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MmNEU3 Sialidase Over-Expression in C2C12 Myoblasts Delays Differentiation and Induces Hypertrophic Myotube Formation

Nadia Papini,[1,2] Luigi Anastasia,[1,2] Cristina Tringali,[1,2] Loredana Dileo,[1] Ivan Carubelli,[1] Maurilio Sampaolesi,[3,4] Eugenio Monti,[5] Guido Tettamanti,[2] and Bruno Venerando[1,2*]

[1]*Department of Medical Chemistry, Biochemistry, and Biotechnology, University of Milan, 20090 Milan, Italy*
[2]*Laboratory of Stem Cells for Tissue Engineering, IRCCS Policlinico San Donato, 20097 San Donato Milanese, Italy*
[3]*Stem Cell Research Institute, University Hospital Gasthuisberg, B-3000 Leuven, Belgium*
[4]*Department of Experimental Medicine, University of Pavia, 27100 Pavia, Italy*
[5]*Department of Biomedical sciences and Biotechnologies, University of Brescia, 25123 Brescia, Italy*

ABSTRACT

Several factors affect the skeletal muscle differentiation process, in particular modifications of cell–cell contact, cell adhesion, and plasma membrane characteristics. In order to support the role of the plasma membrane-associated sialidase NEU3 in skeletal muscle differentiation and to analyse which events of the process are mainly affected by this sialidase, we decided to stably over-express MmNEU3 in C2C12 cells by a lentiviral vector and to investigate cell behavior during the differentiation process. Vitally stained C2C12 and NEU3 over-expressing cells were counted to reveal modifications in differentiation induction. We found that NEU3 over-expressing cells remained proliferative longer than control cells and delayed the onset of differentiation. Expression of p21, myogenic transcription factors, and myosin heavy chain (MHC), assessed by real time PCR, confirmed this behavior. In particular, no MHC-positive myotubes were present in NEU3 over-espressing cells as compared to wild type C2C12 cells at day 3 of differentiation. Moreover, NEU3 over-expressing cells completed the differentiation process very quickly and formed hypertrophic myotubes. Analysis of MAPK/ERK pathway activation showed an increased ERK 1/2 phosphorylation in NEU3 over-expressing cells at the beginning of differentiation. We postulate that sialidase NEU3, decreasing plasma membrane ganglioside GM3 content, affects the EGF receptor and the downstream signaling pathways, promoting proliferation and delaying differentiation. Furthermore NEU3 improves myoblast fusion probably via neural-cell adhesion molecule (NCAM) desialylation. Therefore, this work further supports the central role of NEU3 as a key modulator in skeletal muscle differentiation, particularly in the myoblast fusion step. J. Cell. Biochem. 113: 2967–2978, 2012.

KEY WORDS: SIALIDASE; MYOBLAST; MUSCLE DIFFERENTIATION; NCAM

Skeletal muscle differentiation is a carefully orchestrated process where committed myoblasts undergo a series of consequent events, including withdrawal from the cell cycle, expression of muscle-specific proteins, and ultimately cell fusion to form multinucleated myotubes. Many factors influence this process, such as myogenic basic helix-loop-helix (bHLH) transcription factors (Myf5, MyoD, myogenin, and MRF4), extracellular signaling molecules, and components of extracellular matrix [Yun and Wold, 1996; Krauss et al., 2005]. In particular, factors involved in cell–cell contact and adhesion -such as N- and M-cadherins, integrins,

Abbreviations: 4′-MU-Neu5Ac, 4-methylumbelliferyl N-acetylneuraminic acid; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HPTLC, high performance thin layer chromatography.

The work was performed at Department of Medical Chemistry, Biochemistry, and Biotechnology, University of Milan, 20090 Milan, Italy

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*Correspondence to: Bruno Venerando, Department of Medical Chemistry, Biochemistry and Biotechnology, University of Milan, via F.lli Cervi 93, 20090 Segrate, Milan, Italy. E-mail: bruno.venerando@unimi.it

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ADAMs, neural-cell adhesion molecule (NCAM) and neogenin- play a crucial role in the induction of the myogenic program and in cell fusion step [Yun and Wold, 1996; Krauss et al., 2005; Krauss, 2010]. Moreover, plasma membrane characteristics, composition and organization -such as fluidity, degree of sialylation- can affect skeletal muscle differentiation and especially myoblast fusion [Mukai et al., 2009].

In recent years it has been reported that sialidases, glycohydrolitic enzymes, that catalyze the removal of sialic acid from sialo-glycolipids (gangliosides) and sialo-glycoproteins, are involved in skeletal muscle differentiation. In particular, the lysosomal sialidase NEU1 and the cytosolic sialidase NEU2 play a definite and distinct role in the muscle differentiation process [Sato and Miyagi, 1996; Fanzani et al., 2003; Champigny et al., 2005]. Moreover, we have recently shown that sialidase NEU3 expression increases during skeletal muscle differentiation and that the enzyme down-regulation, although incomplete, is sufficient to totally inhibit C2C12 murine myoblast differentiation and to cause a high responsiveness to apoptotic stimuli [Anastasia et al., 2008]. Indeed the plasma membrane-associated sialidase NEU3 is localized on the extracellular leaflet of cellular plasma membrane [Zanchetti et al., 2007] and is able to modify the ganglioside composition of cell plasma membrane of neighboring cells, showing a high specificity toward gangliosides [Monti et al., 2002; Papini et al., 2004]. Therefore, modulating ganglioside content, NEU3 appears to control transmembrane signaling [Papini et al., 2004; Miyagi et al., 2008].

In order to further investigate the role of NEU3 in skeletal muscle differentiation and to find out if cell membrane remodeling by NEU3 affects this process, we examined the differentiation process in C2C12 cells over-expressing NEU3.

In the present study, C2C12 murine myoblasts were treated with a properly engineered lentiviral vector in order to stably over-express NEU3 sialidase and their behavior along with induced differentiation was investigated. Our results show that NEU3 over-expressing C2C12 cells: (i) continue to proliferate, although exposed to conditions of differentiation by an increased MAPK/ERK pathway activation; (ii) although with delay, they start differentiation and complete it very quickly (2–3 days as compared to 5–7 days required by control cells); (iii) at the end of the differentiation process, they form hypertrophic myotubes, with a myotube diameter about twofold greater than C2C12 wild type cells.

We hypothesize that the modifications of plasma membrane ganglioside pattern as a consequence of NEU3 over-expression—in particular the marked decrease of GM3 content—play the main role in mediating above mentioned effects. Our previous study demonstrated that a strong increase of GM3 level has a dramatic effect on C2C12 cells, causing a complete inhibition of the differentiation process [Anastasia et al., 2008]. So we suppose that the decrease of GM3 content affects the EGF receptor as well as the downstream signaling pathways—in particular the MAPK/ERK pathway—so promoting cell proliferation and delaying the beginning of the differentiation, even though serum was removed from culture medium. Moreover, desialylating greatly NCAM, NEU3 could support myoblast fusion and the formation of hypertrophic myotubes.

Therefore, our data support a pivotal role for NEU3 at the onset of the differentiation process and in the myoblast fusion step.

MATERIALS AND METHODS

MATERIALS

Bovine serum albumine (BSA), 4-methylumbelliferyl N-acetylneuraminic acid (4-MU-Neu5Ac), pepstatin A, aprotinin, leupeptin, trypan blue, Hoechst 33258, colominic acid sodium salt from Escherichia coli, and all cell culture reagents were purchased from Sigma-Aldrich (St Louis, MO). RNeasy Mini Kit was provided from Qiagen (Milan, Italy). iScript cDNA Synthesis kit and iQ SYBR Green Supermix were from Bio-Rad Laboratories (Richmond, VA). Coomassie Protein Assay Reagent, PVDF membrane, SuperSignal West Pico and SuperSignal West Dura Extended Duration Substrate were provided by Pierce Biotechnology (Rockford, IL). pLenti6/V5 vector, Lipofectamine 2000 Reagent, Blasticidin were purchased from Invitrogen Life Technology (Carlsbad, CA). [3-^{3}H]sphingosine (19.8 Ci/mmol) were provided by PerkinElmer (Waltham, MA). High performance silica gel-precoated thin-layer plates (HPTLC Kieselgel 60) were purchased from Merck (Darmstadt, Germany). Ganglioside GD1a was extracted and purified from bovine brain [Sonnino et al., 1996]. Radioactive GD1a containing erythro-C18-sphingosine, isotopically tritium-labeled at position 3, [3-^{3}H(Sph18)]GD1a, was prepared by the dichloro-dicyano-benzoquinone/sodium boro[^{3}H]-hydride method followed by reversed phase HPLC purification (homogeneity 99%, specific radioactivity of 1.2 Ci/mmol) [Sonnino et al., 1984; Sonnino et al., 1996].

CELL CULTURE AND STABLE OVER-EXPRESSION OF NEU3

C2C12 mouse myoblasts were obtained from the American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagle's medium with high glucose (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2, 95% air-humidified atmosphere.

cDNA encoding murine NEU3 was subcloned into lentiviral vector pLenti6/V5 (Invitrogen Life Technology). To generate viral particles, 293FT cells were transfected with 3 μg of the pLenti6/V5 vector and 9 μg of the packaging vector mix (Invitrogen Life Technology) through Lipofectamine 2000 Reagent (Invitrogen Life Technology). Culture medium was collected 2 days after transfection and used to infect C2C12 cells according to the manufacturer's procedure (Invitrogen Life Technology). Infected clones were isolated after selection with 6 μg/ml Blasticidin (Invitrogen Life Technology) and were checked for NEU3 expression. Among these clones we used for our study clone 2, referred to as L-NEU3, which showed the highest expression of NEU3. C2C12 mock cells were also prepared using an empty lentiviral vector. No differences were observed between mock cells and C2C12 wild type.

To assess cell proliferation, a growth curve was performed counting cells after Trypan blue staining. Briefly, 1×10^5 C2C12 and L-NEU3 cells were seeded in 100 mm culture plates and viable cells were counted every 24 h up to 3 days (70% of confluence).

INDUCTION OF SKELETAL MUSCLE DIFFERENTIATION

To induce skeletal muscle differentiation, after reaching 70–80% confluence, C2C12 and L-NEU3 cells were cultured in DMEM supplemented with 2% (v/v) horse serum (HS) (differentiation medium, DM). DM was changed every 2 days and differentiation was completed in 7 days. After differentiation induction, cell proliferation was assessed achieving a growth curve, in brief 4×10^5 C2C12 and L-NEU3 cells were seeded in 60 mm culture plates and viable cells were counted every 24 h up to 4 days. We also counted dead cell number—collecting cell medium after 1, 2, and 3 days of differentiation—and expressed it as a percentage of dead cells, floating in medium, on total cells (sum of dead and viable cells, in suspension and adhering to plate).

SIALIDASE ACTIVITY ASSAY

Proliferating C2C12 and L-NEU3 cells were washed in PBS, harvested by scraping, centrifuged, and resuspended in PBS containing 1 mM EDTA, 1 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin. The cells were lysed by sonication and centrifugation at 800*g* for 10 min at 4°C to eliminate unbroken cells and nuclear components. The crude extract was subsequently, centrifuged at 200,000*g* for 20 min at 4°C on TL100 Ultracentrifuge (Beckman) to obtain a cytosolic and a particulate fraction. Aliquots of the particulate fraction were used to assay NEU3 sialidase activity, according to Papini et al., 2004. Enzimatic activity was determined using 4′-MU-Neu5Ac and [3-^{3}H] GD1a, as substrates. Assays were performed in triplicate with 40 μg of total protein in a final volume of 100 μl and in the presence of 12.5 mM sodium-citrate/phosphate buffer, pH 3.8. In analyses performed using [3-^{3}H] GD1a, final incubaton mixture contained also 0.1% (w/v) Triton X100.

NEU3 enzymatic activity was also assayed using colominic acid as substrate. Incubation was carried out in triplicate in a final volume of 100 μl with 40 μg of total cell lysate proteins and in the presence of 12.5 mM sodium-citrate/phosphate buffer, pH 3.8.

One unit of sialidase activity is defined as liberation of 1 μmol of Neu5Ac/min at 37°C.

METABOLIC LABELING OF CELL SPHINGOLIPIDS

[3-^{3}H]Sphingosine dissolved in ethanol was transferred into a glass sterile tube and dried under a nitrogen stream; the residue was then dissolved in an appropriate volume of pre-warmed (37°C) DMEM + 10% FBS to obtain a final concentration of 3×10^{-8} M (corresponding to 0.4 μCi/100 mm dish). After a 2 h incubation (pulse), the medium was removed and cells were incubated for 48 h with DMEM + 10% FBS not containing radioactive sphingosine (chase), in order to reach a metabolic steady state. This condition was previously established with ad hoc experiments.

At the end of chase, cells were washed and harvested in ice-cold phosphate-buffered saline (PBS) by scraping. The cell suspensions were frozen and lyophilized.

EXTRACTION AND ANALYSIS OF RADIOACTIVE LIPIDS

Total lipids from lyophilized cells were extracted twice with chloroform/methanol/water 20:10:1 (v/v). Lipid extracts dried under a nitrogen stream were dissolved in chloroform/methanol 2:1 by volume and subjected to a two-phase partitioning in chloroform/methanol 2:1 and 20% (v/v) water; the aqueous and organic phases obtained were counted for radioactivity and analyzed by HPTLC. [^{3}H]Sphingolipids of organic phase were separated using the solvent system chloroform/methanol/water 55:20:3 (v/v). The solvent system chloroform/methanol/0.2% aqueous $CaCl_2$ 60:40:9 (v/v) was employed to analyze [^{3}H]gangliosides of aqueous phase. Radioactive lipids were visualized with a Beta-Imager 2000 (Biospace, Paris, France) and identified by comparison with radiolabeled standards. The radioactivity associated with individual lipids was determined with the specific β-Vision software (Biospace, Paris, France).

RNA EXTRACTION, PCR, AND REAL-TIME RT-PCR

Total RNA was extracted from C2C12 and L-NEU3 cells using the RNeasy mini kit (Qiagen), according to the manifacturer's protocol. The iScript cDNA Synthesis Kit (Bio-Rad Laboratories) was used to reverse-transcribe 0.8 μg of RNA. PCR was carried out as follow: 95°C for 2 min (one cycle), 95°C for 30 s followed by 58°C for 30 s and 72°C for 1 min 30 s (32 cycles), finally, 72°C for 5 min. The gene coding for glyceraldeide 3-phosphate dehydrogenase (GAPDH) was taken as housekeeping gene. The primers used are reported in Table I.

Real time PCR was performed by the iCycler thermal cycler (Bio-Rad Laboratories) using cDNA corresponding to 10 ng of total RNA as template. PCR mixture included 0.2 μM primers, 50 mM KCl, 20 mM Tris/HCl pH 8.4, 0.8 mM dNTPs, 0.7 U iTaq DNA Polymerase, 3 mM $MgCl_2$, and SYBR Green (iQ SYBR Green Supermix from Bio-Rad Laboratories) in a final volume of 20 μl. Amplification and real-time data acquisition were performed using the followed cycle conditions: Initial denaturation at 95°C for 3 min, followed by 40 cycles of 10s at 95°C and 30 s at 58°C. The fold change in expression of the different genes in NEU3 overexpressing C2C12 compared with control cells was normalized to the expression of GAPDH mRNA and was calculated by the equation $2^{-\Delta\Delta Ct}$. The expression of different reference genes was monitored in order to

TABLE I. Primer Sequences for RT-PCR

Primers	Forward	Reverse	TM (°C)
GAPDH	5′-CGTGCCGCCTGGAGAAAC-3′	5′-TGGGAGTTGCTGTTGAAGTCG-3′	58
NEU1	5′-AGTGAGCGGCATTCCCTTTG-3′	5′-GGCAATGGTAGTTATTCTGGTTCC-3′	60
NEU2	5′-GCTACAACGAAGCCACCAAC-3′	5′-CAGGGACAGCGATGAAGAA-3′	57
NEU3	5′-CCTCAGTACAGAGATGAGGATG-3′	5′-GCGATGCCCAGGTAATGT-3′	56
NEU4	5′-GCCTGCTTGTTCCTGCTTAC-3′	5′-CAGAGGGCTTCGAGCATTAC-3′	60

choose the housekeeping gene, in particular we established which housekeeping genes were the stably expressed ones in our experimental conditions. Among these GAPDH expression did not change significantly during the differentiation of wild type C2C12 and NEU3 over-expressing cells. Moreover we carried out a validation experiment to verify that the efficiences of the target and reference gene amplifications were approximately equal. The pooled mean ± SD for the ct value of the housekeeping gene for all conditions is shown in Supplementary Materials. The chosen calibrator condition was always wild type C2C12 cells, but at a different day of differentiation or in proliferation, depending on the target gene expression. Calibrator condition was indicated in the figure legends every time. All reactions were performed in triplicate. The primers used are reported in Table II. The accuracy was monitored by the analysis of the melting curves.

CELL STIMULATION WITH EGF

C2C12 and L-NEU3 cells in proliferation were starved overnight in serum-free medium and then stimulated for 15 min with 100 ng/ml EGF.

After stimulation, cells were washed with PBS twice and then lysed for 15 min at 4°C in lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 20 mM NaF, 1 mM Na_3VO_4, 0.5% v/v NP40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A).

Insoluble material was removed by centrifugation at 13,000 × g for 10 min, supernatants were collected and assayed for protein concentration with Coomassie Protein Assay (Pierce). Samples were analysed by immunoblotting.

IMMUNOBLOTTING AND DENSITOMETRY ANALYSIS

One-dimensional SDS–PAGE was performed according to Laemli [1970]. For immunoblotting, proteins separated on 10% (w/v) SDS–PAGE gels were electrophoretically transferred on PVDF membrane in Tris-glicine buffer, using the Mini Transblot System (Bio-Rad Laboratories, Richmond, VA). Primary antibodies were used as follows: anti-EGFR 1:1,000 diluition, (Cell Signalling, Danvers, MA), anti-phospho-EGFR (Tyr 1148) 1:1,000 diluition, (Calbiochem, San Diego, CA), anti ERK1/2 (Zymed, San Francisco), anti phospho ERK1/2 (Cell Signalling, Danvers, MA).

Each membrane was washed four times for 10 min and then incubated with the appropriate secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. For the immunological detection of proteins, the enhanced chemiluminescence system (Pierce Biotechnology, Rockford, IL) was used. The densitometric analysis of blots was performed using Quantity One Software (Bio-Rad Laboratories, Richmond, VA).

IMMUNOFLUORESCENCE STAINING AND MYOTUBE HYPERTROPHY ANALYSIS

C2C12 and L-NEU3 cells were plated onto 6-well culture plates. At different times after exposure to differentiation conditions, cells were washed in PBS, and fixed for 10 min in 4% (w/v) paraformaldehyde in PBS, at room temperature. For permeabilization and blocking, cells were incubated for 30 min in the presence of PBS + 0.2% (v/v) Triton x-100 (TX-100) and 1% (w/v) bovine serum albumine (BSA). Then cells were incubated with a 1:100 diluition of anti-Skeletal Myosin (FAST, clone MY-32, Sigma-Aldrich, St Louis, MO) mouse monoclonal antibody in PBS with 0.2% TX-100 and 1% BSA overnight at 4°C. After incubation, cells were washed three times in PBS and incubated with anti mouse FITC-conjugated secondary antibodies (1:200 diluition) 2 h, at room temperature in the same buffer. After washing in PBS, cells were analyzed under a fluorescent microscope (Olympus IX50) equipped with VarioCam acquisition camera. Cell nuclei were counterstained with Hoechst 33258.

Hypertrophy were assessed by measuring the myotube diameter and the number of myonuclei per myotube.

To quantify the myotube diameter, 15 fields were chosen randomly and, for each field, a minimum of 100 myosin-positive myotubes with more than two myonuclei were measured using the Image Pro Plus software. The diameter per myotube was the mean of five measurements taken along the length of the myotube and averaged from three different experiments [Sharples et al., 2010]. Measurements are expressed as % of C2C12 values.

For myonuclei quantification the mean number of nuclei per myotube (containing more than two myonuclei) was measured at the end of differentiation in five randomly chosen fields per dish. Mean values are a measure of 150 myotubes for each cell line [Park et al., 2005].

STATISTICAL ANALYSES

Data are presented as the means ± standard deviations (S.D.). Statistical analyses were made using unpaired Student's *t*-test. Significance was attributed at the 95% level of confidence (P-value < 0.05).

RESULTS

NEU3 STABLE OVER-EXPRESSION IN C2C12 CELLS

To study the involvement of Neu3 sialidase in skeletal muscle differentiation, C2C12 cells were transduced with a lentiviral vector containing murine NEU3 gene, according to the protocol described in Materials and Methods section. Five stable clones were isolated and NEU3 mRNA levels were checked by Real-Time PCR. Among

TABLE II. Primer Sequences for Real-Time PCR

Primer	Forward	Reverse	TM (°C)
GAPDH	5′-CGTGCCGCCTGGAGAAAC-3′	5′-TGGGAGTTGCTGTTGAAGTCG-3′	60
NEU3	5′- TGCGTGTTCAGTCAAGCC-3′	5′- GCAGTAGAGCACAGGTTAC-3′	56
p21	5′-AAGTGTGCCGTTGTCTCTTC-3′	5′- GTCAAAGTTCCACCGTTCTC-3′	58
MyoD	5′-GCCTGAGCAAAGTGAATGAG-3′	5′-CTTCGATGTAGCGGATGG-3′	56
Myogenin	5′- AGCCACACTGAGGGAGAAG -3′	5′- GTTGAGGGAGCTGAGCAAG-3′	58
MHC	5′-TGAACTGGAGGGTGAGGTAG-3′	5′-TTCGGTCTTCTTCTGTCTGG-3′	58

them, "clone 2" (hereafter named L-NEU3) showed the highest expression of NEU3, displaying a 19-fold increase as compared to C2C12 wild type cells (Fig. 1A), and was chosen for subsequent studies. C2C12 cells were also transduced using the same lentiviral vector without MmNEU3 coding sequence. This mock-transfected cell line was compared with C2C12 wild type and no significant differences were observed (data not shown). Therefore MmNEU3 over-expressing cells (L-NEU3) were correlated to parental C2C12 cells.

The effect of NEU3 over-expression on all other sialidases was also checked: NEU1 expression showed no significant differences as compared to C2C12 cells, and both NEU2 and NEU4 were below detectable levels, as in control C2C12 cells (Fig. 1B).

L-NEU3 cells were tested for NEU3 sialidase activity in the particulate fraction (200,000*g* pellet) using the artificial substrate 4-methylumbelliferyl N-acetylneuraminic acid (4′-MU-Neu5Ac) and ganglioside GD1a, as natural substrate. Over-expressing cells showed a 2.7- and 15-fold activity increase toward 4′-MU-Neu5Ac (L-NEU3 cells specific activity 0.16 mU/mg protein) and GD1a (L-NEU3 cells specific activity 3.07 mU/mg protein), respectively, as compared to C2C12 wild type cells (Fig. 1C).

Cell morphology and proliferation rate were not appreciably affected by NEU3 over-expression (Fig. 1D,E). Cell viability was $\geq$ 98%.

SPHINGOLIPID PATTERN OF NEU3 OVER-EXPRESSING CELLS

The effects of NEU3 over-expression on the sphingolipid pattern of C2C12 cells was studied by feeding both C2C12 and L-NEU3 cells, under normal proliferating conditions, with a radiolabeled precursor, [^{3}H] sphingosine, and analysing the sphingolipid pattern of both cell lines at steady state conditions. At the end of 48 h of chase, cell lipids were extracted, and gangliosides and non-ganglioside sphingolipids were separated and analysed by HPTLC (Fig. 2A,C).

In C2C12 cells, GM3 was the main ganglioside, and represented 75% of the total ganglioside content, followed by ganglioside GD1a (12.3%), GM1 (5.8%), and GM2 (6.9%), in agreement with previous observations [Anastasia et al., 2008]. Instead, L-NEU3 cells showed a remarkable decrease of total ganglioside content (from 81,571 to 50,389 dpm/mg protein); in particular NEU3 over-expression caused a 38% decrease of GM3, that was by far the most abundant ganglioside in both cell types (Fig. 2B). Moreover the content of non-ganglioside sphingolipids increased in NEU3 over-expressing cells

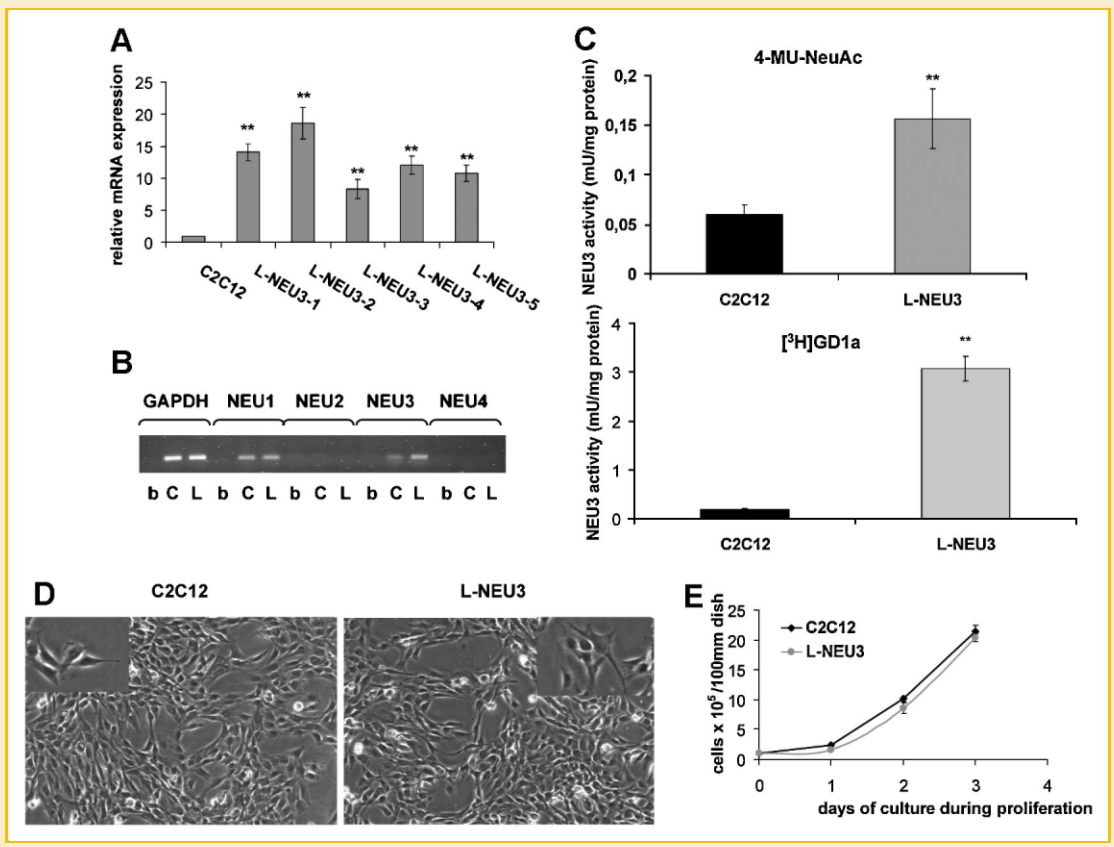

Fig. 1. Overexpression of NEU3 in C2C12 cells. A: Real-time PCR analysis of NEU3 mRNA expression in five stable clones isolated after transfection in comparison with C2C12 cells, the calibrator condition was growing wild type C2C12 cells. B: Sialidase mRNA expression in C2C12 (C) and L-NEU3 (L) cells, (b) RT-PCR reaction blanks. mRNA expression was evaluated by RT-PCR using glyceraldeide 3-phosphate dehydrogenase (GAPDH) gene as house-keeping gene. C: NEU3 sialidase activity in the particulate fraction of proliferating C2C12 and L-NEU3 cells, performed with 4′-MU-Neu5Ac and [^{3}H]GD1a. D: Phase-contrast microphotografts of C2C12 and L-NEU3 cells during proliferation, original magnification 10× (enlargements are shown in insets, magnification 20×). E: Proliferation curves of C2C12 and L-NEU3 cells, cell viability was determined by Trypan blue dye exclusion assay. All the data of A, C, and E panels are the means ± S.D. of five independent experiments, statistical differences were determined by Student's *t*-test: ** $P < 0.001$ compared to C2C12 cells.

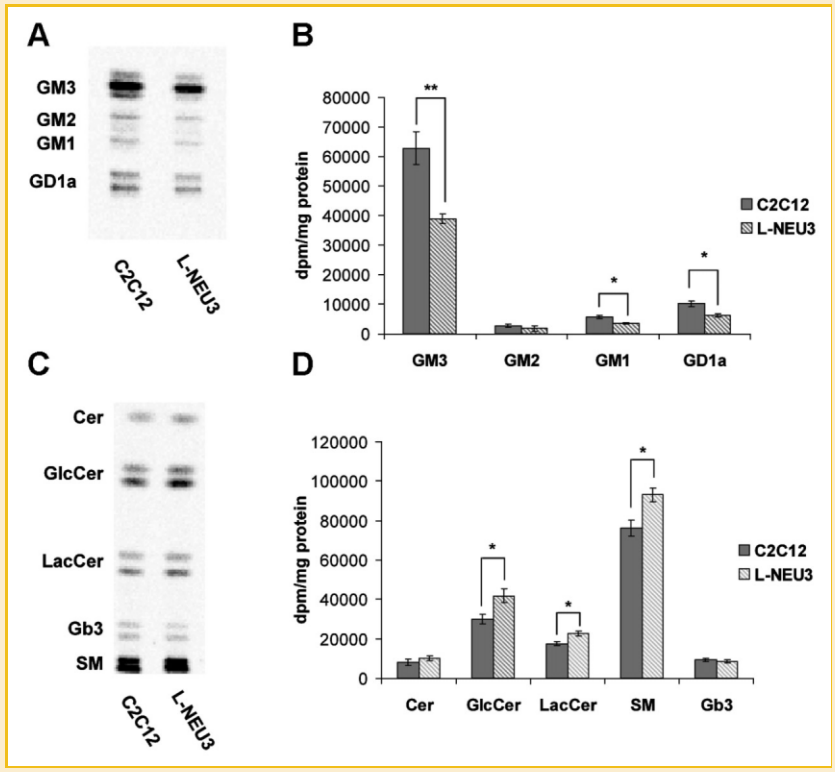

Fig. 2. Sphingolipid profile of C2C12 and L-NEU3 cells. C2C12 and L-NEU3 cells were labeled with [^{3}H] sphingosine for 2 h, after 48 h chase cells were harvested and the sphingolipid pattern of both cell lines were analysed. A: HPTLC separation of gangliosides from C2C12 and L-NEU3 cells using the solvent system chloroform/methanol/0.2% $CaCl_2$ 60:40:9 (v/v). Multiple spots represent different molecular species of gangliosides based on chain length of fatty acid bound to sphingosine. B: Ganglioside content of C2C12 and L-NEU3cells. Data are the means ± S.D. of five independent experiments, statistical differences were determined by Student's *t*-test: $^{*}P < 0.05$ and $^{**}P < 0.001$. C: HPTLC separation of neutral sphingolipids and sphingomielyn (SM) from C2C12 and L-NEU3 cells using the solvent system chloroform/methanol/water 55:20:3 (v/v). D: Neutral sphingolipid and sphingomyelin (SM) content of C2C12 and L-NEU3 cells. Data are the means ± S.D. of five independent experiments, statistical differences were determined by Student's *t*-test: $^{*}P < 0.05$. Multiple spots represent different molecular species of sphingolipids based on chain length of fatty acid bound to sphingosine. Cer, ceramide; GlcCer, glucosylceramide; LacCer, lactosylceramide; SM, sphingomyelin; Gb3, trihexosylceramide.

from 157,400 to 194,720 dpm/mg protein (about 23.6%), with a statistically significant increase of the main components glucosyl-ceramide (Glc-Cer), lactosyl-ceramide (Lac-Cer,) and sphingomyelin (SM) (+36.7%, + 29.4% and +21.6%, respectively) (Fig. 2D).

No significant variation of the minor components, ceramide, and Gb3 was detectable. Noteworthy, in spite of the considerable decrease of GM3, L-NEU3 cells did not show any difference in the expression of GM3 synthase, as compared to C2C12 cells (data not shown).

NEU3 OVER-EXPRESSION MODIFIES THE DIFFERENTIATION PROCESS

To assess possible effects of Neu3 over-expression on skeletal muscle differentiation, subconfluent L-NEU3 and C2C12 cells were shifted to DM to induce differentiation (the first day of incubation in differentiation medium was defined as "day 0 of differentiation"). When differentiation is induced, C2C12 wild type are expected to withdraw from the cell cycle and begin elongation [Andres and Walsh, 1996]. As shown in Figure 3A, after 3 days from differentiation induction, C2C12 cells were aligned and multinucleated myotubes started to be formed. On the contrary, L-NEU3 cells did not respond to the differentiation stimulus, and continued to proliferate until over-confluence. In fact, 3 days after induction no myotube could be found in the culture plates (Fig. 3A). However, later on, L-NEU3 initiated the differentiation process and completed it rapidly within 7 days from the initial induction, as featured by control cells. Notably, after 7 days in DM, both cell lines appeared terminally differentiated, and L-NEU3 cells showed the formation of hypertrophic myotubes, shorter but markedly wider than those of parental cells (Fig. 3A). Prolonged treatment of cells with the differentiation medium (up to 3–4 extra days) did not increase the number of formed myotubes in both cell lines. In other words, in L-NEU3 cells, differentiation was delayed but occurred at a higher rate than in mock cells. Differentiation experiments performed using other clones over-expressing MmNEU3 showed the same results (data not shown).

NEU3 OVER-EXPRESSION DELAYS THE DIFFERENTIATION PROCESS

To confirm that L-NEU3 cells did not promptly respond to the differentiation stimulus and continued to proliferate, we performed a growth curve after differentiation induction. As shown in Figure 3B, after 1 day in differentiation medium, we observed an increase of cell number in both cell line. Two days after differentiation induction, parental C2C12 stopped growth and cell

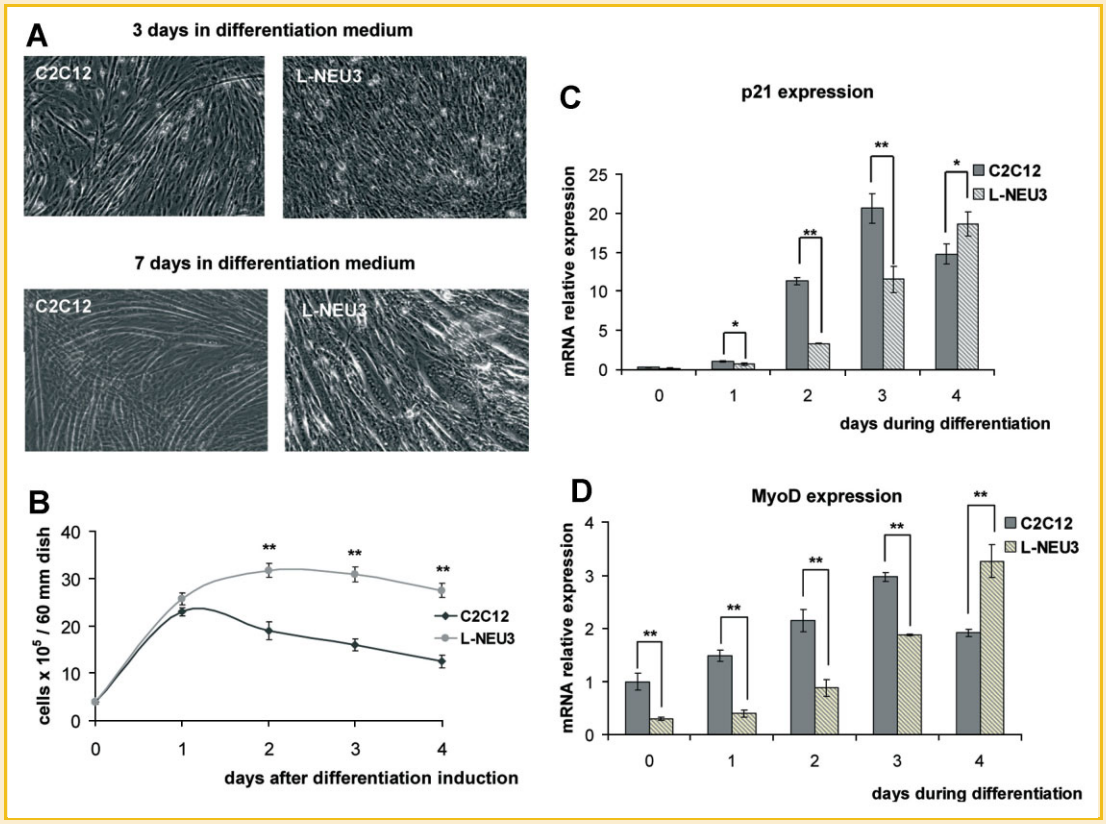

Fig. 3. In differentiation medium NEU3 overexpressing cells keep proliferating and do not start differentiation. A: Phase-contrast microphotografts of C2C12 and L-NEU3 differentiating cells at 3 and 7 days of differentiation. Original magnification 10×. B. Growth curve of C2C12 and L-NEU3 cells after differentiation induction (day 0, 1, 2, 3, and 4 of differentiation). C: p21 mRNA expression assessed by real time PCR in the early stages of differentiation (day 0, 1, 2, 3, 4 of differentiation). The calibrator condition was wild type C2C12 cells at day 1 of differentiation. D: MyoD mRNA expression assessed by real time PCR in the early stages of differentiation (day 0, 1, 2, 3, 4 of differentiation). The calibrator condition was wild type C2C12 cells at day 0 of differentiation. Data are the means ± S.D. of five independent experiments, statistical differences were determined by Student's *t*-test: $^{*}P < 0.05$ and $^{**}P < 0.001$ compared to C2C12 cells at each time point.

number appeared to decrease because some cells died of apoptosis, as usually happens when skeletal muscle differentiation starts [Fernando et al., 2002]. Moreover in the following days, C2C12 cells began to form myotubes. On the contrary, L-NEU3 cell number continued to increase and, then, kept constant up to 3 days after differentiation induction; moreover, no myotubes were formed. After 1, 2, and 3 days in differentiation medium we also collected cell medium and counted dead cells by Trypan blue staining. In wild type C2C12 the highest number of detached dead cells was between day 1 and 2 of differentiation (21.4% of total cells). On the contrary L-NEU3 cells delayed cell death and showed a 16.1% of dead cells after 3 days in differentiation medium.

We measured p21 expression levels by real-time PCR during the first days of differentiation (Fig. 3C). p21 is an inhibitor of cyclin-dependent kinase (CdK) and its expression is induced by MyoD during muscle differentiation [Kitzmann and Fernandez, 2001].

We observed (Fig. 3C) that after induction of differentiation, p21 mRNA expression levels promptly and rapidly increased in C2C12 cells, reaching a maximum at day 3; whereas in L-NEU3 cells the expression started and reached a maximum 1–2 days later. This is in line with the results of the growth curve (Fig. 3B).

Moreover, we determined MyoD expression levels during the early days of differentiation. MyoD is a main regulator of skeletal myogenesis and is crucial for myoblast determination [Yokoyama and Asahara, 2011]. As shown in Figure 3D, we found that, while in C2C12 MyoD expression increased progressively from differentiation induction and reached a maximum at day 3, on the contrary, in L-NEU3 cells MyoD expression was lower as compared to wild type cells, and started to increase only from the second day (Fig. 3D).

Then, we focused our attention on another myogenic transcription factor, myogenin, that is under the control of MyoD. The onset of myogenin expression marks the entry of myoblasts into differentiation and then is followed by the expression of other muscle specific proteins like as sarcomeric myosin [Andres and Walsh, 1996].

In C2C12 cells, myogenin mRNA level started to increase just after 1 day from induction of differentiation with the maximum increase on day 2, then remaining constant (Fig. 4A). Conversely, in L-NEU3 cells, myogenin mRNA expression was very low in the first days from differentiation induction, but rapidly increased after 4 days and then promptly fell down to levels similar to those of parental cells (Fig. 4A). With regard to myosin heavy chain (MHC), a

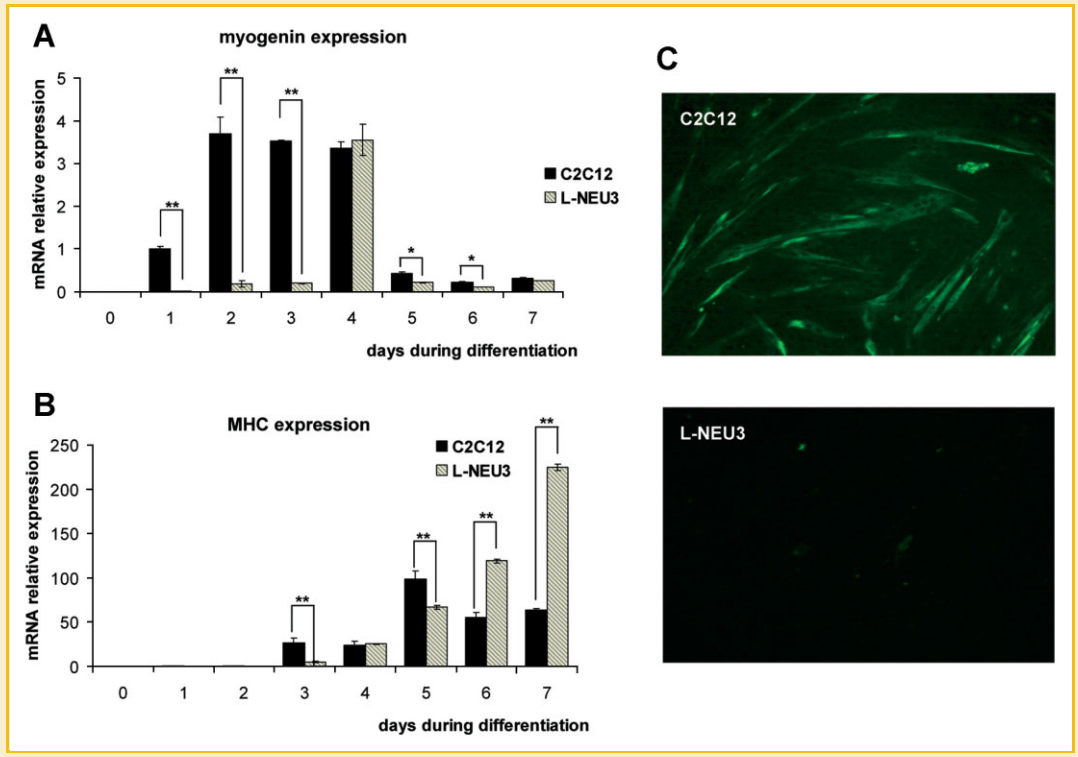

Fig. 4. NEU3 overexpression delays cell differentiation. A: Myogenin mRNA expression by real time PCR during C2C12 and L-NEU3 differentiation. The calibrator condition was wild type C2C12 cells at day 1 of differentiation. B: Real-time PCR analysis of myosin heavy chain (MHC) expression during C2C12 and L-NEU3 differentiation. The calibrator condition was wild type C2C12 cells at day 2 of differentiation. C: Immunofluorescence analysis with anti-MHC antibody (green) in C2C12 and L-NEU3 at the 3rd day of differentiation. Original magnification 10×. Data are the means ± S.D. of three independent experiments, statistical differences were determined by Student's *t*-test: $^{*}P < 0.05$ and $^{**}P < 0.001$ compared to C2C12 cells at each time point. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

marker of terminal skeletal muscle differentiation, real-time PCR analysis showed that C2C12 cells began to clearly express this marker from the third day of induced differentiation; MHC expression reached the maximum value at day 5 of differentiation, then decreased. On the contrary, in -NEU3 cells, at day 3 of differentiation, MHC expression was still low, subsequently increased, achieving much higher levels as compared to C2C12 cells, in particular in the last days of differentiation (Fig. 4B). MHC expression was also analyzed by immunofluorescence: As shown in Figure 4C, at day 3 of differentiation several MHC-positive myotubes were present in parental cells, while none could be detected in L-NEU3 cell culture plates, confirming the delay of muscle differentiation.

NEU3 OVEREXPRESSION ALTERS EGF RECEPTOR ACTIVATION AND SIGNALING

Several studies have shown that GM3 can modulate the function of some growth factors receptors, particularly it can inhibit the activation of the epidermal growth factor receptor (EGF-R) preventing EGF-R autophosphorylation without affecting EGF-R binding [Miljan et al., 2002]. In our previous study [Anastasia et al., 2008], we reported that, in C2C12 cells, NEU3 down-regulation causes an increase in GM3 levels, with a concomitant inhibition of the EGF-R activation, and, eventually, its down-regulation. Thus, we decided to investigate whether the remarkable GM3 decrease observed in L-NEU3 cells has any effect on EGF-R activation. To this purpose L-NEU3 and mock cells were stimulated with 100 ng/ml EGF for 15 min and phosphorylation on tyrosine 1148, one of the main sites of EGF-R autophosphorylation, was assayed. As shown in Figure 5A, EGF stimulation caused a markedly higher phosphorylation (+70%) of EGF-R in L-NEU3 cells, as compared to control cells.

Then, we analysed a key signaling pathway activated by EGF-R, the mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK). This pathway has been reported to induce cell proliferation and protection from apoptosis [Meloche and Pouyssegur, 2007]. First, we analysed ERK 1/2 phosphorylation in proliferating cells after stimulation with EGF. Overnight serum-starved C2C12 and L-NEU3 cells were incubated with 100 ng/ml EGF for 15 min, then, phosphorylated and total MAPK/ERK 1/2 proteins were evaluated by western blot analysis. Western blot with the antibody toward phospho-ERK 1/2 (Thr202/Tyr204) showed an increased activation of ERK1/2 in L-NEU3 cells after EGF stimulation, as compared to wild type cells. Notably, in L-NEU3 cells the band corresponding to phosphoERK2 was completely absent (Fig. 5B). Next, we evaluated ERK1/2 phosphorylation at the beginning of differentiation induction (day 1). Under these conditions, a higher activation of ERK 1/2 was

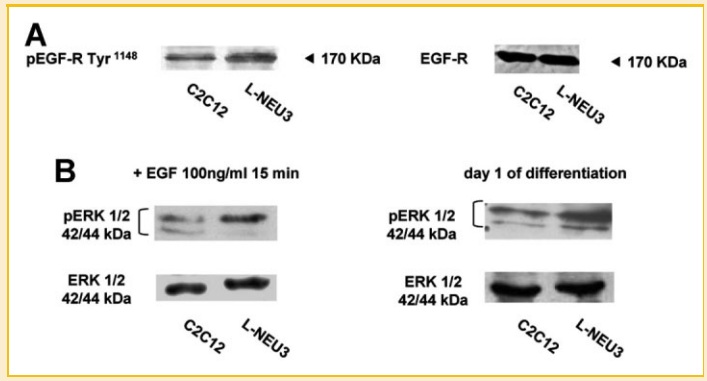

Fig. 5. NEU3 overexpression modifies EGF receptor activation and signaling. A: Western blot analysis of EGF receptor (EGFR) autophosphorylation in C2C12 and L-NEU3 cells using an antibody against the phosphotyrosine 1148 of EGFR. Both cell lines in proliferation were starved overnight in serum-free medium and then stimulated for 15 min with 100 ng/ml EGF. B: Western blot analysis of total cell lysates probed with anti pERK and anti ERK antibodies. Both cell lines were analysed after stimulation with 100ng/ml EGF for 15 min and at day 1 of differentiation.

recorded in L-NEU3 compared to C2C12 cells: Remarkably, both bands of ERK 1/2 were present (Fig. 5B). No significant differences in total ERK expression could be detected in C2C12 and L-NEU3 cells (Fig. 5B).

NEU3 OVER-EXPRESSION CAUSES THE FORMATION OF HYPERTROPHIC MYOTUBES

After 7 days in DM, both C2C12 and L-NEU3 cells were differentiated and showed myotubes expressing MHC (Fig. 6A). Figure 6 B showed C2C12 and L-NEU3 myotubes expressing MHC with nuclei counterstained with Hoechst 33258 at magnification 20× to highlight better the nuclei number. Cell hypertrophy was quantified by measuring myotube diameter and number of nuclei per myotube. As shown in Figure 6C, L-NEU3 cells showed a twofold increase of myotube diameter and a 2.5-fold increase of the mean number of myonuclei present in myotubes as compared to parental cells, clearly indicating hypertrophy. L-NEU3 myotube hypertrophy was confirmed also by the MHC expression level at the end of differentiation (Fig. 4B,C).

Moreover and interestingly, we found that nuclei in L-NEU3 myotubes were not aligned near the plasma membrane as in C2C12 but were frequently arranged in myosacs, containing a lot of nuclei, in the middle of the hypertrophic myotubes (Fig. 6A,B).

Several factors involved in cell–cell and cell–extracellular matrix adhesion were found to regulate myoblast fusion; among these, the NCAM and in particular, the degree of NCAM polysialylation seem to play an important role in vitro [Suzuki et al., 2003]. Indeed, it has been reported that reduction of NCAM polysialylation facilitates myoblast fusion, whereas, in an early stages of differentiation, over-expression of PSA inhibits fusion process [Suzuki et al., 2003]. PSA attached to NCAM is a long linear α2,8-linked polymer of N-acetylneuraminic acid specifically removed by endoneuraminidases [Rutishauser, 1998]. Therefore, to investigate if Neu3 acts on the α2,8 sialyl linkage of this substrate, we determined NEU3 activity toward colominic acid in C2C12 and L-NEU3 cells during differentiation. As shown in Figure 6D, NEU3 sialidase was able to hydrolize this linkage and, in particular, L-NEU3 cells showed a 4.5-fold increase of activity, as compared to controls.

DISCUSSION

Skeletal muscle differentiation is a very complex process in which the cell plasma membrane plays a crucial role. When differentiation is induced, cell–cell adhesion and mutual recognition between cells are essential. During embryo development muscle tissue differentiation is dependent on the phenomenon known as ''community effect'': Cells must contact a number of similar cells to respond to inductive signals in order to undergo a coordinate process of differentiation [Buckingham, 2003]. The ''community effect'' is also necessary for differentiation in vitro, where the establishment of cell–cell contacts provides signaling interactions between similar adjacent cells [Messina et al., 2005]. Moreover, in the final steps of differentiation, myocyte plasma membranes fuse to give rise to polynucleated syncytia [Andres and Walsh, 1996]. On these premises, it seems obvious to expect that enzymes capable of modifying plasma membrane biochemical characteristics would affect skeletal muscle differentiation. It is a consolidated notion that the plasma membrane glycocalix plays a pivotal role in cell–cell interactions and that sialic acid, carried by the glycolipid and glycoprotein component of the glycocalix, is crucial in these phenomena. Thus, we decided to further investigate the possible role of NEU3 sialidase in muscle differentiation and the involvement of this sialidase in particular steps of this process. This enzyme localizes predominantly on the extracellular leaflet of the plasma membrane, and is capable to act also in a ''trans'' mode, that is to modify the ganglioside composition of the plasma membrane of neighbor cells [Papini et al., 2004].

In a previous study, we demonstrated that NEU3 activity is required for C2C12 differentiation, and that its silencing, even if not complete, is sufficient to totally inhibit the differentiation process [Anastasia et al., 2008]. Moreover we found that myoblasts lacking NEU3 are more responsive to apoptotic stimuli. In the same study we hypothesized that the increase of ganglioside GM3, due to NEU3 silencing, was responsible of these effects [Anastasia et al., 2008]. In order to support the role of NEU3 in skeletal muscle differentiation and to determine which differentiation step was mainly affected by NEU3, we decided to over-express the enzyme in C2C12 myoblasts

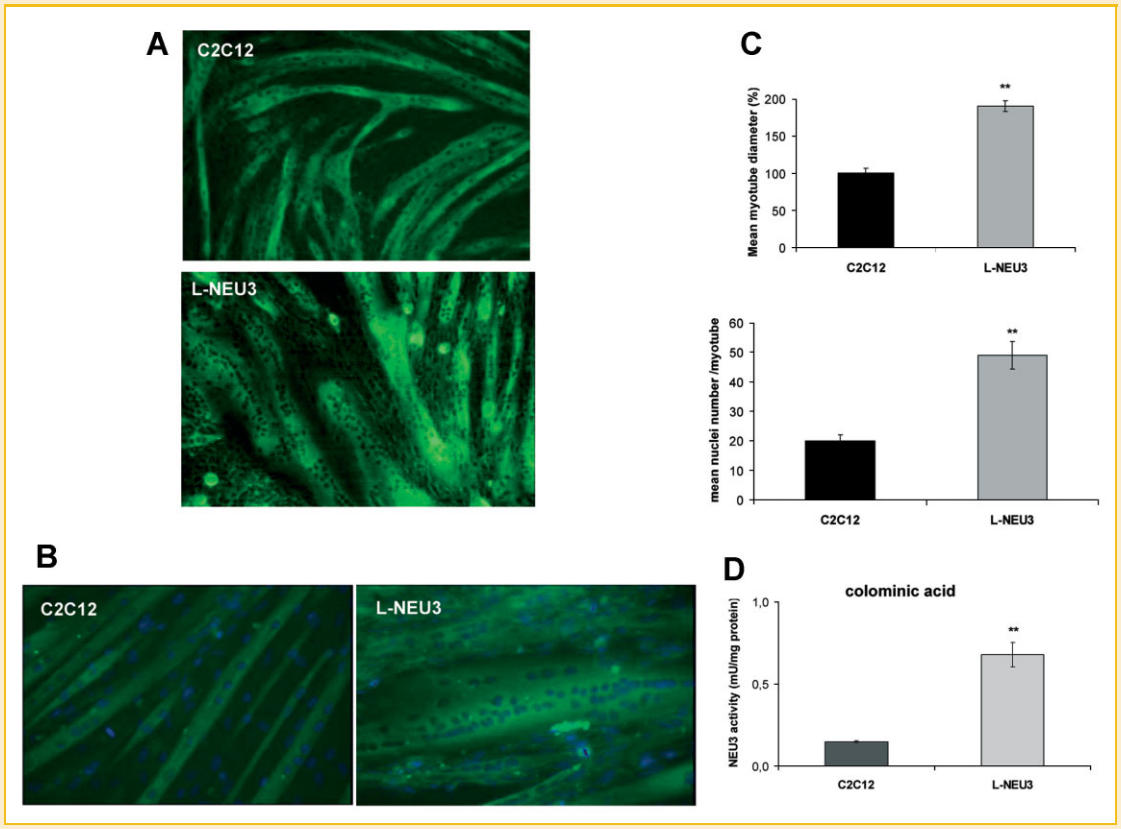

Fig. 6. L-NEU3 cells forms hypertrophic myotubes. A: Immunofluorescence analysis with anti-MHC antibody (green) in C2C12 and L-NEU3 at the 7th day of differentiation. Original magnification 10×. B: Immunofluorescence images showing C2C12 and L-NEU3 myotubes expressing MHC (green) and nuclei counterstained with Hoechst 33258 (blue) at the 7th day of differentiation. Original magnification 20×. C: Myotube diameter and mean number of nuclei per myotube. The average diameter per myotube was measured using the Image Pro Plus software and expressed as % of C2C12 values. Cell nuclei were counterstained with Hoechst 33258. The number of nuclei present in myotubes of five randomly chosen fields was measured at the end of differentiation. D: NEU3 sialidase activity in total cell lysates of C2C12 and L-NEU3 cells at day 5 of differentiation performed with colominic acid. Data represent the means ± S.D. of three independent experiments, statistical differences were determined by Student's *t*-test: ** $P < 0.001$ compared to C2C12 cells. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

and to follow the skeletal muscle differentiation process. NEU3 stably infected myoblasts (L-NEU3) showed no differences in terms of morphology and proliferation as compared to control C2C12 cells (Fig. 1D,E). However, when differentiation was induced by switching cells to 2% (v/v) horse serum, L-NEU3 cells displayed a different behavior.

Indeed upon withdrawal of mitogens, such as serum, from culture medium C2C12 cells promptly blocked proliferation and started to differentiate, whereas L-NEU3 cells continued to proliferate for further 2 days and then started the differentiation process, which they completed within 7 days from initial induction, as well as control C2C12 cells. At the onset of differentiation some myoblasts normally die of apoptosis because they are not able to differentiate [Fernando et al., 2002]. In the first days of differentiation counting of dead cells confimed a delay in L-NEU3 differentiation.

Therefore, NEU3 over-expression caused an initial delay in the differentiation process followed by a much faster differentiation than that occurring in wild type cells. Expression of myogenic transcription factors and proteins that are typically expressed in muscle differentiation, such as MHC, confirmed this trend, in particular, we found a low and delayed MyoD expression in L-NEU3 cells (Fig. 3D). MyoD is a master regulator of skeletal muscle differentiation and drives the transcription of downstream muscle-related genes binding to the E-box sequence (CANNTG) in their promoters [Tapscot, 2005; Yokoyama and Asahara, 2011]. Therefore a decreased or delayed expression of this muscle regulatory factor affects the beginning of the differentiation process and causes a delayed differentiation. It is not surprising that an initial high NEU3 level—as displayed by L-NEU3 cells—seems to prevent cells from exiting from the cell cycle and to promote cell proliferation even though mitogens were absent or decreased, because NEU3 seems to be able to enhance EGF receptor activation, desialylating ganglioside GM3 as well as directly interacting with EGF receptor [Miyagi et al., 2008; Wada et al., 2007]. Indeed NEU3 over-expression commonly occurs in many cancer cells, as it confers cells a higher resistance toward the apoptotic stimuli and a higher proliferation [Miyagi et al., 2008]. It is tempting to speculate that the key molecule in the process is ganglioside GM3 that undergoes a marked decrease after NEU3 over-expression. It has been shown that GM3 inhibits the activation of the EGF receptor by preventing receptor autophosphorylation [Miljan et al., 2002; Coskun et al., 2011]. So NEU3, desialylating GM3 and generating lactosylceramide,

enhances EGF receptor activation and affects the downstream signaling pathways, in particular the MAPK/ERK pathway. To further validate our hypotesis, we tested MAPK/ERK key signaling pathway directly activated by EGF-R, and we observed that ERK is more active in L-NEU3 cells when differentiation was induced (Fig. 5B). The ERK 1/2 pathways has been shown to mediate growth factor-stimulated cell proliferation and coordinately to inhibit differentiation [Rommel et al., 1999] in part by binding of MEK1 to MyoD transcriptional complex and consequently impairing the activity of MyoD [Perry et al., 2001]. Recently, it has been demonstrated that in the early stage of myogenesis the activation of Ras-MAP kinase pathway, in particular the activation of MEK1, inhibits differentiation, whereas a later activation promotes this process [Wu et al., 2000; Jo et al., 2009]. Moreover it was reported that a prolonged or a strong activation of ERK1/2 causes the expression of p21 so leading to cell cycle exit [Meloche and Pouyssegur, 2007]. Therefore, we suppose that in L-NEU3 cells the high activation of ERK 1/2 that occurs in the first day of differentiation can lead to a reduction and a delay of MyoD expression and then, to a delayed p21 induction which ultimately prevents cell cycle arrest (Fig. 3C).

Finally, and most surprisingly, we also observed that NEU3 over-expressing cells formed hypertrophic myotubes with centralized nuclei at the end of the differentiation process (Fig. 6A). We assumed that this effect was not simply caused by the increased myoblast number due to delayed differentiation and over-proliferation of L-NEU3 cells. In order to confirm this hypothesis, we seeded wild type C2C12 cells over-confluent and followed the differentiation process. Wild type C2C12 cells differentiated normally and fused to form myotubes, but did not form hypertrophic myotubes (data not shown). Therefore the formation of hypertrophic myotubes does not merely result from a great deal of myoblasts. Moreover, while C2C12 cells are known to produce myotubes in an asynchronous mode during all the differentiation period, NEU3 over-expressing myoblasts showed a nearly synchronous transition from independent proliferation to aligned differentiation and fusion across the entire population. Therefore we suppose that the differentiation process completes more rapidly in L-NEU3 cells than in control cells, because almost all L-NEU3 cells are likely to be fusion competent, when they start to differentiate. This feature is typical of muscle regeneration and embryonic myogenesis [Chargé and Rudnicki, 2004].

Myogenic cell fusion consists of a strictly regulated series of sequential steps that affect discrete areas of plasma membrane: recognition, adhesion, alignment, breakdown, and fusion of cell membranes are essential. The molecular regulation of this stage of the differentiation process is not fully understood, but a variety of adhesion molecules, that influence it, has been identified, such as N- and M-cadherins, neogenin, and NCAM [Krauss, 2010]. It has been reported that NCAM polysialylation affects myoblast fusion, in particular the overexpression of PSA prevents this differentiation step, whereas abolition of N-linked polysialylation sites on NCAM facilitated it [Suzuki et al., 2003]. Enzymatic activity toward colominic acid confirmed that NEU3 acts on this sialyl linkage and in particular showed that during differentiation in L-NEU3 cells activity toward a linear α2,8-linked polymer of N-acetylneuraminic acid increases as compared to controls (Fig. 6D). Thus we could hypothesize that during differentiation L-NEU3 cells desialylated greatly NCAM promoting myoblast fusion and the formation of hypertrophic myotubes. Moreover the plasma membrane glycocalix plays a pivotal role in cell–cell recognition and interactions and sialic acid, carried by the glycolipid and glycoprotein components of the plasma membrane, is crucial in these phenomena, acting as an antirecognition agent [Schauer, 2009]. So NEU3, by a broad desialylation of cell membrane, could decrease the electronegative charge of the cell surface supporting further myoblast contact and adhesion.

A recent report underlines the role of plasma membrane lipid rafts in the regulation of fusion step during skeletal muscle differentiation. In particular it has been demonstrated that first a dynamic clustering of lipid rafts is essential to provide rigid platforms that cause the accumulation of adhesion molecules at the leading edge of lamellipodia; this plasma membrane organization allows cell recognition and adhesion. Afterward a lateral dispersion of lipid rafts gives rise to ''fusion –competent'' areas with increased membrane fluidity and permits opposed membranes union [Mukai et al., 2009]. NEU3 is associated with caveolin1 and localizes in lipid rafts, moreover this sialidase is able to modify ganglioside pattern of adjacent cells [Wang et al., 2002; Papini et al., 2004]. In particular we showed that in L-NEU3 cells NEU3 sialidase decreased the GM3 content and increase LacCer level of cell membrane. LacCer is a component of lipid rafts and has an important role in raft formation and stabilization, in particular as a result of the less elasticity and the high packing density of this glycosphingolipid. This action is also regulated by the different fatty acid chain lengh of LacCer [Xin-Min et al., 2002]. So this glycosphingolipid could affect the recruitment of adhesion proteins to rafts and the myoblast recognition/adhesion process before the formation of the fusion-competent sites of plasma membrane. Moreover it has been reported that LacCer may modulate raft-related signal transduction, attracting soluble proteins to lipid rafts [Xin-Min et al., 2002]. Therefore, modulating ganglioside plasma membrane content and composition, NEU3 can affect the characteristics and the dynamic clustering/dispersion of lipid rafts and can change myogenic cell ability to fuse.

In conclusion, this work further supports the central role of NEU3 as key modulator in skeletal muscle differentiation. Moreover, it seems clear that NEU3 needs to be strictly regulated during the differentiation process, in particular in the early stages of differentiation. Indeed NEU3 early up-regulation hampers the initiation of the differentiation process, while its increase during the following steps of the process favors and accelerates it. A key role in NEU3 mechanism of action is played by ganglioside GM3, which directly affects signaling pathways involved in muscle differentiation. In the last stage of differentiation, myocyte fusion, NEU3 overexpression can produce a change of myogenic cell fusion competence that finally promotes myotube hypertrophy, probably by NCAM desialylation and plasma membrane ganglioside content alteration.

Further works are needed to clarify the contribution of NEU3 in the regulation of sequential steps of the fusion process and the possibility of a direct interaction between NEU3 and the EGF receptor in the regulation of the differentiation process. Moreover, it

would be of particular interest to assess expression and activity of NEU3 in normal satellite cells to determine the involvement of this sialidase in quiescent state, during self-renewal, and in the transition from quiescence to the activation state and finally to the myogenic differentiation. Experiments along this line are currently in progress in our laboratories.

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