Gene Therapy (2002) 9, 135–146

www.nature.com/gt

RESEARCH ARTICLE

Viral vector-mediated gene expression in olfactory ensheathing glia implants in the lesioned rat spinal cord

MJ Ruitenberg[1], GW Plant[2], CL Christensen[2], B Blits[1], SP Niclou[1], AR Harvey[2], GJ Boer[1] and J Verhaagen[1]

[1]*Graduate School for Neurosciences Amsterdam, Netherlands Institute for Brain Research, Amsterdam, The Netherlands; and* [2]*Red's Spinal Cord Research Laboratory and Collaborative Training and Education Centre (CTEC), Department of Anatomy and Human Biology, University of Western Australia, Perth, Australia*

Implantation of olfactory ensheathing glia (OEG) is a promising strategy to augment long-distance regeneration in the injured spinal cord. In this study, implantation of OEG following unilateral hemisection of the dorsal cervical spinal cord was combined with ex vivo gene transfer techniques. We report, to our knowledge for the first time, that purified cultures of primary OEG are capable of expressing a foreign gene following adenoviral (AdV) and lentiviral (LV) vector-mediated gene transfer. OEG implants subjected to AdV vector-mediated gene transfer expressed high levels of transgenic protein in both intact and lesioned spinal cord at 7 days after implantation. However, the levels of transgene expression gradually declined between 7 and 30 days after implantation in lesioned spinal cord. Infection with LV vectors resulted in stable transduction of primary OEG cultures and transgene expression persisted for at least 4 months after implantation. Genetic engineering of OEG opens the possibility of expressing additional neurotrophic genes and create optimal 'bridging' substrates to support spinal axon regeneration. Furthermore, stable transduction of OEG allows us to reliably study the behaviour of implanted cells and to obtain better understanding of their regeneration supporting properties.

Gene Therapy (2002) **9,** 135–146. DOI: 10.1038/sj/gt/3301626

Keywords: *gene therapy; olfactory ensheathing glia; regeneration; spinal cord injury; viral vectors*

Introduction

Injured axons in the mammalian spinal cord fail to regenerate across the lesion site and do not re-establish synaptic contacts with their targets. As a consequence, severe damage to the spinal cord usually results in long-lasting deficits, involving partial or complete paralysis and loss of sensation below the level of the injury. The development of new strategies to treat such injuries is a major clinical challenge to neuroscience.

The paucity of axon regeneration in the mature central nervous system (CNS) is thought to depend on a critical molecular balance between growth-promoting and -inhibiting cues.[1] Transient and insufficient upregulation of intrinsic growth-associated proteins, eg B-50/GAP-43,[2] decreased neurotrophic factor signalling,[3] and the presence of many inhibitors for axon growth at the lesion site,[4–9] are thought to be responsible for this regenerative failure. As a strategy to overcome the inhibitory nature of the scar, implantation of biological substrates in the lesion site that favour axonal growth has been investigated extensively. Because the peripheral nervous system (PNS) regenerates quite successfully, peripheral nerves and associated cells have been employed as cellular conduits to stimulate CNS regeneration.[10–13] Although some success has been achieved, axonal regeneration was still limited and most regrowing axons were not able to leave the graft and re-enter the CNS tissue distal from the injury. This is likely to be due to many factors, including the inhibitory environment of the CNS, scarring at the distal graft–host interface,[14] and the growth-promoting influence of the graft itself.[15] Over the past few years there has been increasing interest in using another glial cell type, the olfactory ensheathing glia (OEG) cell.[16] Under normal circumstances OEG guide new primary olfactory axons growing from the olfactory epithelium towards their targets, the olfactory bulb glomeruli, where they synapse on, for instance, the dendrites of mitral cells. Since the olfactory bulb is part of the CNS, which is usually regarded as non-permissive tissue for axon growth, OEG are thought to provide a unique cellular boundary between the PNS and CNS. The presence of OEG is thought to allow growing axons of newly generated primary olfactory neurons to cross the transition zone between PNS and CNS and extend processes in the olfactory bulb without inhibition.

The properties of OEG that support axon growth from PNS into CNS tissue are not restricted to the olfactory bulb. This was demonstrated in an experiment where OEG were implanted in the dorsal root entry zone allowing lesioned dorsal root axons to regenerate across

Correspondence: J Verhaagen, Netherlands Institute for Brain Research, Meibergdreef 33, 1105 AZ Amsterdam ZO, The Netherlands
Received 7 November 2001; accepted 28 November 2001

the non-permissive barrier of astrocytes at the PNS–CNS transition zone and to enter the spinal cord.[17] These results indicate that, in contrast to the Schwann cells present in the dorsal root, implanted OEG were able to intermingle with scar tissue creating permissive zones for growing axons. Recent studies have reported encouraging results of regeneration in the injured spinal cord following OEG implantation,[18] accompanied by functional improvement in locomotion behaviour.[19,20] A study using Schwann cell bridges 'capped' with OEG implants in a complete transected spinal cord, showed that the presence of OEG at the lesion site enhanced long-distance axonal regeneration, indicating possible advantages of OEG implants over Schwann cell grafts.[21,22] This was supported by recent evidence showing that OEG can intermingle with co-cultured astrocytes unlike Schwann cells which form islands in the presence of astrocytes with very little interaction.[23] Furthermore, OEG do not induce expression of inhibitory proteoglycans at the glial surface.[23] Although these results demonstrated that OEG implants support regeneration and may have several advantages over the use of Schwann cells in CNS injury paradigms, the number of regenerating axons distal from the injury site was still limited.

In the present study, the possibilities of *ex vivo* viral vector-mediated gene transfer to OEG using adeno-, adeno-associated and lentiviral vector systems were examined. Adenoviral (AdV), adeno-associated viral (AAV) and lentiviral (LV) vector systems are widely used as novel tools for the introduction of foreign genes in neural tissue, either by direct injection of the vector into the nervous system or indirect *ex vivo* gene transfer techniques.[24–34] Transferring genes of interest into OEG would not only allow better monitoring of the behaviour of implanted cells in the injured spinal cord and to study the relationship between OEG and regenerating axons, but would also permit the insertion of additional neurotrophic genes into the cells. This could significantly improve the growth-promoting properties of OEG since limited neurotrophic factor expression and differences in the capacity of various axonal populations to regenerate through OEG implants have been reported.[35–38] Previous studies have reported that delivery of neurotrophic factors using different genetically engineered cellular conduits, such as Schwann cells or fibroblasts, can encourage axon regeneration in the injured spinal cord.[39–42] All studies described increased sprouting of various axonal populations but, in most cases, the majority of regenerating axons was rerouted around the transplant and few fibres were seen distal to the site of injury. OEG implants may be better candidates for neurotrophic factor delivery since they can reduce the problem of regenerating axons to re-enter the distal part of the spinal cord and have advantages over the use of genetically engineered fibroblasts which are non-CNS in origin and may become tumorigenic.

Results

Ex vivo transduction of purified OEG

Three different viral vector systems (AAV, AdV and LV vectors; Figure 1) were tested for their capability to transfer foreign genes into purified cultures of adult rat OEG (Figure 2). Under the specific culture conditions used, virtually no transduction of primary OEG cultures was seen following infection with AAV type 2 (AAV-2) vectors encoding green fluorescent protein (GFP) up to 7 days after infection, even with extremely high multiplicity of infection (MOI) up to 1000. The quality and transducing capability of used AAV-GFP stocks was confirmed on human embryonic kidney (HEK) 293T cells that were infected in parallel with OEG cultures and showed efficient transduction, even at lower MOI (MOI 25), within 24 h following AAV-2 infection (see insert Figure 2). In contrast to AAV-2, AdV and LV vectors encoding either the bacterial marker enzyme β-galactosidase (β-gal; AdV-LacZ) or GFP (LV-GFP), respectively, efficiently transferred the gene of choice into cultured OEG. Many transduced cells expressing high levels of transgenic protein were observed at 3 days after infection. The efficiency of transduction increased in parallel with higher MOI (data not shown), with optimal MOI of 100 and 50 for AdV and LV, respectively. The optimal MOI was defined as the lowest dose of viral vector that was needed to transduce all cultured cells. To ascertain that the insertion of a new gene using viral vector-mediated gene transfer did not interfere with normal cell functioning, AdV vector- and LV vector-infected OEG cultures were phenotypically examined for expression of the general cell markers low-affinity neurotrophin receptor $p75^{NTR}$, Schwann cell marker S-100, and glial-fibrillary acidic protein (GFAP). No changes in cellular phenotype and cell marker expression were found (Figure 3).

AdV vector-mediated transgene expression in OEG implants

Suspensions of AdV-LacZ transduced OEG (MOI 100) were gently injected (1 μl; 100 000 cells) into the spinal cord immediately after unilateral lesion of the cervical (C4) rubrospinal tract (RST), both rostrally and caudally, at 1 mm distance from the lesion site (Figure 4). Analysis of transgene expression 7 days after implantation showed many β-gal-positive cells in the lesion area. All transgene-expressing cells were present in the Hoechst-positive area as expected since OEG were pre-labelled with Hoechst dye before implantation. Adjacent sections stained for $p75^{NTR}$ showed that β-gal-positive cells could be identified as $p75^{NTR}$-positive OEG, and were present in the centre of the lesion as determined by GFAP staining. Although a very low level of diffuse GFAP staining was found in $p75^{NTR}$-purified OEG *in vitro*, the levels of GFAP immunostaining in reactive astrocytes were too high to allow visualisation of co-localisation between GFAP and $p75^{NTR}$ signal in OEG implants (Figure 7a). Analysis at later survival points, 14 and 30 days after implantation respectively, showed a significant decline in the number of β-gal-expressing cells. Nevertheless, the detection of Hoechst-labelled cells implied that implanted OEG were still present at the lesion site. In contrast to the number of transgene-expressing cells, an increased number of $p75^{NTR}$-positive cells was observed compared with 7 days after implantation. Interestingly, at all time-points analysed, a discrepancy was observed in the overlap between Hoechst dye and $p75^{NTR}$ immunoreactivity. Many Hoechst-labelled nuclei were observed outside the $p75^{NTR}$-positive area suggesting leakage of Hoechst label from implanted cells into the surrounding tissue (Figure 4 and 7a). Visualisation of the lesion area using GFAP staining revealed that, based on $p75^{NTR}$

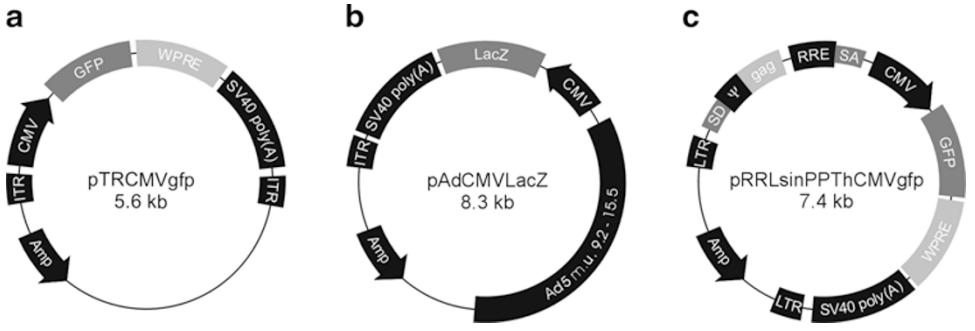

Figure 1 *Schematic drawings of the plasmid vector constructs used in this study. All viral constructs carry an internal cassette encoding either green fluorescent protein (GFP), for both AAV-2 (a) and LV (c) vectors, or the bacterial marker enzyme β-galactosidase (β-gal) in case of AdV vector (b), upstream of the Simian virus 40 (SV40) polyadenylation signal. In all vectors, transgene expression is driven by the cytomegalovirus (CMV) promoter. Both AAV-2 and LV vectors contain the cis-acting woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) to enhance transgene expression. Transgene expression cassettes were cloned in between either the inverted terminal repeats (ITRs) for AdV and AAV-2 vectors or long terminal repeats (LTRs) in case of LV vector. For LV vector, the following cis-acting sequences in the 'proviral' constructs are also labelled: major splice donor site (SD); encapsidation signal (ψ) including the 5′ portion of the gag gene; reverse transcriptase (Rev)-responsive element (RRE); and splice acceptor sites (SA). All plasmid vectors contained an ampicillin resistance gene (Amp) to allow selection for growth in E. Coli bacteria.*

immunoreactivity, implanted OEG remained present at the lesion site, but OEG did not migrate over large distances both rostrally and caudally into the spinal cord as suggested by the distribution of Hoechst labelling. Although intermingling of implanted OEG and reactive astrocytes at the borders of the lesion was observed, no sign of OEG migration at further distances, both proximal and distal from the lesion, was found.

Implantation of AdV-LacZ-transduced OEG in the intact spinal cord resulted in persistent expression of the transgene for at least 30 days (Figure 5). No decrease in expression was observed over time and many β-gal-positive cells were detected up to 30 days after implantation. Transgene-expressing cells were identified as implanted OEG based on Hoechst labelling and $p75^{NTR}$ staining. Staining for GFAP showed mild gliosis at the injection site, but no major tissue damage due to the injection procedure. Again, many Hoechst-positive nuclei displaying no immunoreactivity for both β-gal and $p75^{NTR}$ were detected in the neighbouring tissue surrounding the implant suggesting leakage of Hoechst label.

LV vector-mediated transgene expression in OEG implants

Suspensions of LV-GFP transduced OEG (MOI 50) were injected into the unilaterally lesioned C4 spinal cord as described above. *Ex vivo* transduction of OEG using LV vectors resulted in a persistent GFP expression, at least up to 4 months after implantation (Figure 6). Many GFP-positive cells were detected at survival points of 7, 14, 30 days and 4 months, respectively. Double staining for $p75^{NTR}$ was used to examine whether GFP-positive cells could be identified as implanted OEG. Most GFP-labelled cells were immunoreactive for $p75^{NTR}$, which, together with the characteristic bipolar morphology, showed them to be implanted OEG (Figure 7b). Again, intermingling of OEG and reactive astrocytes did occur, and no migration of OEG, either rostrally or caudally, across the astrocytic barrier into the spinal cord was observed (Figures 6 and 7c). All GFP-labelled cells were detected close to the original injection site, encapsulated by an astrocytic scar. Once more, due to differences in the expression levels of GFAP between OEG and reactive astrocytes, no co-localisation of GFAP and $p75^{NTR}$ signal could be visualised in OEG *in vivo* (Figure 7b). Additional triple stainings were used to visualise GFP-positive implanted OEG, the astrocytic scar, and neurofilament (NF)-positive axonal profiles at the lesion site. At 4 months after lesion, numerous NF-positive axon-like structures were detected in the implant itself, often in close contact with GFP-labelled OEG (Figure 7c).

Discussion

Implantation of OEG in the injured spinal cord has been reported to promote long-distance regeneration and functional recovery following spinal cord injury.[43] In the present study, we have investigated the possibility of *ex vivo* gene transfer to OEG. Three different vector systems were tested and found to behave differently in their capability to transduce OEG. Under the tissue culture conditions used, virtually no transduction of OEG was found following infection with AAV-2 vectors. This observation is in agreement with a previous study showing that Schwann cells, a cell type that is phenotypically related and very similar to OEG, are normally resistant to AAV-2 infection.[44] Moreover, preferential transduction of non-glial cells by AAV-2 vectors has been reported in the CNS.[29] The failure of AAV-2 vectors to transduce primary OEG might be due to the fact that these cells lack membrane-associated heparan sulphate proteoglycan, a component of the receptor for AAV particles.[45] In contrast to AAV-2, efficient transduction of OEG was observed by AdV and LV vectors. No changes in morphology and expression of general cell marker proteins (see Ref. 16) were detected after transduction with these vectors. Following *ex vivo* gene transfer with both AdV or LV vectors, implants of transduced OEG into the site of a spinal cord lesion were $p75^{NTR}$-positive, displayed typical bipolar OEG morphology, and high levels of transgenic protein could be detected during the first week after implantation. These findings suggest that gene transfer with AdV and LV vectors did not interfere with normal cell functioning.

Transgene expression in OEG implants subjected to AdV, but not LV vector-mediated gene transfer gradually decreased during the first month after implantation. Direct toxicity of AdV vectors, immunological rejection

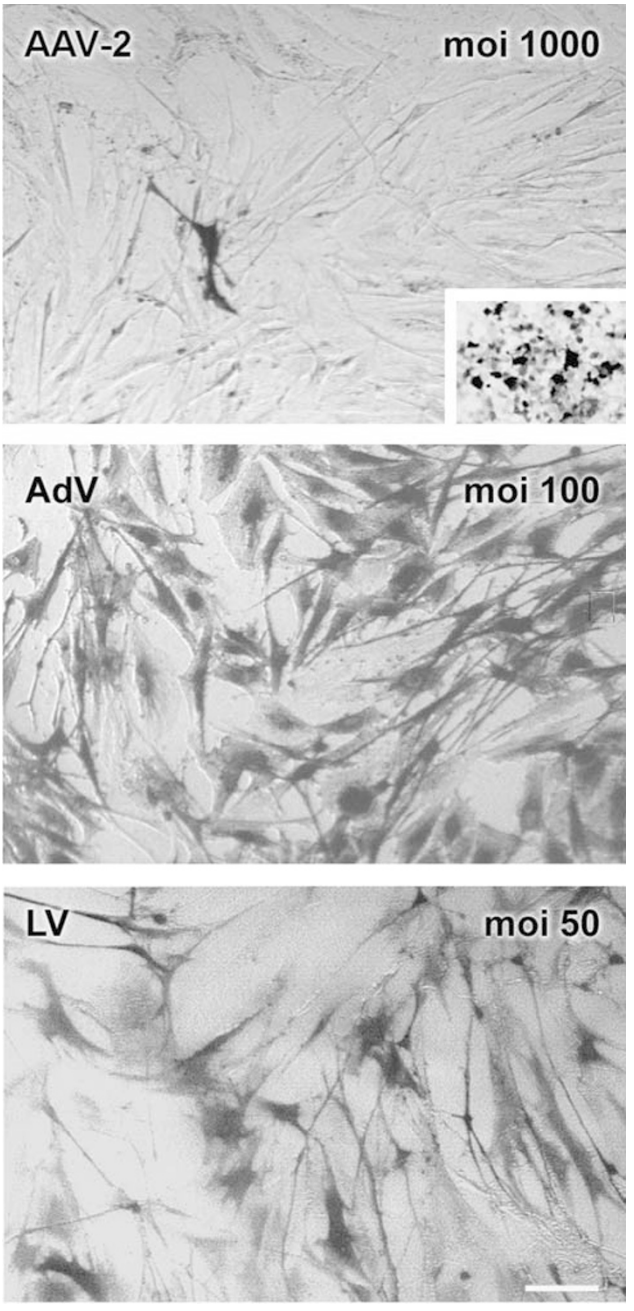

Figure 2 *Transduction of primary OEG cultures. (Top) Immunocytochemistry for GFP at 7 days after infection with AAV-2 vector (MOI 1000). Virtually no transduction of OEG cultures can be observed. In contrast to OEG, infection of HEK 293T cells with the same AAV-GFP (MOI 25; see insert) resulted in efficient transduction within 24 h. (Middle) Enzymatic staining for β-gal protein (X-gal staining) at 3 days following OEG transduction with AdV-LacZ (MOI 100). High levels of AdV-derived β-gal activity are visible in the vast majority of cultured OEG. (Bottom) GFP immunocytochemistry at 3 days following LV vector infection (MOI 50). Nearly all cultured OEG are transduced and express high levels of GFP. Transduced cells display typical bipolar OEG morphology and no signs of cytotoxicity can be observed following infection with AdV and LV vector. Scale bar, 25 μm.*

of implanted OEG, downregulation of cytomegalovirus (CMV) promoter activity, and the loss of recombinant viral genome may have contributed to this loss of transgene expression. Proliferation of implanted OEG at the lesion site may be the most likely cause for the observed decline in transgene expression because no loss of expression was observed in the intact spinal cord. Since first-generation E1-deleted AdV vectors do not integrate into the host genome or autoreplicate, the episomally located vector genome will be lost during the process of mitosis resulting in a concomitant decline in transgene expression.[46] Recently, members of the neuregulin family were described as survival factors for OEG and able to induce a mitogenic response in these cells.[47,48] Interestingly, these neuregulins are highly expressed by reactive astrocytes at the lesion site in the injured CNS.[49] In the intact spinal cord, only minimal proliferation of OEG is expected because of very little astrocytic scarring and contact inhibition. Based on the increase in $p75^{NTR}$ immunoreactivity over time, proliferation of implanted OEG in the lesioned spinal cord seems to occur although the additional staining for $p75^{NTR}$ could also be due to Schwann cells that have been reported previously to invade spinal cord lesion sites.[50] Since there are no cellular markers to distinguish between host Schwann cells and implanted OEG, this possibility cannot be ruled out completely. However, the use of stable LV vector transduced OEG implants, expressing GFP as a specific and reliable marker to visualise implanted OEG, clearly showed that the vast majority of GFP-labelled cells co-expressed $p75^{NTR}$. Immunoreactivity for $p75^{NTR}$ outside the confines of the GFP labelling could only be observed to a minor extent at the lateral edges of the spinal cord, close to the dorsal root entry zone or in the root itself. This demonstrates that there was only very limited invasion of Schwann cells into the injury site which on its own cannot explain the observed increase in $p75^{NTR}$ labelling. Direct cellular toxicity of AdV vectors has been reported after application directly into the CNS,[51] but not using an *ex vivo* approach.[52] It is also unlikely that immunological rejection or an inflammatory response against transduced OEG implants was responsible for the decline in transgene expression because syngenic cells were used for implantation and, as described earlier, no loss of transgene expression was found when AdV-transduced OEG were injected into intact spinal cord. Downregulation of CMV promoter activity in certain areas in the CNS has been suggested previously,[29] and may have contributed to the gradual decline in transgene expression. However, persistent AdV-derived β-gal expression in OEG implanted into intact spinal cord seems to rule out this possibility as does the fact that no loss of CMV promoter-driven GFP expression was found when using LV vectors.

The transient gene expression in OEG implants, using AdV vector-mediated gene transfer, allows the possibility of manipulating the growth-promoting properties of OEG and microenvironment of the lesion during the acute phase following injury. Interestingly, long-term effects on outgrowth of lesioned corticospinal tract axons have been reported following transient expression of neurotrophins.[53] These long-term effects of transient AdV vector-mediated neurotrophic factor expression are most likely the result of priming of axonal outgrowth, that is thought to counteract the inhibitory activity in the lesion environment.[54]

In situations that would require OEG implants displaying prolonged foreign gene expression, eg to promote long-distance regeneration through large contusion lesions, stable transduced OEG may be needed. In agree-

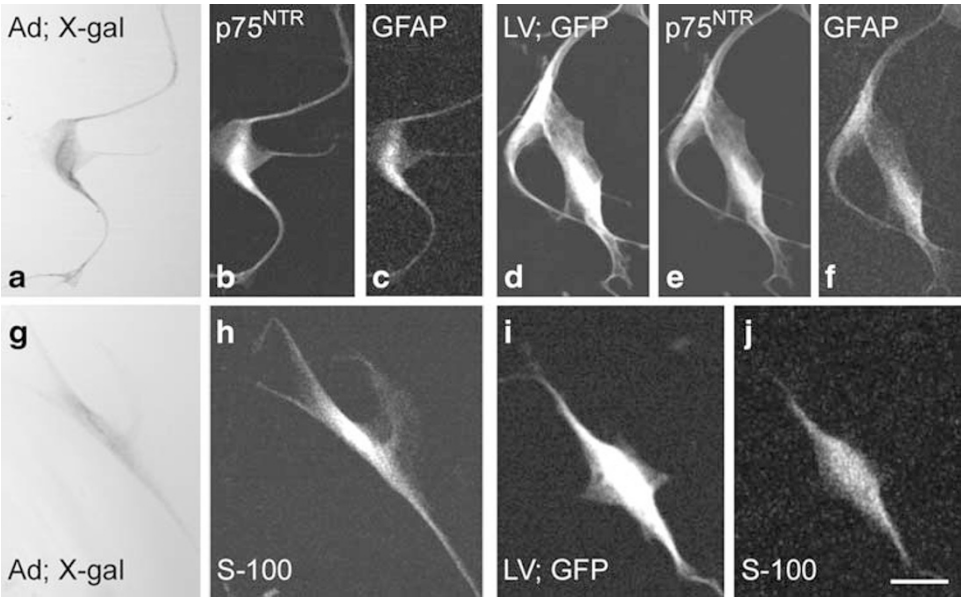

Figure 3 *Phenotypic characterisation of AdV and LV vector-transduced OEG. (a, g) AdV-LacZ vector-transduced OEG (MOI 100; X-gal stained) at 7 days after infection display typical bipolar OEG morphology and still express general cell markers p75^{NTR} (b), low levels of astrocyte marker GFAP (c), and Schwann cell marker S-100 (h). LV transduced OEG (MOI 50) showing high levels of native GFP fluorescence (d, i) are still immunoreactive for p75^{NTR} (e), low levels of GFAP (f), and S-100 (j). No signs of toxicity or loss of OEG marker expression can be observed, indicating that AdV and LV vector-mediated gene transfer does not interfere with OEG phenotype. Scale bar, 12 μm.*

ment with our hypothesis that AdV vector-mediated transgene expression was lost as a consequence of degradation and loss of vector DNA during OEG proliferation, *ex vivo* transduction of OEG using integrating LV vectors resulted in persistent transgene expression for at least 4 months after implantation. Numerous NF-positive axons were detected within the implants and in close association with GFP-labelled OEG. This allows us to start investigating the interactions between implanted OEG and lesioned CNS axons in greater detail. Implantation of stable transduced OEG, using regulatory gene expression if desired,[55] may be a very promising strategy to counteract ongoing degeneration of neural tissue and to stimulate enhanced regeneration.

Using LV-derived GFP as a reliable and specific intracellular marker for implanted OEG, no migration of these cells over larger distances both proximal and distal from the lesion into the spinal cord was observed. Others, using Hoechst dye as a label to track implanted cells, have reported migration of OEG over large distances and hypothesised that this resulted in the long-distance regeneration of lesioned spinal axons through and beyond OEG implants.[20,21,36] However, identification of implanted cells based on Hoechst-labelling has to be interpreted very carefully because of the potential leakage of Hoechst dye from implanted cells into the neighbouring host tissue.[56,57] Our present study, using viral vector-mediated gene transfer, suggests an alternative view which is that this regeneration is in fact not a result of OEG migrating along with regenerating axons. In all rats, GFP-labelled OEG could be detected in the central compartment of the scar tissue, intermingling with GFAP-positive reactive astrocytes surrounding the lesion site, but OEG did not migrate across the astrocytic barrier into the host spinal cord. These results suggest that injected OEG into lesion sites are supportive 'bridging' substrates for CNS axons to cross the hostile scar environment, but that migration of OEG is not necessary for long-distance regeneration. There is evidence showing that intact adult myelinated white matter tracts can be highly permissive for regeneration,[58] supporting the suggestion that OEG migration is not a prerequisite for regeneration beyond the lesion site.

In summary, these results demonstrate that both AdV and LV vectors are useful tools for *ex vivo* gene transfer to OEG and can be exploited in future studies to enhance regeneration in the injured CNS. Furthermore, stable transduction of OEG using LV vectors can be used to reliably track implanted cells, and obtain better understanding of OEG behaviour and signalling in the lesioned spinal cord. As discussed earlier, the use of OEG implants as cellular conduits to stimulate regeneration in the injured CNS may have some advantages over the use of Schwann cells or fibroblasts making them ideal cellular targets for genetic engineering and neurotrophic factor production. Moreover, the recent identification and characterisation of a human olfactory ensheathing cell, with properties very similar to rat OEG,[59] opens possibilities for future clinical applications.

Materials and methods

Experimental animals

Adult female Fischer (F344) inbred rats (175–200 g; Harlan, Oxford, UK) were housed under standard conditions. All animals had free access to water and food. The animal experiments were conducted in accordance with the guidelines of the local animal care committee.

Viral vector production

The AAV-2 vector used in this study was constructed by inserting 'humanized' GFP.[60] Briefly, GFP cDNA was cloned into pcDNA I/AMP yielding the plasmid pc5-

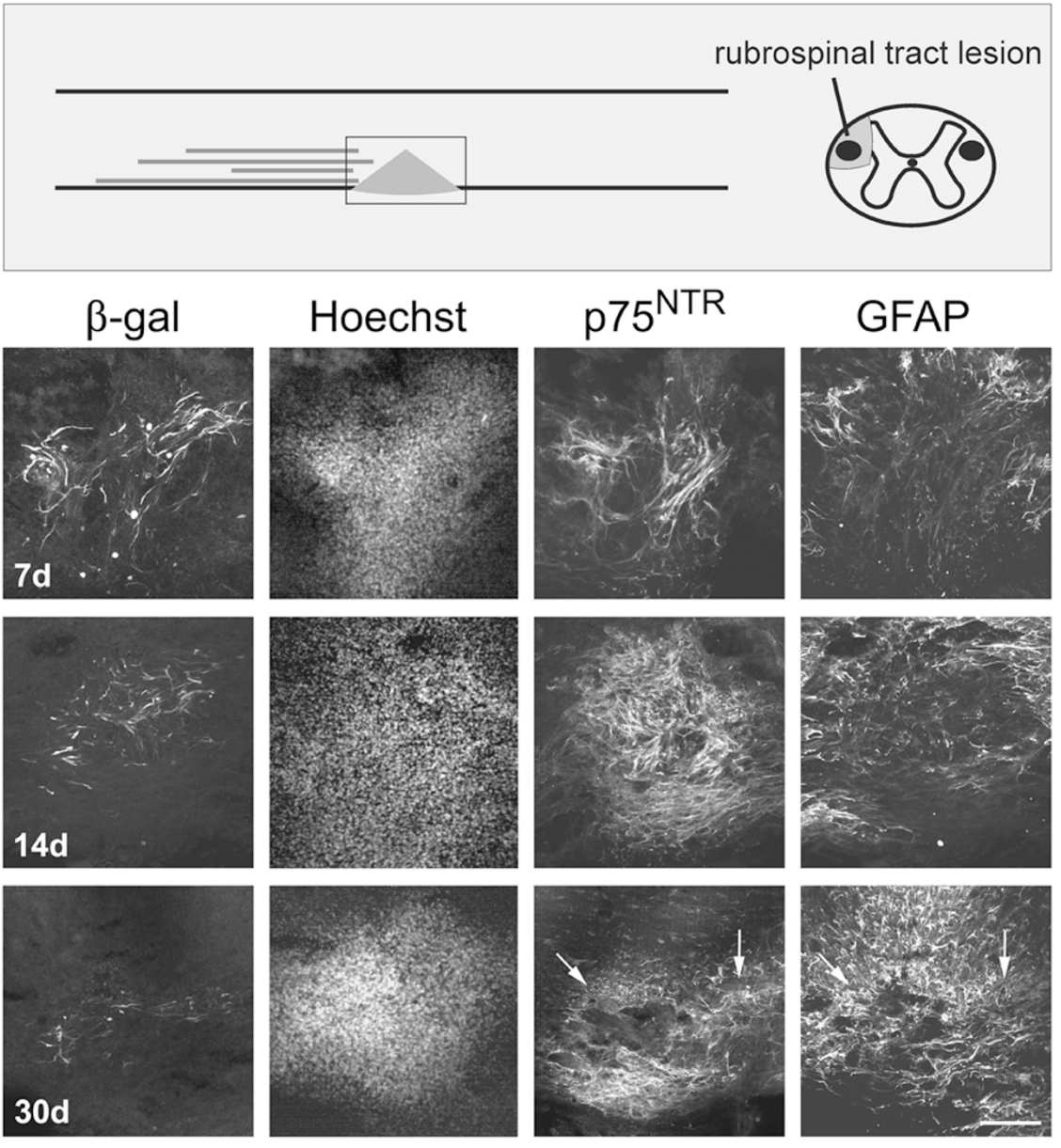

Figure 4 *Analysis of AdV vector-mediated gene expression in OEG following implantation into the lesioned spinal cord. Schematic representations of a C4 RST lesion (top right) and plane of sectioning (top left) are shown. Horizontal sections through the lesion area show numerous implanted OEG expressing high levels of the transgene at 7 days after implantation, as determined by immunohistochemical staining for β-gal. A gradual decline in the number of transgene-expressing cells can be observed in time, and transgenic β-gal expression is almost completely lost at 30 days after implantation. However, the detection of many Hoechst-labelled and p75^{NTR}-positive cells implies that implanted OEG are still present at the lesion site, but have lost transgene expression. Intermingling of p75^{NTR}-positive OEG with reactive astrocytes (GFAP staining) surrounding the lesion area can be easily observed at 30 days after implantation (arrows), but no migration of p75^{NTR}-positive cells into the host spinal cord, either rostrally or caudally, from the lesion is found. Finally, the detection of Hoechst-positive nuclei outside the p75^{NTR}-immunoreactive area suggests redistribution of Hoechst dye from implanted cells to host tissue. Scale bar, 200 μm.*

GFP. Subsequently, pTR-CMV-GFP was constructed by insertion of the transgene expression cassette between the inverted terminal repeats (ITRs) of pTR-UF. Downstream of the GFP cDNA, the *cis*-acting woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) was cloned in to enhance vector-derived transgene expression.[61,62] The construction of the helper plasmid pDG, needed for the production of recombinant AAV-2 vector particles, was described previously.[63] Recombinant AAV-2 particles were produced as described by the method of Hermens and colleagues.[64] Briefly, the vector plasmid pTR-CMV-GFP and the helper plasmid pDG were co-transfected into HEK 293T cells using calcium phosphate precipitation. The medium was replaced after 6 h and the cells were incubated for 48 h at 37°C and 5% CO_2. Next, the cells were dislodged, harvested and freeze–thawed three times to release AAV particles from the cells. Cell debris was removed using low-speed centrifugation. The supernatant was loaded on a Matrix Cellufine-sulphate bead column (Amicon, Danvers, MA, USA). After several washings with 0.1 M phosphate-buffered saline pH 7.4 (0.9% NaCl; PBS), the virus was eluted

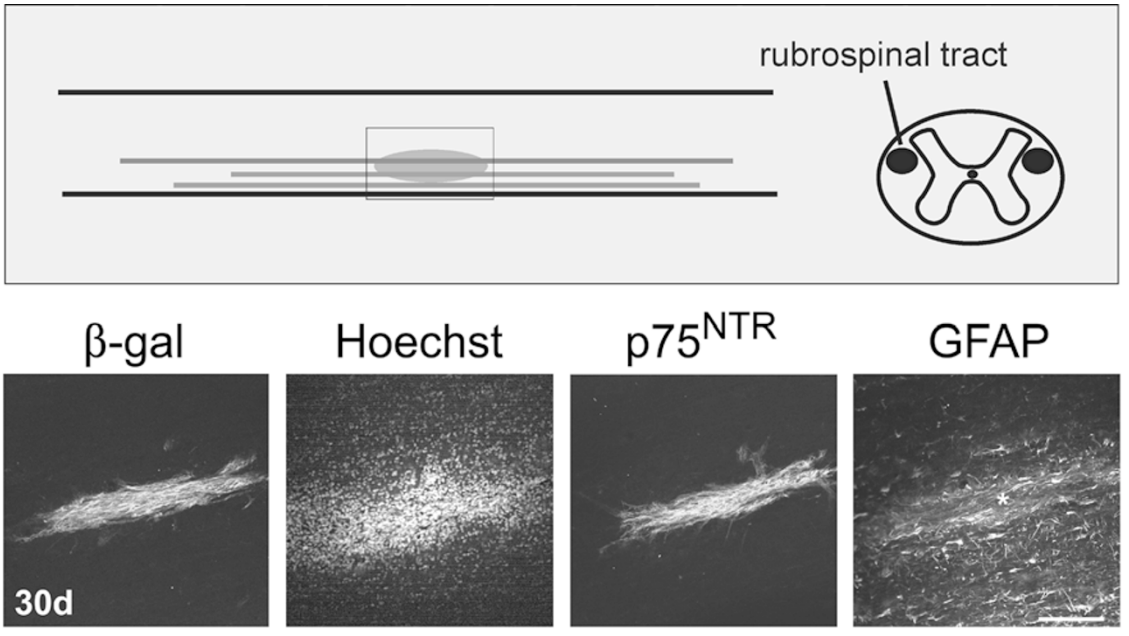

Figure 5 *AdV vector-mediated gene expression in OEG implants in the intact spinal cord. (Top) Schematic representation of OEG implantation close to RST projection and plane of sectioning. Horizontal sections through the site of implantation were analysed for transgene expression, Hoechst labelling and immunoreactivity for p75^{NTR} and GFAP, at 30 days after implantation. In contrast to the lesioned spinal cord, transgene expression persists for at least 30 days after implantation in the intact spinal cord. Numerous cells are detected expressing high levels of β-gal protein and can be identified as implanted OEG based on p75^{NTR} immunoreactivity. Immunohistochemistry for GFAP shows only mild gliosis around the implant (asterisk) due to the injection procedure, but no severe scarring. As in lesioned spinal cord, Hoechst-labelling does not match with the pattern of p75^{NTR} immunoreactivity. Many Hoechst-positive nuclei can be observed in tissue that is not immunoreactive for both β-gal and p75^{NTR}, indicating leakage from implanted cells into the neighbouring tissue. Scale bar, 200 μm.*

from the column with PBS containing 1 M NaCl. Next, viral particles were banded on an Iodixanol (Nycomed Pharm, Oslo, Norway) density gradient using ultracentrifugation and fractions of about 300 μl were collected from the bottom of the gradient. Recombinant AAV-2 stock titres were determined by an infectious unit centre assay,[65] and were in the range of 10^{10} infectious units/ml. To reduce viscosity of the Iodixanol, AAV-2-containing fractions were diluted 10 times with PBS and re-concentrated on a Centricon-100 concentrator (Amicon).

First generation replication-deficient AdV vector stocks encoding the bacterial marker gene β-gal were produced using standard procedures as described previously.[66,67] Briefly, an AdV-targeting plasmid was generated by insertion of an expression unit of the *LacZ* gene, under the control of the CMV promoter, downstream of the adenovirus serotype 5 (AdV-5) ITR (map units 0–1.25) and upstream of the map units 9.2–15.5 of the wild-type AdV genome. The AdV-targeting plasmid was linearised by *Sal*I digestion. The AdV-5 dl309 DNA was digested with *Cla*I and *Xba*I, deleting the E1 gene region of the viral genome. Linearised targeting plasmid was then co-transfected with digested wild-type AdV DNA into the human retinoblastoma 911 producer cells.[68] Plaques were isolated and characterised by PCR for the right recombinant viral clone. Next, purified plaques were expanded on 911 cells, isolated and purified by two rounds of CsCl density gradient centrifugation. Recombinant AdV bands were collected from the gradient, dialysed against TS buffer pH 7.4 (137 mM NaCl, 6 mM KCl, 0.7 mM Na_2HPO_4, 1.1 mM $MgCl_2$, 0.9 mM $CaCl_2$, 25 mM Tris-HCl) and stored in TS buffer containing 10% glycerol at –80°C. Titres of AdV vector stocks were determined using plaque assays on 911 cells and expressed as plaque forming units per ml (p.f.u./ml). Stock titres were in the range of 10^{10}–10^{11} p.f.u./ml.

The production of self-inactivating LV vectors has been described in detail previously.[69] The plasmids needed for the production of GFP-encoding LV vector were generously provided by Drs L Tamagnone and L Naldini (Institute for Cancer Research, University of Torino, Italy). Stocks of LV-GFP were generated by cotransfection of three plasmids, the viral core packaging construct pCMVdeltaR8.74, the VSV-G envelope protein vector pMD.G.2, and the transfer GFP vector pRRLsin-PPThCMV-GFP-wpre into HEK 293T cells.[70,71] In brief, a total of 5×10^6 cells were seeded in 10-cm dishes 24 h before transfection in Iscove's modified Dulbecco culture medium, containing 10% foetal calf serum, penicillin (100 IU/ml), 100 μg/ml streptomycin and 2 mM Glutamax (Sigma, Zwijndrecht, The Netherlands). The culture medium was refreshed 2 h before transfection. Using a modified calcium phosphate method, HEK 293T cells were transfected with 3.5 μg envelope plasmid, 6.5 μg packaging plasmid and 10 μg GFP-expressing gene transfer plasmid per 10-cm dish. The next day, the medium was refreshed. Virion-containing medium was harvested 24 h later, and debris removed from the medium by low-speed centrifugation and filtering through a 0.22-μm cellulose acetate filter. The number of transducing particles was defined by infecting HEK 293T cells and counting the number of GFP-expressing cells after 48 h. Recombinant stock titres were expressed as transducing units (TU) per ml and ranged in the order of 10^7 TU/ml.

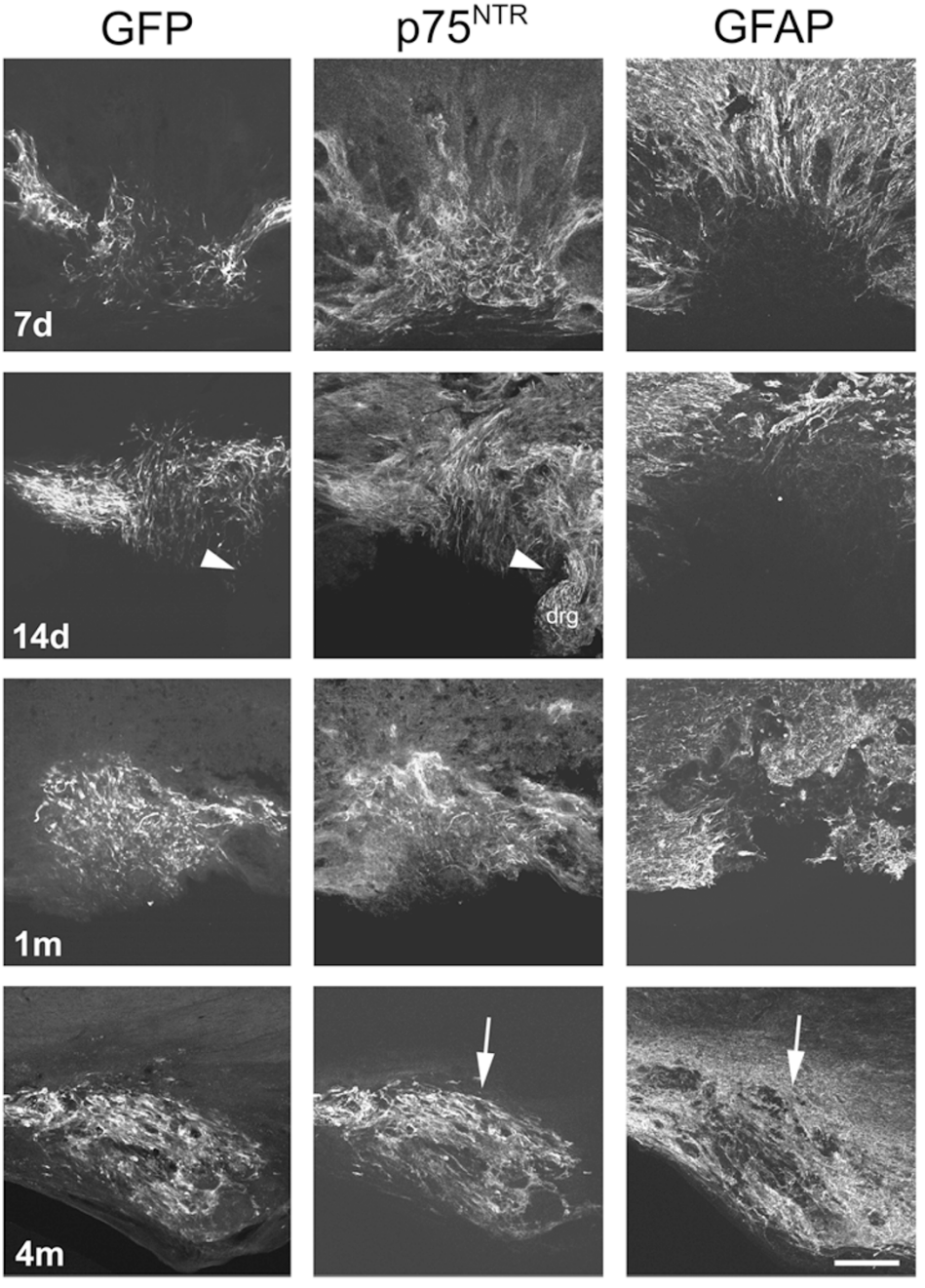

Figure 6 *Analysis of LV vector-mediated gene expression in OEG implants in the lesioned spinal cord. Persistent expression of GFP is found in LV-transduced OEG at least up to 4 months after implantation as determined by native GFP fluorescence. GFP-positive cells are immunoreactive for p75^{NTR} and 'bridging' the lesion gap that is surrounded by a GFAP-positive border of reactive astrocytes. Intermingling of implanted OEG with reactive astrocytes does occur (arrows), but no migration of stable transduced OEG beyond the astrocytic scar is detected. Additional p75^{NTR}-staining, probably Schwann cells, outside the confines of GFP-labelling is observed to some extent at the lateral edges of the spinal cord (arrowheads) close to or in the dorsal roots (drg, dorsal root ganglion). Scale bar, 250 μm.*

OEG cultures and purification

Primary OEG cultures were prepared as described previously.[21] Adult (3–4 months old) female Fischer rats were killed by an overdose of Nembutal (Rhone Merieux, Pinkenba, Queensland, Australia), and the olfactory bulbs dissected and put into Leibovitz's-15 medium (L-15) (Sigma, St Louis, MO, USA). The pia was carefully removed and the olfactory nerve fibre and glomerular layers were dissected away from the rest of the bulb. The tissues were then cut into 1-mm^3 pieces and incubated with 0.25% trypsin (GibcoBRL, Grand Island, NY, USA) containing 50 mg/ml DNase (GibcoBRL) at 37°C for 60 min with continual shaking. Trypsinisation was stopped by adding Dulbecco's modified Eagle's medium (DMEM; Sigma) and Ham's F-12 (D/F-12; Sigma; 1:1 mixture) supplemented with 10% foetal bovine serum (DF-10S; GibcoBRL) and 50 mg/ml gentamicin (GibcoBRL).

The method for the purification of OEG from primary

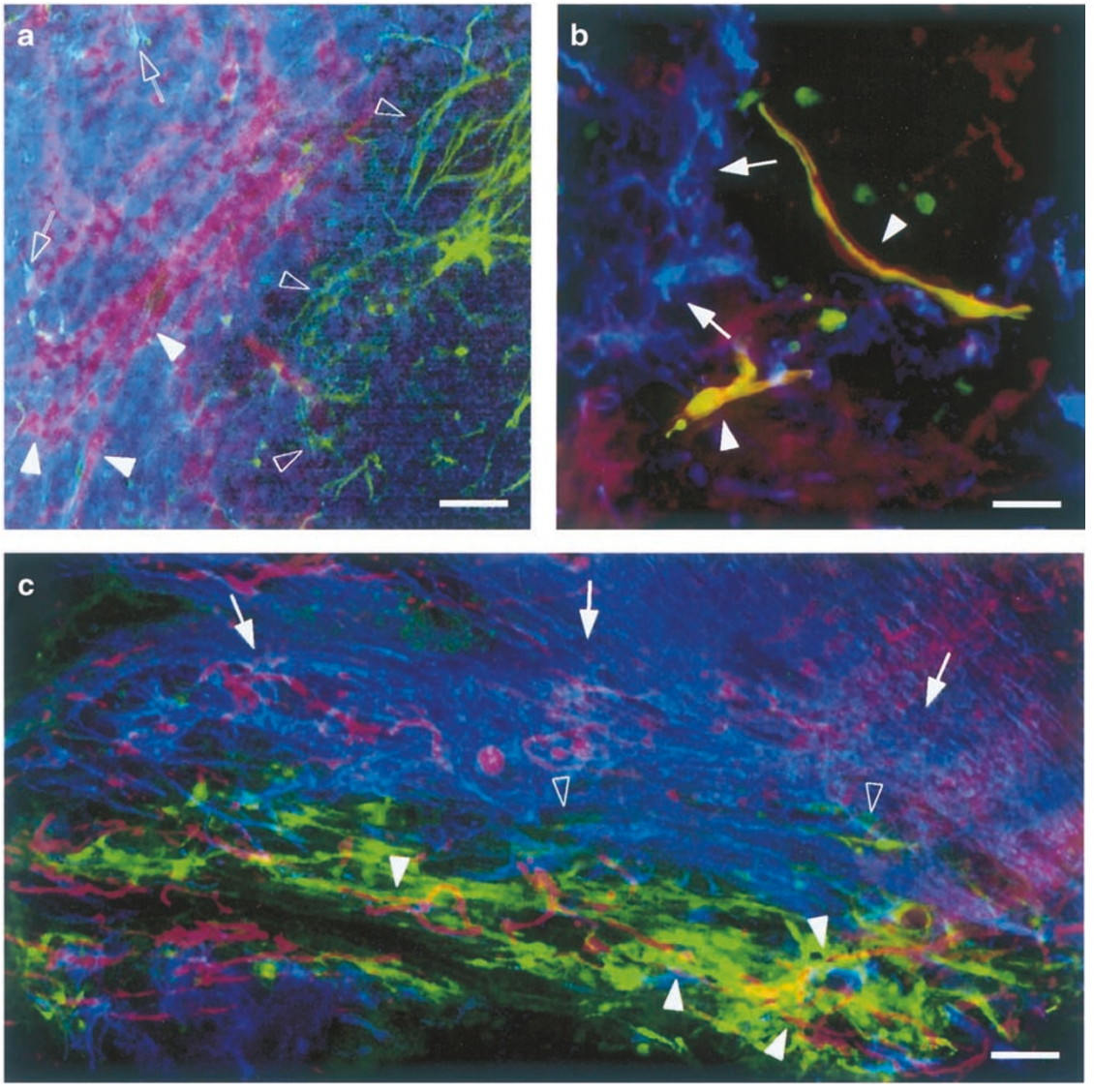

Figure 7 *Characteristics of transduced OEG implants in the lesioned spinal cord. (a) High magnification confocal microscope image of Hoechst (blue)/p75^{NTR} (red)-labelled OEG (arrowheads) at the lesion site, 7 days after implantation. Many Hoechst-positive nuclei can be observed outside the p75^{NTR}-immunoreactive area, suggesting leakage of Hoechst dye from implanted cells into host tissue. GFAP-positive reactive astrocytes (green; open arrowheads) are surrounding the lesion site. Numerous GFAP-positive/p75^{NTR}-negative cells are double labelled for Hoechst dye (open arrows) indicating redistribution of Hoechst dye to host astrocytes. (b) Confocal image of LV vector-transduced OEG (arrowheads) at higher magnification, 1 month after implantation in the lesioned spinal cord. Note that the cytoplasmic GFP signal (green) is co-expressed with membrane-associated p75^{NTR} staining (red), and that the GFAP (blue) is not expressed by implanted OEG. (c) Confocal microscope image of a horizontal section through a LV-GFP-transduced OEG implant, 4 months after injury. Many GFP-labelled OEG (green) are visible at the lesion site, surrounded by a GFAP-positive astrocytic scar (blue; see arrows). Intermingling of GFAP-positive astrocytes and GFP-labelled OEG can be observed (open arrowheads) throughout the whole implant. Numerous NF-positive axons (red; arrowheads) are visible at the lesion site, often in close association with implanted OEG. Scale bar, 75 μm (a), 25 μm (b), 50 μm (c).*

cultures was modified from the original protocol.[21] After 6–7 days, the OEG were separated from the other cells in the primary cultures by immunoaffinity, using an antibody against p75^{NTR}. The cells in primary culture were detached using 0.05% trypsin and 0.02% EDTA (GibcoBRL), centrifuged and washed twice with DF-10S. These cells were suspended in L-15 medium and plated on 100-mm petri dishes that had been pretreated with a series of antibodies as follows. Firstly, 100-mm dishes were incubated overnight at 4°C with a mixture of anti-mouse IgG, IgA and IgM antibodies (1:100; ICN Biomedicals, Seven Hills, New South Wales, Australia) and were washed three times with L-15 medium. The dishes were then incubated with p75^{NTR} antibody (a gift of Dr P Wood, University of Miami School of Medicine, Miami, FL, USA) at 1:5 dilutions in L-15 medium with 5% foetal bovine serum for 2 h at 4°C. The dishes were then washed three times with L-15 medium, and a 10-ml suspension of cells from the olfactory bulbs was plated on to the antibody-treated dishes at a density of 4×10^5 cells per dish for 30 min at 4°C. The dishes were washed five times with L-15 medium to remove unbound cells from the dishes. The bound cells were then removed from the dishes with a cell scraper (Sarstedt, Newton, NC, USA), centrifuged and re-suspended in DF-10S. The cells were then seeded on to poly-L-lysine-coated 100-mm dishes

and fed with DF-10S containing 20 μg/ml pituitary extract and 2 μM forskolin (Sigma).

Ex vivo transduction of OEG cultures

To determine transduction efficiency of cultured OEG using AAV-2, AdV and LV vectors, cells were seeded on to poly-L-lysine-coated 24-well plates at a density of 10^5 cells per well and fed overnight with DF-10S containing 20 μg/ml pituitary extract and 2 μM forskolin. The next day, the medium was replaced with DF-10S containing 2 μg/ml pituitary extract and 0.2 μM forskolin to reduce OEG proliferation. Recombinant AAV-2, AdV and LV vectors encoding the bacterial marker gene β-gal (AdV-LacZ) or GFP (AAV-GFP, LV-GFP) respectively, were added with increasing MOI (AAV-2: MOI 1, 50, 100, 500, 1000; AdV: MOI 1, 5, 10, 25, 50, 100 and 250; LV: MOI 1, 5, 10, 25, 50) to each well. Cultures were analysed for transgene expression 3 or 7 (in case of AAV-2 infection) days after infection by enzymatic staining of β-gal using the 5-bromo-4-chloro-3-indolyl β-D-galactosidase substrate (X-gal staining) or immunocytochemistry for GFP (1:50; rabbit polyclonal antibody; Chemicon, Harrow, UK) using standard procedures with 3,3′-diaminobenzidine tetrachloride (DAB) as a chromophore.

Preparation of transduced OEG suspensions for implantation

Primary cultured OEG were detached by trypsinisation, centrifuged and washed twice in DF-10S. These cells were resuspended in DF-10S, counted and seeded into 60-mm dishes at a density of 10^6 cells per dish. Cells were fed overnight with DF-10S containing 2 μg/ml pituitary extract and 0.2 μM forskolin. The medium was then replaced by medium containing 10^8 p.f.u. AdV-LacZ (MOI 100) or 10^7 TU LV-GFP (MOI 50). The next day, cells were pre-labelled using 10 μg/ml Hoechst 33342 (bis-benzimide; Sigma) for 3 min at 37°C. Cultures were then washed three times with L-15 medium, detached by trypsinisation, centrifuged and washed twice with serum-free D/F-12. Cells were resuspended in D/F-12, counted and pelleted by low-speed centrifugation. Pelleted OEG were carefully resuspended in an appropriate volume of D/F-12 yielding an OEG suspension of 10^5 cells/μl. Viability of OEG suspensions were in the range of 95–100% as determined by trypan blue staining. Suspensions were kept on ice until the end of surgical procedures.

Surgery

Adult female Fischer F344 rats (n = 32; 175–200 g) were deeply anaesthetised intramuscularly with ketamine (100 mg/kg body weight; Nimatek, Eurovet, Bladel, The Netherlands) and xylazine (10 mg/kg body weight; Rompun, Bayer, Leverkussen, Germany). The neck musculature was split at the midline and the cervical spinal cord exposed by dorsal laminectomy of the fourth cervical vertebra (C4). Next, the dura was opened using a small hooked 30-G needle allowing direct access to the spinal cord. For implantation of transduced OEG in lesioned spinal cord, the dorsolateral funiculus of the spinal cord containing the RST was lesioned with a pair of microscissors. First, the midline of the spinal cord and dorsal root entry zone were identified. Next, the left dorsolateral funiculus was cut, as deep as 1 mm from the dorsal surface of the spinal cord (Figure 4). Such a lesion completely disrupted the dorsolateral funiculus, including the RST, and partially lesioned the grey matter. The rostrocaudal extent of the lesion cavity was approximately 0.5–1 mm. Suspended OEG were microinjected 1 mm proximal and distal from the lesion site (1 μl; approximately 10^5 cells) per injection (depth 0.6 mm) using a small glass capillary (tip diameter 60 μm) attached to 10 μl Hamilton syringe needle. In case of OEG implantation in the intact spinal cord, a similar surgical procedure was followed, except for unilateral lesioning of the dorsolateral spinal cord. OEG suspensions were directly injected into the dorsolateral funiculus (1 μl approximately 10^5 cells), in the vicinity of the RST. Injections were performed using a Harvard microinjection apparatus (0.2 μl per min for 5 min). The injector was left in place for 1 min to allow diffusion from the injection site and then carefully removed. Muscles and skin were closed in separate layers. Animals received a post-operative subcutaneous injection of 2 ml physiological salt solution, to compensate for blood loss, and Finadyne (2.4 mg/kg body weight; Schering-Plough, Brussels, Belgium) to suppress pain. At the end of surgery, viability of OEG suspensions was still over 95% as determined by trypan blue staining.

Tissue preparation

Animals were killed at the survival times of 1, 2, 4 and 16 weeks (LV-transduced OEG only) weeks after implantation (n = 4 per time-point). The rats were deeply anaesthetised with sodium pentobarbital (75 mg/kg body weight i.p.; Nembutal, Sanofi Sante, Maassluis, The Netherlands) and transcardially perfused with 100 ml saline followed by 300 ml ice-cold 4% paraformaldehyde (PFA) in PBS. Spinal cords were carefully dissected and post-fixed overnight in 4% PFA solution at 4°C. The tissue was then washed several times with PBS, embedded in 10% gelatine (Difco, Detroit, MI, USA) in PBS, and fixed overnight in 4% PFA solution at 4°C.[72] Consecutive horizontal vibratome sections (40 μm) were cut in PBS and collected in serial vials.

Immunohistochemistry

Vibratome sections were washed three times in Tris-buffered saline pH 7.4 (10 mM Tris/HCl). To enhance tissue penetration and block unspecific antibody binding sites, sections were incubated in PBS containing 0.3% Triton X-100 and 5% foetal calf serum for 1 h at room temperature. Adjacent sections were double immunolabelled for the transgene product β-gal (gal-13, 1:2000; mouse monoclonal antibody, Sigma) or GFP (1:50; rabbit polyclonal antibody, Chemicon), $p75^{NTR}$ (1:50; mouse monoclonal antibody, Roche Diagnostics, Almere, The Netherlands) and GFAP (1:1000; rabbit polyclonal antibody; Dako, Carpinteria, CA, USA). To visualise NF-positive axons, implanted OEG and reactive astrocytes at the lesion site, native GFP fluorescence was used, combined with immunolabelling for NF (1:1000; mouse monoclonal antibody 2H3; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA) and GFAP. For all stainings, primary antibody incubations were performed overnight at 4°C. The next day, sections were washed and incubated with a mixture of fluorophore-conjugated secondary antibodies for 1 h at room temperature: SAR-FITC/DAR-Cy5 (depending on whether native GFP fluorescence was used which interfered with FITC signal) and DAM-Cy3 (all 1:400; Jackson ImmunoResearch Lab-

oratories, West Grove, PA, USA). Sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and coverslipped. Digital photo-images were captured using a Zeiss confocal laser scanning microscope and imported Adobe Photoshop 5.5.

Acknowledgements

Work in the laboratory of J Verhaagen was funded by the Netherlands Organisation for Scientific Research (Pioneer NWO-GMW grant No. 030-94-142) and the Christopher Reeve Paralysis Foundation (research grant No. VBC2-9901-2), and in the laboratory of GW Plant by the National Health and Medical Research Council (research grant No. 990067 to GWP and ARH) and the Neurotrauma Research Program (research grant No. GNT005/006). We thank Dr L Tamagnone and G Gilestro for sharing their expertise on the production of LV vector stocks, and Dr P Wood for providing us with the $p75^{NTR}$ antibody. The 2H3 monoclonal antibody, developed by Drs TM Jessel and J Dodd, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. We are grateful to J Wortel for excellent technical assistance with the animal work, and to G van der Meulen for technical preparation of the figures.

References

1 Horner PJ, Gage FH. Regenerating the damaged central nervous system (review). *Nature* 2000; **407**: 963–970.
2 Fernandes KJ *et al*. Influence of the axotomy to cell body distance in rat rubrospinal and spinal motoneurons: differential regulation of GAP-43, tubulins, and neurofilament-M. *J Comp Neurol* 1999; **414**: 495–510.
3 Liebl DJ, Huang W, Young W, Parada LF. Regulation of Trk receptors following contusion of the rat spinal cord. *Exp Neurol* 2001; **167**: 15–26.
4 Stichel CC *et al*. Inhibition of collagen IV deposition promotes regeneration of injured CNS axons. *Eur J Neurosci* 1999; **11**: 632–646.
5 Bovolenta P, Wandosell F, Nieto-Sampedro M. Characterization of a neurite outgrowth inhibitor expressed after CNS injury. *Eur J Neurosci* 1993; **5**: 454–465.
6 Pasterkamp RJ *et al*. Expression of the gene encoding the chemorepellent semaphorin III is induced in the fibroblast component of neural scar tissue formed following injuries of adult, but not neonatal CNS. *Mol Cell Neurosci* 1999; **13**: 143–166.
7 Pasterkamp RJ, Anderson PN, Verhaagen J. Peripheral nerve injury fails to induce growth of lesioned ascending dorsal column axons into spinal cord scar tissue expressing the axon repellent Semaphorin 3A. *Eur J Neurosci* 2001; **13**: 457–471.
8 Chen MS *et al*. Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. *Nature* 2000; **403**: 434–439.
9 Mukhopadhyay G *et al*. A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron* 1994; **13**: 757–767.
10 David S, Aguayo AJ. Axonal elongation into peripheral nervous system 'bridges' after central nervous system injury in adult rats. *Science* 1981; **214**: 931–933.
11 Xu XM *et al*. Bridging Schwann cell transplants prompt axonal regeneration from both the rostral and caudal stumps of transected adult rat spinal cord. *J Neurocytol* 1997; **26**: 1–16.
12 Guest JD *et al*. The ability of human Schwann cell grafts to promote regeneration in the transected nude rat spinal cord. *Exp Neurol* 1997; **148**: 502–522.
13 Plant GW, Ramon-Cueto A, Bunge MB. Transplantation of Schwann cells and ensheathing glia to improve regeneration in adult spinal cord. In: Ingoglia NA, Murray M (eds). *Axonal Regeneration in the Central Nervous System*. Marcel Dekker: New York, 2000, pp 529–561.
14 Plant GW, Bates ML, Bunge MB. Inhibitory proteoglycan immunoreactivity is higher at the caudal than the rostral Schwann cell graft-transected spinal cord interface. *Mol Cell Neurosci* 2001; **17**: 471–487.
15 Tan MM, Harvey AR. Retinal axon regeneration in peripheral nerve, tectal, and muscle grafts in adult rats. *J Comp Neurol* 1999; **412**: 617–632.
16 Ramon-Cueto A, Avila J. Olfactory ensheathing glia: properties and function (review). *Brain Res Bull* 1998; **46**: 175–187.
17 Ramon-Cueto A, Nieto-Sampedro M. Regeneration into the spinal cord of transected dorsal root axons is promoted by ensheathing glia transplants. *Exp Neurol* 1994; **127**: 232–244.
18 Li Y, Field PM, Raisman G. Regeneration of adult rat corticospinal axons induced by transplanted olfactory ensheathing cells. *J Neurosci* 1998; **18**: 10514–10524.
19 Li Y, Field PM, Raisman G. Repair of adult rat corticospinal tract by transplants of olfactory ensheathing cells. *Science* 1997; **277**: 2000–2002.
20 Ramon-Cueto A, Cordero MI, Santos-Benito FF, Avila J. Functional recovery of paraplegic rats and motor axon regeneration in their spinal cords by olfactory ensheathing glia. *Neuron* 2000; **25**: 425–435.
21 Ramon-Cueto A, Plant GW, Avila J, Bunge MB. Long-distance axonal regeneration in the transected adult rat spinal cord is promoted by olfactory ensheathing glia transplants. *J Neurosci* 1998; **18**: 3803–3815.
22 Franklin RJ, Barnet SC. Olfactory ensheathing cells and CNS regeneration: the sweet smell of success? (review). *Neuron* 2000; **28**: 15–18.
23 Lakatos A, Franklin RJ, Barnet SC. Olfactory ensheathing cells and Schwann cells differ in their in vitro interactions with astrocytes. *Glia* 2000; **32**: 214–225.
24 Hermens WT *et al*. Transient gene transfer to neurons and glia: analysis of adenoviral vector performance in the CNS and PNS. *J Neurosci Meth* 1997; **71**: 85–98.
25 Dijkhuizen PA *et al*. Adenoviral vector-mediated gene delivery to injured rat peripheral nerve. *J Neurotrauma* 1998; **15**: 387–397.
26 Huber AB, Ehrengruber MU, Schwab ME, Brosamle C. Adenoviral gene transfer to the injured spinal cord of the adult rat. *Eur J Neurosci* 2000; **12**: 3437–3442.
27 Boer GJ *et al*. Long-term transgene expression in fetal rat suprachiasmatic nucleus neurografts following *ex vivo* adenoviral vector-mediated gene transfer. *Exp Neurol* 1997; **145**: 536–545.
28 Blits B *et al*. Adenoviral vector-mediated expression of a foreign gene in peripheral nerve tissue bridges implanted in the injured peripheral and central nervous system. *Exp Neurol* 1999; **160**: 256–267.
29 McCown TJ *et al*. Differential and persistent expression patterns of CNS gene transfer by an adeno-associated virus (AAV) vector. *Brain Res* 1996; **713**: 99–107.
30 Peel AL *et al*. Efficient transduction of green fluorescent protein in spinal cord neurons using adeno-associated virus vectors containing cell type-specific promoters. *Gene Therapy* 1997; **4**: 16–24.
31 Naldini L *et al*. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci USA* 1996; **93**: 11382–11388.
32 Blomer U *et al*. Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. *J Virol* 1997; **71**: 6641–6649.
33 Hermens WT, Verhaagen J. Viral vectors, tools for gene transfer in the nervous system (review). *Prog Neurobiol* 1998; **55**: 399–432.
34 Kay MA, Glorioso JC, Naldini L. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics (review). *Nat Med* 2001; **7**: 33–40.
35 Lipson AC, Lindqvist E, Olson L. Neurotrophic activity and

mRNA expression in olfactory ensheathing glia. *Soc Neurosci* 1999; **25**: (Suppl. 1)748 (Abstr. 295.7).
36 Boruch AV *et al*. Neurotrophic and migratory properties of an olfactory ensheathing cell line. *Glia* 2001; **33**: 225–229.
37 Woodhall E, West AK, Chuah MI. Cultured olfactory ensheathing cells express nerve growth factor, brain-derived neurotrophic factor, glia cell line-derived neurotrophic factor and their receptors. *Brain Res Mol Brain Res* 2001; **88**: 203–213.
38 Gudino-Cabrera G *et al*. Limits to the capacity of transplants of olfactory glia to promote axonal regrowth in the CNS. *Neuroreport* 2000; **11**: 467–471.
39 Grill R *et al*. Cellular delivery of neurotrophin-3 promotes corticospinal axonal growth and partial functional recovery after spinal cord injury. *J Neurosci* 1997; **17**: 5560–5572.
40 Tuszynski MH *et al*. Grafts of genetically modified Schwann cells to the spinal cord: survival, axon growth and myelination. *Cell Transplant* 1998; **7**: 187–196.
41 Menei P *et al*. Schwann cells genetically modified to secrete human BDNF promote enhanced axonal regrowth across transected adult rat spinal cord. *Eur J Neurosci* 1998; **10**: 607–621.
42 Plant GW, Horner PJ, Gage FH, Bunge MB. NT-3 prevents degeneration of the corticospinal tract, whereas BDNF transiently induces behavioural improvement in the completely transected spinal cord. *Soc Neurosci* 1999; **25**: (Suppl. 1) 211 (Abstr. 85.7).
43 Raisman G. Olfactory ensheathing cells – another miracle cure for spinal cord injury? (review). *Nat Rev Neurosci* 2001; **2**: 369–375.
44 Girod A *et al*. Genetic capsid modifications allow efficient retargetting of adeno-associated virus type 2. *Nat Med* 1999; **5**: 1052–1056.
45 Summerford C, Samulski RJ. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol* 1998; **72**: 1438–1445.
46 Marienfeld U *et al*. 'Autoreplication' of the vector genome in recombinant adenoviral vectors with different E1 region deletions and transgenes. *Gene Therapy* 1999; **6**: 1101–1113.
47 Pollock GS *et al*. Neuregulin is a mitogen and survival factor for olfactory bulb ensheathing cells and an isoform is produced by astrocytes. *Eur J Neurosci* 1999; **11**: 769–780.
48 Yan H, Bunge MB, Wood PM, Plant GW. Mitogenic response of adult rat olfactory ensheathing glia to four growth factors. *Glia* 2001; **33**: 334–342.
49 Tokita Y *et al*. Regulation of neuregulin expression in the injured rat brain and cultured astrocytes. *J Neurosci* 2001; **21**: 1257–1264.
50 Blakemore WF. Remyelination by Schwann cells of axons demyelinated by intraspinal injection of 6-aminonicotinamide in the rat. *J Neurocytol* 1975; **4**: 745–757.
51 Hermens WT, Verhaagen J. Adenoviral vector-mediated gene expression in the nervous system of immunocompetent Wistar and T cell-deficient nude rats: preferential survival of transduced astroglial cells in nude rats. *Hum Gene Ther* 1997; **8**: 1049–1063.
52 Van Esseveldt KE, Hermens WT, Verhaagen J, Boer GJ. Transgene expression in rat fetal brain grafts is maintained for 7 months after ex vivo adenoviral vector-mediated gene transfer. *Neurosci Lett* 1998; **240**: 116–120.
53 Blits B, Dijkhuizen PA, Boer GJ, Verhaagen J. Intercostal nerve implants transduced with an adenoviral vector encoding neurotrophin-3 promote regrowth of injured rat corticospinal tract fibres and improve hindlimb function. *Exp Neurol* 2000; **164**: 25–37.
54 Cai D *et al*. Prior exposure to neurotrophins blocks inhibition of axonal regeneration by MAG and myelin via a cAMP-dependent mechanism. *Neuron* 1999; **22**: 89–101.
55 Kafri T, van Praag H, Gage FH, Verma IM. Lentiviral vectors: regulated gene expression. *Mol Ther* 2000; **1**: 516–521.
56 Harvey AR. Labeling and identifying grafted cells. In: Dunnett SB, Boulton AA, Baker GB (eds). *Neuromethods, Vol 36: Neural Transplantation Methods*. Humana Press: Totowa, NJ, 1999, pp 319–361.
57 Iwashita Y, Crang AJ, Blakemore WF. Redistribution of bisbenzimide Hoechst 33342 from transplanted cells to host cells. *Neuroreport* 2000; **11**: 1013–1016.
58 Davies SJ *et al*. Regeneration of adult axons in white matter tracts of the central nervous system. *Nature* 1997; **390**: 680–683.
59 Barnett SC *et al*. Identification of a human olfactory ensheathing cell that can effect transplant-mediated remyelination of demyelinated CNS axons. *Brain* 2000; **123**: 1581–1588.
60 Crameri A, Whitehorn EA, Tate E, Stemmer WP. Improved green fluorescent protein by molecular evolution of DNA shuffling. *Nat Biotechnol* 1996; **14**: 315–319.
61 Donello JE, Loeb JE, Hope TJ. Woodchuck hepatitis virus contains a tripartite posttranscriptional regulatory element. *J Virol* 1998; **72**: 5085–5092.
62 Loeb JE *et al*. Enhanced expression of transgenes from adeno-associated virus vectors with the woodchuck hepatitis virus posttranscriptional regulatory element: implications for gene therapy. *Hum Gene Ther* 1999; **10**: 2295–2305.
63 Grimm D, Kern A, Rittner K, Kleinschmidt JA. Novel tools for the production and purification of recombinant adeno-associated virus vectors. *Hum Gene Ther* 1998; **9**: 2745–2760.
64 Hermens WT *et al*. Purification of recombinant adeno-associated virus by iodixanol gradient ultracentrifugation allows rapid and reproducible preparation of vector stocks for gene transfer in the nervous system. *Hum Gene Ther* 1999; **10**: 1885–1891.
65 Salvetti A *et al*. Factors influencing recombinant adeno-associated virus production. *Hum Gene Ther* 1998; **9**: 695–706.
66 Akli S *et al*. Transfer of a foreign gene into the brain using adenovirus vectors. *Nat Genet* 1993; **3**: 224–228.
67 Graham FL, Prevec L. Manipulation of adenovirus vectors. In: Murray EJ (ed.). *Methods in Molecular Biology, Vol. 7: Gene Transfer and Expression Protocols*. Humana Press: Clifton, NJ, 1991, pp 109–129.
68 Fallaux FJ *et al*. Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum Gene Ther* 1996; **7**: 215–222.
69 Naldini L *et al*. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 1996; **272**: 263–267.
70 Zufferey R *et al*. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotechnol* 1997; **15**: 871–875.
71 Follenzi A *et al*. Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat Genet* 2000; **25**: 217–222.
72 Griffioen HA, Van der Beek E, Boer GJ. Gelatin embedding to preserve lesion-damaged hypothalami and intracerebroventricular grafts for vibratome slicing and immunohistochemistry. *J Neurosci Meth* 1992; **43**: 43–47.