Development/Plasticity/Repair

Lamina Propria and Olfactory Bulb Ensheathing Cells Exhibit Differential Integration and Migration and Promote Differential Axon Sprouting in the Lesioned Spinal Cord

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Olfactory bulb-derived (central) ensheathing cell (OB OEC) transplants have shown significant promise in rat models of spinal cord injury, prompting the use of lamina propria-derived (peripheral) olfactory ensheathing cells (LP OECs) in both experimental and clinical trials. Although derived from a common embryonic precursor, both sources of OECs reside in different nervous system compartments postnatally, and their ability to promote regeneration and efficacy after transplantation may differ depending on both their source and mode of transplantation. Here, we have purified green fluorescent protein-expressing LP and OB OECs, assayed their biological differences in vitro, and transplanted them acutely either directly into or rostral and caudal to a dorsolateral funiculus crush. LP and OB OECs exhibit multiple morphological and antigenic similarities in vitro, and, after transplantation, they both attenuate lesion and cavity formation and promote angiogenesis, endogenous Schwann cell infiltration, and axonal sprouting. However, an increased mitotic rate and migratory ability of LP OECs in vitro was reflected in vivo by their superior ability to migrate within the spinal cord, reduce cavity formation and lesion size, and differentially stimulate outgrowth of axonal subpopulations compared with OB OECs. An undesired behavior (autotomy) was also significantly enhanced by LP OEC, over OB OEC, transplantation. These results suggest that LP and OB OECs exhibit intrinsic biological differences that, after transplantation into the lesioned CNS, result in differences in postlesion spinal cord neuropathology and anatomical and behavioral regeneration outcomes that also vary depending on direct versus rostrocaudal transplantation.

Key words: regeneration; olfactory; glia; p75(NGFR); spinal cord injury; rubrospinal; monoaminergic; substance P

Introduction

The abortive regeneration of CNS neurons is in stark contrast to the renewal capacity of the olfactory system (Graziadei et al., 1978, 1979). Reconstitution of the olfactory neuraxis can occur even after central axotomy (Zigova et al., 1992). This axonal growth within the CNS has been partially attributed to olfactory ensheathing cells (OECs), resident in the peripheral and central olfactory neuraxis (Doucette, 1990). For this reason, the therapeutic potential of olfactory bulb-derived (OB) OECs to promote recovery from CNS lesions to descending and ascending spinal tracts (Li et al., 1998; Ramon-Cueto et al., 2000; Lu et al., 2002; Nash et al., 2002; Keyvan-Fouladi et al., 2003), primary afferents (Ramon-Cueto and Nieto-Sampedro, 1994; Li et al., 2003) and remyelination of injured/demyelinated spinal cords (Franklin et al., 1996; Imaizumi et al., 1998; Sasaki et al., 2004) have been tested and yielded variable yet positive outcomes. Lamina propria-derived (LP) OECs have been tested only recently for their ability to promote regeneration after lesion (Lu et al., 2001, 2002; Ramer et al., 2004a,b), despite the ongoing clinical use of LP tissue in spinal cord injury (SCI) treatment (Senior, 2002; Huang et al., 2003).

LP and OB OECs, derived from a common progenitor in the olfactory mucosa (Carter et al., 2004), ensheath and promote the growth of olfactory receptor neuron axons as they exit the olfactory epithelium and extend toward the OB (Williams et al., 2004). As LP OECs enter the nerve fiber layer of the OB, they differentiate into OB OECs, where they associate with astrocytes (Au et al., 2002). Thus, although LP and OB OECs possess similarities in morphology and function in vitro (Au and Roskams, 2003; Jani and Raisman, 2004), subtle differences in ensheathing behavior, their distinct roles in the olfactory neuraxis, and their different maturational states suggest they may exhibit different properties when transplanted after SCI.

Groups have already reported varying regeneration outcomes after dorsal rhizotomy and transplantation of OB versus LP OECs (Ramon-Cueto and Nieto-Sampedro, 1994; Ramer et al., 2004a; Riddell et al., 2004). In these cases, the mode of transplantation and proximity of transplanted cells to the lesion site could influence integration and long-term cell survival of transplanted cells.
To assess how these differences may arise from different OEC sources, methods of transplantation, and assessment measures, it has become important to compare the regeneration-promoting abilities of LP and OB OECs within the same experiment. Here, we have purified transgenic enhanced green fluorescent protein (eGFP)-expressing OECs from the lamina propria and from the nerve fiber layer of the same mice. After establishing their expansion and phenotype in vitro, we acutely transplanted them directly or rostrocaudally into a dorsolateral funiculus crush. Differences examined include interactions with host glia, their ability to promote directional angiogenesis, and their migratory properties within the spinal cord. Furthermore, we have assessed regenerative and sprouting differences between LP and OB OEC-treated animals and demonstrated differences in autotomy resulting from transplantation of these two cell populations.

Materials and Methods

Cell preparation and purification. LP OEC cell culture. GFPunteer LP OECs were harvested from the olfactory mucosa of postnatal day 5 (P5) transgenic mice, homozygous for eGFP [GFP-U-NAGY; generated by Andreas Nagy, University of Toronto, Toronto, Ontario, Canada] by using a protocol provided by Frank Jirik, University of Calgary, Calgary, Alberta, Canada] expressed under the β-actin promoter and driven by the cytomegalovirus enhancer (Au and Roskams, 2003). The entire olfactory mucosa, including turbinates and septum, was dissected from 8–10 pups, mechanically dissociated, and treated with 0.6 mg/ml collagenase D (Roche Products, Indianapolis, IN), 3 U/ml dispase I (Roche Products), 15 μg/ml hyaluronidase (Sigma, St. Louis, MO), 0.5 mg/ml bovine serum albumin (MP Biomedicals, Irvine, CA), and 100 U/ml DNase I (Sigma) for 1 h at 37°C, before centrifugation and plating. Initial plating in MEM–n-valine, 10% fetal bovine serum (FBS), and 100 U/ml penicillin/streptomycin (PS) was followed 4–5 d later by purification using anti-Thy1.1-mediated complement lysis as described previously, to remove contaminating fibroblasts (Au and Roskams, 2003). Cells were replated in DMEM/F-12, 10% FBS, and 100 U/ml P/S and allowed to grow for an additional 4–6 d, when they were again subjected to Thy1.1-mediated complement lysis. LP OECs were plated at a density of 5600 cells/cm² into T75 flasks for transplantation. The time from dissection to transplantation varied from 14 to 16 d in vitro (DIV).

OB OEC cell culture. From the same litter of P5 eGFP transgenic mice, OB OECs were harvested by surgically bisecting the head, removing the meningeal covering of the olfactory bulb, using a scalpel to partition the rostralmost quarter of the olfactory bulb, and extracting the portion of the nerve fiber layer next to the cribriform plate. Nerve fiber layers were dissociated and cultured as described by Ramon-Cueto et al. (1998). Briefly, digestion by 0.1% trypsin for 15 min at 37°C was followed by dissociation by multiple passes through fire-polished pipettes, and the cells were seeded into poly-l-lysine (PLL) (50 μg/ml in 15 mM sodium borate buffer; Sigma) pretreated flasks (1 h at room temperature) containing DMEM/F-12, supplemented with 2 mM l-glutamine, 10% FBS, 50 μg/ml gentamycin, and 100 U/ml P/S. After 5–6 DIV, when cells had grown to ~80% confluency, OB OECs were purified by immunopanning. Detached cells were suspended in DMEM/F-12 with 10% FBS, and 500,000 cells were plated onto each 96 mm Petri dish, pretreated with (1) biotinylated goat-anti rabbit IgG antibody (350 μg/ml, 12 h, 4°C; Vector Laboratories, Burlingame, CA) and (2) rabbit anti-low-affinity nerve growth factor receptor (Sigma, 1 h at room temperature, p75; Chemicon, Temecula, CA). Cells were incubated for 30 min at 37°C, unbound cells were then washed away, and bound cells were detached using a cell lifter. The 30 min immunopanning incubation was used because it produced the greatest purity of OB OEC cultures, with an average of 92% p75positive cells (n = 4). OB OECs were subcultured once more before being plated at a density of 6500 cells/cm² into T75 flasks for transplantation. The time from dissection to transplantation was between 14 and 16 DIV.

Cell harvest for transplantation. Before transplantation, the cells from one T75 flask of LP or OB OECs were detached using 0.25% trypsin/1% EDTA, followed by washing in PBS and resuspension at a concentration of 50,000–60,000 cells/μl in DMEM/F-12. These cells were immediately transplanted into two recipient rats, ensuring that every rat received freshly harvested cells.

Immunocytochemistry: assessment of cell purity. LP OECs. To assess cell purity and to ensure that cells express typical morphology and phenotype before transplantation, GFPuent LP OECs were plated onto glass slides after the second subculture and fixed in 4% paraformaldehyde (PFA) after 2 DIV. Purity of the culture was assessed using the following primary antibodies incubated overnight at 4°C: rabbit anti-p75 (1:1000; Chemicon), anti-mouse S100β (1:1000; Sigma), and anti-galil fibrial acidic protein (GFAP) (1:750; DakoCytomation, Glostrup, Denmark). Slides were then incubated for 1 h at room temperature with Alexa 594 goat secondary antibodies (1:100; Jackson ImmunoResearch, Mississauga, Ontario, Canada) in 2% goat serum, followed by a 10 min incubation in 0.5 μg/ml 4,6-diamidine-2-phenylindole dihydrochloride (DAPI) (Boehringer Mannheim, Mannheim, Germany), and coverslipped in Vectashield mounting media (Vector Laboratories).

OB OECs. Initially, to determine the optimal time and antibody concentrations for immunopanning, OB OEC purity was assessed by plating different panning conditions onto glass slides as described above, staining with rabbit anti-p75 (1:1000; Chemicon), as well as secondary antibodies and DAPI as described above. DAPI nuclei were counted, as well as p75– cells to assess for the enrichment of p75cells. After optimizing cell purification procedures, all OB OEC cell cultures were then subjected to the same slide generation after second subculture and immunocytochemistry as described above for LP OECs.

Cell transplantation. LP or OB OECs cultured for the same DIV were harvested at passage 2 and plated onto Boyden chambers pretreated with PLL (50 μg/ml in borate buffer, 1 h at room temperature) or laminin (1 μg/ml in PBS, 1 h at 37°C) in 24-well plates at a density of 5000 cells/well. The main well contained 2.5% FBS in DMEM/F-12. Cells migrated for 20 h, the top sides of experimental transwells were then swabbed, both sides were swabbed for negative controls, and neither side was swabbed for positive controls. Transwells were fixed in 0.1% glutaraldehyde in 4% PFA in PBS for 30 min at room temperature. Membranes were then stained with DAPI, mounted onto glass slides, coverslipped with Vectashield (Vector Laboratories), and 5× images were collected, encompassing the entire membrane. DAPI– nuclei were counted, and migration values were expressed as a percentage of total cells on positive membranes (experimental minus negative counts divided by the total cells on positive membranes).

Spinal cord injury and cell transplantation. Dorsolateral funiculus crush. Animal procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care and were approved by the Animal Care Committee of the University of British Columbia. Forty-two adult male Sprague Dawley rats (WILKES, Wilmington, MA) were immunosuppressed with cyclosporine A (10 mg/kg i.p., i.p.; Novartis Pharmaceuticals, Mississauga, Ontario, Canada) 2 d before surgery and each day for the duration of the experiment. After anaesthetization with xylazine/ketamine (10 and 70 mg/kg, i.p.), a hemilaminectomy was performed, exposing the left halves of the third and fourth cervical segments. The dura was then cut with microsissors, exposing the spinal cord, and one prong of custom-designed fine surgical forceps was inserted between the gray and white matter, with the other prong outside the dorsolateral funiculus, to a depth of 1 mm. The forceps were then closed for 18–20 s, crushing the dorsolateral funiculus. Previous studies in our laboratory have confirmed that this injury reliably severs all rubrospinal axons (Ramer et al., 2004). Crush treatment was performed blind, because animals were designated to cell transplantation and control groups after crush.

Cell transplantation. LP or OB OEC cell slurry in DMEM/F-12 were drawn into a pulled glass pipette with a diameter of 20 μm in a Hamilton syringe. OECs were stereotactically microinjected either (1) within the lesion site over three injection points, at a depth of 0.7, 1.0, and 1.5 mm, dividing the cell suspension equally between these three points, or (2) 1 mm rostral and caudal (R/C) to the lesion site at a depth of 0.6 and 1.2 mm, dividing the suspension equally between four points (diagrammed in supplemental Fig. 1, available at www.jneurosci.org as supplemental material). A total of 1.5 μl of cell slurry was injected at a rate of 100
image, and the number of intersection points in the lesion site, as defined by an outline of the GFAP scar, was assessed. The scar area was then moved randomly and automatically around the image 100 times to ascertain a baseline value of directionality. The ratio of directionality in the lesion and at the random points is reported. t tests were used when appropriate, or ANOVAs followed by post hoc analysis using Tukey’s tests were used to compare across groups. Graphical data are presented with error bars representing SEM.

Results

To assess their comparative ability to promote regeneration, OECs were purified from the LP or OB of postnatal day 5 mice homozygous for eGFP under the β-actin promoter (Ramon-Cueto and Nieto-Sampedro, 1994; Au and Roskams, 2003). LP (Fig. 1A, C) and OB (Fig. 1B, D) OECs grown for 14 DIV demonstrated similar morphologic and antigenic profiles for S100β, GFAP, and the p75 neurotrophin receptor. LP and OB OECs exhibited both flattened and fusiform morphologies (Fig. 1A, B). OB OECs demonstrated a broader range of p75 expression than LP OECs; some OB OECs were highly p75 positive, whereas the majority demonstrated low p75 levels (Fig. 1B). Despite their antigenic similarity, LP OECs demonstrated a threefold higher expansion rate than OB OECs from passage 1–2 under the same culture conditions.

Purified passage 2 eGFP+ LP and OB OECs cultured for 14 DIV were transplanted either directly into or at sites 1 mm rostral and caudal to a crush of the dorsolateral funiculus. We first addressed differences between LP and OB OECs that may be predicted based on their home environments in the PNS and CNS compartments of the olfactory system such as their ability to migrate within the CNS and preferential interactions with endogenous glia. We tested whether R/C-transplanted LP and OB OECs displayed different patterns of migration within the CNS by assessing their change in distribution from 24 h to 28 d after lesion (Fig. 2). At 24 h after injection, 25 ± 7.7% of all transplanted LP OECs and 30 ± 6.9% of OB OECs were already found at the lesion site, and both types of OECs were similarly arranged in a stream from the rostral injection point to lesion center. Because we could not detect cells between the caudal injection point
Rostrocaudally transplanted rats had more GFP and rostrocaudal transplantation (Fig. 3) exacerbated on laminin with OB OECs migrating 85% less than LP OECs (in a Boyden chamber migration assay, OB OECs migrate 60% less than LP OECs after 20 h on PLL, and the migratory difference is differed significantly between OEC types). Differences in migratory ability of LP and OB OECs (in pixels) at 1 and 0.5 mm rostral/caudal and within the lesion site reveals significantly more GFP cells at 0.5 and 1 mm caudal in OB-treated rats than in LP OEC-treated rats (p \leq 0.05). In vivo, using a Boyden chamber migration assay, OB OECs migrate 60% less than LP OECs after 20 h on PLL, and the migratory difference is exacerbated on laminin with OB OECs migrating 85% less than LP OECs (n = 3; p \leq 0.02, all groups). Data are presented as mean \pm SEM. Scale bars, 250 \mu m.

Lesion site area and cavity formation are decreased by LP versus OB OEC transplantation

Differences in migratory ability may change how effectively LP and OB OECs are able to minimize lesion and cavity formation during the acute phase of SCI. To test whether differential migration correlated with lesion size, we used the GFAP-reactive border as a marker of the perimeter of the lesion area and measured lesion area, cavity area, and the percentage of the lesion site occupied by cavity (Fig. 3A). All groups exhibited an easily defined lesion area containing some evidence of a cavity. However, rats that received direct OB OEC transplants (Fig. 3A) had a significantly increased lesion site area (by approximately twofold to threefold) compared with rats that received the same cells rostrocaudally (Fig. 3B) or with rats that received LP OECs transplanted either directly (Fig. 3C) or rostrocaudally (n = 4 all groups; p \leq 0.02) (Fig. 3D). Rats that received direct or rostrocaudally transplanted LP OECs also developed smaller cavities than rats with direct OB OEC transplants (n = 5, 4, 4; p \leq 0.02 and 0.04, respectively) (Fig. 3F). The greatest significant decrease in cavity formation was in rats that received LP OECs transplanted rostrocaudally (Fig. 3F), which also resulted in a significantly lower percentage of the lesion site occupied by cavity (Fig. 3G), a trend not observed after rostrocaudal OB OEC transplantation (Fig. 3G). The smaller lesion site area in rostrocaudally transplanted LP and OB OEC rats and directly transplanted LP OEC rats was four times smaller than that seen in control animals, which also developed cavities an average of 46 times larger (p \leq 0.01, all groups) than transplanted rats, occupying 93 \pm 3.3% of the lesion site.

Reactive astrogliosis is reduced by rostrocaudal transplantation

The physical barrier of the lesion site and cavity are major obstacles to axonal regeneration (Schwab, 2002). However, the astro-
cytic scar, produced by reactive astrocytes, contains growth-inhibitory molecules that form an additional chemical obstacle to growing axons (Moreau-Fauvarque et al., 2003; Camand et al., 2004). Because both the OEC type and method of introduction significantly altered the lesion site and cavity area, we hypothesized that inhibitory astrogliosis might occur differentially between treatment groups. All groups were tested for the distribution of GFAP-expressing astrocytes (Fig. 3A–D). The intensity of GFAP immunoreactivity surrounding and including the lesion site was normalized to an equally sized defined area of GFAP immunoreactivity contralateral to the lesion site. This controlled for between-animal differences in baseline reactivity and background staining. Direct transplantation of LP and OB OECs produced a reactive astrogliosis similar in extent to direct injection of media (Fig. 3A, C, F). In contrast, rostrocaudal injection of media stimulated the most extensive astrogliosis of all treatment groups, a reaction that was significantly lowered by transplantation of both OEC types rostral and caudal to the lesion (n = 4, 4, 4; LP, p = 0.001; OB, p = 0.01). Rostrocaudal transplantation of both LP and OB OECs also significantly decreased GFAP intensity compared with direct transplantation, with rostrocaudal LP OEC transplantation lessening GFAP reactivity to the greatest extent (n = 4, 4, 5, 4, 4; p = 0.05) (Fig. 3H). The reduced astrocytic reaction observed in rostrocaudal versus direct transplanted rats suggests that this method of transplantation may create a more permissive environment for axon extension (Lakatos et al., 2003), although no additional decrease was observed in CSGP expression between treated groups (data not shown) over that reported previously (Ramer et al., 2004b).

LP and OB OECs similarly recruit endogenous Schwann cells to the lesion site
Interactions between OECs and glia other than astrocytes could also significantly affect regeneration outcomes. Schwann cells (SCs) are highly permissive for regeneration and have been widely used for their ability to promote regeneration and remyelination after CNS lesions (Bunge, 2002). We have suggested previously that part of the mechanism whereby OECs may stimulate regeneration may be their ability to promote the infiltration of endogenous SCs into the spinal cord (Ramer et al., 2004b). However, we do not know whether OB OECs also promote SC infiltration in this lesion paradigm. Immunostaining of all groups for the rat-specific low-affinity nerve growth factor receptor mp75 compared with GFAP and GFP + OECs revealed endogenous cells expressing p75 in areas with OECs within the lesion site (Fig. 4B,D) and, less

Figure 3. Lesion site area and cavity formation are decreased in LP- versus OB-treated rats. Astrogliosis is increased by direct transplantation. Examples of the lesion site area, visualized by anti-GFAP (red pseudocolor) and cavities 28 d after transplantation of GFP + OB OECs directly (A) and rostrocaudally (B) or GFP + LP OECs directly (C) and rostrocaudally (D). A, Exemplar of quantification to assess lesion site area (white line) and cavity formation (yellow line). E, Area of the lesion site is significantly increased in direct OB-treated rats compared with all other groups (n = 4, p ≤ 0.05). F, The area of cavity formed in direct OB is significantly greater than LP direct and LP R/C rats (n = 4, p ≤ 0.05 and p ≤ 0.05). The area of cavity is decreased by rostrocaudal transplantation, with R/C LP OEC transplantation significantly reducing cavity formation compared with all groups (n = 4; p ≤ 0.05). G, R/C LP OEC rats also have the smallest percentage of the lesion site with cavity (n = 4; p ≤ 0.05). H, The reduced astrocytic reaction observed in rostrocaudal versus direct transplanted rats suggests that this method of transplantation may create a more permissive environment for axon extension (Lakatos et al., 2003), although no additional decrease was observed in CSGP expression between treated groups (data not shown) over that reported previously (Ramer et al., 2004b).
frequently, at the injection points in LP- (Fig. 4A) and OB-treated rats (Fig. 4C). We have identified previously these rat p75+ cells as SCs using other markers for rat SCs (Chernousov et al., 1999; Ramer et al., 2004b). Ingrowth of Schwann cells into the spinal cord parenchyma (measured from the GFAP lesion boundary) was more extensive in both OEC-treated groups, in which Schwann cells were found maximally at 350 μm from the lesion site. Schwann cell infiltration was rarely seen in control animals (Fig. 4E) and did not penetrate beyond 80 μm of the lesion boundary. SCs that infiltrated after OEC transplantation associated similarly with LP (Fig. 4B) and OB (Fig. 4C) OECs, as well as with CNS astrocytes (Fig. 4B, D). This suggests that some of the preregenerative effects of LP and OB OEC transplantation may be attributable, in part, to their ability to recruit, activate, or cooperate with endogenous Schwann cells. Axonal sprouting into the lesion site is increased in rats transplanted rostrocaudally with LP OEC animals The results so far suggest distinct biological differences in the ability of OB and LP OECs to promote repair. We next extended these findings to specifically test (1) differences in axonal outgrowth, (2) differences in angiogenesis, and (3) differences in the directionality of these parameters in relation to transplanted cells. This would allow us to establish whether secreted factors released into an SCI lesion area may uniformly promote repair throughout the lesion site or whether the location of the OECs could directly change repair and regeneration parameters. Large-caliber axons expressing NF were frequently seen in LP- (Fig. 5A) and OB (Fig. 5C, D) OECs were transplanted and whether introduction was direct or rostrocaudal. In all animals, some axons sprouted into cavity-free areas of the lesion site, regardless of cell delivery approach, but the distribution of axon growth appeared to differ between treatment groups, in particular, within the lesion site. In rats that received direct transplants of LP and OB OECs, axonal growth was essentially stalled at the caudal lesion boundary (four of four for each group) (Fig. 5B, D). In addition, although some NF+ axons were visible within the lesion site in both direct transplant groups, zones devoid of NF+ axons were apparent, adjacent to the GFAP-immunoreactive border (LP direct, n = 3 of 4; OB direct, n = 4 of 4). Rats that received rostrocaudal LP OEC transplants (Fig. 5A) demonstrated a higher incidence of NF+ axons within the lesion site compared with all other groups. Furthermore, NF+ reactivity appeared uniform throughout the lesion site, including at the astrocytic border, compared with rats that received LP or OB OECs transplanted directly into the lesion. Rats that received rostrocaudal OB OEC transplants demonstrated the lowest incidence of NF+ reactivity within the lesion site (n = 4 of 4) (Fig. 5C). To quantitatively assess the differences in axon growth within the lesion site in all treatment groups, densitometry of NF+ pixels was performed by outlining the GFAP-reactive border and counting NF+ pixels within this area (Fig. 5E). A significant increase in growth within the lesion was promoted by rostrocaudal transplantation of LP OECs compared with all other groups (n = 4; p < 0.001, all groups) (Fig. 5E). However, rats that received OB OECs directly at the lesion site demonstrated significantly more NF+ axons within the lesion compared with rostrocaudal OB OEC transplants (n = 4, all groups; p < 0.05). These results suggest that cell type and delivery method differentially influenced the pattern of growth of sprouting axons and also affected their ability to penetrate the lesion site. Directional angiogenesis is enhanced by OEC treatment Much of the axonal outgrowth reported above, particularly in rats treated by rostrocaudal LP OEC transplantation (Fig. 5A), appeared directed toward the lesion site. We have shown previously that angiogenesis in the lesion site occurs after LP OEC transplantation, and that many axons extend along blood vessel laminae (Ramer et al., 2004b). Furthermore, an angiogenic response correlated with axon growth has also been demonstrated after contusive injury (Loy et al., 2002). Therefore, it is possible that the direction of axon growth may be related to the direction of blood vessel growth, and this could determine whether different transplantation strategies could be used to encourage sprout-
ing axons to extend longitudinally down the spinal cord instead of becoming entangled at the lesion site. To assess the extent and directionality of blood vessel growth, anti-RECA and GFAP detection was used to visualize blood vessels around and within the lesion site (Fig. 6A–E). To analyze directionality, montages of Z-stacked images of RECA-immunoreactive endothelia were assigned a threshold intensity (applied within and across groups), counted, and assigned a best-fit line along their linear axis, using an algorithm programmed in Matlab, to determine a contour map of intersection points. Contour maps for control (G), OB direct (H), OB R/C (I), LP direct (J), and LP R/C (K) show the number of intersections in a given area, as indicated in the color scale bar. The height of the contour map in G–K falling within the lesion site (white outlines in G–K), as indicated by anti-GFAP (green pseudocolor), was compared with the height falling within the same area randomly placed 100 times throughout the image to produce a directionality quotient. Directionality quotients and total number of blood vessels, normalized for tissue area, are presented in Table 1. Scale bars, 250 μm.

**Figure 6.** LP and OB OECs promote directional angiogenesis. Angiogenesis, as recognized by anti-RECA immunoreaction (red), is induced at the lesion site (arrowhead) in OB direct (B) and R/C (C) and LP direct (D) and R/C (E) rats. Directionality was assessed by constructing best-fit lines to RECA-positive structures and assigning normal distributions to each intersection point to determine a contour map of intersection points. Contour maps for control (G), OB direct (H), OB R/C (I), LP direct (J), and LP R/C (K) show the number of intersections in a given area, as indicated in the color scale bar. The height of the contour map in G–K falling within the lesion site (white outlines in G–K), as indicated by anti-GFAP (green pseudocolor), was compared with the height falling within the same area randomly placed 100 times throughout the image to produce a directionality quotient. Directionality quotients and total number of blood vessels, normalized for tissue area, are presented in Table 1. Scale bars, 250 μm.

Scores greater than zero therefore indicate directionality of blood vessels toward the lesion site. In all OEC-treated rats, there were significantly greater numbers of blood vessels than in control animals (Table 1). As well, there was a significant and >1.9-fold increase in directionality of blood vessel growth toward the lesion site in rats that received OEC transplants compared with the random endothelial growth in control animals (Table 1). Rats that were directly transplanted with OB OECs demonstrated the most significant blood vessel growth directly toward the lesion site. Vascularization and enhanced regeneration have been tightly linked in the peripheral nervous system (Hobson et al., 2000), and the increase in blood vessels and their direction of growth in all OEC-treated rats may favor directed axon growth within the CNS.
Autotomy is reduced by OB OEC but elevated by LP OEC transplantation

Transplantation of either LP or OB OECs resulted in the enhanced growth of NFα positive axons within the lesion site; however, it is unclear whether these axons form functional connections, either appropriate or inappropriate, with target neurons. At 27 d after lesion and transplantation, autotomy was apparent in some rats, so we sought to determine whether the degree of autotomy varied between groups because this measure could serve as an admittedly negative behavioral readout for changes in functional connectivity. Autotomy is the self-attack of a (partially) denervated limb, characterized by excessive licking, grooming, and biting of the anesthetic limb, eventually resulting in digit amputation (Kauppila, 1998; Zimmermann, 2001). Autotomy was scored using the methods described by Wall (1979), in which a total of three points were awarded for the attack of each digit: 1 point for pressing CGRPPositive nociceptive/temperature responsive afferents as some growth of dorsal root afferents expressing CGRP- or subP- (Ramer et al., 2004b). We assayed horizontal sections through the lesion site for the presence of these axonal subpopulations using the GFAP stain to identify the boundaries of the lesion site. Montaged, Z-stacked sections were assessed for the total number of positive pixels within the lesion site and within defined areas 0.5 and 1 mm rostral and caudal to the lesion.

We first tested for the presence of different axonal subtypes at 24 h after lesion and transplantation and found that all axons tested were absent from the lesion site and retracted substantially from the rostral and caudal lesion boundaries (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). By 28 d, in rats that received LP OECs (Fig. 8A) and OB OECs (Fig. 8B), subP-positive axons were found within the lesion site but were increased caudal to the lesion site. No significant differences in axonal outgrowth of subtypes were observed between direct and rostrocaudally transplanted rats within LP or OB OEC treatment groups, and both sets of data for each OEC subtype are presented collectively. In OB-transplanted rats, subP reactivity was found rostral to the lesion site (Fig. 8B, inset), whereas less reactivity was observed at this location in LP OEC (Fig. 8A, inset) transplanted rats. subP-positive axons were significantly increased at all points in rats that received OB OECs compared with LP OECs (n = 4 each group; p ≤ 0.05 all levels) (Fig. 8C). This suggests that OB OECs are more effective than LP OECs at promoting the sprouting of subP-positive axons after injury.

CGRP-positive nociceptive/temperature responsive afferents are also responsive to LP OEC transplantation (Ramer et al., 2004b), and their sprouting could impact pain sensation after SCI (Jang et al., 2004). Detection of CGRP-positive axons in rats that received LP OECs (Fig. 8D) and OB OECs (Fig. 8E) revealed similar sprouting patterns between groups, although LP OECs slightly increased sprouting 0.5 mm rostral to the lesion site (n = 4 each group) (Fig. 8F) and demonstrated increased sprouting at the rostral lesion border (Fig. 8D, inset, F). Both types of OEC did, however, promote the growth of CGRP-positive axons over

Table 1. Quantification of blood vessel number and direction after OEC transplantation

<table>
<thead>
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<th>Mean number of blood vessels</th>
<th>Directionality quotient</th>
<th>Fold increase in directionality over control</th>
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<tr>
<td>Control</td>
<td>209 ± 23.91</td>
<td>-0.0287 ± 0.0207</td>
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<tr>
<td>OB direct</td>
<td>302 ± 44.66</td>
<td>0.0767 ± 0.0243</td>
<td>3.6</td>
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<tr>
<td>OB R/C</td>
<td>356 ± 22.71</td>
<td>0.0082 ± 0.0078</td>
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<tr>
<td>LP direct</td>
<td>336 ± 38.77</td>
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<td>LP R/C</td>
<td>368 ± 36.28</td>
<td>0.0233 ± 0.010</td>
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</table>

OEC transplantation enhances angiogenesis and directs blood vessels toward cell-rich areas. Quantification of blood vessel number for control and treated groups and directionality quotients for all groups toward the lesion site were performed using Matlab programming.
control animals, particularly within the lesion site, in which no sparing of CGRP-positive axons was visible at 24 h after lesion. No significant differences in CGRP-positive sprouting were observed between direct and rostrocaudally transplanted rats.

Sprouting of raphespinal 5-HT-positive axons appeared similar in both LP- and OB-treated rats (Fig. 8G,H) and was extensive compared with CGRP + and subP + sprouting; no significant differences were observed between transplant groups (n = 4 for all groups) (Fig. 8J). 5-HT-positive axons also appeared to diminish significantly slightly caudal to the lesion site (Fig. 8G,H, insets). In contrast, detection of TH revealed significantly more TH-positive axons rostral to and within the lesion site in rats that received LP OECs compared with OB OECs (Fig. 8J–L). This was particularly visible at the rostral lesion boundary, in which TH-positive axons were increased in LP-transplanted (Fig. 8J, inset) compared with OB- transplanted (Fig. 8I, inset) rats. Because neither 5-HT- nor TH-positive axons were present within the lesion site 24 h after lesion, the transplanted LP and OB OECs likely promote growth of these axon subtypes.

**Discussion**

OECs are currently considered prime candidates for cell-based therapies to repair the injured CNS (Lakatos and Franklin, 2002). Because some OB OEC transplantation experiments have indicated different degrees of functional recovery from SCI (Ramon-Cueto et al., 2000; Keyvan-Fouladi et al., 2003; Garcia-Alias et al., 2004), cell preparations containing LP OECs are currently being tested for treating human SCI based on the assumption that LP and OB OECs are functionally equivalent (Senior, 2002; Huang et al., 2003; Rabinovich et al., 2003). For both mechanistic and clinical reasons, we have compared the effect of transplanting OB and LP OECs into lesioned spinal cord and determined that they produce significantly different regeneration outcomes. Compared with OB OECs, LP OECs decrease lesion site and cavity area and increase sprouting of NF + and TH + axons but also stimulate autotomy. In contrast, OB OEC transplantation increases lesion site area and cavity formation, promotes sprouting of subP + axons, and promotes the greatest directional angiogenesis. In addition, rostrocaudal compared with direct transplantation suggests that LP and OB OECs have different abilities to migrate and that cell delivery method significantly affects astrogliosis, NF + sprouting, and cavity formation.

The morphological and antigenic profiles of LP and OB OECs in culture (Fig. 1) suggest that both OEC types are very similar. However, their differing roles in PNS and CNS environments in the olfactory system, and expansion capacities in vitro, imply that LP and OB OECs should not be considered functional equals a priori and could behave differently after transplantation. Indeed,
when transplanted rostrocaudally, LP and OB OECs were distributed differently 28 d after lesion: significantly more GFP \(^*\) LP OECs had migrated into the lesion site, whereas GFP \(^*\) OB OECs were located between the caudal injection point and lesion site (Fig. 2). This distribution could be explained by increased death of OB OECs at the lesion site or a greater loss of LP OECs at injection sites. The resemblance in cell distribution of 28 d R/C OB and 7 d R/C/CLP rats, coupled with the increased survival of OB OECs at 28 d, suggests that the difference in cell distribution is primarily a result of intrinsic LP/OB OEC migration differences that resemble those we assayed in vitro (Fig. 2).

The ability of OECs to differentially migrate into a lesion site could have important consequences in SCI, because, in a dorsolateral funiculus lesion, a cavity forms at 24 h after injury when untreated (Ramer et al., 2004b). The greater percentage of cavity in rats that received rostrocaudal OB OEC transplants could thus simply be the result of a retarded migration of OB OECs (Fig. 3). The long-term presence of OECs at the lesion site appears to be a major determinant of reduced cavity size, because rostrocaudal LP OEC transplantation significantly reduced cavity formation compared with direct transplantation (in which fewer LP OECs survive) (Fig. 3).

The decreased lesion site area in rostrocaudally transplanted rats was further reflected in differences in astrocytic reactions after rostrocaudal versus direct transplantation. Increased GFAP reactivity around the lesion site of rats with direct versus rostrocaudal transplants of both OEC types suggests that LP and OB OECs may have similar interactions with astrocytes (Fig. 3). However, the increased GFAP immunoreactivity in rats that received transplants directly into the lesion site suggests that the acute presence of OECs within the lesion either enhances astrocyte division and/or exacerbates astrocytic hypertrophy (Fig. 3). Differences in astrogliosis could arise from (1) unfavorable interactions between OECs and astrocytes, (2) an increased local immune response after direct transplantation, or (3) a favorable interaction between OECs and astrocytes that requires long-term survival of OECs. Increased astrogliosis is unlikely caused by unfavorable OEC–astrocyte interactions, because astrocyte size, shape, and reactivity are not altered in vitro by contact with OB OECs (Lakatos et al., 2000, 2003). In addition, similar numbers of autofluorescent immune infiltrates were detected within the lesion site of rostrocaudally and directly transplanted animals (of the same cell type), arguing that the immune response may contribute to, but is not responsible for, increased astroglia in directly transplanted animals. Therefore, the decrease in GFAP intensity in rostrocaudally transplanted rats may be attributable to a favorable but necessarily prolonged interaction between OECs and astrocytes, because this delivery approach also promotes OEC survival. The association of OECs and astrocytes may synergize to reduce astrogliosis and create a more permissive environment, allowing SC infiltration and axonal extension. SC transplantation has promoted behavioral recovery from SCI (Pearse et al., 2004; Fouad et al., 2005); therefore, their infiltration after OEC transplantation could contribute to or potentiate regeneration regardless of OEC type.

Finally, functional assessments performed after OB OEC transplantation have focused primarily on positive outcome measures (Ramon-Cueto et al., 2000; Keyvan-Fouladi et al., 2003; Garcia-alias et al., 2004), with the assumption that LP OECs would be similarly beneficial. The biological differences observed between LP and OB OECs in vitro (Fig. 2) and after direct and rostrocaudal transplantation (Figs. 2–4) suggest that there may be differences in how OEC type and transplantation approach affect axonal sprouting. Sprouting of NF \(^{+}\) axons within the lesion site was greatest in rats that received rostrocaudal LP OEC transplants, with little axon density decrease across the lesion site (Fig. 5). In contrast, although direct transplantation of LP OECs was effective at drawing axons toward the lesion boundaries, fewer axons were able to penetrate the lesion/host interface. This effect could be a result of both trophic/tropic actions of OECs and changes to the permissiveness of the glial scar/lesion site, because rostrocaudal transplantation also decreased GFAP reactivity and cavity formation (Fig. 3). Therefore, permissiveness and trophic/tropic actions may be maximized by rostrocaudal, but decreased by direct, LP OEC transplantation. However, if only the location of the cell transplant were responsible for differences in NF \(^{+}\) growth, then, based on evidence from LP OECs, one would expect (1) greater NF \(^{+}\) axons within the lesion site in rats transplanted rostrocaudally than directly with OB OECs and (2) the same amount of NF \(^{+}\) growth within the lesion site in rostrocaudal LP- and OB-treated rats. A comparison of only rostrocaudally transplanted LP and OB OEC rats shows that significantly greater NF \(^{+}\) axons are present within the lesion in LP–treated rats (Fig. 5). This suggests that LP and OB OECs might secrete different neurotrophic factors and/or secrete them at different levels.

The significant increase in autotomy in LP OEC versus OB OEC transplant groups further underscores potential functional differences between these two cell types. Although the mechanisms of autotomy are not understood, autotomy is common after denervation or situations in which sensory modalities may be affected, as in our model. At present, it appears that OEC transplantation might have modified the sensory system by facilitating differential growth of different axonal populations or by differentially altering local circuitry. These possibilities imply that a higher degree of plasticity/remodeling may be induced by LP versus OB OECs, which could result in undesired effects. In comparison, recent work with transplanted embryonic stem cells into SCI has revealed the development of allodynia in addition to increased motor function, a feature not tested in most OEC transplantation studies (Hofstetter et al., 2005).

The differential sprouting of some axonal populations further implies that LP and OB OECs secrete different neurotrophic factors and therefore may differently promote recovery (Fig. 8): subp \(^{+}\) axons sprouted significantly more in OB- than LP-treated rats (Fig. 8). However, the most robust difference in sprouting was the growth of TH \(^{+}\) neurons, whose growth was highly promoted by LP OEC transplantation (Fig. 8). Differences in the abilities of LP and OB OECs to promote axon elongation are in accord with their roles in the olfactory neuraxis. Furthermore, although OB OECs express NGF, BDNF, glial cell line–derived neurotrophic factor, artemin, and CNTF in vivo and in vitro (Woodhall et al., 2001; Lipson et al., 2003), their ability to promote outgrowth of peripheral ganglia and CNS explants as well as to promote sprouting within the spinal cord via secreted factors has been variable, whereas their ability to do so via direct cell–cell contact is greater (Sonigra et al., 1999; Lipson et al., 2003; Chuah et al., 2004).

Here, we present the first data directly comparing LP and OB OEC transplantation in a single SCI model and show that there are fundamental differences in intrinsic OEC properties that are retained after they are transplanted into lesioned spinal cord. These differences relate to (1) the ability to migrate and create a permissive regeneration-promoting environment at the lesion site and (2) differential outgrowth-promoting characteristics of LP and OB OECs. Although the maximal benefit to most regeneration parameters was achieved with rostrocaudal LP OEC transplantation, LP OECs may have similar interactions with astrocytes (Fig. 3).
transplantation, this is also associated with autotomy. This sug-
esticates that the vigorous sprouting associated with rostrocaudal LP
OEC transplantation must be controlled so that appropriate con-
nections are favored over inappropriate, and nearby uninjured
tracts are not adversely affected. Thus, rostrocaudal LP OEC
transplantation provides the greatest opportunity to promote re-
generation or reconnect local circuitry after SCI, suggesting that
LP OECs, combined with directed axon guidance stimulation,
may merit additional development as a means to restore motor
and sensory function after spinal cord lesion.

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