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ORIGINAL ARTICLE

Human Neurotrophin Receptor p75NTR Defines Differentiation-Oriented Skeletal Muscle Precursor Cells: Implications for Muscle Regeneration

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Abstract

Satellite cells are resident stem cells of adult skeletal muscle that have roles in tissue repair. Although several efforts have led to the functional characterization of distinct myogenic populations in animal models, the translation of these findings to humans has been limited. Here, we analyzed the expression and function of the neurotrophin receptor p75NTR in human skeletal muscle precursor cells. We combined histological investigations of muscle biopsies with molecular and cellular analyses of primary muscle precursor cells. p75NTR is expressed by most satellite cells in vivo and is a marker for regenerating fibers in inflamed and dystrophic muscle. p75NTR mRNA and protein are also detectable in primary myoblasts, and these levels increase transiently when cell differentiation is triggered. Transcriptome analyses of p75NTR^{high} versus p75NTR^{low} muscle cells showed that p75NTR is the prototype marker for a precursor cell population that has a broad transcriptional repertoire associated with muscle development and maturation. Several in vitro experiments, including receptor blockade and gene silencing in myoblasts, proved that p75NTR specifically regulates myogenesis and dystrophin expression. Taken together, the results indicate that p75NTR is a novel marker of human differentiation-prone muscle precursor cells that is involved in myogenesis in vivo and in vitro.

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INTRODUCTION

Adult skeletal muscle retains muscle stem cells called *satellite cells* that reside below the basal lamina and are directly in contact with the sarcolemma. Adult satellite cells are commonly identified by the expression of the CD56/neural cell adhesion molecule (NCAM) antigen in human muscle (1) and using the Pax7 and Myf5 markers in the mouse (2–4). These cells are mitotically quiescent but enter the cell cycle following muscle injury. After multiple rounds of proliferation leading to the generation of skeletal myoblasts, a portion of these cells maintains Pax7 expression, leaves the cell cycle, and forms the new pool of quiescent satellite cells. The remaining myoblasts upregulate myogenic transcription factors, for example, myogenin (MyoG) and myocyte enhancer factor 2 (MEF2), and differentiate to form multinucleated myofibers (5). Several studies in animal models, such as zebrafish and mice that are mutated in distinct myogenic factors, have led to current knowledge about the steps necessary for myogenesis during embryonic, perinatal, and adult life (6). By contrast, the definition of factors important in regenerative processes in human muscle has remained largely unresolved.

Here, we focused attention on the neurotrophin (NT) receptor p75NTR. Neurotrophins are a family of secreted growth factors, consisting of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT3, and NT4/5. They have a wide range of functions in the development, maintenance, and regeneration of the vertebrate nervous system (7–9). In the neuromuscular system, NTs are mediators of nerve-muscle communication. Postsynaptic muscle fibers may release NTs that are taken up by presynaptic motor nerve terminals that express the appropriate receptors. The NTs are then transported retrogradely to the neuronal cell body, where they may support motor neuron survival (10). Furthermore, NTs potentiate presynaptic release of neurotransmitter (11) and are essential for the maintenance of postsynaptic regions in muscle (11–13). The NTs seem to be involved in skeletal muscle repair; for example, NGF enhances the regenerative

capacity of muscle stem cells in dystrophic muscle (14). Moreover, NGF and BDNF were found to regulate myogenic differentiation of rodent muscle cell lines (15–17). The receptor p75NTR has the ability to bind all NTs with equal affinities. It is a member of the tumor necrosis factor family of receptors and contains an extracellular cysteine-rich domain and a cytoplasmic death domain (7). Signaling is carried out by interacting proteins that are either constitutively associated with or are recruited to the receptor upon ligand binding. The triggered biological responses may be extremely diverse, at times contradictory. In fact, depending on the cellular context, p75NTR has been implicated in the induction of survival or apoptosis, proliferation or differentiation (7). Regarding myocytes, rat Pax7-positive satellite cells display p75NTR in vivo (17), and the receptor may interfere with the differentiation process of rodent myoblasts in vitro (15, 18). Importantly, its blockade by an antagonistic peptide hampers muscle regeneration in a mouse injury model (18). The aim of our study was to clarify the role of p75NTR in human muscle cell biology in vitro and in vivo.

MATERIALS AND METHODS

Patients and Tissues

Muscle biopsies were performed for diagnostic reasons and stored in the institutional tissue bank. Informed consent for biopsy and its storage for research purposes was obtained in all cases. Biopsies were taken from quadriceps femoris muscles in most cases, and specimens with a clear diagnosis, based on clinical, electromyographic, and histological findings were selected.

We examined 13 tissue samples from adults with idiopathic inflammatory myopathies (5 polymyositis [PM], 4 dermatomyositis [DM], 4 inclusion-body myositis [IBM]) and 4 from adult Becker muscular dystrophy [BMD] patients. Histologically normal muscle biopsy specimens from adult individuals were included as controls.

Myoblast Cell Culture

Human primary myoblasts isolated from nonmyopathic tissue specimens were kindly provided by the Telethon-sponsored institutional BioBank. Growth medium consisted of Dulbecco modified Eagle medium (Euroclone, Pero, Italy) containing 20% fetal bovine serum (PAA; M-medical, Cornaredo, Italy), 100 U/mL penicillin, 100 mg/L streptomycin, 292 ng/mL L-glutamine (Euroclone), 100 μg/mL insulin (Sigma, Gallarate, Italy), 25 ng/mL fibroblast growth factor (Peprotech, Tebu BIO, Magenta, Italy), and 10 ng/mL epidermal growth factor (Invitrogen, San Giuliano Milanese, Italy). CD56/NCAM-positive myoblasts were immunoselected by mouse monoclonal anti-human CD56 antibody (clone MY31; BD Biosciences, Buccinasco, Italy) and anti-mouse IgG microbeads (Miltenyi Biotec, Calderara di Reno, Italy). The purity of myoblast preparations was assessed by cytofluorimetric analyses for CD56/NCAM and was greater than 95%. Myoblasts were induced to differentiate in medium containing 2% horse serum (PAA), and fusion index was calculated as the percentage of nuclei within myotubes (with >2 nuclei) with respect to the total number of nuclei.

Immunohistochemistry and Double Immunofluorescence

Six-micrometer-thick tissue sections were fixed with methanol and blocked and stained with the following primary antibodies: mouse monoclonal anti-human p75NTR (clone 74902; R&D Systems, Milan, Italy); mouse monoclonal anti-human CD56 (clone 555514; BD Biosciences); rabbit polyclonal anti-human dystrophin and rat polyclonal anti-human laminin (both provided by Dr. Mora); mouse monoclonal anti-human MyoG (clone F5D; Dako, Milan, Italy); mouse monoclonal anti-human dysferlin (clone HAM1; Novocastra, Florence, Italy); and mouse monoclonal anti-human β-dystroglycan (clone 43DAG1/8D5; Novocastra). Negative stainings consisted of appropriate isotype controls. For immunohistochemistry, EnVision Detection System (Dako) and counterstaining with hematoxylin were used. For double immunofluorescence, Alexa 488–conjugated donkey anti-mouse IgG, Alexa 594-conjugated donkey anti-rabbit IgG, and Alexa 488–conjugated donkey anti-rat IgG (Molecular Probes, Invitrogen) were used. The slides were counterstained with 4′,6-diamidino-2-phenylindole (Sigma) and were mounted with FluorSave (Calbiochem, Milan, Italy). The Zenon technique (Invitrogen) was used to perform staining for confocal imaging with multiple mouse monoclonal antibodies. The same staining protocol was used for immunofluorescence on adherent cells. Fluorescence images were captured with a confocal laser scanning microscope equipped with EZ-C1 Software (Nikon, Sesto Fiorentino, Italy). The ImageProPlus software (Media Cybernetics, Silver Spring, MD) was used for image analysis.

Flow Cytometry

Labeling with mouse monoclonal anti-human p75NTR (clone C40-1457), mouse monoclonal anti-human CD56 (clone MY31), or the corresponding isotype controls (all from BD Biosciences) was followed by detection with phycoerythrin-labeled $F(ab')_2$ fragments goat anti-mouse Ig (Dako). For cell cycle analyses, cells were fixed in 70% ethanol, incubated overnight at 4°C, then stained with propidium iodide solution (50 μg/mL propidium iodide [Sigma], 0.1 mg/mL RNase A [Ambion, Monza, Italy] in PBS–0.05% Triton X-100). Cytofluorometry analyses were performed on FACSCalibur (BD Biosciences). CellQuest software (BD Biosciences) was used for acquisition and FlowJo (Tree Star Inc, Ashland, OR) for data analysis. Thresholds for positivity were set on isotype controls.

Treatment With Anti-p75NTR Blocking Antibody

Myoblasts were exposed to differentiation medium supplemented with 10 μg/mL mouse anti-human p75NTR blocking monoclonal antibody (clone NGFR5; Invitrogen) or purified mouse Ig isotype (BD Biosciences). A second antibody treatment was given the next day.

Selection According to p75NTR Expression

Myoblasts were separated by magnetic cell sorting (Miltenyi Biotech) after incubation with mouse monoclonal anti-human p75NTR antibody (clone C40-1457). Both negative and positive fractions were collected and induced to

differentiate the following day. p75NTR expression was monitored by real-time polymerase chain reaction (PCR) immediately after selection and reached a 10-fold difference in the 2 preparations. Selection was repeated 4 times on the same cell line and reached similar purity.

p75NTR RNA Interference

Small-interfering RNA fragments (siRNA) for p75NTR and nonspecific control were purchased at Eurofins MWG. The siRNA were diluted in 20 nmol/L of Optimem (Invitrogen). Transfection was obtained by Interferin (Polyplus; Celbio, Milan, Italy). Differentiation medium was 2 days after gene silencing. Silencing efficiency in differentiated cultures was monitored between Days 7 and 10 by quantitative PCR. For the proliferation assay, myoblasts were cultured in chamber slides with growth medium containing p75NTR or control siRNA for some days and then labeled with 4′,6-diamidino-2-phenylindole. The number of nuclei/area was counted. For cell cycle analysis, myoblasts were analyzed 3 days after siRNA transfection.

RNA Extraction, cDNA Synthesis, and Real-Time PCR

Total RNA was extracted by TriReagent (Ambion) and reverse transcribed using random hexamer primers and Superscript III reverse transcriptase (all from Invitrogen) following the manufacturers' instructions. Real-time PCR was performed using TAQMan Universal Master Mix (Applied BioSystems, Monza, Italy). Amplification sets for p75NTR were described in Berzi et al (19). Other amplification sets were purchased from Applied BioSystems. Messenger RNA levels of target genes were graphically reported as the percentage of the housekeeping gene Cyclophylin A (*PPIA*). Similar results were obtained with 2 more housekeeping genes (*ALG8* and *PSMB1*).

Microarray Analysis

Total RNA extracted from $p75NTR^{high}$ and $p75NTR^{low}$ cells (4 independent samples for each group, selected as previously described) was used for microarray experiments on Illumina Human_Ref-8_V3 arrays. Quantification and quality analysis of RNA were performed on a Bioanalyzer 2100 (Agilent, Cernusco sul Naviglio, Milan, Italy). Reverse transcription and biotinylated cRNA synthesis were performed using the Illumina TotalPrep RNA Amplification Kit (Ambion) according to the manufacturer's protocol. Hybridization of the cRNAs was carried out on Illumina Human_Ref-8_V3 arrays (Illumina, Son, Netherlands). These arrays contain about 22,000 probes exploring the transcripts contained in the Refseq database. Array hybridization, washing, staining, and scanning in the Beadstation 500 were performed according to standard Illumina protocols. The BeadStudio software (Illumina) was used to analyze raw data grouped by experimental condition. After cubic spline normalization, genes were filtered for detection (detection = 1 in at least 1 experimental group) and selected for statistically significant differential expressions using the Illumina custom test (iterative robust least squares fit, differential score). Stringent criteria were applied: minimal fold change of 1.7 and $p \leq 0.01$ (differential score, ≥ 20). Only 89 probes passed selection (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A202). Microarray data will be deposited at a public repository. Gene Ontology analysis was then conducted using DAVID (20). Hierarchical clustering algorithm (complete linkage) with the default parameters was used to the perform cluster analysis, as implemented in the Mev software. The bioinformatician was not aware of the cell type under analysis. The transcriptome diagram was built using Pathvisio1.1 with plug-ins (21), an open source graphical editor for constructing biological pathways.

Statistical Analysis

The normality of the distribution was assessed by Kolmogorov-Smirnov statistics, and logarithmic transformation of data was applied if necessary. Analysis of variance (in case of normal distribution) or nonparametric Mann-Whitney *U* test (in case of non-normal distribution) was performed to compare means. Paired sample *t*-test was used to compare means at different time points. All p values were 2-sided and subjected to a significance level of 0.05.

RESULTS

p75NTR Is a Marker for Satellite Cells and Regenerating Fibers In Vivo

We examined the localization of the neurotrophin receptor p75NTR in human skeletal muscle by immunohistochemistry and immunofluorescence. This receptor was present on cells in tight contact with mature myofibers, while absent on myofibers themselves (Fig. 1A). Confocal imaging confirmed p75NTR expression on CD56/NCAM-positive satellite cells (Fig. 1B). Quantitative analyses revealed that 88.6% ± 9.5% of CD56/NCAM-positive muscle precursor cells expressed p75NTR in human adult skeletal muscle (Fig. 1C).

Regeneration (as assessed by the number of CD56/NCAM-positive fibers) was only rarely detected in healthy skeletal muscle (Fig. 1D, first column). Therefore, we additionally analyzed regeneration levels in muscle biopsies from inflammatory myopathies (polymyositis PM, dermatomyositis DM, inclusion body myositis IBM), where the pathogenetic process is presumably of autoimmune origin, and Becker muscular dystrophy (BMD), where muscle degeneration is caused by genetic defects in dystrophin gene. Compared with healthy muscle, the inflammatory myopathies exhibited the highest percentages of regenerating fibers, whereas BMD had quite low regenerative levels (Fig. 1D). Importantly, numerous newly forming fibers showed strong p75NTR expression (Fig. 1E, F). No significant differences in the percentage of regenerating fibers displaying p75NTR were found among the different muscle diseases (average ± SD in each group were 81% ± 3% in PM, 49% ± 29% in DM, 83% ± 8% in IBM, 66% ± 18% in BMD). Approximately 70% ± 7.98% (average ± SEM among groups) of CD56/NCAM-positive fibers coexpressed p75NTR, indicating an in vivo role for this molecule in the first phases of cell fusion and differentiation. Overall, these observations support the hypothesis that p75NTR-positive satellite cells constitute a pool of precursor cells critical

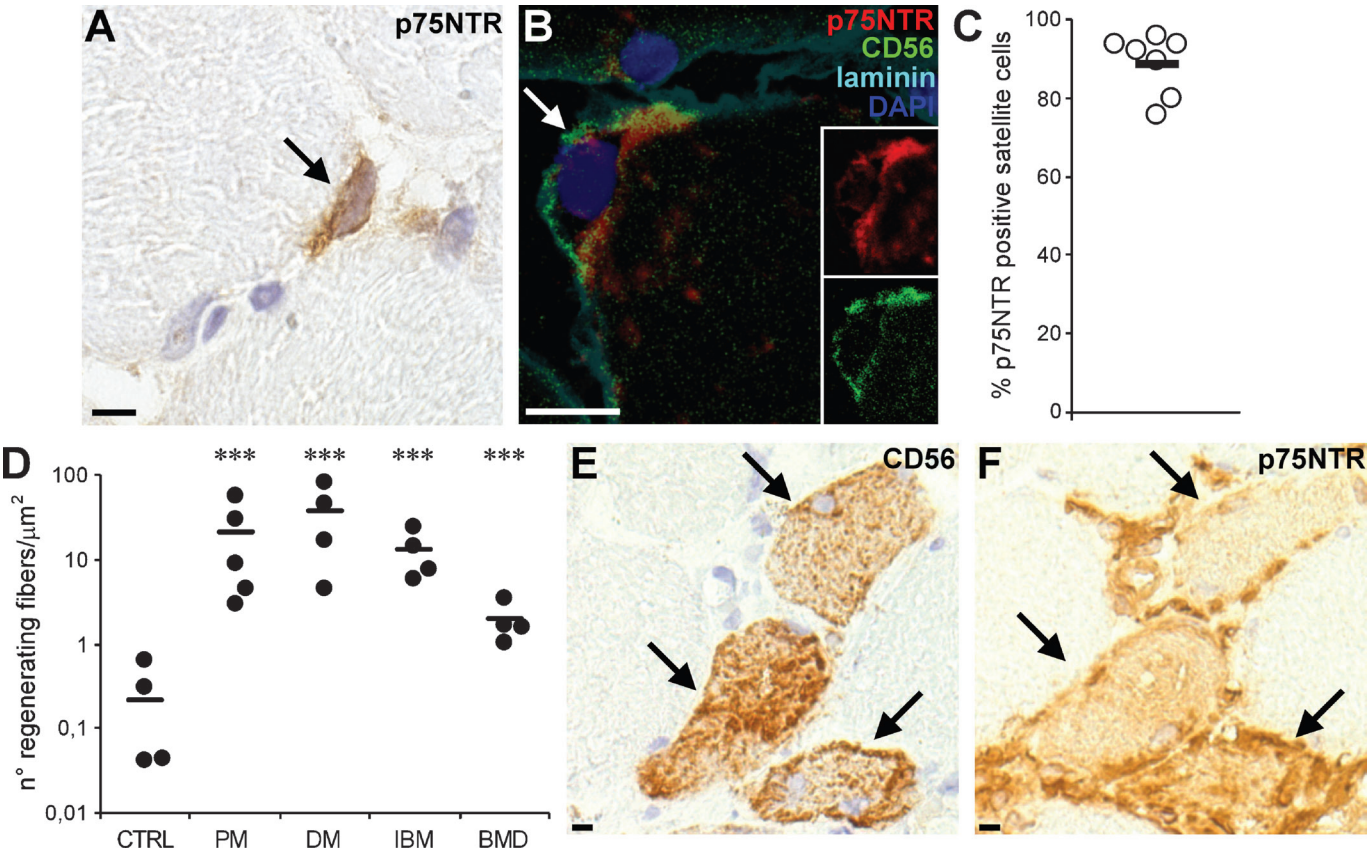


FIGURE 1. p75NTR is a marker for human muscle satellite cells and regenerating fibers. **(A)** p75NTR immunoreactivity in a satellite cell of adult skeletal muscle. **(B)** Confocal imaging for p75NTR and CD56/neural cell adhesion molecule (NCAM) on satellite cells in vivo. Laminin staining was used to define myofibers. In **(A)** and **(B)**, arrows indicate positively stained satellite cells. **(C)** Percentages of CD56/NCAM-positive satellite cells expressing p75NTR in human adult skeletal muscle. Circles represent distinct tissue samples; horizontal bar represents mean. **(D)** Quantification of CD56/NCAM-positive regenerating myofibers. Black bars indicate average values for each group, dots represent distinct tissue samples. **(E, F)** p75NTR expression **(F)** on CD56/NCAM-positive **(E)** regenerating fibers. Stainings were performed on serial sections from a dermatomyositis (DM) muscle. Arrows indicate positively stained fibers. Scale bars = **(A, B)** 5 μm; **(E, F)** 10 μm. ***$p < 0.001$ versus control. CTRL, control; PM, polymyositis; IBM, inclusion body myositis; BMD, Becker muscular dystrophy.

for tissue repair and that p75NTR may sustain regenerative processes in vivo.

p75NTR Is Expressed In Vitro by Human Muscle Precursor Cells and Is Transiently Enhanced During Myogenesis

The analysis was next extended to human muscle cells in vitro. First, primary myoblasts were purified from dissociated muscle cultures based on the expression of the CD56/NCAM marker, and immunofluorescence experiments with antibodies directed to p75NTR were performed. Figure 2A shows that part of human muscle precursor cells displayed the NT receptor under basal conditions. An in vitro model of myoblast differentiation into myotubes was then adopted to verify regulation of the receptor p75NTR during myogenesis. Messenger RNA was isolated at different time points from cells exposed to differentiation medium, and quantitative PCR for p75NTR was performed on relative cDNA. Compared with basal stage (Day 0), p75NTR transcription augmented and reached a peak during the first days of the myogenic process, but returned to initial levels at later time points (Fig. 2B). Cytofluorimetric analyses on distinct cell preparations showed that 20% to 40% of primary myoblasts expressed the receptor under normal growth conditions. Furthermore, myoblasts promptly upregulated p75NTR when exposed to differentiation stimuli (Fig. 2C). From Day 3 on, p75NTR expressing multinucleated myotubes were detected, as demonstrated by immunofluorescence on differentiated cultures (Fig. 2D). Consistent with transcript levels given in Figure 2B, approximately 90% of the myotubes were p75NTR positive at Day 5, whereas only 25% were at Day 14 (Fig. 2E, squares).

p75NTR Defines Differentiation-Oriented Muscle Precursor Cells

Further characterization of p75NTR expressing cells was performed by gene profiling experiments. p75NTR-positive myoblasts were separated from p75NTR-negative myoblasts by magnetic cell sorting, obtaining 2 populations with an approximate 10-fold difference in p75NTR mRNA levels after selection (Fig. 3A). These RNA were used for hybridization

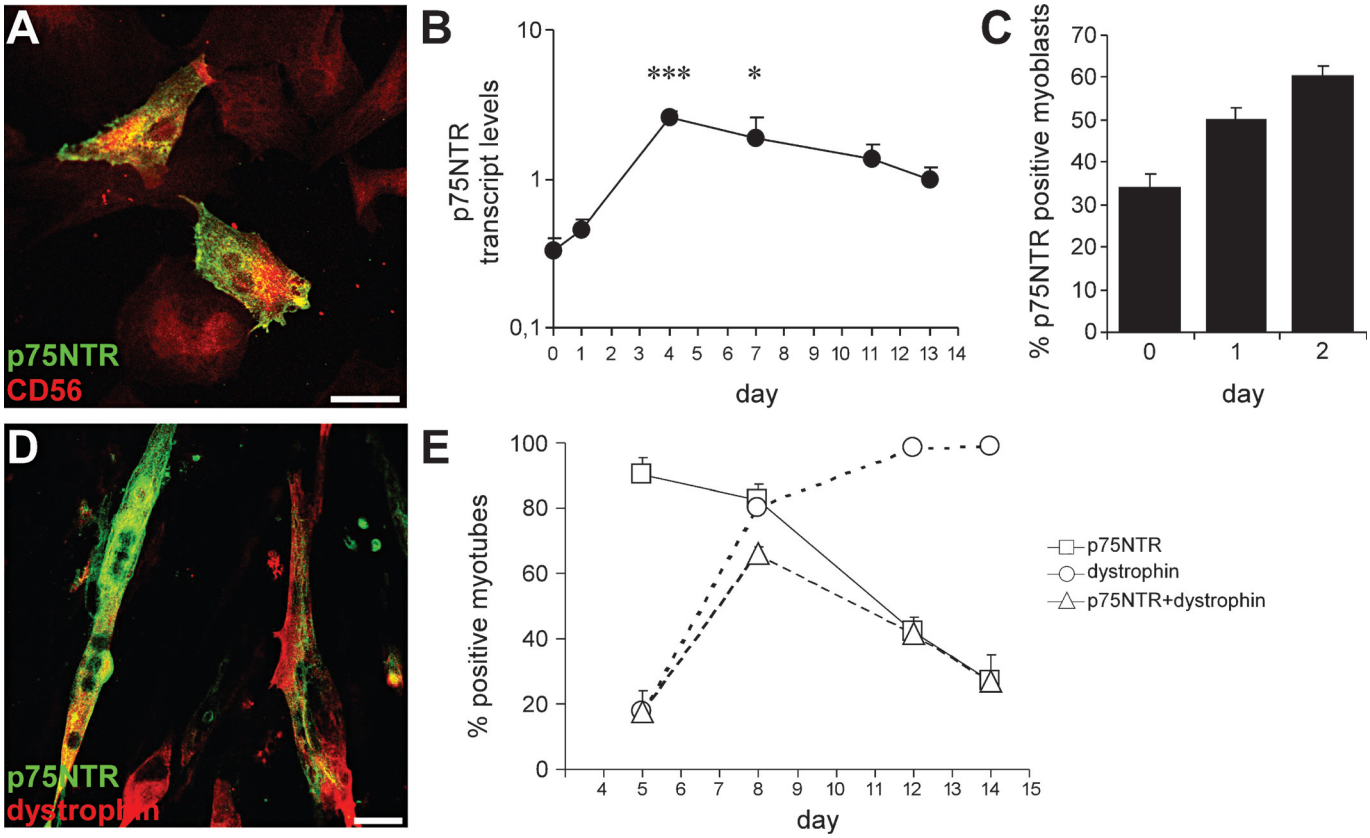
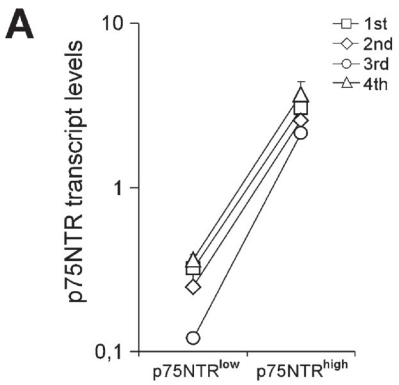


FIGURE 2. p75NTR is expressed in cultured muscle precursor cells and transiently upregulated upon differentiation. **(A)** Double immunofluorescence for p75NTR and CD56/NCAM in cultured myoblasts. **(B)** Upregulation of p75NTR mRNA levels upon differentiation. Quantitative real-time PCR for p75NTR was performed for each time point. For statistics, p75NTR transcript levels at distinct time points were compared with baseline (Day 0). **(C)** Flow cytometry experiments showing percentages of p75NTR-positive mononucleated myoblasts after exposure to differentiation medium. **(D)** Immunofluorescence for p75NTR and dystrophin in myotubes (Day 11). **(E)** Kinetics of p75NTR expression on myotubes and correlation with dystrophin induction. Costaining for p75NTR and dystrophin was performed on differentiating cultures. The percentages of p75NTR (square) or dystrophin (circle) or p75NTR-positive dystrophin-positive (triangle) myotubes were measured at different time points. Experiments were performed in triplicate, and at least 100 myotubes per replicate were counted. Similar observations were obtained in at least 3 independent experiments in at least 2 primary cell lines. Error bars represent SD. Scale bars = **(A, D)** 30 μm. *$p < 0.05$, ***$p < 0.001$.

on standard Illumina microarrays containing approximately 22,000 probes for human genes and the transcriptome of the $p75NTR^{high}$ population was compared with the one of $p75NTR^{low}$ cells. Eighty-nine gene probes passed the significance threshold of 0.01 and displayed a minimal fold change of 1.7 (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A202); most were genes more expressed in $p75NTR^{high}$ than in $p75NTR^{low}$ cells (79 upregulated vs 10 downregulated probes) and related to muscle. In fact, significantly enriched gene ontology categories regarded muscle development ($p < 3.9 \times 10^{-9}$) and contraction ($p < 5.4 \times 10^{-12}$) (Fig. 3B). Diagrammatic representation of the main transcriptome data is given in Figure 3C. Each muscle-related gene product is positioned in the cellular context and is shown as gene symbol contained in an ellipse whose color reflects the extent of fold change. Gene annotations are available in the online interactive tool (http://sites.google.com/site/farinalabbesta/p75-ntr_positive_muscle_pathway,) unzip password: celoria11) that couples our expression data with the main biological databases such as Unigene, Ensembl, NCBI-Genbank. Titin, dysferlin, α and β subunits of the nicotinic receptor, α1 actin, troponin T type 2, and troponin C type 1 were among the upregulated structural genes. Muscle development–related gene products included MyoG, MEF2C, α-enolase, dystrophia myotonica protein kinasetpdel, and CD34. Moreover, cluster analysis of the upregulated genes showed that several muscle-related genes clustered together, suggesting coregulation among them (Fig. 3D). In addition, they were located mainly in the clusters at high fold change (Fig. 3D, upper clusters). For validation, we analyzed transcript levels of 5 upregulated genes (ACTA1, CHRNB1, MEF2C, TNNC1, and MYL4) in $p75NTR^{high}$ and $p75NTR^{low}$ cells by real-time PCR. All of these genes were more expressed in the $p75NTR^{high}$ population ($p < 0.05$ for ACTA1 and CHRNB1; $p < 0.01$ for MEF2C and TNNC1; $p < 0.001$ for MYL4) (Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A203).

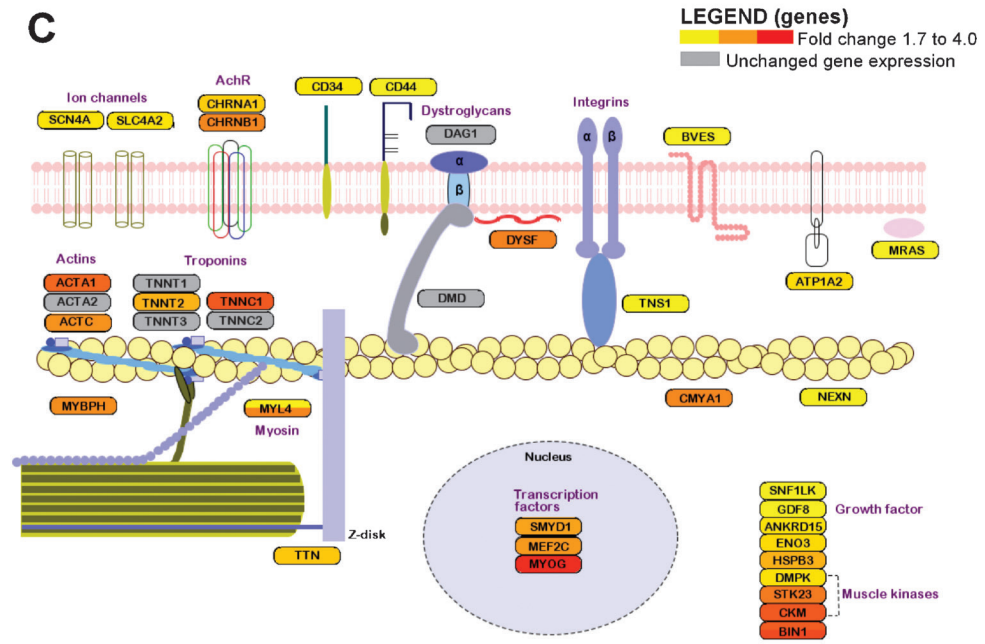
Finally, we validated at the protein level the differential expression for 2 more upregulated genes, namely, MyoG and dysferlin. MyoG is an important nuclear transcription factor regulating terminal differentiation of muscle cells that induces

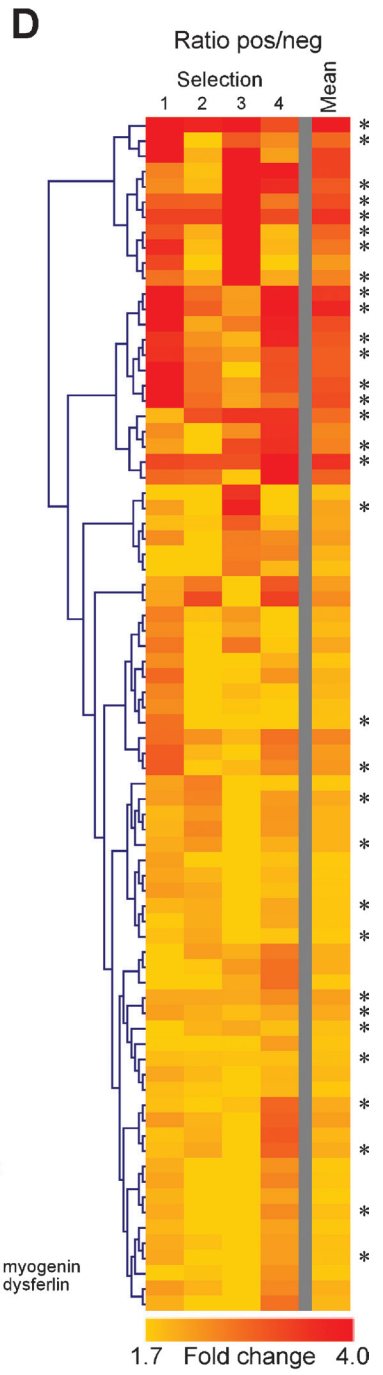
the coordinated expression of several structural genes, including troponins, myosin chains, and titin (22). Dysferlin is a membrane protein essential for correct myofiber structure and function; dysferlin absence causes muscular dystrophy characterized by impaired muscle regeneration (23). Protein detection by immunofluorescence (Fig. 3E, F) and relative quantification in p75NTR-positive or negative myoblasts confirmed that both MyoG and dysferlin were indeed preferentially expressed in p75NTR-positive cells ($p = 0.002$ for both, Fig. 3G). Overall, the transcriptional repertoire displayed by p75NTR-expressing cells suggests that these cells have a strong differentiation potential.

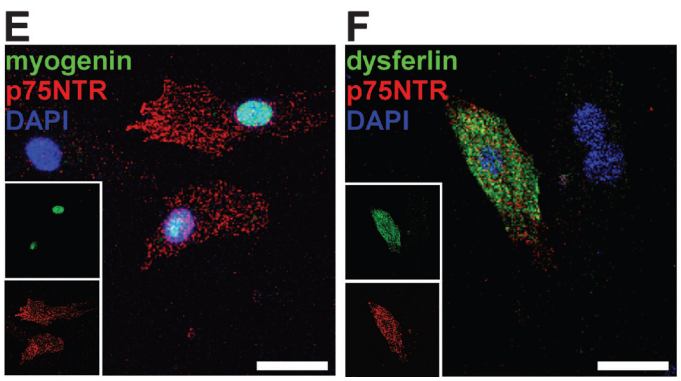


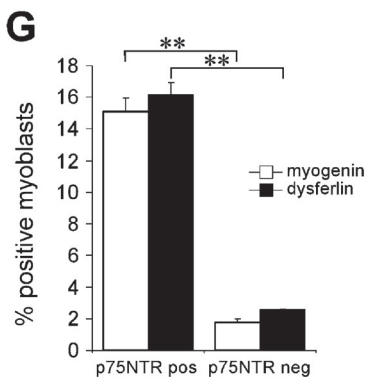
B

Gene Ontology	n° genes	Representative genes	p-value
Muscle Contraction	13	DYSF TTN TNNC1	5.4×10^{-12}
Muscle Development	11	MYOG MEF2C CD34	3.9×10^{-9}









p75NTR Regulates Precursor Cell Differentiation

The functional role of p75NTR in muscle cell differentiation was investigated in vitro. Initially, we hindered p75NTR activity by the addition of an anti-p75NTR blocking antibody and assessed the extent of myogenic fusion. Muscle cells treated with the blocking antibody displayed lower fusion index ($p = 0.001$) than control cells, indicating that p75NTR may influence precursor cell differentiation (Fig. 4A). Next, we separated p75NTR-positive myoblasts from the p75NTR negative cells by magnetic beads and induced in vitro differentiation. Under these conditions, $p75NTR^{high}$ muscle cells formed significantly more myotubes than the $p75NTR^{low}$ population ($p < 0.001$; Fig. 4B). As expected, the addition of the blocking antibody to $p75NTR^{high}$ precursor cells inhibited cell fusion ($p < 0.001$; Fig. 4B, third column). Because fusion was not strongly impaired in $p75NTR^{low}$ cultures, we checked p75NTR levels during differentiation. Interestingly, p75NTR was readily induced in $p75NTR^{low}$ cells and reached values comparable to those in unseparated cells by a few days (Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A204), indicating that differentiation stimuli force p75NTR expression also in p75NTR-negative cells.

Finally, RNA interference experiments for p75NTR were performed. Myoblasts were transfected with p75NTR or scrambled siRNA and were induced to differentiate after 48 hours. The efficiency of silencing in different experiments ranged between 70% and 96%, as assessed by real-time PCR on cultures between Days 7 and 10. The first panel in Figure 4C shows the reduced p75NTR mRNA levels after silencing in a representative experiment. Consistently, 2 genes (*MEF2C* and *dysferlin*) that were enriched in $p75NTR^{high}$ myoblasts displayed lower levels in p75NTR-silenced cultures (Figure, Supplemental Digital Content 4, http://links.lww.com/NEN/A205). Immunofluorescence for p75NTR showed at least 50% reduction in protein expression on myotubes after gene silencing (Fig. 4C, second panel). Most importantly, myoblasts treated with p75NTR siRNA showed a consistent decrease of the fusion index compared with cells treated with control siRNA ($p < 0.001$; Fig. 4C, third panel). Similar results were obtained with a second p75NTR siRNA (not shown).

Because p75NTR signaling might affect several cellular programs, including differentiation, or exert a specific action on myogenesis, we addressed this key point by determining whether p75NTR controlled other cell functions. Proliferation of myoblasts was found to be unaltered in p75NTR-silenced cells versus control cells (Fig. 4D). Furthermore, cell cycle progression did not change upon p75NTR silencing (Fig. 4E). Taken together, consistent with the transcriptome data showing p75NTR as a prototypic marker for differentiation prone muscle precursor cells, these functional data demonstrate that p75NTR specifically regulates myogenesis.

p75NTR Specifically Controls Dystrophin Induction in Myotubes

The kinetics of p75NTR in newly forming myotubes in vitro was compared with that of dystrophin, a fundamental muscle protein induced upon maturation. In contrast to p75NTR, dystrophin was present on few myotubes at Day 5, reached a peak at Day 8, and remained high thereafter (Fig. 2E, circles). Notably, when coexpression of p75NTR and dystrophin was checked (Fig. 2E, triangles) at Day 5, dystrophin started to emerge exclusively on p75NTR-positive myotubes, indicating that sustained expression of p75NTR on multinucleated elements precedes dystrophin induction and might, therefore, regulate it.

We wondered whether p75NTR was needed for dystrophin induction. The dystrophin gene was expressed in myoblasts, but the microarray analysis did not reveal any difference for this molecule between $p75NTR^{high}$ and $p75NTR^{low}$ cells (not shown). Quantitative PCR for dystrophin on cDNA from p75NTR-silenced and control myoblasts confirmed that there was no association between p75NTR and dystrophin levels under basal conditions (not shown). Then, we induced differentiation of p75NTR-silenced myoblasts and measured dystrophin expression on myotubes, as some degree of myogenesis was still present in such cultures. This analysis showed that p75NTR silencing significantly reduced the percentage of dystrophin-expressing myotubes ($p = 0.002$; Fig. 4F), demonstrating that p75NTR may regulate the correct induction of a structural protein, such as dystrophin in differentiated muscle cells. Similarly, the addition of the anti-p75NTR–blocking antibody during differentiation reduced the percentage of dystrophin-positive myotubes (not shown). Regulation of dystrophin by p75NTR was specific because the expression on myotubes of other muscle proteins (e.g. β-dystroglycan and dysferlin) was not altered upon p75NTR silencing (Fig. 4F).

FIGURE 3. p75NTR defines differentiation-oriented muscle precursor cells. **(A)** p75NTR mRNA analysis in myoblasts immediately after immunoselection. Receptor expression was 10-fold higher in $p75NTR^{high}$ than in $p75NTR^{low}$ populations. Selection was repeated 4 times with comparable results. **(B)** Muscle-related Gene Ontology categories were significantly enriched in $p75NTR^{high}$ myoblasts. **(C)** Diagrammatic representation of the p75NTR-associated transcriptome. Each muscle-related gene product is positioned in the cellular context and is shown as gene symbol contained in an ellipse whose color reflects the extent of fold change. **(D)** Fold change–based cluster analysis of upregulated genes. Each column represents the expression ratio between $p75NTR^{high}$ and $p75NTR^{low}$ populations in each of the 4 experiments performed. The last column reports the average ratio of all groups. Muscle-related genes (*) clustered together and were located mainly in the clusters at a high fold change. In **(C)** and **(D)**, gene expression is represented in a color gradient ranging from yellow to red for fold change values from 1.7 to 4.0, respectively. **(E, F)** Double immunofluorescence for p75NTR and myogenin **(E)** or dysferlin **(F)** in cultured myoblasts. **(G)** Percentage of MyoG- or dysferlin-expressing cells among p75NTR-positive and -negative myoblasts. Experiments were performed in triplicate, and at least 150 myoblasts per replicate were counted. Similar observations were obtained in at least 3 independent experiments and were confirmed in 2 primary cell lines. In **(A)** and **(G)**, error bars represent SD. Scale bars = **(E, F)** 30 μm. **$p < 0.01$.

DISCUSSION

Skeletal satellite cells represent a quiescent stem cell population capable of differentiating into mature muscle when there is tissue damage. They are the most efficient cell type in skeletal muscle repair upon acute injury (24). Hence, their characterization in humans may have a profound impact on our understanding of regeneration events in physiological or pathological conditions and on the development of appropriate stem cell–based therapies.

Here, we identified p75NTR as a novel marker for human differentiation-prone muscle precursor cells and demonstrated its involvement in myogenic processes in vitro and in vivo.

Our immunohistochemical experiments demonstrated that this receptor is displayed by satellite cells in human adult skeletal muscle. p75NTR-positive satellite cells have also been detected in rodent muscle (17), but the size of this precursor cell pool has never been determined. We found that in human muscle, most of the satellite cells express p75NTR on the surface, indicating that this molecule is a novel marker for that cell type.

Next, we wondered whether extensive muscle repair as it occurs under pathological settings is associated to p75NTR expression. Two classes of human muscle diseases were analyzed: inflammatory myopathies and Becker muscular dystrophy. The first is a group of acquired disorders of skeletal muscle characterized by inflammation-mediated muscle injury (25). Distinct histological findings define the 3 major inflammatory myopathies: PM and IBM show infiltrates of $CD8^+$ cells attacking major histocompatibility complex class I–positive myofibers, and DM is characterized by microangiopathy caused by complement deposition on capillaries. In IBM, there are complex features of degeneration, such as the formation of rimmed vacuoles containing amyloid deposits (26). Conversely, BMD is a genetic disease and represents a relatively mild form of muscular dystrophy as

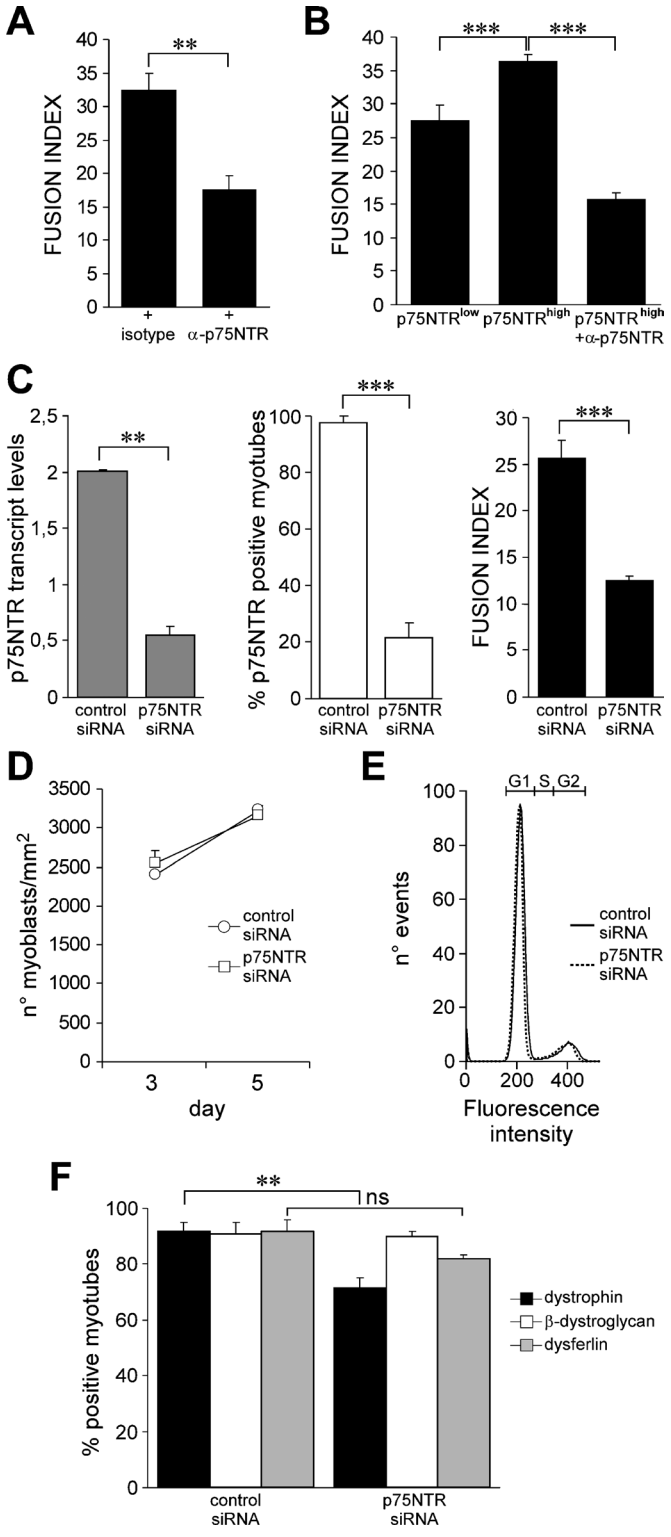


FIGURE 4. p75NTR regulates myogenesis and dystrophin expression in myotubes. **(A)** Fusion index in a blocking experiment with anti-p75NTR antibody or isotype control administered during cell differentiation. **(B)** Extent of myogenesis in $p75NTR^{low}$, $p75NTR^{high}$, and $p75NTR^{high}$ myoblasts treated with anti-p75NTR blocking antibody. In **(A)** and **(B)**, fusion was evaluated at Day 6 after differentiation induction. **(C)** p75NTR silencing in myoblasts and effects on myogenesis. Silencing efficiency and relative fusion index were evaluated at Day 8 after differentiation induction. p75NTR mRNA (first panel) and protein (second panel) levels in p75NTR-silenced myotubes were significantly lower than in control myotubes. p75NTR RNA interference decreased cell differentiation (third panel). **(D)** p75NTR silencing did not alter myoblast proliferation. Small-interfering RNA administration was performed at Day 0; cells were maintained in growth medium for a few days and counted. **(E)** Cell cycle analysis was detected by propidium iodide incorporation in silenced myoblasts. Evaluation was performed at Day 3 after transfection with p75NTR or control siRNA. Cell cycle entry in p75NTR-silenced cells was unchanged. **(F)** Quantification of dystrophin, β-dystroglycan, and dysferlin-positive myotubes in differentiated cultures at Day 8 after differentiation induction. Shown experiments were performed in triplicate, and at least 100 myotubes per replicate were counted. Similar observations were obtained in at least 3 independent experiments. Error bars represent SD. Results in **(A, C, D,** and **E)** were confirmed in 2 primary cell lines. $^{**}p < 0.01$, $^{***}p < 0.001$; ns, not significant.

mutations in dystrophin gene either lead to reduced levels of full-length dystrophin or are deletions that preserve the reading frame generating smaller but somehow still functional dystrophin protein (27). A previous report described p75NTR on regenerating fibers in Duchenne muscular dystrophy (28). Consistently, we found that newly forming fibers in inflammatory myopathies and in BMD express p75NTR, further supporting the notion that this receptor regulates regenerative processes in human skeletal muscle.

The expression of p75NTR on regenerating fibers in animals has not been reported so far, but functional studies in the mouse showed that injection of a p75NTR-blocking peptide in vivo reduces regeneration after cardiotoxin-induced injury (18), and studies on rodent cell lines reported the downregulation of p75NTR during muscle cell differentiation (15–17). Our experiments on human primary muscle cells demonstrated prompt upregulation of p75NTR on myoblasts when they are exposed to differentiating stimuli and robust but transient expression on mature myotubes. This is consistent with the presence of p75NTR in regenerating fibers and its lack of expression on mature myofibers in vivo. Fusion experiments with $p75NTR^{high}$ myoblasts revealed that they differentiate more efficiently than $p75NTR^{low}$ cells. Furthermore, blockade of p75NTR at the protein or at the mRNA level impairs fusion, clearly demonstrating a direct role for this receptor in muscle cell differentiation. Similar results were obtained recently with primary mouse myoblasts, where treatment with a blocking antibody or with an NGF antagonist inhibited fusion (18). On the other hand, p75NTR overexpression in the mouse C2C12 cell line improved myogenesis (15). Of course, the possibility that, in vivo, also p75NTR-negative satellite cells contribute to the formation of new myofibers cannot be excluded. Our results indicate that p75NTR-negative myoblasts upregulate the NT receptor in response to differentiation stimuli, and that p75NTR-positive cells are more efficient in myogenesis than p75NTR-negative cells, suggesting that p75NTR-bearing cells might have a functional advantage in vivo.

Our transcriptome analysis demonstrated that p75NTR-expressing myoblasts display a repertoire of gene products that accounts for their proficiency in promoting differentiation and maturation. Among them, both MyoG and MEF2, two important positive regulators of differentiation (5), are expressed more in p75NTR-positive myoblasts, which also bear increased transcripts for a number of muscle structural proteins.

Taken together, we propose that p75NTR is a novel marker for human differentiation-oriented muscle precursor cells. Stem cell transplantation has been envisaged as a therapeutic option for muscular dystrophies (29), but such application is constrained by the ability of cells to expand in vitro and to preserve regenerative potential. We show that p75NTR-expressing myoblasts have enhanced myogenic properties although maintaining unchanged the proliferation extent. Of note, whereas most satellite cells display the NT receptor in vivo, only a minor percentage of muscle precursor cells expresses it in vitro. As demonstrated, p75NTR is not necessary for proliferation and survival of undifferentiated cells, whereas its expression is finely regulated during myogenesis. It is possible that the culture conditions may favor growth of p75NTR-negative myoblasts because receptor expression is required only during differentiation. In the future, muscle stem cell expansion protocols could be developed to maintain or select the pool of precursor cells expressing the NT receptor.

Another important aspect emerging from our studies is that p75NTR signaling may regulate dystrophin induction. In fact, dystrophin protein is induced exclusively on p75NTR-positive myotubes during the first phases of differentiation, suggesting that the NT receptor is not dispensable for muscle cell maturation. Moreover, p75NTR silencing in differentiating myoblasts leads not only to decreased cell fusion but also to reduced dystrophin expression on myotubes. The effect is specific for dystrophin because 2 other muscle proteins (dysferlin and β-dystroglycan) were not affected by p75NTR blockade. To our knowledge, this is the first description of a pathway controlling dystrophin induction in muscle cells. Clearly, additional studies are required to define which transcriptional and/or posttranscriptional events triggered by p75NTR signaling regulate dystrophin expression. Dystrophin is fundamental not only for muscle structure, but also for myofiber survival (30). In fact, a reduction of dystrophin levels leads to muscle loss, as dystrophin itself triggers signaling molecules that inhibit atrophy pathways (31). Intriguingly, phenotypic elimination of the neurotrophin NGF in adult transgenic mice leads to muscle atrophy, underlying the importance of this trophic stimulus for skeletal muscle homeostasis (32).

We conclude that NTs profoundly tailor muscle regeneration as they may act on precursor and differentiating cells. Regarding the ligands binding p75NTR, the neurotrophins NGF, BDNF, or NT3 have been found in postnatal skeletal muscle or cultured myocytes (15–17, 33–36). Importantly, a recent study on transgenic mice deficient in BDNF expression in skeletal muscle cells demonstrated involvement of this NT in myogenesis in vivo (37). However, the differential autocrine production of such factors by human muscle cells in vitro or in vivo under distinct processes (i.e. regeneration, tissue homeostasis, degeneration) has not been clarified so far, and more comprehensive studies on mechanisms are required.

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REFERENCES

1. Schubert W, Zimmermann K, Cramer M, et al. Lymphocyte antigen Leu-19 as a molecular marker of regeneration in human skeletal muscle. Proc Natl Acad Sci U S A 1989;86:307–11
2. Seale P, Sabourin LA, Girgis-Gabardo A, et al. Pax7 is required for the specification of myogenic satellite cells. Cell 2000;102:777–86

3. Tajbakhsh S, Rocancourt D, Buckingham M. Muscle progenitor cells failing to respond to positional cues adopt non-myogenic fates in myf-5 null mice. Nature 1996;384:266–70
4. Beauchamp JR, Heslop L, Yu DS, et al. Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. J Cell Biol 2000;151:1221–34
5. Le Grand F, Rudnicki MA. Skeletal muscle satellite cells and adult myogenesis. Curr Opin Cell Biol 2007;19:628–33
6. Bryson-Richardson RJ, Currie PD. The genetics of vertebrate myogenesis. Nat Rev Genet 2008;9:632–46
7. Dechant G, Barde YA. The neurotrophin receptor p75(NTR): Novel functions and implications for diseases of the nervous system. Nat Neurosci 2002;5:1131–36
8. Huang EJ, Reichardt LF. Trk receptors: Roles in neuronal signal transduction. Annu Rev Biochem 2003;72:609–42
9. Horner PJ, Gage FH. Regenerating the damaged central nervous system. Nature 2000;407:963–70
10. Yano H, Chao MV. Mechanisms of neurotrophin receptor vesicular transport. J Neurobiol 2004;58:244–57
11. Wang XH, Poo MM. Potentiation of developing synapses by postsynaptic release of neurotrophin-4. Neuron 1997;19:825–35
12. Xie K, Wang T, Olafsson P, et al. Activity-dependent expression of NT-3 in muscle cells in culture: Implications in the development of neuromuscular junctions. J Neurosci 1997;17:2947–58
13. Gonzalez M, Ruggiero FP, Chang Q, et al. Disruption of Trkb-mediated signaling induces disassembly of postsynaptic receptor clusters at neuromuscular junctions. Neuron 1999;24:567–83
14. Lavasani M, Lu A, Peng H, et al. Nerve growth factor improves the muscle regeneration capacity of muscle stem cells in dystrophic muscle. Hum Gene Ther 2006;17:180–92
15. Seidl K, Erck C, Buchberger A. Evidence for the participation of nerve growth factor and its low-affinity receptor (p75NTR) in the regulation of the myogenic program. J Cell Physiol 1998;176:10–21
16. Rende M, Brizi E, Conner J, et al. Nerve growth factor (NGF) influences differentiation and proliferation of myogenic cells in vitro via TrKA. Int J Dev Neurosci 2000;18:869–85
17. Mousavi K, Jasmin BJ. BDNF is expressed in skeletal muscle satellite cells and inhibits myogenic differentiation. J Neurosci 2006;26:5739–49
18. Deponti D, Buono R, Catanzaro G, et al. The low-affinity receptor for neurotrophins p75NTR plays a key role for satellite cell function in muscle repair acting via RhoA. Mol Biol Cell 2009;20:3620–27
19. Berzi A, Ayata CK, Cavalcante P, et al. BDNF and its receptors in human myasthenic thymus: Implications for cell fate in thymic pathology. J Neuroimmunol 2008;197:128–39
20. Dennis G Jr, Sherman BT, Hosack DA, et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol 2003;4:P3
21. van Iersel MP, Kelder T, Pico AR, et al. Presenting and exploring biological pathways with PathVisio. BMC Bioinformatics 2008;9:399
22. Davie JK, Cho JH, Meadows E, et al. Target gene selectivity of the myogenic basic helix-loop-helix transcription factor myogenin in embryonic muscle. Dev Biol 2007;311:650–64
23. Chiu YH, Hornsey MA, Klinge L, et al. Attenuated muscle regeneration is a key factor in dysferlin-deficient muscular dystrophy. Hum Mol Genet 2009;18:1976–89
24. Sherwood RI, Christensen JL, Conboy IM, et al. Isolation of adult mouse myogenic progenitors: Functional heterogeneity of cells within and engrafting skeletal muscle. Cell 2004;119:543–54
25. Dalakas MC, Hohlfeld R. Polymyositis and dermatomyositis. Lancet 2003;362:971–82
26. Needham M, Mastaglia FL. Inclusion body myositis: Current pathogenetic concepts and diagnostic and therapeutic approaches. Lancet Neurol 2007;6:620–31
27. Lim LE, Rando TA. Technology insight: Therapy for Duchenne muscular dystrophy—An opportunity for personalized medicine? Nat Clin Pract Neurol 2008;4:149–58
28. Baron P, Scarpini E, Meola G, et al. Expression of the low-affinity NGF receptor during human muscle development, regeneration, and in tissue culture. Muscle Nerve 1994;17:276–84
29. Darabi R, Santos FN, Perlingeiro RC. The therapeutic potential of embryonic and adult stem cells for skeletal muscle regeneration. Stem Cell Rev 2008;4:217–25
30. Ervasti JM. Dystrophin, its interactions with other proteins, and implications for muscular dystrophy. Biochim Biophys Acta 2007;1772:108–17
31. Acharyya S, Butchbach ME, Sahenk Z, et al. Dystrophin glycoprotein complex dysfunction: A regulatory link between muscular dystrophy and cancer cachexia. Cancer Cell 2005;8:421–32
32. Ruberti F, Capsoni S, Comparini A, et al. Phenotypic knockout of nerve growth factor in adult transgenic mice reveals severe deficits in basal forebrain cholinergic neurons, cell death in the spleen, and skeletal muscle dystrophy. J Neurosci 2000;20:2589–601
33. Murphy RA, Singer RH, Saide JD, et al. Synthesis and secretion of a high molecular weight form of nerve growth factor by skeletal muscle cells in culture. Proc Natl Acad Sci U S A 1977;74:4496–500
34. Liem RS, Brouwer N, Copray JC. Ultrastructural localisation of intramuscular expression of BDNF mRNA by silver-gold intensified non-radioactive in situ hybridisation. Histochem Cell Biol 2001;116:545–51
35. Kust BM, Copray JC, Brouwer N, et al. Elevated levels of neurotrophins in human biceps brachii tissue of amyotrophic lateral sclerosis. Exp Neurol 2002;177:419–27
36. Toti P, Villanova M, Vatti R, et al. Nerve growth factor expression in human dystrophic muscles. Muscle Nerve 2003;27:370–73
37. Clow C, Jasmin BJ. Brain-derived neurotrophic factor regulates satellite cell differentiation and skeletal muscle regeneration. Mol Biol Cell 2010;21:2182–90