter. The plasmid DNA was used for the transfection. After selection and expansion, the cells were injected into mdx mice intramuscularly.*

LacZ and neomycin resistance were isolated and expanded (see Fig. 1 for schematic representation).

Cell culture

The selected transfected muscle-derived cells were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10% horse serum (HS), 0.5% chicken embryo extract (CEE), and 1% penicillin and streptomycin (PS) (all of which were purchased from Gibco BRL, Grand Island, NY, USA). In order to induce differentiation into myotubes, the proliferation medium was switched to fusion medium

(DMEM supplemented with 1% FBS, 1% HS, and 1% PS) for 2-5 days at 37°C.

Myoblast transplantation in mdx mice

C57BL10J mdx/mdx mice (8-10 weeks old) were used as recipients for our experiments. 3×10^5 clonal isolated early myogenic progenitor cells carrying the gene encoding mini-dystrophin, LacZ, and neomycin resistance were injected in the gastrocnemius of mdx mice with a Drummond syringe. In order to investigate the persistence of the transgene expression (mini-dystrophin and LacZ gene), the mice were sacrificed at different time points after injection (5, 15, 30, 45, 60, 75, and 90 days) with the expression of dystrophin and LacZ expression monitored. Three specimens were used to count the number of LacZ positive fibers for 5 to 30 days post-injection, and two specimens were used for 45 to 90 days post-injection. In addition, to survey the immune response, immunostaining against CD4+ and CD8+ activated lymphocytes was performed (see below). The experimental animals were kept in the Rangos Research Center Animal Facility of Children's Hospital of Pittsburgh. The policies and procedures of the animal laboratory were in accordance with those detailed in the guide for the Care and Use of Laboratory Animals published by the U.S. Department of Health and Human Services.

LacZ staining

Transfected cells *in vitro* and cryostat sections of the injected and control muscles *in vivo* were stained for LacZ expression using the following technique: The cells and muscle tissues were fixed with 1% glutaraldehyde (Sigma Chemical Co.) for 1 minute, rinsed twice in phosphate buffered saline (PBS) and incubated in X-gal substrate (0.4 mg/mL 5-bromochloro-3 indolyl-β-D-galactoside [Boehringer-Mannheim, Indianapolis, IN, USA], 1 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$/5 mM $K_3Fe(CN)_6$ in PBS) for 3 hours overnight (37°C). After the LacZ histochemistry, the muscle sections were counterstained with hematoxylin/eosin and visualized by light microscopy (Optiphot microscope; Nikon, Inc., Melville, NY).

Immunohistochemistry for dystrophin & β-galactosidase

Muscle sections were fixed with cold acetone (100%) for 1 minute and rinsed with PBS. The sections were blocked with 10% horse serum (Vector, CA, USA) in PBS for 1 hour. After fixation, the sections were incubated in polyclonal sheep anti-dystrophin (dy10; 1:250) overnight. After subsequent washing, the sections were incubated with a goat anti-sheep antibody conjugated to biotin (Vector, CA, USA) at a dilution of 1:250 for 1 hour. After removal of the secondary antibody, the sections were rinsed in PBS and finally incubated with streptavidin-Cy3 (Sigma, MO, USA) at a dilution of 1:250 for 1 hour. After several rinses in PBS, the muscle sections were mounted in gel mount (Biomeda, Foster City, CA) and observed using fluorescent microscopy (Nikon). The muscle sections were also stained for DAPI to localize the nuclei within the dystrophin-positive myofibers and determine the number of centrally and peripherally located nuclei.

Some muscle sections were also used for co-localization of β-galactosidase and dystrophin, which was performed using the following protocol: The muscle sections were first fixed with cold acetone (100%) followed by a 1 hour blocking step with horse serum (10%), and the dystrophin was detected by immunohistochemistry as described above. The sections were subsequently incubated with a biotinylated anti-β-galactosidase antibody (1/100 in PBS; Sigma) followed by streptavidin conjugated to fluorescein, (1/300 in PBS, Sigma) to stain the β-galactosidase expressing myofibers. After several rinses in PBS, the muscle sections were mounted in gel mount (Biomeda, Foster City, CA) and observed using fluorescent microscopy (Eclipse E 800 Nikon microscope). The co-localization of dystrophin (red) and β-galactosidase (green) was then performed.

Detection of CD4+ and CD8+ activated lymphocytes

The muscle sections were fixed with cold acetone for 10 minutes, and the non-specific binding site was blocked with a goat serum (5%). The sections were incubated with Avidin D blocking solution for 20 minutes, rinsed briefly with PBS, then incubated for 20 minutes with the biotin blocking solution (4 drops per 1 mL of the diluted blocking serum; Vector, CA, USA). A rat monoclonal antibody against CD4 and CD8 (Pharmingen, CA, USA) was incubated for 1 hour at room temperature. The endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 5 minutes. After several rinses in PBS, the sections were incubated with a biotinylated goat anti-rat antibody (Vector, CA, USA, 1/500) for 1 hour. After an additional rinse with PBS, the sections were finally incubated with Vectastain Elite ABC (5 mL PBS plus two drops A and B; Vector, CA, USA) for 30 minutes. The peroxidase activity was revealed using 3',3'-diaminobenzidine (1 mg/mL; Sigma) and hydrogen peroxide (0.03%), and was counterstained with hematoxylin. Muscle sections were then mounted in gel mount (Biomeda) and visualized by light and fluorescent microscopy (Nikon).

Myoblast transplantation in transgenic mdx/β-gal mice

Heterozygotes of transgenic β-galactosidase and mdx mice (transgenic mdx/β-gal mice) (8-10 weeks old) were also used as recipients for this experiment to investigate whether the immune response observed in mdx mice was triggered by the bacterial β-galactosidase. 3×10^5 isolated highly purified mdx muscle-derived cells carrying the gene encoding mini-dystrophin, LacZ, and neomycin resistance were injected in the gastrocnemius of mdx/β-gal mice with a Drummond syringe. In order to investigate the persistence of the transgene expression (mini-dystrophin and LacZ gene), the mice were sacrificed at different time

points after injection (15, 45, and 90 days) with the dystrophin and LacZ monitored.

Results

Clonal isolation of early progenitor myogenic cells expressing mini-dystrophin, LacZ, and neomycin resistance gene

At 10 days post-transfection, colonies appeared and were picked up. The LacZ staining of the colonies showed that 23.9% of them were positive (Fig. 2A) and 76.1% were negative (Fig. 2B). Although the majority of the LacZ-positive colonies included LacZ-negative cells, a few 100% LacZ-positive colonies were found (Fig. 2A). After the colonies were picked up, the rate of LacZ-positive cells decreased with time in cell cultures. Few clones were identified that remained completely LacZ-positive even after 2 months of culturing. Therefore, one of the permanently transfected muscle-derived clones (Fig. 2C) was chosen for our experiments. These muscle cells were capable both of expressing LacZ reporter gene in their undifferentiated state (Fig. 2C) and of fusing into myotubes that expressed mini-dystrophin (Fig. 2D).

Investigation of persistence of transfected cells in the skeletal muscle

These transfected muscle-derived cells were injected in skeletal muscle of mdx mice, and the LacZ and dystrophin expressing myofibers were monitored at several time points post-injection.

In order to investigate the persistence of the transduced myofibers, mice were sacrificed at 5, 15, 30, 45, 60, 75, and 90 days post-injection, and the number of LacZ- and dystrophin-positive myofibers was monitored and compared among the different groups.

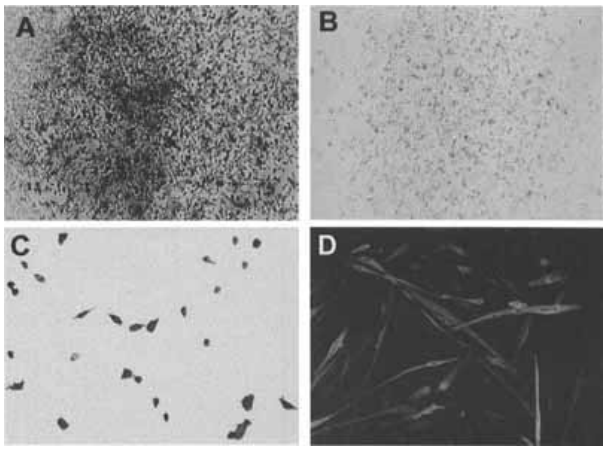


Figure 2. The detection of β-galactosidase and dystrophin in the transfected mdx muscle cells. Some of the selected colonies were expressing β-galactosidase reporter gene (Fig. 2A) while others were found to be β-galactosidase-negative (Figure 2B). The selected colonies that expressed the β-galactosidase reporter gene were expanded. The resulting cells were found capable of expressing the LacZ reporter gene (Fig. 2C) and differentiating into myotubes that therefore expressed dystrophin (Fig. 2D). Magnification: 20X.

Many LacZ- and dystrophin-positive myofibers were detected in the injected muscle at 5 days post-injection (Fig. 3A, B). The number of LacZ- and dystrophin-positive myofibers decreased at 15 days post-injection (Fig. 3E, F) but was stabilized from 30 (Fig. 3I-J) to 75 days (Fig. 3M-N) post-injection. The number of LacZ-positive myofibers was monitored at these time points and is shown in Fig. 4. In fact, 460 ± 273 LacZ-positive myofibers were found at 5 days, which decreased to 92 ± 54 at 15 days post-injection. However, the number of LacZ-positive myofibers did not change significantly between 1 month and 3 months post-transplantation (Fig. 4A). The same decline in the number of dystrophin-positive myofibers as observed with LacZ-positive myofibers was observed between 5 days and 90 days post-transplantation (Fig. 4B).

The injection of transfected dystrophic myoblasts triggered infiltration of lymphocytes

The detection of CD4+ and CD8+ activated lymphocytes at the injected site of the transplanted muscle at 5, 15, 30, and 75 days after injection suggests that the transplantation of the transfected muscle-derived cells

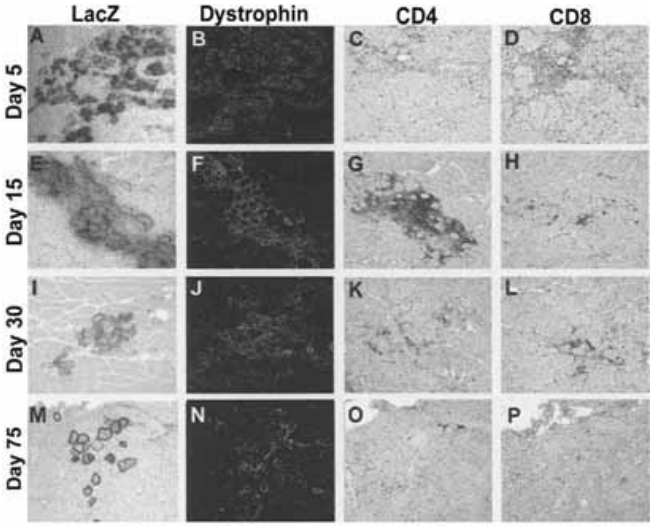


Figure 3. The transfected cells were capable of delivering β-galactosidase and dystrophin in mdx muscle. The transplantation of transfected cells resulted in the production of β-galactosidase (A, E, I, M) and dystrophin expressing myofibers (B, F, J, N) in the injected muscle at 5 (A, B), 15 (E, F), 30 (I, J), and 75 (M, N) days post-injection. The detection of CD4+ (C, G, K, O) and CD8+ activated lymphocytes (D, H, L, P) in the injected muscle at 5 (C, D), 15 (G, H), 30 (K, L), and 75 (O, P) days after injection suggests that the transplantation of the transfected myoblasts triggered an infiltration of CD4 and CD8 activated lymphocytes in the injected muscle. Magnification: 20X.

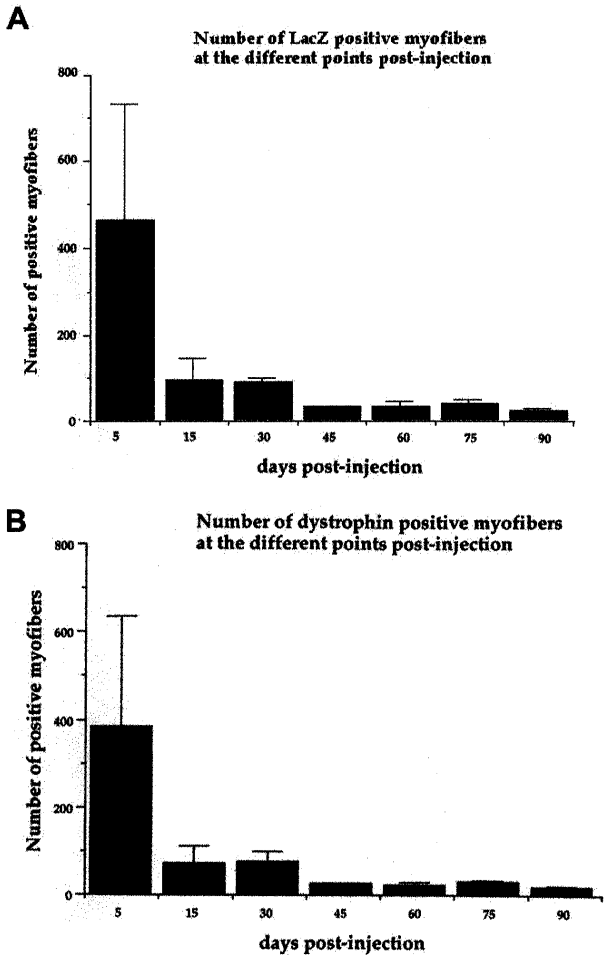


Figure 4. Lack of persistence of β-galactosidase and dystrophin in mdx mice. The number of LacZ- and dystrophin-positive myofibers was monitored at the different time points: 5, 15, 30, 45, 60, 75, and 90 days post-injection (means ± standard deviation).

still triggered an immune response. A few CD4+ and CD8+ activated lymphocytes were found in the injected areas at 5 days post-injection (Fig. 3C, D). Although the number of CD8+ activated lymphocytes did not increase at 15 days post-injection, an increase of CD4+ activated lymphocytes was observed (Fig. 3G, H). However, the number of activated lymphocytes significantly decreased after 30 days (Fig. 3K, L) following injection, suggesting that the immune response is significantly reduced at 1 month post-injection. A few CD4+ and CD8+ activated lymphocytes were still observed in the injected muscle at 75 days post-injection (Fig. 3O, P).

Persistence of gene transfer in transgenic mdx/β-gal mice

These transfected muscle-derived cells were also injected into the skeletal muscle of transgenic mdx/β-gal mice, which display a weak expression of β-galactosidase in myogenic nuclei [12]. In order to investigate the persistence of the transduced myofibers, mice were sacrificed at 15, 45, and 90 days post-injection, and the number of LacZ- and dystrophin-positive myofibers was monitored and compared among the different groups (Fig. 5). We observed that the LacZ- and dystrophin-positive myofibers detected in the injected muscle at 15 days post-injection (A, B) did not decline at 45 (C, D) and 90 days (E, F) post-injection (Fig. 5). A slight infiltration of CD4+ and CD8+ activated lymphocytes was detected in the injected transgenic mdx mice, but the level was very low and similar to that of non-injected mdx mice (not shown).

The co-localization of dystrophin and β-galactosidase at the cell membrane

We observed that mini-dystrophin (Fig. 6C) co-localized with β-galactosidase reporter gene (Fig. 6A, B) at the cell membrane of myofibers. In fact, by superimposing the two

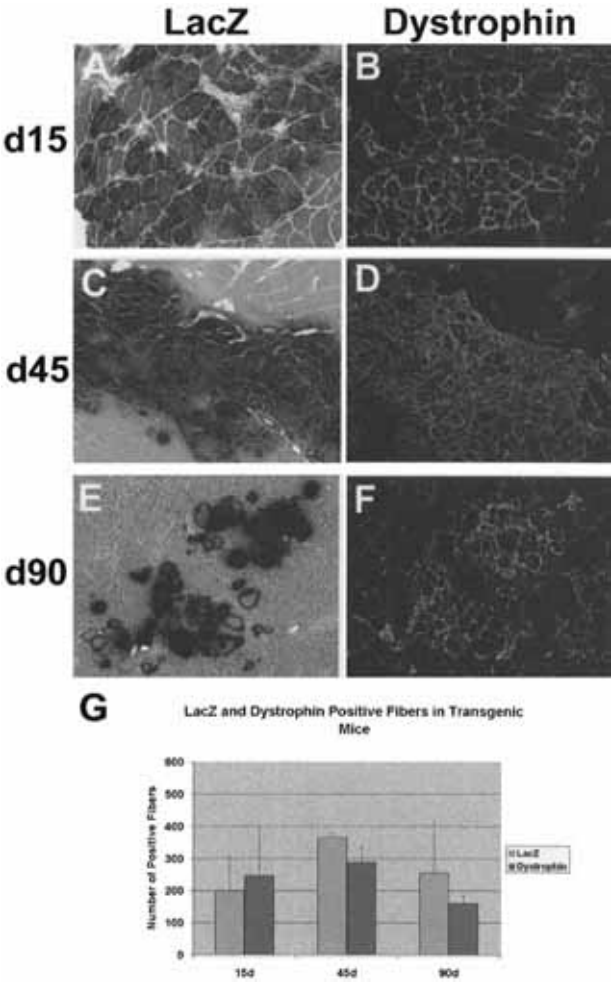


Figure 5. Long-term persistence of β-galactosidase and dystrophin in transgenic mdx/β-gal mice. The transplantation of transfected cells in the transgenic animals resulted in the production of β-galactosidase (A, C, E) and dystrophin expressing myofibers (B, D, F) in the injected muscle at 15 (A, B), 45 (C, D), and 90 (E, F) days post-injection. The number of LacZ-and dystrophin-positive myofibers did not decrease significantly between 15 and 90 days post-injection (G). Magnification: 20X.

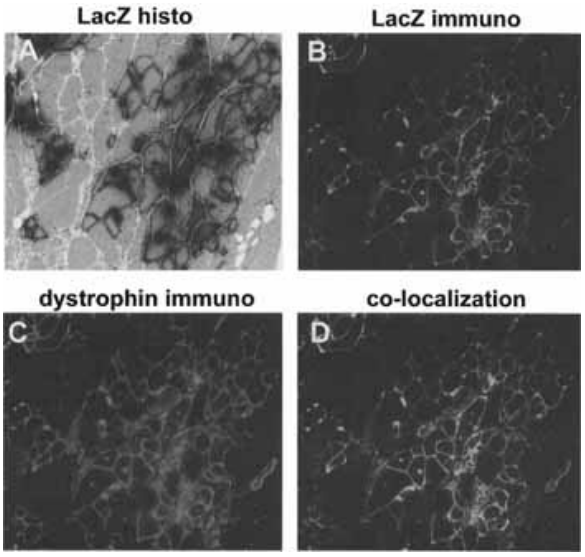


Figure 6. Co-localization of β-galactosidase and mini-dystrophin at the cell membrane of myofibers. The co-localization of dystrophin (C, D) with β-galactosidase (A, B) at the cell membrane (D) of the transfected myofibers suggests that both proteins fused and were transported at the cell membrane. Magnification: 40X.

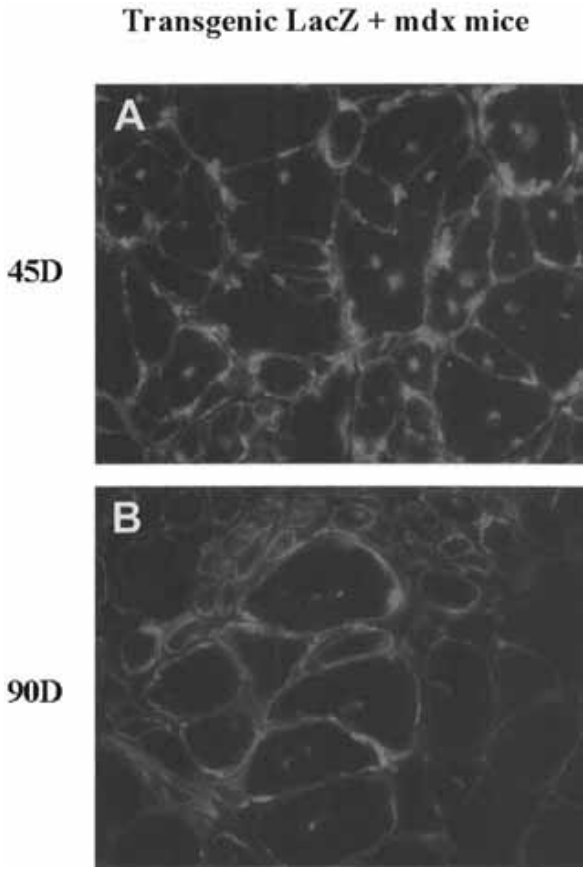
served a perfect co-localization of both proteins (Fig. 6D). This result suggests that the β-galactosidase fuses with the mini-dystrophin and therefore that the complex migrates at the cell membrane.

Functional property of the mini-dystrophin

The number of centro-nucleated dystrophin-positive myofibers was monitored at different time points post-injection in both the mdx and the transgenic mdx/β-gal mice. Although we observed a better persistence of the transfected dystrophin-positive myofibers in the transgenic mice when compared with the mdx mice, a high number of centro-nucleated myofibers was observed in the transgenic mdx/β-gal animals at 45 (Fig. 7A) and 90 days post-injection (Fig. 7B). These results suggest that the mini-dystrophin was incapable of protecting the transfected myofibers against muscle degeneration.

Discussion

For this paper, we tested whether the use of non-viral vectors in an *ex vivo* gene transfer to skeletal muscle may help reduce the immune-rejection problems and consequently lead to long-term persistence of the transgene in the injected muscle. We transfected a population of early myogenic progenitor cells with a plasmid DNA encoding for β-galactosidase, dystrophin and neomycin resistance and found that the transfected cells were capable of expressing dystrophin and LacZ reporter gene *in vitro*. Moreover, the transplantation of the transfected myoblasts is also capable of delivering the transgene in skeletal muscle of mdx mice *in vivo*. Although a major, rapid



decrease in the number of dystrophin-positive myofibers

Figure 7. The partial functional property of the mini-dystrophin. The number of centro-nucleated dystrophin-positive myofibers was monitored at 45 (A, 45D) and 90 days post-injection (B, 90D) in the transgenic mdx/β-gal. We observed a high number of dystrophin-positive that are centro-nucleated myofibers at both time points. These results suggest that the mini-dystrophin was incapable of protecting the transfected myofibers against muscle degeneration. Magnification: 60X.

was observed after implantation, some dystrophin-positive myofibers were found to persist for up to 3 months post-injection. It is likely that the rapid decline of the transduced myofibers early post-implantation is related to the infiltration of CD4+ and CD8+ activated lymphocytes triggered by the β-galactosidase reporter gene since a persistent transgene expression was observed with this method in transgenic mdx/β-gal mice. The salient features of the results presented in this report are as follows:

Isolation of a permanently transfected clonal population of early myogenic progenitor cells

The screening of many muscle cell colonies (500) resulted in the isolation of LacZ-positive and negative colonies. We observed that a large percentage of these colonies were incapable of expressing the LacZ reporter gene. This observation can be attributed to the fact that some cells are

resistant to neomycin but are incapable of expressing the reporter gene due to an alteration of the expression cassette within the transfected cells during cell culturing. Although we investigated and found that these transfected cells contain intact non-viral sequences in their genomes (not illustrated), we cannot exclude the possibility that the expression is not altered.

On the other hand, we observed that a low percentage of these colonies express the LacZ reporter gene, but not in 100% of the cells. In fact, the number of LacZ-negative cells within these colonies tends to increase with the cell culturing. The reason for this phenomenon is similar to that expressed above. Finally, we isolated some colonies that contained only cells expressing the transgene. The expansion of these colonies *in vitro* did not appear to decrease the number of LacZ expressing cells. This clonal cell population also expressed early (desmin and BCL-2) and late myogenic markers (m-cadherin, myogenin, and MyoD) as well as stem cell markers (Sca-1, FLK-1, and CD34). These results suggest the isolation of a new population of early myogenic progenitor cells [49].

The transfected muscle-derived cells can express the neomycin resistance and the β-galactosidase reporter genes as well as differentiate into myotubes that express dystrophin. These results suggest that although these cells have been transfected and selected with a high concentration of geneticin (3 mg/mL), they are still capable of proliferating and differentiating. These data also support the prospect that it is possible to stably transfect muscle-derived cells and obtain clones of transfected cells that express the transgenes due to the integration of the non-viral DNA into the host chromosomes.

The use of these stably transfected cells can deliver dystrophin in mdx skeletal muscle

The transplantation of these transfected myoblasts was capable of delivering the reporter gene and, more importantly, dystrophin in the injected mdx muscle. The co-localization of the LacZ-positive myofibers with the dystrophin expressing myofibers validates that these myofibers were formed by the injected muscle-derived cells and were not revertant dystrophin-positive myofibers. We also observed by immunofluorescence in many myofibers formed by the fusion of the transfected myoblasts that the β-galactosidase was co-localized with the dystrophin at the plasma membrane of the myofibers. Although the mechanism by which β-galactosidase is expressed at the plasma membrane is unclear, we hypothesized that these two transgenes can fuse together and were transported to the cell membrane.

In contrast to our results with adenovirus [24], many dystrophin-positive myofibers were found capable of persisting for 45, 60, 75, and 90 days post-injection. The persistence of a small number of dystrophin-positive myofibers at 3 months post-injection suggests that these myofibers can remain within the injected muscle for a long period of time. The elucidation of the mechanism by which these myofibers persist for a long period of time in the injected muscle will help to develop strategies to alleviate the muscle weakness in DMD patients.

The myoblast-mediated ex vivo gene transfer based on non-viral vectors does not totally eliminate the immune response

Although a small number of transduced myofibers were found to persist for up to 3 months post-injection, a high number of dystrophin-positive myofibers disappeared within the first month post-injection. A significant decrease in transduced myofibers appeared to occur within the first 15 days, but the number of myofibers seemed to remain stable thereafter. Indeed, the remaining myofibers did not decrease in number significantly between 15 and 90 days post-injection.

Concomitant with this disappearance of transduced myofibers, an infiltration of lymphocytes, especially CD4+, was observed at 15 days post-implantation and seemed to decrease at 30-90 days after implantation. The mechanism by which the transduced myofibers triggered an immune response at 15 days, which then decreased and consequently allowed for a long-term persistence of the transduced myofibers, is unknown. The ability of some transfected cells to fuse with pre-existing myofibers and become immunoprotected remains a potential explanation for the persistence of the transduced myofibers at 30-90 days post-implantation. Indeed, we recently observed that muscle cells that fuse exclusively together and/or with host myoblasts lead to a shorter persistence of the reporter gene in contrast to that observed when specific populations of cells fuse preferentially with host myofibers [60, 61].

The presence of the immune response against the transfected cells may be attributed to the bacterial β-galactosidase, which has been found immunogenic in many gene transfer protocols [12]. Indeed, the persistent transgene expression observed with the same approach in transgenic mdx/β-gal mice suggests that the immune response observed in the mdx mice is triggered by the bacterial β-galactosidase. The development of a myoblast-mediated non-viral gene transfer approach using plasmid vectors without β-galactosidase will likely decrease the immunogenicity and therefore allow long-term persistence of a larger number of transfected myofibers.

The ex vivo approach improves the non-viral gene transfer to mature myofibers

It was observed that a poor level of gene transfer occurs with non-viral vectors in mature myofibers. Moreover, an improvement of gene delivery via non-viral vectors was achieved by artificial induction of muscle regeneration and/or the use of dystrophic muscles [16]. These results suggest that the gene transfer by non-viral vectors in mature myofibers, like that observed with viral vectors [10, 24, 72, 73], is at least in part mediated by myoblasts,

which are activated in injured and dystrophic muscles. Indeed, the presence of many myofibers expressing the reporter gene at 5 days post-injection (450 myofibers) suggests that the use of the myoblast-mediated *ex vivo* gene transfer approach allows a better delivery of the non-viral vectors in the skeletal muscle of adult mice.

The limited functional property of the mini-dystrophin

The presence of centro-nucleated dystrophin-positive myofibers was monitored as an index of the functional property of the mini-dystrophin. Although we observed in both the mdx and the transgenic mdx/β-gal mice that some of the dystrophin-positive myofibers were peripherally nucleated, the vast majority of the transfected myofibers were centro-nucleated (Fig.7). This result suggests that the mini-dystrophin is unable to efficiently protect the transfected myofibers against degeneration and therefore that the development of new mutant functional dystrophin is required to achieve an efficient protection of the dystrophic skeletal muscle.

In summary

The *ex vivo* approach can create a reservoir of muscle-derived cells capable of both regenerating and restoring dystrophin to dystrophic muscle and thus presents an important alternative treatment, particularly for DMD patients over 10 years of age whose myofibers have become extremely inefficient in regeneration and may be refractory to direct viral transduction. Although the number of transduced myofibers decreased rapidly after transplantation, some myofibers expressing the transgene were also found in the injected muscle for up to 3 months post-implantation. The myoblast-mediated *ex vivo* gene transfer of non-viral vectors does not totally overcome the immune response, which was observed early post-implantation. Based on our results obtained in the transgenic mdx mice that expressed β-galactosidase, we hypothesized that the deletion of the bacterial β-galactosidase within the plasmid DNA would likely further reduce the immune-rejection problem and therefore improve the long-term persistence of the transgene within the dystrophic muscle. Finally, the myoblast-mediated *ex vivo* gene transfer, like other vectors, can improve the delivery of plasmid DNA in skeletal muscle of 2-month-old mice when compared with the direct injection of the non-viral vector.

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