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Biomarkers expression in rat olfactory ensheathing cells

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1. ABSTRACT

Olfactory Ensheathing Cells (OECs) ensheathe unmyelinated olfactory axons and exhibit antigenic and morphological characteristics both of astrocytes and of Schwann Cells (SCs). As a matter of fact they express an astrocyte-specific marker (GFAP) and low-affinity p75 nerve growth factor receptor (p75 NGFr), S100, as well as adhesion molecules such as laminin and N-CAM like SCs. Immunocytochemical studies reveal that OECs are able to produce different growth and survival factors. *In vitro*, OECs promote axonal growth, probably by secretion of neurotrophic growth factors that support axonal elongation and extension. *In vivo* studies have shown that OECs can form myelin promoting remyelination of damaged axons. In fact, when transplanted, they stimulate extensive sprouting and axonal regeneration of multiple axons. As OECs appear to exert a neuroprotective effect for functional restoration and for neural plasticity in neurodegenerative disorders, they might be considered a suitable approach to functional recovery. These data establish OECs as prime candidates for transplantation, showing some advantages over SC thanks to their different capacity to intermingle with astrocytes after implantation in lesion sites.

2. INTRODUCTION

The mammalian olfactory system is one of the few areas of the Central Nervous System (CNS) that is capable of continuous neurogenesis throughout lifetime (1). Olfactory neurogenesis takes place in the basal cell layer of the olfactory epithelium which is located on the peripheral side of the ethmoidal cribriform plate (2-4). When olfactory receptor neurons (ORNs) die, new neurons are produced by division of basal cells of the deepest layer of epithelium (2,5-7). Upon division and differentiation, ORNs extend their axons through the cribriform plate, within the olfactory nerve and glomerular layer of the olfactory bulbs (OB), where they form synaptic connections with target cells (1,8,6). The ability of ORNs to regenerate throughout life in the adult olfactory system is thought to be due in part to particular glial cells of the olfactory nerve, termed Olfactory Ensheathing Cells (OECs). They wrap up the olfactory nerve along its whole length, from the basal lamina of the epithelium to the olfactory bulb, crossing the peripheral nervous system-central nervous system junction (Figure 1). OECs constitute a common population of glial cells sharing properties with both Schwann cells (SCs) and astrocytes of CNS (6,9-11). Unlike SC, that are derived from neural crests, OECs have a placodal origin (12).

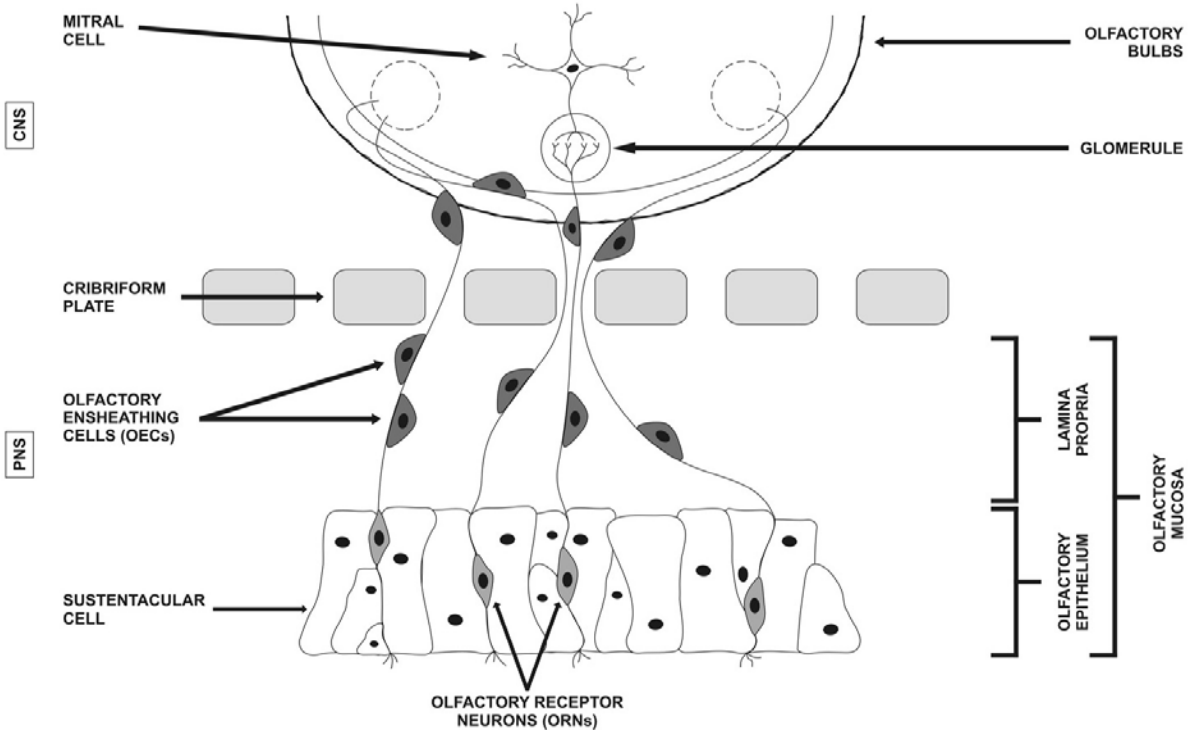

Figure 1. A drawing representing the rat olfactory system. Olfactory Ensheathing cells ensheath olfactory receptor axons through the *lamina propria* in the PNS. Olfactory nerves pass through the cribriform plate into CNS and arrive inside the bulbs. The axons extend into glomeruli to synapse on mitral cells.

During their development they migrate to the OB bud and accompany the small nonmyelinated axons of the ORN (10,13). Lately, OECs have drawn considerable interest because they are glial cells with special properties, as they show exceptional ability to promote regeneration in the injured CNS (14-19). Immunohistochemistry studies have demonstrated that OECs are able to express different markers (20,21) and are a source of growth factors (10,22,23).

Recent reports have revealed that OECs are able to promote remyelination of damaged axons (24,25) synthesizing myelin constituents, such as P0 (21). These findings have stimulated many researches to transplant OECs into transected spinal cord demonstrating their ability to promote regeneration and functional recovery (9,18,26-28). In our opinion, OECs probably have this capability as source of growth factors and adhesion molecules. Moreover, they present distinctive immunohistochemical properties.

This review summarizes recent data on expression of biomarkers in OECs, investigated both *in vivo* and *in vitro,* and their properties.

3. MORPHOLOGICAL AND FUNCTIONAL FEATURES OF OECs

OECs were first described by Golgi and Blanes at the end of 19th century (29,30). They are derived from precursors originating from the olfactory epithelium (12,31,32) and are then distributed within the olfactory nerve and the first two layers of the OB. OECs can be distinguished from other glial types. Compared to astrocytes, they show an electron-denser cytoplasm, and scattered intermediate filaments (6,10,32-34). OECs share properties with both PNS Schwann cells in assisting axon growth and CNS astrocytes in being able to live inside CNS (6,9-11); although expressing same markers, OECs are classified as a different population of glial cells. This is very surprising since OECs are not derived from the neural crest, like SCs, but originate from the olfactory placode.

Many research groups have demonstrated that OECs are an heterogeneous population of cells showing a variety of shapes and sizes and a highly dynamic nature capable of switching from one morphology and back again within an hour (35,36).

In adults, OECs exhibit very distinct morphological shape, acquiring a fusiform morphology, *in vivo.* Moreover, OECs do not wrap individual axons, but enclose packed bundles of unmyelinated axons (1,37,38).

The morphology of OECs *in vitro* is different: it depends on age (embryos, newborn or adult), animal species, and culture conditions (1,12,20,33,37,39). *In vitro*, OECs show two morphological features: they are astrocyte-like with a flat morphology and short oriented processes and Schwann-cell-like with a long fusiform shape and long

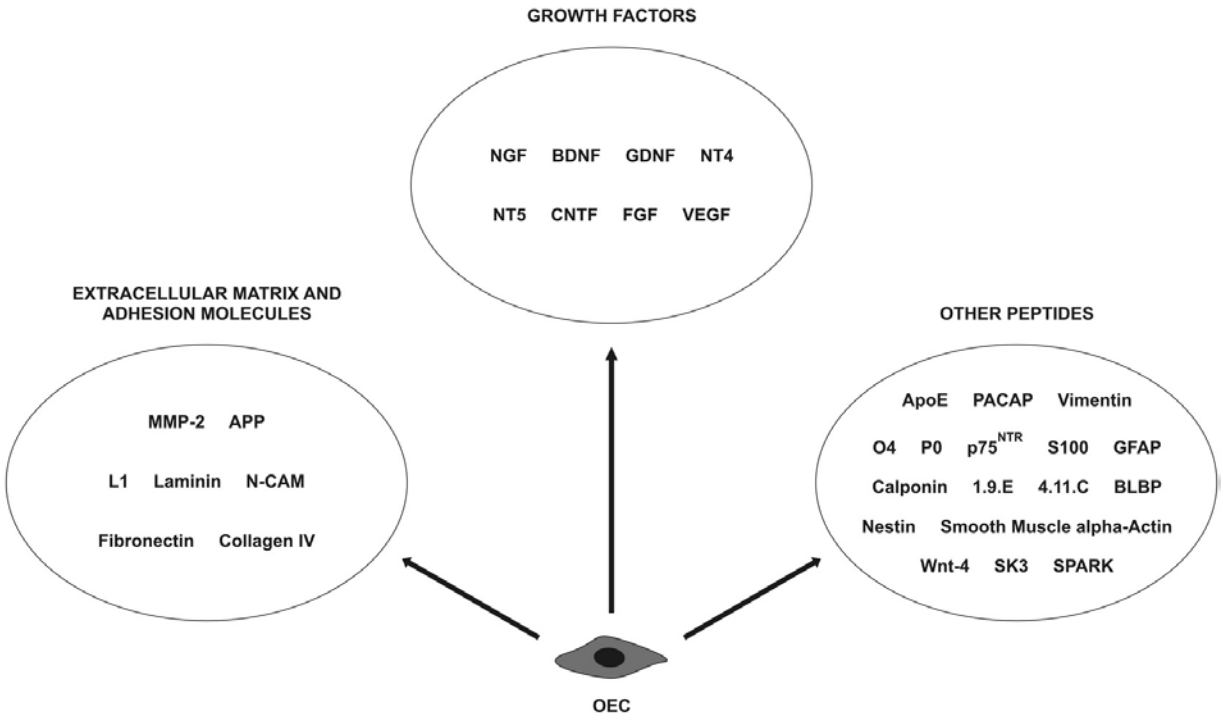

Figure 2. Lists of molecules produced by OECs, divided in: extracellular matrix and adhesion molecules, growth factors and other peptides.

and thin processes (17,20,40). These two different aspects may represent either two types of OECs or different stages of the same cell. A study, in time lapse images, has shown that OECs are able to change from one morphotype to another in less than 1 hour (35). These changes are likely to depend on age and conditions of OECs cultures. Moreover, OECs change in shape both before and after mitosis. During division, OECs retract all processes and assume a spherical shape, then rapidly divide and the two round daughter cells are ready to send out their processes and increase their cellular size (35). When OECs are co-cultured with neurons, they change morphology acquiring a bipolar spindly shape and enfolding axons: it depends on axonal contact (10). Moreover, in co-cultures with dorsal root ganglion neurons, OECs form myelin sheaths around the axons (41).

OECs are located in an area of the CNS capable of continuous renewal throughout its lifetime. In fact, they accompany the small nonmyelinated axons of the ORN creating an adequate environment for their growth (1). This suggests that OECs are able to promote the outgrowth of ORN axons driving them towards their right position in the olfactory bulbs (13). A further functional role of OECs is their capacity of anti-bacterial activity. OECs were, in fact, proved to prevent the turbidity induced in a culture infected with attenuated *Escherichia coli* (42,43).

3.1. Biomarkers expression in OECs

The kind of markers expressed by OECs is variable both *in vivo* and *in vitro*. It depends on different situations, such as the developmental stage, animal species and culture conditions. Immunocytochemical studies were carried out to analyse the expression of molecules by OECs using several antibodies. In this paper we examine the markers expressed by OECs in different conditions, dwelling upon the changes that these cells show during their development (Figure 2). It is known that OECs are a source of growth factors such as NGF, bFGF, BDNF, GDNF (23,44,45), CNTF (46) and they utilize them to communicate with their environment via these trophic molecules (22). Moreover, the expression of NT4, NT5 and neuregulins was found in cultured OECs and in OECs medium (22) while the expression of NT3 was not detected in OECs either *in vivo* or *in vitro*. Consequently OECs and their secreted factors promote functional restoration and neural plasticity.

OECs express adhesion molecules including laminin, L1, N-CAM, fibronectin and collagen IV (20,47) and extracellular matrix molecules such as metalloproteinase-2 (MMP-2)(48) and amyloid precursor protein (APP)(49). These molecules are involved in glia-axon adhesion and laminin acts as a growth-promoting substrate for ORN (4,50). All adhesion molecules are expressed in the OECs membranes at all developmental stages (10). Moreover, a recent study has shown that OECs express high levels of the lipid transporting protein apolipoprotein (ApoE) around the olfactory nerve fascicles, suggesting that ApoE may be involved in axonal growth (51). As mentioned above, OECs are able to secrete many growth factors, including neuregulins that have mitogenic effect and may play a role versus toxic injury of the olfactory mucosa (52). Some authors have found that OECs

express the vascular endothelial growth factor (VEGF) and pituitary adenylate cyclase-activating peptide (PACAP) (53).

OECs express an high number of immunohistochemically detectable markers, such as GFAP, $p75^{NTR}$, S100, 04, vimentin, nestin (10) and neuropeptide Y (54), whose presence depends on age. Vimentin represents a major constituent of OECs intermediate filaments both during development and in adult (20). Furthermore, OECs are positive to nestin in embryonic and postnatal stages (10), while expressing GFAP in the postnatal and adult stages (20,55). Like some glial cells, OECs are positive to 04 protein at all developmental stages. GFAP positive OECs coexpress polysialic acid (PSA): these data were published for the first time by Franceschini and Barnett (20). Some studies have shown that OECs are able to synthesize myelin constituent, P0, that is maintained in the adult and is found in immature SCs and in SCs precursors (56). Thus, adult OECs continue to express P0, whereas mature SCs down-regulate P0 expression (11). *In vivo* and *in vitro* studies have demonstrated that OECs express two monoclonal antibodies, termed 1.9.E and 4.11.C, produced and used by Heredia (57). These antibodies labeled filaments throughout the cytoplasm of OECs reacting with molecules of their cytoskeleton.

Some authors have shown that OECs express brain lipid binding protein (BLBP), a radial glia protein, throughout adulthood, which is a potential indicator of the plastic phenotype of OECs (58).

The expression of molecules depends on culture conditions: when OECs are grown in serum-free astrocytes conditioned medium, two types of OECs are evident: one shows a flattened morphology expressing embryonic N-CAM and GFAP but no low affinity NGF receptor, while the other type shows a spindle shape, expresses low affinity NGF receptor and GFAP, but no embryonic N-CAM (16). Such difference suggests a subdivision of OECs in two different phenotypes, namely: astrocytes-like OECs and SC-like OECs from both neonatal and adult rat olfactory bulbs (20). Recent proteomic studies have demonstrated that calponin, an actin-binding protein, is a specific marker for OECs, allowing to distinguish them from SC and providing the first phenotypic marker for these cells (59). This finding led to some controversy, as other studies have shown that calponin is not expressed by adult rat OECs, but by mesenchymal cells, fibroblasts and meningeal cells, and by astrocytes *in vitro* from olfactory system (60,61). This discrepancy may be due to the different developmental stage. Another motive of discrepancy may be imputed to immunohistochemical procedures, insofar as pre-treatment of OECs cultures with proteinase K used by Ibanez et al. (61) increases the immunostaining for calponin, and the omission of this enzyme may yield false-negative results.

These observations suggest that OECs and SC express many of the same markers, such as p75, S100, GFAP and the lack of a specific biomarker for OECs or SCs makes the distinction between the two cell types difficult. Other proteomic studies have reported that OECs, both *in vitro* and *in vivo*, express smooth muscle alpha-actin, as caldesmon and smooth muscle tropomyosin, both actin-binding proteins associated with smooth muscle cells (62). Since calponin is also an actin-binding protein, the finding by Jahed strengthens previous results (59,63), asserting that OECs are able to express calponin. Moreover, it is known that p75-positive OECs express smooth muscle alpha-actin, while p75 positive SCs do not. This suggests that the co-localization of p75 and smooth muscle alpha-actin could be used as specific marker for OECs both *in vitro* and *in vivo* (62). This observation demonstrates that OECs represent a population of axon-supporting cells showing its own phenotypic characteristic. On this subject, Rodriguez-Gil and Greer (64) have shown that OECs express Wnt-4, a cysteine-rich secreted glycoprotein influencing axon guidance (65) and the persistence of Wnt expression in adult OECs suggests their role of support of ORN axonal growth.

Immunohistochemical analysis reported the presence of SK3 channel (one class of the Ca ions activated K ions channels) in the thin processes of the OECs, suggesting that OECs express SK3 channels (66).

Secreted protein acidic and rich in cysteine (SPARC) is a matricellular protein expressed by OECs and is able to promote axon sprouting and is involved in regulating plasticity and repair in the CNS. OECs are intensely immunopositive for SPARC during embryogenesis, but down-regulate SPARC postnatally (67). The expression of SPARC by OECs supports plasticity phenomena, such as cell migration, regeneration and peripheral myelination. In fact, some experiments showed that after bulbectomy there is an increase of SPARC expression by OECs, helping neurite regrow activity (68). Moreover, SPARC is able to stimulate outgrowth of dorsal root ganglion neurites both *in vitro* and *in vivo*. These data suggest that SPARC is a mediator used by OECs to stimulate endogenous spinal cord repair (69).

3.2. Regenerative properties of OECs

The finding that OECs are a source of multiple trophic factors is very important as they play a decisive role in CNS regeneration (22,48,49,70). During the past decade, OECs have attracted attention since, in view of their remarkable ability to stimulate the directional regrowth of injured axons (71) and showing their functional plasticity (42), they could be used as potential therapeutic agents and provide trophic support to CNS injury (15).

Several studies have demonstrated that OECs play a role in regrowth of newly formed olfactory axons and support axonal growth *in vitro* (19,72), making OECs suitable for regrowing axons. Many researches have focused on implantation of OECs into the region of damaged spinal cord stimulating axonal regeneration, remyelination and functional recovery in animal models of spinal cord injury (SCI)(73). In fact, some authors have shown that transplants of OECs in spinal cord lesion, promote axonal regeneration and functional recovery forming myelin sheaths around demyelinated axons (27,74,75).

Transplantations of OECs into transected spinal cord promote regeneration and locomotor recovery (9,10,26). Moreover, OECs are able to reduce apoptosis of corticospinal tract neurons when transplanted into the dorsal transected spinal cord (76). It was observed that co-transplantation of OECs and fetal ventral mesencephalic cells increased functional restoration in rat model of Parkinson's disease (77). Moreover, adult OECs promote regeneration of lesioned rat optic nerve axons when transplanted between the proximal and distal stumps, stimulating and guiding regeneration of axons (78). OECs promote the recovery of sensorimotor functions in paraplegic rats after transplantation into complete spinal cord transection (18).

3.3. Can OECs form myelin?

In the olfactory system OECs do not express any myelin constituent, but they surround bundles of nonmyelinated axons of the olfactory receptor neurons. Nevertheless some authors report that when OECs were co-cultured with dorsal root ganglion neurons, they were able to form myelin (41). Consequently, some *in vivo* experiments obtained the same result, demonstrating that OECs remyelinated spinal cord axons when transplanted into lesioned areas (79). A few years later, further studies *in vivo* showed that OECs can form myelin, sheathing axons with appropriate diameter in a site of OECs graft subsequent to a lesion of the corticospinal tract (28). To strengthen these results, further reports have shown that OECs express a myelin constituent, P0, which is present in immature SCs and in SCs precursors (21,80). The myelin formed by OECs is typical of the peripheral-type myelin, positive to P0 which is a protein found in peripheral but not in central myelin (79). The ability of OECs to form myelin has been reason of conflicting reports, since recent papers have suggested that SC contaminating OECs are responsible to form myelin, but not OECs (63,81,82). A subsequent study showed that transplantation of OECs expressing Green Fluorescent Protein in lesioned site form myelin around regenerating axons (83). This encourages to believe that OECs are able to form myelin when transplanted in lesioned areas.

Moreover, both SCs and OECs use the same transcription system to form myelin, expressing the factor *desert hedgehog* that has a role in regulating the behaviour of perineurial cells adopting arrangements to form fascicles (84). In fact, many reports have shown that transplants of OECs are able to promote axonal regeneration and functional recovery after spinal cord lesion, by forming myelin sheaths around demyelinated axons (25,74,75). In addition, myelin forming OECs support the development of nodes of Ranvier and the restoration of impulse conduction in demyelinated axons (76).

3.4. Effects of OECs

In vitro studies have reported the ability of OECs to stimulate neuronal survival and neurite sprouting mediated through secretion of growth factors and direct contact between neurons and OECs. In co-culture model, OECs are able to promote neurite sprouting of injured cortical axons by direct cellular contact and secretion of soluble factors (19,85); moreover, they exert a positive effect when co-cultured with rat retinal ganglion cells (36,86). In our recent reports we have demonstrated the positive effect of OECs on the survival and neurite outgrowth of CNS neurons in a co-culture model (87,88).

Several studies have proved that implanted OECs increase the formation of new blood vessels (89). OECs transplantation not only minimizes tissue loss and prevents cavity and scar formation at the lesion site, but stimulates extensive angiogenesis, probably by means of secretion of vascular endothelial growth factor (VEGF) synthesized and expressed by OECs after SCI (53).

The beneficial effects of OECs on functional recovery could be summarized in the following points: 1) stimulation of axonal outgrowth; 2) angiogenesis; 3) migration and interaction with scar tissue; 4) remyelination of spinal cord (90).

3.5. OECs and SC

Several researches have highlighted that OECs and SCs express many of the same phenotypic markers, but they differ in their capacity to intermingle with astrocytes after implantation in the lesion site (91). SCs are known to be incompatible with astrocytes, while OECs are not. This suggests that OECs might be better suited to astrocytes environments and when OECs are transplanted into a lesion area, they are more likely to provide repair-promoting properties. The advantage of OECs over SC depends on a close proximity between OECs and astrocytes that doesn't exist between SC and astrocytes, this compatibility being due to their co-existing within the olfactory system (92). OECs present a greater migratory potential than SCs (28) and do not accumulate, as SCs do, proteoglycans that cause cone collapse (93). Moreover, the difference of expression of molecules such as cadherins, L1, integrins and junctional adhesion molecules in OECs compared to SCs, could contribute to facilitate their interaction with astrocytes. This consideration underlines the advantage that OECs have over SCs: they intermingle with astrocytes, reduce astrocytosis *in vivo* and do not cause any hyperactivity of astrocytes exerting a neuroprotective role by reducing astrocytic gliosis and cystic cavitation (94). Therefore, OECs might represent a suitable cell population than SCs for use in transplantations into the lesioned CNS, to repair the injured or demyelinated spinal cord (91). These observations suggest that remyelination following OECs transplantations proves to be more extensive than after SC transplantation in areas surrounded by astrocytes (25). An *in vitro* study has reported the different influence of SC and OECs on retinal ganglion cells, demonstrating a much better neurite growth in the presence of OECs than SC (95). The reason for this may be that the OECs integrate well with astrocytes, unlike SCs.

In conclusion, OECs prevent cavity formation, modify scar formation, facilitate the invasion of host Schwann cells, and stimulate angiogenesis. Moreover, they create a laminin-rich CNS microenvironment that stimulate extensive sprouting and axonal regeneration of multiple motor and sensory axons creating a permissive glial

environment at the lesion site to promote axonal growth/regeneration of axons (96). These considerations suggest that OECs, in respect of SCs, might be best suited for transplantations into different areas of CNS, remaining one of the most promising strategies for functional recovery in SCI.

4. SUMMARY AND PERSPECTIVES

Current results suggest that OECs 1) restoration of injured axons in the spinal cord; 2) are neuroprotective after lesion, providing to decrease cavity formation; 3) stimulate angiogenesis; 4) when transplanted in the spinal cord stimulate glial-glia relation into the lesioned area; 5) are able to myelinate in demyelinating diseases; 6) integrate well with other cells, such as astrocytes; 7) *in vitro* promote survival and axonal outgrowth. These observations lead to think that OECs might represent promising supporting cells in CNS recovery, make them attractive candidates for neurogenesis, in neurodegenerative disorders, including Alzheimer's (97), Parkinson's (77), Huntington's diseases (98) and spinal cord injury (99,100). Huang et al., (101) have conducted a pilot study showing that OECs transplantation can significantly slow down deterioration of the clinical course of patients with amyotrophic lateral sclerosis. Moreover, OECs, being a source of growth factors and adhesion molecules, show a particular morphological and functional plasticity suggesting that they might be used as potential therapeutic agents facilitating axonal regeneration and functional recovery in the injured nervous system. Therefore, OECs might be considered a clinical alternative to SCs for their ability to integrate into CNS environment after grafting to repair the lesioned spinal cord. All animal experiments suggest that OECs are one of the most promising cell types for repair of SCI. In the future, we hope that OECs could be used for human SCI, clinical trials have begun to use OECs to repair spinal cord lesions in SCI patients (102,103). Some reports contradict these considerations, asserting that implantations of OECs alone are not sufficient for spinal cord repair and require combinations with other synergic therapies (104). A study of Nash et al., (105) has demonstrated that the combination of OECs transplantation with *methylprednisolone*, a drug commonly used in human patients with SCI, promote axonal regeneration. In the future it will be essential to study the molecular mechanisms of OECs to clarify their share in SCI recovery and how their contribution may be employed in human clinical practice.

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Abbreviations: OECs: Olfactory Ensheathing Cells; ORNs: olfactory receptor neurons SC: Schwann Cells; NGF: nerve growth factor; bFGF: basic fibroblast growth factor; GDNF: glial cell derived neurotrophic factor; BDNF: brain derived neurotrophic factor; CNTF: ciliary neurotrophic factor; CM: conditioned medium; CNS: Central Nervous System; SCI: spinal cord injury; VEGF: vascular endothelial growth factor; PACAP: pituitary adenylate cyclase-activating peptide; SPARC: secreted protein acidic and rich in cysteine; PSA: polysialic acid; MMP-2: metalloproteinase-2; APP: amyloid precursor protein .

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