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HNK-1 Epitope-carrying Tenascin-C Spliced Variant Regulates the Proliferation of Mouse Embryonic Neural Stem Cells*[S]

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Neural stem cells (NSCs) possess high proliferative potential and the capacity for self-renewal with retention of multipotency to differentiate into neuronal and glial cells. NSCs are the source for neurogenesis during central nervous system development from fetal and adult stages. Although the human natural killer-1 (HNK-1) carbohydrate epitope is expressed predominantly in the nervous system and involved in intercellular adhesion, cell migration, and synaptic plasticity, the expression patterns and functional roles of HNK-1-containing glycoconjugates in NSCs have not been fully recognized. We found that HNK-1 was expressed in embryonic mouse NSCs and that this expression was lost during the process of differentiation. Based on proteomics analysis, it was revealed that the HNK-1 epitopes were almost exclusively displayed on an extracellular matrix protein, tenascin-C (TNC), in the mouse embryonic NSCs. Furthermore, the HNK-1 epitope was found to be present only on the largest isoform of the TNC molecules. In addition, the expression of HNK-1 was dependent on expression of the largest TNC variant but not by enzymes involved in the biosynthesis of HNK-1. By knocking down HNK-1 sulfotransferase or TNC by small interfering RNA, we further demonstrated that HNK-1 on TNC was involved in the proliferation of NSCs via modulation of the expression level of the epidermal growth factor receptor. Our finding provides insights into the function of HNK-1 carbohydrate epitopes in NSCs to maintain stemness during neural development.

Neural stem cells (NSCs)[4] are undifferentiated neural cells characterized by their high proliferative potential and the capacity for self-renewal with retention of multipotency to differentiate into brain-forming cells, such as neurons, astrocytes, and oligodendrocytes (1–3). Environmental factors of NSCs, such as various growth factors, the extracellular matrix (ECM), and cell adhesion molecules, are known to play important roles in the maintenance of the stem cell population throughout specific cell lineage pathways (4–7).

Glycoconjugates, including glycoproteins, proteoglycans, and glycolipids, are expressed mainly on the cell surface as ECM, and they are known to regulate cell-to-cell communications. Certain glycoconjugates also serve as excellent biomarkers at various stages of the cellular differentiation of NSCs and play important functional roles in determining cell fate (8–12). For example, stage-specific embryonic antigen-1 (SSEA-1), which is well known as a specific maker of undifferentiated cells including mouse embryonic stem cells, is expressed on NSCs and associated with cell migration (8, 10, 13). Recently, we have also demonstrated that cells positive for GD3 ganglioside (NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1′Cer) isolated from mouse brains of various ages possess characteristics of neural stem cells (11).

The human natural killer-1 (HNK-1) carbohydrate epitope (CD57) was originally reported as a specific antigenic determinant for human natural killer cells (14) but is now widely known as an antigen expressed predominantly in the nervous system (15–17). The chemical structure was determined independently as having the basic trisaccharide structure containing a terminal sulfoglucuronyl residue, HSO_3-3GlcAβ1–3Galβ1–4GlcNAc- (18, 19). The minimal carbohydrate determinant reacting to several monoclonal antibodies (*e.g.* Leu-7, NRG50, and a human monoclonal antibody, LT) has been determined to reside in the two terminal carbohydrate units, HSO_3-3GlcAβ1–3Galβ1-, and the sulfate residue is essential for binding (20). In the nervous system, the HNK-1 epitope is present

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1–3.

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[4] The abbreviations used are: NSC, neural stem cell; ECM, extracellular matrix; FNIII domain, fibronectin III domain; GlcAT, glucuronyl transferase; HNK-1, human natural killer-1; HNK-1ST, HNK-1 sulfotransferase; MS/MS, tandem mass spectrometry; PNGase F, peptide N-glycanase F; SSEA-1, stage-specific embryonic antigen-1; TNC, tenascin-C; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium.

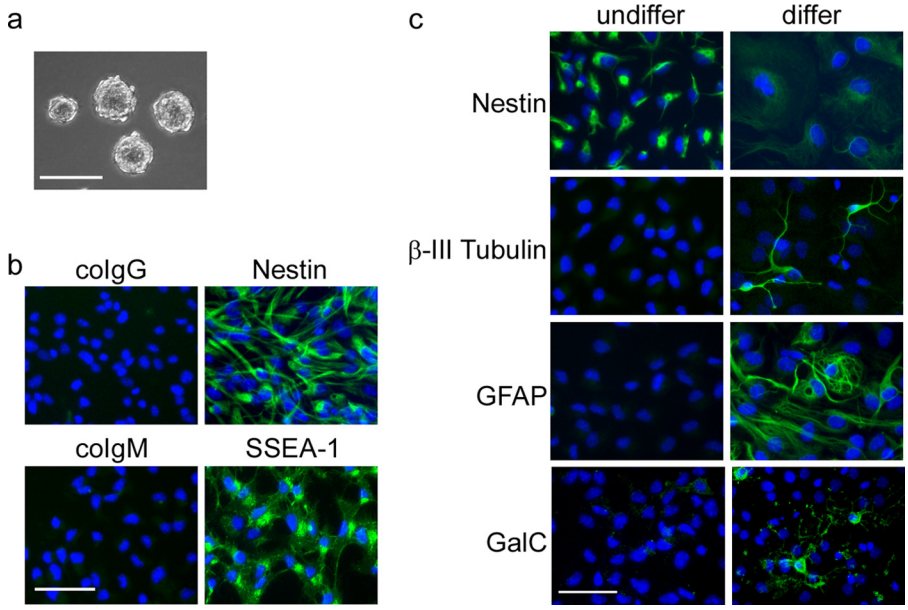

FIGURE 1. **Neural stem cells prepared in the form of neurospheres.** *a*, neurospheres were prepared from the striata of mouse embryos (embryonic day 14.5) and cultured in Neurobasal-A medium supplemented with B27, basic FGF and EGF. *b*, cells forming neurospheres were stained with Rat 401 anti-nestin antibody or AK97 anti-SSEA-1 antibody. Most neurosphere-forming cells were positive for nestin (neural stem cell marker protein; *green*) and SSEA-1 (neural stem cell marker carbohydrate; *green*). *colgG* and *colgM* indicate subclass control IgG and control IgM (BD Biosciences), respectively. *c*, cells forming neurospheres were cultured in undifferentiation condition (*undiffer*) or differentiation condition (*differ*; 0 ng/ml basic FGF and EGF and 1% fetal bovine serum for 5 days) and then stained with antibodies to nestin, β-III tubulin, glial fibrillary acidic protein (*GFAP*), and galactocerebroside (*GalC*). β-III Tubulin$^+$ neurons (*green*), GFAP$^+$ astrocytes (*green*), and GalC$^+$ oligodendrocytes (*green*) are found in the cells cultured in differentiation condition. Nuclei were stained with Hoechst 33258 (*blue*). *Scale bars*, 100 μm in *a*; 50 μm in *b* and *c*.

on a variety of glycoconjugates, including glycoproteins (*e.g.* L1, P0, and neural cell adhesion molecule), ECM proteins (*e.g.* tenascin-R and chondroitin sulfate proteoglycans), and glycolipids (*e.g.* sulfoglucuronic acid-containing glycolipids, such as sulfoglucuronosyl paragloboside and sulfoglucuronosyl lactosaminyl paragloboside). Those molecules are known to play important roles in intercellular adhesion and cell migration (12, 15, 18, 19, 21, 22). Mice deficient in enzymes involved in the biosynthesis of HNK-1 exhibit reduced long term potentiation, defective spatial memory formation, and an increase of hippocampal and cortical beta oscillations (23, 24). These reports clearly indicate important functional roles of HNK-1 in brain functions and neural development. In contrast, there has not been any report describing the expression of HNK-1 in NSCs with the exception of a previous study in which the HNK-1 epitope was found to express on mouse neuroepithelial cells (13). Using a mass spectrometry (MS)-based proteomics approach, we investigated in this study the HNK-1 carbohydrate epitopes in NSCs and identified a protein specifically carrying the HNK-1 epitope and demonstrated its functional role in NSCs. Our study is the first report to describe the expression, the nature of the carrier, and the functional roles of HNK-1 in NSCs.

EXPERIMENTAL PROCEDURES

Materials—NGR50 mouse monoclonal antibody (IgG), prepared from culture supernatants of an NGR50 hybridoma cell line, was used as an anti-HNK-1 antibody (25). Other antibodies used in this study are shown in supplemental Table 1. U0126 was purchased from Sigma-Aldrich.

NSC Culture—NSCs were prepared in the form of neurospheres according to previously described methods with slight modifications (11, 26). In brief, single-cell suspensions prepared from the striata of ICR mouse (Harlan, Indianapolis, IN) embryos (embryonic day 14.5) were cultured in Neurobasal-A medium (Invitrogen) containing B27 serum-free supplement (Invitrogen), L-glutamine (Invitrogen), 20 ng/ml basic fibroblast growth factor (FGF) (Peprotech, Rocky Hill, NJ) and 20 ng/ml epidermal growth factor (EGF) (Peprotech). Neurospheres formed after 5–6 days were collected for passage or analysis. To induce differentiation, the NSCs were cultured for 10 days in Neurobasal-A medium containing B27, L-glutamine (Invitrogen), and 1% fetal bovine serum in the absence of basic FGF and EGF. ICR mice used in this study were treated in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Medical College of Georgia.

Immunocytochemistry—NSCs prepared from neurospheres were plated onto chamber slides (Nalge Nunc International, Naperville, IL) coated with poly-L-ornithine (Sigma-Aldrich) and fibronectin (Sigma-Aldrich) and fixed in PBS containing 4% paraformaldehyde. The NSCs were treated for 2 h with PBS containing 3% fetal bovine serum and 0% or 0.1% Triton X-100 and then stained with primary antibodies such as Rat401 anti-nestin monoclonal antibody (BD Biosciences), AK97 anti-SSEA-1 monoclonal antibody (IgM) (27), anti-β-III tubulin monoclonal antibody (Sigma-Aldrich), anti-glial fibrillary acidic protein polyclonal antibody (DAKO Cytomation, Glostrup, Denmark), anti-galactocerebroside monoclonal antibody (Millipore), MTn12 anti-TNC rat monoclonal antibody (Sigma-Aldrich), and NGR50 anti-HNK-1 monoclonal antibody (25) and secondary antibodies such as anti-mouse IgG antibody conjugated with Alexa Fluor 488 (BD Biosciences), anti-mouse IgM antibody conjugated with Alexa Fluor 488, anti-rabbit IgG antibody conjugated with Alexa Fluor 488, anti-rat IgG antibody conjugated with DyLight488 (Jackson ImmunoResearch, West Grove, PA), and anti-mouse-IgG antibody conjugated with Cy3 (Jackson ImmunoResearch) (supplemental Table 1). Nuclei were stained with 2 μg/ml Hoechst 33258 (Sigma-Aldrich). The stained NSCs were photographed under a Nikon Eclipse TE300 fluorescent microscope (Nikon Instruments, Melville, NY) equipped with a Magnafire digital charge-coupled device camera (Optronics, Goleta, CA).

Western Blot Analysis and Immunoprecipitation—NSCs and cells differentiated from NSCs were lysed in 500 μl of lysis buffer (20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA,

and 1% Triton X-100) using a 1-ml syringe with a 26-gauge needle. The lysates were centrifuged for 10 min at 11,250 × *g* at 4 °C. The resulting supernatant was used for protein analysis. Proteins in this fraction were subjected to 7% or 3–10% gradient SDS-PAGE and subsequently transferred to a polyvinylidene difluoride membrane (Bio-Rad). After blocking with 5% skim milk in 20 mM Tris-HCl (pH 7.6) containing 150 mM NaCl and 0.05% Tween 20, we incubated the membrane with primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies (supplemental Table 1). The protein bands were visualized by Western Lightning Chemiluminescence reagent (PerkinElmer Life Sciences). To remove *N*-glycans on glycoproteins, we incubated a lysate containing 20 μg of proteins with peptide *N*-glycanase F (PNGase F) (50 units; New England BioLabs) for 3 h at 37 °C before being subjected to SDS-PAGE.

For immunoprecipitation, the cell lysates were agitated gently in the presence of control mouse IgG, anti-HNK-1 antibody, or anti-TNC antibody for 1 h followed by incubation with protein A-Sepharose (Pierce) for 3 h at 4 °C. After washing three times with lysate buffer, the immunoprecipitates were subjected to SDS-PAGE and then analyzed by silver staining or Western blotting.

Identification of Glycoproteins by LC-MS/MS Analysis—For liquid chromatography (LC)-MS/MS analysis to identify HNK-1-carrying protein, digestion of the glycoprotein was performed as described previously (28, 29). The digested peptides were reconstituted in 0.1% formic acid and analyzed by a Thermo LTQ linear ion-trap mass spectrometer equipped with a nano-electrospray ionization source and a Finnigan Surveyor LC system (Thermo Fisher Scientific). The peptides were infused directly into the electrospray ionization source through a reverse phase C18 trap column (75 μm × 10.5 cm) equilibrated in 0.1% formic acid at a flow rate of 100 nl/min, and were sequentially eluted with an acetonitrile gradient from 5 to 40% over 60 min. The C18 resin (Magic C18, 5-mm diameter, 100-Å pore size) was purchased from Michrom (Auburn, CA). The spectrometer was operated in a data-dependent mode using a normalized collision energy of 35%. The temperature of the ion transfer tube was set at 200 °C and the spray voltage at 1.8 kV. MS analysis was performed with one full MS scan followed by five MS/MS scans on the five most intense ions from the MS spectrum. The resultant MS and MS/MS data were searched against the NCBI mouse database using the TurboSequest algorithm in the Bioworks software 3.2.

RT-PCR—Reverse transcription-PCR (RT-PCR) was performed as previously described (11, 30, 31). Total RNAs were isolated from cells using TRIzol reagent (Invitrogen). cDNAs were synthesized from the total RNAs as templates using SuperScript III reverse transcriptase (Invitrogen). To discriminate the different TNC spliced variants, PCR was performed using the specific primer pairs (supplemental Table 2) as described previously (30). The other RT-PCR analyses were performed with the following setting: 25–35 cycles of 94 °C for 10 s, 52–55 °C for 30 s, 72 °C for 30 s. The sequences of primers are shown in supplemental Table 2. The PCR products were analyzed by agarose gel electrophoresis using 2% agarose gels containing SYBR safe DNA Gel stain (Invitrogen).

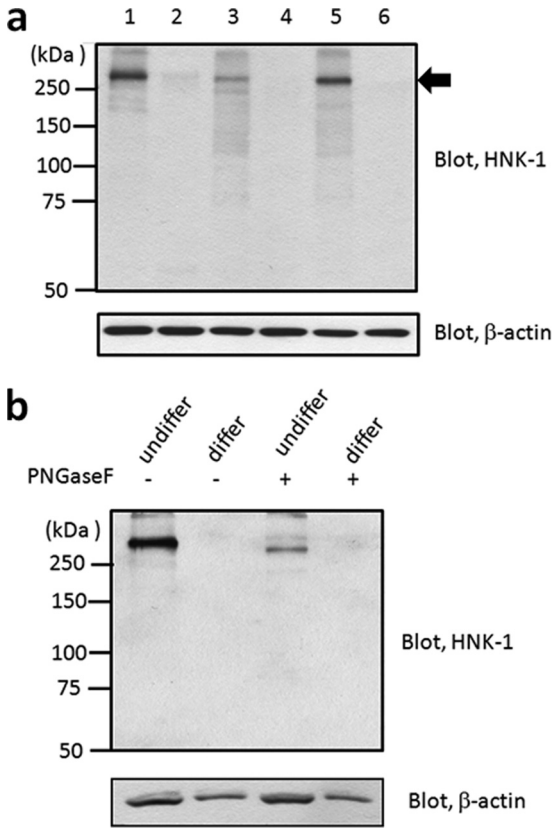

FIGURE 2. **Detection of HNK-1-carrier proteins in NSCs and differentiated cells.** *a*, lysates prepared from primary (*lane 1*), secondary (*lane 3*), and tertiary (*lane 5*) neurospheres and cells differentiated from primary (*lane 2*), secondary (*lane 4*) and tertiary (*lane 6*) neurospheres were analyzed by Western blotting with NGR50 anti-HNK-1 antibody or anti-β-actin antibody (*Blot*). β-Actin was detected as a loading control. *Arrow* indicates a major band positive for HNK-1 with a molecular mass of 280 kDa. *b*, lysates of primary neurospheres and their differentiated cells (containing 20 μg of proteins) were incubated with PNGase F (0 or 50 units/20 μl) at 37 °C for 3 h to remove *N*-glycans and then analyzed by Western blotting with anti-HNK-1 and anti-β-actin antibodies. *undiffer* and *differ* indicate lysates from primary neurospheres and differentiated cells, respectively.

RNA Interference—Negative control double-stranded RNAi and small interfering RNAs (siRNAs) (21-mer) targeting HNK-1 sulfotransferase (HNK-1ST) and TNC were purchased from Qiagen (Valencia, CA). The corresponding target mRNA sequences for the siRNAs are shown in supplemental Table 3. NSCs were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. In brief, NSCs cultured as neurospheres were triturated into single cells and transfected onto a 6-well plate using a reverse transfection method. The mixture containing four kinds of specific siRNA oligomers (24 pmol) was diluted in 400 μl of Dulbecco's modified Eagle's medium and mixed with 4 μl of Lipofectamine RNAiMAX. After incubation of 20 min at room temperature, the siRNA complexes were added into 4 × 10^5 NSCs. The NSCs transfected with negative control siRNA were used as a control. The RNAi results were evaluated by Western blotting with anti-HNK-1 or anti-TNC antibodies.

WST-8 Assay—NSC proliferation was analyzed by a WST-8 assay using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) (32, 33). In brief, siRNA-treated cells were transferred onto 96-well plates at a density of 2 × 10^5 cells/ml (100 μl/well).

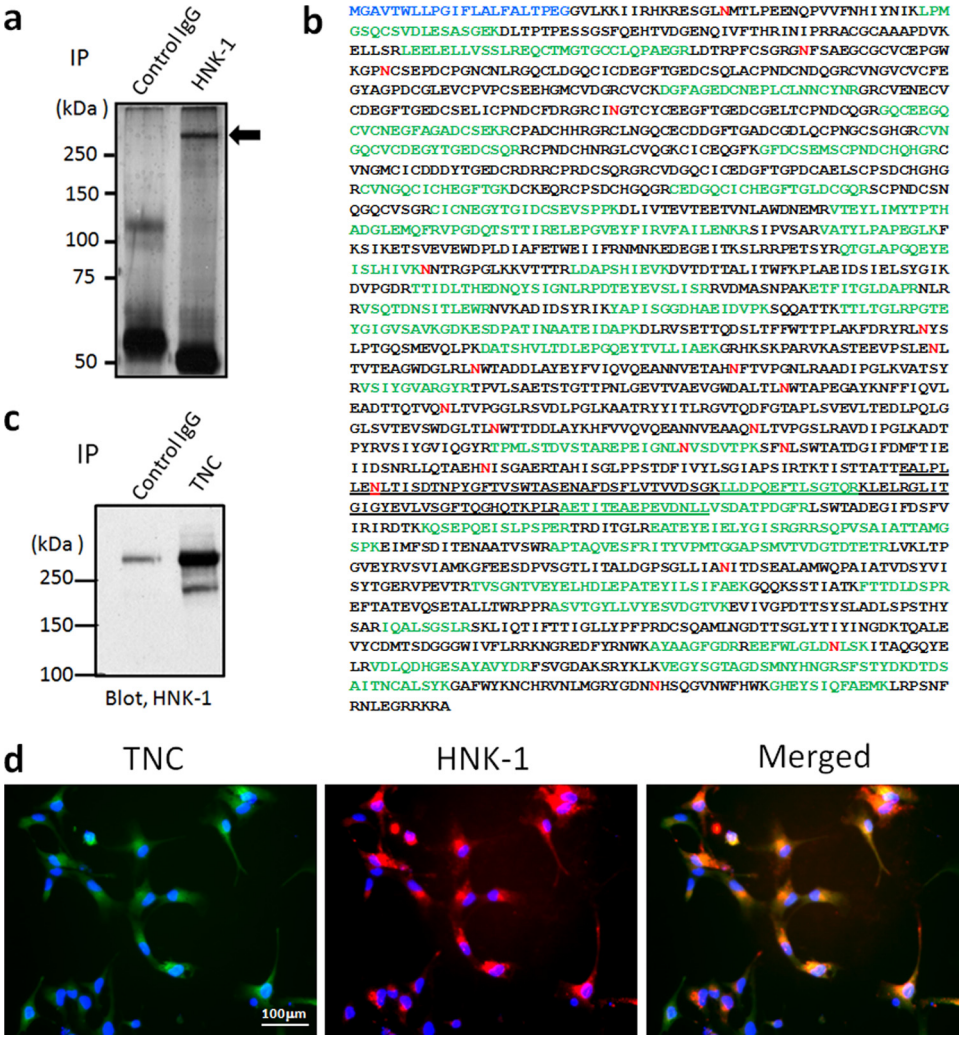

FIGURE 3. **HNK-1 epitopes were carried by TNC in NSCs.** *a*, major HNK-1-carrier protein in secondary neurospheres (*arrow*) was immunoprecipitated with control mouse IgG or anti-HNK-1 antibody (*IP*) and then detected by silver staining. By LC-MS/MS analysis, the major HNK-1-carrier protein was identified as TNC. *b*, amino acid sequence of TNC is shown. The sequences of observed peptides are shown in *green*. The putative signal sequence is shown in *blue*. Twenty potential *N*-glycosylation sites are shown in *red*. The amino acid sequence of the alternatively spliced domain C is *underlined*. *c*, proteins from the secondary neurospheres were immunoprecipitated with control mouse IgG or H300 anti-TNC antibody (*IP*) and then subjected to Western blot analysis with anti-HNK-1 antibody. *d*, NSCs prepared from primary neurospheres were treated with PBS containing 3% fetal bovine serum and 0.1% Triton X-100 and then stained with anti-TNC and anti-HNK-1 antibodies as primary antibodies, and DyLight488-conjugated anti-rat IgG antibody (*green*) and Cy3-conjugated anti-mouse IgG antibody (*red*) as secondary antibodies. Nuclei were stained with 2 μg/ml Hoechst 33258 (*blue*).

After 24, 48, and 72 h of siRNA treatment, the cells were incubated for 4 h with WST-8 solution at 37 °C. The spectrophotometric absorbance of WST-8-formazan produced by the dehydrogenase activity in the living cells was measured at the wavelength of 450 nm (reference, 650 nm) using a Benchmark Plus microplate spectrophotometer (Bio-Rad). The spectrophotometric absorbance measured by this assay is highly correlative with the number of living cells.

TUNEL Assay—Apoptotic cells were detected with the TUNEL (terminal deoxynucleotididyl transferase-mediated dUTP nick-end labeling) assay (34, 35). In brief, the siRNA-transfected cells were plated onto chamber slides and cultured for 3 days. The cells were then fixed in PBS containing 4% paraformaldehyde for 1 h at room temperature and permeabilized in 0.1% sodium citrate containing Triton X-100 for 2 min at 4 °C. The cells were incubated with fluorescein-conjugated TUNEL reaction mixture (Roche Diagnotics) for 2 h at 37 °C.

RESULTS

Detection of a Glycoprotein Carrying HNK-1 in NSCs—In this study, NSCs were isolated from the striata of mouse embryos (embryonic day 14.5) in the form of neurospheres, floating clonal aggregates formed by NSCs *in vitro* (11, 26). The cells forming neurospheres had self-renewal ability, expressed neural stem cell markers such as nestin and SSEA-1, and were capable of differentiating into β-III tubulin$^+$ neurons, glial fibrillary acidic protein$^+$ astrocytes, and galactocerebroside$^+$ oligodendrocytes (Fig. 1). This result clearly demonstrates that the neurosphere-forming cells prepared in this study are NSCs as reported previously (11). To detect glycoproteins carrying HNK-1 in NSCs, we analyzed cell lysates prepared from these NSCs by Western blotting using NGR50, an anti-HNK-1 mouse IgG antibody (25). One major protein band reactive with NGR50, corresponding to an apparent molecular mass of 280 kDa (Fig. 2*a*), was detected in the cell lysates (Fig. 2*a*, *lanes 1*, *3*, and *5*). However, this band disappeared in the lysates of cells differentiated from NSCs (Fig. 2*a*, *lanes 2*, *4*, and *6*). Furthermore, to determine whether HNK-1 epitopes are carried by *N*-glycans or *O*-glycans, we treated the cell lysates with PNGase F and then analyzed the lysates by Western blotting with anti-HNK-1 antibody. After PNGase F treatments, the 280-kDa protein band reactive with the anti-HNK-1 antibody became faintly stained and downward-shifted (Fig. 2*b*, *lanes 3*). Those data indicate that HNK-1 epitopes are expressed in the forms of *N*-glycans as well as *O*-glycans. The major HNK-1-carrier protein, expressed in NSCs with the molecular mass 280 kDa, was further analyzed.

Identification of a Glycoprotein Carrying HNK-1 in NSCs—To isolate the HNK-1-carrier protein, we subjected HNK-1-positive immunoprecipitates prepared from secondary neurospheres with an anti-HNK-1 monoclonal antibody to SDS-PAGE, followed by silver staining (Fig. 3*a*). A piece of the destained polyacrylamide gel containing the HNK-1-positive protein was

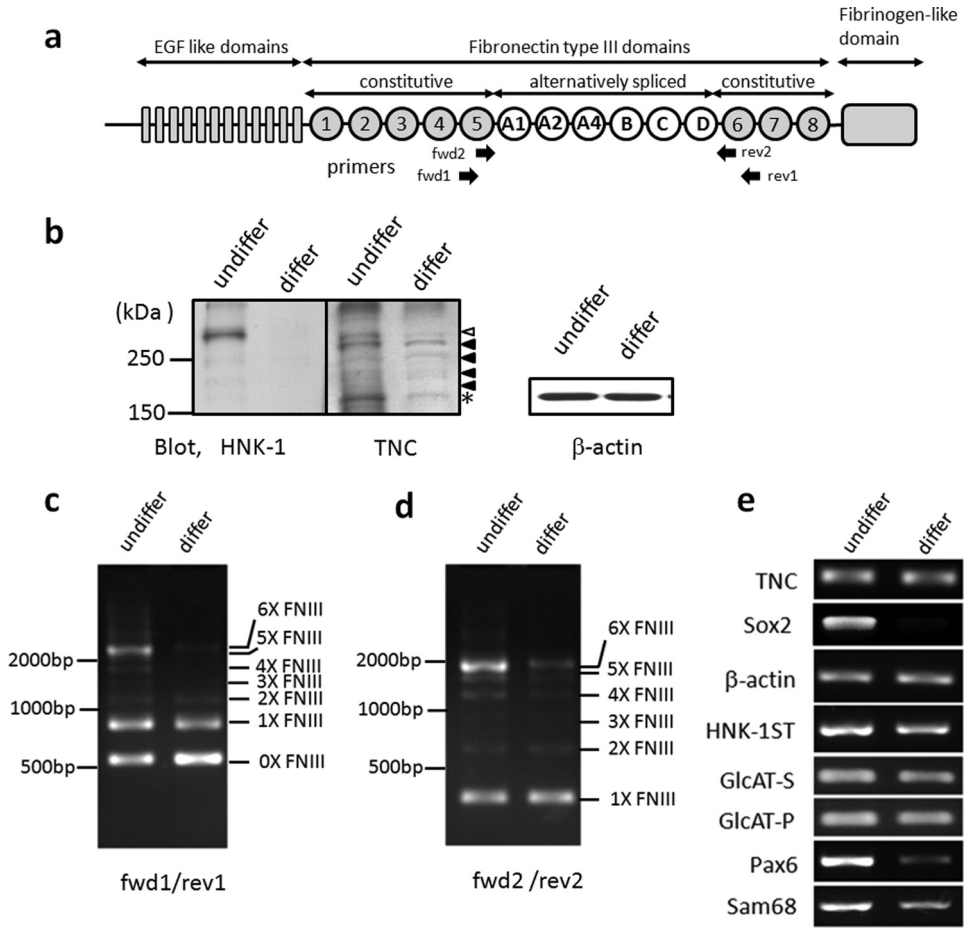

FIGURE 4. **Expression of HNK-1-containing glycans on a larger TNC isoform.** *a*, scheme shows the structural organization of TNC and the localization of the primer pairs used in this study to amplify the splicing domain of TNC. *b*, proteins of NSCs were analyzed by Western blotting with anti-HNK-1, anti-TNC, or anti-β-actin antibodies. Western blot analysis with anti-TNC antibody shows the existence of some alternatively spliced variants in NSCs (indicated by *arrowheads*). The largest TNC variant possesses the HNK-1 epitopes resulting from Western blotting with anti-HNK-1 antibody (indicated by an *open arrowhead*). β-Actin was detected as a loading control. An *asterisk* denotes nonspecific bands. *c* and *d*, isoform expression of TNC was analyzed by RT-PCR using a specific primer pair (shown in *a*) under a condition including primer sequences described previously (30). *e*, mRNA expression of TNC, Sox2, β-actin, HNK-1ST, GlcAT-S, GlcAT-P, Pax6, and Sam68, in undifferentiated (*undiffer*) NSCs and differentiated (*differ*) cells was analyzed by RT-PCR. Sox2 was detected as maker gene of undifferentiated NSCs. β-Actin was used as a control.

excised, digested with trypsin, and then subjected to LC-MS/MS analysis. The data indicated that the 280-kDa protein positive for HNK-1 epitopes is an ECM protein, TNC (Fig. 3*b*). The observed sequence covered 33.7% of the TNC. For confirmation, the TNC in NSCs was immunoprecipitated with anti-TNC antibody and analyzed by Western blotting with anti-HNK-1 antibody. As shown in Fig. 3*c*, HNK-1 epitopes were clearly detected in TNC-positive immunoprecipitates. To evaluate the localization of HNK-1 epitopes and TNC in NSCs, we double-stained NSCs prepared from neurospheres with anti-HNK-1 and anti-TNC antibodies. As shown in Fig. 3*d*, HNK-1 signals were co-localized with TNC in NSCs. This result suggests that HNK-1-carrying TNC is expressed on the surface of NSCs.

Expression of HNK-1-containing Glycans on the Largest TNC Spliced Variant—TNC molecules exist as alternatively spliced isoforms resulting from six kinds of alternatively spliced domains (Fig. 4*a*). Given that HNK-1-carrying TNC was not expressed in cells differentiated from NSCs, we next examined the expression of HNK-1 on alternatively spliced variants of TNC during differentiation by Western blotting with anti-TNC antibody (Fig. 4*b*). By anti-TNC antibody, several spliced variants were detected before and after differentiation. The expression of the largest TNC molecule (see *open arrow*, Fig. 4*b*) is drastically decreased in cells differentiated from NSCs compared with other isoforms. Those data suggested that HNK-1 epitopes were carried by the largest spliced variant in NSCs. To investigate further the expression of TNC variants in NSCs, we conducted an RT-PCR-based approach developed in a previous study (30). By using the two primer pairs designed to span the alternatively spliced domains (Fig. 4*a*), we predicted the sizes of the splicing variants. As shown in Figs. 4*c* and 4*d*, mRNA expression of the largest variant, consisting of 6 fibronectin III (FNIII) domains was down-regulated in cells differentiated from NSCs, despite the absence of any change in the level of mRNA of the TNC during differentiation (Fig. 4*e*). Those data are consistent with the result of the Western blot analysis (Fig. 4*b*). It has been reported that the expression of TNC spliced variants was regulated by a transcription factor, *Pax6* (Paired box gene 6) and an RNA-binding protein, *Sam68* (Src-associated in mitosis, 68 kDa) (36, 37). Accordingly, we analyzed the mRNA levels of *Pax6* and *Sam68* in NSCs before and after differentiation. Both genes were down-regulated after differentiation, especially *Pax6* (Fig. 4*e*). On the other hand, the mRNA levels of three key enzymes involved in the biosynthesis of HNK-1 epitopes, glucuronyl transferase (GlcAT)-P, GlcAT-S, and HNK-1ST showed no changes before and after differentiation (Fig. 4*e*). Taking those data together, the expression of HNK-1 in NSCs was controlled predominantly by TNC spliced variants, probably via the *Pax6* pathway.

Effects of TNC and HNK-ST Knockdown by siRNA on Proliferation of NSCs—In previous reports, TNC is associated with the proliferation of NSCs (38, 39). To evaluate the potential role of HNK-1 on TNC in NSC proliferation, we knocked down TNC or HNK-1 expression by transfecting NSCs with TNC or HNK-1ST siRNAs, respectively. After 72 h of siRNA treatment, cells were collected and analyzed by Western blotting for the expression of HNK-1 epitopes and TNC (Fig. 5*a*). We observed a reduction in TNC and HNK-1 expression on the individual

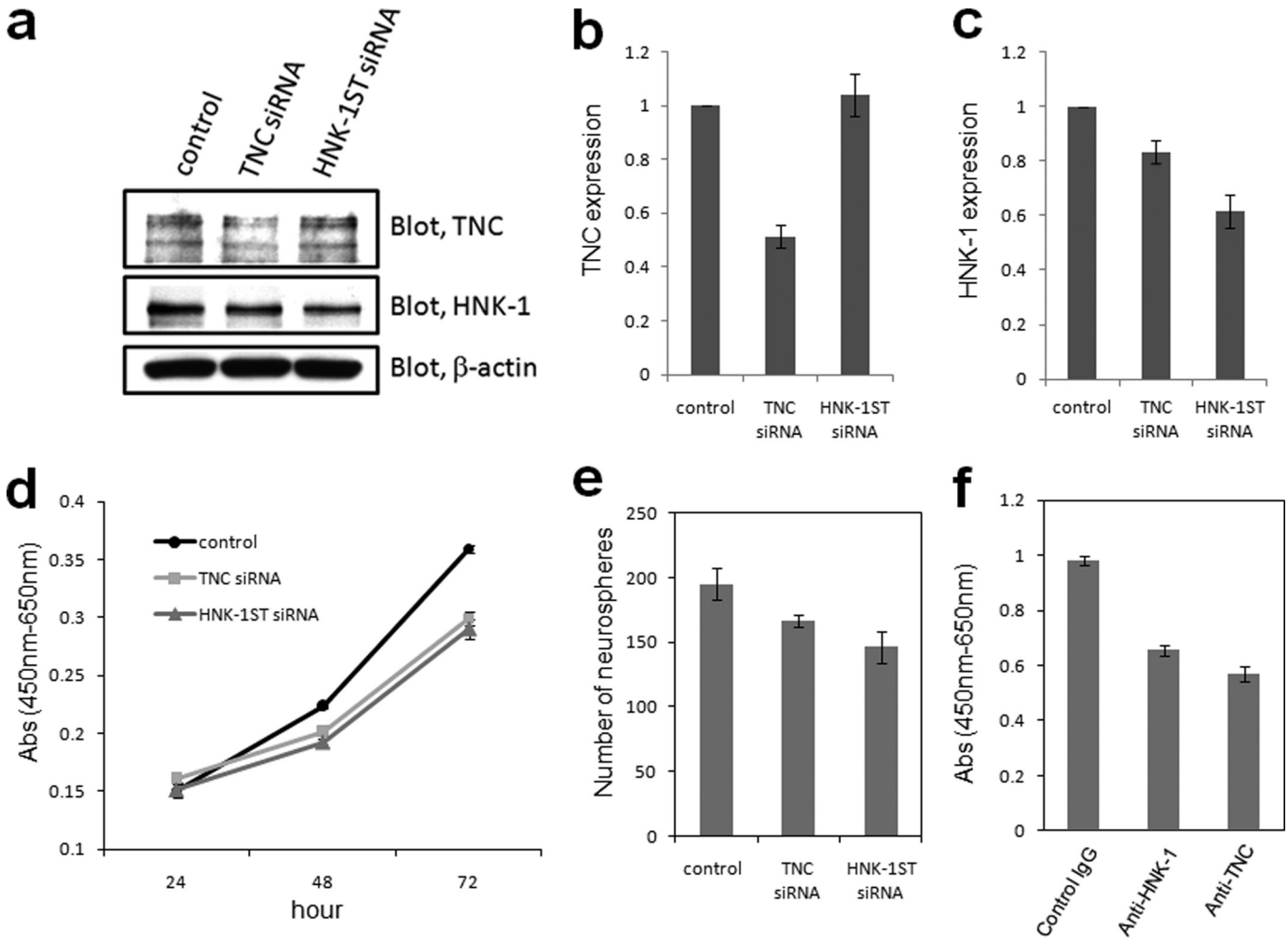

FIGURE 5. **Effect of HNK-1ST or TNC knockdown by siRNA.** *a*, Western blot of NSCs treated with HNK-1ST, TNC, or negative control siRNAs. Samples were collected after 72 h of siRNA treatment and then subjected to Western blot analysis with anti-HNK-1, anti-TNC, and anti-β-actin antibodies. β-Actin was detected as a loading control. Expression levels of TNC (*b*) and HNK-1 (*c*) were measured by densitometric analysis using a scanning densitometer ($n = 3$). *d*, effects of knockdown of TNC or HNK-1ST by siRNA on proliferation of NSCs. NSCs transfected with HNK-1ST, TNC, or negative control siRNAs were cultured as neurospheres for 72 h ($n = 4$). The proliferation rates of neurosphere-forming cells were measured by WST-8 assay at 24, 48, 72 h after siRNA transfection. *e*, number of neurospheres formed by siRNA-transfected NSCs counted at 72 h after lipofection ($n = 4$). *f*, proliferation rates of neurosphere-forming cells in the presence of control IgG, anti-HNK-1 or anti-TNC antibodies (20 μg/ml) estimated by WST-8 assay at 72 h *in vitro* ($n = 3$). The anti-HNK-1 and anti-TNC antibodies inhibit the proliferation of NSCs.

cells transfected with TNC or HNK-1ST siRNAs (Fig. 5, *b* and *c*). Notably, the HNK-1 expression level on the cells transfected with TNC siRNA was decreased (Fig. 5*c*), which is consistent with the result that HNK-1 epitopes are attached to TNC molecules in NSCs. We examined the growth ability of these NSCs transfected with siRNAs. We measured the number of NSCs by a highly sensitive and reproducible method, the WST-8 assay; the spectrophotometric absorbance measured by this assay is known to be highly correlated with the number of living NSCs (32, 33). Transfection of TNC or HNK-1ST with siRNA reduced the number of NSCs (Fig. 5*d*). In addition, it reduced the formation of neurospheres (Fig. 5*e*). In support of those data, antibodies against HNK-1 and TNC also reduced the number of NSCs (Fig. 5*f*). On the other hand, transfection of TNC or HNK-1ST siRNA did not increase the number of NSCs positive for TUNEL, an indicator of cell death accompanied with DNA fragmentation (Fig. 6*a*). In NSCs transfected with TNC or HNK-1ST siRNAs, caspase 3, a critical executioner of apoptosis or programmed cell death signaling, was not activated (Fig. 6*b*). The phosphoinositide 3-kinase-Akt pathway important for cell survival was not repressed (Fig. 6*b*). Therefore, our knockdown analysis revealed that HNK-1 on TNC molecules plays a role in proliferation, but not in cell death or cell survival, of NSCs.

We then analyzed the molecular mechanism underlying the inhibition of NSC proliferation by knockdown of HNK-1. It has been reported that TNC knock-out mice exhibited a delayed expression of the EGF receptor in NSCs (39). Also, in NSCs transfected with TNC or HNK-1ST siRNAs, a significant decrease of EGF receptor was detected (Fig. 7, *a* and *b*). It is known that proliferation of NSCs induced by growth factors is dependent on the Ras-MAPK pathway (5, 7). In fact, EGF-induced proliferation of NSCs was dose-dependently inhibited by U0126, an inhibitor of MAPK kinase (Fig. 7*d*). Interestingly, the activation of extracellular signal-regulated kinase 1 (ERK1) and ERK2 (MAPKs) was slightly inhibited in NSCs transfected with TNC or HNK-1ST siRNAs (Fig. 7, *a* and *c*). These results suggest that HNK-1 on TNC is involved in the proliferation of

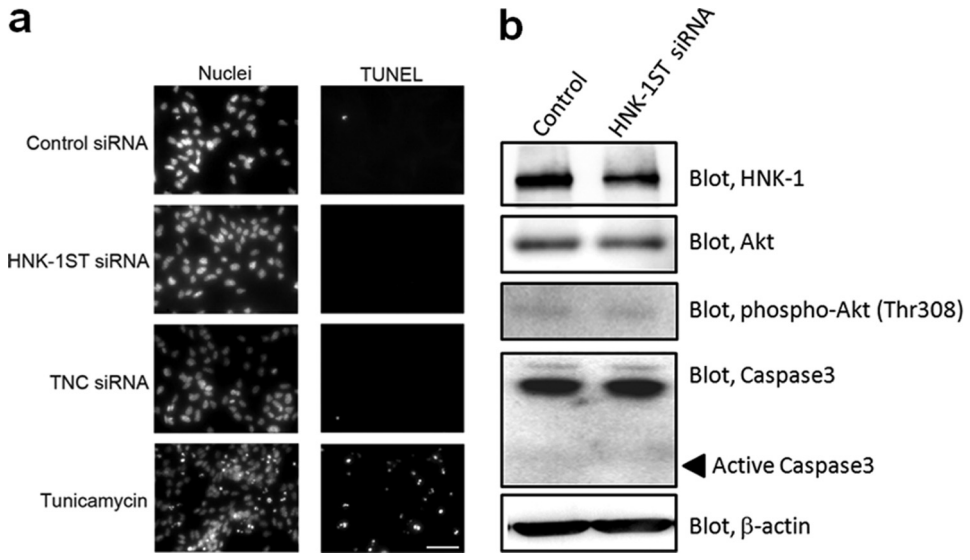

FIGURE 6. **Cell death and cell survival in HNK-1 knockdown NSCs.** *a*, cell death of NSCs transfected with HNK-1ST or TNC siRNA was evaluated by TUNEL assay. Tunicamycin was used as a control inducer of cell death (1 μg/ml for 10 h). There is no difference in the numbers of TUNEL$^+$ cells between control siRNA, HNK-1ST siRNA, and TNC siRNA-transfected cells. *Scale bar*, 25 μm. *b*, activation of cell survival signaling and cell death signaling pathways (phosphoinositide 3-kinase-Akt pathway and caspase pathway, respectively) in NSCs transfected with HNK-1ST siRNA was evaluated by Western blotting with antibodies to Akt, phospho-Akt, caspase 3, and β-actin. The cell survival signaling and cell death signaling pathways in NSCs were not inhibited or activated by transfection with HNK-1ST siRNA.

NSCs by modulating the expression level of EGF receptor and the activation of downstream signaling.

DISCUSSION

The HNK-1 epitopes are predominantly expressed in the nervous system and involved in intercellular adhesion, cell migration, and synaptic plasticity. There has not been any description of the expression of HNK-1 in NSCs, however, except for our study in which we found that HNK-1 epitopes were expressed in NSCs (Fig. 2*a*). Immunocytochemical analysis revealed that most NSCs expressed the HNK-1 epitopes (Fig. 3*d*). Furthermore, the HNK-1 epitopes were ablated in cells differentiated from NSCs (Fig. 2*a*). Those data suggested that HNK-1 can be useful as a suitable biomarker for identifying NSCs.

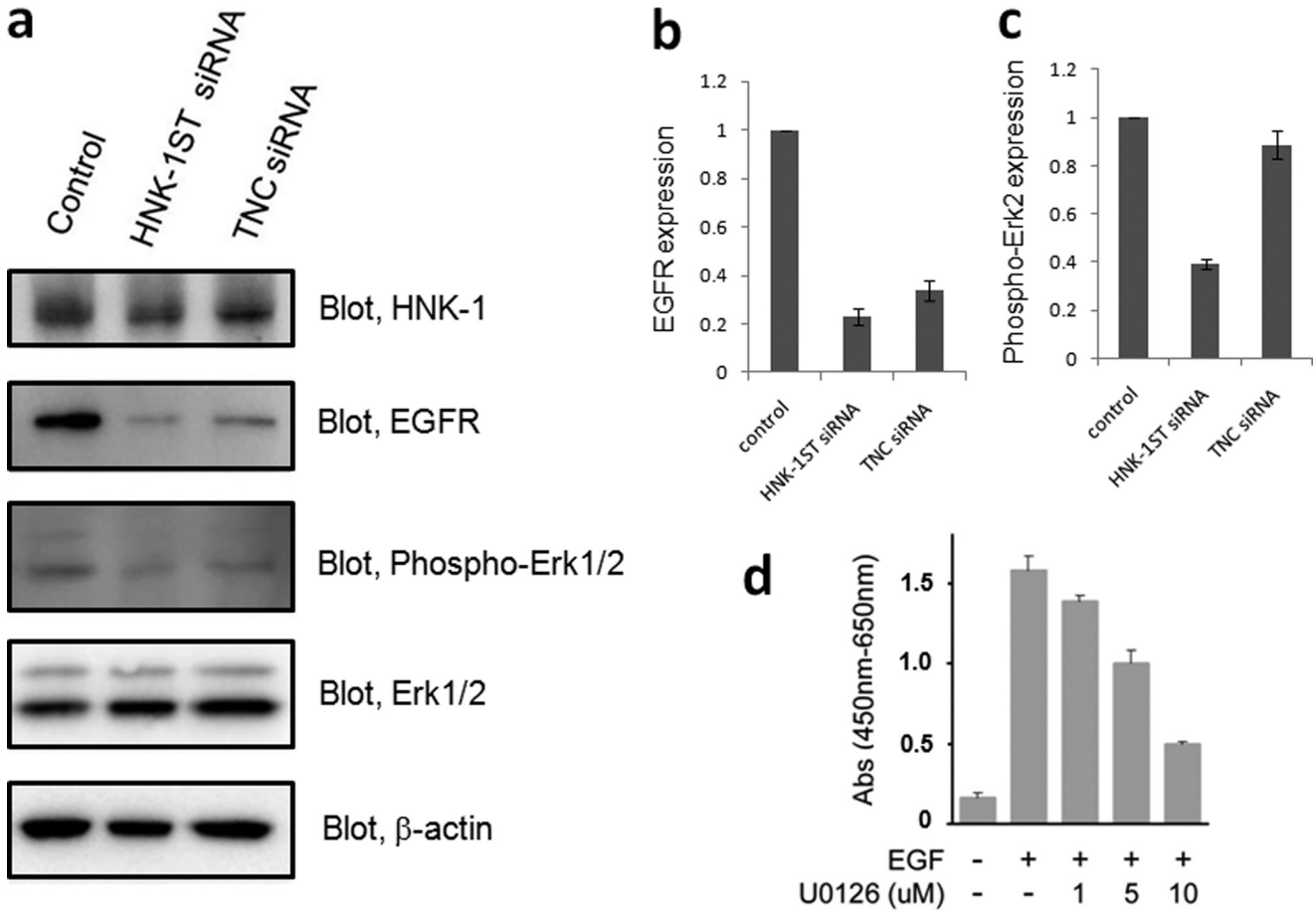

FIGURE 7. **Down-regulation of EGF receptor and inhibition of the Ras-MAPK pathway in NSCs transfected with HNK-1ST or TNC knockdown by siRNA.** *a*, Western blot of NSCs treated with HNK-1ST, TNC, or negative control siRNAs using anti-HNK-1, anti-EGF receptor (*EGFR*), anti-phospho-ERK1/2, anti-ERK, and anti-β-actin antibodies. The expression levels of EGF receptor (*b*) and phospho-ERK2 (*c*) were measured by densitometric analysis using a scanning densitometer ($n = 3$). *d*, importance of the Ras-MAPK pathway in EGF-induced proliferation of NSCs. NSCs were cultured in the presence or absence of EGF (10 ng/ml) and U0126, an inhibitor of MAPK kinase, for 72 h ($n = 3$). The proliferation rates of neurosphere-forming cells were measured by WST-8.

By proteomic analysis using LC-MS/MS, we identified TNC as a major carrier protein of HNK-1 in NSCs. TNC is well known as an ECM protein that is expressed prominently during brain development in the olfactory bulb, ventricular zone, and subventricular zone (38, 40, 41). In mouse brain tissues, TNC possesses six alternatively spliced domains (Fig. 4*a*), leading to a large variety of TNC variants (30). It has been reported that NSCs/neural precursor cells express 20 kinds of TNC isoforms whose expression is regulated by transcription factors *Pax6* and *Sam68* via independent pathways (36, 37). In the present study, HNK-1 carried by TNC was ablated in differentiated cells, due to down-regulation of expression of the largest TNC variant (Fig. 4*b*). Additionally, our RT-PCR analysis indicated that the expression of HNK-1 epitopes is dependent on the existence of the largest spliced variant but not by the enzymes involved in biosynthesis of HNK-1, via predominantly the *Pax6* signaling pathway (Fig. 4, *c*–*e*). A previous report has indicated that HNK-1 epitopes were ectopically located in forebrain tissues of a *Pax6* mutant rat (42). A part of the ectopic expression of HNK-1 might be responsible for down-regulation of the largest TNC resulting from the lack of *Pax6*.

TNC possesses 20 putative *N*-glycosylation sites. By PNGase F treatment (Fig. 2*b*), we demonstrated that HNK-1 epitopes on TNC are carried by *N*-glycans as well as by *O*-glycans. In practice, we detected only two peptides consisting of putative *N*-glycosylation sites. Because the algorithm we used did not identify glycopeptides, the possibility is raised that the undetectable peptides carry *N*-glycans and/or *O*-glycans. In this study, we demonstrated that HNK-1 is expressed in NSCs on the largest TNC variant with six FNIII domains. The TNC variant with five FNIII domains consists primarily of FNIII domains A1, A2, A4, B, and D and lacks domain C, which exhibits the CNS-specific expression resulting from *in situ* hybridization in mouse embryonic day 15 (37, 43). Furthermore, domain C predominantly comprises the largest spliced valiant with six FNIII domains in embryonic day 13 mouse brain (30). Domain C possesses one putative *N*-glycosylation site and several serine or threonine residues as potential *O*-glycosylation sites (Fig. 3*b*). Taking those data together, we expected that domain C is essential for expression of HNK-1 in NSCs. TNC, a multidomain protein, interacts with a number of ECM or cell surface proteins, including proteoglycans and membrane-associated proteins, to modulate cell adhesion, migration, proliferation, and neurite outgrowth (44, 45). Our knockdown analysis revealed that HNK-1 on the largest TNC variant participates in modulation of the proliferation and neurosphere-formation of NSCs (Fig. 5, *d* and *e*). In a previous study, TNC knock-out mice exhibited a delayed expression of the EGF receptor in NSCs both *in vivo* and *in vitro*, which led to a reduction of forming neurospheres in *in vitro* culture (39). We revealed that the HNK-1 or TNC knocked-down cells exhibited down-regulation of EFGR, giving rise to the inhibition of the Ras-MAPK pathway (Fig. 7). Therefore, there is a strong possibility that HNK-1 epitopes are key molecules on TNC to induce the TNC-signaling pathway to regulate cell proliferation.

It has been reported that TNC is expressed in the ventricular zone and subventricular zone in mice throughout embryonic stages to adulthood and is involved in proliferation and migration of neural precursor and neural stem cells (39, 40). The HNK-1 carbohydrate epitope is widely expressed in adult mouse brain regions, including the cortex and hippocampus (24) and mainly expressed in marginal zone along with ventricular zone and subventricular zone in rat embryonic brains (46). These data strongly suggest that the HNK-1-possessing TNC molecules are located in neural stem cells in the ventricular zone and subventricular zone in embryonic and adult brains.

In the nervous system, several receptors for HNK-1 (*e.g.* amphoterin, laminin, and lectican) function through the interaction of HNK-1 (47–49). Those receptors and TNC were located on the ECM of the cell surface. Furthermore, a number of adhesion molecules, such as neural cell adhesion molecule, are composed of many immunoglobulin fold domains, which have a potential of recognizing carbohydrate moieties (22). Based on our data and those reported in the literature, we conclude that possible interactions must exist between the HNK-1 epitopes and their ligand molecules to maintain the stemness and proliferation of NSCs.

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