Neuroscience

Olfactory ensheathing cells are hybrid glial cells that promote neural repair

Reviewed Preprint
v1 • May 21, 2024
Not revised

Patricia E. Phelps, **Sung Min Ha**, **Rana R. Khankan**, **Mahlet A. Mekonnen**, **Giovanni Juarez**, **Kaitlin L. Ingraham Dixie**, **Yen-Wei Chen**, **Xia Yang**

Department of Integrative Biology and Physiology, UCLA, Los Angeles, CA 90095

Abstract

Olfactory ensheathing cells (OECs) are unique glial cells found in both the central and peripheral nervous systems where they support the continuous axonal outgrowth of immature olfactory sensory neurons to their targets. Here we show that following severe spinal cord injury, olfactory bulb-derived OECs transplanted near the injury site modify the normally inhibitory glial scar and facilitate axon regeneration past the scar border and into the lesion center. To understand the mechanisms underlying the reparative properties of such transplanted OECs, we used single-cell RNA-sequencing to study their gene expression programs. Our analyses revealed five diverse subtypes of OECs, each expressing novel marker genes and pathways indicative of progenitor, axonal regeneration and repair, secreted molecules, or microglia-like functions. As expected, we found substantial overlap of OEC genes with those of Schwann cells, but also with astrocytes, oligodendrocytes and microglia. We confirmed established markers on cultured OECs, and then localized select top genes of OEC subtypes in rat olfactory bulb tissue. In addition, we present evidence that OECs secrete both Reelin and Connective tissue growth factor, extracellular matrix molecules which are important for neural repair and axonal outgrowth. Our results support that adult OECs are a unique hybrid glia, some with progenitor characteristics, and that their gene expression patterns indicate diverse functions related to wound healing, injury repair and axonal regeneration.

eLife assessment

This study presents **important** data describing cell states of olfactory ensheathing cells, and how these cell states may relate to repair after spinal cord injury. While the overall framework used for characterizing these cells is **solid**, the quantification and contextualization of results are **incomplete**, given that measurements, significance statistics, and discussion of both previous work and experimental methods that would be necessary to support several claims are not provided. With more thorough quantification and discussion, this work will be of interest to stem cell biologists and spinal cord injury researchers.

https://doi.org/10.7554/eLife.95629.1.sa1

Introduction

Olfactory sensory neurons (OSNs) are remarkable as they undergo life-long neurogenesis within the adult olfactory epithelium (Graziadei & Monti Graziadei, 1985), and their axons are exclusively associated with a specialized type of glia, olfactory ensheathing cells (OECs; (Doucette, 1990). OECs provide the outgrowth and guidance factors needed for OSN axons to reach their glomerular targets and to exhibit regenerative abilities in response to injury (Doucette, 1991; Li et al., 2005; Williams et al., 2004). OECs have received considerable interest due to their ability to support axonal outgrowth even after severe or complete spinal cord transection in rodents, dogs and humans (Granger et al., 2012; Khankan et al., 2016; Ramon-Cueto et al., 2000; Tabakow et al., 2014; Takeoka et al., 2011; Thornton et al., 2018).

The regenerative capacity of adult spinal cord neurons is inhibited by the lesion site environment that includes a reactive glial scar, together with invading meningeal fibroblasts and multiple immune cells (Burda & Sofroniew, 2014; Wanner et al., 2013). The lesion core is composed of non-neural tissue that inhibits axonal outgrowth due to the upregulation and secretion of chondroitin sulfate proteoglycans that impede axon regeneration (Cregg et al., 2014). Studies of severe or complete spinal cord transection in rats followed by the transplantation of OECs provided evidence that the OECs surround axon bundles which regenerate into and occasionally across the injury site (Khankan et al., 2016; Ramon-Cueto et al., 2000; Takeoka et al., 2011; Thornton et al., 2018). In combination with *in vitro* studies, detailed analyses of injury sites suggest that OECs: 1) function as phagocytes to clear degenerating axonal debris and necrotic cells (Khankan et al., 2016; Nazareth et al., 2020; Su et al., 2013), 2) modulate the immune response to injury (Khankan et al., 2016; Vincent et al., 2007), and 3) interact favorably with the glial scar and lesion core that form after injury *in vitro*, (Lakatos et al., 2003; Lakatos et al., 2000) and *in vivo*, (Khankan et al., 2016; Thornton et al., 2018). OEC transplantation following complete spinal cord transection creates an environment that is conducive to neural repair (Khankan et al., 2016; Takeoka et al., 2011; Thornton et al., 2018). OECs secrete molecules that stimulate axon outgrowth such as Brain-derived neurotrophic factor (Bdnf), Nerve growth factor (Ngf), and Laminin (Ruitenberg et al., 2003; Runyan & Phelps, 2009; Woodhall et al., 2001) and enhance cell-to-cell mediated interactions between neurites and OECs in an inhibitory environment (Chung et al., 2004; Khankan et al., 2015; Windus et al., 2007). Khankan et al. (2015) showed that neurites grew 2-3 times longer in growth-inhibitory areas of multicellular 'scar-like' cultures when they were directly associated with olfactory bulb-derived OECs (OB-OECs), but the molecular mechanisms are unknown.

The major question addressed in this study is how OB-OECs perform the diverse functions associated with neural repair. We hypothesize that there exist OB-OEC subtypes, and that their respective gene programs and secreted molecules underlie their multifaceted reparative activities. Here we identify the OB-OEC subtypes and their molecular programs that contribute to injury repair, such as the growth-stimulating secreted and cell adhesion molecules involved in OB-OEC interactions that promote neurite and axonal outgrowth. We used single-cell RNA-sequencing (scRNA-seq) to characterize the immunopurified rat OB-OECs (hereafter called OECs unless stated otherwise) that are similar to those transplanted into our previous rat spinal cord injury studies. We first compared the genes expressed by purified OECs with those expressed by the 'leftover cells', i.e., the cells which are not selected by the panning procedure, to determine if the purification process selected for specific OEC subtypes. After confirming the characteristic OEC markers, our scRNA-seq data revealed five OEC subtypes which expressed unique marker genes and were further characterized and confirmed experimentally. Finally, we examined interesting extracellular matrix (ECM) molecules secreted by OECs, Reelin (*Reln*) and Connective tissue growth factor (*Ccn2*/*Ctgf*), both of which may facilitate axonal outgrowth into the inhibitory injury core environment.

Results

One particularly challenging aspect facing neural repair of spinal cord injury (SCI) is to facilitate axonal outgrowth and eventually regeneration across the inhibitory injury site. A common finding in our studies testing the effects of OEC transplantation following SCI is that OECs modify the injury site so that some axons project into the lesion core surrounded by OECs (Dixie, 2019; Khankan et al., 2016; Takeoka et al., 2011; Thornton et al., 2018). **Figure 1** shows an enlargement of an injury site which contains green fluorescent protein (GFP)-labeled OECs in the glial fibrillary acidic protein (GFAP)-negative injury core between the two GFAP-positive spinal cord stumps. The OECs in the injury core are associated with a few serotonergic-(**Figure 1a-f**) and many neurofilament-labeled axons (**Figure 1g-l**). To learn more about possible mechanisms by which OECs support axon regeneration, we prepared immunopurified OECs and examined their gene expression and diversity with scRNA-seq.

Unbiased scRNA-seq of immunopurified OECs and 'leftover' controls distinguishes OECs, microglia, and fibroblasts

Using scRNA-seq, we sequenced a total of 65,481 cells across 7 samples (n=3 OEC preparations and 4 'leftover' controls) that passed quality control, with an average of 9,354 cells per sample. Cell clusters were visualized using t-distributed stochastic neighbor embedding (tSNE). The tSNE plot showed that cells from imunnopanned OEC samples were separated from the cells from the leftover samples (**Figure 2a**). Cell clustering analysis defined a total of seven clusters (**Figure 2b**), each of which showed expression patterns of marker genes that distinguish one cluster from the others (**Figure 2c**). Based on previously reported cell type marker genes, we found elevated expression of OEC marker genes in clusters 2, 3 and 7, microglia marker genes in clusters 4, 6, and 7, and fibroblast marker genes in clusters 0, 1, and 5 (**Figure 2d**). As expected, many cells enriched in the leftover controls were defined as fibroblast and microglia based on the corresponding known marker genes, whereas cells from the immunopanned OEC samples showed high expression of OEC marker genes (**Figure 2a** vs. **2e**). After cell type assignment, a dot plot clearly depicts distinct expression of cell type-specific marker genes (**Figure 2f**). Our scRNA-seq results support the expected cell type composition following the immunopanning procedure.

We next asked if there were differences between cultures of immunopurified OECs and OECs grown in 'leftover' cultures. While our previous spinal cord injury studies transplanted 90-95% purified OECs, other labs culture and implant less purified OECs and thus many of the neighboring cells, i.e., primarily fibroblasts and microglia, would be included. Our comparison showed that purified OECs express higher levels of *Cryab* (stress protection, inflammation inhibition), *Nqo1* (antioxidant protection, stress adaptation), and *Postn* (cell proliferation, survival, migration) genes (**Figure 2g**), whereas OECs in leftover cultures with fibroblasts and microglia express higher levels of *Apoe* (lipid transport and glial function), *Calcb* (inflammation and pain modulation), and *Mgp* (ECM calcium inhibitor; **Figure 2g**) than purified OECs. These results indicate immunopurified OECs may have better stress response and proliferative and survival capacity than the controls.

Immunofluorescence verification of typical OEC marker genes revealed by scRNA-seq

To validate the OEC markers revealed by scRNA-seq, we re-cultured extra cells not used for sequencing for immunocytochemical confirmation (Suppl. Table 1). Because our adult OECs are immunopurified with anti-nerve growth factor receptor p75 ($Ngfr^{p75}$), we expected and found OEC samples heavily enriched in $Ngfr^{p75}$ in both immunofluorescence (**Figure 3a**) and scRNA-seq

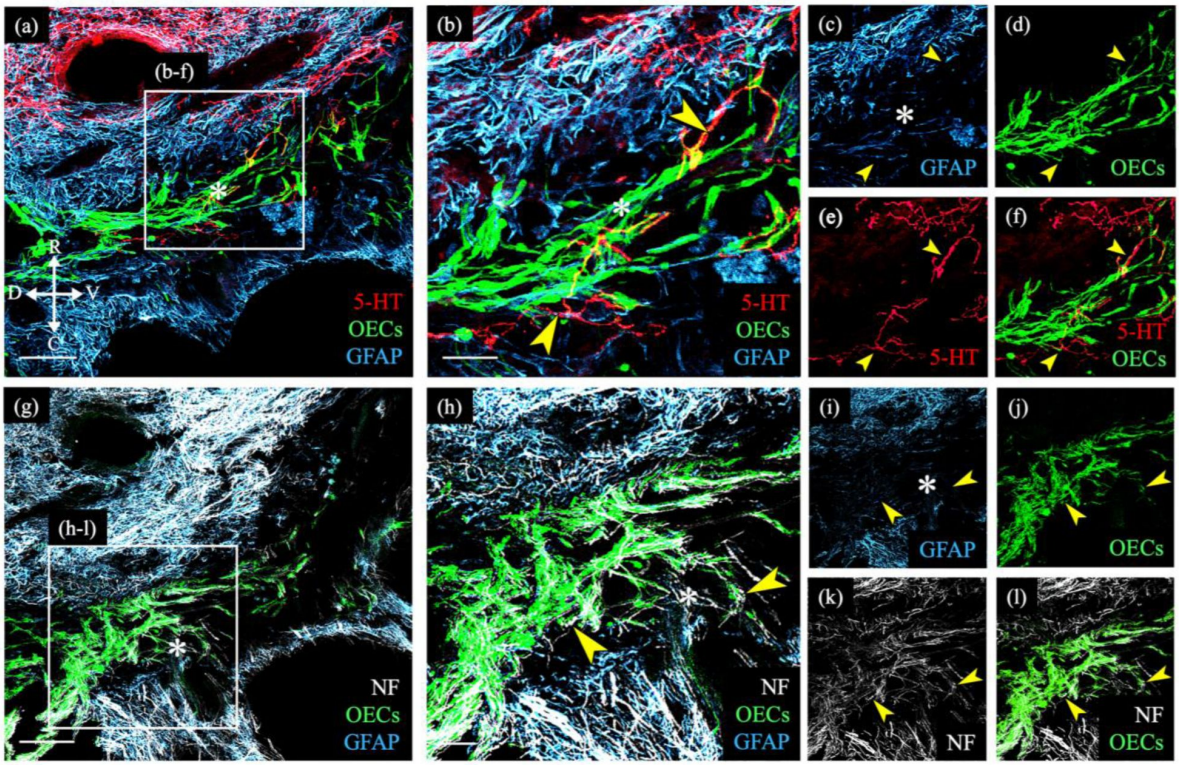

Figure 1.

Transplanted GFP-OECs in the center of the lesion core associate with numerous. axons.

Sagittal sections show rostral and caudal glial scar borders of the injury site which are identified with glial fibrillary acidic protein (GFAP, blue). The GFAP-negative lesion core contains GFP-OECs (green) and is marked with asterisks. Arrowheads mark axons crossing into the lesion core. **(a-f)** Serotonergic axons (5-HT, red) are found in the rostral spinal cord stump and associate with OECs (green) in the lesion core. Single channels for GFAP **(c)**, OECs **(d)**, 5-HT **(e),** and a combination of 5-HT and OECs **(f)**. **(g-l)** Nearby injury site section from the same rat **(a)**. Numerous neurofilament-positive axons (white) are associated with the OECs (green) in the lesion core. Single channels for GFAP **(i)**, OECs **(j)**, neurofilament **(k),** and a combination of neurofilament and OECs **(l)**. Scale bars: a, g = 500 μm; b, h = 100 μm. Reprinted from: Dixie, KL, 2019, *UCLA*. ProQuest ID: Dixie_ucla_0031D_18445.

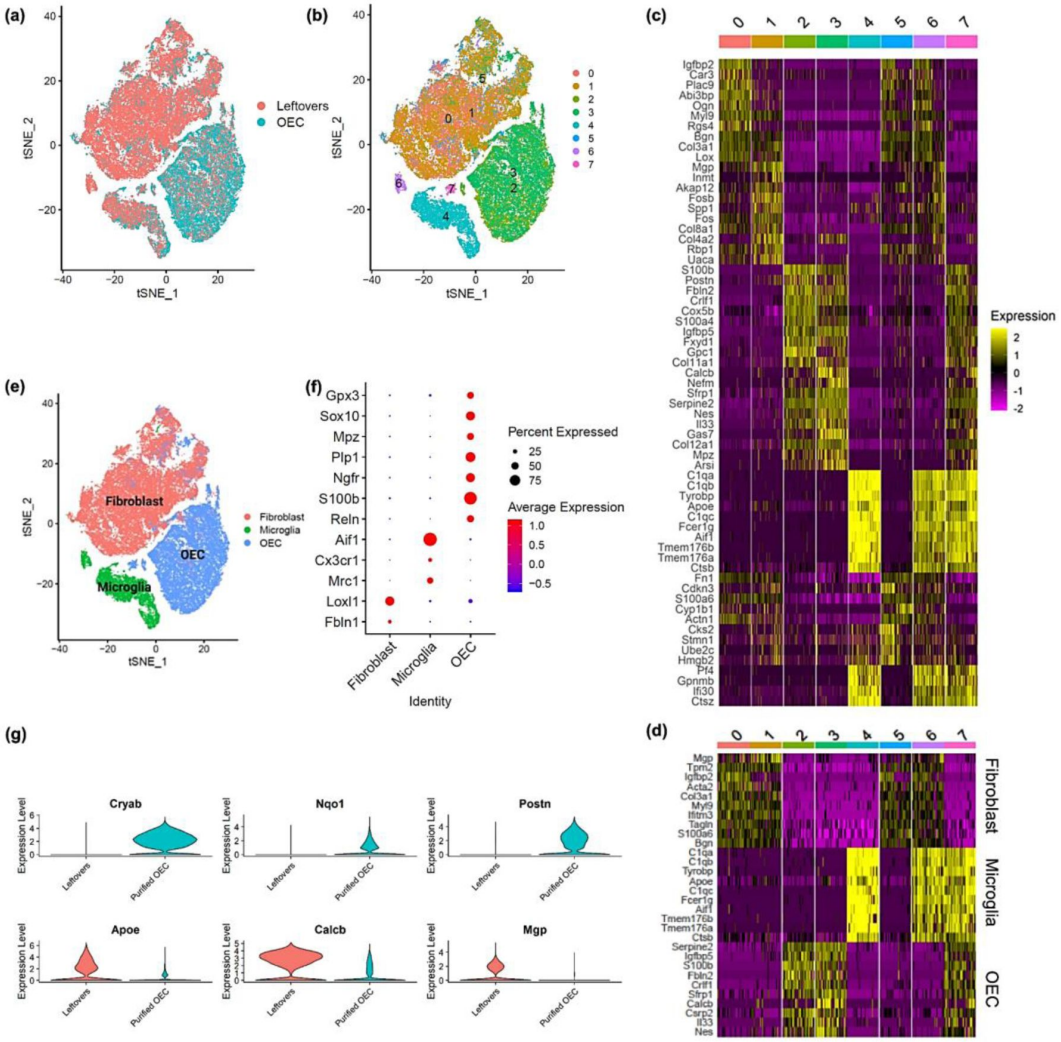

Figure 2.

scRNA-seq results show distinct clusters of OEC and leftover cell samples.

(a) Cells in tSNE plot colored by sample source, with cells from leftover samples in pink and cells from immunopurified OECs in cyan. **(b)** Clustering analysis revealed a total of 7 distinct cell clusters, each indicated with a different color. **(c)** A heatmap showing expression patterns of top marker genes of individual cell clusters. **(d)** Cell clusters showed distinct expression patterns for known cell type markers for fibroblasts, microglia, and OECs. Clusters 0, 1, and 5 had high expression of fibroblast markers; clusters 4, 6, and 7 showed high expression of microglia markers; clusters 2, 3, and 7 showed high expression of OEC markers. Feature plots and violin plots for select marker genes are in Suppl. Figure 4. **(e)** Based on known cell type markers, cell clusters in tSNE plot were labeled with the corresponding cell types. **(f)** The expression of cell-type specific marker genes is depicted in a dot plot. **(g)** Genes that distinguish purified OECs vs OECs in leftover cultures are shown. The top 3 genes were higher in purified OECs, whereas the bottom 3 genes were higher in 'leftover' cultures.

(**Figure 3b**). Next, we detected the high expression of two common glial markers, Blbp (*Fabp7*;**Figure 3c**, **3d**) and S100β (**Figure 3e**, **3f**), in OECs. Sox10 is expressed in the nuclei of neural crest-derived cells such as OECs and Schwann cells, and our cultures showed high expression levels in scRNA-seq and immunocytochemistry (**Figure 3c**, **3g**, **3k**, **3m**). Three cell adhesion molecules, L1-Cam (**Figure 3g**, **3h**; Runyan & Phelps, 2009; Witheford et al., 2013), N-Cam (**Figure 3i**, **3j**), and N-Cadherins (**Figure 3k**, **3l**), are also expressed in purified OEC cultures. In addition, other previously reported OEC genes are now verified by scRNA-seq, including the pro-myelinating Ciliary neurotrophic factor (*Cntf*, **Figure 3n**; Asan et al., 2003; Roet et al., 2011), the laminin receptor α7 integrin (*Itga* 7, **Figure 3o**; (Ingram et al., 2016), and the myelin related gene P0 (*Mpz*,**Figure 3p**; Sasaki et al., 2006). Thus, well-characterized OEC markers were detected by scRNA-seq and most are widely distributed among OEC clusters.

In addition to confirming select genes, we also compared the 209 significant OEC markers identified from our scRNA-seq data with the 309 genes from the meta-analysis of five OEC microarray studies on cultured early-passage OECs versus other tissues (Roet et al., 2011). We found 63 genes overlapping between our OEC markers and the published markers, representing a 15.3-fold overlap enrichment (p-value: 4.3 e-58), further supporting the reliability of our scRNA-seq findings.

Subclustering analysis of OECs revealed refined OEC subtypes and their top marker genes

Next, we extracted OECs and performed a subclustering analysis with OECs alone, and found five distinct subclusters (**Figure 4a**). All subclusters except for cluster 3 expressed previously identified markers of OECs, astrocytes, oligodendrocytes, and Schwann cells (**Figures 4b, c**). Interestingly, cluster 3 showed expression of both microglia and OEC markers. The top 20 genes expressed by each OEC subtype were investigated to evaluate potential functional differences between the subtypes (**Figure 4c**; Suppl. Table 2), and their enriched pathways (**Figure 4d**). Most references for the specific genes within the clusters are found in Suppl. Table 2.

Cluster 0 is rich in matricellular proteins

Pathway enrichment analysis showed that ECM organization and collagen biosynthesis were significantly enriched in the marker genes for the largest OEC subcluster 0 (**Figure 4d**). Two of the top 10 genes are members of the Ccn family of matricellular proteins, *Ccn2*/*Ctgf* (Connective tissue growth factor; **Figure 5a**) and *Ccn3/NOV* (nephroblastoma overexpressed gene; **Figure 5b**). Both are secreted ECM proteins which regulate the activity of growth factors and cytokines, function in wound healing, cell adhesion, and injury repair. The presence of *Ccn3* in OECs is novel, while *Ccn2/Ctgf* is well established (Lamond & Barnett, 2013; Roet et al., 2011). In **figures 5c, c1-2**, we show that *Ccn2* and *Sox10* are highly expressed in our cultured OECs. Because Mokalled et al. (2016) reported that *Ctgfa* is a critical factor required for spontaneous axon regeneration following spinal cord injury site in zebrafish, we asked if GFP-labeled OECs transplanted 2 weeks following a complete spinal cord transection in adult rats, also expressed Ccn2/Ctgf. We found high levels of Ctgf expression in GFP-OECs that bridged much of the injury site and also detected Ccn2 on near-by cells (**Figure 5d, d1-2**). Other top ECM genes are *Serpinf1* (Serine protease inhibitor), *Fbln2* (Fibulin2), *Fn1* (Fibronectin-1), and *Col11a1* (Collagen, type XI, alpha 1; Suppl. Table 2).

The top-ranked marker gene for cluster 0 is *Pmp22* (Peripheral myelin protein 22), a tetraspan membrane glycoprotein that is highly expressed in myelinating Schwann cells, and contributes to the membrane organization of compact peripheral myelin. The third-ranked gene, *Gldn* (Gliomedin) is secreted by Schwann cells and contributes to the formation of the nodes of Ranvier. *Fst* (Follistatin) also plays a role in myelination and is expressed in areas of adult neurogenesis.

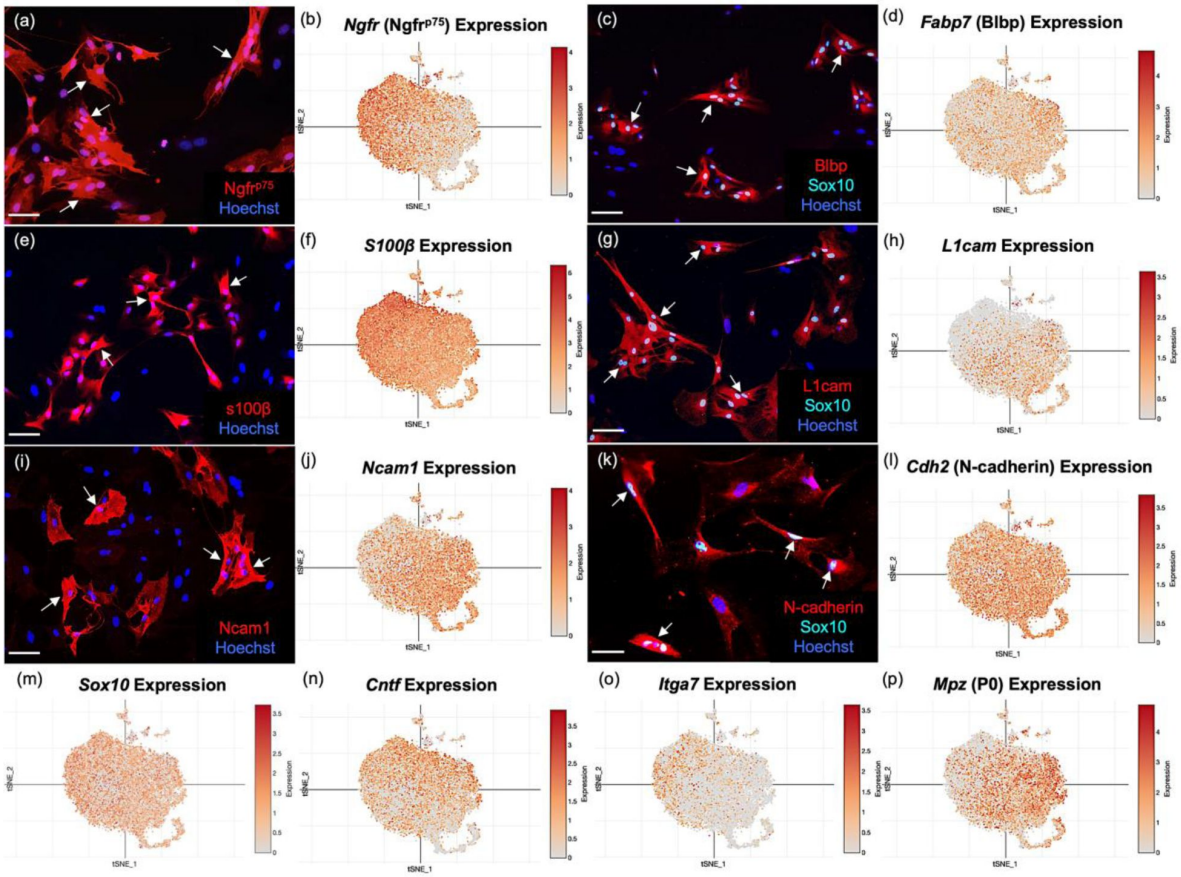

Figure 3.

Well-established OEC markers are revealed by scRNA-seq and immunofluorescence.

OEC cultures were replated from leftover cells prepared for scRNA-seq. Immunolabeled OECs are marked with arrows and all cell nuclei are stained with Hoechst (blue nuclei). tSNE maps of the gene expression in the 5 clusters are shown next to the protein expression in a-l. **(a, b)** Cultured OECs express Ngfr^{p75} protein and *Ngfr^{p75}* gene expression. **(c, d)** Blbp and Sox10 immunoreactive OECs with *Fabp7* gene expression. **(e, f)** S100β-labeled OECs together with *S100β* expression. **(g, h)** L1cam and Sox 10 labeling next to *L1cam* expression. **(i, j)** Ncam1 protein and gene expression**. (k, l)** N-Cadherin and Sox10 markers with *Cdh2* expression. **(m-p)** scRNA-seq data for *Sox10, Cntf, Itga7*, and *Mpz* (references in text). Scale bars: a, c, e, g, i, k = 50 μm.

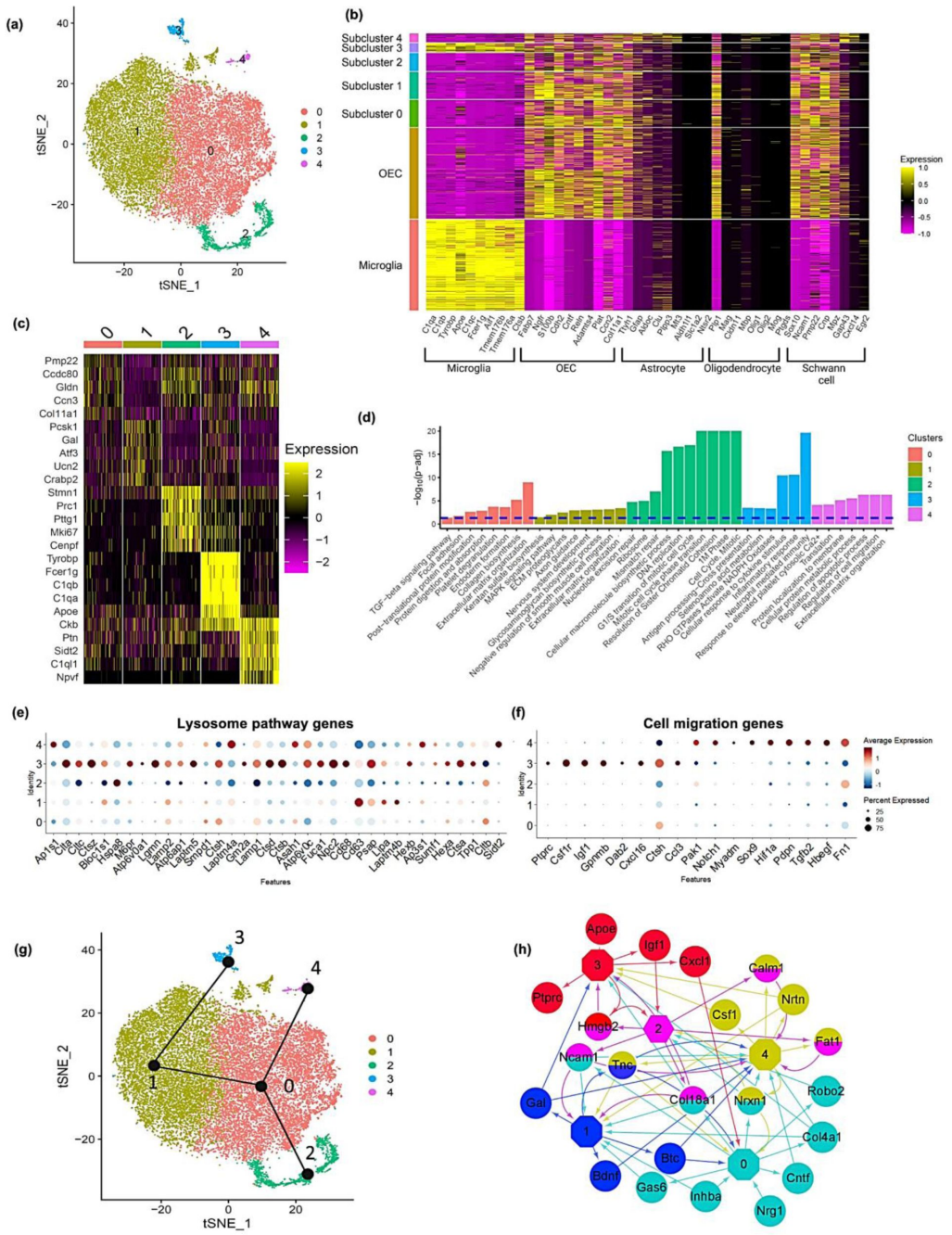

Figure 4.

OEC subclusters, marker genes, and enriched pathways.

(a) Clustering analysis of OECs revealed five separate clusters (0-4). **(b)** Heatmap depicts the expression patterns of known marker genes of glial cell types (x-axis) versus microglia, all OECs and OEC subclusters (y-axis). OEC subclusters express select markers of other glial cell types. **(c)** Heatmap depicts the top five marker genes (y-axis) for each OEC subcluster (x-axis). **(d)** Pathways associated with marker genes of different OEC clusters are shown. The dashed line indicates the false discovery rate (FDR) <0.05 in the pathway analysis. **(e)** Dot plot showing that cluster 3 has higher potential lysosomal function based on lysosome pathway genes than the other clusters. **(f)** Both cluster 3 and 4 express select genes involved in positive regulation of cell migration. **(g)** Trajectory analysis reveals two trajectories, one including subclusters 2, 0, and 4, and another involving subclusters 2, 0, 1, and 3. **(h)** NicheNet ligand-receptor network plot demonstrates intracellular communication between OEC subtypes. Hexagons represent subclusters 0 to 4 and circles indicate ligands secreted from subclusters. Edges (arrows) point to clusters where receptors of the ligands are expressed. Different colors represent different OEC subclusters.

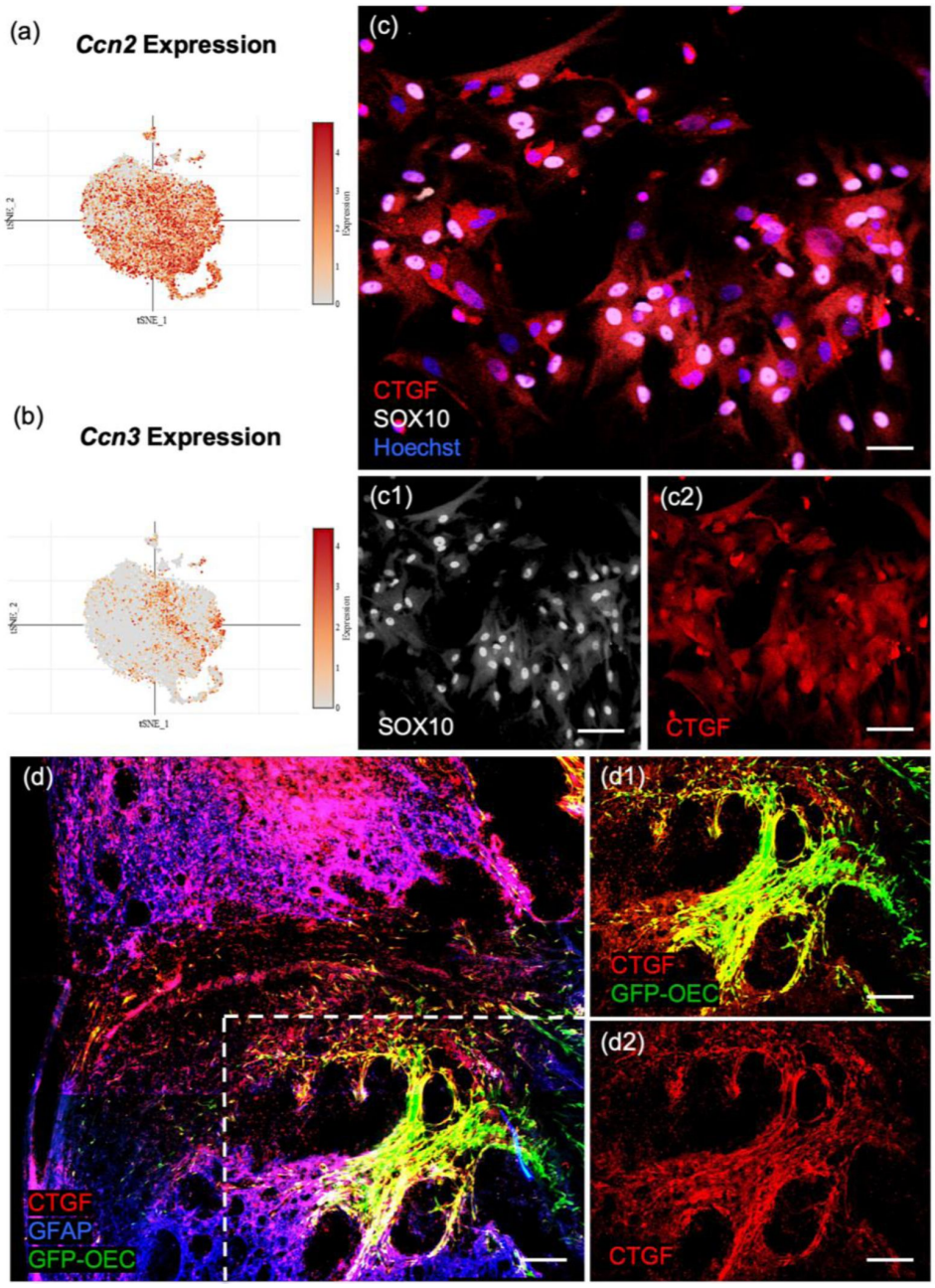

Figure 5.

Confirmation of *Ccn2/Ctgf* (Connective tissue growth factor) in cultured OECs and following implantation after spinal cord injury.

(a, b) The scRNA-seq plots of the 9th (*Ccn2/Ctgf)* and 4th (*Ccn3*/*Nov*) highest-ranked marker genes are strongly expressed in subcluster 0. **(c, c1, c2)** The well-known matricellular protein Connective tissue growth factor (Ctgf) identifies OECs in cell cultures (red, c, c2), and Sox10 nuclear expression (white nuclei, c, c2) confirms they are neural crest-derived cells. **(d, d1, d2)** A sagittal section from a rat that received a complete spinal cord transection followed by Green Fluorescent Protein (GFP)-OEC implantation was fixed 2 weeks postinjury. Glial fibrotic acidic protein (d, Gfap, blue) marks the edges of the borders of the glial scar. GFP-OECs (green) that express Ctgf (red) in the injury site are outlined by the box in d. High expression of Ctgf is detected by GFP-OECs that bridge part of the injury site in d1. The single channel of Ctgf is shown in d2. Scale bars: c-c2 = 50 µm, d-d2 = 250 µm.

Many of the top cluster 0 genes are found in other glial cells: *Fbln2* is secreted by astrocytes, *Ccn2/Ctgf* is expressed by astrocytes and Schwann cells, *Nqo1* (NAD(P)H dehydrogenase, quinone 1 enzyme) is found in Bergmann glia, astrocytes, and oligodendrocytes, and *Marcks* (Myristoylated alanine-rich C-kinase substrate protein) regulates radial glial function and is found in astrocytes and oligodendrocytes. In addition, *Cd200* is expressed by astrocytes and oligodendrocytes, and binds to its receptor, *Cd200R*, on microglial cells. Thus, the OECs in cluster 0 express high levels of genes found in other glia, many of which are related to ECM and myelination.

Cluster 1 contains classic OEC markers

Three of the top five genes in cluster 1, *Pcsk1* (proprotein convertase PC1), *Gal* (neuropeptide and member of the corticotropin-releasing factor family) and *Ucn2* (Urocortin-2) were reported in the meta-analysis by Roet et al. (2011). Galanin is expressed by neural progenitor cells, promotes neuronal differentiation in the subventricular zone and is involved in oligodendrocyte survival.

A well-known oligodendrocyte and Schwann gene associated with myelin, CNP (2-3-cyclic nucleotide 3-phosphodiesterase), was high in OEC cluster 1 in addition to the cytokine *Il11* (Interleukin 11) that is secreted by astrocytes and enhances oligodendrocyte survival, maturation and myelin formation. The chondroitin sulfate proteoglycan Versican (*Vcan*) is another top gene expressed by astrocytes and oligodendrocyte progenitor cells.

Many of the genes and pathways that characterize cluster 1 are involved in nervous system development and axon regeneration (*Atf3*, *Btc*, *Gap43*, *Ngfr* ^{p75}, *Bdnf*, *Pcsk1)*, and are found in other glia (Suppl. Table 2). Interestingly, after nerve injury, *Atf3* (cyclic AMP-dependent transcription factor 3) is upregulated by Schwann cells in the degenerating distal nerve stump and downregulated after axon regeneration is complete (Hunt et al., 2004). *Btc* (Betacellulin), part of the Epidermal growth factor (Egf) family and a ligand for Egfr, also is expressed by Schwann cells after nerve injury in a pattern similar to that of *Atf3*, as is Brain-derived neurotrophic factor (*Bdnf*). In addition, the proprotein convertase PC1 (*Pcsk1*) cleaves pro-Bdnf into its active form in Schwann cells and therefore also contributes to axon outgrowth. Together, the OECs in cluster 1 secrete a number of important growth factors associated with regeneration, including a novel one, *Atf3*, reported here.

Cluster 2 represents a distinctive proliferative OEC subtype

Most top genes in cluster 2 (**Figure 6a-f**) regulate the cell cycle (*Mki67*, *Stmn1*, *Cdk1*, *Cks2*, *Cdkn3*, *Cdca8*, *Cdca3*), are associated with mitosis (*Cenpf*, *Spc24*, *Tpx*, *Ckap2*, *Prc1*, *Ube2c*, *Top2a*), or contribute to DNA replication and repair (*Dut*, *Fam111a*, Suppl. Table 2). The top ranked gene, *Stmn1* (Stathmin 1; **Figure 6e**), is a microtubule destabilizing protein that is widely expressed in other OEC clusters and in areas of adult neurogenesis. Pathway enrichment confirms the proliferative property of cluster 2 with pathways such as G1/S transition of mitotic cell cycle and DNA replication (**Figure 4d**). These results show that there are distinct OEC progenitors in our cultures derived from the outer two layers of the adult olfactory bulb.

MKi67 is a well-known proliferation gene concentrated in cluster 2 (**Figure 6f**). Previous studies suggested the presence of two morphologically distinct OECs – the Schwann-cell-like, spindle-shaped OECs with high Ngfr^{p75} expression and the astrocyte-like, large flat OECs with low Ngfr^{p75} (Franceschini & Barnett, 1996). To determine if the proliferative OECs differ in appearance from adult OECs, we analyzed OECs identified with the anti-Ki67 nuclear marker and anti-Ngfr^{p75}(**Figure 6g-h**). Of the Ki67-positive OECs in our cultures, 24% ± 8% were strongly Ngfr^{p75}-positive and spindle-shaped, whereas 76% ± 8% were flat and weakly Ngfr^{p75}-labeled (n=4 cultures, p= 0.023). Here we show that a large percentage of proliferative OECs are characterized by large, flat morphology and weak Ngfr^{p75} expression.

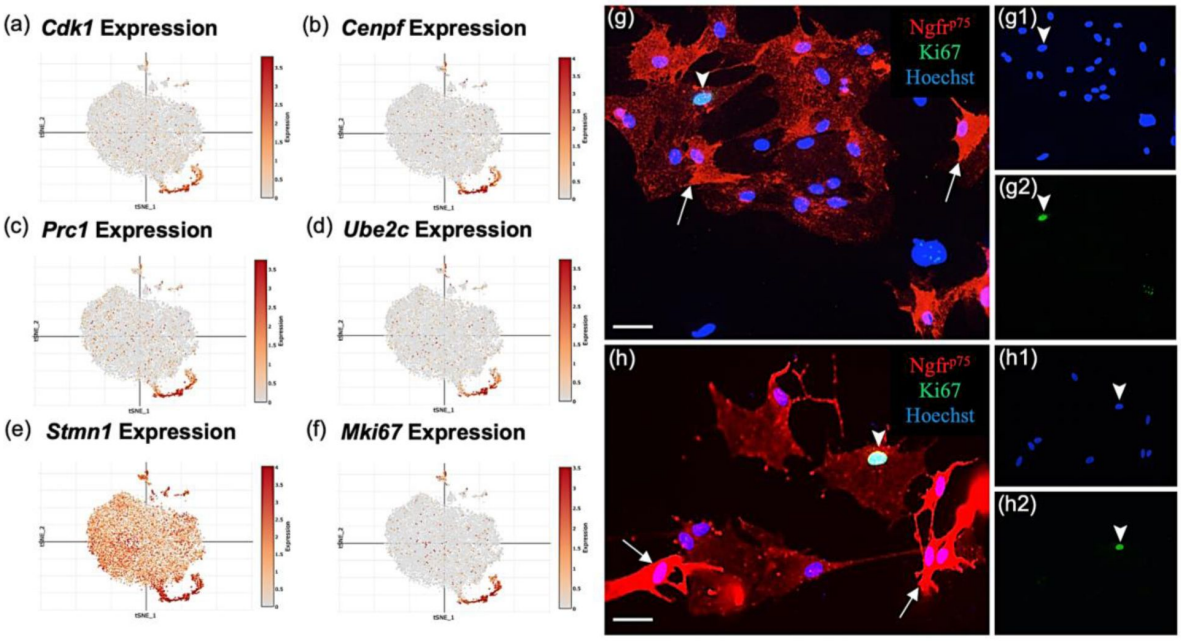

Figure 6.

Subcluster 2 is characterized by cell cycle and proliferative markers.

(a-f) These scRNA-seq plots show high expression of cell proliferation markers in cluster 2, supporting their function as OEC progenitor cells. Only *Stmn1* (Stathmin, a microtubule destabilizing protein) is broadly expressed across all five clusters. **(g-g2, h-h2)** Most OECs in cultures are spindle-shaped and have high Ngfr^{p75} expression (red, white arrows). Cultured OEC progenitors are labeled for Ki67 (g2, h2; green nuclei, arrowheads) and Hoechst (g1, h1; blue nuclei, arrowheads) and have low levels of Ngfr^{p75} expression (red). The Ki67-labeled progenitor cell shown in h and h2 (arrowhead) has low Ngfr^{p75} immunoreactivity and a "flat" morphology. Scale bars: g, h = 50 μm.

Cluster 3 resembles both microglia and OEC

In addition to the typical OEC markers (*Ngfr*^{p75}, *S100b*, and *Sox10*), cluster 3 also expressed numerous microglia markers (**Figure 4b**, Suppl. Table 2). Microglia markers in the top 20 genes include *Tyrobp*, which functions as an adaptor for a variety of immune receptors, *Cst3* (Cystatin C), *Anxa3* (Annexin A3), *Aif1 (*Iba-1), and *Cd68*. OECs are known to endocytose bacteria and degrade axons, and therefore participate in the innate immune function (Khankan et al., 2016; Leung et al., 2008; Nazareth et al., 2015; Vincent et al., 2007). We also observed relatively high expression of genes involved in lysosome functions, such as *Cst3*, *Ctsz*, *Laptm5*, *Ctsb*, and *Lyz2*, compared to those in other OEC clusters (**Figure 4e**). Three additional top genes are involved in the complement system (*C1qA*, *C1qb*, and *C1qc)*, and cluster 3 pathways are highly enriched for inflammatory response and neutrophil immunity (**Figures 4b, d**).

Specialized microglia/macrophage cells are included in cluster 3. Smithson and Kawaja (2010) identified unique monocytes labeled with anti-Iba-1 (*Aif1*) and Annexin A3 (*Anxa3*) in both the olfactory nerve and outer nerve layer of the olfactory bulb, and proposed that they protect the olfactory system against viral invasion into the cranial cavity. We now show that these unique monocytes reported between the bundles of olfactory axons surrounded by OECs (Smithson & Kawaja, 2010), are in fact, a distinct subtype of OECs.

Cluster 4 has characteristics of astrocytes and oligodendrocytes

Cluster 4 is quite small. Its top two marker genes, *Sidt2*, a lysosomal membrane protein that digests macromolecules for reutilization and *Ckb*, a brain-type creatine kinase which fuels ATP-dependent cytoskeletal processes in CNS glia, are found in other OEC clusters. Four top genes, however, appear almost exclusively in cluster 4: 1) *Npvf* (Neuropeptide VF precursor/Gonadotropin-inhibitory hormone) inhibits gonadotropin secretion in several hypothalamic nuclei, and is expressed in the retina, 2) *Stmn2* (Stathmin2) is a tubulin-binding protein that regulates microtubule dynamics, 3) *Vgf* (non-acronymic) is synthesized by neurons and neuroendocrine cells and promotes oligodendrogenesis, and 4) *Mt3* (Metallothionein-3) is a small zinc binding protein associated with growth inhibition and copper and zinc homeostasis (Suppl. Table 2).

Other genes expressed by cluster 4 are either expressed by other glia and/or are ECM molecules. Five genes are associated with both oligodendrocytes and astrocytes *(Ckb, C1ql1, Gria2, Stmn2, Ntrk2*). Among these, the astrocytic gene *C1ql1* is a member of the Complement component 1q family and regulates synaptic connectivity by strengthening existing synapses and tagging inactive synapses for elimination. Astrocytic Thrombospondin 2 (*Thbs2*) also controls synaptogenesis and growth. Other genes reported in astrocytes include *Ptn* (Pleiotrophin), *Igfbp3* (Insulin-like growth factor binding protein 3), and *Mt3*, whereas *Gpm6b* (Glycoprotein M6b) is involved in the formation of nodes of Ranvier in oligodendrocyte and Schwann cell myelin. Cluster 4 has a significant enrichment for genes involved in ECM organization (**Figure 4d**; *Actn1*, *Col81a*, *Bgn*, *Fn1* and *Timp2*), together with clusters 0 and 1, as well as genes involved in positive regulation of cell migration together with cluster 3 (**Figure 4f**; Suppl. Figure 1).

Trajectory and potential interactions among OEC subclusters

Pseudotime trajectory analysis, a widely used approach to predict cell plasticity and lineages based on scRNA-seq data, suggests that there are potential transitions between specific OEC subclusters. Our results show that there are two distinct trajectories (**Figure 4g**). The first trajectory involves clusters 2, 0 and 4, whereas the second involves clusters 2, 0, 1, and 3. Although the directionality of these trajectories is indistinguishable, the fact that cluster 2 is enriched for cell cycle genes and is the converging point of both trajectories, suggests that cluster 2 proliferates into the other OEC clusters. The predicted trajectories based on our scRNAseq data suggest plasticity in the cell clusters.

We also modeled potential autocrine/paracrine ligand-receptor interactions between OEC subclusters using NicheNet (**Figure 4h**). This analysis revealed that clusters 0, 1, and 2 have more ligands going to other OEC clusters, whereas clusters 3 and 4 generally receive ligands from other clusters. The network also showed that clusters 0, 1 and 4 express genes encoding neurotropic factors such as Bdnf, Cntf and Neurturin (*Ntrn*), that are involved in neuronal survival and neurite outgrowth. *Gal* from cluster 1 is a mediator for glia-glia, and glia-neuron communication (Gresle et al., 2015; Ubink et al., 2003) We found that cluster 4 secretes colony stimulating factor 1 (*Csf1*) which also shows potential regulation of cluster 3 through *Csf1r*. Overall, the trajectory and network analyses support potential cell plasticity and lineage relationships and cell-cell communications across OEC subclusters.

Spatial confirmation of defined OEC subclusters within the olfactory nerve layer *in situ*

Here we confirm that some of the top genes derived from cultured OECs clusters are expressed in olfactory bulb sections of 8-10-week-old female Sprague Dawley rats. Our results illustrate the spatial distribution of 2-3 top genes from clusters 0 to 3 in the olfactory nerve layer (**Figure 7**; the corresponding tSNE plots in Suppl. Figure 2 for comparison). In cluster 0 we show high levels of Peripheral myelin protein 22 (Pmp22) in OECs (**Figure 7a**) with the remainder of the olfactory bulb unlabeled. The small protein Gliomedin (Gldn) is a component of Schwann cell microvilli and facilitates the formation of peripheral nodes of Ranvier (Eshed et al., 2005). Here we detect numerous small dot-like structures that overlay the olfactory nerve layer (**Figures 7b, c**). The detection of high levels of Gldn in nonmyelinating OECs without nodes of Ranvier is surprising and suggests that it may also function as a glial ligand associated with olfactory sensory neuron axons. For cluster 1, we examined the expression of the important axonal growth factor Activating transcription factor 3 (Atf3*)* and confirmed that OECs express it widely (**Figure 7d**). Growth associated protein 43 (Gap43), a well-established marker of OECs and immature olfactory sensory neurons, is highly expressed in the olfactory nerve layer and at a lower level in the glomeruli of the olfactory bulb (**Figure 7e**). Nestin (Nes), an intermediate filament associated with neural stem cells, is widely expressed in the olfactory nerve layer (**Figure 7f**).

Compared to the two large clusters, the remaining clusters contain more specialized OECs. The top marker in the proliferative cluster 2 is Stathmin1 (Stmn1), encoding a microtubule destabilizing protein found in areas of adult neurogenesis (Boekhoorn et al., 2014; Suppl. Figure 2). Stathmin 1 immunoreactivity (**Figure 7g**) is detected in all OEC clusters and immature olfactory sensory neurons, and therefore fills the olfactory nerve layer in a pattern similar to that seen in Gap43 (**Figure 7e**). Two markers associated with the cell cycle, Ube2c (**Figure 7h**) and Cdk1 (not shown), identify cluster 2 cells in the olfactory nerve layer, as well as the early progenitor marker Mki67 (**Figure 7i**). Cluster 3 is strongly associated with OEC immune function and the expression of Apolipoprotein E (Apoe), Annexin A3 (Anxa3), and Allograft inflammatory factor 1 (Aif1) were confirmed. Apoe (**Figure 7j**) is detected throughout the olfactory nerve layer and includes some cellular structures. Expression of Anxa3 (**Figure 7k**) and Aif1/Iba1(**Figure 7l**) is similar – distinct small cells labeled in the olfactory nerve layer. Our smallest group, cluster 4, has been difficult to confirm with selected antibodies targeting the top marker genes, likely due to the limited number of cells in this cluster. Overall, our *in-situ* experiments confirmed the presence and distribution of 4 out of the 5 OEC subtypes detected by scRNA-seq.

OECs synthesize and secrete Reelin

Reelin (*Reln*) is detected in OECs in this study (**Figures 2f**, **8a, g**) and reported in previous microarray studies (Guerout et al., 2010; Roet et al., 2011). However, the presence of the *Reln* gene within OECs is disputed in the literature (Dairaghi et al., 2018; Schnaufer et al., 2009). *Reln* codes for a large ECM glycoprotein that is secreted mainly by neurons (D'Arcangelo et al., 1995; Kubasak et al., 2004), but also by Schwann cells (Panteri et al., 2006). The canonical Reelin-signaling pathway, i.e., secreted Reelin binds to the very-low-density lipoprotein receptor

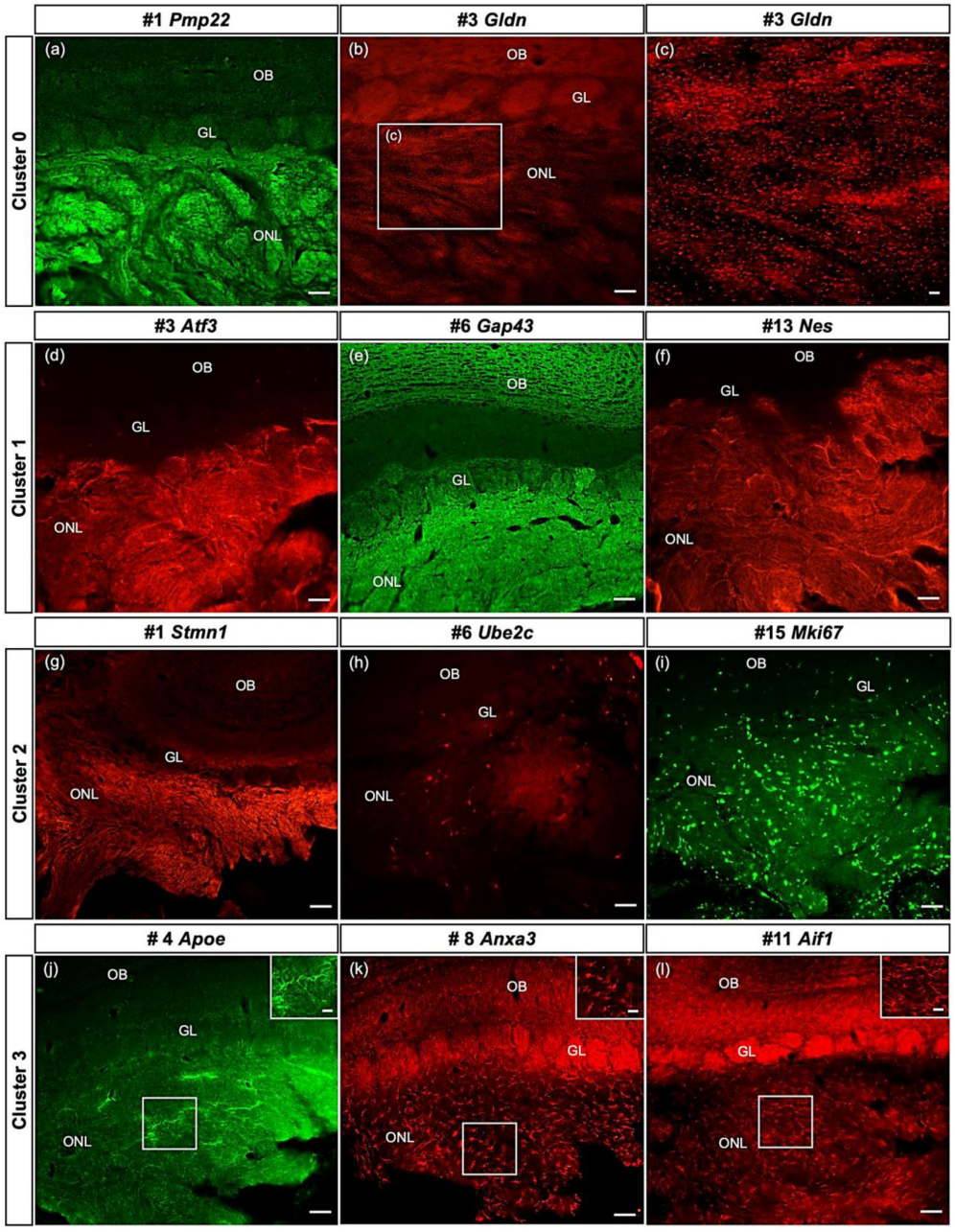

Figure 7.

Spatial confirmation of the defined OECs subclusters within the olfactory nerve layer.

A number of the top 20 genes from this scRNA-seq study of purified OEC cultures are verified in olfactory bulb sections. In all images, the olfactory nerve layer (ONL, layer I) is at the bottom of the image, the glomeruli (GL, layer II) next, and the remainder of the olfactory bulb toward the top. **(a-c)** The top gene in the largest cluster, Peripheral myelin protein 22, is highly expressed throughout the ONL. Gliomedin, the third-ranked gene in cluster 0, is detected as small discrete dot-like structures overlaying the ONL (b, box enlarged in c). **(d-f)** OECs in cluster 1 are immunolabeled by antibodies against the axonal growth factor Atf3 (d) and the intermediate filament Nestin (f). High levels of Gap43 in the ONL (e) are due to expression by OECs and axons of immature olfactory sensory neurons. **(g-i)** Strong immunoreactivity of Stathmin-1 in the ONL reflects labeled axons from immature olfactory sensory neurons and OECs. Ube2c, a G2/M cell cycle regulator, is expressed in a small number of cells in the ONL, whereas Mki67-labeled cells are more widespread. **(j-l)** The immune function of this cluster is confirmed by antibodies against Apoe (j), Anxa3 (k), and Aif1/Iba1 markers expressed by microglia and macrophages. Scale bars: a, b, d-l = 50 μm; c, insets in j-l = 25 μm.

(*Vldlr*) and apolipoprotein E receptor 2 (*Apoer2*) expressed by Disabled-1 (*Dab1*)-positive neurons, is at its highest levels in embryos during neuronal migration, and contributes to neuronal positioning. A recent study claimed that peripheral OECs express high levels of Dab1 and Vldlr (Dairaghi et al., 2018), and that Reelin was expressed only by mesenchymal cells located near the cribriform plate, not in OECs (Dairaghi et al., 2018; Schnaufer et al., 2009). Our scRNA-seq findings of OECs demonstrated low expression levels of *Dab1* and *Apoer2* (**Figure 8b-c**), moderate expression of *Vldlr* (**Figure 8d**) and substantial levels of *Reln* in OEC subclusters 0-3 (**Figure 8a**). Interestingly, OECs also contain proteolytic enzymes that cleave the 400Kd secreted Reelin. Tissue plasminogen activator (tPA) cleaves Reln at its C-terminus (**Figure 8e**), and the matrix metalloproteinase ADAMTS-4 cuts at both the N- and C-cleavage sites of Reln (**Figure 8f**; Krstic et al., 2012).

The confusion about Reelin expression in OECs likely stemmed from the use of standard immunochemical methods to confirm its presence. Our attempts to detect Reelin expression in the adult olfactory nerve layer (ONL) or in cultured OECs were unsuccessful using several antibodies and multiple protocols, despite consistent localization of Reelin within mitral cells of the olfactory bulb. To understand this discrepancy, we examined Reelin expression in embryonic sections which included both the peripheral and OB-OECs. Images of E16.5 $Reln^{+/+}$ and $Reln^{-/-}$olfactory system show Reelin expression in peripheral OECs and in neurons of the olfactory bulb (**Figure 8g**, green), but not on OB-OECs in the ONL or in $Reln^{-/-}$ mice (**Figure 8g, g2, h**). In comparison, Blbp expression is uniformly expressed in both peripheral and OB-OECs (**Figure 8g1, h1**, red). Enlargements of the region where axon bundles from olfactory sensory neurons join the ONL show that Blbp is present in OB-OECs throughout the ONL, but Reelin is detected only on peripheral OECs (**Figure 8g, g1-2**).

To determine if adult rat OB-OECs synthesize and secrete Reelin *in vitro*, we treated primary $Ngfr^{p75}$-purified OECs with or without Brefeldin-A, a fungal metabolite that specifically inhibits protein transport from the endoplasmic reticulum to the Golgi apparatus (Misumi et al., 1986). In Brefeldin-treated primary OEC cultures, spindle-shaped GFP-labeled OECs contain Reelin in their cytoplasm (**Figure 9a**, arrows). Large contaminating Reln-immunonegative cells present in primary cultures serve as an intrinsic control (**Figure 9a**, arrowhead). Reelin expression was also confirmed in Brefeldin-treated $Ngfr^{p75}$-purified OEC cultures (**Figure 9b-c**), a finding which supports our scRNA-seq results.

To further confirm that cultured OECs express and secrete Reelin, we performed western blotting. The G10 antibody recognizes full-length Reelin protein, which is approximately 400 kDa, and its two cleaved fragments at ~300 and ~180 kDa (Lambert de Rouvroit et al., 1999). Both the rat ONL and $Reln^{+/+}$ mouse olfactory bulbs showed the characteristic large molecular weight band at 400 kDa and another at 150 kDa (**Figure 9d**, lanes 1-2, Reln). As expected, these bands were absent in mutant $Reln^{-/-}$ olfactory bulbs (**Figure 9d**, lane 3). Additionally, Reelin-positive bands were present in blots of OEC whole cell lysates (WCL) and OEC conditioned media (CM; **Figure 9d**, lanes 4-8 Reln). A 4-15% gradient gel allowed for the visualization of all three Reelin isoforms (**Figure 9e**). Brain extracts of $Reln^{+/+}$ (positive control) and $Reln^{-/-}$ (negative control) mice confirm the specificity of Reelin detection (**Figure 9e**, lanes 1-2). All three Reelin isoforms at their corresponding molecular weights are present in rat ONL and OEC CM samples (**Figure 9e**, lanes 3-6). These results clearly indicate that cultured OECs derived from the ONL can synthesize and secrete Reelin despite our inability to detect it in the embryonic or adult ONL.

Discussion

Our scRNA-seq study of immunopurified OECs identified five OEC subtypes with distinct gene expression patterns, pathways, and properties of each subtype. The well-established OEC markers, such as *Ngfr* ^{p75}, *S100β*, *Fabp7* (Blbp), and *Sox10*, are widely distributed throughout most clusters

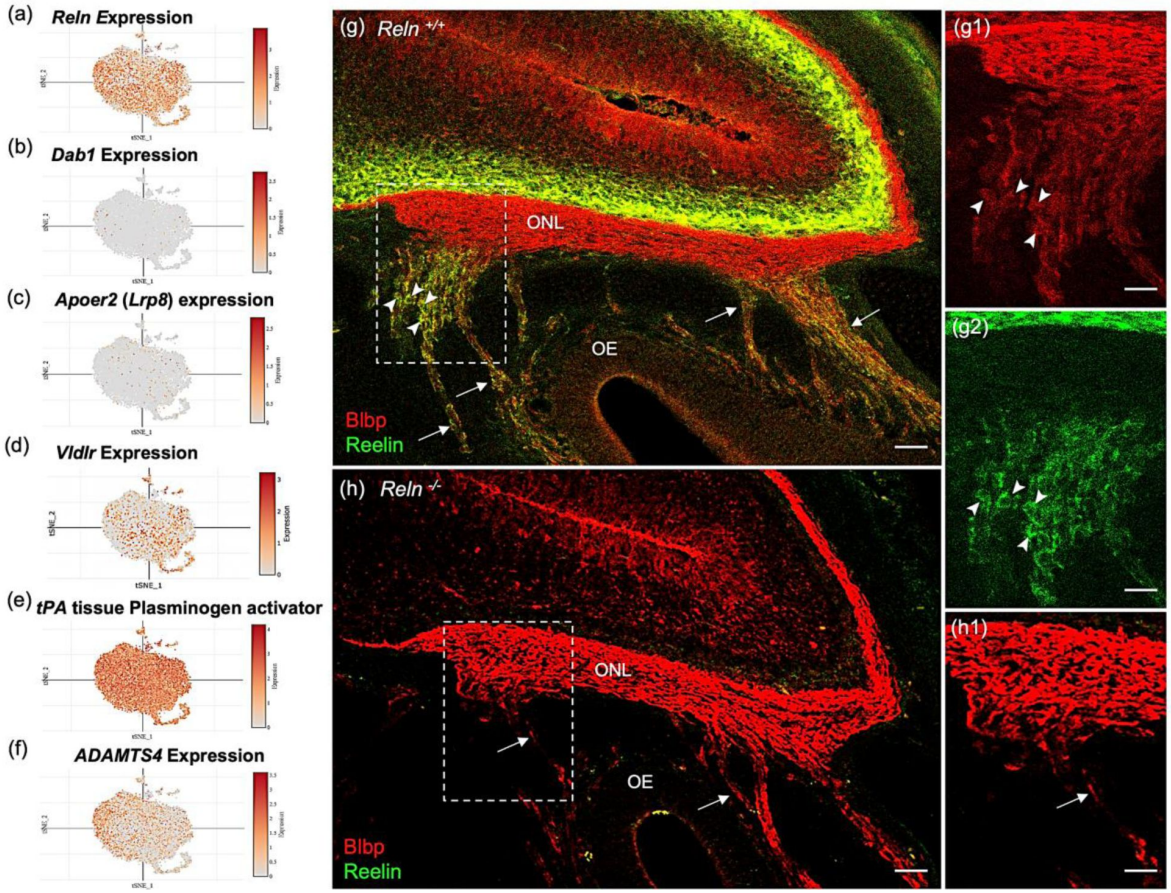

Figure 8.

Reelin-signaling pathway marker genes in OEC clusters and Reelin immunoreactivity in embryonic olfactory system.

(a-f) These six tSNE plots show OEC gene expression levels associated with Reelin signaling. *Reln* is highly expressed in most OEC clusters (a), *Disabled-1* (*Dab1*, b), and *Apolipoprotein E receptor 2* (*Apoer2*, c) show little expression, and *Very-low-density lipoprotein receptor* has moderate expression (*Vldlr*, d). The serine protease tissue Plasminogen Activator (e, *tPA*) is highly expressed in all OEC subsets and cleaves secreted Reelin only at its C-terminus. ADAMTS-4 cleaves both the N- and C-terminals of Reelin, and is expressed at low levels by OECs (f). **(g-g2)** Sagittal sections of the olfactory epithelium (OE) and olfactory bulb from an E16.5 $Reln^{+/+}$mouse immunolabeled for Blbp (g, g1; red) and Reelin (g, g2; green). Axons of olfactory sensory neurons (arrows) are surrounded by peripheral OECs that express both Blbp (g1, arrowheads) and Reelin (g2, arrowheads). High Reln expression is obvious in olfactory bulb neurons (g, g1, green) but the olfactory nerve layer (ONL) only expresses Blbp (g, g2). **(h, h1)** No Reelin is detected in a sagittal section from a $Reln^{-/-}$ mouse, yet Blbp expression in the ONL appears normal. Scale bars: g-h = 50 µm; g1, g2 = 50 µm.

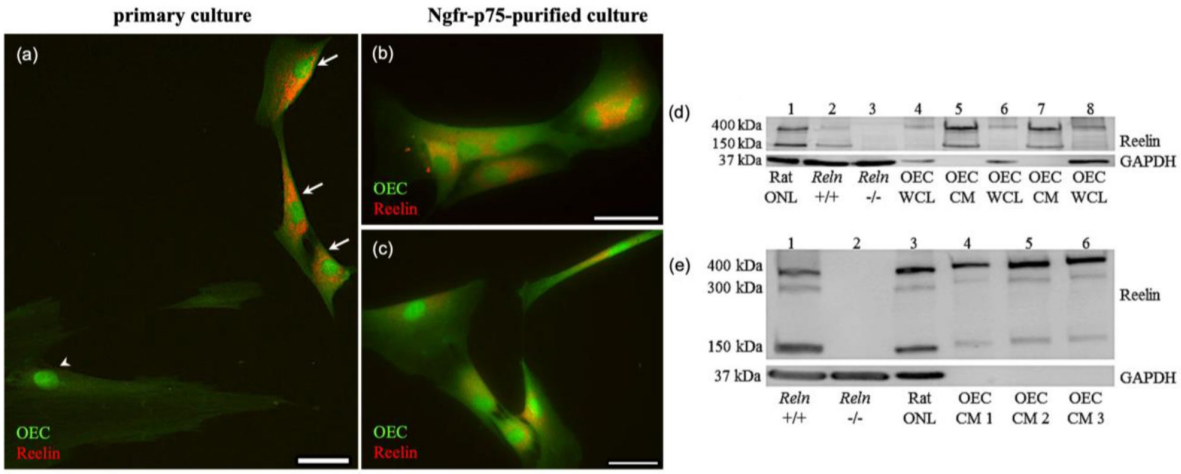

Figure 9.

Reelin is expressed and secreted by olfactory ensheathing cells.

(a-c) Rat OEC cultures were treated with Brefeldin-A to inhibit protein transport and subsequent secretion. Reelin immunoreactivity (red) was detected in GFP-labeled OECs (a, arrows), but not in another cell type (arrowhead) in this primary culture. GFP-labeled OECs which were immunopurified with anti-Ngfr^{p75} also express Reelin (red; b, c). **(d)** Western blot confirms the expression of Reelin in rat olfactory nerve layer and layer II (ONL; lane 1). *Reln*$^{+/+}$and *Reln*$^{-/-}$ olfactory bulbs used as positive and negative controls, respectively (lanes: 2 and 3). OEC whole cell lysates (WCL; lanes: 4, 6, and 8), and OEC conditioned medium (CM; lanes: 5 and 7). **(e)** All three Reelin isoforms (400, 300, and 150 kDa) were visualized using a 4-15% gradient gel. *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mouse cortices were used as controls (lanes: 1 and 2). Reelin was detected in the rat ONL (lane 3) and in three OEC-CM samples (lanes: 4, 5, and 6). GAPDH was the loading control for tissue homogenates (d, lanes 1-4, 6, 8; e, lanes: 1-3). Scale bars: a-c = 40 μm.

derived from cultured OECs. We also experimentally confirmed a number of the top markers in the OEC subtypes on sections of the rat olfactory system and established that OECs secrete both Reelin and Ctgf, ECM molecules reported to be important for neural repair and axon outgrowth. Additional proregenerative OEC marker genes such as *Atf3*, *Btc*, and *Pcsk1* also were identified. Our findings provide an unbiased and in-depth view of the heterogeneity of OEC populations with demonstrated injury repair capacity, and the potential mechanisms that underlie their regenerative and protective properties.

This scRNA-seq analysis confirmed the immunopurification of the OECs compared with the mixture of OECs grown with numerous fibroblasts and microglia. Although some may debate if purified OECs are needed to maximize neural repair, this study suggests that immunopurified OECs have higher expression of anti-stress genes than unpurified controls. Several of our earlier studies, which transplanted similarly purified OECs, also showed evidence of neural regeneration following severe spinal cord injury (Khankan et al., 2016; Takeoka et al., 2011; Thornton et al., 2018). In addition to the purity of OECs, we found significant overlap of the marker genes between our study and those from the meta-analysis of five OEC microarray studies on cultured early-passage OECs versus other tissues (Roet et al., 2011), supporting the reproducibility of our scRNA-seq findings. When we compared our data from cultured OB-OECs to previous scRNA-seq studies of the olfactory system tissue which included peripheral OECs (Durante et al, 2000), or a mixture of peripheral and OB-OECs (Tepe et al., 2018), there was little overlap. Most likely, the substantial differences were due to the dissection areas, techniques used for OEC preparation, the species studied, as well as sex and age of the subjects. It is important to note that our OB-OEC populations were repeatedly shown to induce injury repair, and their subtypes and markers support their diverse reparative functions, whereas the neural repair function of the cell populations in the other scRNA-seq studies is unclear.

OECs represent a hybrid glial cell that promote neural repair

Based on intrinsic gene expression patterns revealed by scRNA-seq data, there is substantial expression of Schwann cell, astrocyte, oligodendrocyte and microglial markers within our five clusters of OECs. We observed transcriptionally distinct clusters that highly expressed markers for proliferation (cluster 2), microglia (cluster 3), and astrocytes and oligodendrocytes (cluster 4). The microglia-like OECs reported by Smithson and Kawaja (2010) resemble our transcriptome-based subtyping of OEC cluster 3 which uniquely identified this subpopulation. Our results further support the activities of OECs in modulating immune responses, based on the expression of genes involved in the endocytosis of bacteria, the regulation of microglia activation, and the expression of numerous lysosomal pathways (Leung et al., 2008). OECs in cluster 4 are distinctive by their expression of several astrocytic markers that reportedly regulate synaptogenesis (Thrombospondin 2) and synaptic connectivity, and remove inactive synaptic connections (Complement component 1q family (Christopherson et al., 2005; Eroglu & Barres, 2010). Our trajectory and network analyses indicate potential relationships between the OEC subclusters, which warrant further experimental testing.

Both OECs and Schwann cells originate from the neural crest, express similar immunological markers, and share many transcriptional similarities (Forni et al., 2011; Vincent et al., 2005). OECs share phenotypic characteristics with myelin-producing Schwann cells and oligodendrocytes, but they more closely resemble Schwann cells (Doucette, 1991; Radtke et al., 2011; Ramon-Cueto & Valverde, 1995). Gliomedin is a required ECM protein that initiates the assembly of nodes of Ranvier along Schwann cells (Eshed et al., 2005), yet despite the fact that OECs do not have nodes of Ranvier, they express high levels of Gliomedin. This novel Gliomedin expression in OECs suggests a possible function as a glial ligand for the unmyelinated axon bundles that OECs ensheath. Following peripheral nerve injury, the loss of contact between axons and myelinating Schwann cells induces a nonmyelinating phenotypic change in Schwann cells (Fu & Gordon, 1997). These dedifferentiated nonmyelinating Schwann cells are proregenerative and upregulate the expression of Glial fibrillary acidic protein (Gfap; (Jessen et al., 1990), $Ngfr^{p75}$

receptor (You et al., 1997), cell adhesion molecules L1 and N-Cam (Martini & Schachner, 1988), and neurotrophic factors, including Bdnf (Meyer et al., 1992) and Glial cell-line derived neurotrophic factor (Trupp et al., 1995). Genes for these proregenerative factors are detected in OECs in the present study, as well as the transcription factor *Mitf*, that controls a network of genes related to plasticity and repair in Schwann cells (Daboussi et al., 2023). Reelin is another developmentally expressed protein detected in immature Schwann cells following sciatic nerve injury (Panteri et al., 2006) that also is found in OECs.

Our *in vitro* and *in vivo* experimental validation of numerous marker genes strongly support their validity. Based on our tissue localization, the two large clusters (0 and 1) were found throughout the olfactory nerve layer, whereas cluster 2 (progenitors) and cluster 3 (microglial) are scattered within the olfactory nerve layer. Future functional studies of these subclusters will benefit from both the marker genes and their localizations uncovered in this study.

Reelin and CTGF are distinctive markers for OECs

Our interest in the function of OEC-secreted Reelin stems from studies using OEC transplants as a therapy following spinal cord injury. OEC transplantation into the spinal cord may provide an exogenous source of Reelin that could phosphorylate the Dab1-containing neurons in the spinal cord, such as sympathetic and parasympathetic preganglionic neurons, and somatic motor neurons, and axons of Dab1-expressing projection neurons, such as the corticospinal pathway (Abadesco et al., 2014; Phelps et al., 2002). Further investigation of the role of OEC-secreted Reelin should increase our understanding of the mechanisms by which OECs mediate repair following spinal cord injury.

Mammals show little evidence of spontaneous axonal regeneration across a spinal cord injury site, in contrast to lower organisms such as fish. Mokalled et al. (2016) reported that glial secretion of Ctgfα/Ccn2 was both necessary and sufficient to stimulate a glial bridge for substantial axon regeneration across the zebrafish transection site. Cell populations reported in the injury site that express Ctgf are ependymal cells, endothelial cells, and reactive astrocytes (Conrad et al., 2005; Mokalled et al., 2016; Schwab et al., 2001). Here we show that OECs contribute to glial bridge formation in rats. The most consistent finding among our severe SCI studies combined with OEC transplantation is the extent of remodeling of the injury site and axons growing into the inhibitory lesion site together with OECs and astrocytes. The formation of a glial bridge across the injury was critical to the spontaneous axon generation seen in zebrafish (Mokalled et al., 2016) and likely contributed to the axon regeneration detected in our OEC transplanted, transected rats (Khankan et al., 2016; Takeoka et al., 2011; Thornton et al., 2018). As spinal cord injury transplant procedures are further enhanced and OEC survival improves, these hybrid glial cells should be re-examined due to their strong proregenerative characteristics.

Conclusion

To understand how immunopurified OECs repair spinal cord injuries, we carried out an unbiased scRNA-seq analysis and found that OECs have a wide range of subtypes including a proliferative state, microglia-like cells and neural regenerative clusters. We demonstrated that these multi-functional OECs are clearly distinguishable from each other by expression of unique genes and pathways within subtypes based on the scRNAseq data and our *in vivo* tissue studies. In addition, these subtypes of OECs produce important ECM genes, including *Reln* and *Ctgf* and many other signaling molecules which are involved in a wide range of intercellular communication as well as axon regeneration. Our single-cell resolution investigation offers a comprehensive molecular landscape of OECs and numerous novel targets for future injury repair and functional/mechanistic studies.

Material and Methods

Animals

All of the experimental procedures used on animals were approved by the Chancellor's Animal Research Committee at UCLA and carried out in accordance with the National Institutes of Health guidelines. Animals were housed in the Terasaki Life Science Building vivarium under standard conditions with free access to food and water.

Sprague-Dawley rats

We bred our male GFP-expressing Sprague-Dawley rats (Perry et al, 1999) with unlabeled female Sprague-Dawley rats (Charles Rivers Laboratory), which were used previously to obtain OECs for two of our spinal cord injury studies (Khankan et al., 2016; Thornton et al., 2018) in order to unambiguously identify the transplanted GFP-positive OECs near the injury sites. A total of 8, 8-10-week-old GFP-labeled female rats were used to obtain GFP-OECs for scRNA-seq. An additional 4, 8-10-week GFP-labeled rats were used to isolate Reelin protein for western blotting and 6, 8-10-week unlabeled female Sprague-Dawley rats were used to confirm gene expression in the five OEC subtypes. An overdose of ketamine-xylazine or pentobarbital was used for euthanasia before either the rat olfactory bulbs were removed or the rats were perfused with 4% paraformaldehyde or 2% PLP (2% Paraformaldehyde; 0.075M Lysine-HCL-monohydride; 0.010M Sodium periodate; 0.1M sodium phosphate) followed by a 1-2 hr postfix.

Reeler mice

To study Reelin expression in OECs, heterozygous ($Reln^{+/-}$) mice originally purchased from the Jackson Laboratory (B6C3Fe-*ala*-$Reln^{rl}$, Bar Harbor, ME) were used to establish a breeding colony. Genotypes of these mice were identified by PCR (D'Arcangelo et al., 1996). To obtain timed pregnancies, $Reln^{+/-}$ mice were bred and checked every morning for plugs. Pregnant mice were heavily anesthetized with sodium pentobarbital (100 mg/kg) and embryos were extracted at embryonic day 16.5. Heads were immersed in 2% PLP, washed in PB, cryoprotected, and embedded in Shandon M1 embedding matrix (Thermo Scientific, 1310).

Preparation of olfactory bulb-derived OEC cultures from rats and mice

Olfactory bulbs were collected from 8-10-week-old GFP-labeled Sprague-Dawley rats or $Reln^{+/+}$ and $Reln^{-/-}$ mice. The leptomeninges were removed to reduce fibroblast contamination. Methods to prepare OEC primary cultures were adapted from Ramon-Cueto et al. (2000) and follow those reported by Runyan and Phelps (2009) for mouse OECs and Khankan et al. (2015) for rat OECs. For scRNA-seq, this protocol was replicated on two separate dates and used a total of 8 adult female Sprague-Dawley rats. Primary cultures were generated from both olfactory bulbs from each rodent. The first two layers of the olfactory bulb were dissected, isolated, and then washed in Hank's balanced salt solution (HBSS, Gibco, Rockville, MD) prior to tissue centrifugation at 365 g for 5 mins. The tissue pellet was resuspended in 0.1% trypsin and HBSS without Ca^{2+}/Mg^{2+} (Gibco, Rockville, MD), then placed in a 37°C water bath, and mixed intermittently for 10 mins. A mixture of 1:1 Dulbecco's modified eagle medium (DMEM) and Ham's F12 (D/F medium, Gibco) supplemented with 10-15% Fetal Bovine Serum (FBS, Hyclone, Logan, UT) and 1% Penicillin/Streptomycin (P/S, Gibco; D/F-FBS-P/S medium) was used to inactivate trypsin prior to centrifugation. Dissociated cells were rinsed, centrifuged 3 times, and plated into 12.5 cm^2 culture flasks pre-coated with 0.05mg/ml poly-L-lysine (PLL, Sigma, St. Louis, MO). Cells were maintained at 37°C for 5-8 days and D/F-FBS-P/S medium was changed every 2 days.

Immunopurification was carried out using hydrophobic petri dishes coated overnight with Biotin-SP-conjugated AffiniPure goat anti-rabbit IgG (1:1000; Jackson ImmunoResearch, West Grove, PA) in 50 mM Tris buffer at 4°C followed by another overnight incubation at 4°C with either mouse anti-Ngfr^{p75} for rat OECs (Suppl. Table 1, Chandler et al., 1984) or rabbit anti-Ngfr^{p75} for mouse OECs (Suppl. Table 1) in 25mM Phosphate-buffered saline (PBS). Dishes were rinsed 3 times with 25 mM PBS and treated with a mixture of PBS and 0.5% Bovine serum albumin (BSA) for 1 hr at room temperature. Prior to the addition of cells, antibody-treated dishes were washed with PBS and DMEM.

In preparation for immunopanning, two pairs of adult rat olfactory bulbs (for scRNA-seq) or four pairs of mouse bulbs (for western blots) were dissociated with 0.25% trypsin-EDTA at 37°C for 3 mins and D/F-FBS-P/S medium was added to inactivate trypsin. Following a medium rinse and centrifugation, resuspended cells were seeded onto pre-treated mouse or rabbit anti-Ngfr^{p75} petri dishes and incubated at 37°C for 10 mins. Unbound rat cells were removed with medium and saved as 'leftover' control cells for scRNA-seq. A cell scraper was used to recover bound cells that were then subjected to a second round of immunopanning in which purified cells from 2 rats on each experimental day were combined yielding 2 samples of purified Ngfr^{p75}-positive rat OECs and 2 samples of 'leftover' control cells. All cells for sequencing were replated into PLL-coated flasks. Immunopurified Ngfr^{p75}-positive mouse OECs were plated on PLL-coated 4-chamber polystrene-vessel culture slides (BD Falcon, San Jose, CA) or used for western blots. Purified OECs and 'leftover' controls were incubated at 37°C with 5% CO_2 for 7-8 days in D/F-FBS-P/S medium supplemented with a mitogen mixture of pituitary extract (20 µg/ml, Gibco) and forskolin (2 µM, Sigma). Media was changed every 2 days and mitogens were withdrawn 2 days prior to harvesting these cells for scRNA-seq.

Brefeldin-A treatment

Rat OECs that contained GFP could be visualized directly. Primary and purified GFP-expressing OEC cultures were rinsed 3 times with D/F medium prior to treatment with Brefeldin-A to block protein transport from the endoplasmic reticulum to the Golgi apparatus and thus block secretion. Brefeldin-A (5 µg/ml; Epicenter, Madison, WI) was mixed with D/F medium and added to OEC cultures for 2 hrs at 37°C while the control cultures received D/F medium only. Cultures were fixed with cold 4% paraformaldehyde in 0.1 M PB for 15 mins at RT, rinsed 3 times with PBS, and stored in PBS with sodium azide at 4°C until immunostaining.

Detection of Reelin expression in rat and mouse OEC cultures, tissue sections and western blots

Cultured rat OECs treated with or without Brefeldin-A were labeled with mouse anti-Reelin G10 and rabbit anti-Ngfr^{p75} (Suppl. Table 1). Culture slides were rinsed with phosphate-buffered saline (PBS; 0.1M phosphate buffer; 0.9% NaCl), blocked with 5% normal goat serum for 1 h, and incubated with the appropriate primary antibodies overnight. The following day, slides were rinsed 3 times with PBS, incubated with species-appropriate Alexa Fluor 594 and/or 647 (1:500, 1:100; Jackson ImmunoResearch) for 1 hr at RT, and then cover slipped with Fluorogel (Electron Microscopy Sciences, Hatfield, PA).

Sagittal sections of the mouse E16.5 olfactory system were cut 40 µm thick, slide mounted, and stored in PB with 0.06% sodium azide. For Reelin and Blbp detection, sections were incubated overnight at RT in goat-anti-Reelin (Suppl. Table 1), rinsed thoroughly, and incubated with donkey anti-goat 488 (1:100; Jackson Immunoresearch, #705-545-0030). Sections were then incubated in rabbit polyclonal anti-Blbp (Suppl. Table 1) overnight followed by 1 hr in donkey anti-rabbit 555 (1:800; Life Technologies, #A31572) and coverslipped with Fluorogel. Confocal images were

obtained from a Zeiss Laser Scanning Microscope (LSM800) with solid-state lasers 488 and 561 nm for double-labeled images. Sections from Khankan et al., 2016 were sectioned sagittally at 25 µm thick.

Tissue harvesting, protein isolation, and western blotting

Olfactory bulbs from adult *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mice and olfactory nerve layers from adult Sprague Dawley rats were freshly dissected as described for primary OEC cultures. Olfactory tissue was collected, placed on ice, and homogenized in Ripa lysis buffer plus protease inhibitor cocktail (Sigma). Protein concentrations of brain and olfactory extracts were determined using the RC DC Protein Assay kit (Bio-Rad, Hercules, CA) as described (Miller et al., 2008). Whole-cell lysates and conditioned medium (CM) were obtained from purified OEC cultures maintained in D/F medium. CM was collected from OEC cultures before cells were incubated with Ripa lysis buffer and collected with a cell scraper. Protein samples were stored at −80°C.

Protein samples (50 µg) were heated to 90–100°C for 3 mins, resolved on 10% SDS-PAGE or 4–15% mini-protean TGX gels (Bio-Rad) run in Tris-Glycine-SDS (TGS) buffer (Bio-Rad) at 60V, and then transferred onto 0.22 µm PVDF/nitrocellulose membranes (Bio-Rad) in TGS with 20% methanol at 25V overnight at 4°C. Precision plus protein standards in dual color (Bio-Rad) were used to determine molecular weight. Membranes were cut, blocked with 5% non-fat dry skimmed milk (Bio-Rad) in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hr at room temperature on a shaker, and then incubated overnight at 4°C TBST plus 2.5% milk containing anti-Reelin G10 or loading control mouse anti-GAPDH antibodies (Suppl. Table 1). Following primary antibody incubation, blots were washed 3 times in TBST for 10 mins while shaking and then probed with horseradish peroxidase-conjugated secondary antibodies anti-mouse IgG (1:2,000 for Reelin, 1:5,000 for GAPDH) in TBST. Immunoblots were developed using a chemiluminescence HRP detection kit (GE Healthcare, Piscataway, NJ,) and imaged with a Typhoon scanner (GE Healthcare).

Single-cell RNA-sequencing

Cell collection and culture

Flasks with purified OECs and leftover cells were rinsed with HBSS and dissociated with trypsin-EDTA as above. Cells were washed in D/F-FBS-P/S medium and further resuspended with siliconized pipettes. Media and cells were washed and centrifuged at 365 *g* for 10 min, and resuspended. After the final wash, cells for sequencing were resuspended in PBS+BSA in volumes of 1200 cells/µL. On each of 2 different culture days, 4 flasks of cells (2 purified OECs and 2 'leftovers') were generated for scRNA-seq, yielding 8 samples in total (4 purified OECs and 4 'leftovers'). To confirm our scRNA-seq results directly with immunocytochemistry, we plated extra cells from the second preparation on PLL-coated coverslips in 24-well plates with 500µL D/F-FBS-P/S medium. Medium was changed at 2 days, cultures were fixed at 3 days in cold 4% paraformaldehyde for 20 mins, washed 3X with PB and stored in PB with 0.06% sodium azide.

scRNA-seq was performed using the 10X Genomics Chromium scRNA-seq system which incorporates microfluidic capture-based encapsulation, barcoding, and library preparation (10X Genomics, Pleasanton, CA). About 10,000 cells per OEC or leftover preparation were loaded into a Chromium Chip B along with partitioning oil, reverse transcription reagents, and a mix of hydrogel beads containing 3,500,000 unique 10X barcodes. Paired-end sequencing was performed on a Novaseq S4 system, using the v3 Illumina platform, at 20-50K reads/cell. The paired-end reads were processed using the Cell Ranger pipeline and *Rattus norvegicus* (Rnor_6.0) from Ensembl as the reference genome (Yates et al., 2020) to produce a gene expression matrix. Among the 8 samples, one OEC sample showed a low fraction of reads and RNA content in cells, which may indicate high level of ambient RNA (Supple. **Figure 3a**). This sample was removed from further analysis, leaving 3 OEC samples and 4 leftover samples for downstream analysis.

scRNA-seq data quality control, normalization, and integration

Gene expression matrix from each of the 7 remaining samples that passed quality control was loaded into the Seurat R package ver 3.0 (Stuart et al., 2019). Cells were called based on the number of genes (200-5000), unique molecular identifiers (UMIs; 5000-20000), and percent of mitochondrial genes (<20%; Suppl. Figure 3b). After quality control, log normalization was performed within each sample using *NormalizeData* function with default parameters. Top 2,000 variable genes were selected using *FindVariableFeatures*. The seven samples were integrated together with *FindIntegrationAnchors* and *IntegrateData* functions which incorporate canonical correlation analysis to align cells with similar transcriptomic patterns across samples.

Cell clustering and cell type identification

The integrated Seurat object was used for principal component analysis (PCA). The top 15 PCs were used to construct the k-nearest neighbor graph, followed by Louvain algorithm to cluster cells based on similar gene expression patterns. Cell clusters were visualized using tSNE plots.

To assign cell type identities to each cluster, marker genes of each cluster were found using *FindAllMarkers* with average log fold change > 0.5 and minimum percent difference > 0.25. Cell cluster marker genes were subsequently compared to known OEC cell type markers curated from previous studies that used different species, sex, age, dissection areas, and cell preparation methods (Durante et al., 2020; Tepe et al., 2018). Cell clusters whose marker genes match known cell type markers were labeled with the corresponding cell type identity. Gene expression heatmaps of top markers were generated to visualize the distinct expression patterns of the marker genes for different cell clusters.

OEC subtype identification

A subset of cells expressing well-known OEC markers (e.g., *Ngfr*^{p75}, *S100β*, and *Sox10*) was labeled as OECs and then selected for further subclustering analysis to identify potential OEC subtypes. To do this, OECs from the original Seurat object were extracted using the *subset* function and the top 10 PCs of each OEC subset were used for clustering analysis and tSNE visualization. Marker genes for each OEC subcluster were identified using the *FindAllMarkerGene* function. Pseudotime trajectory analysis across the OEC subtypes was performed using Slingshot version 1.8.0 (Street et al., 2018).

Pathway enrichment analysis

To annotate the biological functions of the marker genes of individual cell clusters or OEC subclusters, we carried out pathway enrichment analysis using EnrichR ver 3.0, where KEGG, Reactome, and GO Biological Process were used as reference pathway databases (Chen et al., 2013). Top pathways were ranked by multiple-testing adjusted P-values.

Potential cell-cell ligand-receptor interactions across OEC subtypes

In order to characterize potential interactions mediated by secreted ligand-receptor pairs between subtypes of OECs, we implemented NicheNet (Browaeys et al., 2020), which curates ligand-receptor interactions from various publicly available resources. The NicheNet ligand-receptor model was integrated with markers from each OEC subtype to find potentially activated ligand-receptor pairs. Differentially expressed ligands identified by markers from source OEC subtypes and activated receptors identified by NicheNet in target OEC subtypes were used to assess ligand-receptor interaction. Ligand-receptor interaction networks between cell types were visualized using Cytoscape (Shannon et al., 2003).

Confirmation of OEC gene expression *in vitro* and *in vivo*

Immunocytochemistry experiments to confirm gene expression revealed by scRNA-seq were conducted on extra cultured OECs and 'leftover' controls which were replated and cultured for 3 additional days. OECs samples were treated with the following primary antibodies against OEC marker genes identified from scRNA-seq with experimental parameters recorded in Suppl. Table 1; 1) polyclonal and monoclonal anti-Blbp, 2) anti-N-Cadherins, 3) anti-green fluorescent protein, 4) a proliferative marker anti-Ki67, 5) anti-L1, 6) anti-NCAM, 7) monoclonal anti-Ngfr^{p75}, 8) anti-S100β, and 9) anti-SOX10. Prior to adding the primary antibodies, the cells were incubated in 0.3% Triton-X 100 detergent in PBS. Cells were blocked in 5% normal donkey serum (Jackson ImmunoResearch Laboratories, # 017-000-121) with 0.3% Triton-X before adding the primary antibodies and left to incubate at RT overnight on the rotator. Following multiple washes, species-appropriate secondary antibodies with fluorescence for 488, 555, and 647 were used to visualize the immunoreaction (1:200 – 1:500, Jackson ImmunoResearch Labs donkey-anti-chicken-488, # 703-545-155; donkey-anti-rabbit-647, # 711-605-152; donkey-anti-mouse-647, # 715-605-150; Life Technologies, donkey-anti-mouse-555, # A31570; donkey-anti-rabbit-555, # A31572). Cultures were counterstained with Hoechst (Bis-benzimide, 1:500, Sigma-Aldrich, # B2261) and coverslipped with EverBrite medium (Biotium, # 23001).

Experiments were carried out to confirm gene expression of OEC subtype markers in the 8-10-week-old olfactory system of rats. Soft tissues and the jaw were removed from fixed heads that were decalcified in undiluted formic acid bone decalcifier (Immunocal, Decal Chemical Corps, Tallman, NY) for 12 hrs., washed, and then infiltrated with increasing concentrations of 5% to 20% sucrose for 24 hr. Heads in 20% sucrose were heated in a 37°C oven and then placed in a gelatin sucrose mixture for 4 hrs, embedded in warm gelatin sucrose in a plastic mold, allowed to gel, frozen on dry ice and stored at −70°C. Heads were sectioned coronally at 40 µm thick, and mounted in series on 16 glass slides. Several antibodies from each of the 4 OEC clusters were chosen to determine their distribution in olfactory bulb sections. Primary antibodies used are listed in Suppl. Table 1, together with the experimental parameters used. For most antibodies, the olfactory bulb sections first underwent a heat-induced antigen retrieval step with 10mM citric acid at pH 6 for 5 min and then were transferred to 1.5% NDS and 0.1% Triton-X-100 in TBS-BSA (0.1M Tris; 1.4% NaCl; 0.1% BSA) for 1 h followed by overnight incubation in primary antibody at RT. Following multiple washes, species-appropriate secondary antibodies with fluorescence for 488 and 555 were used to visualize the immunofluorescence (IF).

For experiments carried out using a Tyramide Signal Amplification (TSA) kit, sections were first incubated in 1% hydrogen peroxide and 0.1% sodium azide in phosphate-buffered saline PBS for 30 mins followed by 10% NDS and 0.1% Triton X-100 in PBS for 1 hr. Next, the sections were incubated with Avidin-Biotin solution (Vector laboratories; kit #SP-2001) before an overnight incubation with the primary antibody at RT. The following day, sections were washed in PBS followed by TNT buffer (0.1M Tris-HCl; 0.15M NaCl; 0.05% Tween) followed by 1 hr incubation with biotinylated horse anti-goat IgG (1:1000; Vector laboratories; #BA-9500), biotinylated donkey anti-rabbit IgG (1:1000; Jackson Immunoresearch; #711-065-152), or biotinylated donkey anti-mouse IgG (1:1000; Jackson Immunoresearch; #715-065-150) in TSA-specific blocking buffer (TNB; 0.1M Tris-HCl; 0.15M NaCl; 0.5% Blocking reagent; PerkinElmer; #FP1020). Sections were then washed with TNT followed by a 1 h incubation with streptavidin-conjugated horseradish peroxidase (1:1000; PerkinElmer; #NEL750001EA) in TNB, and a 10-min incubation with TSA Plus Fluorescein (1:150; PerkinElmer; #NEL741001KT) or TSA Plus Cyanine 3 (Cy3; 1:100; PerkinElmer; #NEL744001KT). Sections were imaged on an Olympus AX70 microscope with a Zeiss AxioCam HRcRv.2 camera. Images were transferred to Adobe Photoshop for assembly, cropping, and adjustment of brightness/contrast to create the compiled figures.

Morphological analyses of OEC subtypes

Ki67 analysis

To determine if OEC progenitor cells marked with Ki67 immunoreactivity have a distinctive morphology, purified and fixed OEC cultures from 4 rats were processed with anti-Ngfr^{p75}, anti-Ki67 and counterstained with Hoechst (Bis-benzimide, 1:500, Sigma-Aldrich, #B2261). Images were acquired from 7-10 randomly selected fields/sample using an Olympus AX70 microscope and Zen image processing and analysis software (Carl Zeiss). We distinguished the larger, flat 'astrocyte-like' OECs from the smaller, fusiform 'Schwann cell-like' OECs, and recorded their expression of Ngfr^{p75} and Ki67. Cell counts from each field were averaged per rat and then averaged into a group mean ± SEM. A Student t-test was conducted to compare the effect of Ngfr^{p75}-labeled cell morphology and the proliferative marker Ki67. Statistical significance was determined by $p < 0.05$.

Acknowledgements

This work was supported by the National Institute of Neurological Disorders and Stroke: RO1 NS076976 (PEP), R01 NS111378 (XY), R01 NS117148 (XY), and by NICHD of the National Institutes of Health under award number P50HD103557. Additional support came from the UCLA QCBio Collaboratory Postdoc Fellowship (SMH), Eureka fellowship (RRK), MARC U*STAR fellowship T34 GM008563 (GJ), and UCLA Graduate Year Fellowship (KLID). We thank Drs. Julie Miller and Stephanie White for their generosity and considerable assistance with western blots, Drs. Hui Zhong and VR Edgerton for their long-term collaboration on spinal cord injury studies, and Sun Young Lee for excellent technical assistance.

Data availability

Raw scRNA-seq data and expression matrix is available at GEO (accession number GSE215247). The scRNA-seq results also can be viewed interactively at the Single Cell Portal. OEC vs Leftovers: *https://singlecell.broadinstitute.org/single_cell/study/SCP1237/leftovers-and-oecs* . OEC subclusters:*https://singlecell.broadinstitute.org/single_cell/study/SCP1489/oec-subcluster-batch-corrected*

Conflict of Interest

The authors declare no competing interests.

References

Abadesco A. D., Cilluffo M., Yvone G. M., Carpenter E. M., Howell B. W., Phelps P. E (2014) **Novel Disabled-1-expressing neurons identified in adult brain and spinal cord** *Eur J Neurosci* **39**:579–592 https://doi.org/10.1111/ejn.12416

Asan E., Langenhan T., Holtmann B., Bock H., Sendtner M., Carroll P (2003) **Ciliary neurotrophic factor in the olfactory bulb of rats and mice** *Neuroscience* **120**:99–112 https://doi.org/10.1016/s0306-4522(03)00211-2

Boekhoorn K., van Dis V., Goedknegt E., Sobel A., Lucassen P. J., Hoogenraad C. C. (2014) **The microtubule destabilizing protein stathmin controls the transition from dividing neuronal precursors to postmitotic neurons during adult hippocampal neurogenesis** *Dev Neurobiol* **74**:1226–1242 https://doi.org/10.1002/dneu.22200

Browaeys R., Saelens W., Saeys Y (2020) **NicheNet: modeling intercellular communication by linking ligands to target genes** *Nat Methods* **17**:159–162 https://doi.org/10.1038/s41592-019-0667-5

Burda J. E., Sofroniew M. V (2014) **Reactive gliosis and the multicellular response to CNS damage and disease** *Neuron* **81**:229–248 https://doi.org/10.1016/j.neuron.2013.12.034

Chandler C. E., Parsons L. M., Hosang M., Shooter E. M (1984) **A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells** *J Biol Chem* **259**:6882–6889

Chen E. Y., Tan C. M., Kou Y., Duan Q., Wang Z., Meirelles G. V., Clark N. R., Ma'ayan A (2013) **Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool** *BMC Bioinformatics* **14** https://doi.org/10.1186/1471-2105-14-128

Christopherson K. S., Ullian E. M., Stokes C. C., Mullowney C. E., Hell J. W., Agah A., Lawler J., Mosher D. F., Bornstein P., Barres B. A (2005) **Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis** *Cell* **120**:421–433 https://doi.org/10.1016/j.cell.2004.12.020

Chung R. S., Woodhouse A., Fung S., Dickson T. C., West A. K., Vickers J. C., Chuah M. I (2004) **Olfactory ensheathing cells promote neurite sprouting of injured axons in vitro by direct cellular contact and secretion of soluble factors** *Cell Mol Life Sci* **61**:1238–1245 https://doi.org/10.1007/s00018-004-4026-y

Conrad S., Schluesener H. J., Adibzahdeh M., Schwab J. M (2005) **Spinal cord injury induction of lesional expression of profibrotic and angiogenic connective tissue growth factor confined to reactive astrocytes, invading fibroblasts and endothelial cells** *J Neurosurg Spine* **2**:319–326 https://doi.org/10.3171/spi.2005.2.3.0319

Cregg J. M., DePaul M. A., Filous A. R., Lang B. T., Tran A., Silver J (2014) **Functional regeneration beyond the glial scar** *Exp Neurol* **253**:197–207 https://doi.org/10.1016/j.expneurol.2013.12.024

D'Arcangelo G., Miao G. G., Chen S. C., Soares H. D., Morgan J. I., Curran T (1995) **A protein related to extracellular matrix proteins deleted in the mouse mutant reeler** *Nature* **374**:719–723 https://doi.org/10.1038/374719a0

D'Arcangelo G., Miao G. G., Curran T (1996) **Detection of the reelin breakpoint in reeler mice** *Brain Res Mol Brain Res* **39**:234–236 https://doi.org/10.1016/0169-328x(96)00046-0

Daboussi L., Costaguta G., Gullo M., Jasinski N., Pessino V., O'Leary B., Lettieri K., Driscoll S., Pfaff S. L (2023) **Mitf is a Schwann cell sensor of axonal integrity that drives nerve repair** *Cell Rep* **42** https://doi.org/10.1016/j.celrep.2023.113282

Dairaghi L., Flannery E., Giacobini P., Saglam A., Saadi H., Constantin S., Casoni F., Howell B. W., Wray S (2018) **Reelin Can Modulate Migration of Olfactory Ensheathing Cells and Gonadotropin Releasing Hormone Neurons via the Canonical Pathway** *Front Cell Neurosci* **12** https://doi.org/10.3389/fncel.2018.00228

Dixie K. L. I (2019) **Transplantation of olfactory ensheathing cells combined with epidural stimulation and climb training as a long-term treatment for severe spinal cord injury in rodents** *UCLA. ProQuest ID, Dixie_ucla_0031D_18445. Merritt ID: ark:/13030/m5ps32vp*

Doucette R (1990) **Glial influences on axonal growth in the primary olfactory system** *Glia* **3**:433–449 https://doi.org/10.1002/glia.440030602

Doucette R (1991) **PNS-CNS transitional zone of the first cranial nerve** *J Comp Neurol* **312**:451–466 https://doi.org/10.1002/cne.903120311

Durante M. A., Kurtenbach S., Sargi Z. B., Harbour J. W., Choi R., Kurtenbach S., Goss G. M., Matsunami H., Goldstein B. J (2020) **Single-cell analysis of olfactory neurogenesis and differentiation in adult humans** *Nat Neurosci* **23**:323–326 https://doi.org/10.1038/s41593-020-0587-9

Eroglu C., Barres B. A (2010) **Regulation of synaptic connectivity by glia** *Nature* **468**:223–231 https://doi.org/10.1038/nature09612

Eshed Y., Feinberg K., Poliak S., Sabanay H., Sarig-Nadir O., Spiegel I., Bermingham J. R., Peles E (2005) **Gliomedin mediates Schwann cell-axon interaction and the molecular assembly of the nodes of Ranvier** *Neuron* **47**:215–229 https://doi.org/10.1016/j.neuron.2005.06.026

Forni P. E., Taylor-Burds C., Melvin V. S., Williams T., Wray S (2011) **Neural crest and ectodermal cells intermix in the nasal placode to give rise to GnRH-1 neurons, sensory neurons, and olfactory ensheathing cells** *J Neurosci* **31**:6915–6927 https://doi.org/10.1523/JNEUROSCI.6087-10.2011

Franceschini I. A., Barnett S. C (1996) **Low-affinity NGF-receptor and E-N-CAM expression define two types of olfactory nerve ensheathing cells that share a common lineage** *Dev Biol* **173**:327–343 https://doi.org/10.1006/dbio.1996.0027

Fu S. Y., Gordon T (1997) **The cellular and molecular basis of peripheral nerve regeneration** *Mol Neurobiol* **14**:67–116 https://doi.org/10.1007/BF02740621

Granger N., Blamires H., Franklin R. J., Jeffery N. D (2012) **Autologous olfactory mucosal cell transplants in clinical spinal cord injury: a randomized double-blinded trial in a canine translational model** *Brain* **135**:3227–3237 https://doi.org/10.1093/brain/aws268

Graziadei P. P., Monti Graziadei G. A (1985) **Neurogenesis and plasticity of the olfactory sensory neurons** *Ann N Y Acad Sci* **457**:127–142 https://doi.org/10.1111/j.1749-6632.1985.tb20802.x

Gresle M. M. *et al.* (2015) **Galanin is an autocrine myelin and oligodendrocyte trophic signal induced by leukemia inhibitory factor** *Glia* **63**:1005–1020 https://doi.org/10.1002/glia.22798

Guerout N., Derambure C., Drouot L., Bon-Mardion N., Duclos C., Boyer O., Marie J. P (2010) **Comparative gene expression profiling of olfactory ensheathing cells from olfactory bulb and olfactory mucosa** *Glia* **58**:1570–1580 https://doi.org/10.1002/glia.21030

Hunt D., Hossain-Ibrahim K., Mason M. R., Coffin R. S., Lieberman A. R., Winterbottom J., Anderson P. N (2004) **ATF3 upregulation in glia during Wallerian degeneration: differential expression in peripheral nerves and CNS white matter** *BMC Neurosci* **5** https://doi.org/10.1186/1471-2202-5-9

Ingram N. T., Khankan R. R., Phelps P. E (2016) **Olfactory ensheathing cells express alpha7 integrin to mediate their migration on laminin** *PLoS One* **11** https://doi.org/10.1371/journal.pone.0153394

Jessen K. R., Morgan L., Stewart H. J., Mirsky R (1990) **Three markers of adult non-myelin-forming Schwann cells, 217c(Ran-1), A5E3 and GFAP: development and regulation by neuron-Schwann cell interactions** *Development* **109**:91–103 https://doi.org/10.1242/dev.109.1.91

Khankan R. R., Griffis K. G., Haggerty-Skeans J. R., Zhong H., Roy R. R., Edgerton V. R., Phelps P. E (2016) **Olfactory ensheathing cell transplantation after a complete spinal cord transection mediates neuroprotective and immunomodulatory mechanisms to facilitate regeneration** *J Neurosci* **36**:6269–6286 https://doi.org/10.1523/JNEUROSCI.0085-16.2016

Khankan R. R., Wanner I. B., Phelps P. E (2015) **Olfactory ensheathing cell-neurite alignment enhances neurite outgrowth in scar-like cultures** *Exp Neurol* **269**:93–101 https://doi.org/10.1016/j.expneurol.2015.03.025

Krstic D., Rodriguez M., Knuesel I (2012) **Regulated proteolytic processing of Reelin through interplay of tissue plasminogen activator (tPA), ADAMTS-4, ADAMTS-5, and their modulators** *PLoS One* **7** https://doi.org/10.1371/journal.pone.0047793

Kubasak M. D., Brooks R., Chen S., Villeda S. A., Phelps P. E (2004) **Developmental distribution of reelin-positive cells and their secreted product in the rodent spinal cord** *J Comp Neurol* **468**:165–178 https://doi.org/10.1002/cne.10946

Lakatos A., Barnett S. C., Franklin R. J (2003) **Olfactory ensheathing cells induce less host astrocyte response and chondroitin sulphate proteoglycan expression than Schwann cells following transplantation into adult CNS white matter** *Exp Neurol* **184**:237–246 https://doi.org/10.1016/s0014-4886(03)00270-x

Lakatos A., Franklin R. J., Barnett S. C (2000) **Olfactory ensheathing cells and Schwann cells differ in their in vitro interactions with astrocytes** *Glia* **32**:214–225 https://doi.org/10.1002/1098-1136(200012)32:3<214::aid-glia20>3.0.co;2-7

Lambert de Rouvroit C., de Bergeyck V., Cortvrindt C., Bar I., Eeckhout Y., Goffinet A. M. (1999) **Reelin, the extracellular matrix protein deficient in reeler mutant mice, is processed by a metalloproteinase** *Exp Neurol* **156**:214–217 https://doi.org/10.1006/exnr.1998.7007

Lamond R., Barnett S. C (2013) **Schwann cells but not olfactory ensheathing cells inhibit CNS myelination via the secretion of connective tissue growth factor** *J Neurosci* **33**:18686–18697 https://doi.org/10.1523/JNEUROSCI.3233-13.2013

Leung J. Y., Chapman J. A., Harris J. A., Hale D., Chung R. S., West A. K., Chuah M. I (2008) **Olfactory ensheathing cells are attracted to, and can endocytose, bacteria** *Cell Mol Life Sci* **65**:2732–2739 https://doi.org/10.1007/s00018-008-8184-1

Li Y., Li D., Raisman G (2005) **Interaction of olfactory ensheathing cells with astrocytes may be the key to repair of tract injuries in the spinal cord: the 'pathway hypothesis'** *J Neurocytol* **34**:343–351 https://doi.org/10.1007/s11068-005-8361-1

Martini R., Schachner M (1988) **Immunoelectron microscopic localization of neural cell adhesion molecules (L1, N-CAM, and myelin-associated glycoprotein) in regenerating adult mouse sciatic nerve** *J Cell Biol* **106**:1735–1746 https://doi.org/10.1083/jcb.106.5.1735

Meyer M., Matsuoka I., Wetmore C., Olson L., Thoenen H (1992) **Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF mRNA** *J Cell Biol* **119**:45–54 https://doi.org/10.1083/jcb.119.1.45

Miller J. E., Spiteri E., Condro M. C., Dosumu-Johnson R. T., Geschwind D. H., White S. A (2008) **Birdsong decreases protein levels of FoxP2, a molecule required for human speech** *J Neurophysiol* **100**:2015–2025 https://doi.org/10.1152/jn.90415.2008

Misumi Y., Misumi Y., Miki K., Takatsuki A., Tamura G., Ikehara Y (1986) **Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes** *J Biol Chem* **261**:11398–11403

Mokalled M. H., Patra C., Dickson A. L., Endo T., Stainier D. Y., Poss K. D (2016) **Injury-induced ctgfa directs glial bridging and spinal cord regeneration in zebrafish** *Science* **354**:630–634 https://doi.org/10.1126/science.aaf2679

Nazareth L., Lineburg K. E., Chuah M. I., Tello Velasquez J., Chehrehasa F., St John J. A., Ekberg J. A (2015) **Olfactory ensheathing cells are the main phagocytic cells that remove axon debris during early development of the olfactory system** *J Comp Neurol* **523**:479–494 https://doi.org/10.1002/cne.23694

Nazareth L., Shelper T. B., Chacko A., Basu S., Delbaz A., Lee J. Y. P., Chen M., St John J. A., Ekberg J. A. K (2020) **Key differences between olfactory ensheathing cells and Schwann cells regarding phagocytosis of necrotic cells: implications for transplantation therapies** *Sci Rep* **10** https://doi.org/10.1038/s41598-020-75850-8

Panteri R., Mey J., Zhelyaznik N., D'Altocolle A., Fà A. D., Gangitano C. M., Marino R., Lorenzetto E., Buffelli M., Keller F. (2006) **Reelin is transiently expressed in the peripheral nerve during development and is upregulated following nerve crush** *Mol. Cell. Neurosci* **32**:133–142 https://doi.org/10.1016/j.mcn.2006.03.004

Phelps P. E., Rich R., Dupuy-Davies S., Rios Y., Wong T (2002) **Evidence for a cell-specific action of Reelin in the spinal cord** *Dev Biol* **244**:180–198 https://doi.org/10.1006/dbio.2002.0580

Radtke C., Sasaki M., Lankford K. L., Gallo V., Kocsis J. D (2011) **CNPase expression in olfactory ensheathing cells** *J Biomed Biotechnol* **2011** https://doi.org/10.1155/2011/608496

Ramon-Cueto A., Cordero M. I., Santos-Benito F. F., Avila J (2000) **Functional recovery of paraplegic rats and motor axon regeneration in their spinal cords by olfactory ensheathing glia** *Neuron* **25**:425–435 https://doi.org/10.1016/s0896-6273(00)80905-8

Ramon-Cueto A., Valverde F (1995) **Olfactory bulb ensheathing glia: a unique cell type with axonal growth-promoting properties** *Glia* **14**:163–173 https://doi.org/10.1002/glia.440140302

Roet K. C., Bossers K., Franssen E. H., Ruitenberg M. J., Verhaagen J (2011) **A meta-analysis of microarray-based gene expression studies of olfactory bulb-derived olfactory ensheathing cells** *Exp Neurol* **229**:10–45 https://doi.org/10.1016/j.expneurol.2011.03.001

Ruitenberg M. J., Plant G. W., Hamers F. P., Wortel J., Blits B., Dijkhuizen P. A., Gispen W. H., Boer G. J., Verhaagen J (2003) **Ex vivo adenoviral vector-mediated neurotrophin gene transfer to olfactory ensheathing glia: effects on rubrospinal tract regeneration, lesion size, and functional recovery after implantation in the injured rat spinal cord** *J Neurosci* **23**:7045–7058

Runyan S. A., Phelps P. E (2009) **Mouse olfactory ensheathing glia enhance axon outgrowth on a myelin substrate in vitro** *Exp Neurol* **216**:95–104 https://doi.org/10.1016/j.expneurol.2008.11.015

Sasaki M., Black J. A., Lankford K. L., Tokuno H. A., Waxman S. G., Kocsis J. D (2006) **Molecular reconstruction of nodes of Ranvier after remyelination by transplanted olfactory ensheathing cells in the demyelinated spinal cord** *J Neurosci* **26**:1803–1812 https://doi.org/10.1523/JNEUROSCI.3611-05.2006

Schnaufer C., Breer H., Fleischer J (2009) **Outgrowing olfactory axons contain the Reelin receptor VLDLR and navigate through the Reelin-rich cribriform mesenchyme** *Cell Tissue Res* **337**:393–406 https://doi.org/10.1007/s00441-009-0762-5

Schwab J. M., Beschorner R., Nguyen T. D., Meyermann R., Schluesener H. J (2001) **Differential cellular accumulation of connective tissue growth factor defines a subset of reactive astrocytes, invading fibroblasts, and endothelial cells following central nervous system injury in rats and humans** *J Neurotrauma* **18**:377–388 https://doi.org/10.1089/089771501750170930

Shannon P., Markiel A., Ozier O., Baliga N. S., Wang J. T., Ramage D., Amin N., Schwikowski B., Ideker T (2003) **Cytoscape: a software environment for integrated models of biomolecular interaction networks** *Genome Res* **13**:2498–2504 https://doi.org/10.1101/gr.1239303

Smithson L. J., Kawaja M. D (2010) **Microglial/macrophage cells in mammalian olfactory nerve fascicles** *J Neurosci Res* **88**:858–865 https://doi.org/10.1002/jnr.22254

Street K., Risso D., Fletcher R. B., Das D., Ngai J., Yosef N., Purdom E., Dudoit S (2018) **Slingshot: cell lineage and pseudotime inference for single-cell transcriptomics** *BMC Genomics* **19** https://doi.org/10.1186/s12864-018-4772-0

Stuart T., Butler A., Hoffman P., Hafemeister C., Papalexi E., Mauck W. M, Hao Y., Stoeckius M., Smibert P., Satija R. (2019) **Comprehensive integration of single-cell data** *Cell* **177**:1888–1902 https://doi.org/10.1016/j.cell.2019.05.031

Su Z., Chen J., Qiu Y., Yuan Y., Zhu F., Zhu Y., Liu X., Pu Y., He C (2013) **Olfactory ensheathing cells: the primary innate immunocytes in the olfactory pathway to engulf apoptotic olfactory nerve debris** *Glia* **61**:490–503 https://doi.org/10.1002/glia.22450

Tabakow P. *et al.* (2014) **Functional regeneration of supraspinal connections in a patient with transected spinal cord following transplantation of bulbar olfactory ensheathing cells with peripheral nerve bridging** *Cell Transplant* **23**:1631–1655 https://doi.org/10.3727/096368914X685131

Takeoka A. *et al.* (2011) **Axon regeneration can facilitate or suppress hindlimb function after olfactory ensheathing glia transplantation** *J Neurosci* **31**:4298–4310 https://doi.org/10.1523/JNEUROSCI.4967-10.2011

Tepe B., Hill M. C., Pekarek B. T., Hunt P. J., Martin T. J., Martin J. F., Arenkiel B. R (2018) **Single-Cell RNA-Seq of Mouse Olfactory Bulb Reveals Cellular Heterogeneity and Activity-Dependent Molecular Census of Adult-Born Neurons** *Cell Rep* **25**:2689–2703 https://doi.org/10.1016/j.celrep.2018.11.034

Thornton M. A. *et al.* (2018) **Evidence of axon connectivity across a spinal cord transection in rats treated with epidural stimulation and motor training combined with olfactory ensheathing cell transplantation** *Exp Neurol* **309**:119–133 https://doi.org/10.1016/j.expneurol.2018.07.015

Trupp M., Ryden M., Jornvall H., Funakoshi H., Timmusk T., Arenas E., Ibanez C. F (1995) **Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons** *J Cell Biol* **130**:137–148 https://doi.org/10.1083/jcb.130.1.137

Ubink R., Calza L., Hokfelt T (2003) **'Neuro'-peptides in glia: focus on NPY and galanin** *Trends Neurosci* **26**:604–609 https://doi.org/10.1016/j.tins.2003.09.003

Vincent A. J., Choi-Lundberg D. L., Harris J. A., West A. K., Chuah M. I (2007) **Bacteria and PAMPs activate nuclear factor kappaB and Gro production in a subset of olfactory ensheathing cells and astrocytes but not in Schwann cells** *Glia* **55**:905–916 https://doi.org/10.1002/glia.20512

Vincent A. J., West A. K., Chuah M. I (2005) **Morphological and functional plasticity of olfactory ensheathing cells** *J Neurocytol* **34**:65–80 https://doi.org/10.1007/s11068-005-5048-6

Wanner I. B., Anderson M. A., Song B., Levine J., Fernandez A., Gray-Thompson Z., Ao Y., Sofroniew M. V (2013) **Glial scar borders are formed by newly proliferated, elongated astrocytes that interact to corral inflammatory and fibrotic cells via STAT3-dependent mechanisms after spinal cord injury** *J Neurosci* **33**:12870–12886 https://doi.org/10.1523/JNEUROSCI.2121-13.2013

Williams S. K., Franklin R. J., Barnett S. C (2004) **Response of olfactory ensheathing cells to the degeneration and regeneration of the peripheral olfactory system and the involvement of the neuregulins** *J Comp Neurol* **470**:50–62 https://doi.org/10.1002/cne.11045

Windus L. C., Claxton C., Allen C. L., Key B., St John J. A (2007) **Motile membrane protrusions regulate cell-cell adhesion and migration of olfactory ensheathing glia** *Glia* **55**:1708–1719 https://doi.org/10.1002/glia.20586

Witheford M., Westendorf K., Roskams A. J (2013) **Olfactory ensheathing cells promote corticospinal axonal outgrowth by a L1 CAM-dependent mechanism** *Glia* **61**:1873–1889 https://doi.org/10.1002/glia.22564

Woodhall E., West A. K., Chuah M. I (2001) **Cultured olfactory ensheathing cells express nerve growth factor, brain-derived neurotrophic factor, glia cell line-derived neurotrophic factor and their receptors** *Brain Res Mol Brain Res* **88**:203–213 https://doi.org/10.1016/s0169-328x(01)00044-4

Yates A. D. *et al.* (2020) **Ensembl 2020** *Nucleic Acids Res* **48**:D682–D688 https://doi.org/10.1093/nar/gkz966

You S., Petrov T., Chung P. H., Gordon T (1997) **The expression of the low affinity nerve growth factor receptor in long-term denervated Schwann cells** *Glia* **20**:87–100 https://doi.org/10.1002/(sici)1098-1136(199706)20:2

Editors

Reviewing Editor
Alyssa Wilson
Icahn School of Medicine at Mount Sinai, New York, United States of America

Senior Editor
K VijayRaghavan
National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bangalore, India

Joint Public Review:

Summary

This manuscript explores the transcriptomic identities of olfactory ensheathing cells (OECs), glial cells that support life-long axonal growth in olfactory neurons, as they relate to spinal cord injury repair. The authors show that transplantation of cultured, immunopurified rodent OECs at a spinal cord injury site can promote injury-bridging axonal regrowth. They then characterize these OECs using single-cell RNA sequencing, identifying five subtypes and proposing functional roles that include regeneration, wound healing, and cell-cell communication. They identify one progenitor OEC subpopulation and also report several other functionally relevant findings, notably, that OEC marker genes contain mixtures of other glial cell type markers (such as for Schwann cells and astrocytes), and that these cultured OECs produce and secrete Reelin, a regrowth-promoting protein that has been disputed as a gene product of OECs.

This manuscript offers an extensive, cell-level characterization of OECs, supporting their potential therapeutic value for spinal cord injury and suggesting potential underlying repair mechanisms. The authors use various approaches to validate their findings, providing interesting images that show the overlap between sprouting axons and transplanted OECs, and showing that OEC marker genes identified using single-cell RNA sequencing are present in vivo, in both olfactory bulb tissue and spinal cord after OEC transplantation.

Despite the breadth of information presented, however, further quantification of results and explanation of experimental approaches would be needed to support some of the authors' claims. Additionally, a more thorough discussion is needed to contextualize their findings relative to previous work.

(1) Important quantification is lacking for the data presented. For example, multiple figures include immunohistochemistry or immunocytochemistry data (Figures 1, 5, 6), but they are presented without accompanying measures like fractions of cells labeled or comparisons against controls. As a result, for axons projecting via OEC bridges in Figure 1, it is unclear how common these bridges are in the presence or absence of OECs. For Figure 6., it is unclear whether cells having an alternative OEC morphology coincide with progenitor OEC subtype marker genes to a statistically significant degree. Similar quantification is missing in other types of data such as Western blot images (Fig. 9) and OEC marker gene data (for which p-values are not reported; Table S2).

The addition of quantitative measures and, where appropriate, statistical comparisons with p-values or other significance measures, would be important for supporting the authors' claims and more rigorously conveying the results.

(2) Some aspects of the experimental design that are relevant to the interpretation of the results are not explained. For example, OECs appear to be collected from only female rats, but the potential implications of this factor are not discussed.

Additionally, it is unclear from the manuscript to what degree immunopurified cells are OECs as opposed to other cell types. The antibody used to retain OECs, nerve growth factor receptor p75 (Ngfr-p75), can also be expressed by non-OEC olfactory bulb cell types including astrocytes [1-3]. The possible inclusion of Ngfr-p75-positive but non-OEC cell types in the OEC culture is not sufficiently addressed. Such non-OEC cell types are also not distinguished in the analysis of single-cell RNA sequencing data (only microglia, fibroblasts, and OECs are identified; Figure 2). Thus, it is currently unclear whether results related to the OEC subtype may have been impacted by these experimental factors.

(3) The introduction, while well written, does not discuss studies showing no significant effect of OEC implantation after spinal cord injury. The discussion also fails to sufficiently acknowledge this variability in the efficacy of OEC implantation. This omission amplifies bias in the text, suggesting that OECs have significant effects that are not fully reflected in the literature. The introduction would need to be expanded to properly address the nuance suggested by the literature regarding the benefits of OECs after spinal cord injury. Additionally, in the discussion, relating the current study to previous work would help clarify how varying observations may relate to experimental or biological factors.

(a) Cragnolini, A.B. et al., Glia, (2009), doi: 10.1002/glia.20857.
(b) Vickland H. et al., Brain Res., (1991), doi: 10.1016/0006-8993(91)91659-O.
(c) Ung K. et al., Nat Commun., (2021), doi: 10.1038/s41467-021-25444-3.

https://doi.org/10.7554/eLife.95629.1.sa0