From stem cells to Schwann cells
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Targeted differentiation of mouse induced pluripotent stem cells towards Schwann cells

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Submitted
INTRODUCTION

Schwann cells (SCs) are the glial cells of the peripheral nervous system, and are important contributors to proper peripheral nerve development, maintenance, and regeneration. Unlike neurons in the central nervous system (CNS), peripheral neurons have an (albeit limited) capacity to regenerate their axons after injury. This regenerative capacity depends on the interplay between peripheral (motor and sensory) neurons and SCs. It is known from peripheral nerve trauma that SCs are able to phagocytose myelin debris and dedifferentiate from a myelinating to a proliferating precursor phenotype providing guidance and trophic support to newly outgrowing axons. Autologous SC transplantation studies (with or without biodegradable scaffolds) in peripheral nerve gap models have proven that SCs indeed can promote regeneration of peripheral nerves. It is known from peripheral nerve trauma that SCs are able to phagocytose myelin debris and dedifferentiate from a myelinating to a proliferating precursor phenotype providing guidance and trophic support to newly outgrowing axons. Autologous SC transplantation studies (with or without biodegradable scaffolds) in peripheral nerve gap models have proven that SCs indeed can promote regeneration of peripheral nerves.

MATERIAL AND METHODS

Cell culture and CNPase-GFP iPS cell generation

HEK293T cells were maintained in fibroblast medium (consisting of Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum, 2 mM l-glutamin, 100 U/ml penicillin/streptomycin, 2 mM nonessential amino acids and 1 mM sodium pyruvate) and transfected with a lentiviral self-inactivating pRRL.PPT.SF plasmid containing murine Oct4, Klf4, Sox2, c-Myc, and DTomato complimentary DNAs. Cells were co-transfected with pMD2-G and pCMVΔ.R8.91 packaging vectors. Lentiviral supernatant was collected 48 hours post-transfection, filtered through a 0.45 µm filter (Whatman), and concentrated using a 100K Amicon Ultra Centrifugal Filter device (Millipore). CNP-GFP mouse embryonic fibroblasts featuring CNPase driven GFP expression (derived from transgenic mice originally generated by Yuan (Yuan, et al., 2002)) were used for iPS induction. One day before transduction, CNP-GFP fibroblasts were replated onto uncoated 6-wells plates and cultured in fibroblast medium. Around 100,000 CNPase-GFP fibroblasts were transduced with viral supernatant supplemented with 8 µg/ml Polybrene (Sigma-Aldrich), which was replaced by fibroblast medium after 24 hours. Transfection efficiency was evaluated based on fluorescent dTomato expression. Four days post-transduction, cells were dissociated using 0.1% trypsin and seeded onto irradiated mouse embryonic fibroblast feeder layers (IMEF, 20 Gy) in a 1% gelatin coated 6-wells plate, and cultured in mouse embryonic stem cell medium (ES medium) consisting of Knockout-DMEM (KO-DMEM) supplemented with 15% knockout serum replacement, 2 mM L-glutamin, 100 U/ml penicillin/streptomycin, 100 µM β-mercaptoethanol, 2 mM nonessential amino acids, and 1000 U/ml ES-340 leukemia inhibitory factor (LIF). iPS colonies were fragmented 14 days post-transduction, replated onto fresh iMEFs in ES medium and subsequently passaged every 3-4 days.

RT-PCR

For mRNA expression analysis, total RNA from the CNPase-GFP mPS derived Schwann cells was extracted using Trizol Reagent (Sigma-Aldrich) according to the method described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Total RNA (1 µg) was transcribed into cDNA in a PTC-200 Peltier Thermal Cycler (MJ Research) using random hexamers and M-MLV reverse transcriptase (Fermentas). The PCR reaction was performed on 1 µl of cDNA in a Thermal Cycler with Taq DNA polymerase (Fermentas) for 35 cycles with an annealing temperature of 60°C. Primers are listed in Table 1. cDNA was visualized on a 1% TAE-exagarose gel by staining with ethidium bromide.

Differentiation towards neural crest and Schwann cells

For embryoid body (EB) formation CNPase-GFP iPS cells were dissociated using 0.25% trypsin/EDTA and grown for 8 days as suspension cultures in 10 cm low attachment petri-dishes in EB medium consisting of KO-DMEM supplemented with 15% fetal calf serum, 2 mM L-glutamin, 100 U/ml penicillin/streptomycin, 100 µM β-mercaptoethanol, 2 mM nonessential amino acids, of which 4 days with and 4 days without 1*10^-5 M all-trans retinoic acid (ATRA) (-4/4 protocol). EBs were then replated onto 1% gelatin-coated
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6-wells plates and cultured for one additional day in EB medium. For default differentiation, medium was switched to N2 medium consisting of DMEM/F12 supplemented with 1% N2 supplement (R&D Systems), 2 mM L-glutamin, 100 U/ml penicillin/streptomycin, and 1 mM sodium pyruvate for 5 days.

For neural crest induction, medium was switched to neural crest induction medium consisting of N2 medium enriched with 200 ng/ml sonic hedgehog (SHH), 100 ng/ml fibroblast growth factor 8 (FGF8), 20 ng/ml brain-derived neurotrophic factor (BDNF), and 0.2 mM ascorbic acid (AA) (all R&D Systems). After 5 days, cultures were grown in N2 medium supplemented with BDNF, AA and 10 ng/ml FGF2, while SHH and FGF8 were withdrawn. After 14 days, groups of polygonal cells developed which were detached with 0.25% trypsin/EDTA and replated and cultured on Matrigel™ pre-coated glass slides in a 24 wells plate (5-10*10^3 cells/cm²) for 2 days. For specific differentiation towards SCs, medium was switched to N2 medium supplemented with 40 ng/ml neuregulin-1 (NRG-1), 2 µM forskolin and 10 ng/ml FGF2. SCs were collected after 60 days of culture, during which half of the medium was changed every 3 days (for a schematic differentiation protocol see Figure 2A).

Immunocytochemistry

Cell cultures on glass coverslips were fixed with 4% paraformaldehyde, washed with PBS, and pre-incubated in PBS/0.1% Tween 20 (PBS-Tween) containing 5% normal goat serum (NGS) and 3% bovine serum albumine (BSA). Cells were then incubated overnight in primary antibodies diluted in PBS-Tween/1% NGS/1% BSA at 4°C. The following antibodies were used: mouse anti-mouse Oct4 (1:100; Santa Cruz, 5279), mouse anti-mouse Sox2 (1:200; Cell Signaling, 4900S), rabbit anti-Nanog (1:200; Abcam, 80892), mouse anti-mouse SSEA-1 IgM (1:200; Santa Cruz, 21702), mouse anti-mouse desmin (1:200; Dako M0760), mouse anti-mouse GATA (1:200; Santa Cruz, 25310), mouse anti-mouse β-III tubulin (1:400; Abcam 7751), mouse anti-GFP (1:100, Millipore, MAB3580), and rabbit anti-mouse Oct4 (1:100, kindly provided by Dr. D. Meijer, #1909). After washing with PBS, cells were then incubated for 1 hour at room temperature in fluorescently labeled secondary antibodies Alexa Fluor 488-conjugated donkey anti-rabbit (1:300; Molecular Probes, A21206), Alexa Fluor 488-conjugated goat anti-mouse IgM (1:300; Invitrogen, M31601), DyLight 488-conjugated goat anti-rabbit (1:100; Jackson ImmunoResearch), Cy3-conjugated donkey anti-goat (1:300; Jackson ImmunoResearch), and Alexa Fluor 633-conjugated anti-rat (1:300, Molecular Probes, A21094). For nuclear visualization, cells were counterstained with Hoechst 33342 (1:1000; Jackson ImmunoResearch) in PBS. Glass coverslips were rinsed with H2O and mounted in Moviol. Pictures were made using an Axioscope 2 fluorescence microscope (Zeiss) in combination with a mercury lamp (HBO 100) and Leica Application Software. For confocal microscopy, sections were imaged with a Leica TCS SP8 confocal microscope using a PlanApo 63x/1.4 objective. Images were taken sequentially in three channels using 405/638 nm, 488 nm, and 552 nm laser lines with a pinhole setting of 1 AU and a pixel resolution of 106 nm, using Leica LAS AF 3.3 software.

Table 1: Primers used for PCR.

<table>
<thead>
<tr>
<th>Acceptor no.</th>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_013633</td>
<td>Oct4</td>
<td>TCTTCCACAGGCCCCGGCCGTCCT</td>
<td>TGGGCGGACAGCCTGGGGAGATCC</td>
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<tr>
<td>NM_011443</td>
<td>Sox2</td>
<td>TAGAGCTAGACTCCGGGCAGATGA</td>
<td>TTGGCTAATACAGAAGACAGAAGA</td>
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<tr>
<td>NM_010637</td>
<td>Klf4</td>
<td>GCGAACACACAGGGGAGAAGAAC</td>
<td>TCAGCTCCTTCTTCTGCGACACA</td>
</tr>
<tr>
<td>NM_00177352</td>
<td>c-Myc</td>
<td>TCACTACTCGAGGAGGTGGAATCT</td>
<td>AAGTTGAGCAGTAAATATGCGGAAGC</td>
</tr>
<tr>
<td>NM_028016</td>
<td>Nanog</td>
<td>CAGGTGTGTGAGGGTAGCTC</td>
<td>CGGTCTCATGAGTAGCTC</td>
</tr>
</tbody>
</table>

Figure 1: Characterization of CNPase-GFP mouse iPS cells. A: CNPase-GFP iPS colonies in culture and stained for alkaline phosphatase (AP). B: Colonies stained for pluripotency markers showing Oct4, Sox2, SSEA-1, and Nanog expression. C: Default differentiation of dissociated EBs plated on Matrigel™-coated culture dishes gives rise to cells expression markers for different germ layers: Desmin (mesoderm), GATA-4 (endoderm), and βIII-Tubulin (ectoderm). Nuclei were counterstained with Hoechst (blue). Scale bar = 100 µM. D: PCR analysis of ES marker gene expression in CNPase-GFP mouse iPS cells (clone 1).
FACS sorting

CNPase-GFP miPS-derived SCs were dissociated using 0.1% trypsin and analyzed by flow cytometry using a FACS Calibur flow cytometer (Becton Dickinson) and a MoFlo highspeed cell sorter. Data were analyzed with WinMDI 2.8 and FloJo 7.6.5 software.

RESULTS

Characterization of CNPase-GFP IPS cells

We generated IPS cells from CNPase-GFP transgenic mouse fibroblasts. In order to evaluate reprogramming and induced pluripotency, CNPase-GFP IPS cells were compared to mouse ES cells. Like ES cells, CNPase-GFP IPS cells showed positive alkaline phosphatase activity (Figure 1A) and expression of ES cell–associated antigens Oct3/4, Sox2, Nanog, SSEA-1 and SSEA-4 (Figure 1B). To verify capacity for differentiation into cell types derived from each of the three embryonic germ layers, embryoid bodies (EBs) were default differentiated and stained for markers representative of the three germ layers: desmin, GATA and b-III tubulin. Positive staining indicated the capacity of CNPase-GFP IPS cells to differentiate into cell types of the three germ layers (Figure 1C). CNPase-GFP IPS cells were further characterized by means of PCR analysis for pluripotency markers and reprogramming factors. High expression levels of Oct4, Sox2, Klf4, c-Myc and Nanog mRNA confirmed successful induction of pluripotency (Figure 1D).

Differentiation towards Schwann cells

A schematic representation and various stages of the differentiation process are depicted in Figure 2. Undifferentiated CNPase-GFP IPS cells were maintained on irradiated MEFs in the presence of leukemia inhibitory factor (LIF). For neural induction, EBs were formed in the presence of ATRA according to a -4/+4 protocol. After plating on gelatin, EBs started to attach and medium was switched to N2 medium supplemented with appropriate morphogens (SHH, FGF8, BDNF and Ascorbic Acid) to promote neural crest formation. Cells started to migrate out of the colonies and at day 14 of differentiation cells with polygonal neural crest cell morphology started to appear. For neural crest enrichment, neural crest cells were replated on Matrigel™ coated culture dishes, on which they formed clusters of cells. For targeted differentiation towards SCs, they were cultured in the presence of NRG1, FSK, and FGF2 for up to 60
After 20 days cells with a typical SC morphology migrated out of neural crest clusters. In a later stage, a typical parallel longitudinal SC orientation was observed in the cultures; in this stage, cells started to express CNPase strongly (seen as GFP expression; Figure 2J-L). Immunocytochemistry (ICC) was performed to confirm GFP (CNPase) expression as well Oct6 expression (Figure 3A-B). Fluorescent images were analyzed and the numbers of cells expressing GFP and Oct6 were counted (Figure 3C). Of the total of cells, 81.6% ± 29.5% expressed at least one of the two markers (GFP and/or Oct6). Of the differentiated cells, 57.0% ± 25.0% (or 52.4% ± 23.0% of total cells) was double positive for both GFP and Oct6. Of the differentiated cells, 78.4% ± 9.6% (or 61.9% ± 21.9% of total cells) was GFP-positive; 78.6% ± 32.8% (or 72.2% ± 30.1% of total cells) was Oct6-positive. FACS sorting for GFP showed >50% CNPase positivity, confirming the ICC data (Figure 3D).

**DISCUSSION**

iPS cells have been differentiated in vitro into a range of neural cell types relevant for the potential treatment of CNS neurodegenerative diseases such as MS, Parkinson’s disease, or amyotrophic lateral sclerosis (Ogawa, et al., 2011; Dimos, et al., 2008). In the present study we show that CNPase-GFP positive mouse iPS can be effectively differentiated towards Schwann cells (SCs).

Targeted differentiation of iPS cells towards SCs can serve a number of purposes: 1. Patient iPS-derived SCs can be used for the development of in vitro models for immune-mediated neuropathies like Charcot-Marie-Tooth disease, Guillain-Barré syndrome, Schwannomatosis and chronic inflammatory demyelinating polyneuropathy; 2. Peripheral nerve injuries (PNI) are common and can lead to considerable long-term morbidity (Eser, et al., 2009; Novak, et al., 2011). SC transplantation has been shown to improve functional outcome in nerve injury models (Fansa and Keilhoff, 2004a). iPS-derived SCs might be used in autologous cell therapy for the treatment of patients with PNI; 3. Remyelination of the CNS has been established in animal models using cells of the oligodendrocytic lineage (Keirstead, et al., 2005; Totoiu, et al., 2004). It is known that SCs can myelinate axons of the CNS as well (Blakemore, 1977); they have already been used for promoting exogenous myelination in CNS demyelinating diseases like multiple sclerosis (MS) or CNS injury (Duncan, et al., 1981; Pearse, et al., 2004). Therefore, iPS-derived SCs might offer an autologous source of cells for regeneration of the CNS.

Several studies have shown induction of neural crest and subsequent SC differentiation using stromal cell-derived inducing activity (SDIA). Lee et al. (Lee, et al., 2007; Lee, et al., 2010) described a protocol for the differentiation of ES cells into neural crest by firstly recapitulating neuroectoderm formation on MS-5 feeder layers, and subsequently by inducing neural crest formation with the morphogens SHH, FGF8, BDNF and AA. These factors have been shown to initiate dopaminergic patterning of differentiating embryonic stem cells (Chambers, et al., 2009; Lee, et al., 2000; Perrier, et al., 2004). Directed differentiation towards SCs was established using NRG1, forskolin, FGF2 and CNTF. Mizuseki et al. (Mizuseki, et al., 2003) used co-culturing of ES cells on PA6 stromal cells and neural rosette formation to differentiate and enrich p75NTR-positive neural crest cells from non-human ES cells. Liu et al. (Liu, et al., 2012) showed neural crest differentiation from iPS of human origin, as well as subsequent differentiation towards cells expressing GFAP, p75, and Sox9, by means of SDIA.

Although the use of SDIA is an efficient method to differentiate human pluripotent stem cells into neural cells (Kawasaki, et al., 2000), a major drawback is the risk of introducing xenogenic pathogens or antigens in a patient, if clinically applied. Recently, a protocol was published by Menendez et al. for the differentiation of neural crest cells from human pluripotent stem cells without the use of SDIA, however no SC generation was shown (Menendez, et al., 2013). We developed a specific protocol for differentiation of SCs from mouse iPS cells without the use of SDIA. We used EB formation as a neural crest induction model, as others have effectively done before (Kawaguchi, et al., 2010; Zhou and Snead, 2008). For subsequent neural crest and SC differentiation, we have adapted Lee’s protocol used for human embryonic stem cells (Lee, et al., 2007). In the absence of gp130 signaling, ES cells form three...
promotor was used for identification of SCs. CNPase (2',3'-Cyclic nucleotide 3'-phosphohydrolase) is a
to bias differentiation to a GFAP-expressing astrocytic lineage. GFP-expression under the CNPase
and FGF2 are potent mitogens for SCs (Davis and Stroobant, 1990). We left out CNTF as it appeared
while its exact mechanism remains inconclusive (Yu, et al., 2004).

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increase survival and differentiation among neural crest cells (Kalcheim and Gendreau, 1988; Sieber-
(Jeong, et al., 2004; Trumpp, et al., 1999). Neurotrophic factors BDNF and bFGF (FGF2) are known to
neural crest development and migration as well as morphogenesis of neural crest-derived structures
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Blum, 1991). Ascorbic acid is known to promote neuronal differentiation from embryonic stem cells,
while its exact mechanism remains inconclusive (Yu, et al., 2004).

Essential for the induction of the SC lineage is neuregulin-1 (NRG1). Shah et al. (Shah, et al., 1994) showed
that NRG1 commits the differentiation of neural crest progenitor cells towards a glial lineage. Forskolin
and FGF2 are potent mitogens for SCs (Davis and Stroobant, 1990). We left out CNTF as it appeared
to bias differentiation to a GFAP-expressing astrocytic lineage. GFP-expression under the CNPase
promoter was used for identification of SCs. CNPase (2',3'-Cyclic nucleotide 3'-phosphohydrolase) is a
myelin-associated enzyme which is expressed almost exclusively in the two myelinating cell types of
the nervous system (oligodenedrocytes and SCs) (Sprinkle, 1989). In order to distinguish differentiated
SCs from oligodendrocytes, co-expression of Oct6 (Oct6) is a transcription factor expressed in the developing nervous system during embryogenesis and used as a
marker for promyelinating SCs (Jaegle, et al., 2003).

We showed efficient SC generation using the described protocol. Our efficiency typically appeared much
higher compared to e.g. dopaminergic differentiation in previously published protocols. Forskolin,
several groups are working on optimization of in vitro neural crest differentiation; even higher efficiencies
of SC differentiation might be reached by inhibition of SMAD signaling using small molecules such as
SB435142 and LDN-193189, favoring neural crest specification (Chambers, et al., 2012; Kreitzer, et al.,
2013).

Our results show that fibroblast-derived mouse iPS cells can be efficiently differentiated towards cells
with a proper SC phenotype, without the use of stromal cells.

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