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Survival and Integration of Transplanted Olfactory Ensheathing Cells are Crucial for Spinal Cord Injury Repair: Insights from the Last 10 Years of Animal Model Studies

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Abstract

Olfactory ensheathing cells (OECs), the glial cells of the primary olfactory nervous system, support the natural regeneration of the olfactory nerve that occurs throughout life. OECs thus exhibit unique properties supporting neuronal survival and growth. Transplantation of OECs is emerging as a promising treatment for spinal cord injury; however, outcomes in both animals and humans are variable and the method needs improvement and standardization. A major reason for the discrepancy in functional outcomes is the variability in survival and integration of the transplanted cells, key factors for successful spinal cord regeneration. Here, we review the outcomes of OEC transplantation in rodent models over the last 10 years, with a focus on survival and integration of the transplanted cells. We identify the key factors influencing OEC survival: injury type, source of transplanted cells, co-transplantation with other cell types, number and concentration of cells, method of delivery, and time of transplantation after the injury. We found that two key issues are hampering optimization and standardization of OEC transplantation: lack of (1) reliable methods for identifying transplanted cells, and (2) three-dimensional systems for OEC delivery. To develop OEC transplantation as a successful and standardized therapy for spinal cord injury, we must address these issues and increase our understanding of the complex parameters influencing OEC survival.

Keywords

olfactory ensheathing cells, spinal cord injury, cell survival, cell integration, glial cells

Introduction

Olfactory ensheathing cells (OECs) are the glial cells of the primary olfactory nervous system, which consists of the olfactory nerve (cranial nerve I), which extends between the olfactory epithelium at the roof of the nasal cavity and the olfactory bulb in the anterior cranial fossa, and the outer layer of the olfactory bulb termed the nerve fiber layer (NFL) (Fig. 1). The primary olfactory nervous system is unique in that it continuously undergoes regeneration. Even after large-scale injury, it can regenerate as long as the deeper layers of the olfactory bulb, internally to the NFL, remain intact[1–3]. Primary olfactory neurons live for an average of approximately 30 days in rodents (the exact life-span of human primary olfactory neurons is not known), and new neurons continuously arise from progenitors in the olfactory epithelium[4]. The cell bodies of these neurons remain in the epithelium; their dendrites extend into the nasal cavity where they detect odorant molecules. Primary olfactory axons extend basally into the lamina propria, where they fasciculate to form the many branches of the olfactory nerve, which, after traversing the cribriform plate, terminates in the

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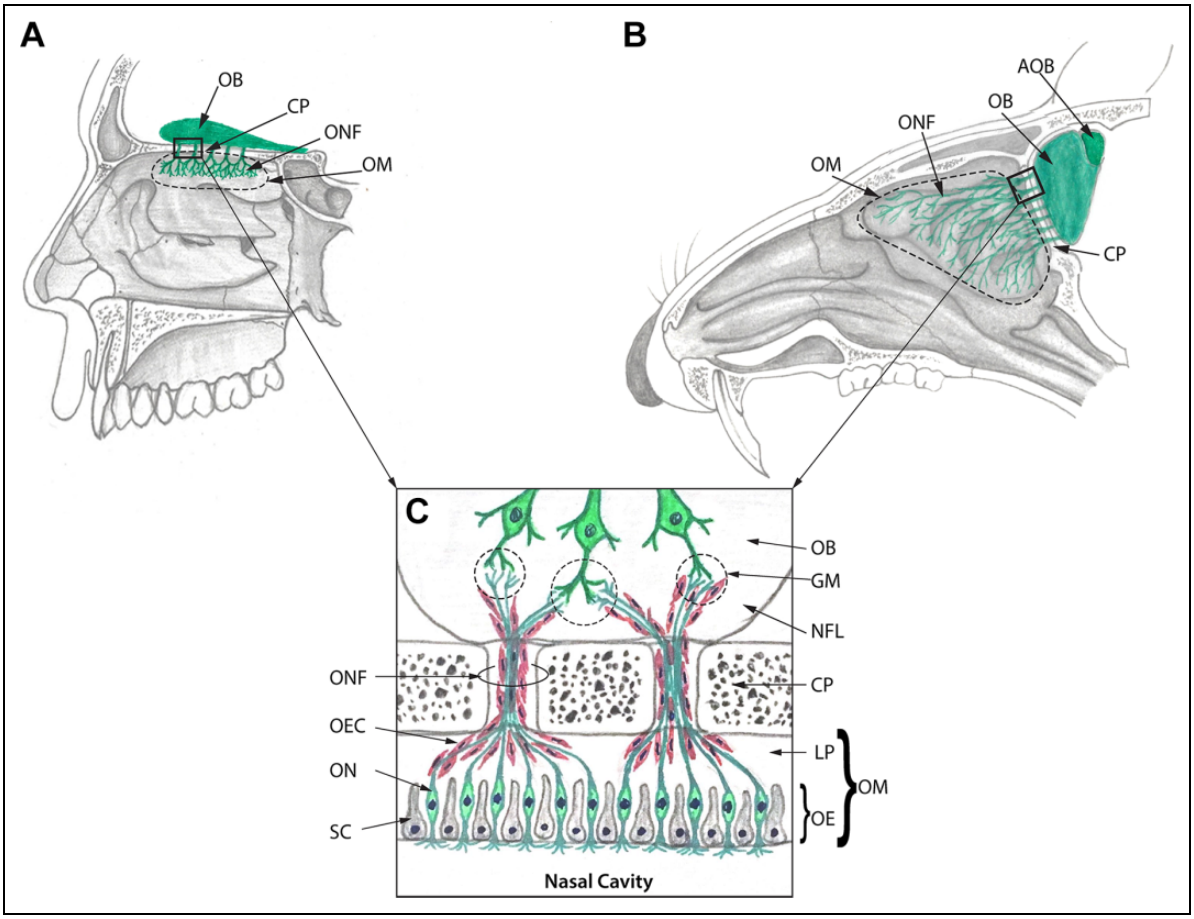

Figure 1. Organization of the olfactory system. Schematic of a sagittal sectional view of (A) the human and (B) rodent nasal cavity with the olfactory nerve and bulb highlighted in green. (C) A histological schematic showing the key cell types of the olfactory system. AOB: accessory olfactory bulb (only present in rodents); CP: cribriform plate; GM: glomeruli; LP: lamina propria; NFL: nerve fiber layer; OB: olfactory bulb; OE: olfactory epithelium; OEC: olfactory ensheathing cells (shown in red); OM: olfactory mucosa (area within the dotted line); ON: olfactory neurons (shown in green): ONF: olfactory nerve fascicles: SC: sustentacular cells.

olfactory bulb (Fig. 1)[1–3]. OECs are considered crucial for the regenerative capacity of the primary olfactory nervous system (reviewed elsewhere[5–8]).

OECs are present throughout the entire primary olfactory nervous system and are in direct contact with the axons of primary olfactory neurons all the way from the lamina propria to the olfactory bulb[9,10] (Fig. 1). OECs are in fact not a homogenous population of cells; sub-populations of OECs exist with distinct anatomical location and functions. In the olfactory nerve, OECs organize primary olfactory axons into fascicles. The axons are not myelinated; instead, relatively large bundles of axons are surrounded by OECs which create a tunnel-like structure[10]. OECs provide structural, neurotrophic, and axonal guidance support for the axons as they extend toward the olfactory bulb[5,11–14]. In the NFL of the olfactory bulb, OECs are thought to be crucial for the sorting of axons to olfactory bulbar targets termed glomeruli, which is dictated by the type of odorant receptor expressed by each axon. Here, OECs are considered to mediate a complex array of de-fasciculation, sorting, and re-fasciculation of axon fascicles according to odorant receptor profile[9,10]. Furthermore, OECs are the main phagocytes in the primary olfactory nervous system, eliminating the large amounts of cellular debris resulting from the constant neuronal regeneration[15–19], as well as eliminating invading microorganisms[15,20,21]. OECs also exhibit a high capacity for migration. During development, OECs migrate ahead of olfactory axons en route to the olfactory bulb[22–24], and after large-scale olfactory bulb injury, the cells migrate into the injury site within the central nervous system (CNS)[25]. Thus, phagocytosis, migration to injury site, axonal guidance, and structural remodeling (reviewed elsewhere[14]) are some of the direct roles that OECs play in axonal regeneration. In addition, OECs have been found to have some indirect effects promoting neural repair and regeneration such as secretion of growth factors, neurotrophins, and basement membrane components[14].

Owing to their ability to promote neural regeneration, as well as their unique migratory properties, transplantation of

Table 1. Summary of Cell Survival Reporting and Quantification. This Table Summarizes the Reporting and Quantification of Cell Survival.

(i) Summary of survival reporting			
Complete quantification	Semi-quantitative analysis	Discussed without quantification	No mention of survival
4[28–31]	2[32,33]	30	30
(ii) Summary of cell survival quantification			
Study	Reported OEC Survival	Quantified at time post treatment	
1[31]	0.6 ± 0.148%	1 week	
	0.473 ± 0.138%	2 weeks	
	0.357 ± 0.122%	13 weeks	
2[29]	6.5 ± 2.5%	4 weeks	
3[28]	2.85 ± 0.73%	4 weeks	
4[30]	0.34 – 1.72%	8 weeks	

OECs has been trialed for spinal cord injury repair in animals and humans with promising but highly variable outcomes. Animal models of spinal cord injury, most of which are rodent models, are invaluable for evaluating and optimizing OEC transplantation toward a therapy. However, the large variability in methodology and outcomes must be addressed to better inform the future directions of the therapeutic use of these cells. The two main issues that warrant attention are (1) cell purity, as OECs are notoriously difficult to purify, in particular OECs isolated from the olfactory mucosa which is the favorable clinical approach (reviewed elsewhere[26,27]), and (2) survival of the transplanted OECs, which is the focus of the current review.

Addressing cell survival is important because increasing the number of OECs that survive the transplantation is likely to directly or indirectly facilitate the regeneration process, and reducing the number of OECs that die will minimize the deleterious effects of the necrotic cells at the transplantation site. But which parameters affect survival of the transplanted OECs? This question has to date been difficult to answer, as quantification of OEC survival at the injury site is complicated because of the lack of specific OEC markers needed to track the transplanted cells. There are, however, certain key factors that are particularly likely to influence cell survival, which we review here. For the review of the literature, publications spanning the last 10 years were searched for the keywords "spinal cord injury" with a combination of the phrases "olfactory ensheathing cells," "OECs," "olfactory ensheathing glia," "OEGs," and "olfactory glia." It is not possible to evaluate OEC survival in living humans who have received an OEC transplant; thus, this review is limited to information arising from animal studies. To date, most animal studies have been performed using rodent spinal cord injury models. Therefore, only studies conducted in rodents were included in this review, while studies involving human trials or other animal models were excluded. Studies limited to *in vitro* experiments, peripheral nerve repair, and review articles were also excluded. It is our belief that the most recent studies would also reflect the collectively generated knowledge of the previously published works, along with the recent most developments in the field, which is why this study only focuses on the studies published over the last 10 years. Studies published more than 10 years before were referred to in cases where the included studies referred to them for specific methodologies.

A total of 66 studies that met the inclusion criteria were included in this review. For each study, details regarding injury model, transplanted cell type (OECs alone, OECs in comparison with or together with other cells), transplantation method, number of transplanted cells, percentages of surviving cells, and survival duration are summarized in Tables 1 and 2, with full details presented in Table 3.

Reporting of Cell Survival

Background. Reporting of cell survival is a crucial parameter to be assessed for evaluating cell transplantation-based therapies, including OEC-mediated spinal cord repair. Although some authors have suggested that the reparative effects of OECs may not require the cells to be permanently present at the injury site[45], more widely discussed properties of OECs such as physically bridging the injury site gap[93] and phagocytosing debris at the injury site[94] do require the OECs to survive after transplantation. While several studies do not quantify cell survival, a few of the studies published before the period mentioned in the inclusion criteria have analyzed the cell survival in depth. One such study reported 2.3 ± 1.4% cell survival after 3 months of transplantation[95]. In this study, two million OECs were injected in a 6 µl suspension over a period of 3 min, at 2 months following a contusion-type injury. Another study analyzed survival of OECs transplanted directly into the injury site as well as at a spinal cord site distant from the injury site in contusion-type injury[96]. According to this study, only 3.1 ± 1.4% of the OECs survived at the injury site at 3 weeks post transplantation when they were injected by themselves. However, when they were injected in the intact spinal cord away from injury site and Schwann cells (SCs) injected in the injury core, the OEC

Table 2. Summary of Transplantation Parameters and Outcomes. This Table Summarizes the Number of Reviewed Publications Across Different Transplantation Parameters, and Their Key Outcomes.

(i) Injury model					
Contusion	Crush	Transection	Hemisection	Other	N/M
20	7	21	6	11	1
(ii) OECs Sources					
OB Alone	OM Alone	Both OB + OM	Cell line	Xenograft	N/M
40	16	2	1	6	1
(iii) Co-transplantation with other cell types					
	MSCs	SCs	NSCs	ESMNs	ASCs	FBs
Used separately	5	5	3	1	1	7
Co-transplanted with OECs	2	3	3	1	1	0
(iv) Cell number/concentration					
total cell number	not mentioned	less than 100,000	100,000–500,000	more than 500,000	
	10	9	34	13	
Concentration	not mentioned	less than 50,000	50,000–100,000	more than 100,000	
cells/μl	12	14	30	10	
(v) Summary of Transplant methods					
Injection	Other+	Both compared			
51	13	2			
(vi) Summary of transplantation time post injury++						
0 Days	1 week	2 weeks	4 weeks	8 weeks	Other	N/M
40	13	6	3	2	7	1

+ Other methods include transplantation of cells encased/embedded in hydrogels; MBL: mucosal pieces, gel sponges or spherical aggregates.
++ out of the 66 studies, 62 performed treatments at one timepoint, two studies did treatments at two timepoints, and two more studies performed treatments at three timepoints, which is why the total number of here appears as 72 here instead of 66.

survival was reported to be 48 ± 6.3%. This indicates that transplanted OECs can survive in healthy spinal cord, while the harsh environment of the injury site reduces survival. The study also reported similar survival rates at 9 weeks after the transplantation.

Recent evidence. Only 36 out of the 66 reviewed studies reported that they assessed cell survival. Out of these 36 studies, only six quantitatively analyzed cell survival, with four studies reporting a fully quantified survival outcome of transplanted cells[28–31]. One study quantified surviving OECs by determining the pixel intensity as seen on fluorescent imaging[32], while another study determined the proportion of surviving cells within the lesion site in relation to the remaining tissue[33]. The remaining 30 studies did not quantify the numbers or percentages of surviving cells. A few studies reported "drastic reduction in survival" or "very low cell survival" 2–8 weeks after transplantation without reporting the quantitative measurements[49,64,66], and in one case the authors reported that none of the transplanted cells survived 7 months after transplantation[36]. Overall, OEC survival was determined at a time post transplantation ranging from 1 week[33,61,64,86] to 1 year[81], with most studies assessing OEC survival between 3 weeks and 3 months after transplantation. Some studies included longer-term determination of survival up to 6 months, 7 months[77,87], or 8 months[68,83,88]. The results regarding cell survival are summarized in Table 1.

In one of the four studies that fully quantified the survival percentages of transplanted OECs[28], female Wistar rats were injured using crush injury and were treated with an allograft of cells obtained from olfactory bulbs of donor Wistar rats with/without embryonic stem-derived motor neurons (ESMN). Quantification of surviving cells at 4 weeks post transplantation reported 2.8 ± 0.73% in OEC only treatment group, and 2.6 ± 1.2% in OECs + ESMN treatment group. It should be noted that these animals were under immunosuppression induced by cyclosporine, to mitigate the chances of rejection of the allograft.

Another study[30] reported on OEC survival in a crush-type injury model in male Wistar rats. Cells for transplantation were obtained from the olfactory mucosa of 4-week-old transgenic Sprague Dawley (SD) rats expressing enhanced green fluorescent protein (GFP) in OECs as well as mesenchymal stem cells (MSCs). Thus, this transplant was an allograft (between rats of different strains). Rats were treated with microinjections of cell suspensions of OECs with/without MSCs 7 days after the injury, and by 8 weeks post-transplantation survival of OECs was estimated to be between 0.34% and 1.72%, while survival of MSCs was estimated to be 0.41–0.96%. These animals also received cyclosporine to prevent graft rejection.

A third study used contusion-type injury in SD rats as injury model[31], into which OECs from the olfactory bulbs of transgenic GFP-expressing SD rats were transplanted (constituting an allogenic graft) using microinjections 1

Table 3. Studies of OEC Transplantation in Rodent Models Included in This Review. The table shows injury model, source of transplanted cells, transplantation method, percentages of cells surviving and duration of study (including the time at which cell survival was studied). n/m: not mentioned. Mentioned, not quantified = studies discussed cell survival, but did not quantify it. Observed, not quantified = studies mention observing cell survival in results, but without quantification. The studies are listed in a descending chronological order by the year of publication.

		Injury model		Transplanted cells					
Reference	Title	Animal	Injury type	Source of cells	Graft type	Transplantation method	Time post injury that transplantation was performed	Survival %	Time at which OEC survival was assessed
Collins et al., 2018[34]	Partial recovery of proprioception in rats with dorsal root injury after human olfactory bulb cell transplantation	Male SD rats	Dorsal Root Avulsion	hOECs (OB)	Xeno-	cells injected in a collagen scaffold	0 days	Mentioned, not quantified	6 weeks post injury
Voronova et al., 2018[35]	Survival and migration of rat olfactory ensheathing cells after transplantation into posttraumatic cysts in the spinal cord	Female Wistar rats	Contusion	OM-OECs	Allo-	1.5×10^5 cells/μl, 10 μl injected = 1.5×10^6 cells	4 weeks	Mentioned, not quantified	4 weeks post transplant
Thornton et al, 2018[36]	Evidence of axon connectivity across a spinal cord transection in rats treated with epidural stimulation and motor training combined with olfactory ensheathing cell transplantation	Female SD rats	Transection	OB-OECs	Allo-	1×10^5 cells/μl, total 4 μl injected 1 mm rostral and caudal = 4×10^5 cells	0 days	Mentioned, as zero % survival	7 months post injury
Carwardine et al., 2017[29]	Transplantation of canine olfactory ensheathing cells producing chondroitinase ABC promotes chondroitin sulphate proteoglycan digestion and axonal sprouting following spinal cord injury	Male athymic nude rats	Crush	OM-OECs, Canine	Xeno-	4×10^4 cells, 2 injections each, immediately rostral and caudal, 1 mm deep in the midline, rate of 0.2 μL/minute = 8×10^4 cells	0 days	4 weeks: 6.5 ± 2.5%	4 weeks post injury
Zhang et al., 2017[37]	The effects of co-transplantation of olfactory ensheathing cells and Schwann cells on local inflammation environment in the contused spinal cord of rats	Female SD rats	Contusion	OB-OECs	Allo-	10^5 cells/μl, 3 μl injected 1 mm rostral and caudal to injury each, 0.8 mm deep, 6 μl = 6×10^5 cells	7 Days	Mentioned but not quantified	6 weeks post transplant

(continued)

Table 3. (continued)

Reference	Title	Injury model		Transplanted cells		Transplantation method	Time post injury that transplantation was performed	Survival %	Time at which OEC survival was assessed
		Animal	Injury type	Source of cells	Graft type				
Zheng et al, 2017[38]	Olfactory ensheathing cell transplantation inhibits P2X4 receptor overexpression in spinal cord injury rats with neuropathic pain	SD rats	n/m	OB-OECs	Allo-	10^5 cells/μl	n/m	Observed, not quantified	4 weeks post injury
Gu M et al., 2017[39]	Feasibility of diffusion tensor imaging for assessing functional recovery in rats with olfactory ensheathing cell transplantation after contusive spinal cord injury (SCI)	Male SD rats	Contusion	OB-OECs	Allo-	5×10^5 cells/μl, 2 μl injected in the lesion= 1×10^6 cells	1 week	n/m	2, 4, 6, 8 weeks post injury
Tang et al., 2017[40]	Ginsenoside Rg1 promotes the migration of olfactory ensheathing cells via the PI3K/Akt pathway to repair rat spinal cord injury	SD rats	Contusion	OB-OECs	Allo-	$5x10^4$ cells/μl, 5 μl injected around the lesion = $2.5x10^5$ cells	0 Days	n/m	4 weeks post injury
Lindsay et al., 2017[41]	Human olfactory mesenchymal stromal cell transplants promote remyelination and earlier improvement in gait co-ordination after spinal cord injury	Male SD rats	Contusion	OM-OECs, Human	Xeno-	$1–2\times10^5$ cells/μl, 30–40 μl injected, until the suspension overflowed = $6–8\times10^6$ cells	3 weeks	Mentioned, not quantified	10 days, 4 weeks, 7 weeks post transplant
Collins et al., 2017[42]	Transplantation of cultured olfactory bulb cells prevents abnormal sensory responses during recovery from dorsal root avulsion in the rat	SD rats	Dorsal Root Avulsion	OB-OECs	Allo-	OECs in matrix, 0.5–1 mm^2, 7 such portions transplanted	0 days	n/m	3 weeks post injury

(continued)

Table 3. (continued)

Reference	Title	Injury model		Transplanted cells		Transplantation method	Time post injury that transplantation was performed	Survival %	Time at which OEC survival was assessed
		Animal	Injury type	Source of cells	Graft type				
Khankan et al., 2016[33]	Olfactory ensheathing cell transplantation after a complete spinal cord transection mediates neuroprotective and immunomodulatory mechanisms to facilitate regeneration	Female SD rats	Transection	OB-OECs	Allo-	5×10^4 cells into four midline injection sites per spinal cord stump = 4×10^5 cells	0 Days	1 week: 79 ± 4%, 2 weeks: 14 ± 7%, 4 weeks: 23 ± 14%, 8 weeks: 0%	1, 2, 4 and 8 weeks post injury
Nategh et al., 2016[43]	Subarachnoid space transplantation of Schwann and/or olfactory ensheathing cells following severe spinal cord injury fails to improve locomotor recovery in rats	Female Wistar Rats	Cord compression	OB-OECs	Allo-	10^4 cells/μl, 5 μl injected into subarachnoid space over 10 seconds with a fine 30-gauge needle = 5×10^5 cells	7 days	n/m	160 days post injury
Gomes et al., 2016[44]	Combination of a peptide-modified gellan gum hydrogel with cell therapy in a lumbar spinal cord injury animal model	Female Wistar rats	Hemisection	OB-OECs	Allo-	2×10^4 cells/μl, 1 μl OECs+ 3 μl ASCs, either in hydrogel or in 4 μl suspension injected rostral to injury = 8×10^4 cells	0 days	n/m	8 weeks post transplant
Li et al., 2016[45]	Functional repair of rat corticospinal tract lesions does not require permanent survival of an immunoincompatible transplant	Female AS rats	RF-induced heat ablation of CST	OB-OECs	Xeno- (C57BL/6 mice)	2.5×10^4 cells/μl, 4 μl injected in lesion = 1×10^5 cells	8 weeks	n/m	8 weeks post transplant
Cloutier et al., 2016[46]	Olfactory ensheathing cells but not fibroblasts reduce the duration of autonomic dysreflexia in spinal cord injured rats	Male Wistar rats	Transection	OM-OECs	Allo-	1×10^5 cells/μl, 4 injections, 0.5 μl each into cord **BEFORE** transection = 2×10^5 cells	0 days	Mentioned, not quantified	9 weeks post injury
Kang et al., 2015[47]	Effectiveness of muscle basal lamina carrying neural stem cells and olfactory ensheathing cells in spinal cord repair	SD rats	Hemisection	OB-OECs	Allo-	1×10^6/ml cell suspension; $1\times1\times1$ mm^3 complex for cells embedded in muscle basement lamina	0 Days	n/m	4 weeks and 8 weeks post injury

(continued)

Table 3. (continued)

Reference	Title	Injury model		Transplanted cells		Transplantation method	Time post injury that transplantation was performed	Survival %	Time at which OEC survival was assessed
		Animal	Injury type	Source of cells	Graft type				
Reginensi et al., 2015[48]	Increased migration of olfactory ensheathing cells secreting the Nogo receptor ectodomain over inhibitory substrates and lesioned spinal cord	Female SD rats	Contusion	OECs (TEG3 cell line)	Allo-	5×10^4 cells/μl, 2 injections 3 μl each, 1 mm lateral to lesion, at 1 mm depth = 3×10^5 cells	0 days (30 min)	n/m	7 days post injury
Torres-Espin et al., 2014[49]	Bone marrow mesenchymal stromal cells and olfactory ensheathing cells transplantation after spinal cord injury – a morphological and functional comparison in rats	Female SD rats	Contusion	OB-OECs	Allo -	5×10^4 cells/μl suspended in L15 medium, injected 3 μl at epicenter, 3 μl caudal and 3 μl rostral = 4.5×10^5 cells in total	0 Days, & 7 days	Mentioned, not quantified	6 weeks post injury
Ibrahim et al., 2014[50]	Comparison of olfactory bulbar and mucosal cultures in a rat rhizotomy model	Female AS rats	Rhizotomy	OB-OECs, OM-OECs	Allo-	a semisolid transplant consisting of the matrix containing the cells	0 days	Mentioned, not quantified	8 weeks post transplant
Torres-Espin et al., 2013[51]	Gene expression changes in the injured spinal cord following transplantation of mesenchymal stem cells or olfactory ensheathing cells	Female SD rats	Contusion	OB-OECs, MSCs	Allo-	5×10^4 cells/μl suspended in L15 medium, injected 3 μl at epicenter, 3 μl caudally and 3 μl rostral = 4.5×10^5 cells	0 Days (30 minutes), & 7 days	Mentioned, not quantified	2 Days & 7 Days post transplant
Mayeur et al., 2013[27]	Potential of olfactory ensheathing cells from different sources for spinal cord repair	Male Fischer rats	Transection	OB-OECs, OM-OECs	Allo-	10^5 cells/μl, 2 injections each rostral and caudal to injury, 1.5 mm from midline = 4 injections of 1μl = 4×10^5 cells	0 days	Mentioned but not quantified	60 days post injury
Barbour et al., 2013[32]	Tissue sparing, behavioral recovery, supraspinal axonal sparing/ regeneration following sub-acute glial transplantation in a model of spinal cord contusion	Female Fischer rats	Contusion	OB-OECs	Allo-	10^5 cells/μl, 5 μl injected in center of the lesion, single injection = 5×10^5 cells	2 weeks	Counted in "pixel density" = 74% decline in 4 months compared with 2 weeks	4 months post transplant

(continued)

Table 3. (continued)

Reference	Title	Injury model		Transplanted cells		Transplantation method	Time post injury that transplantation was performed	Survival %	Time at which OEC survival was assessed
		Animal	Injury type	Source of cells	Graft type				
Toft et al., 2013[52]	A comparative study of glial and non-neural cell properties for transplant-mediated repair of the injured spinal cord	Fisher rats	Transection	OB-OECs, SCs	Allo-	10^5 cells/μl, 1 μl injected on both sides of the central vein = 2×10^5 cells	0 days	n/m	6 weeks post injury
Lang et al., 2013[53]	OECs transplantation results in neuropathic pain associated with BDNF regulating ERK activity in rats following cord hemisection	SD rats	Hemisection	OB-OECs	Allo-	3×10^3 cells/μl, 5 μl injected in a single injection = 1.5×10^4 cells	0 days	Observed, not quantified	1 month post injury
Coutts et al., 2013[54]	Embryonic-derived olfactory ensheathing cells remyelinate focal areas of spinal cord demyelination more efficiently than neonatal or adult-derived cells	Female Fischer rats	Ethydium Bromide + X-irradiation	OB-OECs	Allo-	5×10^4 cells/μl, 1 μl injected at the lesion	1–2 days	n/m	3 weeks post injury
Centenaro et al., 2013[55]	Implications of olfactory lamina propria transplantation on hyperreflexia and myelinated fiber regeneration in rats with complete spinal cord transection	Male Wistar rats	Transection	OM-OECs	Allo-	2–3 mm^2 pieces of mucosal lamina propria used for grafting	0 days, 2 weeks and 4 weeks	n/m	18 weeks post injury
Deumens et al., 2013[56]	Motor outcome and allodynia are largely unaffected by novel olfactory ensheathing cell grafts to repair low-thoracic lesion gaps in the adult rat spinal cord	Female Lewis rats	Hemisection	OB-OECs	Allo-	4×10^5 cells in a scaffold, 2 mm diameter, 2 mm length	0 day	n/m	10 weeks post injury

(continued)

Table 3. (continued)

Reference	Title	Injury model		Transplanted cells		Transplantation method	Time post injury that transplantation was performed	Survival %	Time at which OEC survival was assessed
		Animal	Injury type	Source of cells	Graft type				
Sun et al., 2013[57]	Co-transplantation of olfactory ensheathing cells and Schwann cells combined with treadmill training promotes functional recovery in rats with contused spinal cords	SD rats	Contusion	OB-OECs + SCs	Allo-	OECs 1×10^5 cells/μl, 4 μl in DMEM, 1 mm rostral and caudal to injury, at 4 different depths, 0.5 μl at each point + SCs 10^5 cells/μl × 2 μl in DMEM, in the lesion	14 days	n/m	11 weeks post injury
Luo et al., (2013)[58]	Transplantation of NSCs with OECs alleviates neuropathic pain associated with NGF downregulation in rats following spinal cord injury	Female SD rats	Transection	n/m	n/m	3×10^5 cells/μl, 1 μl in gelatin transplanted in lesion	0 days	Mentioned, not quantified	4 weeks post injury
Li et al., 2012[59]	Differing Schwann cells and olfactory ensheathing cells behaviors, from interacting with astrocyte, produce similar improvements in contused rat spinal cord's motor function	Male SD rats	Contusion	OB-OECs	Allo-	3×10^4/μl × 3 injections 1 μl each, 1 mm rostral, caudal and at lesion site, at 1 mm depth = 9×10^4 cells	7 Days	n/m	3 weeks, 6 weeks post transplant
Yazdani et al., 2012[60]	A comparison between neurally induced bone marrow derived mesenchymal stem cells and olfactory ensheathing glial cells to repair spinal cord injuries in rat	Female Wistar Rats	Crush	OB-OECs	Allo-	2×10^5 cells/μl, 5 μl injected each at injury site, 3 mm rostral and 3 mm caudal to injury = 3×10^6 cells	1 week	Mentioned, not quantified	6 weeks post injury
Roet et al., 2012[61]	Noninvasive bioluminescence imaging of olfactory ensheathing glia and Schwann cells following transplantation into the lesioned rat spinal cord	Female Fischer rats	Dorsal column lesion with a microknife (partial transection)	OB-OECs	Allo-	1.67×10^5 cells/μl, 1 μl injected each, at the lesion, 1 mm rostral and caudal, at 0.8 mm depth = 5×10^5 cells	0 day	n/m	1 week and 14 weeks post injury

(continued)

Table 3. (continued)

Reference	Title	Injury model		Transplanted cells		Transplantation method	Time post injury that transplantation was performed	Survival %	Time at which OEC survival was assessed
		Animal	Injury type	Source of cells	Graft type				
Toft et al., 2012[62]	Transplant-mediated repair properties of rat olfactory mucosal OM-I and OM-II sphere-forming cells	Male Fischer rats	Dorsal column lesion (partial transection)	OM-OECs	Allo-	0.5–1×10^5 cells/μl, 5-8 μl injected	0 days	Mentioned, not quantified	6 weeks post injury
Ziegler et al., 2011[63]	Further evidence of olfactory ensheathing glia facilitating axonal regeneration after a complete spinal cord transection	Wistar Hannover rats	Transection	OB-OECs	Allo-	1×10^5/μl, 4 injections of 0.5 μl into each stump = 4×10^5 cells	0 days	n/m	6 months post injury
Li et al., 2011[64]	Olfactory ensheathing cells can reduce the tissue loss but not the cavity formation in contused spinal cord of rats	Male SD rats	Contusion	OB-OECs	Allo-	3×10^4/μl × 3 injections 1 μl each, 1 mm rostral, caudal and at lesion site, at 1 mm depth = 9×10^4 cells	7 days	Mentioned as "very low" but not quantified	1-6 weeks post transplant
Stamegna et al., 2011[65]	Nasal OEC transplantation promotes respiratory recovery in a subchronic rat model of cervical spinal cord contusion	male SD rats	Contusion	OM-OECs	Allo-	1×10^5/μl, 6 injections, 1ul each, 1 mm rostral, 1.5–2 mm caudal and in the lesion, at 2 mm and 3 mm depths = 6×10^5 cells	15 days	n/m	3 months post transplant
Novikova et al., 2011[66]	Efficacy of olfactory ensheathing cells to support regeneration after spinal cord injury is influenced by method of culture preparation	Female SD rats	Contusion	OB-OECs	Allo-	1×10^5/μl, 1.5–1.6 μl Injected 1 mm rostral & caudal = 1.5–1.6×10^5 cells	0 days	Mentioned as "low" but not quantified	3, 8, 13 weeks post injury
Tharion et al., 2011[67]	Motor recovery following olfactory ensheathing cell transplantation in rats with spinal cord injury	Wistar rats	Contusion	OM-OECs	Allo	10^5 cells/μl, 7–20 μl injected at multiple sites = 0.7–2×10^6 cells	2 weeks	Mentioned, not quantified	10 weeks post transplant
Takeoka et al., 2011[68]	axon regeneration can facilitate or suppress hindlimb function after olfactory ensheathing glia transplantation	female Wistar Hannover rats	Transection	OB-OECs	Allo-	1×10^5 cells/μl, 0.5 μl injected at 1.3, 1.0, 0.8 and 0.5 mm depths in midline rostral and caudal = 4×10^5 cells	0 days	Mentioned, not quantified	8 months post injury

(continued)

Table 3. (continued)

Reference	Title	Injury model		Transplanted cells		Transplantation method	Time post injury that transplantation was performed	Survival %	Time at which OEC survival was assessed
		Animal	Injury type	Source of cells	Graft type				
Centenaro et al., 2011[69]	Olfactory and respiratory lamina propria transplantation after spinal cord transection in rats: Effects on functional recovery and axonal regeneration	Male Wistar rats	Transection	OM-OECs	Allo-	3–4 mm^2 pieces of mucosal lamina propria used for grafting	0 days, 2 weeks and 4 weeks	n/m	18 weeks post injury
Li et al., 2011[70]	Olfactory ensheathing cell transplantation into spinal cord prolongs the survival of mutant SOD1G93A ALS rats through neuroprotection and remyelination	Male SD rats SOD1(G93A)	Genetically induced ALS	OB-OECs	Allo-	2×10^4 cells/μl, 5 μl injected $= 1\times10^5$ cells	100 days age	n/m	40-50 days post injury
Zhang et al., 2011[71]	Scar ablation combined with LP/OEC transplantation promotes anatomical recovery and P0-positive myelination in chronically contused spinal cord of rats	Female LE Rats	Contusion	OM-OECs	Allo-	6×10^6 cells injected in 1–2 μl	6 weeks	Mentioned, not quantified	16 weeks post transplant
Gorrie et al., 2010[72]	Effects of human OEC-derived cell transplants in rodent spinal cord contusion injury	Female athymic rats	Contusion	OM-OECs, Human	Xeno-	1.4×10^5 cells/μl, 5 μl injected in lesion, 1 μl each injected 1 mm rostral and caudal in midline at 0.5 mm depth $= 1\times10^6$ cells	7 Days	Observed, not quantified	6 weeks post transplant
Liu et al., 2010[73]	Analysis of olfactory ensheathing glia transplantation-induced repair of spinal cord injury by electrophysiological, behavioral, and histochemical methods in rats	SD rats	Hemisection	OB-OECs	Allo-	1×10^4 cells/μl, 8 μl injected $= 8\times10^4$ cells	12 hours	n/m	8 weeks post transplant

(continued)

Table 3. (continued)

Reference	Title	Injury model		Transplanted cells		Transplantation method	Time post injury that transplantation was performed	Survival %	Time at which OEC survival was assessed
		Animal	Injury type	Source of cells	Graft type				
Bretzner et al., 2010[74]	Combination of olfactory ensheathing cells with local versus systemic camp treatment after a cervical rubrospinal tract injury	Male SD rats	Crush	OM-OECs	Allo-	1×10^5 cells/μl, 1.5 μl injected total between cranial and caudal sites = 1.5×10^5 cells	0 days	n/m	6 weeks post injury
Amemori et al., 2010[30]	Co-transplantation of olfactory ensheathing glia and mesenchymal stromal cells does not have synergistic effects after spinal cord injury in the rat	Male Wistar rats	Crush	OM-OECs	Allo-	10^5 cells/μl, 1 μl injected each proximal, central and distal to lesion = 3×10^5 cells	7 days	0.34–1.72%	8 weeks post transplant
Ma et al., 2010[75]	Effect of Neurotrophin-3 genetically modified olfactory ensheathing cells transplantation on spinal cord injury	Female SD rats	Contusion	OB-OECs	Allo-	1 x 10^4 cells/μl, 10 μl injected = 1 x 10^5 cells	0 Days	Mentioned, not quantified	8 weeks post injury
Aoki et al., 2010[76]	Limited functional recovery in rats with complete spinal cord injury after transplantation of whole-layer olfactory mucosa	female Wistar Rats	Transection	OM-OECs	Allo-	0.5 mm sized pieces of OM, freshly harvested	2 weeks	Mentioned, not quantified	4 weeks, 8 weeks post transplant
Takeoka et al., 2010[77]	Noradrenergic innervation of the rat spinal cord caudal to a complete spinal cord transection: effects of olfactory ensheathing glia	Female Wistar Hannover rats	Transection	OB-OECs	Allo-	4×10^5 cells injected 1 mm rostral and 1 mm caudal	0 days	n/m	7 months post injury
Kalincik et al., 2010[78]	Olfactory ensheathing cells reduce duration of autonomic dysreflexia in rats with high spinal cord injury	Male Wistar rats	Transection	OM-OECs	Allo-	5×10^5 cells/μl, 2 μl soaked into a porcine gel sponge = 1×10^6 cells	0 days	Mentioned, not quantified	9 weeks post injury
Kalincik at al., 2010[79]	Selected changes in spinal cord morphology after T4 transection and olfactory ensheathing cell transplantation	Male Wistar rats	Transection	OM-OECs	Allo-	5×10^5 cells/μl, 2 μl soaked into a Porcine gel sponge = 1×10^6 cells	0 days	n/m	9 weeks post injury

(continued)

Table 3. (continued)

Reference	Title	Injury model		Transplanted cells		Transplantation method	Time post injury that transplantation was performed	Survival %	Time at which OEC survival was assessed
		Animal	Injury type	Source of cells	Graft type				
Li et al., 2010[31]	Survival and number of olfactory ensheathing cells transplanted in contused spinal cord of rats	SD rats	Contusion	OB-OECs	Allo-	3×10^4 cells/μl, 1 μl injected at 3 sites each = 9×10^4 cells cells	7 days	at 1 week: 536 ± 132.74; at 2 weeks: 426 ± 124.14; 13 weeks: 321 ± 110.24	13 weeks post transplant
Wang et al., 2010[80]	Synergistic effect of neural stem cells and olfactory ensheathing cells on repair of adult rat spinal cord injury	Male SD rats	3/4 transection	OB-OECs	Allo-	1×10^6 cells/μl, 5 μl injected at 2 sites each = 1×10^7 cells	7 days	n/m	12 weeks post injury
Salehi at al., 2009[28]	Repair of spinal cord injury by co-transplantation of embryonic stem cell-derived motor neuron and olfactory ensheathing cell	Female Wistar Rats	Crush	OB-OECs, OECs+ESMN	Allo-	2×10^5 cells/μl, 5 μl injected = 1×10^6 cells, 10 μl injected, 5 μl of each cell = 2×10^6 cells	9 days	28524 ± 7287, 26702 ± 12120	4 weeks post transplant
Muñoz-Quiles et al., 2009[81]	Chronic spinal injury repair by olfactory bulb ensheathing glia and feasibility for autologous therapy	Female Wistar Hannover rats	Transection	OB-OECs	Allo-	1×10^5 cells/μl, 0.5 μl injected at 1.3, 1.0, 0.8 and 0.5 mm depths in midline, rostral and caudal = 4×10^5 cells	1 month and 4 months	n/m	12 months post injury
Su et al., 2009[82]	Reactive astrocytes in glial scar attract olfactory ensheathing cells migration by secreted TNF-α in spinal cord lesion of rat	Male SD rats	Hemisection	OB-OECs	Allo-	1×10^5 cells/μl, 0.5 μl inected at 3 sites in midline = 1.5×10^5 cells	0 days	n/m	10 days post injury
Takeoka et al., 2009[83]	Serotonergic innervation of the caudal spinal stump in rats after complete spinal transection: effect of olfactory ensheathing glia	Female Wistar rats	Transection	OB-OECs	Allo-	4×10^5 cells at 3 sites, 1 mm deep	0 days	n/m	8 months post injury

(continued)

Table 3. (continued)

Reference	Title	Injury model		Transplanted cells		Transplantation method	Time post injury that transplantation was performed	Survival %	Time at which OEC survival was assessed
		Animal	Injury type	Source of cells	Graft type				
Fouad et al., 2009[84]	Transplantation and repair: Combined cell implantation and chondroitinase delivery prevents deterioration of bladder function in rats with complete spinal cord injury	Female Fischer rats	Transection	OB-OECs	Allo-	30 μl suspension injected	0 days	n/m	12 weeks post injury
Yamamoto et al., 2009[85]	Transplanted olfactory mucosal cells restore paw reaching function without regeneration of severed corticospinal tract fibers across the lesion	Female AS Rats	Unilateral CST ablation	OM-OECs	Allo-	2–2.5$\times 10^4$ cells in 4-5 μl suspension = 1$\times 10^5$ cells	8 weeks	Mentioned, not quantified	18 weeks post injury
Lankford et al., 2008[86]	Olfactory ensheathing cells exhibit unique migratory, phagocytic, and myelinating properties in the X-irradiated spinal cord not shared by Schwann cells	Female SD rats	X-irradiation	OB-OECs	Allo-	3$\times 10^4$ cells/μl, 1 μl injected in the lesion as single injection = 3$\times 10^4$ cells	7 days	Mentioned, not quantified	1, 3, 6 weeks post transplantation
Kubasak et al., 2008[87]	OEG implantation and step training enhance hindlimb-stepping ability in adult spinal transected rats	Female Wistar Hannover rats	Transection	OB-OECs	Allo-	4 injections of 5$\times 10^4$ cells each into each spinal cord stump= 4$\times 10^5$ cells	0 days	n/m	7 months post injury
Negredo et al., 2008[88]	Slow- and fast-twitch rat hind limb skeletal muscle phenotypes 8months after spinal cord transection and olfactory ensheathing glia transplantation	Wistar Hannover rats	Transection	OB-OECs	Allo-	10^5 cells/μl, 4 injections, 0.5 μl each, in each stump = 4$\times 10^5$ cells	0 days	n/m	8 months post injury

(continued)

Table 3. (continued)

Reference	Title	Injury model		Transplanted cells		Transplantation method	Time post injury that transplantation was performed	Survival %	Time at which OEC survival was assessed
		Animal	Injury type	Source of cells	Graft type				
Deng et al., 2008[89]	The co-transplantation of human bone marrow stromal cells and embryo olfactory ensheathing cells as a new approach to treat spinal cord injury in a rat model	Female SD rats	Contusion	OB-OECs	Allo-	5×10^4 cells/μl, 5 μl injected around the lesion = 2.5×10^5 cells	0 days (30 min)	Mentioned, not quantified	5 weeks post transplant
Guest et al., 2008[90]	Xenografts of expanded primate olfactory ensheathing glia support transient behavioral recovery that is independent of serotonergic or corticospinal axonal regeneration in nude rats following spinal cord transection	Nude rats	Transection	OB-OECs	Xeno- (Macacs)	1×10^5cells/μl, 0.5 μl injected at four depths (2×10^5 cells/2 μl/stump) = 4×10^5 cells	0 days	Mentioned, not quantified	24 weeks post injury
Bretzner et al., 2008[91]	Undesired effects of a combinatorial treatment for spinal cord injury – transplantation of olfactory ensheathing cells and BDNF infusion to the red nucleus	Male SD rats	Crush	OM-OECs	Allo-	1×10^5 cells/μl, 1.5 μl injected total between cranial and caudal sites = 1.5×10^5 cells	0 days	Mentioned, not quantified	4 weeks post injury
Iwatsuki et al., 2008[92]	Transplantation of olfactory mucosa following spinal cord injury promotes recovery in rats	Female SD rats	Transection	OM-OECs	Allo-	0.5–1 mm pieces of OM, 4–6 pieces inserted in lesion	0 days	Mentioned, not quantified	8 weeks post injury

3D: Three-dimensional; μl: Microliters; AS Rats: Albino Swiss rats, ASCs: Adipose tissue-derived stem cells; CNS: Central nervous system; ESMNs: Embryonic stem cell-derived motor neurons; FBs: Fibroblasts; hOECs: Human olfactory ensheathing cells; mL: mililiters; MSCs: Mesenchymal stem cells; NFL: Nerve fiber layer; N/M: Not mentioned; NSCs: Neural stem cells; OB: Olfactory bulb; OECs: Olfactory ensheathing cells; OM: Olfactory mucosa; PNS: Peripheral nervous system; SCs: Schwann cells; SD Rats: Sprague Dawley Rats

week after injury. The surviving cell proportion was reported to be a fairly constant 0.4–0.6% from 1 week to 13 weeks. No use of immunosuppression was reported.

The last of these four studies reported the use of crush injury in male athymic nude rats[29]. This transplant was a xenograft; the OECs used for treatment were isolated from olfactory mucosa of a euthanized canine. Immediately after the injury, lentiviral-transfected GFP-expressing OECs were injected rostrally and caudally to the injury site. Cell survival analysis was carried out 4 weeks after transplantation; the proportion of surviving cells was estimated to be 6.5 ± 2.5%. No additional immunosuppression was used in this study as the experimental animals were innately immune deficient.

Another study quantified the relative distribution of surviving transplanted cells within the injured spinal cord, rather than determining percentages of surviving cells[33]. In this study, a transection model in female SD rats was used. OECs for treatment were prepared from olfactory bulbs of GFP-expressing transgenic SD rats (allograft) with cells microinjected immediately after transection into four midline injection sites per spinal cord stump. For quantification of OEC distribution, the volume occupied by GFP-labeled cells at the injury site was calculated and divided by the total volume occupied by GFP-labeled cells in the entire spinal cord, which was then reported as a percentage. At 1 week post transplantation, 79 ± 4% of the surviving cells were seen at the injury site, at 2 weeks cell survival was 14 ± 7% and at 4 weeks 23 ± 14%. None of the cells were traced at the injury site at 8 weeks' time post transplantation, except for the group of animals that received immunosuppression. The animals were given cyclosporine for prevention of a graft rejection response. While this study did not assess cell survival in a conventional manner, this was only study among the 66 reviewed studies here that assessed cell distribution over time.

One study quantified OEC survival indirectly by determining the pixel intensity of transplanted cells that expressed a fluorescent tag[32]. In this study, a contusion injury model in female Fischer rats was used. OECs were isolated from olfactory bulbs, also from Fischer rats and transplanted in a suspension containing 100,000 cells/μl, 2 weeks after injury. For the semi-quantitative analysis of cell survival, DsRed pixel density was calculated. The authors observed a 74% decline in the pixel density throughout the rostral–caudal axis of the injury site from 2 weeks to 4 months post transplantation.

None of the studies in which OEC survival was calculated assessed or analyzed the inflammatory status of the injury site. Two studies mentioned intermingling of surviving OECs with reactive astrocytes at the injury site[29,32]. Only one of the studies discussed migration of OECs away from site of injection into the cord tissue and disappearance of the transplanted cells from the lesion site at 8 weeks following transplantation[33].

Most of the studies included in this review did not discuss the survival of transplanted OECs. The reason for this may have been that it can be difficult to track the cells after transplantation, unless a xenograft is transplanted which can be detected with antibodies raised against the donor animal[29]. OECs and SCs are largely indistinguishable from each other immunologically and morphologically using immunohistochemistry in tissues (reviewed elsewhere[5]). After spinal cord injury, endogenous SCs migrate into the injury site (reviewed elsewhere[97]). In addition, a recent study has reported that following a spinal cord injury oligodendrocytes give rise to myelinating cells that they have identified as "Schwann-cell like cells"[98]. Therefore, it is not currently possible to distinguish transplanted OECs from SCs using peripheral glial markers, such as the p75 neurotrophin receptor (p75ntr), which are expressed by both cell types. The only marker sometimes used to distinguish between the two cell types is Leu7, also known as CD57 or HNK-1, which has been suggested to be expressed by SCs but not OECs[99,100]. However, non-myelinating SCs do not express Leu7[101]. The SCs that enter the spinal cord do have the capacity to myelinate axons, but many may have a non-myelinating phenotype. Therefore, Leu7 is not an appropriate marker for distinguishing between OECs and SCs.

Most of the studies that did report cell survival relied on fluorescent proteins expressed by cells taken from genetically modified animals or by cells transfected by lentivirus[29,32,45,54,96]. One older study (not included in this review) reported the use of a nuclear probe, which can be used to specifically identify the transplanted cells[96]. The use of transgenic animals as a source of fluorescence tagged cells for transplantation is not without its own set of issues and challenges, such as variable expression of fluorescent proteins among different cells, differential levels of expression depending in the developmental stages of the animals, possibility of non-specific expression of the protein in closely related cell types, inconsistent fluorescence depending on the environmental changes such as temperature and pH, and variable intensities of expressed fluorescent proteins[102]. These issues may pose a serious question about the reliability of the fluorescent protein for identification of a certain cell type altogether. Using a fluorescent protein can lead to erroneous results as fluorescence can also be expressed from cell debris after a cell has died, or after such debris has been phagocytosed by other cells[19,103]. In some cases, the authors chose to use fluorescence intensity to quantify the surviving OECs[27,32]. This can also further result in false positives as the debris and dead cells tend to express high-intensity fluorescence[104,105]. Use of multiple markers such as a nuclear marker along with a fluorescent protein[28] in combination with high-resolution microscopy to demonstrate that fluorescent cells are alive and exhibit the expected morphology can avoid such issues. Quantification of the correctly identified cells presents another challenge. Many study designs do not specify the details of the quantification method. In some cases, a representative sampling of

sectioned tissue was done by collecting every sixth consecutive section[28]. Here an extrapolation is conducted based on the assumption that the distribution of OECs *in situ* is uniform, which may not occur as the transplanted cells may migrate along discrete tracts within the spinal cord. A three-dimensional reconstruction of the tissue around injury site[96] could prove useful in avoiding such issues. A more frequent sampling can be done to reduce the extrapolation needed. Furthermore, if cells are transplanted from a male to female animal, labeling for the Y chromosome may be feasible[96].

Cell Survival Depends on Injury Model

Background. Many transplanted cells die due to inflammation in the injured spinal cord, as the inflammatory process that ensues after an injury creates a hostile environment[33]. Injury disrupts the blood–brain barrier and allows macrophages to enter the injury site, and tissue damage at the injury site activates local microglia. The increased macrophage activity makes survival and integration of grafted cells even more challenging[106]. Astrocytes react to spinal cord injury by actively proliferating and migrating to the lesion to form a scar known as the astroglial (astrocytic) scar. The scar aides the injured cord by securing it structurally, but it also impedes axonal outgrowth and repair mechanisms owing to its dense configuration and hostile microenvironment[107,108]. The intraspinal cell transplantation process itself warrants further manipulation of the scar at the injury site, which may trigger another inflammatory reaction further elevating the hostility of host tissue and thus adversely affecting the survival of transplanted cells. For these reasons, the injury model used strongly influences survival of the transplanted cells and functional outcomes. Transection-type injury is caused by a sharp cutting trauma and results in little peri-lesional secondary injuries. In contrast, contusion-type injuries are caused by blunt compressing trauma to the cord, and results in widespread secondary injuries, ultimately leading to a substantially more pronounced immune response involving macrophages and microglia[109]. In addition to the type of injury, level of injury can also affect the cell survival post transplantation. Similar to the types of injury, the inflammatory responses differ between cervical and thoracic injuries[110].

Recent evidence. All the 66 papers reviewed here have used rats as the experimental injury model and transplant recipient. Compression- or contusion-type injury was the most commonly used injury mode, comprising 27 out of the 63 studies. Transection injury models were used in 21 studies, and hemisection in six out of the 66 studies. Three more studies used partial transection to induce spinal cord injury[61,62,80], and another study employed unilateral ablation of cortico-spinal tract[85]. Two studies used X-irradiation of the spinal cord along with ethidium bromide-induced demyelination[54,86]. One study used rhizotomy as the injury model[50]. Two more studies described the use of dorsal root avulsion[34,42]. Another paper used radiofrequency-induced heat ablation of the cord tissue[45]. One further study investigated the role of OECs for their possible beneficial effects in a genetically induced amyotrophic lateral sclerosis (ALS) model[70]. One paper did not describe the details regarding injury model or the transplantation method[38]. These results are summarized in Table 2, section (i).

The host immune system plays a critical role in the fate of transplanted cells. This is particularly important in spinal cord injury where the innate and adaptive immune systems are activated in response to an injury, creating an environment that is hostile for the transplanted cells. Transection-type injuries are more precise and cause minimal accumulation of cell debris in comparison to contusion and crush injuries. Due to a wider range of tissue damage and ruptured blood vessels, contusion and crush-type injuries lead to a more severe activation of immune system than transection[33,111,112]. The best survival percentage as reported following a crush injury in the reviewed literature was $6.5 \pm 2.5\%$ out of the 80,000 transplanted cells at 4 weeks post transplantation[29]. In contrast, another study using a similar injury model also assessed at 4 weeks post transplantation reported $\sim 3\%$ survival out of one million transplanted cells[28]. Another study using crush injury reported a mere $\sim 1\%$ survival out of 300,000 transplanted cells at 8 weeks post transplantation[30]. The lowest survival rates were reported following a contusion-type injury, which were less than 0.5% at 2 weeks (and declining further at 13 weeks) from the originally transplanted 90,000 cells[31]. The only study that reported on cell survival after a transection-type injury used a semi-quantitative analysis[33]. Instead of quantifying percentages of surviving cells, this study reported on the relative distribution of transplanted cells between the injury site and surrounding tissue. As outlined earlier, at 1 week post injury, approximately 80% of the surviving cells were found directly within the injury site. This percentage was drastically reduced to $\sim 15\%$ after 2 weeks, followed by a small but significant increase again at 4 weeks post transplantation. It is possible that cells either migrated back into the lesion core over time, or that cells exhibited better survival or proliferation at the injury site than within surrounding areas. The reported enhancement in survival of OECs following cyclosporine treatment[33] would suggest that the host immune response is a key factor for survival of OECs after transplantation. The inflammatory environment at the injury site, which changes over time, may correlate with survival or migration of transplanted cells.

Source of OECs can Affect Cell Survival In Vivo

Background. The second important factor is the source of the transplanted cells. OECs can be acquired from the olfactory mucosa (which contains the olfactory epithelium and underlying lamina propria with OECs), or from the olfactory bulb (which contains the NFL with OECs). When cultured *in*

vitro, the different subtypes of OECs can retain their functions; OECs from the peripheral nervous system directionally organize axons in fascicles, whereas OECs from the olfactory bulb induce a disorganized axonal growth, consistent with their *in vivo* roles[113]. This indicates that mucosal OECs may be better for spinal cord injury repairs, as organized axonal extension is favorable. Obtaining cells from the mucosa is also favorable from a clinical point of view, as the mucosa is easy to access and there is no damage to the olfactory bulb in the CNS.

Most studies to date have been conducted on rodent animal models, using either human or rodent OECs for transplantation. The transplanted cells were an allograft (donor and recipient are the same species but may or may not be the same strain) in most of the cases, and a xenograft (donor and recipient are different species) in some cases. Most, but not all, of the transplantations performed in humans to date have used autologous transplanted cells from the olfactory mucosa[114–120]. Despite the risks, olfactory bulb OECs have also been transplanted in humans with spinal cord injury; these cells were autografts[121], or allografts[122,123]. In one of these studies, autologous transplantation of olfactory bulb-derived OECs led to a remarkable functional improvement in a patient with complete spinal cord injury[121]. Using autologous OECs drastically reduces the risks related to graft rejections. Expansion of OECs from biopsies is, however, time-consuming and therefore donor cell transplantation may be warranted for immediate use in acute-phase treatment for spinal cord injury. Thus, it is essential to take into account graft-versus-host responses in understanding why OECs sometimes do not survive.

Recent evidence. The sources of OECs can be discussed from two aspects: the species of origin, and the anatomical location of their origin. The reviewed papers report multiple sources of OECs. In 42 of the review papers, OECs were isolated from the rat olfactory bulb (OB), while 18 studies used OECs from the rat olfactory mucosa (OM). Two of these papers compared olfactory bulb- and mucosa-derived OECs[27,50]. One recent study used a cell line (TEG3 cell line) as the source of OECs[48]. Thus, 59 of the studies used allogenic transplants (58 studies used primary cells; one study used cell line). Two studies using OB-OECs out of the 42 reported cell survivals to be $\sim$2.85% at 4 weeks[28] and $\sim$0.6% at 1 week[31], respectively, as previously detailed. Two more studies using OB-OECs performed semi-quantitative survival analysis, one of which reported cell surviving up to 4 weeks without immunosuppression and 8 weeks with immunosuppression[33], whereas the other study detected fluorescent signal emanating from OECs for up to 4 months after transplant in 64% of the animals[32]. Another 15 studies out of the 42 OB-OEC studies observed cell survival without statistical quantification, with the longest recorded survival time of 8 months after transplant[68]. The information regarding sources of cells is summarized in Table 2, section (ii).

The one study that quantified OM-OEC survival reported the survival between 0.34% and 1.72% at 8 weeks[30]. Another 13 studies out of the 18 using OM-OECs also reported presence of OECs qualitatively for up to 10 weeks after transplantation[67]. Among the six xenograft studies, one paper used mouse OB as OEC source[45], another used canine OECs purified from the OM[29]. Four studies used primate-sourced cells. OECs from the OBs from macaques (Cercopithecinae primates) were used in one study[90], and in two studies, the authors transplanted human OECs purified from the OM into rat injury models[41,72], while another study reported using human OB-derived OECs[34]. In the latter case, cells were taken from 15 patients who had undergone surgeries for anterior skull base fractures due to trauma or cancer, and whose OBs were not salvageable. The remaining one study did not describe the source of OECs[58]. In summary, the transplantations were most commonly allogenic, and the OB was the most common anatomical source of OECs. It is notable that all the studies that transplanted xenografts either used immunosuppressive drugs or used athymic nude rats as recipients[29,90]. Some studies conducting allogenic transplantation also used immunosuppression.

As reviewed here, 59 of the 66 included studies have used allogenic transplants, but six of the studies have reported the use of xenografts along with the use of immunosuppression to prevent or mitigate graft rejection. Xenografts, though not without their benefits, do present with new complications, especially for assessment of cell survival and requirement for immunosuppression. In the animal studies performed to date, the OB was the most common source of OECs (42 out of 66 studies). Only two of the papers compared olfactory bulb- and mucosa-derived OECs[27,50]. One of the two studies used a dorsal root rhizotomy model in rat, and found that bulb-derived OECs demonstrated greater clinical benefit at bridging the injury site to help regenerate severed axons across the gap, than mucosa-derived OECs[50]. However, the other study[27] concluded that mucosa-derived OECs exhibit better survival and integration in the transection injury site than bulb-derived OECs. This finding may be attributed to the different intrinsic functional properties of OECs isolated from distinct anatomical regions[7,113].

In the light of this recent evidence, more studies comparing OB-derived and OM-derived OECs for repair of spinal cord injuries may be required. Studies comparing the two populations side by side, on the same injury model, would provide more robust conclusions to determine if one of the populations is better than the other, or if a mixture of the two is optimal. However, the accumulated evidence suggests that OM-derived OECs appear to be surviving and inducing spinal cord repair. This may be the most important point from a clinical aspect, as mentioned before, since OM-derived cells can be harvested from the patients for an autologous transplant or can be harvested from donors without risking intrusive brain surgeries which are needed to harvest the OB-derived OECs.

Co-transplantation with other Cell Types may be Beneficial

Background. In their native environment, mucosal OECs are in close proximity with a number of other cell types such as primary olfactory neurons, olfactory fibroblasts, and SCs, whereas bulbar OECs are mostly close to astrocytes and OB neurons. Intercellular dynamics between OECs and other cell types may, therefore, have a large impact on their survival, proliferation, and neuro-reparative function post transplantation, as shown in several past studies[124–128]. Older studies have also shown that co-transplantation with SCs may have a beneficial effect on OEC survival[96]. Fibroblasts, the commonly found contaminating cells with OM-OECs, can also have some effect on cell survival *in vivo* since their survival time differs from that of OECs[33]. Hence, different culture conditions prior to transplantation may result in unintentional co-transplantation of cells affecting their survival. However, due to high variance in the reported culture protocols[26], it is not practical to comment on their impact on cell survival in this review.

Recent evidence. Many of the studies used other cell types either for co-transplantation with OECs or as a comparison to OECs. For co-transplantation studies, SCs (peripheral nerve glia) and neural stem cells (NSCs) were the most commonly used cells, followed by MSCs. Three studies used SCs for co-transplantation with OECs[37,43,57], two of which also used SCs as a separate treatment group[37,43], whereas three more studies used SCs separately, for a comparison between the two cell types[32,52,59]. Only one of the three studies co-transplanting OECs and SCs discussed cell survival[37], mentioning that the cells were observed for up to 6 weeks following transplantation in the injury site. The same study also concluded that this co-transplantation showed some synergistic effects on cell migration and reduced reactive astrogliosis. Two studies co-transplanted MSCs with OECs[30,89] and three studies transplanted the two cell types separately and compared the outcomes[49,51,60]. One of these studies observed 0.34–1.72% cell survival (discussed in detail above) which did not improve upon co-transplantation with MSCs[30]. The other study reported the transplanted cells surviving up to and beyond 2 weeks (no quantification was reported), and proposed a synergistic benefit of co-transplanting OECs with MSCs[89]. Three studies reported co-transplantation of OECs together with NSCs[47,58,80], one study used ESMNs[28], and one adipose tissue-derived stromal/stem cells[44] (ASCs). Only one out of the three studies that co-transplanted NSCs with OECs commented on cell survival, and concluded that co-transplantation could improve cell survival for NSCs[58]. The study the co-transplanted ESMNs and OECs performed a detailed quantification of cell survival and is discussed above[28]. This study also concluded that co-transplantation with OECs improved cell survival for ESMNs significantly. None of the other studies listed above discussed effects of co-transplantation on cell survival. Seven studies used fibroblasts (FBs) as a separate comparison treatment group, five of which used isolated and cultured FBs[33,36,43,46,52], while the other two studies used pieces of respiratory lamina propria to deliver the FBs[55,69]. Fibroblasts have been often included as a comparison group since they are one of the commonly found contaminating cells in OM-OEC cultures. In summary, six studies used peripheral glia (SCs), seven studies used connective tissue supporting cells (FBs), and 10 studies reported using various stem cells (MSCs five studies, NSCs three studies, ESMNs one study, and ASCs one study). These details are summarized in Table 2, section (iii).

Overall, the reviewed literature does not conclusively suggest that the co-transplanted cell types have any significant influence over OEC survival *in vivo.* Conversely, however, the studies using stem cells such as MSCs[30,89], NSCs[47,58,80], and ESMNs[28] reported some benefit from co-transplantation with OECs in comparison to being transplanted alone. However, most of these studies focused more on the effects of OECs on the survival of the co-transplanted stem cells rather than the other way around, given the natural function of OECs as the supportive cells in the olfactory system. Some of the studies concurred that co-transplantation of these stem cells along with OECs resulted in a synergistic effect leading to better stem cell survival and improved functional outcomes with less undesirable side effects such as hyperalgesia[28,58,80,89]. However, one of the studies did not find any added advantages of co-transplanting OECs and MSCs[30]. Due to the low number of studies reporting on cell survival, a conclusion regarding the potential benefit of co-transplantation cannot currently be drawn.

Cell number and Concentration Affect OEC Survival

Background. As OECs are dependent on cell–cell contact for survival and neural regeneration[113,129,130], cell density becomes another important factor for cell survival following transplantation in an injured cord. *In vitro* studies of OEC behavior suggest that these cells also need intercellular contact to survive[130], migrate[113,130], and promote axonal extension[129]. Widespread cell–cell contact can only be established if there are enough cells in the transplant, which is therefore a key factor regulating OEC survival and integration into the host tissue. Cell concentration can have an effect on dispersion and survival of the transplanted cells. Under such conditions, cells are likely to be damaged physically, drastically reducing cell survival[131].

Recent evidence. The number of transplanted cells ranged from 15,000 cells[53] in total to as high as 10 million cells[80]. Most of the studies (34 out of 66), however, reported the total number of transplanted cells in the range of approximately 100,000–500,000 cells. Nine studies in total reported less than 100,000 cells for treatments, whereas 11 studies

reported having used more than one million cells for treatment. In addition to the total number of cells transplanted, the concentration of cells in suspension is also a very important factor to consider. The reported concentration ranged between 1,000 cells/μl[47] to 3,000,000–6,000,000 cells/μl[71]. Most studies (30 studies), however, used a cell concentration of 50,000–100,000 cells/μl. Several studies used pieces of lamina propria from OM, or a matrix embedded with cells, and were hence unable to report either the total number or the concentration of cells[34,42,50,55,69,76,92]. Another study only reported the transplanted volume to be 30 μl but did not mention the concentration or the total number of transplanted cells[84]. Three studies only reported the total number of cells, without reporting the concentration and the transplanted volume[56,77,83]. One study reported transplanted cell number per injection[87]. A summary of these parameters can be found in Table 2, section (iv).

OECs are dependent on cell–cell contact for survival and many biological functions relating to neural regeneration[113,129,130]. Thus, it is highly likely that when transplanted *in vivo*, the number of cells and the cell concentration in the injected suspension are important factors for OEC survival and integration into the host tissue. Too low density would cause the cells to disperse throughout the tissue, resulting in cell loss from the injury site and lack of cell–cell interaction. On the contrary, very high density can result in increased shear stress and physical damage to the cells during injection procedure, as shown for other cell types[131,132]. Studies using higher cell suspension densities have reported very low survival rates. In the study with the highest reported survival rate to date (6.5 $\pm$ 2.5% at 4 weeks), a cell density of 40,000 cells/μl was used. However, in another study a lower cell concentration of 30,000 cells/μl resulted in a much lower survival rate of 0.473 $\pm$ 0.138% at 2 weeks[31]. At higher densities, survival appears to be lower; in one study, a cell density of 200,000 cells/μl resulted in 1.03 $\pm$ 0.35% cell survival after 8 weeks, and another report showed that a density of 100,000 cells/μl resulted in 0.6 $\pm$ 0.148%, 0.473 $\pm$ 0.138%, and 0.357 $\pm$ 0.122% at 1 week, 2 weeks, and 13 weeks. All these three studies have used crush injury as their injury model.

Method of Transplantation: A Three-dimensional Construct may be Warranted

Background. Another important factor is the transplantation method. The majority of the studies published so far have transplanted OECs in suspension via injections. Due to the very fragile nature of the cord tissue and constant flow of the cerebrospinal fluid, cells injected in a suspension are likely to be washed away or migrate to the wrong location and therefore have less chance to integrate into the host tissue[30]. However, cells transplanted with a scaffold or nerve bridge formation may have a better chance of remaining at the transplantation site and integrating within the injured cord tissue[121,133].

Recent evidence. Out of the 66 studies included in this review, 51 used injections of cell suspensions for transplantation. Another 13 studies used some form of a three-dimensional construct or scaffold to transplant the cells, such as mucosal pieces[55,69,76,92], matrix[42,50] (unspecified, endogenous in origin), gelatin sponge[58,78,79], muscle basement lamina[47], collagen scaffold[34,56], or hydrogel[44] (unspecified). One more study used lamina propria as well as cell suspension injections for comparison[71], and another study reported the use of a spherical cell aggregate in comparison with suspension injections[62]. The reported volumes for transplantation ranged from 1 μl[54,58,86,134] to 8 μl[37,49,62,73], except two studies, in which the volume injected was 30 μl[84] and 7–20 μl[67], respectively. Most of these studies used injections of cell suspensions at multiple sites and/or depths, except for three studies where the cells were injected via a single injection[32,53,86]. All the different methods used are summarized in Table 2, section (v).

Most of the studies included in this review used cells injected into the injury site in a suspension. Since the spinal cord is a highly fragile structure lacking in mechanical strength, cells injected in a suspension form are likely to "leak away" from the cord tissue, or to migrate to a location distant from the injury site. A single injection containing the cells causes minimal manipulation of the injury site, limiting collateral damage; however, injections given at multiple sites provide a controlled method for disseminating the treatment to a wider injury site and allow for a larger volume to be injected. Either way, cells injected in a suspension form depend entirely upon the OECs' ability to migrate toward the injury site to arrive at the lesion, integrate, and survive. To date, cell survival has not been compared between single and multiple OEC injections, and thus the benefit of multiple injections remains unknown. Another important difference is that the single injections are usually made in the lesion core and multiple injections are usually made at and around the lesion site. Cells injected directly in the injury core face the hostile environment and may migrate away or die (as implied in Khankan et al.[33]), whereas cells injected around the injury site need to migrate toward the injury site, which may cause delay in the reparative changes and reduce the number of surviving cells that manage to ultimately reach the injury site. A three-dimensional structure or a scaffold such as a nerve bridge may resolve these issues, and use of a matrix that can hold the cells in place would likely result in an increased survival rate[34,42,50]. Yet, the use of matrices also has obstacles that need to be overcome, in particular the ability of cells to receive sufficient growth-supporting factors deep within the matrix/scaffold. Perhaps creating a system in which cells can form stable cell–cell contacts prior to transplantation will aid cell survival. As an example, we have recently developed a novel a self-assembling three-dimensional cell construct termed a naked liquid marble, in which cells rapidly form stable cell–cell contacts and the cells aggregate into stable three-dimensional structures[135]. Thus, the naked liquid marble culture of OECs, or

other three-dimensional constructs, may improve cell survival and functional outcomes after transplantation. Future experimental works are needed to confirm this hypothesis.

Time Between Injury and Transplantation is Important for OEC Survival

Background. The final factor to consider is the time post injury at which OECs are transplanted. After a spinal cord injury, the inflammatory environment within the injury site varies significantly depending on the time elapsed; several phases of immune and inflammatory responses have been thoroughly described in the literature[106,112,136]. The direct mechanical spinal cord injury is rapidly followed by secondary injury (within 30 min of injury in humans), caused by free radicals, glutamate excitotoxicity, and inflammation. The secondary injury, which consists of several phases each characterized by distinct hemodynamic and inflammatory characteristics, leads to pronounced death of neural cells and often causes the injury to extend to higher spinal segments[107]. The inflammatory response subsides over time (the exact time-frame varies between species[137]) and chronic inflammation gradually takes over. The acute inflammatory response is mediated by innate immune cells (invading macrophages, neutrophils, and resident microglia[137,138]). These cells specialize in phagocytosis and removal of debris, and secrete a range of pro-inflammatory cytokines. Acute inflammation in spinal cord injury is directly correlated with apoptotic cell death and impairment of regeneration (reviewed elsewhere[139]). The chronic inflammatory response is primarily mediated by adaptive immune cells (B and T lymphocytes) and induces further degeneration and death of neural cells[137,138], but also has roles in repair[140]. Chronic inflammation in spinal cord injury is also accompanied by immune and endocrine dysfunction (reviewed elsewhere[39]). The constant turnover of the inflammatory cells creates a dynamic internal milieu at the injury site, which has a profound influence over survival of the transplanted cells. OECs, however, are thought to be immunomodulatory and may reduce harmful inflammation[141].

Recent evidence. The timepoint post injury at which transplantation is performed also constitutes a significant factor for variability in outcome. The inflammatory status, and therefore also the immune response that the grafted cells are likely be exposed to, varies greatly with time post injury, ultimately affecting cell survival. Of all the reviewed studies, 39 studies reported to have transplanted cells immediately following spinal cord injury (within 30 min) and one study reported a lag period of 12 h[73]. The treatment period in these 40 studies is considered as acute phase in this review. A further 13 studies reported a waiting period of 1 week, 6 studies reported 2 weeks' delay before treatment, 3 studies reported 4 weeks,[35,45,85] and 2 more studies reported 8 weeks[45,85] of waiting period before treatment. Out of these, two studies compared treatments at the same day as injury (zero days) and 1 week[49,51]. One of the studies[51] focused on gene expression analysis of the injury site, and observed a rapid rejection of transplanted cells over the first few days after transplantation. The other study[49], however, observed some cell survival (not quantified) at 1 week after the transplantation which reduced markedly after the second week. At the same time, the reduction in cell survival during the second week after transplantation was observed to be greater in the acute-phase treatment animals. Two more studies compared treatments across zero days, 2 weeks and 4 weeks[55,69]. Both these studies did not assess cell survival but rather focused on functional outcome in terms of behavioral recovery, and structural repairs. Other studies reported variable waiting periods between injury and treatments such as 1–2 days[54], 9 days[28], 15 days[65], 3 weeks[41], 1 month and 4 months[81], and 6 weeks[71]. One of these studies[28] quantified cell survival in depth, which is discussed previously. Two of these studies observed cells surviving at the injury site[41,71]. The remaining three did not comment on the cell survival. The study investigating OEC transplantation in genetically induced ALS treated the rats at 100 days of age to allow the lesions characteristic of ALS to develop prior to transplantation[70]. The studies suggest that overall cell survival and desirable effects of transplants are best if the transplantation is done sooner rather than later following an injury, and cell survival drops drastically in the initial days after treatment. However, the sub-acute transplantation may provide some protection against the rapid cell death following transplantation. The information regarding treatment timings after injury is provided in Table 2, section (vi).

The literature describes several phases of immune and inflammatory responses in a spinal cord following a spinal cord injury[106,112], with an acute inflammatory response following the injury, which is replaced by a chronic inflammatory reaction over time. Cells transplanted in the acute phase of spinal cord injury must survive through the acute inflammatory response, but survival can also be limited in the chronic phase. Lymphocytes, the primary mediators of chronic inflammation, are also the cells responsible for graft rejection. Perhaps, cells transplanted in the sub-acute injury phase (4–14 days in mice[142]) have a better chance of long-term survival than cells transplanted in the acute[49,51] or chronic phase[69]. Despite this, many studies focus entirely on acute-phase transplantation (36 out of 63 studies, four more studies focus on a range of treatment phases including the acute phase), which may be due to practical reasons. Acute-phase treatments can be performed during the same surgical procedure as the injury, whereas delayed treatments require a second set of invasive surgery. However, the need to generate clinically relevant therapies means that animal studies should attempt to reflect the likely human scenario. In humans, the earliest time that cells could be transplanted into the injured spinal cord would be at a minimum several hours after injury, since the patient would need transport to the hospital and stabilization of the injury site. This is assuming, of course, that a bank of donor cells was available and

could be prepared within hours of notification. However, in most hospital settings it is more likely that cell transplantation would occur some days after injury. If using autologous cell transplantation, then sufficient cells number could not be generated for several weeks after injury. While studies examining efficacy of OEC transplantation immediately after injury may provide critical information about the biology of the injury system, perhaps delayed transplantation may provide more clinically relevant outcomes.

Conclusion

To date, a limited number of studies of OEC transplantation into the CNS have assessed or quantified cell survival. Survival of the transplanted cells is crucial for successful outcomes following OEC transplantation into the injured spinal cord. It is therefore essential to define the factors that are most critical for survival of the transplanted cells. Many of the reports on OEC transplantation into the injured CNS do not report on cell survival, or do not quantify surviving cells. The reason for this is that it can be very difficult to track the transplanted cells over time. Tracking and quantification methods must be improved; for example, it is essential to determine differences in protein expression between OECs and SCs and to use new innovative methods for labeling transplanted cells such as nuclear probes, in combination with advanced microscopy. Review of the literature on all studies of OEC transplantation into the injured rodent spinal cord over the last 10 years revealed the following factors to likely influence OEC survival after transplantation: (1) injury type, (2) OEC source, (3) co-transplantation with other cell types, (4) number/concentration of transplanted cells, (5) transplantation method, and (6) time between injury and cell transplantation. To determine the influence of each of these factors on the ability of transplanted cells to survive over time, robust and reliable quantification of cell survival is necessary in the future. Out of these, injury type, time between injury and transplantation, and OEC source (in particular allografts versus xenograft) relate to immune responses and inflammation. OEC source and cell number/concentration are likely to be crucial for cell–cell interactions, which promote survival.

Overall, the likely survival of transplanted OECs into various models of spinal cord injury is low, and new approaches to improve cell survival are needed. If cell survival and integration can be enhanced, then improved functional outcomes could be achieved. Therefore, new methodologies in which cells are transplanted in three-dimensional constructs which protect the transplanted cells and/or provide stable cell–cell contracts are likely to enhance the therapeutic potential of OEC transplantation.

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