Grafted murine induced pluripotent stem cells prevent death of injured rat motoneurons otherwise destined to die

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Abstract

Human plexus injuries often include the avulsion of one or more ventral roots, resulting in debilitating conditions. In this study the effects of undifferentiated murine iPSCs on damaged motoneurons were investigated following avulsion of the lumbar 4 (L4) ventral root, an injury known to induce the death of the majority of the affected motoneurons. Avulsion and reimplantation of the L4 ventral root (AR procedure) was accompanied by the transplantation of murine iPSCs into the injured spinal cord segment in rats. Control animals underwent ventral root avulsion and reimplantation, but did not receive iPSCs. The grafted iPSCs induced an improved reinnervation of the reimplanted ventral root by the host motoneurons as compared with the controls (number of retrogradely labeled motoneurons: 503 ± 38 [AR+iPSCs group] vs 48 ± 6 [controls, AR group]). Morphological reinnervation resulted in a functional recovery, i.e. the grafted animals exhibited more motor units in their reinnervated hind limb muscles, which produced a greater force than that in the controls (50 ± 2.1% vs 11.9 ± 4.2% maximal tetanic tension [% ratio of operated/intact side]). Grafting of undifferentiated iPCSs downregulated the astroglial activation within the L4 segment. The grafted cells differentiated into neurons and astrocytes in the injured cord. The grafted iPSCs, host neurons and glia were found to produce the cytokines and neurotrophic factors MIP-1a, IL-10, GDNF and NT-4. These findings suggest that, following ventral root avulsion injury, iPSCs are able to induce motoneuron survival and regeneration through combined neurotrophic and cytokine modulatory effects.

Introduction

Following an injury or various diseases afflicting the cell body or axon of the motoneuron, spinal motoneurons become increasingly vulnerable to glutamate cytotoxicity (McNaught and Brown, 1998; Spalloni et al., 2013). Avulsion of one or more ventral roots, which typically occurs in plexus injuries, induces the death of motoneurons in consequence of the excessive amounts of glutamate loaded onto the affected neurons (Carlstedt, 2008). Glutamate activates the N-methyl-D-aspartate receptors, leading to an excessive influx of Ca^{2+} into the damaged cells and to the death of the vast majority of the affected motoneurons (Koliatsos et al., 1994; Nogradi and Vrbova, 2001; Nogradi et al., 2007). Damaged motoneurons do not die immediately after the injury, and the window of 10-12 days between the injury and the motoneuron death can be successfully used to rescue the injured motoneurons (Nogradi et al., 2007; Pinter et al., 2010]. Reimplantation of the avulsed ventral root is the primary requirement for regeneration of the motor axons, but in a typical situation few motoneurons are able to survive and reinnervate peripheral targets without additional neuroprotective support (Carlstedt et al., 1990; Carlstedt, 2008; Nogradi and Vrbova, 1996). These neuroprotective strategies involve the use of neurotrophic factors, Na^{+} channel blockers/glutamate release inhibitors and native or genetically enginereed stem cells (Blits et al., 2004; Eggers et al., 2008; Eggers et al., 2013; Nogradi and Vrbova, 2001; Novikov et al., 1997; Wu et al., 2003). However, only Na^{+} channel blockers/glutamate release inhibitors, such as riluzole have proved effective from the aspects of inducing both motoneuron survival and functional reinnervation, while neurotrophic factors and stem/progenitor cell therapies have failed to induce the axonal outgrowth of otherwise surviving motoneuron populations (Blits et al., 2004; Eggers et al., 2013; Pinter et al., 2010). The use of embryonic stem cells is otherwise complicated by ethical concerns.

Yamanaka et al. have reported that murine and human skin fibroblasts can be reprogrammed to a pluripotent state through application of the transcription factors KLF4, SOX2, OCT4 and c-MYC (Okita et al., 2007; Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Due to the availability of species- or individual-specific induced pluripotent stem cells (iPSCs) not only does this suggest a new therapeutic potential for cell rescue, but the ethical concerns can be minimized, too. Indeed, recent studies have shown that iPSCs or their derivatives have neuroprotective effect on certain populations of central nervous system (CNS) neurons and are able, to a certain extent to replace lost neurons or glial cells within the CNS (Chen et al., 2010; Fujimoto et al., 2012; Hargus et al., 2010; Tsuji et al., 2010).

Here, we investigated whether mouse iPSCs grafted into the injured rat spinal cord immediately after ventral root avulsion and reimplantation are able to rescue injured motoneurons and induce reinnervation of the reimplanted ventral root by the regenerating motor axons. A further aim of this study was to reveal the differentiation pattern of the grafted iPSCs and the mechanism of action by which they induced motoneuron survival and functional reinnervation in the target skeletal muscles.

Materials and Methods

Cell culture

Murine embryonic fibroblasts (MEFs) obtained by a standard protocol from a murine fetus 13 days *post coitum* were cultured in FM medium (DMEM-GlutamaxI supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin) (Robertson, 1987). The MEFs at passage one were used to generate iPSCs,- and those at passage three to generate feeder cells treated with mitomycin C (Sigma-Aldrich, St Lake, MO, USA).

iPSCs and enhanced green fluorescent protein (eGFP) containing iPSCs (eGFP-iPSCs) were cultured in embryonic stem cell medium (ESC medium: DMEM-GlutamaxI supplemented with 15% ESC qualified FBS (SLI Ltd, West Sussex, UK), 1 mM β-mercaptoethanol, 1% non-essential amino acids (Sigma-Aldrich, St Lake, MO, USA), 10 U/ml penicillin, 10 µg/ml streptomycin and 1000 U/ml leukemia inhibitory factor (ESGRO, Chemicon/Millipore, Billerica, MA, USA).

Generation of iPSCs and eGFP-iPSCs

The generation of iPSCs was described in detail elsewhere (Nemes et al., 2014). Briefly: MEFs were seeded at $5x10^4$ cells per well in a 6-well plate coated with gelatin in FM medium. On the following day, the medium was replaced with ESC medium supplemented with valproic acid (final concentration 1 mM) and the purified recombinant reprogramming protein cocktail (Oct4, Sox2, Klf4 and c-Myc) at a final concentration of 8 µg/ml. After a 12-h incubation, the medium was changed to normal ESC medium and the cells were cultured for 36 h. The cells were treated 4 times with the protein cocktail at intervals of 48 h. On day 9, the cells were transferred to feeder-coated

10-cm dishes and cultured further until ESC-like colonies appeared. The medium was refreshed every second day.

Under the control of the elongation factor-1-alpha (EF1α) promoter, the eGFP reporter protein was introduced into the iPSCs by lentiviral transduction. The eGFP-expressing iPSC colonies were identified under a fluorescent microscope and picked individually. Only those iPSC colonies were used for transplantation that uniformly expressed the eGFP.

Neural differentiation assay

Differentiation of mouse iPSCs and eGFP-iPSCs towards neural lineages was performed via embryonic body (EB) formation in suspension in accordance with the previously published protocol (Bibel et al., 2004) Briefly, iPSCs and eGFP-iPSCs were dissociated with 0.05% trypsin and $4.5x10^6$ cells in 15 ml medium were seeded onto a non-adherent 10 cm bacterial dish (Greiner) coated with 2-Hydroxyethyl methacrylate (2-HEMA, Sigma-Aldrich) and cultured at 37°C±2°C, 5±1% CO_2 for 8 days. The medium was refreshed every second day. From day 4, the differentiation medium was supplemented with retinoic acid (5 µM final concentration). On day 8, the EBs were dissociated with 0.25% Trypsin; and $2x10^5$ cells/cm^2 in Medium A (3 mg/ml Glucose, 3 mg/ml AlbuMaxI, 10 U/ml Penicillin, 10 µl/ml Streptomycin, 1% N2 supplement and 10 ng/ml bFGF (freshly added) in DMEM/F12) were seeded on polyornithine/laminin coated coverslips. The medium was refreshed on day 9. From day 10, the cells were cultured in Medium B (50% DMEM/F12, 50% Neurobasal Medium, 1 mM GlutaMaxI, 3 mg/ml AlbuMaxI, 10 U/ml Penicillin, 10 µg/ml Streptomycin, 0.5% N2 supplement and 1% B27 supplement). The cells were cultured until day 12.

Ventral root avulsion–reimplantation (AR procedure) and transplantation of iPSCs

All the operations were carried out under deep ketamine-xylazine anesthesia (ketamine hydrochloride, 110 mg/kg body weight; xylazine (Rompun) 12 mg/kg body weight) with sterile precautions. To maintain the body temperature at 37.0±0.5 °C, the rats were kept on a heating pad (Supertech Ltd, Pécs, Hungary) during the surgery. Laminectomy was performed at the level of T13–L1, the dura was opened and the left L4 ventral root was pulled out, leaving the dorsal roots intact. The cut end of the ventral root was then inserted into the ventrolateral part of the spinal cord and 3 x 10^5 iPSCs were injected into the caudal part of the L4 segment (AR+iPSCs group). The spinal cord was covered with the remaining dura, the wound was closed and the animals were allowed to recover. In the control experiments (AR group), the L4 ventral root was avulsed and reimplanted without iPSC grafts. Animals were examined after a survival time of 3, 7, 12, 16 or 21 days or 3 months. Appropriate care was taken to minimize pain and discomfort.

Retrograde labeling

Three months after the surgery 5 animals per group and 5 intact animals were deeply anesthetized as described above. On the operated side the ventral ramus of the left L4 spinal nerve was cut and the proximal stump of the nerve was covered with a few Fast Blue crystals. Four days after the application of this fluorescent dye, the animals were reanesthetized and were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer.

RNA isolation and cDNA synthesis

Spinal cord samples were stored in RNAlater (Qiagen) at -20 °C. For tissue lysis, the samples were

placed in 2-ml Eppendorf tubes, and 600 µl of RLT buffer and a 5-mm stainless steel bead were added to each tube. The tissues were lysed for 4 min at 30 Hz by a TissueLyser instrument (Qiagen). The lysates were spun for 3 min at 13.000 rpm. Total RNA was purified by using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration and purity were measured by Nanodrop (Thermo Scientific). 1 µg of RNA was digested with RQ1 DNase (Promega) and reverse-transcribed with SuperScript III RT enzyme and oligo$(dT)_{20}$ primers (Life Technologies).

Semiquantitative polymerase chain reaction (PCR)

For quantification of the PCR products, 4 spinal cord samples from 4 different treatment groups were analyzed (avulsed, non-injected spinal cords 3 and 7 days after operation; and avulsed, iPSC-injected spinal cords 3 and 7 days after the operation). The spinal cord cDNA was amplified by using gene-specific primers that recognize both murine and rat gene products. The primer sequences used are listed in Supplementary Table 1. The reaction was carried out in GoTaq Green Master Mix (Promega) in Veriti Thermal Cycler (Applied Biosystems) with an initial denaturation step at 95° C for 2 min, and 22-38 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s and extension at 72 °C for 30 s. The PCR products were run in ethidium bromide-containing 1.5% agarose gels, and pictures were taken with the Gel Logic 1500 Imaging System (Kodak). The intensities of the bands were quantified with ImageJ Software (imagej.nih.gov/ij). The band intensity of each gene product was normalized to that of the housekeeping gene GAPDH. The data for the replicates (n=4) were averaged, and the standard errors of the mean (SEM) are depicted as error bars in the diagrams.

Immunohistochemistry

For the cultured iPSCs, cells were permeabilized in PBS containing 0.1% Triton X-100 for 5 minutes and blocked in 5% bovine serum albumin for 1 hour at room temperature (RT). The cells were than incubated 1 hour at RT with the following primary antibodies: monoclonal anti-nestin (Developmental Studies Hybridoma Bank, 1:100), rabbit neuronal class III β tubulin antibody (Covance, 1:2000), mouse anti Oct 4 (Santa-Cruz; 1:100), mouse anti-SSEA1 (Santa Cruz; 1:100), goat anti-Sox2 (Santa Cruz; 1:50), goat anti-Nanog (R&D Systems; 1:50). The primary antibodies were visualized with AlexaFluor 647 donkey anti-rabbit IgG (H+L), AlexaFluor 594 donkey anti-mouse (H+L) and Alexa Fluor 594 donkey anti-goat IgG (H+L) (1:2000). Finally the cells were incubated with DAPI (4',6-diamidino-2-phenylindole; 0.2 µg/ml final concentration) in PBS for 20 minutes at RT.

For the spinal cords, 25 µm transverse sections were cut on a cryostat (Leica CM 1850, Leica GmbH, Germany) and mounted onto gelatin-coated glass slides. Nonspecific binding sites were subsequently blocked with 3% normal donkey, goat or horse serum. Primary antibodies were incubated overnight at 4 °C, washed, and then incubated with fluorescent-conjugated secondary antibodies for 1 h at room temperature. The following primary antibodies were used: anti-rabbit brain-derived nerve growth factor (BDNF), anti-neuron-specific beta III tubulin, anti-rabbit glial-derived nerve growth factor (GDNF) (1:200; all from Abcam), anti-mouse GFAP (1:100; Santa Cruz Biotechnology, Inc, CA), chicken anti-green fluorescent protein polyclonal (GFP) (1:2000; Chemicon), biotinylated *Griffonia (Bandeira) Simplicifolia* isolectin B4 (for microglia/macrophages, 1:200; Vector), anti-rat IL-1-alpha, anti-rat IL-6, anti-rat IL-10, anti-rat TNF-alpha, anti-rat MIP-1 alpha (1:200; all from Abbiotec, San Diego, CA), goat anti-choline acetyltransferase (1:200; Millipore, Billerica, MA), anti-mouse M6 (mouse-specific neuron marker, 1:400; DSHB, Iowa), anti-mouse M2 (mouse-specific astrocyte marker, 1:400; DSHB, Iowa City,

IA), anti-mouse MOG (mouse-specific oligodendrocyte marker, 1:50; R&D Systems, Minneapolis, MN) anti-rabbit neurotrophin 4/5 (1:200; Abbiotec, San Diego, CA) and SSEA-1 (stage-specific mouse embryonic antigen, 1:400; DSHB, Iowa City, IA). Secondary antibodies were used as follows: Alexa Fluor 594 donkey anti-rat, Alexa Fluor 546 goat anti-rabbit, Alexa Fluor 546 donkey anti-goat, Alexa Fluor 488 goat anti-chicken, streptavidin Alexa Fluor 405 conjugate, streptavidin Alexa Fluor 546 conjugate, streptavidin Alexa Fluor 488 conjugate (1:400; all from Invitrogen), biotinylated anti-goat IgG (H+L) and biotinylated anti-mouse IgG (H+L). To validate TNF-alpha and IL-1-alpha immunohistochemistry, Mg^{3}HeLa cells transfected with a TNF-alpha plasmid (kind gift from Ernő Duda, at the Biological Research Centre, Szeged, Hungary) and rat testis were used, respectively.

Fluorescent signals were detected in an Olympus BX50 epifluorescence microscope equipped with a DP70 digital camera (Olympus Ltd, Tokyo, Japan). Confocal microscopic images were obtained with an Olympus FluoView® FV10i compact confocal microscope.

Quantification of differentiation process of the grafted iPSCs

To quantify the differentation of grafted iPSCs, we processed cross sections of spinal cord containing grafted cells for immunohistochemistry 3, 7, 10, 12, and 16 days after the transplantation. For each cell marker, we randomly selected three tissue sections that were 100–150 µm apart. At 120x magnification of the confocal microscope (square area of 102 µm x 102 µm), we first counted the number of GFP/DAPI-positive cells (for SSEA-1) and DAPI-positive cells (for M2 and M6) in 3 random fields per section. Next, we determined the number of the GFP-positive cells colabeled with SSEA-1. We only counted those GFP-positive cell bodies that contained a nucleus (identified with DAPI). Then, the percentage of co-labeled GFP/cell marker was calculated for each rat (n=4). For M2 and M6, we determined the number of M2- or M6-positive cells as percentage of

DAPI-positive cells in the graft. Quantification of grafted cells that produced factors (NT-4, GDNF, IL-10 and MIP-1a) was performed as described for the SSEA-1.

Cell counts

The numbers of retrogradely labeled cells were determined on 25-µm-thick serial cryostat sections. To avoid double counting of neurons present in two consecutive sections, the retrogradely labeled neurons were mapped with the aid of an Olympus (Olympus Ltd, Tokyo, Japan) drawing tube, and their locations were compared with those of labeled neurons in the previous section (Nógrádi et al., 2007; Pintér et al., 2010). All the sections from the L4 motoneuron pool were used.

Quantification of astrocyte and microglia/macrophage densities

To assess the densities of GFAP-positive astrocytes and GSA-B_4-positive microglia/macrophages in injured and grafted ventral horns, we photographed injured, grafted and intact, randomly chosen frames of the ventral horns from each rat at a primary magnification of 10x, using an Olympus FluoView® FV10i confocal microscope at distances of 0.5, 1, 1.5 and 2 mm rostrally and caudally to the reimplanted ventral root (n=5/group). To quantify the densities of GSA-B_4-positive microglia/macrophages in the graft, we randomly selected three tissue sections being 100–150 µm apart and photographed the graft from each rat at a primary magnification of 40x, using an Olympus FluoView® FV10i confocal microscope. Using ImageJ Software (NIH), we measured the relative density of GFAP and GSA-B4 immunoreactivity in the samples. The background/autofluorescence of unstained samples as reference intensity was then subtracted from the intensity of the injured, grafted and intact ventral horns and from the grafts to determine the final density. The GFAP and GSA-B4 intensity of the injured and grafted ventral horns and that of the graft was then divided by the intensity of the GFAP and/or GSA-B4 reactivity of the contralateral intact ventral horns. As

GSA-B4 labels the endothelial cells of some capillaries, the density of the capillaries was deducted from the total densities. Furthermore, automatic thresholding was performed for each image by using the NIH ImageJ software to determine the threshold for the specific signal. After the threshold had been set, the density above the threshold was quantified.

Muscle tension recording

The animals that were selected for tension recording (n=5 both in the control and in the grafted group) were anesthetized with ketamine-xylazine at the end of the 3-month survival period and the tibialis anterior (TA) and extensor digitorum longus (EDL) muscles of both the reinnervated and the contralateral control hindlimb were prepared for tension recording. These muscles were chosen for tension recording as the motoneurons innervating them are situated mainly in the L4 spinal segment. The muscles of the contralateral leg were regarded as suitable controls because their tension increased with age in a similar way to that of the muscles in normal, unoperated animals. The distal tendons were dissected free and attached to strain gauges, and the exposed parts of the muscles were kept moist with Krebs' saline solution. Isometric contractions were then elicited from the muscles by stimulating the ventral ramus of the L4 spinal nerve with bipolar electrodes. The length of each muscle was adjusted so as to produce maximal twitch tension. Single twitch and tetanic (40-100 Hz) contractions were displayed and recorded on a computer; all the additional recording hardware and software were developed by Supertech Ltd (Pécs, Hungary; system "Kellényi"). Maximal tetanic tension was achieved at a stimulation frequency of about 100 Hz. An estimate of the numbers of motor axons supplying the muscles was obtained by subjecting the L4 spinal nerve to stimuli of increasing intensity and recording the stepwise increments in the twitch contractions.

Statistical analysis

The paired T test was used to compare the groups of data. Data are reported throughout the manuscript as mean ± S.E.M. The differences of multiple groups were computed according to one-way ANOVA followed by Tukey's *post hoc* test. Probability values of $p < 0.05$ were regarded as significant for all analyses.

Results

Characterization of the eGFP-iPSC line

The pluripotency of the parental iPSC line was verified by in vitro and in vivo methods (Nemes et al., 2014). The expression pattern of different markers was verified by immunocytochemistry. The cells expressed the major pluripotency markers: SSEA1, Oct4, Sox2 and Nanog (Fig. S1A).

As no difference in the morphology and the growth dynamics between the parental cell line and the eGFP-iPSCs was observed, we verified the neural differentiation capability of the eGFP-iPSCs. The transduced cells were positive for nestin, the neural progenitor cell marker, and for neural class III β-Tubulin (Tuj1), a neuron marker (Fig. S1B). During the in vitro differentiation we could not detect significant loss of the eGFP signal.

iPSC grafts improve motoneuron survival and reinnervation of denervated muscles

The ability of iPSCs to rescue damaged motoneurons otherwise destined to die was investigated in long-term studies, allowing the animals to survive for 3 months after the avulsion injury. The number of resident motoneurons in the intact L4 motoneuron pool was first assessed by retrograde labeling of the ventral ramus of the L4 spinal nerve. The average number of retrogradely labeled

motoneurons was 1164±29. The motoneurons were localized mainly in the lateral motoneuron column of the L4 spinal segment. Three months after the avulsion and reimplantation in the control animals (the AR group, whose L4 ventral root was avulsed and reimplanted, but which received no iPSC graft), 48±6 retrogradely labeled motoneurons were detected. In contrast, a significantly higher number of reinnervating motoneurons (503 ± 38) was observed when the iPSCs were transplanted immediately after ventral root reimplantation (AR+iPSCs animals; Figs. 1B,C).

In order to determine the proportion of surviving motoneurons that were able to send their axons into the reimplanted L4 ventral root, the number of choline acetyltransferase (ChAT)-positive motoneurons labeled with the fluorescent retrograde tracer Fast Blue (FB) was compared with the number of surviving ChAT-positive motoneurons in the injured L4 segment on the operated side. In the control rats, the percentage of reinnervating FB-positive motoneurons within the surviving ChAT-positive motor pool was only 14 ± 1.8%, whereas in the grafted animals this ratio was much higher, at 61 ± 3.3%, indicating that more than half of the surviving motoneurons in the AR+iPSCs group were able to maintain a long axon in the L4 reimplanted ventral root and peripheral nerve pathways (Fig. 1D).

The question of whether the iPSC grafts promoted the survival of resident motoneurons was investigated by comparing the number of ChAT-positive motoneurons on the operated side with that on the intact side. In the AR group, the proportion of surviving motoneurons on the operated side was 40 ± 2,5%, whereas in the AR+iPSCs animals it was 72 ± 5.4%, indicating that the treatment with iPSCs significantly improved the survival of the injured motoneurons (Fig. 1E).

Functional reinnervation was confirmed by muscle force measurements on the reinnervated muscles 3 months after the avulsion injury. In the AR rats that did not receive a graft, only 5 ± 0.6 motor units were detected in the EDL, and 6 ± 1 motor units in the TA muscle (Figs. 1F,H). In contrast, in the animals that also received an iPSC graft, 15 ± 0.8 axons were found to innervate the EDL

muscle, and 17 ± 0.7 reached the TA muscle via the reimplanted ventral root (Figs. 1F,H). The presence of a greater number of motor units in the grafted rats allowed the production of a larger force by the reinnervated muscles. In the control rats, the maximal tetanic tension (% ratio of operated side/intact side) was 11.9 ± 4.2% for the EDL muscle and 6 ± 2.2% for the TA muscle, while the grafted animals produced significantly more tension (50.0 ± 2.1 % for the EDL muscle and 53.2 ± 4.1 % for the TA muscle; Figs. 1G,I).

Differentiation of grafted iPSCs in the host cord

The grafted iPSCs formed a large cluster in the L4 segment 3 and 7 days after transplantation and expressed the embryonic stem cell marker stage-specific embryonic antigen-1 (Figs. 2A and S2A). The grafted iPSCs did not exhibit any sign of differentiation on day 3 postoperatively, i.e. no mouse-specific glial or neuronal markers were expressed by the grafted cells. On day 7 after grafting the transplanted iPSCs started to differentiate into neurons and astrocytes, but not oligodendrocytes (Figs. 2B,C). The iPSC-derived neurons expressed various neuronal markers (M6, a mouse-specific neuronal marker, and approximately two-thirds of these expressed beta III-tubulin too; Fig. S2B). The iPSC-derived astrocytes expressed the mouse-specific astrocyte marker M2, and only a few M2-positive graft-derived astrocytes were additionally GFAP-positive (Fig. S2C). The grafted cells had not migrated away from the graft 7 days after transplantation. The graft-derived beta III-tubulin-positive neurons and GFAP-positive astrocytes could mainly be detected at graft–host interface, but the less differentiated M6-positive neurons and M2-positive astrocytes appeared within the center of the graft too (Figs. 2B,C).

By this time, the eGFP expression of the majority of the iPSC-derived neurons and astrocytes had decreased or ceased, suggesting that the GFP expression pattern diminishes with differentiation. A few iPSC-derived neurons and astrocytes did retain the ability to express eGFP strongly, but these

cells were found only within the center of the graft, not at the graft–host interface (Figs. 2B,C). Quantification of the differentiation pattern of the grafted iPSCs showed that 3 days after transplantation approximately 50% of the iPSCs expressed the SSEA-1 marker, but this ratio was halved by day 7 after transplantation (Fig. 2D). The differentation of the grafted iPSCs continued with time and on postoperative days 10, 12 and 16, the grafted cells had lost their eGFP expression completely and decreasing numbers of iPSC-derived neurons and astrocytes were found in the injured cord (Figs. 3A-D).

On day 7 after transplantation 29.6 ± 4.1% of the grafted cells was identified as M2-positive astrocytes and 31.4 ± 3.8% as M6-positive neurons, whereas by day 16 after transplantation these numbers decreased to 3.7 ± 1.1% (M2) and 3.5 ± 0.6% (M6), respectively (Fig. 3E).

Microglia/macrophage reaction against iPSC-derived neurons and astrocytes

First we determined whether significant numbers of host cells invaded the territory of the grafted iPSCs, a co-labeling with DAPI nuclear staining and GFP immunohistochemistry was performed. We found that the percentage of the GFP+/DAPI+ cells was 92.5 +-2% in 7-day-old grafts, indicating that either few host cells invaded the graft or not all the grafted cells were able to maintain their GFP expression pattern by this survival time.

The differentiation of the grafted cells resulted in an altered antigen expression pattern on the cell surface. On day 7 increased activation of microglia/macrophages could be observed around the graft in the host cord (Fig. 4A). Numerous GSA-B4-positive cells were identified in the graft but these cells were positive for GFP (Fig. 4B). At this time point the grafted cells started to differentiate to M2-positive astrocytes and M6-positive neurons located at the graft-host interface. (Fig. 4C,D). Not surprisingly this differentiating cells were intermingled with GSA-B4-positive macrophages suggesting that differentiation of the grafted cells is accompanied by an attraction of macrophages

to the cell differentiation zone (Fig. 4C,D). On days 10 and 12 after the avulsion injury, strong reactions of the activated microglia/macrophages (GSA-B4-positive cells) could be detected in the injured ventral horn and in the graft (Figs. 4E,F and Figs 5A,B). Microglia cells appeared around the graft-derived neurons and astrocytes (Figs. S3A,B), suggesting that the presence of antigens on the cell surface plays a very important role in cell recognition and elimination by macrophages. On day 16 after grafting, the microglial activity was significantly decreased in the graft and in the injured ventral horn, and there was a marked reduction in the number of grafted cells, too (Figs. 5C-E). A strong reaction of the activated microglia was detected only close to the pial surface (Figs. 5C,D), where the remaining M2 and M6-positive cells were located. On day 21 after grafting, the transplanted cells could no longer be found in the spinal cord and the microglial reaction had diminished (not shown). Quantification of microglia/macrophage reaction revealed a dramatic increase 10 and 12 after transplantation in the graft with significantly lower densities of GSA-B4-positive macrophages at days 7 and 16 postoperatively (Fig. 5E).

Transplantation of iPSCs delays injury-related astrocyte reaction

Astrocytes and microglia play a pivotal role in the removal of degenerating motoneurons after an avulsion injury. The glial reactions were therefore analyzed in the injured spinal cords of the AR and the AR+iPSCs animals in the first 7 postoperative days, a period known to be critical for the injured motoneurons. The densities of astrocytes and microglia were measured rostrally and caudally to the injury in the injured ventral horn. On day 3, a significantly lower density of microglia was detected only 1 mm rostrally and 0,5 mm caudally to the central site of the avulsion/reimplantation injury (Figs. 6A,B). On day 7 after the injury, no significant difference in microglial density was detected between the experimental groups (Figs. 6E,F). On the other hand, on day 3 after the avulsion injury the astroglial reaction was significantly weaker in the AR+iPSCs

group than in the control (AR) animals (Figs. 6C,D). By day 7 postoperatively, the astroglial reaction had increased in the grafted animals and the astrocyte density closely approached or even reached that in the injured hemicord of the AR animals (Figs. 6G,H).

Determination of neurotrophic factors and cytokines in the affected segment

A semiquantitative PCR analysis was performed to determine the factors that act in the grafted spinal cords. Neurotrophic and immune factors known to play roles in spinal cord injuries (Klusman and Schwab, 1997) and their experimental treatment. mRNA levels of both mouse and rat gene products were evaluated in samples taken from the L4 segment of the AR and the AR+iPSCs animals and from iPSC cultures. No differences were found between the AR and the AR+iPSCs animals (n=4 in each group) in the mRNA levels of BDNF, IL-4, IL6, IL-18, and NT-3 (data not shown) on days 3 and 7 following surgery, but the mRNA levels of NT-4, GDNF, IL-1a, IL-10, MIP-1a and TNF-a were significantly higher in the samples of the the AR+iPSCs animals than in those from the AR animals at one or both postoperative time points (Figs. 7A-F). However, only the mRNA levels of NT-4 and IL-10 were significantly higher in the AR+iPSCs animals on both postoperative days 3 and 7 as compared with the levels in the AR animals (Figs. 7A,C). Of the 6 factors, IL-10 was the only one that demonstrated no detectable mRNA level in control animals (Fig. 7C) and only NT-4 mRNA could be proven to be produced by the native murine iPSCs (Fig. 7A).

Protein expression pattern of neurotrophic factors and cytokines in the grafted cells and in the injured ventral horn

To test whether the mRNAs of these factors are translated into protein production, the protein expressions of these cytokines and neurotrophic factors were studied with immunohistochemistry

through the use of mouse/rat-specific antibodies.

On day 3 after grafting, NT-4, GDNF, IL-10 and MIP-1a were found to be expressed in the AR+iPSCs animals (Figs. 7G-N). NT-4 expression was confined to the grafted iPSCs (Fig. 7K), whereas the other 3 factors were expressed by both the transplanted iPSCs and the host tissue, with seemingly similar expression intensity in the graft and host tissues (Figs. 7L-N).

On postoperative day 7, the protein expressions of NT-4, GDNF, IL-10 and MIP-1a were maintained. NT-4 was expressed weakly and its expression was restricted to the center of the graft as it was not detected in the host tissue (Fig. 8A). The expression of GDNF was stronger than that of NT-4 in the graft and could be detected in the host tissue too (Fig. 8B). The strong immunoreactivity of IL-10 and MIP-1a was found both in the graft and in the host tissue (Figs. 8C,D). Surprisingly, IL-1a and TNF-alpha were expressed in the host tissue, but not in the grafted iPSCs (Figs. 8E,F).

We examined the protein production in the injured ventral horns both of the AR and the AR+iPSCs animals with a view to determining the cellular origin of these factors in the host tissue. Only the astrocytes in the ventral horns of AR+iPSCs animals expressed GDNF on days 3 and 7 after the injury (Figs. S4A,B). IL-10 was expressed both by the astrocytes and by the neurons on days 3 and 7 in the AR+iPSCs group (Figs. S4C,D). MIP-1a was expressed by the neurons, the astrocytes and some microglia cells on days 3 and 7 postoperatively in the AR group (Figs. S5A,C and S6A,C). Similarly, on day 3 postoperatively, the neurons, the astrocytes and a few microglia cells expressed MIP-1a in the AR+iPSCs animals (Fig. S5B), while the expression of this cytokine had become restricted to the neurons and the astrocytes by postoperative day 7 (Figs. S5D and S6D). On postoperative day 7, TNF-alpha was produced only by the astrocytes and the microglia in the AR+iPSCs animals (Figs. S7A,B), while IL-1a was weakly expressed by the astrocytes of the grafted animals (Fig. S8).

The percentage of GFP/DAPI-positive grafted cells expressing the different neurotrophic factors and cytokines was determined 3 and 7 days after transplantation. Quantification of the protein expression patterns of the grafted iPSCs showed that all the four factors were expressed by more than 60% of the grafted cells 3 days after transplantation (Fig. 8G, NT-4: 62.2 ± 2.6%; GDNF: 66.9 ± 4.2%; IL-10: 70.1 ± 4.7% and MIP-1a: 69.1 ± 10%). Significant decrease in the percentage of secreting cells was observed in the cases of NT-4 and IL-10 (43.1± 5.6% vs 49.5 ± 4.2%) by day 7 postoperatively, whereas only moderate decrease in the expression of GDNF and MIP-1a was seen (57.2 ± 15.7% vs 58.8 ± 1.9%).

Discussion

The transplantation of iPSC-derived neural progenitor cells is of great promise as concerns the promotion of functional recovery, particularly in the acute and sub-acute phases after a CNS injury. (Fujimoto et al., 2012; Nori et al., 2011; Nutt et al., 2013). As far as we are aware, this is the first study that has provided evidence that transplanted murine iPSCs are able to rescue large numbers of motoneurons after an avulsion injury, these motoneurons being otherwise destined to die. This survival of the injured motoneurons resulted in the functional reinnervation of the denervated hind limb muscles, i.e. the rescued motoneurons were able to send their axons via the reimplanted ventral root and peripheral nerve pathways and reinnervate the target muscle fibers. These beneficial effects were achieved despite the fact that xenogeneic cells were transplanted into the rat spinal cord. It may be assumed that the grafted cells exerted their effect during the first week after grafting, while they were undifferentiated and the damaged motoneurons survived and were susceptible for the paracrine effects of the stem cells. It could be argued that the molecular therapeutic principles gained from these experiments and not the cross-species transplantation can be translated and introduced into the therapeutic arsenal of human plexus injuries. Moreover, it

should be noted that the very long distances that the regenerating axons of rescued motoneurons should travel in humans may limit the success of such a strategy.

Studies by Carlstedt and others have provided evidence that some of the injured motoneurons survive after an avulsion injury and their axons grow along the reimplanted ventral root and peripheral nerve pathways (Carlstedt et al., 1986; Nogradi and Vrbova, 1996; Nogradi and Vrbova, 2001). The spontaneous reinnervating capacity of the damaged motoneurons is limited and thus they are not able to achieve satisfactory reinnervation of the target muscles when they have to regenerate their axons along lengthy predegenerated nerve pathways (Eggers et al., 2010; Nogradi and Vrbova, 2001). The application of riluzole, a compound possessing combined Na^+ channel-blocking and presynaptic glutamate release-inhibiting effects, proved to induce a successful reinnervation of denervated muscles, involving up to 67% of the injured L4 motoneuron pool (Nogradi and Vrbova, 2001; Nogradi et al., 2007). A number of studies have revealed that damaged motoneurons can also be rescued by locally applied neurotrophic factors or by grafted stem or neural progenitor cells, producing neurotrophic factors after avulsion-induced motoneuron injury (Blits et al., 2004; Eggers et al., 2008; Hell RC, 2009; Su H, 2009). However, motoneurons that were rescued by a mechanism induced purely by neurotrophic factors were usuallly not able to grow their axons into the reimplanted ventral root and produce functional reinnervation of peripheral targets.

Recent work from our laboratories has shown that intraspinal or intraradicular application of the clonal neuroectodermal stem cell line NE-GFP-4C induced not only the survival of the injured motoneurons after a ventral root injury but also extensive reinnervation of the denervated hindlimb muscles by the regenerating axons (Pajenda et al., 2013).

In the present study, we transplanted murine iPSCs produced by a novel recombinant protein method (Nemes et al., 2014). In the first week after grafting, only minor phenotypic changes were

observed, and this period of slow differentiation and migration of the grafted cells coincided with the intense gene expression profile of neurotrophic factors and cytokines and with the critical period of motoneuron vulnerability, when motoneurons can be rescued.

Interestingly, during this period of days after grafting, the majority of the iPSC-derived neurons lost the eGFP signal, and those which still expressed the fluorescence reporter protein tended to b located in the center of the graft rather than at the host–graft interface. By day 16, all the iPSC derivatives had lost the eGFP expression.

One explanation for the eGFP signal loss might be that, during the *in vivo* differentiation of the grafted cells, the EF1α promoter is gradually and/or partially silenced. During the *in vitro* neural differentiation, we did not observe diminution of the eGFP signal, suggesting that, in the course of the *in vitro* and *in vivo* differentiation of the eGFP-iPSCs, the signalling pathways are modulated differently by the microenvironment in which the differentiation takes place.

After the early phase, the grafted iPSCs underwent a relatively fast differentiation process, accompanied by an intense and increasing microglial response, resulting in rapid elimination of the grafted iPSCs by day 21 after grafting. It may be argued that this fast cell differentiation and the features of the recombinant protein-based iPSC production result in the early presence of cell surface antigens, leading to the rapid elimination of the grafted cells by the host immune system.

Following ventral root avulsion, the first 10-12 days is the most critical period for the survival of rat motoneurons (Koliatsos et al., 1994). In this short period, injured motoneurons can respond to rescuing strategies if treatment is started within 10 days after avulsion (Nogradi et al., 2007). The grafted iPSCs produced two neurotrophic factors, NT-4 and GDNF, and the cytokines IL-10 and MIP-1-alpha in the first 7 days after grafting. Moreover, injured ventral horn neurons and glial cells express various cytokines and neurotrophic factors, indicating a strong signaling and modulatory process in the injured ventral horn. The role of GDNF in motoneuron survival is well established.

Various studies have shown that the application of GDNF induced motoneuron survival following ventral root avulsion and reimplantation, accompanied by sprouting of the regenerating axons and nerve coil formation in the ventral root, but the injured motoneurons failed to send their axons into the reimplanted root (Blits et al., 2004; Eggers et al., 2008; Wu et al., 2003). Recent results from our laboratories have shown that, spatiotemporally limited engineered expression of the neurotrophic factors BDNF and/or GDNF results in relatively good motoneuron survival and target reinnervation (Pajenda et al., 2014). Others have suggested a synergistic effect between GDNF and riluzole on the functional recovery following ventral root avulsion (Bergerot et al., 2004). NT-4/5 is reportedly a high-affinity ligand for the TrkB receptor (Minichiello et al., 1998). Signaling through the TrkB receptor activates numerous intracellular pathways that may promote cytoplasmic and nuclear events that induce neuronal survival, but the precise role of NT4/5 in neuronal (more specifically, motoneuron) survival needs to be confirmed.

On the other hand, the grafted iPSCs produced the anti-inflammatory IL-10 and the pro-inflammatory MIP-1-alpha. IL-10 is known to initiate various signaling cascades through the IL-10 receptor in spinal cord neurons *in vitro*, thereby preventing the cell death otherwise induced by glutamate excitotoxicity (Bachis et al., 2001; Zhou et al., 2009a; Zhou et al., 2009b). The application of IL-10 after a spinal cord injury reportedly prevented neuronal death by inhibiting caspase-3 cleavage (Jackson et al., 2005; Zhou et al., 2009a).

In addition to the expression of the above factors by the grafted and host cells, the pro-inflammatory cytokines IL-1-alpha, and TNF-alpha were produced only by the cells of the host spinal cord but not in the grafted cells. The mechanisms of action of IL-1-alpha, MIP-1-alpha and TNF-alpha in the CNS are not yet fully understood (Aldskogius, 2011; Glezer et al., 2007; Juttler et al., 2002). Recent data provided evidence that IL-1-alpha and TNF-alpha are neuroprotective against excitotoxicity-induced cell death in the CNS via distinct pathways (Carlson et al., 1998; Carlson et

al., 1999). Moreover, the administration of IL-1-beta, IL-6 and TNF-alpha to the injured murine spinal cord induced decreased levels of microglial activation and tissue loss (Klusman and Schwab, 1997). MIP-1-alpha acts as a pro-inflammatory cytokine in the peripheral immune system, and its role in regulation/signaling in the injured CNS is not well understood. It could be argued that the co-expression of these two, seemingly antagonistic groups of cytokines may result in an enhanced effect on the downregulation of microglial and astroglial reactivity. It was surprising to find that the humoral secretion pattern of the grafted iPSCs shows both similarities and differences when compared with the expression pattern of grafted NE-GFP-4C neuroectodermal stem cells in the same injury model (Pajer et al. 2014)**.** The NE-GFP-4C cells produced 5 modulatory cytokines (IL-1-alpha, IL-6, IL-10, TNF-alpha and MIP-1-alpha), but not neurotrophic factors and this cytokine signalling resulted in an even more significant motoneuron survival and regeneration. It may be thought, that apart from different molecular composition of the graft secretome, other factors, including the rapid differentiation of iPSCs and the early presence of surface antigens may contribute to the differential effects on motoneuron survival and reinnervation of peripheral targets.

Conclusions

Our data suggest that grafted iPSCs induce a firm reinnervation by surviving motoneurons otherwise destined to die via complex regulatory and signaling mechanisms, that involve both cytokines and neurotrophic factors. These mechanisms appear to involve interactions between the grafted stem cells and the host tissue. It further seems evident that these motoneuron-rescuing mechanisms are very fine-tuned as they are able to rescue injured motoneurons and enhance regeneration of their axons without inducing axonal sprouting and aberrant reinnervation.

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Figure legends

Figure 1. Reinnervation of hind limb muscles by motoneurons rescued by iPSCs.

A. Schematic diagram of the surgical procedure, including the avulsion and reimplantation of the L4 ventral root and the transplantation of iPSCs. **B.** Images of retrogradely labeled reinnervating (FB-positive) and surviving (ChAT-positive) motoneurons taken from spinal cords of AR and AR+iPSCs animals. **C-E.** Individual twitch contractions of motor units (MUs) and tetanic curves are shown. **D.** The extensor digitorum longus (EDL) and the tibialis anterior (TA) muscles of AR+iPSCs rats are innervated by significantly higher numbers of motor axons than are the muscles of the AR animals. The higher numbers of reinnervating motor axons produced stronger maximal tetanic muscle contractions in the AR+iPSCs animals. Error bars, S.E.M., $p<0.05$. Scale bar in **A:** 100 μm.

Figure 2. Differentiation of grafted iPSCs and their derivatives

A. Expression of embryonic stem cell marker stage-specific embryonic antigen-1 by the grafted iPSCs 7 days after transplantation. **B** and **C.** iPSC-derived astrocytes (M2) and neurons (M6) could be detected mainly at the graft–host interface. Arrows show GFP and M6 colocalized cells in the graft. **D.** The majority of the grafted iPSCs (52.5 ± 2.5%) expressed SSEA-1 3 days after transplantation. Significantly decreased SSEA-1 expression was detected (25.9 ± 4.8%) 7 days after transplantation. * indicates significant difference between the 3 days and 7 days data ($p<0.05$), n=4 at each time point. **A:** 100 μm, in the enlarged inset: 20 μm; in **B** and **C:** 100 μm, in the enlarged inset: 50 μm; in **D:** 200 μm, in the insets: 20 μm.

Figure 3. Derivatives of grafted iPSCs disappear from the spinal cord

A, B, C and D. On days 7, 10, 12 and 16 after grafting, iPSC-derived neurons and astrocytes, which have

lost their GFP expression were detected. A continuous decrease of M2-positive astrocytes and M6-positive neurons is seen along with the survival and differentiation of the grafted cells. On day 16 after grafting, very few iPSC-derived neurons and astrocytes were found in the injured cord. **E.** Quantification M2- and M6-positive cells 7, 10, 12 and 16 days after transplantation. * = significant difference between 7, 10, 12 and 16 days data; ** = significant difference between 10 and 12 days data, $p<0.05$, n=4 at each time point. Scale bar in **A:** 250 µm **B:** 200 µm, in the insets **D**: 20 µm.

Figure 4. Microglia/macrophage reaction in the graft on days 7 after transplantation

A and **B.** On day 7 after transplantation, few GFP-positive grafted cells displayed GSA-B4 reactivity. Arrows point to the GFP/GSA-B4 co-labeled grafted cells in **B.** **C** and **D.** Activated microglia/macrophages appeared around the iPSC-derived M2- and M6-positive cells 7 days postoperatively. **E** and **F.** Ten days after transplantation the activated microglia/macrophages showed very strong density within the graft and in the ventral horn. (g = graft, h = host cord) Scale bar in **A,B:** 100 µm **C: 25** µm and **E:** 100 µm and 25 µm; and in **F:** 20 µm

Figure 5. Microglia/macrophage reaction in the graft on days 12 and 16 after transplantation

A and **B.** On days 12 after transplantation, the activated microglia/macrophages showed very strong density within the graft and in the ventral horn. **C** and **D.** It can be seen that 16 days after grafting very few iPSC-derived neurons and astrocytes were detected and the density of activated microglia/macrophages had decreased in the ventral horn, but increased around the surviving graft-derived cells. **E.** Quantification of density of GSA-B4 reactivity within the graft. Significant increase of the GSA-B4 density is seen 10 and 12 days after transplantation compared with the 7 days and 16 days data. * = significant difference between the 10, 12 and the 7, 16 days data; ** = significant difference between 7 and 16 days data, $p<0.05$, n=4 at each time point. Scale bar: in **A:** 100 µm and 25 µm; and in **B:** 20 µm

Figure 6. Astrocytic and microglial/macrophage reactions in injured ventral horns of the AR and the AR+iPSCs groups.

A-D. Microglial/macrophage (**A, B:** GSA B4-positive) and astroglial (**C, D:** GFAP-positive) densities are shown in spinal segment L4 of the AR and the AR+iPSCs animals 3 days after transplantation. **E-H**. Microglial (**E, F:** GSA B4-positive) and astroglial (**G, H:** GFAP-positive) densities are shown in spinal segment L4 of the AR and the AR+iPSCs animals 7 days after grafting. Asterisks indicate the significant differences in density between AR and iPSC animals at various distances from the site of avulsion. Error bars, S.E.M., $p<0.05$. **B, F, D** and **H**. Examples of transverse sections 500 µm cranially to the avulsion site processed for GFAP immunohistochemistry or GSA B4 isolectin histochemistry. Scale bar in **B,D,F,H:** 100 µm.

Figure 7. **Elevated mRNA levels (survival times 3 and 7 days) and immunohistochemical detection (survival time 3 days) of neurotrophic factors and cytokines**

A-F. Relative gene expression levels of various cytokines in the ventral horns of the AR and the AR+iPSCs animals and in native (non-grafted) cultured protein-based murine iPSCs. There were significant higher mRNA levels of all factors of the AR+iPSCs animals by 7 days after grafting relative to the AR data. Only

the NT-4/5 mRNA levels of the grafted L4 segments were significantly different relative to their controls at 3 days. IL-10 was produced only in the grafted cords, and the native murine iPSCs produced only NT4/5 in detectable amounts. **G-J.** Immunohistochemical detection of the factors produced by the grafted cells and the host cord at 3 days after transplantation. Note the ventral location of the graft. Insets are confocal images taken at various optical thickness settings. **K-N.** High-magnification epifluorescence photographs taken from the graft–host interface. Only NT4/5 is not detectable in the host cord around the graft, while the other 3 factors are expressed by both the host and the grafted tissue (g = graft, h = host). Data are presented as means ± S.E.M, $p<0.05$. One-way ANOVA analysis with Tukey's *post-hoc* test was used. Scale bar in **G-J**: 250 µm and 100 µm in insets; in **K-N**: 50 µm.

Figure 8. Immunohistochemical detection of the produced factors 7 days after transplantation.

A. NT-4/5 expression pattern is shown in the grafted cells. **B-D.** GDNF, IL-10 and MIP-1a are expressed by the grafted iPSCs (graft=g) and host (h) tissue. Framed areas are enlarged in **A'-D'**. **E** and **F.** Protein expression of IL-1a and TNF-alpha were detected only in the host tissue. G. Secretion pattern of the neurotrophic factors NT-4/5 and GDNF and that of the cytokines IL-10 and MIP-1a shows more than 60% of the DAPI+/GFP+ grafted cells express these factors on day 3 with a significant decrease by day 7 in the cases of NT-4/5 and IL-10. * = significant difference between 3 and 7 days data; $p<0.05$, n=5 at each time point. Scale bar in **A-D**: 200 µm; in **A'-D'** and in **E-F**: 150 µm.

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