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Crucial roles for olfactory ensheathing cells and olfactory mucosal cells in the repair of damaged neural tracts.

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Abstract

Olfactory ensheathing cells, the glial cells of the olfactory nervous system, exhibit unique growth-promoting and migratory properties that make them interesting candidates for cell therapies targeting neuronal injuries such as spinal cord injury. Transplantation of olfactory cells is feasible and safe in humans; however, functional outcomes are highly variable with some studies showing dramatic improvements and some no improvements at all. We propose that the reason for this is that the identity and purity of the cells is different in each individual study. We have shown that olfactory ensheathing cells are not a uniform cell population and that individual subpopulations of OECs are present in different regions of the olfactory nervous system, with strikingly different behaviours. Furthermore, the presence of fibroblasts and other cell types in the transplant can dramatically alter the behaviour of the transplanted glial cells. Thus, a thorough characterisation of the differences between olfactory ensheathing cell subpopulations and how the behaviour of these cells is affected by the presence of other cell types is highly warranted.
Repairing spinal cord injury by transplanting OECs.

Neural injuries are almost always serious with life-changing consequences. Injuries to the central nervous system (CNS) are particularly devastating as central neural tracts cannot regenerate themselves after injury. Therefore, many serious spinal cord injuries lead to permanent disability. The main problem arising after injury to the CNS is the fact that newly sprouting axons cannot find their target due to the presence of a glial scar, inflammatory mediators, inhibitory factors and reactive oxygen species (Hulsebosch, 2002; Jones et al., 2003). To date, there are no therapies that can address this problem, but one methodology that has shown encouraging results for spinal cord injury is to transplant glial cells, the supporting cells of the nervous system which are crucial for the survival of all types of neurons, and for extension of axons.

Transplantation of the glial cells of the olfactory nervous system, olfactory ensheathing cells (OECs) has shown promise in both animals and humans with spinal cord injury. In humans, transplantation of OECs that had been purified from the nasal cavity of patients with spinal cord injury (autologous transplantation) showed that the procedure was safe in a Phase I clinical trial (Feron et al., 2005; Mackay-Sim et al., 2008). In animals, transplanted OECs have been shown to survive and actively migrate into the injury site (Boruch et al., 2001; Deng et al., 2006), reduce scar and cavity formation (Ramer et al., 2004; Li et al., 2011), to improve locomotion and restore moveability of the hindlimbs (Gorrie et al., 2010; Takeoka et al., 2011) and restore breathing and climbing ability (Li et al., 2003) even after complete transections of the spinal cord (Takeoka et al., 2011; Ziegler et al., 2011). While migration of transplanted OECs is often limited, some studies showed that OECs have migrated much further than the immediate injury site which may be due to their attraction to reactive astrocytes (Chuah et al., 2011). Other studies have shown that OEC transplantation leads to
no functional improvement (Centenaro et al., 2013; Deumens et al., 2013) and that migration in the injured spinal cord can be quite limited. In a canine model of spinal cord injury, OEC transplantation resulted in some improvements of fore-hind limb coordination, but no changes in functionality of the long tracts in the spinal cord, which was attributed to limited OEC migration (Granger et al., 2012).

Approaches that combine transplanted glia with growth factors have also been trialled. Transplantation of OECs in combination with glial cell-derived neurotrophic factor (GDNF) into the injured optic nerve has been shown to promote regrowth of axons, beyond the results seen with OECs alone, however, the mechanisms behind the GDNF-mediated effect remain unknown (Liu et al., 2010). It is also possible to genetically modify OECs to express significant levels of GDNF, which in turn further promotes nerve repair (Cao et al., 2004). Transplanted OECs can also stimulate Schwann cells endogenous to the injury site to in turn promote neuronal survival and growth and potentially also remyelination (Ramer et al., 2004; Zhang et al., 2011). Taken together, these findings show that the roles of OECs need to be considered within a multicellular environment.

One variable factor likely to account for some of the differences in functional outcomes is the source of OECs. We have shown that the behaviour of OECs is strikingly different between individual populations of OECs isolated from distinct regions of the olfactory nervous system (Windus et al., 2007; Windus et al., 2010; Windus et al., 2011). Another reason for discrepancy is the purity of the cells. While some studies have found that inclusion of olfactory fibroblasts in transplants may be beneficial (Li et al., 2008; Teng et al., 2008; Li et al., 2012), others have suggested that purified OECs yield better functional outcomes (Toft et al., 2013). Thus, the most important question in the field that needs to be addressed is which
combination of cells from the olfactory mucosa yields the optimal functional outcomes in spinal cord injury repair? To answer this question, the characteristics of different OEC subpopulations and the effects of olfactory fibroblasts and other cell types on OEC behaviour and resultant axonal extension must be addressed.

What are olfactory ensheathing cells?
Primary olfactory receptor neurons arise from stem cells residing in the epithelium of the nasal cavity. Their axons extend through the basal side of the epithelium and converge into fascicles (axon bundles), constituting the highly branched olfactory nerve which ends in the olfactory bulb within the brain. Olfactory neurons only live for 1-3 months after which they undergo apoptosis and are replaced by new neurons originating from precursor cells in the nasal epithelium (Mackay-Sim and Kittel, 1991). Thus, the primary olfactory nervous system constantly regenerates itself throughout life. OECs arise from neural crest to populate the olfactory placode (Barraud et al., 2010). They then differentiate and migrate together with the extending axons of primary olfactory neurons that are forming the developing olfactory nerve. Thus, unlike other glia, OECs are unique in that they migrate from the peripheral nervous system into the central nervous system. Here, OECs always extend ahead of the pioneer olfactory axons, leading to the assumption that OECs guide axons along a defined path (Valverde et al., 1992; Tennent and Chuah, 1996; Chehrehasa et al., 2010; Windus et al., 2011). In contrast, the dominant glial type in the rest of the peripheral nervous system, Schwann cells, are known to follow paths already consisting of axons during development (Jessen and Mirsky, 2005).

In the olfactory nerve, OECs surround large bundles of axons with the cells bodies of the OECs located mainly on the exterior of the fascicles with the lamellipodial penetrating
between the axons (Fig. 1, Movie 1). In contrast, Schwann cells in the neighbouring trigeminal nerve wrap up individual or small groups of axons (Fig. 1E). The olfactory nerve passes from the peripheral nervous system through the cribriform plate to enter the central nervous system. Within the nerve fibre layer, the outer layer of the olfactory bulb, the primary olfactory axons defasciculate, sort out into specific subtypes and then project to their correct topographical targets (Fig. 1B). The OECs are intermixed amongst the axons with distinctly different arrangement and morphology (Movie 2) compared to OECs in the peripheral nerve (Movie 1). The OECs within the nerve fibre layer are thought to mediate sorting and guidance of the axons to their targets within the olfactory bulb. The nerve fibre layer consists of an outer and an inner layer, both populated by OECs. In the outer nerve fibre layer, OECs are thought to facilitate defasciculation of the mixed bundles of axons which then extend into the inner nerve fibre layer, where the OECs are instead involved in sorting and refasciculation of axons based on which type of odorant receptor they express. Therefore, OECs in the peripheral nerve are most likely to promote cell-cell adhesion and extension of axons in fascicles, whereas OECs in the olfactory bulb contribute to more complex and variable cellular behaviours (Fig. 1D) (Au et al., 2002; Ekberg et al., 2012).

**Why is transplantation of OECs a promising approach for repairing damaged nerves?**

The main challenge in repairing injuries to the central nervous system is for newly sprouting axons to find their target, despite the presence of physical barriers and inflammatory/inhibitory mediators. Novel therapies for repairing damaged neural tract therefore need to address this particular problem. While the use of artificial substrates can promote axon growth (Chehrehasa et al., 2006) it is likely that a combination of glia and growth factors is needed.
To date, the majority of trials using transplanted glial cells have been using either Schwann cells or OECs, or in some instances, a combination of both. After transplantation, Schwann cells myelinate individual axons (Li et al., 2007), but do not migrate far into the injury site and do not integrate well with other cells in the CNS (Andrews and Stelzner, 2007; Franssen et al., 2009). In contrast, OECs fasciculate axon bundles rather than individual axons (Fig. 2) and integrate within both peripheral and central neural tracts. This property can be attributed to the fact that they are normally present at the PNS-CNS interface in the olfactory nervous system. Indeed one of the properties that makes OECs such an attractive candidate for transplant therapies in comparison to other glia, such as Schwann cells, is their ability to freely associate with other cell types, in particular with astrocytes (Lakatos et al., 2003). The ability of OECs to myelinate axons is widely debated in the field, with some studies showing that transplanted OECs can myelinate axons under certain conditions (Babiarz et al., 2010), but others studies suggesting that they cannot (Li et al., 2007). OECs are now considered crucial to the unique ability of the olfactory system to continuously regenerate itself both under normal physiological conditions and after injury (Graziadei and Graziadei, 1979; Farbman and Squinto, 1985; Mackay-Sim and Kittel, 1991). Therefore the contribution of OECs to olfactory neural regeneration is thought to be worthy of translation into repair of other regions of the nervous system.

OECs produce numerous factors that may aid nerve repair such as nerve growth factor (NGF), vascular endothelial growth factor (VEGF), GDNF, fibroblast growth factor (FGF), insulin-like growth factor (IGF) as well as cell adhesion and extracellular matrix molecules (Chuah and Teague, 1999; Woodhall et al., 2001; Woodhall et al., 2003; Vincent et al., 2005; Mackay-Sim and St John, 2011). OECs also produce carbohydrate-binding molecules, galectins (St John and Key, 1999) that are likely to promote fasciculation or be involved in
guidance of axons that express cell surface carbohydrates (St John and Key, 2001; St John et al., 2006; Lineburg et al., 2011). All of these molecules can aid the growth and regeneration of neurons.

OECs also express molecules that can be beneficial to repairing an injury site and reducing astrocytic scar tissue, and they express matrix metalloproteinases MMP-2 and MMP-9 (Gueye et al., 2011) which enable OECs to migrate and penetrate the extracellular matrix.

Within their endogenous environment OECs are known to phagocytose axonal debris that arises from the turnover of olfactory receptor neurons (Su et al., 2013; Ekberg and St John, unpublished data). Further, we have recently shown that they can phagocytose bacteria (*E. coli* and *B. thailandensis* in vitro) (Panni et al., 2013). Thus, OECs that are transplanted into an injury site are likely to contribute to the immune response to remove cellular debris and foreign matter.

Together, these various characteristics of OEC biology make them excellent candidates for neural repair therapies, however, the use of OECs needs to be considered within a complex multicellular environment.

**OECs: a heterogeneous population of cells**

One of the problems in making comparisons of published experiments is that different sources of OECs have been used in different studies. OECs exist in the peripheral nervous system (the nasal epithelium) and in the central nervous system (the olfactory bulb in the brain) (Fig. 1) and cells that have been purified from either of these regions have been transplanted into an injury site in the spinal cord. We have demonstrated that peripheral
OECs have distinctly different behaviours and effects on axon growth compared to central OECs (Windus et al., 2010) but it is yet to be determined which subpopulation of OECs has the most potential for neural regeneration therapies. In addition, the purity of the cells varies depending on the protocols used and the methods used for identification of OECs. Unless there is 100% purity of OECs, other cell types are included in the transplanted cell mix but it is unknown what the contributions of the different cells are, whether they are crucial to the role of OECs and if they enhance or impede regeneration. These variations in the source and purity of the cell preparations are likely to lead to variations in therapeutic outcomes.

Apart from their different proposed roles for olfactory axon guidance in vivo, it is clear that OEC populations also differ in their molecular composition. In the peripheral nerve and the outer nerve fibre layer of the olfactory bulb, OECs express high levels of S100ß and the p75 neurotrophin receptor (p75ntr) (Au et al., 2002; Windus et al., 2010). In contrast, OECs of the inner nerve fibre layer do not express p75ntr and only low levels of S100ß (Au et al., 2002; Windus et al., 2010). Instead, OECs in the inner nerve fibre layer express neuropeptide Y (NPY), which is not expressed at all by OECs in the outer nerve fibre layer and the olfactory nerve (Ubink and Hokfelt, 2000; Windus et al., 2010).

OECs show a remarkable plasticity when cultured in vitro (Pixley, 1992); the morphology and molecular composition is highly dependent on culture conditions (Vincent et al., 2003; Huang et al., 2008). OECs can change morphology very rapidly; when studied with time-lapse imaging, OECs can transition from being bipolar to flattened within an hour after changing the composition of the culture medium (Fig. 1F-G) (Huang et al., 2008). Here, the bipolar, spindle-shaped OECs migrate three-fold faster than the OECs with a flattened morphology (Huang et al., 2008) so morphology may reflect a different functional state. We
have developed transgenic mice in which OECs express a bright fluorescent red protein (S100β-DsRed mice), from which we have purified individual OEC populations and studied their behaviour (Windus et al., 2007; Windus et al., 2010). We compared peripheral OECs from the olfactory nerve, and central OECs from the olfactory bulb. We found that OECs from the olfactory nerve adhered to and migrated in close contact with each other, whereas bulb OECs were only loosely associated and showed complex mixed behaviours involving both adhesion and repulsion (Windus et al., 2010). These findings reflect the proposed roles of OECs in vivo: in the olfactory nerve, OECs are thought to mediate fasciculation of axons, and in the bulbs, OECs are suggested to mediate a variety of functions including defasciculation, sorting and refasciculation of olfactory axons. We also showed that OECs from the olfactory bulb can be further divided into individual subpopulations with distinct characteristics depending on their location within the bulb (dorsal/ventral, rostral/caudal) (Windus et al., 2010). Thus, OECs are not a uniform population of cells; at least three subpopulations are now clearly defined with strikingly different behaviours and proposed roles in the olfactory nervous system. What is yet to be addressed in detail is whether the subpopulations of OECs have distinct characteristics following transplantation and if so which subpopulation is best for neural repair therapies?

**Purification of OECs for transplantation**

The variations in the outcomes in terms of anatomy and functionality in the different studies of neural repair can be attributed to both the anatomical source of the OECs and to the cell purity. Each individual laboratory optimises their own protocols for dissection, purification and culturing of OECs. For example, “peripheral OECs” from the olfactory epithelium and lamina propria have been used in many studies (Ramer et al., 2004) as they are easily dissected from the nasal cavity and therefore are most relevant to straightforward autologous
transplantation in humans (Mackay-Sim et al., 2008). Alternatively, “central OECs” are often acquired from the entire nerve fibre layer of the olfactory bulb (Li et al., 1997; Ramon-Cueto, 2000; Lopez-Vales et al., 2006; Li et al., 2007), however, other groups have used a more defined population of central OECs from a distinct region of the olfactory bulb, that is, rostral (Lankford et al., 2008) or ventral (Guest et al., 2008). In some studies, the term “central OECs” has been used without further explanation of their anatomical origin (Teng et al., 2008).

When OECs are isolated from any site within the olfactory nervous system, in particular the olfactory mucosa, they are “contaminated” with many other cell types (Fig. 2). The olfactory epithelium is populated by olfactory neurons, OECs, neural stem cells, other progenitors and sustentacular cells. Within the lamina propria immediately beneath the epithelium, olfactory receptor axons are ensheathed by OECs which are themselves surrounded by fibroblasts (Fig. 2). In close proximity to the olfactory nerve bundles are the trigeminal nerve bundles in which Schwann cells wrap up the trigeminal axons (Fig. 1E). In rodents, the accessory olfactory nerve is also present (Fig. 2) and the role/benefit of accessory OECs is not known, but these could also contaminate “main OEC” cultures. The purification of OECs often relies on the use of immunostaining for the marker p75ntr, but OECs within both the main olfactory nervous system and the accessory olfactory systems express p75ntr, as do Schwann cells of the trigeminal nerve which also innervates the nasal cavity. Further complicating the identification of cells is the fact that cultured fibroblasts have very similar morphology to OECs and have been shown to express p75 in vitro (Garcia-Escudero et al., 2012) under some conditions. Thus cultures of cells from the olfactory mucosa which are reported to contain bipolar cells that express p75ntr may potentially include OECs from the main and accessory olfactory system, Schwann cells and fibroblasts.
One example that highlights the variability in functional outcomes after transplantation of OECs from the olfactory mucosa is a recent study investigating the method in a canine model. Here, transplantation had quite positive therapeutic outcomes with some dogs regaining considerable function and others showing no improvement (Granger et al., 2012). The variability in the results is likely due in part to the natural variations in the injuries that the dogs sustained, but may also be due to the cellular mix that was used. The purity of the cells was reported to be around 50% p75 positive, but with considerable variation between preparations (+/- 30%). If fibroblasts in culture can also express p75 under certain conditions (Garcia-Escudero et al., 2012) then without further markers the identity of the cells becomes less clear.

It is also possible that combining OECs with other cell types may be beneficial in terms of functional outcomes. In the olfactory nerve fascicles, OECs and fibroblasts together form tunnel-like structures through which bundles of axons extend (Li et al., 2005) and fibroblasts have been shown to promote the proliferation of OECs in the olfactory bulb (Yui et al., 2011). After transplantation into the injured corticospinal tract, OECs and fibroblasts in conjunction form a “bridge” that creates a path for regenerating axons (Li et al., 1998). Similarly, OECs and fibroblasts transplanted together can enhance regeneration of nigrostriatal dopaminergic axons (Teng et al., 2008). Indeed, it has been proposed that the combination of fibroblasts together with OECs is needed for maximal therapeutic potential (Raisman and Li, 2007). In support of this idea, it has been shown that fibroblasts can dramatically enhance the migration and organisation of Schwann cells at an injury site (Parrinello et al., 2010), which may confer superior capacity for neural regeneration.
If Schwann cells or fibroblasts are included in the semi-purified “OEC” mix of cells, differences in the proliferation rates could result in the cultures being overrun by one or other cell type. Without precise tracking of the relative proportions of the cells while in culture and after transplantation it would difficult to replicate therapeutic outcomes as variations in the contributions of the different cell types could alter the regenerative capacity of the cell transplant therapy. At present, there are not other markers or purification protocols that clearly separate the different glia or fibroblasts and thus there is limited capacity to improve the purification techniques. Thus, we need to consider both the source of OECs in terms of subpopulations, and their behaviours in a multicellular environment containing other cell types, in particular fibroblasts. Recently the regenerative ability of OECs, Schwann cells and fibroblasts has been examined in the spinal cord of rat and the results showed that the purified glia performed better than fibroblasts (Toft et al., 2013). In particular, the use of dissociated mucosa resulted in higher levels of hypertrophy compared to the purified glia preparations which indicates that the use of undetermined mixed cell preparations may not be ideal (Toft et al., 2013).

**Human spinal cord injury and autologous olfactory ensheathing cell transplantation**

The promising studies using animal models have led to the use of OECs in human spinal transplant therapies. In 2005, a clinical trial showed that transplantation of OECs from the nasal cavity into the spinal cord of the same individual (autologous transplantation) is feasible and safe in humans (Feron et al., 2005). The study was a Phase I/IIa clinical trial; this type of trial is generally conducted to determine whether a procedure is safe (Phase I) and to initiate evaluation of efficacy/functional outcomes in a small group of patients (IIa). OECs were isolated from the nasal epithelium of spinal cord injury patients, cultured and transplanted into the injury site. The quantity of OECs transplanted ranged from 12-20
million, and the patients were males aged 18-55 who had all sustained complete thoracic paraplegia 18-32 months prior to transplantation. Patients were monitored for 3 years using a range of standard methods for evaluating anatomical and functional outcomes such as magnetic resonance imaging (MRI), AIS scores (which determines completion of a spinal cord injury, ranging from A to E; a score of A shows that the entire spinal cord is severed with little hope of regaining motor control below the site of injury), FIM/SCIM scores (which determines the degree of disability/independence) and others. The results demonstrated that the procedure is safe; at 3 years, there were no alterations in MRIs, and no adverse effects in any of the patients. While the patient number was too low (3 individuals/group) for any real meaningful evaluation of functional improvements, one patient exhibited increased sensation to light touch and pin prick tests, suggesting an increased sensation in the zone of partial preservation (Mackay-Sim et al., 2008).

**Using olfactory mucosa (mixed cell source) for neural regeneration in humans**

Since this study, autologous transplantation of nasal mucosa (containing OECs, but also other cell types and potentially including neural stem cells) has been performed in several clinics; however, the procedure does not yet follow formal clinical trial protocols. Together, these reports represent 32 cases of autologous transplantation of olfactory mucosa in spinal cord injury patients. The outcomes suggest that the transplantation was well tolerated, although 15% of patients showed meylomalacia (softening of the spinal cord primarily caused by bleeding) or syringomyelia (development of a fluid-filled cavity) that could have been caused by the procedure. AIS scores showed improvement in 13 patients (40%) and decreased in only one patient. So far, these studies show that the procedure is relatively safe (Lima et al., 2010) and may improve therapeutic outcomes, although no real conclusions can be drawn until a proper Phase II clinical trial is conducted.
The way forward

The main challenge is the fact that the purity and identity of the cells transplanted has not yet been fully addressed in any study. As discussed above “nasal mucosa” contains not only OECs, but also Schwann cells, fibroblasts, olfactory stem cells and other cell types. Furthermore, as we have shown, OECs consist of many different subpopulations with strikingly different behaviours (Windus et al., 2010). Thus, the differences in functional outcomes are most likely attributable to the fact that each transplant contained an individual composition of cells. In order to improve reproducibility, it is therefore critical to determine how the presence of other cell types, in particular fibroblasts and olfactory stem cells, affect the behaviour of OECs, and to determine how individual subpopulations of OECs modulate axonal extension.
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Figure legends

Figure 1. Olfactory ensheathing cells are the glia of the olfactory system. (A) A sagittal view of an OMP-ZsGreen mouse head showing the olfactory system (Ekberg et al., 2011). Axons of olfactory receptor neurons express ZsGreen fluorescent protein. Axons project from the nasal cavity in the peripheral nervous system into the olfactory bulb within the central nervous system. (B-C) A coronal view of the olfactory system in a OMP-ZsGreen mouse crossed with a S100β-DsRed mouse. Axons express ZsGreen (shown in B) and OECs express DsRed (shown in C); chondrocytes also express DsRed. OECs are present in the peripheral olfactory nerve nerve bundles and in the nerve fibre layer surrounding the exterior of the olfactory bulb. Boxed area in C is shown in D. (D) OECs (red) help fasciculate olfactory receptor axons (green) in the lamina propria (LP) which underlies the olfactory epithelium (OE). When axons (green) enter the nerve fibre layer (NFL) of the olfactory bulb, OECs (red) help axons defasciculate, sort out and project to their targets. (E) Within the lamina propria, olfactory nerve bundles are adjacent to trigeminal nerve bundles. (F-G) Cultured OECs can alter morphology rapidly and sometimes exhibit the bipolar morphology but then switch to the flattened morphology. Scale bar is 1000 μm in A, 500 μm in B-C, 100 μm in D, 8 μm in E, 5 μm in F-G.

Figure 2. The olfactory mucosa. The olfactory mucosa consists of numerous cells types. Within the outer olfactory epithelial layer are the stem cells (horizontal basal cell and globose basal cell) that give rise to immature olfactory receptor neurons which mature and migrate apically and are supported by sustentacular cells. The axons of the receptor neurons penetrate through the basement membrane into the lamina propria where they are ensheathed by OECs which together with fibroblasts form the olfactory nerve bundles. Also located within the
lamina propria are bundles of the accessory olfactory nerve which are surrounded by accessory OECs, as well as the trigeminal nerve bundles which are surrounded by Schwann cells.

**Movie 1. Morphology of peripheral OECs.** Three-dimensional reconstruction of Ds-Red OECs surrounding an olfactory nerve bundle within the lamina propria. The cell bodies of the OECs are largely restricted to the exterior of the bundles with the lamellipodia penetrating into the bundles.

**Movie 2. Morphology of central OECs.** Three-dimensional reconstruction of Ds-Red OECs within the nerve fibre layer of the olfactory bulb. The cell bodies of the OECs have no distinct arrangement and the lamellipodial protrude in all directions.