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Research Report

Effects of human OEC-derived cell transplants in rodent spinal cord contusion injury

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ABSTRACT

Numerous reports indicate that rodent olfactory ensheathing cells (OECs) assist in spinal cord repair and clinical trials have been undertaken using autologous transplantation of human olfactory ensheathing cells (hOECs) as a treatment for spinal cord injury. However, there are few studies investigating the efficacy of hOECs in animal models of spinal cord injury. In this study hOECs were derived from biopsies of human olfactory mucosa, purifed by culture in a serum-free medium containing neurotrophin-3, genetically labelled with EGFP, and stored frozen. These hOEC-derived cells were thawed and transplanted into the spinal cord injury site 7 days after a moderate contusion injury of the spinal cord at thoracic level T10 in the athymic rat. Six weeks later the animals receiving the hOEC-derived transplants had greater functional improvement in their hindlimbs than controls, assessed using open field (BBB scale) and horizontal rung walking tests. Histological analysis demonstrated beneficial effects of hOEC-derived cell transplantation: reductions in the volume of the lesion and the cavities within the lesion. The transplanted cells were located at the periphery of the lesion where they integrated with GFAP-positive astrocytes resulting in a significant reduction of GFAP staining intensity adjacent to the lesion. Although their mechanism of action is unclear we conclude that hOEC-derived cell transplants improved functional recovery after transplantation into the contused spinal cord, probably by modulating inflammatory responses and reducing secondary damage to the cord.

1. Introduction

Traumatic spinal cord injury is a complex, progressive event leading to long-term motor and sensory deficits, and dysfunction of the autonomic nervous system. In the spinal cord there is cell death, severing of axons, demyelination, inflammation and the formation of cystic cavities (Sekhon and Fehlings, 2001). There is limited capacity of the human spinal cord to repair itself, so there is a need for therapeutic strategies to protect and repair the injured spinal cord. Olfactory ensheathing cells (OECs) are a unique type of glial cell that share some properties with astrocytes and Schwann cells but have

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Abbreviations: OEC, olfactory ensheathing cell; GFAP, glial fibrillary acidic protein; BBB, Basso Beattie Bresnahan locomotor rating scale; pFB-hrGFP, green fluorescent protein

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intrinsic properties that make them ideal candidates for cellular grafts into injured spinal tissue (Chuah and Au, 1994; Fairless and Barnett, 2005; Farbman and Squinto, 1985; Ramon-Cueto and Valverde, 1995).

Many studies have demonstrated that OECs are therapeutic when transplanted into the injured spinal cord in rodent models including restoration of motor function (Keyvan-Fouladi et al., 2003; Kubasak et al., 2008; Li et al., 2003; Li et al., 1997; Lopez-Vales et al., 2007), improvement of respiratory function (Li et al., 2003) and restoration of bladder function (Pascual et al., 2002). Transplanted OECs induced axon regeneration (Keyvan-Fouladi et al., 2003; Li et al., 1997, 1998, 2007; Lopez-Vales et al., 2007; Plant et al., 2003; Ramon-Cueto et al., 1998), remyelination of axons (Barnett et al., 2000; Imaizumi et al., 1998; Kato et al., 2000), reduced lesion size, cystic cavitation and astrocytic gliosis (Lopez-Vales et al., 2006; Ramer et al., 2004; Takami et al., 2002; Verdu et al., 2001) and ameliorated electrical conduction through the injury site (Imaizumi et al., 1998, 2000; Lopez-Vales et al., 2006, 2007; Toft et al., 2007).

Most previous studies have investigated the therapeutic properties of rat OECs although human OECs remyelinated spinal cord axons after demyelinating lesions (Barnett et al., 2000; Kato et al., 2000) and primate OECs induced locomotor recovery after transection injury in the rat (Guest et al., 2008). There are no previous studies of human OEC transplantation into the contused rat spinal cord, a model of injury that mimics aspects of many common human injuries. Most previous studies, including the human and primate reports, obtained OECs from the olfactory bulbs within the cranial cavity. This is not a clinically favoured site for sourcing autologous OECs because of the morbidity associated with surgical access and destruction of the olfactory bulbs to obtain the cells. OECs can be obtained from the olfactory mucosa in the nose (Bianco et al., 2004) and OECs from this source were therapeutic when transplanted into the transected spinal cord in rat (Lu et al., 2001, 2002). hOECs derived from the olfactory mucosa were similar to rat nasal OECs in proliferation, survival and migration after transplantation into the normal spinal cord of athymic rats (Deng et al., 2006). For autologous transplants, OECs are relatively easy to obtain from the human nose by biopsy of the olfactory mucosa, without affecting olfactory function (Feron et al., 1998). A Phase I clinical trial demonstrated that transplantation of autologous OECs was safe in human paraplegia (Feron et al., 2005; Mackay-Sim et al., 2008).

The aim of this study was to establish whether OECs obtained from the human mucosa and genetically labelled with green fluorescent protein are able to produce similar behavioural outcomes to that reported for rat OECs. While the remyelination potential of human bulbar OECs has been documented, this is the first time locomotor improvements following human mucosal OEC transplants have been investigated. We used the traumatic contusion model (MASCIS impactor) which results in a complex central lesion with damage to spinal cord tissue and blood vessels, and ongoing secondary injury and cyst formation (Basso et al., 1996) similar to human spinal injury (Sekhon and Fehlings, 2001). The use of athymic rats (Rolstad, 2001) limits xenograft rejection of the hOECs, and delaying transplantation until a week after injury reduces exposure of the grafted cells to the acute inflammatory phase.

2. Results

2.1. Sample

Twenty animals (9 receiving hOECs and 11 controls) were used in the final analysis. Three animals were excluded from the study because the compression rate during spinal cord contusion and/or the initial BBB score was more than two standard deviations from the group mean and one animal was excluded due to post-operative complications. The mean compression rate for the hOEC transplanted group was 0.36±0.02 m/s compared to 0.38±0.04 m/s for the control group. There was no difference between the groups ($t=0.658$, df 18, $p=0.52$). The lesion locations were comparable for the transplanted and non-transplanted group lesions. The positioning of the impactor head between the T9 and T11 vertebrate prevents much variation in lesion location between animals. There was a small decrease in body weight (<10%) in the first week after the surgery that then increased during the 6-week post-contusion period to above baseline levels. All rats were spontaneously voiding urine by 2 weeks post-contusion surgery. The cell transplantation procedure 1 week after spinal cord contusion did not cause any noticeable adverse effects. Despite obvious locomotor deficits, all animals were alert, healthy and mobile after surgery.

2.2. Phenotyping of cells

Prior to transduction with pFB-hrGFP, cells were identified immunocytochemically with antibodies against glial fibrillary acidic protein (GFAP; Dako, Denmark), S100 (Dako, Denmark), $p75^{NTR}$ (Neubody, AUS) and R-phycoerythrin-conjugated anti-HNK1 (Sigma). The percentages of S100-positive and GFAP positive cells were >95%, nearly all cells were also $p75^{NTR}$ immunoreactive and there was no staining of NHK 1 antibody (Feron et al., 2005) indicating the culture was highly enriched for OEC. However immunophenotyping of these cells just prior to transplantation showed no expression of any of these markers. For reasons addressed in Discussion, we consider the most likely explanation for this loss of expression is due to plasticity of the OECs which is well known to occur in vitro (Barnett et al., 2000; Vincent et al., 2003, 2005; Moreno-Flores et al., 2003), rather than replacement of all the OECs by another immunonegative cell type. We therefore have used the term hOECs to describe these transplants, but acknowledge their identity is unconfirmed.

2.3. Behavioural assessments

2.3.1. Open field assessment

During the 6 weeks following hOEC transplantation or medium injection there was an increase in BBB scores for both groups (Fig. 1A) with a significantly greater improvement in the hOEC transplant group, (two way ANOVA repeated measures, F (1,18)=9.6, $p=0.006$). Bonferroni post hoc tests show significant increases in the OEC group by 4 weeks post

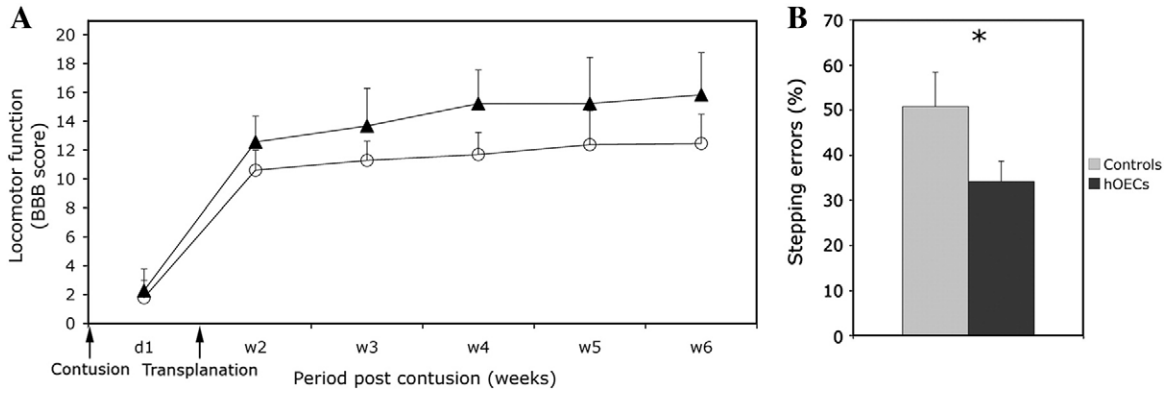

Fig. 1 – Human OEC transplantation improved locomotor behaviour. (A) hOECs transplantation (triangles) at 1 week after contusion injury significantly improved locomotor recovery measured using the BBB open field scores compared to controls (circles), (repeated measures ANOVA, (F (1,18)=9.6, p=0.006)). Bonferroni post hoc tests show significant increases in the OEC group by 4 weeks post injury. (B) hOEC transplantation (dark bar) resulted in fewer stepping errors than the controls (light bar) during the horizontal ladder task, *p=0.05. (Scores shown as means and SEM).

injury. The BBB scores obtained by the hOEC transplanted animals at 6 weeks (15.8±2.9) indicate better hind-fore limb coordination, paw placement and trunk stability compared to the controls (12.45±2.0).

2.3.2. *Horizontal Ladder Rung walking test*

Six weeks after transplantation most animals were plantar stepping with weight support and most were capable of crossing the horizontal ladder. In general the animals performed poorly in this task and the hindlimb deficits appeared more pronounced than in the open field test. Each animal showed variability in completing the task with many displaying spasms or jumps in movement, especially after a stepping error. Only consecutive steps completed after the rat had stabilised itself were scored. The hOEC-treated group made significantly fewer stepping errors in this task (34.2±20%) than the control group (50.8±15.5%), (t=2.12, df 15, p=0.05) (Fig. 1B).

2.4. *Histological analysis*

2.4.1. *Lesion size*

This moderate contusion resulted in a typical "oval" shaped injury with a large centrally located lesion containing necrotic tissue, cellular debris, macrophages, cystic cavities and trabeculae of tissue (Fig. 2A–E). Surrounding the lesion there was relative sparing of tissue but extensive diffuse axonal injury in the white matter. The terminal bulbs of axons were identifiable by their smooth rounded appearance and eosinophilic staining in H&E stained sections (Fig. 2C), and were clearly identified in the peripheral white matter with NF-200 staining (Fig. 5D). Spinal cords with hOEC transplants had smaller lesions with less cavitation (Fig. 2B, E). The overall percentage of lesion area was significantly less for the hOEC transplanted spinal cords (37.2±6.6%) compared to the controls (51.04±5.4%), (t=5.1, df 18, p<0.001). The dorsal part of the cord sustained the most injury and there was no difference in severity for hOEC transplanted animals and controls at this level. At each of the middle levels through the cord the hOEC transplanted animals showed consistently smaller lesion sizes (Fig. 3A). Within the lesion, the overall percentage of cavity area was significantly reduced for hOEC groups (3.1±2.7%) compared to the controls (15.5±15.3%), (t=2.2, df 18, p=0.038). Cavitation was highest in the middle sections of the spinal cord for both groups, with the controls showing significantly greater cavitation than hOEC transplanted animals at the central level (Fig. 3B). The highest percentage of spared tissue was seen toward the ventral part of the spinal cord for both groups, however overall there was differential sparing in the hOEC-transplanted animals (35±6.24%) compared to the controls (25.9±1.1 %), (t=-2.1, df 18, p=0.054), most apparent in the central and ventral parts of the cord (Fig. 3C).

2.4.2. *hOEC survival*

Six weeks after transplantation into the lesion site the hOECs were clearly visible within the lesion (Fig. 4A), aligned in a rostro-caudal direction (Fig. 4B) and clustered at the periphery of the lesion both at the lateral white matter border and near the rostral and caudal grey matter. Except for a few individual cells (Fig. 4C) hOECs did not migrate from the lesion into surrounding spinal cord tissue.

2.4.3. *Immunohistochemistry*

Six weeks after transplantation, GFAP staining intensity was increased immediately adjacent to the lesion in controls (Fig. 5A) and to a lesser extent in hOEC transplanted spinal cords (Fig. 5B). Quantification of the GFAP staining intensity showed an overall 17% reduction in GFAP intensity in hOEC transplanted rats. The control animals showed a 27±9.5% increase in GFAP staining intensity adjacent to the lesion compared to an increase of 10±6.6 % in the hOEC transplanted animals (t=4.5, df 18, p=0.001). This pattern was consistent throughout the spinal cord, and all but the ventral section showed significant differences (Fig. 3D). High power examination at the edge of the lesion showed close association between some hOECs and GFAP positive astrocytes at the host-graft interface (Fig. 5C). Occasional single hOECs were seen migrating short distances towards the white matter, and astrocytic processes were seen extending towards the centre

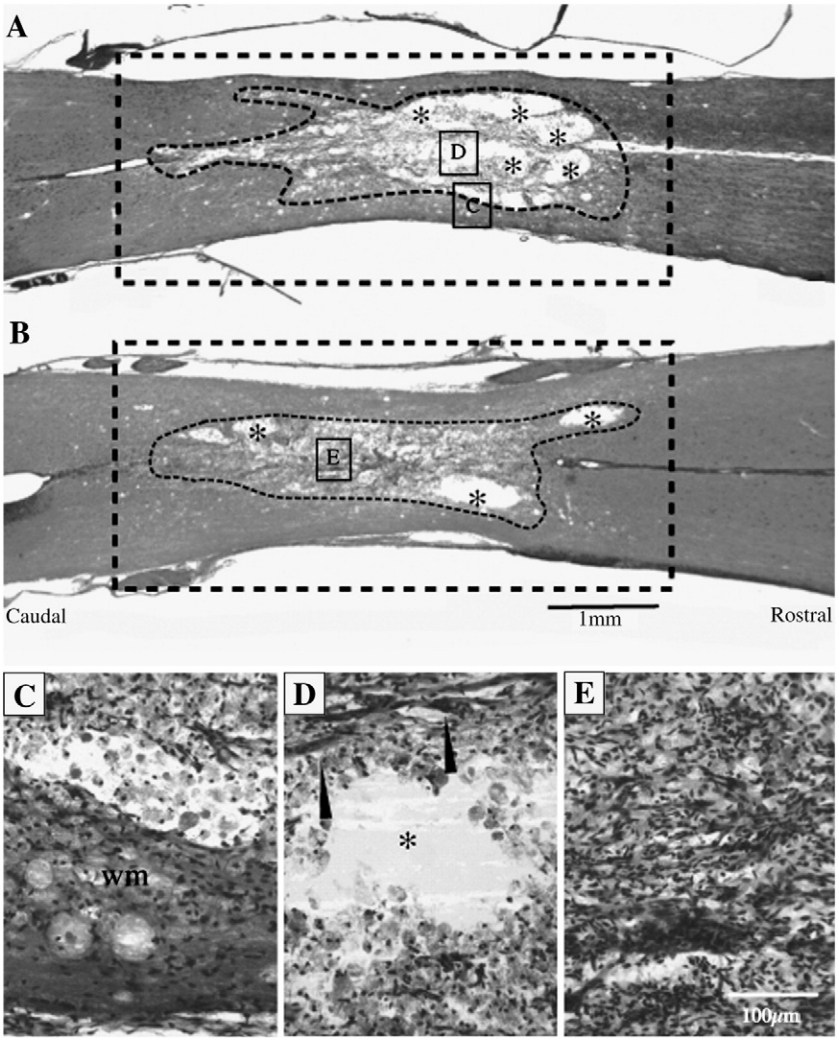

Fig. 2 – Human OEC transplantation and the lesion site. (A, B) Horizontal sections of spinal cord stained with H&E showing the lesion site 5 weeks after (A) control medium injection or (B) hOEC transplantation. Large dotted box shows the total area within which the spinal cord was quantified. Smaller dotted region shows the lesion area with cavities (*) and cell debris. (C–E) High power images from the boxed areas in A and B. (C) The penumbra of the lesion showing the peripheral white matter (wm). (D) The centre of the lesion in the control animal containing cell debris, macrophages, and trabeculae of spinal tissue extending into the lesion (long arrows). (E) The centre of the lesion in the hOEC transplanted animal.

of the lesion. GFAP staining was also seen in trabeculae of tissue fragments within the lesion centre.

NF200 staining identified large axons and terminal bulbs in the white matter adjacent to the lesion (Fig. 5D). hOECs were located in close proximity to axons at the caudal and rostral ends of the lesion but few of these axons continued into the epicentre. The hOECs appeared to be aligned in the same direction as axons and confocal examination confirmed a close association between some hOECs and NF200-positive axons (Fig. 5E) but it was not possible to determine if hOECs were enwrapping axons.

High numbers of large autofluorescent cells with macrophage morphology were evident in the lesion centre of hOEC transplanted (Fig. 4A, D–F) and control spinal cords. Staining with ED1, a specific marker for rat myeloid cells, confirmed these were macrophages or microglia (Fig. 5F, G) and demonstrated there was also an extensive infiltration of ED1-positive cells in the white matter throughout the spinal cord segment. There was no apparent difference in the amount or pattern of macrophage staining between hOEC transplanted and control spinal cords.

3. Discussion

In this study we show that hOEC-derived cells from the olfactory mucosa induced significant locomotor and histological improvements after transplantation into the contused spinal cord of the rat. Compared to controls the hOEC transplanted animals had significantly better hindlimb locomotor function in the open field test, fewer stepping errors in the horizontal ladder task, reduced spinal cord lesion volume and reduced cavitation within the lesion. There was also evidence for reduced astrogliosis at the lesion site.

3.1. *Human OECs from olfactory mucosa*

The cells used in this study are derived from the human olfactory mucosa. OEC cultures from this source are potentially contaminated by Schwann cells associated with the trigeminal nerve whose branches innervate olfactory epithelium and olfactory bulb (Schaefer et al., 2002), or other cell types located in the olfactory mucosa, including precursor cells and stem cells (Lindsay et al., 2010). A comprehensive review article by Kawaja et al. (2009) describes the technical challenges in isolating, purifying and phenotyping OECs from olfactory bulb and mucosal tissue cultures. Several antigenic markers, including calponin and smooth muscle actin, have been reported to identify OECs but not Schwann cells (Boyd et al., 2006; Jahed et al., 2007) but these have not been verified independently and at present there is no method to unequivocally distinguish between OECs and Schwann cells in vitro. We used a serum-free medium containing NT3 to selectively propagate OECs (Bianco et al., 2004). This medium does not support long-term culture of Schwann cells (Cheng et al., 1998) although NT3 supports survival of 50% Schwann cells in the first 3 h of culture (Meier et al., 1999) and 20-30% in the first 24 h (Lobsiger et al., 2000). Furthermore, no cells stained positive for HNK 1, a marker that recognises human myelinating Schwann cells (Levi et al., 1994). Throughout our paper we have referred to the transplanted cells as hOECs and while we consider these cell preparations to be highly enriched for OECs it is possible that the transplants contain other, as yet unidentified, cell types from the olfactory mucosa. The loss of expression of GFAP, S100 and P75 in these cells during their preparation (EGFP labelling, frozen storage and subsequent revival and expansion) is a matter of concern and one that will be further investigated by phenotyping cells at each step of their preparation using a more comprehensive panel of antibodies (Lim et al., 2010; Garcia-Escudero, et al., 2010). Previously, hOECs grown under the same NT3 culture conditions, but without the subsequent labelling and storage steps maintained their GFAP+, S100+, p75-NGF+ phenotype (Feron et al., 2005). OECs are very plastic in vitro and the expression of markers varies with culture conditions (Barnett et al., 2000; Vincent et al., 2003, 2005) and species (Bock et al., 2007; Smithson and Kawaja, 2009). p75-NGF can be reduced following incubation in 10% fetal calf serum without loss of regenerative capacity (Moreno-Flores et al., 2003). It is possible that the hOECs have lost their immunological phenotype due to the particular protocol we used. The less likely possibility also exists that OECs (and Schwann cells) are completely absent from the cell culture immediately prior to transplantation, and it is made up entirely of a different cell population. A recent review (Lindsay et al., 2010) outlines the complexity and heterogenicity of cells located in the human olfactory mucosa. If this is the case here it is critical to identify these cells as we have demonstrated their potential for improving function after spinal cord injury.

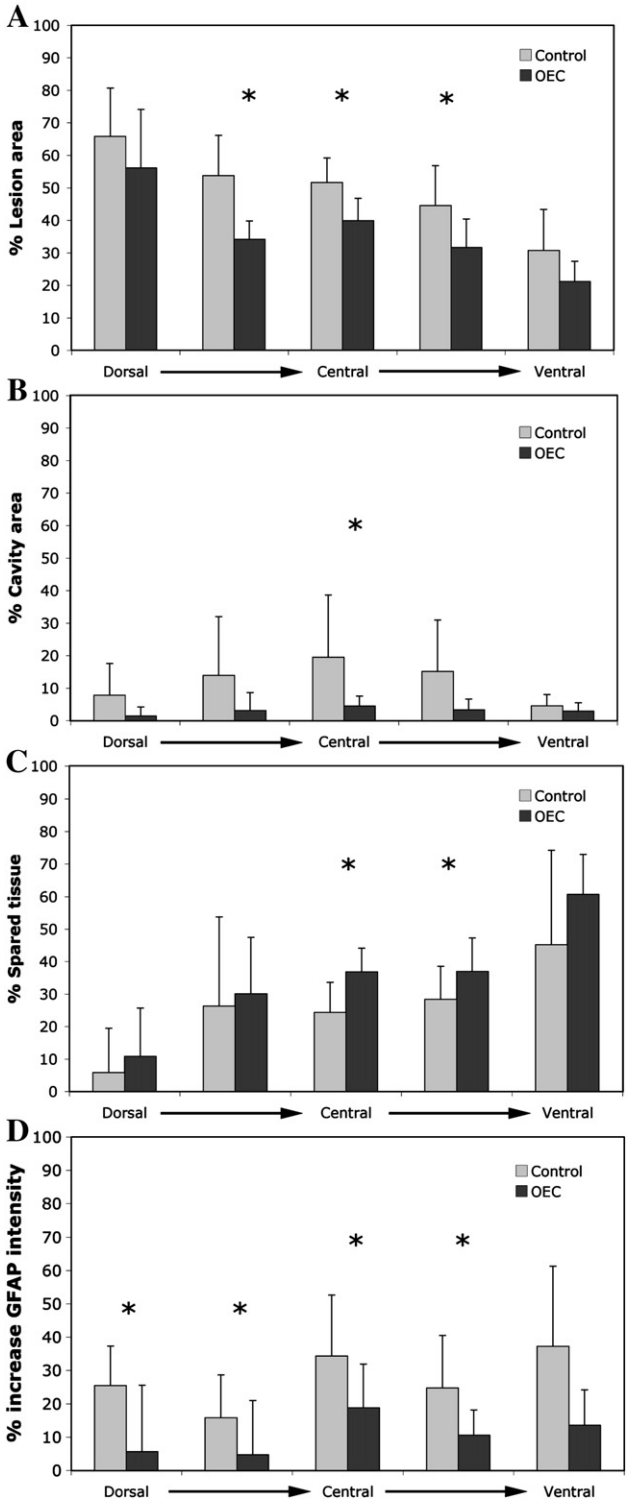

Fig. 3 – Transplanted hOECs reduced lesion size, cavity area and increased tissue sparing. Measurements, expressed as percentages, taken throughout the spinal cord from the dorsal (left) to the ventral (right) surface. (A) Lesion area is highest in the dorsal spinal cord and transplanted hOECs reduced lesion size centrally. (B) The cavity area within the lesion was highest in the middle of the spinal cord and reduced in the hOEC transplanted animals compared to the controls. (C) Tissue sparing increased from dorsal to ventral cord, in both groups, however there was differential sparing of tissue for the hOEC transplanted animals. D) hOEC transplants reduced the GFAP intensity adjacent to the lesion. *$p<0.05$.

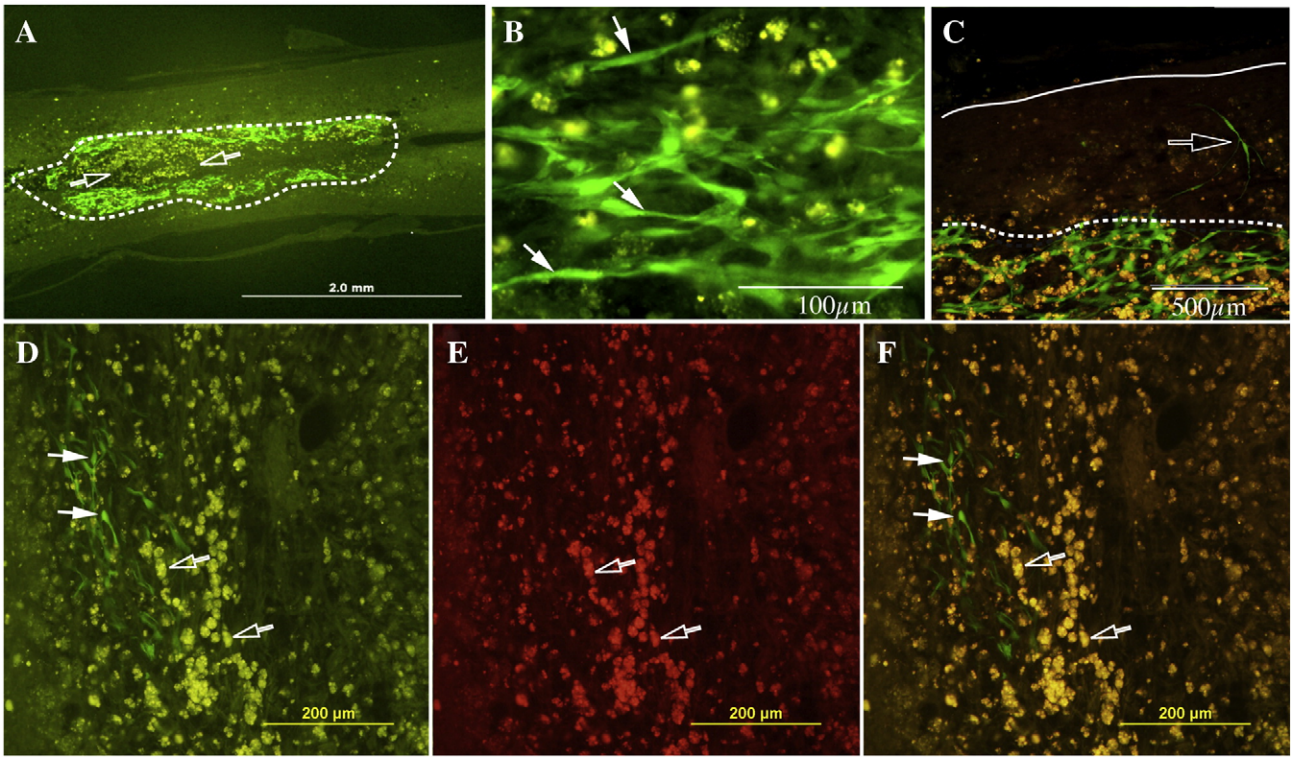

Fig. 4 – Transplanted hOECs remained within the lesion. Horizontal sections of spinal cord 5 weeks after hOEC transplantation. (A) GFP labelled hOECs (green) are located within the lesion, especially close to the periphery of the lesion (dotted line). Yellow cells (arrows) are autofluorescent macrophages seen in similar numbers in hOEC transplanted and control sections. (B) High power image of the GFP labelled hOECs (green) showing their morphology and rostro-caudal orientation. (C) Rarely, hOECs (open arrow) were seen in the parenchyma surrounding the lesion (dotted line shows lesion edge, solid line shows spinal cord edge). D, E, F are images of the same section viewed with (D) green filter only, (E) red filter only and (F) merged, demonstrating the autofluorescent cells (open arrows, yellow) in the center of the lesion, with hOEC (closed arrows, green) clearly distinguishable. These autofluorescent cells were also seen in the control sections.

Clinical application of OECs requires an accessible source of these cells. In the majority of experiments OECs were obtained from the rodent olfactory bulb and a method has been devised for unilateral bulbectomy through the frontal bone in primate (Rubio et al., 2008). OECs have been obtained from post-mortem brain (Barnett et al., 2000) but a clinically preferable source is the olfactory mucosa, accessible through the external naris (Feron et al., 1998; Bianco et al., 2004) because there is little morbidity and it allows autologous transplantation (Feron et al., 2005; Mackay-Sim et al., 2008). This is the first study in which hOECs were transplanted into the contusion model of spinal cord injury and the results show more marked post-transplantation improvements in hindlimb locomotion compared to rodent OEC transplantation in similar models (Barakat et al., 2005; Pearse et al., 2004; Takami et al., 2002). This raises the possibility that hOECs may be subtly different from rodent OECs as indicated by recent in vitro studies of rodent, canine, primate and human OECs which show interspecies differences in proliferation and responses to growth factors (Barnett et al., 2000; Krudewig et al., 2006; Rubio et al., 2008). Another source of variation between studies might be the different methods of cell culture used in different laboratories.

There is variability in the reported survival of OECs after transplantation into the spinal cord. The use of genetic reporter GFP provides unequivocal visualisation after transplantation (Guest et al., 2008; Pearse et al., 2007; Sasaki et al., 2007) and hOECs survived for 6 weeks in our study. The timing of hOEC transplantation in this study was delayed until 7 days after contusion injury because this had been reported to be more effective than transplantation at the time of injury (Plant et al., 2003). The toxicity of the lesion site to transplanted OECs was demonstrated by differences in the survival rate: 69% at 3 days to less than 5% by 3–9 weeks after transplantation into the epicentre of a contusion lesion compared to a 60% survival rate when transplanted away from the lesion (Pearse et al., 2007). In vitro experiments further demonstrate that apoptosis of OECs was significantly increased when co-cultured with acutely injured spinal cord explants compared to delayed injury explants (Woodhouse et al., 2005).

3.2. *hOECs improved locomotor ability compared to controls*

As expected, our results showed that the injury was most severe at the dorsal surface of the spinal cord where the impact occurred. Tissue examined at deeper levels throughout

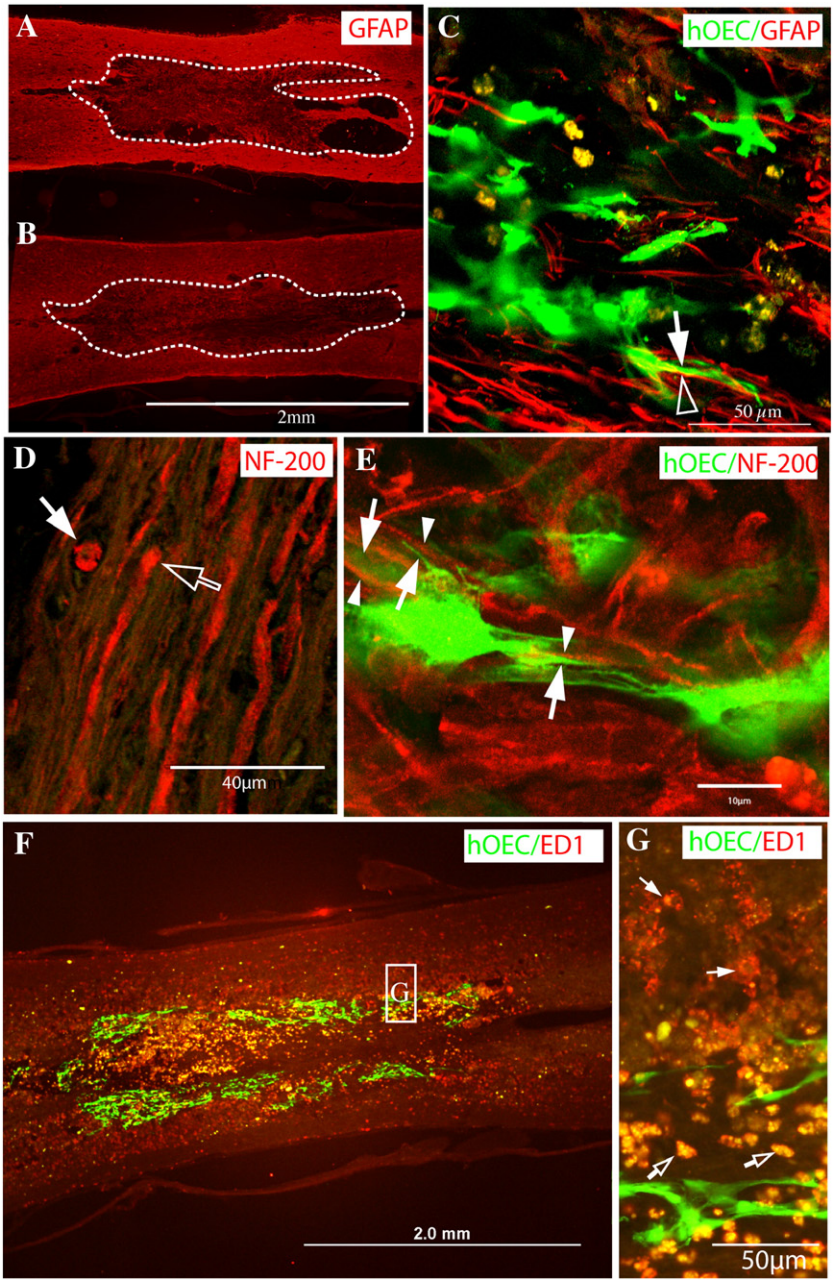

Fig. 5 – Cellular interactions of transplanted hOECs. Horizontal sections of spinal cord tissue 5 weeks after hOEC transplantation. (A) High levels of GFAP staining (red) immediately adjacent to the lesion (dotted line) in a control spinal cord. (B) Reduced GFAP staining intensity adjacent to the lesion in a hOECs transplanted spinal cord. (C) At the lesion interface hOECs (green, arrow) and astrocytic process (red, open arrowhead) were seen in close proximity. (D) NF-200 staining of axons in the peripheral white matter showing large axons with apparent retraction balls (open arrow) and terminal bulbs (closed arrow). (E) Confocal image showing hOECs (green, arrows) located in close association with NF200-positive axons (red, arrowheads) at the periphery of the lesion. (F) Longitudinal section of the spinal cord lesion from a hOEC transplanted rat immunoreacted with ED1 to identify macrophages/microglia. (G) High power image from (F) showing ED1-positive cells (red) co-localising with autofluorescent cells (yellow) within the lesion centre (open arrows) and ED1-positive macrophages/microglia that do not display autofluorescence in the white matter (closed arrows).

the spinal cord showed that injury severity lessened further away from the impact site with the most spared tissue located ventrally as described by Basso et al. (1996). In accordance with this, most animals in our study could sustain weight support in stance or some degree of weight supported stepping by 2 weeks after injury and this improved over the following 4 weeks. As reflected in their higher BBB scores, hOEC transplanted animals had more "consistent" hind-fore limb

co-ordination compared to "occasional" or "frequent" for the control groups, and more parallel paw placement, compared to a rotated placement for the controls. These differences in locomotor ability were more apparent on the horizontal ladder task (Metz and Whishaw, 2002) compared to the open field assessment (Basso et al., 1995).

Interestingly there was differential sparing of tissue for the hOEC transplanted animals compared to the controls particularly in the central to ventral parts of the cord. In the rat, descending spinal tracts located in this region include the medullary and pontine reticulospinal tracts, the medial and lateral vestibulospinal tracts and raphespinal fibres projecting from the raphe obscurus and pallidus nuclei (Watson et al., 2009). These tracts are involved in the maintenance of posture and balance during walking and the initiation and maintenance of locomotion (Fouad and Pearson, 2004). Plasticity or regeneration of these fibre tracts has been reported to improve functional hindlimb recovery following spinal lesions (Ballermann and Fouad, 2006; Ito et al., 1999; Lopez-Vales et al., 2007; Lu et al., 2002; Boido et al., 2009). While the dorsal columns and dorsolateral tissue containing the corticospinal and rubrospinal tracts appear to be markedly damaged in our injury model, the transplanted hOECs may be promoting survival or plasticity of axons in the lateral, ventrolateral and ventral parts of the cord, leading to the locomotor improvements seen.

3.3. *hOECs reduced histological measures of injury compared to controls*

We have shown that hOECs from olfactory mucosa reduce lesion size and cavity area within the lesion. This is consistent with findings after transplantation of mouse mucosal OECs into a rat dorsolateral spinal cord injury (Ramer et al., 2004), and rat olfactory bulb OECs in a moderate contusion lesion (Plant et al., 2003; Takami et al., 2002), but not in other studies of rat olfactory bulb OECs transplanted into contusions (Barakat et al., 2005; Pearse et al., 2007). In the present study, the hOEC graft appeared continuous with the host tissue when stained with H&E, in agreement with the findings of Takami et al. (2002). However fluorescence immunochemistry demonstrated that cavity-filling cells were heterogeneous, with the hOECs congregated at the periphery of the lesion as described by others (Barakat et al., 2005; Pearse et al., 2007). The presence of hOEC at the edge of the lesion was associated with reductions in GFAP intensity adjacent to the lesion indicating that hOECs have ameliorated the injury-induced astrogliosis. In co-culture with astrocytes OECs reduce astrogliosis in a "wound injury" model (O'Toole et al., 2007).

A previous study reported histological and behavioural recovery after OEC transplantation into the contusion injury of the spinal cord (Plant et al., 2003). That study used OECs obtained from the adult rat olfactory bulb, with similar histological recovery as shown here for hOECs from the olfactory mucosa. Plant et al. (2003) also observed improved locomotor ability and demonstrated significant axonal sparing/regeneration when OECs were transplanted into the lesion site 7 days after the contusion.

Demonstration of efficacy in contusion injuries is an important pre-clinical outcome because contusions are the most common type of human injury and all other studies of OEC transplantation have used either small discrete spinal cord lesions or complete spinal cord transections (Guest et al., 2008; Keyvan-Fouladi et al., 2003; Li et al., 1997; Lopez-Vales et al., 2007; Lu et al., 2002).

3.4. *Mechanism of hOEC action*

We demonstrate here significant improvements in functional and histological outcomes after transplantation of hOECs into the contusion site but there was no evidence that the OECs migrated into the surrounding parenchyma to remyelinate descending or ascending axons nor there was there evidence for axons coursing through the lesion/transplant site. We conclude that the hOECs may have been neuroprotective, probably mediated by growth factors and cytokines released into the surrounding tissues. OECs secrete soluble factors, including neurotrophins, that promote neurite sprouting in vitro and in the injured spinal cord (Chuah et al., 2004; Chung et al., 2004; Woodhouse et al., 2005). Many studies have demonstrated the regenerative and anti-apoptotic effects of neurotrophic factors in spinal cord repair (Bregman et al., 2002; Jin et al., 2002; Lo et al., 2008; Vavrek et al., 2006). Recent gene expression analyses have shown that OECs also express a large range of genes involved in wound repair, extracellular matrix formation, cell adhesion (Franssen et al., 2008; Kafitz and Greer, 1998; Vincent et al., 2005) and inflammatory responses (Vincent et al., 2005).

The reduction of astrogliosis in the hOEC transplanted animals suggests another potential mechanism of action, namely, modulation of the inflammation in the spinal cord. Inflammation is an important process that removes cellular debris after injury but, when left unresolved, activated neutrophils and macrophages/microglia can continue to release toxic cytokines and destroy healthy tissue, leading to spreading secondary damage. This is a major problem following spinal cord injury (Fitch et al., 1999). The reduction of astrocytes at the lesion site is consistent with a reduced inflammatory response to the injury but the issue requires a deeper analysis of inflammatory cells and molecules. For example, there was no obvious difference in macrophage/microglia density at the lesion in the present study but a time-course analysis would better reveal dynamic changes during the early stages and wound and repair responses. Lopez-Vales et al. (2004) has reported an earlier higher recruitment of microglia/macrophages by rat OEC within in the first 2 weeks post-injury compared to DMEM injected animals, with significant improvements in function and tissue sparing.

4. Experimental procedures

4.1. *Biopsy and cell culture of human OECs*

Olfactory mucosa was obtained by biopsy from human volunteers during routine nasal surgery (septoplasty or turbinectomy) under general anesthesia (Feron et al., 1998). All biopsies were obtained with informed consent using a protocol approved by the human ethics committees of Griffith University and the Brisbane Private Hospital, Brisbane, Australia, in accordance with the guidelines of the

National Health and Medical Research Council of Australia. The biopsies, from the posterior septum close to the cribiform plate, were placed immediately on ice in Dulbecco's modified Eagle's medium/Ham F12 (DMEM/F12; JRH) and hOECs were cultured as described (Bianco et al., 2004). Briefly, the biopsied tissue was incubated for 45 min at 37 °C in Dispase II (2.4 U/ml in Puck's solution; Boehringer, Mannheim, Germany), after which the lamina propria was separated from the epithelium with a microspatula and washed in Hank's balanced salt solution (HBSS; JRH). The lamina propria was then cut into smaller 40 μm pieces using a McIlwain tissue chopper (Brinkman Instruments, Inc., Westbury, New York) and incubated in collagenase (0.25%; type I; Sigma, St. Louis, MO) in DMEM/F12 for 10 min at 37 °C. The tissue was mechanically dissociated into single cells by trituration and then washed in HBSS. The cells were pelleted by centrifugation and resuspended in DMEM/F12 supplemented with foetal calf serum (FCS, 10%) and plated into poly-L-lysine-coated tissue culture flasks (1 μg/cm^2; Sigma). Cells were grown in this medium at 37 °C/5% CO_2 for 48 h, after which the medium was changed to DMEM/F12 supplemented with neurotrophin-3 (50 ng/ml; Alamone Labs, Jerusalem, Israel), a process that enhances OEC proliferation (Bianco et al., 2004). To create a cell line that overexpresses human recombinant green fluorescent protein (hrGFP), cells were transduced with the pFB-hrGFP retroviral supernatant (Stratagene, Catalogue #972002) according to the manufacturer's instructions. Fluorescence activated cell sorting (FACS) was then performed to isolate a near >99% pure population of cells expressing hrGFP. After FACS, cultures were grown to approximately 80-90% confluence and harvested for cyropreservation by incubation in Trypsin-EDTA (0.25% Trypsin, 1 mM EDTA; JRH) solution for 5 min at 37 °C/5% CO_2 followed by centrifugation to pelletise the cells. The cell pellet was then re-suspended in cryo-storage medium comprising of 90% FCS and 10% dimethyl sulfoxide (DMSO) at 4 °C. Aliquots of cell suspension (0.5 ml each) were dispensed into cryotubes (Nunc) and placed immediately on ice before being placed in liquid nitrogen for long-term storage. To revive cryopreserved cells, cryotubes were removed from liquid nitrogen and placed immediately into a 37 °C water bath to thaw after which 1 ml of pre-warmed (37 °C) DMEM/F12 + 10% FCS was added drop-wise to a final volume of 1.5 ml. The cell suspension was then transferred slowly into 10 ml of pre-warmed DMEM/F12 + 10% FCS and centrifuged at 200*g* for 10 min. The pellet was re-suspended in DMEM/F12 + 10% FCS and cultured as previously described prior to use in transplantation experiments. hOEC cultures were transported to the University of New South Wales on the day prior to the experiment by courier at ambient temperature. Prior to transplantation cells were harvested, counted using the vital dye Trypan Blue, and re-suspended at a concentration of 15×10^4 cells/μl in DMEM/F12 + 10% FCS.

4.2. *Animals*

24 inbred athymic rats (CBH-rnu/Arc, female, 8–9 weeks old) were obtained from the Animal Resources Centre (Perth, Australia). They were housed in sterilized cages equipped with an air filter and sterile bedding materials and maintained on 12:12-h light–dark cycle, with free access to sterilized water and irradiated food throughout the experiment. All animal procedures were performed with approval from the University of New South Wales Animal Care and Ethics Committee.

4.3. *Spinal cord injury*

Rats were anesthetized with ketamine:xylazine (100:10 mg/kg, IP) and placed on a homeothermic blanket (body temperature maintained at 37 °C ± 1 °C). An incision was made in the dorsal midline skin from the mid to the low thoracic region and subsequent layers of connective tissue and muscle were separated to expose the spinal column. A bilateral laminectomy was performed on the T10 vertebrae. Animals were subjected to a moderate contusion injury using the MASCIS weight drop device developed at New York University (12.5 mm, 10 g,). The impact height, velocity and compression were recorded and the compression rate calculated. Rats were excluded from the study if they had a compression rate more than two standard deviations from the group mean. The muscles and the skin were sutured closed in layers. Post-operatively animals were given antibiotics (cephalothin sodium 100 mg/mL, 0.1 ml), analgesics (carprofen 50 mg/mL, 0.01 ml) and supplementary fluid (Hartman's replacement solution 2 ml) twice daily for 3 days. Bladders were expressed manually until the normal voiding response returned. Antibiotics were continued until the urine was clear.

4.4. *hOEC transplantation*

One week after the spinal cord contusion injury, animals were re-anesthetized as above and the T10 lesion site re-exposed. The vertebral column was stabilized using clamps to the T9 and T11 spinous process and a 5-μl syringe (26G, SGE, Australia) attached to a micromanipulater was used to inject hOECs in solution into the spinal cord lesion. A total of 1×10^6 hOECs were suspended in 7 μl of DMEM/F12 + 10% FCS. 5 μl of hOEC solution was injected into the centre of the lesion over 5 min. 1 μl was injected 1 mm rostral and 1 μl was injected 1 mm caudal on the midline at a depth of 0.5 mm. Control animals received the same volume of culture media, but without cells, injected into the same sites. After injection, syringes were held in place for a further 3 min to reduce leakage on removal.

4.5. *Behavioural assessments*

Each animal was familiarised with the open field apparatus and pre-trained on the horizontal ladder prior to surgery. Following the spinal cord contusion the animals were assessed in the open field apparatus at day 1 post-contusion and then weekly for 6 weeks. Animals were also tested on the horizontal ladder at 6 weeks post-contusion. All sessions were recorded using digital cameras linked to a FileMaker Pro database (FileMaker Pro 7.0 v3, FileMaker Inc, 1984–2007, Ca) for 2 min (open field) or for three passes of the horizontal ladder. Scoring was conducted by 2 independent observers blinded as to the treatment group using the digital recordings which could be analysed frame by frame when necessary. Averaged scores were used as the final score for each trial.

4.5.1. *Open field assessments*

The BBB rating scale (Basso et al., 1995) was used for open field assessments. Briefly, this rating scale assesses hindlimb movement on a 21-point scale while animals move freely within a 90-cm diameter open field. The BBB rating scale incorporates ankle, knee and hip movements initially, then weight support and plantar stepping, and finally coordinated limb movements, paw rotation and trunk stability. Animals were assessed on the first day after contusion surgery to test for consistency of the lesion and thereafter assessed weekly. Animals were excluded from the study if their BBB score at day 1 was greater than two standard deviations from the group mean.

4.5.2. *Horizontal ladder rung walking test*

For a more challenging assessment of hindlimb function rats were also tested on a horizontal ladder rung walking apparatus using the methods described by Metz and Whishaw (2002). Two 100-cm perspex sheets constituted the side walls of the apparatus which were separated by 10 cm and connected at their bases by a series of 3 mm metal rungs set in an irregular pattern. The ladder was elevated to a height of 20 cm above a mirror angled at 45° to provide a ventral view of the hindlimbs. A dark home cage was suspended at the end of the ladder to encourage locomotion. Performance was scored using a 7-category scale. For each step the foot placement on the rung was rated according to its position and placement accuracy; (0) Total miss, (1) Deep slip, (2) Slight slip, (3) Replacement, (4) Correction, (5) Partial placement and (6) Correct placement. Scores of 0, 1 or 2 were considered 'errors", i.e. an error represented a total miss or any type of foot slip. The percentage of steps with errors was calculated for each animal during 3 passes of the apparatus and averaged for the group.

4.6. *Histological analysis*

Six weeks after hOEC transplantation each animal was injected with sodium pentobarbitone (Lethobarb; 0.15 ml/100 g, IP) and perfused transcardially with physiological saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). A segment of spinal cord extending 5 mm rostral and caudal to the injury site was collected. The tissue was post fixed in the same fixative for 2 h and then cryoprotected in a solution of 30% sucrose in 0.1 M phosphate buffer with 0.01 % sodium azide until sectioning. Sections were cut at a thickness of 30 μm from the dorsal to ventral surface of the spinal cord (approx 1.8 mm in total depth). All the sections were mounted on a series of 10 so that adjacent sections were on consecutive slides. Each slide therefore contained 6–7 sections of spinal cord at 300-μm intervals from the dorsal to ventral surface. For each animal, spinal cord sections were aligned anatomically using the central canal and each cord was assessed using 5 dorsal to ventral sections. For some animals the very dorsal and/or the very ventral sections were not complete so these were excluded from the analysis. One slide was stained with haematoxylin and eosin (H&E); one was mounted in Fluromount (Dako) for examination of hOECs expressing GFP. Three other slides were used for immunohistochemistry for GFAP, NF200 and ED1. Sections were incubated in phosphate buffered saline with triton-X (PBST) for 10 min and in 5% normal goat serum for 30 min before overnight incubation in primary antibody at 4 °C; Rabbit anti-GFAP (1/1000, Dako, Denmark), Mouse anti-Neurofilament 200 (1/1000, Sigma, MO) and Rabbit anti-ED1 (1/2000, AbD Serotec, UK). Sections were rinsed in 3 changes of PBST before incubation in either AF 568-conjugated Goat anti-mouse IgG (1/200, Molecular Probes, Invitrogen, CA), or TRITC-conjugated Swine anti-rabbit IgG antibody (1/200, Dako, Denmark) for 1 h at room temperature. All sections were counterstained with Hoechst (33342, Invitrogen, CA) for 10 min to visualize the cell nuclei. Sections were then rinsed in 3 changes of PBST and cover slipped using Fluromount mounting media (Dako, Denmark).

4.6.1. *Lesion size*

Low-power digital images of the H&E stained sections (Fig. 2) were used for injury quantification. Each section was captured using a PixelLink video camera mounted on an Olympus BH-2 microscope. One observer blind as to the group made all the measurements using Image J analysis software (Rasband, 1997–2009).

The following measurements were made on each horizontal section: the spinal cord area, measured in a 5 mm length of tissue centred on the epicenter of the lesion (thick dashed lines in Fig. 2) and the lesion area, defined by the area of extensive tissue disruption, loss of neurons, and a rim of pale staining tissue (thin dashed lines in Fig. 2). High power microscopy was used to verify tissue injury; cavity areas within the lesion, which were non-cellular areas selected by grey scale intensity using Image J software. Also measured was the width of the spinal cord at a line drawn perpendicular to the midline at the epicenter of the lesion and the width of spared tissue on the same line as above, with the left and right sides summed.

Measurements were expressed as the percentage of lesion area in the spinal cord area, the percentage of cavity area in the lesion area, and the percentage of spared tissue in the width of the spinal cord.

4.6.2. *Immunohistochemistry*

hOECs exhibited strong green fluorescence. All other immunohistochemistry was carried out using red fluorescent secondary antibodies. GFAP staining intensity was measured as a mean grey scale value using NIH Image. The increase in GFAP intensity adjacent to the lesion was calculated from the mean grayscale value of GFAP staining adjacent to the lesion divided by the mean grayscale value of GFAP staining 5 mm distal to the lesion. The percentage increase in GFAP intensity in each spinal cord was averaged for each group at each level of spinal cord tissue. Sections of uninjured spinal cord showed no differences in GFAP staining intensity across the section using this method. Primary antibody was omitted for negative controls. Sections stained with NF200 were examined using confocal microscopy to determine if hOECs were co-localised with NF 200 positive axon profiles. Antibodies against ED1 were used to confirm macrophage/microglia infiltration.

4.7. *Statistical methods*

For the open field test, two way ANOVA repeated measures was used to compare BBB scores for the hOEC transplanted and

control groups over the 6-week period with Boneronni post hoc tests. For the horizontal ladder test and histological measurements, *t*-tests were used to compare the groups at 6 weeks post contusion. The statistical packages SPSS v17.0 (SPSS released 23/08/08, Chicago, SPSS Inc.) and Prism 5 (GraphPad Software, Inc, CA) were used for statistical analysis and $\alpha = 0.05$ was used as the criterion for statistical significance.

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