

Olfactory Ensheathing Cells: Characteristics, Genetic Engineering, and Therapeutic Potential

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ABSTRACT

Injured neurons in the mammalian central nervous system (CNS) do not normally regenerate their axons after injury. Neurotrauma to the CNS usually results in axonal damage and subsequent loss of communication between neuronal networks, causing long-term functional deficits. For CNS regeneration, repair strategies need to be developed that promote regrowth of lesioned axon projections and restoration of neuronal connectivity. After spinal cord injury (SCI), cystic cavitations are often found, particularly in the later stages, due to the loss of neural tissue at the original impact site. Ultimately, for the promotion of axonal regrowth in these situations, some form of transplantation will be required to provide lesioned axons with a supportive substrate along which they can extend. Here, we review the use of olfactory ensheathing cells: their location and role in the olfactory system, their use as cellular transplants in SCI paradigms, alone or in combination with gene therapy, and the unique properties of these cells that may give them a potential advantage over other cellular transplants.

Key words: gene therapy; neurotrophic factors; olfactory ensheathing glia; regeneration; spinal cord

INTRODUCTION

A QUARTER OF A CENTURY has passed since Richardson et al. (1980) published their pioneering experiments on peripheral nerve grafting in the injured central nervous system (CNS). A study that revolutionized scientific thinking about the (in)ability of adult CNS neurons to regenerate following axotomy. Up to then, neurons in the adult CNS were thought to lack the intrinsic capacity to regenerate their axons after injury. Severed central axons fail to regenerate across the injury site and to re-

establish synaptic contacts with target neurons, a process required to restore communication between the higher brain centers and affected areas of the body. Consequently, depending on the severity and vertebral level, spinal trauma normally leads to long-term deficits in voluntary motor and sensory performance as well as impairments in the autonomic nervous system functioning. Most human injuries to the spinal cord are characterized by the presence of one or more cystic cavitations at the original impact site (Bunge et al., 1993). For the promotion of axonal regrowth in these situations, some form of

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transplantation will be required to provide lesioned axons with a scaffold along which they can extend.

Since the original publication by Richardson et al. (1980), an ever growing number of papers have been published using some form of (neural) transplantation for the promotion of axonal regrowth and spinal cord repair. Apart from Schwann cell-containing grafts, a variety of other cellular bridging materials have been trialed, for example, pieces of embryonic spinal cord, engineered fibroblasts, neural stem cells, bone marrow-derived cells, and olfactory ensheathing cells (Bunge and Pearse, 2003; Mykатыn et al., 2004; Reier, 2004). Here, we will specifically focus on the use of olfactory ensheathing cells (OECs), that is, their location and role in the olfactory system, use as cellular transplants in spinal cord injury (SCI) paradigms, alone or in combination with gene therapy, and the unique characteristics they possess, which may give them potential advantages over Schwann cells and other cellular conduits.

PRIMARY OLFACTORY SYSTEM AS A SOURCE OF OLFACTORY ENSHEATHING CELLS

The adult primary olfactory system is a unique part of the mammalian nervous system that has preserved the capacity to continuously regenerate during adulthood (Schwob, 2002). Olfactory receptor neurons, located in the olfactory mucosa of the nose, are liable to wear as a consequence of respiration and have an average lifespan of 6–8 weeks (Carr and Farbman, 1993). Throughout life, dying olfactory receptor neurons are replaced by newborn cells that differentiate from a stem cell layer at the base of the epithelium (Weiler and Farbman, 1997). These new, differentiating odorant receptor progenitors ascend their axons via discrete bundles (*fila olfactoria*) into the olfactory bulb, which is part of the CNS. The ability of growing primary olfactory axons to navigate through a CNS environment is thought to relate to the glial composition and cytoarchitecture of the olfactory bulb (Doucette, 1990, 1991), in particular the presence of OECs (Goodman et al., 1993; Ramon-Cueto et al., 1993; Kafitz and Greer, 1998; Ramon-Cueto and Avila, 1998).

OEC progenitors are thought to originate from the olfactory placode and can be seen during early embryogenesis migrating along with olfactory axons towards the developing olfactory bulb (Doucette, 1989; Chuah and Au, 1991; Valverde et al., 1993; Treloar et al., 1996; Chuah and West, 2002). These observations suggest a unique developmental origin of OECs compared to other nervous system macroglia. In the adult, OECs are found

in both the peripheral and central compartments of the primary olfactory system, that is, the olfactory epithelium and bulb (Fig. 1). In the nasal cavity, OECs reside in the lamina propria, where they closely associate with afferent axon bundles of olfactory receptor neurons (Doucette, 1991; Field et al., 2003). In the cranium, OECs are found in the nerve layer of the first cranial nerve that covers the surface of the olfactory bulb. Similar to Schwann cells, OECs upregulate low-affinity nerve growth factor receptor p75 following experimental injury (Gong et al., 1994; Turner and Perez-Polo, 1994) and, deafferented OECs are thought to provide a cellular matrix for both new and regenerating primary olfactory axons (Williams et al., 2004). Thus, these unique cells, which share common characteristics with both CNS and peripheral nervous system (PNS) glia, enfold growing axons in the bulb and are thought to play a key role in supporting regeneration of olfactory axons. These features have turned OECs into attractive transplantation candidates to foster axonal growth in other areas of the CNS.

OLFACTORY ENSHEATHING CELL TRANSPLANTS

Several spinal cord lesion models, involving either dorsal root, specific tract, or partial spinal cord lesions (Ramon-Cueto and Nieto-Sampedro, 1994; Li et al., 1997, 1998; Navarro et al., 1999; Nash et al., 2002; Gomez et al., 2003; Li et al., 2003; Ruitenberg et al., 2003, 2005; Verdu et al., 2003; Andrews and Stelzner, 2004; Chuah et al., 2004; Li et al., 2004; Polentes et al., 2004; Ramer et al., 2004a,b; Riddell et al., 2004), photochemical damage (Garcia-Alias et al., 2004), complete spinal cord transection (Ramon-Cueto et al., 1998, 2000; Lu et al., 2001, 2002; Cao et al., 2004; Fouad et al., 2005) or contusive injury (Takami et al., 2002; Plant et al., 2003), have been used to test the potential of these ensheathing cells to promote axonal regeneration outside the olfactory bulb. The first evidence that OECs were able to support regenerative growth of lesioned axons in the damaged spinal cord was obtained in an experimental model for dorsal root injury. Following rhizotomy and subsequent microsurgical anastomosis of the dorsal root stump to the spinal cord, engraftment of OECs at the interface of the PNS and CNS allowed sensory axons of the dorsal root, which are normally refractory to regenerate, to cross the non-permissive barrier of the dorsal root entry zone and to re-enter the spinal (Ramon-Cueto and Nieto-Sampedro, 1994). These findings were confirmed in a later study by Navarro et al. (1999) and, more recently, in a separate report by Li et al. (2004). Three other independent reports, however, have not been able

to reproduce these results (Gomez et al., 2003; Ramer et al., 2004a; Riddell et al., 2004). It is unclear whether these discrepancies represent true differences in outcome or whether they are related to experimental differences in transplant preparation, grafting procedures and source of transplanted OECs (i.e., lamina propria vs. olfactory bulb). Several studies have also demonstrated that transplantation of OECs at sites of SCI induced modest regrowth of a variety of propriospinal and descending axon projections, including fibers of raphespinal, coeruleospinal, and/or corticospinal (CST) origin (Li et al., 1997, 1998; Ramon-Cueto et al., 1998, 2000; Cao et al., 2004) as well as a certain degree of functional recovery (Li et al., 1997, 1998, 2003; Ramon-Cueto et al., 2000; Lu et al., 2001, 2002; Keyvan-Fouladi et al., 2003; Plant et al., 2003; Ruitenberg et al., 2003; Cao et al., 2004). Some controversy, which remains to be elucidated, has arisen on the regenerative response of the CST, since others have found that regrowth of lesioned CST axons was restricted to a modest sprouting response at the proximal injury and OEC transplantation site (Takami et al., 2002; Plant et al., 2003; Ruitenberg et al., 2005). Comparative studies will need to be undertaken to assess whether these discrepancies represent true differences in outcomes or whether they are related to inter-experimental variables (e.g., the injury model of choice and transplantation procedures). Bridging of the completely transected spinal cord via Schwann cell-seeded guidance channels successfully joined the two spinal stumps together but did not allow regenerating axons to exit the graft (Xu et al., 1997; Plant et al., 2001a). Interestingly, transplantation of OECs at both ends of the Schwann cell bridge enabled axons to grow across the grafted area and to regenerate for long distances (up to 1.5–2.5 cm) into host spinal cord (Ramon-Cueto et al., 1998). These remarkable observations suggest that the presence of OECs positively influenced the growth-impeding properties of the neural scar. Originally, it was hypothesized that OECs chaperone regenerating axons through the spinal cord, perhaps isolating them from the hostile CNS environment, which would explain the remarkable potential of these cells to support regenerative growth. Based on Hoechst labelling prior to transplantation, OECs were reported to migrate over large distances in host spinal cord (Ramon-Cueto et al., 1998; Boruch et al., 2001). However, possible leakiness of Hoechst label from transplanted cells and subsequent redistribution to host tissue has been observed (Iwashita et al., 2000; Ruitenberg et al., 2002), indicating that this dye is not a reliable marker to track transplanted cells in host tissue. Two studies have used OEC transplants that were pre-labelled with superparamagnetic iron oxide, allowing visualization of labelled cells *in vivo* by MRI imaging (Dunning et al., 2004; Lee et al.,

2004). Although this technique allows live imaging of transplanted cells, a potentially confounding factor in studying the migratory behaviour of transplanted cells with this technique is the uptake of superparamagnetic iron oxide from dead cells by macrophages. *Ex vivo* viral transduction of OECs with green fluorescent protein (GFP) has unequivocally demonstrated that transplanted cells do migrate towards the lesion epicenter but not over large distances rostral or caudal from the injury site (Ruitenberg et al., 2002; Li et al., 2003, 2004). These results have been confirmed in xenografting experiments using OECs from GFP mice (Ramer et al., 2004a,b). Taken together, these findings suggest that the regeneration-supporting capacity of OECs is more likely related to a different interaction with scar-associated cells. It appears that OECs have the capacity to intermingle with cells that are related to the neural scar such as reactive astrocytes (Lakatos et al., 2000; Verdu, 2001) and meningeal fibroblasts (Ramon-Cueto et al., 1998). OEC transplants have been shown to not dramatically upregulate inhibitory proteoglycan expression at the graft–host interface (Lakatos et al., 2003), a phenomenon that has been described for Schwann cell transplants (Plant et al., 2001a). This could create a more permissive microenvironment in the scar, allowing axonal elongation through and beyond the site of injury. Interestingly, in a recent publication by Fouad et al. (2005), a combinatory approach of OEC or Schwann cell transplantation with chondroitinase ABC treatment, in order to reduce the inhibitory proteoglycan component in the glial scar, further improved axonal regeneration through these grafts as well as functional recovery.

A few reports have described Schwann cell-like myelination of spinal axons by transplanted OECs (Franceschini and Barnett, 1996; Franklin et al., 1996; Li et al., 1998; Barnett et al., 2000; Kato et al., 2000), but these findings were inconclusive as unpurified OECs cultures were used for transplantation (Li et al., 1998) and no reliable labels were used to identify transplanted cells. *In vitro*, pure cultures of adult OECs failed to produce myelin under conditions that triggered Schwann cells to do so (Plant et al., 2002). The latter observation may suggest the presence of another cell type that supports myelination following certain transplant preparation protocols, or the existence of a separate signalling cascade that is needed by these cells to produce compact myelin. Recently, two independent, conflicting reports were published studying the *in vivo* myelination potential of embryonic or adult OECs. These cells, expressing the LacZ reporter gene or GFP, respectively, were transplanted into the spinal cord in an attempt to obtain conclusive evidence whether these cells were capable of myelination (Boyd et al., 2004; Sasaki et al., 2004). In the first study

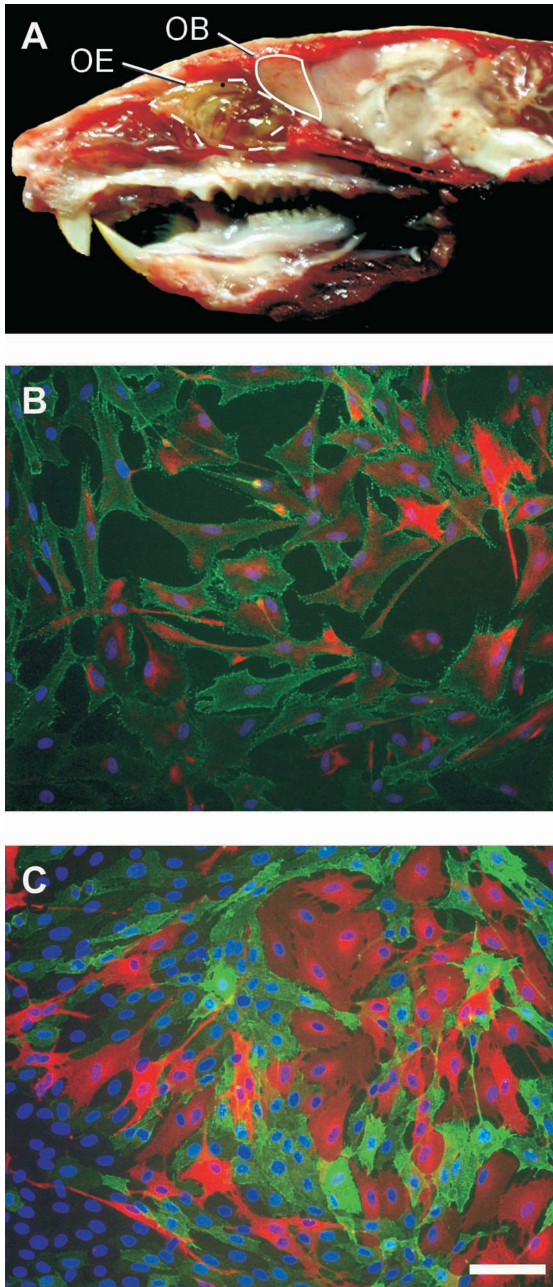


FIG. 1. The primary olfactory system as a source for olfactory ensheathing cells (OECs). (A) Sagittal preparation through the head of an adult rat. Lamina propria-derived OECs can be obtained following biopsies from the olfactory epithelium (OE) and underlying structures in the nasal cavity (dashed line), or from the olfactory nerve layer of the olfactory bulb (OB; outlined area). (B) Pure cultures of OECs, expressing low-affinity nerve growth factor receptor p75 (green) and glial fibrillary acidic protein (GFAP; red), can be isolated from the OB nerve layer following immunopanning for p75, as described in Plant et al. (2002). (C) Immunopurification of p75-expressing cells (red) from adult epithelial preparations significantly enriched OEC numbers but no pure cultures could be obtained. Significant numbers of contaminating cells—i.e., Thy-1 expressing fibroblasts (green) and another yet unidentified population of cells (nuclear Hoechst dye; blue)—were still present in these cultures (Ruitenber and Plant; unpublished data).

bellied OECs reportedly led to some remyelination of spinal axons by OECs (Sasaki et al., 2004), although the authors did not confirm their findings in a standardized *in vitro* myelination model as used by Devon and Doucette (1992) and Plant et al. (2002).

Taken together, both the olfactory bulb (Plant et al., 2001b) and lamina propria can be used as a source of OECs for transplantation purposes (Au and Roskams, 2003; Bianco et al., 2004). Transplantation of OECs remains one of the most promising strategies available to augment axonal regeneration and functional recovery after SCI. There are, however, conflicting reports on the potential of these cells to promote regeneration of specific pathways (e.g., corticospinal axons), as well as on their ability to myelinate axons in the damaged CNS. Careful evaluation of the literature does not provide a fundamental unifying difference between conflicting reports (i.e., isolation and transplantation procedures, as well as source, purity, and age of transplanted cells). Further analysis is therefore required in comparative studies addressing these issues using OEC transplants from embryonic, postnatal and adult rats as well as standardization of OEC isolation and culturing protocols (Plant et al., 2001b; Barnett and Roskams, 2002).

COMBINING GENE THERAPY AND OEC TRANSPLANTATION

Recent studies have demonstrated that OECs express several molecules that are known to promote axonal growth (Ramon-Cueto and Avila, 1998), including NGF, BDNF, GDNF, and NT-4/5 (Boruch et al., 2001; Woodhall et al., 2001). The expression of neurotrophins by

by Boyd et al. (2004), OEC processes were found in close association with axon bundles but failed to myelinate severed axons. These findings contrast earlier observations *in vitro* where unlabelled embryonic OECs were believed to myelinate dorsal root ganglia (DRG) neurites (Devon and Doucette, 1992). The observed remyelination of CNS axons was due to unlabelled host Schwann cells, which may have been recruited in increased numbers to the OEC transplantation site (Takami et al., 2002; Ramer et al., 2004b). Intriguingly, transplantation of adult GFP-la-

OECs may contribute to and, in part, explain the underlying mechanism of the growth-promoting properties of OECs. When compared to Schwann cells, however, lower levels of neurotrophins seem to be produced by OECs. For instance, endogenous BDNF expression by OECs (0.15 ng/day/10⁵ cells) (Woodhall et al., 2001) appears approximately threefold lower when compared to Schwann cells (5 ng/day/10⁶ cells) (Menei et al., 1998). This suggests that other yet unidentified differences in protein expression, which remain to be explored, contribute to the remarkable regeneration-supporting properties of OECs compared to other glial cell types. Additionally, this opens the possibility for genetic engineering of OECs in order to elevate neurotrophin expression, which could significantly improve regenerative growth through these grafts.

Over the last decade, a number of genetically engineered cells have been transplanted into the injured CNS to study their neuroprotective and neurite regrowth promoting effects (Hendriks et al., 2004). To date, only three studies have been published using OEC transplants that were engineered to secrete neurotrophic factors (Ruitenber et al., 2003, 2005; Cao et al., 2004). In an initial collaborative study by our laboratory and the Neuroregeneration Laboratory (Netherlands Institute for Brain Research, Amsterdam, The Netherlands), we examined if adenoviral (AdV) vector-mediated expression of the neurotrophins BDNF and NT-3 by transplanted OECs could induce improved regenerative growth of lesioned RST axons through these grafts (Ruitenber et al., 2003). *Ex vivo* transduction of primary OECs with AdV vectors results in robust but transient expression of the transgene for a period of approximately 30 days following transplantation in the lesioned spinal cord (Ruitenber et al., 2002). In all experimental animals, the left lateral funiculus of the cervical spinal cord was lesioned. Spinal cord injured animals then received an OEC transplant that was *ex vivo* transduced with an AdV vector encoding BDNF and/or NT-3, or a marker protein, respectively. Additional control groups received either no transplant or a graft of non-transduced OECs. Monitoring of the hind limb function during horizontal rope walking revealed enhanced recovery in rats that received a transplant of OECs transduced with a neurotrophin-encoding AdV vector. Analysis of RST regeneration showed that AdV vector-mediated production of BDNF, but not NT-3, from transplanted OECs significantly improved sprouting of RST axons without promoting robust long-distance regeneration.

Consequently, this could not have accounted for the functional improvements as no anatomical restoration of the rubrospinal pathway was obtained. Volumetric analysis of lesion size, measured in all experimental animals,

revealed that lesions were smaller in all OEC-transplanted rats but only significantly reduced in size in animals that received OEC grafts secreting the neurotrophins BDNF and NT-3. Interestingly, lesion volumes were directly correlated to the functional performance of experimental animals. These findings suggest that neurotrophin-mediated tissue preservation can have a beneficial role in the functional outcome after SCI, which stresses the importance of limiting secondary damage to the spinal cord in the initial stages after injury. In a more recent publication, we also studied the regenerative response of the dorsal CST projection following injury and NT-3 production from AdV vector-transduced OEC transplants (Ruitenber et al., 2005). Similar to earlier findings by Blits et al. (2000), transient production of NT-3 from engineered cells resulted in long-distance maintenance/regeneration of CST axons in these animals, at least up to 1 cm caudal to the injury and transplantation site as revealed by anterograde tracing of the lesioned CST projection. No such effect was observed in any of the control groups which received an injection of transplantation medium only, or a transplant of OECs cells that were either non-infected or transduced with an AdV vector encoding a marker gene. All OEC grafts reduced secondary tissue loss, which resulted in a significant sparing of spinal cord tissue at the lesion site commensurate with other studies (Takami et al., 2002; Plant et al., 2003; Ruitenber et al., 2003; Ramer et al., 2004b). No additional preservation of spinal tissue by AdV vector-derived NT-3 was detected in this particular experimental model. Different regenerative responses of CST and RST motor pathways were observed following AdV vector-mediated expression of BDNF and NT-3, indicating that individual pathways may require different, specific conditions for optimal regrowth, which correlates with receptor expression studies in relevant brain areas (Liebl et al., 2001). Nevertheless, transplantation of these engineered cells did provide proof of principle that genetic engineering increased growth of lesioned CNS axons through these transplants. Persistent transduction of OECs has been achieved following lentiviral (Ruitenber et al., 2002) and retroviral (Boyd et al., 2004; Cao et al., 2004) transduction. To date, the publication by Cao et al. (2004) is the only study that has investigated the effects of chronic neurotrophic factor expression on spinal axon regeneration. *In vitro*, transduced OECs had an approximate 300-fold increase in the levels of GDNF expression. Transduced cells appeared normal, and no change in the expression of antigenic markers was observed, suggesting that GDNF production did not seem to alter cell phenotype or functioning. Following transplantation of these engineered OECs into the spinal cord, high levels of GDNF expression were detected for up to 8 weeks after

injury. Neurofilament staining revealed robust ingrowth of axons into areas containing GDNF-secreting OECs when compared to control cell transplants. Furthermore, retrograde tracing suggested augmented regrowth of CST and RST axons. Interestingly, analysis of anterograde CST tracing showed vigorous axon sprouting at the lesion and transplantation area in animals that received GDNF secreting OEC transplant, but no significant differences in CST axon numbers were found further distally. These observations could indicate that chronic expression of GDNF from OEC transplants entrapped regenerating axons due to high neurotrophic factor content at the site of engraftment. A similar phenomenon was recently described in the spinal cord after direct adeno-associated viral (AAV) vector-mediated gene transfer of GDNF to ventral motoneurons (Blits et al., 2004), and stresses the importance of vector development with regulatory transgene expression (Blesch et al., 2001).

CONCLUSION

Combination of OEC transplantation with neurotrophin gene therapy is emerging as a promising strategy to promote regeneration of lesioned spinal cord axons in rats. With the development of non-toxic gene therapy systems such as lentiviral (LV) vectors, robust and long-term transgene expression in transplanted cells following *ex vivo* gene transfer is now a realistic option (Ruitenberget al., 2002; Cao et al., 2004). Further improvements are required, however, to develop more sophisticated strategies that promote strong directional regrowth of targeted pathways in the spinal cord, resulting in the reconnection of neural networks. Advanced tissue engineering procedures will hopefully lead to the development of cellular conduits that are more effective in the sparing of spinal cord tissue and the promotion of regeneration. Although there is much optimism about the clinical potential for OEC transplantation in the treatment of CNS trauma, surprisingly little is known about the basic biology of these cells and the mechanism of action by which they promote axonal growth.

Questions that need answering include the following: (1) Are there specific markers that can be used to identify OECs (and subtypes) (Franceschini and Barnett, 1996)? (2) What genes do OECs express that are beneficial to regeneration (Woodhall et al., 2003)? (3) Do they also express inhibitory molecules or guidance cues? (4) Related to the last point, what differences between OECs and Schwann cells allow integration and intermingling of OECs with host astrocytes where Schwann cells seem to aggregate and induce increased neural scar formation (Lakatos et al., 2003; Barnett et

al., 2004)? With the development of genome-wide screening techniques (i.e., microarray technology), it is now scientifically feasible to perform large-scale analysis of differences in gene expression between different cell types. In an initial attempt to start addressing these questions, we have compared OEC and Schwann cell transcriptomes using mRNA, isolated from purified, primary cell cultures that were obtained from adult rats as described in Plant et al. (2002) and hybridized it against the rat genome high-density oligonucleotide array U34 (Affymetrix) (Fig 2A). From a total number of just over 8800 genes present, expression of approximately 4800 genes appeared absent or could not be reliably be detected in both cell types. Another 3236 genes were found to be expressed by both cell types. From the total pool, 745 genes seemed differentially expressed between the cells—that is, 450 genes expressed exclusively in OECs, whereas a total of 295 gene transcripts seemed solely present in Schwann cells (Fig. 2B). To identify potential key molecules in OECs that could account for their unique capability to intermingle with scar-associated cells and to stimulate regenerative axonal growth, we have confined our studies to those molecules that were exclusively expressed by OECs, in particular, cell surface and secreted proteins. We have so far confirmed differential expression of a subset of these genes in independent RNA samples from separate OEC cultures using RT-PCR (Fig. 2C). Similarities in OEC characteristics *in vivo* and *in vitro* have yet to be ascertained. Our ongoing research now focuses on the expression of these molecules *in vivo* and the role that these molecules may play during development and regeneration of the primary olfactory system. It is hoped that these experiments will increase our understanding of OEC biology and provide further insight into novel molecules that are produced by these cells which could play a crucial role during CNS regeneration.

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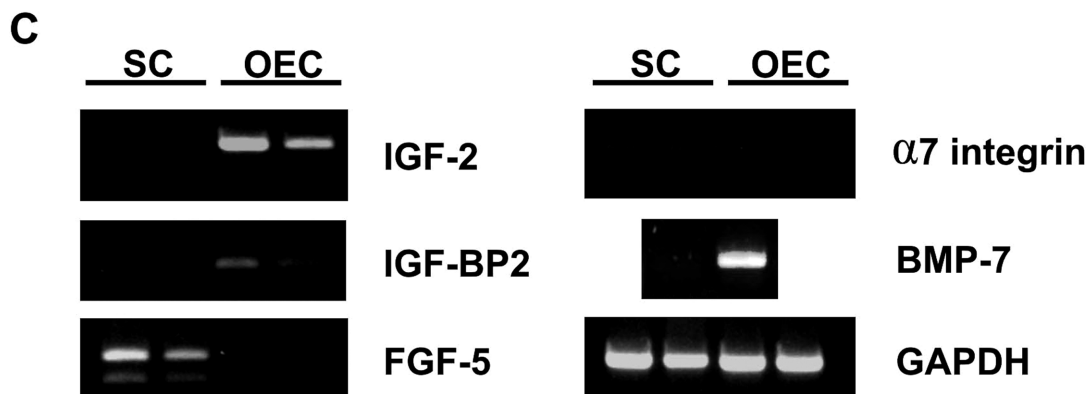
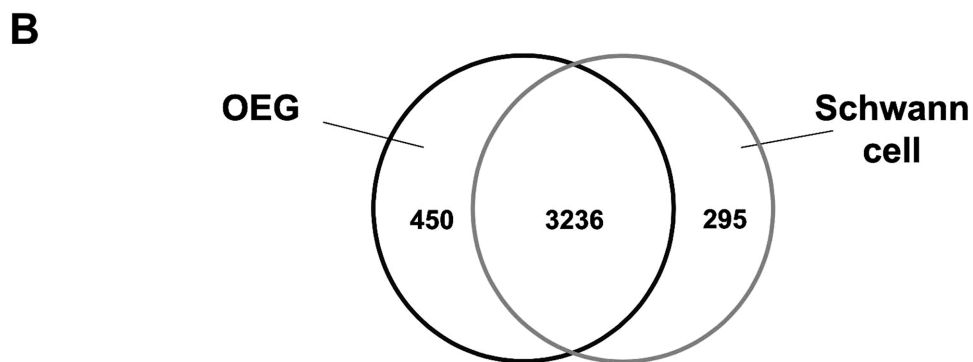
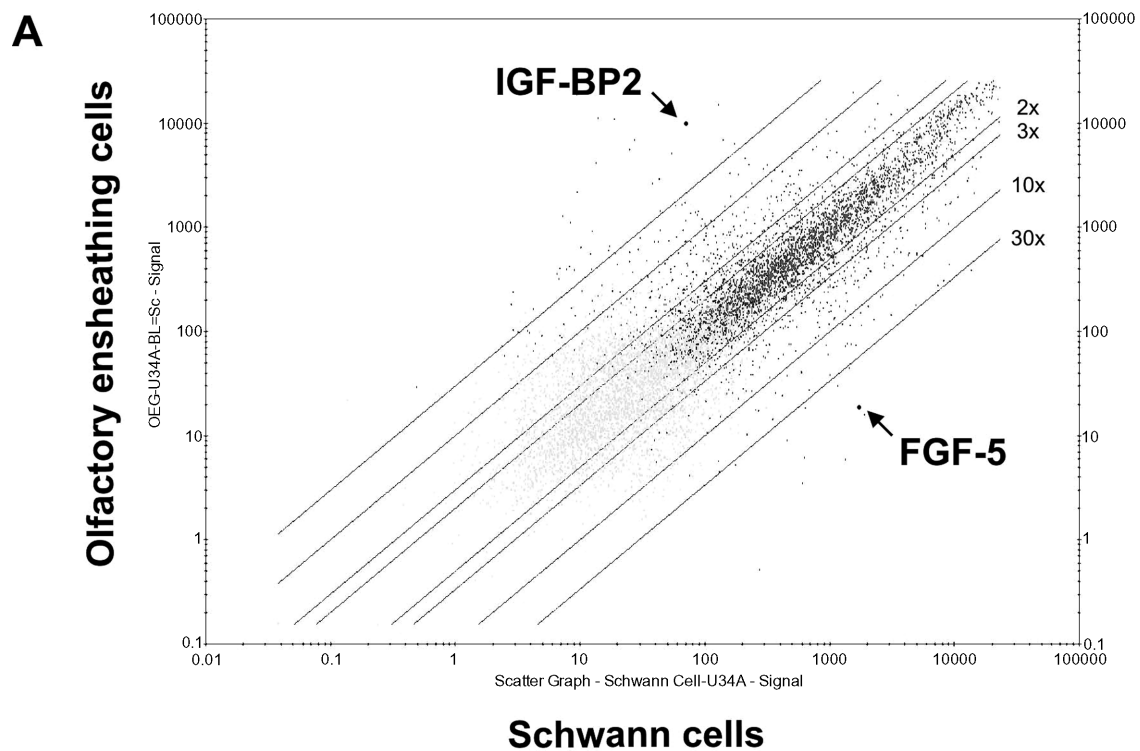


FIG. 2. Preliminary data on comparative gene expression profiling between olfactory ensheathing glia cells (OEG) and Schwann cells (SC). **(A)** Scatter plot of gene expression data following hybridization of OEG and Schwann cell transcriptome against Affymetrix U34 oligonucleotide arrays. **(B)** Venn diagram showing the overlap in gene expression between OEGs and Schwann cells. A total of 450 genes seemed selectively expressed in OEGs, whereas 295 gene transcripts were only detected in RNA preparations from Schwann cells. **(C)** Confirmation of differential expression of a subset of genes, including several growth factors, between OEGs and Schwann cells using reverse transcriptase-polymerase chain reaction (RT-PCR).

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