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Sialidase NEU4 is involved in glioblastoma stem cell survival

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The human sialidase, NEU4, has emerged as a possible regulator of neuronal differentiation and its overexpression has been demonstrated to promote the acquisition of a stem cell-like phenotype in neuroblastoma cells. In this paper, we demonstrated that glioblastoma stem cells (GSCs) isolated from glioblastoma multiforme (GBM) cell lines and patients' specimens as neurospheres are specifically marked by the upregulation of *NEU4*; in contrast, the expression of *NEU4* is very low in non-neurosphere-differentiated GBM cells. We showed that *NEU4* silencing by miRNA or a chemical inhibitor of its catalytic activity triggered key events in GSCs, including (a) the activation of the glycogen synthase kinase 3β, with the consequent inhibition of Sonic Hedgehog and Wnt/β-catenin signalling pathways; (b) the decrease of the stem cell-like gene expression and marker signatures, evidenced by the reduction of *NANOG*, *OCT-4*, *SOX-2*, *CD133* expression, ganglioside GD3 synthesis, and an altered protein glycosylation profile; and (c) a significant decrease in GSCs survival. Consistent with this finding, increased NEU4 activity and expression induced in the more differentiated GBM cells by the NEU4 agonist thymoquinone increased the expression of *OCT-4* and *GLI-1*. Thus, *NEU4* expression and activity appeared to help to determine the molecular signature of GSCs and to be closely connected with their survival properties. Given the pivotal role played by GSCs in GBM lethality, our results strongly suggest that NEU4 inhibition could significantly improve current therapies against this tumour.
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Glioblastoma multiforme (GBM) is the most lethal and least successfully treated brain tumour,[1] with a median survival of 15 months.[2] Many studies have revealed that GBM includes a heterogeneous mixture of both cancer stem cells that possess the property of self-renewal, as well as more differentiated cancer cells.[3,4] GBM stem cells (glioblastoma stem cells (GSCs)) are believed to be responsible for GBM development, progression, recurrence, and therapeutic resistance.[5,6] GSCs share the expression of many markers, such as CD133 and nestin (*NES*),[7] and core signalling pathways, such as Sonic Hedgehog (SHH)-GLI, Notch-1,[8] and Wnt/β-catenin,[9] with normal neural stem cells. These signalling pathways are known to sustain the long-term self-renewal and propagation of GSCs.[8,9] To identify GSCs features, recent papers have focused on the glycosylation status of some markers including CD133.[10,11] Furthermore, several glycoprotein markers involved in the differentiation status of GSCs were identified through a multi-lectin affinity chromatography and quantitative glycoproteomics approach.[12] Regarding glycolipids, b-series gangliosides such as GD2 (Klassen *et al.*[13]) and GD3 (Yanagisawa *et al.*[14] and Nakatani *et al.*[15]) have been previously demonstrated to be highly expressed in normal neural stem cells. In mouse neural stem cells, GD3 interacts with epidermal growth factor receptor (EGFR) stimulating its downstream signalling to support cell self-renewal capability.[16] During mammalian brain development, the levels of simple gangliosides, that is, GD3 and GM3, decrease, while the synthesis of a-series gangliosides (GM1 and GD1a) increases.[17] As the ganglioside pattern of neural stem cells seems to reflect their undifferentiated status and their self-renewal ability, it could be hypothesized that GSCs could also display an altered synthesis of gangliosides. The stage-specific embryonic antigenic-1 (CD15), a glycosylated epitope carried by glycoproteins and by glycolipids was associated with GSCs.[18] Sialidases are enzymes critically involved in the control of protein and lipid glycosylation and altered in cancer.[19–23] Sialidases (EC 3.2.1.18) catalytically remove sialic acid[24] and, among many crucial cell events, in the nervous system they are involved in neuronal differentiation, neuritogenesis, and axonal growth.[25–27] Among the four sialidases described so far, that is, NEU1, NEU2, NEU3, and NEU4, the latter appeared to be intriguingly related to the fate of neural cells. The level of *NEU4* gene expression decreases during the development of the mouse brain and during retinoic acid-induced neuronal differentiation.[28] Moreover, we previously reported that the *NEU4* overexpression enhances an undifferentiated stem cell-like phenotype and cell proliferation in human neuroblastoma cells[22] and, recently, it has been demonstrated that mouse neural stem cells highly express *NEU4*.[29]

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Abbreviations: GBM, glioblastoma multiforme; GSC, glioblastoma stem cell; SHH, Sonic Hedgehog; EGFR, epidermal growth factor receptor; PTCH1, protein patched homolog 1; TQ, thymoquinone; DANA, 2,3-deydro-2-deoxy-N-acetylneuraminic acid; GSK-3β, glycogen synthase kinase 3β

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Despite these findings, the role of sialidases in GBM pathogenesis remains to be firmly established. We first analysed sialidase expression in GSCs and we identified a significant increase of *NEU4* expression in comparison with more differentiated GBM cells. Then, through *NEU4* silencing and its chemical inhibition in GSCs, we demonstrated that (a) *NEU4* is connected to the inhibition of glycogen synthase kinase-3β (GSK-3β) and, therefore, to the activation of the downstream pathways SHH and Wnt/β-catenin; (b) *NEU4* expression is associated with the expression of the transcriptional factors, *GLI-1*, *NANOG*, *OCT-4*, and *SOX-2*; and (c) the silencing or chemical inhibition of NEU4 changes the entire glycosylation pattern of proteins and lipids, making it more similar to that of differentiated GBM cells and drastically reduces GSCs survival.

Results

Sialidase *NEU4* expression is higher in GSCs isolated from GBM cell lines than in non-neurospheres differentiated GBM cells. Human GBM cell lines U87MG, U138MG, and T98G were cultivated in selective medium, for 4 weeks, to promote the enrichment of GSCs, as previously reported.[30] As shown in Figure 1a, after an initial steady phase of about 4 days, some U87MG and U138MG cells began to grow in suspension forming typical aggregates, referred to as neurospheres (U87MG duplication rate: 7 days; U138MG duplication rate: 14 days) (Figure 1b). We demonstrated that neurospheres formed by both U87MG and U138MG cells were highly enriched by GSCs through the evaluation of the expression of the stem cell markers *CD133* (5.6-fold increase in U87MG cells and 4-fold increase in U138MG cells, after 4 weeks) and nestin (9.5-fold increase in U87MG cells and 1.8-fold increase in U138MG cells, after 4 weeks) (Figures 1c and d). After 4 weeks, the expression of these two markers did not further increase. Moreover, the GSC phenotype was further confirmed by functional assays of Hoechst 33342 dye exclusion and self-renewal (serial neurosphere formation) (data not shown). Instead, T98G cells were not able to survive in the selective medium, and after 14 days all cells died (Figure 1a). Accordingly, CD133 and nestin expression did not significantly increase (Figure 1e), indicating the absence of GSCs among T98G cells able to survive in these culture conditions. In parallel, we also determine the expression of sialidases *NEU1*, *NEU3*, and *NEU4* in all three GBM cell lines and in neurospheres isolated from them. The cytosolic sialidase *NEU2* was not expressed. *NEU1* and *NEU3* expression did not significantly change between U87MG-adherent cells and GSC-enriched neurospheres, or between U138MG-adherent cells and GSC-enriched neurospheres. Instead, *NEU4* expression that was scarcely detectable in U87MG and in U138MG cells, increased by 14-fold comparing U87MG- and U138MG-adherent cells with the corresponding GSCs, after 4 weeks in selective medium (Figures 1f and g). We did not record the increment of *NEU4* expression in T98G cells cultivated in the same selective medium (Figure 1h).

***NEU4* silencing impairs U87MG-GSCs survival.** To determine the role of sialidase NEU4 and the significance of its increase in GSCs, after 4 weeks of culture in selective medium, GSCs isolated from U87MG cells (referred to as U-GSCs) were transfected with pcDNA 6.2-GW/EmGFP-miR carrying a miRNA specifically designed towards *NEU4*. The silencing effect was verified by real-time PCR (-60% as *NEU4* mRNA expression) (Figure 2a). *NEU4* silencing strongly impaired U-GSC survival: in fact, 6 days after the end of selection, mock U-GSCs began to grow ($+25\%$); instead, 70% of *NEU4* silencing U-GSCs (referred to as iNEU4 U-GSCs) died and, after 12 days, almost the 87% of cells died (Figure 2b). The clonogenic potential was determined performing limiting dilution assays: neurospheres were readily formed after 5 days by mock U-GSCs but not by iNEU4 U-GSCs (Figure 2c). Hoechst 33342 staining of mock U-GSCs and iNEU4 U-GSCs revealed the condensation of chromatin in iNEU4 U-GSCs, indicating an apoptotic phenotype (Figure 2d). The configuration of key proteins involved in cell cycle control revealed that iNEU4 U-GSCs were mainly blocked in the G2 phase: in fact, phospho-RB (Ser807/811) was not detected in contrast to mock U-GSCs, cyclin B1 was markedly accumulated, and inactive phospho-CDC2 (Tyr15) decreased (63%) ($P<0.01$) (Figure 2e). Moreover, we detected a marked downregulation of activated AKT (45%; $P<0.01$) and the appearance of the active fragment derived from the cleavage of caspase 3 in iNEU4 U-GSCs (Figure 2e), confirming the inhibition of pro-survival pathways and, instead, the parallel activation of signalling linked to cell death.

***NEU4* silencing reduces the activation of signalling pathways related to stemness in U87MG-GSCs.** Through the investigation of the molecular pathways altered by *NEU4* silencing in GSCs and mainly related to their survival and maintenance, we identified a 74% decrease of phospho-glycogen synthase kinase-3β (GSK-3β) (Ser9) (inhibitory phosphorylation) in iNEU4 U-GSCs (Figure 3a) ($P<0.001$). This event was clearly related to many important subsequent effects: first, we detected a decrease in the mRNA expression of *SHH* (82%), protein patched homolog 1 (*PTCH1*) (70%), and *GLI-1* (41.7%), clearly related to the activation of SHH pathway, and β-catenin (55.6%) and *axin 2* (82%), clearly related to the activation of Wnt/β-catenin pathway, in iNEU4 U-GSCs (Figure 3b). Activated GSK-3β is a well-known inhibitor of both SHH[31,32] and Wnt/β-catenin[33] signalling pathways. As SHH and Wnt/β-catenin pathways have a central role in GSCs behaviour by promoting an embryonic stem cell-like gene expression signature,[34,35] we investigated the expression of the known stem cell markers *NANOG*, *OCT-4*, and *SOX-2*. As shown in Figure 3c, in iNEU4 U-GSCs, *NANOG* expression decreased by 17%, *OCT-4* by 71.5%, and *SOX-2* by 42%, confirming the partial inhibition of the signalling pathways that fuel stem cell growth. Accordingly, the expression of the stemness marker *CD133* decreased in iNEU4 U-GSCs (30%) (Figure 3c). Interestingly, also the content of the EGFR, which is usually constitutively activated in GSCs, decreased in iNEU4 U-GSCs (-74%; $P<0.001$) (Figure 3d).

***NEU4* silencing alters the sialo-glycoconjugate profile of U87MG-GSCs.** As gangliosides and sialo-glycoproteins could be potential substrates of NEU4, we investigated sphingolipid pattern of GSCs after *NEU4* silencing. It was

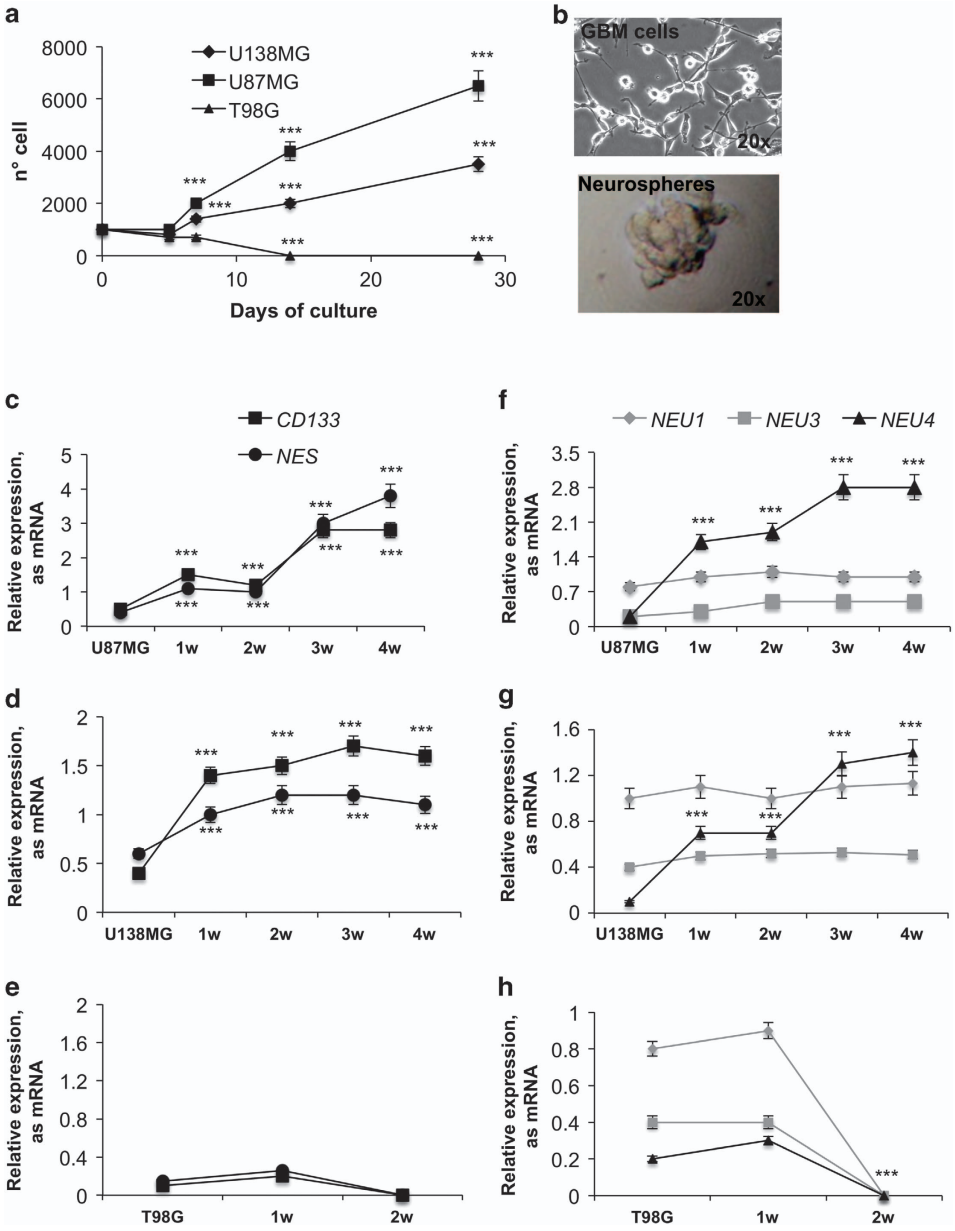

Figure 1 *NEU4* expression in GBM cell lines and GSCs. (**a**) Growth curves of U87MG, U138MG, and T98G GBM cells cultivated in DMEM F12 medium plus supplements for up to 4 weeks. Values are the mean ± S.D. of four independent experiments. Significance is based on the Student's *t*-test: ***$P<0.001$ (values compared with that on Day 0). (**b**) Microphotographs of representative GBM cells and neurospheres formed by U87MG cells after 4 weeks of culture in the selective medium. Original magnification × 20 (Olympus Ix50). (**c**) Real-time PCR analysis of CD133 and nestin expression in U87MG cells; (**d**) U138MG cells; (**e**) T98G cells during for weeks of culture in DMEM F12 medium plus supplements. (**f**) Real-time PCR analysis of *NEU1*, *NEU3*, and *NEU4* sialidases expression in U87MG cells; (**g**) U138MG cells; (**h**) T98G cells during 4 weeks of culture in DMEM F12 medium plus supplements. Values are the mean ± S.D. of four independent experiments. Significance is based on the Student's *t*-test: ***$P<0.001$ (values compared with starting cell lines)

evaluated by metabolic labelling with [3-^{3}H]sphingosine. After a 2-h pulse followed by a 24-h chase, a metabolic steady state was obtained. The ganglioside profile of iNEU4 U-GSCs was clearly different from that of mock U-GSCs (Figures 4a and b). The content of all ganglioside, except GM3 and GM2, decreased in iNEU4 U-GSCs; in particular, GD1a, GD3, and GM1 underwent a 29, 74, and 55% decrease, respectively (Figure 4b). It is worth noting that these modifications were associated with alterations concerning the expression of GD3 synthase (−59%) (Figure 4c), which is regulated at a transcriptional level by *Sp1* factors (−52.3% in iNEU4 U-GSCs) (Figure 4d). Significantly, after *NEU4* silencing, GD3 synthase expression was reduced to the level shown by more differentiated U87MG cells (Figure 4d).

Among neutral sphingolipids, in iNEU4 U-GSCs, we detected the decrease of lactosylceramide (LacCer) (−30%), which is considered a pro-survival sphingolipid, and the parallel increase of ceramide (Cer) (+50%), which is known as a pro-apoptotic sphingolipid;[36] glycosylceramide (GlcCer) did not change (Figures 4e and f).

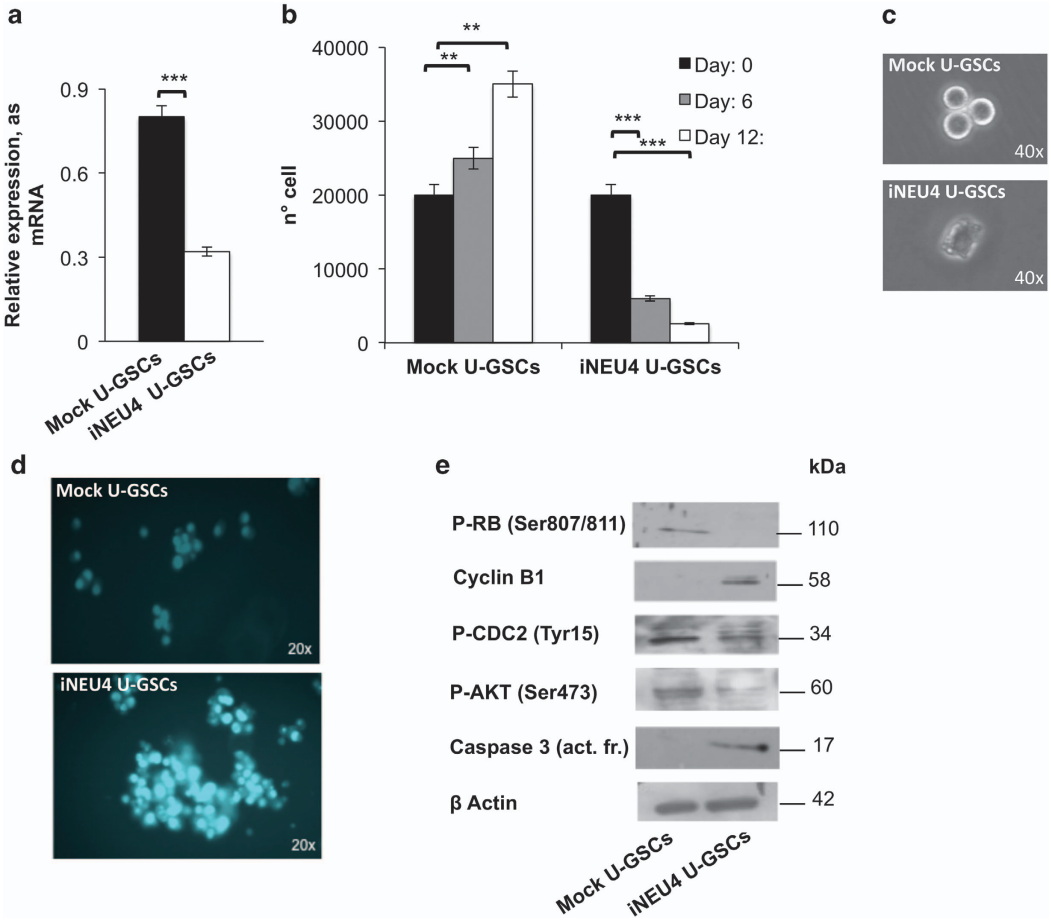

Figure 2 Effects of *NEU4* silencing on U-GSCs survival. (**a**) Real-time PCR analysis of *NEU4* sialidase expression in mock and iNEU4 U-GSCs. (**b**) Cell viability assessed through Trypan Blue after *NEU4* silencing. (**c**) Neurosphere assay assessed after 6 days after *NEU4* silencing. Original magnification $\times$ 20 (Olympus Ix50). (**d**) Hoechst 33342 staining in mock and iNEU4 U-GSCs. Pseudo-colouring approach used for this image. (**e**) Western blot analysis of P-RB (Ser 807/811), cyclin B1, P-CDC2 (Tyr15), P-AKT (Ser473), and active caspase 3 in mock and iNEU4 U-GSCs. β-Actin was used as loading control. Values are the mean $\pm$ S.D. of four independent experiments. Significance is based on the Student's *t*-test: $^{**}P<0.01$, $^{***}P<0.001$

We also investigated the α2-3 sialoglycoprotein profile of mock U-GSCs and iNEU4 U-GSCs (Figure 5a). Overall, the level of sialylation displayed by iNEU4 U-GSCs appeared to be reduced in comparison with mock U-GSCs with the exception of some glycoproteins of $\sim$50–60 kDa, which showed an increased content of sialic acid (Figure 5a). As for sphingolipids, it should be underlined that other enzymes involved in the sialylation of glycoproteins changed their expression following *NEU4* silencing. Interestingly, α2-3 silayltransferase (*ST3GalIII*), which encodes a α2-3 sialyltransferase, was significantly increased as mRNA expression in U-GSCs than in U87MG cells ($+$70%), but decreased in iNEU4 U-GSCs ($-$70% in comparison with mock U-GSCs) reverting to the levels found in more differentiated U87MG cells (Figure 5b).

Chemical inhibition or activation of NEU4 changes the expression of stem cell-like genes. To further corroborate the effects recorded after *NEU4* silencing in U-GSCs, we tested the effects of a selective inhibitor of NEU4 previously described[37] and referred to as C.6 below. We first checked the inhibition level caused by C.6 in assays of U-GSC sialidase activity. The assay was not selective and therefore included contributions of NEU1, NEU3, and NEU4 activities (NEU2 expression was not detected). As shown in Figures 6a, C.6 induced only a 16% inhibition of the total sialidase activity, likely to be due to its selectivity for NEU4.[37] Under similar conditions, the nonspecific inhibitor, DANA (2,3-deydro-2-deoxy-N-acetylneuraminic acid), inhibited the 37% of total sialidase activity. We next cultivated U-GSCs in the presence of C.6 for 4 days and recorded the effects induced on the expression of stem-cell marker genes; C.6 treatment of U-GSCs decreased the expression of β-catenin ($-$28%), *GLI-1* ($-$68%), *NANOG* ($-$75%), and *OCT-4* ($-$84%) (Figure 6b), and the amount of phospho-GSK-3β (Ser9; 66.4%; $P<0.01$) (Figure 6c), reproducing the same effects seen for *NEU4* silencing (Figures 3a–c). Moreover, the proliferation and survival capabilities of U-GSCs were impaired by C.6 treatment, similar to our observations in iNEU4 U-GSCs (Figure 2b); after 4 days of treatment, we observed that the initial number of U-GSCs was reduced by 44%, in contrast to non-treated U-GSCs that increased their number by 94% in the same period of culture (Figure 6d).

In addition, we tested the effects of thymoquinone (TQ) on U87MG cells. TQ derived from the black cumin oil has been

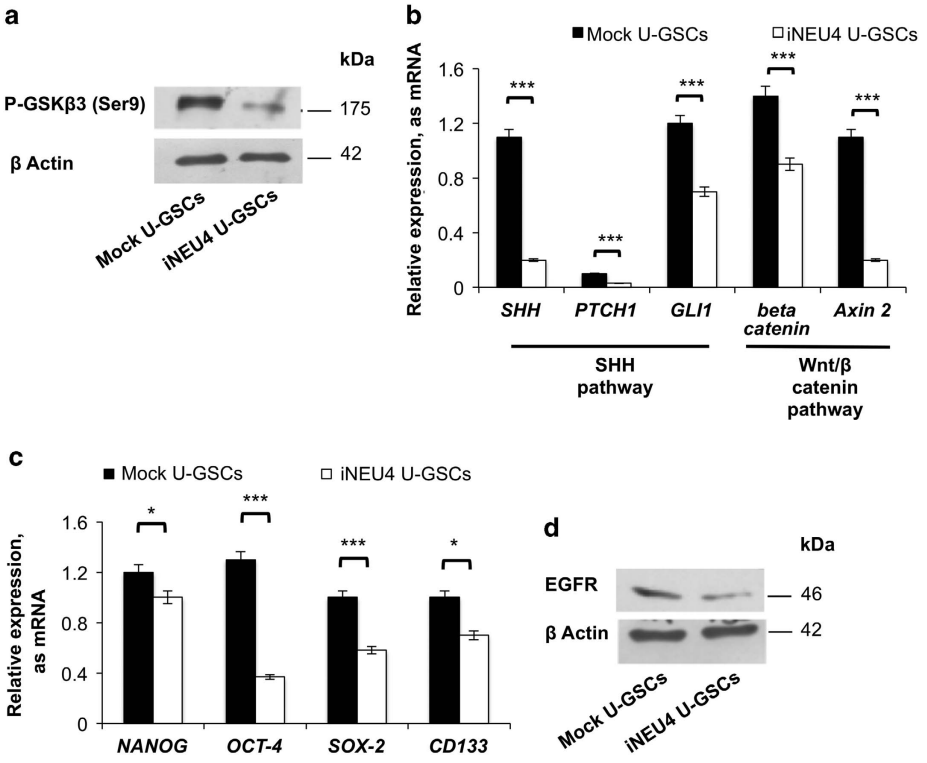

Figure 3 Effects of *NEU4* silencing on signalling pathways related to stemness. (**a**) Western blot analysis of P-GSK-3β (Ser9) in mock and iNEU4 U-GSCs. β-Actin was used as loading control. (**b**) Real-time PCR analysis of *SHH*, *PTCH1*, *GLI-1*, β catenin, *axin 2*, and (**c**) *NANOG*, *OCT-4*, *SOX-2*, and *CD133* in mock and iNEU4 U-GSCs. (**d**) Western blot analysis of EGFR in mock and iNEU4 U-GSCs. β-Actin was used as loading control. Values are the mean ± S.D. of five independent experiments. Significance is based on the Student's *t*-test: $^*P<0.05$, $^{***}P<0.001$

reported to be an agonist of NEU4 sialidase activity in live cells.[38] Consistent with this, the expression of *GLI-1* and *OCT-4* increased by 775% and 700%, respectively, after treatment with TQ (Figure 6e).

***NEU4* high expression characterizes GSCs isolated from human surgical specimens and regulates the activation of the stemness pathways.** In order to confirm our data in cells directly isolated from patients and, therefore, to exclude the possibility that they could be related to long-term cultures of GBM cell lines, we assayed sialidase expression in GBM tissues and in GSCs isolated from six human surgical specimens, as previously described.[39,40] These GBM-derived GSCs were demonstrated to be endowed with long-term self-renewal, high *in vivo* tumorigenic potential, multipotency, and increased ability to establish and expand GBM-like tumours in serial transplantation.[39] Among sialidases, *NEU4* expression underwent the most significant change, increasing 40.3-fold in GBM-derived GSCs in comparison with GBM tissues. Instead, *NEU1* expression showed a slight decrease in GSCs (1.6-fold) and *NEU3* increased (13.4-fold) (Figure 7a).

In order to confirm the role of NEU4 in regulating the 'stemness' pathways emerged from the studies performed in U87MG cells, we silenced *NEU4* in GSCs isolated from a surgical specimen (L0627 cells[39]). We achieved a 50% *NEU4* silencing as mRNA. As observed for U87MG GSCs, *NEU4* silencing strongly impaired LO627 GSC survival; in fact, 6 days after the end of selection mock LO627 cells began to grow ($+48\%$); 18% of *NEU4* silencing LO627-GSCs (referred to as LO627 iNEU4) died and, after 12 days almost 52% of LO627 iNEU4 cells died (Figure 7b). Moreover, C.6 treatment of LO627 cells reduced the initial number by 14%, in contrast to non-treated LO627 cells that increased their number by 100% after 4 days of culture (Figure 7c). Similar to U87MG cells, we identified in LO627 iNEU4 cells the decrease in mRNA expression of genes related to the SHH pathway (*SHH*: 83.5%; *GLI-1*: 59%), to the Wnt/β catenin pathway (β catenin: 30%; *axin 2*: 40%), and to 'stemness' transcriptional factors and markers (*NANOG*: 30%; *OCT-4*: 40%; *SOX-2*: 40%; *CD133*: 30%) (Figure 7d). Accordingly, C.6 treatment of LO627 cells reduced the expression of *GLI-1* (37%), *NANOG* (37%), *OCT-4* (20%) (Figure 7e), and phospho-GSK-3β (Ser9) content (65%; $P<0.01$) (Figure 7f).

Discussion

The sialidase NEU4 is the most recently identified sialidase isoenzyme;[41] its physiological role inside the cell is still largely cryptic. Some recent papers have identified a strong involvement of NEU4 in neuronal differentiation and, in particular, its expression appeared to be elevated during the immature stage of neuronal cells or in undifferentiated neuroblastoma cells.[22,28,29,42] The data presented here are the first to demonstrate that *NEU4* expression is high in GSCs isolated from both GBM cell lines and patients, and that its

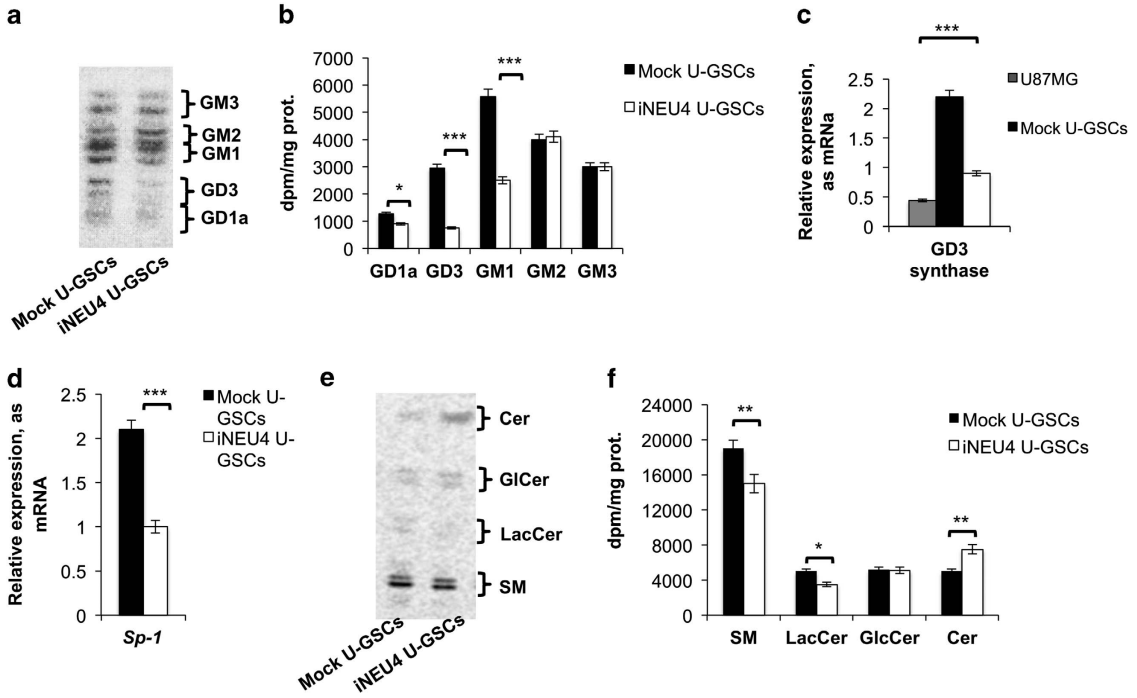

Figure 4 Modifications induced by *NEU4* silencing on U-GSC sphingolipid pattern. (**a**) HPTC separation of mock and iNEU4 U-GSCs gangliosides. The doublets are due to the heterogeneity of the ceramide moiety. Solvent system: chloroform/methanol/0.2% aqueous $CaCl_2$ 60 : 40 : 9 (v/v). The image was acquired by radiochromatoscanning (Beta Imager 2000). (**b**) The ganglioside content of mock and iNEU4 U-GSCs. (**c**) Real-time PCR analysis of GD3 synthase and (**d**) *Sp1* expression in mock and iNEU4 U-GSCs. (**e**) HPTLC separation of mock and iNEU4 U-GSCs neutral sphingolipids. Solvent system: chloroform/methanol/H_2O 55 : 20 : 3 (v/v). The image was acquired by radiochromatoscanning (Beta Imager 2000). (**f**) The neutral sphingolipid content of mock and iNEU4 U-GSCs. The values are the mean ± S.D. of five independent experiments. Significance is based on the Student's *t*-test: $^{**}P<0.01$, $^{***}P<0.001$

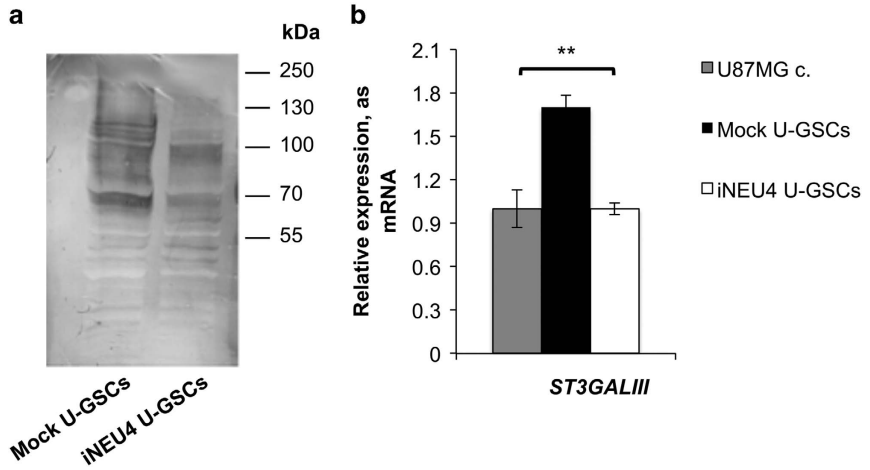

Figure 5 Modifications induced by *NEU4* silencing on U-GSC sialoglycoprotein pattern. (**a**) The α2-3 sialoglycoprotein profile was assessed using western blotting and MAA (*Maackia amurensis* agglutinin) lectin staining of mock and iNEU4 U-GSCs. An equal content of protein was loaded in each lane. (**b**) Real-time PCR analysis of *ST3GalIII* expression in mock and iNEU4 U-GSCs. The values are the mean ± S.D. of four independent experiments. Significance is based on the Student's *t*-test: $^{**}P<0.01$

upregulation appears to be intimately correlated with the capability to form neurospheres. Cells that were not able to form neurospheres, such as T98G cells, did not show *NEU4* upregulation. In contrast, in the bulk of more differentiated non-neurosphere GBM cells and in GBM surgical specimens, *NEU4* expression is very low. NEU4 appears to be closely interconnected with the key pathways activated in GSCs responsible for their stemness and self-renewal properties. In fact, through silencing in U87MG-derived GSCs, we demonstrated that NEU4 activity affects the activation status of GSK-3β, inducing its inhibition (phosphorylation) when NEU4 is fully expressed. GSK-3β inhibition has been previously shown to significantly reduce the stem-like $CD133^+$ fraction of a human GBM cell line, as well as the expression of the stem-cell markers, *SOX-2* and nestin, while, in parallel, it increased the expression of differentiation markers, including glial fibrillary

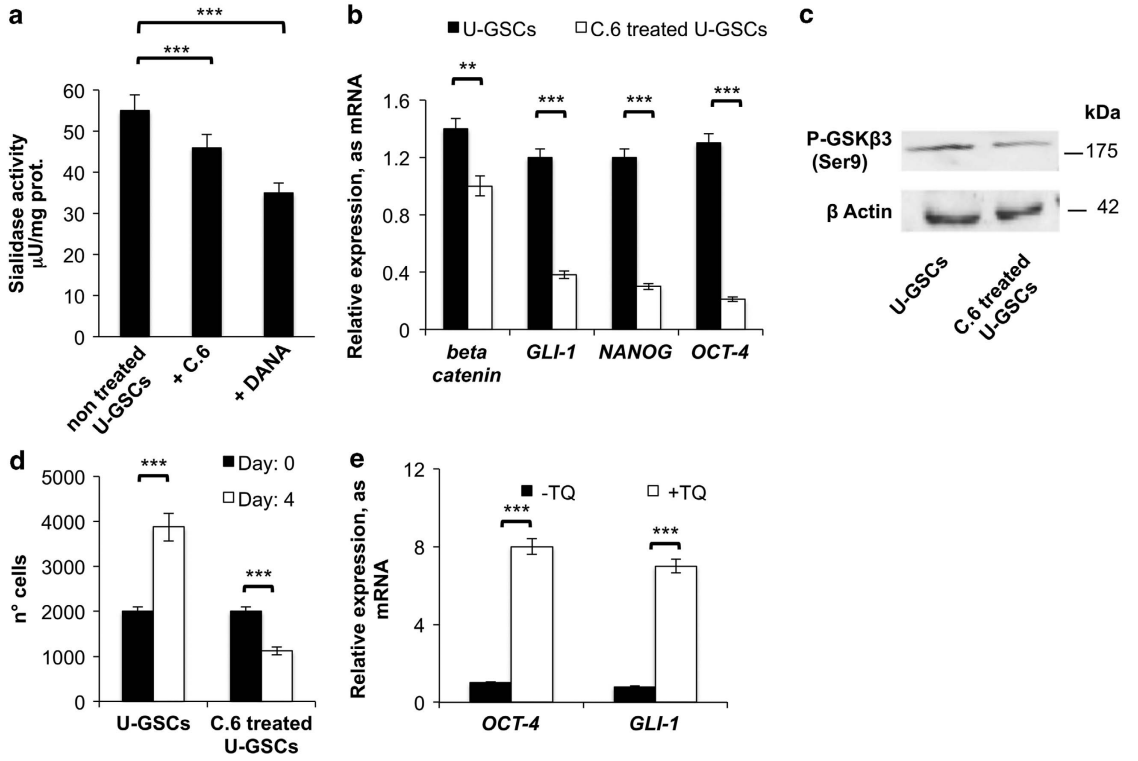

Figure 6 Effects induced by the chemical inhibition of NEU4 in U-GCS or activation in GBM cells. (**a**) *In vitro* sialidase activity of U-GSCs assayed towards 4-MU-NeuAc as substrate, with the inhibitors C.6 (20 μM) and DANA (20 μM). (**b**) Real-time PCR analysis of β-catenin, *GLI-1*, *NANOG*, and *OCT-4* expression after i*n vivo* treatment of U-GSCs with C.6 (0.7 mM) for 4 days. (**c**) Western blot analysis of P-GSK-3β (Ser9) in C.6-treated and non-treated U-GSCs. β-Actin was used as loading control. (**d**) Cell viability assessed through Trypan Blue after 4 days of GSCs treatment with C.6 (0.7 mM). (**e**) Real-time PCR analysis of *OCT-4* and *GLI-1* expression in U87MG GBM cells after 30 min of TQ treatment (0.3 mM). The values are the mean ± S.D. of four independent experiments. Significance is based on the Student's *t*-test: $^{**}P<0.01$, $^{***}P<0.001$

acidic protein, β-tubulin III and 2′, 3′-cyclic nucleotide 3′-phospohodiesterease.[43] GSK-3β inhibition by administration of lithium or through specific inhibitors such as SB216763 has been demonstrated to impair neurosphere formation.[43] The focal role had by GSK-3β in U-GSCs could be easily explained by interactions with the main signalling pathways, which sustain GSC self-renewal and propagation, SHH/GLI and Wnt/β-catenin.[44,45] Importantly, *NEU4* silencing radically changed the molecular signature of U-GSCs, decreasing the expression of *SHH*, *PTCH-1*, and *GLI-1* (key components of the SHH pathway) of β-catenin and *axin 2* (key components of the Wnt/β-catenin pathway), and of genes regulated by them, such as *NANOG*, *OCT-4*, and *SOX-2*. We found that the expression of *CD133* decreased, possibly because this gene is subjected to the transcriptional control of *OCT-4* and *SOX-2*.[46] In addition, the level of EGFR was significantly reduced. EGFR and β-catenin signalling are known to interact: intratumoral administration of β-catenin siRNA into subcutaneous gliomas in nude mice has been shown to transcriptionally downregulate EGFR.[47] Therefore, we hypothesized that the decrease of β-catenin induced by *NEU4* silencing could be responsible for the reduction in EGFR content. EGFR strongly regulates proliferation and migration of neural stem cells. GSCs constitutively activate EGFR, leading to the activation of AKT. Numerous studies have revealed the crucial role of the EGFR signalling cascade in the maintenance of GSCs, wherein EGF is capable of promoting neurosphere formation and self-renewal.[48] Moreover, CD133 has been demonstrated to interact with the phosphoinositide 3-kinase 85 kDa regulatory subunit, resulting in the preferential activation of AKT in GSCs.[49] Therefore, we speculate that the decrease of EGFR and CD133 in *NEU4*-silenced U-GSCs may act in synergy to reduce AKT signalling, and thus its pro-survival drive. The molecular revolution of U-GSCs phenotype subsequent to *NEU4* silencing involved also the expression of GD3 synthase (ST8SiaI) that is clearly related to the presence of ganglioside GD3. GD3 is present in neural stem cells where it interacts with EGFR, stimulating its activation and its downstream signalling to maintain the self-renewal capability.[16] In this study, we demonstrated that a high expression of GD3 synthase leads to a subsequent higher content of GD3 as a hallmark of GSCs. In normal neural stem cells, GD3 is linked to their self-renewal.[15] GD3 synthase upregulation in GSCs appeared to be linked to the overexpression of *Sp-1* factors,[50] which, in turn, could be related also to EGFR activation.[51] *NEU4* silencing reverted the upregulation of GD3 synthase, decreasing the expression of *Sp-1*. We demonstrated that another feature of GSCs is the upregulation of *ST3GalIII*, which is involved in the *de novo* synthesis of carbohydrate antigens on different glycoproteins, and in GSCs it could be responsible for an altered pattern of glycosylation, already previously described.[52] *NEU4* silencing decreased *ST3GalIII* expression in U-GSCs, possibly decreasing the expression of *Sp-1* factors that have been

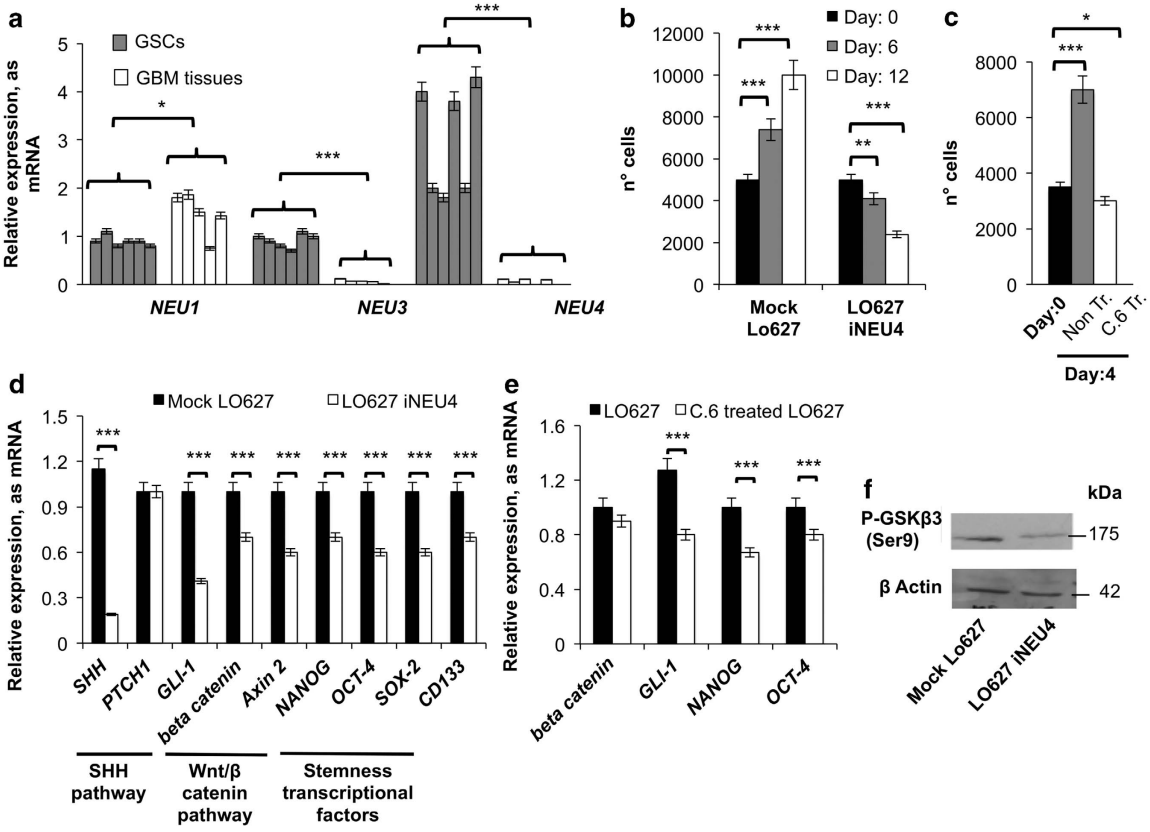

Figure 7 Effects of *NEU4* silencing and chemical inhibition in LO627 cells. (**a**) Real-time PCR of *NEU1*, *NEU3*, and *NEU4* expression in GSCs isolated from six different surgical specimens and in five GBM tissues. Mean values of sialidase expression in GSCs and in GBM tissues were compared. (**b**) Cell viability assessed through Trypan Blue after *NEU4* silencing in LO627 cells and (**c**) after 4 days of LO627 cells treatment with C.6 (0.7 mM). (**d**) Real-time analysis of *SHH*, *PTCH1*, *GLI-1*, β-catenin, *axin 2*, *NANOG*, *OCT-4*, *SOX-2*, and *CD133* in mock and iNEU4 LO627. (**e**) Real-time PCR analysis of β-catenin, *GLI-1*, *NANOG*, and *OCT-4* expression after *in vivo* treatment of LO627 cells with C.6 (0.7 mM) for 4 days. (**f**) Western blot analysis of P-GSK-3β (Ser9) in C.6-treated and non-treated LO627 cells. β-Actin was used as loading control. Significance is based on the Student's *t*-test: $^{*}P<0.05$; $^{**}P<0.01$, $^{***}P<0.001$

demonstrated to regulate the transcription of this gene.[53] In this way, *NEU4* silencing changed the glycosylation pattern of GSCs. Summing up, *NEU4* silencing induced a sequence of interrelated molecular events, summarized in Figure 8, which significantly changed GSCs signalling and phenotype, leading to a significant decrease of their maintenance, as recorded by the inhibition of pro-survival pathways (AKT), the activation of caspase-3, the increase of the pro-apoptotic molecule, ceramide, and the decrease of the anti-apoptotic glycolipid, LacCer. Consequently, *NEU4* silencing impaired the survival of U-GSCs inducing a block in the G2 phase and cell death.

Importantly, the effects induced by *NEU4* silencing on signalling pathways related to U-GSCs self-renewal and propagation, and on cell proliferation were confirmed in LO627 cells, that is, human short-term GSCs isolated from a surgical specimen, further confirming the key role had by NEU4.

Thus, in this emerging model, NEU4 appears to be intrinsically connected to the stem cell-like signalling of GSCs. As a further evidence of this, the treatment of U87MG GBM cells with TQ, an agonist of NEU4 activity,[38] strongly enhanced the expression of the stem cell-like gene expression signature. Unfortunately, it is still unclear how NEU4 could interact with GSK-3β. On the basis of these data and our previous results that appeared to exclude the action of NEU4 on gangliosides and that identified, on the other hand, its efficient recognition of glycoproteins,[22] we strongly suspect that also in GSCs NEU4 primarily acts on glycoproteins, because ganglioside profile did not change accordingly to *NEU4* silencing. In fact, we recorded a decrease of GD1a and GD3, while GM3 remained unchanged after *NEU4* silencing, instead of an increase, as expected, if these gangliosides were really substrates of NEU4 in GSCs. These ganglioside modifications could be related to alterations induced in the expression of other biosynthetic enzymes such as GD3 synthase, as discussed above. Notably, inspection of the sialo-glycoprotein pattern of *NEU4*-silenced GSCs showed dramatic changes from control GSCs. We identified some bands from 50 to 65 kDa that were more sialylated in *NEU4*-silenced GSCs. These sialoglycoproteins could therefore interact with GSK-3β, modulating its activation status based on the level of sialylation. On the other hand, in literature, several examples of sialoglycoproteins, including mucins, able to interact with GSK-3 have been described.[54]

Finally, we tested the effects of a previously identified specific inhibitor of NEU4 (Albohy *et al.*[37]) on both GSCs isolated from U87MG cell lines and in LO627 cells. We found that chemical inhibition of the enzyme resulted in similar effects to *NEU4* silencing. Treatment of GSCs with the C.6

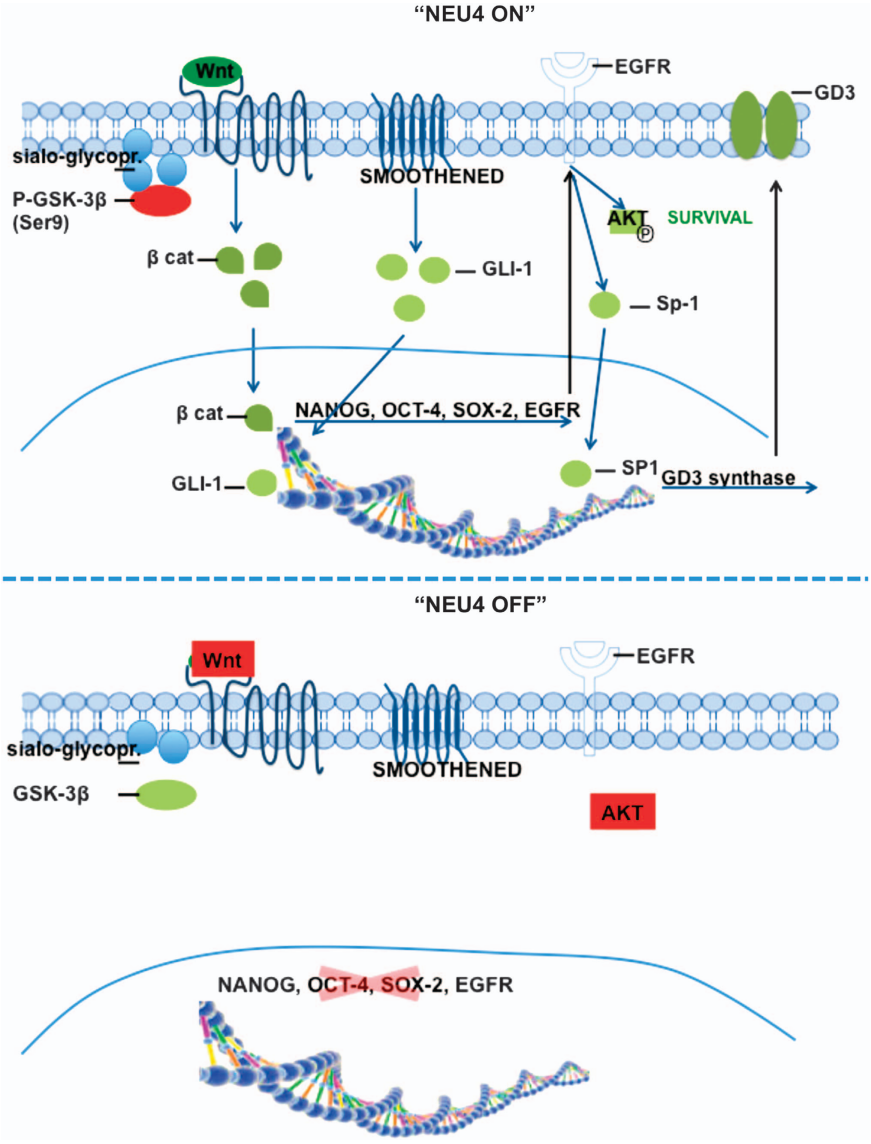

Figure 8 Schematic picture of pathways regulated by NEU4 in GSCs

inhibitor markedly resembled *NEU4* silencing, as it reduced the expression of β-catenin, *GLI-1*, *NANOG*, and *OCT-4* stem-cell markers. Most significantly, we observed that treatment of GSCs with the NEU4 inhibitor, C.6, impaired cell proliferation and survival. Together, these finding confirms that the specific inhibition of NEU4 can be explored to suppress the growth and stem-cell phenotype of GSCs, and suggest that this strategy could form the basis of future therapeutic strategies for GBM.

Materials and Methods

Cell cultures. U87MG, U138MG, and T98G cells were purchased from ECACC (Sigma Aldrich, St. Louis, MO, USA) and cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum and 2 mM glutamine (Sigma Aldrich). GSCs were isolated from U87MG, U138MG, and T98G cells by plating 2×10^3 cells/cm^2 in the selective medium DMEM/F12 (Sigma Aldrich) plus 10 ng/ml basic fibroblast growth factor (Millipore, Billerica, MA, USA), 20 ng/ml EGF (Sigma Aldrich), B27 supplement minus vitamin A (Life Technology, Carlsbad, CA, USA), and 2 mM glutamine, and culturing them for 3 weeks, at 37 °C, 5% CO_2.

GSCs isolated from human post-surgical specimens and GBM tissues isolated from patients admitted at S. Raffaele Hospital (Milan, Italy) were provided by Dr R Galli. LO627 cells were cultured as previously described.[39]

***NEU4* silencing in GSCs isolated from U87MG cells (U-GSCs) and in LO627 cells.** To stably silence *NEU4*, an miRNA targeting the human *NEU4* gene sequence was designed employing the BlockIT RNAi Designer software (Life Technologies) and inserted into the pcDNA 6.2-GW/EmGFP-miR vector (Life Technologies). U87-GSCs (5×10^5) were transfected overnight, using Lipofectamine LTX and Plus Reagent (Life Technologies), according to the manufacturer's procedure. To stably silence *NEU4* in LO627 cells, we employed the BlockIT lentiviral PolII miR RNAi Expression System (Life Technologies) and infected cells at an MOI of 5 according to the manufacturer's instructions. Silenced U-GSCs and LO627 cells were isolated after selection with 10 μg/ml blasticidin for a week.

Chemical inhibition and activation of sialidase NEU4. The NEU4-specific inhibitor, 5-acetamido-9-[4-hydroxymethyl-1,2,3-triazol-1-yl]-2,3,5,9-tetradeoxy-D-glycerol-D-galacto-2-nonulopyranosonic acid, referred to as compound 6 (C.6) here, was synthesized by Albohy *et al.*[37], as previously reported. C.6 (0.7 mM) was added to 4×10^5 U87-GSCs or LO627 cells in the presence of Lipofectamine LTX (Life Technologies) to allow the entry of the inhibitor inside the

cells. In parallel, control cells were treated only with Lipofectamine LTX. Cells were collected after 4 days.

In order to stimulate NEU4 sialidase expression and enzymatic activity, 6×10^5 U87MG cells were treated with 0.3 mM TQ (Sigma Aldrich) for 30 min at 37 °C.

Cellular death assays. To determine the cell growth and viability, 5×10^4 mock U-GSCs, *NEU4*-silenced U-GSCs, mock LO627, *NEU4*-silenced LO627, C.6-treated U87-GSCs and C.6-treated LO627 cells were seeded in 96-well culture plates. Six (for *NEU4*-silenced cells) or four (for C.6-treated cells) days later, viable cells were counted with Trypan blue.

To test the clonogenic potential of mock and *NEU4*-silenced U-GSCs, a limiting dilution assay was performed plating 10–20 cells/well after neurosphere dissociation and counting cells in neurospheres newly formed after 4 days.

For Hoechst 33342 staining, 5×10^4 mock and *NEU4*-silenced U-GSCs were washed twice with PBS and incubated for 15 min with 10 μg/ml Hoechst 33342 in the dark at room temperature. The cells were then observed using an inverted fluorescence microscope (IX50 Olympus, Tokyo, Japan) and imaged.

Real-time PCR. Real-time PCR was performed as previously reported.[19] Primer sequences were the following: *NEU1*: forward primer, 5′-CCTGGATATTG GCACTGAA-3′, reverse primer, 5′-CATCGCTGAGGAGACAGAAG-3′; *NEU2*: forward primer: 5′-TGCGCAGAGGAGACTACGA-3′, reverse primer, 5′-GTCTGC GCGTCATACAAGG-3′; *NEU3*: forward primer, 5′-GGCTTGTTTGGGTGTTTGTT-3′, reverse primer, 5′-TTTTGAATTGGCTTGGGTTC-3′; *NEU4*: forward primer, 5′-AC CGCCGAGAGTGTTTTGG-3′, reverse primer, 5′-CGTGGTCATCGCTGTAGAA GG-3′; *NANOG*: forward primer, 5′-GGTCCCAGTCAAGAAACAGA-3′, reverse primer, 5′-GAGGTTCAGGATGTTGGAGA-3′; *OCT-4*: forward primer, 5′-AGGAGA AGCTGGAGCAAAA-3′, reverse primer, 5′-GGCTGAATACCTTCCCAAA-3′; *CD133*: forward primer, 5′-CTAGCCTGCGGTCATCTCTC-3′, reverse primer, 5′-AGGCCATCCAAATCTGTCCT-3′; *NES*: forward primer, 5′-CCAAGAACTGGC TCAGGAAA-3′, reverse primer, 5′-TCTCCCTGCTCTACCACCTC-3′; *ST3GALIII*: forward primer, 5′-CATCATCGTGGGCAATGGAG-3′, reverse primer, 5′-TCAAAG CCTTTCACTGGTGC-3′; β catenin: forward primer, 5′-CATCATCGTGGGCAATG GAG-3′, reverse primer, 5′-GCACGAACAAGCAACTGAAC-3′; *GLI-1*: forward primer, 5′-TGAGCTGGACATGCTGGTT-3′, reverse primer, 5′-GATTCAGGCTCA CGCTTCTC-3′; *SHH*: forward primer: 5′-CCCCAATTACAACCCCGACA-3′, reverse primer, 5′-AGTTTCACTCCTGGCCACTG-3′; *PTCH1*: forward primer: 5′-TGCCATTTCTCGCTCTTGGT-3′, reverse primer: 5′-GAATTGGGATTAACGCG GCC-3′; *axin 2*: forward primer: 5′-GGCTCCAGAAGATCACAAAG-3′, reverse primer: 5′-TATGGAATTTCTTCCCCACA-3′; GD3 synthase: forward primer, 5′-CC CTGAACCAGTTCGATGTT-3′, reverse primer, 5′-CAGCATAATTCGGCAAAGGT-3′; *SOX-2*: forward primer, 5′-GCACATGAACGGCTGGAGCAACG-3′, reverse primer, 5′-TGCTGCGAGTAGGACATGCTGTAGG-3′; β actin: forward primer, 5′-CGACAGGATGCAGAAGGAG-3′, reverse primer, 5′-ACATCTGCTGGAAGGT GGA-3′.

Western blotting. Proteins (25 μg) were separated on 10% SDS-PAGE, and transferred onto PVDF membrane. The following antibodies were used for the assays: anti-β actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-P-AKT (Ser473), anti-EGFR, anti-cleaved caspase 3, anti-P-RB (Ser807/811), anti-cyclin B1, P-CDC2 (Tyr15) and anti-GSK-3β (Ser9) (Cell Signaling, Danvers, MA, USA). Blots were quantified using GS-700 calibrated densitometer and the Quantity One software (Bio-Rad, Hercules, CA, USA).

Metabolic labelling of cell sphingolipids. Sphingolipid analysis was carried out through cell metabolic labelling with [3-^{3}H]sphingosine (PerkinElmer, Waltham, MA, USA), as previously reported.[20] Ganglioside and neutral sphingolipid extracts were analysed by HPTLC carried out with the solvent systems chloroform/methanol/0.2% $CaCl_2$ 55 : 45 : 6 (v/v) and chloroform/methanol/water 110 : 40 : 6 (v/v), respectively.

Sialoglycoprotein analysis. Proteins (30 μg) of mock and *NEU4*-silenced U-GSCs were separated on a 10% SDS-PAGE gel and transferred onto a PVDF membrane. Sialoglycoproteins were visualized employing the DIG Glycan Differentiation kit (Roche Applied Science, Indianapolis, IN, USA) as previously reported.[22] Densitometric analysis was performed using Quantity One software.

Sialidase activity. The sialidase activity present in the particulate fraction of U-GSCs was assayed using 4-MU-NeuAc (4-methylumbelliferyl-N-acetylneuraminic acid) (Sigma Aldrich) substrate, as previously reported.[42] To test the inhibitory effect of C.6 or that of DANA, the inhibitors (20 μM) were added to the enzymatic mixture. One unit of sialidase activity is defined as the amount of enzyme liberating 1 μmol of product per min.

Statistical analysis. The values are presented as the mean ± S.D. The statistical analyses were performed using Student's *t*-test.

Conflict of Interest

The authors declare no conflict of interest.

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