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Designing Olfactory Ensheathing Cell Transplantation Therapies: Influence of Cell Microenvironment

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Abstract

Olfactory ensheathing cell (OEC) transplantation is emerging as a promising treatment option for injuries of the nervous system. OECs can be obtained relatively easily from nasal biopsies, and exhibit several properties such as secretion of trophic factors, and phagocytosis of debris that facilitate neural regeneration and repair. But a major limitation of OEC-based cell therapies is the poor survival of transplanted cells which subsequently limit their therapeutic efficacy. There is an unmet need for approaches that enable the *in vitro* production of OECs in a state that will optimize their survival and integration after transplantation into the hostile injury site. Here, we present an overview of the strategies to modulate OECs focusing on oxygen levels, stimulating migratory, phagocytic, and secretory properties, and on bioengineering a suitable environment *in vitro*.

Keywords

OECs, microenvironment, stimulation, cell transplantation, neural repair

Introduction

The olfactory system has a unique neurogenic niche in which olfactory sensory neurons are replaced throughout an individual's lifespan. Because the olfactory neuroepithelium is exposed to the external environment, there is a constant turnover of olfactory neurons, and newborn olfactory neurons are supported and guided by specialized glia called olfactory ensheathing cells (OECs). OECs are located in the lamina propria underlying the olfactory mucosa and surround the axons of the olfactory sensory neurons from the epithelium up into the nerve fiber layer of the olfactory bulb[1–3]. Thus, OECs can be easily obtained from an intranasal biopsy of the olfactory mucosa including the lamina propria. OECs share morphological and molecular features with both central nervous system (CNS) glia such as astrocytes, and peripheral glia such as Schwann cells[4–6]. They support the continual regeneration of neurons by acting as a suitable substrate, and by migrating in tandem or ahead of emerging olfactory axons[7–9]. OECs are also considered to be the primary innate immunocytes in the olfactory system. They are a dynamic cell population that can be stimulated from a resting state to a phagocytic state, and they are capable of clearing bacteria and axonal debris[10,11]. Due to their numerous properties, the transplantation of OECs to repair injuries in other regions of the nervous system, particularly spinal cord injury (SCI), is being explored by many research groups.

An injury to the spinal cord is devastating and often an irreversible event that usually triggers multiple deleterious processes such as delayed and progressive cell death, ischemia, hypoxia, inflammation, and extensive scarring[12]. This complex injury site microenvironment is pro-apoptotic and anti-regenerative[13]. To overcome these inhibitory factors, OECs have been trialed extensively for SCI repair because of their versatile and favorable biological functions which can ameliorate the environment of the injury site and promote regeneration. OECs can offer neuroprotection, enhance neurite outgrowth, provide axonal

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guidance cues, and promote remyelination in animal models and in humans (reviewed in Gómez et al.[14]). Despite several completed clinical trials with transplantation of OECs demonstrating safety and efficacy, the recovery outcomes in patients are often variable. While there are multiple factors contributing to the variable recovery outcomes such as differences in cell source, cell purity, cell delivery techniques, and assessment of functional readouts (reviewed in Kawaja et al.[15], Miah et al.[16], Yao et al.[17]), a consistent observation across studies has been the poor survival of transplanted cells, with the reported survival rates of transplanted OECs being as low as 0.3% to 3% in animal models[18–21]. To compensate for this massive cell loss post-transplantation, excess cells are transplanted into the injury site. However, this approach comes with limitations as it introduces additional cytotoxic products (apoptotic corpses) at the injury site without any improvement in viability. In addition, it is not always feasible to produce a surplus of cells for autologous therapies due to the limitations in cell production from a small biopsy source material. While the majority of our knowledge on OEC biology comes from using rodent olfactory tissues, OECs have also been isolated and purified from different species such as dogs, pigs, primates, and humans[22–26]. There are fundamental inter-species differences in the control of OEC proliferation and their response to different growth factors (reviewed in Wewetzer et al.[27]). To overcome the complexities in culturing OECs from different species, it is important to identify and maintain cells under optimal conditions that favor cell proliferation and rapid expansion while maintaining cell-specific properties such as morphology, antigen expression, and phagocytosis. For OECs to be used clinically for cell transplantation, it will be imperative to produce sufficient purified cells in a short timeframe *in vitro*. Therefore, it is critical to test protocols for cell isolation, purification, and expansion for OECs obtained from individual species to predetermine optimal culture conditions, rather than assume cells from different species will respond similarly.

Strategies need to be designed that enable the *in vitro* production of OECs in a state that will optimize their survival and integration after transplantation into the hostile injury site. However, commonly used *in vitro* models for cell expansion do not reflect the conditions of the injury site and this critical aspect of the OEC transplantation therapy is mostly unexplored. By implementing pretreatment strategies for the culture of OECs in an environment mimicking the host site before transplantation, their phagocytic, secretory, and migratory capacity can be improved to enhance viability and neural regeneration at the transplantation site. This review focuses on the following themes prior to cell transplantation of OECs: (1) homeostatic/hypoxic preconditioning and (2) priming/activating cells, and (3) bioengineering a suitable microenvironment.

Homeostatic/Hypoxic Preconditioning of OECs

Oxygen availability is a fundamental requirement for cellular function, and decreased oxygen levels can induce cellular stress. Under homeostatic conditions, cells require oxygen levels between 2% and 9% (14.4–64.8 mm Hg), whereas lower oxygen levels 0.5% to 2% (<10 mm Hg) are considered hypoxic[28]. Standard cell culture practice involves culturing cells in liquid medium incubated at atmospheric oxygen levels of 21% which is considerably higher than physiological oxygen levels. Continued exposure to oxygen concentration above physiological levels can lead to premature senescence of primary cells[29,30]. It is likely that cells are physiologically adapted to their anatomic niche conditions. By culturing cells *ex vivo* under higher oxygen levels and then transplanting them *in vivo* to homeostatic or hypoxic conditions, the cells may require significant re-adaptation which may confer additional cellular stress. This may be a contributing factor to the poor survival of OECs after transplantation. Hence, there is a need for *in vitro* approaches to mimic the low oxygen conditions that the cells experience in their tissue-specific niche and the transplantation site.

To gain insight into the potential for homeostatic oxygen or hypoxic preconditioning where cells are cultured under low oxygen conditions, it is useful to examine how other cells respond, such as mesenchymal stem cells (MSCs; Table 1). The aims of homeostatic/hypoxic preconditioning are to improve the viability of the cell product and the therapeutic properties of the transplanted cells. Culturing bone marrow–derived mesenchymal stem cells (BM-MSCs) in hypoxic conditions has been shown to increase proliferation, multipotency, and the secretion of cytoprotective molecules[49,52]. This has partly been attributed to the provision of oxygen levels similar to the resident cellular niche. The cellular niche encompasses the local microenvironment that includes both cellular and acellular components that nourish and regulate the functions of cells. Oxygen levels in the niches of mesenchymal and neural stem cells are 2% to 8% and 1% to 8%, respectively[53,54].

Olfactory mucosa–mesenchymal stem cells (OM-MSCs) are a type of Nestin-positive stem cells identified[55] in the olfactory mucosa that have the potential to differentiate into smooth muscle cells, adipocytes, osteocytes, and neurons and show similar antigenic profile to BM-MSCs[56,57]. The OM-MSCs secrete anti-inflammatory cytokines and have been shown to improve myelination of rat spinal cord cell cultures[58]. Due to these favorable properties, OM-MSCs are an alternative source of MSCs for autologous cell transplantation. OM-MSCs and OECs are resident within the same niche, the highly cellular lamina propria (reviewed in Lindsay et al.[59]). The application of conditions tested on OM-MSCs to OECs can be an appropriate strategy to recreate an optimized microenvironment for the culture and expansion of OECs, and to improve their efficacy for cell transplantation.

Table 1. Effect of Hypoxia on Therapeutic Potential of Mesenchymal Stem Cells.

Cell type	Hypoxia method	Oxygen levels and duration	Potential therapeutic application	Observations	References
Human OM-MSCs	Not mentioned	3% (48 h)	Ischemic disease	Hypoxia generated OM-MSCs extracellular vesicles promote paracrine HIF-1α, VEGF signaling for angiogenesis, and enhanced proliferation and migration of human brain microvascular endothelial cells	Ge et al.[31]
Human OM-MSC	92% N2	3% (48 h)	Intracerebral hemorrhage	Preconditioning of OM-MSC in hypoxia delays senescence and aids in the therapeutic efficacy of OM-MSCs in intracerebral hemorrhage model. microRNA-326 (miR-326) expression was significantly increased in the hypoxia OM-MSCs.	Liu et al.[32]
Human OM-MSC	Not mentioned	Below 0.5% O_2	Cerebral ischemia/ reperfusion injury	OM-MSCs attenuated apoptosis and oxidative stress in ischemic stroke models and improved neurologic deficits in rats	He et al.[33]
Human OM-MSC	94% N2	1% (48 h)	Cerebral ischemia/ reperfusion injury	Hypoxia preconditioned OM-MSCs alleviate pyroptosis and apoptosis of microglial cells by HIF-1α activation	Huang et al.[34]
Human OM-MSC	Not mentioned	3%	Parkinson's disease	OM-MSCs differentiated into dopaminergic neurons at physiological oxygen level of 3%. Increase in β-tubulin and Tyrosine hydroxylase expression	Zhuo et al.[35]
Human OM-MSC	Not mentioned	3% (48 h)	Cerebral ischemia	Hypoxia reduced gene expression at 5% serum of VEGF, GDNF, BDNF, and NGF and increased expression of Matrix metalloproteinase-2 and BDNF at 20% serum conditions	Yuan et al.[36]
Human BM-MSCs	Anaerobic chamber	2% O_2 (48 or 72 h)	Spinal cord injury repair	*In vitro* hypoxic pretreatment enhanced cell survival of transplanted BM-MSCs after spinal cord injury	Luo et al.[37]
Human BM-MSCs and porcine BM-MSCs	HypOxystation	1%, 2%, or 5% for short term (48 h) and long term (10 days)	Acute respiratory distress syndrome	At 2% hypoxia, MSCs exhibited increased proliferation, self-renewal, and modulation of inflammatory genes. Potential to obtain MSCs with augmented function for therapeutic application	Antebi et al.[38]
Human BM-MSC	Hypoxic C-chamber	1% (24 h)	BM-MSC stem cell therapy	Hypoxia induced HIF-1α enhanced the migration of BM-MSC through activation of matrix metalloproteinase-2	Choi et al.[39]
Human BM-MSCs	94% N2	5% O_2	BM-MSC stem cell therapy	Hypoxia increased proliferation and differentiation of BM-MSCs in both young and old healthy donors depending on age and culture conditions	Mohd et al.[40]

(continued)

Table 1. (continued)

Cell type	Hypoxia method	Oxygen levels and duration	Potential therapeutic application	Observations	References
Human BM-MSC and Mouse BM-MSC	94% N2	1% for 24 h	Upscaling MSC production for cell therapies	Hypoxia increased the size and number of neurospheres generated from BM-MSCs	Mung et al.[41]
Human BM-MSCs	Hypoxic C-Chamber connected to ProOx Model 21 controller	2% O_2	Improving *in vitro* culture conditions for clinical application	Efficient expansion of BM-MSCs at 2% O_2 compared with 20% O_2. Increased cell proliferation and cellular metabolism	Dos Santos et al.[42]
Mouse BM-MSCs	94% N2 Hypoxic cell incubator	1% (48 h)	Spinal cord injury repair	Hypoxic preconditioning increased exosome production and the exosomes promoted functional recovery following SCI in mice by shuttling miR-216a-5p	Liu et al.[43]
Mouse BM-MSCs	ProOx-C-Chamber	1.5% O_2 (48 h)	Pulmonary fibrosis	Hypoxic preconditioning promoted cell proliferation, expansion, and reduced hydrogen peroxide induced cytotoxicity. Improved survival and lung function in bleomycin-induced pulmonary fibrotic mice was also observed	Lan et al.[44]
Mouse BM-MSCs	ProOx C-chamber system	0.1%–0.3% O_2 (24 h)	Ischemic stroke in mice	Intranasally delivered hypoxic preconditioned BM-MSCs showed enhanced homing to ischemic region and improved sensorimotor recovery in treated mice	Wei et al.[45]
Mouse BM-MSC	94% N2	1%	Neovascularization and microvascular network remodeling	Enhanced cell migration and three-dimensional capillary-like structure formation in Matrigel. Increased expression of angiogenesis related markers	Annabi et al.[46]
Rat BM-MSCs	90% N2 Incubator chamber	5% O2	Wound healing	Hypoxic pretreatment in combination with curcumin enhanced cell survival, mitochondrial fusion, and accelerated wound healing in a mice wound model	Wang et al.[47]
Rat BM-MSCs	92% N2	3% O_2 (24 h)	Spinal cord ischemia/ reperfusion injury	Hypoxic preconditioning improved protective effects of BM-MSCs on neurological function, tissue damage, and inhibited apoptosis	Wang et al.[48]
Bovine BM-MSCs	93% N2 HypOxystation	2% O_2 (1 week)	Musculoskeletal tissue regeneration	Hypoxic preconditioning promoted BM-MSCs survival and extracellular matrix production in low oxygen and nutrient limited *in vitro* microenvironment	Peck et al.[49]
Human UC-MSCs	94% N2	1% O_2 (72 h)	Ischemia	Hypoxic stimulation increased production of microvesicles. These microvesicles promoted new vessel formation	Zhang et al.[50]
UC-MSCs	Various levels of N2 gas was used	1.5%, 2.5%, and 5% (72 h)	Stem cell therapy	Hypoxia induced high metabolism rate at 1.5% and 2.5% O_2 in UC-MSCs, reduced cell death, and increased cell proliferation	Lavrentieva et al.[51]

OM-MSC: olfactory mucosa–mesenchymal stem cell; HIF-1α: hypoxia-inducible factor 1-alpha; VEGF: vascular endothelial growth factor; GDNF: glial-derived neurotrophic factor; BDNF: brain-derived neurotrophic factor; NGF: nerve growth factor; BM: bone marrow; SCI: spinal cord injury; UC: umbilical cord.

Similar to BM-MSCs, hypoxic preconditioning of OM-MSCs resulted in increased secretion of neuroprotective paracrine factors against cerebral ischemia/reperfusion injury. Interestingly, hypoxic OM-MSCs were able to inhibit microglial cell death following cerebral ischemia/reperfusion injury *in vitro*. This anti-pyroptotic and anti-apoptotic effect of OM-MSCs on microglia was mediated by regulating expression levels of hypoxia-inducible factor 1-alpha (HIF-1α), a key transcription factor regulating cellular response to hypoxia[34,33]. Preconditioning of OM-MSCs resulted in marked increase of HIF-1α, and silencing HIF-1α in OM-MSCs affected cell viability and resulted in acceleration of apoptosis[32]. A hypoxic environment could also promote differentiation of OM-MSCs to dopaminergic neurons by upregulation of HIF-1α and activation of tyrosine hydroxylase[35,60]. Thus, it is clear that MSCs respond in various ways to low oxygen conditions and hence the effect of low oxygen conditions should be considered for OECs.

Survival of OECs in culture and at the transplantation site can be compromised by a lack of oxygen and nutrients to support their viability. *In vitro* sensitivity of OECs to hypoxia and serum deprivation was tested by Pellitteri et al., in neonatal mouse OEC cultures. OEC proliferation and survival were reduced when exposed to a combination of hypoxia and serum starvation[61]. Addition of basic fibroblast growth factor, a mitogen for OECs[62], could improve survival and proliferation of OECs from hypoxia or serum deprivation. Intriguingly, the growth rate of primate OECs was unaffected by environmental oxygen concentration in contrast to rodent OECs which appeared to overcome replicative senescence when cultured in low oxygen conditions[25].

A recent study[63] investigated the therapeutic effects of exosomes from human umbilical cord–derived MSCs on OECs in hypoxic conditions for sciatic nerve regeneration in rats. Treating OECs with exosomes resulted in improved viability, proliferation, and migration of OECs, and increased the secretion of brain-derived neurotrophic factor (BDNF) thereby resulting in improved functional recovery in injured rats. Notably, extracellular vesicles derived from hypoxia-preconditioned OM-MSCs (3% O_2) could promote HIF-1α–vascular endothelial growth signaling in human brain microvascular endothelial cells via miR-612 upregulation and downregulation of *TP53*, a component of cellular stress responses, resulting in enhanced angiogenesis in *in vitro* tube formation assays[31].

While these studies make a case for preconditioning cells to low oxygen conditions before transplantation into a "hostile" hypoxic environment, the adoption of low oxygen pretreatment to a clinical setting will be contingent on the protocol consistency. It will be critical to predetermine the vulnerability and responses of the OEC cellular product to hypoxic stress, the duration and percentage O_2 of low oxygen exposure, and ultimately the ideal conditions to improve cell survival and integration at the transplantation site.

Pretransplantation Cell Priming

The inflammatory environment and the inhibitory extracellular matrix at the injury site in the CNS result in poor growth conditions for both the endogenous and transplanted cells[64,65]. OECs offer a potential therapeutic benefit as they can modulate the inflammatory environment, remove cell and myelin debris, and offer neurotrophic and physical support to regenerating axons (Fig. 1, reviewed in Yao et al.[17], Brosius Lutz and Barres[66], and Fregnan et al.[67]). Many aspects of OECs and their cellular interactions for pro-regenerative functions have been studied *in vitro* using assays for neurite outgrowth, interaction with astrocytes, debris clearance, and phagocytosis assays[14]. One avenue to further improve the therapeutic efficacy of OECs is to enhance their activities. Thus, there is a need for the design of approaches to activate or train OECs to attain a functionally relevant phenotype *in vitro* and to retain or enhance their relevant function *in vivo* after transplantation.

Different approaches have been tested to stimulate the secretion of growth factors, and to enhance the migratory and phagocytic capabilities of OECs. The main objectives of cell priming or preconditioning cells by exposure to an activating/priming agent *in vitro* are to augment their potential therapeutic properties and to better prepare the cells to face the conditions at the transplantation site.

Soluble signaling cues. OECs secrete many neurotrophic molecules such as neurotrophin-3 (NT-3), nerve growth factor (NGF), glial-derived neurotrophic factor (GDNF), BDNF, neurotrophins-4/5 (NT-4/5), and vascular endothelial growth factor (VEGF)[68–70], These molecules can also counteract the diffusion of inhibitory molecules from neuronal debris by phagocytosing debris.

To optimize the functional outcomes from OEC transplantation, cell modulation with different neurotrophins has been tested (reviewed in Rosner et al.[71] and Wright et al.[72]). NT-3 is an interesting candidate as it can promote both the proliferation and survival of OECs, and also different groups have shown that local application of NT-3 at the injury site was favorable for regeneration after SCI[73–75]. To achieve long-term and site-specific delivery of NT-3 to the injury site, OECs genetically modified to secrete high amounts of NT-3 were transplanted to the injured spinal cord, and these cells could significantly improve axonal outgrowth[73,76]. A recent study explored the effect of NT-3 in a rat model of SCI and showed that NT-3 could inhibit the mitogen-activated protein kinase (MAPK) signaling pathway[77]. Similarly, NGF and BDNF play a neuroprotective role by modulating the MAPK/mitogen-activated extracellular signal-related kinase (MEK) pathway[78,79].

The Wingless-related integration site (Wnt) signaling pathway influences multiple aspects of neural development from cell proliferation, cell fate specification, and neuronal morphogenesis to cell death (reviewed in Ciani and Salinas[80]).

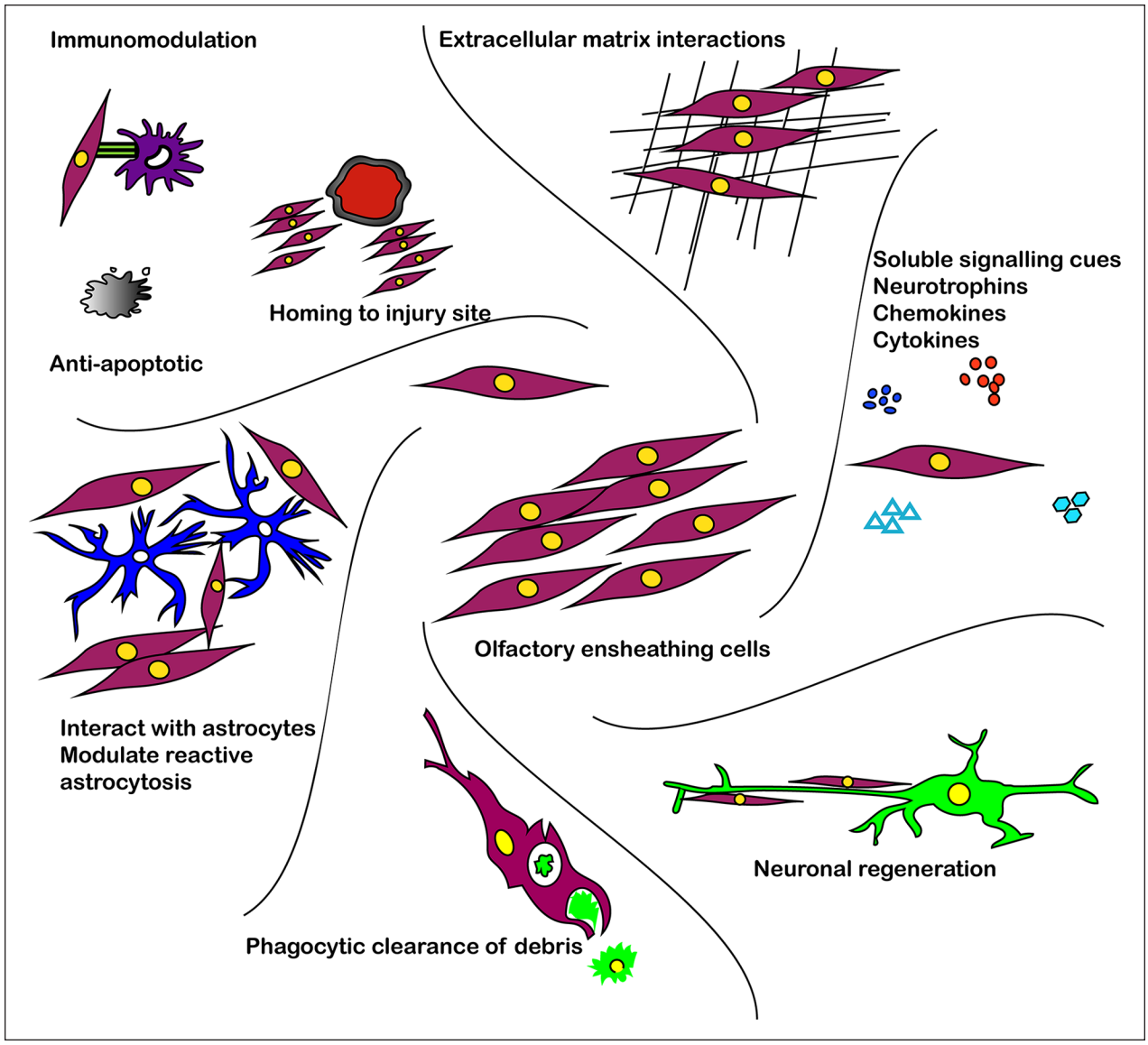

Figure 1. Schematic of the various biological roles of olfactory ensheathing cells that favour neural regeneration. The therapeutic effects of olfactory ensheathing cell transplantation for neural repair are attributed to their biological roles such as phagocytosis of debris, interaction with astrocytes, neurotrophic support, immunomodulation, and neuronal regeneration.

A specialized subgroup of OECs in the inner nerve layer of the olfactory bulb was identified using Wnt reporter mice, and Wnt signaling was implicated in appropriate olfactory axonal targeting and in neural regeneration[81–83]. Notably, the activation of Wnt signaling could promote self-renewal of olfactory epithelial stem cells and neuronal differentiation. Furthermore, Wnt signaling activation is critical for the regeneration of adult olfactory epithelium after methimazole induced injury[84]. Activation of canonical Wnt signaling was shown to be both necessary and sufficient to drive the transition of horizontal basal stem cells from a resting to an activated neurogenic state in the uninjured epithelium[85]. Recently, it was reported that Wnt-activated OECs can stimulate neural stem cell proliferation and neuronal differentiation in neonatal mouse OECs. Interestingly, the conditioned medium from Wnt-activated OECs was sufficient to stimulate proliferation of neural stem cells determined by an increase in Ki67 and Sox2 double positive cells, and it could also promote the differentiation of neural stem cells into β-tubulin III positive neurons[86].

There is growing evidence supporting a paracrine/secretory effect of transplanted cells such as MSCs and OECs on neural regeneration (reviewed in Makridakis et al.[87]). These studies indicate that there may not be a need for homing of large cell numbers to the injury site to observe an effect. Secreted signaling cues could be sufficient to drive cellular responses, and there is potential for using activation or stimulation of the cells as another approach to enhance

therapeutic potency of transplanted cells by improving their function and their resistance to inflammatory conditions. Little is known about the immunomodulatory properties of OM-MSCs. To address this, Jafari et al., compared the cytokine secretion of stimulated OM-MSCs and adipose-derived MSCs by short-term priming protocols to stimulate Toll-like receptors. Interestingly, OM-MSCs had significantly higher levels of immunosuppressive cytokines interleukin-8, transforming growth factor beta (TGF-β) and C-C motif chemokine ligand 5 secretion in comparison with adipose tissue–derived MSCs even before any treatment[88]. We recently reported that OECs produced less pro-inflammatory cytokines compared with Schwann cells and macrophages when exposed to necrotic bodies and in a pro-inflammatory microenvironment[89]. The secretome of OM-MSCs has been reported previously[90] and the results showed that the secreted proteins were mainly associated with neurotrophy, cell growth, angiogenesis, cell differentiation, and apoptosis. In cerebral ischemia reperfusion injury, models, OM-MSCs were shown to downregulate reactive oxygen species and lipid peroxidation levels, and eventually reduce neuronal apoptosis[33]. Recently, extracellular vesicles derived from OECs were shown to display neuroprotective effects on neural progenitor cells and promoted peripheral nerve regeneration in rats[91,92].

Overall, these studies suggest that OECs can be stimulated *in vitro* to enhance the activity, function, and secretome of OECs which can then exert various benefits to other cell types. However, a robust analytical approach is required to identify the "ideal" activators for OECs and to measure the immunosuppressive potential of activated OECs in an inflammatory environment.

Migration. Transplanted cells will encounter a complex and unfavorable environment during their migration as they are faced with different cell types such as reactive astrocytes, activated microglia, invading fibroblasts, inflammatory molecules, and debris at the injury site. These interactions have the potential to modulate the transplanted cells and affect their ability to migrate. It is likely that OECs transplanted at the site of SCI will be surrounded by glial-fibrillary acidic protein-positive cells, possibly reactive astrocytes[93], and these astrocytes can limit OEC migration. For instance, tumor necrosis factor alpha (TNFα) is secreted by reactive astrocytes at the site of injury and can modulate OEC migration in a dose-dependent fashion, blocking tumor necrosis factor receptor 1 alpha (TNFR1α). This can result in the reduced migration of olfactory bulb OECs[94]. Despite the odds being stacked against migration and integration at the injury site, OECs have been shown to migrate with the regenerating axons[95] and interact with astrocytes[5,96]. These migratory properties of OECs, along with their ability to interact with astrocytes at the injury site and modulation of the inflammatory environment, are thought to contribute toward favorable neural repair in the CNS[5,94,97,98]. Moreover, OECs can also downregulate the translocation of nuclear factor kappa beta (NFκB) in astrocytes, an important response implicated in astrocyte activation. Insulin-like growth factor-1, secreted by OECs is considered a key contributor to the modulation of astrocytes activation by OECs by potentially preventing the translocation of NFκB to astrocyte nuclei[99].

Different candidates have been tested to stimulate OEC migration with the objective of improving neural repair outcomes. We have shown previously that OEC migration is characterized by lamellipodial waves that appear to direct intercellular interactions. The lamellipodia migration of OECs could also be enhanced by GDNF which further mediates the motility of axons[100,101]. Integrin alpha-7 has been reported to play an important role in the migration of adult OECs without directly affecting neurite regeneration[102]. Fibulin-3, Slit2, and NogoA have been shown to inhibit OEC migration, and interestingly they are also often found to be overexpressed in the scar tissue at lesion sites[103–105]. Similarly, lysophosphatidic acid (LPA) is produced at the injury site, and has been reported to promote migration and proliferation of OECs via extracellular signal-regulated kinase (ERK1/2) signaling[106] while also facilitating the homing of OECs to the injury site[107]. We recently showed that liraglutide, a glucagon-like peptide-1 receptor agonist, could stimulate OEC migration by reducing time in arrest, upregulating laminin-1, and activating the ERK pathway[108]. Another approach to augment OEC migration at the site of injury is to genetically modify cells. One such study was to modify OECs to express Nogo receptor ectodomain. These modified cells migrated longer than non-modified cells both *in vitro* and post-transplantation in a rat model of SCI. The myelin mediated inhibition of OEC migration could be partly overcome by treatment with NEP1-40 peptide or antibodies against Nogo receptor[109].

These studies further support the notion that stimulating migration of OECs is feasible, and perhaps incorporating cells with enhanced migratory properties should be a consideration when designing OEC-based cell therapies for neural repair.

Phagocytosis. The persistence of cellular and myelin debris at the site of CNS injury impedes neural regeneration[110]. Effective stimulation of OEC phagocytic activity is another avenue to promote debris clearance and thereby improve neural regeneration. Accumulating evidence from our group and other studies has helped identify different compounds that can increase OEC phagocytosis, including curcumin[111], curcumin with lipopolysaccharide (LPS)[112], natural products 2-methoxy-1,4-naphthoquinone[113], the serrulatane diterpenoids 3-acetoxy-7,8-dihydroxyserrulat-14-en-19-oic acid, and 3,7,8-trihydroxyserrulat-14-en-19-oic acid[114]. The anti-inflammatory cytokine TGF-β has also been implicated in increasing OEC phagocytosis[115].

Curcumin elicits pleiotropic effects in OECs in a dose-dependent manner. In assays where neurons are co-cultured

with OECs and neuronal debris, increased clearance of debris was observed in the presence of LPS and curcumin stimulus[112] or TGF-β[115], and this in turn promoted neuronal survival. Strikingly, pretreatment with curcumin resulted in improved functional recovery and axon growth in a rat model of SCI. Cells stimulated by curcumin exhibited increased expression of phosphatidylserine receptor suggestive of increased phagocytosis and secreted more growth factors *in vivo* at the injury site[116]. Recently, it was shown that when activated by curcumin and LPS, OECs had pro-angiogenic effects such as promoting proliferation, migration, and vessel formation of vascular endothelial cells likely by modulating the phosphatidylinositol 3-kinase/protein kinase B pathway[117].

Compared with Schwann cells, OECs appear to have more favorable neural repair characteristics. In addition to producing less pro-inflammatory cytokines compared with Schwann cells in a pro-inflammatory environment, we have also demonstrated that OECs phagocytosed more myelin debris than Schwann cells[89]. More data are clearly needed to understand how OECs interact with the immune and nervous systems, and how debris clearance is coordinated between OECs and professional phagocytic cells at the injury site.

Overall, these studies show that OECs are responsive to stimulation and the potential exists that these various activities can be manipulated to further enhance the therapeutic benefits of OECs after transplantation. To create a microenvironment suitable to drive axonal regeneration, we need to develop and test approaches to activate and train OECs *in vitro* to maximize their functions *in vivo*. Systematic analysis of the priming agents and optimizing the duration of priming to modulate therapeutic efficacy will be the key to achieving efficient cell therapy outcomes with minimum cell dosage and side effects.

Bioengineering a Suitable Microenvironment

Another challenge in the application of cell therapies for SCIs is the retention of biological functions of transplanted cells. For cells to function consistently as "living drugs," we must aim to recreate or mimic their *in vivo* niche in a dish and to standardize cell production protocols[118]. The factors that directly or indirectly affect the cell behavior such as extracellular matrix, neighboring cells, signaling cues, and mechanical forces caused by movement of physiological fluids, all constitute the microenvironment of a cell.

OECs are conventionally cultured *in vitro* and expanded as adherent monolayers under conditions commonly used for mammalian cells. However, access to nutrition and oxygen is not uniform and well-controlled under these conditions, and intercellular interaction is unnatural when cells are adhered to a dish. Moreover, the properties of these cells are dependent on factors such as cell density and time in culture. Cells are also reliant on direct contact with the surrounding extracellular matrix and neighboring cells for maintenance and regulation of their biological function. So, two-dimensional adherent culture conditions are not ideal, and there is a disparity between what the cells require for performing their biological roles and what is provided *in vitro*. There is a need for developing models mimicking both the resident cellular niches and the transplantation niche.

Rapid advances in materials science have led to the use of different biomaterials with the aim of promoting functional tissue repair at the site of injury[119,120]. Provision of three-dimensional (3D) support has been shown to improve efficacy of BM-MSCs after transplantation by mimicking the cellular niche, and creating a conducive and stable environment for axonal regeneration and cell survival (reviewed in Zhou et al.[121]).

Different biomaterials have been trialed in combination with OECs with varying success (Table 2). These biomaterials function as carriers for the cells and as structural scaffolds for axonal regrowth. The minimum prerequisites for a suitable biomaterial are biocompatibility, biodegradability, and adaptive mechanical properties. Despite the application of fabricated and synthetic 3D scaffolds such as fibrin and polymer-based scaffolds for nerve repair, there remains a need for biologically relevant scaffolds or scaffold-free 3D culture techniques. It is expected that decellularized scaffold-based tissue constructs could be directly transplanted for the regrowth of axonal tracts and to hasten the neural regeneration *in vivo*[144]. Decellularization is the process of creating an acellular extracellular matrix scaffold by removal of the cellular components of living tissues. These acellular scaffolds are subsequently used to provide structural and spatial support, cytokine support, and integration through cell surface molecules[145]. Spinal cord decellularized scaffolds have been shown to promote axonal regeneration and functional motor recovery in the hind limbs of rats with SCI[146–148]. Decellularized scaffolds seeded with OECs showed good biocompatibility with adherent and proliferating OECs observed in the scaffold, and when transplanted into rat spinal cord, the decellularized scaffold + OEC group could promote axonal regeneration and showed significant motor function recovery after 3 weeks of injury[149]. However, decellularized materials which have a fixed architecture restrict to some degree the movement and interactions of cells that are seeded into the 3D construct. Thus, the resultant cell relationships may not reflect a more natural arrangement that may occur if the cells had a less restrictive environment.

To simplify the final cell product that is transplanted and to minimize potential adverse effects, our research has focused on the development of stable 3D constructs that are substrate and scaffold-free and can be cultured in standard cell culture medium. We recently reported two 3D spheroid culture systems: floating liquid marbles and the naked liquid marbles[150,151]. In the naked liquid marble system, OECs cultured within a liquid drop on a superhydrophobic surface can form spheroids within hours. This rapid formation of spheroids is advantageous as short-term cultured OECs have

Table 2. Studies Using Olfactory Ensheathing Cells–Seeded Scaffolds.

Study	Cell types	Scaffold type	Outcome	References
Partial recovery with dorsal root injury in rats Injury model: Rat unilateral four root dorsal transection injury (C6, C7, C8, and T1 region)	Human olfactory bulb OECs	3D collagen scaffold	4.8 mg/ml collagen with 1×10^6 cells gave an optimal cellular network of OECs. Microglial activation in the deep dorsal horn of cervical C7 and C8 level or axonal loss in C3 level was observed in the responder rats; 30% errors observed in climbing performance of control rats compared with rats with OEC transplants	Collins et al.[122]
OEC collagen grafts do not improve spinal trauma-induced motor deficits. Injury model: Rat 2 mm long unilateral low—thoracic hemisection cavities (T13 region)	Rat olfactory bulb OECs	2 mm long cylindrical collagen scaffolds with diameter of 2 mm	Implantation of collagen scaffold seeded with OECs did not improve or worsen motor outcomes and allodynia following thoracic SCI hemisection in rats	Deumens et al.[123]
Phenotypic study of rat OECs on 3D collagen scaffolds	Rat olfactory bulb–derived OECs	The average pore size of the 3D collagen scaffold was 20–100 μm in diameter	3D collagen scaffold is biocompatible with OECs and scaffolds yielded 67% more OECs compared with monolayer culture. Also, spindle-like bipolar morphology of OEC was retained on 3D collagen scaffolds	Wang et al.[124]
In vitro biocompatibility of OECs with biomimetic silk scaffold	Rat olfactory bulb OECs	*Bombyx mori* porous silk scaffold	Water-rinsed silk fibroin scaffolds were biocompatible with OECs, favored cell proliferation and secretion of neurotrophic factors	Wentao et al.[125]
Optimal diameter of scaffold helps in guiding growth and migration of OECs	Rat olfactory bulb OECs	SFS	300 nM SFS is biocompatible for culture and unidirectional migration of OECs	Shen et al.[126]
Microencapsulation of transplanted OECs reduce pain post sciatic nerve injury L4–L5 dorsal root ganglia C chronic sciatic nerve compression injury	Rat olfactory bulb OECs	Cell suspension was mixed 1:1 with 1.5% alginic acid	Purinergic receptor P2X2/3 expression is elevated in chronic constriction injury (CCI) models. Microencapsulation of OECs reduced pain after sciatic nerve injury	Zhao et al.[127]
Potential biomaterials functioning as cell carriers for neuro transplantation	Rat olfactory bulb OECs	2% alginate, alginate-0.025% fibronectin hydrogel 500–800 μm alginate and matrigel preparation	Alginate-fibronectin increased proliferation of OECs but significantly lower than with matrigel. Neurite outgrowth of OECs was increased in alginate-fibronectin hydrogel compared with alginate alone	Novikova et al.[128]
Neuroregenerative properties of OECs in multi-layered conductive nanofibrous conduits Injury model: 8 mm transected sciatic nerve in rats	Rat olfactory bulb OECs	Single-walled carbon nanotube/poly (L-lactic acid) (SWCNT/PLLA) scaffolds	OEC-seeded nerve conduits transplanted to the transected rat sciatic nerve improved axonal growth and peripheral nerve regeneration	Kabiri et al.[129]
Long-distance axon regrowth in presence of OECs, olfactory nerve fibroblasts and biomaterials Injury model: Rat 2 mm long dorsal hemisected (T11/T12 region) SCI model	Rat olfactory bulb OECs/ ONF	Poly(D, L)-lactide matrices	Lack of OEC/ONF migration from the rostral/caudal site of injection to injury site and poor cell survival on biomatrices due to low seeding numbers of OEC/ONF and incompatibility of biomatrices. Modest locomotory function seen in swing speed, stride length in hind limbs, and axonal regrowth after OEC/ONF transplantation	Deumens et al.[130]
Enhanced neural regeneration with OECs in PLGA scaffolds Injury model: Rat 2 mm wide complete transected (T9–T10 region) SCI model	Rat olfactory bulb OECs	PLGA pore size 300–500 μM	Enhanced locomotor function, axon myelination, neuronal protection, and decreased astrogliosis post SCI in PLGA and OEC combination compared with PLGA or untreated groups	Wang et al.[131]

(continued)

Table 2. (continued)

Study	Cell types	Scaffold type	Outcome	References
Directionality and bipolarity of OECs on electrospun nanofibers	Rat OECs	PLGA	Nano composite electrospinning fibers of 237 nm diameter favored bipolarity and unidirectional migration of OECs	Kueh et al.[132]
PLGA with OECs for bridging sciatic nerve defects in rats Injury model: 7 mm sciatic was resected to 10 mm nerve defect	Rat olfactory bulb OECs	100 μM diameter PLGA (with 85:15 carboxyl end)	A combination of PLGA and OECs can improve the functional and structural outcome in defective sciatic nerve but the sciatic functional index cannot be recovered in more serious injuries	Li et al.[133]
OECs combined with chitosan decreased neuropathic pain. Injury model: Chronic sciatic nerve compression injury in rats	Rat olfactory bulb OECs	Chitosan	OEC-seeded chitosan scaffolds can inhibit Purinergic receptor (P2X7R) overexpression and reduce neuropathic pain	Zhang et al.[134]
Electrical stimulation of OECs using conductive polymers	Rat olfactory bulb OECs	0.4 mm Polypyrrole/ chitosan polymers	Polypyrrole/chitosan membranes supported cell adhesion and proliferation even without electrical stimulation. Stimulation increased secretion of neurotrophic factors	Qi et al.[135]
3D printed polycaprolactone/polypyrrole conducting scaffolds aid neurite outgrowth	Human OE-MSCs	PCL/polypyrrole (PPy) conducting scaffolds	OE-MSCs on scaffolds showed increased differentiation to Schwann-like cells, increased secretion of NGF and BDNF, and increased neurite outgrowth but conductivity of scaffold had no effect on cell attachment, proliferation, viability, and distribution	Entezari et al.[136]
Interactions between Schwann cells (SCs) and OECs with starch/polycaprolactone scaffold	Rat olfactory bulb OECs and sciatic nerve Schwann cells	SPCL	OECs and SCs are biocompatible with SPCL. Improved growth, proliferation, and migration of cells was observed in long-term culture	Silva et al.[137]
Comparison of scaffolds for migration and growth of glial cells	Rat OECs	PCL and C/PCL	C/PCL biomaterial made scaffold is better suited for cell proliferation, migration, and neurite outgrowth	Schnell et al.[138]
Characterization of OECs cultured on polyurethane/polylactide scaffold	Rat olfactory bulb OECs	PU/PLDL scaffold	Different ratio of PU to PLDL did not alter phenotype of OECs but proliferation rate depended upon equal ratio of polymers	Grzesiak et al.[139]
BioPEGylation of PHB-polyethylene glycol (PHB-*b*-DEG) hybrid polymers promotes healthy nerve cell and migration	OECs	Polyhydroxybutyrate-polyethylene glycol	bioPEGylated PHB supported OEC migration, promoted cell proliferation and attachment. No cytotoxicity response in OECs	Chan et al.[140]
Compatibility of OECs with a self-assembling peptide scaffold	Rat olfactory bulb OECs	A new peptide hydrogel scaffold GRGDSPmx	On the new scaffold, OEC proliferation was increased, cells showed less apoptosis and maintained spindle-shaped morphology	Zhang et al.[141]
Albumin scaffold seeded with adipose-derived stem cells and OECs for spinal cord injury repair	Adipose-derived stem cells and rat OECs	Serum-derived albumin scaffold	Rats treated with cell-seeded scaffolds showed improved locomotor skills and presence of cells expressing neuronal markers at injury site	Ferrero-Gutierrez et al.[142]
Improved locomotor behavior in rats after delayed cell transplantation into transected spinal cord Injury model: Rat rostrocaudally 3–4 mm complete transected (T9–T11 region) SCI model	Rat olfactory lamina propria	Three to five 1 mm^2 lamina propria pieces/ Gelfoam	Olfactory lamina propria grafts result in gradual improvement in locomotor recovery and axonal regeneration	Lu et al.[143]

OEC: olfactory ensheathing cell; SFS: silk fibroin scaffolds; SCI: spinal cord injury; ONF: olfactory nerve fibroblasts; PLGA: poly (lactic-*co*-glycolic-acid); OE-MSC: olfactory ecto–mesenchymal stem cell; PCL: poly-ε-caprolactone; NGF: nerve growth factor; BDNF: brain-derived neurotrophic factor; SPCL: starch-based polycaprolactone scaffold; C/PCL: collagen/Poly-ε-caprolactone; PU/PLDL: polyurethane/polylactide; PHB: polyhydroxybutyrate.

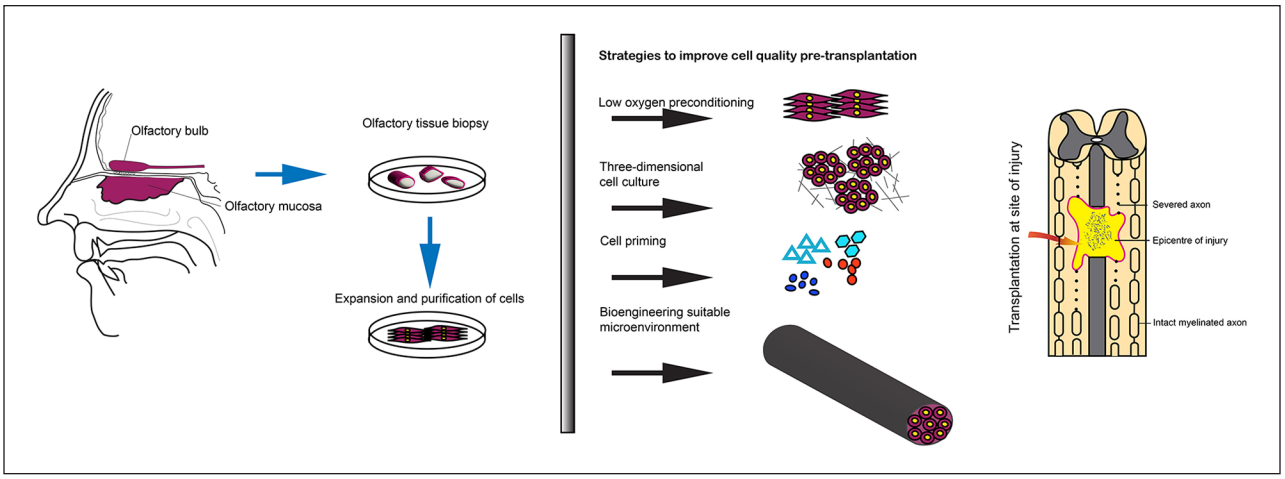

Figure 2. Schematic overview of the different strategies to improve cells pretransplantation. Olfactory ensheathing cells are isolated and purified from biopsies of olfactory mucosa or olfactory bulb tissue. The cells can be modulated by exposure to low oxygen, stimulated to improve migratory and phagocytic properties, and cultured in three-dimensional constructs prior to transplantation at the site of spinal cord injury.

better effects on the neural survival and axonal growth[152]. Furthermore, we could customize the size of the spheroid using vibration at different frequencies[153] or by changing cell density.

A major advantage of culturing cells in 3D spheres is that it closely mimics the *in vivo* environment and can recapitulate the cellular interactions and cell-matrix interactions. Importantly, our ability to culture OECs in 3D in this naked liquid marble system revealed two critical attributes of this process: (1) unrestricted movement of cells within liquid marbles enabled natural arrangement of cells reminiscent of their *in vivo* organization and (2) cells retained their migration properties from spheroids when transferred to a two-dimensional culture plate. Due to the naked liquid marble system resulting in 3D cell constructs that closely mimic the *in vivo* environment, it is suitable for a range of *in vitro* studies of OECs which may better reflect cell function and responses.

3D bioprinting is a bespoke approach to address the variable nature of SCIs wherein personalized tissue scaffolds suitable to match an individual's injury site can be generated. For instance, Joung et al.[154] reported a 3D spinal cord tissue-like platform where multiple neural progenitor cells could be placed within a printed scaffold. More recently, a novel bio-ink containing hydroxypropyl chitosan, thiolated hyaluronic acid, vinyl sulfonated hyaluronic acid, and matrigel was used for the fabrication of a tissue scaffold to mimic the white matter of spinal cord[155]. The feasibility of printing primary cultured OECs was demonstrated by Othon et al.[156], where using biological laser printing several lines of OECs could be printed through a multilayer hydrogel scaffold.

In summary, integration of emerging technologies such as 3D bioprinting in combination with scaffold-free models has the potential to create highly complex environments for the recreation of cellular and transplantation niches thereby facilitating the use of predictive and biologically relevant *in vitro* models.

Conclusion

The microenvironment of the injured spinal cord is unfavorable for the survival of transplanted cells. In this review, we have discussed potential strategies to precondition and stimulate OECs for transplantation to improve their survival and to enhance their therapeutic potential (Fig. 2). When cells are isolated from their native environment, expanded *in vitro*, and then transplanted back *in vivo* to a harsh injury environment, the therapeutic potency of the cells is not well-preserved, possibly due to changes in the microenvironment of the cells. Preconditioning OECs *in vitro* may improve their migration, phagocytic, and immunomodulatory abilities. Understanding how the manipulation of different stimuli, such as oxygen levels, signaling cues, and 3D culture parameters of cells, can affect the behavior of OECs should be a consideration in the design of cell transplantation therapies. Future studies should focus on the development of robust *in vitro* models that can activate and retain biological properties of the cells by mimicking conditions of the tissue-specific microenvironment. This will help to improve the overall reliability of cell-based therapies and to unlock the therapeutic capabilities of OECs for neural repair.

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Ethical Approval

This study was approved by our institutional review board.

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

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