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Reprogramming induced neurons from olfactory ensheathing glial cells: A feasible approach for spinal cord injury repair

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Every year, around the world, between 250,000 and 500,000 people suffer a spinal cord injury (SCI). SCI is a devastating medical condition that arises from trauma or disease-induced damage to the spinal cord, disrupting the neural connections that allow communication between the brain and the rest of the body, which results in varying degrees of motor and sensory impairment. Disconnection in the spinal tracts is an irreversible condition owing to the poor capacity for spontaneous axonal regeneration in the affected neurons. This is due to several causes: (i) intrinsic neuronal deficits in the expression of genes involved in axon regrowth/regeneration; (ii) the presence of inhibitory factors as well as the lack of trophic factors for neuroprotection and regeneration in the affected area; and (iii) a physical impediment due to the formation of the glial scar (Varadarajan et al., 2022).

Recent therapeutic studies for SCI have focused on cell transplantation in the damaged site using stem cells (Okano and Sipp, 2020). The mechanisms through which this cell type promotes repair include neuroprotection, immunomodulation, axon regeneration, and neuronal relay formation. In this regard, an innovative approach has been to reprogram somatic cells into induced pluripotent stem cells and differentiate them into neurons for cell replacement therapy (Ahuja et al., 2020). However, induced pluripotent stem cell technology has suffered from some drawbacks as demonstrated by tumorigenesis, indeterminate differentiation, or genomic instability (Lee et al., 2013). An alternative technique to bypass this downside is cellular direct conversion: to reprogram somatic cells into terminally differentiated cells without going through a stem cell stage (Bocchi et al., 2022). The induced neurons (iNs) are either generated *in vitro* and subsequently transplanted at the site of the lesion or reprogramming is induced from glial cells located at the affected area (Bocchi et al., 2022).

The choice of the cell type to be reprogrammed is a major challenge for the potential applications of iNs, as it not only must be susceptible to efficient reprogramming but easily accessible for a therapeutic approach. Astrocytes are one of the main cell sources for direct conversion to neurons as they share a common neural origin, and they are ubiquitously distributed throughout the central nervous system. In this instance, most of the glia-to-neuron conversion research has been carried out using virus-mediated ectopic expression of transcription factors, known for their essential contribution during neurogenesis in development, mainly NEUROG2, NEUROD1, and ASCL1 (Bocchi et al., 2022).

A promising candidate for direct conversion to neurons is olfactory ensheathing glia (OEG) due to its reported capacity to promote central nervous system regeneration (Gómez et al., 2018). OEG is located in the mammalian olfactory system (olfactory mucosa and olfactory bulb) and provides a pro-regenerative environment for olfactory sensory neuronal axons (**Figure 1**). In the adult lifetime, olfactory sensory neurons are constantly renewed and OEG is responsible for facilitating axonal growth from the neuroepithelium to the olfactory bulb, where olfactory axons synapse with mitral and tufted cells. OEG neuroregenerative capacity has been tested in an *in vitro* model of axotomized rat retinal ganglion neurons and *in vivo* in rat models of SCI. Its reparative ability is due to a combination of several factors (Gómez et al., 2018): expression of membrane-bound and secreted adhesion molecules that promote axonal growth; secretion of proteases that decrease reactivity and size of the glial scar; also, they secrete neurotrophic factors and cytokines that play a relevant role in neuroprotection and repair in the damaged zone. Furthermore, these cells can easily be obtained from the olfactory mucosa of patients by biopsy so autologous therapies can be performed and avoid post-transplant rejections.

Generation of induced neurons from human adult olfactory ensheathing glia: Sun et al. (2019) reported that mouse OEGs could be directly reprogrammed into neuronal cells by the single transcription factor NEUROG2. Our aim was to go a step further and to generate induced neurons from an adult, human starting cell type, overcoming the differences between the direct conversion of mouse and human cells into neurons (Portela-Lomba et al., 2024). Additionally, its mucosal origin provides accessibility to the somatic cell source, potentially facilitating clinical applications.

We first screened the transcription factors NEUROG2, NEUROD1, and ASCL1 to convert human mucosa olfactory ensheathing glia (hmOEG) to induced neurons (hmOEG-iNs) and assessed their reprogramming competence by immunostaining with neuronal markers (Tuj1, NeuN). Ectopic expression of NEUROD1 induced neuronal markers expression and a morphological change towards a neuronal phenotype; by contrast, no other candidate genes triggered the conversion of hmOEG into neuronal cells. Indeed, NEUROD1 transfected hmOEG cells showed the development of neuronal-like extensions along time and the positive staining of mature neuronal markers such as the axonal marker MAP1B/Neurofilament H (Smi31) and the presynaptic neuronal marker synapsin (**Figure 1**). Our results suggested that hmOEG to neuron conversion was conducted without transitioning through a neuroprogenitor stage, as was evidenced by the lack of expression of SOX2, a characteristic marker of neuronal precursors, during the first days of induction.

We determined the functionality of hmOEG-iNs by performing electrophysiological assays using the patch-clamp technique: recording of hmOEG-iNs revealed a sodium current sufficient to trigger a train of action potentials. The neuronal identity of hmOEG-iNs was assessed by analyzing the expression pattern of different neuronal subtype markers: we detected hmOEG-iNs that expressed the glutamatergic neuron marker vGLUT1 and the GABAergic neuron marker GAD67 (glutamate decarboxylase 67) but no cells stained positive for cholinergic or dopaminergic neuronal markers.

To assess that the committed reprogramming could take place *in vivo*, we engrafted transduced hmOEG cells in mouse hippocampus one week after infection. Two and three months after transplantation, these cells survived within the brain parenchyma and expressed neuronal markers (Tuj1, MAP2, and NeuN), suggesting that they had been converted into neurons (**Figure 1**).

Challenges and future directions: A concerning issue in direct conversion is the low reprogramming efficiency, accompanied by the death of those cells that do not make it to the iN fate, suggesting that the role of neurogenic factors is context-dependent. Thus, several conditions can be adjusted to enhance cell maturation and efficiency.

For example, during neuronal reprogramming the converting cells increase the production of reactive oxygen species, leading to elevated lipid peroxidation and causing the converting neurons to predominantly die via ferroptosis, a reactive oxygen species-dependent form of regulated cell death (Gascón et al., 2016). It would be worth testing the combination of *NEUROD1* with *BCL2*, an anti-apoptotic gene, which also prevents ferroptosis, to assess the efficiency of hmOEG to neuron conversion.

Transcriptional repressors prevent the induction of neurogenic cascades. For instance, the RE-1 transcription repressor complex (REST) is expressed in non-neuronal cells where it represses neuronal genes. Accordingly, early ablation of *REST* in hmOEG could improve *NEUROD1*-induced neuronal reprogramming efficiency (Masserdotti et al., 2015). Additionally, characterization of the epigenetic status of the neurogenic gene *NEUROD1* during hmOEG to neuron reprogramming is mandatory, as the efficiency conversion of the starting cell type will depend on the transcriptional accessibility of target genes. Therefore, co-factors that remove epigenetic barriers to reprogramming (e.g., repressive DNA methylation and histone modifications) can enable a neuronal gene expression program and improve transcription factor-mediated neuronal direct conversion.

After *NEUROD1* lentiviral induction *in vitro*, hmOEG successfully integrates in brain tissue, and neuronal fate commitment is maintained in an *in vivo* environment. However, a fraction of the ectopic hmOEG-NEUROD1 cell population was phagocytosed by microglia, in an environment of intense astroglial reactivity around the injection site, despite the use of NOD-SCID immunosuppressed mice. Although unexpected, rejection of autologous induced pluripotent stem cell grafts has already been reported and this immune response is suggested to be triggered by epigenetic alterations present in the reprogramming cell lines (Liu et al., 2017).

With the same purpose, to enhance and facilitate neuronal direct reprogramming, some authors have turned to the use of small molecules that target neurogenic signaling pathways (Wang et al., 2022) (i.e.: CHIR99021 (GSK3 inhibitor), SB431542 (TGFβ receptor inhibitor), Forskolin (PKA activator), SAG (Hedgehog activator). In a previous work, although we screened different combinations of small molecules that presumably enhanced neural reprogramming, only partial reprogramming was achieved in our starting OEG population (Portela-Lomba et al., 2023).

Although we have had promising results, we need to increase the reprogramming efficiency to allow the engraftment of hmOEG-iNs in a clinically relevant model of SCI and examine its effectiveness *in vivo*. For such purpose, we need to get insight into the mechanisms responsible for OEG direct reprogramming, so we can modify them at will and increase the number of functional OEG-induced neurons (**Figure 1**).

Conclusion and final remarks: In conclusion, we succeeded in directly converting olfactory ensheathing glia from adult hmOEG to neurons. We showed that the cells under study maintained the characteristic neuroregenerative properties of OEG and, after transduction of the single neurogenic transcription factor NEUROD1, they exhibited morphological and immunolabeling neuronal features, fired action potentials, and expressed glutamatergic and GABAergic markers. Additionally, after engraftment of transduced hmOEG cells in the mouse hippocampus, these cells maintained their neural commitment, showing specific neuronal labeling.

Thereby, if we add to the neuroregenerative

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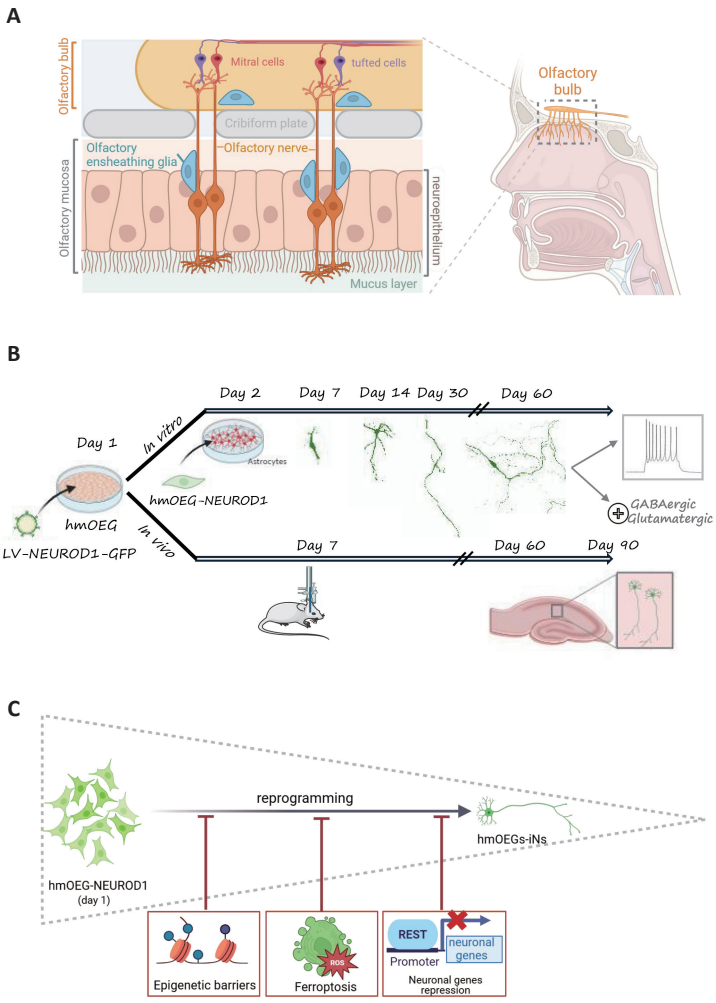

Figure 1 | **OEG to neuron reprogramming.**
(A) OEG is located in the mammalian olfactory system and provides a pro-regenerative environment for olfactory sensory neuronal axons. (B) After induction of hmOEG with the neurogenic transcription factor NEUROD1, cells exhibited morphological and immunolabeling neuronal features, fired action potentials, and expressed glutamatergic and GABAergic markers. After engraftment of transduced hmOEG cells in the mouse hippocampus, these cells survived within the brain parenchyma and expressed neuronal markers. (C) To enhance reprogramming efficiency it will be necessary to characterize and adjust the epigenetic and metabolic landscape of hmOEG-iNs, as well as the activity of neuronal genes repressors. Created with BioRender.com. hm-OEG: Human mucosa olfactory ensheathing glia; hmOEG-iNs: human mucosa olfactory ensheathing glia induced neurons; LV-NEUROD1-GFP: lentivirus with the gene coding for the transcription factor NEUROD1 and the green fluorescent protein; OEG: olfactory ensheathing glia.

capacity of OEG cultures, the conversion to neurons of a fraction of their population through reprogramming techniques, the engraftment of OEG and OEG-induced neurons (OEG-iNs) could enhance neural repair at the damaged site in SCI. This is due to the intrinsic axonal regenerative capacities of OEG, combined with the presence of OEG-iNs acting as a relay in the damaged zone. Therefore, long distance connectivity between the nervous tracts would be accomplished, allowing functional recovery.

In the future, we will go in depth into the mechanisms of neural direct reprogramming and we will apply this knowledge to increase the regeneration of axonal tracts, herein enhancing the recovery of motor and sensitive functions after SCI, if successful. But this will be another story.

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