# High Efficiency Myogenic Conversion of Human Fibroblasts by Adenoviral Vector-mediated *MyoD* Gene Transfer

## An Alternative Strategy for Ex Vivo Gene Therapy of Primary Myopathies

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## Abstract

**Ex vivo gene therapy of primary myopathies, based on autologous transplantation of genetically modified myogenic cells, is seriously limited by the number of primary myogenic cells that can be isolated, expanded, transduced, and reimplanted into the patient's muscles. We explored the possibility of using the *MyoD* gene to induce myogenic conversion of nonmuscle, primary cells in a quantitatively relevant fashion. Primary human and murine fibroblasts from skin, muscle, or bone marrow were infected by an E1-deleted adenoviral vector carrying a retroviral long terminal repeat–promoted *MyoD* cDNA. Expression of *MyoD* caused irreversible withdrawal from the cell cycle and myogenic differentiation in the majority (from 60 to 90%) of cultured fibroblasts, as defined by activation of muscle-specific genes, fusion into contractile myotubes, and appearance of ultrastructurally normal sarcomagenesis in culture. 24 h after adenoviral exposure, *MyoD*-converted cultures were injected into regenerating muscle of immunodeficient (severe combined immunodeficiency/beige) mice, where they gave rise to β-galactosidase positive, centrally nucleated fibers expressing human myosin heavy chains. Fibers originating from converted fibroblasts were indistinguishable from those obtained by injection of control cultures of *lacZ*-transduced satellite cells. *MyoD*-converted murine fibroblasts participated to muscle regeneration also in immunocompetent, syngeneic mice. Although antibodies from these mice bound to adenoviral infected cells in vitro, no inflammatory infiltrate was present in the graft site throughout the 3-wk study period. These data support the feasibility of an alternative approach to gene therapy of primary myopathies, based on implantation of large numbers of genetically modified primary fibroblasts massively converted to myogenesis by adenoviral delivery of *MyoD* ex vivo. (*J. Clin. Invest.* 1998. 101:2119–2128.)** **Key words: myogenesis • cell therapy • muscular dystrophy • gene transfer • b-HLH myogenic factors**

## Introduction

Gene therapy of monogenic primary myopathies, such as Duchenne muscular dystrophy (DMD),[1] is based on the assumption that delivery of a transgene replacing the defective or missing protein in at least a fraction of the skeletal muscles would result in phenotypic correction of the defect, and amelioration of the clinical symptoms (1). The ex vivo approach to gene therapy of DMD is based on transplantation of autologous myogenic (e.g., satellite) cells engineered to express a functional dystrophin gene product. Previous attempts of transplanting myogenic cells from compatible donors in DMD patients have faced a number of problems (reviewed in 1), raising doubts on the feasibility of the entire myoblast transplantation approach. The use of genetically modified autologous myogenic cells should avoid most of the immunological problems associated with the allogeneic approach, while still providing enough protein product to allow normal or seminormal function by the newly formed fibers. Evidence that genetically corrected cells could reverse the clinical phenotype if delivered in sufficient numbers and at the right time to dystrophic muscle comes from observations of genetic normalization in muscle fibers of female DMD carriers with skewed inactivation of the X chromosome carrying the normal dystrophin allele (2). In the mouse model of muscular dystrophy, muscle fibers genetically engineered to produce a dystrophin or minidystrophin protein have been indeed shown to reverse the abnormal phenotype (reviewed in 1). Such evidence is still lacking in a human muscle context, although the general concept that human satellite cells can be transduced by retroviral vectors, and stably express a transgene after fusion into regenerating muscle fibers has been provided in human/immunodeficient mouse models (3, 4). Satellite cells obtained from Duchenne and Becker muscular dystrophy patients can be transduced with an efficiency comparable to that of normal cells, despite their reduced proliferative potential and life span in culture (4). However, the low recovery of satellite cells from dystrophic muscle biopsies and, therefore, the difficulty in ob-

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*Received for publication 14 August 1997 and accepted in revised form 24 February 1998.*

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/98/05/2119/10 $2.00
Volume 101, Number 10, May 1998, 2119–2128
http://www.jci.org

1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; DMD, Duchenne muscular dystrophy; LTR, long terminal repeat; MLC1F, myosin light chain fast 1; MLC3F, myosin light chain fast 3; *nLacZ*, β-galactosidase with a nuclear localization signal; *scid/bg*, severe combined immunodeficiency/beige; TA, tibialis anterior.

taining reasonable numbers of genetically modified cells, is clearly a limiting factor in devising an ex vivo gene therapy strategy for these patients. The problem becomes almost insoluble in advanced patients, where the number and growth potential of myogenic cells has been exhausted by the continuous degeneration/regeneration cycles (5).

It has been known for a decade (6) that forced expression of *MyoD* and related members of the myogenic family of basic helix-loop-helix transcription factors, would activate myogenesis in nonmuscle cells; this has been related to a possible default tendency of embryonic cells towards myogenesis (discussed in 7, 8). The efficiency of this process is directly related to the lineage relationship with myoblasts, being highest in fibroblasts, lower in chondroblasts, and virtually null in hepatocytes or amnioblasts (9). In keeping with these observations, we and others have reported recently that fibroblasts undergo myogenic differentiation when cocultured with myogenic lines (C2C12) or primary cells, but not with other cell types (10–12). It is thus possible that a proportion of adult cells of mesodermal origin conserve a bi- or multipotential state of determination throughout life, thereby enhancing the regenerative capacity of the tissue in which they reside (13).

Taking the above observations into consideration, we addressed the issue of whether the phenomenon of myogenic conversion of primary fibroblasts could be exploited in practical terms to provide an alternative source of transplantable myogenic cells. The rationale of this approach is that fibroblasts are not affected in their growth capacity by the dystrophic defects, and can be easily obtained from clinically accessible sites, expanded in vitro without significant limitation, and transduced at high efficiency by currently available technology. Genetically modified fibroblasts converted to myogenesis before implantation would represent an alternative, very abundant source of autologous myogenic cells for ex vivo gene therapy. Crucial to the success of this strategy is the efficiency of myogenic conversion. Conversion mediated by coculture or nonviral delivery of *MyoD* is limited to a small percentage of the target cell population, and is of little usefulness from a practical point of view. In fact, expression of *MyoD* severely impairs growth capacity, preventing the possibility of further expansion of converted cells in culture. In this report, we show that an adenoviral vector expressing *MyoD* under the control of a viral promoter can induce massive myogenic conversion of human and murine primary fibroblasts in culture, with an overall efficiency largely exceeding 50%. We report the successful validation of the whole experimental strategy in an in vivo model. Early passage primary human fibroblasts have been expanded in culture, transduced by a retroviral vector expressing a reporter gene, converted to myogenesis by adeno-delivered *MyoD*, and injected into regenerating muscles of severe combined immunodeficiency/beige (*scid/bg*) recipient mice, where they form apparently normal fibers at an efficiency comparable to that of satellite cells. The ex vivo exposure to the adenoviral vector causes humoral (anti-Ad5) but not cellular immune response against treated cells, as assayed in immunocompetent mice transplanted with converted syngeneic fibroblasts.

## Methods

*Reagents.* RPMI 1640, FCS, horse serum, and trypsin were obtained from GIBCO Laboratories (Grand Island, NY). Collagenase-dispase, dexamethasone, bromodeoxyuridine (BrdU; Boehringer Mannheim, Mannheim, Germany) geneticin (G418), β-mercaptoethanol, and fluorochrome-conjugated secondary antibodies were obtained from Sigma Chemical Co. (St. Louis, MO). Basic FGF and Dotap were obtained from Boehringer Mannheim; DiI (1,1′, dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate) was obtained from Molecular Probes (Eugene, OR). Anti-BrdU monoclonal antibody and [$^{14}$C]thymidine were obtained from Amersham Intl. (Slough, UK). Anti-Leu, anti-CD4, and anti-Mac3 rat anti–mouse leukocyte antigen antibodies were obtained from PharMingen (San Diego, CA).

*Cell cultures.* Fetal fibroblasts were isolated from skin and skeletal muscle of C3H mouse embryos (15–17 d) or legally aborted human fetuses (8–12 wk), as described (4). Murine, genetically marked fibroblasts, were isolated from *MLC3F/nlacZ* transgenic mice, expressing a nuclear β-galactosidase gene under the transcriptional control of the myosin light chain 3 fast promoter, which restricts expression to skeletal and cardiac muscle (14). Human and murine (from C3H or transgenic mice) satellite cells were isolated as previously described (4). Human adult dermal or muscle fibroblasts were obtained from tissues of patients undergoing posttraumatic surgery. Tissues were finely minced with scissors, digested with 2 mg/ml dispase, 0.1 mg/ml collagenase in PBS for 45 min at 37°C, washed in RPMI medium, and pipetted to obtain a single-cell suspension. Murine bone marrow fibroblasts were obtained by resuspending and plating cells flushed from the long bones of 8–10-wk-old *MLC3F/nlacZ* mice. Human bone marrow was obtained from healthy donors. After removal of nonadherent cells, fibroblasts were cultured in growth medium (see below) supplemented with 2 ng/ml bFGF.

All cells were grown in RPMI supplemented with 15% FCS, 1% gentamycin, and 0.3 mM β-mercaptoethanol (growth medium). Myogenic differentiation was induced by shifting the cells to RPMI supplemented with 2% horse serum (differentiation medium). Fibroblasts were purified by subculture (at least two rounds) in growth medium. Removal of myogenic cells was routinely verified by immunocytochemical staining of a cell aliquot subcultured for 5 d in differentiation medium.

In some experiments, cells were prelabeled with 0.5 μCi/ml of [$^{14}$C]thymidine (Amersham Intl.) for 24 h, and then exposed to the adenoviral vector expressing *MyoD* (see below); survival was measured by counting residual cpm incorporated.

*Cell transduction and transfection.* Fibroblasts (from 3rd to 10th passage) were infected as described (4) with a replication-defective retroviral vector (LBSN) expressing a cytoplasmic β-galactosidase gene under the long terminal repeat (LTR) promoter, and/or with an Ad5-derived, E1A-deleted adenoviral vector expressing the full-length murine *MyoD* cDNA under the transcriptional control of Rous sarcoma virus LTR (15). For nonviral transduction, $10^5$ cells were plated on 60-mm tissue culture dishes and transfected by standard calcium-phosphate or lipofectamine (Dotap) with 10 μg of the PMC11 plasmid, containing the *MyoD* cDNA under the control of the cytomegalovirus promoter. Alternatively, $2 \times 10^6$ cells were electroporated with 6 μg of the same plasmid in growth medium at 120V, 960 μF. After transduction, cells were grown for 24 h in growth medium, and then either shifted to differentiation medium for 3–4 d or injected in vivo (see below).

*Immunocytochemistry.* Immunofluorescence analysis was carried out as described (16) using the following antibodies: MF20, a monoclonal antibody that recognizes all sarcomeric myosins (17); a rabbit antiserum against sarcomeric proteins (18); an anti-*MyoD* polyclonal antibody (19); BD5, a monoclonal antibody that recognizes slow myosin heavy chains (20); a rabbit polyclonal antibody against human fetal myosin (21); an anti-BrdU monoclonal antibody; and anti-leu, anti-CD4, and anti-Mac3 rat anti–mouse leukocyte antigens antibodies. Briefly, cell cultures or cryostat sections were fixed with 4% paraformaldehyde for 10 min at 4°C, washed 3× in PBS, and incubated at 4°C with the primary antibodies in PBS + 1% BSA. Cultures labeled with BrdU were treated for 10 min at room temperature with 2 M HCl and washed 3× with PBS before incubation with the anti-

BrdU antibody. After the first incubation, cells or sections were washed 3× with PBS + 1% BSA and incubated for 1 h at room temperature with rhodamine-conjugated goat anti–mouse Ig or with a fluorescein-conjugated goat anti–rabbit Ig (1:100 dilution). Cultures or sections were then washed, mounted in 75% glycerol/PBS (pH 7.5), and observed under a Zeiss Axiophot epifluorescence microscope.

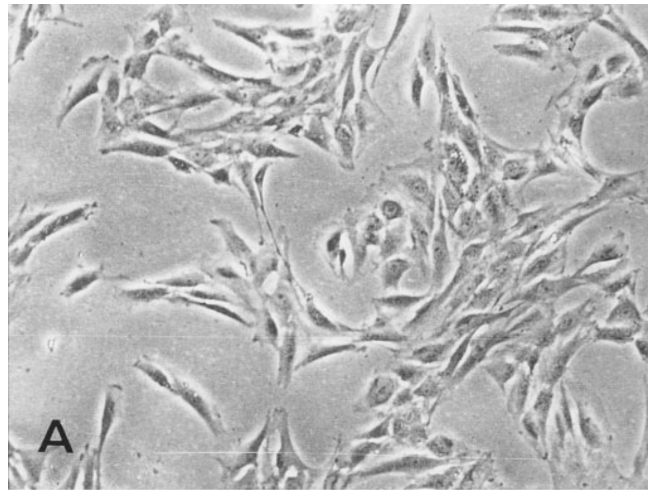
*Electron microscopy.* Cell cultures were washed in PBS, fixed in 2% glutaraldehyde in 0.1 M Millonig's buffer (pH 7.4) for 1 h at 4°C, postfixed for 1 h in 1% buffered osmium tetroxide, and dehydrated in graded alcohol. Cells were treated with propylene oxide, embedded in Epon 812, and cut into ultrathin sections. The ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Zeiss 109 electron microscope.

*RNA and DNA analysis.* Total RNA was extracted by the method of Chomczynski et al. (22), run in 15-μg aliquots on 1% agarose/formaldehyde gels, and transferred by capillary Northern blotting to nylon membranes (Hybond-N; Amersham Intl.). Filters were cross-linked for 2 h at 80°C under vacuum, and hybridized to [$^{32}$P]-labeled probes for *MyoD* and *myogenin* (23), myosin light chain 1 fast (MLC1F) and M creatine kinase (MCK) (24), and acetylcholine receptor (ACh) α-subunit (25) under standard conditions (26). Southern dot blot analysis with a probe recognizing *Alu* sequences was performed on DNA samples extracted from the tibialis anterior (TA) of immunodeficient (*scid/bg*) mice previously injected with human cells (see below) as reported (4).

*Implantation of MyoD-converted fibroblasts into mouse muscles.* Transduced and converted human or murine fibroblasts (1 or 2 × $10^6$ cells) were trypsinized, resuspended in 20–50 μl of PBS, and injected into a single site of the regenerating tibialis anterior muscle of either syngeneic (C3H) or immunodeficient (*scid/bg*) mice which had received a 30-μl injection of $10^{-5}$ M cardiotoxin (Latoxan) 48 h earlier. For some experiments, myogenically converted mouse fibroblasts were labeled with DiI dye 1 h before injection in vivo (a 0.5% solution of DiI in absolute ethanol was diluted just before use in 0.3 M sucrose to a final concentration of 0.05%). Mice were killed after various periods of time, and muscles were cryosectioned and stained for β-galactosidase activity or processed for immunohistochemistry. At the time of killing, serum was collected from C3H mice and reacted with adeno-infected or control cells at various dilution, followed by a fluorescein-conjugated anti–mouse IG secondary antibody.

## Results

*Adenoviral vector-mediated delivery of MyoD induces massive myogenic conversion of primary fibroblasts.* We initially compared different methods of conversion to myogenesis primary fibroblasts by using third-passage late fetal dermal fibroblasts from *MLC3F/nLacZ* transgenic mice. Conversion of these cells can be easily and quantitatively monitored by counting the number of β-gal$^+$ nuclei at the end of each experimental treatment. Infection with a recombinant adenoviral vector expressing the murine *MyoD* cDNA for 3 h at a (moi) (plaque-forming units/cell) of 2,000 induced a massive myogenic conversion of the fibroblast culture, which reached 83% of the total cell population. In comparison, cocultivation with wild-type satellite cells (12), treatment with 1 μM dexamethasone (27), and transfection by calcium-phosphate, lipofectamine, or electroporation of the same plasmid expressing *MyoD*, induced myogenic conversion in a low percentage of the population, ranging from 1% for dexamethasone treatment to 14% for electroporation (data not shown). This result likely reflects the different transduction efficiency of the various methods, as a similar experiment with a *lacZ* reporter gene gave comparable results (not shown).



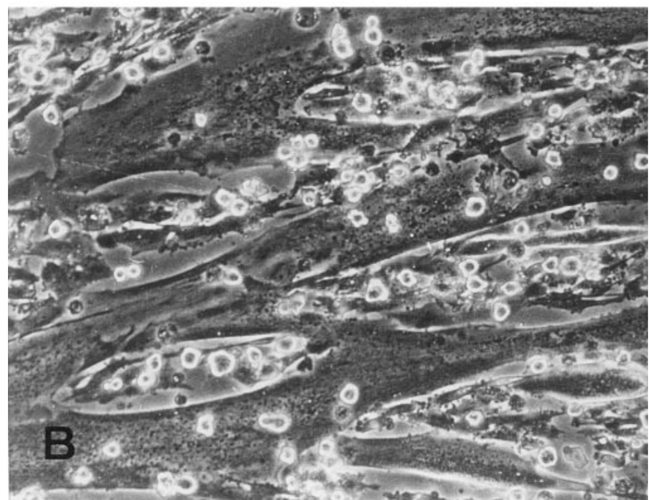




*Figure 1.* Phase-contrast microphotographs of human fetal dermal fibroblasts before (*A*) and 5 d after (*B*) exposure to the *MyoD* adenoviral vector at an moi of 2,000.

Efficiency of conversion increased linearly by increasing the moi from 500 and reached a plateau at ~ 2,000. At this concentration, cytotoxicity, resulting in cell death in culture, was < 30% of the plated cells, in agreement with previous observations (15). Similar results were obtained with human muscle or dermal fibroblasts (scored by immunostaining with MF20 antimyosin antibody), which were best converted (50–70% in different experiments) at an moi of 2,000. In both murine and human fibroblasts, efficiency of conversion did not vary with the number of passages in culture (we compared 3rd, 6th and 10th passages); however, when the cells reached senescence, they could no longer be converted at all. Phase-contrast appearance of human fetal skin fibroblasts before and after adeno-*MyoD*–induced conversion is shown in Fig. 1 (*A* and *B*).

*Dermal fibroblasts are converted more efficiently than fibroblasts of other origin.* Previous studies have shown that fibroblasts of different histological origin have a different potential for myogenic conversion induced by coculture with satellite cells (12). Therefore, we compared the relative efficiency of

*Table I. Efficiency of Myogenic Conversion of Human and Murine Fibroblasts of Different Origin*

| | Murine | | | Human | |
|---|---|---|---|---|---|
| | Fetal | Neonatal | Adult | Fetal | Adult |
| Dermis | 70 | 51 | 56 | 65 | 42 |
| Muscle | 43 | 42 | 44 | 40 | 32 |
| Bone marrow | ND | 7 | 6 | ND | 6 |

Fibroblasts were isolated, expanded, infected with the *MyoD* adenoviral vector for 3 h at an moi of 2,000, cultured for an additional day, and then induced to differentiation (see Methods). The cultures were fixed and immunostained with MF20 antimyosin antibody. Efficiency of conversion was measured as percentage of cells which expressed sarcomeric myosin heavy chains. Values shown are the average of two separate experiments, each performed in triplicate. *ND*, Not done.

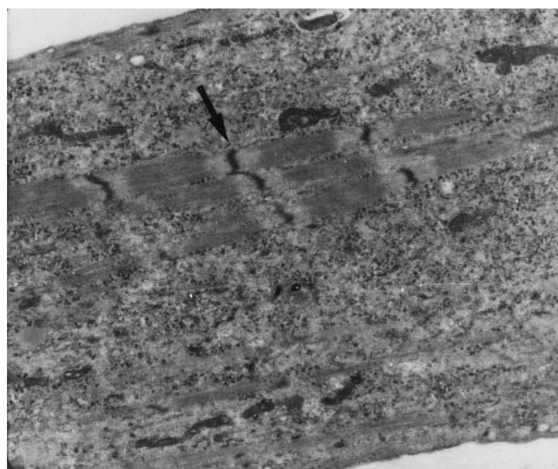


*Figure 2.* Electron microscopy of a 5-d-old culture of myotubes derived from adeno-*MyoD*–converted adult human dermal fibroblasts, showing well developed sarcomeres. The arrow indicates newly formed Z lines.

the *MyoD* adenoviral vector in inducing conversion of human as well as murine primary fibroblasts obtained from different tissues. When possible, cells from late fetal, neonatal, and adult tissues were compared to evaluate any age-related differential response to *MyoD*-induced conversion. A quantitative analysis of the results is presented in Table I, which shows that fibroblasts isolated from the dermis (both human and murine) convert better than fibroblasts isolated from skeletal muscle (range of 42–70 versus 32–44%), whereas adherent cells from bone marrow have the lowest propensity to myogenic conversion (6–7%). Conversion efficiency declines with age, although much less dramatically than the myogenic cell growth potential (5, 28). Finally, we observed that murine fibroblasts tend to convert better than their human counterparts (42–70 versus 32–65), although the difference was modest. These data indicate that despite some species and age-related variation, dermal and muscle fibroblasts appear as a good target for *MyoD*-induced myogenic conversion, whereas bone marrow stromal cells do not.

*Converted fibroblasts express a myogenic phenotype in vitro.* To compare the adeno-*MyoD*–induced phenotype with that of primary myogenic cells, we analyzed by immunocytochemistry, electron microscopy, and gene expression analysis a number of muscle-specific markers in human and murine-converted dermal fibroblasts, and in differentiating satellite cells of comparable age. All differentiated myotubes, derived from fusion of converted murine newborn dermal fibroblasts, express fast embryonic myosin heavy chains, although a fraction of them also express slow myosin heavy chains (data not shown), as observed in myotubes derived from newborn satellite cells (21). Similarly, myotubes derived from human fetal dermal fibroblasts show a well organized initial sarcomerogenesis, with aligned sarcomeres and patterned Z lines (Fig. 2), as reported for myotubes derived from primary myogenic cells, which never complete sarcomagenesis in vitro (29). To verify whether the introduction of *MyoD* in primary fibroblasts would also activate transcription of genes responsible for membrane and metabolic muscle-specific functions, we measured by Northern blotting the expression of RNAs coding muscle-specific proteins such as the ACh α-subunit, MCK, as well as *MyoD*, *myogenin*, and MLC1F chosen as positive controls. No detectable level of any of these messages was detected in unconverted fibroblasts; on the other hand, these mRNAs were expressed at comparable levels in myotubes derived from converted fibroblasts and in myotubes derived from satellite cells of corresponding age (Fig. 3). Thus, under all the parameters analyzed in vitro, myotubes originating from converted fibroblasts cannot be distinguished from those derived by primary myogenic cells.

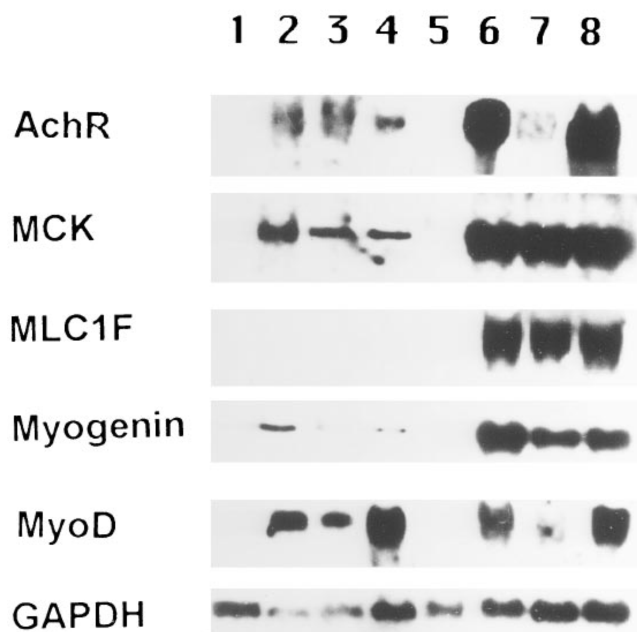



*Figure 3.* Northern blot analysis of total RNA extracted from human (lanes *1–4*) and murine (lanes *5–8*) myotubes derived from adeno-*MyoD*–converted dermal fibroblasts (lanes *2* and *6*), adeno-*MyoD*–converted muscle fibroblasts (lanes *3* and *7*), control fibroblasts (lanes *1* and *5*), and differentiated myotubes (lanes *4* and *8*). Filters were hybridized with probes for the α-subunit of the acetylcholine receptor (*AchR*), muscle creatine kinase (*MCK*), myosin light chain 1 fast (*MLC1F*), *Myogenin*, *MyoD*, and glyceraldehyde phosphodehydrogenase (*GAPDH*). Note absence of these messages in control fibroblasts despite overloading in the corresponding lanes (*1* and *5*).

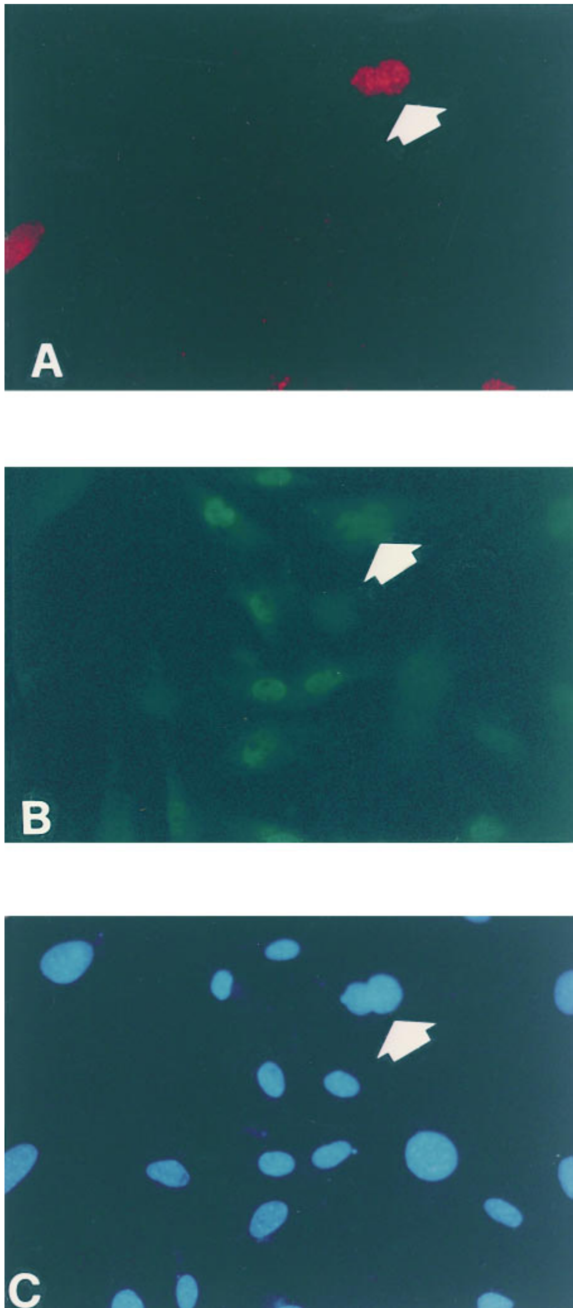
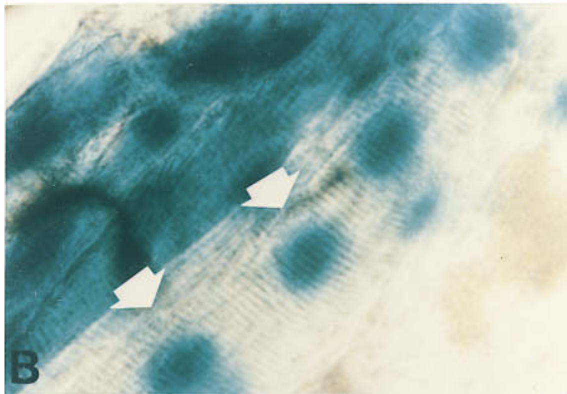



*Figure 4.* Effect of adeno-delivered *MyoD* gene expression on DNA synthesis of murine dermal fibroblasts. Cells were exposed to 10 μM BrdU for 12 h, and analyzed 24 h after adenoviral infection by double staining with an anti-BrdU monoclonal antibody (*A*), and with a polyclonal antibody against *MyoD* (*B*). Nuclei are stained by Hoechst dye (*C*). The arrow shows a $BrdU^+/MyoD^+$ double-labeled nucleus.

*DNA synthesis in MyoD-converted fibroblasts.* We next investigated whether fibroblasts exposed to the *MyoD* adenoviral vector would still maintain the capacity to divide, as *MyoD* overexpression has an antiproliferative effect (30). Murine and human fetal skin fibroblasts were infected with the *MyoD* adenoviral vector for 3 h at an moi of 2,000 in serum-free medium, then cultured in growth medium containing 10 μM BrdU, fixed at 12, 24, 48, and 72 h after infection, and double-stained with anti-*MyoD* and anti-BrdU antibodies. As shown in Fig. 4, 24 h after exposure to the adenoviral vector, only a few cells expressing *MyoD* had incorporated BrdU, whereas no double-positive cells were detected at 48 and 72 h (data not shown). Expression of *MyoD*, therefore, blocks cell division in converted fibroblasts.

*Myogenically converted murine fibroblasts participate in muscle regeneration and form normal fibers in vivo.* We next investigated whether myogenically converted fibroblasts would participate in the formation of new fibers during the process of muscle regeneration. To this purpose $10^6$ adeno-*MyoD*–converted late fetal dermal fibroblasts from *MLC3F/nLacZ* mice were injected into the TA muscle of *scid/bg* mice, in which muscle regeneration had been induced previously by a single injection of cardiotoxin. Mice were killed after 1, 2, 4, and 8 wk, and muscle regeneration analyzed by histochemistry. No staining whatsoever was observed in regenerating muscles injected with culture medium only (not shown). Conversely, β-gal-staining fibers were observed in the area of regeneration in 19 out of 24 injected muscles during the entire study. Fig. 5*A* shows a whole-mount β-gal stain of a TA muscle from a mouse killed 2 wk after the injection of converted fibroblasts, extensively regenerated by *lacZ*-positive fibers. Fig. 5 *B* shows a higher magnification of the same area, in which a cluster of β-$gal^+$ nuclei is aligned within fully developed fibers with apparently normal arrays of sarcomeres. A transverse section at 2 wk (Fig. 5 *C*) showed the presence of centrally located β-$gal^+$ nuclei, surrounded by a large number of β-$gal^-$ nuclei (revealed by Hoechst staining in Fig. 5 *D*) typically present in regenerating muscles. Despite variations among different experiments, the β-$gal^+$ nuclei remained present in roughly similar numbers at different times after the injection, although the total cellularity of the tissue was clearly reduced with the maturation of regenerated muscle. A quantitative analysis of these data is reported in Table II, which also compares the results obtained with *MyoD*-converted fibroblasts to those obtained with satellite cells. Converted fibroblasts gave rise to clusters of β-$gal^+$ aligned nuclei within 1 wk from injection, with no further significant increase or decrease in number at 2, 4, or 8 wk. In contrast, satellite cells from the same transgenic mice, injected in comparable numbers, gave rise to fewer clusters of β-$gal^+$ aligned nuclei at 1 wk, but their number increased considerably at 2 wk and at a slower rate thereafter. These experiments indicate that *MyoD*-converted fibroblasts may participate in muscle regeneration, and are able to form muscle fibers indistinguishable from those originating from primary myogenic (satellite) cells, although with a different kinetics and an overall reduced efficiency. This difference can be explained probably by the growth arrest induced by the *MyoD* transgene, which results in precocious and synchronous differentiation of the converted fibroblasts after injection, whereas satellite cells retain their proliferative potential, and may give rise to successive rounds of proliferation/differentiation in vivo, also explaining a final colonization twice more efficient. As already reported for satellite cells (and here confirmed) the overall survival of injected cells and the efficiency of colonization were found to be very low for *MyoD*-converted fibroblasts. However, most of β-$gal^+$ nuclei were found at the site of injection and along the track of the needle, whereas fewer β-$gal^+$ nuclei were found scattered through the muscle. Therefore, to evaluate efficiency of colonization in the site of injection, we counted the number of both β-$gal^+$ and total nuclei in



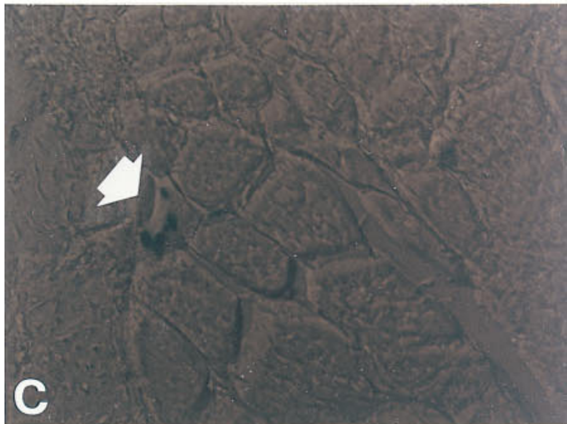



*Figure 5.* In vivo differentiation of adeno-*myoD*–converted murine dermal fibroblasts from *MLC3F/nLacZ* mice 14 d after implantation into regenerating muscles of *scid/bg* mice. (*A*) Whole-mount β-gal staining of a TA muscle injected with $2 \times 10^6$ converted fibroblasts. (*B*) Higher magnification of a few partially dissected fibers showing β-gal$^+$ nuclei (*arrow*) aligned within well developed sarcomeres. (*C*) Transverse section of a similar sample, showing β-gal$^+$ nuclei inside newly formed fibers, surrounded by β-gal$^-$ nuclei (revealed by Hoechst staining in *D*) both inside and surrounding muscle fibers.

*Table II. Survival of MyoD-converted Fibroblasts and Satellite Cells from MLC3F/LacZ Mice after Injection into Regenerating TA Muscle of scid/bg Mice*

| | *n* of β-gal$^+$ nuclei/injected TA muscle ( ) % of injected cells | | *n* of β-gal$^+$ nuclei/site of injection (200 μm$^2$) ( ) % of donor nuclei | |
|---|---|---|---|---|
| Time | *MyoD*-fibroblasts | Satellite cells | *MyoD*-fibroblasts | Satellite cells |
| 0 | $1.0 \times 10^6$ (100) | $1.0 \times 10^6$ (100) | | |
| 1st wk | $2.1 \times 10^3$ (0.2) | $0.5 \times 10^3$ (0.05) | 8/242 (3.3) | 2/256 (0.8) |
| 2nd wk | $1.8 \times 10^3$ (0.18) | $1.9 \times 10^3$ (0.19) | 7/127 (5.5) | 6/123 (4.8) |
| 4th wk | $1.6 \times 10^3$ (0.16) | $3.3 \times 10^3$ (0.33) | 6/99 (6.0) | 8/105 (8.5) |
| 8th wk | $1.9 \times 10^3$ (0.19) | $3.6 \times 10^3$ (0.36) | 7/56 (12.5) | 12/60 (13.1) |

20 μl containing $10^6$ cells was injected into a single site of regenerating TA of *scid/bg* mice. At the indicated times, mice were killed and successive 15-mm cryostat sections were prepared from the whole TA. One of every five slides was X-gal stained for β-gal activity. Nuclei were counterstained with Hoechst. All sections were scored for the total number containing β-gal$^+$ nuclei and the obtained values were multiplied by five to calculate the total *n* of β-gal$^+$ nuclei per injected muscle. In addition, areas of 200 μm$^2$ containing β-gal$^+$ nuclei were selected and the *n* of both + and total nuclei was counted. Note that the increase of the ratio of β-gal$^+$/total nuclei is due to the decrease of mononucleated cells, which progressively occurs during regeneration.

areas of 200 μm$^2$ containing β-galactosidase positive nuclei. Table II shows that the overall efficiency of colonization in these selected areas rose from a low percentage to ~ 10% of the nuclei, due to the decrease of mononucleated cells, which progressively occurs during regeneration.

*Genetically modified, converted human fibroblasts form muscle fibers expressing the transgene in vivo.* Fibroblasts isolated from human fetal skin were expanded and transduced in vitro with a high-titer stock of the retroviral vector LBSN, carrying a *lacZ* gene encoding a cytoplasmic form of β-gal (4). Transduction efficiency, estimated by the number of β-gal–staining cells in culture, ranged between 40 and 70%, making further selection of the transduced cells (e.g., G418 resistance) unnecessary. Transduced fibroblasts were exposed to the adeno-*MyoD* vector at an moi of 2,000, and then injected into the regenerating TA muscles of *scid/bg* mice ($10^6$ cells/muscle in a single injection). In our experience, the *scid/bg* immunodeficient background is the most tolerant to xenotransplantation of human cells, particularly in the muscle tissue (our unpublished observations). The efficiency of myogenic conversion of transduced fibroblasts was checked by allowing part of the cell culture to differentiate in vitro. On average, ~ 70% of the fibroblasts underwent myogenic conversion in these conditions, and most myotubes (> 90%) scored positive for cytoplasmic

β-gal expression (not shown). After 1, 2, and 4 wk, mice were killed and the TA serially sectioned and stained for β-gal activity. 2 wk after injection, β-gal$^+$ fibers were observed in 7 out of 8 injected muscles (Fig. 6 *A*). Higher magnification clearly revealed fibers accumulating the reporter gene product at variable levels (intensity of blue staining; Fig. 6 *B*), suggesting a variability in the proportion of injected/host cells in the β-gal$^+$ fibers. The presence and relative contribution of human cells in the newly formed fibers was confirmed by immunocytochemical analysis using an antibody that recognizes human but not mouse fetal myosin heavy chains, which showed patches of intensive expression of human muscle-specific proteins in the regenerating areas (Fig. 6 *C*). In general, the average number of β-gal$^+$ fibers per muscle obtained by injecting human converted fibroblasts was lower than that observed after injection of murine fibroblasts, although comparable to that observed in experiments in which *lacZ*-transduced human satellite cells were used (4). This indicates that human cells are, as expected, less efficient than murine ones in colonizing a mouse muscle. However, and more important, human converted fibroblasts perform like primary myogenic cells in the same in vivo background, in terms of both number and size of newly formed fibers. To better quantify the survival of human myogenically converted fibroblasts in mouse muscle, we also performed dot-blot Southern analysis with an Alu probe, as previously described (4). To this purpose, DNA was extracted from TA muscles of *scid/bg* mice 2 wk after the injection of *MyoD*–converted human fibroblasts (after the same experimental protocol described above). As a positive control, the same number of human satellite cells was injected in similarly treated contralateral TA. The results of the dot-blot (not shown) indicated that ~ 0.1% of the DNA extracted from TA injected with converted fibroblasts was of human origin; on the other hand, this value rose to 0.3–0.5% in the case of the sample injected with human satellite cells. Because histochemical analysis had revealed that virtually all the β-gal staining was inside muscle fibers, we can conclude that the observed values faithfully reflect the percentage of human nuclei incorporated into regenerated muscle fibers. The difference between satellite cells and fibroblasts may be due to incomplete myogenic conversion of fibroblasts and to the absence of cells with self-renewal potential in this population.

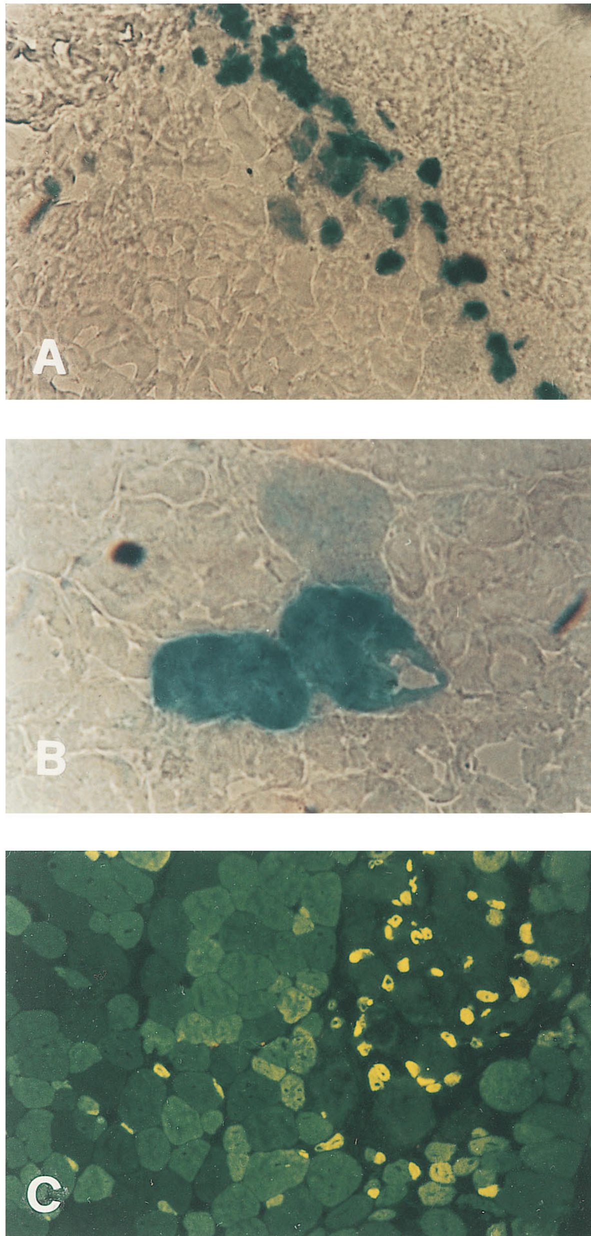



*Figure 6.* In vivo differentiation of adeno-*MyoD*–converted human dermal fibroblasts transduced in vitro with the LBSN retroviral vector, and injected into regenerating TA muscles of *scid/bg* mice. (*A*) Transverse cryostat section of a TA muscle 14 d after injection of $2 \times 10^6$ LBSN-transduced, *MyoD*-converted fibroblasts. (*B*) Higher magnification showing three β-gal$^+$ fibers. (*C*) Immunofluorescent labeling with an anti–human neonatal myosin heavy chain antibody.

*Immune response against cells exposed ex vivo to the adenoviral vector.* One of the major problems associated with the in vivo use of adenoviral vectors in immunocompetent recipients is the induction of a significant, T cell–mediated immune response against both the viral proteins and the product of the transgene(s). In our model, no viral particle is injected directly in vivo. However, we did inject cells that had been exposed to an adenoviral vector in vitro shortly before administration. Replication-defective adenoviral DNA is expected to be rapidly diluted, and eventually lost, in cells in active cell division. Expression of the *MyoD* transgene, however, blocks cell division almost immediately in our converted fibroblasts, thus preventing the possibility of diluting the adenoviral vector by continuous culture. To test whether an immune response can be raised by cells expressing a defective adenoviral genome in vivo, we injected $2 \times 10^6$ C3H murine skin fibroblasts converted to myogenesis by exposure to the adeno-*MyoD* vector in the regenerating muscle of syngeneic mice. Cells were labeled with DiI before injection and not with a *lacZ* transgene to avoid a possible immune reaction against the β-gal protein. Animals were killed 7, 14, and 21 d after the injection, and muscles were analyzed for the presence of immune infiltrate by immunofluorescence using antibodies against cell surface markers of murine leukocytes and macrophages (see Methods). No significant immune infiltrate was detected for up to 3 wk after injection around the labeled cells (Fig. 7, *A*, *D*, and *G*), even though by this time all treated mice had developed antibodies against Ad5 proteins, which recognize adeno-infected but not control C3H fibroblasts (data not shown). These experiments indicate that fibroblasts converted in vitro by ex-

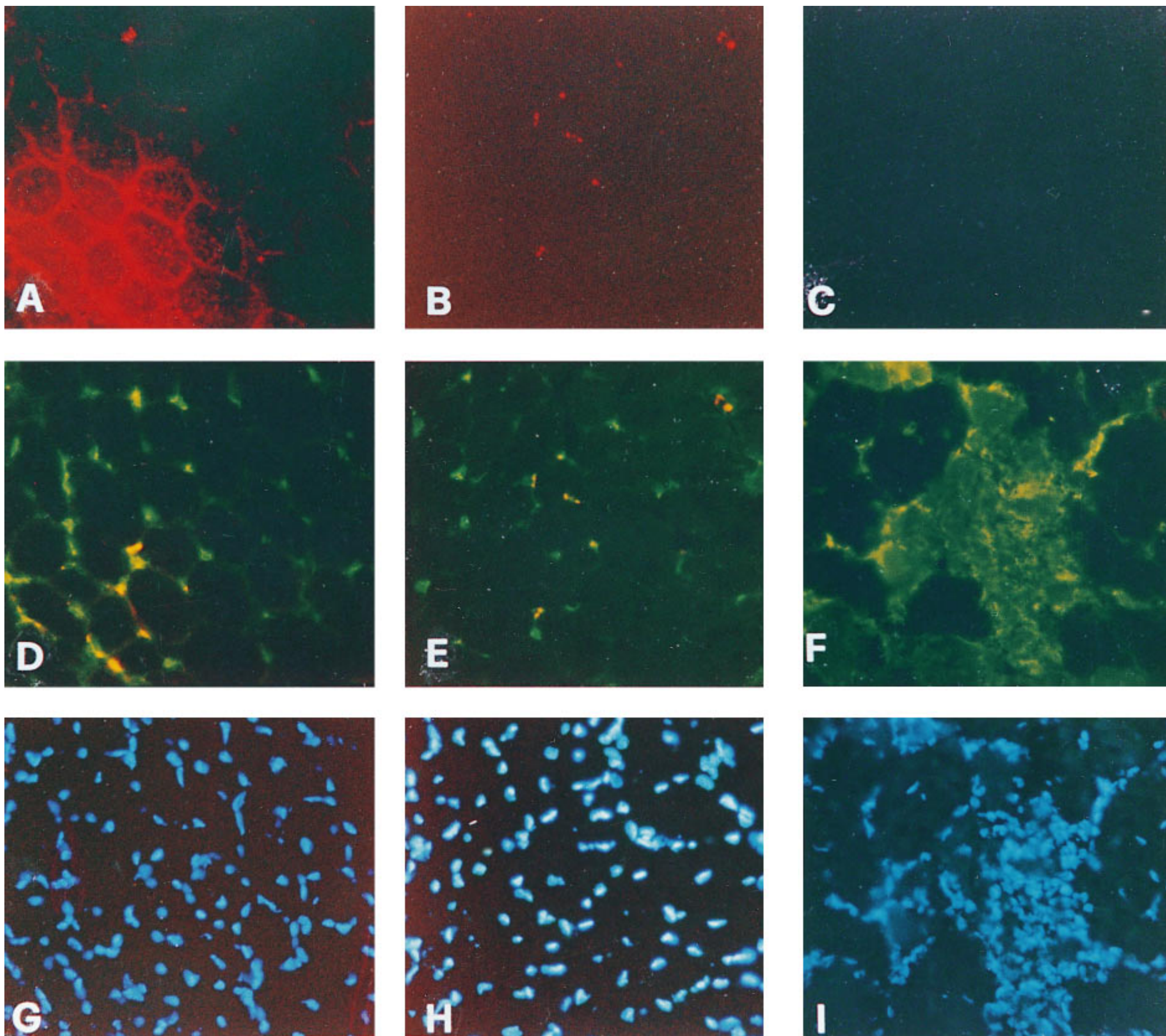



*Figure 7.* Absence of an immune infiltrate around fibers derived from injection of DiI-labeled, adeno-*MyoD*–converted fibroblasts into the TA muscle of immunocompetent C3H mice. (*A*, *D*, and *G*) Transverse cryostat section of the regenerating TA muscle 14 d after the injection of $2 \times 10^6$ cells. (*B*, *E*, and *H*) Transverse cryostat section of the contralateral, control TA muscle. (*C*, *F*, and *I*) Transverse cryostat section of the regenerating TA muscle 14 d after the injection of 2,000 moi of concentrated *MyoD*-adenoviral vector. (*A–C*) Localization of DiI positive fibers derived from DiI-labeled injected cells in *A* but not in *B* or *C*. (*D–F*) Immunofluorescent analysis of the same sections stained with a pan-leucocyte anti-Leu antibody. (*G–I*) Hoechst nuclear staining.

posure to an adeno-*MyoD* vector, and administered in vivo by intramuscular injection, do not elicit, and are therefore unlikely to be eliminated by, a cell-mediated cytotoxic immune response. In contrast, direct injection of adenoviral vector into regenerating muscle induced a strong immune infiltrate (Fig. 7, *C*, *F*, and *I*), as already reported (31).

## Discussion

The data reported in this paper suggest an alternative therapeutic strategy for primary myopathies. They show that it is possible to isolate, expand in vitro, and transduce with a retroviral vector human and murine skin fibroblasts, and then massively convert them to myogenesis by transient expression of *MyoD* delivered though an adenoviral vector. By a variety of morphological, immunocytochemical, and biochemical criteria, converted cells are indistinguishable from primary myogenic cells of the same species and age. They also form apparently normal fibers when injected into regenerating skeletal muscle of immunodeficient mice.

A comparison among different tissue sources, age, and species revealed that dermal fibroblasts convert more efficiently than muscle fibroblasts, whereas bone marrow stromal cells are converted only to a modest extent. This difference may be related to the embryological origin of these mesenchymal cells. In fact, dermal fibroblasts derive from the dermomyotome, which also gives rise to myoblasts, whereas limb muscle fibroblasts derive from the somatopleura. The origin of bone marrow stromal cells is still unclear but very unlikely somitic (32). As predicted, murine cells convert slightly better than human cells, and the efficiency of conversion declines overall with age. However, it is crucial to stress that this reduction is minimal

when compared to the dramatic age-dependent reduction of growth potential of primary myogenic cells, particularly when derived from myopathic patients. This point bears important implications for ex vivo therapy: among the many problems still facing this approach, the impossibility of obtaining large numbers of genetically corrected cells appeared as one of the most, if not the most, difficult to overcome. Although alternative strategies are being devised, involving for instance immortalization by conditional mutants of the SV40 large-T antigen (33–35), conversion of fibroblasts appears so far the simplest and safest solution. Fibroblasts can be isolated from a patient by a skin biopsy, expanded in vitro (their growth potential is uncompromised by the myopathy), and efficiently transduced with a retroviral vector expressing a therapeutic gene. When the required number of cells is obtained, and before the occurrence of senescence, fibroblasts can be converted to myogenesis by transient expression of *MyoD*, and then implanted into the muscles of the same patient. One main advantage of this strategy is the fact that transient expression of foreign *MyoD* activates endogenous *MyoD*, therefore, irreversibly committing cells to myogenesis, and rendering permanent expression of the transgene unnecessary. Furthermore, the number of cells that can be genetically manipulated is in this case extremely high.

Immunological problems, related to the use of a first generation adenoviral vector, do exist but do not appear as dramatic as in the case of direct virus injection (31). Cells are exposed to the adenoviral vector in vitro while actively dividing. No viral integration should occur under these conditions but, by the time they are injected, the cells apparently still contain significant amounts of viral particles and proteins. This cannot be avoided since expression of *MyoD* has a strong antiproliferative effect on infected cells, which stop dividing and cannot be subcultured until viral antigens are diluted out. Indeed, when injected with adeno-converted syngeneic cells, immunocompetent mice develop antibodies which recognize adeno-infected but not normal cells. Yet, no immune cellular infiltrate was detected around fibers containing adeno-converted cells. At least in part, this may be due to the fact that by the time the humoral immune response is activated, viral antigens are probably no longer present on fibers derived by the adeno-converted fibroblasts. Most important, however, the ex vivo strategy overcomes one of the major factors causing immune rejection of muscle fibers transduced by direct injection, i.e., the concomitant transduction of immune cells in vivo. Transduction of professional antigen-presenting cells has most likely a key role in mounting a strong, T cell–mediated cytotoxic immune response against adeno-infected cells. In fact, this response has been shown to be essentially directed against the product of the transgene carried by the adenoviral vector, whereas mainly soluble antibodies are produced against viral proteins and these cause no significant damage to transduced cells or fibers (31). Our data are in agreement with these reports, as the adeno-MyoD vector was not injected in vivo, and, anyway, contained no foreign antigen for the recipient mice. Nevertheless, a second injection of adeno-infected cells into an immunized host could still face an anti-adeno neutralizing antibody response, and indeed preliminary experiments failed to detect labeled fibers in the muscle of immunocompetent mice 2 wk after a second injection of adeno-*MyoD*–converted fibroblasts. Thus, clearly, immunological problems are still facing this experimental approach, although these could be in principle overcome by improving the technology of *MyoD* delivery to the fibroblast cultures. For instance, second- or third-generation adenoviral vectors, currently tested for delivery of minidystrophin genes in vivo (36, 37), might be used for *MyoD*-mediated conversion in vitro, and in principle also in vivo. Indeed, *MyoD* might be expressed by the same vector carrying the *dystrophin* gene, with the double advantage of transferring the therapeutic gene in both myogenic and nonmyogenic cells while simultaneously recruiting the latter to myogenesis. This may be particularly useful in the first phases of myopathies such as DMD, where fibroblast infiltration may be diverted from the default fibrotic fate, and rather pushed into the formation of dystrophin-containing muscle fibers. Alternatively, more efficient nonviral transient transduction methods might be developed and used to express *MyoD* into fibroblasts in culture. This strategy is currently being developed in our laboratory.

In conclusion, this work shows that it is possible to exploit the unique capacity of *MyoD* to activate myogenesis in fibroblasts to create a virtually infinite source of autologous myogenic cells in patients whose normal myogenic cells have been depleted, or are close to the exhaustion of their growth potential.

Other problems associated with myoblast transplantation, such as local delivery and survival of reimplanted cells (see reference 1), and long-term expression of the retrovirally delivered gene, remain to be faced also with this kind of ex vivo therapeutic strategy. For example, we know that *MyoD*-converted fibroblasts are initially lost from the injected muscle with kinetics similar to that of primary myogenic cells (Lattanzi, L., and J.R. Beauchamp, unpublished observations). However, recent results show that survival can be dramatically increased by immunosuppressive drugs and by antibodies that inhibit phagocyte adhesion to target cells (38, 39).

In conclusion, we believe that a rapid advance in our understanding of the biology of mesenchymal and myogenic cells, together with better pharmacological control of the inflammatory, immune, and vascular reaction in the host tissue, will be instrumental in devising strategies with an increasing chance of success for these types of genetic diseases.

## Acknowledgments

We thank A. Stoppacciaro (Università La Sapienza, Rome, Italy) for helpful discussion and advice on the histological analysis, and G. Arcese (Università La Sapienza), G. Campus (Università di Sassari; Italy), and C. Fieri (Clinica Columbus, Rome, Italy) for providing human tissues and cells. We also thank S. Alemà (Istituto Biologia Cellulare CNR, Rome, Italy), G. Butler-Brown (Université Rene-Descartes, Paris, France), M. Buckingham (Institut Pasteur, Paris, France), J. Boulter (Salk Institute, La Jolla, CA), D. Fischman (Cornell University, Ithaca, NY), S. Hauschka (University of Washington, Seattle, WA), S. Schiaffino (Università di Padova, Padova, Italy), and W. Wright (University of Texas Southwestern Medical Center, Dallas, TX) for the gift of antibodies and plasmids.

This work was supported by grants from Telethon, EC BIOTECH-2 project, Fondazione Cenci Bolognetti, and A.I.R.C.

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