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Treating spinal cord injury in rats with a combination of human fetal neural stem cells and hydrogels modified with serotonin

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Currently, there is no effective strategy for the treatment of spinal cord injury (SCI). A combination of biomaterials and stem cell therapy seems to be a promising approach to increase regenerative potential after SCI. We evaluated the use of a cell-polymer construct based on a combination of the conditionally immortalized spinal progenitor cell line SPC-01_GFP3, derived from human fetal spinal cord tissue, with a serotonin-modified poly(2-hydroxyethyl methacrylate) hydrogel (pHEMA-5HT). We compared the effect of treatment with a pHEMA-5HT hydrogel seeded with SPC-01_GFP3 cells, treatment with a pHEMA-5HT only and no treatment on functional outcome and tissue reconstruction in hemisected rats. Prior to transplantation the cell-polymer construct displayed a high potential to support the growth, proliferation and differentiation of SPC-01 cells *in vitro.* One month after surgery, combined hydrogel-cell treatment reduced astrogliosis and tissue atrophy and increased axonal and blood vessel ingrowth into the implant; however, two months later only the ingrowth of blood vessels remained increased. SPC-01_GFP3 cells survived well *in vivo* and expressed advanced markers of neuronal differentiation. However, a majority of the transplanted cells migrated out of the lesion and only rarely remained in the hydrogel. No differences among the groups in motor or sensory recovery were observed. Despite the support of the hydrogel as a cell carrier *in vitro*, and good results *in vivo* one month postsurgery, there was only a small effect on long term recovery, mainly due to the limited ability of the hydrogels to support the *in vivo* growth and differentiation of cells within the implant. Further modifications will be necessary to achieve stable long term improvement in functional outcome.

Key words: **s**pinal cord injury, hemisection, neural stem cells, SPC-01, hydrogel, serotonin

INTRODUCTION

Spinal cord injury (SCI) is one of the most common traumatic injuries with lifelong consequences. No successful treatment has yet been developed. Current surgical techniques and pharmacotherapy help to stabilize the environment but are unable to restore lost function and only minimally alleviate on-going side effects (Fehlings et al. 1976, Pointlart et al. 2000, Kwon et al. 2004). After the primary insult, nonselective massive cell death and on-going secondary processes render the microenvironment unsuitable for endogenous regeneration (Fitch and Silver 2008, Oyinbo 2011)

One of the promising approaches to treatment is the application of stem cell therapy due to the "rescue" and possible "replacement" effect of such cells in the lesion environment (Nandoe Tewarie et al. 2009, Ruff et al. 2012). Currently, among the many stem cell types used in spinal cord injury research, neural stem/ progenitor cells appear to display the ability to maintain a potential for both the rescue and the replacement effects.

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Moreover, several publications have already proven the effect of transplanted neural stem cells (NSCs) on functional outcome in different types of animal models (Cummings et al. 2005, Karimi-Abdolrezaee et al. 2006, Hooshmand et al. 2009, Nandoe Tewarie et al. 2009). In the search for a stable and less ethically controversial source of neural stem cells, advances in molecular biology now enable the reprogramming of stem cells and the derivation of genetically modified neural precursor cell lines. Conditionally immortalized cell lines can be easily expanded *in vitro*, and after transplantation, these cells survive and differentiate under *in vivo* conditions better than primary cultures of NSCs. One of the possibilities is to establish a conditioned cell line by the insertion of the *c-myc* gene-regulated hormone response element responding to the presence of 4-hydroxy tamoxifen (cMycER^{TAM} technology) (Pollock et al. 2006). This technology has helped to obtain several different fetal neural lines derived from the human striatum, cortex and spinal cord (Jung et al. 2010). One of these cortical cell lines, CTX0E03, has proven its effectiveness and is currently being evaluated as a treatment for stroke in a phase I clinical trial (Pollock et al. 2006, http://www.reneuron.com/the-pisces-clinical-trial-in-disabled-stroke-patients). Based on these results, three cell lines of fetal NSCs derived from fetal human spinal cord – SPC-01, SPC-04 and SPC-06 – were described, and their properties in regards to their potential use in cell therapy for SCI were examined. One of these cell lines, SPC-01, is further explored in the present study. In our previous work, the SPC-01 line was described as a ventral-specific subtype precursor able to create NKX6.1^{+}/LHX3^{+}/CHX10^{+} V2a interneurons and Isl1^{+}/ChAT^{+} motoneurons under specified condition *in vitro*. SPC-01 cells robustly survived in a spinal cord injury environment for a 4 month period and differentiated into relevant neuronal subtypes, including ChAT^{+} motoneurons *in vivo* (Price and Cocks 2011, Cocks – unpublished results). Grafted cells in the lesion produced, and stimulated the production of, several trophic factor and thus supported recovery not only by protecting the remaining tissue, but also by facilitating the sprouting of endogenous GAP43^{+} axons, which led to a significant improvement of locomotor and sensory function recovery (Amemori et al. 2011, Amemori et al. – unpublished results) as early as 2 months after transplantation.

In recent years it has become evident that combination strategies will play an important role in SCI treatment and that a multimodal approach will have a better chance to lead to functional improvement and tissue regeneration, particularly in chronic lesions where posttraumatic cavities are fully developed. When used in combination with biocompatible hydrogels and/or trophic factors, transplanted stem cells have a better chance to survive in the hostile lesion environment (Blesch et al. 2002, Fouad et al. 2005, Sykova et al. 2006). The hydrogel surface can be modified to increase the specific ingrowth of neural elements and facilitate stem cell differentiation (Lieb et al. 2005, Aizawa et al. 2008, Hejcl et al. 2009, Kubinova et al. 2009, 2010, Buzanska et al. 2009, Zychowicz et al. 2011). For hydrogel surface modifications, multifunctional molecules, such as the neurotransmitter serotonin, which can support the attachment of implanted or endogenous cells and positively influence and facilitate neuronal differentiation, can be used (Khurshid et al. 2010, Platel et al. 2010, Peng et al. 2012).

In our study we assessed the effect of treating a spinal cord hemisection in rats with a combination of the conditionally immortalized human neural stem cell line SPC-01, derived from fetal spinal cord, and a poly(2-hydroxyethyl methacrylate) (pHEMA) hydrogel modified with serotonin molecules (pHEMA-5-HT). We were particularly looking at the implant's ability to serve as a cell carrier and 3D matrix for *in vitro* growth and differentiation and the effect of implantation on tissue reconstruction and sensory-motoric recovery after SCI.

METHODS

Chemicals for hydrogel synthesis

2-Aminopropyl methacrylamide hydrochloride was purchased from PolysciencesLtd. (Warrington, PA, USA). All other chemicals were purchased from Sigma-Aldrich Ltd. (Prague, Czech Republic).

Hydrogel synthesis

The porous hydrogels were synthesized by the radical polymerization of 2-hydroxyethyl methacrylate (835 mg, 6.42 mmol), [2-(methacryloyloxy)ethyl](trimethyl)ammonium chloride (57 mg, 0.274 mmol), ethylene glycol dimethacrylate (10 mg, 0.050 mmol) and 6 mg (0.033 mmol) of 2-aminopropyl methacrylamide

hydrochloride in poly(ethylene glycol) (M_w=400, 1.900 g) using azobis(isobutyronitrile) (4 mg) as an initiator and sodium chloride (5.01 g) as a porogen. The polymerization was carried out at 70°C in a sealed steel tube for 16 h and the gel 1 was then washed with water (3 × 24 h). All of the following reactions (see Fig. 1) were carried out at room temperature. Gel 1, containing protonated primary amino groups, was then converted to free base 2 and swollen by immersion into a solution of ethyldiisopropylamine (1 wt. % in methanol, 24 h) and washed with methanol (2 × 24 h). The free base-containing gel 2 was reacted with poly (ethylene glycol) diglycidyl ether (M_w=526, 10 wt. % in methanol, 48 h), and the resulting epoxide-containing gel 3 was washed with methanol (2 × 12 h). The epoxides were then reacted with a solution of serotonin hydrogen oxalate (200 mg) and triethylamine (2.00 g) in methanol (200 mL) for 3 days. The resulting serotonin-containing gel 4 was then washed with methanol (3 × 24 h) and phosphate buffered saline, pH 7.4 (PBS, 14 × 24 h). The gel was stored in phosphate-buffered saline (PBS) under argon at +5°C.

Hydrogel seeding with the SPC-01 cell line

The SPC-01 line was derived from 8-week-old fetal spinal cord tissue and conditionally immortalised with the 4-hydroxy tamoxifen (4OHT) inducible cMyc (cMycER^{TAM}) using an MMLV type of retrovirus. The product of the cMycER^{TAM} gene in the presence of 4OHT stimulates cell proliferation. In the absence of 4OHT, cell growth arrests, and the cells differentiate into neurons and astrocytes (Price and Cocks 2011, Cocks – unpublished results, Pollock et al. 2006). The SPC-01 cells were cultured in DMEM/F12 supplemented with 3% human albumin, 50 mg/ml apotransferine, 8.1 mg/ml putrescune DiHCL, 10 mg/ml human recombinant insulin, 20 µg/ml progesterone, 200 mM L-glutamine, 20 µg/ml sodium selenit, 10 µg/ml hrFGF, 10 µg/ml hrEGF, 45 penicillin/streptomycin at 50 U/ml (GIBCO) and 1 mM 4OHT as an anti-apoptotic factor. SPC-01 cells were seeded on the HEMA hydrogels. The gels with the cells were cultured for 28 days. The medium was changed every two days, and every seven

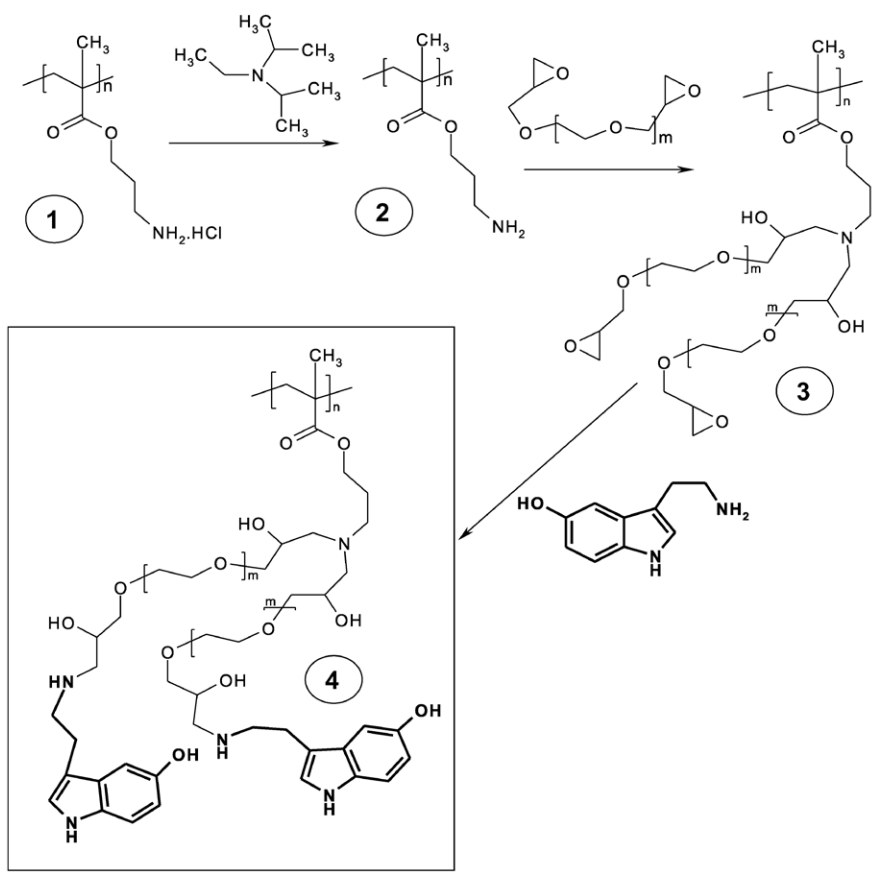

Fig. 1. Synthesis of the serotonin-containing hydrogel

days several samples of the gels with cells were chosen for immunohistochemical analysis. After 28 days the gel implants were used for implantation. In some of the *in vitro* and in *in vivo* experiments, SPC-01_GFP3 cells (transfected with a GFP construct using a lentiviral vector) were used.

Hydrogel implantation

Two-month-old male Wistar rats (*n*=27) (Velaz, Prague, Czech Republic) with body weight between 300–330 g underwent a hemisection at the Th8 level. The animals were separated into three groups: A group (*n*=11) treated with a pHEMA-5HT hydrogel seeded with SPC-01 cells and two control groups bridged with a pHEMA-5HT hydrogel alone (*n*=7) or only hemisected, then left untreated (*n*=9). The animals were injected intraperitoneally with pentobarbital for anaesthesia (solution of 1 g/100 ml, 6 ml/1 kg of animal weight), Antibiotics (gentamicin 0.05 ml i.m.), and Atropine (0.2 ml s.c. 1:5) to prevent salivation during the surgery. The surgery was carried out using an operating microscope (Zeiss) at 15–25× under aseptic conditions. A linear 1–2 cm skin incision at the Th8-9 level was performed. The paravertebral muscles were detached from the lamina, and a laminectomy at Th8 was performed. Then, a 2 mm length of the dura mater was separated from the midline to the right edge of the spine, followed by a lateral hemisection. During the hemisection procedure, a cavity 1 mm^3 in size was created. The hydrogels with /without SPC-01 cells were properly adjusted to fill the cavity volume in order to avoid mechanical stress and prevent cavity formation. The dura was sutured with Dafilon 10/0 thread (BBraun, Aesculap, Tuttlingen, Germany). From the muscles to the skin the layers were sutured separately (Hejcl et al. 2009). Following the hemisection procedure, cyclosporin A (10 mg/kg, Sandimmun, Novartis) and MPSS (1.7 mg/kg, solu-medrol, Pfizer) were injected once a day; after one month, the frequency decreased to once every two days. To prevent infection, the animals were housed in pairs with food and water *ad libitum* in a system of internally ventilated cages (IVC, Tecniplast). This study was performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) regarding the use of animals in research and was approved by the Central Commission for Animal Protection of the Academy of Sciences of the Czech Republic in Prague.

Behavioral assessment

The behavioral part of the experiment included a 12 week period postinjury. The animals were tested by two blind observers 5 days before the injury in order to obtain presurgical control data and then once a week starting 7 days after surgery. Hind limb performance was evaluated using the Basso–Beattie–Bresnahan (BBB) open-field locomotor test (Basso et al. 1995). Sensory sensitivity (Thermal nociception) was examined by measuring the latency of hind limb withdrawal from a thermal stimulus using a plantar test device (Ugo Basile, Italy) as described previously (Urdziková et al. 2006.)

Immunocytochemistry

Cells seeded on hydrogel cubes were washed in PBS (pH 7.2) and fixed with 4% paraformaldehyde in PBS for 30 minutes. Prior to immunostaining, the fixed cells were twice washed in PBS. Permeabilisation and blocking were carried out in a blocking buffer consisting of 0.1% Triton (Chemicon 2170-S), 5% goat serum, and 1mg/ml bovine serum albumin in TRISS buffer for 20 min. To identify SPC-01 cells, antibodies directed against neuronal-cell adhesive molecule (N-CAM Chemicon AB5032), neurofilaments 70 (Chemicon MAB1615), nestin (Chemicon MAB5320), synaptophysin (Chemicon MAB5258), myelin/oligodendrocyte-specific protein (MOSP Millipore MAB328), CNPase (Abcam ab109758) and βIII-tubulin (S-SigmaT-8660) were used. To visualize primary antibody reactivity, appropriate secondary antibodies were used: goat anti-mouse IgG conjugated with Alexa-Fluor 488 or 594 (molecular probes, Invitrogen A11029, A11032). The samples were washed three times with PBS and mounted with vectashield (Vector H-1000) on a glass slide.

Immunohistochemistry

Rats were sacrificed one or three months after surgery. Anesthetized animals were perfused with 4% paraformaldehyde in 0.1M PBS (pH 7.4). After perfusion, a 3 cm piece of the spinal cord containing the center of the lesion was removed from the body, and the spinal cord, along with the bone, was left in 4% paraformaldehyde in 0.1 M phosphate buffer overnight. Then, the spinal cord was removed from the bone and post-fixed in the same fixative for 1 week. The fixed spines were immersed in

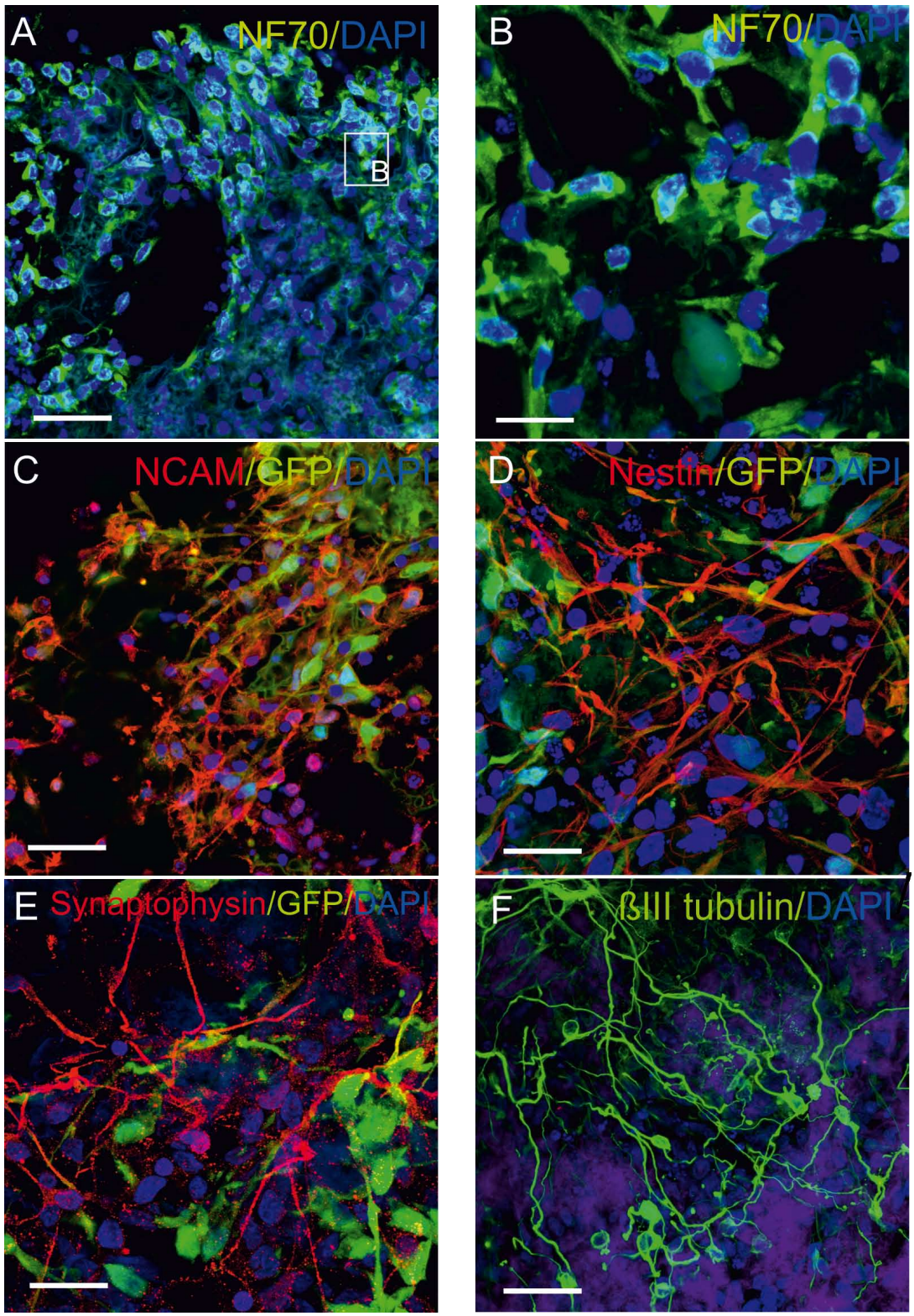

Fig. 2. SPC-01 human fetal neural stem cells twenty-eight days after seeding on a pHEMA-5HT hydrogel. The HEMA hydrogel, containing serotonin molecules, was grown through to confluence by the SPC-01 cells. The cells showed positivity for the neuroectodermal markers neurofilaments 70 (A) – higher magnification (B), neuronal-cell adhesive molecule (C), nestin (D), synaptophysin (E) and βIII-tubulin (F). Scale bar is 40 μm (A, C, D, E, F) and 20 μm (B).

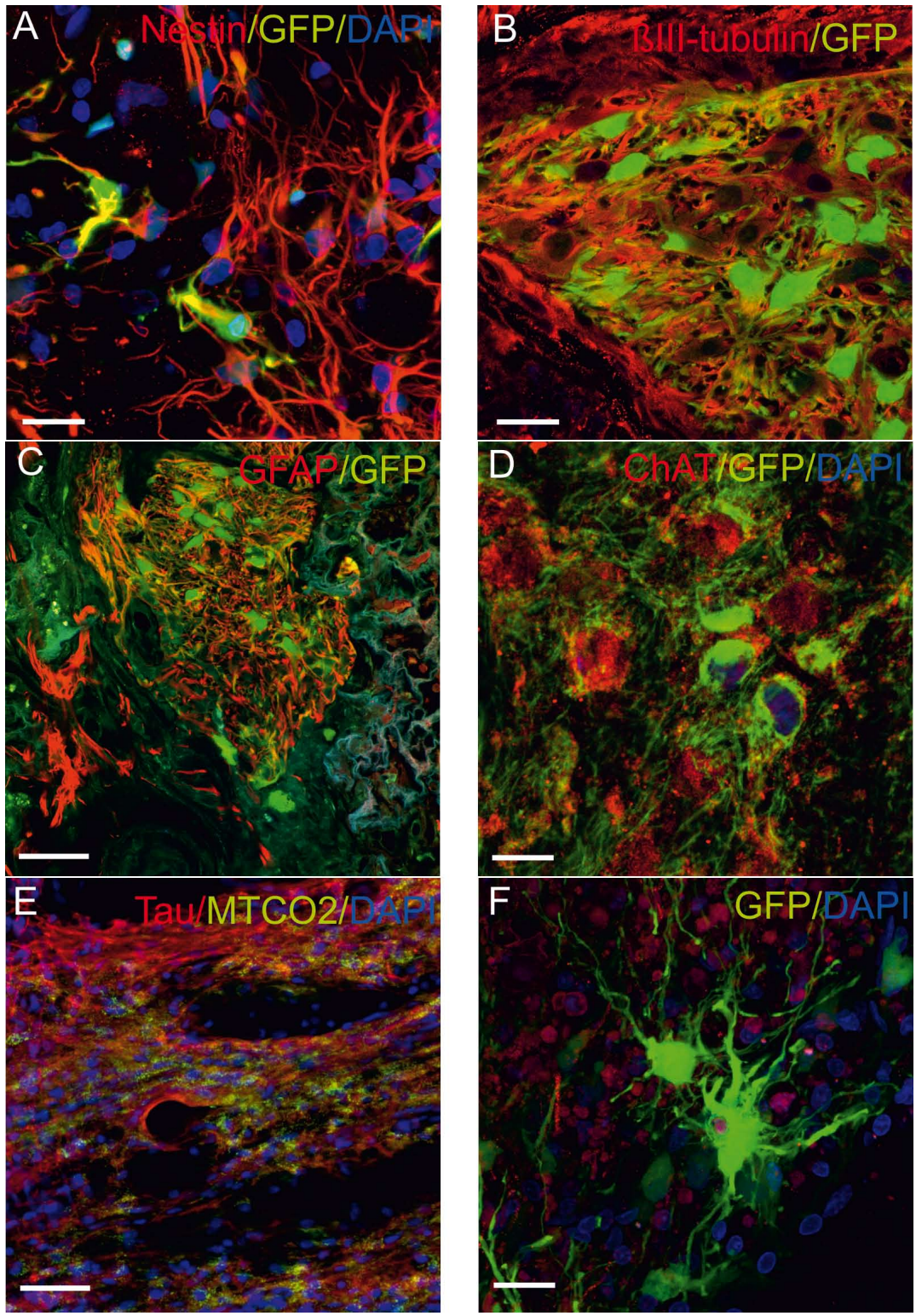

Fig. 3. SPC-01_GFP3 cells seeded on a pHEMA-5HT hydrogel one month after implantation into a spinal cord hemisection cavity. The cells survived well, mostly at the hydrogel-tissue border and in the peripheral part of the implanted hydrogel. The implanted cells were positive for the neuronal markers nestin (A) and βIII-tubulin (B) as well as for GFAP (C) ChAT (D) and Tau (E). The implanted cells also showed an astrocyte- like morphology (F). Scale bar is 50 µm (C, E), 20 µm (A, B), and 10 µm (D, F).

PBS with 30% sucrose. Frozen spinal sections (40 µm) were cut through the area of interest. To identify SPC-01_GFP3 cells in the spinal cord, antibodies directed against βIII-tubulin (S-Sigma T-8660), glial fibrillary acidic protein (GFAP- Cy3 Sigma C-9205), choline acetyltransferase (ChAT, ab68779 Abcam), mitochondrially encoded cytochrome c oxidase II (MTC02, ab109739 Abcam), nestin (Chemicon MAB5320) and Tau (A0024 Dako Cytomation) were used. To assess the interaction between the implant and the surrounding lesioned tissue, hematoxylin-eosin staining and antibodies directed against GFAP (GFAP- Cy3 Sigma C-9205), neurofilaments 160 (Sigma N5264) and the endothelial cell marker RECA1 (abcam ab9774) were used. To visualize primary antibody reactivity, appropriate secondary antibodies were used: goat anti-mouse IgG conjugated with Alexa-Fluor 488 or 594 and goat anti-rabbit IgG conjugated with Alexa-Fluor 594 (molecular probes, Invitrogen, A11029, A11032, and A11012). Since all of the antibodies cross-react with rat proteins and since the cells can lose GFP during their differentiation, for colocalization studies we used not only GFP, but also human-specific markers such as MTC02, which does not cross-react with rat cells. The samples were washed three times with PBS and mounted with vectashield (Vector H-1000) on a glass slide

Fluorescence and confocal microscopy

The samples were examined using a spectral confocal microscope (LSM 5 DUO, Zeiss) equipped with an Ar/HeNe laser or a ZEISS AXIO Observer D1 microscope (Carl Zeiss, Germany). For confocal microscopy, 405 nm (DAPI), 560 nm (gamAF594), and 488 nm (Ar, gamAF488) lasers were used to visualize the neural markers expressed by differentiated SPC-01_GFP3 cells *in vitro* and *in vivo*. The Observer D1 microscope was used to visualize the effects of the cell and hydrogel implant on the microstructural reconstruction of the surrounding damaged tissue and the implant's *in vivo* ability to support host tissue ingrowth.

Quantification of microstructural changes

For evaluating spinal tissue atrophy, the surface areas of the tissue on hematoxylin-eosin slices were

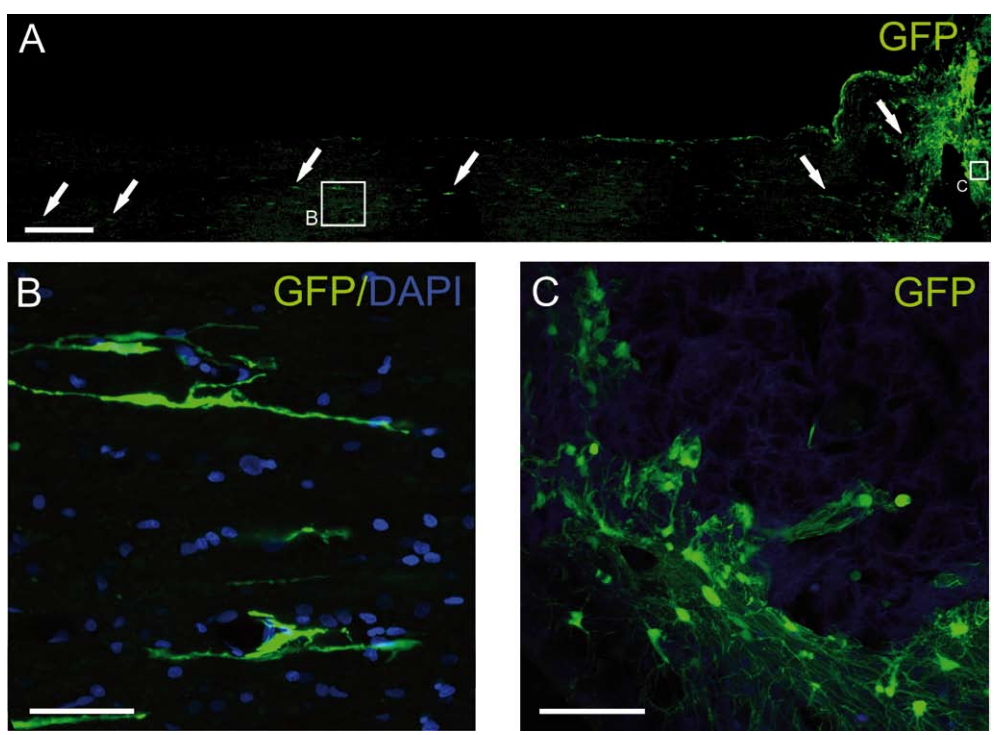

Fig. 4. Implanted SPC-01_GFP3 cells seeded on a pHEMA-5HT hydrogel in a spinal cord hemisection cavity three months after implantation (A). The majority of the implanted cells migrated out of the hydrogel (B). Other cells remained along the hydrogel-tissue border, leaving the center of the hydrogel only sparsely populated (C). Scale bar is 300 µm (A), 100 µm (C), and 50 µm (B).

averaged using image J software; the analysis was performed at 25× magnification.

To evaluate the degree of astrogliosis in the tissue surrounding the hydrogel, an analysis based on the size of the area displaying high fluorescence intensity for GFAP positivity was made at 200× magnification. Five specific locations surrounding the main lesion cavity were measured to determine the extent of GFAP positivity. The measurements were carried out on every 6th slice from the spinal cord of each treated and control animal at the terminal point of the experiment.

To evaluate the ingrowth of axons into the hydrogel and hydrogel revascularization, an analysis based on the size of the area displaying high fluorescence intensity for NF160 or RECA positivity was made at 200× magnification. Three specific locations inside the hydrogel were measured to determine the extent of NF160 or RECA positivity. The measurements were made on every 6th slice from the spinal cord of each treated and control animal at the endpoint of the experiment. All of the fluorescent analyses were carried out using Axiovision 4.8 software (Zeiss).

Statistical evaluation

In the immunohistochemical analysis, the *F*-test was used to determine variability within the groups. For analyzing differences in the histological data among the groups and to compare two different time points in the histological data, Student's *t*-test was used [one side, two choices, for the same or different data variability (regarding the *F*-test results)]. In order to distinguish the effect of treatment on behavioral recovery, one way ANOVA was used at each time

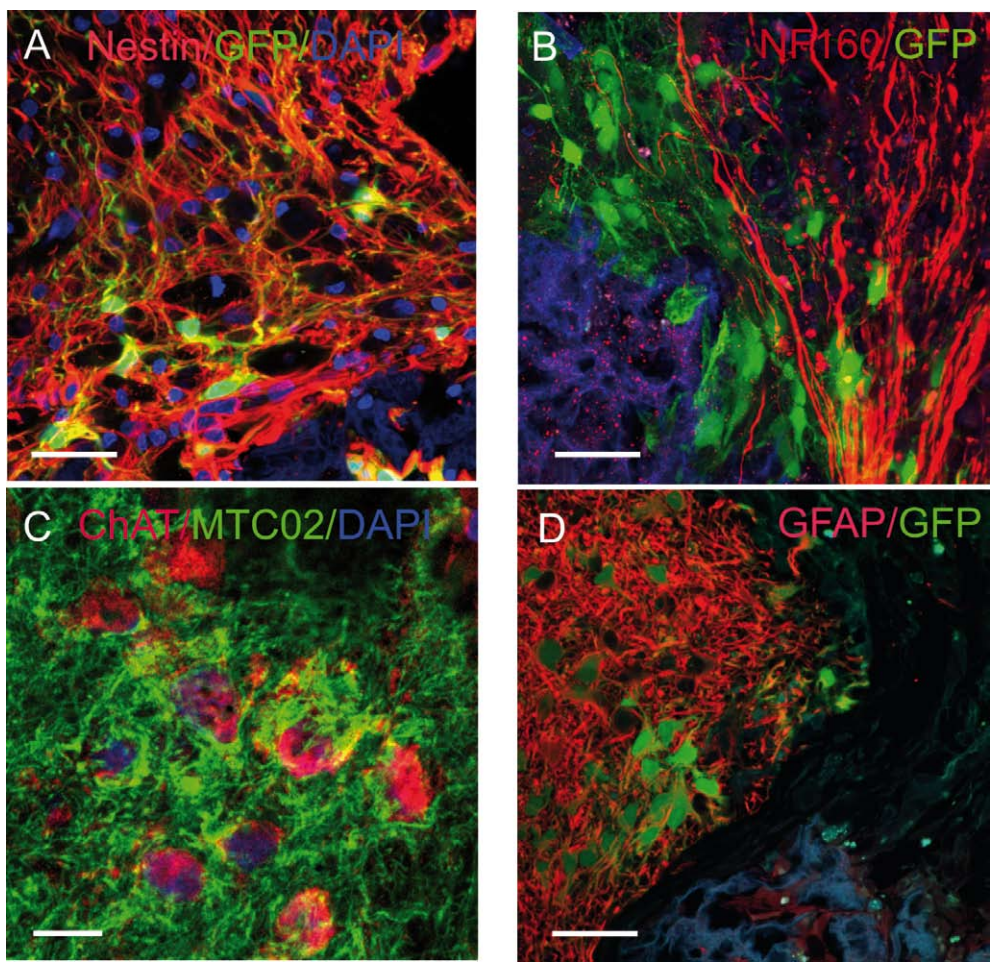

Fig. 5. Differentiation of SPC-01_GPF3 cells and their interaction with endogenous neural cells three months after the implantation of a cell-polymer construct. The implanted cells that remained in the vicinity of the hydrogel-tissue border were positive for nestin (A) and ChAT (C). They interacted with host NF160-positive axons (B) and integrated into the GFAP-positive scar (D). Scale bar is 40 μm (A,B), 10 μm (C), and 80 μm (D).

point. The standard error of the mean was calculated in order to better assess the data variability.

RESULTS

SPC-01 culture

Prior to transplantation, we evaluated the ability of SPC-01 cells to grow and differentiate on pHEMA-5HT hydrogels *in vitro*. After 28 days of culturing, the populations of SPC-01 as well as SPC-01_GFP3 cells on the pHEMA-5HT hydrogels were nearly confluent. Despite the presence of 4OHT, both cell lines differentiated into a neural phenotype. The cells were positive for several markers of neural differentiation, including neurofilaments 70 (Fig. 2A, in detail B), neuronal-cell adhesive molecule (Fig. 2C), nestin (Fig. 2D), synaptophysin (Fig. 2E) and βIII-tubulin (Fig. 2F). No CNPase- or MOSP-positive cells were detected. These results show that the pHEMA-5HT hydrogel can support the expansion and growth of spinal progenitor cells in a 3D artificial environment. Serotonin molecules can facilitate the neural differentiation of these cells even in the presence of 4OHT, which under standard culture conditions maintains SPC-01 cells in a proliferative and immature state.

SPC-01 proliferation and differentiation *in vivo*

One month after the implantation of the cell-polymer construct, the SPC-01_GFP3 cells survived very well in the lesion area. The majority of them remained in the peripheral part of the hydrogel and at the hydro-

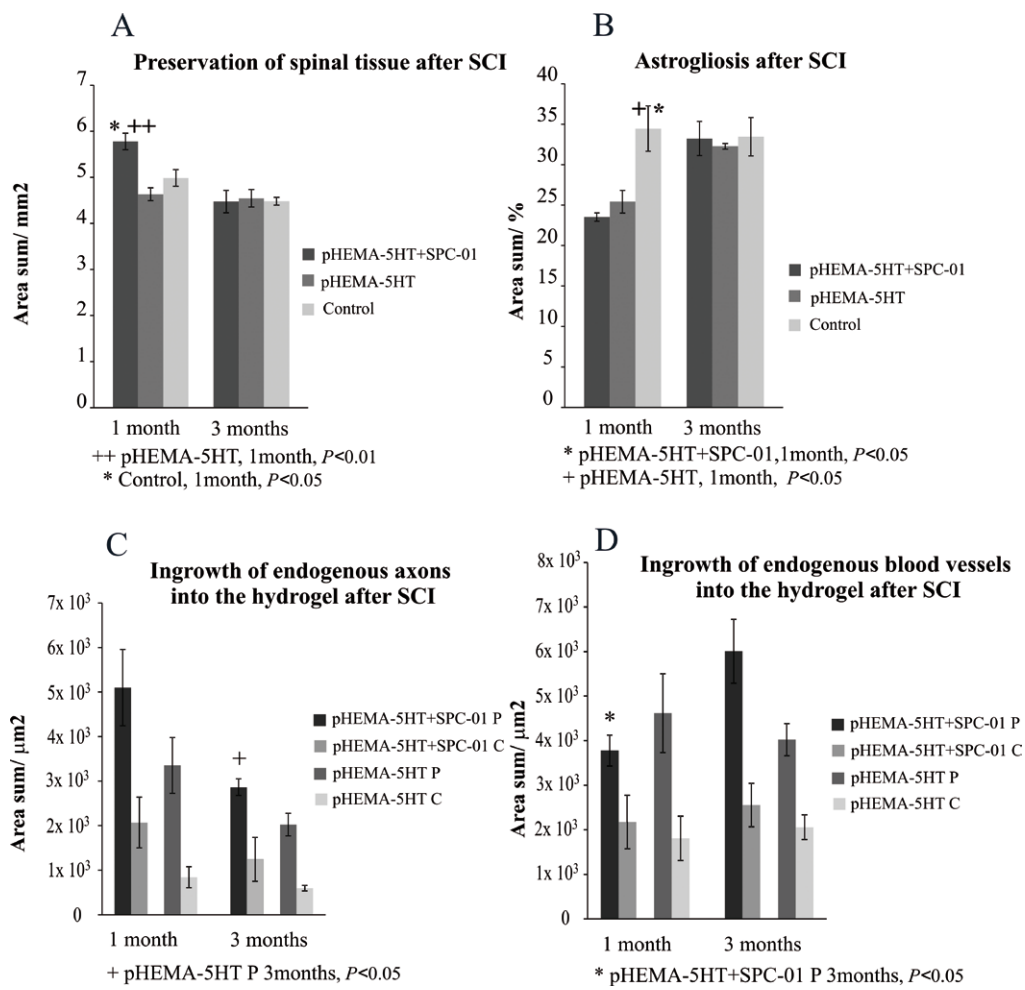

Fig. 6. Quantification of the effect of treatment on the host tissue. The evaluation of the spared spinal tissue (A) and the extent of astrogliosis (B) in lesioned animals and in animals treated with combined therapy or hydrogel only, one and three month after surgery. The ingrowth of host axons (C) and blood vessels (D) into the hydrogel or cell-polymer implant one and three months after injury. The peripheral (P) and central (C) parts of the implant were evaluated separately.

gel tissue border; a few cells were dispersed at a distance from the lesion area. The implanted cells were nestin-positive (Fig. 3A); however, they expressed several markers of advanced neural differentiation including βIII-tubulin (Fig. 3B), GFAP (Fig. 3C), ChAT (Fig. 3D) and Tau (Fig. 3E). Some of the grafted SPC-01 cells showed an astrocyte-like morphology (Fig. 3F).

Three months after the implantation of the cell-polymer construct, the grafted cells still survived well in the tissue (Fig. 4A); however, the majority of them migrated into the spinal cord stump, some of them as far as 4 mm rostrally from the hydrogel-tissue border (Fig. 4A, B). We did not observe such long distance migration towards the caudal end of the spinal cord. Other cells remain in the hydrogel-tissue border leaving the center of the hydrogel populated only sparsely (Fig. 4A, C). The implanted cells were losing their GFP expression, therefore we had to detect the cells also by specific staining for human mitochondria (MTC02). The implanted cells were still positive for nestin (Fig. 5A), while other cells were positive for NF 160 (Fig. 5B), ChAT (Fig. 5C) and GFAP (Fig. 5D). The majority of the implanted cells showed interconnectivity with endogenous tissue elements in the spinal cord tissue, and some of them remained primarily in clusters on the edge of the hydrogel. GFAP-positive cells formed part of the glial scar. We did not observe any colocalization of GFP or MTC02 with oligodendrocytic markers, such as CNPase or MOSP.

Spinal cord tissue atrophy

To assess the effect of treatment on protecting against tissue atrophy, the extent of spared tissue on the hydrogel border was evaluated. On haematoxylin-eosin stained slices 1 month after surgery, a difference in the area sum of the remaining spinal tissue between the cell-polymer group (5.78 ± 0.18 mm^2) and both other groups, control (4.99 ± 0.18 mm^2, $F_{3,4}$=1.47 P<0.35, t_7=2.39 P<0.05) and hydrogel-only (4.63 ± 0.14 mm^2, $F_{3,3}$=2.00 P<0.29, t_6=3.43 P<0.01), was observed (Fig. 6A). No statistical difference in the amount of remaining tissue was observed among the groups 3 months after surgery.

Glial scar

On GFAP-Cy3 stained spinal tissue, a significant decrease in the extent of astrocyte marker positivity (GFAP) was observed in both the SPC-01_GFP3-seeded pHEMA-5HT hydrogel group ($F_{3,3}$=3.80 P<0.15, t_6=2.07 P<0.05) and the pHEMA-5HT hydrogel group ($F_{3,3}$=28.61 P<0.01, t_3=2.76 P<0.05) compared to the control animals (Fig. 6B) one month after surgery. No differences between the animals treated with a hydrogel alone and those treated with a hydrogel seeded with cells were detected. No statistically significant difference in astrocyte remodeling of the glial scar was observed among the groups 3 months after surgery.

Ingrowth of endogenous tissue elements into the hydrogel

We evaluated the implant's suitability for endogenous axon and blood vessel ingrowth. The hydrogel was partially populated by connective tissue elements. In hydrogels in which clusters of SPC-01_GFP3 cells were present at the border of the hydrogel, an insignificant trend towards an increase of axonal ingrowth into the peripheral and central zones of the polymer was detected one month after surgery. A significant long term effect of SPC-01_GFP3 cells on axonal infiltration was observed two months later (seeded vs. un-seeded hydrogel, $F_{3,4}$=2.40 P<0.21, t_7=1.99 P<0.05) (Fig. 6C). A long term effect of SPC-01_GFP3 cells was observed on the vascularization of the peripheral parts of the hydrogel. We observed a gradual increase in blood vessel infiltration (SPC-01 cell-seeded pHEMA-5HT hydrogel 1 m. vs. 3 m. $F_{4,3}$=4.82 P<0.11, t_7=2.15 P<0.05) (Fig. 6D), and at the end of the experiment a strong trend towards an increased attraction of blood vessels into the hydrogel was observed in the combined therapy group ($F_{4,3}$=3.30 P<0.18, t_7=1.86 P<0.06).

Behavioral testing

No significant differences in the average hindlimb locomotor score or in the average latency of hindlimb withdrawal were observed among any groups over a twelve week period.

DISCUSSION

In the present study we used a 01 clone of human fetal neural stem cells (SPC-01) seeded on poly hydroxyethyl methacrylate-based hydrogels modified with serotonin molecules to treat a lateral hemisection of the spinal cord in adult rats. We demonstrated the synergic effect of serotonin-modified pHEMA hydro-

gels on the growth and differentiation of neural stem cells *in vitro* in the presence of 4OHT and the paracrine effect of the implanted cells on the protection of the remaining tissue and the ingrowth of host axons and blood vessels. The polymer itself reduced astrogliosis one month after injury. The SPC-01 cells survived well, proliferated and differentiated into relevant neuronal subtypes *in vivo*. However, we did not observe any long term effect that would lead to tissue reconstruction and the improvement of locomotor and sensory function.

The initial aim of utilizing a pHEMA hydrogel modified on its surface with serotonin molecules was to support the attachment of seeded SPC-01 neural precursors and to accelerate their differentiation process, since serotonin is considered to be a positive regulator of adult neurogenesis in the subventricular zone (Platel et al. 2010) and inhibiting serotonin uptake can promote neurogenesis and protect the viability of neural stem cells (Peng et al. 2012). In our work the serotonin molecules were covalently bound on the hydrogel with only a slight chance of being released into the injured spinal cord.

After 28 days of *in vitro* co-cultivation, the modified pHEMA hydrogels were filled with SPC-01 neural precursors that, due to the presence of the serotonin molecules on the hydrogel surface, started to differentiate despite the presence of 4OHT and the absence of additional differentiating factors in the medium. The cells were positive for several markers of neural differentiation, attached well to the surface of the hydrogel and grew through it within 4 weeks. We performed *in vitro* experiments with SPC-01 cells as well as with SPC-01_GFP3 cells with no difference in cell behavior. The pHEMA-5HT hydrogel appeared to be an ideal material for further *in vivo* study and an excellent 3D cell carrier for transfer into the injured tissue.

The differentiation of implanted SPC-01_GFP3 cells continued *in vivo*, and one month after implantation the cells were positive for relevant markers of neuronal differentiation, including those for neurons (Tau) and motoneurons (ChAT), or for GFAP, a marker of astrocytes. A portion of these cells displayed an astrocyte- like phenotype morphology and showed interconnectivity with endogenous tissue elements. At the end of the experiment the cells remained positive for the same markers (Nestin, GFAP, NF160 or ChAT), but they demonstrated a more mature phenotype. In our previous work we have shown that SPC-01 cells, when transplanted into a balloon-induced spinal cord compression lesion, differentiated and matured very slowly. Two months after grafting they were positive only for nestin and GFAP, and only 4 months after grafting did they express Islet2, Tau, and Chat, more mature neuronal markers. In none of our studies did we observe the expression of oligodendrocytic markers. This might be due to the original p2 and pMN sub-domains from which the SPC-01 cell line was derived, since these sub-domains give rise to two main lineages of interneurons, V2a and V2b, as well as to motoneurons during development (Price and Cocks 2011). In the present study the implanted cells were more mature already one month after surgery, pointing out the effect of the pHEMA-5HT hydrogel as a material accelerating their differentiation. Our previous work had also shown that SPC-01 cells were able to spare the white matter and prevent tissue atrophy due to the expression of neurotrophins while stimulating the production of host neurotrophic factors as a result of their robust survival (17% of grafted cells) in the center of the lesion (Amemori et al. 2011, Amemori et al. – unpublished results). It is well known that the production of NGF, NT3 and BDNF by either implanted or by endogenous cells is crucial for neuronal cell survival, proliferation and tissue reconstruction after SCI (Jakeman et al. 1998, Jones et al. 2001, Blesh et al. 2002). In our experiments, one month after implantation a supportive effect of SPC-01 cells in protecting the remaining tissue was found in the combined therapy group. On the other hand, the presence of the hydrogel itself decreased the extent of astrogliosis one month after injury. A similar effect on glial scar reduction was observed in the case of pHPMA-based hydrogels (Woerly et al. 2004). This effect was not seen 3 months after injury, possibly due to the fact that the SPC-01_GFP3 cells migrated out of the hydrogel and differentiated into GFAP-positive astrocytes, therefore contributing to the GFAP positivity found using immunohistochemistry.

The implanted hydrogels were partially penetrated by endogenous axons and capillaries, and the SPC-01 cells increased the ingrowth of host axons into the implant. All of these results were apparent one month after surgery, i.e. at the time when the majority of the transplanted cells were still present in the center of lesion and along the hydrogel-tissue border. The efficacy of the serotonin molecule as an attractant proved to be sufficient for new tissue ingrowth and the differentiation of the implanted cells. However, for long term cell attachment and survival in the hydrogel, further modification of the hydrogel surface, such as the

addition of primary AA sequences of extracellular matrix proteins attached to the biomaterial, might be beneficial (Lieb et al. 2005). The decreased effectiveness of serotonin from a long-term perspective may also be caused by the eventual enzymatic degradation of serotonin bound to the hydrogel surface, which may require the use of synthetic serotonin analogs-agonists that are resistant to enzymatic degradation.

During the time of the experiment, over a period of 3 months, the majority of the implanted SPC-01 cells migrated up to 4 mm from the center of the lesion, while only a minority remained in the peripheral part of the hydrogel and along the hydrogel-tissue border. This fact prevents the prolongation of the initial effect of paracrine stimulation and thus the functional reconstruction of the injured tissue. Despite the fact that at the end of the experiment the hydrogel material was still supporting the ingrowth of endogenous tissue elements, and despite the synergic effect of SPC-01-derived neural precursors on attracting endogenous blood vessels and axons into the seeded hydrogel, no further reduction in tissue atrophy or astrogliosis was observed.

The possible reasons why the treatment did not affect the behavioral recovery are more complex. The major reason, already described above, is that the initial paracrine effect became less effective due to the inability of the pHEMA-5HT hydrogel to support the proliferation of the implanted cells and their accumulation in the central part of the hydrogel. Those cells dispersed along the spinal cord and thus could not contribute to the cumulative effect described in our previous work (Amemori et al. 2011, Amemori et al. – unpublished results). Further, the hemisection model of SCI is not the ideal model for the assessment of functional outcome due to large intra-group variability, which can be caused by variability in the number of spared axons along the incision line in the central part of the spinal cord lesion.

Most *in vivo* studies have focused on the initial acute and sub-acute intervals following SCI, up to one month survival, and thus describe mostly the paracrine effect of the treatment without analyzing its effect on long term reconstruction in chronic injury (Bakshi et al. 2004, Piantino et al. 2006, Austin et al. 2012, Moradi et al. 2012, Niapour et al. 2012). In our experiment, a significant effect of the treatment during the acute and subacute phases was found as well. However, instead of a long-term effect on tissue reconstruction within the implant leading to behavioral recovery, we observed vanishing effects as well as the prevalence of typical endogenous repair processes, such as glial scar formation and tissue atrophy. It is therefore obvious that for assessments of spinal cord regeneration, long term experiments are necessary.

CONCLUSIONS

We have shown that serotonin-modified pHEMA hydrogels can serve as a supportive environment to stimulate and accelerate the differentiation of implanted SPC-01_GFP3 cells both *in vitro* and *in vivo*. SPC-01_GFP3 cells seeded on a pHEMA-5HT hydrogel migrated out from the polymer center and survived well at the cell-hydrogel border as well as in the spinal cord tissue. The treatment resulted in an initial reduction of tissue atrophy and glial scar formation, probably due to a paracrine effect, and long term support of the infiltration of the implanted material by host blood vessels and axons. However, the hydrogel did not provide ideal long term support for the continued growth and differentiation of the cells within the implant, most probably due to the aggressive spinal cord injury environment. Further surface modifications will be necessary to achieve long term tissue reconstruction after spinal cord injury.

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