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# Overexpression of Polysialylated Neural Cell Adhesion Molecule Improves the Migration Capacity of Induced Pluripotent Stem Cell-Derived Oligodendrocyte Precursors

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**Key Words.** Remyelination • Pluripotent stem cells • Cell transplantation • Oligodendrocyte • Differentiation

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## ABSTRACT

Cell replacement therapy aiming at the compensation of lost oligodendrocytes and restoration of myelination in acquired or congenital demyelination disorders has gained considerable interest since the discovery of induced pluripotent stem cells (iPSCs). Patient-derived iPSCs provide an inexhaustible source for transplantable autologous oligodendrocyte precursors (OPCs). The first transplantation studies in animal models for demyelination with iPSC-derived OPCs demonstrated their survival and remyelinating capacity, but also revealed their limited migration capacity. In the present study, we induced overexpression of the polysialylating enzyme sialyltransferase X (STX) in iPSC-derived OPCs to stimulate the production of polysialic acid-neuronal cell adhesion molecules (PSA-NCAMs), known to promote and facilitate the migration of OPCs. The STX-overexpressing iPSC-derived OPCs showed a normal differentiation and maturation pattern and were able to downregulate PSA-NCAMs when they became myelin-forming oligodendrocytes. After implantation in the demyelinated corpus callosum of cuprizone-fed mice, STX-expressing iPSC-derived OPCs demonstrated a significant increase in migration along the axons. Our findings suggest that the reach and efficacy of iPSC-derived OPC transplantation can be improved by stimulating the OPC migration potential via specific gene modulation. STEM CELLS TRANSLATIONAL MEDICINE 2014;3:1100–1109

## INTRODUCTION

Transplantation of young myelin-forming cells has been considered a therapeutic option to compensate for lost or nonfunctional oligodendrocytes and to restore myelin sheets in congenital (e.g., vanishing white matter disease) or acquired (e.g., multiple sclerosis [MS]) demyelinating disorders, because no effective pharmacological approach to induce remyelination is available. Many studies in experimental animals have demonstrated the feasibility of such a cell graft approach. However, the lack of a suitable, clinically acceptable source for transplantable autologous oligodendrocyte precursor cells (OPCs) has obviated any aspiration for a clinical application, so far. The ground-breaking discovery of induced pluripotent stem cells (iPSCs) [1] has provided an unprecedented tool to generate large numbers of autologous OPCs from a few somatic cells (e.g., isolated from a small skin biopsy). Reprogramming patient-derived somatic cells to pluripotency and subsequent differentiation into an oligodendrocytic cell lineage has revitalized the idea to test cell transplantation as a clinical approach for treating demyelinating disorders.

Recently, Wang et al. [2] demonstrated the ability of iPSC-derived OPCs to myelinate and rescue a mouse model of congenital hypomyelination. Our laboratory has transplanted iPSC-derived OPCs into a mouse model, mimicking local demyelination such as occurs in MS. We could demonstrate that iPSC-derived OPCs survived and remyelinated the corpus callosum around the injection site [3]. We noticed that the stereotactically injected iPSC-derived OPCs, remarkably, had only a limited potential to migrate along the axon bundles of the corpus callosum. For cell replacement therapy, it is crucial that iPSC-derived OPCs be able migrate freely all the way to lesion sites after transplantation [4]. Thus, it would be desirable to manipulate the transplanted OPCs such that they have an increased migration potential that does not interfere with their survival.

The process of migration and adhesion in OPCs is mainly mediated by adhesion molecules, with a major role for the neural cell adhesion molecule (NCAM) [5, 6]. NCAM is a member of the immunoglobulin family and has the ability to bind long homopolymers of sialic acid. These polysialic acid (PSA) residues are attached to the core glycan of the fifth Ig domain [7, 8] and promote

migration owing to reduced homophilic NCAM-NCAM interactions and cell-cell adhesions [9]. The PSA residues are attached to NCAM via two different enzymes: polysialyltransferase (PST) or sialyltransferase X (STX) [7, 10]. PSA-NCAM is expressed on demyelinated axons, activated astrocytes, Schwann cells, and OPCs, and it has been associated with OPC and Schwann cell migration [11]. In addition, a strong correlation has been demonstrated between the disappearance of PSA-NCAM on both axons and OPCs and the initiation of the myelinating process [9, 12]. PSA-NCAM is of high importance for the migration of OPCs but forms an inhibiting factor in myelination and needs to be downregulated on initiation of myelination [9, 13].

Overexpression of PSA-NCAM on iPSC-derived OPCs via transduction of these cells with PST or STX could be a useful tool to accomplish better migration after transplantation. The concept has been tested with *STX*-transduced Schwann cells implanted after spinal cord injury or implanted in the brain after lysophosphatidyl-choline lesioning [14, 15]. Indeed, the grafted STX-overexpressing Schwann cells demonstrated significantly increased migration and, importantly, silenced STX expression on the start of myelin-basic protein (MBP) expression and the initiation of remyelination of axons.

In the present study, we aim to induce overexpression of STX in iPSC-derived OPCs via lentiviral transduction and compare their migratory capacity with control iPSC-derived OPCs in vitro and in vivo. Moreover, we want to establish the downregulation of PSA-NCAMs in the iPSC-derived OPCs on maturation into oligodendrocytes (i.e., on the start of MBP expression).

## MATERIALS AND METHODS

### Generation of iPSCs

For the generation of iPS cells, E14 mouse embryonic fibroblasts (MEFs) were isolated from C57BL/6 mice. These fibroblasts were reprogrammed using polycistronic lentivirus harboring Oct4, Sox2, Klf4, and cMyc coding sequences [16]. In brief, MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) until passage 3. For lentiviral particle production, HEK293T cells were transfected (Fugene-HD; Roche, Almere, The Netherlands, http://www.roche-applied-science.com) with a polycistronic lentiviral plasmid, together with packaging and envelope plasmids (Addgene, Cambridge, U.K., http://www.addgene.org). Lentivirus containing supernatant was collected and mixed with 8 $\mu$g/ml polybrene (Sigma-Aldrich, Zwijndrecht, The Netherlands, http://www.sigmaaldrich.com), filtered through an 0.45-$\mu$m filter (Whatman, The Netherlands, http://www.whatman.com) and directly added to MEFs. Three days after transduction, the cells were trypsinized and replated on irradiated mouse embryonic fibroblasts (iMEFs) and cultured in embryonic stem cell (ES) medium. One week after replating of infected MEFs, the first ES-like colonies started to appear. The colonies were picked and propagated on iMEFs in ES medium (knockout DMEM [KO-DMEM], 15% knockout serum replacement, 1% nonessential amino acids [all Invitrogen, Breda, The Netherlands, http://www.invitrogen.com]; 2 mM L-glutamine, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin [all PAA, Cölbe, Germany, http://www.paa.com]; 100 $\mu$M 2-mercaptoethanol, supplemented with 1,000 U/ml leukemia inhibitory factor [LIF; Millipore, Amsterdam, The Netherlands, http://www.millipore.com]). Subsequent passages were done using trypsin-based dissociation. To analyze the differentiation

potential of our iPS cells, they were cultured as embryoid bodies (EBs) for 8 days and transferred onto gelatin-coated coverslips for another 5 days. The cells were then fixed and stained for markers of the three germ layers.

### Reverse Transcriptase-Polymerase Chain Reaction

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of the expression of the major pluripotency transcription factors (Oct4, Klf4, Nanog, Sox2, and cMyc) was performed on mRNA collected from pooled MEFs, the mouse iPSCs, iPSC-derived EBs, and mouse ES cells. The forward and reverse primers used are listed in Table 1. Each RT-PCR analysis was done in triplicate. For analysis of STX overexpression, quantitative RT-PCR (qRT-PCR) was applied using primers complementary to the human STX coding sequence.

### Oligodendrocyte Differentiation of iPSCs

The procedure to differentiate mouse iPSCs into an oligodendrocytic cell lineage has been previously described [3]. Mouse iPS medium (KO-DMEM, 15% knockout serum replacement, 1% nonessential amino acids [all Invitrogen], 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, 1% L-glutamine [all PAA], 100 $\mu$M $\beta$-mercaptoethanol), supplemented with 1,000 U/ml LIF (Millipore), was used to keep the iPSCs in a pluripotent state. Trypsin-based dissociation was applied to start the cultivation of embryonic bodies on nonadherent dishes. These were coated with 1% bovine serum albumin in phosphate-buffered saline (PBS) to prevent early adhesion of the cells. Embryonic bodies were propagated for 8 days in mouse EB medium (KO-DMEM, 15% fetal calf serum [FCS; PAA], 1% L-glutamine, 1% nonessential amino acids, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 100 $\mu$M $\beta$-mercaptoethanol). For differentiation toward a neural stem cell (NSC) stage, the cells were plated on laminin-coated adherent dishes and cultured in serum free N2 medium (DMEM/F-12 [Invitrogen], 1% N2 supplement [PAA], 1% L-glutamine, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, 1% nonessential amino acids, and 2 $\mu$g/ml heparin [Sigma-Aldrich]), supplemented with 20 ng/ml epidermal growth factor (EGF, Invitrogen) and 20 ng/ml basic fibroblast growth factor (bFGF; Invitrogen) for 7–10 days. After dissociation of the cells with Accutase (Sigma-Aldrich), the cells were transferred onto coverslips coated with poly-L-ornithine hydrobromide (Sigma-Aldrich) and laminin (Sigma-Aldrich). The cells were then differentiated to OPCs in serum free N2 medium supplemented with 10 ng/ml platelet-derived growth factor (PDGF, PeproTech, Rocky Hill, NJ, http://www.peprotech.com) for 4 days and purified by expression of the receptor PDGF receptor-$\alpha$ (PDGFR$\alpha$) using FACsorting (supplemental online Fig. 1). Continuing differentiation of the iPSC-derived OPCs to mature oligodendrocytes was induced by culturing in serum free N2 medium supplemented with 10 ng/ml triiodothyronine (T3; Sigma-Aldrich) and 10 ng/ml neurotrophin 3 (NT3; R&D Systems Inc., Minneapolis, MN, http://www.rndsystems.com) for an additional 7 days. Culturing took place at 37°C, 5% $CO_2$, and 95% humidity.

### Coculture of iPS-Derived OPCs With Dorsal Root Ganglion Neurons

In order to examine the myelinating capacity of IPS-derived OPCs in vitro, we cocultured them with rat dorsal root ganglion (DRG) neurons. DRGs were explanted from 15-day-old Wistar

**Table 1.** Primers used for reverse transcriptase-polymerase chain reaction

| Transcription factor | Forward primer | Reverse primer |
|---|---|---|
| Oct4 | TCTTTCCACCAGGCCCCCGGCTC | TGCGGGCGGACATGGGGAGATCC |
| Nanog | CAGGTGTTTGAGGGTAGCTC | CGGTTCATCATGGTACAGTC |
| Sox2 | TAGAGCTAGACTCCGGGCGATGA | TTGCCTTAAACAAGACCACGAAA |
| Klf4 | GCGAACTCACACAGGCGAGAAACC | TCGCTTCCTCTTCCTCCGACACA |
| cMyc | TGACCTAACTCGAGGAGGAGCTGGAATC | AAGTTTGAGGCAGTTAAAATTATGGCTGAGC |
| GAPDH | CATCAAGAAGGTGGTGAAGC | ACCACCCTGTTGCTGTAG |
| hSTX | TGTTGAGTGGGTCAACGAGC | TGTTGGTCAGCCAGTATCCG |

Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

rat embryos and dissociated in a solution containing papain (1.2 U/ml, Sigma-Aldrich), L-cysteine (0.24 mg/ml, Sigma-Aldrich), and DNase I (40 mg/ml, Roche) for 1 hour at 37°C. The dissociated cells were plated at a density of 60,000 cells per 13-mm coverslip (VWR International, Radnor, PA, http://www.us.vwr.com) precoated with poly-L-lysine (5 $\mu$g/ml, Sigma-Aldrich) and growth factor reduced Matrigel (1:40 dilution; BD Biosciences, San Diego, CA, http://www.bdbiosciences.com). DRG neurons were cultured for 21 days in DMEM (Gibco, Grand Island, NY, http://www.invitrogen.com; supplemented with 10% FCS, Bodinco BV, Alkmaar, The Netherlands, http:/www,bodinco.nl; L-glutamine and penicillin/streptomycin, Invitrogen) in the presence of nerve growth factor (100 ng/ml, Serotec Ltd., Oxford, U.K., http://www.serotec.com). The cells were pulsed four times for 2 days with fluorodeoxyuridine (10 $\mu$M, Sigma-Aldrich) to remove contaminating proliferating cells, in particular fibroblasts and Schwann cells. The purity of the DRG culture was microscopically confirmed. Subsequently, 50,000 iPS-derived OPCs were seeded onto coverslips containing the DRG neurons with extensive axonal outgrowth. The following day, the medium was changed to N2 with 30 ng/ml T3 and 10 ng/ml NT3. OPCs were cocultured with the DRG neurons for 14 days with a medium change every second day. After that period, the cells were fixed and subjected to immunocytochemical staining for MBP and neurofilaments.

The myelination efficiency of STX-transfected iPS-derived OPCs and control empty vector iPS-derived OPCs in the DRG cocultures was quantified by analysis of immunofluorescence images, 15 per coverslip, using ImageJ software. In brief, in merged images of the red channel (neurofilament) and green channel (MBP), yellow areas (indicating myelinated axons) were measured and divided by the area covered only by green (representing the oligodendrocytes and their extensions); this quotient was used as an indication of the myelination efficiency. Myelination efficiency data derived from the cocultures with control (empty vector) iPS-derived OPCs were normalized to 1.

### Transfection With STX Gene

To induce overexpression of PSA-NCAMs in iPSC-derived neural stem cells (NSCs), a lentiviral vector was applied, introducing the gene encoding for the human polysialyltransferase ST8SIA2 (STX). HEK 293 (human embryonic kidney) cells were transduced with the lentiviral vectors pLenti–GIII–CMV-hST8SIA2 (encoding for STX and puromycin resistance) and pLenti–GIII–CMV Blank (encoding for puromycin resistance) as a control (both from Applied Biological Materials Inc., Richmond, British Columbia, Canada, http://www.abmgood.com), using Fugene HD (Promega, Madison, WI, http://www.promega.com). Two hours before transfection, the

culture medium (standard medium: DMEM, 10% FCS, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin) of 80% confluent HEK cells was refreshed to ensure high transfection efficiency. The viral supernatant of these cells was collected 48 hours after transfection and passed through a 0.45-$\mu$m pore Acrodisc filter (Whatman) to remove cell debris. Centrifugation (2,000$g$, 20 minutes) of the filtrate in an Amicon Ultra tube (Millipore) concentrated the virus in 200 $\mu$l of standard medium. The lentiviral vectors were diluted in N2 medium supplemented with EGF, bFGF and 8 $\mu$g/ml hexadimethrine bromide (Polybrene, Sigma-Aldrich). The medium of the targeted NSCs was replaced with this virus-containing N2 medium for 24 hours. Successfully transduced NSCs were selected by culturing the cells in N2 medium supplemented with EGF, bFGF, and 1 $\mu$g/ml puromycin for 4 days. The cells were cultured in N2 medium supplemented with bFGF and EGF with ongoing puromycin selection at 0.5 $\mu$g/ml.

### Western Blot Analysis

iPS-derived OPCs from the control and STX-transfected cell lines were analyzed for expression of NCAM and PSA-NCAM. OPCs were lysed in RIPA+ (RIPA buffer plus protease inhibitors, Sigma-Aldrich) lysis buffer, and samples were sonicated and centrifuged for 10 minutes at 4°C at maximum speed. The supernatant was collected and the protein concentration determined using RC DC protein assay (Bio-Rad, Hercules, CA, http://www.bio-rad.com). A protein amount of 60 $\mu$g was diluted in 6$\times$ sample buffer (0.625 M Tris, pH 6.8, 20% SDS, 87% glycerol, 2-mercaptoethanol, 0.1% bromophenol blue) boiled at 95°C for 5 minutes and placed on ice before loading on a 7.5% acrylamide gel. The proteins were separated at 110 V. For blotting, a nitrocellulose membrane was applied according to manufacturer's instructions. Blocking was mediated for 1 hour in 5% nonfat dry milk in PBS-Tween (PBS-T). For detection of NCAMs, membranes were incubated in rabbit anti-NCAM antibody (AB5032, Millipore) (1:3,000 dilution); for detection of PSA-NCAM, we used mouse anti-PSA-NCAM (MAB5324, Millipore) (1:2,000 dilution), all in PBS-T with 5% nonfat dry milk. After incubation overnight at 4°C, the membranes were incubated for 1 hour at room temperature in biotinylated secondary antibodies (all from Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). For PSA-NCAM, we used goat anti-mouse IgM (BA-2020; dilution 1:100) and for NCAM, goat anti-rabbit IgG (BA-1000, Vector Laboratories) The membranes were then incubated with streptavidin-biotinylated horseradish peroxidase complex (RPN1051; Amersham, GE Healthcare, Little Chalfont, U.K., http://www.gehealthcare.com), diluted 1:5,000 in PBS for 15 minutes at room temperature. Proteins were detected using the ECL Prime system

(RPN2232; Amersham, GE Healthcare) according to the man-ufacturer's protocol.

## In Vitro Migration Assay

To quantify the migratory potential of the iPS-derived OPCs, a standardized scratch-migration assay was performed. Three different OPC suspensions (i.e., cells overexpressing STX gene, cells transduced with an empty vector, and nontransduced cells) were seeded, each in 4 uncoated wells of a 12-well tissue culture plate and incubated in N2 medium complemented with PDGF (10 ng/ml) at 37°C overnight. After incubation, a standard gap across the seeded cell suspensions was created by a scratch with a 10-$\mu$l pipette tip. Afterward, the medium was refreshed to remove the debris (dead and floating cells). Photographs were taken at 0, 24, 48, and 72 hours after scratching to record and quantify the spontaneous migratory activity of the OPCs to bridge the gap. For quantification, the cells were pseudocolored in the imaging software (ImageJ), and the percentage of the colored area in the photographs was calculated. The average values for the different measurement points of each of the experimental groups were normalized to the values at 24 hours after scratching, which were set as 1. To ensure the involvement of PSA-NCAM in the observed results, additional PSA cleavage experiments were performed with addition of endoneuraminidase-N (EndoN; ABC-abc0020; ABC Scientific, Los Angeles, CA, http://www.abcscientific.com) at 1:5,000 dilution for 12 hours before introducing the scratch. To exclude the possibility that an increase in cell number in the gap could be ascribed to increased cell proliferation, we counted the total number of cells present in a well at 0, 24, 48, and 72 hours after scratching.

## Transplantation of iPSC-Derived OPCs in Cuprizone Mouse Model

In previous studies, we have shown that transplanted mouse iPSC-derived OPCs are able to remyelinate corpus callosum axons that were demyelinated after mice were given a diet of 0.2% (wt/wt) cuprizone [3]. These stereotactically injected OPCs demonstrated a limited migration along the axonal bundles of the corpus callosum. To examine the effect of increased expression of PSA-NCAMs on the migration of implanted mouse iPSC-derived OPCs, we stereotactically injected the modified OPCs in the corpus callosum, as previously described. C57Bl/6 mice were given a diet of 0.2% (wt/wt) cuprizone (Sigma-Aldrich), a copper chelator. This diet leads to selective oligodendrocyte death, followed by demyelination of axons, mainly in the corpus callosum within 4–6 weeks [17, 18]. During short-term exposure (<6 weeks), dying oligodendrocytes are replaced by OPCs located in the corpus callosum and adjacent tissues. However, continuing the cuprizone diet for a longer period results in depletion of the pool of endogenous OPCs in the corpus callosum and, finally, to its complete demyelination [19]. At 9 weeks after the start of the cuprizone diet, the mice were divided into 2 groups: 1 group (n = 6) received a suspension of iPSC-derived OPCs transduced with an empty vector, and 1 group (n = 6) was injected with a suspension of STX-expressing iPSC-derived OPCs. Additionally, to identify the implanted cells afterward in brain sections, they were transduced with lentivirus encoding for enhanced green fluorescent protein (GFP) before OPC differentiation. On the day of transplantation, the cells were harvested with Accutase (Sigma-Aldrich), counted, and resuspended in PBS at a concentration of 25,000 cells per

microliter. Next, 4 $\mu$l of cell suspension was injected into the corpus callosum of C57BL/6 mice using the following stereotactic coordinates (in reference to the Bregma point): +0.98 mm (anteroposterior axis), −1.75 mm (lateromedial axis), −2.25 mm (vertical axis) [20, 21]. Next, suspensions of 100,000 cells in 4 $\mu$l PBS were slowly injected into the corpus callosum of ketamine-anesthetized mice using a 10 $\mu$l Hamilton injection syringe (22s/2"/3) (80365; Hamilton Co., Reno, NV, http://www.hamiltoncompany.com). Every injection was done within a standardized window (i.e., 5-minute injection time and 2-minute deposition rest) before needle retraction to prevent potential variation in the effect of shearing forces. After implantation of the iPSC-derived OPCs, the mice were taken off the cuprizone diet and given the normal diet again to avoid degeneration of the implanted OPCs by cuprizone. Mice with implantation of control and STX-transfected iPSC-derived OPCs were perfusion fixated at 3 weeks.

## In Vivo Migration Assay

The distances along which the GFP-labeled implanted cells migrated in the demyelinated corpus callosum were measured based on images of a brain section taken at the site of injection. In brief, for every image, the center of the injection site was determined, and the distances from that point to the GFP-labeled cell bodies were measured. The results are presented as the mean ± SEM. For each condition/section, the migration of the 75 most distant cells was measured.

## Immunocytochemistry

Immunocytochemistry was used to confirm the expression of the transfected genes, to identify the reprogrammed and differentiated cell types in culture, and to recognize and characterize iPSC-derived OPCs after transplantation in the corpus callosum of cuprizone fed mice. Cell cultures were fixed on coverslips for 15 minutes with 4% paraformaldehyde (Sigma-Aldrich) and then washed 3 times with plain PBS. Nonspecific binding sites were blocked for 1 hour in blocking solution (PBS plus 0.1% Triton, supplemented with 5% normal goat serum [NGS] and 2% FCS). Primary antibodies (Table 2) were diluted in PBS plus 0.1% Triton plus 1% FCS plus 1% NGS and applied overnight at 4°C. After incubation, the cells were washed three times in PBS. Secondary antibodies and Hoechst (1:1,000) were diluted in PBS plus 0.1% Triton and applied for 1 hour at room temperature in the dark. After washing in PBS three times, the cells were mounted on glass slides using Mowiol (Sigma-Aldrich).

The mice that received stereotactic cell grafts were perfused transcardially with 4% paraformaldehyde under isoflurane anesthesia. The brains were excised and sectioned on a cryostat for detection and immunohistochemical analysis of the cell implants. Implanted cells were identified by their expression of GFP. To analyze the differentiation of the implanted cells, the primary antibodies listed in Table 2 were used. Subsequently, various fluorescent secondary antibodies were used to visualize the specific primary immunoreaction product in single and double immunohistochemical stains.

## RESULTS

### iPSC-Derived NSCs and STX Transfection

Using lentiviral transduction of the E14 mouse embryonic fibroblasts with the Yamanaka pluripotency factors, we were able to

**Table 2.** Antibodies used for immunohistochemistry

| Antibody | Company | Catalog number |
|---|---|---|
| NCAM | Millipore | AB5032 |
| Nestin | Millipore | MAB353 |
| PSA-NCAM | Millipore | MAB5324 |
| MBP | AbD Serotec | MCA409S |
| PDGFR$\alpha$ | Santa Cruz Biotechnology | sc-338 |
| NF | Millipore | MAB1615 |
| GFP | Developmental Studies Hybridoma Bank | GFP-G1 |
| Oct4 | Santa Cruz Biotechnology | sc-5279 |
| Nanog | Abcam | ab80892 |
| Sox2 | Cell Signaling | #4900S |
| SSEA1 | Santa Cruz Biotechnology | sc-21702 |
| SSEA4 | Developmental Studies Hybridoma Bank | MC-813-70 |

Abbreviations: GFP, green fluorescent protein; MBP, myelin-basic protein; NCAM, neural cell adhesion molecule; NF, neurofilament; PDGFR$\alpha$, platelet-derived growth factor receptor-$\alpha$; PSA-NCAM, polysialic acid-neuronal cell adhesion molecule; SSEA, stage-specific embryonic antigen.

generate several clones of iPSCs. These iPSCs were characterized according to their expression of crucial endogenous pluripotent transcription factors and pluripotency markers, at the mRNA and protein level (supplemental online Fig. 2). We verified the presence of all three germ layers after the formation of embryoid bodies (data not shown).

We have chosen the intermediate NSC stage (Fig. 1A) in the iPSC-OPC differentiation protocol for lentiviral transduction to induce overexpression of STX and subsequent PSA-NCAM production in the OPCs, because this method resulted in the highest efficiency and expression level (data not shown). Selective differentiation and survival of proliferating nestin-positive NSCs (Fig. 1A) was achieved by culturing enzymatically dissociated 8-day-old iPSC-derived EBs in N2 medium in the presence of bFGF and EGF. We transduced these iPSC-derived NSCs with a lentiviral vector containing the gene encoding for STX and a separate control cell population with an empty vector using cell selection via puromycin resistance. Most NSCs demonstrate a high level of NCAM expression but only very low levels of PSA-NCAM (Fig. 1). After establishing successful *STX* gene transfection with qRT-PCR (Fig. 1B), we were able to demonstrate high levels of PSA-NCAM in *STX*-transfected NSCs (Fig. 1C, 1D) in contrast to nontransfected or iPSC-derived NSCs transduced with an empty vector (Fig. 1E, 1F). To verify the specificity of the NCAM polysialylation by STX, we treated the transfected NSCs with EndoN for 24 hours and observed a complete reduction in the expression of PSA-NCAM (Fig. 1G).

### Differentiation of *STX*-Transfected iPSC-Derived NSCs Into Functional OPCs

Using the previously published protocol to differentiate iPSCs into an oligodendrocytic cell lineage [3], we continued to differentiate the *STX*-transfected iPSC-derived NSCs into OPCs. A similar pattern of OPC differentiation was observed for the *STX*-transfected iPSC-derived NSCs as for the nontransfected control

NSCs (supplemental online Fig. 3). The resulting *STX*-transfected iPSC-derived OPCs showed high expression of PSA-NCAM (Fig. 2A), in contrast to the minimal expression in the nontransfected control NSCs after OPC differentiation (Fig. 2B). No differences in morphology and differentiation were observed between the groups. It is well known and functionally essential that during the in vivo maturation of OPCs and the start of myelin formation, oligodendrocytes downregulate STX activity and stop PSA-NCAM formation to allow contacting axons. In our cultures, we observed that nontransfected, as well as *STX*-transfected, OPCs, silenced PSA-NCAM expression the moment they started to express MBP (Fig. 2C, 2D), indicating that STX activity in the transfected iPSC-derived OPCs was regulated similarly to control OPCs during functional maturation.

To examine whether STX-overexpressing iPSC-derived OPCs would show a similar in vitro myelination activity as empty vector transduced iPSC-derived OPCs, we cocultured similar numbers of them on coverslips containing a standardized number of rat DRG sensory neurons that had formed an extensive network of long axons in 2 weeks of preculture (Fig. 2E, 2F). We quantified the myelination efficiency for both OPC populations and found no significant differences between them (Fig. 2G). However, the *STX*-transfected OPCs seemed to be more dispersed over the DRG coculture, reflecting increased migration activity compared with the controls, which stayed more clustered.

### PSA-NCAM and iPSC-Derived OPC Migration In Vitro

To examine the effect of STX-overexpression and the subsequent presence of PSA-NCAM on the spontaneous migration activity of iPSC-derived OPCs in vitro, we plated *STX*-transfected iPSC-derived OPCs, parallel to the relevant control (nontransduced or empty vector-transduced) iPSC-derived OPCs in 12-well plates. As an additional control, we treated *STX*-transfected iPSC-derived OPC with EndoN for 12 hours before plating. Endoneuraminidase is an enzyme that cleaves PSA from the PSA-NCAM. In a modification of the widely used wound healing in vitro assay, we induced a gap of standardized dimensions by scratching through the middle of the plated cell cultures and examined the spontaneous bridging of the different plated iPSC-derived OPC populations after 3 days (Fig. 3A–3C). *STX*-transfected iPSC-derived OPCs showed significantly increased migratory activity compared with the control OPCs, an effect that was abolished when the cells were treated with endoneuraminidase (Fig. 3D). To exclude the possibility that the increase in cell number bridging the gap was due to an increase in proliferation, we counted all the cells at the start and at different stages of the migration assay. No differences in the proliferation rate were observed among the various iPS-derived OPC populations (Fig. 3E)

### PSA-NCAM and iPSC-Derived OPC Migration In Vivo

Feeding mice with a diet containing 0.2% cuprizone led to complete demyelination of the corpus callosum after 6 weeks. Stereotactic injection of iPSC-derived OPCs after 9 weeks of the cuprizone diet at a standard location in the corpus callosum was used to examine the migratory behavior and remyelination along the nude axon bundles and the effect of STX overexpression on that. As has been demonstrated in previous studies [3], injected mouse iPSC-derived OPCs survived and repopulated the corpus callosum (Fig. 4). At 3 weeks after implantation, most of the injected iPSC-derived OPCs had matured into
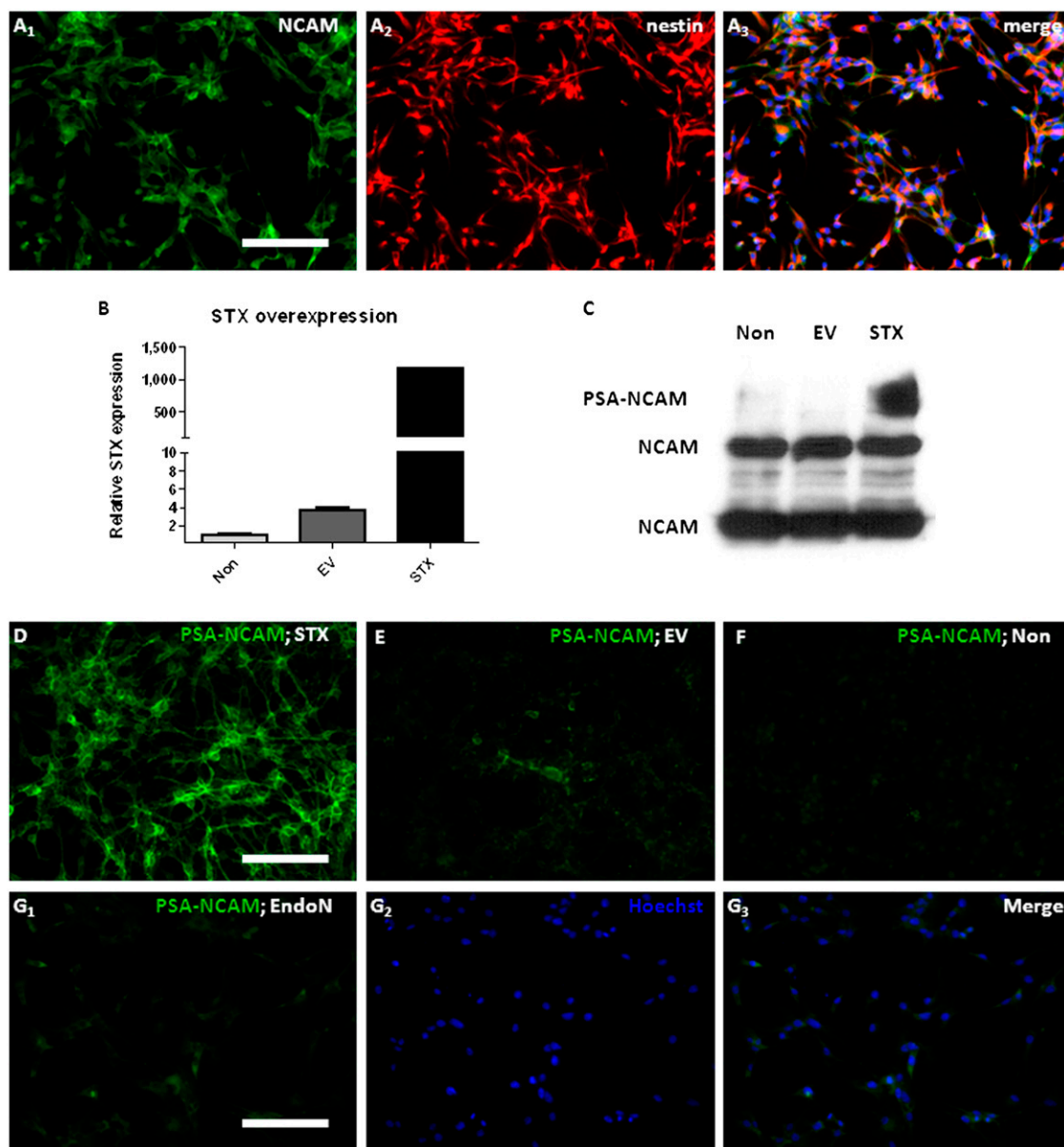
**Figure 1.** STX overexpression in iPSC-derived neural stem cells (NSCs). **(A₁–A₃):** Expression of NCAM and nestin in iPSC-derived NSCs merged with Hoechst nuclear staining. **(B):** Quantitative polymerase chain reaction-normalized STX expression in control NSC groups (i.e., nontransfected, EV-transfected, and STX-overexpressing NSCs). **(C):** Western blot showing NCAM and PSA-NCAM protein expression in control and *STX*-transfected iPSC-derived NSCs. **(D–F):** PSA-NCAM immunostaining of control and STX-overexpressing iPSC-derived NSCs. **(G₁–G₃):** PSA-NCAM immunostaining of STX-overexpressing NSCs after 24 hours of EndoN treatment. Scale bar = 100 $\mu$m. Abbreviations: EndoN, endoneuraminidase-N; EV, empty vector; NCAM, neural cell adhesion molecule; Non, nontransfected; PSA-NCAM, polysialic acid-neural cell adhesion molecule; STX, sialyltransferase X.

MBP-expressing oligodendrocytes, although immature OPCs and some nestin-positive implanted cells could be detected (supplemental online Fig. 4). A similar pattern of survival and maturation was observed with the control and *STX*-transfected iPSC-derived OPCs. However, considerable differences were seen in the location and spread of the implanted iPSC-derived OPCs (Fig. 4B). The iPSC-derived OPCs expressing STX showed a significant increase in migration along the axons (Fig. 4C) compared with the control iPSC-derived OPCs (Fig. 4D) and had repopulated the corpus callosum along its entire width at the unilateral side of the injection. No difference in total cell survival between the control and *STX*-transfected iPSC-derived OPCs could be observed (data not shown).

## DISCUSSION

In this study, we demonstrated that forced overexpression of the polysialic acid transferase STX in iPSC-derived OPCs significantly increased their migration potential in vitro and in vivo compared with control iPSC-derived OPCs, which are only able to form low levels of PSA-NCAM [12].

Importantly, the *STX*-transfected iPS-derived OPCs retained the capacity to downregulate PSA-NCAM formation at the moment they reached the myelin-forming stage, in line with previous findings of *STX*-transfected Schwann cells [8, 12, 15]. The presence of PSA-NCAM has been shown to interfere with the
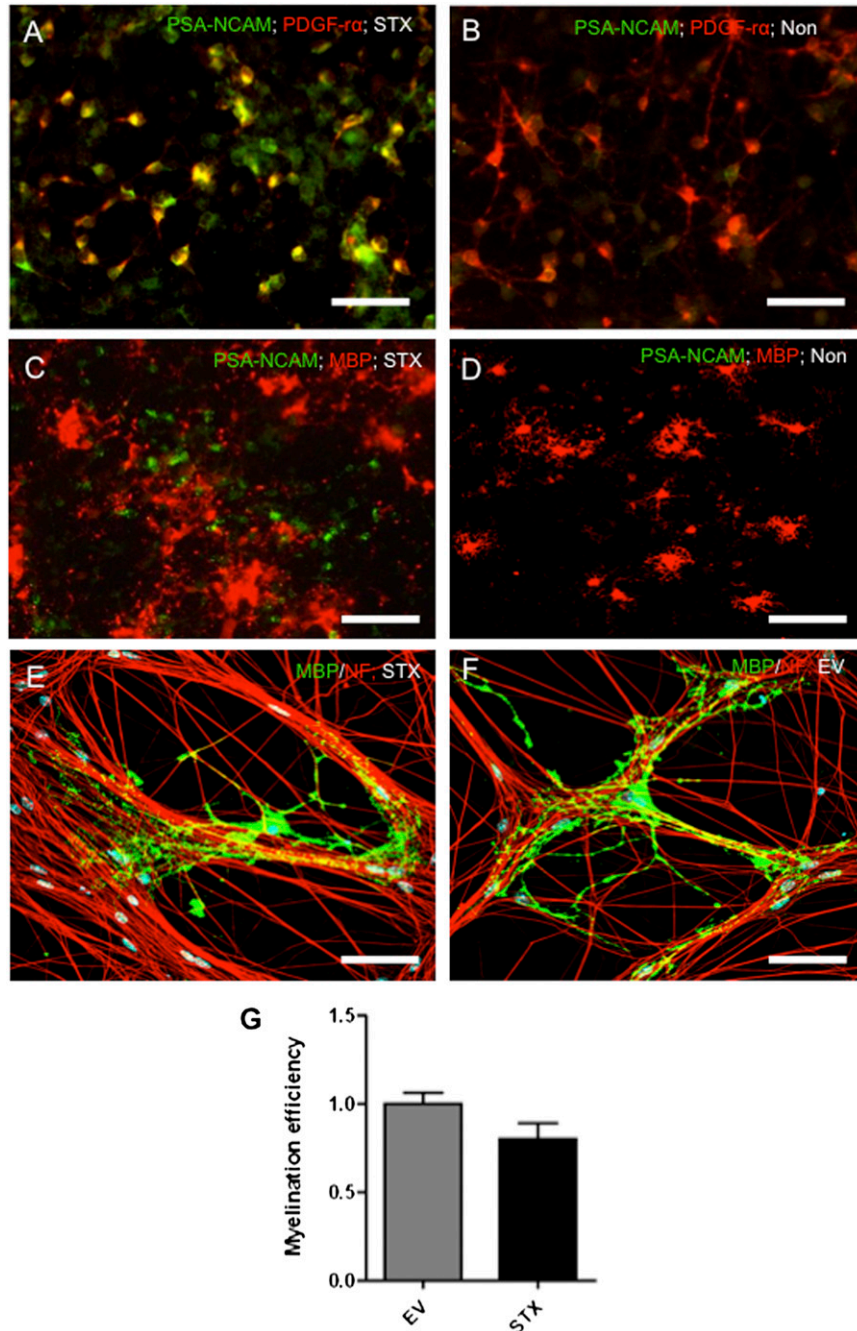
**Figure 2.** Differentiation of STX-overexpressing iPSC-derived neural stem cells into oligodendrocytes. **(A):** Most of the PDGFR$\alpha$-positive (red) *STX*-transfected iPSC-derived oligodendrocyte precursors (OPCs) express PSA-NCAM (green). **(B):** Nontransfected iPSC-derived OPCs show only very low levels of PSA-NCAM. **(C):** During differentiation into mature oligodendrocytes, *STX*-transfected iPSC-derived OPCs start to express MBP (red), which is accompanied by complete downregulation of STX activity and loss of PSA-NCAM. Only the still undifferentiated *STX*-transfected iPSC-derived OPCs between the MBP producing cells still express some PSA-NCAM. **(D):** Nontransfected iPSC-derived OPCs show a similar pattern and no PSA-NCAM immunostaining could be detected after the start of MBP expression. **(E, F):** Two-week-old coculture of rat dorsal root ganglion (DRG) neurons (NF = red) with STX-overexpressing iPSC-derived OPCs **(E)** or control (transfected with EV) iPSC-derived OPCs **(F)**. Bars = 100 $\mu$m. **(G):** Graph indicating myelination efficiency (see Materials and Methods section) of STX-overexpressing iPSC-derived OPCs (STX) or control (transfected with empty vector) iPSC-derived OPCs after 2 weeks of coculture with rat DRG neurons; no significant differences were found (Student's *t* test). Graph bar indicates mean $\pm$ SD. Abbreviations: EV, empty vector; MBP, myelin-basic protein; NF, neurofilament; Non, nontransfected; PDGFR$\alpha$, platelet-derived growth factor receptor-$\alpha$; PSA-NCAM, polysialic acid-neuronal cell adhesion molecule; STX, sialyltransferase X.

proper maturation of OPCs and to inhibit the actual myelination of axons [8, 9, 12, 13]. It has been proposed that axonal factors can induce the downregulation of PSA-NCAM [8, 12]; however, apparently also in the absence of axons, maturing iPSC-derived OPCs stop PSA-NCAM formation the moment they produce one of the major myelin proteins, MBP. Whether this is accomplished via epigenetic silencing of STX expression, via an MBP-dependent feedback loop, at the translational
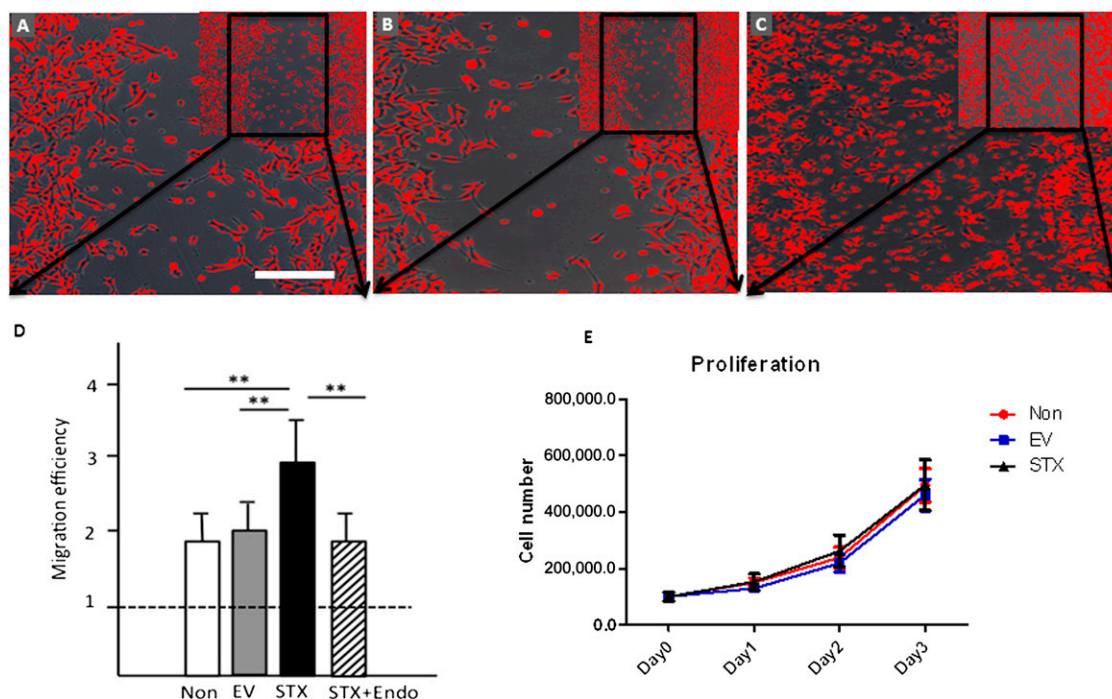
**Figure 3.** Comparison of in vitro migration capacity of STX-overexpressing iPSC-derived oligodendrocyte precursors (OPCs) and controls. **(A–C):** Representative photographs of the migration assay of nontransfected **(A)**, EV-transfected **(B)**, and *STX*-transfected OPCs **(C)** 48 hours after standard gap introduction showing increased migratory capacity of STX-overexpressing OPCs. The cells were pseudocolored red (bar = 100 $\mu$m). **(D):** Migration efficiency of different OPC groups represented as gap area bridged by the iPSC-derived OPCs at day 3. Data at day 1 was normalized to 1 for each group. Two-way analysis of variance; ∗∗, $p < .01$; $n = 3$. **(E):** Proliferation curves of the three different iPSC-derived OPC populations during the migration assay, showing no significant differences. Abbreviations: EV, empty vector; Non, nontransfected; STX, sialyltransferase X; STX+Endo, sialyltransferase X-transfected, endoneuraminidase-treated.

level, or via increased breakdown of PSA-NCAM remains to be elucidated.

Our data imply that *STX*-transfected iPSC-derived OPCs have the ability to repopulate and remyelinate a wider area in the brain after injection compared with unmodulated iPS-derived OPCs. Obviously, the migratory activity of OPCs within the brain does not depend only on the composition of the extracellular matrix. Apart from physical barriers (e.g., the presence of an astrocytic scar), the concentration and gradient of chemotactic signals play a crucial role, as does the interaction with activated microglia and the neuroinflammatory factors they release. In the cuprizone mouse model, oligodendrocyte degeneration and myelin destruction result in a massive recruitment and activation of microglia in the corpus callosum [17, 22]. Olah et al. [23] performed genome-wide gene expression analysis of microglia in the corpus callosum during demyelination and remyelination in the mouse cuprizone model. Remyelination in this model spontaneously occurs after an episode of toxin-induced primary demyelination and normal diet is restored. They provided evidence for the existence of a microglia phenotype that supports remyelination already at the onset of demyelination and persists throughout the remyelination process. During demyelination, these microglia appear to be involved in the phagocytosis of myelin debris and apoptotic cells; during remyelination, they express a cytokine and chemokine repertoire, enabling them to activate and recruit endogenous OPCs to the lesion site and deliver trophic support [23]. Although we did not include the final stage of actual remyelination in our experiments, the iPSC-derived OPCs that we injected during this stage might have benefited from the presence of this remyelination supporting microglia, after overcoming the activation of microglia at the site of injection as a response to the tissue damage induced by the stereotactic needle. Similar experiments in mice depleted from microglia (e.g., by treatment with colony stimulating factor 1 receptor inhibitors [24]), could help our understanding of the effect of microglia on the migration of implanted OPCs.

As described for most intracerebral cell grafting studies in experimental animals, most injected cells do not survive the traumatic injection procedure and the transition to a hostile environment. Only those cells that will finally succeed in specific functional integration will survive over time. Most of the iPSC-derived OPCs that survived in the corpus callosum migrated and matured toward a functional oligodendrocyte stage, although immature iPSC-derived OPCs could still be detected. Occasionally, nestin-positive cells could be observed between the surviving implanted iPSC-derived OPCs (supplemental online Fig. 4). Either these cells dedifferentiated into NSC-like cells, such as has been described before in vitro [25], or the cell implants contained some PDGFRα-expressing NSCs. Additional purification steps using more specific membrane markers for OPCs and/or NSCs should be included to eliminate the contamination of these NSCs. Recently, it was found that NSCs within intracerebral neuronal grafts secrete chemoattractants for their neuronal progeny, preventing extensive migration of these neurons into the host brain [26]. It is as yet unclear whether the presence of NSCs in our iPSC-derived OPC grafts similarly interfered with migration of the OPCs. In none of our implantation experiments was teratoma formation by contaminating iPSCs observed.
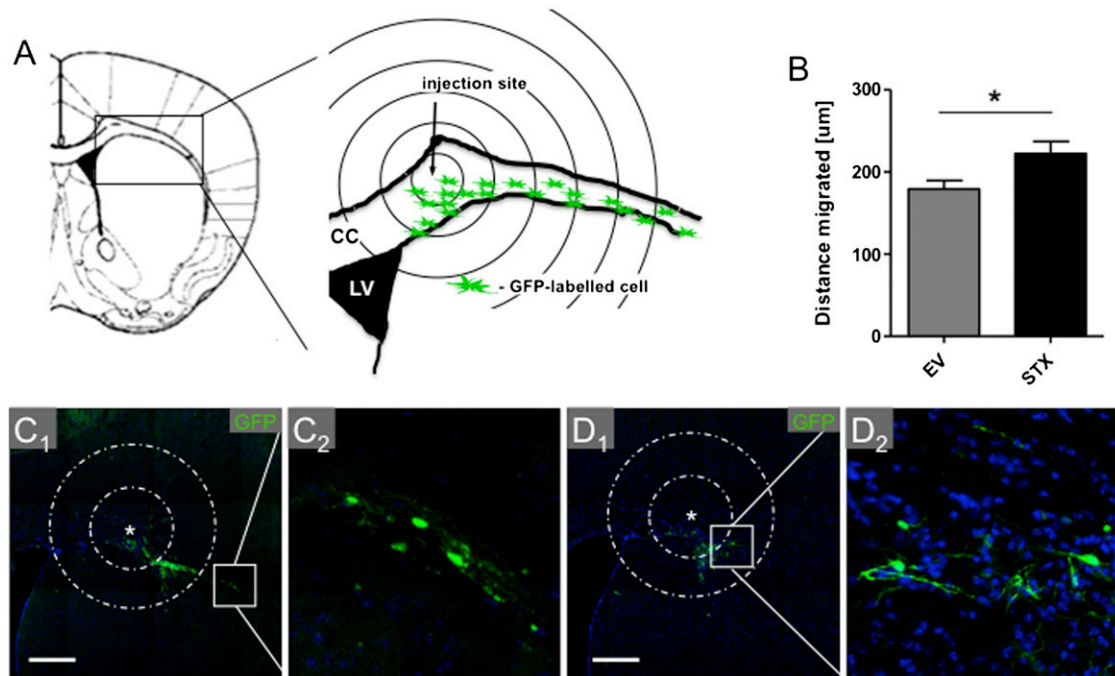
**Figure 4.** Implantation of GFP-labeled iPSC-derived oligodendrocyte precursors (OPCs) into the CC of cuprizone-fed mice. **(A):** Schematic drawing of the injection site of GFP-labeled cells that migrate along the axons in the CC. The measuring scale (50-$\mu$m steps) that we used to determine the distance traveled is indicated. **(B):** Average distance traveled from injection site by control (EV) and STX-overexpressing iPSC-derived OPCs. *t* test; *n* = 4; $*$, $p < .05$. **(C, D):** Representative photographs of GFP-labeled STX-overexpressing OPCs **(C1, C2)** and control (EV) OPCs **(D1, D2)** injected at the site indicated by an asterisk. The measuring scale is projected on top (inner scale radius 100 $\mu$m; outer scale radius 200 $\mu$m). Although the STX-overexpressing OPCs could migrated up to a distance of 300 $\mu$m (area magnified in **C2**), most of the control (EV) OPCs stayed within the 200-$\mu$m radius (area magnified in **D2**). Scale bars = 100 $\mu$m. Abbreviations: CC, corpus callosum; EV, empty vector; GFP, green fluorescent protein; LV, lateral ventricle; STX, sialyltransferase X.

## CONCLUSION

Our findings indicate that the reach and efficacy of iPSC-derived OPC transplantation in brain tissue near demyelinated areas can be improved by stimulating the OPC migration potential via the forced expression of PSA-NCAM.

## AUTHOR CONTRIBUTIONS

M.C.: conception and design, collection of data, data analysis, manuscript writing; L.L. and K.B.: collection of data, data analysis, manuscript writing; E.B. and S.C.: conception and design, manuscript writing, final approval.

## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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