Transplantation of neural stem progenitor cells from different sources for severe spinal cord injury repair in rat

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\begin{abstract}
Neural stem progenitor cell (NSPC) transplantation has been regarded as a promising therapeutic method for spinal cord injury (SCI) repair. However, different NSPCs may have different therapeutic effects, and it is therefore important to identify the optimal NSPC type. In our study, we compared the transcriptomes of human fetal brain-derived NSPCs (BNSPCs), spinal cord-derived NSPCs (SCNSPCs) and H9 embryonic stem-cell derived NSPCs (H9-NSPCs) in vitro and subsequently we transplanted each NSPC type on a collagen scaffold into a T8-9 complete SCI rat model in vivo. In vitro data showed that SCNSPCs had more highly expressed genes involved in nerve-related functions than the other two cell types. In vivo, compared with BNSPCs and H9-NSPCs, SCNSPCs exhibited the best therapeutic effects; in fact, SCNSPCs facilitated electrophysiological and hindlimb functional recovery. This study demonstrates that SCNSPCs may be an appropriate candidate cell type for SCI repair, which is of great clinical significance.
\end{abstract}

\section{Introduction}

Spinal cord injury (SCI) can lead to serious motor, sensory and autonomic dysfunction, for which there is currently no efficacious treatment [1,2]. Neural stem progenitor cell (NSPC) transplantation has been widely studied and may serve as an appropriate multi-pronged strategy to therapeutically target pathological processes during SCI evolution [3]. NSPCs can differentiate into neurons, astrocytes, and oligodendrocytes both in vitro and in vivo, replenishing lost cells at the lesion site. The most commonly reported mechanisms by which NSPCs repair SCIs are neuroprotection, axon regeneration and sprouting, myelin regeneration, immunomodulation, and neuronal relay formation [2]. The most frequently used NSPC types are human fetal brain-derived NSPCs (BNSPCs), spinal cord-derived NSPCs (SCNSPCs), induced pluripotent stem cell-derived NSPCs, and embryonic stem cell (ESC)-derived NSPCs. However, each of these NSPC types may have different effects in SCI restoration due to differences in cell proliferation, differentiation, and gene expression. Previous in vitro studies have shown that NSPCs from the human cortex proliferate faster than those from the spinal cord in long-term culture [4-6]. In another study, Watanabe et al.
compared the differentiation ability of forebrain-derived NSPCs and SCNSPCs isolated from green fluorescent protein (GFP) Sprague-Dawley (SD) rats in vitro at embryonic day 14 (E14). They transplanted the forebrain NSPCs and SCNSPCs into a rat T10 spinal cord contusion model, examined the in vivo differentiation capability, and evaluated whether forebrain or SCNSPCs could contribute to behavioral recovery of SCI rats. They found that forebrain NSPCs could differentiate into more neurons and fewer oligodendrocytes than SCNSPCs both in vitro and in vivo. Furthermore, neurons differentiated from forebrain NSPCs secreted excitatory glutamate and inhibitory γ-aminobutyric acid (GABA) in vitro and glycine in vivo. In contrast, neurons derived from SCNSPCs could secrete neurotransmitters (such as inhibitory glycine) in addition to glutamate and GABA in vitro, but not in vivo. However, no significant differences were observed in motor function restoration between rats treated with forebrain NSPCs and SCNSPCs in the contusion model [7].

In addition to differences in proliferation or differentiation abilities, recent studies have shown that BNSPCs, SCNSPCs or pluripotent stem cell-derived NSPCs have various effects on host axon regeneration in vivo. In 2016, Kadoya et al. transplanted NSPCs from either the telencephalon, hindbrain, or spinal cord of E14 rats into adult rat C4 corticospinal tract (CST) lesion areas. They found a clear divergence in CST projection regeneration capacity, with more axons penetrating into NSPC spinal cord grafts than telencephalic or hindbrain NSPC grafts [8]. They also grafted SCNSPCs isolated from a human fetus at 11 gestational weeks or H9 ESC-derived NSPCs (H9–NSPCs) expressing midbrain markers into a rat C4 CST lesion model. In that model, CST axons successfully regenerated into SCNSPC graft rather than rostral-fated H9-NSPC graft. Kadoya also generated two NSPC cell types from human induced pluripotent stem cells: one with rostralized forebrain specificity and the other with caudalized spinal cord specificity. Kadoya grafted these cells into rat C4 CST lesions. After six weeks, CST axons regenerated into the caudal-fated NSPC graft rather than the rostral-fated NSPC graft. They therefore concluded that injured spinal cord “replacement” with homologous spinal cord NSPCs could enable remarkable regeneration of corticospinal projections both within and beyond the SCI lesion region. Recently, researchers in the same lab performed translational profiling analyses to further investigate the mechanism underlying CST regeneration [9]. They discovered that implantation of E12 mouse SCNSPCs into an injured C5 mouse dorsal column site enabled host CST neurons to regrasp to an embryonic transcriptional growth state, thus restoring forelimb function to the SCI mouse.

Recently, Zou et al. transplanted NSPCs derived from a human fetal brain at 10 gestational weeks or the thoracic spinal cord into adult SD rat T8-10 spinal cords in a complete transection model, and compared the therapeutic effects in each group. Compared with BNSPC group, the SCNSPC group displayed better therapeutic outcomes, including higher grafted cell survival rate, a lower apoptosis rate, and stronger proliferation ability. SCNSPCs can differentiate into more mature neurons and extended longer axons. SCNSPCs transplantation also promotes locomotor functions in SCI rats [10]. Kelly et al. reported that there were many differences in gene expression profiles of BNSPCs and SCNSPCs. Using microarray technology, they identified 229 differentially expressed genes between mouse cortical- and spinal cord-derived neurons. They also derived a population of LeX negative NSPCs in neurospheres from the embryonic mouse spinal cord but not the cortex [11].

In addition to fetal brain- or spinal cord-derived NSPCs, some studies have also tested transplantation of pluripotent stem cell-derived NSPCs in SCI animals [8,12]. However, compared with brain- or spinal cord-derived NSPCs, pluripotent stem cell-derived NSPCs have divergent immunogenicity and immunomodulatory potentials, which may influence their treatment effects [13]. Liu et al. exhibited that human fetal SCNSPCs and human ESC-derived NSPCs share similar leucocyte antigen, co-stimulatory and adhesion molecules under equivalent conditions. Nevertheless, these cell types differ in immunogenicity and immunomodulatory potentials; SCNSPCs have lower immunogenicity than ESC-derived NSPCs. In response to stimulation with inflammatory factors, SCNSPCs do not cause the proliferation of peripheral blood mononuclear cells, but ESC-derived NSPCs do. Moreover, SCNSPCs have a stronger immunomodulatory ability. After proliferation of peripheral blood mononuclear cells, SCNSPCs could secrete more transforming growth factor β-1 (TGF-β1), which is involved in the maintenance of immune tolerance and T cell homeostasis [13-18]. Several studies have also analyzed tumorigenicity and immune rejection of pluripotent stem cell-derived NSPCs after transplantation into SCI model [19-22].

In this study, we compared the transcriptomes of human BNSPCs, SCNSPCs, and H9–NSPCs for the first time. Subsequently, we transplanted the three types of NSPCs into a rat T8-9 complete spinal cord transection model. Combining in vitro transcriptome data with in vivo results, we demonstrate that SCNSPC transplantation could be a promising strategy for SCI repair.

2. Materials and methods

2.1. Ethics

All animal experiments were performed in accordance with the Chinese Ministry of Public Health Guide and the US National Institutes of Health (NIH) Guide for the care and use of laboratory animals. The use of human fetal tissue was approved by the Reproductive Study Ethics Committee of Nanjing Drum Tower Hospital and Nanjing Medical University (2018-223-01). Informed consent was provided by the donors. Human fetal tissues were acquired after legal pregnancy termination.

2.2. Human fetal NSPCs culture

Human fetal tissue at gestational 13 weeks was obtained from the Nanjing Drum Tower Hospital after a normal termination in accordance with nationally approved ethical and legal guidelines. Primary cells were isolated from the whole brain or the complete spinal cord. Tissues were harvested with a scalpel and cut into small pieces in a Petri dish (Corning, USA). Cells were then transferred into a 50 mL centrifuge tube (Corning, USA) and digested at 37 °C with 5 mL accutase (Sigma-Aldrich, USA) for 5 min at 25 °C. After centrifugation, cells were re-suspended in 10 mL proliferation medium and transferred to a 10 cm Petri dish (Corning, USA) precoated with 125 μg laminin (BioLamina, Sweden). The proliferation medium was composed of the following: serum-free modified N2 medium containing 1% non-essential amino acid (Gibco, USA), 1% sodium pyruvate (Gibco, USA), 1% penicillin-streptomycin (Gibco, USA), 15 mM HEPEs (Sigma-Aldrich, USA), 2% B27 (Gibco, USA), 25 mg/L recombinant human insulin (Yeasen, Shanghai, China), 100 mg/L human plasma apo-transferrin (Millipore, USA), 15 g/L glucose (Sigma-Aldrich, USA), 20 mM lactate (Sigma-Aldrich, USA), 20 mM pyruvate (Sigma-Aldrich, USA), 30 mM sodium selenite (Sigma-Aldrich, USA), 100 μM putrescine (Sigma-Aldrich, USA), 40 ng/mL epidermal growth factor (Peprotech, USA), 20 ng/mL basic fibroblast growth factor (Peprotech, USA), 20 ng/mL leukemia inhibitory factor (Peprotech, USA), 10 ng/mL neurotrophins-3 (Peprotech, USA) and 1.83 μg/L heparin (Sigma-Aldrich, USA) in a 2:1 mixture of DMEM (Gibco, USA) to DMEM/F12 (Gibco, USA) medium.

The medium was changed for each sample every other day. BNSPCs and SCNSPCs reached the primary culture stage at 15 days, and were then passaged approximately every 5 and 7 days, respectively, when they grew to 80% confluence. During the process of passaging, BNSPCs and SCNSPCs were digested with accutase, then replated onto new 10 cm petri dishes precoated with laminin at a density of 1 × 10⁶ cells per
2.3. Human H9–NSPCs culture

H9-ESCs were induced to differentiate into H9–NSPCs as previously reported [23]. Briefly, H9-ESCs cultured in E8 medium were passaged in a six well plate coated with Matrigel (BD Biosciences, USA). H9-ESCs at about 30% confluency were cultured in basic medium containing 1 μM compound C (Selleck, USA). After three days, 100 nM retinoic acid was added to the basic medium. After another three days, 100 ng/mL SHH and 100 nM retinoic acid were both added to the basic medium without compound C. After another three days of differentiation, H9-ESCs had almost differentiated into H9–NSPCs. Basic medium was composed of 50% Advanced DMEM/F12 (Invitrogen, USA), 50% Neurobasal (Gibco, USA), 1 × N2 (Gibco, USA), 1 × B27 (Gibco, USA), 1% GlutaMAX (Gibco, USA), 1% NEAA (Gibco, USA), 1% penicillin-streptomycin (Gibco, USA). H9–NSPCs were then cultured on plates coated with Matrigel in basic medium containing 100 nM retinoic acid (Selleck, USA) and 0.5 μM small purmorphamine (Selleck, USA).

2.4. Differentiation assay

We carried out the differentiation experiments of three types of NSPCs for 7 days in vitro. The basic differentiation medium of NSPCs and SCNSPCs were proliferation medium minus growth factors. Basic differentiation medium was composed of the following: serum-free modified N2 medium containing 1% non-essential amino acid (Gibco, USA), 1% sodium pyruvate (Gibco, USA), 1% penicillin-streptomycin (Gibco, USA), 15 mM HEPEs (Sigma-Aldrich, USA), 2% B27 (Gibco, USA), 25 mg/L recombinant human insulin (Yeasen, China), 100 mg/L human plasma apo-transferrin (Millipore, USA), 15 g/L glucose (Sigma-Aldrich, USA), 20 nM progesterone (Sigma-Aldrich, USA), 30 mM sodium selenite (Sigma-Aldrich, USA), 100 μM putrescine (Sigma-Aldrich, USA) in a 2:1 mixture of DMEM (Gibco, USA) to DMEM/F-12 (Gibco, USA) medium. For neuronal differentiation, 1 μM DAPT (Selleck, USA), 10 ng/mL brain-derived neurotrophic factor (Peprotech, USA), and 10 ng/mL glial cell line-derived neurotrophic factor (Peprotech, USA) were added to the basic differentiation medium. For astrocytic differentiation, only 1 μM DAPT (Selleck, USA) was added to the basic differentiation medium. In addition, 1 μM DAPT (Selleck, USA), 10 μM bionin (Selleck, USA), and 40 ng/mL Tridihothoronyne (Selleck, USA) were added for oligodendrocyte differentiation [27,28].

H9–NSPCs were differentiated in the medium composed of the following: 50% Advanced DMEM/F12 (Invitrogen), 50% Neurobasal (Gibco, 12,348–017), 1 × N2 (Gibco, 17,502–048), 1 × B27 (Gibco, 17,504–044), 1% GlutaMAX (Gibco, 35,050–061), 1% NEAA (Gibco, 11,140–050), 1% penicillin/streptomycin (Gibco, 15,140–122), 10 ng/mL brain-derived neurotrophic factor (Peprotech, USA), and 10 ng/mL glial cell line-derived neurotrophic factor (Peprotech, USA). For neuronal, astrocytic and oligodendrocytic differentiation, the same small molecules were used for BNSPCs and SCNSPCs directional differentiation were added to the basic differentiation medium of H9–NSPCs.

2.5. Lentiviral transduction

To track the transplanted BNSPCs, H9–NSPCs and SCNSPCs in vivo, we infected cells with lentivirus expressing GFP (Beijing Mijia Technology Co., Ltd) during proliferation culture in vitro. Specifically, approximately 10^6 BNSPCs, H9–NSPCs or SCNSPCs were seeded in a 10 cm Petri dish at the sixth passage and 5 mL proliferation medium containing 50 μL GFP lentivirus was added. After 12 h, the proliferation medium was removed and 10 mL of fresh proliferation medium was added. About 80–90% of BNSPCs, H9–NSPCs, and SCNSPCs were successfully transfected with GFP.

2.6. Immunocytochemistry

Human BNSPCs, SCNSPCs, and H9–NSPCs cultured on plates were fixed in 4% parafomaldehyde (PFA) for 10–15 min at room temperature (RT). After washing three times with phosphate buffer saline (PBS), fixed samples were permeabilized with 0.2% Triton X-100 and 5% donkey serum in PBS for 40–60 min. Primary antibodies (Table S1) diluted in blocking solution were applied overnight at 4 °C. After rinsing with PBS three times, samples were incubated with donkey Alexa Fluor-conjugated secondary antibodies (1:500; Invitrogen) for 30 min at RT. Nuclear counterstaining was conducted with Hoechst 33,342. Images were taken with a Vert. A1 fluorescence microscope (Zeiss, USA) or a confocal microscope (Leica, USA).

2.7. Seeding of BNSPCs, H9–NSPCs, and SCNSPCs on the collagen scaffold

To promote the cell adhesion on collagen scaffold, we prepared 10 μg/mL laminin with PBS, and the collagen scaffolds were immersed into the solution at 37 °C for 2 h. The collagen scaffolds were then rinsed in PBS once and the excess PBS was removed by a sterilized filter paper. NSPCs were digested and the density of cell suspension was adjusted to 10^6 cells/10 μL, and were carefully dropped onto collagen scaffolds. Collagen scaffolds loaded with NSPCs were put in the cell incubator at 37 °C for 2 h. NSPCs began to extend axons and adhere to the porous collagen scaffolds, then sufficient proliferation medium was added in the cell culture plate for subsequent long-term culture.

2.8. Ultrastructure observations

Collagen scaffold were produced as previously reported [10]. Briefly, deproteinized bovine skin was immersed in 0.5 M acetic acid solution at 4 °C. Samples were then homogenized in a blender for 15 min and neutralized with 4 M NaOH to obtain a uniform collagen solution. Dialysis and lyophilization yielded the collagen scaffold. To generate an aligned porous structure, the collagen scaffold were cut into 0.2 × 0.5 × 0.5 cm blocks, then infiltrated with 40 mL MES (pH 6.5) containing 0.6 mg/mL N-hydroxysuccinimide and 1 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. Pellets were then washed.

For scanning electron microscope (SEM), untreated collagen scaffolds or those loaded with cells were washed three times with PBS, fixed in 2.5% glutaraldehyde for ~90 min, dehydrated with a graded ethanol series, then freeze-dried for 2 days. Dried samples were coated with gold and examined via SEM.

2.9. RNA sequencing (RNA-seq)

Total RNA was collected from each sample (namely one human fetal BNSPC sample, one human SCNSPC sample, and one human H9–NSPC sample) with the RNeasy mini kit, then stored at −80 °C. RNA integrity was assessed with the Agilent Bioanalyzer 2000 (Agilent, USA). TrueSeq stranded mRNA-seq libraries were prepared from 1 μg of total RNA per sample with an Illumina mRNA-seq kit (RS-122-2103) and sequenced on an Illumina NovaSeq platform, generating an average of ~20 million paired-end 150-bp reads per sample (range = 18.2–26.8 million reads). Reads were mapped to the human genome GRCh38 using stringtie [29] with default parameters. Between 86.9% and 94.1% (average: 90.0%) of the reads mapped uniquely to the human genome. Total counts of read fragments to candidate gene regions were derived with the stringtie, using the refSeq gene model (GRCh38) as a reference, and used as a basis for the quantification of gene expression. Only genes with read counts above 30 were used for subsequent analysis. Unsupervised hierarchical clustering was performed with the hclust function in R. Samples were clustered using uncentered Pearson correlation and average linkage. Corrected gene counts values were obtained with DESeq2 [30] and differentially expressed genes were selected using a threshold of P <
A SD rat T8-9 complete spinal cord transection model was established using a microsissor. The surgical procedure was slightly modified from a previously described method [32]. Briefly, all surgeries were carried out under intraperitoneal anesthesia with a 10% chloral hydroxlate solution (500 mg/kg body weight) and we employed the analgesic WNT7A sense 5′-CAGAGACACGAACAAGAAGG-3′; 5′-GGATGAGCCTGTTAG ACCGTGACG-3′; 3′-ACTCCAGATCATACAGCTTTGACCGACACGT-5′; 3′-TTTCTCAACAGGGGCAGCA 5′-ACAAGCAGGAGCTTCTCGACTTCACCA 3′; 5′-CACCTCAATCCTGGTGTCGCA 3′; 5′-GTTCGGCTT TTTGGGTGGATT 3′, POUSF2 antisense 5′-TACGAAGAGGGCCACACA 3′; DHH sense 5′-CGACCGCAACAAATGGTGGT 3′, DHH antisense 5′-ATGCTGTTTGACCAGACGAT 3′; OLIG2 sense 5′-CTTCAAGGGCCGCTTGACTTTGATG 3′, OLIG2 antisense 5′-GAAGGCGGTGGCAGTAGAAGG 3′; SOX10 sense 5′-AAAAGCAGGCGACGTCAA 3′, SOX10 antisense 5′-ACCTTGCTTCAGACGCTCCA 3′; KCNJ10 sense 5′-AGACGGGCGAAACAATGTAG 3′, KCNJ10 antisense 5′-CCACTTCTGTTGGAGGACC 3′; LRRK2 sense 5′-TAATGTGGGAGGTATGGGC 3′, LRRK2 antisense 5′-TCCCCACCTTCCACCAACAGG 3′; WNT7A antisense 5′-CCCCGGGATACAGCAGAAT 3′, WNT7A antisense 5′-CGTGGGACCTTTACATTCCAGC 3′; GAD1 sense 5′-TGTTGTTGGTGAAGCCGATA 3′, GAD1 antisense 5′-ACCGTGTAGCAGTGA TTC. 2.11. Surgery processing

Animals were randomly assigned into four experimental groups accepting different treatments: scaffold group which received transplantation of collagen scaffolds (scaffold group, n = 10); the BNSPCs group which received transplantation of collagen scaffolds loaded with 10^6 BNSPCs (scaffold + BNSPCs group, n = 11); the H9–NSPCs group which received transplantation of collagen scaffolds loaded with 10^6 H9–NSPCs (scaffold + H9–NSPCs group, n = 10); and the SCNSPCs group, which received transplantation of collagen scaffolds loaded with 10^6 SCNSPCs (scaffold + SCNSPCs group, n = 11).

2.12. Behavioral assessments

The hindlimb walking ability of SCI Rats was assessed weekly post-injury by two observers blinded to the experiments. The Basso, Beattie & Bresnahan (BBB) locomotor rating scale was used to measure hindlimb walking ability and evaluate the motion amplitude, fineness and coordination of the three hindlimb joints of rats.

2.13. Electrophysiology assessment

Cortical motor evoked potentials (CMEPs) were examined at 1 month post-injury (mpi) using Keypoint 9033A07 (Alpine bioMed ApS, Denmark). Rats were anesthetized with 10% chloral hydrate solution dissolved in saline (500 mg/kg body weight), and placed in a prone position during the testing process. One stimulus electrode was placed into the subcutaneous tissue 1 cm behind the intersection of the cranial midline and eyebrow, and the other electrode was placed into the contra-lateral subcutaneous tissue. The recording electrode was inserted into the muscle of one hindleg. The grounding electrode was placed subcutaneously near the L1 spinal segment. Multiple pulse stimulation was used to produce a CMEP wave with the following parameters: 45 mA, 0.1 ms and 2 Hz. Each test was repeated at least five times to ensure waveform stability.

2.14. Histology and immunohistochemistry

Animals were deeply anesthetized with 10% chloral hydrate (500 mg/kg, body weight) and transcardially perfused with 0.9% NaCl physiological saline solution or PBS followed by perfusion with 4% PFA. Immediately after perfusion, spinal cords were dissected, post-fixed overnight at 4 °C, and transferred to 20% sucrose and 30% sucrose overnight at 4 °C separately. Horizontal spinal cord sections of ~2 cm in containing the injury site were sectioned on a cryostat set at -20 °C. Immunohistochemistry was performed to analyze survival, proliferation, maturation, differentiation, synapse formation, and integration of grafted cells. Cryosections were permeabilized with PBS containing 0.2% Triton X-100 and 5% normal donkey serum for 40 min at RT; blocking buffer was then removed and replaced with primary antibodies (Table S1). Frozen sections were incubated with appropriate primary antibodies as follows: human Nestin (mouse, NEUROMICS at 1:150; mouse, GeneTex at 1:500 to label NSPCs); SOX2 (rabbit, GeneTex at 1:500 to label NSPCs); TUJ1 (mouse, Millipore at 1:500; mouse, GeneTex at 1:300; rabbit, Abcam at 1:500; rabbit, Cell Signaling Technology at 1:300 to label immature neurons); GFAP (rabbit, Abcam at 1:1000 to label astrocytes); GFP (rabbit, Abcam at 1:800; mouse, Abcam at 1:800; chicken, GeneTex at 1:500 to label GFP-expressing grafted human BNSPCs, SCNSPCs or H9–NSPCs); NF (rabbit, Sigma-Aldrich at 1:500 to label mature neurons); NeuN (rabbit, Abcam at 1:500 to label nuclei of mature neurons); S100 (rabbit, Abcam at 1:500 to label Schwann cells); MBP (rabbit, Abcam at 1:500 to label mature oligodendrocytes); SYP (mouse, Millipore at 1:500 to label presynaptic cell membrane); PSD95 (rabbit, Abcam at 1:400 to label postsynaptic cell membrane); GABA (mouse, Sigma-Aldrich at 1:200 to label inhibitory GABAergic neurons); GAD65/67 (rabbit, Sigma-Aldrich at 1:500 to label inhibitory GABAergic neurons); VGLUT1 (guinea pig, Sigma-Aldrich at 1:500 to label glutamatergic terminals). After incubated with primary antibodies at 4 °C overnight, the next day, sections were washed in PBS for three times, incubated with Alexa Fluor-conjugated secondary antibody (X21202, 488 donkey anti-mouse; A10037, 568 donkey anti-mouse; A21206, 488 donkey anti-rabbit; A11011, 568 goat anti-rabbit; A11039, 488 goat anti-chicken; A11075, 568 goat anti-guinea pig; AB150177, 488 donkey anti-sheep) at a concentration of 1:500 in PBS for 1 h at RT, then washed in PBS three times. For nuclear staining, sections were mounted onto glass microscope slides and cover-slipped.
with mounting medium containing DAPI to stain the nuclei. Sections were then imaged with an Observer. Z1 confocal microscope (Zeiss, USA) or an SP8 confocal microscope (Leica, USA).

2.15. Statistical analysis

All experiments were repeated at least three times. The mean values and standard deviations were first calculated, and data points that were outside the mean ± two standard deviations were eliminated as outliers. Two-group comparisons were tested for statistical significance with a two-tailed Student’s t-test. Multiple-group comparisons were tested with one-way repeated-measures analysis of variance (ANOVA) followed by post-hoc analyses. Statistically significant differences were called at p < 0.05. * represented P value < 0.05, ** represented P value < 0.01, *** represented P value < 0.001.

3. Results

3.1. Phenotypic characterization of human BNSPCs, H9–NSPCs and SCNSPCs

We first characterized BNSPCs, H9–NSPCs and SCNSPCs in vitro (Fig. 1A). Primary human BNSPCs and SCNSPCs were isolated from the entire brain or spinal cord segment, respectively, of a human fetus at 13 gestational weeks. These cells could be passaged for at least 12 generations. BNSPCs or SCNSPCs used for identification were from the six generations. These cells could be passaged for at least 12 generations. H9–NSPCs could be passaged for four to five generations. H9–NSPCs used for identification were from the second or third generation. Immunofluorescence staining and quantitative analysis showed that BNSPCs and SCNSPCs possessed similar expression profiles. Approximately 85.7% ± 5.1% of BNSPCs expressed Nestin (Fig. 1B and C) and 88.7% ± 5.7% expressed SOX2 (Fig. 1D and E), both of which are NSPC markers. 95.3% ± 3.9% of BNSPCs expressed TUJ1, a marker for neural progenitors and immature neurons (Fig. 1F and G). In SCNSPCs, ~90.1% ± 8.2% expressed Nestin, 90.6% ± 5.5% expressed SOX2, and 89.1% ± 7.3% expressed TUJ1. Compared with BNSPCs and SCNSPCs, there was a significant decrease in the number of H9–NSPCs expressing Nestin (17.8% ± 4.5%, p < 0.01 vs. BNSPCs and SCNSPCs), SOX2 (49.1% ± 9.2%, p < 0.01 vs. BNSPCs and SCNSPCs) and TUJ1 (16.6% ± 3.1%, p < 0.01 vs. BNSPCs and p < 0.05 vs. SCNSPCs). We also found that expression of the astrocyte marker GFAP differed greatly between BNSPCs, H9–NSPCs, and SCNSPCs. Immunostaining results showed that no BNSPCs was positive for GFAP (Fig. 1H). A significantly higher percentage of SCNSPCs and H9–NSPCs expressed GFAP compared to BNSPCs (13.2% ± 3.7%, p < 0.01, and 7.2% ± 1.9%, p < 0.05, respectively) (Fig. 1I). SEM images revealed many tiny pores and longitudinally ordered structures inside the collagen scaffold (Fig. 1J-L). Culturing of BNSPCs, H9–NSPCs or SCNSPCs showed that cells could extend long axons on the collagen scaffold (Fig. 1M-O). Cell viability was measured by AO/PI staining. BNSPCs, H9–NSPCs, and SCNSPCs cultured on collagen scaffolds were all alive after cultured for 2 weeks (Fig. 1P-R). We also noticed that after BNSPCs, H9–NSPCs, or SCNSPCs were cultured in the differentiation condition for 7 days in vitro, they all could differentiate into neurons, astrocytes or oligodendrocytes (Supplementary Fig. 1).

Fig. 1. Characterization of human fetal BNSPCs, H9–NSPCs, and SCNSPCs, and morphology of collagen scaffolds loaded with BNSPCs, H9–NSPCs, and SCNSPCs. (A) Schematic diagram of the workflow for isolation and culturing of human fetal BNSPCs, H9–NSPCs, and SCNSPCs. Each cell type was immunofluorescence stained and pictures were captured under an inverted microscope. Immunofluorescence staining with (B) the NSPCs marker Nestin, (D) the NSPCs marker SOX2, (F) the immature neuron marker TUJ1, (H) the astrocyte marker GFAP. Scale bar = 200 μm. Quantification of percentages of (C) Nestin+, (E) SOX2+, (G) TUJ1+, (I) GFAP+ cells. * p < 0.05, ** p < 0.01. (J) Macroscopic image showing the collagen scaffold structure with a diameter of 4 mm. Scale bar = 2 mm. Scanning electron microscope images showing (K) cross-sectional morphology of a collagen scaffold, Scale bar = 25 μm, (L) longitudinal sectional morphology of a collagen scaffold, Scale bar = 100 μm, and culturing (M) BNSPCs, (N) H9–NSPCs, (O) SCNSPCs on collagen scaffolds for 2 weeks. Scale bar = 20 μm. Confocal images of AO/PI staining of (P) BNSPCs, (Q) H9–NSPCs, and (R) SCNSPCs cultured on collagen scaffolds for 2 weeks. Scale bar = 100 μm.
3.2. SCNSPCs had more highly expressed genes related to nerve function than other cell types

We examined the transcriptome of BNSPCs, H9–NSPCs, and SCNSPCs (Fig. 2A). The PCA and clustering results of three groups of NSPCs were exhibited in the Supplementary Fig. 2. There were 15,479 genes expressed by all three types of NSPCs (Fig. 2B). In contrast, 821 genes were expressed only in BNSPCs, 1026 were expressed only in H9–NSPCs, and 1068 were expressed only in SCNSPCs. We then analyzed the 50 most highly expressed unique genes in each NSPC type (Fig. 2C and D). GO enrichment analysis revealed that the 50 most highly expressed unique genes in BNSPCs were related to brain development and BMP signaling, whereas the 50 most highly expressed unique genes in H9–NSPCs were associated with organ differentiation and hormone metabolism; those in SCNSPCs were involved in pattern specification process and regionalization (Fig. 2D).

We then identified differentially expressed genes between SCNSPCs and the other two types of NSPCs. With a threshold of log2 (fold change) > 1, there were 3802 differentially expressed genes between SCNSPCs and BNSPCs and 5446 between SCNSPCs and H9–NSPCs (Fig. 2E and F). Among the two sets of differentially expressed genes, 2461 were differentially expressed in both BNSPCs and H9–NSPCs compared with SCNSPCs. GO enrichment analysis of these 2461 overlapping differentially expressed genes indicated that they were associated with extracellular matrix organization, extracellular structure organization, axonogenesis, axon guidance, neuron projection guidance, synapse organization, indicating great differences in gene expression related to nerve cells characteristics between SCNSPCs and the other two types of NSPCs (Fig. 2G).

Next, we focused on 11 nerve-related functions, namely neuron differentiation, myelination, axon ensheathment and oligodendrocyte differentiation, synapse organization, neurotransmitter secretion,
glutamatergic synapse, dopaminergic synapse and GABAergic synapse, axon extension, and axonogenesis. Compared with BNSPCs and H9–NSPCs, SCNSPCs had a greater number of highly expressed genes (Fig. 3 and Supplementary Fig. 4).

We then analyzed only very highly expressed genes by increasing the threshold value to screen for genes that were expressed 10 times higher in BNSPCs or H9–NSPCs compared to SCNSPCs or vice versa. This narrowed the scope of differentially expressed genes in BNSPCs, H9–NSPCs, and SCNSPCs to be analyzed. With the increased threshold, SCNSPCs still had a greater number of highly expressed genes related to each of the 11 functions. After analysis of transcriptome data, we carried out RT-qPCR to confirm if the selected genes expression level has certain difference. We verified the following genes by RT-qPCR: DAB1, ASCL1, TRIM67, POU3F2, DHH, OLIG2, SOX10, KCNJ10, LRRK2, WNT7A, GAD1. The results showed that the expression levels of these genes were basically consistent with the results of transcriptome data, and in SCNSPCs most of them were higher expressed than BNSPCs or H9–NSPCs (Supplementary Fig. 3).

Therefore, based on the RNA-seq and RT-qPCR data we hypothesized that SCNSPCs may exhibit some advantages in these nerve-related functions. However, additional experiments were needed to verify this hypothesis.

3.3. SCNSPCs had increased survived and differentiated into more neurons in SCI rats

To track transplanted BNSPCs, H9–NSPCs, or SCNSPCs in vivo, we infected these cells with GFP-labeled lentivirus before transplantation. The resulting GFP-tagged BNSPCs, H9–NSPCs, and SCNSPCs combined with collagen scaffolds were transplanted into rats with T8-9 complete spinal cord transection to investigate their therapeutic effects. Rats in each group were sacrificed at one mpi to determine survival and differentiation rates of the grafted cells (Fig. 4A and Supplementary Fig. 5). Histological analysis of transplanted GFP-BNSPCs, GFP-H9-NSPCs, or GFP-SCNSPCs at one mpi indicated that more SCNSPCs than BNSPCs or H9–NSPCs survived after transplantation into SCI site. Quantitative analysis of immunohistochemistry staining revealed that a significantly larger percentage of grafted GFP+ cells survived in scaffold + SCNSPCs group (40.2% ± 6.9%) than in the scaffold + BNSPCs group (29.3% ± 2.9%, p < 0.05) or the scaffold + H9–NSPCs group (20.1% ± 3.2%, p < 0.05) (Fig. 4B and C). We also found a significant increase in NeuN and GFP double-positive immunostained mature neurons in the injured site in the scaffold + SCNSPCs group (9.1% ± 2.3%) compared with the scaffold + BNSPCs group (4.5% ± 0.8%, p < 0.05) or the scaffold + H9–NSPCs group (0% ± 0%, p < 0.01) (Fig. 4D). This indicated that grafted GFP-SCNSPCs differentiated into a higher number of mature neurons than grafted GFP-BNSPCs or GFP-H9-NSPCs did. We
found that some GFP+ grafted SCNSPCs extended axons robustly into the host spinal cord (Supplementary Fig. 6). Notably, some NF+ mature nerve fibers that were not co-labeled with GFP also emerged in the injured site in the NSPC transplantation group; these neurons were therefore not differentiated from grafted cells, but instead originated from endogenous mature neurons in the rat host. Quantitative analysis showed significantly more NF+ nerve fibers in the injured area in the scaffold + SCNSPCs group (15.8% ± 2.5%) compared to the scaffold + BNSPCs group (8.6% ± 1.9%, p < 0.05) or the scaffold + H9–NSPCs group (3.4% ± 0.8%, p < 0.01) (Fig. 4E–G). These data showed that engrafted GFP-SCNSPCs had better rates of survival and differentiation into mature neurons than the other two cell types. Additionally, these in vivo immunohistofluorescence staining results were consistent with the in vitro RNA-seq data, which revealed that 10 genes were expressed 10 or more times higher in SCNSPCs than in BNSPCs or H9–NSPCs (Fig. 4H). These genes have been reported as having vital roles in regulating differentiation of NSPCs. For example, achaete-scute family bHLH transcription factor 1 (ASCL1) could increase NSPCs differentiation towards neurons [33], and DAB1 could inhibit differentiation of NSPCs towards astrocytes [34]. Some engrafted GFP+ cells were not mature neuronal cells. We therefore also carried out immunofluorescence staining of the NSPCs marker Nestin, SOX2, and the proliferation marker Ki67 (Supplementary Fig. 7 and Supplementary Fig. 8). As expected, many engrafted GFP+ cells were immunostained for Nestin and Ki67, but no GFP+ cells were SOX2-positive.

3.4. SCNSPCs at the lesion site could be myelinated

Intact central nervous system (CNS) nerve fibers are wrapped with a fatty substance called myelin. Signal transduction along neighbouring neurons lacking myelin tends to be very slow. However, myelination could greatly increase the transduction speed [35]. Myelination may therefore reflect the degree of SCI repair to some extent. Thus, we tested the myelination and axon ensheathment of transplanted GFP-BNSPCs, GFP-H9–NSPCs, or GFP-SCNSPCs. Immunohistofluorescence staining and quantitative analysis of S100β showed a remarkable percentage of GFP+ /S100β+ double-immunostained cells in the injured region of the scaffold + SCNSPCs group (36.7% ± 5.1%) compared with the scaffold + BNSPCs group (0% ± 0%, p < 0.01) and the H9–NSPCs group (20.1% ± 3.9%, p < 0.05). This indicated that more grafted GFP+ cells became wrapped and myelinated by Schwann cells in the scaffold + SCNSPCs group (Fig. 5A–C). Furthermore, H9–NSPCs and SCNSPCs could be myelinated by p75NTR positive Schwann cells (Supplementary Fig. 9). We also measured the marker MBP, which is a vital constituent of the myelin sheath of Schwann cells and oligodendrocytes in the CNS, to verify the myelination and axon ensheathment of grafted GFP+ cells [35].
Quantitative analysis revealed that ~23.5% ± 5.6% of grafted GFP+ SCNSPCs were co-labeled with MBP in the scaffold + SCNSPCs group, whereas only 6.7% ± 1.5% of grafted GFP+ BNSPCs were MBP+ (p < 0.01 vs. SCNSPCs) and no MBP+ signals were detected in the scaffold + H9–NSPCs group (p < 0.01 vs. SCNSPCs) (Fig. 5D–F). We also checked myelin protein zero (MPZ) and myelin proteolipid protein (PLP) expression at lesion site in each group. The results showed that H9–NSPCs and SCNSPCs could be myelinated by Schwann cells which were immunostained for MPZ (Supplementary Fig. 10) and all three NSPCs could be myelinated by oligodendrocytes which were immunostained for PLP (Supplementary Fig. 11) [36,37]. The RNA-seq data were consistent with immunohistofluorescence staining results; SCNSPCs showed a relatively higher capacity for myelination and differentiation into oligodendrocytes (Fig. 5G–I). Specifically, SCNSPCs showed higher expression of POU3F2, a member of the neural cell-specific class III POU domain transcription factors (Fig. 5H). POU3F2 reportedly has a significant effect in controlling myelination in Schwann cells [38]. SCNSPCs also showed high expression of KCNJ10 (also known as Kir4.1); KCNJ10 is a vital gene that is responsible for the major K+ conductance of oligodendrocytes and is critical for myelination [39]. Desert hedgehog (DHH), which encodes a secreted protein that is a key component of Schwann cell modulation of nerve perineurium formation [40], was also highly expressed in SCNSPCs.

3.5. SCNSPCs formed synapses and secreted neurotransmitters in the injured region

In the CNS, a synapse is a tiny gap at the end of a neuron that allows transmittance of an electric pulse between adjacent neurons. Having demonstrated that transplanted GFP+ cells could differentiate into

![Fig. 5.](image-url)
neurons and were myelinated by Schwann cells in the injured site, we sought to determine whether these grafted cells could form synapses. We used two common synaptic markers to identify synapse formation: SYP, a presynaptic marker localized to synaptic vesicles, and PSD95, which is a postsynaptic scaffolding protein in excitatory neurons [41,42].

Significantly more grafted GFP+ SCNSPCs formed SYP+ synapses (7.9% ± 1.5%) than BNSPCs (0% ± 0%, p < 0.01) or H9–NSPCs (1.1% ± 0.2%, p < 0.01) (Fig. 6A–C). Additionally, 21.5% ± 3.6% of grafted GFP+ SCNSPCs were co-immunostained with PSD95 at the damaged site, whereas no GFP+/PSD95+ double-immunostained signals were observed in the scaffold + BNSPCs group or the scaffold + H9–NSPCs group (Fig. 6D–F). Consistent with these results, the in vitro RNA-seq data showed that some vital genes associated with synapse organization were also highly expressed in SCNSPCs (Fig. 6G). For example, Wnt family member 7A (WNT7A), which promotes synaptic, and leucine-rich repeat kinase 2 (LRRK2), which regulates synapse morphology, were both expressed more highly in SCNSPCs than in BNSPCs or H9–NSPCs [43,44]. These findings indicated that, compared with grafted GFP-BNSPCs or GFP-H9-NSPCs, GFP-SCNSPCs had a greater ability to promote synapse formation in the SCI region.

Neurotransmitters are endogenous chemical messengers in the CNS. They participate in diverse biological processes by transmitting signals between neurons or from neurons to muscles [45]. GABA+ inhibitory neurotransmitters, GAD65/67+ inhibitory neurotransmitters, and VGLUT1+ excitatory neurotransmitters were found almost exclusively in the damaged area of the scaffold + SCNSPCs group (11.9% ± 3.1%, 5.7% ± 0.8%, and 6.2% ± 1.3%, respectively) (Fig. 6H and I). No GABA, GAD65/67 or VGLUT1 signals were present in the scaffold + BNSPCs group. Although no GABA+ signals were observed in scaffold + H9–NSPCs group, several grafted GFP+ cells were double immunostained with GAD65/67 (1.1% ± 0.2%; p < 0.05 compared to the scaffold + SCNSPCs group) or VGLUT1 (4.0% ± 0.9%). RNA-seq data confirmed the immunohistofluorescence staining result (Fig. 6J).

Glutamate decarboxylase 1 (GAD1) is the gene encoding GAD67, which is the main rate-limiting enzyme for GABA synthesis [46,47]. GAD1 was highly expressed in SCNSPCs in vitro. These results demonstrated that

Fig. 6. GFP-SCNSPCs formed synapses and secreted neurotransmitters in the SCI region at 1 mpi in T8-9 severe spinal cord transection model rats. Immunofluorescence staining of (A) SYP+ presynaptic cells, (D) PSD95+ postsynaptic cells, and (H) GABA+ inhibitory neurotransmitters, GAD65/67+ inhibitory neurotransmitters, VGLUT1+ excitatory neurotransmitters in the lesion site. Scale bar = 50 μm. Quantification of percentage of GFP+ cells (B) and (E) in the lesion site. *p < 0.05, **p < 0.01, ***p < 0.001. Quantification of percentage of (C) GFP+/SYP+ cells in GFP+ cells, *p < 0.05, **p < 0.01, ***p < 0.001. (F) GFP+/PSD95+ cells in GFP+ cells, ****p < 0.001, and (I) GABA+, GAD65/67+ and VGLUT1+ cells in the lesion site. *p < 0.05, **p < 0.01, ***p < 0.001. Expression of genes related to (G) synapse organization, and (J) neurotransmitter secretion of BNSPCs, H9–NSPCs, and SCNSPCs as determined by RNA sequencing.
grafted GFP + SCNSPCs were able to generate more functional neurons because they not only formed more synapses, but also secreted more inhibitory and excitatory neurotransmitters.

3.6. SCNSPCs transplantation promoted electrophysiological signaling and motor function recovery in SCI rats

Electrophysiology tests and hindlimb movements are two vital indexes used to evaluate SCI recovery (Fig. 7A and B). Hindlimb walking ability was evaluated using BBB score. At one mpi, BBB score was higher in the scaffold + SCNSPCs group (5.4 ± 1.2) than in the scaffold + BNSPCs group (3.3 ± 0.9) or the scaffold + H9–NSPCs group (3.7 ± 0.9) (Fig. 7C). The BBB score for the scaffold + SCNSPCs group, near six, indicated that rats could move all three hindlimb joints, with great movement of one joint and slight movement of the other two. In contrast, rats in the scaffold + BNSPCs and scaffold + H9–NSPCs group could move only two hindlimb joints.

In the CMEPs test, the scaffold + SCNSPCs group again exhibited the best recovery effect. The CMEPs results demonstrated responsive waves in all rats with cell implantation after SCI (Fig. 7D), but the average signal-to-response latency was significantly shorter in the scaffold + SCNSPCs group (5.12 ± 0.36 ms) than in the scaffold + BNSPCs group (6.18 ± 0.28 ms, p < 0.001) or in the scaffold + H9–NSPCs group (8.14 ± 1.57 ms, p < 0.001) (Fig. 7E). The amplitude of the hindleg CMEPs waves of rats in each group. Quantification of CMEPs evaluation. (A) Schematic diagram of rat CMEPs evaluation. (B) BBB scores of rats in each group. *p < 0.05 vs. scaffold group, **p < 0.01 vs. scaffold group, ***p < 0.001 vs. scaffold + BNSPCs group, ^p < 0.05 vs. scaffold + H9–NSPCs group. (D) CMEPs waves of rats in each group. Quantification of (E) latent periods and (F) amplitude for rats in each group. **p < 0.01, ***p < 0.001.

4. Discussion

Animals with SCI can be treated with different types of NSPCs to promote recovery [3]. However, it is important to identify an optimal type of NSPCs for this purpose. The most commonly used NSPCs include those derived from the brain or spinal cord, or pluripotent stem cells. Although these all reportedly have some therapeutic effects for SCI, there are controversies associated with transplantation of pluripotent stem cell-derived NSPCs that limit their applications. For instance, ESCs are isolated from the inner cell mass of a developing blastocyst, which involves destruction of the blastocyst and thus raises ethical concerns [48]. Moreover, such cells may form teratomas or tumors [49,50] or be rejected by the host immune system [22,52].

To date, there have been no studies comparing the transcriptome of BNSPCs, SCNSPCs, and pluripotent stem cell-derived NSPCs, nor have there been comparisons of the effects of transplanting these cell types into severe spinal cord transection models. In the present study, we performed RNA-seq to compare differences in gene expression between human BNSPCs, SCNSPCs, and H9–NSPCs in vitro for the first time. Subsequently, we treated rat T8–9 severe spinal cord transection models with each of the three types of GFP-NSPCs combined with collagen scaffolds to examine the therapeutic effects of each cell type and identify the most suitable type for SCI repair.

With respect to neuron differentiation, the RNA-seq data revealed that SCNSPCs had higher expression of more genes related to neuronal differentiation of NSPCs (e.g., ASCL1, TRIM67, and DAB1) than the other two cell types. ASCL1 can regulate electric field-induced neuronal differentiation through the PI3K/Akt pathway [33]. TRIM67 can induce neuronal differentiation of NSPCs through modulating Ras signaling via degradation of 80K–H [51]. However, DAB1 suppresses astroglial differentiation in NSPCs [34]. In vivo result were consistent with the
RNA-seq data. Several NF+ mature nerve fibers and NeuN+ mature neurons emerged in the damaged region in the GFP-SCNSPCs transplantation group. NeuN+ neurons were co-labeled with GFP+ cells, indicating that these population of mature neurons were derived from grafted GFP-SCNSPCs. However, the NF+ mature axons were not co-labeled with GFP. There are several possible explanations: these nerve fibers could have been endogenous host axons that migrated from the surrounding intact spinal cord; they may have differentiated from host Nestin+ NSPCs; or they could have transdifferentiated from other cells [53]. Several NF+ mature axons were also observed in the GFP-BNSPCs group, but few NF+ or NeuN+ mature neurons were seen in the GFP-H9-NSPCs group.

There were also differences between cell types with respect to myelination. The RNA-seq data further demonstrated that SCNSPCs had high expression of genes encoding the pro-myelination transcription factors OLG2 [54] and SOX10 [55]. In scaffold + SCNSPCs transplantation group, there were significantly more S100β+ and MBP+ signals detected among GFP+ grafted cells in the damaged zone. In contrast, very few MBP+ and S100β+ cells were detected in the GFP-BNSPCs and GFP-H9-NSPCs groups, respectively. The in vitro and in vivo findings suggested that grafted GFP-SCNSPCs were myelinated by Schwann cells or oligodendrocytes at higher levels than GFP-BNSPCs or GFP-H9-NSPCs. This result was consistent with previous findings. For example, during development, myelination occurs earlier in human spinal cord neural cells than in brain neural cells; significant myelination of human spinal cord nerve fibers occurs at the second trimester, and expression of key markers of myelination rapidly increase between 15 and 22 gestational weeks [56]. However, Zhang et al. recently reported that human spinal cord NSPCs expressed myelination genes at 13 gestational weeks [57].

With regard to synapse organization, the RNA-seq results showed that LRRK2 (which regulates synapse morphology) and WNT7A (which modulates synapse strength) were most highly expressed in SCNSPCs. LRRK2 functions by interacting with downstream effectors at the pre- and post-synaptic compartments, such as eukaryotic initiation factor 4E-binding protein (4E-BP) and the microRNA machinery, both of which negatively regulate protein synthesis [44]. WNT7A plays an important role in promoting dendritic spine growth and synaptic strength via Ca2+/Calmodulin-dependent protein kinase II [43]. In the GFP-BNSPCs and GFP-H9-NSPCs engraftment groups, the presynaptic marker SY− and the postsynaptic marker PSD95+ signals were detected in the damaged zone in the GFP-SCNSPCs group. Neurotransmitter secretion was also assessed. The RNA-seq data showed high expression of GAD1 in the SCNSPCs group. GAD1 encodes the enzyme GAD67, which generates the calming neurotransmitter GABA. At one mpi, we observed GABA+, GAD65/67+, and VGLUT1+ cells at the lesion site only in the GFP-SCNSPCs transplantation group. Formation of more synapses and higher neurotransmitter secretion suggested that GFP-SCNSPCs transplantation could facilitate functional neuron formation in the injured area.

We also found that human fetus-derived NSPCs played a greater role than the other two cell types in improving adverse microenvironment in the injured region. The transcriptome data showed that SCNSPCs highly expressed more genes related to glutamate receptor and neurotransmitter secretion than the other two cell types (Supplementary Fig. 12). After CNS injury, a large amount of glutamate is released, causing cell excitotoxicity. At this time, the transplanted NSPCs cell membranes, with selective glutamate receptor and neurotransmitter channels, may take up the glutamate concentration at the lesion site, thus indirectly improving the local microenvironment [58]. Pleiotropin (PTN) regulates microglia-mediated neuroinflammation and is thus related to CNS injury repair. Here, PTN expressed ~10 times higher in SCNSPCs than BNSPCs and ~25 times higher than in SCNSPCs than H9-NSPCs. These results indicated that SCNSPCs may have superior SCI repair effects by improving the microenvironment. The microenvironment of the injured spinal cord is intricate, and microenvironment imbalance is a key cause of poor regeneration after SCI. Microenvironment imbalance can be defined as a decrease in promoting factors and an increase in inhibitory factors at the tissues, cell, and molecular levels [58]. Tissue imbalances involve ischemia, glial scar formation, and myelination; cellular imbalances involve transformation phenotypes of microglia and macrophages and differentiation of endogenous stem cells; and molecular imbalances involve cytokines, neurotrophic factors, and chemokines. Our results both in vitro and in vivo indicated that GFP-SCNSPCs transplantation could promote myelination of grafted cells, which could partially decrease tissue-level imbalances; grafted GFP-SCNSPCs mobilized a small number of endogenous mature neurons to migrate to lesion site, and promoted neuronal differentiation of endogenous NSPCs or transdifferentiation of other cells into neurons, both of which ameliorated cellular-level imbalances. Fetal-derived NSPCs transplantation into a rat cervical SCI model has been reported to reduce the number of pro-inflammatory M1 macrophages, thus attenuating the chronic immune microenvironment at cellular level [59]. At molecular level, SCNSPCs secrete more TGF-β1 (a factor involved in maintenance of immune tolerance and T cell homeostasis) than ESC-derived NSPCs in response to inflammatory factors stimulation [13-18].

In conclusion, our in vitro transcriptome data and in vivo cell engraftment results both suggested that grafted GFP-SCNSPCs were better able than GFP-BNSPCs or GFP-H9-NSPCs to differentiate into neurons at the SCI lesion site and to be wrapped with Schwann cells and myelinated. Additionally, GFP-SCNSPCs generated more functional synapses and some endogenous mature axons emerged at the lesion site. Based on these findings, we hypothesized that grafted GFP-SCNSPCs would likely form more synapses with endogenous mature nerve fibers at the lesion site or in the rostral and caudal intact spinal cord over a longer observation time. Synapses that were formed during the current observation time may have acted as neural relays to partially reconnect the interrupted neuronal circuit, thus promoting electrophysiological signaling and hindlimb motor function in SCI rats. In addition, improvement of the microenvironment may be a necessary factor for SCI repair. We here showed that SCNSPCs may be considered a suitable alternative donor cell type for SCI repair and for future clinical translational research.

The limited sample sources used here could be expanded in future experiments. We used human brain and spinal cord tissue from a fetus as 13 gestational weeks to generate NSPCs, but NSPCs produced from fetuses at other gestational ages may lead to different results. The application of more embryonic stem cell or induced pluripotent stem cell lines to produce NSPCs should also be considered. In addition, other sequencing methods beyond RNA-seq, such as an assay for transposase-accessible chromatin with high throughput sequencing, may yield valuable data. These future experiments would allow more comprehensive selection of an optimal NSPCs donor type for therapeutic effect in SCI repair.

5. Conclusion

NSPC transplantation has been regarded as a promising therapeutic method for SCI repair. However, different NSPCs may differ in therapeutic effects. Identification of an optimal NSPC type is therefore an important consideration. We here compared the transcriptome of human fetal BNSPCs, SCNSPCs, and H9-NSPCs in vitro, and subsequently transplanted each type of NSPCs combined with collagen scaffold into a rat T8-9 complete SCI model. The in vitro data showed that SCNSPCs had more highly expressed genes that are involved in nerve related functions than the other two cell types. Compared with BNSPCs and H9-NSPCs, SCNSPCs also exhibited the best therapeutic effects in vivo, facilitating electrophysiological and hindlimb functional recovery. This work suggests that SCNSPCs may be an appropriate candidate cell type for SCI repair and provides a clinically significant potential strategy for SCI treatment.
Credit authorship contribution statement

Bai Xu: Conceptualization, Methodology, Investigation, Writing - original draft, revision & editing. Man Yin: Conceptualization, Methodology, Formal analysis, Investigation. Yaming Yang: Methodology, Resources, Investigation. Yunlong Zou, Wenbin Liu and Zhan Wang: Resources. Liyang Qiao and Jixiang Zhang: Methodology, Investigation. Yuyu Wu: Resources, Investigation. He Shen, Bing Chen and Ya Shi: Supervision. Minhan Sun and Xianming Wu: Formal analysis. Weiyuan Liu, Weiwei Xue and Qi Zhang: Methodology. Yongheng Fan: Investigation. Falong Lu: Resources, Methodology. Yannan Zhao and Zhifeng Xiao: Conceptualization, revision & editing, Supervision. Jianwu Dai: Conceptualization, Supervision, Funding acquisition.

Ethics approval and consent to participate

All animal experiments were performed in accordance with Chinese Ministry of Public Health Guide and US National Institutes of Health Guide for the care and use of laboratory animals. The use of human fetal tissue was agreed by the Reproductive Study Ethics Committee of Nanjing Drum Tower Hospital and Nanjing Medical University (2018-223-01). Informed consent was provided by the donors. The aborted human fetal tissue was acquired after legal termination of pregnancy.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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