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Expression of polysialylated neural cell adhesion molecules on adult stem cells after neuronal differentiation of inner ear spiral ganglion neurons

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ABSTRACT

During brain development, polysialylated (polySia) neural cell adhesion molecules (polySia–NCAMs) modulate cell–cell adhesive interactions involved in synaptogenesis, neural plasticity, myelination, and neural stem cell (NSC) proliferation and differentiation. Our findings show that polySia–NCAM is expressed on NSC isolated from adult guinea pig spiral ganglion (GPSG), and in neurons and Schwann cells after differentiation of the NSC with epidermal, glia, fibroblast growth factors (GFs) and neurotrophins. These differentiated cells were immunoreactive with mAb's to polySia, NCAM, β-III tubulin, nestin, S-100 and stained with BrdU. NSC could regenerate and be differentiated into neurons and Schwann cells. We conclude: (1) polySia is expressed on NSC isolated from adult GPSG and on neurons and Schwann cells differentiated from these NSC; (2) polySia is expressed on neurons primarily during the early stage of neuronal development and is expressed on Schwann cells at points of cell–cell contact; (3) polySia is a functional biomarker that modulates neuronal differentiation in inner ear stem cells. These new findings suggest that replacement of defective cells in the inner ear of hearing impaired patients using adult spiral ganglion neurons may offer potential hope to improve the quality of life for patients with auditory dysfunction and impaired hearing disorders.

1. Introduction

Regeneration of sensory neurons can occur in the inner ear of human and adult animals [1,2]. In the vestibular organ and the spiral ganglia of adults, cells showing the identical phenotypic characteristics of neural stem cells (NSCs) have been identified [3]. These adult NSCs form neurospheres that are characterized by self-renewal and can differentiate into neurons and glia cells [1].

In the proliferation and differentiation of neural stem cells, distinct cell surface glycoconjugates including, glycoproteins, glycosphingolipids, and extracellular matrix molecules play a critical role in regulating development and differentiation [4,5]. A well-studied member of this class of cell surface glycoproteins is the polysialic acid (polySia) that posttranslationally modifies neural cell adhesion molecules (NCAMs). The polySia glycan is an extended, linear homopolymer consisting of α2,8-ketosidic linked residues of N-acetylneuraminic acid (Neu5Ac; Sia). Their degree of polymerization (DP), or chain length, can exceed 400 Sia residues when accurately determined in the absence of acid hydrolysis [6,7]. Thus, polySia is a large polyanionic "space filling" molecule that functions as an anti-adhesive glycan on cell–cell and cell–matrix interactions [7–10].

The predominant protein carrier of polySia in brain is NCAM, although this glycotope is also expressed on a restricted set of other glycoproteins including the α-subunit of the sodium channel in adult rat brain [11], neuropilin-2 in human dendritic cells [12] and on CD36, a human milk glycoprotein [13]. Autopolysialylation of the two mammalian polysialyl transferases, ST8Sia II (STX) and ST8Sia IV (PST), has also been described [14,15]. More recently, the synaptic CAM, SynCAM1 (*Cadm1*), which is expressed selectively on NG2 glia cells, has been reported to be polysialylated and to modulate SynCAM 1 function [9,16].

The embryonic (E) or heavily polysialylated form of NCAM is a cell surface glycoprotein that modulates many key functional interactions between cells, including cell–cell and cell–matrix adhesion, neural migration, neurite outgrowth, fasciculation, axon

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path finding, synaptic plasticity, cell signaling/cytokine response and myelination [9,17–19]. More recent studies showed polySia was expressed on human and murine leukocytes and to regulate immune responses [6]. It is also expressed on human dendritic cells where it modulates T-lymphocyte–dendritic cell interactions [12]. While expression of the polysialylated form of NCAM is usually restricted to early stages of embryonic and postnatal development [10,20], it is persistently expressed in selective regions of adult brains that are associated with synaptic plasticity and neurogenesis, including the hippocampus, hypothalamus, dentate gyrus and olfactory bulb. A further important role for the polySia glycan is its support of dynamic changes associated with peripheral nerve regeneration [21].

Due to its polyanionic charge, the polySia chains that modify N-linked glycans on NCAM prevents both the homophilic and heterophilic binding interactions between NCAM expressing cells [9,22]. In this context, polySia functions as an anti-adhesive glycotope preventing cell adhesion and cell migration. As such, re-expression of polySia on a number of adult human cancers allows the detachment of these cells from their original tumor site, thereby aiding their malignant potential by facilitating metastatic spread [10,23–26].

The cell surface expression of polySia is developmentally regulated in both the central nervous system (CNS) and the peripheral nervous system (PNS). Thus, growth and migration of nerve cells, fasciculation, synapse formation, and myelination processes occur as noted above [10,11,27,28]. Polysialylated NCAM is also a known neurological marker in neural stem cells formed in the CNS and is involved in their migration and differentiation [29,30]. While these latter studies focused primarily on differentiation of CNS cells, there is a dearth of information on the role of adult neural stem cells present in the inner ear. Accordingly, the objective of this study was to determine if polySia–NCAM was expressed on adult NSC isolated from guinea pig spiral ganglion, and in neurons and Schwann cells differentiated from these stem cells. Our new findings show that polysialylated NCAM is expressed on these adult stem cells, and in neurons and Schwann cells after differentiation with epidermal, glia, fibroblast growth factors (GFs) and neurotrophins. The results suggest a functional role for polySia in neuronal differentiation in inner ear stem cells, a finding that has not been previously reported.

2. Materials and methods

2.1. Isolation and culturing of guinea pig spiral ganglion neurons

Spiral ganglion neurons were isolated from adult guinea pigs weighing ca. 300 g. The animals were first anesthetized with pentobarbital by infusion via the intra-abdominal route. Spiral ganglia were obtained by dissection and transferred to a tube containing Dulbecco's modified Eagle's media (DMEM; Gibco, Carlsbad, CA, USA, 41966). The rest of procedures follow as described in Rask-Andersen et al. [1].

2.2. Immunofluorescent histochemical staining of neurospheres differentiated from cultured spiral ganglion neurons

To examine the neurospheres obtained from the cultured spiral ganglia for cell proliferation, they were transferred to a 6-well slide, the surface of which was protected with a cover slide. 3.5 μL of 10 mM bromodeoxyuridine (BrdU; ABD Serotec; Raleigh, NC, USA; Cat. No. MCA 2060) were added and the cultures incubated for 4–12 h at 37 °C in 5% CO_2 atmosphere.

After incubation, the differentiated cells were embedded in 4% paraformaldehyde (Sigma, St. Louis, MO, USA) and washed with PBS. For the first 5 min, cells were incubated with 0.2% Triton-X100 in PBS (Junsei Chemical, Tokyo, Japan) followed by 2 M HCl for 1 h at room temperature and then for 5 min with 0.1 M $Na_2B_4O_7$ (Sigma, St. Louis, MO, USA) before rinsing with PBS. Blocking was carried out using 10% goat serum (Vector Lab, Inc., Peterborough, England,) for 5 h at room temperature.

For the double antibody staining experiments, antibodies specific for BrdU, Nestin (1:200; Chemicon; Cat. No. MAB 353), polysialylated NCAM (1:100; Miltenyl Biotec, Bergisch Gladbach, Germany, Cat. No. 130–093-274), non-polysialylated NCAM (1:100; Abacam, Cat. No. ab8233), β-III tubulin (1:100; Abcam, Cat. No. ab6046), and S-100 (1:100; Sigma, St. Louis, MO, USA) were used. Cells were added to PBS containing 10% goat serum and incubated with the primary antibodies at 4 °C for 12 h, before rinsing with PBS. The secondary antibodies, including the cyanine-conjugated antibody, goat anti-rat IgG antibody (Alexa Fluor-red, Cambridge, England), sheep anti-mouse Cy2 (Cy2-green color for nestin (Jackson ImmunoResearch, Baltimore, MD USA), NCAM (Abcam,), β-III tubulin, (Abcam), and goat anti-rabbit fluorescein isothiocyanate (FITC, green color for S-100; (Abcam, diluted 1:200) were added and incubated in PBS – 10% goat serum. After rinsing with PBS, mounting was carried out using the mounting medium for fluorescence (Vector Lab, Inc.). For nuclear staining, 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc.) was used. For nuclear staining, whole mounting was done using DAPI-conjugated mounting medium (VECTASHIELD, Burlingame, CA, Cat. No. H01200).

2.3. Immunohistochemical staining of cells differentiated from spiral ganglion stem cell neurospheres

Cells that were differentiated from the adult cultured spiral ganglion stem cells were prepared for immunohistochemical staining using antibodies specific for NCAM (1:100; Sigma), polysialic acid (1:100; Miltenyl Biotec (as above), β-III tubulin (1:300; Chemicon, Millipore, Billerica, MA USA), and S-100 (1:100; DAKO, Glostrup, Denmark). After sub-culturing for one week in the cell differentiation medium, the cells were repeatedly washed with PBS by re-suspension before embedding in 4% paraformaldehyde in PBS (10 min).

For the staining of neurons and Schwann cells obtained after differentiation of the neurospheres as described above, the primary antibodies were diluted in PBS containing 1% bovine serum albumin (BSA). After addition of the primary antibody, cells were incubated for 1 h at room temperature before rinsing three times for 5 min each with 1% saponin in PBS. The secondary antibodies were added and incubated at room temperature for 30 min, before adding the avidin-horseradish peroxidase (Vectastatin ABC kit, (Vector Laboratories) for visualization in the light microscope.

3. Results

3.1. Growth of adult neural stem cells isolated from guinea pig spiral ganglion neurons

After isolation of the adult neural stem cells (NSC) from guinea pig spiral ganglia (GPSG) and three sub-passages in a growth medium containing epidermal growth factor (EGF) and fibroblast growth factor (FGF), cell colonies with a round-shaped phenotype characteristic of neurospheres were obtained. These neurospheres were capable of self-renewal upon sub-culturing. The population of neurospheres expressed both polysialylated and non-polysialylated NCAMs, markers known to be associated with neural stem cells (Fig. 1) [31,32].

To examine cells that were differentiated from these neurospheres, differentiation was induced by growth in a culture medium containing brain derived neutrophic factor (BDNF), glia-derived neutrophic factor (GDNF), and neutrophic factor 3 (NT-3). After induction and growth for 1 week, differentiated cells co-expressed both β -III tubulin, a marker for nerve cells, S-100, a marker for neuro glial cells and were positive for BrdU. Taken together, these findings support the conclusion that these cells were derived from the differentiated neurospheres (Fig. 2).

3.2. Expression of polysialic acid and NCAM on cells derived from the differentiated adult neural stem cells

Immunohistochemical staining of the differentiated neurons showed they all co-expressed polysialylated NCAM. NCAM expression was most evident over the axon or growth cone and the cell body of the neuron (Fig. 3A). Strong expression of the polySia glycan was readily apparent in the cell body of the neurons. There was an attenuated level of polySia expression relative to NCAM in those cells in which the axons were generated (Fig. 3B and C).

Immunohistochemical staining of the Schwann cells showed that NCAM was expressed in approximately one-third of the cells. As shown in Fig. 4A, the extent of this expression was lower than in the neurons. As shown in Fig. 4B, however, the extent of NCAM expression was greater in the axons that were at the junction between neurons and Schwann cells. Only low levels of polySia expression was detected on the differentiated Schwann cells (Fig. 4C). This finding supports the earlier seminal studies of Jung-nickel, Grothe and colleagues showing that the persistent expression of polySia in Schwann cells impairs the recovery of peripheral nerve regeneration. It is also consistent with earlier studies that during peripheral nerve development polySia is expressed on growing axons but its expression is attenuated in mature, myelinated axons and that in adults, polySia is restricted to a subset of small unmyelinated axons [21].

4. Discussion

The proliferation, self-renewal, differentiation, and survival of neural stem cells are determined by multiple growth factors and signals produced by cells in the surrounding environment. In these signal transduction pathways, a number of important cell surface glycoconjugates are essential molecular players. Chief among the adhesive and signal transduction glycans are the polysialylated NCAM glycoproteins that decorate the cell surface of both precursor stem cells and some mature neuronal cells within the central nervous system [5].

As shown by Stoenica et al. in 2006 [33] polysialylated NCAM is required for synaptic plasticity in the dentate gyrus of mice. PolySia–NCAM also regulates the formation of neuritic processes in other regions of the adult brain where neural regeneration occurs, including the subventricular zone, olfactory bulb, and hippocampus [33]. PolySia–NCAM is persistently expressed in these regions. This represents a critically important process in the growth and development of the brain. Expression of polySia–NCAM is also involved in increasing the plasticity of nerve cells in both axonal regeneration and stimulating the number of neuronal progenitor cells [5,34]. PolySia has also been shown to enhance the migration of stem cells and thereby increase repair within the nervous system [35,36]. The over expression of the polySia glycan has also been shown to delay differentiation of premature cells in the CNS [4].

While expression of polySia–NCAM on NSC and on cells differentiated from the CNS are well known, there is a paucity of information regarding the expression and function of this polysialylated glycan in the peripheral nervous system. For example, there has

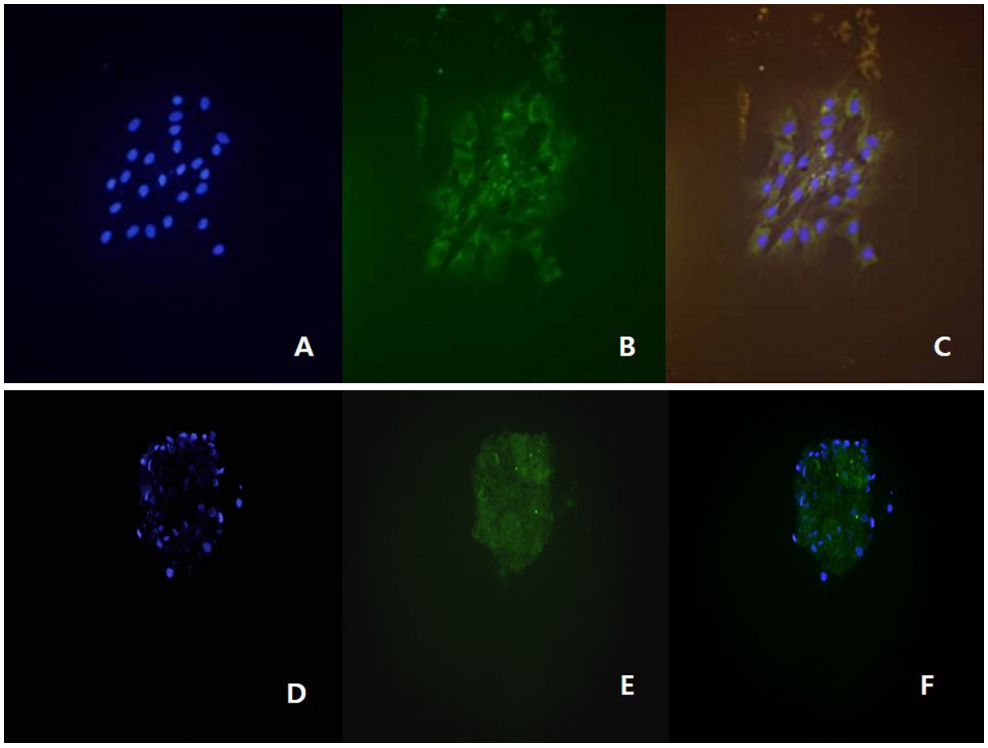

Fig. 1. *Immunofluorescent staining of* neural cell adhesion molecules (NCAM, A, B, C) & polysialic acid (polySia, D, E, F) expressed on cultured adult neural stem cells derived *from guinea pig spiral ganglion neurons.* (A, D) IF image of BrdU stained neurospheres, a marker for cell proliferation (control). (B) IF image of neurospheres stained with an anti-NCAM antibody specific for detecting the protein moiety of NCAM. (E) IF image of neurospheres stained with an anti-polysialic acid antibody specific for detecting the polySia moiety of NCAM. (C, F) IF images that merge panels A + B and D + E. (Image magnifications, ×400 for A, B, C/×200 for D, E, F).

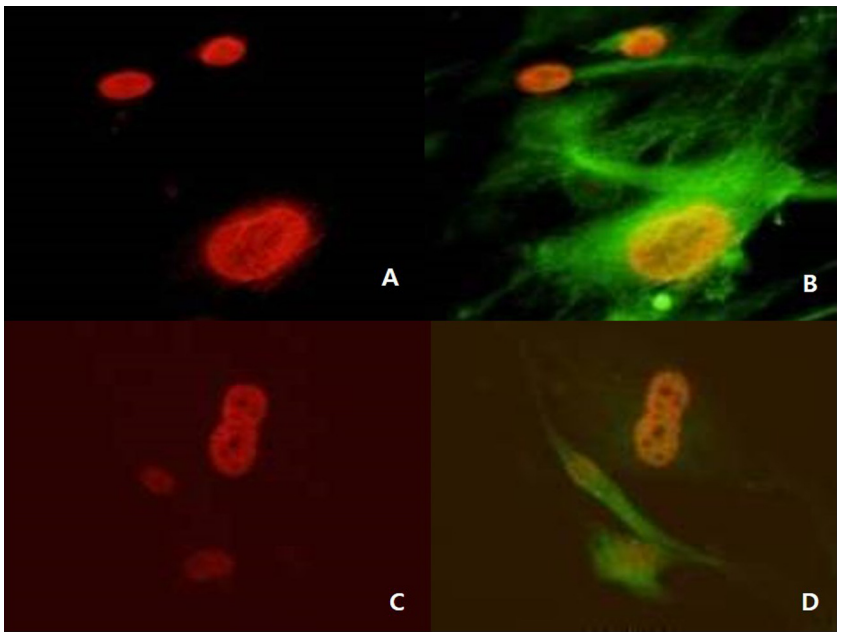

Fig. 2. Immunofluorescent (IF) staining of neurons and Schwann cells derived from the neurospheres after cellular differentiation. (A, C) IF image of neurons after staining with BrdU, a marker for cell proliferation (control). (B) IF image of neurons after staining with BrdU and β-III tubulin, a marker for detection of neural cells. (D) IF image of Schwann cells after staining with BrdU and S-100, a cell marker for neuro glial cells. (Image magnifications, ×400).

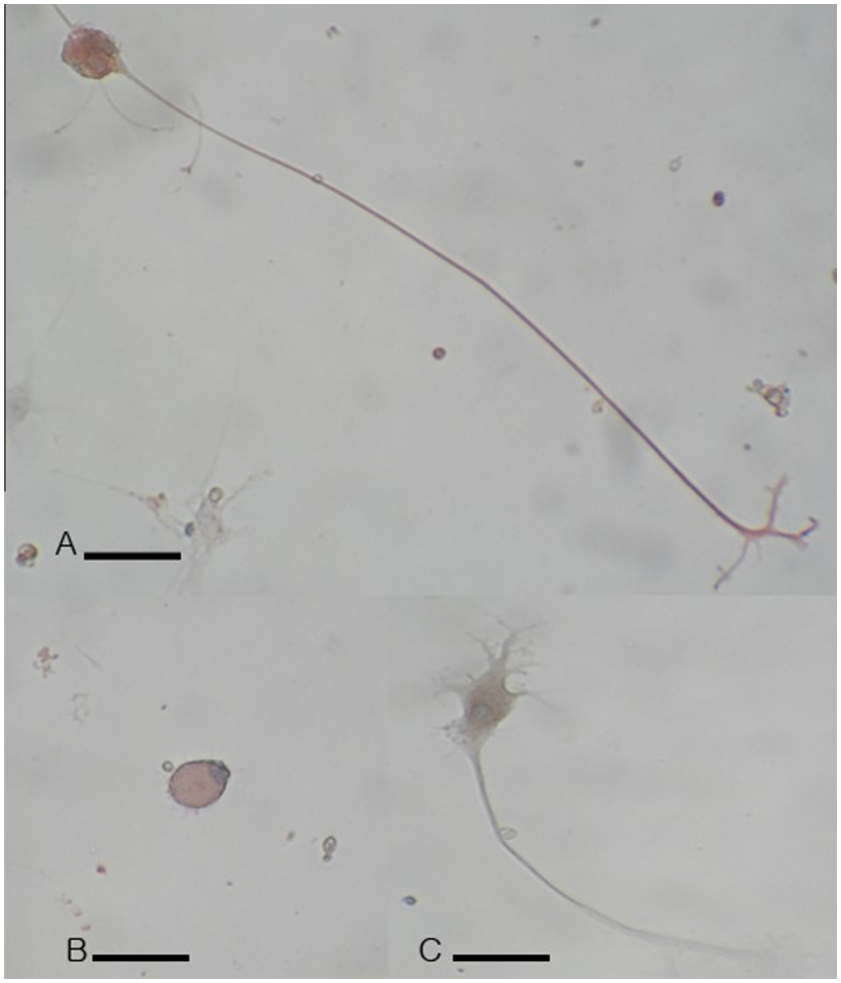

Fig. 3. Immunocytochemical staining of neurons derived from the neurospheres after differentiation with anti-NCAM and anti-polySia antibodies. (×200; scale bar, 60 μm) (A) ICC staining of neurons with an anti-NCAM antibody showing strong NCAM expression in the cell body, axon and growth cone. (B, C) ICC staining of neurons with an anti-polySia antibody showing expression of polySia in the early stage of development of the neuron, and which is strongest in the cell body.

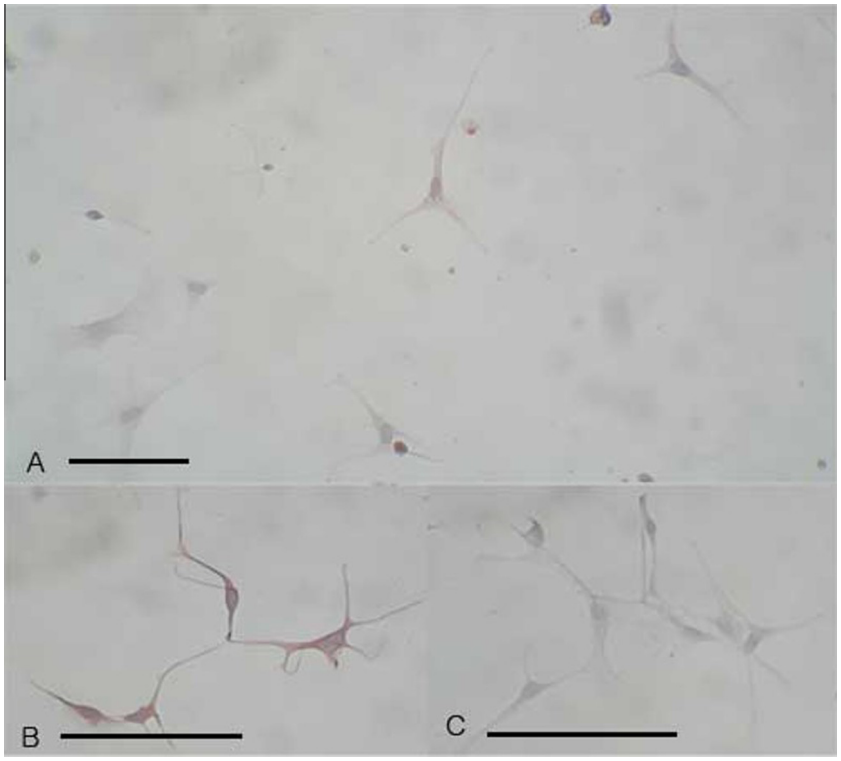

Fig. 4. Immunocytochemical staining of Schwann cells derived from the neurospheres after differentiation with anti-NCAM and anti-polySia antibodies. (×200; scale bar, 100 μm). (A) ICC staining of Schwann cells with anti-NCAM. Approximately one-third of the Schwann cells express NCAM. The level of NCAM expression on Schwann cells is lower compared with the level expressed on neurons. (B) ICC staining of Schwann cells with anti-NCAM. These cells show relatively strong expression of NCAM at points of cell–cell contact. (C) ICC staining of Schwann cells with an anti-polySia antibody. Schwann cells obtained from neurospheres after differentiation have undetectable levels of polySia.

been skepticism even regarding the presence of stem cells in the inner ear because of the irreversibility of hearing loss in human and mammals. It has been reported, however, that regeneration of new hair cells occurs in the damaged vestibular organ [37,38]. In 2003, Li et al. isolated stem cells from the macula utriculi of adult mice. Further, several studies have reported the isolation and growth of neural stem cells from the olfactory organs, the organ of Corti, and spiral ganglia [1,39]. However, despite studies showing the presence of stem cells in the inner ear, no studies have been carried out to determine if polySia–NCAM is expressed on these stem cells, and/or on their differentiated neuronal cells.

In the present study, we showed that neurospheres isolated from adult guinea pig spiral ganglia synchronously express BrdU, a marker for proliferating cells, nestin, a marker for neuro-derived stem cells and polysialylated-NCAM. Expression of the polySia glycan appeared relatively stronger than NCAM, possibly due to the greater sensitivity of the anti-polySia antibody to detect the polySia glycan, compared with the anti-NCAM antibody to detect the protein moiety. Based on the findings that NCAM was expressed on both nerve and Schwann cells differentiated from the neurospheres, this result suggests that NCAM alone is not a reliable marker for detection of neural stem cells differentiated from inner ear spiral ganglia. In contrast, polySia is a sensitive marker, since it is expressed in the early embryonic stage of neuronal and neuro glial cell development [40]. In addition, our present studies have showed that polySia was expressed principally during early development on axons generated from the nerve cells, as well as on the neurospheres. On this basis, we conclude that polySia is a sensitive biomarker for adult neural stem cells derived from inner ear spiral ganglion neurons.

As reported herein, the attenuated level of polySia expression on Schwann cells, and the relative stronger expression of NCAM depends on both the embryonic stage of differentiation and the intercellular contacts associated with myelination, fasciculation, and the formation of ganglion. These differences in the level of polySia and NCAM expression are in accord with studies showing that polySia expression is reduced in mature myelinated axons and is restricted to a subset of myelinated axons in adults during peripheral neuron development [21]. The molecular underpinning of this observation may relate to a different conformational change in NCAM structure resulting from the developmentally regulated loss of polySia that increases its adhesive properties of NCAM [8,10,20,31,34,35].

In studies relevant to oligodendrocytes, cells that are involved in myelination of the CNS, polySia has been reported to be a negative regulator since myelination is also promoted by the developmental loss of this glycan [41]. In several pathologic conditions, including multiple sclerosis, polySia is re-expressed in axons that are demyelinated [29,30,42]. In the present study, the polySia glycan was shown to be abundantly expressed on neurons during the early stage of neuronal development and attenuated on neurons and Schwann cells differentiated from the adult stem cells. Based on these findings, we conclude that the methods we used to induce neuronal differentiation in adult stem cells in the neurosphere population were effective in eliciting differentiation. We conclude, therefore, that our current experimental model will be useful for carrying out a detailed molecular analyses of the regulation of polySia expression in peripheral nerve cells, and to potentially elucidate its role as a negative regulator mediating various functions association with cell adhesion and cell migration.

In summary, we have shown that neural stem cells isolated from adult guinea pig spiral ganglia differentially express polySia and NCAM. We also discovered that the polySia moiety of NCAM was abundantly expressed on neurons principally during the early

stages of development. In contrast, its expression was markedly reduced on neurons and Schwann cells differentiated from the adult neural stem cells, thus showing that the polySia glycan is a marker for neural stem cells in the peripheral nervous system, a finding that, to our knowledge, has not been previously reported. As described herein, NCAM and polySia, which are differentially expressed depending on the degree of development, both play a critical role in the adhesion, myelination, fasciculation, and the formation of ganglions, including spiral ganglia in the PNS.

Conflict of interest

None declared.

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