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NEU4L sialidase overexpression promotes β-catenin signaling in neuroblastoma cells, enhancing stem-like malignant cell growth

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Neuroblastoma (NB) is a frequently lethal tumor that occurs in childhood and originates from embryonic neural crest cells. The malignant and aggressive phenotype of NB is strictly related to the deregulation of pivotal pathways governing the proliferation/differentiation status of neural crest precursor cells, such as *MYCN*, Delta/Notch and Wnt/β-catenin (*CTNNB1*) signaling. In this article, we demonstrate that sialidase NEU4 long (NEU4L) influences the differentiation/proliferation behavior of NB SK-N-BE cells by determining hyperactivation of the Wnt/β-catenin signaling pathway. *NEU4L* overexpression in SK-N-BE cells induced significant increases in active, nonphosphorylated β-catenin content, β-catenin/TCF transcriptional activity and β-catenin gene target expression including *MYCN*, *MYC*, *CCND2* (cyclin D2) and *CDC25A*. In turn, these molecular features strongly modified the behavior of *NEU4L* SK-N-BE overexpressing cells, promoting the following: (1) an enhanced proliferation rate, mainly due to a faster transition from G1 to S phase in the cell cycle; (2) a more undifferentiated cell phenotype, which was similar to stem-like NB cells and possibly mediated by an increase of the expression of the pluripotency genes, *MYC*, *NANOG*, *OCT-4*, *CD133* and *NES* (nestin); (3) the failure of NB cell differentiation after serum withdrawal. The molecular link between NEU4L and Wnt/β-catenin signaling appeared to rely most likely on the capability of the enzyme to modify the sialylation level of cell glycoproteins. These findings could provide a new candidate for therapeutic treatment.

Introduction

Neuroblastoma (NB) is the most common childhood malignancy and arises in the peripheral sympathetic nervous system from neural crest-derived cells.[1] Half of all children with NB present features that characterize their tumors as high risk with poor survival, despite therapy.[2,3] The genomic aberrations, which usually characterize the disease, lead to blockade of the normal sympathoadrenal differentiation, giving rise to different clinical stages that reflect maturation arrest at different steps.[4] Thus, features such as enhanced proliferation, plasticity and high migratory capacity might mirror the failure of NB cells to progress beyond the stage of development at which these events are normal.[4] The emblematic deregulation of signal molecules such as MYCN, Trk, hASH1, Delta/Notch, Wnt/β-catenin appears to represent the aberrant appropriation of key pathways that normally support the expansion of neural crest precursor cells during the developmental program.[4–6]

In addition to these pivotal signaling cascades, the expression of characteristic sialoglycoconjugates (gangliosides and sialoglycoproteins) drastically changes during neural development and is crucial in directing cell fate toward tumorigenesis.[7] This is not surprising, as many sialoglycoconjugates control signal transduction and cell behavior.[8,9] In this regard, the polysialic acid-neural cell adhesion molecule (PSA-NCAM) and the ganglioside GD3 are well-known sialoglycoconjugates that have been identified in the neural crest and are involved in its evolution.[7,10] PSA-NCAM expression is elevated in advanced stages or fatal courses of NB.[11] In line

Key words: sialidase, neuroblastoma, β-catenin, cancer stem cells, sialoglycoproteins

Abbreviations: FBS: fetal bovine serum; MTC: mock transfected cells; 4-MU-NeuAc: 4-methylumbelliferyl-*N*-acetyl-D-neuraminic acid; NB: neuroblastoma; NEU4L: NEU4 long; NEU4S: NEU4 short; PSA-NCAM: polysialic acid-neural cell adhesion molecule; RER: rough endoplasmic reticulum; TCF: T-cell factor; SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis; PVDF: polyvinylidene difluoride; HPTLC: high performance thin layer chromatography

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with this, sialidases (EC 3.2.1.18), which catalytically remove sialic acid,[12] are involved in crucial cell events and neuronal differentiation through the modulation of membrane gangliosides and the subsequent stimulation of neuritogenesis and axonal growth.[13,14] In mammals, four sialidases (NEU1, NEU2, NEU3 and NEU4) are present. They are involved in several key physiological events,[12] and their deregulation is recognized to be related to cancer transformation.[15–20] In particular, the increase in *NEU3* expression inhibits NB cell growth in favor of differentiation.[21,22]

Among the four isoenzymes, *NEU4* is unique because it is expressed as two forms, NEU4 long (NEU4L) and NEU4 short (NEU4S), which differ in an N-terminal 12-amino acid sequence that strongly influences enzyme localization.[23,24] *NEU4* expression in adult tissues is very low and is restricted to brain, liver, kidney and colon.[25] The physiological role played by NEU4 remains fairly elusive even if recent data increasingly link this sialidase to the fate of neural cells. The expression of murine Neu4 is dynamically regulated during the development of the mouse brain and negatively regulates neuritogenesis during the differentiation of Neuro2a cells.[26] These data suggest that the enzyme could be involved in the specification of the fate of neural cells and that alterations of its expression could therefore be important for cancer transformation.

Starting from these premises, we overexpressed *NEU4L* in SK-N-BE cells and analyzed the consequent changes in their phenotype in order to identify a possible role of NEU4L in NB pathogenesis. Our results demonstrated that *NEU4L* overexpression caused the following: (1) increase of the proliferation rate; (2) hindering of the start of the neuronal differentiation program induced by serum deprivation; (3) activation of the Wnt/β-catenin pathway and subsequent stimulation of genes involved in the control of proliferation (*MYC*, *MYCN* and *CCND2*) and pluripotency/self-renewal (*NANOG*, *OCT-4*, *MYC*, *CD133* and *NES*); (4) the establishment of a new cell sialoglycoprotein pattern.

Material and Methods

Cell culture and stable overexpression of NEU4L in SK-N-BE cells

The human NB cell line SK-N-BE was purchased from ECACC (Sigma Aldrich, St. Louis, MO) and cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (FBS) and 2 mM glutamine (Sigma Aldrich). cDNA-encoding human *NEU4L* was subcloned into the pcDNA3.1myc-his mammalian expression vector (Invitrogen Life Technology, Carlsbad, CA) and transfected into 1×10^6 SK-N-BE cells using Lipofectamine 2000 reagent (Invitrogen Life Technology). Stable mock (transfected with the vector alone) and *NEU4L* transfectants were isolated after selection with 400 μg/ml geneticin (Invitrogen Life Technology).

To induce differentiation, 6×10^5 mock transfected cells (MTC) and *NEU4L* overexpressing SK-N-BE cells were cultivated in 1% FBS RPMI 1640 medium for up to 6 days. Neurite length was assessed using an inverted microscope (Olympus IX50) and the software Image ProPlus.

To stimulate the Wnt signaling pathway, 6×10^5 MTC cells were treated with 10 mM LiCl in 1% FBS RPMI 1640 for 3 days.

Cell sub-fractionation

NEU4L overexpressing cells (2×10^7) were harvested in PBS containing 1 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin (Sigma Aldrich) and lysed using a Dounce homogeniser. Nuclei and unbroken cells were separated by centrifugation at 800*g* for 10 min at 4°C. Mitochondria were isolated using the Mitochondria isolation kit for mammalian cells (Pierce Biotechnology, Rockford, IL) following the manufacturer's protocol. Lysosomes were isolated after the centrifugation of cell lysates devoid of nuclei at 12,000*g* for 10 min at 4°C and separated using a self-generated iodixanol (Optiprep) (Sigma-Aldrich) gradient (20% iodixanol starting concentration; 270,000*g*, 3 hr, 4°C). The rough endoplasmic reticulum (RER) was isolated using the endoplasmic reticulum isolation kit (Sigma-Aldrich) following the manufacturer's instructions. Plasma membranes were isolated after centrifugation of cell lysates devoid of organelles at 200,000*g* for 20 min at 4°C; the resulting supernatant will be referred to as "cytosol."

To analyze the subcellular localization of β-catenin, fractionated nuclear and cytosolic protein lysates from MTC and *NEU4L* overexpressing cells were isolated using the NE-PER Extraction Reagents kit (Pierce Biotechnology).[27]

Sialidase activity assay

The sialidase activity present in the particulate fraction of MTC and *NEU4L* overexpressing cells was assayed using 4-methylumbelliferyl-*N*-acetyl-D-neuraminic acid (4-MU-NeuAc), [^{3}H]GD1a,[20] and fetuin (Sigma Aldrich) at pH 3.2 or 7.0, as previously reported.[28] One unit of sialidase activity is defined as the amount of enzyme liberating 1 μmol of product per min.

Action of NEU4L on endogenous PSA-NCAM

Mixtures of MTC and *NEU4L* overexpressing cells (100 μg, as protein) were mixed with 0.1 ml of 0.5 M citric acid/sodium phosphate at pH 7.0, and water was added to a final volume of 100 μl. The resulting suspensions were incubated at 37°C for 10, 30 and 60 min. At the end of the indicated times, the samples were separated on an 8% SDS-PAGE gel and transferred onto a PVDF membrane; the sialylation degree of PSA-NCAM was determined using the antibody anti-PSA-NCAM (Millipore, Billerica, MA).

Detection of changes in mitochondrial potential

Changes in mitochondrial potential were assayed using 1×10^4 MTC and *NEU4L* overexpressing cells, using the Mitochondria staining kit (Sigma-Aldrich).

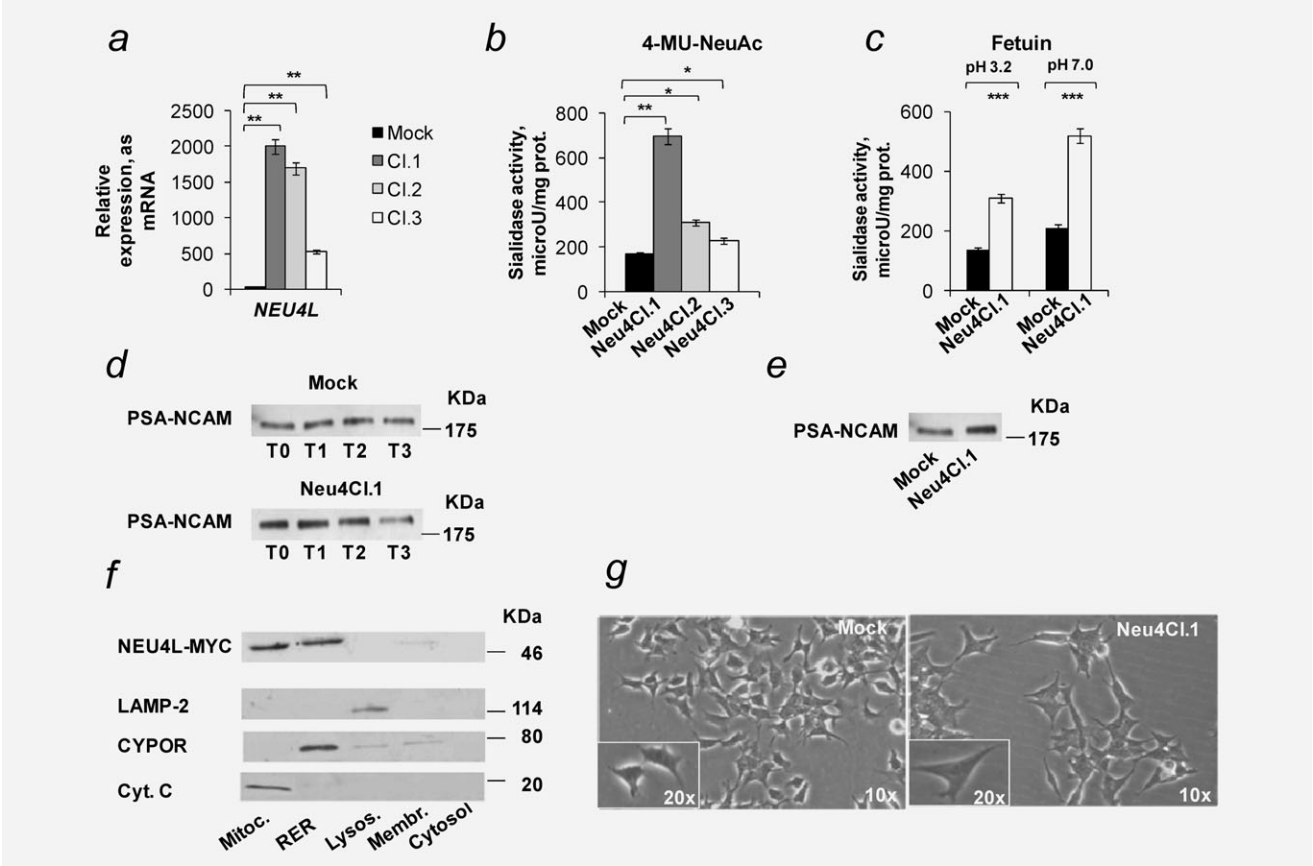

Figure 1. Overexpression of NEU4L in SK-N-BE cells. (*a*) *NEU4L* expression assessed using real-time PCR in three selected clones. Data are expressed as fold-change relative to MTC. (*b*) Sialidase activity of MTC and *NEU4L* overexpressing clones assayed with 4-MU-NeuAc at pH 3.2 and (*c*) fetuin at pH 3.2 and 7.0. Four replicate experiments were performed. (*d*) Sialidase activity of MTC and NEU4Cl.1 cells toward PSA-NCAM at pH 7.0 (TO, starting time; T1, 10 min; T2, 30 min; T3, 60 min of incubation). (*e*) PSA-NCAM content assayed by Western blot in MTC and Neu4Cl.1 cells. Three replicate experiments were performed. (*f*) Subcellular localization of NEU4L-MYC in Neu4Cl.1 cells determined after cell subfractionation and Western blot using an anti-MYC epitope antibody. Three replicate experiments were performed. (*g*) Phase contrast microphotographs of MTC and Neu4Cl.1 cells. Original magnification 10× (Olympus IX50). Significance is based on the Student's *t*-test: $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

Glycoprotein analysis

Membrane and cytosolic proteins (30 μg) of MTC and *NEU4L* overexpressing cells were separated on a 10% SDS-PAGE gel and transferred onto a PVDF membrane. Sialoglycoproteins were visualized using the DIG Glycan Differentiation kit (Roche Applied Science, Indianapolis, IN) as previously reported.[19] Densitometric analysis was performed using the Gelanalyzer software.

TOPflash assay

MTC and *NEU4L* overexpressing cells (6×10^4/well) were seeded into 24-well plates and transiently transfected with the TOPflash (Millipore) and pRL-TK (constitutively encoding *Renilla* luciferase) (Promega, Madison, WI) vectors using Lipofectamine LTX Reagent (Invitrogen Life Technologies). Twenty-four hours after transfection, the activities of firefly luciferase (TOPflash) and *Renilla* luciferase (pRL-TK, used as an internal control for transfection efficiency) were measured using the Dual Luciferase assay kit (Promega). Firefly luciferase activity was normalized against that of *Renilla* luciferase.

Statistical analyses

Values are presented as the mean ± SD. Statistical analyses were performed using the Student's *t*-test and compared MTC data with data from each clone.

Results

NEU4L overexpression in SK-N-BE cells

To stably overexpress *NEU4L*, SK-N-BE cells were transfected with the corresponding cDNA. In three selected clones, *NEU4L* showed a 500–2,000-fold increase in mRNA content compared to that observed in MTC (Fig. 1*a*), and the catalytic activity toward the artificial substrate 4-MU-NeuAc increased from 1.3- to 4.1-fold (Fig. 1*b*). Sialidase *NEU1* and *NEU3* expression levels were unchanged after *NEU4L* overexpression (Supporting Information Fig. 1*a*). After transfection,

the mRNA expression ratio of *NEU4L* relative to total *NEU4* (*NEU4L* mRNA/*NEU4* mRNA) was 0.98 ($p < 0.01$). *NEU4L* overexpression was particularly effective in clone 1 (2,000-fold increase in mRNA expression and 4.1-fold increase in catalytic activity). This clone, indicated as Neu4Cl.1, was used for most studies.

To confirm that transfected NEU4L was active against sialoglycoproteins, the sialidase activities of MTC and Neu4Cl.1 cells were assayed using fetuin (α2-3 linked sialoglycoprotein) as the substrate. The activity was greater in Neu4Cl.1 than MTC cells at pH 3.2 (2.2-fold) and pH 7.0 (2.4-fold) (Fig. 1*c*).

The increase of sialidase activity recorded after *NEU4L* overexpression was lower than the parallel increase of mRNA expression; this discrepancy could be related to the action of the enzyme not being fully efficient toward the substrates used in the assay and to reduced *in vitro* activity of NEU4, as previously reported.[26] Moreover, it should be noted that the *in vitro* sialidase assay is not specific for NEU4L but is also affected by the activities of NEU1 and NEU3, which are higher than that of NEU4L, at least in MTC (Supporting Information Fig. 1*b*). Therefore, in MTC, sialidase activity assayed *in vitro* is almost entirely due to NEU1 and NEU3 sialidases.

Additionally, NEU4L was active against α2-8 linked sialoglycoproteins; after the cell homogenates of MTC and Neu4Cl.1 cells were incubated at pH 7.0, we detected the progressive desialylation of endogenous PSA-NCAM only in Neu4Cl.1 cells (13% after 30 min and 38% after 60 min; $p < 0.01$) (Fig. 1*d*). However, even if PSA-NCAM constitutes an *in vitro* substrate of NEU4L, Neu4Cl.1 cells showed a higher content of PSA-NCAM (+25%; $p < 0.01$) (Fig. 1*e*). No activity was detected toward α2-6 sialoglycoproteins and toward gangliosides GD1a and GM3 (data not shown).

The expression of NEU4L as a fusion protein linked to the epitope MYC allowed us to track the localization of the enzyme; to this purpose, the Neu4Cl.1 cell homogenate was fractionated and assayed for NEU4L-MYC distribution. Over 50% of the enzyme was recovered in the mitochondrial fraction, enriched for cytochrome C; the remaining aliquot was detected in the RER fraction, along with a high recovery of NADPH-cytochrome P450 oxidoreductase (CYPOR), which was used as a marker. NEU4L-MYC was completely absent in the lysosomal, plasma membrane and cytosolic fractions (Fig. 1*f*). The localization of NEU4L in the mitochondria did not appear to alter the functionality of these organelles and did not induce changes in membrane potential (Supporting Information Figs. 2*a* and 2*b*). After *NEU4L* overexpression, SK-N-BE cells acquired a different morphology than that of the MTC, appearing considerably larger (2.5-fold; $p < 0.05$) and more adhesive to the plate (Fig. 1*g*).

NEU4L overexpression accelerates the proliferation rate

Growth curve assays showed that after 4 days of culture, there were significantly more viable cells among *NEU4L* overexpressing cells than in control cells, and in particular, Neu4Cl.1 cells grew 1.7-fold faster than MTC (Fig. 2*a*) (population doubling times: MTC, 28.8 hr; Neu4Cl.1cells, 15 hr; Neu4Cl.2 cells, 20.4 hr; Neu4Cl.3cells, 28 hr). Accordingly, [^{3}H]thymidine incorporation increased by 43% (Fig. 2*b*), and an MTT assay recorded that the proliferation was faster by 1.7-fold (Fig. 2*c*) in Neu4Cl.1 cells than in MTC. A similar trend was observed in Neu4Cl.2 cells; Neu4Cl.3 appeared to proliferate like MTC (Figs. 2*a* and 2*c*). The increment in the phosphorylated forms of RB and CDK2 recorded in Neu4Cl.1 cells (+67 and +33%, respectively; $p < 0.01$) (Fig. 2*d*) suggested that the increase in the proliferation rate might result from a faster transition of the cell cycle from the G1 to the S phase.

We further assessed the anchorage-independent growth potential of MTC and Neu4Cl.1 cells. Neu4Cl.1 cells were able to form more (+64%; $p < 0.05$) and larger colonies in soft agar than were MTC (colony forming efficiency: MTC, 15%; Neu4Cl.1 cells, 24.7%) (Fig. 2*e*).

NEU4L overexpression delays neuronal differentiation induced by serum reduction

As do many NB cells,[29] nontransfected SK-N-BE cells grown in 1% FBS differentiate toward the neuronal lineage. Accordingly, after just 24 hr in 1% FBS, MTC tended to establish a dense network of neurites and undergo morphological changes, such as the reduction of the soma; after 3 days, their morphology was dramatically transformed (Fig. 3*a*). In contrast, serum reduction did not suffice to trigger the same process in Neu4Cl.1 cells because the morphological changes, and above all, the neurite outgrowth occurred at lower levels than for the MTC (Fig. 3*a*). After 3 days of culture, the proportion of MTC that bore neurites at least twice the length of the soma diameter rose to 55%, compared with 12% for the Neu4Cl.1 cells (Supporting Information Fig. 3*a*). Accordingly, in MTC, neurite outgrowth was associated with an increased expression of protein markers for neuronal differentiation,[30,31] c-SRC and NCAM (+116 and +38%, respectively, after 3 days of culture in 1% FBS medium, in comparison to Neu4Cl.1 cells; $p < 0.01$) (Fig. 3*b*). The expression, as mRNA, of the neuron specific enolase (*NSE*)[32] increased only in MTC; this occurred immediately, during the first day of treatment (5-fold after 1 day, 6.5-fold after 2 days), and then stabilized after 3 days. In Neu4Cl.1 cells, *NSE* did not increase (Supporting Information Fig. 3*b*). This behavior was confirmed also by Neu4Cl.2 cells (data not shown).

Reducing the serum level to 1% halted the proliferation of Neu4Cl.1 cells (Fig. 3*c*), as revealed in an MTT assay performed after 3 days of culture; in contrast, MTC only slowed their proliferation rate (doubling time in 1% FBS: 68.6 hr). In Neu4Cl.1 cells, serum reduction quickly blocked the molecular machinery that drives cell cycle progression (Fig. 3*d*); although this event was detected also in MTC, it occurred later and in a more gradual manner. After 3 days, a 64% decrease (compared to the initial value; $p < 0.05$) of the phosphorylated form of CDK2 was detected in Neu4Cl.1

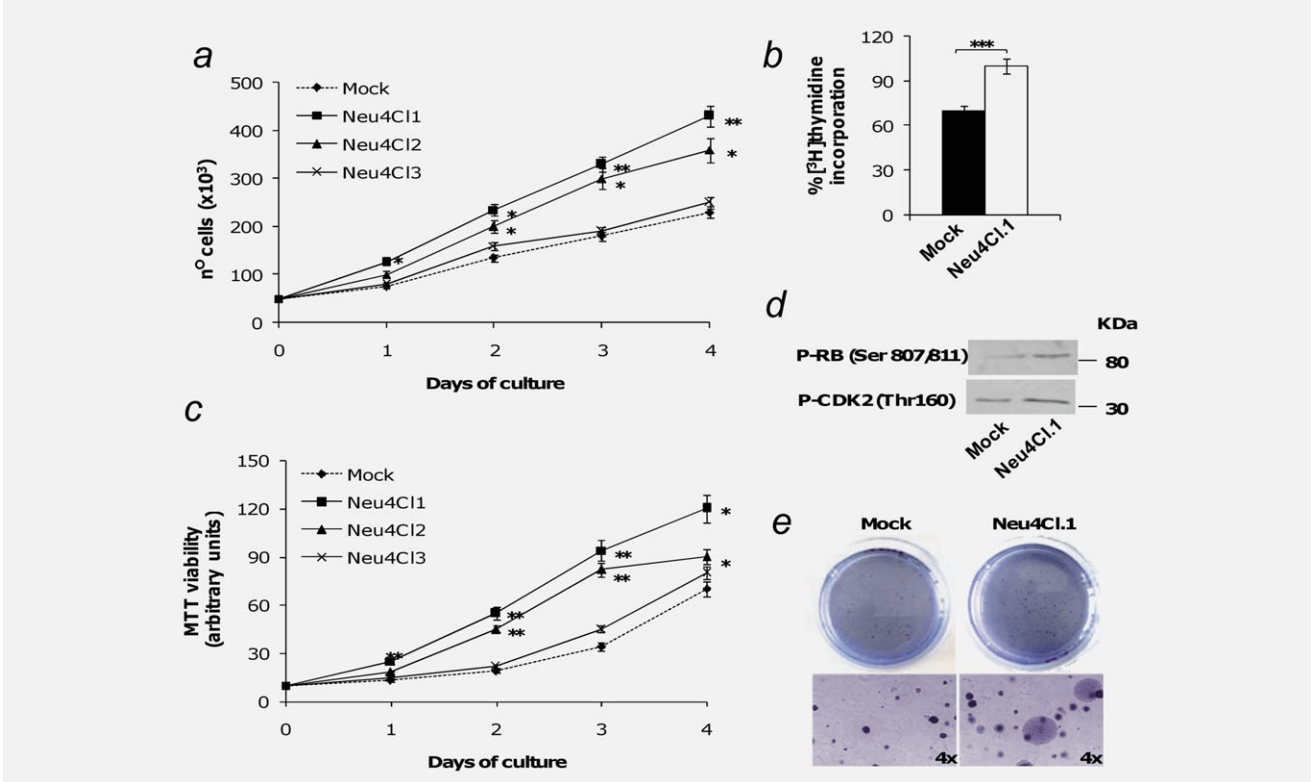

Figure 2. Effects of NEU4L overexpression on the proliferation rate of SK-N-BE cells. (*a*) Growth curves of MTC and *NEU4L* overexpressing clones. (*b*) Incorporation of [^{3}H]thymidine in MTC and Neu4Cl.1 cells. (*c*) MTT assay of MTC and *NEU4L* overexpressing clones. Three replicate experiments were performed. (*d*) Western blot analysis of total MTC and Neu4Cl.1 cell lysates probed with anti-P-RB (Ser807/811) and anti-P-CDK2 (Thr160) antibodies. Four replicate experiments were performed. (*e*) Images and microphotographs of colonies formed by MTC and Neu4Cl.1 cells in the soft-agar assay after 2 weeks of incubation at 37°C. Original magnification ×4 (Olympus IX50). Three replicate experiments were performed. Significance is based on the Student's *t*-test: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cells; this value compared with a 22% decrease (compared to the initial value; $p < 0.05$) recorded in MTC. Correspondingly, the phosphorylation status of RB also decreased at higher levels in Neu4Cl.1 cells (Fig. 3*d*).

Cell cycle analysis showed a marked accumulation in G1 phase (87.86% Neu4Cl.1 cells *vs.* 72.28% MTC) after 3 days of culture in 1% FBS medium (Fig. 3*e*). Already after 1 day, 63.41% of Neu4Cl.1 cells and 51.51% of MTC were in G1 phase, and after 2 days, 84.18% of Neu4Cl.1 and 63.22% of MTC were in G1 phase (Supporting Information Fig. 3*c*); these results further proved that in Neu4Cl.1 cells, proliferation was blocked soon after serum reduction. The significance of the differences between these values was assessed using Student's *t*-test ($p < 0.01$). Although Neu4Cl.1 cells were blocked in G1 phase and unable to differentiate (unlike MTC), they survived, and no sign of apoptosis such as chromatin condensation was detected (Supporting Information Fig. 3*d*). After 6 days of culture in 1% FBS, replacement of the medium with one containing 10% FBS drastically restimulated the proliferation machinery of Neu4Cl.1 cells, as if the cells were woken from a quiescent state. CDK2 was markedly activated and, correspondingly, RB was significantly phosphorylated (Fig. 3*d*), inducing cell cycle progression and a progressive transition into the S phase (Fig. 3*e*). In contrast, medium replenishment had lesser effects in MTC (Figs. 3*d* and 3*e*).

Activation of Wnt/β catenin signaling in NEU4L overexpressing cells

To investigate the molecular mechanism underlying the changes induced in the SK-N-BE cell control of proliferation/differentiation by *NEU4L* overexpression, we focused our attention on those pathways ruling these events in neural crest progenitors.[4–6,33] Our data demonstrated the upregulation of β-catenin signaling in all *NEU4L* overexpressing clones. In particular, Neu4Cl.1 cells expressed the nonphosphorylated, active form of β-catenin, at higher levels than did MTC (+100%; $p < 0.01$) (Fig. 4*a*), and a more elevated nuclear localization was observed (+121%; $p < 0.001$) (Fig. 4*b*). Cytosolic β-catenin increased slightly in Neu4Cl.1 cells compared with MTC (+12%; $p < 0.05$) (Fig. 4*b*). The β-catenin-TCF luciferase reporter vector, TOPflash, was used to assess whether β-catenin-TCF transcriptional activity was also

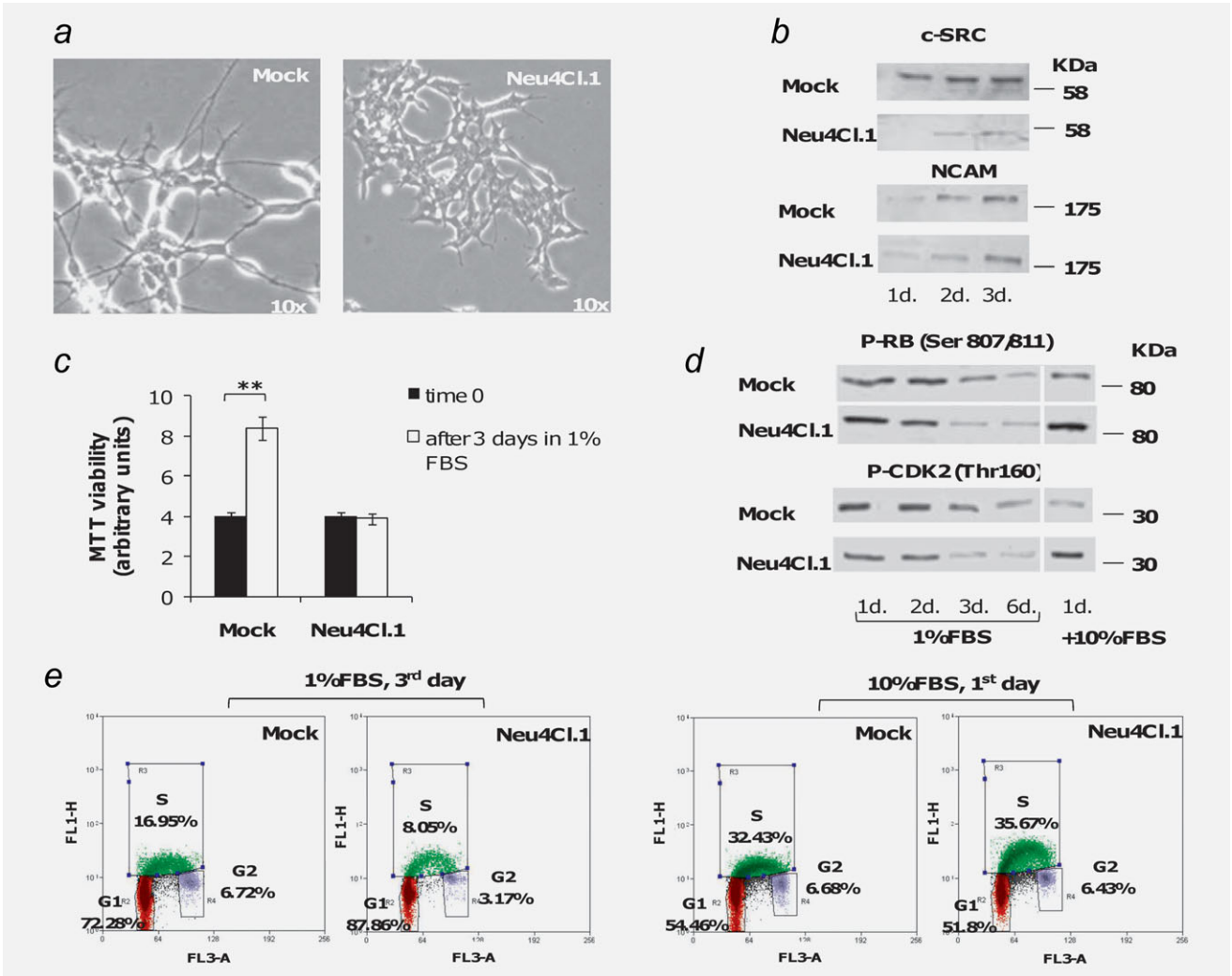

Figure 3. Effects of serum reduction treatment on MTC and Neu4Cl.1 cells. Cell differentiation assessment: (*a*) phase contrast micrographs of MTC and Neu4Cl.1 cells after 3 days of culture in 1% FBS medium. Original magnification ×10 (Olympus IX50). (*b*) Western blot analysis of c-SRC and NCAM (the antibody used is specific for the protein, not for the sialylation level) in total MTC and Neu4Cl.1 cell lysates during 3 days of culture in 1% FBS. Five replicate experiments were performed. Cell proliferation assessment: (*c*) proliferation of MTC and Neu4Cl.1 cells was assessed after 3 days of culture in 1% FBS medium using an MTT assay. Three replicate experiments were performed. (*d*) Western blot analysis of P-RB (Ser807/811) and P-CDK2 (Thr160) in MTC and Neu4Cl.1 cells cultivated in 1% FBS medium for 1, 2, 3 and 6 days and after replenishment with 10% FBS medium. Four replicate experiments were performed. (*e*) Cell cycle analysis performed on MTC and Neu4Cl.1 cells using propidium iodide and BrdU staining after 3 days of culture in 1% FBS medium and after replenishment with 10% FBS medium. Three replicate experiments were performed. Significance is based on the Student's *t*-test: $^{**}p < 0.01$.
[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

enhanced. The results demonstrated higher (+107%) β-catenin transactivation activity in Neu4Cl.1 cells than in MTC (Fig. 4*c*). Moreover, we ascertained marked upregulation, as mRNA, of some β-catenin target genes[34] linked to proliferation: *MYCN*, *MYC*, *AXIN2* and *CCND2* mRNAs increased by 2.3-fold (Neu4Cl.3 cells *vs.* MTC) to 4.2-fold (Neu4Cl.1 *vs.* MTC), 1.8-fold (Neu4Cl.3 cells *vs.* MTC) to 11-fold (Neu4Cl.1 *vs.* MTC), 1.8-fold (Neu4Cl.1 cells *vs.* MTC) to 2-fold (Neu4Cl.2 cells *vs.* MTC) and 1.8-fold (Neu4Cl.3 cells *vs.* MTC) to 5-fold (Neu4Cl.1 cells *vs.* MTC), respectively (Fig. 4*d*). *CDC25A* mRNA showed a small but significant increase only in Neu4Cl.1 (1.5-fold *vs.* MTC) (Fig. 4*d*). Recently, *CDC25A* has been recognized as a β-catenin target gene in sarcoma cells;[35] however, it has also been demonstrated that *CDC25A* and the level of Wnt activation did not uniformly correlate, possibly due to the complex nature of the transcriptional and translational regulation of this gene.[35]

Because the Wnt/β-catenin pathway is intimately connected to the circuitry of pluripotency and self-renewal in stem cells, we assessed the expression of typical genes also expressed by neural crest precursor cells. As shown in Figure 4*e*, Neu4Cl.1 and Neu4Cl.2 cells upregulated *CD133* (2.7-fold

and 1.4-fold, respectively, *vs.* MTC) and *NANOG* (3-fold and 2.1-fold, respectively, *vs.* MTC). Moreover, Neu4Cl.1 significantly upregulated *NES* (2.5-fold *vs.* MTC) and *OCT-4* (1.8-fold *vs.* MTC). The increased expression of these genes was not significant in NeuCl.3 cells.

LiCl treatment blocks the differentiation of SK-N-BE cells induced by serum reduction

To determine whether the upregulation of β-catenin signaling could cause the delay of neuronal differentiation triggered by the reduction in FBS levels to 1%, as observed in *NEU4L* overexpressing cells, MTC were treated with 10 mM LiCl, which activates β-catenin (a 71% increase of nonphosphorylated β-catenin was detected in LiCl-treated MTC compared to nontreated MTC; $p < 0.05$; Fig. 5*a*) through the inhibition of GSK3[36] (a 36% increase of phospho-GSKβ3 (Ser9) was detected in LiCl-treated MTC compared to nontreated MTC; $p < 0.05$; Fig. 5*a*). In the presence of LiCl, neurite outgrowth was inhibited (−71%; $p < 0.05$) in MTC cells grown for 3 days in 1% FBS medium (Figs. 5*b* and 5*c*). Accordingly, LiCl inhibited the increase of c-SRC (−44%; $p < 0.01$) and NCAM (−25%; $p < 0.05$) (Fig. 5*d*). Interestingly, thymidine incorporation was decreased by 25% in MTC treated with LiCl (Fig. 5*e*).

NEU4L overexpression promotes modifications of the sialoglycoprotein profile

To identify a molecular connection between *NEU4L* overexpression and β-catenin signaling activation, we examined the modifications that occurred in sialosphingolipids and sialoglycoproteins in *NEU4* overexpressing cells. The sialosphingolipid pattern was explored through metabolic labeling using [3-^{3}H]sphingosine as a precursor. We did not find any significant difference in the ganglioside or neutral sphingolipid pattern between Neu4Cl.1 and MTC (Figs. 6*a* and 6*b*). These results were further confirmed by the extraction and the analysis of endogenous gangliosides and neutral sphingolipids (data not shown), and similar results were recorded for the other two clones. Conversely, we detected marked desialylation of membrane α2-3-linked sialoglycoproteins of ∼90 kDa (−23%; $p < 0.01$) (Fig. 6*c*) and of cytosolic α2-3-linked sialoglycoproteins of 58 and 50 kDa (−37%; $p < 0.001$) (Fig. 6*d*) in Neu4Cl.1 cells, compared to MTC. Regarding sialoglycoproteins containing α2-6-linked sialic acid residues, no significant differences between Neu4Cl.1 and MTC were detected (Supporting Information Fig. 4). These results were also confirmed in Neu4Cl.2 cells and to a lesser extent in Neu4Cl.3 cells (data not shown).

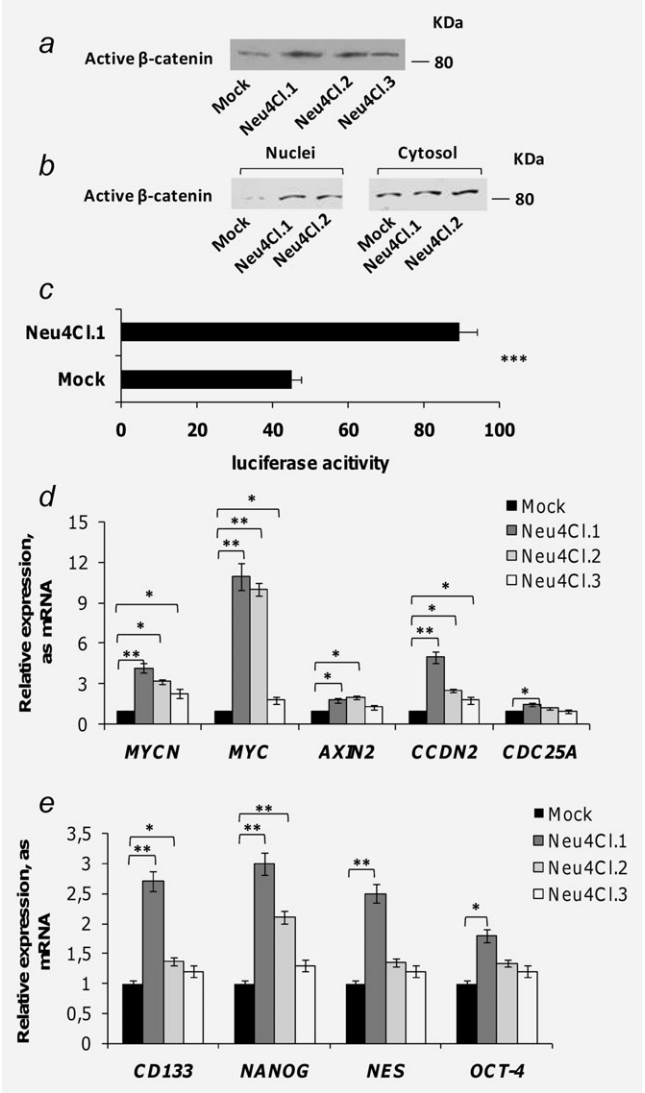

Figure 4. Activation of Wnt/β-catenin signaling in SK-N-BE cells after NEU4L overexpression. (*a*) Western blot analysis of active, nonphosphorylated β-catenin in MTC and *NEU4L* overexpressing clones. (*b*) Western blot analysis of active β-catenin content in the nuclear and cytosolic fractions of MTC, Neu4Cl.1 and Neu4Cl.2 cells. Three replicate experiments were performed. (*c*) TOP-flash assay of MTC and Neu4Cl.1 cells. Firefly luciferase activity was normalized against *Renilla* luciferase. Three replicate experiments were performed. Real-time PCR analysis of (*d*) *MYCN*, *MYC*, *AXIN2*, *CCND2* and *CDC25A* and (*e*) of *CD133*, *NANOG*, *NES* and *OCT-4* mRNA content in MTC and *NEU4L* overexpressing clones. Data are expressed as the fold-change relative to MTC. Four replicate experiments were performed. Significance is based on the Student's *t*-test: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Discussion

NB pathogenesis is connected with an aberrant embryonal development of primitive neural crest cells that normally give rise to the sympathetic nervous system.[4,6,37] NB profits from many genes that control neural crest growth and differentiation, misappropriating their expression to support tumor growth and/or a status of dedifferentiation.[4] Among the signaling pathways governing neural crest cell fate and known to be important to NB onset, the Wnt/β catenin system has recently attracted increasing interest.[27,34,38,39] β-catenin plays a key role in premigratory neural crest development by controlling the maintenance and expansion of neural crest stem cells.[39] Transcriptional regulators such as *MYC* and *MYCN*,

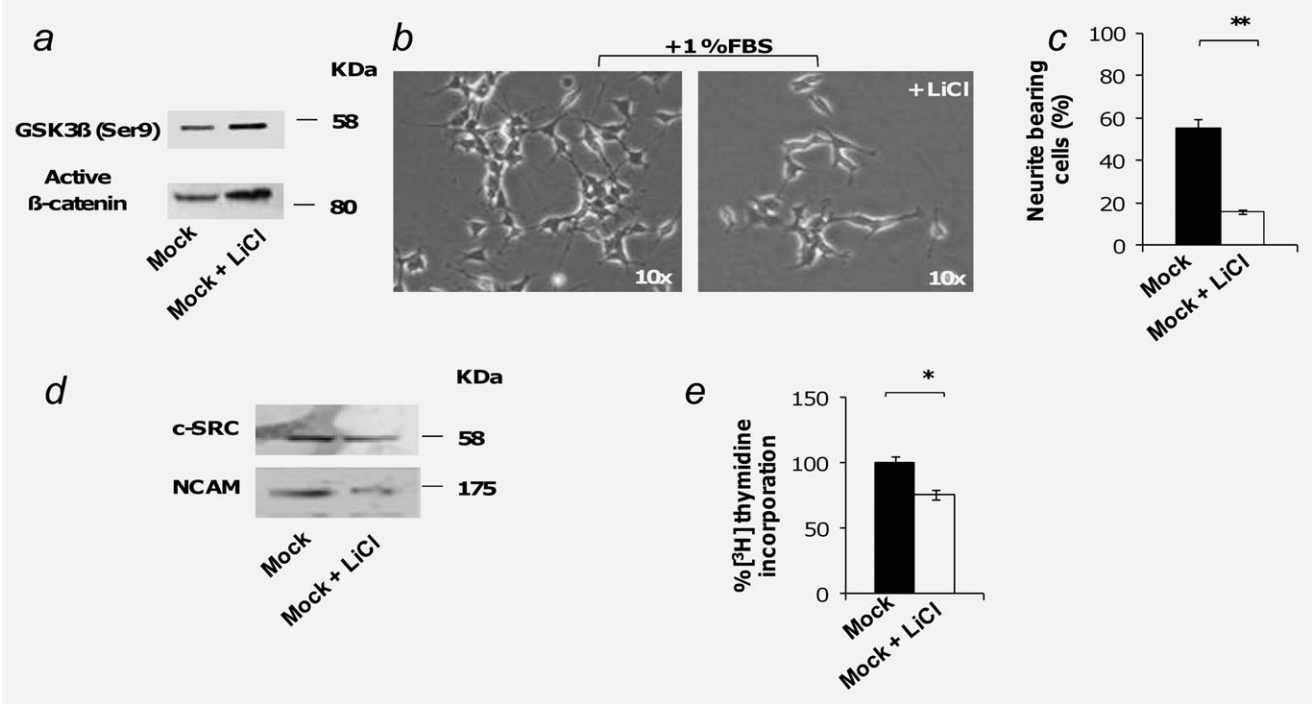

Figure 5. Effects of LiCl treatment on SK-N-BE cell differentiation and proliferation under serum starvation. (*a*) Western blot analysis of active, nonphosphorylated β-catenin and phospho-GSK3β (Ser9) in untreated and LiCl-treated MTC. Four replicate experiments were performed. (*b*) Phase contrast micrographs of untreated and LiCl-treated MTC. Original magnification 10× (Olympus IX50). (*c*) Quantification of untreated and LiCl-treated MTC bearing neurites at least twice as long as the size of the soma after 3 days of culture in 1% FBS medium. (*d*) Western blot analysis of c-SRC and NCAM in untreated and LiCl-treated MTC after 3 days of culture in 1% FBS medium. Four replicate experiments were performed. (*e*) Proliferation of untreated and LiCl-treated MTC after 3 days of culture in 1% FBS medium assessed using [^{3}H] thymidine incorporation. Four replicate experiments were performed. Significance is based on the Student's *t*-test: $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

which regulate cell cycle, differentiation and the apoptosis of neuroblasts, are directly induced by β-catenin. Novel data proved that deregulation of the Wnt signaling pathway is critical to NB development,[27] but the specific mechanisms that activate this pathway in NB remain elusive.[39] In this report, we demonstrated that the increase of active β-catenin is a key effect subsequent to *NEU4L* sialidase overexpression in SK-N-BE cells. This event enhanced β-catenin/TCF transcriptional activity that in turn, increased the expression of β-catenin gene targets, such as *MYCN*, *MYC*, *CCND2*, *CDC25A*, *NANOG* and *OCT-4*. The stimulation of this peculiar signaling axis could be related to the appearance of at least two effects. First, *NEU4L* overexpressing cells exhibited enhanced proliferation rates, mainly due to a faster transition from the G1 to the S phase, as a classical effect of Wnt/β-catenin activation and increased *MYCN* and *MYC* expression.[34] Second, the cell differentiation status was affected; morphological changes consisting of a larger and more adhesive appearance, which were recorded in *NEU4L* overexpressing cells, evoked features observed in stem-like NB cells.[40] Moreover, in contrast to MTC, which differentiated along a neuronal lineage within 3 days when subjected to a mild stimulus such as decreasing the level of FBS to 1%, *NEU4L* overexpressing cells failed to undergo differentiation in the same conditions, at least for the same time interval as that needed for MTC to differentiate. To confirm that these last events could be associated with Wnt/β-catenin activation, we treated MTC with 10 mM LiCl.[36] LiCl-treated MTC were unable to trigger neuronal differentiation in terms of neurite outgrowth and the appearance of markers (c-SRC and NCAM) under serum deprivation. Moreover, the proliferation of LiCl-treated MTC was blocked more significantly than nontreated MTC, in a similar way to *NEU4L* overexpressing cells.

These findings, when considered together with the increase in the expression of *CD133* and nestin, which are reported to be markers of NB stem cells,[40,41] suggest that *NEU4L* overexpression allowed NEU4Cl.1 cells to revert to an undifferentiated phenotype through the reacquisition of stemness properties. The increased content of PSA-NCAM observed in Neu4Cl.1 cells compared with MTC could be related to these events; in fact, PSA-NCAM levels are related to neuronal plasticity and advanced NB.[42,43] This last result appears to conflict with the evidence that PSA-NCAM is recognized by NEU4L *in vitro*; however, because PSA-NCAM is exposed on the external cell surface,[44] it is very improbable that the enzyme could interact and act on this protein *in*

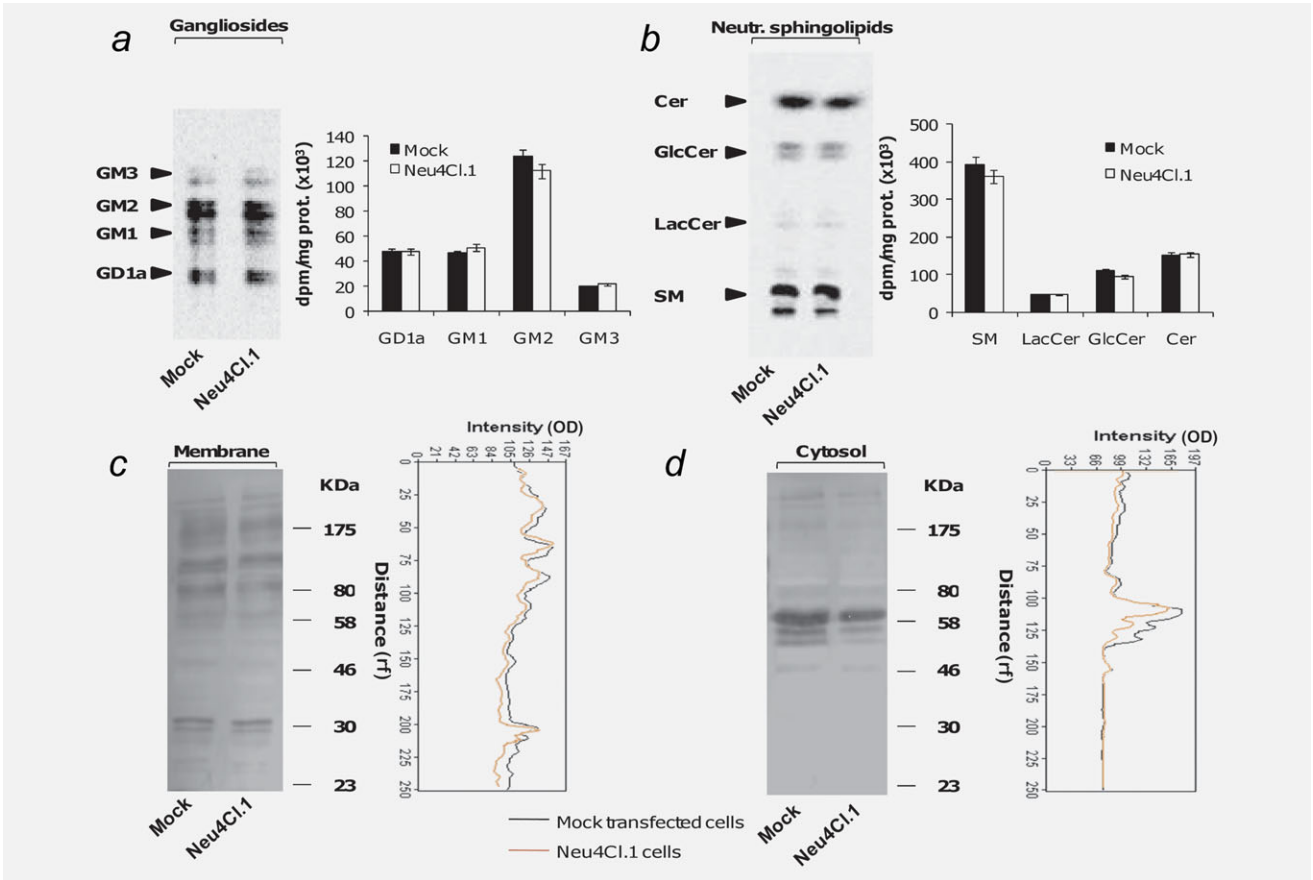

Figure 6. Sphingolipid and sialoglycoprotein profile of MTC and Neu4Cl.1 cells. HPTLC of (*a*) gangliosides and (*b*) neutral sphingolipids extracted from MTC and Neu4Cl.1 cells after metabolic labeling with [^{3}H] sphingosine. Doublets are due to heterogeneity of the ceramide moiety. Solvent system used for ganglioside HPTLC: chloroform/methanol/0.2% aqueous $CaCl_2$ 60:40:9 (v/v); solvent system used for neutral sphingolipid HPTLC: chloroform/methanol/H_2O 55:20:3 (v/v). Image acquired by radiochromatoscanning (Beta Imager 2000). The data, expressed as dpm/mg protein, represent the mean ± SD of four experiments. α2-3 Sialoglycoprotein profile assessed using Western blot and *Maackia amurensis* agglutinin lectin staining of (*c*) membranes and (*d*) cytosolic proteins of MTC and Neu4Cl.1 cells. An equal content of protein was loaded in each lane. Three replicate experiments were performed. Significance is based on the Student's *t*-test: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

vivo. Therefore, the sialylation level of NCAM in Neu4Cl.1 cells did not appear to be directly under the control of NEU4L activity. As a possible outcome of the enhanced proliferation and reacquisition of an undifferentiated phenotype, *NEU4L* overexpressing cells appeared to be endowed with greater malignant potential, expressed as a higher colony-forming capacity in soft agar.

The dependency of the observed effects on the level of *NEU4L* overexpression was demonstrated by using several clones; an increase of at least 1.8-fold in catalytic activity was required to achieve the modified phenotype. In addition, it must be excluded that the transfection procedure selected for different pre-existent clones in MTC and *NEU4L* overexpressing cells because MTC showed degrees of Wnt/β-catenin signaling activation and target gene expression that were similar to those observed in nontransfected SK-N-BE cells. Neu4Cl.3 cells exhibiting a low level of *NEU4L* overexpression demonstrated morphology and behavior very similar to MTC, further demonstrating the necessity of *NEU4L* overexpression for the events observed to occur. Moreover, the expression ratio of *NEU4L* relative to total *NEU4* was nearly 1 in all of the clones, excluding any possible involvement of NEU4S in the phenomena observed.

It is likely that NEU4L can interact with the Wnt/β-catenin signaling system because of its capacity to desialylate sialoglycoproteins. NEU4L efficiently recognized sialoglycoproteins *in vitro* and at neutral pH. Moreover, *in vivo*, NEU4L overexpression markedly modified the sialylation level of some cytosolic and membrane sialoglycoproteins. Interestingly, the majority of the components of the Wnt/β-catenin axis are glycoproteins;[34] thus, we hypothesize that the sialylation level of one or more of these factors could act as an "on/off switch" cue depending on NEU4L activity. In particular, the structural characterization of human Dickkopf-1 (DKK1), which is a crucial inhibitor of the Wnt signaling pathway, demonstrated that it carries long sialylated antennae that might be correlated with its functionality.[45] DKK1 was previously found to inhibit NB cell proliferation.[46] The

hypotheses that DKK1 might be a direct substrate of NEU4L and that NEU4L might modulate its functionality are currently under investigation. The RER is currently considered a crucial point of Wnt trafficking;[34] the identification of NEU4L in the RER suggests that the enzyme might interact with the members of this pathway mainly in this location. Recently, a splice variant of *WNT13* (*WNT13B*) was identified in mitochondria,[47] but it is not expressed in NB cells; therefore, an action of the mitochondrial fraction of NEU4L toward proteins belonging to this pathway directly in mitochondria is not likely.

In contrast to previous published data,[48] NEU4L expressed by SK-N-BE cells was not able to recognize ganglioside substrates *in vitro* and *in vivo*.

In summary, we defined sialidase NEU4L as potentially contributing to highly aggressive NB through the promotion of the Wnt/β-catenin signaling pathway and a multiplicity of subsequent events that support the growth of undifferentiated, highly proliferative cells. The identification of the decisive role of NEU4L expression in NB genesis and outcome could indicate a possible novel and interesting approach for therapy.

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