Unique *in vivo* properties of olfactory ensheathing cells that may contribute to neural repair and protection following spinal cord injury

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Abstract

Olfactory ensheathing cells (OECs) are specialized glial cells that guide olfactory receptor axons from the nasal mucosa into the brain where they make synaptic contacts in the olfactory bulb. While a number of studies have demonstrated that *in vivo* transplantation of OECs into injured spinal cord results in improved functional outcome, precise cellular mechanisms underlying this improvement are not fully understood. Current thinking is that OECs can encourage axonal regeneration, provide trophic support for injured neurons and for angiogenesis, and remyelinate axons. However, Schwann cell (SC) transplantation also results in significant functional improvement in animal models of spinal cord injury. In culture SCs and OECs share a number of phenotypic properties such as expression of the low affinity NGF receptor (p75). An important area of research has been to distinguish potential differences in the *in vivo* behavior of OECs and SCs to determine if one cell type may offer greater advantage as a cellular therapeutic candidate. In this review we focus on several unique features of OECs when they are transplanted into the spinal cord.

Introduction

Olfactory ensheathing cells (OECs) and Schwann cells (SCs) share a number of morphological and molecular markers, but have different embryonic origins: OECs are derived from the olfactory placode and Schwann cells from the neural crest. While SCs typically present as spindle shaped cells in culture, OECs have both a spindle and a flattened morphology. Both cell types express p75, GFAP and S100, and cell adhesion molecules such as L1 and N-CAM. Moreover, both express extracellular matrix molecules such as fibronectin and laminin (reviewed by Wewetzer et al., 2002). Both cell types are also a source of trophic factors including NGF, BDNF, GDNF, CNTF, FGF and VEGF, but the expression of these factors may vary depending on culture conditions or the particular *in vivo* microenvironment (Au and Roskams; 2003; Chuah and West, 2002; Oudega and Xu, 2006; Ramon-Cueto and Avilia, 1998). While both OECs and SCs have high expression of p75 in culture, they show low p75

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expression in stable *in vivo* conditions; p75 is drastically upregulated after olfactory receptor or peripheral axonal injury, respectively (Wewetzer et al., 2005; Taniuchi et al., 1988). Thus, while of different embryonic origin both cell types have remarkable similarity in terms of their moleclular markers.

There is an extensive literature on transplantation of OECs and SCs into injured spinal cord. Both cell types have been shown to improve functional outcome in various spinal cord lesion models. A comprehensive and critical review of this work can be found in Franssen et al. (2007). Given the remarkable similarities between many aspects of OECs and SCs, and the potential clinical importance of using either of these cell types as a cellular clinical tool in spinal cord injury (SCI), we focus here on differences in the in vivo fate of OECs and SCs transplanted into the injured spinal cord.

1. OECs build unique cellular tunnels through which axons regenerate across a spinal cord transection lesion site

A unique feature of axonal regeneration following OEC transplantation into transected spinal cord is the occurrence of groups of regenerated axons within the transection lesion site with a peripheral pattern of myelination surrounded by a fibroblast-like cell that forms a "tunnel" around small clusters of myelinated axons (Li et al., 1997; Sasaki et al., 2004: See Figure 1A-C). These tunnels have not been reported following SC transplantation into spinal cord transection lesions (Imaizumi et al., 2000a,b) or into demyelinating lesions (Lankford et al., 2002). Li et al. (1998) referred to the surrounding fibroblast-like cells as "A" cells and the myelin-forming cells as "S" cells, and suggest that both can be derived from the donor OECs (Fig 1D,E). While transplanted SCs can myelinate spinal cord axons (Blakemore and Crang, 1985; Baron-Van Evercooren et al., 1992; Honmou et al., 1996; Kohama et al., 2001) and are in some cases associated with improved functional outcome following transplantation into the injured spinal cord (Takami et al., 2002), the "A" and "S" cell organization appears to be unique to OEC transplantation (Li et al., 1997, 1998; Imaizumi et al., 2000a,b).

This cellular "tunnel" organization through which axons can regenerate in the spinal cord is not observed following endogenous repair or following transplantation of SCs (Imaizumi et al., 2000a). These clusters of myelinated axons growing through the cellular tunnels were variable from animal to animal and confined mostly to and near the lesion zone. Thus, the hypothesis was developed that the fibroblast-like A cell of the transplanted OEC population formed a tunnel-like structure which provided a permissive environment through which transected spinal cord axons could regenerate across a lesion site (Li et al., 1997; Figure 2D,E). Axons regenerating across the spinal cord transection site within these OEC tunnels may be shielded from myelin inhibitory proteins present on oligodendroctyes. The myelinated axons within the tunnel have a peripheral pattern of myelin including P0 expression, and when GFP-expressing cells are used for transplantation both endogenous and transplanted OECs are able to myelinate the regenerated axons (Sasaki et al., 2004; 2006a). The relative advantage of this unique *in vivo* property of OECs is not fully understood, but clearly represents a distinct difference between OECs and SCs in the structural changes that occur in spinal cord transection lesions after transplantation.

The tunnel-like configuration is not observed when OECs are transplanted into a demyelinated lesion (Imaizumi et al., 1998). Rather, the demyelinated axons are remyelinated by GFP-identified OECs with a peripheral pattern of myelin (Sasaki et al., 2006). Since axons are in continuity in demyelination models, it may be that the S-type cells dominate and form myelin and the A-type cells die-back or do not participate in repair. Thus, it may be that only when OECs associate with transected axons they may form a cellular bridge through which axons can regenerate.

It has been argued that OECs are not the myelinating cell in the above studies and that contaminating SCs may be responsible for the remyelination (Rizek and Kawaja, 2006). These investigators suggest that OECs, but not SCs, express the muscle fiber actin binding protein calponin (Boyd et al., 2006), and that most cells in OEC culture preparations are p75[+] and calponin[-], thus suggesting a contamination in OEC cultures of Schwann cells. A strong counter argument to SC contamination accounting for the remyelination by OECs, is the Franklin et al. (1996b) study where they used an OEC cell line and achieved remyelination. More recently this group demonstrated that calponin was present in the olfactory fibroblast meningeal cells, but not in the adult OECs (Ibanez et al., 2007), thus strengthening the argument that remyelination following OEC transplantation was not the result of SC contamination.

2. OECs, but not SCs, migrate extensively in the X-irradiated spinal cord and establish a unique phagocytic phenotype

Although SCs and OECs share many morphological, antigenic, and proliferative characteristics (Ramon-Cueto and Avila, 1998; Wewetzer et al., 2002), OECs exhibit properties that suggest a greater potential for migration within the CNS (Bartolomei and Greer, 2000; Lakatos and Franklin, 2002; Franklin, 2002; Lu and Ashwell, 2002). OECs intermingle more freely with co-cultured astrocytes than SCs (Lakotas et al., 2000, Van den Pol and Santarelli, 2003), adhere less strongly to astrocyte monolayers (Fairless et al., 2005), do not induce astrocytic hypertrophy (Lakatos et al., 2000) *in vitro*, and elicit smaller increases in GFAP and chondroitin sulphate proteoglycan expression after transplantation than SCs (Lakatos et al., 2003; Garcia-Alias et al., 2004; Andrews and Stelzner, 2007). The morphology and antigen expression of adult OECs also appear to be more plastic than SCs, changing in response to culture media composition (Alexander et al., 2002), cell density (Sonigra et al., 1999), and axon contact (Ramon-Cueto et al., 1993). Like SCs, OECs phagocytize cellular debris after injury *in vivo* (Chuah et al., 1995) or *in vitro* (Wewetzer et al., 2005), but unlike SCs, OECs also exhibit microglia-like cytokine responses to bacterial proteins (Vincent et al., 2007). Taken together, these observations imply that OECs produce a more complex array of responses to local environmental conditions.

Are there differences in migration properties between SCs and OECs in vivo? When transplanted into chemically demyelinated lesions in the spinal cord, both OECs and SCs migrate and myelinate axons within the lesion site (Blakemore, 1977; Harrison, 1980; Honmou et al., 1996; Kato et al., 2000; Franklin, 2003; Akiyama et al., 2004; Sasaki et al., 2006a,b). However, OECs, SCs and oligodendrocyte progenitor cells (OPCs) show poor to limited survival and migration after transplantation into normal white matter (Franklin et al., 1996a; O'Leary and Blakemore, 1997; Iwashita et al., 2000; Hinks et al., 2001; Lankford et al., 2008). Hinks et al. (2001) made the interesting observation that focal X-irradiation of the spinal cord a week prior to transplantation selectively facilitates migration of OPCs, but not SCs (Iwashita et al., 2000; Lankford et al., 2008). The OPCs migrated extensively throughout both white and grey matter in the spinal cord. The dosage of X-irradiation used in these studies does not produce obvious gross structural changes in the spinal cord over the course of the study nor are there obvious functional deficits. However, NG2^{+} OPCs are lost in the X-irradiation zone. The precise changes induced by the irradiation that provide for a permissive environment for the enhanced migration of OPCs is not known. Hinks et al. (2001) suggest that the killing of endogenous OPCs by the irradiation opens structural niches to which the transplanted OPCs can migrate.

While SCs do not migrate in the normal or X-irradiated spinal cord, we recently found that OECs migrate extensively in both white and grey matter of the X-irradiated spinal cord similarly to OPCs (Lankford et al., 2008). Within the X-irradiated spinal cord, the OECs appeared randomly distributed within their area of dispersion (Fig 3). The cells had relatively

small cell bodies (less than 25 μm) and gave rise to variable numbers of branched processes (Fig. 3A,B). Although in culture the OECs were p75$^+$, the distributed cells were p75$^-$ suggesting a phenotypic change *in vivo*. Interestingly the OECs showed weak OX-42 staining on their processes and were phagocytic. Phagocytic debris can be seen in electron micrographs of identified GFP-expressing OECs that have been transplanted into the X-irradiated spinal cord (Lankford et al., 2008). A possibility is that the OECs migrate to areas of killed OPCs and phagocytize these cells. While not proven this possibility is supported by the observation that there is no other clear cell loss than the OPCs following the irradiation protocol. These observations indicate that the dispersed OECs developed a unique *in vivo* phenotype as compared to OECs in culture. Moreover this also demonstrates a clear difference between the *in vivo* properties of OEC and SC populations.

3. OECs preloaded into the X-irradiated spinal cord can repair demyelinated lesions induced several weeks later

Virtually all experimental cell transplantation approaches to improve function after SCI or demyelination, utilized transplantation of cells after lesion induction. Given that in the irradiated spinal cord OECs migrate over a broad region and possibly reoccupy OPC progenitor zones, we asked the question as to whether OECs could be "pre-loaded" into the spinal cord and if they would respond to subsequent myelin injury (Lankford et al., 2008). The spinal cords were irradiated and OECs injected into the dorsal funiculus one week later. Two weeks later, a time point when the OECs are dispersed throughout white and grey matter, we made a small focal demyelinating lesion by microinjection of ethidium bromide into the dorsal funiculus. After three additional weeks the spinal cords were prepared for histology. The dispersed OECs moved into the delayed demyelinated lesion (Fig. 3A). Interestingly the GFP identified cells remyelinated host axons with a peripheral-like pattern of myelin (Fig. 3B-E). Thus under these experimental conditions the OECs could be "pre-loaded" into a relatively intact spinal cord, and mobilize for myelin repair when lesions were induced several weeks later. These results revealed a clear difference between the migratory properties of OECs and SCs in the X-irradiated spinal cord and demonstrated that engrafted OECs can participate in repair of subsequent delayed lesions.

Concluding Remarks

Transplantation of OECs or SCs into demyelinated or traumatic spinal cord injuries can improve functional outcome. There has been controversy as to whether one cell type is more beneficial than the other. Because OECs and SCs in culture share many phenotypic properties such as the low affinity NGF receptor (p75) expression as outlined above, distinctions between these two cell types have been difficult to clearly delineate. In this review we outlined two clear differences in the *in vivo* fate of transplanted OECs into the spinal cord: 1) the formation of cellular tunnels by OECs in the transected spinal cord through which axons can regenerate across the lesion site and 2) the ability of OECs, but not SCs to migrate within the X-irradiated spinal cord. At a minimum these observations suggest that in spite of the numerous similarities of the two cell types differences are indeed present between them. The extent to which these differences may impact their potential as cellular therapy candidates in SCI is not as yet established.

A key issue with cellular transplantation approaches to remyelinate CNS axons is the inability of axons to migrate within normal white matter (Hinks et al., 2001). If myelinating cells were transplanted into a focal multiple sclerosis lesion it is unlikely that cells could migrate from one focal lesion to another, thus limiting repair potential. The studies by the Franklin and Blakemore groups (Iwashita et al., 2000; Hinks et al. 2001) established that OPCs could migrate through normal (no myelin disruption) white matter in the X-irradiated spinal cord and home to a demyelinated lesion site some distance away. While pre-X-irradition may not be practical

as a means of enhancing cellular mobility for myelin repair, their X-irradiation studies provide important proof of principle that enhancing the mobility of myelin forming cells through normal white matter could have potential therapeutic benefits. They subsequently demonstrated that SC precursors derived from embryonic tissue can migrate through normal white matter (Woodhoo et al., 2007). Better understanding of the molecular mechanisms that allow myelin forming cells to migrate through normal adult white matter will be important in potentially engineering cells for myelin repair.

A consequence of X-irradiation used to kill tumors is post-radiation myelopathy and necrosis. There has been interest in a potential cell replacement therapy to treat post-radiation myelopathy and necrosis (Wong and van der Kogel, 2004; Rezvani et al., 2001). Chari et al. (2006) point out that the levels of X-irradiation needed to allow extensive migration of transplanted OPCs will result in post-radiation necrosis possibly from delayed endothelial cell damage suggesting that oligodendrocyte replacement to achieve remyelination alone would not be an effective thereapy. While our animals showed minimal behavioral deficits and pathological damage to the spinal cord over the course of our studies, by 4-6 months nearly all animals with the same level of X-irradiation develop severe post-radiation necrosis and paralysis (unpublished results). A cellular approach for reducing post-radiation necrosis would in principle require a pluripotent cell that could protect or replace damaged epithelial cells, as well as possibly contribute to remyelination and neural protection. Interestingly, Rezvani et al. (2001) injected neural stem cells into the X-irradiated spinal cord and found a reduction in the number of animals that developed paralysis. OECs have been shown to provide neuroprotection possibly by the release of neurotrophic factors (Sasaki et al., 2006b) and it will be interesting to determine if they or other cell types can modulate the course of post-radiation necrosis. It will be of importance to determine if cells injected just after X-irradiation can migrate and survive within the X-irradiated spinal cord and respond to subsequent injury (delayed cell loss) by either providing protection or by cellular repair.

While controversy exists in terms of the relative benefits of OECs and SCs in contributing to improved functional outcome when transplanted into spinal cord injury sites in animal models, it clear that both cell types have been reported to improve functional outcome (See Franssen et al., 2007 for an overview). Moreover, in spite of the numerous similarites between the two cell types there are differences in their *in vivo* fate when transplanted into spinal cord. What advantages these differences may have is unclear and future comparative work assessing OECs and SCs as cell therapy tools will be critical.

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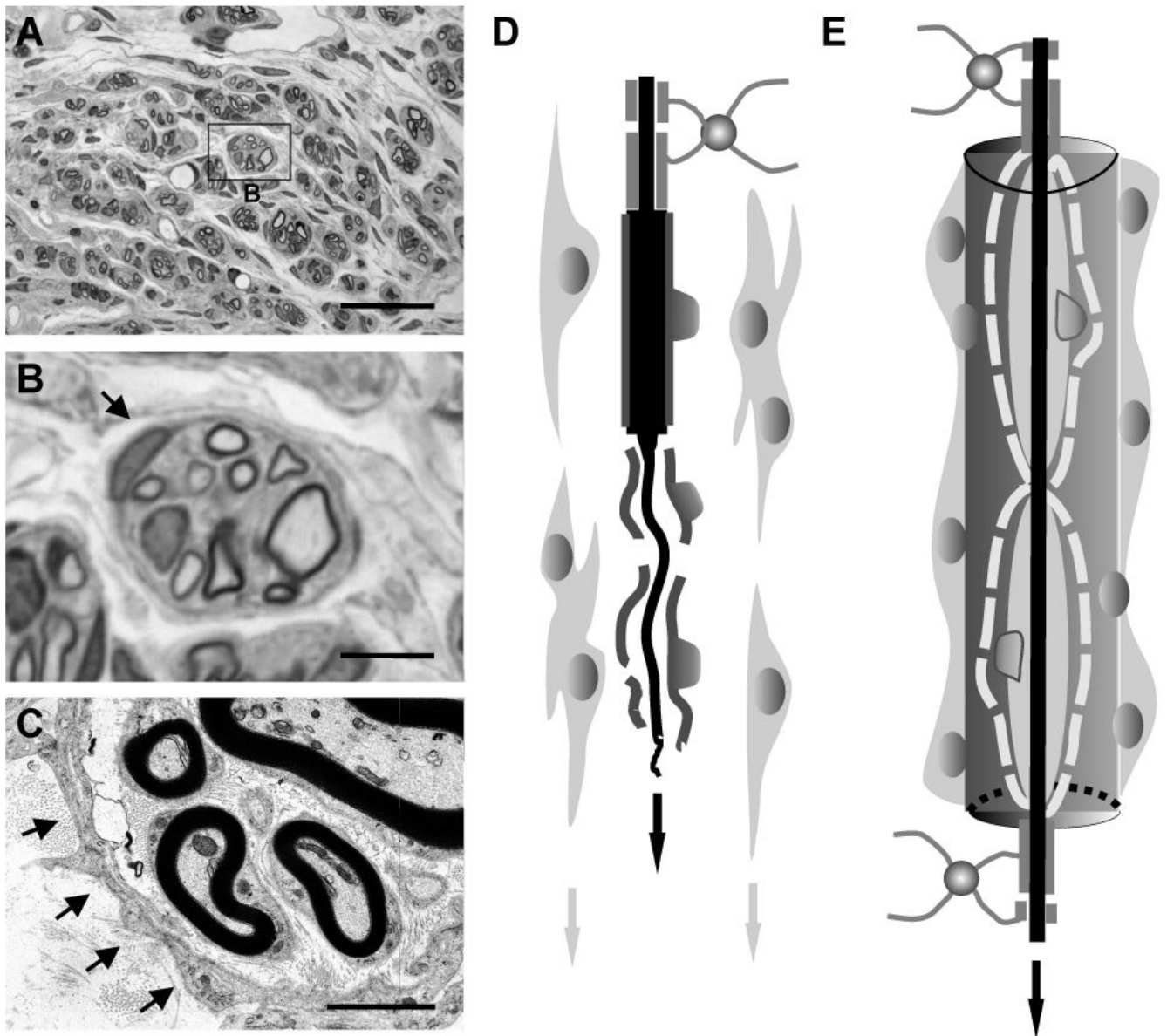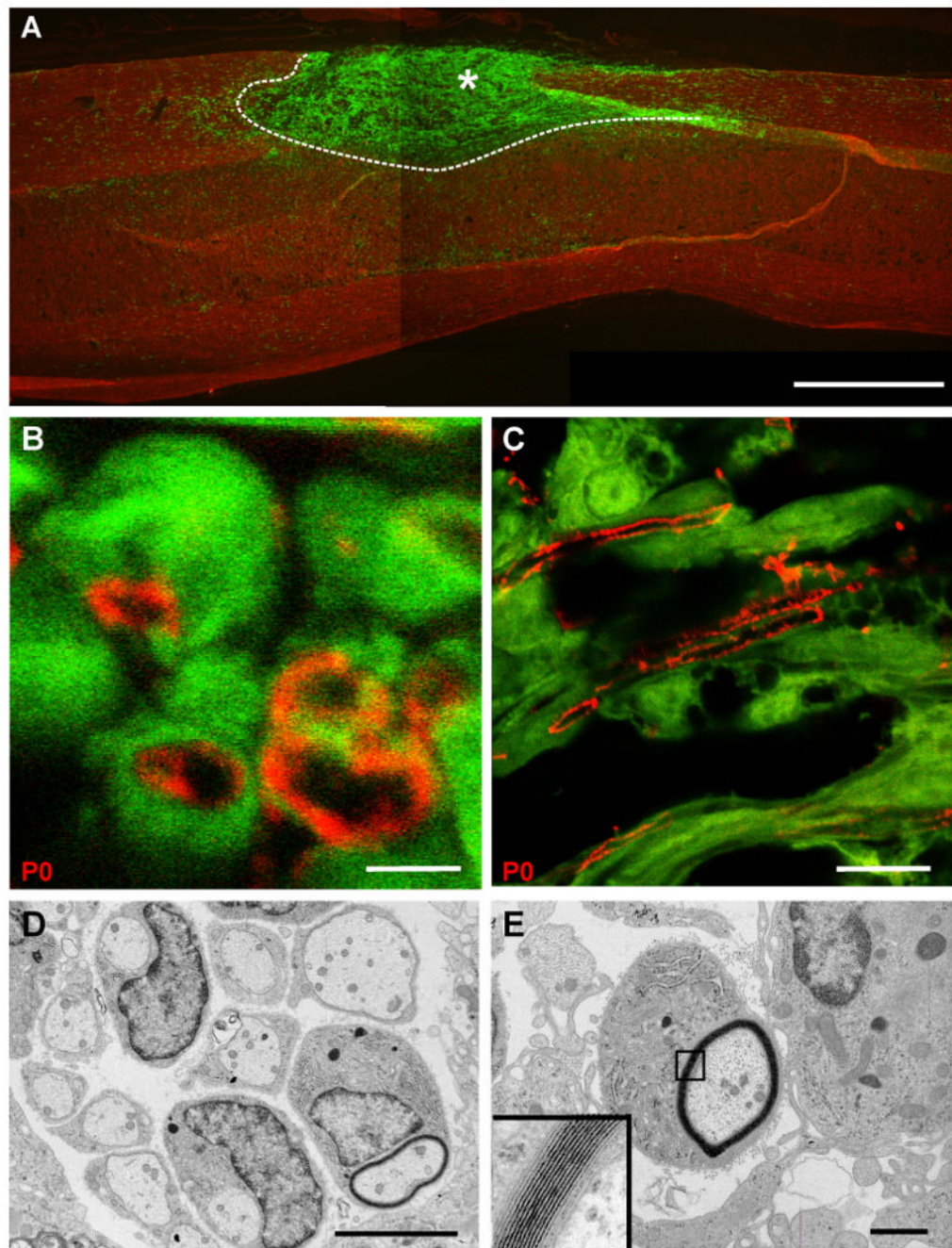
Figure 1. Transplantation of OECs into a transected (dorsal hemisection) spinal cord results in the formation of cellular "tunnels" across the transection site

Axons can regenerate through these cellular tunnels and are myelinated (A,B). The electron micrograph in C shows a cytoplasmic process of a cell forming a cellular "tunnel" through which axons regenerate. Raisman (2001) proposed that two phenotypes of OECs may be present. One type (A cell) forms the tunnel through which axons regenerate and the other type (S cells) can myelinate the regenerated axons within the cellular tunnel (D, E). Scale bars: A, B, C = 30 μm, 6 μm and 2 μm repectively. A-C have been modified from Sasaki et al., 2004.
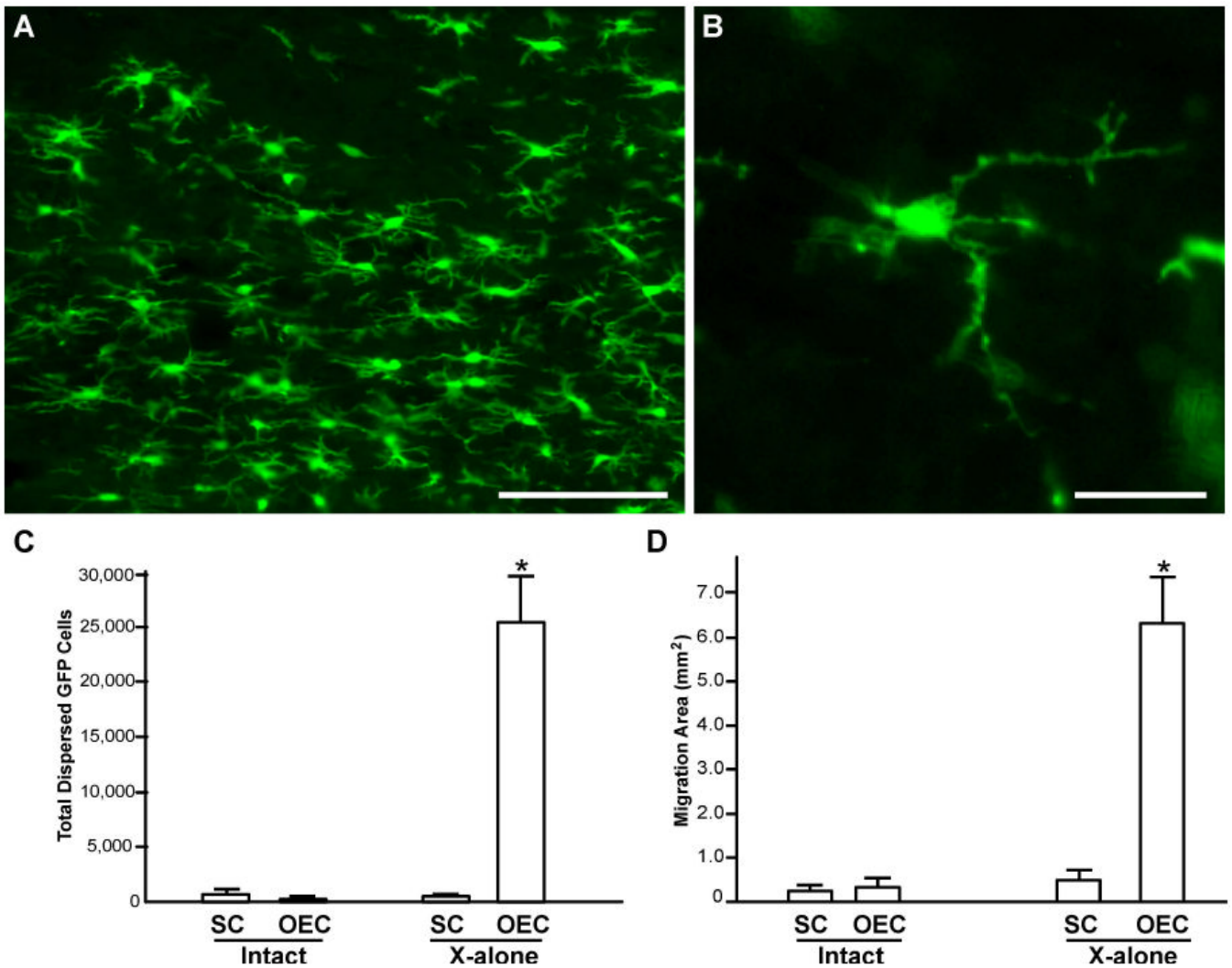
Figure 2. Distribution and morphology of dispersed OECs in X-irradiated spinal cord
A. Fluorescent micrograph showing distributed GFP-expressing OECs transplanted a week
after spinal cord X-irradiation. The micrograph was obtained three weeks after OEC
transplantation. B. Higher power micrograph showing typical small cell bodies and branched
processes. C-D. Bar graphs showing the total number (C) and migration area (D) of dispersed
transplanted SCs and OECs in intact and X-irradiated spinal cord at three weeks post-
transplantation indicate that OECs, but not SCs survive and distribute extensively in the X-
irradiated spinal cord. A=200μm, B= 25μm. Modified from Lankford et al., 2008.

Figure 3. Localization of OECs following induction of a delayed demyelinated lesion
Three weeks after OEC transplantation into the X-irradiated spinal cord a focal demyelinated lesion was made in the dorsal funiculus. A. Sagittal section three weeks after lesion induction. Note the high density of green cells (*) within the demyelinated lesion. Red is GFAP staining. Dashed lines in A and C indicate boundary of lesioned area. Within the lesion P0 myelin is associated with surrounding green GFP cytoplasm of the transplanted OECs (B coronal section; C longitudinal section). Electron microscopy further indicates the myelinated and premyelinating profiles within the demyelinated lesion. Scale bars: A=1 mm, B=5μm, C=10μm; D=3μm, E=1μm. Modified from Lankford et al., 2008.