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Olfactory ensheathing cells genetically modified to secrete GDNF to promote spinal cord repair

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Summary

Olfactory ensheathing cell (OEC) transplantation has emerged as a very promising therapy for spinal cord repair. In this study, we tested the ability of genetically modified OECs to secrete high levels of glial cell line-derived neurotrophic factor (GDNF) to promote spinal cord repair. The GDNF gene was transduced into OECs using a retroviral-based system. The engineered OECs were first characterized by their ability to express and secrete biologically active GDNF *in vitro*. After implantation into the spinal cord of adult rats with complete spinal cord transection, OEC survival and GDNF production were examined. The locomotor functions of animals were assessed and axon regeneration was evaluated at the morphological level. To our knowledge, we report for the first time that the genetically modified OECs are capable of producing GDNF *in vivo* to significantly improve recovery after spinal cord injury (SCI). This work combined the outgrowth-promoting property of OECs with the neuroprotective effects of the additionally overexpressed neurotrophic factors and opens new avenues for the treatment of SCI.

Keywords: spinal cord injury; olfactory ensheathing cell; glial cell line-derived neurotrophic factor; transplantation; gene therapy

Abbreviations: CM = conditioned medium; CSN = corticospinal neuron; CST = corticospinal tract; DMEM = Dulbecco's modified Eagle medium; GDNF = glial cell line-derived neurotrophic factor; GDNF = glial cell line-derived neurotrophic factor; GFAP = glial fibrillary acidic protein; GFP = green fluorescent protein; HRP = horseradish peroxidase; LTR = long terminal repeat; NF = neurofilament; OECs = olfactory ensheathing cells; PBS = phosphate-buffered saline; RSN = rubrospinal neuron; SCI = spinal cord injury

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Introduction

Spinal cord injury (SCI) is one of the most devastating forms of trauma experienced by humans. Approximately half of the patients have complete cord injury with no preservation of voluntary motor or sensory function below the level of injury (Tator *et al*., 1990). Development of powerful strategies to treat SCI is still a major clinical challenge, although recent dramatic progress in cellular transplantation, gene therapy and molecular treatment has heightened the optimism about future cures for such injuries (Grill *et al*., 1997*a*, *b*; Giehl *et al*., 1997; Li *et al*., 1997; Rapalino *et al*., 1998; Jones *et al*., 2001; Blits *et al*., 2002; Schwab, 2002).

Olfactory ensheathing cells (OECs) permit growing axons from neurons of the nasal cavity olfactory mucosa to re-enter the olfactory bulb of the brain and form synapses with second-order neurons (Doucette, 1984). Recent studies have shown that implantation of rodent and human OECs appears to be one of the most promising strategies to promote long-distance regeneration in the injured spinal cord (Li *et al*., 1997; Barnett *et al*., 2000; Kato *et al*., 2000; Ramon-Cueto *et al*., 2000). Yet the number of axons that regrow and reconnect is still insufficient (Gudino-Cabrera *et al*., 2000).

Neurotrophic factors were originally identified as critical mediators of neuronal survival and nerve fibre outgrowth during development. The beneficial effects of neurotrophic factors on neuronal protection and repair in the CNS have been well documented (Tuszynski, 1999; Jones *et al*., 2001).

Although more than 30 neurotrophic factors are known, fewer than six of them have been investigated as potential treatments for lesioned spinal cords in the animal model (Schwab, 2002). Brain-derived neurotrophic factor and neurotrophin-3 have been studied extensively to find whether they have a role in promoting regeneration of spinal motor pathways (Jones *et al*., 2001; Liu *et al*., 2002; Tuszynski *et al*., 2003; Zhou *et al*., 2003). Brain-derived neurotrophic factor was reported to promote rubrospinal tract (RST) regeneration, but has limited effects on corticospinal tract (CST) regeneration (Schnell *et al*., 1994; Tetzlaff *et al*., 1994). Neurotrophin-3, which has been found to augment the growth of corticospinal axons after spinal cord injury, has been showed to promote the death of some corticospinal neurons (Giehl *et al*., 2001). More recent studies also show that removal of NT-3 or blocking TrkC activity could enhance myelination of Schwann cells, which is known to be very important for functional recovery (Chan *et al*., 2001; Cosgaya *et al*., 2002). The glial cell line-derived neurotrophic factor (GDNF), originally identified as a trophic factor for midbrain dopaminergic neurons (Lin *et al*., 1993), has been found to be the most potent trophic factor for motoneurons (Henderson *et al*., 1994; Li *et al*., 1995; Oppenheim *et al*., 1995). Fibroblasts expressing GDNF were able to directly promote axon elongation of primary cultured cortical neurons (Paratcha *et al*., 2003). In a similar culture system, cortical neurons growing on a monolayer of fibroblasts expressing brain-derived neurotrophic factor were characterized by a great number of shorter and more branching neurites, suggesting that GDNF may have a more potent effect in stimulating axonal growth in these cells (Paratcha *et al*., 2003). Furthermore, GDNF was able to induce motor axon outgrowth across the surrounding white matter in the organotypic spinal cord culture model (Ho *et al*., 2000). When applied into the spinal cord, GDNF was able to exert a trophic effect on corticospinal neurons and promote long-term survival after axotomy (Giehl *et al*., 1997). Moreover, GDNF has recently been shown to exert behavioural and anatomical neuroprotection following SCI (Watabe *et al*., 2000; Cheng *et al*., 2002). Injections and pumps can be used to deliver neurotrophic factors to the lesion site. However, these methods do not achieve long-term, localized, high-dose neurotrophic factor delivery. An alternative approach that achieves long-term and site-specific delivery of neurotrophic factors to the injured spinal cord is *ex vivo* gene therapy (Tuszynski, 1997). Schwann cells, fibroblasts and intercostal nerve grafts genetically engineered to express neurotrophic factors have been reported (Grill *et al*., 1997; Menei *et al*., 1998; Tuszynski *et al*., 1998; Blits *et al*., 1999, 2000; Liu *et al*., 1999*a*, *b*; Blesch *et al*., 2001). These studies described increased sprouting of various axonal populations but, in most cases, the majority of regenerating axons were rerouted around the transplant and few fibres were seen distal to the site of injury.

Implants of OECs may be better for neurotrophic factor delivery since this may be helpful for regenerating axons to re-enter the distal part of the spinal cord (Li *et al*., 1997; Ramon-Cueto *et al*., 2000) and is advantageous over the use of genetically engineered fibroblasts, which are non-CNS in origin and may become tumorigenic. The feasibility of transplanting genetically modified OECs into the intact and injured spinal cord was extensively described by Ruitenberg and colleagues (Ruitenberg *et al*., 2002). Therefore, upgrading of the growth-promoting properties of OEC by having them secrete additional neurotrophic factors may be a valuable strategy for promoting spinal cord repair (Blits *et al*., 2002; Ruitenberg *et al*., 2002), since limited neurotrophic factor expression of OECs has been reported (Boruch *et al*., 2001; Woodhall *et al*., 2001).

In the present study, we tested the ability of genetically modified OECs to secrete a high level of GDNF in order to promote spinal cord repair. The GDNF gene was transduced into OECs using a retroviral system. The engineered OECs were first characterized by their ability to express and secrete biologically active GDNF *in vitro*. After implantation into the spinal cord of adult rats with complete spinal cord transection, OEC survival and GDNF production were examined. The locomotor functions of animals were assessed, and axon regeneration was evaluated at the morphological level. To our knowledge, we report for the first time that the genetically modified OECs are capable of producing GDNF *in vivo* to significantly improve recovery after SCI.

Materials and methods

Primary culture and purification of OECs

Primary olfactory bulb cultures were set up from adult Sprague–Dawley rats (2.5 months old). The modified protocol of Ramon-Cueto and colleagues (Ramon-Cueto *et al*., 1998) was used. Briefly, the olfactory nerve layer was peeled away from the rest of the olfactory bulb, then dissociated with 0.25% trypsin and 0.03% collagenase and incubated at 37°C for 30 min. The cells were then washed with D/F_{12}–10% fetal bovine serum and scattered and plated on uncoated dishes twice, each for 12 h at 37°C in 5% CO_2. The cell suspension was collected and 5 ml (approximately 300 000 cells) was plated on 100 mm Petri dishes for 45 min at 37°C in 5% CO_2. These dishes were then incubated with anti-rabbit immunoglobulin G antibody, 1.5 μg polyclonal rabbit p-75 NGFR antibody (Santa Cruz Biotechnology), for 12 h at 4°C and then with phosphate-buffered saline (PBS)–5% bovine serum albumin for 4 h at room temperature. PBS was used to wash the dishes after each step. Unbound cells were washed off and the attached cells were collected using a cell scraper and then seeded into poly-L-lysine-treated 25 mm^2 flasks, and incubated with D/F_{12}–10% fetal bovine serum containing as mitogen 2 μM forskolin (Sigma) and 20 μg/ml pituitary extract (Sigma). The purity of the cultured OECs was determined by comparing the number of Hoechst-labelled nuclei with the number of p-75 NGFR immunoreactive cells under a microscope.

Retroviral preparation and infection of OECs

The cDNA encoding rat preproGDNF was isolated by using the RT-PCR method with total RNA extracted from rat brain. Specific

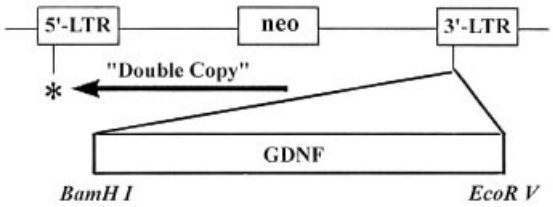

Fig. 1 Structure of double-copy retroviral vector pN2A-GDNF. The original Moloney murine leukaemia virus-based retroviral vector pN2A contains a GDNF gene in the U3 region of the 3′-LTR. 'neo' is a G418/neomycin resistant gene. 'Double copy' indicates that in the infected cell the transduced gene could be duplicated and transferred to the 5′-LTR (asterisk). Placement of the foreign gene outside the retroviral transcriptional unit, eliminating or at least reducing the negative effects of the retroviral transcriptional unit, was able to improve the expression of the foreign gene (Hantzopoulos *et al*., 1989).

primers (forward, 5′-GGAAGCTTATGAAGTTATGGGATGTCG-3′; reverse, 5′- GAGGATCCTCAGATACATCCACACC-3′) for PCR were designed to amplify preproGDNF cDNA that yielded 652 bp amplified products. Then GDNF cDNA was inserted into U3 area of the 3′ long terminal repeat (LTR) of the replication-defective recombinant retroviral pN2A (Hantzopoulos *et al*., 1989) by EcoRV and *Bam*HI enzyme sites (pN2A-GDNF). As shown in Fig. 1, the unique feature of this double-copy vector is that the transduced gene inserted in the 3′-LTR is capable of duplication and transfer to the 5′-LTR in infected cells, which can improve the expression of transduced genes. The construct was confirmed by restriction analysis and sequencing.

Lipofectamine (Gibco)-packaged pN2A-GDNF was used to transfect the packaging cell line, PA317 (Miller and Buttimore, 1986). The neomycin analogue G418 was used to isolate resistant colonies. Viral supernatants from these colonies were titred on NIH 3T3 cells as described previously (Whittemore *et al*., 1994). The cell line (PA317-GDNF2) with the highest titre (8×10^4 colony-forming units/ml) was used to infect the purified adult rat primary OECs.

The most effective method for infecting dividing OECs was a 2 h pretreatment with 8 pg/ml polybrene followed by overnight infection with the recombinant retrovirus in medium containing mitogens. The same procedure was repeated the next day, but polybrene was added for only l h. Four to five days after the last infection, the OECs were selected with 200 μg/ml G418. Once the selected population had grown in a stable manner, the cells were expanded using the same selection medium, with a maximum total number of passages of six or seven, to produce enough OECs for experiments.

Characterization of GDNF OECs

Primary cultures of adult rat normal OECs and GDNF OECs were fixed for 10 min with 4% paraformaldehyde. After washing in PBS and blocking with 1% bovine serum albumin for 30 min, the cultures were incubated overnight at 4°C with the rabbit polyclonal anti-human GDNF antibody (1/200; Promega), anti-p75NGFR rabbit antibody (5 pg/ml, Santa Cruz Biotechnology), monoclonal mouse anti-glial fibrillary acidic protein (GFAP) (1 : 1000; Sigma) and anti-S-100 mouse antibody (1 : 2000; Sigma) diluted in PBS containing 1% bovine serum albumin. The next day the cultures were first washed in PBS and then incubated for 40 min at 37°C with fluorescein isothiocyanate-labelled secondary antibodies (Promega). They were then washed and examined with an Olympus BX-50 fluorescence microscope.

Assay of GDNF production

The amount of GDNF secreted by GDNF OECs was measured by enzyme-linked immunosorbent assay (ELISA) using the GDNF Emax ImmunoAssay System (Promega). According to the manufacturer's instructions, the ELISA plates (96 wells) were coated with an anti-GDNF monoclonal antibody (pH 8.2) overnight at 4–8°C. Plates were then blocked for 1 h at room temperature with blocking buffer. GDNF standards ranging from 0 to 1000 pg/ml were prepared using recombinant GDNF. Conditioned medium (CM) was obtained using 2×10^6 cells in 2 ml medium, but with only 1% fetal bovine serum for 24 h, then added to the wells (100 μl) undiluted or diluted 1 : 10 or 1 : 100. Samples and standards were incubated at room temperature for 6 h on a shaker. The plate was then incubated sequentially with chicken anti-human GDNF polyclonal antibody overnight at 4°C, horseradish peroxidase (HRP)-conjugated anti-chicken antibody (1 : 5000) at room temperature for 2 h, and the enzyme substrate tetramethylbenzidine for 15 min at room temperature. PBS was used to wash the plates after each step. The enzyme reaction was stopped by adding 100 ml of 1 M phosphoric acid per well and the absorbance was measured at 450 nm. Sample values were calculated from the standard curve in the linear range.

The biological activity of the secreted GDNF was tested using a PC12 cell line, which stably expresses GDNF receptor GFRα1 and Ret (Chen *et al*., 2001). Briefly, 2×10^4 PC12-GFRα-Ret cells were added to each well of a 24-well plate (Costar) that had been coated with poly-L-lysine. After attachment, the cells were exposed to CM from OECs or GDNF OECs. PBS was used as negative control and 100 ng/ml GDNF was the positive control. GDNF was prepared as previously described (Chen *et al*., 2000). Five days later, the effect of GDNF on cell differentiation was determined. Cells possessing one or more neurites of a length more than twice the diameter of the cell body were scored as positive. Each value is the mean ± SEM sampled from three independent experiments.

Hoechst labelling of OECs

When cells reached confluence, monolayers of purified OECs or GDNF OECs were incubated at 37°C in 1.5 μg/ml of the nuclear fluorochrome bisbenzimide (Hoechst 33342; Sigma) for 15 min. After several washes in Dulbecco's modified Eagle medium (DMEM), cells were trypsinized and collected for transplantation.

Surgical procedures

Animal care and use followed recommended NIH guidelines. Female adult Sprague–Dawley rats were anaesthetized with 2% pentobarbital sodium (0.2 ml/kg) intraperitoneally. Laminectomy was performed to expose the dorsal surface of the T_{8-9} segment, followed by a transection at T_8 using microscissors. The distal stump was carefully lifted up, allowing verification of complete transection. Rats then received stereotaxic injections into four sites of the midline of both cord stumps (ventral funiculus, grey commissure, dorsal CST, gracile fasciculus) at 1 mm from the transaction site (Ramon-Cueto *et al*., 1998) using sterile glass needles. In total, 41 animals were operated: (i) eight animals received a transection with no grafted OECs, and each site received 0.5 μl DMEM; (ii) 15 received a graft of normal OECs, each site

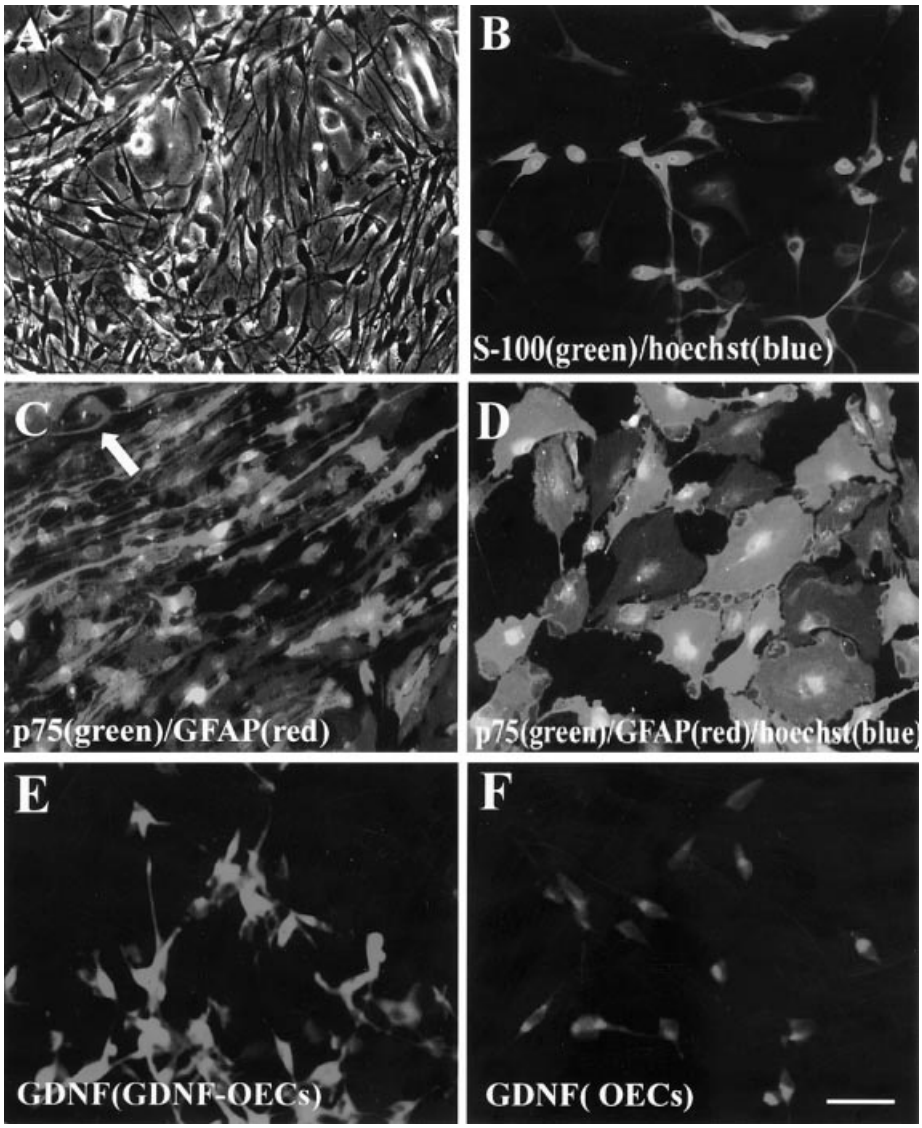

Fig. 2 Characterization of a GDNF-overexpressing OECs cell line. (**A**) Phases of GDNF OECs in primary cultures. (**B**) GDNF OECs double-stained with S-100 and Hoechst. (**C**, **D**) GDNF OECs were characterized as intensively immunostaining for p75NGFR and weakly staining for GFAP. A small number of GFAP-positive cells that did not express p75NGFR were probably astrocytes (arrow in **C**). GFAP expression was much stronger in astrocytes than in OECs. Immunostaining for GDNF was much stronger in GDNF OECs (**E**) than in normal OECs (**F**). Scale bar = 100 μm. This figure can be viewed in colour as supplementary material at Brain Online.

receiving 0.5 μl OEC suspension containing about 50 000 cells; and (iii) 18 received a graft of GDNF OECs, each site receiving 0.5 μl cell suspension containing about 50 000 cells. Postoperatively, rats were kept at 22–25°C on highly absorbent bedding, injected with cefazolin sodium (40 mg/day) for up to 1 week, and received bladder expression twice daily until normal function returned.

RT-PCR analysis

Two months after cell transplantation, animals of the OEC or GDNF OEC group were euthanized with a lethal dose of pentobarbital sodium and $T_{7–9}$ spinal cord tissue was collected. Total RNA was isolated from the tissue using TRIzol (Gibco BRL) and the RNA concentration was measured photometrically. After RNA extraction, the samples were digested with RNase-free DNase I, and cDNA was synthesized using a Dmniscript TM PT kit (Qiagen). For PCR, specific primers (forward, 5′-AATATGCCCGAAGATTATCC-3′; reverse, 5′-GTTTAGCGGAATGCTTTCTT-3′) were designed to amplify GDNF cDNA to yield 466 bp amplified products. To quantify the RT-PCR, β-actin (primers: forward, 5′-AAGATTTGG-CACCACACTTTCTAC-3′; reverse, 5′-CACGGTTGGCCTTAGG-GTT-3′) was co-amplified with GDNF. Forty picomoles of each primer and 1 μg DNA were used for PCR, which was carried out in a programmable heating block using cycles consisting of denaturation at 95°C for 1 min followed by annealing at 55°C for 1 min and DNA extension at 72°C for 1 min. After 30 cycles of PCR, samples were electrophoresed on 1.5% agarose gel. Gels were stained with ethidium bromide and photographed under ultraviolet light.

Retrograde tracing with HRP

Twelve animals (three controls, four from the OEC and six from the GDNF OEC group) were used for HRP retrograde tracing. Eight weeks after surgery, an aqueous suspension of 30% HRP (Sigma; RZ

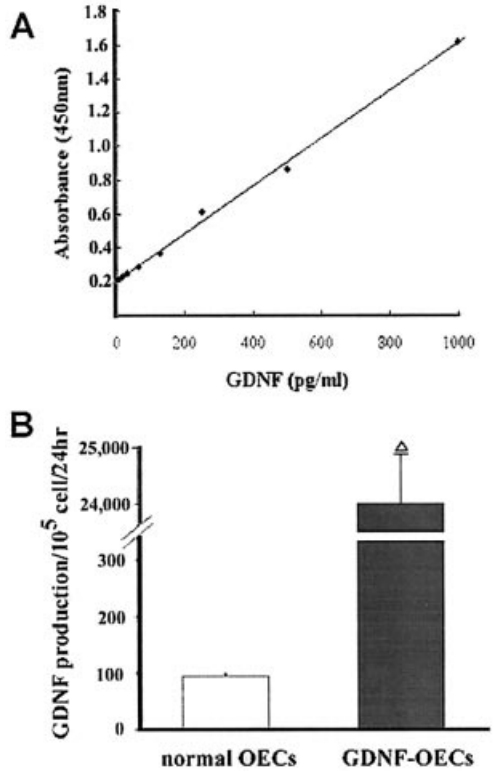

Fig. 3 Detection of *ex vivo* GDNF secretion from normal OECs and GDNF OECs by ELISA. (**A**) GDNF standard curve obtained using the GDNF Emax ImmunoAssay System. (**B**) Bar graph comparing *ex vivo* GDNF secretion between normal OECs and GDNF OEC groups. GDNF production by control uninfected OECs was 95 pg/ml per 10^6 cells, whereas GDNF OECs produced an average of 25 ng GDNF/10^6 cells/24 h. $^*P < 0.01$ versus normal OEC group.

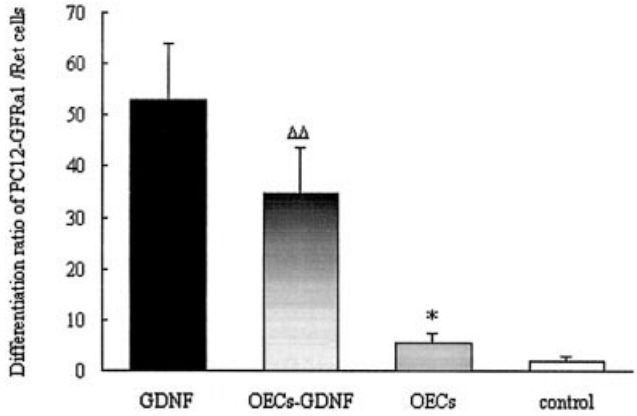

Fig. 4 Bar graph comparing differentiation ratios of PC12-GFRα1-Ret cells among groups. PC12-GFRα1-Ret cells were incubated for 5 days with DMEM (control), GDNF 50 ng/ml, CM obtained from GDNF OECs or uninfected OECs. $^*P < 0.05$ versus control group; $^{\Delta\Delta}P < 0.01$ versus OEC supernatant.

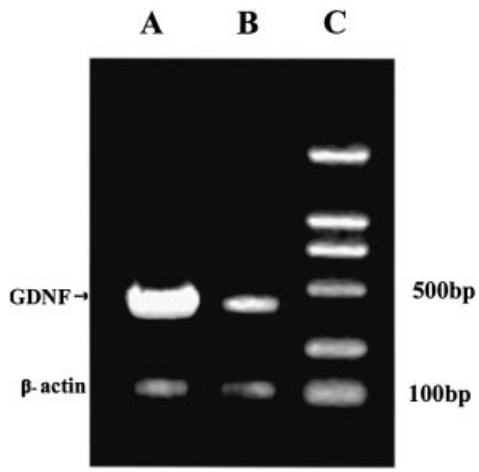

Fig. 5 Expression of GDNF mRNA transcripts was detected 8 weeks after operation by RT-PCR analysis. Lane A, GDNF OEC group; lane B, OEC group; lane C, marker. The upper GDNF band is around 460 bp and the control actin band is around 100 bp. In rats injected with GDNF OECs, expression of GDNF mRNA in the spinal cord was dramatically increased.

>3.0) and 2% dimethyl sulphoxide (Sigma) was injected bilaterally three or four segments caudal to the transplant to avoid diffusion of HRP into the transplant. After injection, the surgical exposure was closed and the animals were maintained for 36 h before being perfused by buffered 1% paraformaldehyde and 1.25% glutaraldehyde. The brain and spinal cord were removed and stored in 20% sucrose in 0.1 M PBS at 4°C overnight. Then the sensory motor cortex in the forebrain and the magnocellular portion of the red nucleus in the midbrain were cut transversely and serially at 30 μm. Every third section in the red nucleus or cortex was collected and stained with tetramethylbenzidine (Sigma) and hydrogen peroxide according to the method of Mesulam (Mesulam, 1978). After counterstaining with neutral red, the sections were observed under a light microscope. The distal spinal cord including the transected area was cryosectioned sagittally to ensure the injection was confined and that there was no spread of dye to the transplant. Animals that did not measure up to this criterion were eliminated from the study.

For identification of neurons in the red nucleus and cortex, the caudalmost section through the nucleus magnocellularis and sensory motor cortex where HRP-labelled neurons could be observed was designated as the first section for analysis. The numbers of HRP-labelled and neutral red-labelled rubrospinal neurons (RSNs) and corticospinal neurons (CSNs) on both sites were counted separately using a digitizing tablet and PC-based software (Metamorph). The criterion for a CSN was an HRP-filled pyramidal shape >4 μm in diameter. For the RSN, only neurons with a clearly visible nucleus were counted.

Antegrade tracing with biotinylated dextran amine

Antegrade tracing of CST fibres from the motor cortex was performed following Ramon-Cueto *et al*. (2000). Briefly, animals were anesthetized and two holes were drilled in the cranium to expose both sensorimotor cortices. A 10% solution of biotinylated dextran amine (BDA; molecular weight 10 000; Molecular Probes) was injected bilaterally in eight sites of each sensorimotor cortex (0.5 ml/site) to cover the entire hindlimb region. Eighteen days later, rats were killed and spinal sections were incubated with fluorescein-conjugated streptavidin to visualize BDA-containing corticospinal axons. To quantify CST fibres we followed a method described previously (Blits *et al*., 2000). To quantify the number of CST fibres

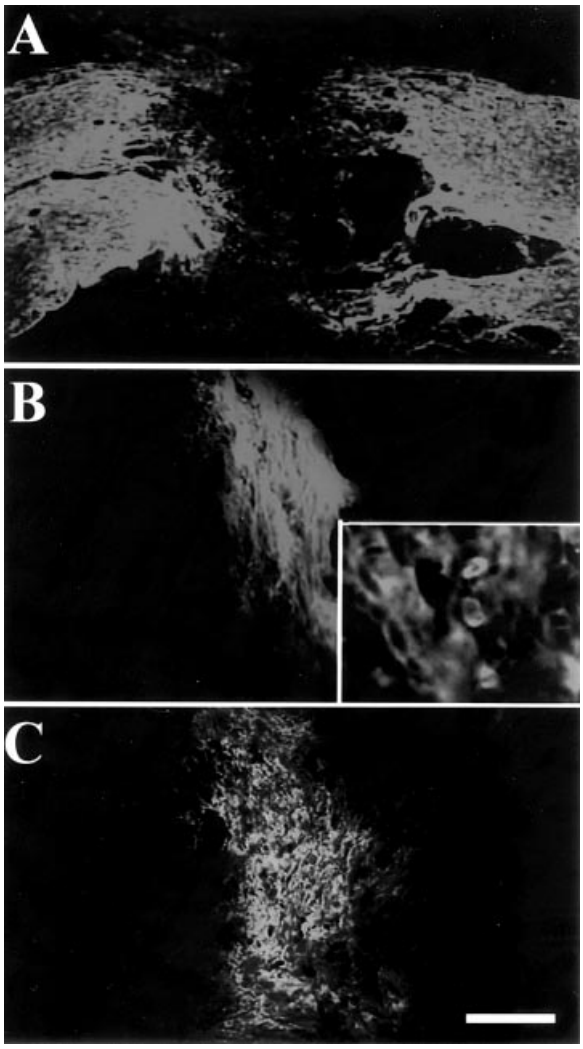

Fig. 6 Transgenetic gene expression of OEC implants *in vivo*. The OECs were infected with the recombinant retrovirus containing GFP and implanted in the lesion of the spinal cord. (**A**) The lesion gap is surrounded by an intensively GFAP-positive (red) border of reactive astrocytes. (**B**) Persistent expression of GFP (green) is found in OECs up to at least 2 months after implantation, as determined by native GFP fluorescence. Inset shows the magnified image. (**C**) Most GFP-positive OECs are immunoreactive for p75 NGFR (blue). Additional p75NGFR staining outside the confines of GFP labelling could be due to the Schwann cells that have invaded the spinal cord, as suggested by Ruitenberg *et al*. (2002). Scale bar = 150 μm. This figure can be viewed in colour as supplementary material at Brain Online.

in the animals that survived for 8 weeks, the centre of each lesion (between the proximal and distal scar) was identified microscopically and determined as point 0. From this point, fibre counting was performed on the sagittal sections and at 2.5 and 4.5 mm proximal and distal to the centre of the lesion.

Immunohistochemistry

Rats were perfused with 4% paraformaldehyde in 0.1 M ice-cold phosphate buffer. The spinal cord was removed, postfixed for 5 h and placed in 30% sucrose/PBS before preparing 30 μm sagittal cryosections. Hoechst-labelled OECs were visualized with a fluorescent microscope equipped with a 365 nm excitation filter and a 420 nm emission filter. For immunofluorescence, sections were permeabilized and blocked with 0.3% Triton X-100/10% normal goat serum in 0.1 M PBS for 15 min. Primary antibodies were then applied to the sections overnight at 4°C. Each section was double-labelled with mouse monoclonal immunoglobulin G against neurofilaments (Sigma; 1 : 200) and anti-p75NGFR. The following day, sections were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse and rhodamine-conjugated goat anti-rabbit (Promega) secondary antibodies. Slides were washed, mounted, and examined by Olympus fluorescent microscopy.

Neurofilament (NF) immunohistostaining was quantitatively determined in 3 mm wide strips of spinal tissue through the lesion centre. Slides were viewed and photographed with an Olympus photo microscope (BX70). The photographs were digitized with a video image analysis system (Metamorph) in conjunction with a computer. After background correction, the grey levels of each slide were automatically detected. Then the mean of grey levels for all slides from each animal were obtained and statistical analysis was performed.

Functional recovery

Functional tests were performed before operation and 2 h, 3 days and 1, 2, 3, 4, 5, 6, 7 and 8 weeks after operation. Locomotor activity was evaluated using the open-field walking scoring system. One animal at a time was allowed to move freely inside a circular plastic tray (90 cm diameter, 24 cm wall height) for 5 min. Behavioural recovery was scored according to the BBB (Basso, Beattie, Bresnahan) scale (Basso *et al*., 1996), which is composed of 21 different criteria of the movement of the hind limb from complete paralysis to complete mobility. As a second test for hind limb function, animals were subjected to an inclined plane test (Rivlin and Tator, 1977). The maximum angle at which the animal could maintain a stable position for 5 s on the inclined plane was recorded. Before each evaluation, we carefully examined the rats for perineal infection, wounds in the hind limbs, and tail and foot autophagia.

Results

Characterization of GDNF OECs

The GDNF OECs in primary cultures, which had a spindle-like morphology with two or three processes or a flat appearance (Franceschini and Barnett, 1996; Li *et al*., 1998; Gudino-Cabrera *et al*., 2000; Wewetzer *et al*., 2002), displayed S-100 immunoreactivity (Fig. 2A and B). Immunofluorescent staining demonstrated that the purity of GDNF OECs was more than 92%. To further ascertain that insertion of a new gene using a viral vector-mediated gene transfer did not interfere with normal cell functioning, GDNF OEC cultures were phenotypically examined for the expression of general cell markers: the low-affinity neurotrophin receptor p75NGFR, the Schwann cell marker S-100, and GFAP. As shown in Fig. 2C and D, GDNF OECs were characterized as having intensive immunostaining for p75 NGFR and weak immunostaining for GFAP. A small amount of GFAP-positive cells that did not express p75NGFR were probably astrocytes, as GFAP expression in astrocytes was much stronger than that in OECs. As expected, immunostain-

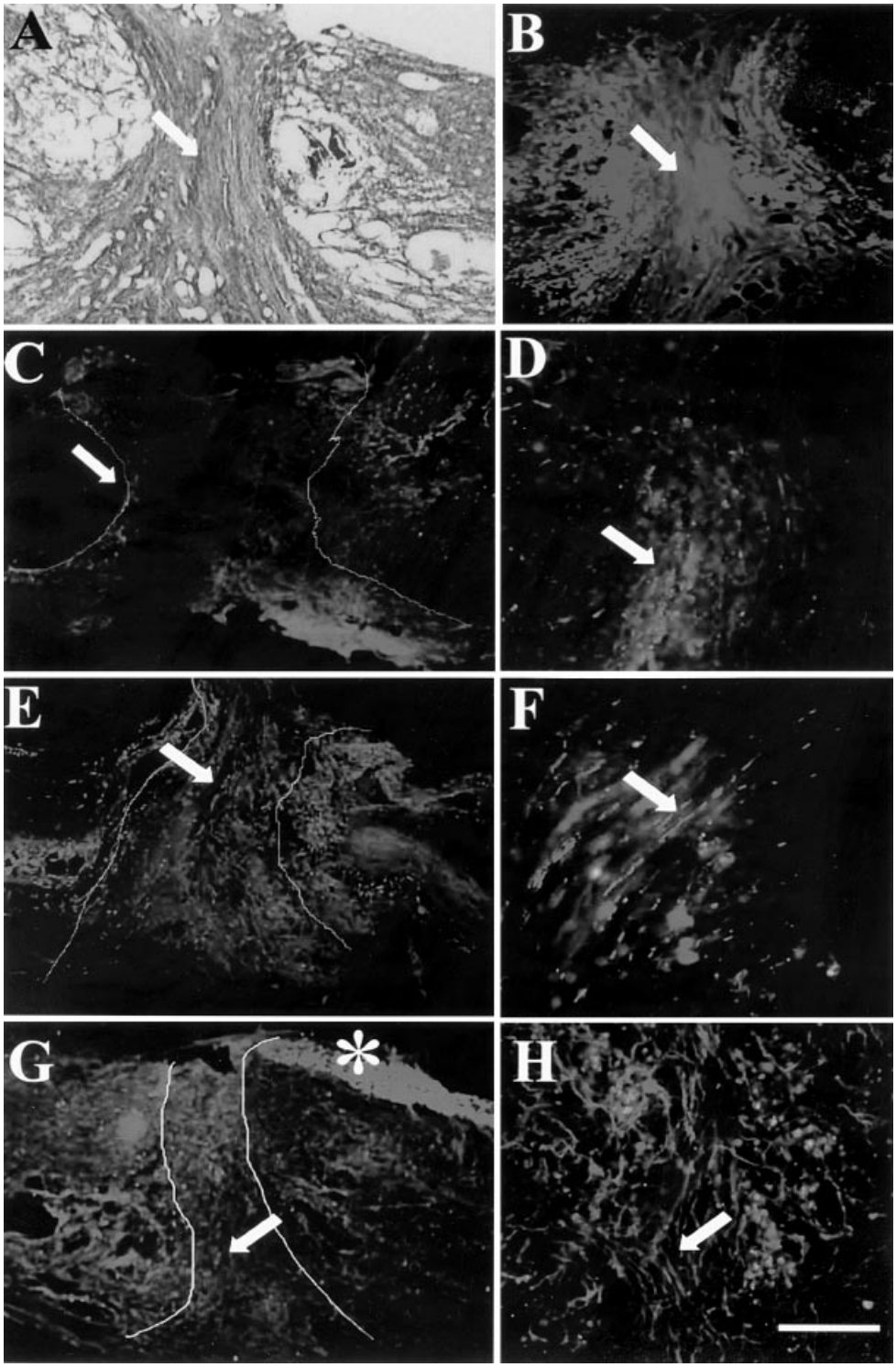

Fig. 7 NF immunostaining through the spinal cord lesion 8 weeks after injury. (**A**) Haematoxylin and eosin staining demonstrated that spinal cord transection resulted in an obvious traverse scar at the T_8 lesion epicentres (arrow) and neuronal necrosis, reactive gliosis and cavitation in adjacent rostral and caudal regions. (**B**) GFAP immunochemistry shows glial scar (arrow) in spinal cord lesion. (**C**, **D**) In control animals, NF immunofluorescence at the lesion site (outlined in **C**) displayed mostly scattered profiles, and many NF-immunoreactive fibres were stopped at the host–scar interface (arrows). (**E**, **F**) In the normal OEC group, elongated NF-positive axons (arrows) were present throughout the lesion (outlined in **E**). (**G**, **H**) In the GDNF OEC group, a dramatically increased amount of NF-positive fibres (arrows) was found in the lesion site (outlined in **G**); some of them were derived from the invaded dorsal root (asterisk). The axonal profiles within the centre of the lesion site had a variety of orientations. Scale bars = 100 μm in **A**, **C**, **E**, **G**; 25 μm in **B**, **D**, **F**, **H**. This figure can be viewed in colour as supplementary material at Brain Online.

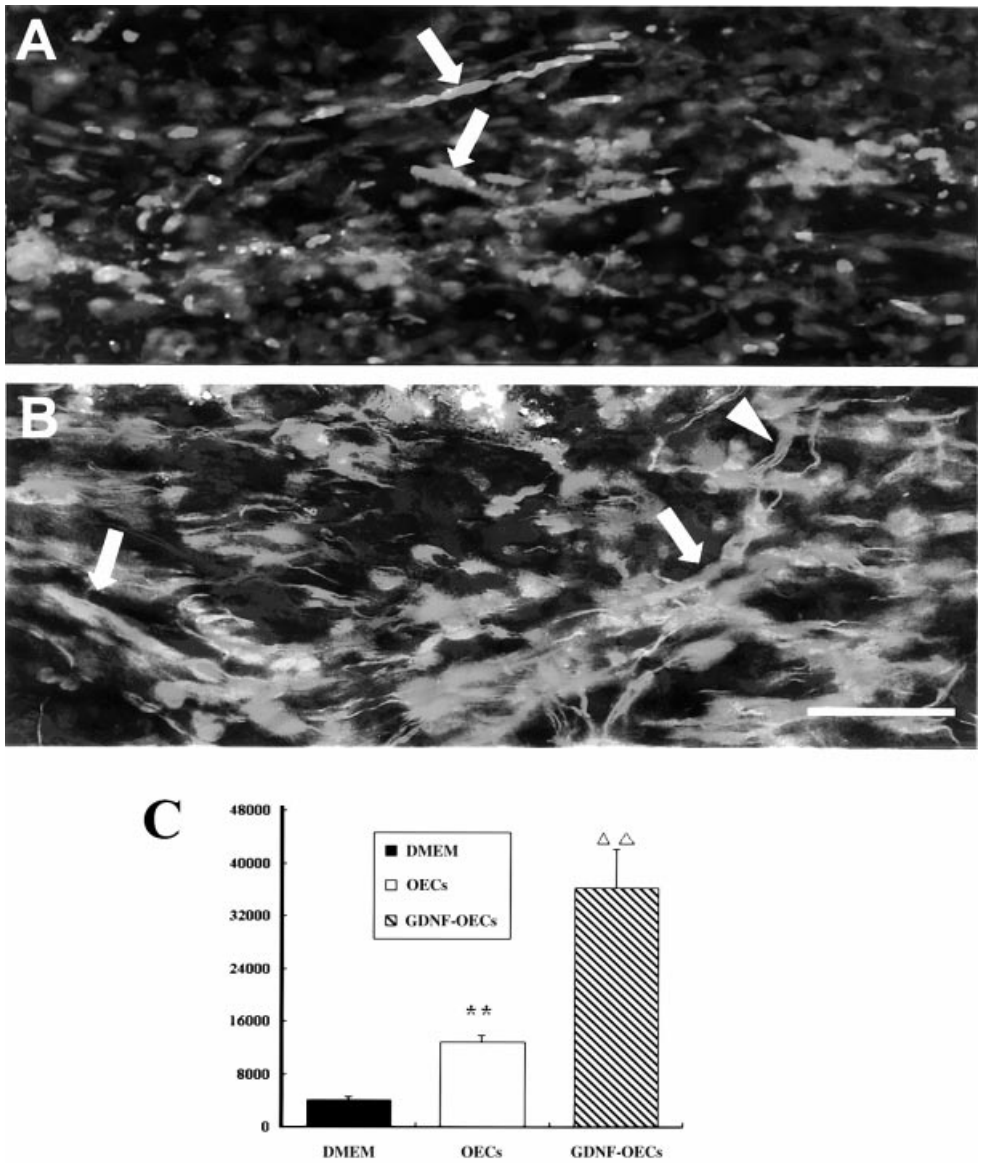

Fig. 8 High-magnification microscope images of NF-positive axons sprouting through the lesion centre. (**A**) Normal OEC group. (**B**) GDNF OEC group. Implanted OECs are indicated by Hoechst (blue)/p75NGFR (red) labelling. Note numerous NF-positive axons (green) growing through the injury site 8 weeks after injury, often in close association with implanted OECs or GDNF OECs (arrows). In the GDNF OEC group, NF-positive axons were often found in bundles (arrowhead). Scale bar = 20 μm. The bar graph shows grey levels of NF activity in the lesion in the different groups 8 weeks after surgery. NF immunoactivities were significantly stronger in animals receiving GDNF OECs than in those receiving normal OECs ($P < 0.01$) and significantly stronger in animals receiving OECs than in those receiving DMEM. $^{**}P < 0.01$ versus DMEM group; $^{\Delta\Delta}P < 0.01$ versus OEC group. ANOVA test followed by least significant difference test, $n = 5$. This figure can be viewed in colour as supplementary material at Brain Online.

ing for GDNF was much stronger in GDNF OECs than in normal OECs (Fig. 2E and F).

Ex vivo GDNF secretion and biological effect

The amount of GDNF secreted by GDNF OECs was determined by ELISA with detection sensitivity to 31.2 pg/ml of GDNF. GDNF production by uninfected OECs, was estimated to be 95 pg/ml per 10^6 cells, whereas GDNF OECs produced an average of 25 ng GDNF/10^6 cells/day (Fig. 3).

The biological activity of the secreted GDNF was examined using PC12-GFRα-Ret cells. After 72 h of culture in CM from normal OECs, 5.4 ± 1.87% of cells differentiated. However, in CM from GDNF OECs, cell differentiation was six-fold greater than that of normal OECs (Fig. 4). This bioassay confirmed that GDNF secreted from GDNF OECs was biologically active and capable of promoting PC12-GFRα-Ret cell differentiation.

Transgenetic gene expression of OECs in vivo

Eight weeks after surgery, RT-PCR was used to measure the mRNA level of GDNF in the injured spinal cord (Fig. 5). In injured spinal cord injected with GDNF OECs, significantly

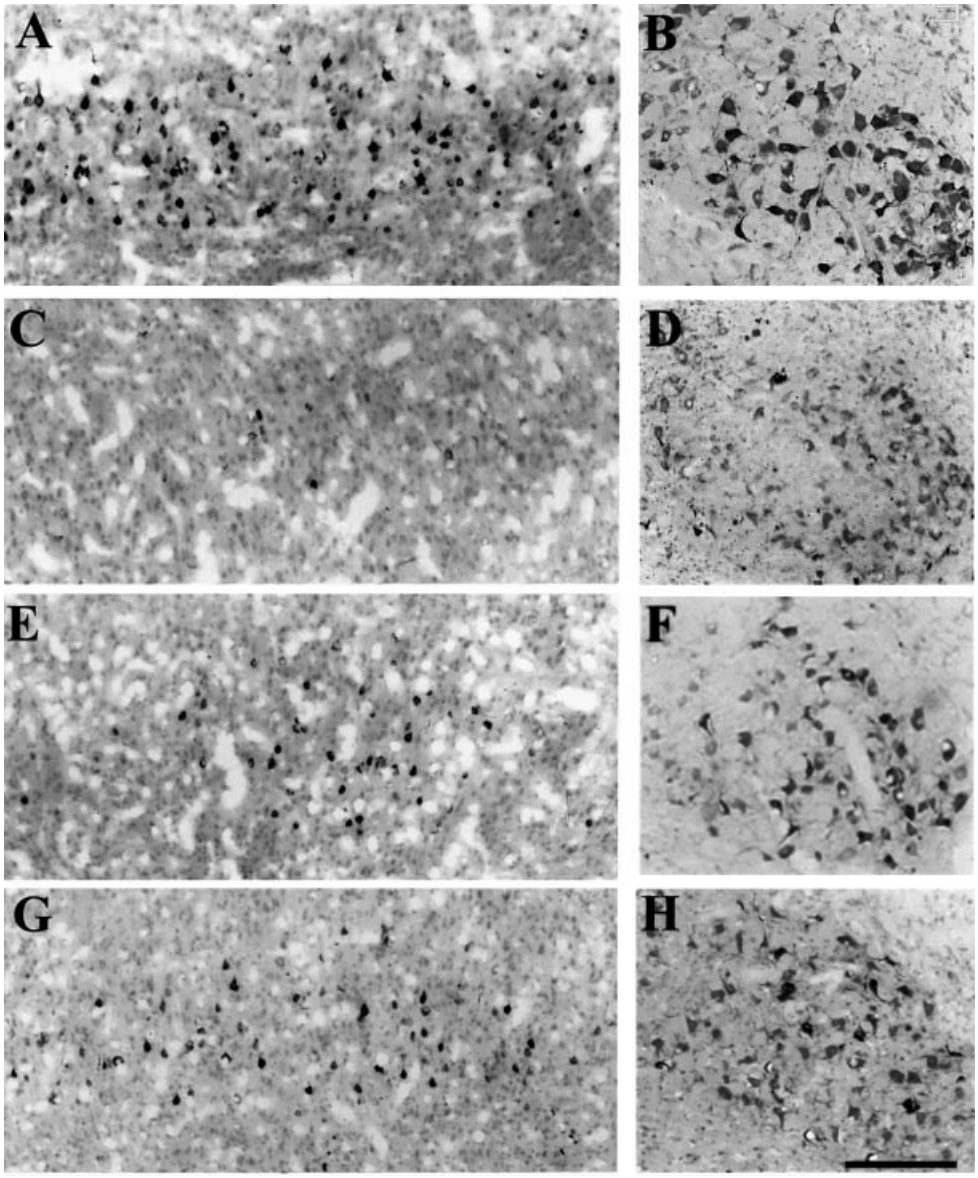

Fig. 9 Photomicrographs showing HRP retrograde tracing of CSN (**A**, **C**, **E**,**G**) and RSN (**B**, **D**, **F**, **H**) 8 weeks after thoracic transection. The dark purple-staining cells are HRP-positive. (**A**, **B**) Sham-operated animal. (**C**, **D**) DMEM group. (**E**, **F**) OEC group. (**G**, **H**) GDNF OEC group. In the DMEM group (control), few HRP-labelled neurons were observed in the CSN or RSN. In animals receiving OECs only, a few labelled cells were observed. Numerous HRP-labelled neurons were found in the GDNF OEC group. Scale bar = 200 μm. This figure can be viewed in colour as supplementary material at Brain Online.

higher levels of GDNF mRNA expression were detected compared with that in the spinal cord of rats injected with normal OECs. This result demonstrated that *ex vivo* transduction of OECs with retrovectors resulted in persistently increased GDNF expression *in vivo*, up to at least 2 months after implantation.

To further confirm the expression of the transgene and the location of OECs in the lesion, OECs were infected with the recombinant retrovirus containing green fluorescent protein (GFP), then implanted in the lesion of spinal cord. As shown in Fig. 6, 2 months after implantation, GFP-labelled OECs were visualized as a dense mass of elongated, brightly fluorescent cells extending from the lesion site. Most GFP-positive implants were immunoreactive for p75NGFR. These results confirmed that the implanted OECs were capable of surviving in the injured spinal cord for at least 2 months after implantation. The OECs were gathered in the lesion gap, which was surrounded by an intensively GFAP-positive border of reactive astrocytes. We could not find OECs across the astrocytic barrier into the spinal cord. These

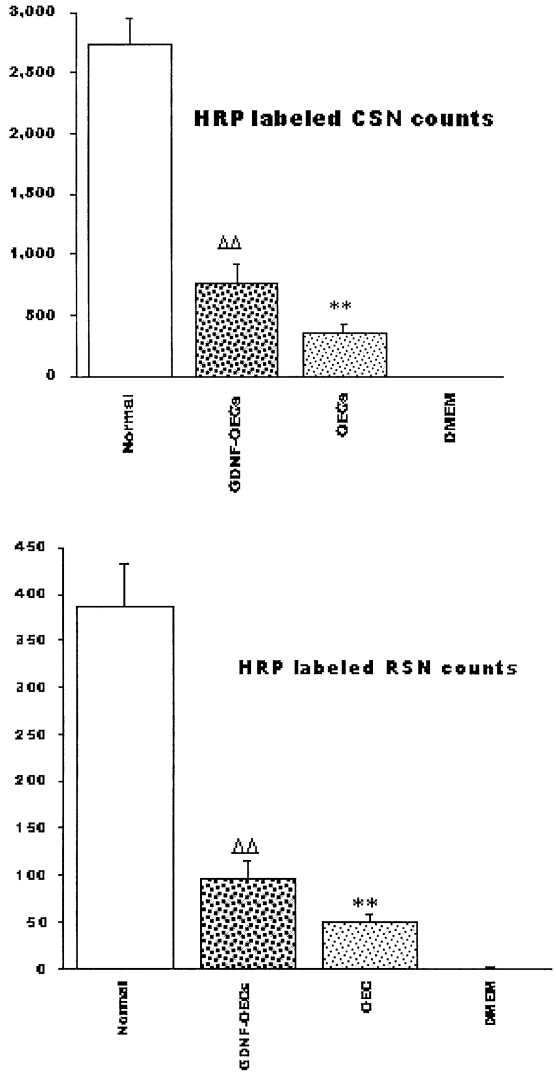

Fig. 10 Bar graph showing the numbers of HRP-labelled RSN and CSN among animal groups 8 weeks after surgery. In animals receiving GDNF OECs, there were significantly more RSN and CSN labelled than in those receiving normal OECs ($P < 0.01$). More RSN and CSN were labelled in animals receiving OECs than in those receiving DMEM ($P < 0.01$). $^{**}P < 0.01$ versus DMEM group; $^{\Delta\Delta}P < 0.01$ versus OEC group. ANOVA test followed by the least significant difference test, $n = 3$–5.

results are in good agreement with previous report by Ruitenberg and colleagues (Ruitenberg *et al*., 2002).

Axonal regeneration

Spinal cord transection lesion was characterized by an obvious traverse scar at the T_8 lesion epicentres and neuronal necrosis, reactive gliosis and cavitation in adjacent rostral and caudal regions (Fig. 7A and B). In control animals, 2 months after lesion only scattered NF-positive fibres were found in the central scar and many NF-immunoreactive fibres were stopped at the host–scar interface (Fig. 7C and D). As shown in Fig. 7E and F, elongated NF-positive axons are present throughout the lesion in normal OEC group. Dramatically increased amounts of NF-positive fibres were found in the lesion site in the GDNF OEC group, and some of them were derived from the invaded dorsal root (Fig. 7G and H). High-magnification microscopy showed numerous NF-positive axons growing through the lesion, often in close association with implanted OECs or GDNF OECs (Fig. 8). In the GDNF OEC group, NF-positive axons were often found in bundles. Statistical analysis revealed significantly higher grey levels of NF immunoactivity in animals receiving GDNF OECs than in those receiving normal OECs.

Figure 9 shows RSN and CSN labelling after injections of HRP into the low thoracic and upper lumbar region. HRP retrograde staining showed that most labelled RSN were located in the ventral–lateral portion of the magnocellular nucleus of the red nucleus (Fig. 9B, D, F, H). Neurons in the cortex (Fig. 9A, C, E, G) were smaller than in the RSN. As shown in Fig. 10, the number of HRP-labelled RSN and CSN were counted in all groups of animals. On average, in normal animals, ~386 and ~2737 neurons were labelled on both sides of the RSN and CSN, respectively. After thoracic transection, few HRP-labelled RSN or CSN were detected in the DMEM group. With treatment of normal OECs, a few cells (RSN, 50; CSN, 355) labelled with HRP were detected. OEC transplantation may have provided a permissive environment that allowed a small percentage of axotomized neurons to regenerate into the caudal spinal cord ($P < 0.01$ versus DMEM group). The highest regeneration ratio was in the GDNF OEC group (approximately 97 in RSN and 776 in CSN were labelled with HRP). These numbers were significantly higher than those in the normal OEC group ($P < 0.01$).

To further confirm the axonal regeneration, antegrade tracing experiments were performed. Figure 11 shows the images for BDA-traced corticospinal axons 8 weeks after spinal cord injury. In the control group, the transected CST showed little regeneration response in the segment rostral to the lesion centre. However, in the normal OEC group a few CST fibres were found to have formed termination bulbs and some fibres had grown through the lesion and reached the segment distal to the lesion centre. Quantitative analysis revealed that more BDA-traced CST fibres were found in the segment near the injury side in the GDNF OEC group. Interestingly, in a distal segment 4.5 mm away from the lesion centre, no significant differences were found between the OEC and GDNF OEC groups (Fig. 12).

Behavioural assessment

Figure 13 shows the behavioural results during the 8-week assessment period for each group of animals. All injured rats manifested complete hind limb paralysis immediately after injury. The BBB scores were in the range of 0–2 in the control animals. Following transplantation of the OECs, hind limb functional recovery increased gradually; 8 weeks after transplantation, all 15 animals displayed BBB scores greater than that achieved by any of the eight controls ($P < 0.01$).

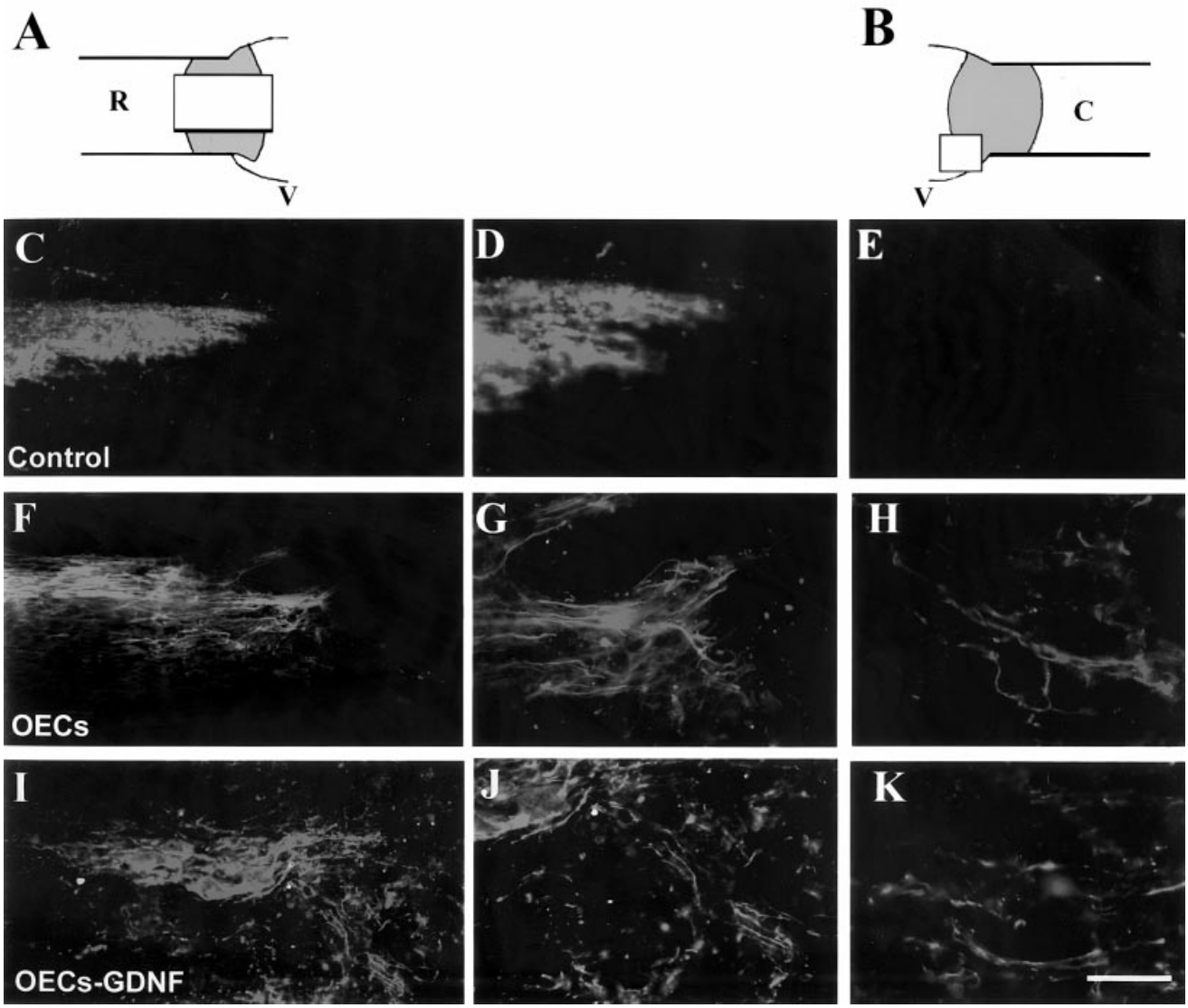

Fig. 11 BDA-traced corticospinal axons in the lesion 8 weeks after implantation. (**A**) Diagram of a sagittal spinal cord section containing the rostral part of the injury region. The locations of the images shown in **C**, **F** and **I** are represented (box). R = rostral; V = ventral. (**B**) Diagram of a sagittal spinal cord section containing the caudal part of the injury region. The locations of the images shown in **E**, **H** and **K** are represented (box). C = caudal. (**C**, **D**, **E**) In the control group, the transected CST showed little regeneration response at the rostral end. Few fibres were found to grow through the lesion and to reach the segment distal to the lesion centre. (**F**, **G**, **H**) In the OEC group, a few CST fibres were found to have formed termination bulbs and some fibres had grown through the lesion and reached the segment distal to the lesion centre. (**I**, **J**, **K**) In the GDNF OEC group, very robust fibre growth and termination bulb formation were observed, and many BDA-labelled axons were seen at the caudal end of the lesion. Scale bar = 50 μm in **C**, **F**, **I**; 100 μm in **D**, **G**, **J**; 150 μm in **E**, **H**, **K**. This figure can be viewed in colour as supplementary material at Brain Online.

Five of the 15 experimental animals from the OEC group could support their body weight on their hind limbs; the other 10 animals had ankle, knee and hip movements in one or both legs but did not obviously bear weight. The GDNF OEC group regained more functional recovery than the normal OEC group 8 weeks after transplantation ($P < 0.01$); 10 of the 18 rats treated with GDNF OECs could walk in a coordinated manner.

Differences in score on the inclined plane test among the three groups were also significant. From 2 weeks after operation, rats in the OEC group began to show more functional recovery than those in the control group ($P < 0.05$) and at 5 weeks after implantation animals in the GDNF OEC group had a higher score than those in the OEC group ($P < 0.05$).

Discussion

Combining transplantation and gene therapy is perhaps one of the most powerful strategies to promote CNS repair. As implantation of OECs in the injured spinal cord has been reported to promote long-distance regeneration and func-

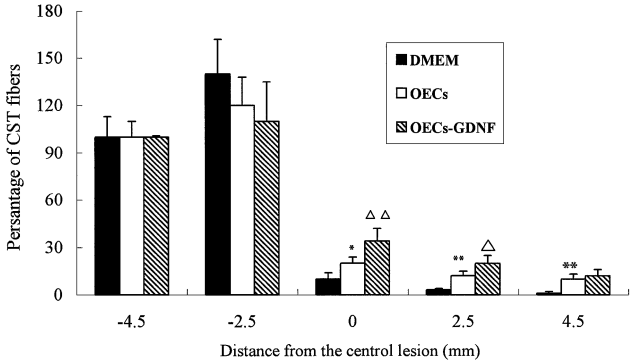

Fig. 12 Quantitative comparison of CST regrowth in the spared grey matter of the transected spinal cord 8 weeks after implantation. To compensate for variations in actual tracing efficiency by the cortical BDA injections, the fibre number counted 4.5 mm proximal from the centre of the lesion was set to 100% and all data were calculated as relative percentages. In the control group, few CST fibres were observed in the distal spinal cord. In the normal OEC group, a few CST fibres had grown through the lesion and reached the segment distal to the lesion centre. In the GDNF OEC group, however, more CST regrowth was found in the segment near the injury side. $^{*}P < 0.05$, $^{**}P < 0.01$ versus DMEM group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ versus OEC group. ANOVA test followed by least significant difference test, $n = 5$.

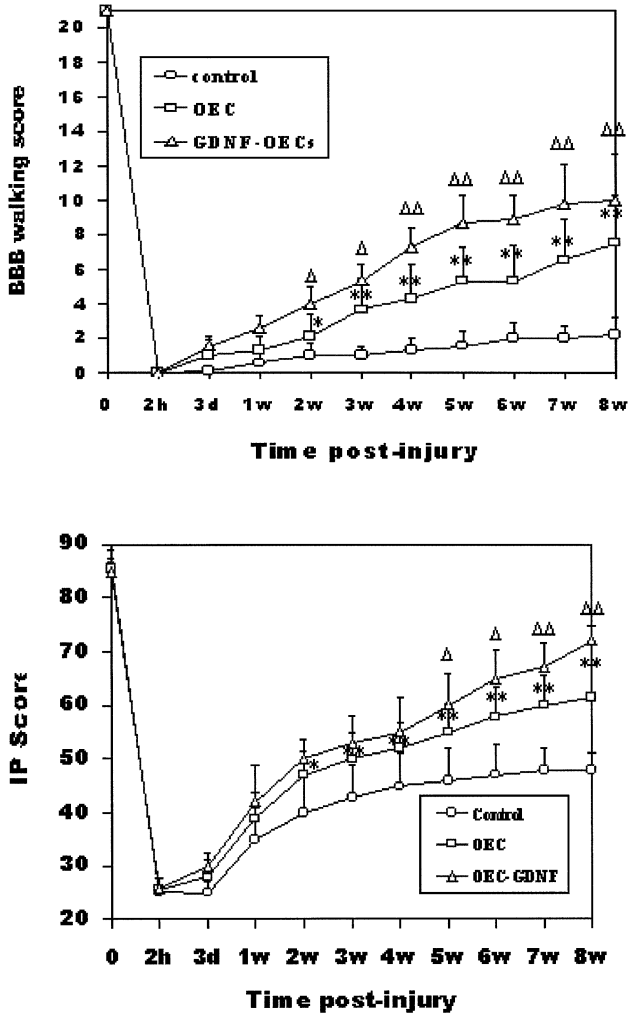

Fig. 13 Functional analysis of hindlimb movements following SCI among animal groups, using the BBB behavioural assessment (*top*) and the inclined plane (IP) test (*bottom*) $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ versus OEC group. ANOVA test followed by least significant difference test, $n = 8$–18.

tional recovery following SCI (Li *et al*., 1997; Ramon-Cueto *et al*., 2000), there is much interest in upgrading OECs to enhance the regenerative properties of these cells. Recently, researchers have begun to transfect OECs to express fluorescent markers for tracing experiments (Ruitenberg *et al*., 2002) or to use transgenic animals expressing a xenogeneic protein as a source for modified OECs (Imaizumi *et al*., 2000). In the present study, OECs were genetically modified to overexpress exogenous neurotrophic factor and were transplanted into the transected spinal cord.

Ex vivo gene therapy is a valuable approach to the achievement of long-term and site-specific delivery of therapeutic agents in the CNS. Both retroviral and adenovirus vectors have been widely used for gene transfer. Retrovectors integrate with high efficiency and contain no viral genes so they can mediate long-term expression and avoid host cellular immune responses (Robbins *et al*., 1998). In the present study, high levels of transgenic GDNF mRNA could be detected even 2 months after implantation. Tracing the GFP OECs confirmed that the implanted, genetically modified OECs were capable of surviving and expressing the foreign gene in the spinal cord lesion. The sustained high-level expression of GDNF in OEC implants allows the possibility of manipulating the growth-promoting properties of OECs and the microenvironment at the lesion site not only during the acute but also during the chronic phase following injury. Retrovectors are limited by the viral LTR sequence, which may interfere with the expression of various gene cassettes. No changes in morphology and expression of general cell marker proteins were detected after transduction of OECs by retroviral vectors in this study. Following *ex vivo* gene transfer, implants of transduced OECs into the site of a spinal cord lesion were p75NGFR-positive and displayed typical bipolar morphology, suggesting that gene transfer with retroviral pN2A-GDNF vectors did not interfere with normal cell functioning.

GDNF was originally identified as a potent trophic factor for midbrain dopaminergic neurons (Lin *et al*., 1993; Beck *et al*., 1995; Tomac *et al*., 1995). It was subsequently found that GDNF also strongly supports the survival of motoneurons both *in vitro* and *in vivo* (Henderson *et al*., 1994; Li *et al*., 1995; Oppenheim *et al*., 1995; Yan *et al*., 1995; Houenou *et al*., 1996). When applied into the spinal cord, GDNF exerts a trophic effect on corticospinal neurons and promotes their long-term survival after axotomy (Giehl *et al*., 1997). In the present study, we demonstrate that treatment with OECs with a modified GDNF gene could stimulate an increase in the regeneration of corticospinal or rubrospinal axons in adult rats after spinal cord transection compared with treatment

with normal OECs. The HRP retrograde labelling studies clearly demonstrated that injured axons had indeed regenerated through the transection site. Numerous HRP-labelled neurons were detected 8 weeks after SCI in the GDNF OEC group. However, in the normal OEC group, less than half of the HRP-labelled neurons were detected. BDA antegrade tracing experiments confirmed that more CST fibres had grown through the lesion and reached the segment distal to the lesion centre in the GDNF OEC group than in the normal OEC group. NF immunochemical labelling also showed large number of fibres in the lesioned spinal cord following GDNF OEC injections. These results suggest that GDNF *ex vivo* gene delivery might enhance the growth-promoting properties of OECs after SCI. There are several possible explanations for this phenomenon. First, GDNF elicits a chemotropic effect, directing the growth of axons to regions with the highest concentration of growth factor. Secondly, GDNF may increase the survival ratio of RSN and CSN after axotomy, which results in an increased number of regenerated corticospinal or rubrospinal fibres. Moreover, recent studies have also shown that cultured OECs express GDNF receptor GFRα1 (Woodhall *et al*., 2001); therefore, high levels of GDNF secreted from GDNF OECs may have trophic effects on themselves.

The locomotor functions of animals were evaluated using the inclined plane method of Rivlin and BBB scale. The BBB scoring system differs from other locomotor scoring systems in several respects. First, the score is not a summation of component behaviours. Each BBB score requires fulfilment of a unique set of criteria. Secondly, the scores encompass many behavioural traits and represent a detailed characterization of rat locomotor function. Thirdly, the scores are based on observations of rat recovery from SCI. The ordering of the scores assumes progressive recovery and that each recovery stage represents better locomotion than the preceding stage (Basso *et al*., 1996). Using the BBB scale and the inclined plane method, we demonstrated a progressive recovery over time among three groups. The functional recovery in the GDNF OEC group showed statistically significant improvements compared with the normal OEC group. This result is in accordance with the morphological experiments. Significant improvements of locomotor function were also achieved in the GDNF OEC and normal OEC groups compared with the control group. The enhanced recovery of function in the GDNF OEC group may not have been mainly due to the enhanced outgrowth of a still very limited number of nerve fibres, according to our retrograde and antegrade tracing results. After spinal cord transection, rats from the control group always had severe tissue loss next to the transection centre. A positive correlation of the increased tissue sparing with higher locomotor scores after SCI has been reported previously (Basso *et al*., 1996). It is conceivable that the transplanted OECs have a beneficial effect on tissue sparing, as shown by other investigators in recent publications (Takami *et al*., 2002; Plant *et al*., 2003). Moreover, GDNF secreted by the OEC may also counteract the tissue loss, as the GDNF administration was reported to increase tissue sparing in a contusion model (Cheng *et al*., 2002). Furthermore, GDNF secreted by the OEC may have stimulated the repair or survival of spinal motor neurons, which may have contributed to the functional recovery (Watabe *et al*., 2000; Cheng *et al*., 2002).

In summary, the present study shows that the growth-promoting properties of OECs were significantly improved when these cells were genetically modified to secrete an increased level of GDNF. Genetic engineering of OECs opens up new possibilities for future clinical applications in SCI.

Note added in proof

As this manuscript was under revision Ruitenberg *et al*. (2003) published their paper which addresses the point of the effect of genetically modified OECs on tissue sparing. Their results may help to support the notion that the behavioural effect observed in the present study could be partly explained in this way.

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