In Vitro and In Vivo Tetracycline-Controlled Myogenic Conversion of NIH-3T3 Cells: Evidence of Programmed Cell Death After Muscle Cell Transplantation

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Ex vivo gene therapy of Duchenne muscular dystrophy based on autologous transplantation of genetically modified myoblasts is limited by their premature senescence. MyoD-converted fibroblasts represent an alternative source of myogenic cells. In this study the forced MyoD-dependent conversion of murine NIH-3T3 fibroblasts into myoblasts under the control of an inducible promoter silent in the presence of tetracycline was evaluated. After tetracycline withdrawal this promoter drives the transcription of MyoD in the engineered fibroblasts, inducing their myogenesis and giving rise to β-galactosidase-positive cells. MyoD-expressing fibroblasts withdrew from the cell cycle, but were unable to fuse in vitro into multinucleated myotubes. Five days following implantation of engineered fibroblasts in muscles of C57BL/10J mice we observed a sevenfold increase of β-galactosidase-positive regenerating myofibers in animals not treated with antibiotic compared with treated animals. After 1 week the number of positive fibers decreased and several apoptotic myonuclei were detected. Three weeks following implantation of MyoD-converted fibroblasts in recipient mice, no positive "blue" fiber was observed. Our results suggest that transactivation by tetracycline of MyoD may drive an in vivo myogenic conversion of NIH-3T3 fibroblasts and that, in this experimental setting, apoptosis plays a relevant role in limiting the efficacy of engineered fibroblast transplantation. This work opens the question whether apoptotic phenomena also play a general role as limiting factors of cell-mediated gene therapy of inherited muscle disorders.

Key words: Inducible promoter; Myogenesis; Cell transplantation; Muscular dystrophy; Apoptosis

INTRODUCTION

Dystrophin deficiency at the muscle membrane leads to Duchenne muscular dystrophy (DMD), a severe muscle-wasting disease that is inevitably fatal in early adulthood (16). Transplantation of normal myoblasts into dystrophic muscles has been used in DMD with little success (15,18,19,21). This limited success may be due to several problems, in particular the difficulty of producing large amounts of myoblasts capable of fusion to form new muscle fibers and the immune response of the host against the injected myoblasts. To avoid rejection problems, autologous transplantation of the patient's corrected myoblasts has been proposed but the low proliferative capacity of DMD myoblasts limits such procedure.

The four known myogenic regulatory factors (MRF)

of the MyoD family (MyoD, Myogenin, MRF4, and Myf-5) are basic helix–loop–helix transcription factors able to activate muscle gene expression, suggesting a significant role in determination and differentiation of skeletal muscle (26,40). During muscle development in vivo, MyoD and Myf-5 are responsible for mediating primary activation, defining the myoblast state, positioning the proper number of cells in the muscle-forming regions of the body, and for receiving inhibitory signals from the environment. Myogenin and MRF4 are activated by MyoD or Myf-5 and are used for bypassing negative control in vivo, and for activating most downstream muscle genes by direct interaction with transcriptional enhancers (39).

The forced expression of any of MyoD family genes in nonmuscle cultured cells can convert them to a skele-

tal muscle phenotype. MyoD is able to activate muscle gene expression by binding to a specific DNA sequence, called E-box, after heterodimerization with E47 (a myogenesis-related protein) (7). In the presence of fetal bovine serum (FBS) in proliferative medium, myogenic differentiation is inhibited through the intracellular expression of inhibitor of differentiation (Id) proteins. Id protein binds E47, displacing MyoD; if FBS is withdrawn from the medium, E47 is released from Id and is free to bind MyoD, promoting differentiation (1). MyoD~E47 is an artificial dimer where MyoD is covalently bound to E47 by a tether. This construct is not sensitive to the inhibition induced by the factors present in FBS (25). The most evident disadvantage in vitro of the forced MyoD-induced differentiation program is the irreversible withdrawal from the cell cycle, which limits the absolute quantity of recovered transfected cells. A possible solution to the MyoD-dependent inhibition of proliferation is the utilization of inducible promoters driving the expression of MyoD~E47 construct.

In the present article, we describe a system that not only allows differential control of MyoD~E47 activity but produces a muscle-specific gene expression in a very high number of cells starting from nonmuscle cells such as NIH-3T3 mouse fibroblasts. This system makes use of a tetracycline-regulated transcriptional activator (tTA) consisting of the *E. coli* Tn10 tetracycline repressor (tetR) fused with the transactivation domain of herpes simplex virus VP16 protein (12). The tTA hybrid protein acts as a transcriptional activator only in the absence of tetracycline, stimulating a minimal human cytomegalovirus promoter ($P_{hCMV*-1}$ promoter) fused to tetracycline operator sequence (tetO). We set up an in vitro expression system where tetO sequences are followed by MyoD~E47 cDNA; only in the absence of tetracycline was the tTA protein able to bind to the tetO sequence, promoting the forced transcription of exogenous MyoD~E47. Therefore, MyoD~E47 gene expression in NIH-3T3 stably transfected cells can be repressed during administration of tetracycline, whereas removal of the antibiotic induces in the same cells a myogenic process.

The aim of this work was to investigate the in vitro and in vivo induction of forced myogenesis in a tetracycline-dependent manner, the long-term survival of the system, and the feasibility to obtain an infinite number of cells able to produce a muscle-specific gene expression starting from nonmuscle cells previously genetically modified. The limits of this type of approach are also discussed.

MATERIALS AND METHODS

Plasmids

All nucleic acid manipulations were performed following standard protocols (31). Vector pUHD10.3 contains a multiple cloning site (MCS) immediately downstream to the $P_{hCMV*-1}$ promoter. Therefore, any cDNA or gene inserted into one of the sites in the MCS will be responsive to the tTA regulatory protein. Vector pUHD-15.1neo contains the tTA regulatory protein under the control of a cytomegalovirus promoter and the gene encoding neomycine resistance (41). Vector pUHC13.3 expresses luciferase gene under the control of the $P_{hCMV*-1}$ promoter. Plasmids pUHD10.3, pUHD15.1neo, and pUHC13.3 were generous gifts of Prof. H. Bujard (Universitat Heidelberg, Germany). Plasmid pUHD10.3MyoD~E47 was obtained by inserting a *Eco*RI/*Cla*I fragment encoding MyoD~E47 tethered dimer from the vector pECEMyoD~E47 into pUHD10.3 previously linearized with *Eco*RI. Vector pMRF4Z contains the regulatory sequences of mouse MRF4 driving the expression of *E. coli* β-galactosidase or lacZ (27). Vector pMRF4(300)Z was constructed as follows: a fragment spanning bases −300 to +43 of the upstream region of mouse MRF4 was amplified and gel purified; then the fragment, containing an engineered *Bam*HI site at its 5′ end and a *Xho*I site at its 3′ end, was ligated in substitution of the original 6.5-kb insert into pMRF4Z. Plasmids pECE MyoD~E47 and pMRF4Z were kind gifts of Dr. Wold. Vector pY3 confers resistance to the toxin hygromycin B (3). The plasmids used for transfections were purified by Plasmid Maxi Kit (Quiagen).

Cell Culture and Transfection

The NIH-3T3 Swiss Albino mouse fibroblasts cell line was purchased from the American Type Culture Collection (Rockville, MD). The growth medium (GM) for the cells was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. DMEM supplemented with 5% horse serum was used as differentiation medium (DM).

For stable and transient transfections cells were seeded at 2.5×10^5 on 60-mm tissue culture dishes and transfected 24 h after using standard calcium-phosphate technique. In the first step cells were transfected with 10 μg of pUHD15.1neo (Fig. 1). Selective medium containing 300 μg/ml geneticin was supplied to the cells 24 h after transfection, and resistant colonies appeared after 15–20 days. Isolated colonies were picked, transferred to multiwell plates, and expanded. The levels of activity of tTA were analyzed in 48 geneticin-resistant clones by transient transfection with 1 μg of pUHC13.3. Cells were split into two 35-mm dishes and tetracycline (Tc) was added into one dish to a final concentration of 2 μg/ml. Luciferase expression was assayed 48 h after transfection with Promega luciferase assay system. Protein concentration of each cellular lysate was determined according to the Lowry method. The luminometer readings in the luciferase assay divided by the optical density at
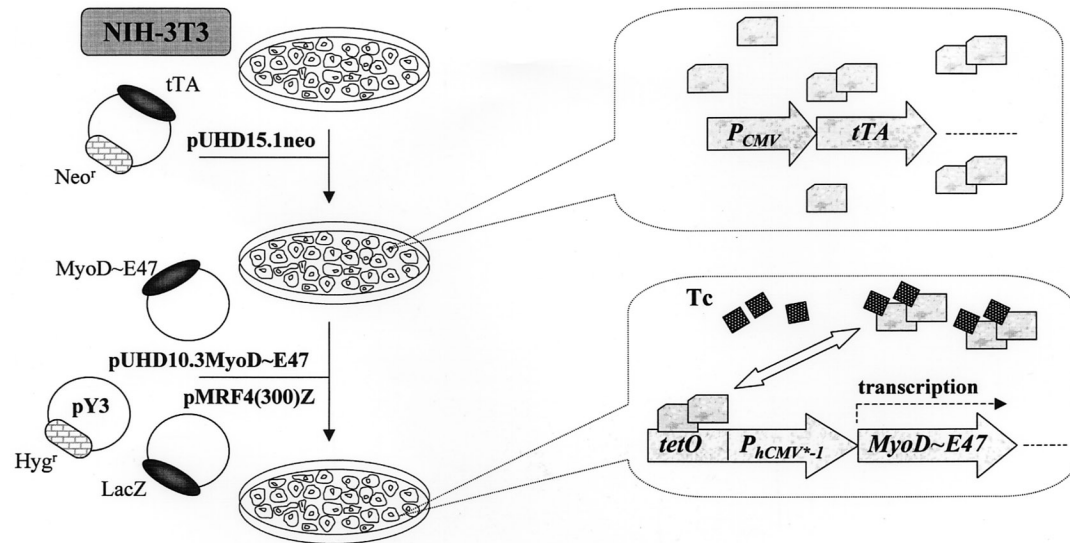
Figure 1. Schematic illustration representing the experimental design of the tetracycline-controlled myogenic conversion in vitro.

750 nm in the protein assay were plotted as the luciferase activity.

As second step, the best positive clone obtained was expanded and cotransfected with the vectors pUHD10.3 MyoD~E47, pMRF4Z(300), and pY3 (Fig. 1). At the moment of the transfection, 2 μg/ml Tc was added to the medium. Twenty-four hours after transfection, hygromycin B (Boehringer Mannheim) was added to a final concentration of 60 μg/ml and stably transfected colonies were obtained within 2 weeks.

More than 35 stable resistant clones were isolated, expanded, and assayed for β-galactosidase expression. For each single clone 1.5×10^5 cells were plated on 35-mm dishes. Twenty-four hours after plating GM was replaced with differentiating medium with or without Tc. The X-gal staining was performed in the presence of Tc and at 2, 7, and 10 days after Tc removal. The doubling time of growing and differentiation of stably transfected cells was calculated by plating 1.5×10^5 cells of each cell type on 35-mm dishes in GM and/or in DM in the presence and in the absence of Tc. The cells were counted with an hemocytometer. Data were expressed as mean values ± SD.

For the immunofluorescence analysis, 10^4 cells were seeded on coverslips and 24 h later GM was replaced with differentiating medium with or without Tc. Immunofluorescence analysis were performed in the presence of Tc and at 3, 7, and 10 days after Tc removal.

Immunocytochemistry

Immunofluorescence analysis was carried out using the following antibodies: a monoclonal antibody that recognizes slow myosin heavy chain (clone WB-MHCS,

Novocastra), an anti-desmin monoclonal antibody (clone DE-R11, Novocastra) and an anti-dystrophin monoclonal antibody Dys1 (anti-dystrophin rod-domain, clone DY4/6D3; Novocastra).

Cells were fixed with 4% paraformaldehyde for 10 min at 4°C and permeabilized with 0.01% Triton X-100. Cells were then incubated with primary antibodies diluted 1:20 overnight at 4°C. After washing with phosphate-buffered saline (PBS), cells were incubated with a fluorescein-conjugated goat anti-mouse Ig (1:100 dilution) (Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature. Cells were then washed, mounted in 50% glycerol/PBS (pH 7.4), and observed under a Zeiss Axiophot fluorescence microscope.

In Vitro X-Gal Histochemistry

Stable transfected cells were rinsed twice in PBS, fixed in 0.25% glutaraldehyde, and then incubated in a 0.5 mg/ml solution of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for 3 h. The blue cells were observed by phase-contrast light microscopy.

Cell Transplantation

For in vivo tetracycline-dependent modulation, C57BL/10J wild-type mice were used. Animals were immunosuppressed with daily IP injections of 2.5 mg/kg FK506 (Fujisawa Pharmaceutical Co. LTD, Osaka, Japan) from the day before transplantation to the day of sacrifice. The mice were anesthetized with 10 mg/ml ketamine. The skin was opened to expose the tibialis anterior (TA) muscles and cell injections were performed using a glass micropipette (Drummond Scientific, Broomall, PA). To induce muscle regeneration a

small longitudinal cut injury was made with a surgical blade before injections. The right TA muscle of all animals was injected with 2×10^6 transfected cells (suspended in a final volume of 10 μl) kept in GM in the presence of Tc, while the left TA muscle was injected with saline solution. The in vivo myogenic conversion was repressed, adding 800 μg/ml doxycycline hydrochloride (dox), a Tc derivative, to drinking water (containing 1% sucrose) starting 48 h before cell injection. Water containing doxycycline was changed every 3 days and mantained in light-protected bottles.

Cells were transplanted in 3-month-old mice; 9 out of 18 animals received dox. Three animals for each group were killed 5, 7, and 21 days after cell transplantation.

Tissue Sectioning and Analysis

Animals were killed by IP pentobarbital administration. The TA muscles were removed, frozen in liquid nitrogen-cooled isopentane, and sectioned in transverse serial sections. β-Galactosidase- and dystrophin-positive fibers were counted in 12-μm serial sections obtained at every 100 μm throughout the muscle. LacZ-positive myofibers were counted from adjacent hematoxylin-eosin stained sections and expressed as percentage of the total fibers. The presence of lymphocyte function associated antigen-1 (LFA-1)-positive cells was revealed as described (13).

In Vivo LacZ Staining

The cryostat sections were transferred to gelatin-coated glass slides. The sections were then fixed by dipping the slides in a cold (4°C) solution of 0.25% glutaraldehyde in PBS, pH 7.4, for 15 min, rinsed twice for 5 min in PBS, and stained overnight at 37°C with X-Gal at a concentration of 1 mg/ml in 5 mM $K_3Fe(CN)$, 5 mM $K_4Fe(CN)$, 1 mM $MgCl_2$ in PBS. The slides were rinsed using PBS and examined microscopically for the presence of β-gal-labeled ("blue") myofibers (9).

mRNA Analysis

Total RNA was prepared using the RNAzol™ B isolation kit (Biotecx Laboratories, Inc.). RNA (3 μg) was reverse-transcribed for 1 h at 42°C using random hexamers and the GeneAmp RNA PCR kit (Perkin Elmer). The cDNAs were analyzed by polymerase chain reaction (PCR) on a thermal cycler (Perkin Elmer). Each amplification contained equal amount of cDNA, 1.5 mM $MgCl_2$, 30 pmol of each primer, 5% dimethyl sulfoxide, 200 μM each dNTP (Boehringer Mannheim), 2 units of Taq DNA polymerase (Amplitaq, Perkin Elmer) in 1× PCR buffer (Perkin Elmer). Control reactions were performed on cDNAs synthesized without reverse transcriptase. No product was observed, indicating that the

amplification was not due to contaminating genomic DNA (data not shown). The following primers were used:

LacZ/for: 5′-GTC GTT TAC AAC GTC GTG ACT-3′
LacZ/rev: 5′-ATG GGC GCA TCG TAA CCG TGC-3′
Act/A1: 5′-TCC TGC GTC TGG ACC TGG-3′
Act/A2: 5′-CCA TCT CTT GCT CGA AGT-3′

Primers LacZ/for and LacZ/rev amplified a 272-bp region of E. coli β-galactosidase gene. The amplification was performed during 5 min at 94°C for the first cycle, denaturation at 94°C for 30 s, annealing at 56°C for 45 s, extension at 72°C for 45 s for the subsequent 30 cycles, and a final extension at 72°C for 10 min. Primers Act/A1 and Act/A2 generated a 175-bp fragment on the mouse β-actin cDNA. Amplification conditions for β-actin were as follows: an initial denaturation at 94°C for 5 min was followed by 24 cycles of denaturation at 94°C for 30 s, annealing at 65°C (2 cycles) and 55°C (22 cycles) for 45 s, extension at 72°C for 30 s, and a final extension at 72°C for 10 min. PCR products were electrophoresed on a 3.5% agarose gel (1% agarose, 2.5% NuSeive) and photographed under ultraviolet light after staining with ethidium bromide.

In Situ Hybridization

Probe Labeling. A 1.3-kb fragment encoding β-galactosidase was excised from the vector pCMVβ (Clontech) after double digestion with EcoRI and NotI. The cDNAs were gel isolated and digoxigenin labeled by random priming according to the manufacturer's instructions (Dig-DNA labeling and detection kit from Boehringer Mannheim).

Preparation of Tissue Sections. Briefly, 8-μm cryostat sections were cut and transferred onto slides, fixed in 4% paraformaldehyde in 0.1 M PBS for 10 min at room temperature, washed 3 × 4 min in 0.1 M PBS, and treated for 10 min in 0.1 M triethanolamine and 0.25% acetic anhydride. The slides were washed in 2× SSC (1× SSC = 0.3 M sodium chloride and 0.03 M sodium citrate, pH 7.4) for 5 min, dehydrated through graded ethanol before air drying. Prehybridization was performed by adding 100 μl of hybridization solution (SSC 5×, 50% formamide, 1× Denhardt's solution, 10% dextran sulfate, and 40 μg/ml sheared salmon sperm DNA) without cDNA probes and by incubating slides in a humidified chamber at room temperature for 2 h.

Hybridization. Eighty microliters of hybridization solution containing 50 ng/ml of Dig-labeled cDNA probe was placed on each slide. Slides were covered with parafilm and transferred to a humidified chamber at 42°C for 18 h.

Immunological Detection System. After hybridization the sections were washed with 2× SSC for 1 h at room temperature, followed by 0.2× SSC for 30 min at 50°C. Slides were next washed for 5 min in buffer 1 (100 maleic acid, 150 mM NaCl, pH 7.5), incubated for 30 min in buffer 1 containing 0.5% blocking reagent, then incubated for 2 h in 1:2000 dilution of an anti-digoxigenin antibody conjugated with alkaline phospatase (Boehringer) in buffer 1. After washing for 15 min in buffer 1 containing 1 mM levamisole, slides were incubated in color developing solution (35 μl of bromo-chloro-indolyl phosphate, 45 μl of nitroblue tetrazolium in 10 ml of 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, and 1% Tween 20) for 18 h at room temperature. Reactions were stopped in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). Slides were washed in 100 mM PBS, mounted in aqueous medium, and viewed under a light microscope.

In Situ DNA Fragmentation

The TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) method was performed for identification of DNA fragmentation. This technique was applied using a commercial kit (In Situ Cell Death Detection Kit AP from Boehringer Mannheim). Briefly, 8-μm cryostat sections were air dried for more than 1 h and then fixed for 30 min in 4% paraformaldehyde in 100 mM PBS, pH 7.4. Cells were permeabilized with 0.1% Triton X-100 in 100 mM sodium citrate, pH 5.2, for 2 min at 4°C and rinsed with PBS. Sections were incubated in a prewarmed humidified chamber at 37°C for 1 h with 50 μl of a reaction mixture containing terminal transferase and fluorescein-labeled nucleotides. Sections were then incubated at 37°C for 1 h with an alkaline phosphatase-labeled anti-fluorescein antibody (Boehringer Mannheim). After washing in PBS, the filtered chromogenic substrate (NBT-X-Phosphate) was converted by alkaline phosphatase in a humidified chamber at room temperature for 15 min. Slides were mounted in glycerol gelatin (Sigma).

RESULTS

Evaluation of tTA Regulatory Protein Activity in NIH-3T3 Transfected Cells

In the first series of experiments, vector pUHD15.1-Neo, encoding tTA regulatory protein and G418 resistance genes, was stably introduced into NIH-3T3 cells. The expression levels of tTA were analyzed in 48 independent geneticin-resistant clones by transient luciferase expression assay performed in the presence and in the absence of tetracycline. The aim was to establish a stable cell line that gives high levels of luciferase activity when Tc was absent and the lowest possible background expression in the presence of Tc. A wide range of clonal variation of the efficacy of Tc-controlled gene expression among different tTA-expressing clones was observed. The levels of inducible expression of the reporter gene obtained in 12 out of 48 clones are shown in Figure 2. We found that the 12 clones fell into three distinct categories: 1 clone did not express the luciferase gene (clone P10); 4 clones expressed the luciferase gene with an extremely low background in the presence of 2 μg/ml Tc and >100-fold higher in the absence of Tc (clones P4, P6, P9, and P11); the other 7 clones had a high maximal luciferase expression in the absence of Tc but the basal expression level was >10-fold higher than the previous four clones.

Myogenic Conversion in Transfected Cells

Clone P4 was selected for its optimal modulation of tTA expression and cotransfected with the vectors pUH-D10.3MyoD~E47, pMRF4Z(300), and pY3. Preliminary results had shown that cotransfection with only pMRF4Z(300) and pY3 was not able to induce β-gal expression in 10 hygromycin B-resistant clones, indicating a direct specific transactivation of the reporter gene by MyoD. As parameter of a correct inducible MyoD~E47 gene expression, β-galactosidase positivity was evaluated in 36 independent hygromycin B-resistant clones. A wide range of clonal variation in terms of β-galactosidase positivity in a Tc-dependent manner was observed. Forty-eight hours after the Tc removal, 17 out of 36 resistance clones showed a β-galactosidase positivity, but 5 out of 17 presented a homogeneous weak positivity also in the presence of Tc. Clones A3, A14 (Fig. 3A, B) and A25 showed a highly homogenous β-galactosidase expression 48 h after the Tc removal (>95% of blue cells) and this positivity was confirmed after 7 and 10 days.

The three positive clones were also characterized in terms of differentiative potential, keeping the cells in DM and analyzing the expression of muscle-specific proteins in the presence and in the absence of Tc (Table 1). The clones A3 and A25 showed, after 3 days, a weak positivity for anti-desmin antibody (10% of the cells); after 10 days about 30% of the cells were desmin positive and about 5% of the cells were dystrophin and myosin positive.

After 3 days, clone A14 showed positivity for anti-desmin antibody (30% of the cells), which increased in time: after 10 days 65% of the cells were desmin positive (Fig. 3C). Clone A14 was also positive for dystrophin and for myosin (8% of the cells).

In the presence of Tc, none of these clones showed positivity in immunofluorescence detections. As shown in Figure 3D, after 10 days of culture in DM without Tc, A14 cells were able to form few myotubes but the majority of differentiated blue cells remained strictly
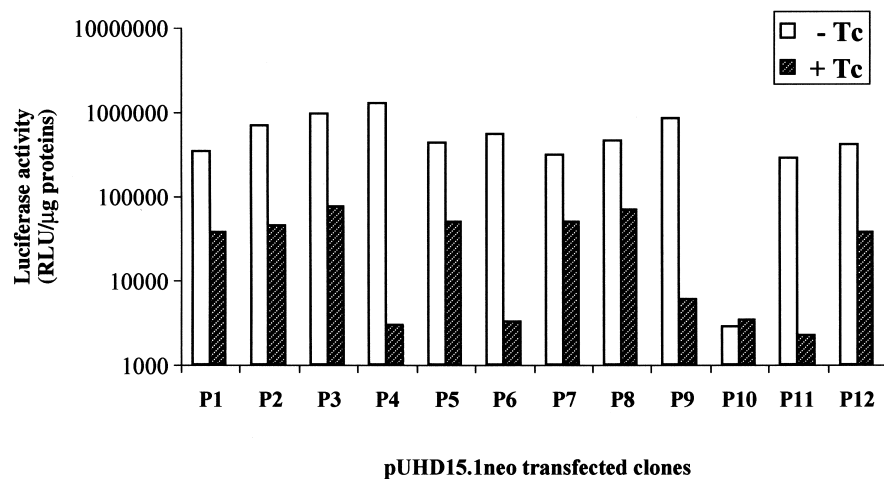
Figure 2. Luciferase activities of 12 different stable tTA-expressing clones following transient transfection with plasmid pUHC13.3. After transfection all clones were grown for 48 h in the absence of Tc (− Tc) or in the presence of 2 µg/ml Tc (+ Tc) before being assayed. The data represent the mean values of four different determinations.
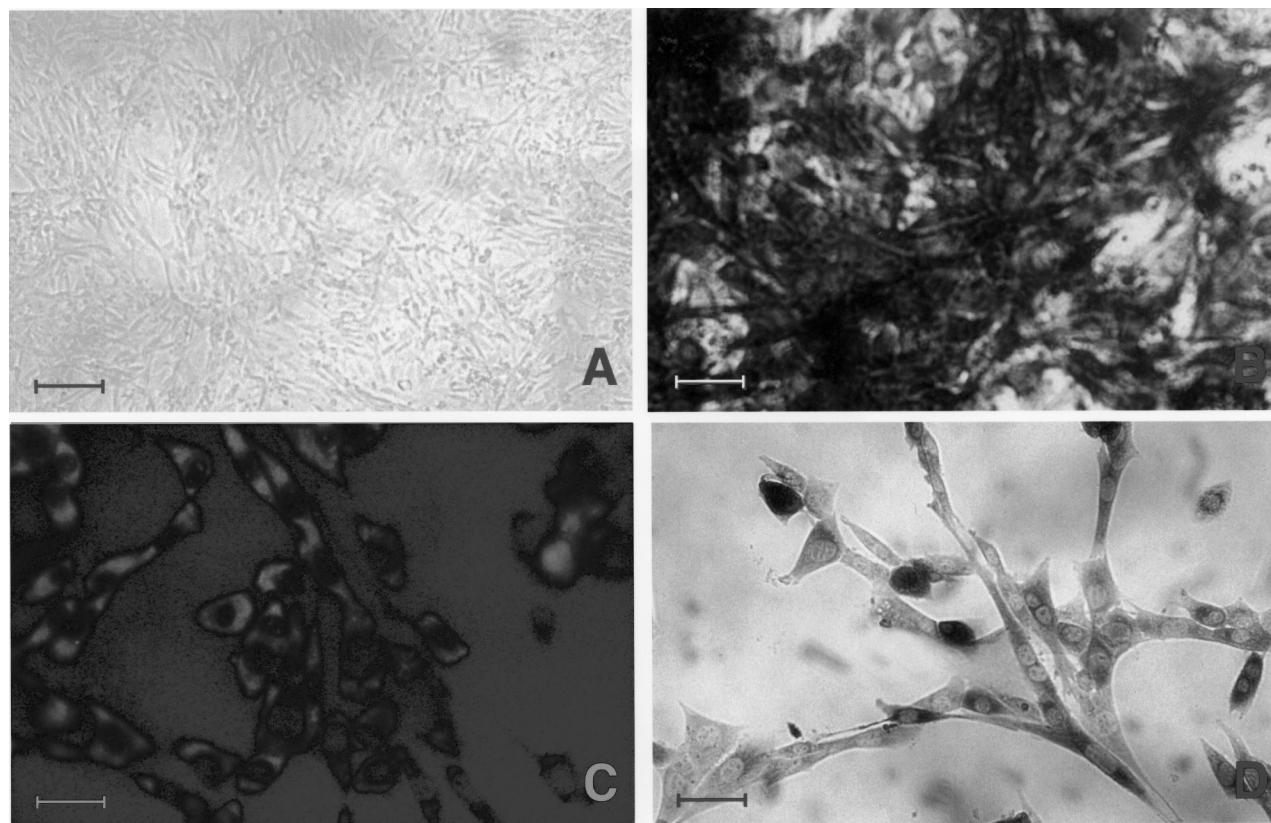
Figure 3. Inducible β-gal activity in clone A14 cells: X-gal staining reveals no blue cells under not induced condition (A, scale bar 200 µm) but almost 95% positive cells upon induction (B, scale bar 100 µm). In vitro differentiation of the same cells kept in DM for 10 days without Tc by immunostaining for anti-desmin antibody (C) and by staining for X-Gal (D) (scale bar 50 µm). In (D) few myotubes and prevalently mononucleated differentiated "blue" cells are shown.

mononucleated. Analysis of growth curves showed a progressive slowing of proliferation with the withdrawal of tetracycline and the consequent expression of myogenic factors.

The doubling time of clone A14 cells was determined in the presence and in the absence of Tc in DM. In the presence of Tc, this clone showed the same cellular growth as untransfected NIH-3T3 cells (18.2 ± 1.4 and 18.3 ± 1.9 h, respectively). Forty-eight hours after the withdrawal of Tc the doubling time was 28.7 ± 2.5 h; after 4 days it was 36.2 ± 2.7 h. Doubling time remained stationary in time (8–10 days after withdrawal of Tc). Similar results were obtained with clones A3 and A25.

To determine the stability of the inducible system, clone A14 cells were maintained in GM for 4 months in the presence of Tc; β-galactosidase expression was analyzed after a total of 30 passages. Total RNA was extracted from cells maintained in DM for 3 days in the presence and in the absence of Tc, and LacZ expression was evaluated by the RT-PCR method. A high level of inducibility was observed (Fig. 4). The hygromycin B-resistant clone A14 was designated for in vivo experiments.

Dox-Dependent Induction of Myogenesis In Vivo

Mice were sacrificed 5, 7, and 21 days after transplantation of clone A14 cells. The in vivo MyoD-mediated myogenic conversion and muscle fusion in the area of cell injection were analyzed by hematoxylin-eosin (Fig. 5A) and X-gal (Fig. 5B) staining. This area was about 300 μm^2 and contained regenerating myofibers and cellular infiltrations as confirmed by the presence of LFA-1-positive cells. The cell infiltration decreased rapidly 7 days after transplantation and was not significantly different from the left TA muscles treated with saline solution.

To detect β-gal-positive myofibers, 25 sections were analyzed for each muscle. No β-gal-positive myofibers were observed in regenerating muscles injected with saline solution (data not shown). Fibers of the same section exhibited different levels of LacZ activity, some fibers being more intensely stained than others. Five days after transplantation the percentage of β-galactosidase-positive myofibers was $0.8 \pm 1.2\%$ (corresponding to 15

± 22.8 fibers/section) for the animals that received dox and $5.7 \pm 1.8\%$ (corresponding to 108 ± 34.2 fibers/section) for the animals that did not ($p < 0.05$, Student t-test). Seven days after cell injection, the percentage of β-gal-positive muscle fibers was significantly decreased in both groups compared with the results obtained after 5 days ($0.3 \pm 0.7\%$ corresponding to 6 ± 13.3 fibers/section in the presence of dox and $1.9 \pm 1.6\%$ corresponding to 36 ± 30.4 fibers/section when dox was absent).

Figure 5B shows the incision area in which a cluster of β-gal-positive myofibers are aligned within regenerating fibers with apparently normal structures. The presence of β-gal-positive myofibers was confirmed in adjacent sections by in situ hybridization method (Fig. 5C). The lacZ expression pattern observed by in situ hybridization using a digoxigenin-labeled probe was identical to the X-gal staining pattern. Only in a few cases was LacZ mRNA expressed in β-gal-negative myofibers. The β-gal-positive fibers were found at the site of injections and along the track of the incision area.

Mature peripherally nucleated myofibers were observed 21 days after transplantation within injected areas. No β-gal-positive fibers were detected at this time point.

Apoptotic Myonuclei Detected by In Situ DNA Labeling

To provide more information about the lack of X-gal expression, transplanted TA muscles were tested by the TUNEL method. A minimal positive reaction was detected in nuclei of doxycycline-treated muscles while a large number of myonuclei became positive in muscles that did not received dox (Fig. 6A, C). The peak concentration of apoptotic nuclei was observed in mice sacrificed 7 days after cell injection.

At this time, the mean value of positive myonuclei observed in the animals that did not received dox was 44.3 ± 8.1 apoptotic nuclei/mm^2 while in the animals treated with dox the mean was 12.5 ± 4.1 apoptotic nuclei/mm^2. In the left TA muscles injected with a saline solution, a mean value of 11.2 ± 2.6 apoptotic nuclei/mm^2 was detected. The difference in apoptotic nuclei between dox- and non-dox-treated muscles was statistically significant ($p < 0.05$, Student t-test). The TA muscles from mice not treated with dox showed foci of in-

Table 1. Phenotypic Characterization of Geneticin- and Hygromycin B-Resistant Clones

	"Blue" Cells (− Tc)	Desmin (− Tc)	Dystrophin (− Tc)	Myosin (− Tc)	Doubling Time	
					(+ Tc)	(− Tc)
A3	>95%	30%	5%	5%	17.6 ± 2.4	37.2 ± 2.2
A25	>95%	30%	5%	5%	18.7 ± 1.9	37.4 ± 1.6
A14	>95%	65%	8%	8%	18.2 ± 1.4	36.2 ± 2.7
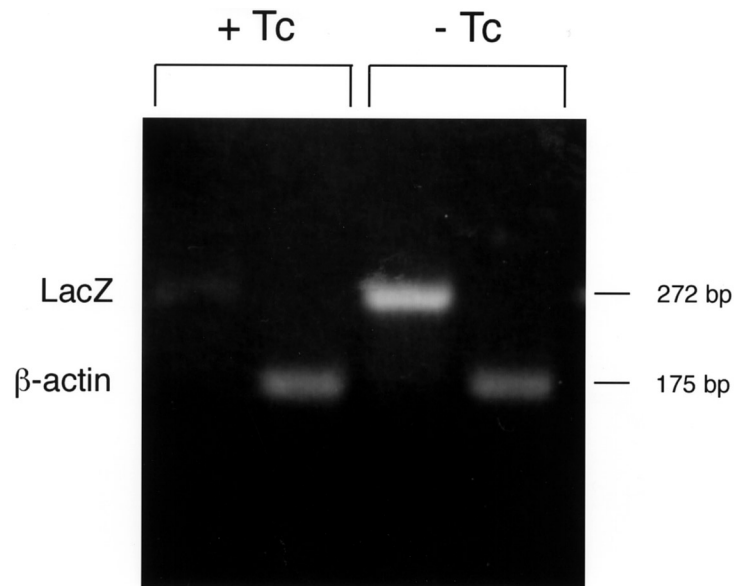
Figure 4. RT-PCR analysis of total RNA extracted from clone A14 cells after 30 passages in culture and maintained in DM for 3 days in the presence and in the absence of Tc. A high level of inducibility in lacZ expression (272-bp bands) was observed. β-Actin expression (175-bp bands) shows equivalent amounts of cDNA between treated and untreated clone A14 cells.

flammation in the injected areas with the presence of infiltrating LFA-1-positive cells (Fig. 6B, D), macrophages, other mononuclear cells, and small regenerating myofibers with centrally located myonuclei. Apoptotic nuclei were observed in these areas of the muscle but could not be clearly identified as myonuclei. Those nuclei were not included in counts.

Many apoptotic nuclei were localized at the periphery of the injection site and belonged to LFA-1-negative cells. A few muscle fibers that displayed histological signs of regeneration, such as central nuclei, were also observed to contain apoptotic nuclei.

DISCUSSION

The lack of dystrophin causes Duchenne muscular dystrophy, a progressive muscle fiber degenerative disease resulting in muscle weakness and premature death. Presently there is no treatment for DMD, but gene replacement is considered as an attractive potential therapy. The expression of functional dystrophin into muscle fibers was obtained by myoblast-mediated gene transfer (14,24,37). Injected myoblasts are able to cross basal lamina and become an integral part of mature host muscle fibers (2). Ex vivo gene therapy based on autologous transplantation of genetically modified myoblasts is limited by their low proliferative capacity (due to premature senescence). Nonmuscle cells, such as fibroblasts, amnyocites, or chorionic-villus cells, are not encumbered by exhaustion of their proliferating potential;

they are easily obtained in significant quantities and can be stably converted into myoblasts by transfection with a MyoD construct, thus offering a potential source of cells viable for ex vivo gene transfer.

In this work, we explored this possible alternative therapeutic strategy for primary myopathies such as DMD. The myoblast conversion of the murine NIH-3T3 cell line was obtained through the expression of inducible MyoD. The use of a system in which the time of myogenic conversion is pharmacologically regulated allowed us to overcome the cell growth reduction due to the induction of the myogenic determination program. The transactivation of a MRF4-driven reporter gene was used to verify the MyoD expression, taking advantage of the physiological interaction between MyoD and the promoter/enhancer complex of MRF4. The myogenic phenotype of converted fibroblasts was assayed by immunohistochemical characterization of muscle-specific proteins.

In general, studies using inducible expression in stable cell lines overcome some of the inherent limitations of transient transfection studies, including variable transfection efficiencies and the long-term stability of the system. However, with most regulated expression systems, inactivation is usually not complete and the inducers (i.e., heat shock, metal ions, and hormones) often can activate endogenous promoters or affect other cellular processes. The ability to control the expression of
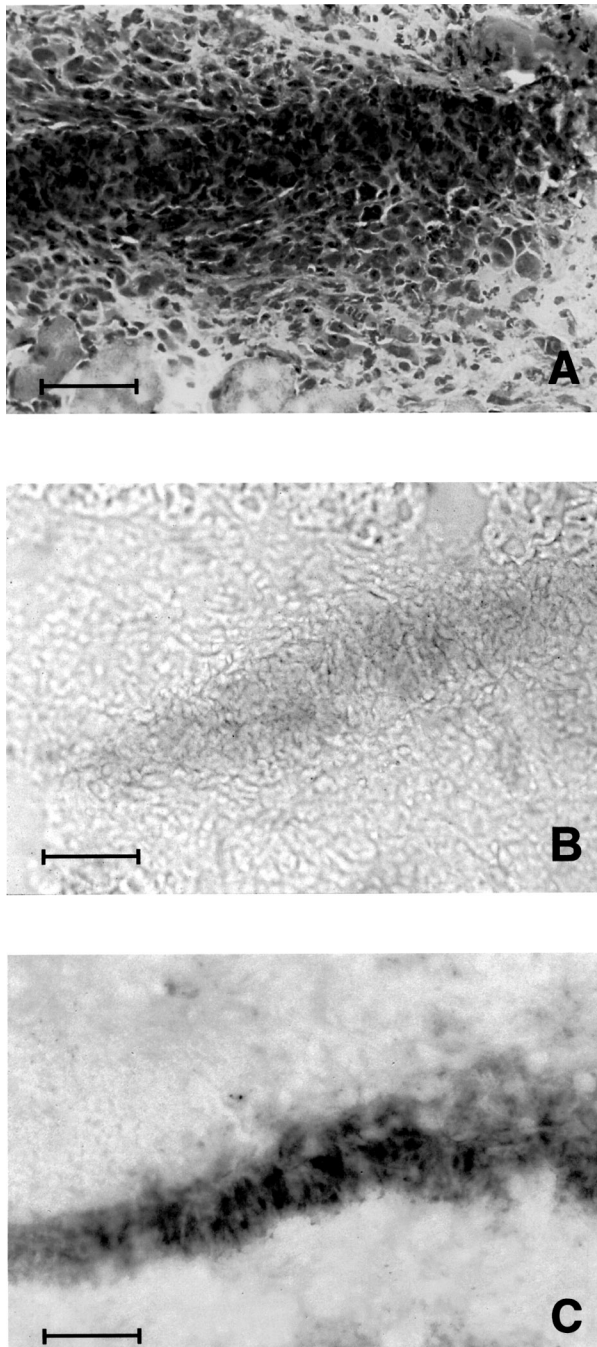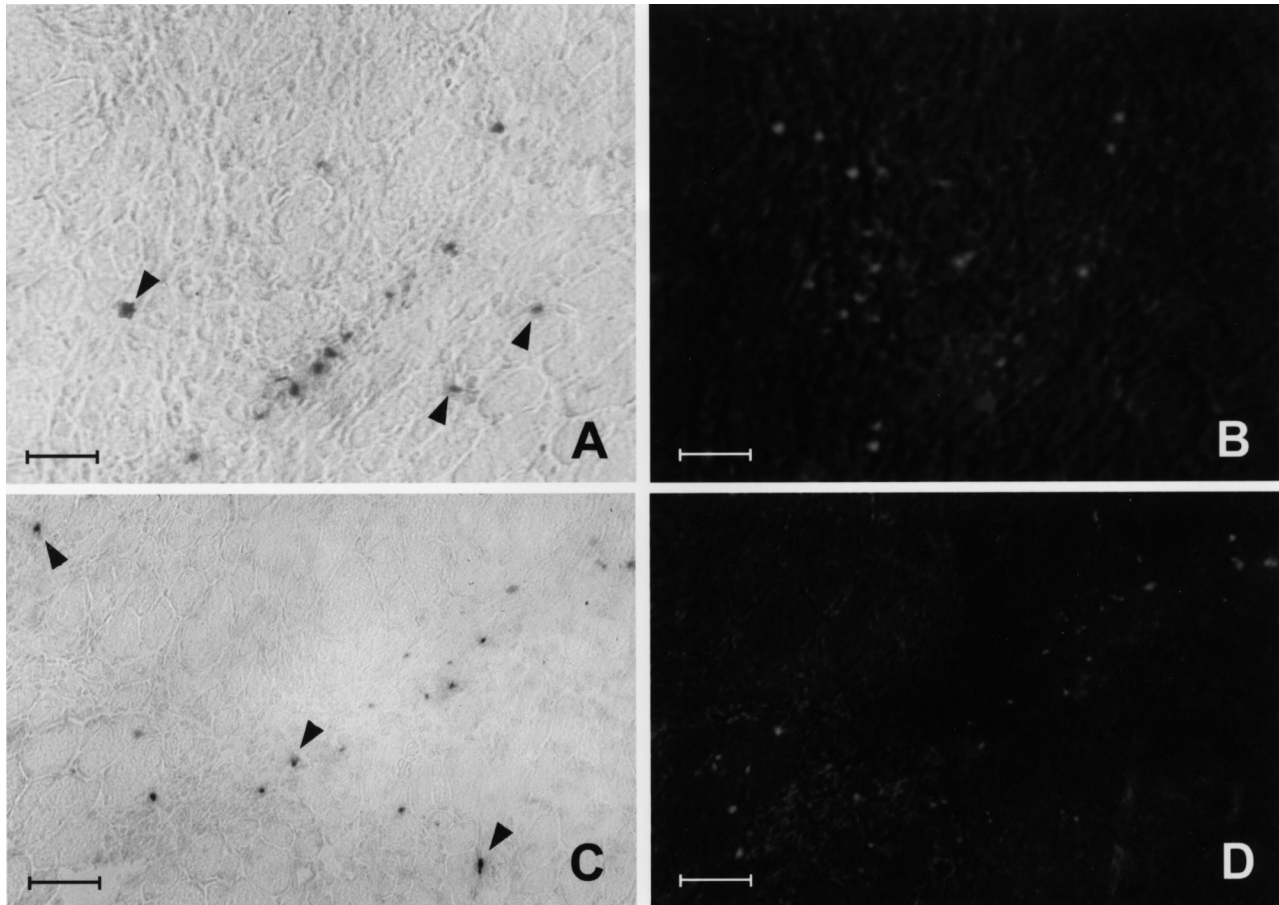
Figure 5. Serial sections of TA muscle from C57BL/10J mouse 5 days after cell transplantation stained for hematoxylin-eosin (A) and for X-gal (B). The presence of lacZ mRNA in "blue" X-gal-positive myofibers was confirmed in adjacent section by in situ hybridization method (C). Scale bar: 40 μm.

a cloned gene in a homogeneous population of stably transfected cells provides a powerful tool for studying protein function. An alternative approach is to use prokaryotic promoters and their activators such as the tetracycline-regulated gene expression system derived from the *E. coli* tetracycline resistance operon. The transactivator protein (tTA) stimulates transcription from a promoter containing tetracycline operator sequences (tetO), but it is prevented from interacting with the tetO by micromolar concentrations of tetracycline (11). This system allows high levels of expression in the activated state and essentially complete inactivation by tetracycline or its derivatives, such as doxycycline, that are not toxic for eukaryotic cells and animals (29). In our model the tetracycline-inducible system permits an examination of phenotypic and morphological changes that are associated with changes in the synthesis of MyoD protein.

In order to establish an optimal controllable system, the cells used for in vitro and in vivo experiments are the result of two sequential transfections and two different screening steps. Initially, starting from NIH-3T3 cell line, a careful selection of the clones expressing tTA was performed by transient transfection with the reporter gene luciferase linked to a tTA -responsive promoter. Then the tTA-expressing cell line (clone P4) was used to establish the stable cell line expressing MyoD~E47 cDNA. Isolated clones had been screened for their ability to express the gene product at a high level and in a regulated manner by comparing expression obtained in the presence and in the absence of tetracycline. The correct inducible activation of MyoD is revealed by the expression of the reporter gene β-galactosidase. We observed a wide range of clonal variation in terms of β-gal positivity, due to different numbers and several integration sites of the plasmids into the cells. The screening steps for obtaining resistance clones allowed us to isolate one clone (clone A14) in which MyoD expression was tightly regulated after Tc removal and with a high homogenous pattern (more than 95% of the cells). After integration in NIH-3T3 cells, these exogenous genes remained unmodified and were stably expressed over time.

We also characterized the forced myogenic conversion of selected fibrogenic cells by immunohistochemical reactions with specific myogenic markers such as anti-desmin, anti-myosin heavy chain, and anti-dystrophin antibodies. We observed a lower positivity for muscle proteins in spite of the very high level of β-gal positivity. The heterogeneous efficiency of MyoD-driven myogenic conversion has been demonstrated. Several studies have shown how the efficiency of myogenic conversion of nonmuscle cells is directly related to the relationship with myoblast lineage (5). The potential for

Figure 6. Serial sections of TA muscle from C57BL/10J mouse not treated with dox 7 days after cellular transplantation stained for in situ DNA end-labeling (A, C) and immunostained for LFA-1 (B, D). Arrows show LFA-1-negative apoptotic nuclei. (A, B) scale bar 35 μm; (C, D) scale bar 50 μm.

myogenic conversion is high in fibroblasts in which terminal differentiation and myotube formation can be easily observed and lower in other cells types (chondroblasts, hepatocytes, and amnioblasts), which are refractory to myogenic conversion in spite of exogenous MyoD expression (33). Also, the histological origin plays a role in the potential for myogenic conversion. A study that compared the relative efficiency of MyoD to induce myogenic conversion of human and murine primary fibroblasts isolated from different tissues demonstrated that fibroblasts from the dermis converted better than fibroblasts from skeletal muscles, whereas in the same study adherent cells from bone marrow presented the lowest propensity to myogenic conversion (20). Conversion efficiency declined with age and murine fibroblasts seemed to convert better than their human counterparts.

In the absence of tetracycline, clone A14 cells performed a permanent withdrawal from cell cycle as shown in the analysis of growth curves. These data confirmed

the activation of forced myogenic process. In spite of the expression of specific muscle proteins, we observed a low in vitro myotube formation; cells grown in DM appeared to become aligned, but the majority of differentiated cells remained strictly mononucleated, failing to fuse in multinucleated cells. This type of phenotype has been recently described as characteristic of NIH-3T3 cells converted by exogenous expression of MyoD family members (30). Analysis of muscle regulatory and structural gene expression failed to provide an explanation for the in vitro fusion defectiveness. A possible cause of this phenotype has been attributed to the failure to accumulate the transcripts encoding muscle-specific isoforms of the β1D integrin subunit and the transcriptional factor MEF2-D1b2. However, the dissociation of terminal differentiation and myogenic conversion also has been previously observed in myoblasts in particular conditions, such as the C_2C_{12} cells transformed by the v-*myc* oncogene (6). The mechanisms involved in the fusion of myotubes are still poorly defined. Our data con-

firm the suggestion that myotube formation can be genetically dissociated from MyoD-dependent transcriptional activation; muscle-specific gene expression can be considered necessary but not sufficient to commit differentiated myocytes to myotubes.

Another primary goal of this study was to verify whether tetracycline-dependent regulation could be demonstrated in an experimental model relevant to gene therapy. In vivo applications of the tetracycline-inducible system have been documented in transgenic mice. Long-term expression has been described following the transplantation of retrovirally transduced primary cells in animals (4). In addition to the suitable control system, the successful transplantation of engineered cells required an excellent survival of the implanted cells and the fusion of forced converted myoblasts with host muscle fibers.

Selected derivates of NIH-3T3 cells (clone A14) were transplanted in skeletal muscles of normal mice. Five days after transplantation, a negligible β-galactosidase gene expression was observed in TA muscles when animals were treated with doxycycline in the drinking water. However, a statistically significant increase of X-gal-positive regenerating myofibers was found in muscle cross sections from normal mice not treated with doxycycline, suggesting the possibility of an in vivo-inducible MyoD-mediated myogenic conversion. The presence of β-galactosidase mRNA detected by in situ hybridization technique has shown the same tissue localization, supporting the specificity of X-gal staining. The blue cells were localized as clusters of lacZ-positive cells around the site of injection and along the track of the incision area, confirming the minimal cellular movement due to their limited migratory capacity in the host muscle. The number of positive blue cells decreased significantly 7 days after transplantation and disappeared completely after 3 weeks. The lack of in vivo β-gal expression is probably also due to the presence of a high level of cell infiltration inside the injection area (35).

In agreement with other reports in which the low positive efficiency in terms of long-term survival of transplanted myoblasts was due in part to rapid and massive death of the donor cells soon after injection (8,17,34), several apoptotic myonuclei were observed in the mouse TA muscles, with a peak at 7 days after cellular transplantation. Even if the role of apoptosis in the muscle is not clear yet, apoptotic events have been demonstrated to occur in differentiated myocardial muscle, neonatal and adult skeletal muscle of dystrophic mice in vivo, and skeletal myoblasts in response to different stimuli (23,28,32,36). In vitro experiments have demonstrated the role of muscle cellular apoptosis on normal and dystrophin-deficient myoblasts in regulating their cellular proliferation (10). We observed a significant increase of

apoptotic nuclei in the group of animals not treated with doxycycline compared with treated muscles, suggesting the possibility that apoptotic events occurred more frequently in the forcedly converted myogenic cells. Nevertheless, the positivity to DNA fragmentation was mainly observed inside the site of cellular implantation where a high number of LFA-1-positive infiltrates and small regenerating myofibers with centrally located myonuclei were present. It is likely that cytokines locally secreted by inflammatory cells may induce programmed cell death. The positive apoptotic myonuclei were also localized near the site of injection in peripherally located myofibers. This implies that the apoptotic events observed in animals not treated may be considered as a direct consequence of the in vivo-induced myogenic conversion. Our data are in agreement with the evidence that MyoD-positive cells (i.e., activated satellite cells and proliferating myoblasts in culture) are able to undergo apoptosis (22,38).

In conclusion, we have focused on two different aspects. First was the possibility to regulate the myogenic conversion of nonmuscle cells such as fibroblasts in a tetracycline-dependent manner; the feasibility of the system has been demonstrated also through in vivo experiments. Second, we have shown one of the most important limits of ex vivo gene therapy of primary myopathies based on the forced conversion of fibroblast to myoblasts. There is for that situation very poor long-term survival of a high number of donor cells transplanted into the muscle of the hosts. In particular, we have examined the possibility that apoptotic events might play a role in the death of transplanted cells. This problem will have to be further addressed in the perspective of the cell-mediated gene therapy of inherited muscle disorders.

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