TERM

ORIGINAL ARTICLE

Transplantation of PSA-NCAM-Positive Neural Precursors from Human Embryonic Stem Cells Promotes Functional Recovery in an Animal Model of Spinal Cord Injury

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Abstract

BACKGROUND: Spinal cord injury (SCI) results in permanent impairment of motor and sensory functions at and below the lesion site. There is no therapeutic option to the functional recovery of SCI involving diverse injury responses of different cell types in the lesion that limit endogenous nerve regeneration. In this regard, cell replacement therapy utilizing stem cells or their derivatives has become a highly promising approach to promote locomotor recovery. For this reason, the demand for a safe and efficient multipotent cell source that can differentiate into various neural cells is increasing. In this study, we evaluated the efficacy and safety of human polysialylated-neural cell adhesion molecule (PSA-NCAM)-positive neural precursor cells (hNPCs^PSA-NCAM+) as a treatment for SCI.

METHODS: One hundred thousand hNPCs^PSA-NCAM+ isolated from human embryonic stem cell-derived NPCs were transplanted into the lesion site by microinjection 7 days after contusive SCI at the thoracic level. We examined the histological characteristics of the graft and behavioral improvement in the SCI rats 10 weeks after transplantation.

RESULTS: Locomotor activity improvement was estimated by the Basso–Beattie–Bresnahan locomotor rating scale. Behavioral tests revealed that the transplantation of the hNPCs^PSA-NCAM+ into the injured spinal cords of rats significantly improved locomotor function. Histological examination showed that hNPCs^PSA-NCAM+ had differentiated into neural cells and successfully integrated into the host tissue with no evidence of tumor formation. We investigated cytokine expressions, which led to the early therapeutic effect of hNPCs^PSA-NCAM+, and found that some undifferentiated NPCs still expressed midkine, a well-known neurotrophic factor involved in neural development and inflammatory responses, 10 weeks after transplantation.

CONCLUSION: Our results demonstrate that hNPCs^PSA-NCAM+ serve as a safe and efficient cell source which has the potential to improve impaired motor function following SCI.

Keywords Human pluripotent stem cell · Human embryonic stem cell · PSA-NCAM-positive neural precursor · Spinal cord injury · Transplantation

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1 Introduction

Spinal cord injury (SCI) causes extensive neuronal and glial cell death. It leads to axon demyelination and the loss of axonal connections, resulting in permanent impairment in motor and sensory functions below the lesion site [1]. Spontaneous recovery from SCI is limited owing to the unfavorable environment for axon regrowth in the damaged area [2]. Thus far, there is no therapeutic option for improving functional recovery after SCI other than cell transplantation. Cell replacement therapy may be a potential therapeutic strategy to restore neuronal connectivity and remyelinate damaged axons. Several studies have shown that the transplantation of stem cells or their derivatives could promote locomotor recovery [3–8]. In particular, transplantation of neural precursor cells (NPCs) derived from human embryonic stem cells (hESCs) could lead to partial recovery of impaired neuronal circuits or remyelination of damaged axons and thus, the restoration of locomotor function in injured animals [9].

ESC-derived NPCs can be continuously cultured in the presence of some growth factors, such as basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) [10, 11], and are capable of differentiating into all neural lineages, including neurons, astrocytes, and oligodendrocytes [12]. Because of these properties, NPCs are considered a useful source of cell replacement therapy for neurodegenerative diseases, including SCI. Despite the therapeutic potential for cell therapy to treat SCI, NPCs are known to have tumorigenic potential. There have been several reports of tumorigenesis after NPC transplantation, although undifferentiated pluripotent stem cells (PSCs) were not detected [11, 13–15]. To overcome this issue in previous studies, we obtained highly pure populations of NPCs from hESCs [12] by sorting with a monoclonal antibody recognizing polysialylated-neural cell adhesion molecule (PSA-NCAM) and reported the sorted NPCs had no tumorigenicity [16, 17].

In this study, we describe the potential of highly pure, non-tumorigenic PSA-NCAM + -hNPCs (hNPCs$^{PSA-NCAM+}$) as a therapeutic cell source for SCI treatment. In a contusive SCI rat model transplanted with hNPCs$^{PSA-NCAM+}$, the behavioral recovery of the hindlimbs was significantly increased. Immunohistochemical analysis showed that transplanted hNPCs$^{PSA-NCAM+}$ differentiated into neural lineage cells and did not form non-neural tissues or teratomas. Based on these results, hNPCs$^{PSA-NCAM+}$ may be an effective and clinically applicable source to treat motor dysfunctions induced by SCI.

2 Materials and methods

2.1 Cell culture and differentiation of hNPCs$^{PSA-NCAM+}$

hESCs (WA09, WiCell, Madison, WI, USA) were cultured and differentiated into NPCs following as previously described protocols [12, 18]. Briefly, undifferentiated hESCs were maintained with mitotically inactivated feeder cells (STO; ATCC, Manassas, VA, USA) in ES medium (DMEM/F12 medium supplemented with 20% Knock-out Serum Replacement (KSR; Invitrogen, Carlsbad, CA, USA), $1 \times$ non-essential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma, St Louis, MO, USA) with 4 ng/ml bFGF (Peprotech, Rocky Hill, NJ, USA). To induce neural differentiation, hESC colonies were cultured as embryoid bodies (EBs) in the presence of 5 μM dorsomorphin (Sigma) and 5 μM SB431542 (Calbiochem, San Diego, CA, USA) for 4 days and then placed on a matrigel-coated (Corning, Corning, NY, USA) culture dish in $1 \times$ N2-containing (Invitrogen) medium supplemented with 20 ng/ml bFGF and 100 μg/ml insulin for an additional 5 days. Once neural rosettes appeared, they were mechanically isolated and passed onto a matrigel-coated culture dish after gentle trituration. The cells were expanded in N2B27 medium (composed of DMEM/F12 medium, $1 \times$ N2 and $1 \times$ B27 (Invitrogen)) supplemented with 20 ng/ml bFGF for another week. PSA-NCAM-positive cells were isolated using Anti-PSA-NCAM-MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) from the expanded neural rosette cells according to the manufacturer's instructions. The isolated hNPCs$^{PSA-NCAM+}$ were seeded on a matrigel-coated (Corning) culture dish at a density of ~ 2–3×10^5 cells per cm^2.

2.2 Spinal cord injury and cell transplantation

Generation of contusive SCI model and cell transplantation were performed as previously described [8]. Briefly, adult male rats (Sprague–Dawley, 200–225 g, Orient Bio, Gyeonggi-do, Korea) were anesthetized under masked-inhalation anesthesia (enflurane: induction 3%; maintenance 2%), and laminectomy was performed at the T9 vertebral level. The exposed dorsal surface of the spinal cord was subjected to a weight-drop impact by dropping a 10 g weight rod from a 25 mm height onto the cord surface using the NYU weight-drop impactor. Manual bladder compression was performed until the animals were urinating independently. Animals that did not recover bladder function were excluded from further analysis.

One week after SCI, the rats received hNPCs$^{PSA-NCAM+}$ transplantation or vehicle injection. With the rats under

masked-inhalation anesthesia, the spinal cord was re-exposed at the SCI level, and a tiny hole was opened in the dura mater over the epicenter of the injury site. The fine tip of a glass capillary (850 μm in O.D. with 100 μm tip diameter) filled with hNPCs$^{PSA-NCAM+}$ (1×10^5 cells per 5 μl) or PBS vehicle. The hNPCs$^{PSA-NCAM+}$ or PBS vehicle were administered into the spinal cord for 30 s. The capillary was left in place for 1 min and then slowly withdrawn to avoid any outflow of the cell suspension. For immune suppression, the animals received cyclosporine-A (10 mg/kg, i.p.) every day beginning on the first day prior to transplantation and continuing until the second week after transplantation. All surgeries and experiments were done with the approval of the Institutional Animal Care and Use Committee of Yonsei University College of Medicine, Seoul, Korea (4-2015-1097).

2.3 Behavioral assessment

The improvement of locomotor activity for contusive SCI rats who received either hNPC$^{PSA-NCAM+}$ transplantation (n = 9) or PBS vehicle injection (n = 7) was examined. The rats were trained as described by Basso, Beattie and Bresnahan (BBB), which ranged from complete paralysis (score = 0) to normal locomotion (score = 21) [19]. The rats were gently adapted to the open field, a molded-plastic circular enclosure with a smooth, non-slip floor (90 cm diameter, 30 cm wall height). Once a rat walked continuously in the open field, three blinded examiners conducted a 5-min, preoperative testing session using the BBB locomotor rating scale. Postoperative testing was performed on days 1, 4, and 7 after SCI and then weekly for 10 weeks after transplantation. The BBB scores collected from both hindlimbs were averaged for analysis.

2.4 Immunocytochemistry and flow cytometry

Cells were fixed in 4% paraformaldehyde-PBS solution and permeabilized with 0.3% Triton X-100-PBS solution. After blocking with 2% BSA-PBS solution for 1 h at room temperature, the cell was incubated overnight at 4 °C with primary antibodies: SOX1 (1:200, Millipore, Billerica, MA, USA), Nestin (1:1000, Millipore), and Midkine (1:200, R&D Systems, McKinley, MN, USA). Appropriate fluorescence-tagged secondary antibodies from Molecular Probes (Eugene, OR, USA) and Vector Laboratories, Burlingame, CA, USA) were used for visualization. Cells were mounted in DAPI mounting medium (Vector Laboratories), and images were obtained using an Olympus IX71 microscope equipped with a DP71 digital camera or Olympus FSX100 system.

To evaluate the purity of the isolated hNPCs$^{PSA-NCAM+}$ after 3 ∼ 4 passages, the cells were dissociated into single cells and incubated in 1% BSA-PBS solution. The cells were incubated with Anti-PSA-NCAM antibody (1:300, Millipore) and Alexa-Flour 488-conjugated anti-mouse IgG/IgM (Molecular Probes) for secondary antibodies. Flow cytometry was performed using FACSCalibur (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (BD Biosciences).

2.5 Immunohistochemistry

In order to evaluate the characteristics of the transplanted cells *in vivo*, we performed histological analyses after 10-week behavioral test. The animals were anesthetized with urethane (1.5 g/kg, i.p.) and perfused with PBS and 4% paraformaldehyde in PBS. After dissection of spinal cord including lesion site, the spinal cord post-fixed for 4–6 h with 4% paraformaldehyde, immersed overnight in 30% sucrose in 0.1 M PBS, and embedded in an OCT compound (Tissue-Tek, Torrance, CA, USA). Fixed cord segments were cut into 10 μm coronal sections using the cryostat. Every tenth cord section was collected, mounted onto gelatin-coated slides, and stored at − 70 °C before performing immunostaining. Sections incubated overnight at 4 °C with primary antibodies: Tuj1(1:200, BioLegend, San Diego, CA, USA), NeuN (1:200, Millipore), NG2 (1:200, Millipore), Glial fibrillary acidic protein (GFAP, 1:500, Millipore), Nestin (1:400, Millipore), Ki67 (1:150, Leica Biosystems, Buffalo Grove, IL, USA), human nuclear antigen (HNA, 1:100, Millipore), midkine (1:200, R&D System). After visualization with appropriate fluorescence-tagged secondary antibodies, sections were mounted in DAPI mounting medium (Vector Laboratories), and images were obtained using an Olympus IX71 microscope equipped with a DP71 digital camera or Olympus FSX100 system.

2.6 Conditioned medium collection from hNPCs$^{PSA-NCAM+}$ and antibody array

In order to analyze secretome and identify soluble factors responsible for the therapeutic effects of hNPC$^{PSA-MCAM+}$, conditioned medium was collected from hNPC$^{PSA-MCAM+}$ under serum-free DMEM with low glucose supplemented with $1 \times$ ITS (insulin, transferrin, and selenium, Invitrogen) during last 24 h of culture, centrifuged at 1,000 × g for 10 min at 4 °C to remove cell debris, filtered through a membrane with a pore size of 0.22 μm in diameter (Millipore), and then frozen in aliquots at − 80 °C before antibody array. The cytokines of conditioned medium from 2 independent batches were profiled by Cytokine Profiling antibody array (SCK100, Full Moon BioSystems, Sunnyvale, CA, USA) following the manufacturer's instruction, normalized against the blank (medium) and data were

analyzed using ExDEGA v.3.2.1 software (eBiogen, Seoul, Korea).

2.7 Enzyme-linked immunosorbent assay (ELISA)

The concentrations of Midkine, GDNF (both from Aviva, San Diego, CA, USA), VEGF and TGF-β (both from R&D Systems) in conditioned medium were measured by ELISA following the manufacturer's instructions. Optical densities were measured at 450 nm using a microplate reader (model 680, Bio-Rad, Hercules, CA, USA). The experiment was repeated at least 4 times.

2.8 Statistical analysis

The results from the behavioral tests were analyzed with a two-way repeated measures analysis of variance (ANOVA). When the ANOVAs identified significant differences, pair-wise comparisons between mean values were

performed using post hoc Tukey's tests. Significance was set at $p < 0.05$. The data are presented as the mean $\pm$ SEM.

3 Results

3.1 Generation of hNPCs$^{PSA-NCAM+}$ from hESCs

The overall scheme for hNPC$^{PSA-NCAM+}$ differentiation is shown in Fig. 1A. NPCs were differentiated from hESCs using a previously reported protocol with slight modifications [16]. The hNPCs$^{PSA-NCAM+}$ isolated with magnetic-activated cell sorting (MACS) were cultured through several passages. Subsequently, the high purity of the hNPCs$^{PSA-NCAM+}$ was verified using FACS analysis (Fig. 1B, C). Immunostaining results indicated that the hNPCs$^{PSA-NCAM+}$ expressed markers of human neural stem
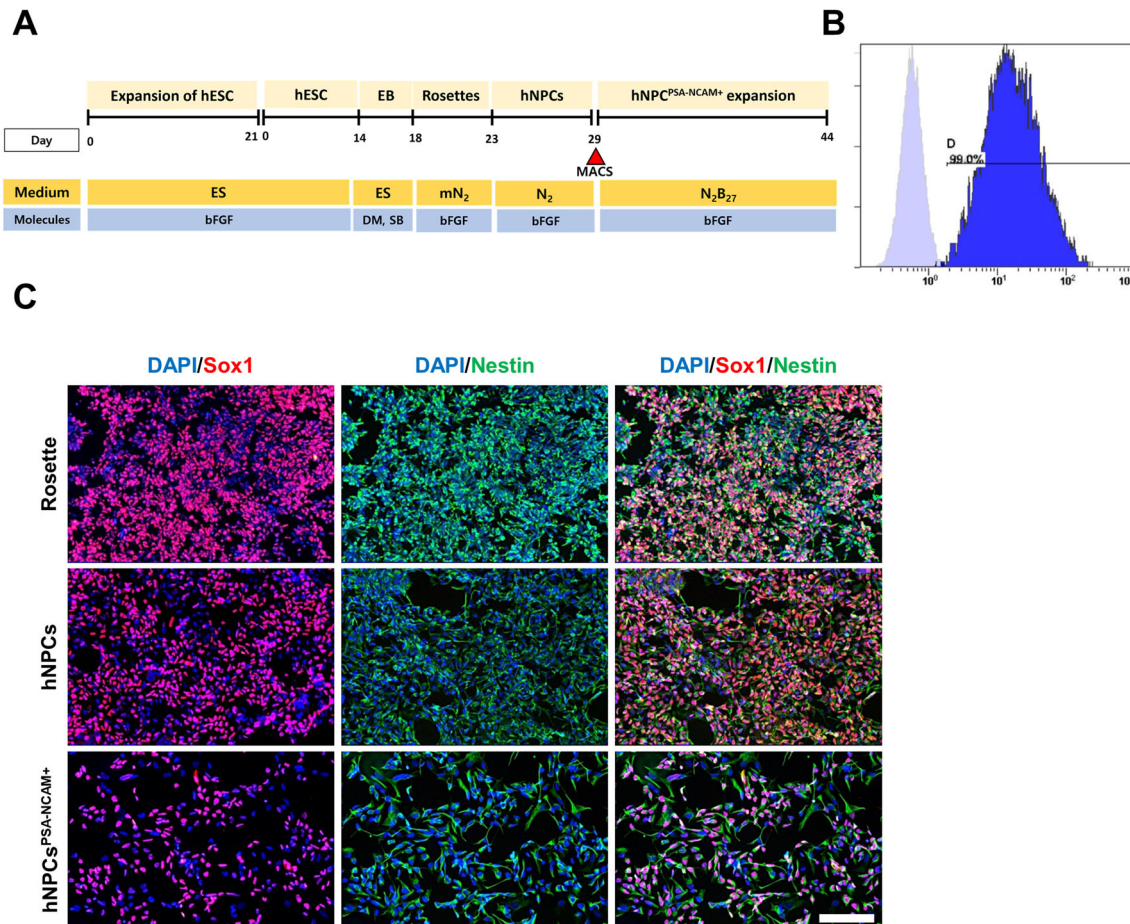

Fig. 1 Differentiation and characterization of hNPCs$^{PSA-NCAM+}$ from human embryonic stem cells. A Schematic diagram of the differentiation conditions for hNPCs$^{PSA-NCAM+}$. B Flow cytometry analysis for PSA-NCAM in hNPCs$^{PSA-NCAM+}$. After several passages, the majority were still in the PSA-NCAM + form and had not differentiated into other cells. C Immunocytochemistry analysis of parental neural rosettes, rosette-derived NPCs, and hNPCs$^{PSA-NCAM+}$. hNPCs$^{PSA-NCAM+}$ expressed markers for NPCs like their parental cells and had the characteristics of NPCs. Scale bar: 50 μm
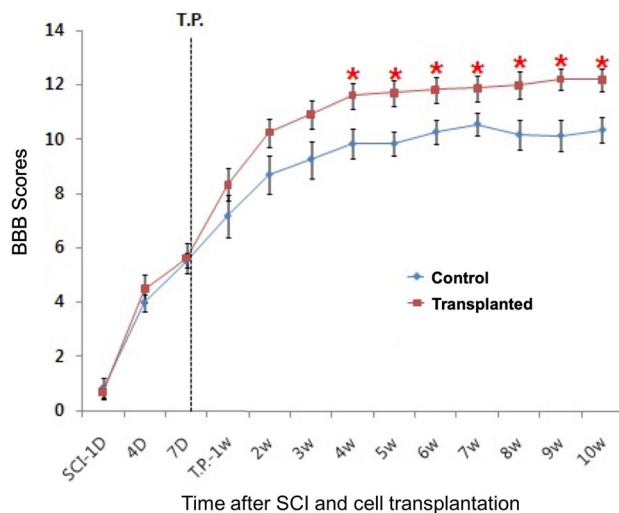
Fig. 2 Recovery of hindlimb locomotor function 10 weeks after hNPC$^{PSA-NCAM+}$ transplantation. The BBB score was assessed for each rat in the two groups: rats receiving cell transplants or PBS, 1 week after SCI. In the behavioral test, the results showed a significant improvement in the hNPC$^{PSA-NCAM+}$-transplanted SCI rats compared with the control group from 4 weeks after cell transplantation. Error bars indicate s.e.m. *$p < 0.05$

cells like their parental sources (neural rosettes and unsorted hNPCs).

3.2 Improvement of locomotor activity in SCI rats after transplantation of hNPCs$^{PSA-NCAM+}$

To test the therapeutic potential of hNPCs$^{PSA-NCAM+}$ for treating SCI, we transplanted hNPCs$^{PSA-NCAM+}$ into the lesion sites of SCI rats and measured locomotor activity according to the Basso–Beattie–Bresnahan (BBB) locomotor rating scale. After SCI, the rats had completely lost the function of both hindlimbs (BBB score = 0) (Fig. 2). However, SCI rats showed a behavioral improvement through spontaneous recovery with some movement of the hip, knee, and ankle joints (BBB score = 4–6). At 1 week post-SCI, the rats received hNPCs$^{PSA-NCAM+}$ (n = 9) or PBS vehicle (n = 7) into the lesion site. The control group spontaneously recovered up to a BBB score of ∼ 10 by 4 weeks after PBS treatment; they showed weight-supported stepping using the dorsum of the foot. However, the locomotor activity of the cell transplant group improved more rapidly than that of the control group. Although there was no statistical significance, hNPCs$^{PSA-NCAM+}$-transplanted rats showed higher BBB scores than the control group during the first 3 weeks after transplantation.

Four weeks after transplantation, hNPCs$^{PSA-NCAM+}$-transplanted rats showed significantly higher BBB scores than the control group with maintenance until the 10th week of the last behavioral evaluation ($p < 0.05$), indicating that the hNPCs$^{PSA-NCAM+}$ transplantation significantly enhanced locomotor recovery in the SCI rats.

3.3 In vivo differentiation of hNPCs$^{PSA-NCAM+}$ 10 weeks after transplantation

Next, we examined the differentiation of the hNPCs$^{PSA-NCAM+}$ in the grafts within the injured spinal cords by immunostaining for the following neural lineage cell markers: neuron-specific class III β-tubulin (Tuj1) and neuronal nuclei (NeuN) for neurons, neural-glial antigen 2 (NG2) for oligodendrocytes, and glial fibrillary acidic protein (GFAP) for astrocytes. Ten weeks after transplantation, the grafts showed neural differentiation of hNPCs$^{PSA-NCAM+}$ within the injured spinal cords. All lineage markers were co-labeled with human nuclear antigen (HNA) and observed throughout the injection area (Fig. 3A), suggesting that the hNPCs$^{PSA-NCAM+}$ successfully differentiated into neural lineage cells *in vivo* and became engrafted into the host despite the harsh environment of the damaged tissue. We also identified undifferentiated and proliferating cells by the immunoreactivities of Nestin and Ki67. Nestin-positive cells were still detected in the graft, but Ki67-positive cells were not detected (Fig. 3B), indicating that some transplanted cells remained in undifferentiated, nonproliferating states even 10 weeks after transplantation. Collectively, our data show that the hNPCs$^{PSA-NCAM+}$ could differentiate into neural lineage cells in the lesion site, which might help to improve recovery from contusion injuries.

3.4 Expression of midkine in hNPCs$^{PSA-NCAM+}$ in vitro and in vivo

Although there was no statistical significance, behavioral recovery from the early stage after transplantation increased more in the transplanted rats than in the control group. Since this period was insufficient for the transplanted hNPCs$^{PSA-NCAM+}$ to engraft into the host or differentiate into neural lineage cells, these effects might have resulted from factor(s) secreted by the transplanted cells rather than the transplanted cells themselves, and several reports have shown that NPCs secrete neurotrophic factors, which are involved in neuroprotection and

Fig. 3 Histological analysis for *in vivo* differentiation of transplanted hNPC$^{PSA-NCAM+}$ in the injured spinal cord 10 weeks post-transplantation. **A** Coronal section image of a graft showing several types of surviving cells derived from the transplants. Scale bar: 50 μm (upper panel), 100 μm (lower panel). **B** Some cells remained undifferentiated (Nestin) but few cells were in a proliferating state (Ki67). DAPI for nuclear staining (blue), human nuclear antigen for hNPC$^{PSA-NCAM+}$ (red). Scale bar: 100 μm
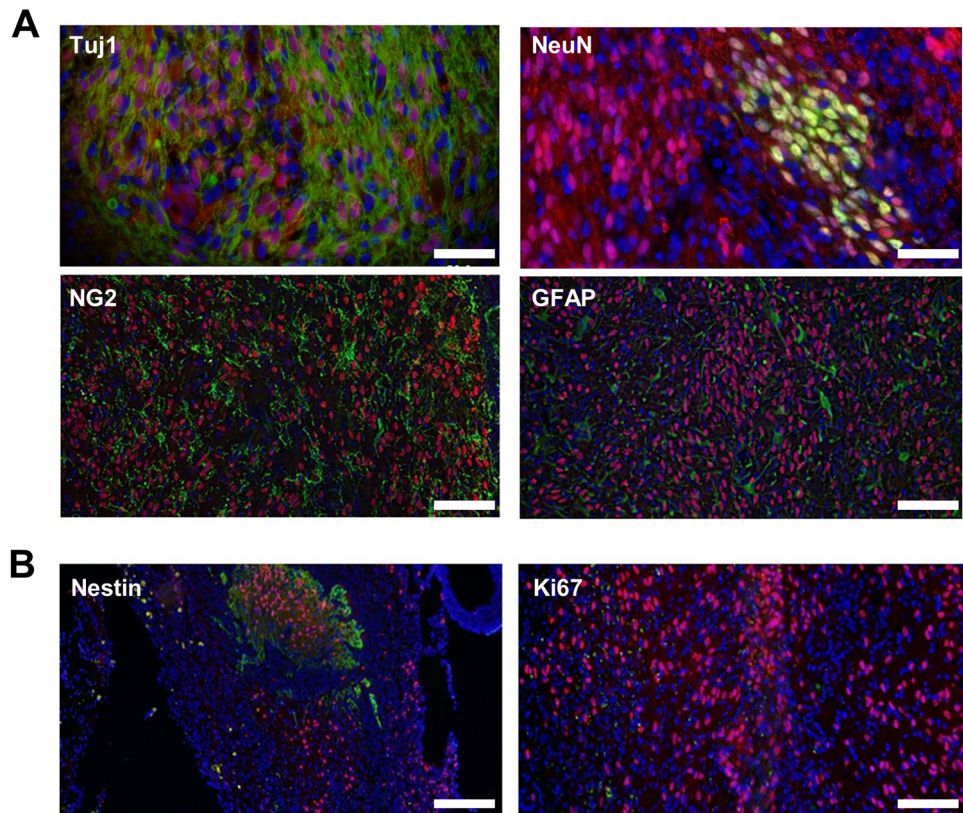
neuroregeneration under pathogenic conditions [20–22]. To identify the high-quality candidate biomarkers and/or therapeutic factor(s) secreted by the hNPCs$^{PSA-NCAM+}$, the protein profile of the hNPCs$^{PSA-NCAM+}$-conditioned medium was analyzed by antibody array. Of the 310 antibodies used for profiling human cytokines and biomarkers, the top 10 proteins in the hNPCs$^{PSA-NCAM+}$-conditioned medium compared to that of the blank (serum free medium) were midkine, TIMP metallopeptidase inhibitor 1 (TIMP-1), insulin-like growth factor-binding protein 7 (IGF-BP7), matrix metalloproteinase 2 (MMP-2), matrix metalloproteinase 10 (MMP-10), vascular endothelial growth factor (VEGF), endostatin, fibroblast growth factor 5 (FGF-5), follistatin, and Melanoma-derived growth regulatory protein (MIA) (Fig. 4A). Glial cell-derived neurotrophic factor (GDNF) and transforming growth factor-β (TGF-β) were also detected, but at low levels. Midkine was highly abundant in the antibody array, which was also validated by enzyme-linked immunosorbent assay (ELISA), while VEGF and TGF-β were at moderate levels and GDNF was in trace amounts (Fig. 4B).

Midkine is a well-known neurotrophic factor, which is expressed in NPCs and plays an important role in regulating neural development and inflammatory responses [23–25]. Midkine is also expressed under pathogenic central nervous system (CNS) conditions, such as cerebral infarct or SCI [26–28], and known to be involved in repairing damaged nerve tissue [25, 27]. We performed immunostaining to confirm the expression of midkine in hNPCs$^{PSA-NCAM+}$ or its derivatives *in vitro* and *in vivo*. Most of the hNPCs$^{PSA-NCAM+}$ strongly expressed midkine *in vitro* (Fig. 5A), and some of the transplanted cells still strongly expressed midkine 10 weeks after transplantation *in vivo* (Fig. 5B, C). These results suggested that the behavioral improvement after cell transplantation might be due to neurotrophic factors secreted from hNPCs$^{PSA-NCAM+}$ and cell replacement.
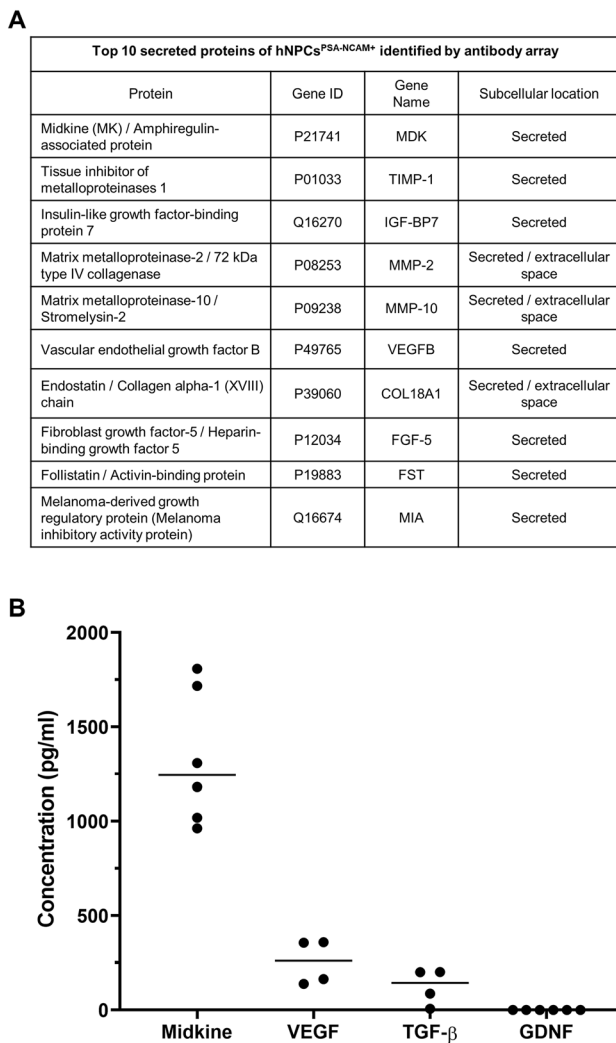
A

Top 10 secreted proteins of hNPCs^PSA-NCAM+ identified by antibody array			
Protein	Gene ID	Gene Name	Subcellular location
Midkine (MK) / Amphiregulin-associated protein	P21741	MDK	Secreted
Tissue inhibitor of metalloproteinases 1	P01033	TIMP-1	Secreted
Insulin-like growth factor-binding protein 7	Q16270	IGF-BP7	Secreted
Matrix metalloproteinase-2 / 72 kDa type IV collagenase	P08253	MMP-2	Secreted / extracellular space
Matrix metalloproteinase-10 / Stromelysin-2	P09238	MMP-10	Secreted / extracellular space
Vascular endothelial growth factor B	P49765	VEGFB	Secreted
Endostatin / Collagen alpha-1 (XVIII) chain	P39060	COL18A1	Secreted / extracellular space
Fibroblast growth factor-5 / Heparin-binding growth factor 5	P12034	FGF-5	Secreted
Follistatin / Activin-binding protein	P19883	FST	Secreted
Melanoma-derived growth regulatory protein (Melanoma inhibitory activity protein)	Q16674	MIA	Secreted

B

Fig. 4 Major secreted proteins of the hNPC^PSA-NCAM+ identified by antibody array and ELISA. **A** Top 10 secreted proteins identified in the hNPC^PSA-NCAM+ conditioned medium by antibody array. **B** Secretion of key cytokines in the conditioned medium of hNPC^PSA-NCAM+ by ELISA. Horizontal bars indicate the median values of 4 or 6 independent experiments

4 Discussion

We showed that the hNPCs^PSA-NCAM+ isolated from hESC-derived NPCs were well-integrated and successfully differentiated into three neural lineage cells after transplantation into traumatically injured rat spinal cords. After transplantation of hNPCs^PSA-NCAM+, the impaired locomotor activity of SCI rats recovered more than that of the control group. Our *in vivo* results also showed that all neural lineage makers co-labeled with HNA were frequently observed in the cell injection site. In addition, there was no evidence of tumor formation induced by transplantation. These results demonstrate that hNPCs^PSA-NCAM+ are effective and safe cell sources with the

therapeutic potential to improve motor dysfunction caused by SCI.

SCI is a fatal event that leads to a permanent impairment of motor and sensory functions resulting from massive cell death in the lesion site, the loss of neural connections, and demyelination of spared axons [1]. NPCs could differentiate into neural cells, such as neurons, oligodendrocytes, and astrocytes, making them an excellent therapeutic candidate for regeneration and protection of neurons at SCI sites [29]. Ten weeks after transplantation, we confirmed that the transplanted hNPCs^PSA-NCAM+ had differentiated into neurons, astrocytes, and oligodendrocytes (Fig. 3). Transplant-derived neural cells are thought to contribute to behavioral recovery by relaying damaged neural connections. These relays occur when the host axon extends beyond the injury and synapses with the graft-derived neurons [29–32]. Loss of myelin occurs early after SCI [33, 34], followed by apoptosis of oligodendrocytes [35]. Because demyelination and loss of oligodendrocytes with cell death lead to an impaired neural conduction, replacement of lost oligodendrocytes with cell transplantation and myelination may lead to the recovery of impaired locomotion after SCI [4, 8, 36].

The therapeutic benefits of NPC transplantation include not only the cell replacement effect but also the effect of substances secreted from the cells [37–40]. We identified some cells that remained undifferentiated within the transplanted site. These undifferentiated hNPCs^PSA-NCAM+ might strongly secrete trophic factors at the transplanted site, thus reducing secondary damage following SCI. We confirmed the expression of midkine, a representative cytokine secreted from NPCs, *in vitro* and *in vivo* (Fig. 4). Midkine is strongly expressed in the nervous system during the embryonic stage [41] and observed at the beginning of neurogenesis [42]. Midkine promotes neurite extension, survival, and migration of embryonic neurons [43–45] and repair of the injured nervous system [23, 25]. This potential mechanism could explain the immediate and early effects after NPC transplantation before the integration of grafted cells into the host.

Despite the potential of using NPCs as a treatment for SCI, NPCs have the risk of tumor formation even in the absence of undifferentiated PSCs [11, 13–15]. We previously reported that hNPCs^PSA-NCAM+ do not generate non-neural tissues or teratomas induced by NPCs [16, 17]. Similarly, in this study, no non-neural tissues or mesodermal tumors were found during the 10 weeks after hNPCs^PSA-NCAM+ transplantation, confirming that hNPCs^PSA-NCAM+ are safer than other cells derived from stem cells.

In conclusion, we suggest that hNPCs^PSA-NCAM+ are a safe and effective therapeutic source for the treatment of SCI. hNPCs^PSA-NCAM+ are non-tumorigenic cells that can
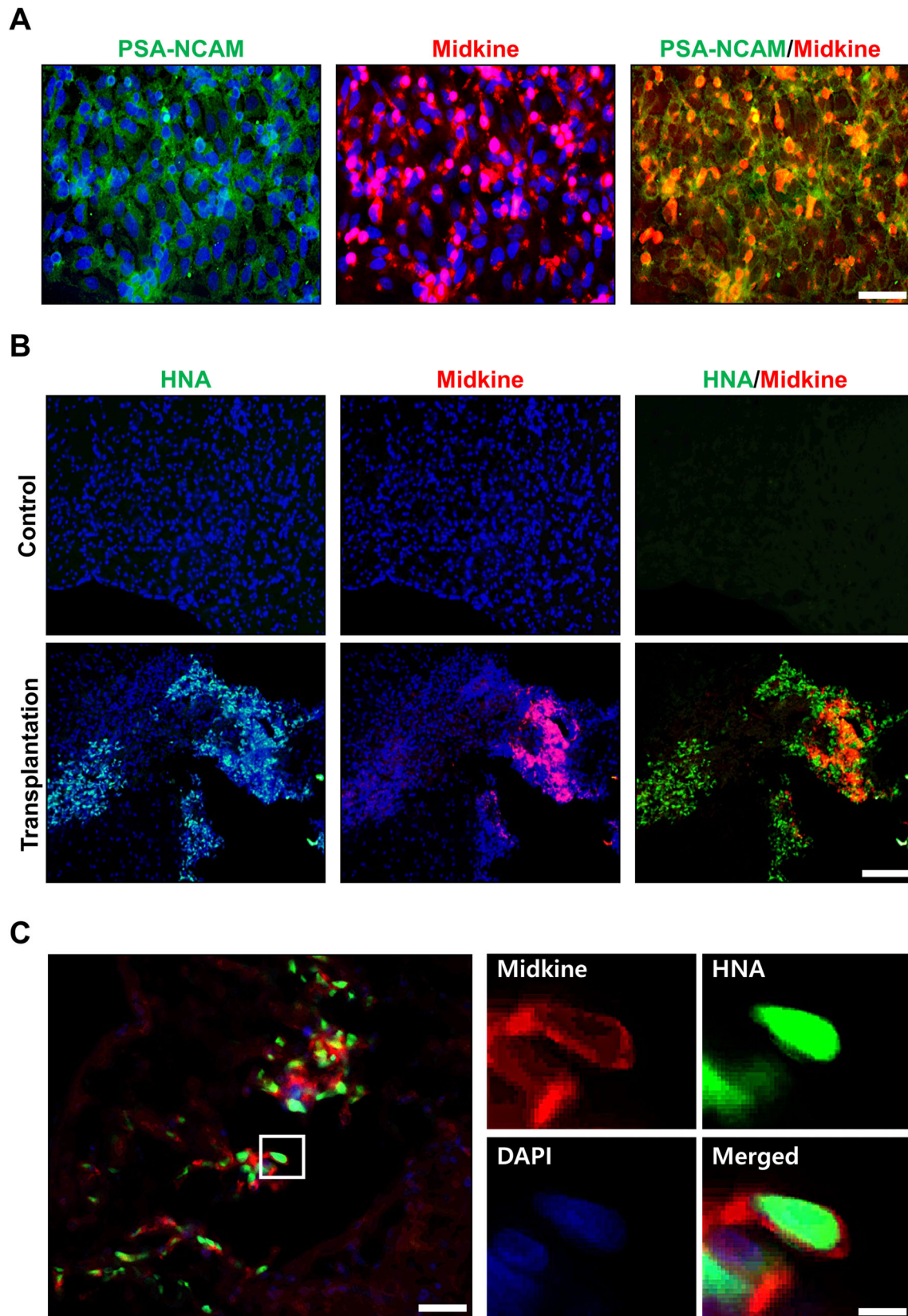
◀**Fig. 5** Expression of midkine in hNPC$^{PSA-NCAM+}$ *in vitro* and *in vivo*. **A** hNPCs$^{PSA-NCAM+}$ expressed midkine *in vitro*. Scale bar: 50 μm. **B, C** Some of the transplanted hNPCs.$^{PSA-NCAM+}$ continued to express midkine in the injured spinal cord 10 weeks after transplantation *in vivo*. Scale bar: 100 μm (B), 50 μm (C, left panel) 10 μm (C, right panel)

differentiate into various neural lineage cells after transplantation and successfully induce functional recovery through direct or indirect mechanisms. We expect this knowledge to lead to the development of new treatment methods for SCI.

Author's contribution D.-H. Kim Ph.D.: Conception and design, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing. H.-J. Cho MS: Collection and/or assembly of data, Data analysis and interpretation. C.-Y. Park Ph.D.: Collection and/or assembly of data, Data analysis and interpretation. M.S. Cho Ph.D.: Conception and design, Data analysis and interpretation, Manuscript writing and Final approval of manuscript. D.-W. Kim Ph.D.: Conception and design, Data analysis and interpretation, Manuscript writing and Final approval of manuscript.

Declarations

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