

Nerve Growth Factor Improves the Muscle Regeneration Capacity of Muscle Stem Cells in Dystrophic Muscle

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ABSTRACT

Researchers have attempted to use gene- and cell-based therapies to restore dystrophin and alleviate the muscle weakness that results from Duchenne muscular dystrophy (DMD). Our research group has isolated populations of muscle-derived stem cells (MDSCs) from the postnatal skeletal muscle of mice. In comparison with satellite cells, MDSCs display an improved transplantation capacity in dystrophic *mdx* muscle that we attribute to their ability to undergo long-term proliferation, self-renewal, and multipotent differentiation, including differentiation toward endothelial and neuronal lineages. Here we tested whether the use of nerve growth factor (NGF) improves the transplantation efficiency of MDSCs. We used two methods of *in vitro* NGF stimulation: retroviral transduction of MDSCs with a CL-NGF vector and direct stimulation of MDSCs with NGF protein. Neither method of NGF treatment changed the marker profile or proliferation behavior of the MDSCs, but direct stimulation with NGF protein significantly reduced the *in vitro* differentiation ability of the cells. NGF stimulation also significantly enhanced the engraftment efficiency of MDSCs transplanted within the dystrophic muscle of *mdx* mice, resulting in the regeneration of numerous dystrophin-positive muscle fibers. These findings highlight the importance of NGF as a modulatory molecule, the study of which will broaden our understanding of its biologic role in the regeneration and repair of skeletal muscle by muscle-derived cells.

OVERVIEW SUMMARY

Growth factors play important roles as signaling molecules throughout postnatal development, adult life, and aging. This study investigated the effect of nerve growth factor (NGF) on the regeneration of the skeletal muscle of dystrophic (*mdx*) mice. Transplantation of muscle-derived stem cells (MDSCs) either stimulated with or genetically engineered to express NGF resulted in the regeneration of significantly more dystrophin-positive myofibers than did transplantation of control (nontreated) MDSCs. NGF did not alter the marker profile or proliferation behavior of MDSCs; however, MDSCs stimulated with NGF exhibited reduced *in vitro* differentiation, which may at least partially explain their improved regeneration capacity. These findings underscore the importance of NGF during skeletal muscle tissue remodeling and indicate that this molecule can improve the muscle regeneration capacity of muscle stem cells.

INTRODUCTION

DUCHENNE MUSCULAR DYSTROPHY (DMD) is a progressive muscle disorder characterized by dystrophin deficiency that results in initial necrosis of muscle fibers, which in turn leads to progressive muscle weakness (Duchenne, 1868; Hoffman *et al.*, 1987a; Gorospe and Hoffman, 1992). Researchers have localized dystrophin in the sarcolemma of myofibers (Arahata *et al.*, 1988; Bonilla *et al.*, 1988; Watkins *et al.*, 1988), where it is thought to play a role in maintaining plasma membrane integrity and stability (Hutter *et al.*, 1991; Menke and Jockusch, 1991; Petrof *et al.*, 1993). Because dystrophic muscle has a heightened susceptibility to structural damage and a decreased capacity to undergo self-repair, early death commonly occurs as a result of congestive cardiac and/or respiratory failure.

Despite the extensive efforts of researchers to develop an effective way to deliver dystrophin in dystrophic muscle (e.g.,

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cell and gene therapy), there is no known treatment that substantially slows the course of DMD and rescues the diseased muscle tissue. Because of the unavailability of successful gene therapy, researchers have attempted to use a variety of other approaches to impede the predictable, progressive decline in muscle strength; the most successful of these approaches involves the use of steroids. Several randomized trials focused on corticosteroids (mainly prednisone and deflazacort) have shown that steroids improve muscle function and strength by prolonging ambulation and preventing scoliosis in boys with DMD (Biggar *et al.*, 2004; Bushby *et al.*, 2004; Yilmaz *et al.*, 2004). Although the mechanism is not clear, corticosteroids have antiinflammatory and membrane-stabilizing effects (Alman, 2005) and act as catabolic agents, reducing muscle fiber size in the skeletal muscle of *mdx* mice and making the fibers less susceptible to contraction-induced muscle damage (Lynch *et al.*, 2000). However, because not all clinical trials have generated conclusive results showing the efficacy and safety of long-term steroid use, the utility of these drugs remains unknown (Lynch, 2001; Campbell and Jacob, 2003; Manzur *et al.*, 2004).

Advances in clinical management also have significantly improved the life expectancy of DMD patients, their functional ability, and their quality of life. These advances include the use of respiratory muscle training, noninvasive ventilation (Smith *et al.*, 1988; Bach *et al.*, 1991), a mechanical in-exsufflator for pediatric patients (Miske *et al.*, 2004), and low-resistance exercise and voluntary active exercises such as swimming and hydrotherapy, which increase muscle strength and endurance (for review, see Eagle, 2002). In addition, physical therapy can help relax joint contractures and early stabilization of the spine can maintain patient balance and slow the progression of respiratory compromise (Harper *et al.*, 2004).

Like the muscles of humans with DMD, the muscles of *mdx* mice are dystrophin deficient because of a point mutation in the dystrophin gene (Hoffman *et al.*, 1987b); this genetic similarity makes the *mdx* mouse an excellent genetic and biochemical model for DMD. Unlike the muscles of humans with DMD, however, the muscles of *mdx* mice maintain active and efficient regeneration that compensates for the repeated cycles of degeneration. Although the limb skeletal muscle of *mdx* mice continues to undergo relatively efficient regeneration regardless of the age of the animals, muscles in the diaphragms of *mdx* mice of all ages and in the postural-tonic muscles (such as the soleus) of old mice (more than 52 weeks) exhibit many pathologic features identical to those observed in the muscle of DMD patients, including fiber atrophy; frequent splitting, necrosis, and progressive fibrosis; and severe loss of tissue compliance (Stedman *et al.*, 1991; Pastoret and Sebille, 1993, 1995).

Although lack of dystrophin leads to progressive muscle degeneration, the evolution of DMD is likely dependent on other factors, such as insufficient expression of growth-associated proteins. After skeletal muscle damage, quiescent myogenic stem cells, which are normally embedded in the basal lamina of the muscle fibers, are activated; these cells migrate toward the damaged area, where they undergo a cycle of proliferation, fusion, and differentiation that culminates in the generation of myofibers that replace the damaged ones (Grounds, 1991). Various growth factors appear to regulate skeletal myoblast proliferation and differentiation, play a role in different stages of muscle regeneration, and enhance the healing process (Allen *et al.*,

1990; Florini *et al.*, 1991; Trippel, 1997). In addition to stimulating cell proliferation, growth factors can support cell survival and regulate critical intracellular signal transduction pathways (Weiss and Schlessinger, 1998) under conditions that otherwise lead to apoptotic death.

To date, the list of growth factors known to affect the behavior of skeletal muscle cells or be expressed in skeletal muscle tissue is extensive. However, few studies have investigated the role of nerve growth factor (NGF) during skeletal muscle regeneration, and its exact mechanism of action is poorly understood. In addition to acting as a target-derived factor for developing neurons, NGF has an autocrine effect on myoblast proliferation and fusion (Rende, 1998; Ou *et al.*, 1999; Rende *et al.*, 2000). Moreover, adult knockout mice expressing a neutralizing antibody against NGF display a severe dystrophy and reduced muscle mass (Capsoni *et al.*, 2000; Ruberti *et al.*, 2000). Evidence suggests that NGF acts by binding to the high-affinity tyrosine kinase receptor (TrkA) and the low-affinity p75-neurotrophin receptor (p75^{NTR}). TrkA is found in developing adult rat myoblasts (Wheeler *et al.*, 1998) and during differentiation of muscle cells (Rende *et al.*, 2000). NGF and p75^{NTR} are widely expressed in myoblasts, human myocyte cultures, and regenerating myofibers in the muscle of DMD patients (Zhao *et al.*, 1991; Baron *et al.*, 1994).

Previous studies in our laboratory have revealed that the delivery of human recombinant NGF protein by direct intramuscular injection improves muscle recovery (Kasemkijwattana *et al.*, 2000; Menetrey *et al.*, 2000). However, the efficiency of direct intramuscular injection of growth factors varies depending on the type of injury encountered and is limited by the need to maintain high-enough concentrations to achieve a therapeutic effect. In addition, the use of growth factor proteins to promote healing is severely hindered by the difficulty of ensuring their delivery to the injured site (Evans and Robbins, 1994), their short biologic half-lives (Evans and Robbins, 1994; Robbins and Ghivizzani, 1998), and the rapid clearance of these molecules from the bloodstream. For the study reported here, we used a combination of muscle-derived stem cell (MDSC)-based gene therapy and direct stimulation with NGF protein to examine the effects of NGF on the proliferation and differentiation capacity of MDSCs *in vitro* and their regeneration efficiency in *mdx* muscle *in vivo*.

MATERIALS AND METHODS

Animals

mdx mice (C57BL/10SCsn DMD^{mdx/J}) were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal protocols used for these experiments were approved by the Institutional Animal Care and Use Committee of Children's Hospital of Pittsburgh (protocol 3/02).

Cell isolation and culturing

A previously described modified preplate technique (Qu-Petersen *et al.*, 2002) was used to obtain MDSCs from normal (C57BL/6J) 3-week-old female mice. Cells were cultured at an initial density of 450 cells/cm² in flasks coated with collagen type I (Sigma-Aldrich, St. Louis, MO) and were maintained in

proliferation medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with 10% horse serum, 10% fetal bovine serum [FBS], and 1% penicillin–streptomycin; all GIBCO-brand reagents from Invitrogen, Carlsbad, CA) containing 0.5% chick embryo extract (Accurate Chemical, Westbury, NY). After 2 days of growth (confluency < 50%), the cells were trypsinized, counted, and replated to generate the quantity of cells needed for each experiment.

Generation of retroviral vector expressing NGF

NGF cDNA was amplified from plasmid pSP72NGFpA (kindly provided by P. Robbins, University of Pittsburgh, Pittsburgh, PA) with primers NGF1 (agg cgg cgg ccc acc atg ctg tgc ctc aag cca gtg aaa) and NGF2 (tca aga tct tca gcc tct tct tgt agc ctt cct) and *Pfu* DNA polymerase (Stratagene, La Jolla, CA). The polymerase chain reaction (PCR) product was cut with restriction enzymes *NotI* and *BglIII* and cloned into the same two sites of retroviral vector pCLX. The vector DNA was converted into a replication-defective retrovirus by cotransfection (by calcium phosphate precipitation) into packaging cell line GP-293 (Clontech, Palo Alto, CA) with a plasmid, pVSVG, that expressed vesicular stomatitis virus glycoprotein as the viral envelope (Peng *et al.*, 2002). Conditioned medium containing retroviral vector was stored at -80°C until use.

Stimulation or retroviral transduction of MDSCs with NGF

MDSCs were plated at 20–30% initial confluency and either stimulated with NGF (100 ng/ml; Sigma-Aldrich) for 7 days (S-MDSCs) or retrovirally transduced (in the presence of Polybrene [8 $\mu\text{g}/\text{ml}$]) with the CL-NGF vector to express NGF (E-MDSCs) at a multiplicity of infection of 5. Normal MDSCs neither stimulated nor transduced served as the control group (C-MDSCs). E-, S-, and C-MDSCs were expanded for 1 week in proliferation medium (20% serum) at an initial density of 8×10^4 cells per well on collagen type I-coated 6-well plates, where they remained for 48 hr; the proliferation medium then was replaced with low-serum medium (2% FBS), in which the cells were cultured for an additional 24 hr. Tissue culture supernatant was collected and spun at 1200 rpm for 5 min at 4°C , and the level of functional NGF secreted by the cells in the tissue culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA) (NGF E_{max} ImmunoAssay System kit; Promega, Madison, WI) performed as detailed in the manufacturer's instructions.

Cell characterization by flow cytometry

Flow cytometry was used to analyze the expression of the cell surface markers CD34 and stem cell antigen-1 (Sca-1). Cultured cells were trypsinized, spun, washed in a buffer solution made of phosphate-buffered saline (PBS) (Dulbecco phosphate-buffered salt solution 1 \times ; Mediatech, Herndon, VA) containing 0.5% bovine serum albumin (BSA; MP Biomedicals, Irvine, CA) and 0.1% sodium azide (Sigma-Aldrich), and counted. After trypsinization, the cells were maintained on ice for the remainder of the procedure. The cells were then divided into aliquots and spun into a pellet. A blocking solution containing a 1:10 dilution of mouse serum (Sigma-Aldrich) in the buffer so-

lution described above supplemented with 1 μl of Fc block (rat anti-mouse CD16/CD32; BD Biosciences Pharmingen, San Diego, CA) was used to resuspend each pellet, and the suspensions were incubated for 10 min on ice. Predetermined, optimal amounts of both direct and biotin-conjugated rat anti-mouse monoclonal antibodies (CD34 and Sca-1) were each placed in tubes for 30 min. Each experimental tube received fluorescein isothiocyanate (FITC)-conjugated CD34 and biotin-conjugated Sca-1. A separate cell portion received equivalent amounts of isotype control antibodies. After several rinses, all fractions (including the controls) were labeled with streptavidin–allophycocyanin (APC) for 20 min. Just before analysis, 7-amino-actinomycin D (7-AAD) was added to each tube to exclude nonviable cells from the analysis. All antibodies, including APC and 7-AAD, were purchased from BD Biosciences Pharmingen. At least 10,000 live cell events were analyzed by flow cytometry (FACSAria cytometer using FACSDiva software; BD Biosciences Immunocytometry Systems, Mountain View, CA).

Myogenic marker expression analyzed by immunocytochemistry

A fraction of each cell group was evaluated by immunofluorescence staining for expression of the myogenic proteins desmin and Pax-7. Analysis was performed on methanol-fixed cells that were blocked with 5% goat serum in PBS for 1 hr. The cells were incubated for 1 hr with the following primary antibodies: mouse IgG anti-desmin (diluted 1:250; Sigma-Aldrich) and mouse anti-Pax-7 (diluted 1:50; R&D Systems, Minneapolis, MN). After being rinsed thoroughly with PBS, the cells were incubated for 30 min with the secondary antibody, biotinylated goat anti-mouse IgG (diluted 1:250; Vector, Burlingame, CA). To fluorescently label the antigenic binding, the cells were washed and incubated with streptavidin–Cy3 (diluted 1:500; Sigma-Aldrich) for 10 min; nuclei then were counterstained with 4',6-diamidino-2-phenylindole (DAPI, diluted 1:10⁴; Sigma-Aldrich) in PBS. All dilutions were in 5% goat serum in PBS at room temperature. Negative control staining was performed according to an identical procedure, with omission of the primary antibody. Northern Eclipse software (version 6.0; EMPIX Imaging, Mississauga, ON, Canada) was used to quantify the percentage of myogenic cells as the ratio of cells that strongly expressed desmin or Pax-7 to the total number of nuclei in 10 randomly chosen fields at $\times 200$ magnification.

Cell division and myogenic differentiation

For analyses of population doubling time (PDT) and cellular division time (DT), cells from each group were plated at an initial density of 500 cells per well in a collagen type I-coated 12-well plate. A novel microscope imaging system (CytoWorks; Automated Cell, Pittsburgh, PA) equipped with a $\times 20$ objective was used to acquire images at 10-min intervals over a period of 4 days. DT was measured for each treatment condition by selecting and tracking 100 cells directly from a time-lapsed video record; the time lapse between cytokinesis events was recorded as the length of the cell division cycle. The average PDT was calculated by fitting an exponential trend line to several measured data points. PDT was estimated by using the software package SigmaStat 2.0 (SPSS, Chicago, IL) to per-

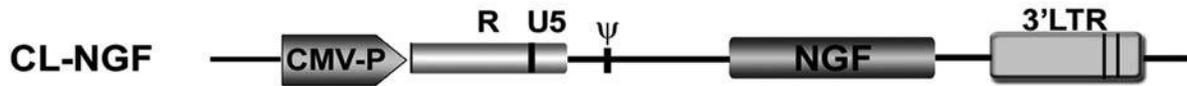


FIG. 1. Schematic of the retroviral vector expressing NGF. The vector CL-NGF carries a 3' long terminal repeat (LTR), virus packing signal (ψ), and human NGF cDNA driven by the cytomegalovirus promoter (CMV-P).

form nonlinear regression to generate the best fit to the curve. The fraction of daughter cells that were dividing (α) was calculated by measuring PDT and DT and solving the rearranged Sherley model to obtain the correlation coefficient (R^2) for the nonlinear regression (Sherley *et al.*, 1995; Deasy *et al.*, 2002), a value that indicates how well the data actually fit the model (such that $0 < R^2 < 1.0$).

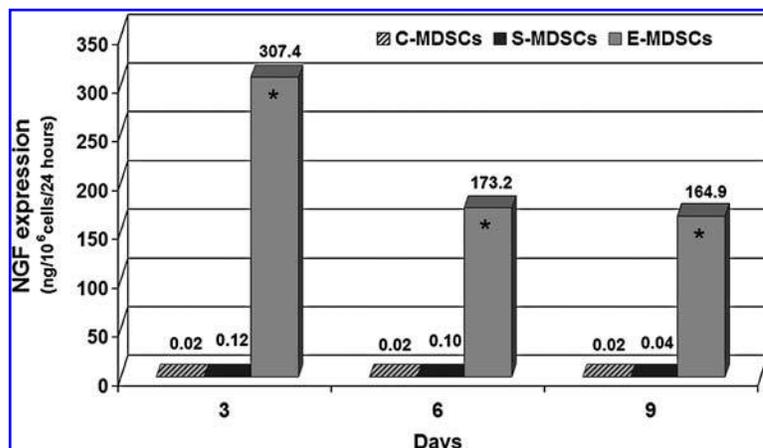
Myogenic differentiation was evaluated by immunocytochemical staining for MyoD and fast myosin heavy chain (MyHC) expression. MyoD is a marker of early myogenic differentiation, whereas MyHC is a marker of terminal myogenic differentiation. E-, S-, and C-MDSCs were plated in collagen type I-coated 24-well plates in high-serum DMEM (described earlier). To induce differentiation, proliferation medium was replaced with differentiation medium (low serum: DMEM supplemented with 2% FBS and 1% penicillin-streptomycin). Two sets of experiments were performed to permit assessment of the rate of differentiation at multiple time points. In the first set of experiments, cells from each group were seeded at a density of 500 cells/cm². The cultures were shifted into differentiation medium after 3 days, and the cells were stained for MyoD and MyHC at 2, 4, 6, and 8 days. In the second set of experiments, cells from each group were seeded at a density of 1000 cells/cm². The cultures were shifted into differentiation medium 1 day after seeding, and the cells were stained for MyoD and MyHC at 3, 5, 7, 9, and 11 days. The differentiation medium was replaced every 2 days. Immunocytochemistry staining for MyHC was performed as described earlier for myogenic markers with monoclonal mouse anti-MyHC (diluted 1:250; Sigma-Aldrich) as the primary antibody. For immunofluorescence staining of MyoD, cultured cells were fixed with 2% paraformaldehyde in PBS containing 1% Triton X-100 and were blocked in 5% horse serum in PBS for 1 hr. The cells then were incubated for 3 hr with anti-MyoD monoclonal antibody clone 5.8A (diluted 1:150; BD Biosciences

Pharmingen) in the same blocking solution. The cells were exposed to a secondary biotinylated anti-mouse IgG (diluted 1:200; Vector Laboratories, Burlingame, CA) in PBS for 1 hr and to streptavidin-Cy3 (diluted 1:500; Sigma-Aldrich) for 15 min. Nuclei were counterstained with DAPI. All procedures were performed at room temperature. Negative control staining was performed by an identical procedure, but the primary antibody was omitted. For each group and time point, 10 randomly chosen representative fields were evaluated at $\times 200$ magnification to determine the degree of differentiation (percent ratio of MyoD- or MyHC-expressing nuclei to total number of nuclei), an indicator of differentiation efficiency.

Myofiber regeneration in vivo

A total of $(2-3) \times 10^5$ C-, E-, or S-MDSCs was injected into the gastrocnemius muscle of 6- to 8-week-old male *mdx* mice. Mice in the E-MDSC group and their controls were immunosuppressed by subcutaneous injection of tacrolimus ($C_{44}H_{69}NO_{12} \cdot H_2O$), previously known as FK506 (2.5 mg/kg mouse body weight per day; Astellas Pharma US, Deerfield, IL) beginning on the day of cell transplantation and continuing until the day of euthanization; this immunosuppression protocol is described elsewhere (Kinoshita *et al.*, 1994). Ten to 14 days after transplantation, the gastrocnemius muscles were harvested, flash frozen in liquid nitrogen-cooled 2-methylbutane, and serially sectioned (10 μ m). For dystrophin staining of the cryopreserved tissue, acetone-fixed, horse serum-blocked sections were exposed to a rabbit anti-dystrophin antibody (diluted 1:1000, kindly provided by T. Partridge, Imperial College of Medicine, London, UK) for 3 hr, as previously described (Lu *et al.*, 2003). Sections then were washed in PBS and incubated with biotinylated anti-rabbit IgG antibody for 1 hr. Next, the sections were washed again and incubated with streptavidin-Cy3 (diluted 1:300; Sigma-Aldrich) for

FIG. 2. Quantitative detection of the NGF protein in cell supernatant by ELISA. Two different methods of NGF stimulation were used in this study: retroviral transduction of MDSCs with the CL-NGF vector to induce expression of human NGF (E-MDSCs) and direct stimulation of MDSCs with NGF protein (100 ng/ml) for 7 days (S-MDSCs). For both groups, normal MDSCs that were neither stimulated nor transduced served as controls (C-MDSCs). ELISA results revealed that, in comparison with S- and C-MDSCs, E-MDSCs produced a significantly higher level of NGF 3, 6, and 9 days after transduction ($n = 3$, $*p < 0.05$).



20 min. All incubations were at room temperature. Fluorescence microscopy was performed and digital images were acquired with a Nikon Eclipse E800 microscope equipped with a Spot digital camera and Northern Eclipse software (version 6.0; EMPIX Imaging). Muscle regeneration was assessed by manually counting the number of dystrophin-positive myofibers in an area containing the largest graft and calculating the regeneration efficiency index (RI: the number of dystrophin-positive fibers in the host muscle per 10^5 donor cells) for ease of both comparison and graphical display (Jankowski *et al.*, 2002).

Myofiber analysis

Northern Eclipse software and the same images used to calculate the RI were used to perform dimensional analysis of regenerated dystrophin-positive myofibers. Using a threshold to distinguish the immunofluorescence signal from the background signal, this software determines the area of each fiber and provides quantitative measurements of the number of pixels occupied by each individual fiber. The fiber area distribution (FAD) of 4000 individual myofibers per group was measured by determining the total number of pixels occupied by each fiber—a number that was easy to convert to square micrometers with analysis software. Hematoxylin and eosin (H&E) staining of noninjected regions of the same grafts was used to detect the boundaries of the host *mdx* myofibers.

Statistical analysis

Differences with $p < 0.05$ were considered statistically significant. All values are given as the mean \pm standard deviation of the mean (SD). The Student *t* test or the Mann–Whitney rank sum test (where appropriate) was used for direct comparisons between treatment and control groups. Multiple group compar-

isons were made through the use of one-way analysis of variance (ANOVA) and Student–Newman–Keuls pairwise comparisons to determine the significance level. Nonparametric distributions were also detected, and comparisons were made using Kruskal–Wallis one-way ANOVA on ranks with the Tukey test for pairwise comparisons or the Dunnett test for comparing treatment groups with a single control group.

RESULTS

Quantitative detection of NGF

We used an ELISA to measure the levels of NGF secreted by MDSCs *in vitro*. After transduction with retroviral NGF (schematic shown in Fig. 1), genetically engineered MDSCs (E-MDSCs) were able to synthesize, process, and secrete active human NGF. The level of NGF secreted by E-MDSCs reached 307.4 ± 42.5 ng/ 10^6 cells per 24 hr 3 days after transduction and continued to be high 6 and 9 days after transduction (173.2 ± 11.1 and 164.9 ± 28.2 ng/ 10^6 cells per 24 hr, respectively, $n = 3$, $p < 0.05$). In contrast, NGF-stimulated MDSCs (S-MDSCs) and control (nontreated) MDSCs (C-MDSCs) on average secreted NGF at barely detectable levels at the three time points (0.09 ± 0.17 and 0.02 ± 0.37 ng/ 10^6 cells per 24 hr, averages for S-MDSCs and C-MDSCs, respectively; Fig. 2).

Proliferation kinetics

To investigate the cellular response of MDSCs to NGF, we examined cellular division time (DT) and population doubling time (PDT). Using images acquired every 10 min for a period of 4 days, we measured the DT of the dividing cells ($n = 100$ cells per group) by recording the lapsed time between cytokinesis

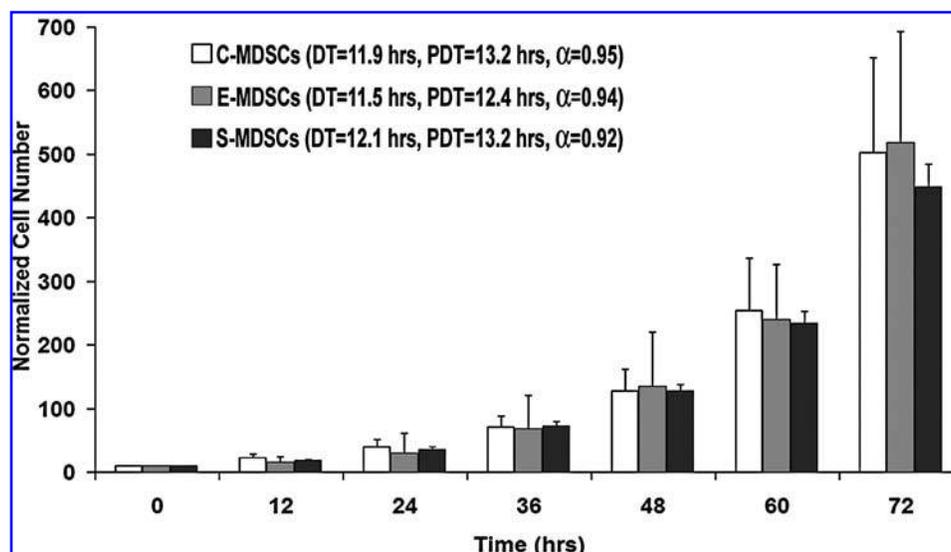


FIG. 3. Proliferation kinetics of MDSCs. Shown is the independent variable, time (t), versus the dependent variable, normalized cell number. Comparison of the C-, E-, and S-MDSC groups ($n = 100$ cells per group, $p = 0.053$) revealed no differences in the average cellular division time (DT) or population doubling time (PDT). We fit the experimental data sets to the model equations by using nonlinear regression to estimate the mitotic fraction (α) (i.e., the fraction of daughter cells that was actively dividing). The mean mitotic fraction remained relatively constant among the groups (approximately 0.94).

events. We fit experimental data sets for C-, E-, and S-MDSCs to the Sherley model equations by using nonlinear regression with the correlation coefficient $R^2 > 0.90$ to estimate mitotic fraction (α) (i.e., the fraction of daughter cells that are actively dividing). Our data suggest that the average DTs (C-MDSCs, 11.9 hr; E-MDSCs, 11.5 hr; and S-MDSCs, 12.1 hr) were not significantly different in the various groups ($p = 0.053$, Kruskal–Wallis analysis on ranks) and that the PDTs of the different cell groups were also quite similar (11–13 hr). Moreover, we observed a strong association for the three groups (as indicated by $R^2 = 0.99$) but no difference in the estimated α (C-MDSCs, 0.95; E-MDSCs, 0.94; S-MDSCs, 0.92) (Fig. 3). These results indicate that neither NGF transduction nor NGF stimulation significantly altered the proliferation kinetics of MDSCs.

In vitro stem cell and myogenic marker expression profiles

Flow cytometry revealed similar expression of CD34 and Sca-1 by C-, E-, and S-MDSCs (Fig. 4A). More than 70% of the

cells in each group were positive for both stem cell markers, and we observed no significant difference between the groups (in terms of stem cell marker expression) after 2 weeks of *in vitro* expansion ($n = 3, p = 0.655$). This marker stability suggests that NGF did not affect the stem cell marker expression of MDSCs *in vitro*.

We also used immunofluorescence staining to assess expression by the cells of two myogenic proteins: Pax-7 and desmin. As shown in Fig. 4B, there was no significant difference in the expression of Pax-7 by the various groups of treated and nontreated MDSCs ($n = 4, p = 0.148$). Whereas E- and C-MDSCs expressed similar levels of desmin, the S-MDSC group contained significantly more desmin-expressing cells ($n = 4, p < 0.05$).

In vitro myogenic differentiation

After cultivating the cells under low-serum conditions and at various densities, we performed immunohistochemical staining for MyoD and fast myosin heavy chain (MyHC) expression to analyze myotube formation in the three groups (Fig. 5A–C). To

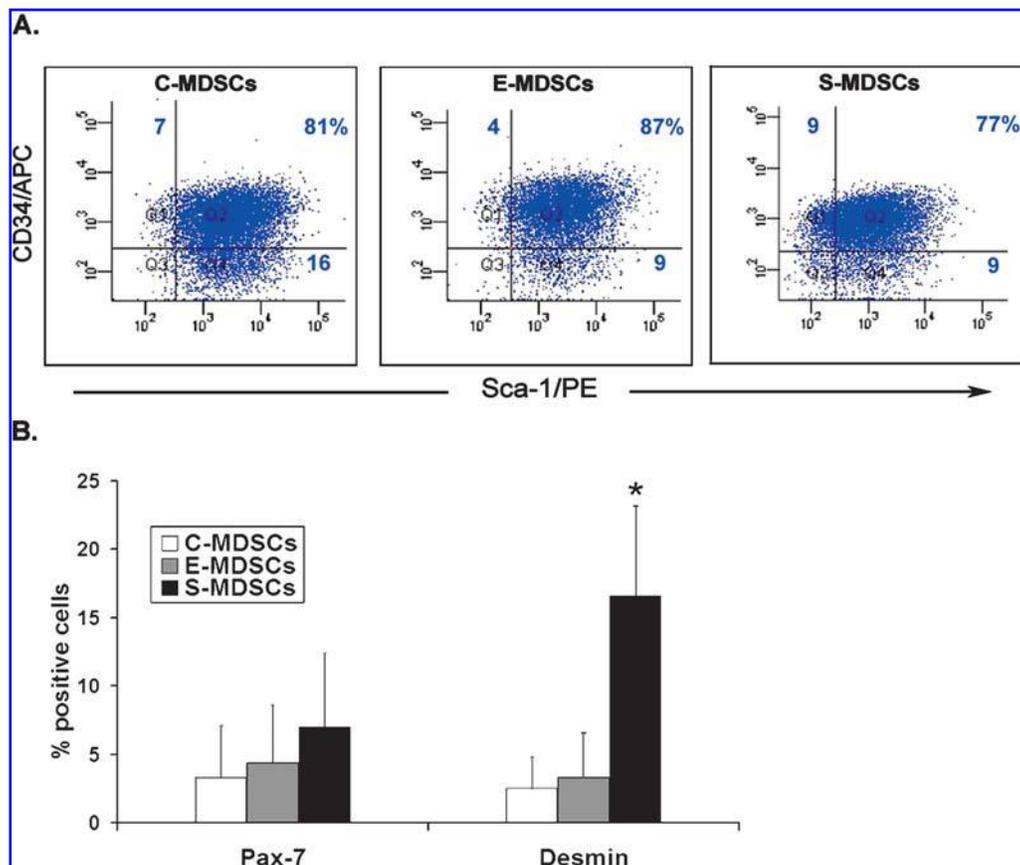


FIG. 4. Marker profile analysis of MDSCs. (A) Flow cytometry revealed high expression levels of the stem cell markers CD34 and Sca-1 (>70%) by the control cells (C-MDSCs) and 7 days after either retroviral transduction of the cells with the CL-NGF vector (E-MDSCs) or stimulation with NGF (S-MDSCs). After 2 weeks of *in vitro* expansion, the groups exhibited no significant differences in marker profiles ($n = 3, p = 0.655$). Data were collected by performing logarithmic amplification on 5000 cells and excluding cell debris by combining forward and side scatters and are presented as dot plots (the percentage of cells in each relevant quadrant is indicated in the upper right-hand corner). (B) A myogenic differentiation assay revealed low levels of Pax-7 and desmin expression. There were no significant differences in Pax-7 expression between the treated groups and the control and no significant difference in desmin expression between E-MDSCs and C-MDSCs; however, direct stimulation of MDSCs with NGF resulted in significantly more desmin-positive cells in the S-MDSC group ($n = 4, *p = 0.05$).

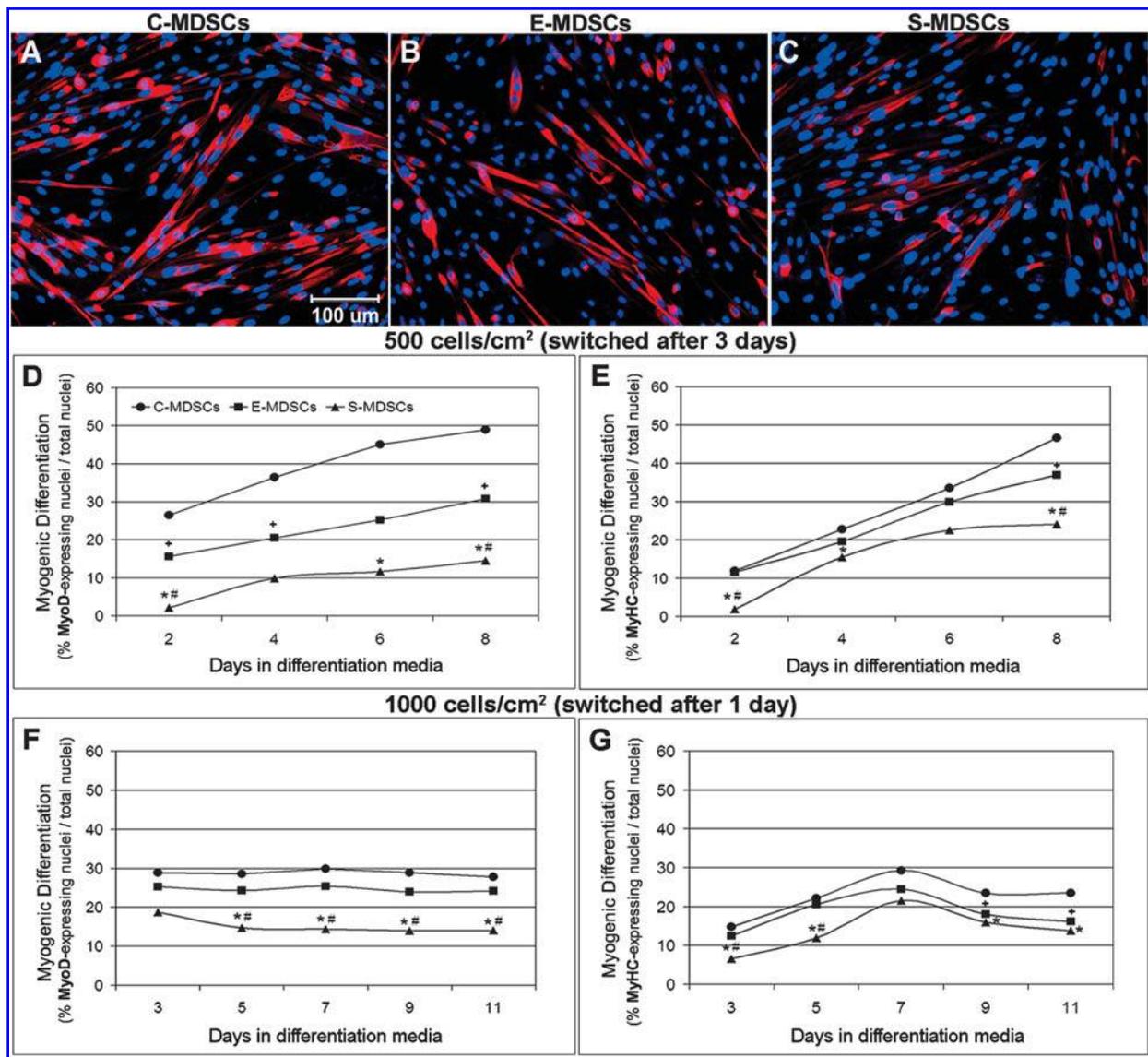


FIG. 5. Time course of myogenic differentiation *in vitro*. Representative images of MyHC-positive C-, E-, and S-MDSCs (Cy3; red) overlaid on nuclear counterstain (DAPI; blue) are shown (A–C). Scale bar: 100 μ m. The degree of differentiation (percent ratio of MyoD- or MyHC-expressing nuclei to total nuclei) is indicated both for cells seeded at an initial density of 500 cells/cm², switched to differentiation medium after 3 days, and stained at 2, 4, 6, and 8 days for MyoD (D) and MyHC expression (E) and for cells seeded at an initial density of 1000 cells/cm², switched to differentiation medium the next day, and stained at 3, 5, 7, 9, and 11 days for MyoD (F) and MyHC expression (G). Comparison of E-MDSCs (solid squares) with C-MDSCs (solid circles) revealed that the differentiation efficiency of the E-MDSCs appears to lie between the values for C-MDSCs and S-MDSCs at all time points under both experimental conditions; however, in comparison with C-MDSCs, S-MDSCs seeded at either density (solid triangles) consistently exhibited a lower degree of differentiation ($*p < 0.05$ [S-MDSCs versus C-MDSCs], $+p < 0.05$ [E-MDSCs versus C-MDSCs], and $\#p < 0.05$ [E-MDSCs versus S-MDSCs], Kruskal–Wallis one-way ANOVA on ranks) (D–G). Each graph portrays results that are representative of three independent experiments.

determine the effect of cell confluency or cell differentiation, we seeded the cells at a density of either 500 or 1000 cells/cm² and switched the cells to differentiation medium 3 days or 1 day after initial seeding. Under these experimental conditions, S-MDSCs consistently showed significantly lower rates of differentiation efficiency than C- and E-MDSCs, which indicates that S-MDSCs had a decreased ability to fuse and form multinucleated myotubes (Fig. 5D–G: $*p < 0.05$ [S-MDSCs versus C-MD-

SCs], $+p < 0.05$ [E-MDSCs versus C-MDSCs], and $\#p < 0.05$ [E-MDSCs versus S-MDSCs], Kruskal–Wallis one-way ANOVA on ranks). The differentiation efficiency of the E-MDSCs appears to lie between the values for C-MDSCs and S-MDSCs at all time points in both experimental conditions. These results were consistent regardless of initial cell seeding density or assay duration. Each graph portrays results that are representative of three independent experiments.

Muscle regeneration

We evaluated the ability of the three groups of cells (C-, E-, and S-MDSCs) to regenerate dystrophic skeletal muscle by transplanting 3×10^5 cells from each cell group into the gastrocnemius muscles of 8-week-old male *mdx* mice. The dystrophin-positive grafts of E- and C-MDSCs are shown in Fig. 6A and B. The average RI of E-MDSCs was significantly larger than that of C-MDSCs (435.6 ± 85.5 versus 197.5 ± 53.8 , $n = 8$ mice per group, $p < 0.001$; Fig. 6C). Differences in the size of the dystrophin-positive grafts generated by S-MDSCs and C-MDSCs were even more dramatic, as shown in Fig. 7A and B, respectively. Our analysis revealed that the average RI of S-MDSCs was 3-fold higher than that of C-MDSCs (852 ± 203.3 versus 266.8 ± 137.4 , $n = 11$ mice per group, $p < 0.001$; Fig. 7C). In addition, the RI of S-MDSCs was statistically higher than that of E-MDSCs ($p < 0.001$).

FAD analysis of grafts generated by the three groups of MDSCs revealed that more than 50% of the dystrophin-positive myofibers ($n = 5000$ myofibers) in each group had areas of $0\text{--}100 \mu\text{m}^2$; in this range, no statistically significant differences existed among the treatment groups compared with C-MDSCs ($p = 0.678$; Fig. 8). We also analyzed noninjected areas of *mdx* muscle and found that most of the myofibers had areas greater than $1000 \mu\text{m}^2$. These findings indicate that

C-, E-, and S-MDSCs generated new dystrophin-positive myofibers primarily through the fusion of donor cells with one another, as indicated by the small size and centronucleation of the regenerated myofibers (Fig. 8B).

DISCUSSION

Although the precise role that NGF plays in the various steps of muscle regeneration remains largely unknown, our observations indicate that NGF can improve the ability of MDSCs to regenerate the skeletal muscle of *mdx* mice. Whereas the results of our *in vitro* experiments demonstrate that neither retro-NGF transduction nor direct stimulation with NGF protein changes the marker profile of MDSCs or their proliferation dynamics, direct stimulation with NGF protein appears to reduce the myogenic differentiation of MDSCs *in vitro*.

The process of muscle regeneration in normal and dystrophic muscle depends on locally produced cytokines and growth factors (DiMario *et al.*, 1989; Anderson *et al.*, 1991; Clarke *et al.*, 1993; Trippel, 1997). Skeletal muscle injuries induce a well-established sequence of cellular events that result in the release of growth factors that stimulate quiescent satellite cells and other muscle precursor cells to enter the cell cycle, proliferate,

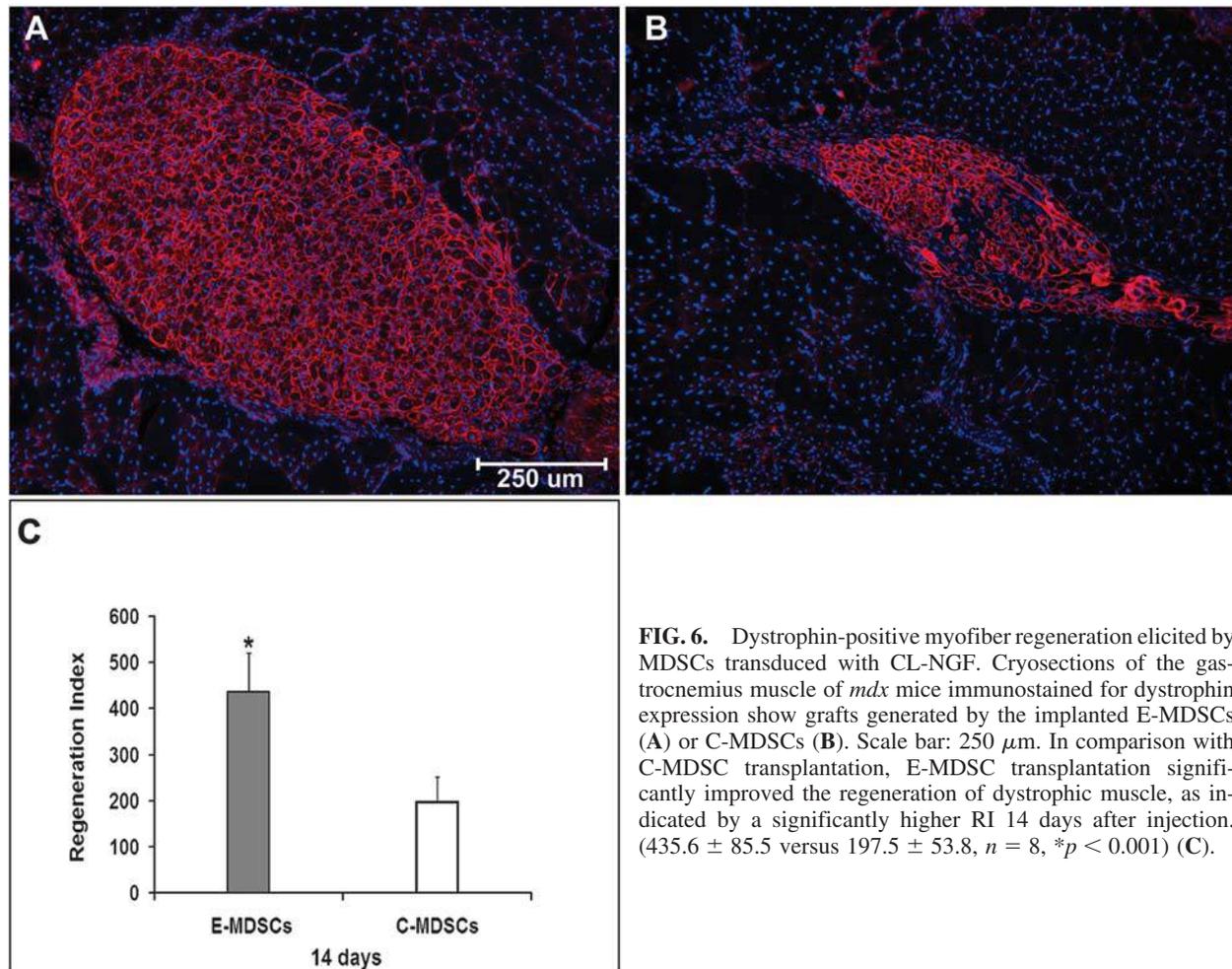
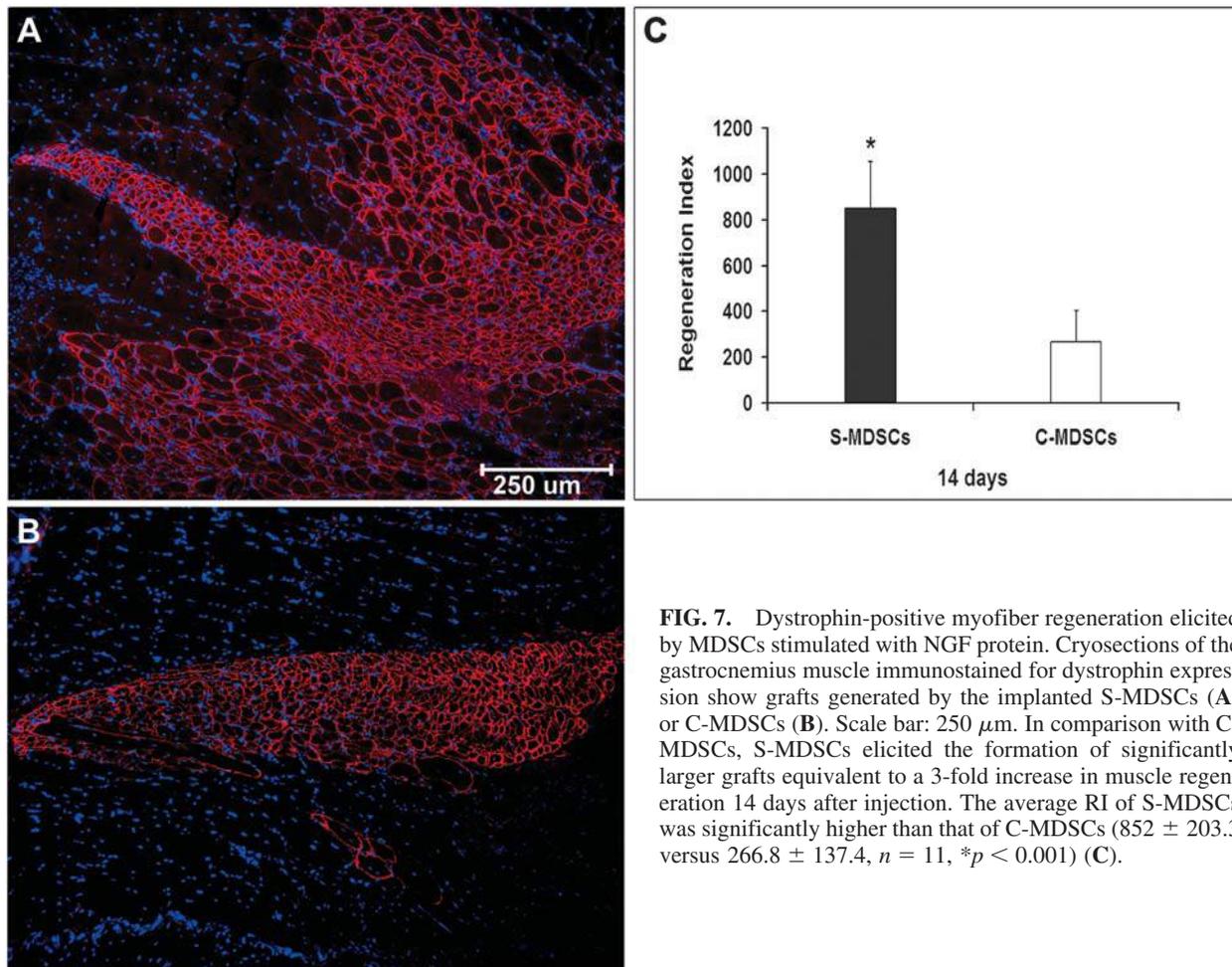


FIG. 6. Dystrophin-positive myofiber regeneration elicited by MDSCs transduced with CL-NGF. Cryosections of the gastrocnemius muscle of *mdx* mice immunostained for dystrophin expression show grafts generated by the implanted E-MDSCs (A) or C-MDSCs (B). Scale bar: $250 \mu\text{m}$. In comparison with C-MDSC transplantation, E-MDSC transplantation significantly improved the regeneration of dystrophic muscle, as indicated by a significantly higher RI 14 days after injection. (435.6 ± 85.5 versus 197.5 ± 53.8 , $n = 8$, $*p < 0.001$) (C).



and eventually fuse to form newly regenerated myofibers and restore muscle architecture (White and Esser, 1989; Grounds, 1991). Some studies suggest that NGF, the best characterized neurotrophic factor, plays an important role in restoring innervation in many tissues after injury. For example, NGF is integral to the survival of sensory and peripheral neurons that control contraction of smooth muscle (Kushima *et al.*, 1992; Shitaka and Saito, 1994; Acosta *et al.*, 2001) and axonal regeneration in skeletal muscle (McCallister *et al.*, 2001).

A prior study performed in our laboratory has shown that MDSCs stimulated with NGF or vascular endothelial growth factor (VEGF) acquire the expression of 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), a myelin-associated enzyme, and von Willebrand factor (vWF), an endothelial cell marker (Qu-Petersen *et al.*, 2002), findings that indicate the potential differentiation of MDSCs toward neurogenic and endothelial lineages, respectively. These results suggest that the release of local environmental cues (1) can trigger the differentiation of muscle stem cells toward nonmyogenic lineages after transplantation of the cells into dystrophic muscle and (2) may contribute to the regeneration of functional muscle tissue by enhancing vascular and neuronal supplies.

Here we investigated whether MDSCs retrovirally transduced to express NGF display different proliferation behavior

or altered myogenic differentiation *in vitro* and improved regeneration of dystrophic muscle *in vivo*. We used MDSCs rather than satellite cells because MDSCs exhibit a superior transplantation capacity in skeletal muscle and the ability to undergo multilineage differentiation (Jankowski *et al.*, 2002; Qu-Petersen *et al.*, 2002). The *ex vivo* gene transfer approach enables constant delivery of lower, more physiological doses of proteins that, if delivered by direct injection, would be quickly degraded by natural processes. Retroviral vectors infect dividing cells with high efficiency (Salvatori *et al.*, 1993) and elicit long-term, stable expression of the gene of interest by becoming integrated within the host cell genome; such vectors are already being used in clinical settings (Anderson, 1992; Miller *et al.*, 1992). Our results indicate that MDSCs transduced with a retrovirus vector continuously delivered high levels of NGF for up to 9 days in culture while maintaining their typical stem cell marker profile (CD34 positive and Sca-1 positive). After injection into the skeletal muscle of *mdx* mice, E-MDSCs proliferated and differentiated to generate dystrophin-positive muscle fibers that formed a large graft as early as 14 days after injection.

The proliferation dynamics of the treated MDSCs, including PDT, DT, and α , were similar irrespective of the treatment method (i.e., stimulation or transduction). However, S-MDSCs exhibited higher regeneration efficiency *in vivo* than did E-MDSCs or

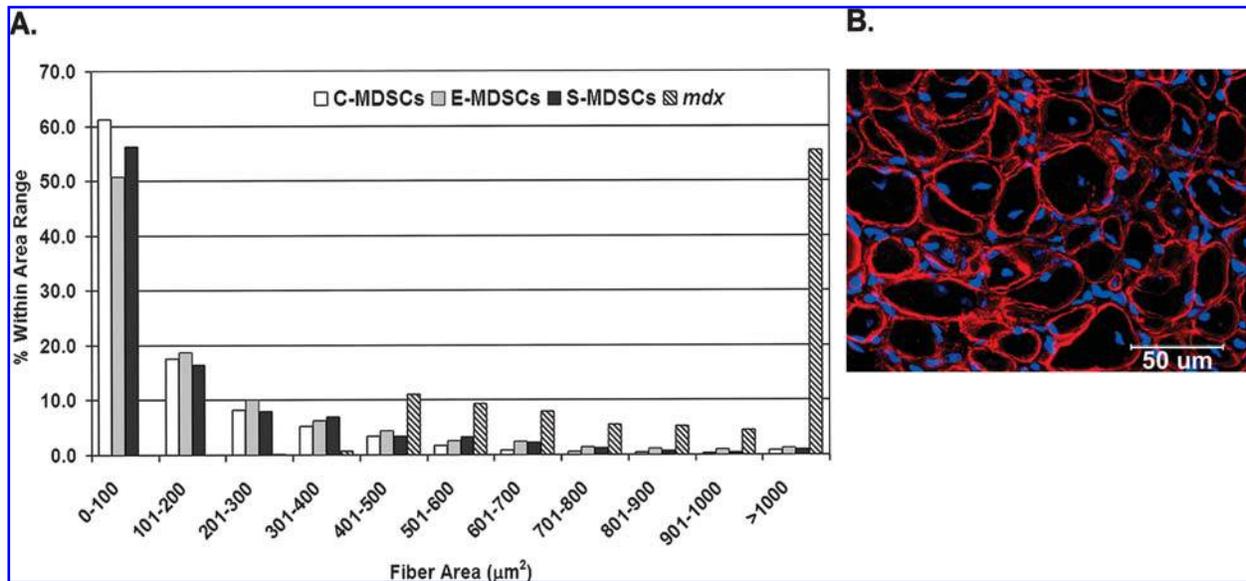


FIG. 8. Fiber area distribution (FAD) of newly generated (dystrophin-positive) and host myofibers. The overall distribution shows that most dystrophin-positive myofibers observed in the MDSC groups had areas of 0–100 μm², whereas most host *mdx* myofibers (control) had areas greater than 1000 μm² (A). In addition, a high-magnification image of a small portion of an E-MDSC graft, which is representative of the grafts observed in all MDSC groups, shows a large number of dystrophin-positive centronucleated myofibers (B). Five thousand myofibers in treated (dystrophin-positive) and noninjected areas were analyzed in each group.

C-MDSCs. Our examination of NGF secretion by the S- and E-MDSCs *in vivo* revealed no differences in the immunoreactivity of the cells to NGF (data not shown). However, it is important to note that the immunopositivity exhibited by the MDSCs could be due to both NGF from other sources in the muscle and NGF secreted by the injected cells. We postulate that the enhanced muscle regeneration exhibited by S-MDSCs compared with E- and C-MDSCs *in vivo* might be due to the lower degree of S-MDSC differentiation. Some research suggests that muscle cells with decreased fusion characteristics have increased regeneration capacities in skeletal muscle (Jankowski *et al.*, 2002). This could explain our observation that S-MDSCs differentiated into myotubes less effectively than did C- and E-MDSCs *in vitro* and exhibited the greatest regeneration efficiency *in vivo*. The higher proliferation ability of S-MDSCs before fusion *in vivo* might have allowed them to remain in an undifferentiated state longer, which would result in enhanced regeneration.

We were unable to uncover a cellular mechanism that would explain the relationship between the lower expression of the myogenic differentiation markers MyoD and MyHC by S-MDSCs and their higher expression of the myogenic proliferation marker desmin. We posit that exposure to exogenous NGF myogenic differentiation conditions (low serum level) reduces differentiation but permits proliferation. S-MDSCs and E-MDSCs initially seeded at higher densities and switched to differentiation medium 3 days after seeding showed lower fusion kinetics *in vitro* than did similarly cultured C-MDSCs.

Reddypalli and associates (2005) reported that signaling mediated by p75^{NTR}, the low-affinity receptor for NGF, plays an important role in normal muscle development; indeed, the ab-

sence of p75^{NTR} in mutant mice leads to impaired muscle force. They have also found that p75^{NTR}-mediated phosphorylation of Akt plays an important role in myoblast survival and that endogenous activation of Akt is most likely mediated by secreted NGF. The significance of the autocrine signaling by NGF is underscored by its downregulation before myoblast differentiation (Rende *et al.*, 2000). Because Akt mediates cell survival and p75^{NTR} activation can promote phosphorylation of Akt, we posit that NGF activates Akt through p75^{NTR} in MDSCs and thus promotes the survival of the MDSCs and their transition into differentiated states. On the basis of these findings, we believe that stimulated MDSC expression of NGF at optimal levels (i.e., levels that closely mimic biologic conditions) leads to endogenous activation of Akt and subsequent autocrine signaling of NGF. Consequently, high levels of NGF expression (as exhibited by E-MDSCs) likely reflect changes such as downregulation of p75^{NTR} and/or the TrkA receptor for NGF that reduce overall binding affinities for NGF. The results of future experiments should facilitate elucidation of the signaling mechanisms and the link between the *in vitro* and *in vivo* settings used to study muscle regeneration.

The FAD shows that most of the individual dystrophin-positive myofibers in all the MDSC groups had areas of 0–100 μm², whereas most of the host *mdx* myofibers had areas of >1000 μm². Nuclear staining with DAPI revealed that most of the small myofibers observed in the MDSC grafts were centronucleated. In combination, these results suggest that injection of C-, E-, or S-MDSCs led to the formation of new dystrophin-positive myofibers via the fusion of donor cells with one another rather than the fusion of donor cells with host muscle fibers. Studies conducted by Toti and associates (2003) show that only regenerated myofibers

in the muscles of DMD patients consistently express NGF; dystrophic myofibers or healthy myofibers do not show NGF immunoreactivity. In addition, the authors report that regenerated myofibers in muscle biopsy specimens from DMD patients are small and round, and occur mostly in clusters. These observations led them to hypothesize that, in addition to its autocrine function, NGF has a paracrine effect on neighboring regenerated myofibers. Our observations of myofibers generated by the MDSCs transplanted into the skeletal muscle of *mdx* mice in this study, particularly E-MDSCs, suggest a similar phenomenon.

Our study results show that NGF plays an important role in enhancing muscle regeneration by MDSCs. The performance of additional research focused on NGF will substantially enhance our understanding of the mechanism underlying this effect and, in so doing, may lead to the improvement of cell and gene therapy strategies for the treatment of DMD and other muscular dystrophies.

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