STEM CELLS

REGENERATIVE MEDICINE

Co-Transplantation of Adipose Tissue-Derived Stromal Cells and Olfactory Ensheathing Cells for Spinal Cord Injury Repair

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ABSTRACT

Patients suffering from spinal cord injury (SCI) still have a dismal prognosis. Despite all the efforts developed in this area, currently there are no effective treatments. Therefore, cell therapies have been proposed as a viable alternative to the current treatments used. Adipose tissue-derived stromal cells (ASCs) and olfactory ensheathing cells (OECs) have been used with promising results in different models of SCI, namely due to the regenerative properties of the secretome of the first, and the guidance capability of the second. Using an in vitro model of axonal growth, the dorsal root ganglia explants, we demonstrated that OECs induce neurite outgrowth mainly through cell-cell interactions, while ASCs' effects are strongly mediated by the release of paracrine factors. A proteomic analysis of ASCs' secretome revealed the presence of proteins involved in VEGF, PI3K, and Cadherin signaling pathways, which may be responsible for the effects observed. Then, the cotransplantation of ASCs and OECs showed to improve motor deficits of SCI-rats. Particular parameters of movement such as stepping, coordination, and toe clearance were improved in rats that received the transplant of cells, in comparison to non-treated rats. A histological analysis of the spinal cord tissues revealed that transplantation of ASCs and OECs had a major effect on the reduction of inflammatory cells close the lesion site. A slight reduction of astrogliosis was also evident. Overall, the results obtained with the present work indicate that the cotransplantation of ASCs and OECs brings important functional benefits to the injured spinal cord. STEM CELLS *2018;36:696–708*

SIGNIFICANCE STATEMENT

Spinal cord injury (SCI) still has no cure and cellular transplantation has been considered a valid alternative for SCI treatment. This study shows that adipose tissue-derived stromal cells (ASCs) and olfactory ensheathing cells (OECs) induce neurite outgrowth of dorsal root ganglia explants through distinct mechanisms. The cotransplantation of ASCs and OECs into a rat model of SCI led to functional improvements. Additionally, there was a reduction of inflammatory cells close to the injury site and ASCs were integrated in the spinal cord tissue, 8 weeks post-injury. Overall, the transplantation of ASCs and OECs is a promising strategy for SCI repair.

INTRODUCTION

Spinal cord injury (SCI) is one of the most devastating conditions of the CNS for which there is still no cure. Sensorial and motor function deficits, cardiac and respiratory complications, sexual and urinary dysfunctions, and sometimes depression are among the consequences of SCI. The treatment to this condition is mainly based on palliative care. However, there are new therapies currently under investigation aiming to promote functional recovery after SCI through different neurorestorative mechanisms [1, 2]. From those, cellular transplantation emerges as a promising tool, either by replacing the damaged nervous tissue or alternatively potentiating endogenous neuronal regeneration by exerting a neurotrophic and/or neuroprotective role.

In this context, mesenchymal stromal cells (MSCs) have gained increased notoriety, much due to their supportive character [3]. These cells, first characterized by Friedenstein et al. (1974) [4], are identified by their multipotency,

adhesion to plastic, and positive/negative expression of some specific markers [5]. Adipose tissue-derived stromal cells (ASCs) are a type of MSCs derived from an abundant and accessible source (adipose tissue). Moreover, ASCs have shown to possess an immunomodulatory profile [6, 7], in addition to a neurotrophic role, providing protection, survival, and differentiation of different cells and tissues [8, 9]. All these effects have been associated to ASCs' secretome, that is, the set of molecules that these cells secrete to the extracellular milieu [6, 10, 11]. ASCs have been applied to animal models of SCI with promising results [12, 13]. In a cervical SCI model, transplanted ASCs modulated the structure of the glial scar and stimulated axonal sprouting [12]. In a thoracic compression injury model, ASCs were capable of promoting functional recovery in addition to tissue preservation and axonal regeneration [13]. Regarding human clinical trials, the autologous transplantation of ASCs, obtained from lipoaspirates, into SCI patients was free of serious adverse events and some patients showed mild improvements in motor and sensory scores [14].

Olfactory ensheathing cells (OECs) have also gathered particular attention due to their potential for SCI treatment [15]. OECs are a specific type of cells present in the olfactory system that support the constant neuronal regeneration in the transition between the PNS and the CNS [16]. Considering OECs' role in their native olfactory system, Ramon-Cueto and colleagues [17, 18] transplanted these cells into different models of CNS injury in rats, demonstrating improved axonal regeneration after a lesion. Following their pioneering work, different preclinical studies managed to reproduce the beneficial effects of OECs grafts, including partial recovery of motor function [19–21], even under a chronic SCI lesion [22]. Among several interesting properties, OECs are capable of: (a) bridging the lesion site, by interacting with endogenous astrocytic processes [23], (b) myelinating previously demyelinated regions of the spinal cord [24], (c) having a phagocytic activity, removing degenerating axons [25, 26], and (d) modulating the glial scar as well as promoting angiogenesis [27]. Transplantation of OECs in humans has also already been tested [28, 29]. The use of these cells was shown to be safe and in some cases patients presented improvements in motor and light touch scores [30].

In addition to the beneficial properties above described, ASCs and OECs have the advantage of being good candidates for autologous transplantation, avoiding ethical concerns. Moreover, previous work from our group showed that ASCs and OECs present positive paracrine interactions, with improved proliferation and metabolic activity of both cells when seeded in an indirect coculture system [31]. In this work, we aimed to assess the regenerative potential of the combined application of ASCs and OECs either in an in vitro model of axonal regeneration as well as in an in vivo model of SCI.

Materials and Methods

Cell Isolation and Culture

Human ASCs were isolated according to Dubois et al. [32] with a collaboration with LaCell LLC. The culture of these cells is described in Supporting Information. OECs were harvested and cultured according to a previously described protocol [33]. Details about the culture of these cells are described in Supporting Information.

Secretome Collection

For the secretome used in in vitro experiments, ASCs at sixth passage (P6) were seeded at 4,000 cells per cm^2 density in cell culture flasks, with their normal growth medium (Supporting Information). Seventy-two hours after seeding, the media was removed, and cells were washed three times with phospate-buffered saline (PBS) without Ca^{2+}/Mg^{2+} (Invitrogen, USA), followed by neurobasal medium (Invitrogen) with 1% penicillin/streptomycin (pen/strep, Invitrogen). Then, fresh neurobasal media (with 1% pen/strep) was added to the cells. Twenty-four hours later, this media was collected (now called conditioned media [CM]), filtered (0.2 μm pore diameter), and frozen in liquid nitrogen for later application.

OECs isolated and purified as described in supporting information were seeded at 40,000 cells per cm^2 density in fibronectin-coated cell culture flasks, with their normal growth medium (Supporting Information). Then, the same conditioning protocol was followed as for ASCs, in order to collect OECs' secretome.

As a control, flasks without cells but with neurobasal media (with 1% pen/strep) were kept for 24 hours at the same conditions, before being filtered and frozen in liquid nitrogen.

Proteomic Data Analysis

Proteomic data of ASCs' secretome, previously obtained from Pires et al. [34] and newly obtained OECs proteomic data were analyzed using two distinct approaches. In the first approach, the Protein Analysis Through Evolutionary Relationships (PANTHER) classification system (http://pantherdb.org) was used to identify the signaling pathways related with ASCs and OECs secreted proteins. Then, the main hits obtained were grouped according to their main function and represented in a pie chart. In the second approach, a PANTHER overrepresentation test was performed to highlight the most representative Reactome pathways. This test was performed using the following criteria: entire human database as background list; applying a Binomial distribution test with Bonferroni correction for multiple comparisons and a cut-off of .05 *p*-value.

Dorsal Root Ganglia Isolation and Culture with ASCs/OECs

OECs were first isolated and purified as described in supporting information. They were seeded at 20,000 cells per cm^2 density on fibronectin-coated 24 well plates, with their normal growth medium. Seventy-two hours after seeding, ASCs were seeded on top of OECs cultures (in coculture group) or on fibronectin-coated 24 well plates, at 7,500 cells/cm^2 density. The growth medium of OECs was used for this step, since previous experiments (nonpublished data) showed that ASCs proliferation and metabolic viability were not altered in this medium. Twenty-four hours later, dorsal root ganglia (DRG) explants were isolated according to Allodi et al. [35] and placed on top of all experimental groups (ASCs and OECs alone, or coculture). Thoracic DRG explants were isolated from P5-P7 rats and cleaned to remove peripheral processes. Then they were placed over the experimental conditions under study. The medium was changed to DRGs normal growth medium:

neurobasal medium supplemented with 6 mg/ml D-glucose (Sigma, USA), 1% pen/strep, 2% B27 (Invitrogen) and 2 mM L-Glutamine (Invitrogen). Some DRG explants were additionally placed on a control group without cells, cultured with normal growth medium. The cultures were kept for 4 days, with daily medium exchanges. After fixation, an ICC for neurofilament (NF) was performed and samples imaged with an Olympus IX81 fluorescence microscope. The analysis was made using the software ImageJ (NIH).

DRG Isolation and Culture with ASCs/OECs Secretome

Collagen hydrogel droplets were used as a matrix for DRGs adhesion and growth. The matrix was prepared by mixing rat tail collagen type I (3.61 mg/ml; 89.6% [vol/vol]; BD Biosciences, USA) with DMEM concentrated medium (10×; 10% [vol/vol]; Invitrogen) and a solution of $NaHCO_3$ (7.5% [wt/vol]; 0.4% [vol/vol]). After mixing, collagen droplets were incubated for 2 hours at 37°C, 5% CO_2 (vol/vol). Then, DRGs were isolated and placed on top of previously prepared collagen droplets with their normal growth medium. Twenty-four hours later, this medium was replaced with the CM from ASCs, OECs, or the control group. In the moment of media addition, both secretomes, as well as the control, were further supplemented with B27, L-Glutamine, and Glucose. DRGs were kept in culture for 5 days and media were replaced once, at day 3. After 5 days, samples were fixed and subjected to an ICC for NF. Images were obtained through confocal microscopy (Olympus FV1000).

Immunocytochemistry

ICC protocols were used to identify DRG neurite projections in culture, applying the mouse anti-NF 200 kDa antibody (1:200, Millipore, USA). Details can be found in Supporting Information.

DRG Outgrowth Analysis

To calculate the area occupied by the neurites, Neurite J plugin for ImageJ was applied [36]. Initially, the body area of the explant was defined and then the threshold contrast was adjusted to highlight the neurites formed from the DRG body. After determining the scale, the plugin automatically converted the images to 8 bits. Finally, using the menu "analyze particles" the software automatically calculated the areas occupied by the neurites, using the dark background as contrast. In order to calculate the number of intersections, Neurite J plugin was used.

In Vivo Proof of Concept

Animals and Groups. Eight-weeks-old female Wistar rats (Charles River, France), housed in light and temperature controlled rooms and fed with standard diet, were used in the in vivo studies. Handling was performed for 3 days before the surgeries. Animals were divided in three distinct groups according to the respective treatment/procedure: (a) Animals subjected to SCI with no treatment (SCI, $n = 5$), (b) SCI animals treated with a transplantation of ASCs and OECs (Cells, $n = 5$), and (c) Animals with laminectomy only, without SCI (Sham, $n = 5$). In the cells-treated group, a total of 40,000 OECs and 40,000 ASCs were injected per animal, divided by two injections, 2 mm rostral and 2 mm caudal to the lesion, using a Hamilton syringe.

Spinal Cord Injury Surgery. All animals were anesthetized by intraperitoneal injection of a mixture (1.5:1) of ketamine (100 mg/ml, Imalgene/Merial, France) and medetomidine hydrochloride (1 mg/ml, Domitor/Pfizer, USA). Once anesthetized, fur was shaved from the surgical site and the skin disinfected with ethanol 70% and chlorohexidine. Then, a dorsal midline incision was made from T7-T13 and the paravertebral muscles retracted. A laminectomy was performed at T10 level, in which the spinous processes were removed and the spinal cord exposed. A unilateral defect (hemisection) on the left side of the spinal cord was done, removing 2–3 mm of nervous tissue. After the respective treatment, paravertebral muscles and skin were closed with Vicryl sutures (Johnson and Johnson, USA). The incision of SCI control animals was closed after injury without treatment. Post-operative care was given to all SCI rats (protocol described in Supporting Information).

Behavioral Analysis

BBB Test. To evaluate motor behavior the Basso, Beattie, Bresnahan Locomotor Rating Scale (BBB) [37] was used every week, starting exactly 1 week post-injury, up to a total of 8 weeks. Locomotion of the affected hindlimb is rated by two blinded observers in a 4-minutes test.

Activity Box Test. General locomotion activity was measured by assessing the number of rearings and distance travelled in a closed arena, 5 and 8 weeks post-injury [38]. The arena (43.2 cm × 43.2 cm) has transparent acrylic walls (Med Associates Inc., USA) and is placed in a brightly illuminated room. Animals started the test at the arena's center and were given 5 minutes to explore it.

Swimming Test. Eight weeks after the injury, spontaneous motility was also measured through a swimming test. Each animal was placed at the border of a pool with 170 cm of diameter, having a central platform with 12 cm of diameter. Each animal had three trials to find the platform, with each trial having a maximum of 2 minutes. The average velocity of these animals was determined through an infra-red camera, associated to the VideoTrack software (Viewpoint, France).

Immunohistochemistry. After obtaining the spinal cord sections (detailed in Supporting Information) immunohistochemistry (IHC) protocols were performed. The following primary antibodies were used: (a) mouse anti-CD11b/c (Pharmingen, USA), (b) rabbit anti-rat glial fibrillary acidic protein (GFAP, Dako, Denmark), (c) mouse anti-NF (Millipore), (d) rabbit anti-tyrosine hydroxylase (TH, Millipore), and (v) mouse anti-nuclei antibody (HuNu, Millipore). IHC and staining quantification protocols can be found in Supporting Information.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software). Differences among groups were assessed by: (a) one-way ANOVA test in results presented in Figures 1B, 1C, 3B, and 5C, and 6, (b) two-way ANOVA in results presented in Figures 1D, 3C, 4, and 5A, 5B. One or two-way ANOVA tests were followed by the Bonferroni post-hoc test. A p-value of $\leq .05$ was set as the criteria for statistical significance.

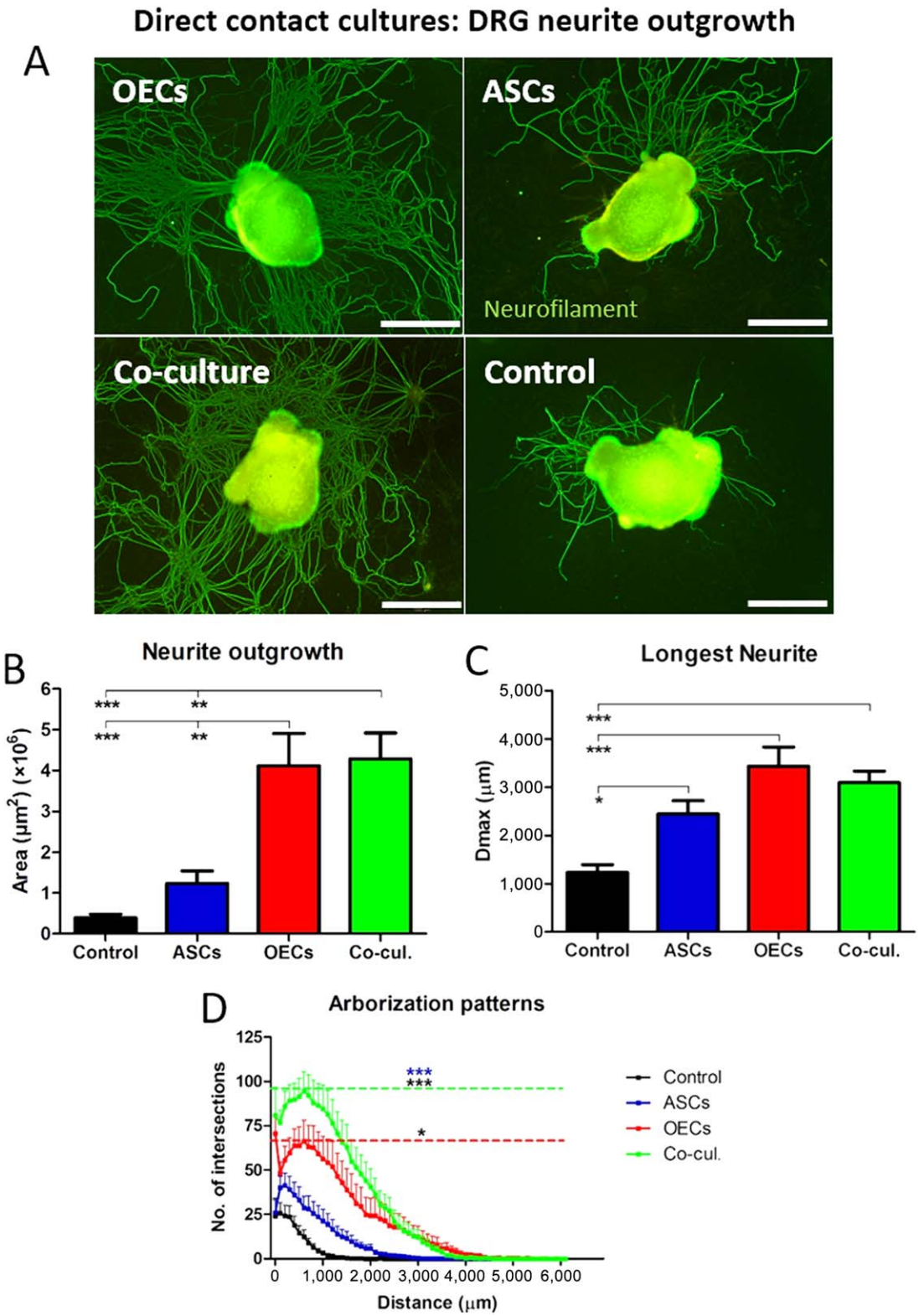

Figure 1. Two-dimensional direct contact cultures of ASCs, OECs, or both with dorsal root ganglia (DRG) explants. In direct culture conditions, OECs have a higher impact on neurite outgrowth in comparison to ASCs. **(A):** Representative fluorescence microscopy images of DRG explants and their neurites stained with neurofilament (in green), for each of the groups under evaluation. **(B):** Quantification of the neurite area with ImageJ software. **(C):** Quantification of the distance of the longest neurite (Dmax) with Neurite J plugin. **(D):** Analysis of the arborization patterns produced by each experimental group. Scale: 1 mm; Values are shown as mean ± SEM ($n = 3$ independent experiments); *, $p \leq .05$; **, $p \leq .01$; ***, $p \leq .001$. In graph (D), the differences between groups are highlighted according to their group colors. Abbreviations: ASCs, adipose tissue-derived stromal cells; OECs, olfactory ensheathing cells.

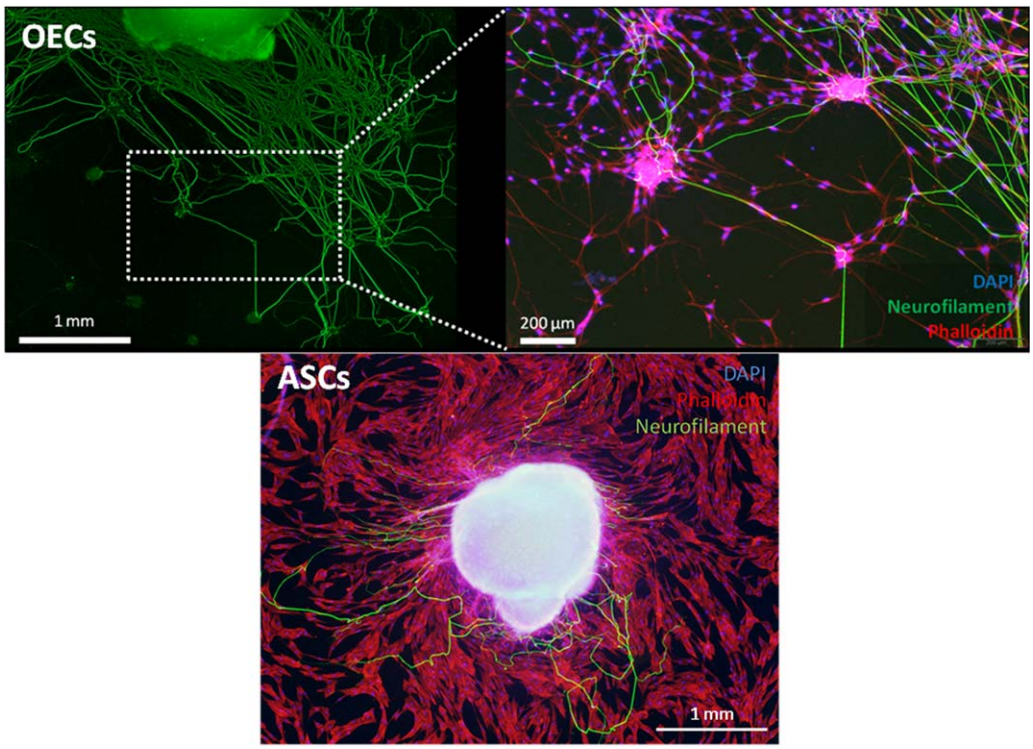

Figure 2. Culture of dorsal root ganglia (DRG) explants with OECs or ASCs. Neurites (in green) formed by the DRG follow the orientation of OECs (in red, upper right panel). In the case of ASCs (lower panel), it seems that the neurites formed do not follow the direction and orientation of cells as clearly as in OECs cultures. Abbreviations: ASCs, adipose tissue-derived stromal cells; OECs, olfactory ensheathing cells.

Results

Effects of Direct Coculture of ASCs/OECs with DRGs

In order to assess the effects of both cell types on neuritogenesis, we started by performing direct cultures of both cells (alone or in combination) with DRG explants. After 4 days of culture, it was visible a clear effect of OECs (or coculture of OECs with ASCs) on neurite outgrowth from the explants (Fig. 1A). The area occupied by the neurites cultured with OECs ($4.1 \pm 0.8 \times 10^6$ μm^2) was similar to the area of the neurites from the coculture group ($4.3 \pm 0.6 \times 10^6$ μm^2), but significantly higher in comparison to ASCs group ($1.2 \pm 0.3 \times 10^6$ μm^2) or the control group ($0.4 \pm 0.1 \times 10^6$ μm^2) (Fig. 1B). After analyzing the arborization pattern of each experimental group, it was possible to observe that coculture and OECs groups present significantly more intersections (Fig. 1D), which is a synonym of higher ramification of the neurites formed. In addition, the neurites formed in these groups extended to longer distances than ASCs group (OECs: $3,433 \pm 400$ μm; Coculture: $3,093 \pm 239$ μm; ASCs: $2,446 \pm 274$ μm); or the control ($1,231 \pm 161$ μm) (Fig. 1C).

After a more detailed analysis to the cellular disposition and neurite formation it becomes clear that newly formed neurites followed very closely OECs direction and orientation, which was not so evident in cultures with ASCs (Fig. 2). These results might indicate that the influence of OECs on neurite outgrowth, under a direct contact paradigm, is higher in comparison to ASCs. In particular, OECs seem to have a guidance capacity, favoring the growth of neurites toward OECs-enriched regions.

Effects of ASCs/OECs Secretome on DRGs Neurite Formation

Using the same in vitro model, we cultured DRG explants without the presence of the cells, but in contact with the factors secreted by ASCs or OECs. In this context, and after 5 days in contact with ASCs or OECs secretome, the only group that presented relevant neurite formation was the conditioned media (CM) of ASCs (total area: $32,675 \pm 11,869$ μm^2). OECs secretome and the control did not induce any significant neurite extension (Fig. 3A–3C). In order to characterize and explore the role of ASCs' and OECs' secretome on the effects observed, a set of proteomic data previously obtained from ASCs, was re-analyzed [34] and a new proteomic analysis was performed for OECs secretome (Supporting Information Tables 2 and 3). The proteins identified in the secretome of ASCs may be involved in several different pathways (Fig. 3D), from which we highlight the VEGF and Cadherin signaling pathways, as well as the PI3 kinase pathway, which are responsible for a myriad of cellular processes, including neurite outgrowth and neuronal protection. Proteins involved in neuronal communication pathways are also present in ASCs' secretome. On the other hand, even though OECs secretome also contains proteins related to neurite outgrowth pathways, these seem less relevant (Fig. 3D). For example, the VEGF signaling pathway is absent of the main hits analysis for signaling pathways. A complementary analysis, based on a PANTHER overrepresentation test, also highlights the importance of reactome pathways associated with neurite outgrowth (Supporting Information Table 1).

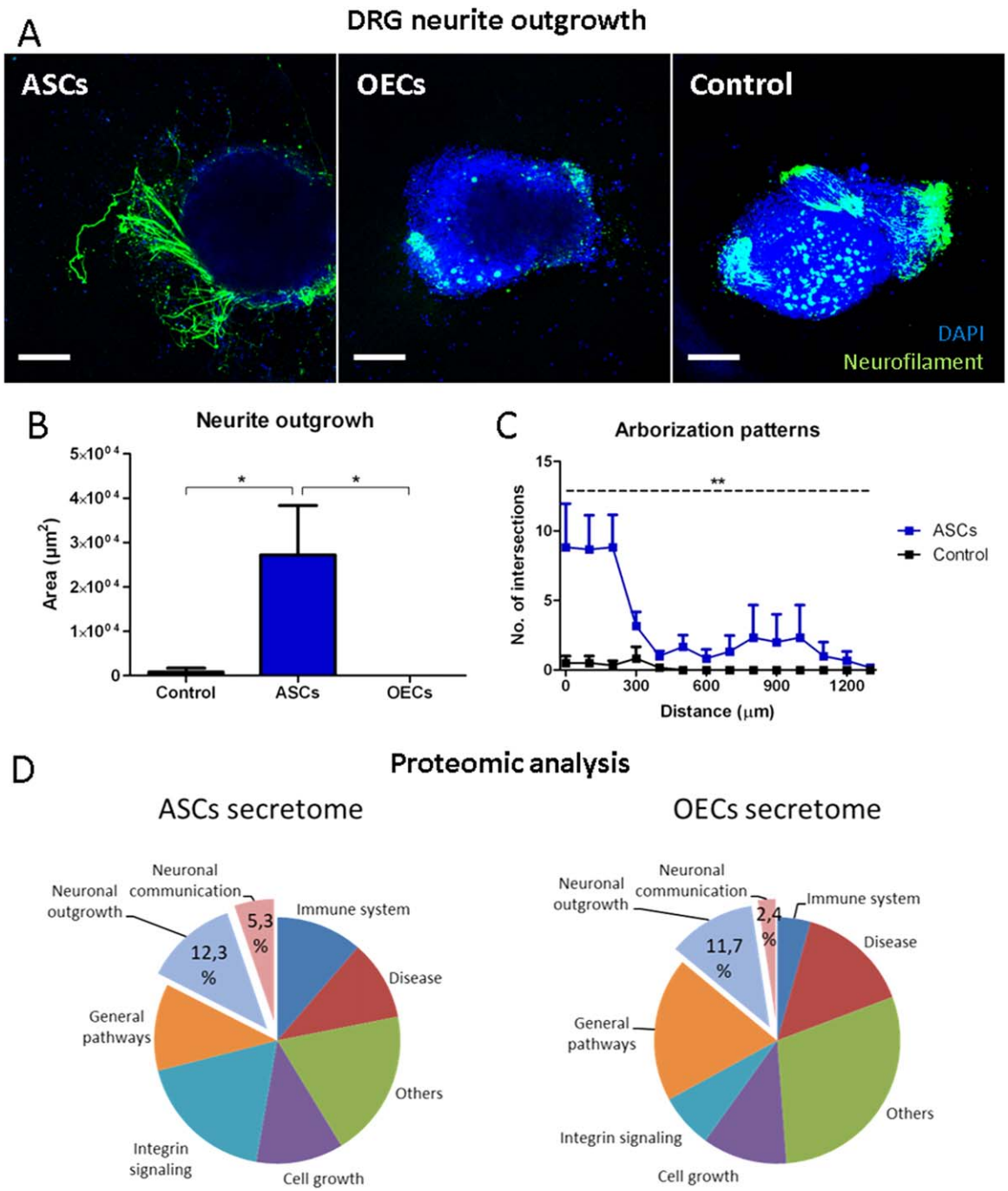

Figure 3. Cultures of dorsal root ganglia (DRG) explants with the secretome derived from different cell sources and proteomic analysis. **(A):** Representative confocal images of DRG explants and their neurites stained in green. **(B):** Quantification of the neurite area with ImageJ software. **(C):** Analysis of the arborization patterns produced by each experimental group. Only ASCs' secretome was able to induce neurite outgrowth on DRG explants, while OECs secretome and the control did not present a significant neurite formation. **(D):** Proteomic analysis performed for ASCs' and OECs' secretome. The proteins identified are involved in several different pathways, some of which are responsible for processes such as neuronal outgrowth and neuroprotection. Scale: 100 μm; Values are shown as mean ± SEM ($n = 6$ replicates); *, $p \leq .05$; **, $p \leq .01$. Abbreviations: ASCs, adipose tissue-derived stromal cells; OECs, olfactory ensheathing cells.

ASCs and OECs Treatment Leads to an Improvement of Locomotion in SCI Rats

Taking into account the results obtained in vitro, we decided to move to an in vivo proof of concept, by transplanting both cell types into SCI animals. We divided the animals in three different groups: SCI rats without treatment (hemisection–HS group), SCI rats transplanted with ASCs/OECs (Cells group), and noninjured rats (laminectomy only–Sham group).

After 8 weeks, the BBB analysis revealed that animals treated with cells displayed improved motor outcomes, when comparing to nontreated animals (Fig. 4B, 4C).

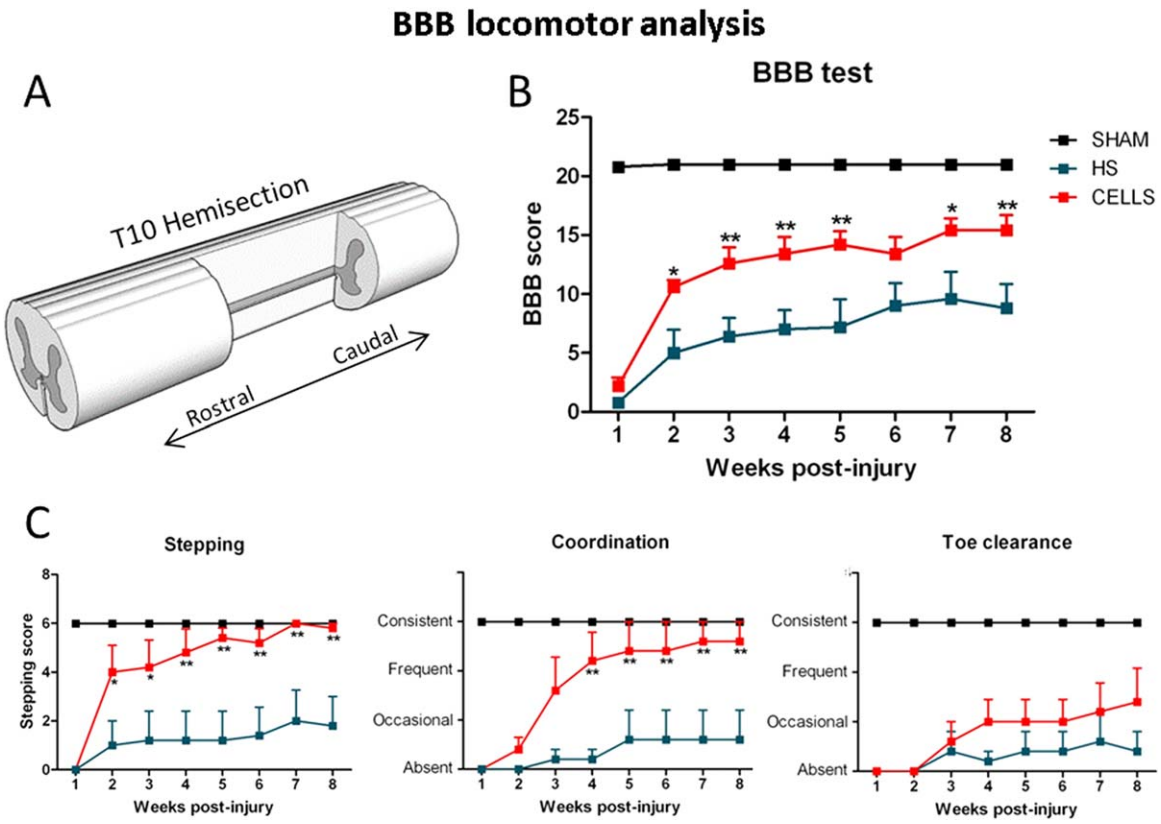

Figure 4. Locomotor recovery assessed by the BBB test, during 8 weeks of follow-up. **(A):** Schematic representation of the T10 left hemisection injury induced. **(B):** Mean BBB score of the different experimental groups. **(C):** Evaluation of three different parameters used in the BBB subscore: stepping, coordination, and toe clearance. Cells-treated animals presented significant improvements of BBB score, in different time points, in comparison to nontreated (HS) animals. Values are shown as mean ± SEM ($n = 5$); *, $p \leq .05$; **, $p \leq .01$. Abbreviations: BBB, Basso, Beattie, Bresnahan Locomotor Rating Scale; HS, hemisection.

In particular, the mean BBB score was statistically different at 2, 3, 4, 5, 7, and 8 weeks post-injury (Fig. 4B). In addition, sham animals did not present motor deficits during this period. After a detailed analysis on the BBB test, we concluded that the main differences between Cells and HS groups were in the stepping capability, coordination, and toe clearance (Fig. 4C). Transplanted rats presented higher stepping scores, meaning that were capable of doing plantar steps while nontreated rats did mostly dorsal steps. They also showed more frequent coordinated steps and a tendency to perform better in toe clearance. No differences were observed in parameters such as the position of the paw, tail position, and trunk instability (data not shown).

No significant differences between cells-treated and nontreated rats were found in the total distance measurements (Fig. 5A) and in the number of rearings (Fig. 5B), both evaluated at zero and 8 weeks post-injury. However, it is interesting to notice that in rearing behavior, sham animals are only significantly different in comparison to the nontreated group (HS), at 8 weeks post-injury, a fact that can be interpreted as indicative of the relevance of the ASCs/OECs based therapy.

A similar result was observed in the swimming test (Fig. 5C) performed 8 weeks post-injury. HS group was not significantly different from Cells' transplanted group; however, sham animals presented a significantly higher average velocity in water, only when compared with nontreated animals.

Transplantation of ASCs and OECs Reduces the Infiltration of Inflammatory Cells

Eight weeks after the injury, IHC against CD11b/c revealed that the area occupied by inflammatory cells in regions close to the injury site was significantly increased in the HS group ($18{,}765 \pm 3{,}932\ \mu m^2$) in comparison to the levels of the sham group ($5{,}590 \pm 1{,}606\ \mu m^2$), and to rats cotransplanted with ASCs and OECs ($7{,}548 \pm 525\ \mu m^2$, Fig. 6A, 6B). This indicates that ASCs/OECs treatment was apparently preventing the infiltration of inflammatory cells, which partially explains the functional results obtained. In the contralateral side to the lesion, there were no differences between groups in the area of CD11b/c (data not shown). IHC against GFAP revealed a similar trend, although there were no significant differences among groups (Fig. 6A, 6C). Nevertheless, injured rats present higher levels of astrogliosis than sham animals, while treatment with cells provides an evident reduction in those values.

Transplanted ASCs Were Present in the Spinal Cord Eight Weeks after Transplantation

An IHC against HuNu was performed in order to identify transplanted ASCs, since it is a specific marker for human cells. In fact, results showed that ASCs were still present at the spinal cord, 8 weeks post-transplantation (for all

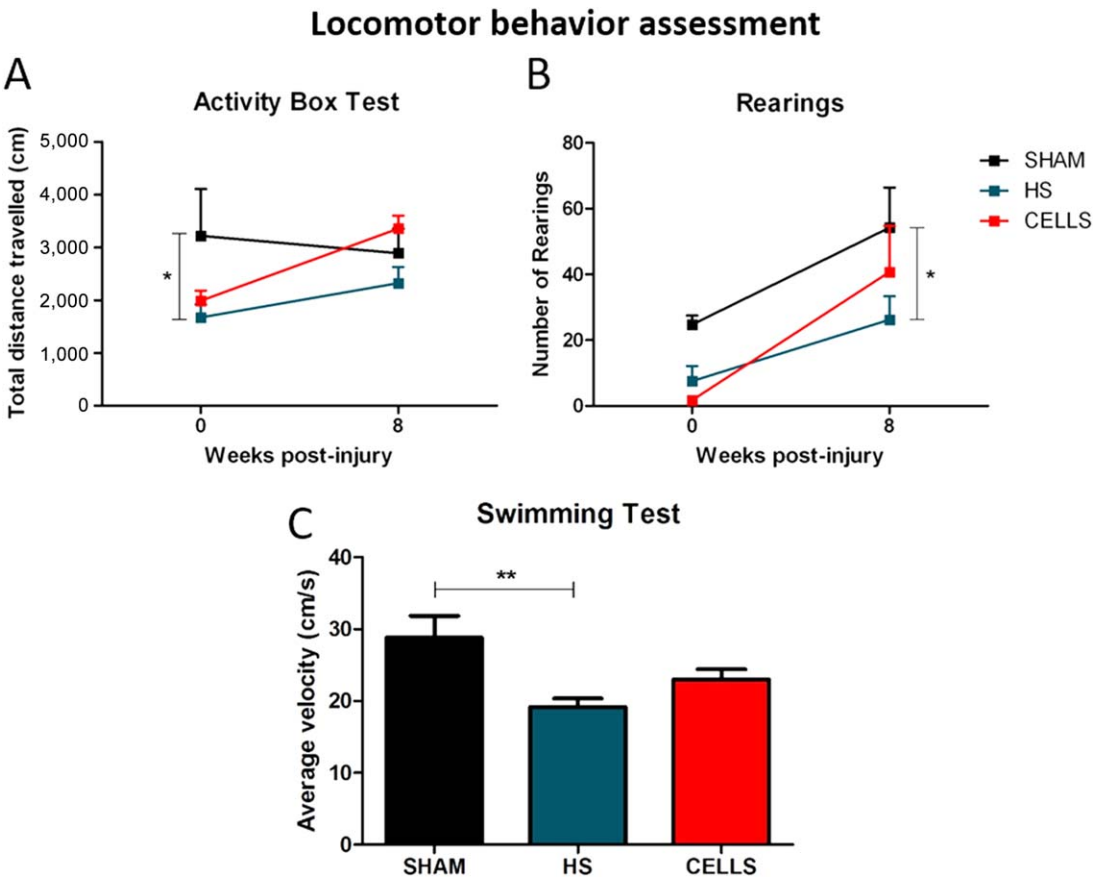

Figure 5. General locomotor activity measured by the activity box test and swimming test. Average distance travelled by each group **(A)** and total number of rearings **(B)** in the activity box test, both at zero and 8 weeks post-injury. There are no significant differences between cells-treated and nontreated animals. **(C):** Average velocity for each experimental group measured in a swimming test, 8 weeks post-injury. The transplantation of ASCs and OECs did not lead to an improvement of the average velocity in water of injured animals. Values are shown as mean ± SEM ($n = 5$); *, $p \leq .05$; **, $p \leq .01$. Abbreviation: HS, hemisection.

transplanted animals). These cells could mostly be found in regions close to the injury site, or where there was high infiltration of cells (Fig. 7).

Discussion

The use of cellular therapies in SCI research has been widely explored [39]. Several different types of cells have already been tested [40] and even numerous clinical trials have been done or are still ongoing [14, 41, 42]. The big advantage of the cells used in our therapeutic approach is their accessibility and easy translatability to the clinics. Despite of the promising results demonstrated by the solo transplantation of ASCs or OECs in other works [12, 13, 20, 22], the combined administration of these two specific cell populations has rarely been tested. In addition, the mechanisms by which they act remain elusive. In order to understand ASCs and OECs influence on a specific phenomenon such as neurite outgrowth, we started by using DRG explants as a model of axonal regeneration [35].

From the data obtained, we can conclude that OECs provide better support to guided neurite outgrowth than ASCs, producing massive neurite arborizations (Figs. 1, 2). On the other hand, when we used the secretome of the cells, the only one capable of inducing neurite formation was ASCs CM (Fig. 3). This could mean that both ASCs and OECs have the potential to induce neurite formation, but their main mechanism of action might be different. For instance, in a study from Leaver et al. [43] olfactory ensheathing glia (OEG) also induced significant increases in the number and extension of neurites from retinal explants, in that case in comparison to Schwann cells (SCs). This enhanced growth was also suggested to be contact-mediated since conditioned media from OEG did not present the same effects [43]. Another example of contact mediated increase of axonal outgrowth induced by OECs includes DRG axons grown on myelin substrates [44]. In our study, the secretome of OECs did not reveal to be a growth promoting substrate for axonal outgrowth of DRG explants, even though there is a substantial body of literature supporting the relevance of OECs' secreted factors in axonal regeneration context [45–47]. The contact-mediated ability of OECs might be partially due to the action of metalloproteinases (MMPs) such as MMP-2, MMP-3, and MT1-MMP, which seem to play a role on cellular motility and on their neurotrophic properties [48]. Meanwhile, the secretome of ASCs has been characterized by our group and others and it is known to contain several axonal-growth factors, such as nerve growth factor (NGF), glia-derived nexin, or semaphorin 7A, as well as factors related to neuronal differentiation such as pigment epithelium-derived factor [6, 10, 34, 49]. Some of these

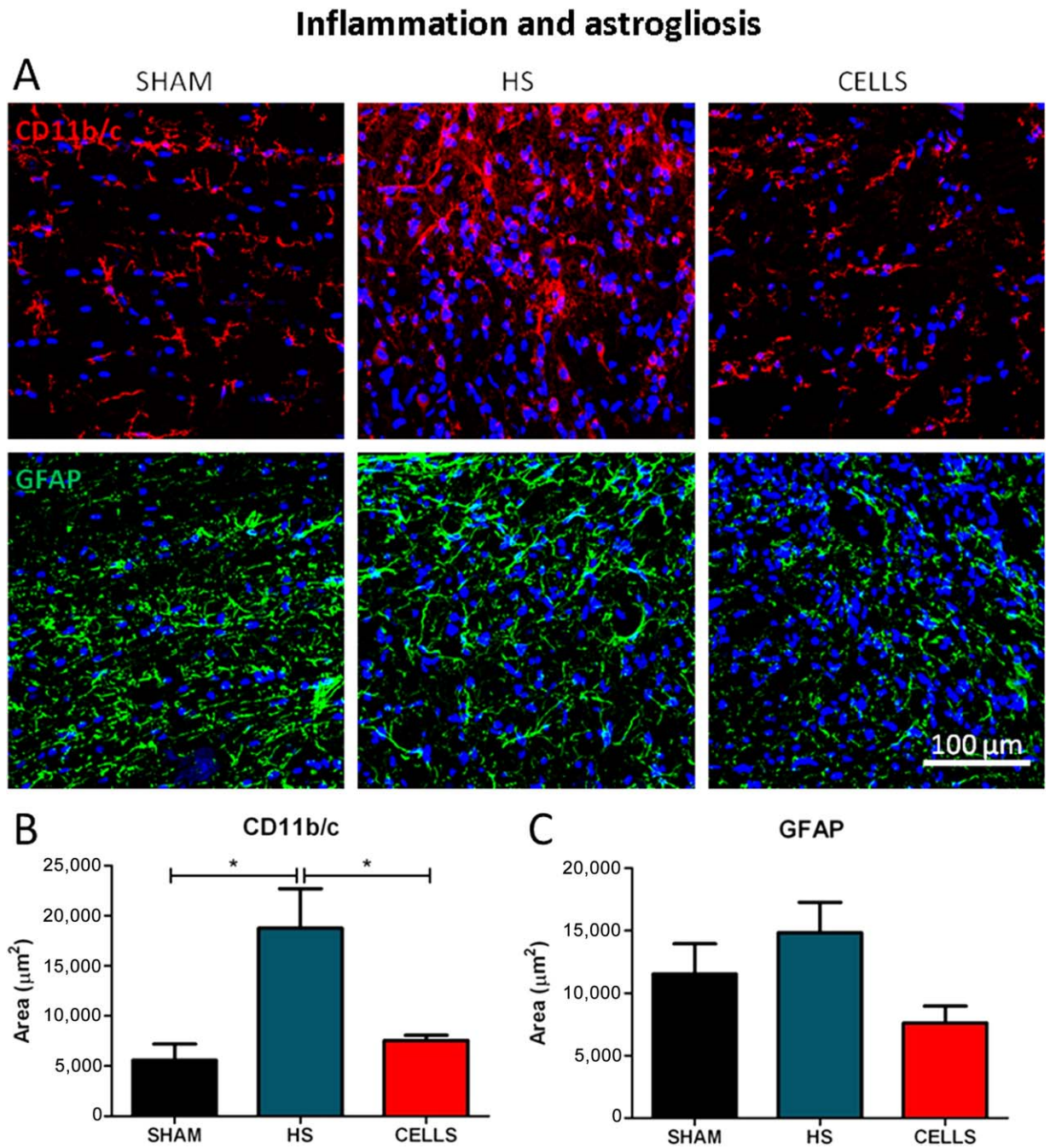

Figure 6. Immunohistochemistry results for CD11b/c and GFAP markers. **(A):** Representative confocal images of spinal cord longitudinal sections, immunostained for CD11b/c (upper panel) and GFAP (lower panel). **(B):** Quantification of CD11b/c and **(C)** GFAP markers' area. Injured animals (HS group) have significantly increased levels of CD11b/c, while cells-treated animals present values close to those of sham animals. Values are shown as mean ± SEM ($n = 5$); *, $p \leq .05$. Abbreviations: GFAP, glial fibrillary acidic protein; HS, hemisection.

might be responsible for the effects observed in DRG explants. Furthermore, after exploring our proteomic data [34], we could find in ASCs' secretome proteins associated to several different signaling pathways. From those, we highlight the VEGF signaling pathway for instance, which has a key role in angiogenesis [50] but also has been shown to promote adult neurogenesis and neuronal cell migration by stimulating endothelial cells in vascular niches to release cues for neural stem cells [51]. The cell cycle regulator PI3K pathway also plays a crucial role in neuronal cell survival and proliferation being stimulated by growth factors such as NGF or brain-derived neurotrophic factor [52, 53]. Finally, cadherin signaling pathway is involved in central processes such as cell adhesion, neurogenesis and synaptic plasticity [54–56] and importantly it has been demonstrated to promote neurite outgrowth by facilitation of fibroblast growth factor (FGF) receptor signaling [57]. Several other pathways are highly associated with our secretome, namely the hypoxia response via hypoxia-inducible factor, as well as the endothelial growth factor (EGF) signaling pathway, or the FGF signaling pathway, which in general are

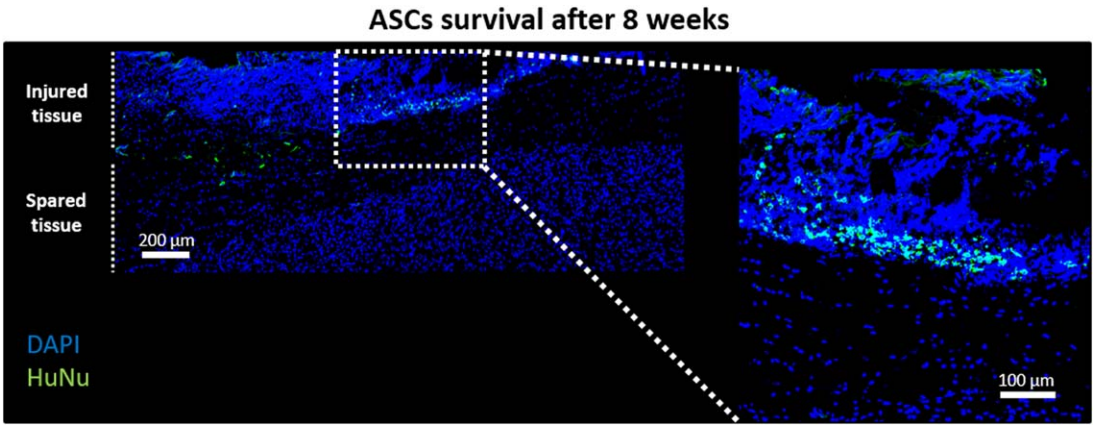

Figure 7. Confocal images depicting adipose tissue-derived stem cell ([ASCs] stained for HuNu, in green) present at the site of injury. ASCs might have migrated from the injection sites to the border of the hemisection injury.

implicated in neurite outgrowth, neurogenesis, neuroprotection and in many cellular processes that may contribute to SCI regeneration. Proteins involved in neuronal communication pathways, such as the dopamine receptor-mediated signaling pathway, are also present in ASCs secretome. Moreover, by exploring reactome pathways (Supporting Information Table 1) we could highlight the possible role of neural cell adhesion molecule 1 (NCAM1) pathway in ASCs mediated-neurite outgrowth. ASCs secretome was enriched in proteins directly or indirectly related to NCAM1. This molecule modulates neuronal cell adhesion, survival, and synaptic plasticity among others. We found 14-3-3 protein which blocks neuronal apoptosis [58]; fibronectin, a main component of the ECM, responsible for cell adhesion through integrin activation [59]; or spectrin, implicated in the normal morphology of neuron cell bodies and neurites [60]. Additionally, the semaphorin receptor protein neuropilin-1 (in its soluble form [61] or released through exosomes [62]), which is involved in axonal guidance mechanisms, was also present in our sample [63]. On the other hand, our proteomic analysis of OECs secretome shows in fact that different proteins were present, and consequently associated to numerous signaling pathways. Nevertheless, none of them showed to be sufficient to induce neurite outgrowth of DRG explants. The absence of the VEGF pathway, together with a reduction of neuronal communication pathways or integrin signaling, could be responsible for the lack of effects observed.

Considering the in vitro results herein obtained, we opted to transplant ASCs and OECs together, so we could take advantage from the contact-mediated regenerative capacity of OECs and at the same time, benefit from the neurite outgrowth and neuroprotective properties associated to ASCs secretome. In this study, the acute transplantation of ASCs and OECs led to functional improvements, most evident in the BBB test. This was associated with a marked decrease of CD11b/c marker and a slight decrease of GFAP in areas close to the lesion. The effect on microglial/inflammatory cells is supported by an in vitro experiment in which ASCs and OECs secretome reduced significantly the number of CD11b/c cells in culture, revealing that the factors secreted by both types of cells might mediate this mechanism (Supporting Information Fig. 2). We did not see differences in any other markers analyzed, namely NF for axons or tyrosine hydroxylase for specific dopaminergic neurons (Supporting Information Fig. 1). This excludes a direct effect of OECs on neuronal rewiring, and supports the idea that OECs might have worked as "feeders" supporters for ASCs growth, as previously shown by our group in in vitro experiments [31]. Therefore, the functional improvements obtained were not directly connected to preservation or regeneration of the neuronal circuitry, but more linked to modulation of the injury environment. In fact, a reduction of inflammation and astrogliosis has been previously associated with improvements in locomotor function [64, 65].

In the histological analysis, we could detect transplanted ASCs, 8 weeks after injury. Their distribution seems to be restricted to regions close to the injury site, or in areas of visible cellular infiltration. In this sense, ASCs were able to migrate from the injection sites to the injury site and were integrated into the spinal cord tissue. The survival of ASCs 2 months after transplantation is an important finding because this means that ASCs were able to exert their paracrine-based effect over longer periods of time. As previously mentioned, ASCs secrete immunomodulatory molecules, which associated with their long survival may explain the decrease observed on inflammation. Unfortunately, we could not trace transplanted OECs. These cells share many markers with SCs and it is known that SCs infiltrate the spinal cord after a lesion [66], thus we could not distinguish between these two types of cells. Nevertheless, cultures of OECs before transplantation presented around 90% purity levels, based on ICC against p75 positive cells (Supporting Information Fig. 3).

Overall, our results unveil a beneficial effect of transplanting both ASCs and OECs after a SCI. However, many issues remain to be clarified regarding the application of these cell populations in SCI. In the case of OECs, the influence of the specific region from where they are isolated, the purity of the cultures, and the existence of subtypes of OECs [16] are just some examples of the variety existing in the literature. In addition, for both ASCs and OECs it will be important to understand which are the main mechanisms and molecules mediating their effects, so one can take the maximum advantage of these cells. Finally, it will be interesting to test the transplantation of ASCs and OECs after the establishment of the lesion, in a subchronic or chronic phase, or even combine ASCs/OECs with a biomaterial or scaffold that can enhance their survival in the hostile environment existing after a SCI.

Conclusion

Cellular therapies for SCI treatment are one of the most explored strategies in preclinical research. The choice of a cell population suitable for transplantation needs to take into account not only its potential therapeutic effect but also its translatability into a clinical setting. In this context, ASCs and OECs could be an interesting solution.

From this work, it is possible to conclude that both ASCs and OECs have the potential to induce neurite outgrowth in a DRG explant model, even though their main mechanism of action might be different. If on one side, OECs seem to provide physical support and guidance for neurite outgrowth and elongation, ASCs on the other hand may secrete different molecules that potentiate neurite formation and growth, assuming a more paracrine role on neuritogenesis. In addition, their combined administration into an animal model of SCI resulted in significant improvements of locomotor behavior and a marked reduction of inflammatory cells. Neurite outgrowth was not observed at the lesion site, which may suggest a more supportive role for OECs in vivo, instead of a rewiring of the neuronal network. Importantly, ASCs could be detected 2 months post-transplantation. In summary, the acute transplantation of ASCs and OECs after a SCI is a promising and easily translatable approach that with the proper refinements, could lead to a significant amelioration of patients suffering from SCI.

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Author Contributions

E.D.G. and S.S.M.: collection and/or assembly of data, data analysis and interpretation, manuscript writing; R.C.A-S., F.G.T., A.O.P., S.I.A., and H.L.-A.: collection and/or assembly of data; B.M.: collection and/or assembly of data, manuscript writing; J.M.G. and A.C.L.: provision of study material or patients; N.S.: manuscript writing; N.A.S.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; A.J.S.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

J.G. discloses employment at LaCell LLC; Patents pending to LaCell LLC and Tulane University; Consultant/Advisory role with Obatala Sciences. J.G. is also a co-owner of LaCell LLC and Obatala Sciences Inc. The other authors indicated no potential conflicts of interest.

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