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Effect of Cell Adhesion Molecules on the Neurite Outgrowth of Induced Pluripotent Stem Cell-Derived Dopaminergic Neurons

Su-Ping Peng,^{1,2} Melitta Schachner,¹ Erik Boddeke,² and Sjef Copray²

Abstract

Intrastriatal transplantation of dopaminergic neurons has been shown to be a potentially very effective therapeutic approach for the treatment of Parkinson's disease (PD). With the detection of induced pluripotent stem cells (iPSCs), an unlimited source of autologous dopaminergic (DA) neurons became available. Although the iPSC-derived dopaminergic neurons exhibited most of the fundamental dopaminergic characteristics, detailed analysis and comparison with primary DA neurons have shown some aberrations in the expression of genes involved in neuronal development and neurite outgrowth. The limited outgrowth of the iPSC-derived DA neurons may hamper their potential application in cell transplantation therapy for PD. In the present study, we examined whether the forced expression of L1 cell adhesion molecule (L1CAM) and polysialylated neuronal cell adhesion molecule (PSA-NCAM), via gene transduction, can promote the neurite formation and outgrowth of iPSC-derived DA neurons. In cultures on astrocyte layers, both adhesion factors significantly increased neurite formation of the adhesion factor overexpressing iPSC-derived DA neurons in comparison to control iPSC-derived DA neurons. The same tendency was observed when the DA neurons were plated on postnatal organotypic striatal slices; however, this effect did not reach statistical significance. Next, we examined the neurite outgrowth of the L1CAM- or PSA-NCAM-overexpressing iPSC-derived DA neurons after implantation in the striatum of unilaterally 6-hydroxydopamine (6-OHDA)-lesioned rats, the animal model for PD. Like the outgrowth on the organotypic striatal slices, no significant L1CAM- and PSA-NCAM-enforced neurite outgrowth of the implanted DA neurons was observed. Apparently, induced expression of L1CAM or PSA-NCAM in the iPSC-derived DA neurons cannot completely restore the neurite outgrowth potential that was reduced in these DA neurons as a consequence of epigenetic aberrations resulting from the iPSC reprogramming process.

Introduction

THE SPECIFIC AND PROGRESSIVE loss of dopaminergic (DA) neurons in the substantia nigra (SN) is a major hallmark of Parkinson's disease (PD) (Perlow, 1987). At the time point that patients are diagnosed with PD, the loss of SN DA neurons is usually already more than 60%, resulting in a more than 80% depletion of dopamine in the striatum. Neither DA neuron loss nor striatum denervation is reversible. However, intrastriatal transplantation of DA neurons and the subsequent restoration of the striatal dopamine level have proven to be an effective symptom-reducing treatment for PD (Freed et al., 1992; Lindvall et al., 1990).

Today, induced pluripotent stem cell (iPSC)-derived DA neurons are considered the most promising source for DA

neuron grafts for PD patients. iPSCs are obtained by the reprogramming of somatic cells, such as skin fibroblasts (Takahashi and Yamanaka, 2006; Takahashi et al., 2007), which can be collected from the PD patient via a small skin biopsy. The autologous origin of the iPSC-derived DA neuron graft makes immunosuppression superfluous. Because iPSCs have an unrestricted capacity for self-renewal, an unlimited number of cells can be obtained for transplantation purposes.

Various strategies have been developed to differentiate the pluripotent cells toward a ventral midbrain (VM) DA neuron fate (Chambers et al., 2009; Kawasaki et al., 2000; Perrier et al., 2004). DA neurons derived from iPSCs show properties resembling primary VM DA neurons, such as the expression of the specific markers *Lmx1A*, *Nurr1*, *TH*, *Pitx3*, and *Girk2*

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(Yang et al., 2008), the spontaneous or stimulated release of dopamine, and spontaneous action potential firing with a slow pace (1–10 Hz) (Kriks et al., 2011; Zhang et al., 2010). However, when grafted into the striatum of 6-hydroxydopamine (6-OHDA) unilaterally lesioned rats, the animal model for PD, the neurites from the iPSC-derived DA neurons appear to be restricted within the injection tracks (Yang et al., 2008) or only shortly extending from the site of the graft (Kriks et al., 2011).

Recently, we performed an in-depth comparative analysis of the expression profile of pure mouse iPSC-derived DA neurons with that of pure mouse primary DA neurons (Roessler et al., 2014). We used *Pitx3*-green fluorescent protein (GFP) mice to enable the isolation of pure iPSC-derived DA neurons and pure primary VM DA neurons with GFP-based fluorescence-activated cell sorting (FACS). Besides comparative global gene expression mapping, we performed comprehensive DNA methylation profiling by reduced representation bisulfite sequencing (RRBS). The mouse iPSC-derived DA neurons largely appeared to adopt characteristics of their *in vivo* counterparts in morphology, dopamine production, global gene expression, and CpG island (CGI) methylation profiles, although some fibroblast genes still appeared to be expressed (Roessler et al., 2014). However, we also found deviations in CGI methylation for a subpopulation of genes that clearly effected predominantly the expression of genes associated with functional annotations, such as “nervous system development,” “neurogenesis,” “neuron differentiation,” and neurite outgrowth” (Roessler et al., 2014).

It is likely that these iPSC reprogramming “artefacts” are responsible for the limited outgrowth and survival of iPSC-derived DA neurons after implantation in the rat PD model. To achieve significant functional improvement in this animal model, a substantial reinnervation of the affected putamen and caudate is essential. In contrast to pluripotent stem cell-derived DA neurons, primary fetal VM DA neurons have been shown to be able to reinnervate the grafted striatum extensively (Stromberg et al., 1986), providing the proof-of-principle of the beneficial functionality of DA neuron grafts in PD patients (Lindvall et al., 1990; Piccini et al., 2000). Even when 10 times more tyrosine hydroxylase-positive (TH⁺) cells were implanted, pluripotent stem cell-derived DA neurons gave rise to a much lesser extent of striatal reinnervation than primary fetal VM DA neurons (Kirkeby et al., 2012).

An approach to improve the outgrowth of iPSC-derived DA neurons and to overcome the neurite outgrowth restrictions due to the iPSC-reprogramming could be the forced overexpression of cell adhesion molecules that are highly expressed in the developing fetal brain, in particular L1 cell adhesion molecule (L1CAM) and polysialylated neuronal cell adhesion molecule (PSA-NCAM). L1CAM and PSA-NCAM are crucial cell adhesion molecules for embryonic neuron development and axon guidance. During the fetal development of VM DA neurons, the PSA-NCAM content increases strikingly in both the developing striatum and the mesencephalon, with TH⁺ cells clearly outlined by PSA-NCAM (Shults et al., 1992). L1CAM is also expressed in fetal VM DA neurons and particularly mostly enriched in the TH⁺ fibers in the medial forebrain bundle (MFB) (Torre et al., 2010). It has been demonstrated that specific stimulation of the

L1CAM signaling pathway in VM tissue grafts by L1 antibody (Neomarkers, Lab Vision, Fremont, CA, USA) resulted in a significantly greater area of graft-derived innervation compared to control grafts (Marchionini et al., 2003).

In the present study, we aimed to examine the effect of L1CAM or PSA-NCAM (by transduction of the gene encoding for STX, the enzyme that adds PSA onto NCAM) overexpression on the neurite outgrowth of mouse iPSC-derived DA neurons *in vitro* as well as *in vivo* after transplantation in the rat PD model. By generating iPSCs from the fibroblasts of *Pitx3*^{GFP/+} transgenic mouse, we were able to obtain a pure population of DA neurons after their specific differentiation using FACS.

Materials and methods

iPSC culture and DA neuron differentiation

Mouse *Pitx3*^{GFP/+} iPSC clones CI, CII, and CIII (passages 15–52) were maintained on γ -irradiated mouse embryonic fibroblasts in ES Medium [Knockout DMEM, 15% knockout serum replacement, 1% nonessential amino acids (all Invitrogen, Breda, The Netherlands, www.invitrogen.com); 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin (all PAA Laboratories, Cölbe, Germany, www.paa.com); 100 μ M β -mercaptoethanol) supplemented with 1000 U/mL leukemia inhibitory factor (LIF; Millipore, Amsterdam, The Netherlands, www.millipore.com)].

The DA differentiation protocol for mouse iPSCs was based on the MS5 protocol (Barberi et al., 2003). iPSC colonies were dissected manually and plated on MS5 cells in KSR Medium [Dulbecco's modified Eagle medium (DMEM), 15% knockout serum replacement, 100 μ M β -mercaptoethanol, 100 U/mL penicillin, 100 μ g/mL streptomycin] from day 2 to 5. Sonic hedgehog (SHH; 200 ng/mL, PeproTech, Rocky Hill, NJ, USA) and fibroblast growth factor-8 (FGF8; 100 ng/mL, PeproTech) were added to the medium from day 6 to 8 of differentiation. Medium was changed into N2 medium [DMEM/F12 (Invitrogen), 2% N2 supplement (PAA Laboratories), 2 mM L-glutamine, penicillin/streptomycin, 1% sodium pyruvate (Invitrogen)] supplemented with SHH, FGF8, combined with FGF2 (100 ng/mL, PeproTech) from day 9 to 11. Followed by a final differentiation step in the presence of brain-derived neurotrophic factor (BDNF; 20 ng/mL, PeproTech), glial cell-derived neurotrophic factor (GDNF; 20 ng/mL, PeproTech), transforming growth factor- β 3 (TGF β 3; 1 ng/mL, PeproTech), ascorbic acid (200 μ M), and dibutyryl cyclic adenosine monophosphate (dbcAMP; 100 μ M) until the appearance of dopaminergic neuronal cell types.

RT-PCR and qPCR

Total RNA was extracted from transduced iPSCs or differentiated iPSCs using a standard RNeasy Kit (QIAGEN, Venlo, The Netherlands). Primers were used for L1, STX, Nestin, Pax6, Map2, β -III-tubulin, Nurr1, *Pitx3*, TH, and DAT. Primer sequence information is presented in Table 1. Relative gene expression levels were determined by qPCR real-time PCR (LightCycler) using the QuantiTectTM SYBR[®] Green PCR (QIAGEN) LightCycler[®] Kit according to the manufacturer's instructions. For each reaction, 0.1 ng of total RNA was used as input. Relative

TABLE 1. PRIMERS FOR qPCR

Primer	Forward	Reverse
<i>Pax6</i>	CGGAGTTATGATACCTACACCC	GTGAAATGAGTCCTGTTGAAGTG
<i>Nestin</i>	AGGTGGGCAGCAACTGGCA	TCAGCCTCCAGCAGAGTCCTGT
<i>Map-2</i>	CTTCGGCTTATTAACCAACCA	AGTAACAATTTGTACCTGACCC
<i>β-III-tubulin</i>	TGTTCAAACGCATCTCGGAG	TCCATCTCATCCATGCCCTC
<i>Nurr1</i>	AGGGAACCTGCACTTCGGCGG	GCCCCGTCAGATCTCCTTGTCGG
<i>Pitx3</i>	CTTCCAGAGGAATCGCTACCCT	CTGCGAAGCCACCTTTGCACAG
<i>DAT</i>	GTTTAGGTTTAGTGTTTTTG	TCTTAATCCTTACCTACCTCCA
<i>TH</i>	AGAAGAGCCGTCTCAGAGCA	GGGCATCCTCGATGAGACT
<i>GADPH</i>	CATCAAGAAGGTGGTGAAGC	ACCACCCTGTTGCTGTAG
<i>Human L1CAM</i>	TACAACGTCACCTACTGGCG	AGGATCACGGAGGTGGTGTT
<i>Mouse L1CAM</i>	CCTGCCTGCTCATAACAGATTC	CAGCGGAACCTCCACTTGGG
<i>Human ST8SIA2</i>	GGCAGAGGTACAATCAGATCAGC	ATGTCTCCATTTGGACGAGGC
<i>Mouse ST8SIA2</i>	AGGCAGAGGTACAATCAGATCA	GAGAGAGCGTCTGGTTGTGTC

expression levels were normalized to the housekeeping gene hydroxymethylbilane synthase (*HMBS*).

Quantitative dopamine determination by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The dopamine production by the iPSC-derived DA neurons was analyzed as described earlier (van de Merbel et al., 2011). We analyzed 100 μ L of supernatant from terminally DA-differentiated iPSCs under stimulation by 56 mM KCl.

Immunostaining

Cell cultures of iPSC-derived cells were fixed with 4% paraformaldehyde (PFA) for 15 min and washed three times with phosphate-buffered saline (PBS) before staining. Antigen retrieval was performed on brain cryosections with a 10 mM sodium citrate solution for 10 min heated in a microwave. Immunostaining was performed for the following markers—neuronal markers: Rabbit anti-tyrosine hydroxylase (1:500; cat. no. AB152, Chemicon, Millipore, Amsterdam, The Netherlands), mouse anti-GFP (1:500; cat. no. MAB3580, Millipore, Billerica, MA, USA), mouse anti-L1CAM (1:400; cat. no. ab24345, Abcam), mouse anti-PSA-NCAM, and immunoglobulin M (IgM, 1:400; cat. no. MAB5324, Millipore). Samples were incubated for 24 h with primary antibodies diluted in PBS with 0.05% Triton at 4°C, and primary antibodies were detected with appropriate secondary antibodies coupled to Alexa Fluor 594 or Alexa Fluor 488 (1:800; Jackson ImmunoResearch, West Grove, PA, USA) for 2–3 h at room temperature. Samples were counterstained for 10 min with Hoechst to visualize cell nuclei.

Fluorescence-activated cell sorting

Differentiated iPSC-derived cells were dissociated using the Papain Dissociation Kit (cat. no. LK003150, Worthington Biochemical, Lakewood, NJ, USA). Dissociated cells were collected in colorless DMEM (Gibco) with BDNF, ascorbic acid, GDNF, TGF β 3, and dbcAMP (see above), and sorted on a MoFlo-XDP or MoFlo-Astrios sorter (Beckman-Coulter, Brea, CA, USA) with a 100- μ m nozzle at a pressure of 15–17 psi based on GFP expression. Cells were collected in N2 medium with BDNF, ascorbic acid, GDNF, TGF β 3, and dbcAMP. Cells were centrifuged at 300 relative centrifugal force (rcf) for 10 min and resuspended in PBS.

Gene transduction

Sorted GFP⁺ iPSC-derived DA neurons were transduced with lentiviral vectors containing genes encoding for human L1CAM, human STX (ST8Sia II), and puromycin resistance under the elongation factor-1 α (EF-1 α) promoter; an empty vector was used as control.

Lentiviral particles were produced by co-transfecting human embryonic kidney (HEK) 293T cells (Invitrogen, Carlsbad, CA, USA) with the lentiviral vector pCMV Δ 8.91 and vesicular stomatitis virus G protein (VSV-G) plasmid using Fugene (FuGENE-HD; Roche, Almere, The Netherlands) in a six-well plate following the procedure provided by the manufacturer. The ratio used was 3 μ L of FuGENE to 1 μ g of DNA for each well. After overnight transfection, culture medium was changed to N2 medium; 24 h later, media containing virus and 4 μ g/mL Polybrene (Sigma-Aldrich, Zwijndrecht, The Netherlands) were mixed and filtered through a 0.45- μ m filter (Whatman, The Netherlands). The viral particles were further concentrated 100-fold by ultracentrifugation (20,000 $\times$ g, 20 min). The pellet was resuspended in N2 medium.

For transduction, N2 medium containing lentiviral particles was added to the resuspended sorted cells in a pre-lubricated EP tube. After 4 h of transduction, cells were washed twice by adding N2 medium, centrifuged at 300 rcf for 10 min, and resuspended in PBS for grafting in 6-OHDA rats or in N2 medium for culturing on an astrocyte layer (4000 cells/well) or on organotypic striatal slices (400 cells/slice). Three days after transduction, the cells were incubated with 0.5 μ g/mL puromycin for 24 h for selective survival of only transduced cells. After 1 week of culturing, samples were taken for qPCR analysis or for immunocytochemistry staining.

Culture of primary astrocytes

Brain tissue was explanted from P1–P3 mice, meninges were peeled off, and the brain tissue was chopped into small pieces and incubated with TE buffer at 37°C for 30 minutes. Glia medium [DMEM, with 10% FBS, 1% sodium pyruvate (Invitrogen), 100 U/mL Pen/Strep] was added to stop the digestion, and tissue was pipetted into cell suspension and cultured at 37°C under 5% CO₂. Medium was changed every 3–4 days. The flasks of cell culture were shaken at

150 rpm, 37°C for 1 h, for three times every other day to remove microglia. The remaining astrocytes were passaged into a 24-well culture plate as monolayer for sorted iPSC-derived DA neurons.

Culture of organotypic striatal slices

Organotypic striatal slice cultures were performed with a modification of a previously published method (Cavaliere et al., 2010). Striata were dissected from P1–P3 mice, cut on a vibratome coronally at 250 μ m, and collected in ice-cold F12 medium. Slices were cultured at the liquid–air interface on Millicell CM Cell Culture Inserts (Millipore) and maintained in Neurobasal Medium supplemented with 0.5% B27 supplement, 2 mM L-glutamine, 25% horse serum, and 25 mg/mL gentamicin at 35°C under 5% CO₂. Medium was changed every other day.

Neurite tracing and analysis

Neurite tracing was performed with simple neurite tracer in the software program Fiji (http://fiji.sc/Simple_Neurite_Tracer). The total neurite length represented the sum of the lengths of all neurites of one TH⁺ neuron. The number of primary neurites per neuron represented the number of neurites directly extending out from a TH⁺ soma. The number of branches per neuron comprised the total number of neurites traced minus the number of primary neurites. The effective area comprised the area of the polygon created by linking up the far ends of all neurites of one neuron using the polygon selection option in Fiji.

Lesions, rotation behavior, and transplantation surgery

Adult female Sprague-Dawley (Harlan) rats (180–230 grams) were housed under standard conditions with free access to food and water. Rats were anesthetized with ketamine (90 mg/kg) and xylazine (4 mg/kg). Unilateral retrograde destruction of dopaminergic neurons in the substantia nigra was induced by two stereotaxic injections of 2.5 μ L of 6-OHDA (3 mg/mL in 0.2% ascorbic acid and 0.9% saline, Sigma) in the MFB of the nigrostriatal pathway (Stereotaxic coordinates 1, AP –4.0, ML –0.8, and V –8.0; toothbar set at +3.4. Coordinates 2, AP –4.4, ML –1.2, and V –7.8; toothbar set at –2.4). The unilateral destruction of the nigrostriatal pathway was confirmed by the recording of D-amphetamine (5 mg/kg)-induced rotation behavior 2 weeks postlesion. Amphetamine-induced rotation scores were obtained using Rota-8 software [University Medical Center Groningen (UMCG)]. Rats that rotated more than 5 rpm were included. Under ketamine/metomedidine anesthesia, 2–3 $\times 10^4$ cells were transplanted stereotactically into the striatum of each animal (coordinates, AP +0.9 mm, ML –2.6 mm, and V –4.0 mm; toothbar set at 0.0); control rats received a similar injection with mouse embryonic fibroblasts (MEFs).

Rotation behavior was evaluated 4 and 8 weeks after grafting. Daily subcutaneous (s.c.) injections of cyclosporine A (15 mg/kg; Sigma-Aldrich) were given, starting 24 h before cell grafting (double dosage) and continued until the rats were sacrificed and perfusion-fixed at 9 weeks after cell grafting.

All animal experiments were carried out according to the Dutch Regulations for Animal Welfare. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Groningen.

Tissue fixation and histology

Under ketamine/metomedidine anesthesia, rats were perfused transcardially with 4% PFA in PBS. Brains were explanted, postfixed in 4% PFA for 24 h, and soaked in a 20% sucrose solution for 1 day. They were sectioned (14- μ m sections) on a cryostat after embedding in O.C.T. compound (Sakura Finetek, Torrance, CA, USA).

Statistical analysis

The data presented of neurite outgrowth of DA neurons on astrocyte or organotypic slices were from three independent experiments ($n=3$). In each independent experiment, 8–15 cells were analyzed in each group. All tests were performed and analyzed in a blinded manner. Throughout the text and in the figures, all values are expressed as the mean $\pm$ standard error of the mean (SEM). SPSS analysis of difference in means between two groups of independent samples was performed using the Student *t*-test. Differences in means among multiple data sets were analyzed with one-way analysis of variance (ANOVA) by Tukey post hoc analysis. In all analyses, *p* values of less than 0.05 were considered significant.

Results

Dopaminergic neuron differentiation of iPSC lines

iPSCs were generated from embryonic fibroblasts isolated from Pitx3^{GFP/+} transgenic mice. The lineage-specific differentiation of three iPSC clones into midbrain DA neurons was performed according to previously described protocols (Barberi et al., 2003). The appearance of Pitx3-expressing DA neurons in the differentiating iPSCs could be detected by their expression of GFP; at day 20 of differentiation, most of the Pitx3-GFP⁺ cells co-expressed TH (Fig. 1A). GFP expression under control of the Pitx3 promoter allowed purification of the differentiated DA neurons with FACS (Roessler et al., 2014). The highest yield of GFP⁺ cells (1.78%) after sorting from iPSC clone III (CIII) is shown in Figure 1B. mRNA was collected and analyzed with RT-PCR. The purified Pitx3-GFP⁺ cells of iPSC clone CIII appeared to express the proper profile of DA neuron markers, *i.e.*, both neuronal markers (Map2 and β -III-tubulin) and midbrain DA neuron markers (Nurr1, Pitx3, TH, and DAT) (Fig. 1C). Measurement of KCl stimulated secretion of dopamine revealed dopamine release in the clone CIII-derived Pitx3-GFP⁺ cells (Fig. 1D).

Overexpression of L1CAM and PSA-NCAM in iPSC-derived DA neurons

The sorted Pitx3-GFP⁺ DA neurons were transduced with a lentiviral vector containing the genes encoding human L1CAM or STX; an empty vector was used as a control. The transduced cells were washed thoroughly before they were plated onto primary astrocyte layers. The expression of L1CAM and STX was analyzed using qPCR. There was endogenous expression of mouse L1CAM and mouse STX in DA neurons transduced with control vectors; the level of endogenous L1CAM and STX expression in astrocytes appeared to be very low (Fig. 2A, C). One week after transduction, specific expression of human L1CAM mRNA could be detected at a four times higher level in the L1CAM

FIG. 1. Dopaminergic differentiation of 3 *Pitx3*^{GFP/+} iPSC clones. (A) The appearance of *Pitx3*-expressing DA neurons in the differentiating iPSCs could be detected by their expression of GFP; at day 20 of differentiation most of the *Pitx3*-GFP⁺ cells co-expressed TH as demonstrated with immunostaining. (B) Three clones (CI, CII, CIII) of iPSCs reprogrammed from mouse embryonic *Pitx3*^{GFP/+} fibroblasts were differentiated into VM DA neurons. At day 20 of differentiation, the *Pitx3*-GFP⁺ cells of each iPSC clone were sorted. (C) mRNA expression for neural precursor (NP) markers, neuronal (N) markers, and midbrain dopaminergic neuron (DA) markers was analyzed using RT-PCR. (D) Secretion of dopamine by the 3 *Pitx3*-GFP⁺ cell cultures upon KCl stimulation was measured with high-performance liquid chromatography (HPLC). It is clear that the *Pitx3*-GFP⁺ cells differentiated from iPSC clone III best fulfilled the criteria of DA neurons. Color images available online at www.liebertpub.com/cell

vector-transduced group (Fig. 2A). L1CAM was also detected by immunofluorescent staining both in the soma and along the neurite of DA neurons in both groups (Fig. 2B). The expression of human STX was about 10 times higher in the STX vector-transduced group in comparison to the control vector group (Fig. 2C). Immunostaining showed the presence of PSA-NCAM both in the soma and the neurite of DA neurons in both groups (Fig. 2D). Both L1CAM and PSA-NCAM were not detectable in the primary astrocyte layer (Fig. 2C, D).

L1CAM and PSA-NCAM overexpression stimulates neurite outgrowth of iPSC-derived DA neurons in vitro

Transduced DA neurons were plated onto a primary astrocyte layer to create an optimal supporting environment for outgrowing neurons, enabling a clear analysis of the effects of L1CAM and PSA-NCAM overexpression on cell morphology. DA neurons co-cultured on top of a primary astrocyte layer had an oval or trigonal soma of approximately similar sizes (Fig. 3A). Tracings made of DA neurons from the control group and the L1CAM overexpressing group revealed bipolar or tripolar neurite formation, whereas DA neurons in the PSA-NCAM group occasionally also had five or six primary neurites (Fig. 3B). However, this difference was not significant among the three groups (control vs. L1CAM, 0.79; control vs. PSA-NCAM, 0.17). The morphology of the

neurites in both L1CAM and PSA-NCAM groups was much more complex (Fig. 3A), with more branches than in the control group (Fig. 3C). The longest neurite from each TH⁺ DA neuron was also measured. Both L1CAM and PSA-NCAM overexpressing cells extended their neurite significantly longer than the control ones (Fig. 3D). With longer neurites and more branches, the total neurite length in these two groups was significantly longer (Fig. 3E) and covered a larger area (Fig. 3F).

Effect of L1CAM and PSA-NCAM on neurite outgrowth of iPSC-derived DA neurons in organotypic slices

To provide an environment mimicking the striatal *in vivo* situation, transduced iPSC-derived DA neurons from all three groups (L1CAM or PSA-NCAM overexpressing and controls) were placed onto mouse organotypic striatal slices and cultured for 1 week. TH⁺ DA neurons were either clustered as groups or scattered on the slice (Fig. 4A). Analysis of the number of primary neurites revealed no significant differences between the three groups (control vs. L1CAM, 0.48; control vs. PSA-NCAM, 0.43) (Fig. 4C). In general, all DA neurons on the striatal slices grew much longer neurites than those cultured on the astrocyte layers (Fig. 4B vs. Fig. 3D). This may be due to the high level of cell adhesion molecules such as L1CAM and PSA-NCAM already present in postnatal organotypic striatal slices

FIG. 2. Overexpression of hLICAM and hSTX in iPSC-derived DA neurons. (A and C) Transduction of the iPSC-derived DA neurons with hLICAM, hSTX, or control (empty) vectors resulted, after culturing for 1 week on an astrocyte cell layer, in the overexpression of hLICAM and hSTX. The expression of human and mouse LICAM or human and mouse STX mRNA was analyzed by qPCR. (B and D) Expression of LICAM and PSA-NCAM was verified by co-immunostaining of LICAM and PSA-NCAM with TH. Color images available online at www.liebertpub.com/cell

FIG. 3. Overexpression of L1CAM and STX stimulated the outgrowth of iPSC-derived DA neurons on an astrocyte layer. (A) iPSC-derived DA neurons overexpressing L1CAM or STX were cultured on a primary astrocyte layer for 1 week. Immunostaining for TH shows the morphology of DA neurons from the three groups (including controls). Tracings of exemplary neurons are shown. The tracings were used to determine the number of primary neurites (B), the number of branches (C), the length of the longest neurite per neuron (D), the total neurite length (E), and the effective area (area linked-up by the far end of neurites from one neuron) (F). Data are presented as mean $\pm$ SEM, $n = 10$ to 15 per group. (*) $p \leq 0.05$, (**) $p \leq 0.001$, (***) $p = 0.000$, one-way ANOVA, Tukey post hoc analysis. Ctrl, control. Color images available online at www.liebertpub.com/cell

FIG. 4. Outgrowth of iPSC-derived DA neurons on organotypic striatal slices. (A) iPSC-derived DA neurons overexpressing L1CAM or PSA-NCAM or control ones were cultured on organotypic striatal slices from P1–P3 mice. Soma and neurites of DA neurons were stained with TH. (B) The longest neurite from scattered DA neurons or cells on the edge of clustered DA neurons were measured. (C) The number of primary neurites per cell was counted. Data were presented as mean $\pm$ SEM, $n = 15$ per group. Color images available online at www.liebertpub.com/cell

(Fig. 5), and the maximum neurite outgrowth of scattered DA neurons was promoted.

Due to the neuronal clustering and the severe intertwining of the neurites, it was difficult to trace all branches from one neuron. Therefore, we only traced the longest neurite distinguishable from each neuron (mostly scattered DA neurons). On average, the longest neurite from each neuron in the L1CAM and PSA-NCAM group is longer than the control group; however, no significant differences were observed (control vs. L1CAM, 0.45; control vs. PSA-NCAM, 0.31) (Fig. 4B). Remarkably, clustered DA neurons of the L1CAM and PSA-NCAM groups tended to extend their neurites in a parallel direction (Fig. 4A), unlike those

of the control group (Fig. 4A). Moreover, the neurites in L1CAM and PSA-NCAM group were much longer than in control group. This phenomenon may point to the fact that L1CAM- and PSA-NCAM-overexpressing DA neurons are more responsive to patterning cues in the striatal slice.

Implantation of iPSC-derived DA neurons in 6-OHDA lesion rats

Sorted control iPSC-derived DA neurons and iPSC-derived DA neurons overexpressing L1CAM or PSA-NCAM were transplanted into unilaterally 6-OHDA lesioned rats, according to the procedure described before (Roessler et al., 2014).

FIG. 5. Organotypic striatal slice culture. Striatal slices were cut from the striatum of P1–P3 mice with a thickness of 250 μm . The slices were cultured for 2 weeks before sorted DA neurons were layered on them. Striatal slices were characterized by immunostaining for the neuronal marker β -III-tubulin (A), the microglia marker Iba1 (B), the astrocyte marker glial fibrillary acidic protein (GFAP) (C), and the cell adhesion molecules LICAM (D), and PSA-NCAM (E). Color images available online at www.liebertpub.com/cell

Two weeks after 6-OHDA injection, rats that rotated more than 5 rpm after amphetamine injection were included in the experiments. At 8 weeks after implantation, a small reduction in the number of rotations was observed in the groups (Fig. 6A). Immunohistochemical examination of the implanted striata revealed the survival of only, on average, 423 (± 108) implanted TH⁺ neurons in the denervated striatum. The presence of only a very small number of DA neurons may account for the small reduction in rotation behavior.

Apparently, the subsequent stressful procedures of FACS and viral transduction had made the DA neurons vulnerable for the stress associated with the implantation procedure, resulting in the survival of only about 2% of the injected cells. Accurate analysis of the three-dimensional outgrowth

of the newly formed neurites extending from the surviving individual implanted neurons showed that the maximal length of the neurites in each of the groups of implanted iPSC-derived DA neurons did not exceed 500 μm (Fig. 6B). As observed in the cultures on the striatal organotypic slices, no significant differences were found between the neurite outgrowth of the control and the LICAM- or PSA-NCAM-expressing grafted DA neurons, respectively, 468 ± 108 , 492 ± 64 , and $477 \pm 73 \mu\text{m}$.

Discussion

Considering the option of a cell replacement therapy for PD, iPSCs are presently the most promising source for an

FIG. 6. Intrastratial transplantation of iPSC-derived DA neurons in unilaterally 6-OHDA-lesioned rats. (A) Amphetamine-induced rotation of the 6-OHDA-lesioned rats transplanted with the iPSC-derived DA neurons at 4 or 8 weeks after grafting showed a small nonsignificant reduction after 8 weeks; mean $\pm$ SEM, $n = 3$. (B) Immunohistochemical staining for TH shows the intact contralateral side of the unilaterally lesioned rat striatum with the punctuate dopaminergic terminals (1), which is completely gone at the ipsilateral side after dopaminergic denervation by 6-OHDA lesioning of the SN DA neurons (2); in this denervated striatum a cluster of TH⁺ implanted neurons can be detected at the side of implantation. Individual neuronal cell bodies (arrow) from these clusters extend their thin, curly neurites in the striatum (3 and 4). Bar, 100 μm (1 and 3) or 50 μm (2 and 4). Color images available online at www.liebertpub.com/cell

unlimited number of autologous DA neurons to be grafted intracerebrally. The *in vitro*-differentiated, iPSC-derived DA neurons have been demonstrated to adopt the characteristics of true (VM) DA neurons as assessed by cell type-specific marker expression, electrophysiology, and dopamine release (Hargus et al., 2010; Kriks et al., 2011). However, comparison of the genome-wide expression profile of iPSC-derived DA neurons to primary DA neurons showed a subset of hypermethylated genes that were downregulated in iPSC-derived VM DA neurons. Gene annotation analysis showed that these genes were involved in nervous system development, neuron differentiation, neurogenesis, and neurite outgrowth (Roessler et al., 2014). This may explain the fact that iPSC-derived DA neurons are not as efficient as primary DA neurons in re-innervating the DA depleted striatum in PD 6-OHDA rats and in giving rise to functional recovery.

Extensive striatum-wide reinnervation is an absolute prerequisite for a DA neuron replacement therapy for PD to be successful. Yet the short neurites extending from grafted iPSC-derived DA neurons appeared to be restricted within a short range around the injection track (Hargus et al., 2010; Wernig et al., 2008). In the present study, we attempted to restore this hampered neurite outgrowth potential of iPSC-derived DA neurons by forcing their overexpression of L1CAM and PSA-NCAM, both crucial adhesion molecules for axon guidance during embryonic neuronal development. It should be noted that our comparative genome-wide expression profiling did not reveal differences in the expression of genes encoding the formation of L1CAM and PSA-NCAM between the primary and the iPSC-derived DA neurons. iPSCs were reprogrammed from fibroblasts of *Pitx3*^{GFP/+} mice (Maxwell et al., 2005) and subsequently differentiated into DA neurons. Inducing overexpression of L1CAM and PSA-NCAM in DA neurons derived from iPSCs appeared to be cumbersome.

Our first approach was to transduce the relevant genes in the iPSCs and to establish stably transduced iPSC lines that were subsequently differentiated into DA neurons. However, L1CAM expression was lost during differentiation and could only be detected in the undifferentiated cells. This is in accordance with previous observations on transgene silencing when transduced stem cells differentiate into specialized cell types (Hamaguchi et al., 2000; Vroemen et al., 2005). We decided to perform lentiviral *hL1CAM* and *hSTX* gene transduction on a suspension of *Pitx3*-GFP DA neurons sorted by FACS and were able to show stable upregulation of both *hL1CAM* and *hSTX* in the transduced iPSC-derived DA neurons.

These L1CAM and PSA-NCAM overexpressing iPSC-derived DA neurons allowed us to evaluate the effect of these molecules on neurite outgrowth *in vitro* on top of a feeder layer of astrocytes (which did not express these molecules). We demonstrated that, indeed, the neurites in both THE L1CAM and PSA-NCAM groups showed a much more complex, branched morphology and overall length than those in the control group. When the iPSC-derived DA neurons were cultured on top of organotypic slices of postnatal striatum, similar differences were observed, but these differences were less pronounced and not statistically significant. Because the postnatal striatal slices appeared to be already rich in L1CAM and PSA-NCAM content, we speculate that this may have obscured the effects of growth promotion of iPSC-derived DA neurons by the transduced adhesion factors. Only the pattern of outgrowth of the L1CAM or PSA-NCAM

overexpressing iPSC-derived DA neurons appeared different from the control ones, indicating a different response of these cells to the cues in the substrate.

We intended to examine the effect of L1CAM and PSA-NCAM on the neurite outgrowth of iPSC-derived DA neurons in the denervated striatum of 6-OHDA-lesioned PD rats. In our previous studies (Roessler et al., 2014), we already had demonstrated that iPSC-derived DA neurons transplanted in this rat model do survive and show some outgrowth. The subsequent stressful procedures of FACS and lentiviral transduction made the DA neurons vulnerable to the stress associated with the implantation procedure, resulting in the survival of only about 2% of the injected cells. Low survival (<3%) of implanted iPSC-derived DA neurons have been described before (Doi et al., 2014; Rhee et al., 2011), and sorting before transduction has been shown to further reduce the survival rate to less than 0.06% (Hargus et al., 2010).

Although our *in vitro* experiments clearly revealed the outgrowth-stimulating ability of L1CAM and PSA-NCAM, pronounced outgrowth stimulation could not be detected in the few surviving L1CAM and PSA-NCAM overexpressing iPSC-derived DA neurons in the denervated striatum of the unilaterally 6-OHDA-lesioned rats. This is in contrast to recent findings by Battista et al. (2014), who demonstrated that PSA-NCAM overexpression was able to enhance neurite outgrowth of stably transduced mESC-derived DA neurons *in vivo*. Moreover, experimental studies with the spinal cord injury model clearly demonstrated that L1CAM (Chen et al., 2007) and PSA-NCAM (Zhang et al., 2007) were able to enhance axon regeneration and extension. It is possible that the sorting, lentiviral transduction of the sorted DA neurons, and implantation protocol affected the viability and health of the surviving DA neurons, interfering with the potential growth-promoting effects of the adhesion factors.

In conclusion, we have demonstrated that L1CAM and PSA-NCAM significantly stimulate the neurite outgrowth of iPSC-derived DA neurons, both neurite extension and branching *in vitro*. However, we failed to demonstrate significant neurite outgrowth stimulation by L1CAM and PSA-NCAM in iPSC-derived DA neurons co-cultured on striatal organotypic slices and after implantation in the 6-OHDA-denervated striatum. Apparently, under these conditions, induced expression of L1CAM- or PSA-NCAM in the iPSC-derived DA neurons cannot completely restore the neurite outgrowth potential that was reduced in these DA neurons as a consequence of epigenetic aberrations resulting from the iPSC-reprogramming process.

Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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