



Considering the Cellular Composition of Olfactory Ensheathing Cell Transplants for Spinal Cord Injury Repair: A Review of the Literature

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Olfactory ensheathing cells (OECs) are specialized glia cells of the olfactory system that support the continual regeneration of olfactory neurons throughout adulthood. Owing to their pro-regenerative properties, OECs have been transplanted in animal models of spinal cord injuries (SCI) and trialed in clinical studies on SCI patients. Although these studies have provided convincing evidence to support the continued development of OEC transplantation as a treatment option for the repair of SCI, discrepancies in the reported outcome has shown that OEC transplantation requires further improvement. Much of the variability in the reparative potential of OEC transplants is due to the variations in the cell composition of transplants between studies. As a result, the optimal cell preparation is currently a subject of debate. Here we review, the characterization as well as the effect of the cell composition of olfactory cell transplantation on therapeutic outcome in SCI. Firstly, we summarize and review the cell composition of olfactory cell preparations across the different species studied prior to transplantation. Since the purity of cells in olfactory transplants might affect the study outcome we also examine the effect of the proportions of OECs and the different cell types identified in the transplant on neuroregeneration. Finally, we consider the effect of the yield of cells on neuroregeneration by assessing the cell dose of transplants on therapeutic outcome.

Keywords: cell transplantation, glial cells, olfactory ensheathing cells, cellular composition, neuroregeneration, spinal cord injury

INTRODUCTION

Neurogenesis in the mammalian olfactory system has been well established through early *in vitro* and *in vivo* studies in rodents and other species (Graziadei and Graziadei, 1979a; Graziadei and Okano, 1979c; Graziadei et al., 1980; Schwob et al., 1995; Kornack and Rakic, 2001; Hahn et al., 2005). Neurogenesis occurs in the olfactory epithelia (OE) where short lived olfactory sensory neurons (OSNs) are replaced in order to preserve olfactory function throughout life, though despite ongoing neurogenesis, neuronal regeneration has been shown to decline with advancing age (Kondo et al., 2010; Holbrook et al., 2011; Mobley et al., 2014). The continual regeneration of

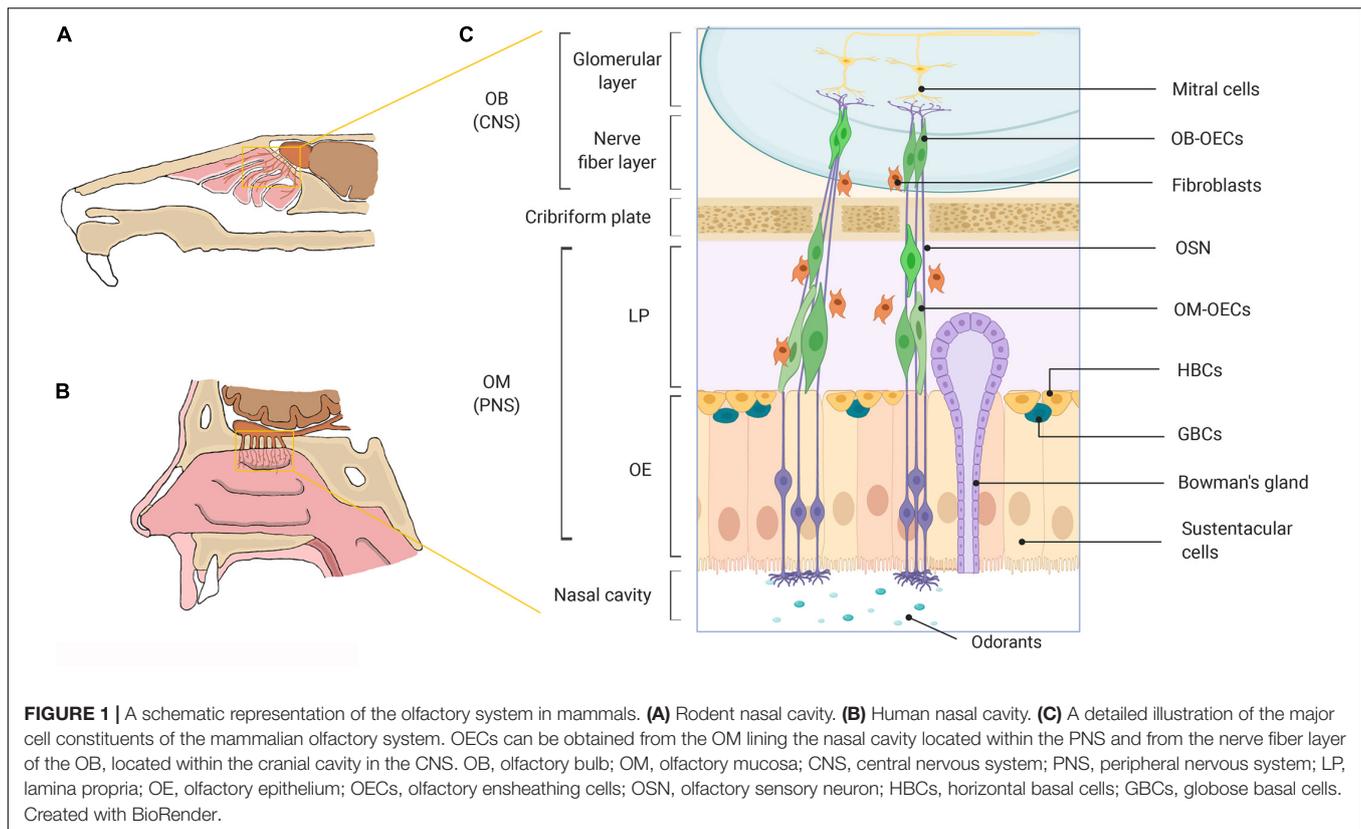
OSNs either during normal cell turnover or after injury to the olfactory nerve is maintained by multipotent and/or neuronal precursor cells (globose basal cells, GBCs and horizontal basal cells, HBCs) in the basal layer of the OE (Graziadei and Graziadei, 1979b; Mackay-Sim and Kittel, 1991; Leung et al., 2007; Holbrook et al., 2011; Chen C. R. et al., 2014; Schwob et al., 2017; **Figure 1**). Regeneration of OSNs is thought to be highly dependent on specialized glial cells called olfactory ensheathing cells (OECs) which, exhibit unique neurotrophic properties. OECs play a vital role in supporting and guiding newly formed olfactory sensory axons from their origin in the peripheral nervous system (PNS) environment of the olfactory mucosa (OM) to establish synaptic connections with their targets in the normally non-permissive central nervous system (CNS) environment of the olfactory bulb (OB) to facilitate odor detection (Raisman and Li, 2007; Roet and Verhaagen, 2014). In the olfactory nerve OECs provide structural support by ensheathing large bundles of non-myelinated olfactory sensory axons to form a conduit for newly generated axons to grow through as they extend all the way from the lamina propria (LP) toward the nerve fiber layer of the OB (Doucette, 1990, 1991; Tennent and Chuah, 1996; Field et al., 2003; Raisman and Li, 2007; Choi et al., 2008a). Olfactory ensheathing cells further support neural regeneration by secreting a range of neurotrophic and guidance factors (Woodhall et al., 2001; Lipson et al., 2003; Chung et al., 2004), phagocytosing axonal debris and/or invading pathogens (Leung et al., 2008; Panni et al., 2013; Su et al., 2013; Nazareth et al., 2015) and integrating and interacting with other cell types such as CNS glia and astrocytes (Chuah et al., 2011). Owing to the strong pro-regenerative properties of OECs, as well as their unique migratory properties (Tennent and Chuah, 1996; Ekberg et al., 2012), OECs have been investigated for transplant-mediated repair of CNS injury with promising but highly variable outcomes.

Transplantation of OECs in experimental animal models of spinal cord injuries (SCI) has been extensively studied and together with studies in humans have provided convincing evidence to support the continued development of OEC transplantation as a treatment option (Feron et al., 2005; Mackay-Sim et al., 2008; Granger et al., 2012; Tabakow et al., 2013, 2014; Watzlawick et al., 2016; Nakhjavan-Shahraki et al., 2018). Transplantation of OECs in animal studies has been shown to have a wide range of therapeutic effects by promoting axonal regeneration, restoring function, reducing cavity and scar formation, remyelinating axons (a function they do not serve in the nasal cavity), modulating neuroinflammation and promoting neuronal survival and plasticity (Li et al., 1997, 2003; Ramon-Cueto et al., 1998, 2000; Lu et al., 2002; Plant et al., 2003; Ramer et al., 2004; Lopez-Vales et al., 2006; Lankford et al., 2008). A systematic meta-analysis of 62 experimental studies in rodent models of SCI found OEC transplants improved functional recovery with an effect size of 19.2%, after correcting for publication bias and missing data (Watzlawick et al., 2016). The efficacy of OEC transplants was further corroborated in a recent systematic meta-analysis of 933 animals (control group = 464 and treatment group = 469), which confirmed that OEC transplantation significantly improves motor function recovery in animals after SCI (Nakhjavan-Shahraki et al., 2018).

However, despite the successes reported in published studies of regeneration within the CNS, a number of studies have been unable to confirm these positive results (Takami et al., 2002; Deumens et al., 2006; Lu et al., 2006; Steward et al., 2006). Conflicting evidence in reported outcomes has led to doubts about the efficacy of OECs for CNS repair.

The variation in the reparative potential of OEC transplants is not fully understood and may be due to several factors including differences in SCI model, cell delivery techniques, adequate assessment of functional outcome measures and perhaps most importantly the considerable differences in the cell purity and yield of cell transplants between studies. OEC cultures generated *in vitro* from the olfactory region are derived from either the OM or OB and typically contain a heterogeneous mix of cell types including olfactory nerve fibroblasts (ONFs) and other accessory cells, along with OECs (Lindsay et al., 2010; Chen L. et al., 2014; Yao et al., 2018). The yield of OECs, often measured as percentage proportion differs between the two olfactory regions, with higher yields being generated *in vitro* from OB biopsies than OM biopsies (Jani and Raisman, 2004; Kueh et al., 2011; Mayeur et al., 2013; Ibrahim et al., 2014). However, for clinical application, autologous OM transplants are the preferred source as mucosal biopsies are easily accessible via less invasive intranasal endoscopy, avoiding the requirement for intrusive intracranial surgery to harvest biopsies from the OB (Choi et al., 2008b; Andrews et al., 2016; Holbrook et al., 2016). In addition to the anatomical source, the proportions and types of cells present in olfactory transplants is greatly influenced by the *in vitro* methods/conditions used to isolate and culture OEC preparations (Kawaja et al., 2009; Ekberg and St John, 2014; Reshamwala et al., 2020). As there is a lack of consensus with regards to the optimal cell culture protocol for OECs, it is likely that variations in cell preparations lead to variability in treatment outcomes.

The optimal cell preparation is still a subject of debate. OEC purification methods from the bulb and mucosa have been developed to separate OECs from the many potentially “contaminating” cells (Kawaja et al., 2009). Although, a number of groups have reported beneficial effects by transplanting purified OECs (Ramon-Cueto and Nieto-Sampedro, 1994; Toft et al., 2013), several studies have demonstrated unpurified, mixed cell preparations of OECs and other cells from the olfactory niche might be important for neural repair. Raisman et al. have argued that ONFs are a crucial component of OEC transplants in facilitating the formation of organized OEC bridges through which axons can regenerate across injured tissue. Unpurified primary OB cultures containing 50:50 ratio of OECs and ONFs were suggested as the optimal proportion of cell types needed for neural repair (Li et al., 1998, 2003; Keyvan-Fouladi et al., 2003). The reported benefits of additional cells in olfactory cell transplants based on rodent studies has led to the transplantation of unpurified mixed cell cultures in human studies, with varying degrees of neurological and neurophysiological improvement being observed (Tabakow et al., 2013, 2014). While, studies in humans have provided vital data about the safety and feasibility of olfactory cell transplantation (Yao et al., 2018) clinical application of OEC transplants for human transplantation, will



require an understanding of what constitutes the human cell cultures and the importance of additional cell types in the cultures for SCI repair.

Cell based therapies have been a major focus in the development of new treatments for SCI. While numerous cell types have been investigated, OEC transplantation remains among the most widely studied and has emerged as a promising treatment for SCI repair (Tetzlaff et al., 2011; Assinck et al., 2017; Willison et al., 2020). However, given the variability in outcomes, the therapy still requires improvement. To help guide the development of OEC transplantation for SCI repair, the objective of the present review was to summarize and review the current literature pertaining to the cellular characteristics and composition of OEC cell preparations for transplantation in the treatment of SCI across the different species studied. Specifically, with a focus on the effect of three main parameters on neuroregeneration and repair in traumatic SCI: proportion of OECs in cell preparations, proportion of additional cells in the transplant (namely, fibroblasts) and total cell number transplanted. These are important factors that must be optimized in the clinical translation of OEC transplants for human SCI.

METHODS

Search Strategy

A literature search was conducted using PubMed database for original studies related to the therapeutic application of olfactory

bulb and mucosa cell preparations in traumatic models of SCI that were published from January 1990 up until 11th April 2021. The search terms are reported in **Table 1**. The titles and abstracts, and when not explicit full texts were screened to select studies in SCI models. The methods and results sections of these publications were read to identify studies that met the pre-defined inclusion criteria. Only studies that measured the proportions of OECs and/or mentioned the presence of additional cell types in bulb and mucosal cultures in SCI repair were included. Studies that included the application of unpurified (primary) and purified OEC cultures in combined treatments (e.g., with cells, scaffolds, and drugs, etc.) to measure synergistic effects were also included, as long as the proportions of cells in olfactory tissue culture were reported. All the studies included in the analysis of this review are summarized in **Supplementary Table S1**. Studies that mentioned the presence of OECs and its cell constituents but did not measure the proportions of these cells were excluded along with studies that transplanted complete non-dissociated pieces of olfactory tissue. Studies that evaluated the therapeutic application of bulb and mucosal OECs and other cell types exclusively in *in vitro* models and non-traumatic models of SCI were excluded. Reviews and case reports were also excluded.

Data Extraction

The data from those studies that fitted the criteria were extracted into a table format to include the following information: (1) reference details (PubMed ID), (2) OEC culture source (donor species and olfactory tissue source), (3) recipient species, (4)

TABLE 1 | PubMed search.

("olfactory mucosa"[MeSH Terms] OR ("olfactory"[All Fields] AND "mucosa"[All Fields]) OR "olfactory mucosa"[All Fields]) OR ("olfactory bulb"[MeSH Terms] OR ("olfactory"[All Fields] AND "bulb"[All Fields]) OR "olfactory bulb"[All Fields])	AND	("olfactory ensheathing cells"[All Fields] OR "OEC"[All Fields] OR ("olfactory"[All Fields] AND "ensheathing"[All Fields] AND "cells"[All Fields]) OR "olfactory ensheathing glia"[All Fields] OR "OEG"[All Fields] OR ("olfactory"[All Fields] AND "ensheathing"[All Fields] AND "glia"[All Fields]))	AND	("1990/01/01"[PDAT]: "3000/12/31"[PDAT])
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injury model, (5) the total number of cells transplanted, (6) characterization antibodies used to identify the cells in culture, (7) cell purification methods (if applied), (8) purity of the final cell preparation, i.e., proportions of OECs in percentage and where mentioned additional cells, (9) method to determine purity, (10) the reported structural/functional outcome measures such as, locomotor recovery, axonal regeneration, remyelination, tissue sparing, and sensory preservation etc. The studies were identified either as positive or negative studies based on the resulting outcome measure following the application of OM and OB cell culture transplants. Positive studies were identified on the basis of any reported improvements in any measure of functional or histological outcome and negative studies were identified by the lack of therapeutic effect after transplantation. Where a single publication included more than one experiment in relation to, i.e., OEC source, injury and purification methods, the data was extracted and treated as an independent experiment. For the analysis of cellular composition, where a percentage range of values for OEC and other cell types was given, the midpoint of the range was selected for analysis. Where the composition was marked as >X%, the X% value was selected for analysis; where the compositions were given as separate percentage values within the same experiment, these were treated as separate experiments.

RESULTS AND DISCUSSION

Study Details

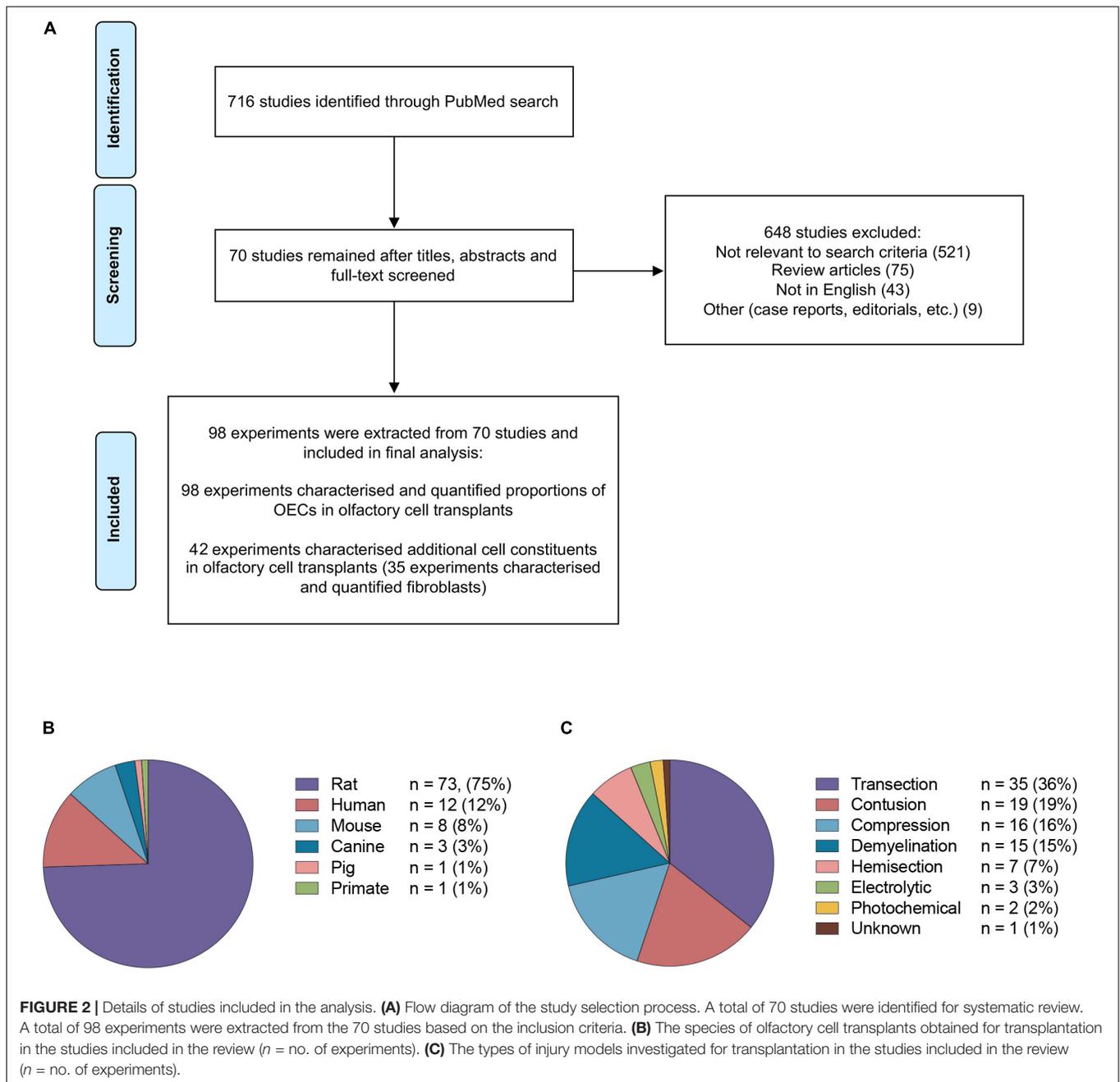
The study selection process is summarized in **Figure 2A**. The PubMed search strategy yielded a total of 716 studies using the search terms outlined. Of the studies that were identified, 648 were excluded based on either title, abstract or after full text screening. Ultimately, 70 publications matched the search criteria and were included for the final analysis. When the same study compared different experimental groups in relation to OEC transplant source, OEC proportions, injury and purification methods these were treated as separate experiments. Consequently, a total of 98 experiments were extracted from 70 publications.

The majority of the data collected from these studies related to rodent olfactory derived cultures ($n = 82$, 83.7%). In comparison, the composition of cells in human OEC cultures ($n = 12$, 12.2%) and large animal model OEC cultures ($n = 5$, 5.1%) were reported less often (**Figure 2B**). Seventy-eight (79.6%) experiments used allogeneic transplants, 13 used xenogeneic (13.3%) and the remaining studies applied autologous transplantation ($n = 7$, 7.1%). The type of spinal cord injury in published studies varied considerably (**Figure 2C**). The most common injury models in

the studies were transection in 35 (35.7%) experiments, contusion in 19 (19.4%), compression in 16 (16.3%), demyelination in 15 (15.3%), hemisection in 7 (7.1%), electrolytic in 3 (3.0%) and photochemical in 2 (2.0%) experiments, and in one study it was unclear which SCI model was applied (Zheng et al., 2017). It should be noted that in addition to the heterogeneity in injuries amongst the studies, there was also considerable heterogeneity with regards to the time of application of cells post injury, injection volumes, concentration of cells, number and localization of injections. The impact of these factors in OEC transplantation will not be discussed here as it has been reviewed elsewhere (Watzlawick et al., 2016; Nakhjavan-Shahraki et al., 2018). All but one study (Tabakow et al., 2014) in the review included a control or a comparison group.

Composition of Olfactory Cell Transplants

To understand the influence of the types of cells present in the olfactory transplant in addition to OECs, we reviewed experiments reporting the characterization of the cell composition prior to transplantation. The characterization and quantification of cells in olfactory transplants was mostly limited to OECs ($n = 98$, 100%). Additional cells in the transplant were described less often ($n = 42$, 42.9%), with fibroblasts/meningeal cells ($n = 35$, 35.7%) being characterized most often. Despite the complexity of the cell mixture, overall we found that OEC transplantation studies did not focus on characterizing the additional cell types in the cell transplant, ($n = 19$, 19.4%), summarized in **Supplementary Table S2**. The characterization and quantification of additional cell constituents in transplants was mostly limited to OB transplants with the majority of studies describing rat derived cell preparations ($n = 15$), and included two studies describing canine and two studies describing human OEC cell transplants. The additional cell types identified in these transplants included astrocytes ($n = 6$), endothelial cells ($n = 3$), macrophages/microglia ($n = 2$), oligodendrocyte progenitors ($n = 2$), oligodendrocyte precursors ($n = 1$), Schwann cells ($n = 3$) and stem cells/neuronal cells/connective tissue ($n = 1$). It is generally accepted that OM-OEC transplants will contain several cell types compared to OB-OEC transplants (Lindsay et al., 2010; Holbrook et al., 2011, 2016; Chen C. R. et al., 2014; Borgmann-Winter et al., 2015), but despite this only 3 out of 19 studies discussed the presence of accompanying cells in OM transplants, of which the characterization was very limited. Granger et al. (2012) found that cells in addition to OECs and fibroblasts made up ~2% of the transplant in canine unpurified OM cell transplants however, they did not immun characterize



these cells. Others (Kalincik et al., 2010b; Stamegna et al., 2011) described using HNK-1 marker to label myelinating purified rat Schwann cell transplants. Both studies reported rat OM-OEC cell transplants were negative for HNK-1 but speculated the possibility of non-myelinating Schwann cell contamination, which lacks or downregulates the HNK-1 epitope *in vitro*.

None of the studies included in the review discussed the influence of additional cells in SCI repair, other than fibroblasts (discussed in section “Proportions of Fibroblasts in the Mixed Olfactory Cell Transplants”) and coupled with a lack of sufficient number of studies characterizing and quantifying the proportions of different cell types within the OEC cell transplant

it was not possible to assess the impact of additional cells in olfactory cell transplants. It is possible that the different cellular components of olfactory transplants may not have been reported as the majority of studies transplanted highly purified preparations containing $> 70\%$ OECs, $n = 67$ (68.4%), of which 48 (71.6%) of experiments contained $>90\%$ OECs. However, some studies found that even in highly purified transplants additional cells remained, albeit in small amounts. To date, a number of methods have been developed to identify and purify heterogeneous cultures to obtain highly purified OEC cultures. Such methods include but are not limited to differential adhesion, immunopanning, FACS and selective media [reviewed elsewhere

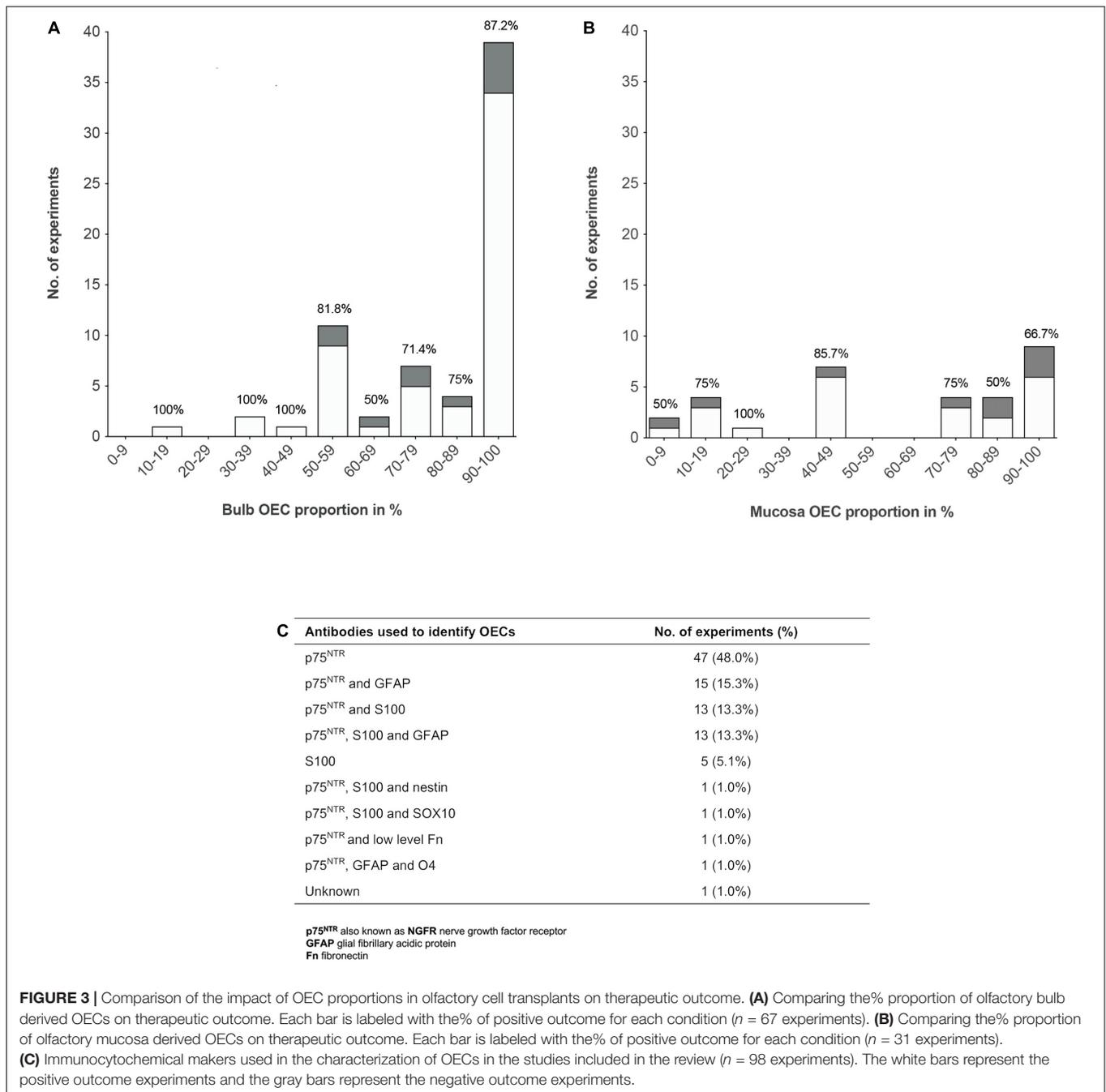
(Kawaja et al., 2009)]. Due to the paucity of data it is not known how purification methods might affect the therapeutic efficacy of OECs and the cell constituents of OEC cultures. One study included in the analysis of the review investigated the influence of the cell culture preparation of purified OECs prior to transplantation and found that the method of cell purification and the age of cells in culture have influence on the growth-promoting and neuroprotective effects of OECs after transplantation into the injured spinal cord (Novikova et al., 2011). There are almost as many descriptions of OEC preparations as there are laboratories exploring their biology. The purification method and length of culture are likely to be of importance and further research is required to identify the methods for preparing cells in culture prior to their implantation that yield the best outcome for SCI repair. The lack of phenotypic and functional characterization of the subpopulations of cells of OEC cultures across the studies reviewed are also likely due to a general lack of specific *in vitro* markers making the identification and quantification of the additional cell types more challenging (Yao et al., 2018). The most commonly used marker to identify OECs, p75 neurotrophin receptor is also expressed *in vitro* by Schwann cells (Chen C. R. et al., 2014), astrocytes (Coutts et al., 2013) and lamina propria mesenchymal stem cells (MSCs) (Lindsay et al., 2013, 2017) which may be present in OM derived cell transplants under certain conditions. Additionally, Thy1.1 marker was not only used to characterize fibroblasts (Lankford et al., 2008; Toft et al., 2013; Ibrahim et al., 2014), but was also used to characterize stem cells, neuronal cells and connective tissue (Toft et al., 2007). Of the studies identifying additional cells in the transplants the cell types described varied greatly in characterization and proportions between studies, these differences are likely due to be influenced by several factors including (1) source and site of biopsy tissue (either bulbar or mucosal), (2) interspecies and intraspecies (age, bodyweight and sex of animals) differences, (3) cell culture conditions – time in culture, reagents, passage number, purification techniques applied, (4) the storage of olfactory tissue prior to *in vitro* cell culture, (5) the marker(s) used to identify cell types. To develop the optimal olfactory cell transplant will require an understanding of the influence of each of these factors.

Proportions of Olfactory Ensheathing Cells in Olfactory Cell Transplants

To analyze the proportion of OECs in olfactory cell transplants in SCI, data from 98 experiments were extracted from the 70 studies analyzed (Figure 3). All studies identified cells through immunocytochemical staining. One study quantified the percentage proportions of cells through flow cytometry, while the remaining studies performed manual cell counts of positively stained cells from micrograph images. The majority of experiments (92, 93.9%) used p75^{NTR} either on its own or in combination with other markers to identify OECs. The antibody markers used to identify OECs are summarized in Figure 3C.

The results demonstrated a tendency for transplanting OB derived cell preparations ($n = 67$, 68.4%) over OM derived

cell preparations ($n = 31$, 31.6%). Overall, the data showed that more experiments on transplantation of OB and OM in SCI model reported therapeutic benefits ($n = 56$, 83.6% and $n = 22$, 71.0%, respectively) than an absence of beneficial effects ($n = 11$, 16.4% and $n = 9$, 29.0%, respectively). What is striking in Figure 3A is the general preference for transplanting highly pure cell preparations containing > 90% proportion of OB-OECs ($n = 39$, 39.8%) compared to mixed cell transplants. Over 86% of these studies reported neurological improvement after transplantation, which included axonal remyelination, axonal regeneration, electrophysiological and functional recovery and reduction of glial scar formation amongst other improvements. One study included in the analysis compared the effect of varying proportions of primary (unpurified) OB and OM transplants (containing 70 and 15% OECs, respectively) and purified OB and OM transplants (containing 97 and 98% OECs, respectively) with a medium only transplant control group in a complete transection injury model in rats (Mayeur et al., 2013). This study demonstrated that while all olfactory treated groups were equally capable of inducing electrophysiological improvement, reducing astrocytic reactivity in the lesion and glial scar formation, only primary OB and purified OB and purified OM transplants were capable of promoting axonal regrowth and inducing functional recovery compared to the control group. The absence of neural repair when transplanting primary OM transplants was reported to be due to low yields of OECs and/or inhibition of repair by contaminating cells present in the primary OM (Mayeur et al., 2013). Similarly, another study reported pure preparations of OB-OECs (containing > 97% OECs) provided optimal regenerative and integrative properties into the transected rat spinal cord compared to unpurified mixed OM cell transplants (containing 12% OECs) and mixed OB cell transplants (containing 50% purified OB-OECs and 50% purified OB-fibroblasts, both populations were purified separately and combined in a mixed suspension for transplantation). Although unpurified OM-OECs and mixed OB-OECs/OB-fibroblasts transplants were capable of supporting axonal regeneration and preventing cavity formation within the lesion, these mixed cell transplants also resulted in longer lesion lengths, greater astrocytic reaction and extensive scarring. The differences in host transplant reactivity were confirmed by transplanting the cell preparations into the normal, non-lesioned spinal cord (Toft et al., 2013). Overall, the results might indicate that higher proportions of OECs in the transplant lead to greater neurological improvement. However, not all studies reported therapeutic improvement after transplanting highly purified olfactory cell preparations. Both Barakat et al. (2005) and Pearse et al. (2007) reported limited survival of OECs, limited axonal regeneration and limited functional recovery in a contusion injury model in rats after transplanting OB cell preparations containing > 94% OECs transplanted directly into the lesion site. Pearse et al. (2007) suggested the absence of recovery was due to the poor survival of highly purified OEC transplants within the harsh environment of the injury site, as only ~3% of OECs survived at 3 weeks after transplantation. In contrast, a 3 fold increase in survival of OECs was observed, when purified OECs were co-transplanted in the injury site in a 1:1 ratio



with purified Schwann cells, which resulted in modest but significant locomotor recovery compared to injury-only control animals (Pearse et al., 2007). Likewise, Lakatos et al. (2003) found poor remyelination of demyelinated axons using highly purified transplants containing ~94% OB-OECs. An estimated 3 fold increase in the degree of remyelination was observed when unpurified OEC preparations containing ~69% OB-OECs, were transplanted in a CNS demyelination injury model in rats, compared to that achieved with purified OECs alone (discussed in further detail below). Additionally, in a single-blinded phase I human clinical trial using a matched group of

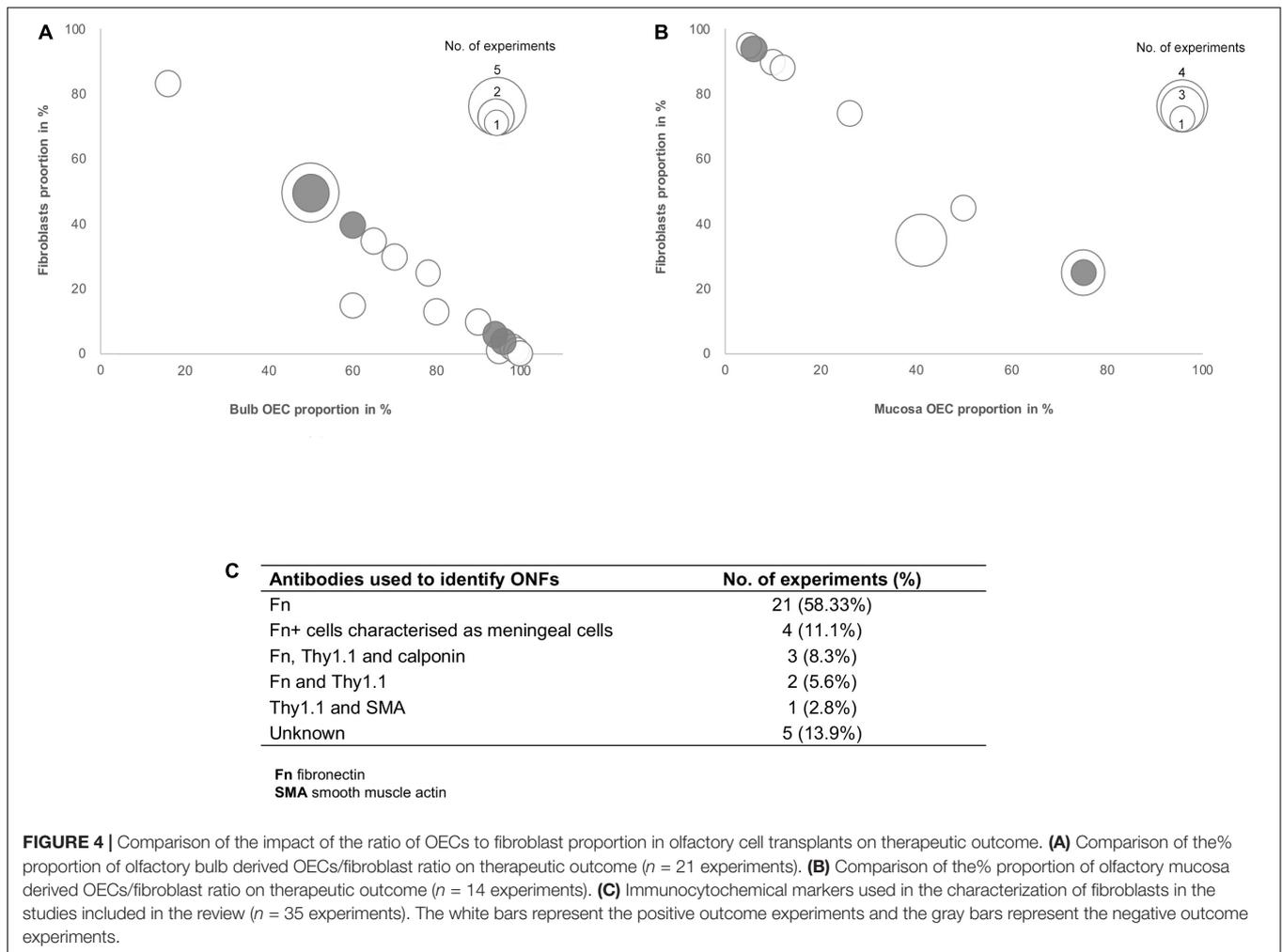
injury-only controls, Feron et al. (2005) tested the safety and feasibility of autologous OEC transplants in the injured spinal cord. Three patients with chronic thoracic SCI, characterized as American Spinal Injury Association (ASIA) category A on the Impairment Scale, were injected with high proportions of OM-OECs (containing > 80% OECs). At 3 years after transplantation no significant functional recovery was observed except in one patient who showed an improvement in light touch and pin prick sensitivity (Mackay-Sim et al., 2008). The study confirmed the safety and feasibility of the procedure as no adverse events occurred post-transplantation.

Although, the data reveals a preference for transplanting highly purified populations of both bulb and mucosa derived OECs rather than mixed cell preparations, both **Figures 3A,B** show successful transplant-mediated functional repair was possible with low proportions of OECs in mixed cell transplants. Notably, the data collected shows mixed cell transplants containing < 50% OECs may be equally capable of promoting neurological improvement as highly purified transplants containing > 90% OECs. Both of these transplant groups showed that 83% of studies (24 out of 29 studies) using transplants containing < 50% OECs, as well as 83% of studies (40 out of 48 studies) using transplants containing > 90% OECs resulted in favorable outcomes. Unpurified mucosal cell transplants containing OEC proportions as low as 5% were capable of restoring paw reaching function in 5 out of 6 rats in a CST lesion model, in comparison to 8 lesioned, non-transplanted rats, which showed no functional recovery for up to 18 weeks after the lesion (Yamamoto et al., 2009). A study by Granger et al. (2012) also demonstrated that autologous OM cell transplants containing low proportions of OECs (~10% OECs) were equally capable of improving locomotor function in companion dogs with chronic clinical SCI, as those receiving transplants containing higher proportions of OECs (~80% OECs, proportions extrapolated from **Supplementary Data**). Additionally, a Phase I non-randomized controlled clinical trial using unpurified suspensions, described as a mix of autologous OECs and ONFs from the OM was transplanted in three patients with chronic thoracic SCI and compared to three unoperated, injured control patients (all patients graded ASIA A) (Tabakow et al., 2013). One year after transplantation functional improvement was observed in 2 out of the 3 patients receiving transplants with lower proportions of OECs, 10 and 12% OECs, which resulted in conversion from ASIA grade A to ASIA C and ASIA B, respectively. The third patient transplanted with a higher proportion of OECs, 25.7% OECs, remained at ASIA A but showed improved motor and sensory function of the first spinal segments below the injury. No improvements were observed in the control group. Following on from this study, the same group transplanted unpurified suspensions, described as autologous bulbar OEC/ONFs containing OEC proportions as low as 16% in a single patient with chronic thoracic transection injury (Tabakow et al., 2014) – similar injury to that of patient denoted T1 in the author's 2013 study (Tabakow et al., 2013). Considerable improvements were observed in the patient's sensory and motor function, as the patient converted from ASIA A to ASIA C within 11 months after transplantation. However, in contrast to the 2013 study (Tabakow et al., 2013) there were several differences in the study setting that may have contributed to the neurological recovery observed in the patient. OB cells were transplanted in a combinatorial treatment that included a peripheral nerve graft taken from the patient's sural nerve, removal of inhibitory scar tissue in the lesion site and an intensive rehabilitation schedule. Additionally, others have noted, following surgery there appeared to be a possible decompression of a cyst which may have contributed to the recovery observed, together with the untethering of dural adhesions (Guest and Dietrich, 2015).

We were unable to find evidence for an optimal proportion of OECs that leads to neurological recovery. However, looking at the trends in the data collected successful transplant-mediated repair appeared to be possible using both low and high proportions of OECs in olfactory transplants. The studies reviewed also indicate that the proportions of OECs within the transplant cell population might not be linearly related to the neurological improvements observed. It is possible that instead a threshold proportion of OECs exists, which might be quite low that leads to repair. Nevertheless, while some studies suggest low proportions of OECs in mixed cell transplants are capable of promoting neurological recovery in both animals and humans, more studies are required to confirm the efficacy of such cell preparations, as a clear study bias toward testing cultures with high proportions of OECs makes it difficult to draw conclusions about the efficacy of cultures with lower OEC proportions. It is also possible that cells other than OECs present within the mixed cell transplant may have a beneficial effect on repair (discussed in more detail below). Clinical studies have shown that human OEC cell transplants are well tolerated in the spine with neurological improvements being observed in some patients. We identified 4 clinical studies that identified and quantified the proportions of OECs in the cell preparation prior to transplantation. These studies mostly transplanted autologous cell transplants (Feron et al., 2005; Tabakow et al., 2013, 2014) with one using allogenic cell transplants (Chen L. et al., 2014). While human clinical studies have demonstrated the safety and feasibility of OEC transplantation, investigations reporting the composition of OEC transplants are limited to a handful of studies with various proportions and yields being transplanted. Further investigation is required to determine the cell type and proportions that lead to repair, once this is identified we can develop clinically relevant methods for OEC cell transplants.

Proportions of Fibroblasts in the Mixed Olfactory Cell Transplants

Several studies included in this review suggested that the effects of olfactory cell transplants might not solely depend on the OEC component of the transplant and additional cell populations within the mixed cell transplant may be of importance in supporting the reparative properties of olfactory transplants. Fibroblasts in the transplant cell population were most often described and different groups suggested different optimal proportions of the combination of OECs and fibroblasts in the transplant. To analyze the relationship between proportions of OECs and fibroblasts in olfactory cell transplants, data from 35 experiments were extracted from the 22 studies that characterized and quantified fibroblasts (**Figure 4**). All studies included in the analysis identified cells through immunocytochemical staining and quantified the percentage proportions of cells through manual cell counts of positively stained cells from micrograph images (**Figure 4C**). The majority of experiments used fibronectin either on its own or in combination with other markers to identify fibroblasts ($n = 31$, 88.6%), the characterization of OECs is described above. It should be noted that a study included in the analysis in **Figure 4**, described cells



immunoreactive for fibronectin as meningeal cells, these cells were cultured from rat OB (Lakatos et al., 2003) and were often described by others as OB fibroblasts (Li et al., 2003; Andrews and Stelzner, 2004; Bretzner et al., 2008).

The data reviewed suggests there is no clear relationship between the proportions of fibroblasts in transplants and neurological improvements in the injured spine. The trend in the data shows cell preparations containing 50% OECs and 50% fibroblasts appear to be transplanted most often ($n = 7$, 20%). These studies are mostly positive, with 78% ($n = 7$) reporting neurological improvement after transplantation, indicating that fibroblasts might play a role in supporting the reparative properties of olfactory transplants. Studies by the Raisman group, consistently reported unpurified OB derived transplants which were characterized as containing a mix of 50% OECs and 50% fibroblasts, as optimal for the promotion of functional repair in rat SCI models (Keyvan-Fouladi et al., 2003; Li et al., 2003, 2004; Ibrahim et al., 2014). The mechanisms underlying the therapeutic effect of transplants containing 50% OECs with 50% ONFs was demonstrated to be necessary for the formation of a sheath similar to their physiology *in situ*, where ONFs form an outer perineurial-like wrapping around bundles of OECs

ensheathing a highly organized tissue bridge consisting of an aligned array of nerve fibers (Li et al., 1998). Keyvan-Fouladi et al. (2003) reported unpurified OB transplants, when transplanted 2 months after partial injury to the corticospinal tract (CST) in rats, were capable of restoring directed forepaw retrieval, compared to non-transplanted (injury only) rats, which failed to show return of retrieval for up to 6 months after the injury. In support of the use of unpurified OB transplants, Ibrahim et al. (2014) concluded that superior reparative effects, such as axonal regeneration and forepaw grasping observed in a rhizotomy injury model in rats, are attributed to transplants containing equal proportions of OECs and fibroblasts in unpurified OB cell transplants. In contrast, unpurified OM transplants which were largely composed of fibroblasts and low proportions of OECs (~95% fibroblasts and ~5% OECs), failed to exert regenerative effects in the same injury model. However, other studies included in the analysis have challenged the claim that 50:50 mixture of OECs/fibroblasts in the transplant is the optimal composition for transplant-mediated repair of the injured spine and have instead suggested the regenerative properties of OECs are enhanced when OECs form the substantial majority of the cells in the transplant. Toft et al. (2013) reported transplantation

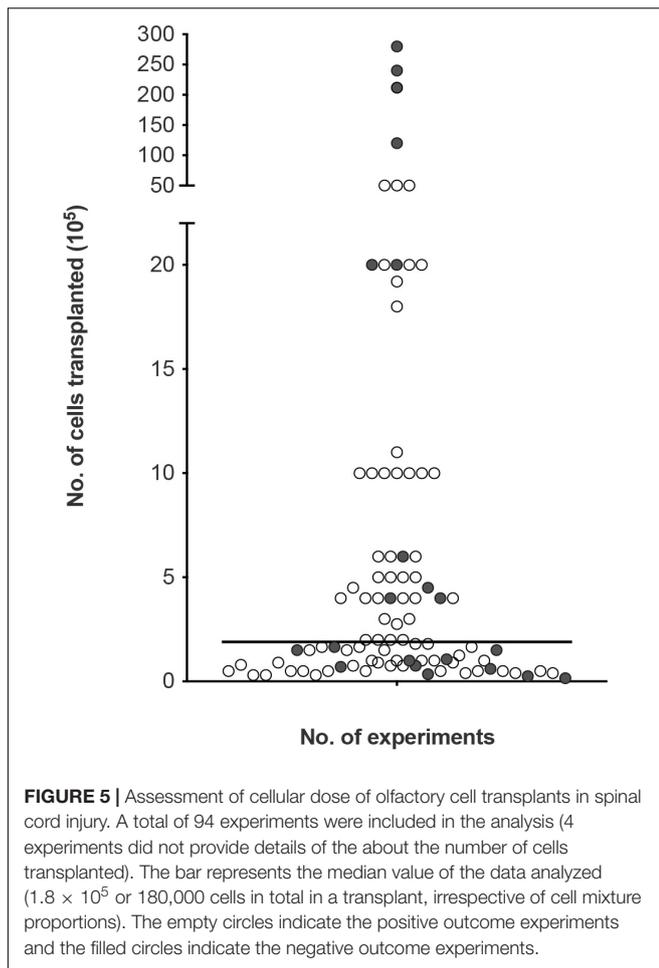
of 50:50 mixture of OB-OECs/OB-fibroblasts (each population was purified separately and then combined for transplantation) resulted in unfavorable responses, such as longer lesion length, marked astrocytic response and extensive scarring compared to highly purified OB-OEC transplants, containing > 97% OECs (discussed above). Similarly, Lakatos et al. (2003) demonstrated cell transplants containing equal proportions of OB-OECs and OB-fibroblasts (each population was purified separately and then combined for transplantation) had poor remyelinating capacity in a CNS demyelination injury model in rats. Instead they found transplants containing 70% purified OB-OECs combined with 30% purified ONFs resulted in approximately 2.5 times greater remyelination in comparison. Histological examination revealed when ONFs were present as a component of unpurified OB transplants (containing ~60% OECs) they adopted an arrangement similar to that described by Li et al. (1998) the fibroblasts loosely encircled bundles of OECs containing myelinated axons creating a bridge, while transplants containing 70% purified OB-OECs combined with 30% purified OB-fibroblasts largely resulted in compacted clumps or cords of cells. The formation of OEC bridges may not necessarily be a requirement for repair as remyelination was equally possible using both unpurified and purified transplants (70:30 OECs and fibroblasts). Additionally, remyelination using these preparations was approximately 3-fold greater compared to that achieved with purified OECs alone. Andrews and Stelzner (2007) also acknowledged the importance of including fibroblasts, suggesting that the inclusion of at least 20% fibroblasts in OEC transplants enhanced the survival of OECs within the lesion compared to the limited survival of highly purified Schwann cells in a dorsal column crush injury model in rats.

Most human OEC studies consisted of clinical investigations and literature regarding human OEC xenotransplants is fragmentary. We identified only three studies reporting results of human OEC in rat models of SCI. There were no reports of human OEC transplantation in large animal models of SCI. Collins et al. (2018) transplanted unpurified human OB-OECs containing 90% OECs, along with fibroblasts and MSCs (characterization and quantification not stated), embedded within a collagen scaffold in a transection injury model. They found that half of the treated group recovered proprioception while the other half did not. In their previous study (Ibrahim et al., 2009) using the same injury model but using rat OB-OEC transplants (containing 50% OECs and 50% fibroblasts) they found greater (70% of rats) recovery of proprioception and fewer (30% of rats) lacking recovery amongst the rats receiving treatment. It was suggested the partial functional recovery using human OB-OECs could be due to higher proportions of OECs and fewer fibroblasts in the OB transplant. They also found that transplanted human OB-OECs showed limited migration and were predominantly located at the lesion site, which was similarly described in another study using highly purified human OM-OECs transplanted into the spinal cord of immunodeficient rats (Deng et al., 2006). Deng et al. (2006) compared the proliferation, survival and migration of highly purified human and rat OM-OECs transplants containing 100% OECs in a hemisection injury model. They found both rat and human

OM-OECs rapidly stopped dividing following implantation and showed similarly minimal survival and migration using highly purified transplants in injured animals compared to intact animals. Human OECs have also been shown to share similar repair properties to rat OECs in terms of remyelination. Barnett et al. (2000) demonstrated human OB-OECs containing 30–66% OECs (transplants also contained fibroblasts and endothelial cells, proportions were not stated), were capable of remyelinating demyelinated CNS axons in rat spinal cord. Additionally, they demonstrated that human OECs growth factor requirements were similar to those of rat OECs. Whilst human OECs might share many properties with their rat counterpart, further investigation is required to determine the *in vivo* repair properties of human OECs, which are still poorly defined.

The studies reviewed indicate that the regenerative properties of OECs are enhanced when they are co-transplanted with fibroblasts which have been reported to facilitate a favorable environment by promoting axonal regeneration, remyelination, survival of OECs and functional recovery after SCI. Furthermore, olfactory fibroblasts in the transplant have been shown to provide important growth factors and a permissive substrate that stimulates the proliferation of OECs (Yui et al., 2011). Looking at the trend in the data we were unable to infer a relationship between the ratio of OECs and fibroblasts in the transplant and outcome. The observed benefits of co-transplanting OECs with fibroblasts in rodent studies has led to human clinical trials omitting purification procedures, resulting in modest functional improvement using both OM and OB cell preparations either on their own or in combinatorial treatment respectively, as described above (Tabakow et al., 2013, 2014). Despite the potential for complexity of cell cultures the precise cell types have been rarely well characterized in clinical studies of both OB and OM transplants. A number of different cell types exist alongside OECs *in situ*, in mixed cell transplants it might be possible that cells other than OECs contribute to CNS repair, particularly when derived from the mucosa (Yao et al., 2018). Schwann cells might contaminate OEC transplants as branches of the trigeminal nerve are present in the OM and OB. Defining Schwann cell contribution is difficult because Schwann cells and OECs both express the same markers *in vitro* (Chen C. R. et al., 2014, Chen L. et al., 2014). Additionally, using different cell culture protocols to OECs, MSCs cultured from the lamina propria of human OM have been shown to promote CNS remyelination both *in vitro* and *in vivo* in rat models of SCI (Lindsay et al., 2013, 2017). Conversely, it is also possible that without purification undesirable cells might remain in the cell transplant. Dlouhy et al. (2014) reported a patient with SCI developed a spinal cord tumor mass 8 years after intraspinal transplantation of autologous undissociated OM tissue. It is worth noting that this transplant was neither characterized nor cultured prior to transplantation and other clinical studies using dissociated OM cell preparations have been shown to be relatively safe (Feron et al., 2005; Mackay-Sim et al., 2008; Tabakow et al., 2013).

Nevertheless, regulatory requirements for clinical application of OEC transplants in SCI will require a high level of therapeutic specification and reproducibility. The future development of human OEC transplants will therefore require identification



of the cell types and proportions in the transplant that leads to repair. If we are to use OEC transplants for human transplantation, we need to understand what constitutes the human cell cultures and the importance of additional cell types in the cultures for SCI repair.

Cell Dosage in Transplants

To look at the potential effect of the cell dose in olfactory cell transplants rather than the proportions, we reviewed the number of experiments reporting the therapeutic outcome compared to the absolute number of transplanted cells (i.e., the total number of cells, not limited to OEC component of the cell cultures, **Figure 5**). The number of cells transplanted ranged from 15,000 to 28 million cells across the different species. The trend in the data collected shows the number of cells required for therapeutic repair might be low as the majority of the studies reviewed, transplanted a total of 500,000 cells or less $n = 67$ (71.3%), of which 79.1% ($n = 53$) reported neurological improvement. In comparison, transplantation of greater than 500,000 cells resulted in slightly fewer studies reporting neurological improvement, 74.1% ($n = 20$) of studies. We found the median dose of cells transplanted was $\sim 200,000$ cells.

Very few studies looked at the effect of cell dose on neurological improvement. Although, $\sim 200,000$ cells might be sufficient for transplant-mediated repair it might not be the optimal dose of cells. A recent study included in the analysis of this review, compared different dosages of OM cell preparations in a contusion injury model in rats. Treatment groups included 200,000, 500,000, 1 million and >1 million cells. While, all transplanted groups led to higher functional recovery compared to the control injury-only group, 500,000 transplanted cells showed the best improvement in hind-limb motor recovery among the treated groups (Muniswami and Tharion, 2018). This study suggests the efficacy of olfactory transplants may be dose dependent but more studies comparing the dose of cells in the same experimental paradigm are required to determine the optimum cell dose. It is possible the cell dose might be contingent upon the size of the species and the size of the lesion. Furthermore, there was no clear relationship between studies reporting a lack of therapeutic benefit with the total number of cells transplanted. This may be due to a myriad of confounding factors, the most obvious being the great variability in cell culture techniques between different laboratories.

Effective cell doses for human SCI have not been established. Since the majority of our data collected relates to studies in rats, the effective dose in human cell transplantation will likely be higher. In clinical studies neurological improvements were observed when the number of cells transplanted ranged from 500,000 (Tabakow et al., 2014) to ~ 2 million cells (Tabakow et al., 2013). Increasing the number of cells did not appear to be linked with more positive results. When three patients with thoracic paraplegia received 1.8, 1.9, and 21.2 million cells, functional improvements were only observed in the two patients who received the lower cell doses. The third patient receiving the higher cells dose in comparison showed minimal functional improvement (Tabakow et al., 2013). In another clinical study, 12, 24, and 28 million cells were transplanted in three paraplegic patients. No significant functional recovery was observed 3 years after transplantation except in the patient who received the lower cell dose showed some improvement in light touch and pin prick sensitivity (Feron et al., 2005; Mackay-Sim et al., 2008). It may be the case that the capacity for OEC transplant-mediated repair is reduced/minimal using higher cell numbers in human. This is consistent with a meta-analysis conducted by Watzlawick et al. (2016) who demonstrated that cell numbers above the optimum dose of 180,000 cells were associated with reduced locomotor outcome in rat models of traumatic SCI. The declining benefit-risk ratio linked to higher cell doses was suggested to be due to the increasing neurotoxic effects exerted by the requirement for higher volumes of injections. Higher cell doses are likely to require larger injection volumes and the delivery of such volumes have the potential to cause further neurological injury to the already injured spinal cord due to the build-up of fluid pressure (Guest et al., 2011). Nonetheless, with so few studies transplanting high cell numbers it would be difficult to claim that higher cell numbers in the transplant are associated with lack of neurological improvement in humans but if a ceiling to the beneficial effect exists it requires further

investigation. Additionally, a recent review (Reshamwala et al., 2019) suggested the survival of OECs after transplantation was likely influenced by the number/concentration of transplanted cells. It was suggested that survival was highest when using transplants containing low cell densities. Higher cell densities were linked to negative outcomes possibly due to the increased shear stress and physical damage to the cells during injection procedure. Approaches to improving cell survival will likely lead to improvements in functional outcomes.

Limitations of the Review

The main limitation of the present study is the great heterogeneity in methodology (cell culture techniques, animal injury models, etc.) and outcome measures (axon regeneration, myelination, and motor recovery, etc.) across studies, making accurate comparisons challenging. In order to compare results from such a variety of sources the different outcome measures were combined into a general classification of positive and negative outcomes. It is possible that this binary classification may mask treatment related effects on specific outcomes but without the standardization of methodology and outcome measures accurate interpretation will always be difficult. Additionally, the design of our study meant that statistical analysis was not possible, the trends in the data was measured instead. In summary, the review provides a broad understanding of the characterization of OEC transplants used for the treatment of SCI and highlights some of the outstanding questions that need to be resolved to maximize treatments for human SCI.

CONCLUSION AND RECOMMENDATIONS

Over the years the olfactory system has been proposed as a useful tissue to harvest cells for transplant-mediated repair of SCI. In this review we summarized the current literature relating to the composition, purity and cell dose of OEC cultures used for transplant-mediated repair of SCI. Overall, we found that OEC transplants are not very well characterized and crucial questions about the therapeutic cellular composition, purity and dose remain unanswered. Unless olfactory cell transplants are composed of 100% OECs, additional cells will remain in the transplants and it is presently unknown what effect variations in the cell types and proportions will have *in vivo* and if they contribute to therapeutic outcome. The future development of human OEC transplants for clinical application in SCI

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repair will require characterization and identification of the regenerative components of OEC transplants as well as the purity and cell dose. These are important factors that must be optimized in the clinical translation of any cellular strategy. A better understanding of the components of OEC transplants that leads to repair will further strengthen the effectiveness of cell transplants for the treatment of SCI and improve the clinical outcomes.

A wealth of preclinical data generated using rodent studies has been valuable in demonstrating the benefits of olfactory tissue culture transplantation. However, this review highlights the lack of studies involving human OECs. Human OECs should be studied in their own right, and for ethical reasons, OM is the more likely candidate for initial transplantation in patients. Future studies should therefore endeavor to characterize and identify the regenerative cell types in the OM and look to find optimal cell culture mixes in more clinically relevant injury models such as spinal cord contusion and compression. These cell types should be tested *in vitro*, such as in neurite outgrowth or organotypic slice culture models, and *in vivo*, to identify the active components and estimate the minimum number of cells required for clinical effectiveness. The active cell types should then be cultured and expanded under Good Manufacturing Practice guidelines to allow future clinical application.

AUTHOR CONTRIBUTIONS

DC provided the idea for the review. MM searched the literature, prepared the figures, and structured and wrote the manuscript. DC and PF contributed to editing and approval of the final manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fncel.2021.781489/full#supplementary-material>

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