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Cell-lineage Regulated Myogenesis for Dystrophin Replacement: a Novel Therapeutic Approach for Treatment of Muscular Dystrophy

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ABSTRACT

Duchenne muscular dystrophy (DMD) is characterized in skeletal muscle by cycles of myofiber necrosis and regeneration leading to loss of muscle fibers and replacement with fibrotic connective and adipose tissue. The ongoing activation and recruitment of muscle satellite cells for myofiber regeneration results in loss of regenerative capacity in part due to proliferative senescence. We explored a method whereby new myoblasts could be generated in dystrophic muscles by transplantation of primary fibroblasts engineered to express a micro-dystrophin/eGFP (µDys/eGFP) fusion gene together with a tamoxifen-inducible form of the myogenic regulator MyoD [MyoD-ER(T)]. Fibroblasts isolated from *mdx<sup>4cv</sup>* mice, a mouse model for DMD, were efficiently transduced with lentiviral vectors expressing µDys/eGFP and MyoD-ER(T) and underwent myogenic conversion when exposed to tamoxifen. These cells could also be induced to differentiate into µDys/eGFP-expressing myocytes and myotubes. Transplantation of transduced *mdx<sup>4cv</sup>* fibroblasts into *mdx<sup>4cv</sup>* muscles enabled tamoxifen-dependent regeneration of myofibers that express micro-dystrophin. This lineage control method therefore allows replenishment of myogenic stem cells using autologous fibroblasts carrying an exogenous dystrophin gene. This strategy carries several potential advantages over conventional myoblast transplantation methods including: 1) the relative simplicity of culturing fibroblasts compared with myoblasts, 2) a readily available cell source and ease of expansion, and 3) the ability to induce MyoD gene expression *in vivo* via administration of a medication. Our study provides a proof of concept for a novel gene/stem cell therapy technique and opens another potential therapeutic approach for degenerative muscle disorders.
INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked, recessive lethal genetic disorder caused by mutation of the dystrophin gene (1, 2). DMD is the most common neuromuscular disorder affecting approximately 1 in 3,500 newborn males (3). Affected boys are usually diagnosed between 3 to 5 years of age (4). Early symptoms of delayed walking and gait disturbance rapidly progress to general muscle and especially proximal weakness. By age 12, most patients use a wheelchair for mobility and often develop severe scoliosis and limb contractures. While improved clinical management has extended the life expectancy of patients in recent years, most still die in their mid to late 20’s from respiratory and/or cardiac failure (5). Current treatment options for DMD patients focus primarily on relief of symptoms and prevention of complications, as there is no effective treatment (6).

In DMD, mutation of the dystrophin gene results in little or no production of a functional dystrophin protein (1, 2, 7). It is postulated that dystrophin along with the dystrophin-glycoprotein complex (DGC) plays an important role in protecting muscle fibers from eccentric contraction-induced injury (8-10). The absence of dystrophin results in membrane instability and repeated tears in the sarcolemma with calcium entry into the muscle cell (11). The resulting cascade of events lead muscle fibers to undergo cycles of degeneration and regeneration until the repair capacity is no longer sufficient, and muscle fibers are replaced by adipose and fibrous connective tissue (12, 13).

In various strains of mdx mice, models for DMD, viral vector mediated gene replacement \textit{via} delivery of therapeutic micro-, mini- or full-length dystrophin genes has been shown to
prevent dystrophic changes in neonatal and young adult mice (14-18). Recent studies also suggest that such vectors can stop further progression of the dystrophic phenotype and bring back some strength in old mdx mice (19, 20). Patients with DMD, even as young boys, have fibrotic changes and fatty deposition in their affected muscles suggesting that muscle repair capacity may be already overtaxed (12). In addition, as muscle weakness progresses, patient muscles gradually lose myofibers and it becomes increasingly difficult to isolate myoblasts that retain a robust ability to proliferate when cultured in vitro (21). Therefore, even if viral vectors were able to target the remaining muscle fibers and myogenic precursors, there may be insufficient residual regenerative capacity to bring back significant muscle mass and strength in older patients.

Strategies to treat DMD by transplantation of wild type (allogeneic) myoblasts have previously been unsuccessful, in large part due to immune responses against the donor myoblasts (22-26). Genetic modification of cells with subsequent transplantation back into the target organ (ex vivo gene therapy) represents an alternative treatment option for patients with DMD. However, this approach is hampered by the difficulty in isolating sufficient numbers of myoblasts from a patient’s own muscle that can be transduced with a dystrophin gene and expanded in culture without undergoing proliferative senescence, losing myogenic capacity or losing the ability to express muscle genes following terminal differentiation in vivo (21, 27-29). Nonetheless, the idea of using genetically modified autologous cells as a myogenic source still holds the advantage of potentially avoiding most of the immunological problems associated with the allogeneic approach. There are indications that several different types of stem cells besides
satellite cells can contribute to muscle regeneration and/or reconstitution of muscle satellite cells (30, 31). However, a major limitation for stem cell therapy is the availability of an adequate cell source. In addition to difficulties in harvesting homogeneous stem cell populations, maintaining these stems cells in the appropriate developmental stage also poses great challenges. Another issue may be tied to the multipotency of a stem cell. Due to the possibility of a stem cell being able to take various developmental pathways to becoming different cell types, there is the attendant difficulty of ensuring that a stem cell ultimately differentiates into the desired cell type for therapeutic purposes.

In this study, we evaluated fibroblasts as a source of myogenic progenitors as a therapeutic potential in the treatment of muscle disorders. In 1998, Lattanzi and coworkers demonstrated the feasibility of adenoviral mediated MyoD gene delivery to cultured fibroblasts as a potential source for muscle fiber generation in vitro. These authors showed that myoblasts converted from fibroblasts with MyoD in culture are able to form muscle fibers in vivo after intra-muscular transplantation (32). Huard and coworkers also showed that cultured myoblasts generated from MyoD transduced fibroblasts could form myofibers in vivo, but at a very low efficiency (33). To assess its feasibility as a potential therapeutic approach in DMD, we have investigated myogenic conversion of transplanted primary mdx fibroblasts using a tamoxifen-inducible MyoD-ER(T) gene in combination with a therapeutic gene, micro-dystrophin/eGFP, under the control of the muscle specific human skeletal $\alpha$-actin promoter. We have also used a lentiviral vector to stably transduce primary mdx$^{4cv}$ fibroblasts ex vivo to allow for greater expansion of cells prior to transplantation back into the mdx muscles.
Here we show that temporally inducible MyoD-ER(T) expression results in regulated myogenic conversion of cultured and transplanted primary fibroblast both in vitro and in vivo. Fibroblasts converted into the myogenic lineage in vivo can differentiate into dystrophin-expressing myofibers. Thus, our study demonstrates proof of principle that temporally controlled in vivo myogenic conversion of transplanted fibroblasts is feasible and opens the possibility that introduction of a drug-inducible MyoD gene with a therapeutic gene could lead to not only rescue of existing muscle fibers but may also facilitate repopulation of the muscle mass in myopathies.

RESULTS

Characterization of the MyoD-ER(T) vectors

A mouse MyoD cDNA and a second modified to contain the human estrogen receptor (for 4-hydroxytamoxifen, see methods) were initially tested by plasmid transfection into 293D cells. Western analysis of the cell lysates showed both MyoD and MyoD-ER(T) bands, at 44 kDa and 64 KDa, respectively, indicating production of the appropriate protein products. The MyoD protein loaded in lane 1 serves as a positive control (Fig. 1A). Lentiviral vectors used in this study are illustrated in Figure 1B.

In vitro myogenic conversion of 10T1/2 fibroblasts

To confirm that lentiviral mediated MyoD-ER(T) expression was inducible and functionally effective in initiating myogenic conversion of fibroblasts in vitro, 10T1/2 embryonic fibroblast
cell lines were infected with a variety of vectors. The 10T1/2 cells were sequentially transduced with a reporter gene-carrying virus (Lv-CMV-\textit{nlacZ}) at an MOI of 10 and with either Lv-CMV-MyoD or Lv-CMV-MyoD-ER(T), each at an MOI of 20. Clones expressing both the reporter gene and MyoD or MyoD-ER(T) were isolated, challenged with 1 µM of 4-hydroxytamoxifen (4OH-tamoxifen) for 24 hours then switched to myogenic differentiation medium (Fig. 2A and B). Almost all of the cells expressing MyoD-ER(T) fused into multinucleated myotubes within 48 hrs of culturing in differentiation media (Fig. 2B). No myotubes were observed in 10T1/2 cell cultures that had not been transduced with Lv-CMV-MyoD-ER(T) (Fig. 2A), nor were myotubes found in cultures not exposed to 4OH-tamoxifen (data not shown). In contrast, cells transduced with the L-CMV-MyoD vector underwent spontaneous differentiation into myocytes and myotubes, limiting the ability to expand the cell population. These results suggest that the MyoD-ER(T) protein may be less active than MyoD, allowing proliferation and expansion of the transduced cells as non-terminally differentiated myoblasts. Findings were similar with mouse 3T3 cells (data not shown). 10T1/2 cells transduced with both the \textit{lacZ} and the MyoD-ER(T) vectors were also intramuscularly transplanted into the TA muscles of 2-day-old C57Bl/6 mice after 24 hrs of 4OH-tamoxifen exposure. At 14 days after cell transplantation, TA muscles were harvested, fixed, and stained with X-gal. Nuclear-targeted β-galactosidase was clearly observed in some of the muscle fibers (Figures 2C-E). These data showed that 10T1/2 fibroblasts transduced with Lv-CMV-MyoD-ER(T) and exposed to 4OH-tamoxifen undergo myogenic conversion and are capable of forming myofibers \textit{in vivo} after transplantation into mouse muscle.
**In vivo myogenic conversion of primary fibroblasts from dystrophic mdx mice**

Primary cultured fibroblasts isolated from tails of mdx<sup>4cv</sup> mice were transduced with a lentiviral vector expressing a micro-dystrophin/enhanced green fluorescent protein fusion protein (Lv-HSA-\(\mu\)Dys/eGFP) alone or with Lv-CMV-MyoD-ER(T) (Fig. 1B). To monitor the transduction efficiency and function of MyoD-ER(T) as well as the muscle-specificity of the human \(\alpha\)-skeletal actin (HSA) promoter, some of the cells were exposed to 1 \(\mu\)M 4OH-tamoxifen for 24 hours. In cells transduced with the MyoD-ER(T) and receiving 4OH-tamoxifen, myotube formation was noted approximately 48 hours after switching to differentiation media (Fig. 3, right). These myotubes strongly expressed the skeletal muscle isoform of myosin heavy chain (MHC; Fig. 3, bottom panel) a skeletal muscle differentiation marker. In those cells infected with both Lv-HSA-\(\mu\)Dys/eGFP and Lv-CMV-MyoD-ER(T), expression of \(\mu\)Dys/eGFP was observed in approximately 70% of the multinucleated myotubes (Table 1). Cultures not exposed to tamoxifen displayed no myotube formation or \(\mu\)Dys/eGFP expression (Fig. 3), although a small number of MHC positive cells were observed (Table 1). These results show that MyoD-ER(T) was active in initiating myogenesis in the primary fibroblasts and subsequently in inducing multinucleated myotube formation, and confirm that the HSA promoter used to drive expression of \(\mu\)Dys/eGFP retained its muscle-specificity in the context of a lentiviral vector integrated into fibroblasts.

The doubly transduced mdx<sup>4cv</sup> primary fibroblasts as well as those transduced only with Lv-HSA-\(\mu\)Dys/eGFP were cultured and expanded for 1-2 weeks before transplantation into TA
muscles of \textit{mdx}^{4cv} mice. Two weeks after intra-muscular transplantation of \(1.0 \times 10^6\) fibroblasts, during which daily intra-peritoneal injections of 4OH-tamoxifen were performed, the TA muscles were harvested and cryosectioned. A second cohort was analyzed two weeks later. Numerous \(\mu\text{Dys/eGFP}\) positive fibers (160.3 +/- 53.9, \(n=8\)) were found in transplants using MyoD-ER(T) transduced cells coupled with 4OH-tamoxifen treatment, while essentially no positive fibers were detected in muscles injected with cells not expressing MyoD-ER(T) or in animals not receiving 4OH-tamoxifen (Fig. 4A and B). Fibroblasts from the transplantation were observed in the negative control groups. There were significant differences between transplants receiving the MyoD-ER(T) and \(\mu\text{Dys/eGFP}\) transduced cells combined with 4OH-tamoxifen administration as compared with the other three negative control groups: no 4OH-tamoxifen administration, 1.16 +/- 0.41, \(n = 8\); 4OH-tamoxifen administration but cells not transduced with the MyoD vector, 0.89 +/- 0/40, \(n = 5\); cells not transduced with either vector, 1.00 +/- 0.50, \(n = 4\); with \(P < 0.05\), \(P < 0.05\), and \(P < 0.05\), respectively (Fig. 4B). Four weeks after transplantation and two weeks of 4OH-tamoxifen administration, \(\mu\text{Dys/eGFP}\)-positive fibers were still present (41.7 +/- 14.6, \(n = 6\)). Again, essentially none were detected in the negative control groups that did not receive 4OH-tamoxifen treatment (0.17 +/- 0.17, \(n = 6\), \(P < 0.05\))(Table 1).

Histological analysis revealed 2 classes of \(\mu\text{Dys/eGFP}\) expressing fibers in transplanted muscles. Many fibers were of normal size, suggesting that transplanted cells had fused with pre-existing myofibers (Fig. 4A). However, we also observed numerous clusters of small-caliber \(\mu\text{Dys/eGFP}\)-positive fibers (diameters < 15 \(\mu\text{m}\)), as detected using anti-GFP
antibody (Figures 4B and 5). By analyzing serial sections we found dystrophin-positive fibers spread out over an area at least 1.5 mm past the site of injection (Fig. 5B). To identify whether the small caliber fibers represented newly generated myotubes, we looked for developmental myosin heavy chain (dMHC) expression. As shown in Figure 5A, almost all of these small, µDys/eGFP-positive fibers expressed dMHC. These studies indicate that transplanted fibroblasts expressing MyoD-ER(T) were converted to myogenic cells in vivo under the control of 4OH-tamoxifen, and subsequently followed the myogenic program by forming new myotubes and myofibers and also fusing into existing muscle fibers.

**DISCUSSION**

Clinical features of DMD result from the absence of a single protein, dystrophin, which ultimately leads to skeletal muscle loss and gradual replacement by fibrotic and fatty tissue. Treatment of DMD in older patients might therefore require replenishment of myogenic progenitor cells or the muscles themselves. In this study we focused on fibroblasts as a potential source of myogenic progenitors. Studies have shown that transfection of fibroblasts with the muscle specific transcription factor MyoD causes differentiation of the fibroblasts into myoblasts (34). In addition, it appears that the efficiency of myogenic conversion is strongly linked to the lineage relationship with muscle cells: highest in fibroblasts, lower in chondroblasts, and null in hepatocytes (35). Other studies demonstrate that fibroblasts of different histological origins can spontaneously convert to myogenic phenotypes both in vitro and in vivo, albeit at a very low efficiency (36, 37). Of practical importance is the relative ease
with which fibroblasts can be isolated, cultured, expanded and harvested. These advantages have allowed fibroblasts to be safely used in other clinical settings (38). Although primary fibroblasts cannot be passaged indefinitely due to the onset of proliferative senescence, 20-30 divisions are feasible. Thus, fibroblasts represent an intriguing cellular target for \textit{ex vivo} cell/gene therapy and myogenic conversion \textit{in vivo}.

We have investigated the feasibility of converting fibroblasts to myogenic cells using an inducible MyoD-ER(T) gene, with an estrogen receptor component responsive to tamoxifen medication for expression, in conjunction with a lentiviral gene delivery method. This strategy provides additional advantages beyond those outlined above for fibroblasts as a potential cell source for myogenesis. The use of MyoD-ER(T) takes advantage of temporally controlled expression of MyoD \textit{in vivo} through administration of a medication already in clinical use, tamoxifen. In addition, we have chosen to utilize a retroviral method to stably transduce the fibroblasts. Previously, others have used adenoviral delivery systems (32). However, the lentiviral system with MyoD-ER(T) may be better suited because it takes advantage of the relative ease with which fibroblasts can be expanded compared with myoblast cultures. Once the fibroblasts are stably transduced with MyoD-ER(T), they can be expanded using simplified culture conditions prior to transplantation back into the target organ. Myoblasts themselves can be difficult to expand greatly in culture and have a tendency to give rise to differentiation defective variants (28, 39). Additionally, the use of MyoD-ER(T) prevented fibroblasts from prematurely differentiating into post-mitotic myocytes, as happens with unregulated MyoD, and therefore their proliferative capabilities are preserved.
To assess its feasibility as a potential therapeutic approach for treatment of DMD, we have coupled the use of MyoD-ER(T) in fibroblasts with a therapeutic gene/reporter, micro-dystrophin/eGFP, under the control of the muscle specific human skeletal α-actin (HSA) gene promoter, in the mdx<sup>4cv</sup> animal model. This combination allows for both specificity and control of the therapeutic gene expression by first having the fibroblasts undergo myogenic conversion, then having the muscle specific promoter express the therapeutic gene. Initiation of the myogenic conversion is controlled by exogenous administration of the medication tamoxifen.

We demonstrated that MyoD-ER(T) activity was efficiently controlled by 4OH-tamoxifen administration both <em>in vitro</em> and <em>in vivo</em>. MyoD-ER(T) was able to transform primary cultured fibroblasts from mdx<sup>4cv</sup> mouse into myogenic cells, both <em>in vitro</em> and <em>in vivo</em>. Our study also showed that systemically administered tamoxifen achieved adequate local intramuscular dosage and was capable of efficiently activating MyoD-ER(T). These initial transplantation studies serve as a model for <em>ex vivo</em> autologous myogenic transplantation by demonstrating the potential use of primary mdx fibroblasts to generate myofibers expressing micro-dystrophin in the mdx<sup>4cv</sup> mouse. These μDys-positive muscle fibers were found as both newly formed myotubes and also as fully mature myofibers. The latter likely arose by fusion between transplanted fibroblasts that converted <em>in vivo</em> to the myogenic lineage and subsequently fused with pre-existing, dystrophin-negative myofibers. However, we cannot exclude the possibility that the dystrophin-positive myofibers arose from maturation of nascent myotubes. Given that we observed no μDys-positive myofibers without tamoxifen activation, the fibroblasts themselves appear incapable of efficient fusion into myofibers without the myogenic conversion initiated by
MyoD-ER(T). Evidence that the small caliber dystrophin-positive cells were nascent myotubes comes from both their morphology and that they were immunoreactive with antibodies for the developmental isoform of myosin heavy chain. These nascent myotubes likely formed from fusion of exogenous, converted fibroblasts either with themselves or with endogenous myotubes or activated satellite cells. The larger µDys-positive myofibers did not express developmental myosin heavy chain.

A curious result in our study was a loss in the number of dystrophin-positive myotubes between the 2 and 4 week time points, while the number of dystrophin-positive myofibers remained stable. This data could indicate that the nascent myotubes are unable to mature. It is unclear whether converted fibroblasts that fuse only with themselves or with endogenous myocytes would be able to develop normal myotendinous or neuromuscular junctions, without which maturation into normal myofibers would be blocked. This scenario is likely to be an issue for any type of myogenic stem cell transplantation, and it may be important to optimize transplant methods for fusion of myoblasts and nascent myotubes with preexisting, innervated myofibers. Alternatively, this data could mean that the small myotubes are being converted to myofibers, and that there is a slow loss of both myotubes and myofibers due to an immune response against transplanted cells, or that an immune response is directed specifically at myotubes, which, unlike myofibers, express MHC class I (40, 41). The histological data were suggestive of an inflammatory response in transplanted muscles, although there is a certain background of immune cell infiltration in untreated mdx muscles (Fig. 4A;(42)).

Several aspects of the current protocol could have contributed to such an inflammatory
response. The genetically modified fibroblasts used for transplantation expressed two exogenous proteins that could potentially act as foreign antigens. The first was the micro-dystrophin/eGFP fusion that was used as both a therapeutic and a marker protein. Micro-dystrophin by itself has not elicited an immune response in previous studies, and the eGFP moiety is not essential for the strategy other than to simplify monitoring of transplanted cells (19, 20). The second exogenous protein was the MyoD-ER(T) fusion protein. While mouse MyoD was employed in this study the estrogen receptor sequence was human and it also carried the single amino acid substitution that confers responsiveness to tamoxifen (43). Use of the murine sequence might alleviate concerns about ER antigenicity. Another potential trigger of inflammation might be the transplanted fibroblasts, as in vitro cell culturing can produce cell surface composition changes that can be recognized as foreign. Modified culture or cell washing conditions that avoid bovine serum may eliminate this concern, and we note that others have not observed inflammatory responses when transplanting murine fibroblasts (32). As with other retroviral mediated gene therapies, one risk of using lentiviral mediated gene delivery is tumorigenesis or other perturbations of cellular function due to random integrations (44). In this study, we did not observe any tumor formation after transplantation in either MyoD-ER(T) positive or negative cells transduced with Lv-HSA-μDys/eGFP. However, these potential side effects and risks are important considerations for future studies.

This is the first report demonstrating the use of MyoD-ER(T) to initiate myogenesis in vivo using fibroblasts. Other recent studies have shown that retroviral-mediated gene transfer can be used to convert mouse and human fibroblasts into pluripotent cells with embryonic stem
(ES) cell phenotypes (45-47). Converting fibroblasts into ES cells raises the possibility of a ready source of stem cells for tissue engineering of numerous tissues, including striated muscle. Our studies suggest that it may be simpler to convert fibroblasts directly into a myogenic lineage for skeletal muscle engineering, although it is tempting to speculate whether a combination of these approaches might yield a progenitor cell lacking the proliferative limitations of primary fibroblasts while retaining a preference for differentiation down a myogenic pathway. Similarly, several other cell types have been studied in recent years as a source of myogenic precursors, including mesenchymal stem cells (48), pericytes (49), mesoangioblasts (50), and AC133+ cells (51), and it will be important to address the potential benefits of genetically modifying these other cell types prior to transplantation.

The studies in this manuscript represent a proof of principle that fibroblasts can be used to support muscle regeneration in vivo. A number of aspects of the approach could clearly be modified to increase the efficiency of the method. These include exploring other routes of delivery besides direct local intra-muscular implantation, particularly delivery via the vasculature (50), and modifying the genes that are delivered via lentiviruses. A particularly exciting potential for this approach is the use of systemic gene delivery methodologies to potentially allow for transduction and myogenic conversion of fibroblasts in vivo (16). Such an approach would eliminate the need to isolate and culture fibroblasts in vitro prior to transplantation, and could enable the generation of new myogenic stem cells directly within muscles that have undergone extensive wasting and fibrosis due to years of dystrophic degeneration.
MATERIAL AND METHODS

Lentiviral vector constructions and preparations

The MyoD-ER(T) construction methods have been described previously (52). Briefly, the full-length mouse MyoD cDNA was isolated and cloned from a previously described adenoviral vector (32). A cDNA for the modified estrogen receptor responsive to tamoxifen and 4-hydroxytamoxifen, ER(T) (43), was cloned using a PCR method with the primer pair including an internal ClaI restriction enzyme site: (sense) 5’-ACGGCATCGATTCTGCTGGAGACATGAGAGCT-3’, and (anti-sense) 5’-GCGCCATCGATGACTGTGGCAGGGAAACCCT-3’, and then inserted into the NarI site (aa 173) in the middle of the mouse MyoD cDNA. The final clone was verified by DNA sequencing.

Lentiviral vector construction and viral preparation followed methods described elsewhere (18). Briefly, transfer vectors were constructed by inserting the corresponding coding sequences into the polylinker of the pRRL-cPPT-CMV-X-PRE-SIN vector (53). The MyoD and MyoD-ER(T) as well as the reporter nlacZ vectors were constructed as previously described (18). VSV-G-pseudotyped lentiviral vectors were produced by cotransfecting into 293D cells (a kind gift from Dr. Dusty Miller), and purified by ultracentrifugation as described (53). The titer of viruses containing the reporter gene was measured by transducing NIH3T3 cells with serial dilutions of vector preparations, and also by quantitative RT-PCR methods which were adjusted with flow cytometry (FACS) data of well-known titered control GFP vector (Lv-MSCV-eGFP, data not shown) (54).
Cell culture

The confirmation of biological activity of MyoD-ER(T) was tested on NIH3T3 cells (ATCC: CRL-1658) and C3H/10T1/2 (ATCC: CCL-22) with 1 µM of 4-hydroxy-tamoxifen (4OHT, Sigma, St. Louis, MO) for 24 hours following a switch to differentiation media containing DMEM plus 2% horse serum. For primary fibroblast cultures methods were followed and modified from those previously described (29). Briefly, tail tips (about 1.5 mm) from 2 – 3 days old mdx4cv mice were cut and washed in 70% ethanol and 1x phosphate buffered saline (PBS) then minced with small bent scissors for 15 minutes and digested with 0.2 % Collagenase II and 1.2 U/ml of Dispase (both Worthington Biochemical Corporation, NJ) for 45 min. The finely minced tissues were then sparingly plated onto 100 cm tissue culture dishes, with fresh DMEM plus 10% Fetal calf serum and pen/strep for 5-7 days under a standard cell culture incubation environment. After stable colony formation was observed, the media was exchanged and large tissues were removed. The cells were then maintained in growth medium, with fresh media exchanged every 2-3 days. At day 14, after culture in DMEM/10% FCS, when cells were approximately 70-90% confluent, they were trypsinized and seeded onto new plates with fresh media. These cells were then stained with Col I/III antibody (1:400, Calbiochem, San Diego, CA) following Alexa 594 conjugated anti-rabbit IgG (H+L) (1:1200, Molecular Probes, Eugene, OR) to confirm that they were fibroblasts (data not shown).
**Lentiviral transduction**

Lentiviral vectors for transduction contained MyoD-ER(T) and nlacZ or micro-dystrophin/eGFP (µDys/eGFP). For 10T1/2 cells, 1x 10⁶ cells were transduced by Lv-CMV-nlacZ with or without Lv-CMV-MyoD-ER(T) with an MOI of 20 for each vector. For primary cultured mdx⁴cv fibroblasts, 1x 10⁶ cells were transduced by Lv-HSA-µDys/eGFP with or without Lv-CMV-MyoD-ER(T) at an MOI of 20. The transductions were confirmed with X-gal staining, anti-GFP antibody to detect expression of µDys/eGFP expression, anti-MyoD antibody to confirm the lentiviral transduction, and/or anti-myosin heavy chain (MHC) antibody to detect myogenic differentiation. Once cells became subconfluent, MyoD-ER(T) was activated by diluting 1 mM 4-hyroxytamoxifen (4OH-tamoxifen) suspended in ethanol to 1 µM in culture medium and incubating the cells for 48 hrs. The medium was then switched to DMEM/2% horse serum to stimulate differentiation and fusion of the myogenic converted fibroblasts into multi-nucleated myotubes. These myotubes were immunostained with monoclonal anti-MHC antibody (F59; Developmental Studies Hybridoma Bank, University Iowa, Iowa City, IA) followed by secondary anti-rabbit IgG–Alexa594 (Molecular Probes, Eugene, OR).

**Animal experiments**

C57Bl/6Ros.Cg-Dmd<sup>mdx<sub>4cv</sub></sup> (mdx<sub>4cv</sub>) and C57Bl/6 strains of mice were used. Animal experiments were approved by our Institutional Animal Care and Use Committee and were conducted in accordance with guidelines for humane treatment of laboratory animals. 10T1/2 cell harvest and centrifugation followed established protocols, and cells were resuspended in 30
µl of the PBS solution without Ca\(^{2+}\) and Mg\(^{2+}\) before injection into the tibialis anterior (TA) muscles. The cultured \(mdx^{4cv}\) primary fibroblasts were trypsinized, diluted in 2% fetal calf serum, then centrifuged. After centrifuging and decanting, cells were resuspended in medium (or PBS) with 10% mouse serum. After final centrifugation, the cells were resuspended in an appropriate volume of PBS (without Ca\(^{2+}\) and Mg\(^{2+}\)) at a concentration of 1x 10\(^6\) cells/30 µl. The cells were kept on ice prior to injection. Each TA muscle of 6-7 week old \(mdx^{4cv}\) mice was injected with 30 µl of cell suspensions. The preparation of 4OH-tamoxifen as a ligand of MyoD-ER(T) has been described (55). Briefly, the 4OH-tamoxifen (1 mg/100 µl suspended in 5% ethanol and 95% peanut oil) was given once a day for a total of 14 days, from 1 day prior to transplantation to day 13 post-transplantation. The animals were sacrificed at either 14 or 28 days after transplantation.

**Muscle sections, immunostaining and statistical analysis**

The muscles were carefully dissected and cryopreserved using Optimal Cutting Temperature compound and liquid nitrogen cooled isopentane, then muscle sections of 10 µm thickness were prepared using a cryostat. Immunostaining against dystrophin and GFP proteins were performed as previously described (18). Briefly, the dilution ratios of the antibodies were as follows: anti-GFP antibody, 1:2000 (Molecular Probes, Eugene, OR); anti-N terminal dystrophin, 1:600 (56); and mouse monoclonal anti-developmental MHC (dMHC), 1:40 (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK). Secondary antibodies included anti-rabbit IgG–Alexa488 (for GFP) and –Alexa594 (for dystrophin, both Molecular Probes,
Eugene OR), and anti-mouse IgG–Alexa594 (for dMHC). Muscle sections were then mounted with VECTASHIELD containing 500 ng/ml 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, Vector Labs, Burlingame, CA).

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44. Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M.P., Wulffraat, N.,


LEGENDS TO FIGURES

Figure 1. Construction of lentiviral vectors expressing MyoD-ER(T), nlacZ, and micro-dystrophin/eGFP. (A) Left: A mutated estrogen binding site region (amino acids [aa] 282 to 595) from the estrogen receptor cDNA was inserted in-frame into the NarI site (aa 173) in the middle of the MyoD cDNA. This fusion gene was inserted into a plasmid containing the CMV promoter to drive expression of the MyoD-ER(T) fusion protein. Right: Western analysis of MyoD-ER(T) and MyoD expression in 293 cells transfected with CMV-MyoD or CMV-MyoD-ER(T): lane 1 was loaded with purified MyoD protein and shows a band at about 44 kDa.; lane 2 is the cell lysate from the CMV-MyoD-ER(T) transfected cells and shows a fusion protein at about 64 kDa; lanes 3 and 4 show negative (no-transfection) and positive controls (CMV-MyoD expression cassette was transfected). (B) The CMV-MyoD-ER(T) expression cassette was inserted into a lentiviral backbone (14). Lv-CMV-nlacZ, expressing a nuclear localizing β-galactosidase was also used as a reporter vector. The micro-dystrophin-enhanced green fluorescent protein (µDys/eGFP) fusion protein cDNA was generated by recombinant PCR and cloned into a lentiviral vector cassette under the control of the muscle specific human skeletal α-actin (HSA) gene promoter. The eGFP portion was included to easily distinguish transduced fibers from revertant fibers in mdx muscles. The titer of each virus stock was estimated at 3.0x10^8, 5.0 x10^8, and 3.0 x10^8 transducing units (TU)/ml. RRE, HIV Rev response element; cPPT, HIV-1 central polypyrimidine tract; PRE, human hepatitis virus post-transcriptional regulatory element.
Figure 2. Myogenic conversion of mouse embryonic 10T1/2 cells *in vitro* and in mouse muscles. The mouse embryonic 10T1/2 cells were doubly-transduced with Lv-CMV*nlacZ* and Lv-CMV-MyoD-ER(T). The cells were imaged before (A) and after (B) exposure to 1 μM of 4OH-tamoxifen (Sigma, St. Louis, MO). In the presence of tamoxifen, the cells turned into myoblasts and fused into myotubes that expressed lacZ (B) and myosin heavy chain (not shown). (C) Shows an entire hind limb of a C57Bl/6 mouse transplanted at 3 days of age with 10T1/2 fibroblasts that had been doubly-transduced with Lv-CMV*nlacZ* and Lv-CMV-MyoD-ER(T) then treated for 24 hours with 4OH-tamoxifen prior to transplant. Analysis was 2 weeks post-transplant. Low magnification view (C), high magnification view (D). (E) shows a dissected single myofiber isolated from the mouse shown in (C) and stained for β-galactosidase activity.

Figure 3. Efficient myogenic conversion of fibroblasts with MyoD-ER(T) controlled by 4OH-tamoxifen *in vitro*. Primary cultured fibroblasts isolated from *mdx* *mdx* mouse tail preps were transduced with Lv-HSA-μDys/eGFP alone or together with Lv-CMV-MyoD-ER(T). When cultured in the presence of 1 μM 4OH-tamoxifen (Sigma St. Louis, MO), cells co-transduced with both vectors fused into multinucleated myotubes expressing μDys/eGFP (green, anti-GFP staining) and myosin heavy chain (red; antibody F59). No myotubes were observed in cultures that were not incubated with the MyoD vector, and very few were observed in cultures not exposed to tamoxifen (see also Table 1). Bar represents 50 μm.
Figure 4. MyoD-ER(T) control of cell fate in vivo. (A) Primary \( mdx^{4cv} \) fibroblasts in culture were transduced with the lentiviral vectors Lv-HSA-\( \mu \)Dys/eGFP [\( \mu \)Dys/eGFP] and Lv-CMV-MyoD-ER(T) [MyoD-ER(T)] then transplanted into the TA muscles of 6-7 week old \( mdx^{4cv} \) mice. These animals were treated with or without 4OH-tamoxifen by intra-peritoneal injections daily for 14 days, then sacrificed 14 or 28 days after transplantation. Indicated on the left are the vectors used to infect the cells prior to each transplant, and whether or not the mice were injected with tamoxifen (tamox). No treatment refers to a control where the fibroblasts were not infected and no tamoxifen was administered to the mice. At the top is indicated the imaging method: eGFP, green fluorescence; Dystrophin, immunofluorescent labeling of dystrophin using antisera against the N-terminal actin-binding domain; H&E, hematoxylin and eosin staining. Myofibers expressing \( \mu \)Dys/eGFP were abundant in muscles injected with \( mdx^{4cv} \) fibroblasts that had been co-infected with both the dystrophin and the MyoD vectors only when 4OH-tamoxifen was administered to the mice (top row). Few positive myofibers were present after transplanting cells that were not co-infected with the MyoD vector (upper middle), when no 4OH-tamoxifen was administered (lower middle), or when using cells that were not transduced (bottom row). There were also no small fiber groupings observed in the three negative controls. As shown in the left and middle columns, micro-dystrophin and eGFP signals co-localized along the sarcolemma of positive fibers. This co-staining revealed that transplanted fibroblasts had fused into myofibers that expressed the micro-dystrophin/eGFP fusion protein from the muscle-specific \( \alpha \)-skeletal actin promoter (HSA). Lv-CMV-MyoD-ER(T) transduction and 4OH-tamoxifen administration to mice were required
for this efficient myogenic conversion event. Bar represents 50 µm. (B) The numbers of positive fibers at two weeks and four weeks after transplantation are shown as bar graphs. Blue bars show the total number of µDys/eGFP positive myofibers, green bars show positive fibers larger than 15 µm in diameter. Only the MyoD-ER(T) transduced and 4OH-tamoxifen exposed group showed significant numbers of positive myofibers. The left graph shows data from all four groups at 2 weeks, the right graph shows the MyoD-ER(T) transduced groups at both 2 and 4 weeks. There are significant differences between the treatment group and the three different negative controls: without MyoD-ER(T) (p< 0.001), without 4OH-tamoxifen (p< 0.001), and without either (p< 0.001).

**Figure 5.** New myofiber formation from transplanted fibroblasts via MyoD-ER(T) controlled myogenic conversion. TA muscle sections from mice injected two weeks earlier with MyoD-ER(T) transduced fibroblasts and treated with 4OH-tamoxifen. (A) Many of the small caliber myofibers were positive for micro-dystrophin/eGFP and also positive for the developmental isoform of myosin heavy chain (dMHC), used here as a marker for nascent myotubes and myofibers. The middle panel shows the merged picture, where yellow signals show newly generated fibers formed by transplanted fibroblasts marked with micro-dystrophin/eGFP. The middle right panel shows negative and positive controls for dMHC staining. No primary antibody control staining gave weaker background signals on interstitial spaces, the negative control, and the regenerating fibers were dMHC positive, the positive control is shown in the inset. Bar represents 50 µm. The right panel shows a high
magnification view of µDys/eGFP and dMHC positive fibers. The green signals localized along the sarcolemma and red signals in the sarcoplasm. Bar represents 10 µm. (B) The small micro-dystrophin/eGFP-positive fibers grouped longitudinally over at least 1.5 mm in the transplanted TA muscles. Four serial sections spaced 0.5 mm apart show the groupings of small micro-dystrophin/eGFP positive fibers in a transplanted *mdx*<sup>4cv</sup> TA muscle. Bar represents 50 µm.

**Table 1.** Percent MHC positive cells in fibroblast cultures transduced with lentiviral vectors

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<th>LV-µDys/eGFP</th>
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<th>Std error</th>
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ND: none detected

NA: not applicable
Figure 1
Figure 3
Figure 4A
Figure 4b
Figure 5