Biomaterials 105 (2016) 38–51

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Combination of a peptide-modified gellan gum hydrogel with cell therapy in a lumbar spinal cord injury animal model

Eduardo D. Gomes [a, b], Sofia S. Mendes [a, b], Hugo Leite-Almeida [a, b], Jeffrey M. Gimble [c], Roger Y. Tam [d, e], Molly S. Shoichet [d, e], Nuno Sousa [a, b], Nuno A. Silva [a, b], António J. Salgado [a, b, *]

[a] Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal
[b] ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal
[c] Center for Stem Cell Research and Regenerative Medicine, Tulane University, 1430 Tulane Avenue, SL-99, New Orleans, LA, 70112, USA
[d] Department of Chemical Engineering and Applied Chemistry, 200 College St., University of Toronto, Toronto, Ontario, M5S 3E5, Canada
[e] Institute of Biomaterials and Biomedical Engineering, 160 College St., University of Toronto, Toronto, Ontario M5S 3E1, Canada

ARTICLE INFO

Article history:
Received 21 April 2016
Received in revised form
9 July 2016
Accepted 16 July 2016
Available online 20 July 2016

Keywords:
Spinal cord injury
Regenerative medicine
Adipose tissue-derived Stromal/Stem Cells
Olfactory Ensheathing Cells
Extracellular matrix like hydrogels

ABSTRACT

Spinal Cord Injury (SCI) is a highly incapacitating condition for which there is still no cure. Current clinical approaches are mainly based on palliative care, so there is a need to find possible treatments to SCI. Cellular transplantation is regarded with great expectation due to the therapeutic potential of cells such as Adipose tissue-derived Stromal/Stem Cells (ASCs) or Olfactory Ensheathing Cells (OECs). Both are accessible sources and present positive paracrine and cell-to-cell interactions, previously reported by our group. Additionally, biomaterials such as hydrogels have been applied in SCI repair with promising results. We propose to combine a GRGDS-modified gellan gum hydrogel with ASCs and OECs in order to promote SCI regeneration. *In vitro*, ASCs and OECs could be co-cultured within GG-GRGDS hydrogels inducing a more robust neurite outgrowth when compared to controls. *In vivo* experiments in a hemisection SCI rat model revealed that the administration of ASCs and OECs encapsulated in a GG-GRGDS hydrogel led to significant motor improvements when compared to both control (SCI) and hydrogel alone (GG-GRGDS) groups. This was accompanied by a decreased infiltration of inflammatory cells and astrocytes, and by an increased intensity of neurofilament. These results suggest evident gains induced by the encapsulation of ASCs and OECs in GG-GRGDS based hydrogels.

1. Introduction

Spinal Cord Injury (SCI) is a highly debilitating condition for which there is still no cure. SCI individuals usually have life-long loss of function and reduced quality of life. Its incidence internationally varies from as low as 2.3 to as high as 83 per million inhabitants every year [1]. The secondary events occurring after the primary injury increase the complexity of the disease, which hinders SCI treatment [2]. The current medical approaches after a spinal cord trauma are limited, consisting of the stabilization of the spine, decompression of the cord and eventually the administration of anti-inflammatory drugs [2]. Therefore, the development of novel therapeutic strategies targeting this condition is imperative.

Amongst the different approaches suggested so far, cellular based therapies have been one of the most frequently explored. From the different sources of cells currently being tested, Adipose tissue-derived Stromal/Stem Cells (ASCs) and Olfactory Ensheathing Cells (OECs) have shown promising results [3,4]. For instance, intraspinal transplantation of murine ASCs in a SCI animal model, one week after injury, promoted the protection of denuded axons probably by preventing oligodendrocytes' degeneration and by participating in the regeneration of the myelin sheath [5]. In addition, ASCs transplantation also induced evident gains in motor performance [5]. These beneficial outcomes have been mostly related with the nature of the ASC's secretome, that is, the panel of molecules secreted by these cells to the extracellular

* Corresponding author. Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal.
E-mail address: asalgado@ecsaude.uminho.pt (A.J. Salgado).

http://dx.doi.org/10.1016/j.biomaterials.2016.07.019
0142-9612/

milieu. In fact several reports have shown that ASC's secretome contains important neuroregulatory molecules such as Nerve Growth Factor (NGF), Brain-derived Neurotrophic Factor (BDNF), Glial cell line-derived Neurotrophic Factor (GDNF), Vascular Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF), basic Fibroblast Growth Factor (bFGF), Insulin-like Growth Factor 1 (IGF1), Transforming Growth Factor Beta 1 (TGF-β1), among others, that are able to modulate neuronal and glial survival and differentiation [6–8]. Moreover, the molecules secreted by ASCs have also been shown to modulate the response of the immune system [7,9]. Alternatively, OECs are mainly characterized by participating in the growth and guidance of primary olfactory neurons. Their common origin with Schwann cells may explain some similarities observed between these two cell types, namely the capacity of OECs to surround olfactory axons, form fascicular processes and synthesize peripheral-like myelin [10]. The potential OEC transplantation as a therapy for CNS damage has already been explored *in vivo*. About two thirds of the experimental studies using these cells reported improvements in behavior outcome [11]. For instance, murine OECs were able to remyelinate axons in spinal cord injured rats [12,13], leading also to functional improvement of electric conduction in previously demyelinated axons [13]. Therefore, it is considered that OECs can create a permissive environment for axonal regeneration, in the usually hostile milieu of the damaged CNS [14]. For all these reasons, autologous transplantation of OECs in SCI patients has already been performed. In one clinical trial, results showed that autologous OECs are safe after three years post-transplantation [15,16].

ASCs and OECs present themselves as promising candidates for SCI cell therapy, mostly because they are easily accessible (ASCs can be obtained in large quantities from lipoaspirates while OECs can be safely isolated from nasal biopsies) and can be applied in an autologous manner, avoiding ethical concerns and the need for immunosuppression. By combining both, we envision taking advantage of the beneficial properties of each cell type simultaneously; namely, the neuronal regeneration guided and supported by OECs, which can be boosted by the paracrine effects of ASCs. Furthermore, previous work from our group showed that the secretome of rat-derived OECs has a positive effect on MSCs from different sources, but more evidently on human ASCs by increasing their proliferation and metabolic activity. Similarly, the secretome of ASCs also proved to be particularly beneficial for OECs in the same parameters [17]. These results reinforce the potential benefits of the combined use of these cells.

Therefore, the objective of the present work was to assess if the combined delivery of human ASCs and murine OECs into the injury site of a rat lumbar hemisection model, was able to induce motor and histological improvements in the injured rats. For this purpose and in order to efficiently deliver both cell populations, a hydrogel-based biomaterial (gellan gum, chemically grafted with a fibronectin-mimetic GRGDS peptide – GG-GRGDS) was used. Hydrogels are particularly appealing to be used as vehicles for cell transplantation because not only can they enhance cell survival, but they can also be designed to match the mechanical properties and water content of the CNS [18–21]. Our proposed chemically-modified gellan gum hydrogel is a biocompatible and biodegradable natural polysaccharide composed of repeating units of glucose, glucuronic acid and rhamnose [22,23] and is FDA approved [24]. Its physical properties allow the injection *in situ* and the filling of cavities provoked by the injury, in a minimally invasive manner. Moreover when chemically grafted with the fibronectin-mimetic peptide GRGDS (GG-GRGDS), this biomaterial was shown to have enhanced cell adhesion and proliferation [25,26], which lead to significant improvements of the metabolic activity and secretome of encapsulated cells [25,26].

2. Materials and methods

2.1. Cell isolation and culture

Human Adipose tissue-derived Stromal/Stem Cells (ASCs) were isolated according to the protocol described by Dubois et al. [27] from human lipoaspirates obtained from consenting donors under an institutional review board approved protocol at LaCell LLC. These cells were cultured and maintained in α-MEM (Invitrogen, USA), with 10% Fetal Bovine Serum (FBS, Biochrom AG, Germany) and 1% antibiotic-antimycotic solution – penicillin-streptomycin (Invitrogen, USA) at 37 °C and 5% CO_2 (v/v).

The animal care committee of the research institute approved all the animal protocols in accordance with standardized animal care guidelines [28]. Olfactory Ensheathing Cells (OECs) were harvested from olfactory bulbs of neonatal (P5-P7) Wistar-Han rats, according to the protocol previously described [29]. Briefly, upon dissection, the meninges and blood vessels were removed and the tissue was digested with collagenase type I (2.5 mg/ml, Sigma, USA) for 30 min at 37 °C, with agitation. The digested tissue was mechanically dissociated with a 5 ml pipette and centrifuged at 1000 rpm for 5 min. Then, the tissue was resuspended and subjected to a second mechanical dissociation using a P1000 micropipette. After a second centrifugation, cells were resuspended and seeded on uncoated plates for two consecutive periods of 24 h. It is expected that most of the fibroblasts and astrocytes attach in the first and second periods, respectively. After this purification step, the remaining cells were seeded on fibronectin coated surfaces (for 2D direct co-cultures) or encapsulated in GG-GRGDS hydrogel (for 3D co-cultures). Coating was done overnight with a 1 mg/ml fibronectin solution (Sigma, USA). Cells were cultured in DMEM/F12 (Invitrogen, USA) with 10% FBS and 1% antibiotic-antimycotic solution at 37 °C and 5% CO_2 (v/v). OECs were additionally enriched with Bovine Pituitary Extract (5.36 μg/ml, Invitrogen, USA) and Forskolin (1.4 μg/ml, Sigma, USA).

2.2. 2D direct co-cultures

In order to assess the potential positive or negative interactions between OECs and ASCs, a direct co-culture system with these two cell types was used. After isolation and purification (described in section **2.1**), OECs were seeded (60 000 cells/cm^2) on fibronectin coated coverslips. 24 h later, ASCs were seeded (10 000 cells/cm^2) over the OECs culture. Cells were allowed to grow in OECs culture medium (DMEM/F12 with supplements) since previous experiments showed its suitability for the culture of ASCs (data not shown). Medium was changed once, after three days of culture. Following one and seven days of incubation both cell growth and morphology was assessed by immunocytochemistry (ICC). OECs (60 000 cells/cm^2) and ASCs (10 000 cells/cm^2) monocultures were used as controls. Cell counts were performed by taking ten representative micrographs per sample, and the mean number of cells per field was determined for each sample of each group ($n = 3$).

2.3. Synthesis of GG-GRGDS hydrogel

The synthesis of GG-GRGDS hydrogel was performed according to the protocols described by Silva et al. [25]. Briefly, gellan gum (Sigma, USA) was first dissolved in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (100 mM, pH 5.5, Sigma, USA) at 37 °C. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM, Sigma, USA) and furfurylamine (Acros Organics, Belgium) were then added in a 4:1 M ratio (of each reagent relative to the –COOH groups in gellan gum) and stirred at 37 °C for 48 h. The solution was then dialyzed (Mw cutoff 12–14 kDa, Spectrum

Labs, USA) alternately against distilled water and PBS (0.1 M, pH 7.2) for five days. Finally, water was removed by lyophilization to obtain furan-modified gellan gum (furan-GG) as a white powder.

Immobilization of maleimide-modified GRGDS peptide (mal-GRGDS, Anaspec, USA) to furan-GG was performed via Diels-Alder chemistry between the maleimide functional group of the peptide with the furan group of the gellan gum [25]. Furan-GG was first dissolved in MES buffer (100 mM, pH 5.5) at 37 °C (1.2 mg/ml). Mal-GRGDS was then added in a 5:1 maleimide:furan molar ratio and vigorously stirred for 48 h. The solution was then dialyzed (Mw cutoff 12–14 kDa) alternately against distilled water and PBS (0.1 M, pH 7.2) for five days. Finally, the water was removed by lyophilization to obtain GRGDS-modified Gellan Gum (GG-GRGDS) as a white powder. The amount of peptide immobilized on the hydrogel was calculated by amino acid analysis. In brief, this method involved acid hydrolysis of the peptide with 6 N HCl for 24 h, followed by derivatization with phenylisothiocyanate (PITC). The derivatized hydrolyzates were then quantified using reverse phase HPLC. As a control, a known amount of mal-GRGDS was incubated with unmodified gellan gum, and the peptide concentration was quantified by amino acid analysis.

2.4. GG-GRGDS 3D hydrogel preparation

GG-GRGDS lyophilized powder was sterilized by exposure to UV lights for 15 min, a method previously used without affecting the material properties [26]. Then, GG-GRGDS was dissolved in ultra pure water, at 1% (w/v) concentration and heated at 40 °C overnight, in order to obtain a homogenous solution. Before encapsulating the cells, $CaCl_2$ at 0.3% (w/v) was added [to obtain a final concentration of 0.03% (w/v) of $CaCl_2$ in solution] to enable the ionic crosslinking of the hydrogel. GG-GRGDS hydrogel used for the *in vitro* 3D cultures was mixed with unmodified GG (1% w/v) in a 1:1 ratio, while for the *in vivo* studies only the modified hydrogel was used.

2.5. 3D cell culture – OECs and ASCs

Direct co-cultures of OECs and ASCs encapsulated within the GG-GRGDS hydrogel were performed with the objective of assessing the interactions of these cells in a 3D environment. Moreover, this experiment was also crucial to determine if the hydrogel was suitable for the growth of both cell types and a proper vehicle for cellular transplantation.

OECs and ASCs were obtained as described in **2.1** and GG-GRGDS hydrogel was prepared according to **2.4**. Both cell types were resuspended in complete DMEM/F12 medium and encapsulated into the hydrogel, by mixing the pellets with the appropriate volume of hydrogel. A total of 30 000 ASCs and 30 000 OECs were encapsulated in 100 μl of GG-GRGDS and monocultures of each cell type were used as controls, using the same cellular densities as co-cultures. Cells were allowed to grow for seven days, with medium changes every two days. After seven days of incubation, phalloidin and DAPI were used to assess cellular growth and morphology. Immunocytochemistry against human nuclear antigen (HNA) was used to specifically identify ASCs. Samples were analyzed by confocal microscopy using a confocal point-scanning microscope, Olympus FV1000.

2.6. 3D cultures – DRG explants in co-culture with GG-GRGDS/ASCs-OECs

Dorsal Root Ganglia (DRG) explant organotypic cultures can be used as a model of axonal regeneration as they are able to produce extensive neurite outgrowth. The explants were dissected from the cervical and thoracic regions of the spine of neonatal rat pups (P5) and placed in cold HBSS (1x) without Ca^{2+}/Mg^{2+} (Invitrogen, USA). After cleaning the remnants of peripheral nerve processes, DRGs were placed on the top of GG-GRGDS hydrogels with or without encapsulated cells. Four groups were performed: 1) hydrogel alone; 2) hydrogel + ASCs; 3) hydrogel + OECs; and 4) hydrogel + ASCs/OECs. The encapsulation of cells was performed 24 h before DRG isolation and co-culture. A total of 30 000 cells were encapsulated in 50 μl of GG-GRGDS (15 000 ASCs and 15 000 OECs in co-culture group). In the first 24 h cells were maintained in complete DMEM/F12 medium to promote cellular growth. After DRGs isolation and seeding onto GG-GRGDS hydrogels, the medium was changed to neurobasal medium (Invitrogen, USA) supplemented with 6 mg/ml D-glucose (Sigma, USA), 1% penicillin-streptomycin, 1x B27 (Invitrogen, USA) and 2 mM L-Glutamine (Invitrogen, USA). Medium was changed every two days and cultures were kept in a humidified atmosphere at 37 °C, 5% CO_2 (v/v). Following seven days of co-culture, DRGs were fixed and subjected to an immunocytochemistry for neurofilament, a major component of axonal cytoskeleton. Imaging was performed using an Olympus IX81 fluorescence microscope and images were analyzed using the ImageJ software (NIH).

2.7. Immunocytochemistry (ICC) and phalloidin/DAPI staining

ICC protocols were used to identify OECs/ASCs in 2D and 3D co-cultures, as well as DRGs neurite projections in 3D co-cultures. Rabbit anti-NGF receptor (p75) antibody (1:100, Millipore, USA) was used to identify OECs and mouse anti-Human Nuclear Antigen antibody (HuNu, 1:100, Millipore, USA) for ASCs. Mouse anti-neurofilament 200 kDa antibody (1:200, Millipore, USA) was used to identify neurite outgrowth from DRG explants. Controls were made by the omission of the appropriate primary antibody. Briefly, samples were fixed with a solution of 4% paraformaldehyde (PFA, Panreac, Spain) in PBS for 20 min (in 2D cultures) or 45 min (in 3D cultures) at room temperature (RT). Then, after three washes with PBS, cell membranes were permeabilized (with exception of p75 antibody) with 0.3% Triton X-100 (Sigma, USA) for 5 min (2D) or 10 min (3D). Non-specific binding sites were blocked using a solution of 10% Fetal Calf Serum (FCS, Biochrom AG, Germany) in PBS for 1 h (2D) or 1 h 30 min (3D). The respective primary antibodies were then added for 1 h (2D) or 48 h (3D), after which cells were exposed to the specific secondary antibody for 1 h (2D) or 18 h (3D). Alexa Fluor 488 goat anti-rabbit was used for OECs and Alexa Fluor 488 goat anti-mouse for ASCs and DRG neurites (both from Invitrogen, USA). Cell nuclei were then counterstained with 1 μg/ml of DAPI (Invitrogen, USA) for 15 min (2D) or 30 min (3D). Imaging was performed using an Olympus BX61 fluorescence microscope.

Phalloidin and DAPI staining protocol was performed in 3D co-cultures of ASCs and OECs. Cells were subjected to a 45 min period of fixation with 4% PFA at RT. Then, cell membranes were permeabilized with 0.3% Triton X-100 for 10 min and the cells were washed three times with PBS. A PBS solution with 0.1 μg/ml phalloidin (Sigma, USA) and 1 μg/ml DAPI (Invitrogen, USA) was then added to the cells for 45 min at RT. Imaging was performed using a confocal point-scanning microscope, Olympus FV1000.

2.8. DRGs neurite extension analysis with image J software

After obtaining the micrographs of DRG explant cultures through fluorescence microscopy, the pictures were opened with the Image J software. Firstly, the scale was defined and then, with the help of drawing tools, the area occupied by the neurite arborization was shaped, as well as the area occupied by the DRG. After the software automatically calculated both areas, the area occupied

by the DRG was subtracted from the total area of the neurite arborization, in order to obtain only the area occupied by the arborization extension. To calculate the length of the longest neurites Neurite-J plugin was used [30]. Briefly, with this plugin we defined the area of the DRG body and then concentric rings with a 25 μm interval were automatically created. The length of the longest neurite was defined as the length of the last ring intersecting neurites (Fig. 1).

2.9. In vivo testing

2.9.1. 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 three days before the surgeries. Animals were divided in five different groups according to the respective treatment/procedure (initial n = 5/6): 1) Animals subjected to SCI with no treatment (SCI, n = 6); 2) SCI animals treated with GG-GRGDS implanted at the injury site (GG-GRGDS, n = 6); 3) SCI animals transplanted with an injection of ASCs/OECs, rostral to the injury site (ASCs/OECs, n = 6); 4) SCI animals treated with local administration of ASCs and OECs encapsulated in GG-GRGDS (GG-GRGDS+ASCs/OECs, n = 6); and 5) Animals with laminectomy only, no SCI (Sham, n = 5). Due to health complications after the SCI, a total of seven animals died or were sacrificed during the post-operatory period, one from the SCI group (final n = 5), three from the ASCs/OECs group (final n = 3) and three from GG-GRGDS + ASCs/OECs group (final n = 3). In the groups applicable, a total of 4 μl of GG-GRGDS per animal were implanted at the injury site, using a micropipette. Hydrogel formulation was done as described in section **2.4**. Both cell types were encapsulated within the hydrogel, as described in section **2.5**. Rats treated with cells received around 60 000 ASCs and 20 000 OECs, either encapsulated in 4 μl of GG-GRGDS placed at the injury site, or through an intraspinal injection 2 mm rostral to the injury site, suspended in 4 μl of DMEM/F12.

2.9.2. 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 T8-L4 and the paravertebral muscles retracted. A laminectomy was performed at L1 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, spine stabilization was performed as previously described [31]; paravertebral muscles and skin were then closed with Vicryl sutures (Johnson and Johnson, USA). The incision of SCI control animals was closed after injury without treatment.

2.9.3. Post-operative care

Following SCI surgery, rats were kept under heat lamps and received subcutaneous injections of vitamins (Duphalyte/Pfizer, USA), 0.9% NaCl, the anti-inflammatory drug carprofen (5 mg/ml, Rimadyl/Pfizer, USA), the analgesic butorphanol (10 mg/ml, Butomidor/Richter Pharma AG, Austria) and the antibiotic enrofloxacin (5 mg/ml, Baytril/Bayer, Germany), besides atipamezole (5 mg/ml, Antisedan/Pfizer, USA) a drug used in order to revert anesthesia. Bladder evacuation was done manually twice a day. Then, during

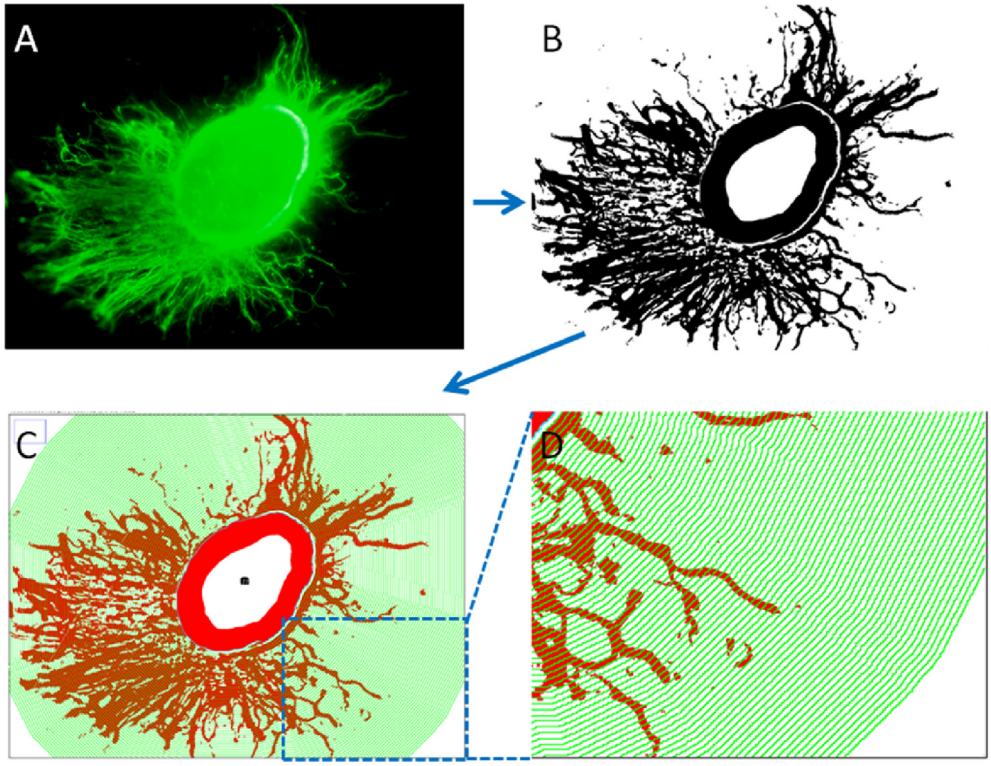

Fig. 1. Quantification process used for determining the length of the longest neurite using the Neurite-J plugin [30] for Image J software. Briefly, the micrographs obtained after fluorescence microscopy (A) were transformed into 8-bits images (B). Then, the area occupied by the DRG body as well as the neurites were defined through threshold adjustments. Finally, the software automatically drew concentric rings with 25 μm interval (C–D) and the distance of the last ring intersecting neurites was defined as the length of the longest neurite.

the first week post-injury, rats received daily subcutaneous injections of all the above mentioned components with the exception of atipamezole. Carprofen administration was stopped three days post-injury. Throughout the treatment and recovery period, animals were examined for symptoms of illness or potential reaction to the treatment. The diet was changed to a diet with higher caloric-content (Mucedola 4RF25, Italy) and the food was presented to the rats in the cage.

2.9.4. Motor behavior analysis by BBB score

The motor behavior of all rats was assessed by the Basso, Beattie, Bresnahan Locomotor Rating Scale (BBB) [32] on day three post-injury and every week, starting exactly one week post-injury, up to four weeks. The test was then repeated eight weeks after injury. The BBB is a 21-point scale designed to assess hindlimb locomotor recovery following spinal cord injury. In summary, animals were placed in an open arena with no obstacles and allowed to move freely. During the 4 min of the test, two observers (blinded to the animal treatment) evaluated the animal locomotion profile, recording and attributing a score to the animal performance.

2.9.5. Activity box test

The activity box test was used to assess general locomotor behavior of SCI rats, by measuring the amount of rearing activity and the total distance travelled by the animals, eight weeks after injury [33]. The test was performed in a square arena (43.2 cm × 43.2 cm) with transparent acrylic walls (Med Associates Inc., USA) placed in a brightly illuminated room. Animals started the test at the arena's centre and were given 5 min to explore it. The total distance travelled in the arena and the number of rearings were automatically registered by the equipment sensors.

2.9.6. Histological analysis

Rats were deeply anesthetized by an intraperitoneal injection of 200 mg/ml sodium pentobarbital (Eutasil, Ceva Saúde Animal, Portugal) and perfused through the ascending aorta with 0.9% NaCl followed by 4% PFA. A rough dissection of the spine and spinal cord was performed, centered on the site of hemisection and the tissues were fixed in 4% PFA overnight. A more detailed dissection of the spinal cord was then done and the tissues were carefully placed on a solution of saccharose at 30% (w/v). After 24 h, 2.5–3 cm length of spinal cord tissues, centered on the lesion, were involved in frozen section medium (Neg-50, Thermo Scientific, USA), frozen with liquid nitrogen and stored at −20 °C. Later on, longitudinal and transversal cross sections of 10 and 20 μm thickness were performed using a Leica CM1900 cryostat.

2.9.7. Immunohistochemistry (IHC)

Spinal cord cross sections were initially permeabilized with 0.2% TBS-T for 10 min. Then, the slides were blocked with a solution of 5% FBS in PBS for 30 min. After that, the samples were incubated overnight with the following primary antibodies: i) mouse anti-CD11b/c (Pharmingen, USA); ii) rabbit anti-rat GFAP (Dako, Denmark); iii) mouse anti-neurofilament (Millipore, USA) and iv) mouse anti-nuclei antibody (HuNu, Millipore, USA). On the next day, samples were incubated for 2 h with the respective secondary antibodies, alexa fluor 488 goat anti-rabbit for GFAP, alexa fluor 594 goat anti-mouse for CD11b/c and alexa fluor 488 goat anti-mouse for neurofilament and HuNu (all from Invitrogen, USA). All samples were counterstained with DAPI (Sigma, USA) for 10 min. Between steps, 3–5 washes with PBS were performed. Finally, the slides were mounted in Immu-Mount® (Thermo Scientific, USA) and observed at a confocal point-scanning microscope, Olympus FV1000. All images were treated using ImageJ software.

2.9.8. HuNu positive cells quantification

Spinal cord sections from all animals were screened for the presence of HuNu, a marker for human cells (ASCs). In animals were this marker was detected, three micrographs per section were taken at 400× magnification, in a total of three sections per animal. Then, the total number of HuNu positive cells per image was quantified, and the average number of cells per field was calculated for each section and for each animal.

2.9.9. Antibodies' area analysis with image J software

Six micrographs per animal were taken at the ipsilateral side of rostral and caudal regions surrounding the 3 mm gap created with the hemisection injury.

After obtaining micrographs through confocal microscopy, the photos were opened with the Image J software. Before starting the analysis, the scale was determined. Then, the images were converted to 8 bits and were processed in the menu “make binary”. Finally, using the menu “analyze particles” the software automatically calculated the areas occupied by each marker, using the dark background as contrast. The mean value of the six micrographs per animal was considered for analysis.

2.10. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, USA). Differences among groups were assessed by: 1) one way ANOVA test in results presented in Figs. 4B–C, 5C–D, 6C and 7B–C; 2) two-way ANOVA in results presented in Figs. 2B and 5A; 3) students' *t*-test in results from Fig. 8C. One or two-way ANOVA tests were followed by the Bonferroni post-hoc test. A p-value of $\leq$0.05 (95% confidence level) was set as the criteria for statistical significance.

3. Results

3.1. 2D direct co-culture of Adipose tissue-derived Stromal/Stem Cells (ASCs) and Olfactory Ensheathing Cells (OECs)

In order to assess the interactions between ASCs and OECs, direct co-cultures were performed. Immunocytochemistry after seven days revealed that cellular morphology was not affected (Fig. 2A). In fact, both cell types maintained their typical morphology, despite being in a co-culture system.

Regarding cell numbers, in the first 24 h (at day 1) both cell types presented more cells in co-culture, when compared to mono-culture conditions (for ASCs: 9.7 ± 1.9 vs 3.4 ± 0.3 cells/field; for OECs: 18.7 ± 2.4 vs 9.0 ± 3,1 cells/field, $p = 0.063$, Fig. 2B). However, these differences disappeared after seven days of co-culture and the number of cells was similar in mono- and co-cultures (ASCs: 30.2 ± 5.6 vs 26.1 ± 18.8 cells/field; OECs: 41.0 ± 2.7 vs 48.0 ± 4.3 cells/field, Fig. 2B). These results indicate that both cell types can grow together and may benefit from cell-cell interactions, particularly in the first hours of co-culture.

3.2. GG-GRGDS synthesis and 3D co-culture of ASCs and OECs

In order to synthesize GG-GRGDS hydrogel, we used Diels-Alder “click” chemistry to immobilize maleimide-containing GRGDS peptides with furan-modified gellan gum. Functionalization of GG with furan was previously performed by our group [25], in which the degree of furan substitution to GG was calculated to be approximately 27%. Maleimide-modified GRGDS peptide was then reacted with furan-GG in a 5:1 M ratio. After dialysis to remove unbound peptide, quantification by amino acid analysis of the immobilized peptide was calculated to be 189 nmol of GRGDS per

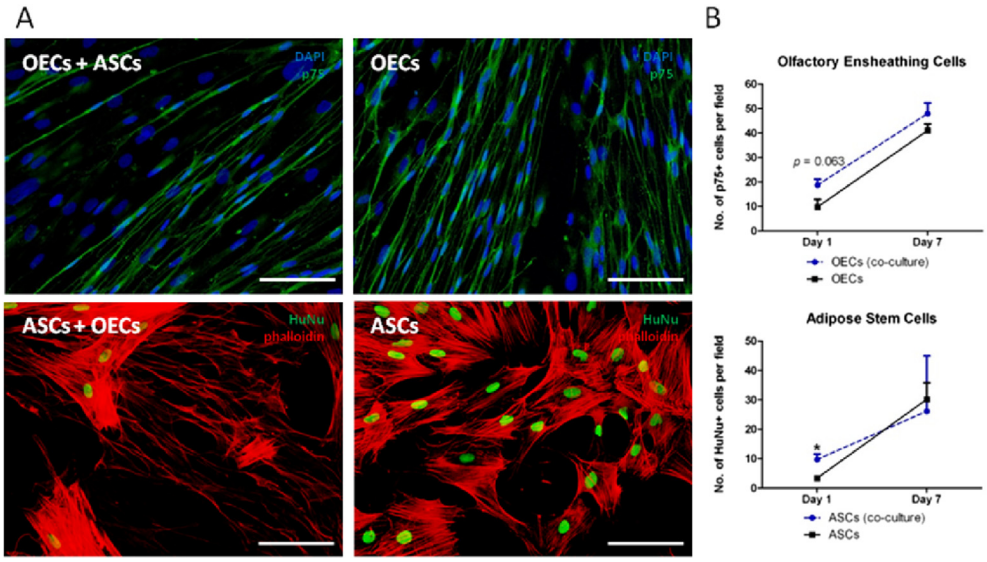

Fig. 2. 2D direct co-cultures of ASCs and OECs. Cellular morphology (A) after seven days was not affected by being in a co-culture system. Cell numbers (B) were increased after one day in co-culture but no differences were seen after seven days. Scale bar: 100 μm. Values are shown as mean ± SEM (n = 3); *p < 0.05.

mg of GG (Fig. 3A).

The encapsulation of ASCs and OECs inside the GG-GRGDS hydrogel was a critical step to demonstrate the suitability of this biomaterial as a vehicle for cellular transplantation. In this sense, both cell types were cultured in GG-GRGDS hydrogels, either alone or in co-culture. As it is visible on Fig. 3B–D, ASCs and OECs were able to grow within the hydrogel matrix, extending their processes and presenting their typical morphology. The co-culture of both cell types was also successfully performed as they were evenly distributed in the hydrogel, maintaining their cellular morphology identical to monoculture conditions (Fig. 3D). Moreover, it was possible to observe the beneficial impact of the GRGDS modification on the cell morphology, as both ASCs and OECs encapsulated in regular gellan gum hydrogels were unable to extend any cellular processes (Fig. 3E).

3.3. In vitro axonal outgrowth assessment through DRG explants

DRG explant organotypic cultures were used to evaluate the degree of axonal/neurite outgrowth promoted by ASCs and OECs when encapsulated in the GG-GRGDS hydrogel. For this purpose, ASCs and/or OECs were firstly encapsulated inside GG-GRGDS hydrogels. Then, following a period of 24 h to induce cellular growth, DRG explants were placed on the top of hydrogels with or without encapsulated cells. After seven days of co-culture, both the DRG total neurite extension area and the length of the longest neurite were significantly increased in the group combining hydrogel and ASCs ($4.8 \pm 0.7 \times 10^6$ μm^2 and 1644.4 ± 171.7 μm, respectively), in comparison to the group of hydrogel alone ($2.2 \pm 0.3 \times 10^6$ μm^2 and 1017.5 ± 79.6 μm, Fig. 4). GG-GRGDS + ASCs group also induced a significant increase in the neurite area in comparison to GG-GRGDS + OECs group ($4.8 \pm 0.7 \times 10^6$ vs $2.4 \pm 0.4 \times 10^6$ μm^2). The encapsulation of OECs did not translate into a significant increase of the neurite arborization obtained, since there were no differences to the group of hydrogel alone. Despite a positive trend towards an increase of neurite area and length, the group of co-culture of ASCs/OECs did not present significant differences in comparison to hydrogel alone. These results point out to a major role of ASCs on neurite induction, in this particular case of co-culture with hydrogel and DRGs. The importance of peptide engraftment in the gellan gum (GG) hydrogel was once again demonstrated. DRGs cultured on regular GG were unable to extend any neurite (Fig. 4A). For this reason, the quantification of neurite extension and the longest neurite were not performed in this group.

3.4. In vivo functional and histological assessment following SCI

A left lumbar hemisection SCI model (L1 level) in eight-weeks-old Wistar female rats was used in order to assess the efficacy of the proposed therapy. Immediately after injury, animals were treated either with a transplantation of ASCs and OECs, a combination of GG-GRGDS hydrogel with ASCs/OECs, or alternatively with the hydrogel alone. Non-treated animals (HS group) or animals without injury (Sham, only subjected to laminectomy) were used as controls. At day 3 post-injury, every week up to four weeks and then one month later, at eight weeks, rats were subjected to the BBB test in order to evaluate the locomotor behavior of their hindlimbs. Notably, rats treated with ASCs/OECs encapsulated in the GG-GRGDS presented significant locomotor improvements, either in comparison to non-treated rats (6.3 ± 2.6 vs 1.8 ± 0.8 at the BBB score eight weeks post-injury, for instance) or to GG-GRGDS-treated animals (6.7 ± 1.2 vs 2.8 ± 0.9 at BBB score, in the four weeks time point, Fig. 5A).

These BBB values mean that on average, rats treated with the combination of hydrogel and cells were able to extensively move two joints and slightly move one joint of the hindlimb, while non-treated animals were only able to extensively move one joint. Significant locomotor improvements were also seen at two, three and four weeks post-injury (Fig. 5A). At eight weeks, rats were also submitted to a more general test of locomotion in an open field arena. Regarding the total distance travelled in the activity box test, GG-GRGDS + ASCs/OECs-treated animals travelled significantly higher distances (2901.7 ± 343.8 cm) in comparison to non-treated and GG-GRGDS-treated ones (1817.7 ± 264.8 and 2011.9 ± 190.7 cm, respectively, Fig. 5C). This group of animals was even able to travel similar distances to sham animals (2675.1 ± 218.0 cm). However, concerning rearing behavior, there were no differences between the four injury groups (Fig. 5D).

Animals were then sacrificed and their spinal cords processed to

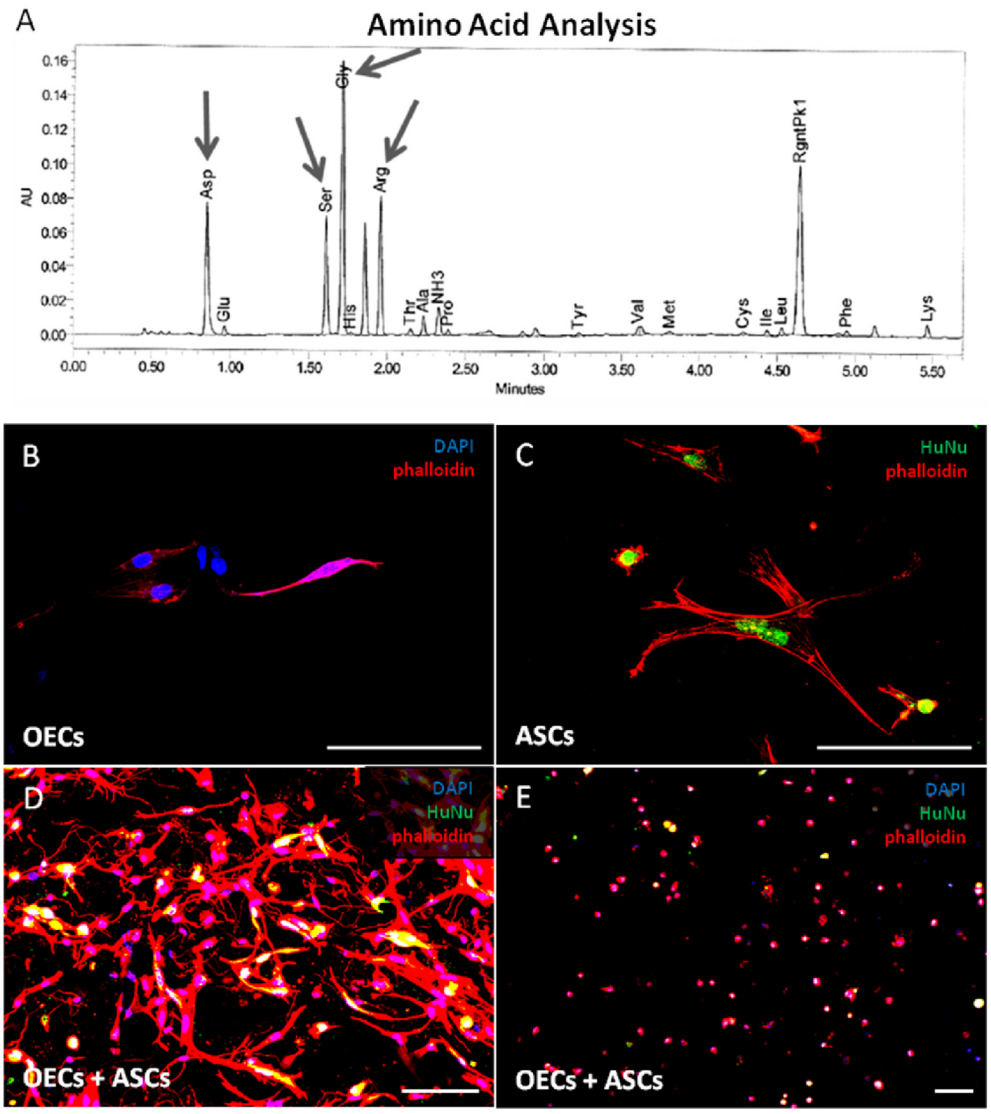

Fig. 3. Immobilization of the GRGDS peptide into the GG hydrogel and its effects on the growth of ASCs and OECs. (A) Amino acid analysis was used to quantify the amount of peptides immobilized to GG hydrogels (approximately 189 nmol GRGDS/mg of GG, grey arrows point to each amino acid). (B–E) Confocal images of ASCs and OECs cultures encapsulated in (B–D) GG-GRGDS hydrogels and (E) in unmodified GG. Cells grown in GG-GRGDS presented their typical morphology either in mono- or co-cultures. In contrast, cells grown in regular GG do not show cellular extensions into the hydrogels. Scale bar: 100 μm. Images are representative of n = 3 independent experiments.

perform histological analysis. Levels of axonal regeneration/preservation were estimated through immunostaining for neurofilament (NF). Both GG-GRGDS and GG-GRGDS + ASCs/OECs revealed significantly higher levels of NF expression area at the lesion site ($1.9 \pm 0.3 \times 10^4$ and $2.3 \pm 0.2 \times 10^4$ μm^2, respectively), when compared to non-treated animals ($0.8 \pm 0.03 \times 10^4$ μm^2, Fig. 6C).

After immunohistochemistry for CD11b/c and GFAP (markers of inflammatory cells and astrocytes respectively), it was possible to observe an increase in expression of both markers after injury in the non-treated group, when compared to sham group (HS group: $7.7 \pm 3.3 \times 10^4$ and $11.8 \pm 7.4 \times 10^4$ μm^2, for CD11b/c and GFAP respectively; Sham group: $0.4 \pm 0.1 \times 10^4$ and $1.0 \pm 0.5 \times 10^4$, for CD11b/c and GFAP respectively, Fig. 7). Nevertheless, there were not statistically significant differences between all groups after quantification (Fig. 7B and C). Treatment with GG-GRGDS + ASCs/OECs promoted the greatest decrease of CD11b/c and GFAP expression ($1.5 \pm 0.8 \times 10^4$ and $1.1 \pm 0.5 \times 10^4$ μm^2, respectively) reaching levels close to those of Sham animals (Fig. 7B and C).

Finally, following an IHC for Anti-Nuclei Antibody (HuNu), it was possible to identify transplanted ASCs in the spinal cord tissue eight weeks after injury (Fig. 8). ASCs delivered through intraspinal injection, or within GG-GRGDS hydrogel were mainly found in regions close to the injury site. After quantification of the cells positive for the HuNu marker, we could find more ASCs when transplanted within the hydrogel (48.5 ± 7.0 cells per field) than when transplanted intraspinally (32.7 ± 7.0 cells per field), however this difference was not statistically significant.

4. Discussion

In the present work, we have developed a combinatorial approach to SCI repair based on the use of ASCs and OECs with GG-GRGDS hydrogels. Although the use of this hydrogel in CNS regenerative medicine approaches has been limited, it has been shown to present mechanical, as well as thermal and conduction properties very similar to soft tissues like the spinal cord [34]. Moreover, previous studies from our laboratory have shown that these GRGDS modified GG hydrogels are able to support the growth of multiple cell types including neural stem/progenitor cells (NSPCs), OECs and MSCs [25,26]. Before encapsulating the

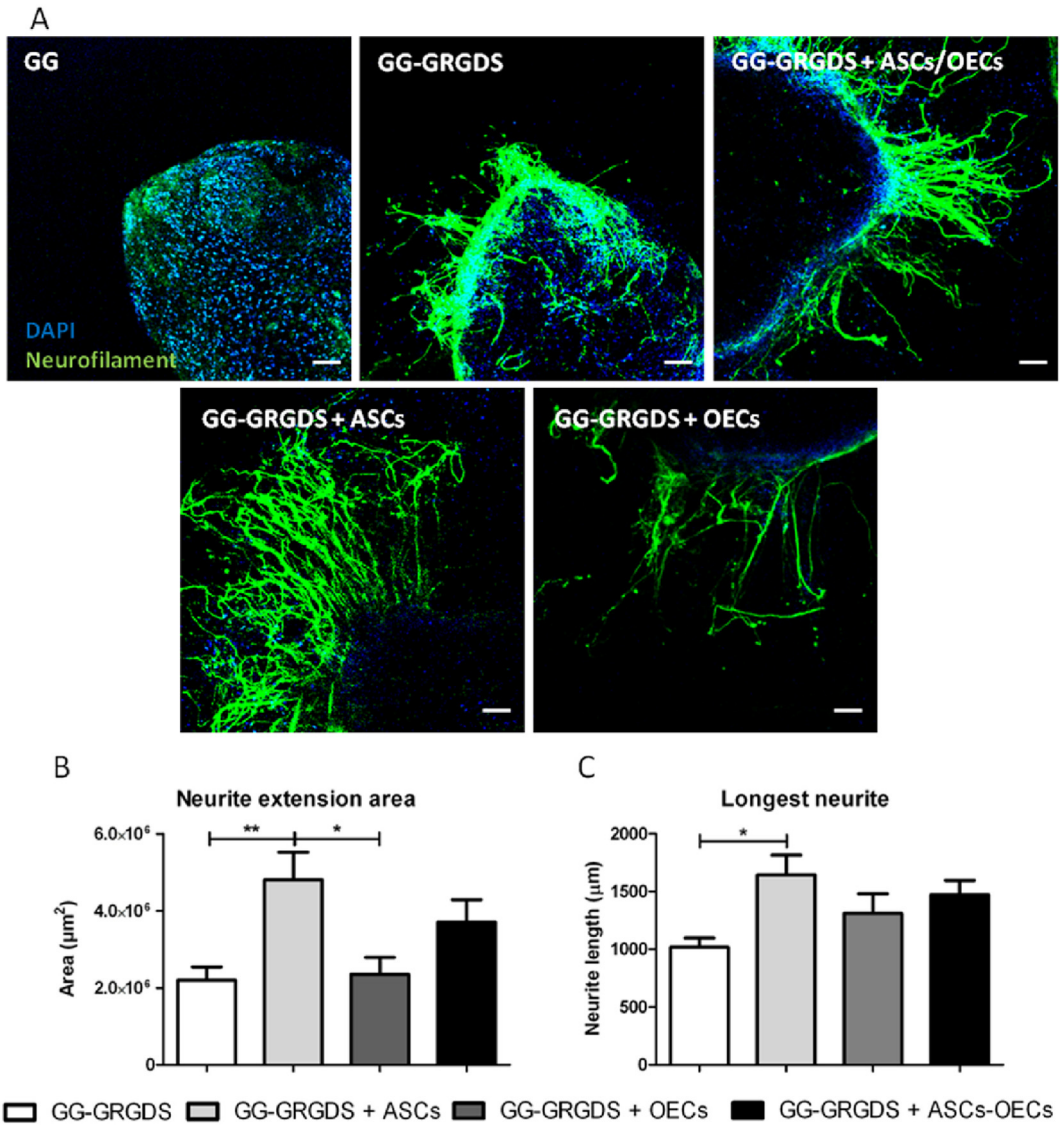

Fig. 4. Effects of GG-GRGDS hydrogel encapsulated with ASCs and/or OECs on neurite outgrowth of DRG explants, after 7 days. (A) Representative confocal images of neurites from DRGs (stained with neurofilament) cultured in the presence or absence of cells. (B) Quantification of neurite extension area and (C) quantification of the longest neurite. GG-GRGDS with ASCs promoted a significant increase of neurite extension area in comparison to hydrogel alone and hydrogel with OECs. Moreover, hydrogel with ASCs also induced a significant increase in the length of the longest neurite, in comparison to hydrogel alone. DRGs were unable to grow on unmodified gellan gum (GG). Scale: 100 μm. Values are shown as mean ± SEM (n = 8/10); $^{*}p < 0.05$, $^{**}p < 0.01$.

cells, we tested whether both cell types were capable of growing in direct contact cultures. Different cellular densities were used (1:6 ratio ASCs-OECs) because OECs need a minimal number of 60 000 cells/cm^2 to properly grow, while on the other hand, at this density ASCs would reach confluence in less than 48 h. After seven days of co-culture, cellular morphology of ASCs and OECs was similar to monoculture conditions, which reveals that direct cell contact did not have a significant impact on the size and morphology of the cells. In addition, both cell types presented more cells in the first 24 h of co-culture in comparison to monocultures. This might indicate a beneficial interaction between ASCs and OECs even under direct cell-cell contact. However, this difference was lost after seven days of co-culture, probably because cells reached confluence and could not expand over those numbers. The results herein obtained, together with the beneficial effects previously observed in indirect co-cultures [17] led us to choose these cells to combine with GG-GRGDS hydrogel. As shown in Fig. 3, ASCs and OECs grew within the 3D hydrogel matrix, extending their processes and presenting their typical morphology. Previous works from our group have already shown the suitability of GG-GRGDS hydrogel for promoting cellular growth and survival of OECs [25], ASCs [35] as well as MSCs from other sources [26]. The presence of GRGDS peptides is essential, as cells encapsulated in regular gellan gum do not present their normal morphology, instead having a round shape (Fig. 3E). Moreover, DRGs were unable to extend their neurites when cultured on regular GG (Fig. 4A). Both experiments reinforce the value of our peptide functionalization procedure.

Dorsal Root Ganglia explants have been used as a model of axonal regeneration *in vitro* [36]. To test our therapeutic approach, DRGs were cultured with ASCs and/or OECs encapsulated on the GG-GRGDS hydrogel. In this case the same cellular densities (15 000 cells/50 μl) were used for both cells (1:1) since there is less proliferation in 3D matrices and, at the same time, we could compare more precisely each cell type effect on DRG explants. ASCs induced a significant increase of DRG neurite extension area, as well

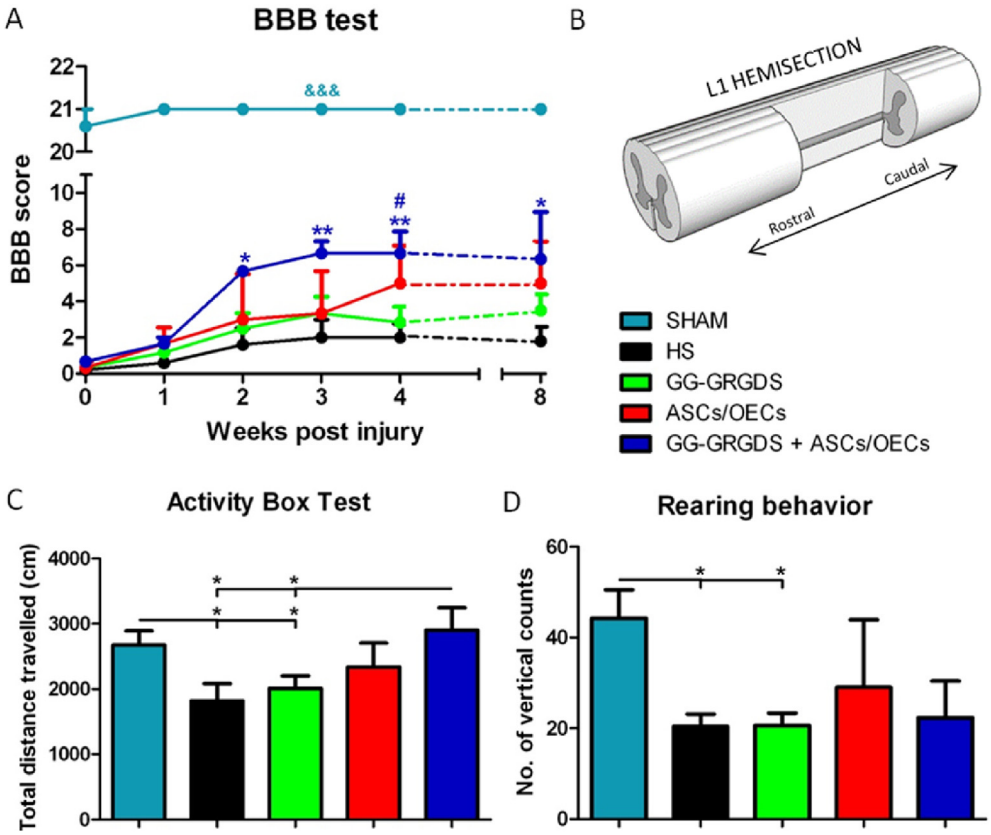

Fig. 5. In vivo locomotor evaluation of SCI rats. (A) BBB test performed during a follow-up of eight weeks post-injury. (B) Schematic representation of the hemisection injury induced in SCI animals at L1 level. (C) Activity box test and (D) rearing behavior evaluated in the open field arena, eight weeks after the lesion. Animals treated with the combination of GG-GRGDS and ASCs/OECs presented significant motor improvements in comparison to non-treated animals (HS group) and animals treated with GG-GRGDS only, both in the BBB test and in the total distance travelled on the activity box test. Values are shown as mean ± SEM. In graph A, (*) represents differences in comparison to HS group, (#) represents differences to GG-GRGDS group and (&) represents differences between sham group and all the others; one symbol $p < 0.05$, two symbols $p < 0.01$; three symbols $p < 0.001$.

as an increase in the length of the longest neurite, when compared to hydrogel alone and hydrogel + OECs cultures. From the literature it is known that ASCs have a potent neuroregulatory secretome, expressing growth factors that are able to mediate neurite/axonal outgrowth. From these the most representative are NGF, BDNF, and GDNF [6,7]. On the other hand, OECs are known to guide axonal migration through a direct interaction with neuronal processes of neighboring axons [10]. This capacity is partially related with the expression of different matrix metalloproteinases (MMPs) by OECs. Particularly MMP-2, MMP-3 and MT1-MMP seem to contribute to OECs motility across the extracellular matrix and their neurotrophic properties [37]. However, in this experiment most of the cells were not in direct contact with DRG explants, which were seeded on the top of hydrogel droplets. In this sense, the effect of the secretome, that is, the molecules secreted by the encapsulated cells, seemed to be more relevant than direct cell-cell interactions. As we have seen in 2D experiments with DRG explants [38], the secretome of ASCs induces higher neurite arborization levels, while in direct contact cultures, OECs provide a better support for neurite outgrowth than ASCs. Overall these findings are a strong indicator of the positive effects exerted by ASCs (mainly through secreted molecules) and OECs (direct cell-cell contact) on a particular phenomenon, neurite/axonal outgrowth, which is essential for the reestablishment of the functionality of the injured spinal cord.

In this study a left hemisection injury was performed at the L1 level, which results in a very aggressive injury. This happens because the motor central pattern generator (CPG) present at the lumbar region [39,40] is partially affected, which explains the low motor recovery profile observed in these animals (Fig. 5A), especially compared to hemisection injuries performed at the thoracic level [31]. Despite the aggressiveness of the injury, the combined treatment with GG-GRGDS and ASCs/OECs was capable of inducing significant motor improvements in the paralyzed hindlimbs of SCI rats, as assessed by the BBB test. The combination of cellular therapy with the hydrogel proved to be important in this recovery, since GG-GRGDS treatment or ASCs/OECs alone did not induce significant improvements in comparison to non-treated animals. Nevertheless, no major differences were found between groups with treatment. This means that our therapeutic strategy can be refined in order to further improve the functional outcomes obtained. Similar results were observed in the activity box test, since animals treated with the combinatorial strategy travelled longer distances than hydrogel- and non-treated groups. No differences between treatments were observed with respect to the number of vertical counts. Rearing is a type of behavior highly dependent on weight-support capacity on the hindlimbs and none of the groups reached the mean score of nine in the BBB scale, which is indicative of weight-support ability. Another study using the same combination of cells, but seeded on a serum-derived albumin scaffold, showed that rats treated with the combinatorial approach also presented improved locomotor skills over untreated animals [41]. Histologically, those rats presented reduced glial scar formation and more cells expressing markers of neurons and axons at the lesion site [41]. Our functional results can be partially explained by the CD11b/c and GFAP levels observed. Despite not being statistically different between groups, there was a trend for rats treated

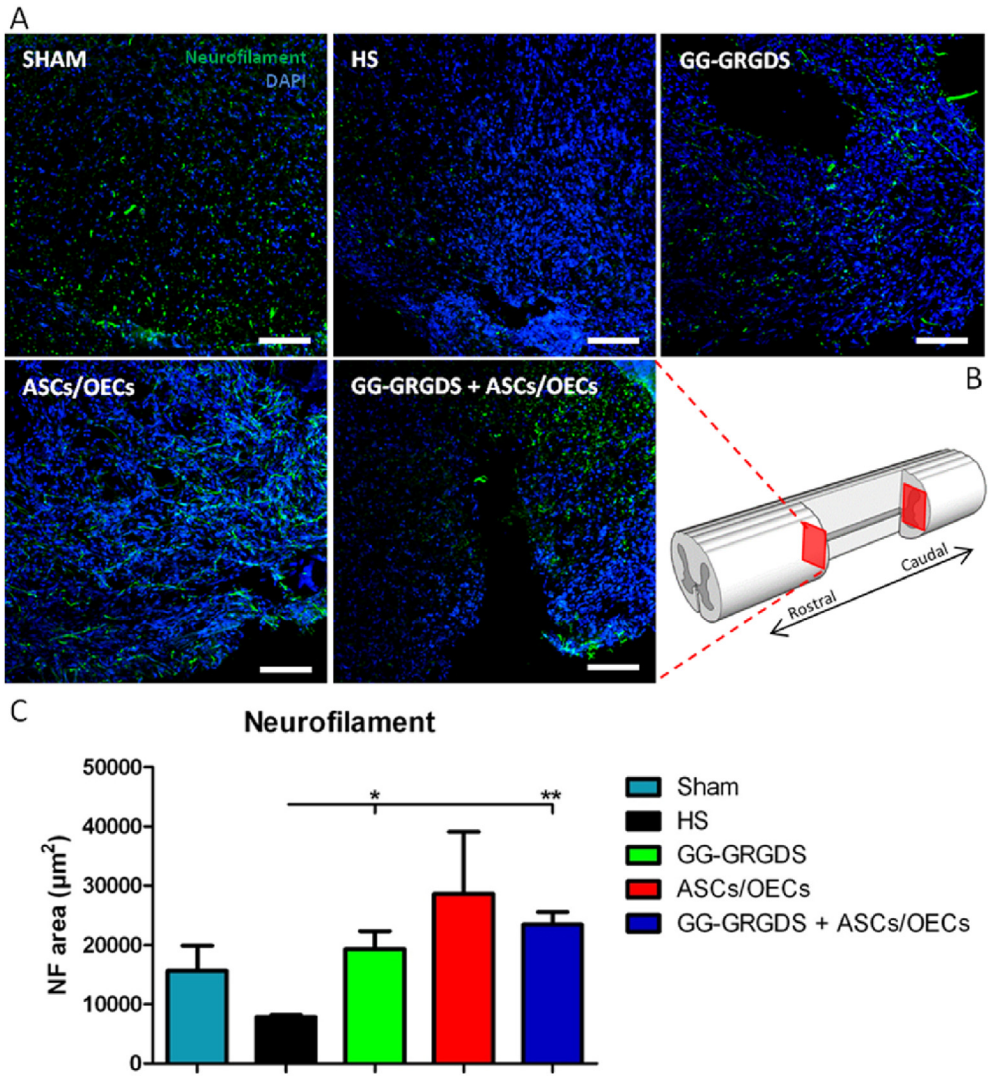

Fig. 6. Representative confocal images of the immunostaining for neurofilament; scale: 100 μm. (B) Schematic representation of the regions analyzed by confocal microscopy to determine axonal regeneration/preservation. (C) Quantification of expression areas of neurofilament marker. The treatment with the combination of GG-GRGDS and ASCs/OECs induced a significant increase in neurofilament area. Values are shown as mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$.

with GG-GRGDS and ASCs/OECs to have reduced CD11b/c and GFAP expression areas, close to the lesion site. These results point out that the combinatorial treatment resulted in a less prominent inflammatory status and less astrogliosis, two features that have been associated with better motor performances in different studies [42,43]. The combined treatment also resulted in an increased area of neurofilament expression, close to the lesion site, a fact that could be the result of an improved preservation of the remaining neuronal network and/or the creation of an environment propitious to neuroregeneration. In any case, the combination of these three major histological alterations (i.e. increased neurofilament, decreased astrogliosis and inflammation) can partially account for the functional improvements obtained with the combinatorial strategy herein tested. Eight weeks after injury, we could not find any trace or evidences of the hydrogel presence at the injury site. From previous experiments we know that GG has a slow degradation rate (for a hydrogel) and it can be found at least four weeks following injury [22]. Thus the present study indicates that its full degradation happens for periods between four-eight weeks. In this case it may have been cleared by body fluids with time; however it must have remained at the injury site long enough, in order to allow cellular integration within the injured tissue (Fig. 8). Moreover, we have previously demonstrated that this hydrogel integrates very well within the spinal cord tissue and it does not cause any kind of inflammatory reaction [22], which goes along with the anti-inflammatory profile found in GG-GRGDS-only transplanted animals (Fig. 7C). The lesion epicenter was predominantly filled with connective tissue in all groups and there was no infiltration of blood vessels, astrocytes, axonal ingrowth or any other type of cells. The hydrogel did not bridge the injury epicenter, but was important to prevent the infiltration of cells in the tissues close to the lesion. This effect was even more visible in the group combining hydrogel and cells (Fig. 7). It is also noteworthy the presence of ASCs two months following injury (Fig. 8), which indicates that cells were capable of integrating the injured tissue, possibly exerting a long-lasting effect. A higher number of ASCs was transplanted in comparison to OECs (3:1 ratio, ASCs-OECs) due to the results obtained from DRG explants regarding their regenerative potential. Both ASCs transplanted through intraspinal injection or delivered within the hydrogel could be found in the spinal cord tissue. There was no significant difference in the mean number of HuNu positive cells per field

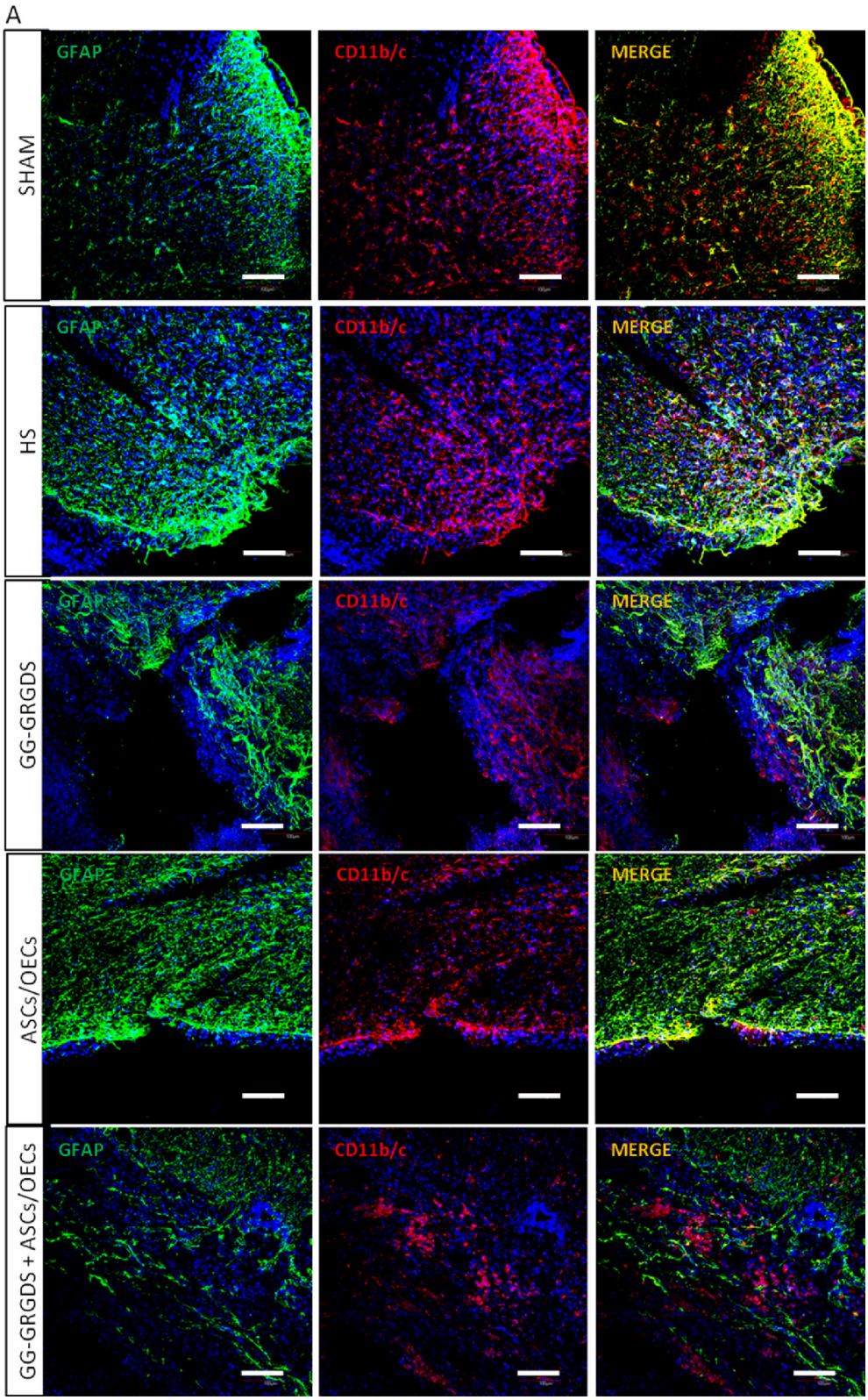

Fig. 7. (A) Representative confocal images of the immunostaining for GFAP, CD11b/c and the respective merge; scale: 100 μm. The analysis was performed at the beginning and at end of the gap produced by the hemisection (represented in Fig. 6B). (B–C) Quantification of the area expressing GFAP (B) and CD11b/c (C) markers. Although there are no significant differences between groups, there is a trend for rats treated with GG-GRGDS + ASCs/OECs presenting less expression areas of both markers in comparison to non-treated animals. Values are shown as mean ± SEM (n = 3).

between groups with hydrogel + cells and cells only (48.5 ± 7.0 vs 32.7 ± 7.0, respectively). This means that GG-GRGDS had a marginal impact on ASCs survival after transplantation. However, it is interesting to notice that the BBB score from animals of the

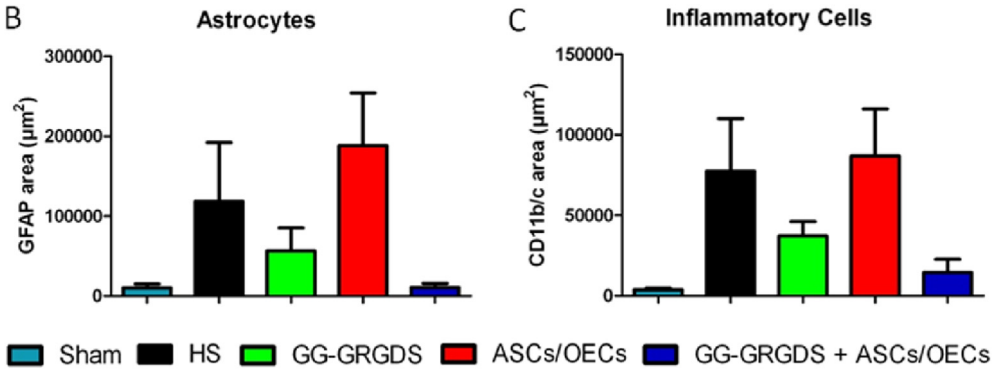

Fig. 7. (*continued*).

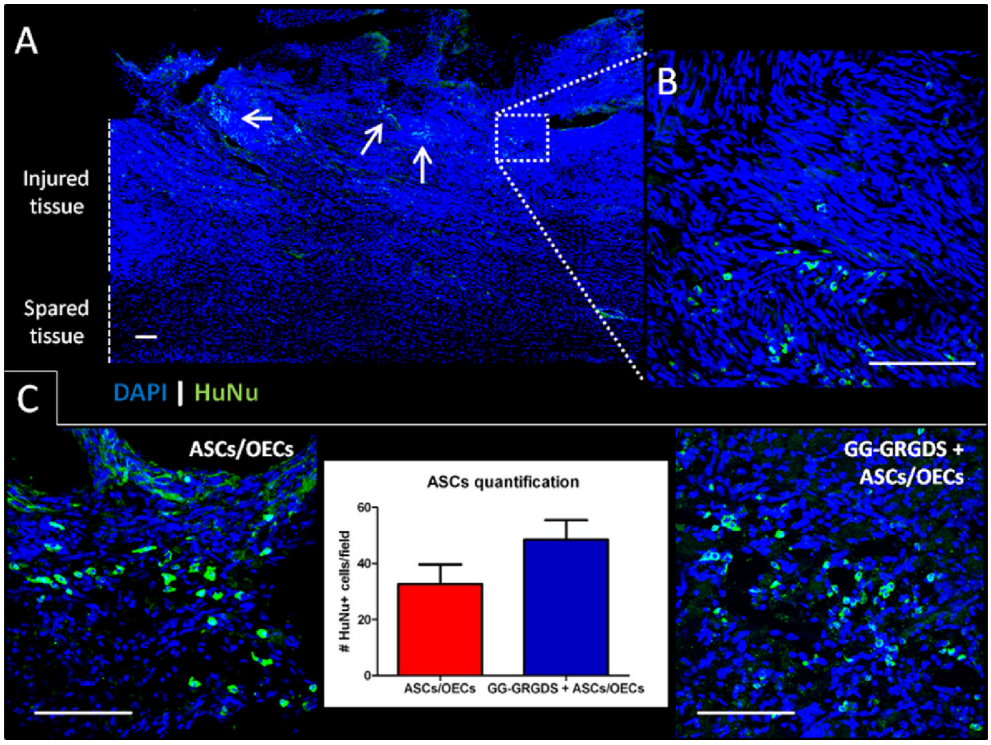

Fig. 8. Representative confocal microscopy images from longitudinal sections of spinal cord tissues of rats transplanted with GG-GRGDS with cells or only cells, eight weeks after lesion. (A) ASCs identified with HuNu (specific marker for human cells – represented by the white arrows) were mainly found in regions close to injury site and the adjacent tissues, in both transplanted groups. (B) Magnification detail of HuNu positive cells dispersed in tissue adjacent to the lesion. (C) Quantification of HuNu positive cells per field in each group with transplanted cells. The number of HuNu positive cells is higher in animals where ASCs were transplanted encapsulated in GG-GRGDS hydrogels, although this difference is not significant. Scale bar: 100 μm; values are shown as mean ± SEM (n = 3).

hydrogel + cells group was higher in the first time points evaluated, reaching a plateau at the third week post-injury. This fact could be related with the combined effects of cell survival provided by the hydrogel's physical and structural support at earlier time points. Regarding the fate of OECs, we could not identify these cells, since there is no specific marker for them. Most of the markers are shared with Schwann Cells and these cells can easily invade the CNS tissue after an injury [44]. All together, these data support the use of GG-GRGDS with ASCs and OECs as a possible candidate for SCI repair studies. Even though the results are promising, we should be careful in their interpretation, due to the low final number of animals. Nevertheless, one advantage of using a tissue engineering approach like the one herein presented is that it can work as a baseline for further refinements. For instance, GG-GRGDS hydrogel can be tested for additional modifications, such as the engraftment of other peptides also suitable to neuronal migration and differentiation [45]. In addition, the hydrogel may also be used as a depot for neurotrophic factors [46] and drug delivery [47]. By addressing spinal cord injuries with this kind of integrated view, we believe meaningful functional improvements to SCI patients may be obtained in the future.

5. Conclusions

The present work revealed that ASCs and OECs are able to grow in direct contact cultures, either in 2D or 3D surfaces, without significant alterations in their morphology and proliferation. Moreover, GG-GRGDS based hydrogels proved to be suitable for the culture of these two cell types. Additionally the combination of hydrogel and the cell populations herein tested, particularly ASCs,

promoted an increased neurite/axonal outgrowth, using an *in vitro* model of axonal regeneration. Finally, combinatorial treatment with GG-GRGDS hydrogel and ASCs/OECs resulted in significant motor and histological improvements of SCI rats. Overall, our results suggest that the conjugation of GG-GRGDS hydrogel and cells with therapeutical potential, such as ASCs and OECs, is a promising therapy for the repair of Spinal Cord Injuries.

Disclosures

The authors declare no conflict of interest.

Acknowledgements

We want to acknowledge Fábio Teixeira, Miguel Carvalho and Ana Brandão for the help with the animal surgeries and post-operative care. We also want to acknowledge Professor Jeffrey Gimble at the Tulane University Center for Stem Cell Research and Regenerative Medicine and LaCell LLC (New Orleans, Louisiana, USA) for kindly providing the ASCs used in this study. Financial support from Prémios Santa Casa Neurociências – Prize Melo e Castro for Spinal Cord Injury Research; Portuguese Foundation for Science and Technology [Doctoral fellowship (SFRH/BD/103075/2014) to E. D. Gomes; IF Development Grant to A. J. Salgado; Post-Doctoral fellowship (SFRH/BPD/97701/2013) to N. A. Silva]; This article has been developed under the scope of the projects NORTE-01-0145-FEDER-000013, supported by the Northern Portugal Regional Operational Programme (NORTE 2020), under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (FEDER); This work has been funded by FEDER funds, through the Competitiveness Factors Operational Programme (COMPETE), and by National funds, through the Foundation for Science and Technology (FCT), under the scope of the project POCI-01-0145-FEDER-007038.

References

[1] E.M. Hagen, T. Rekand, N.E. Gilhus, M. Gronning, Traumatic spinal cord injuries—incidence, mechanisms and course, Tidsskr. Nor. Laegeforen 132 (2012) 831–837.
[2] N.A. Silva, N. Sousa, R.L. Reis, A.J. Salgado, From basics to clinical: a comprehensive review on spinal cord injury, Prog. Neurobiol. 114 (2014) 25–57.
[3] K. Menezes, M.A. Nascimento, J.P. Goncalves, A.S. Cruz, D.V. Lopes, B. Curzio, M. Bonamino, J.R. de Menezes, R. Borojevic, M.I. Rossi, T. Coelho-Sampaio, Human mesenchymal cells from adipose tissue deposit laminin and promote regeneration of injured spinal cord in rats, PLoS One 9 (2014) e96020.
[4] R. Lopez-Vales, J. Fores, X. Navarro, E. Verdu, Chronic transplantation of olfactory ensheathing cells promotes partial recovery after complete spinal cord transection in the rat, Glia 55 (2007) 303–311.
[5] D. Arboleda, S. Forostyak, P. Jendelova, D. Marekova, T. Amemori, H. Pivonkova, K. Masinova, E. Sykova, Transplantation of predifferentiated adipose-derived stromal cells for the treatment of spinal cord injury, Cell Mol. Neurobiol. 31 (2011) 1113–1122.
[6] C.A. Ribeiro, J.S. Fraga, M. Graos, N.M. Neves, R.L. Reis, J.M. Gimble, N. Sousa, A.J. Salgado, The secretome of stem cells isolated from the adipose tissue and Wharton jelly acts differently on central nervous system derived cell populations, Stem Cell Res. Ther. 3 (2012) 18.
[7] A.J. Salgado, R.L. Reis, N.J. Sousa, J.M. Gimble, Adipose tissue derived stem cells secretome: soluble factors and their roles in regenerative medicine, Curr. Stem Cell Res. Ther. 5 (2010) 103–110.
[8] J. Rehman, D. Traktuev, J. Li, S. Merfeld-Clauss, C.J. Temm-Grove, J.E. Bovenkerk, C.L. Pell, B.H. Johnstone, R.V. Considine, K.L. March, Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells, Circulation 109 (2004) 1292–1298.
[9] L. Cui, S. Yin, W. Liu, N. Li, W. Zhang, Y. Cao, Expanded adipose-derived stem cells suppress mixed lymphocyte reaction by secretion of prostaglandin E2, Tissue Eng. 13 (2007) 1185–1195.
[10] J. Babiarz, N. Kane-Goldsmith, S. Basak, K. Liu, W. Young, M. Grumet, Juvenile and adult olfactory ensheathing cells bundle and myelinate dorsal root ganglion axons in culture, Exp. Neurol. 229 (2011) 72–79.
[11] W. Tetzlaff, E.B. Okon, S. Karimi-Abdolrezaee, C.E. Hill, J.S. Sparling, J.R. Plemel, W.T. Plunet, E.C. Tsai, D. Baptiste, L.J. Smithson, M.D. Kawaja, M.G. Fehlings, B.K. Kwon, A systematic review of cellular transplantation therapies for spinal cord injury, J. Neurotrauma 28 (2011) 1611–1682.
[12] R.J. Franklin, J.M. Gilson, I.A. Franceschini, S.C. Barnett, Schwann cell-like myelination following transplantation of an olfactory bulb-ensheathing cell line into areas of demyelination in the adult CNS, Glia 17 (1996) 217–224.
[13] T. Imaizumi, K.L. Lankford, S.G. Waxman, C.A. Greer, J.D. Kocsis, Transplanted olfactory ensheathing cells remyelinate and enhance axonal conduction in the demyelinated dorsal columns of the rat spinal cord, J. Neurosci. 18 (1998) 6176–6185.
[14] S.C. Barnett, L. Chang, Olfactory ensheathing cells and CNS repair: going solo or in need of a friend? Trends Neurosci. 27 (2004) 54–60.
[15] A. Mackay-Sim, F. Feron, J. Cochrane, L. Bassingthwaighte, C. Bayliss, W. Davies, P. Fronek, C. Gray, G. Kerr, P. Licina, A. Nowitzke, C. Perry, P.A. Silburn, S. Urquhart, T. Geraghty, Autologous olfactory ensheathing cell transplantation in human paraplegia: a 3-year clinical trial, Brain 131 (2008) 2376–2386.
[16] F. Feron, C. Perry, J. Cochrane, P. Licina, A. Nowitzke, S. Urquhart, T. Geraghty, A. Mackay-Sim, Autologous olfactory ensheathing cell transplantation in human spinal cord injury, Brain 128 (2005) 2951–2960.
[17] N.A. Silva, J.M. Gimble, N. Sousa, R.L. Reis, A.J. Salgado, Combining adult stem cells and olfactory ensheathing cells: the secretome effect, Stem Cells Dev. 22 (2013) 1232–1240.
[18] J.W. Austin, C.E. Kang, M.D. Baumann, L. DiDiodato, K. Satkunendrarajah, J.R. Wilson, G.J. Stanisz, M.S. Shoichet, M.G. Fehlings, The effects of intrathecal injection of a hyaluronan-based hydrogel on inflammation, scarring and neurobehavioural outcomes in a rat model of severe spinal cord injury associated with arachnoiditis, Biomaterials 33 (2012) 4555–4564.
[19] A.J. Mothe, R.Y. Tam, T. Zahir, C.H. Tator, M.S. Shoichet, Repair of the injured spinal cord by transplantation of neural stem cells in a hyaluronan-based hydrogel, Biomaterials 34 (2013) 3775–3783.
[20] M.I. Gunther, N. Weidner, R. Muller, A. Blesch, Cell-seeded alginate hydrogel scaffolds promote directed linear axonal regeneration in the injured rat spinal cord, Acta Biomater. 27 (2015) 140–150.
[21] Y.H. Yang, Z. Khan, C. Ma, H.J. Lim, L.A. Smith Callahan, Optimization of adhesive conditions for neural differentiation of murine embryonic stem cells using hydrogels functionalized with continuous Ile-Lys-Val-Ala-Val concentration gradients, Acta Biomater. 21 (2015) 55–62.
[22] N.A. Silva, A.J. Salgado, R.A. Sousa, J.T. Oliveira, A.J. Pedro, H. Leite-Almeida, R. Cerqueira, A. Almeida, F. Mastronardi, J.F. Mano, N.M. Neves, N. Sousa, R.L. Reis, Development and characterization of a novel hybrid tissue engineering-based scaffold for spinal cord injury repair, Tissue Eng. Part A 16 (2010) 45–54.
[23] H. Grasdalen, O. Smidsrød, Gelation of gellan gum, Carbohydr. Polym. 7 (1987) 371–393.
[24] R. Mao, J. Tang, B.G. Swanson, Texture properties of high and low acyl mixed gellan gels, Carbohydr. Polym. 41 (2000) 331–338.
[25] N.A. Silva, M.J. Cooke, R.Y. Tam, N. Sousa, A.J. Salgado, R.L. Reis, M.S. Shoichet, The effects of peptide modified gellan gum and olfactory ensheathing glia cells on neural stem/progenitor cell fate, Biomaterials 33 (2012) 6345–6354.
[26] N.A. Silva, J. Moreira, S. Ribeiro-Samy, E.D. Gomes, R.Y. Tam, M.S. Shoichet, R.L. Reis, N. Sousa, A.J. Salgado, Modulation of bone marrow mesenchymal stem cell secretome by ECM-like hydrogels, Biochimie 95 (2013) 2314–2319.
[27] S.G. Dubois, E.Z. Floyd, S. Zvonic, G. Kilroy, X. Wu, S. Carling, Y.D. Halvorsen, E. Ravussin, J.M. Gimble, Isolation of human adipose-derived stem cells from biopsies and liposuction specimens, Methods Mol. Biol. 449 (2008) 69–79.
[28] L.F.M.v. Zutphen, V. Baumans, A.C. Beynen, in: Principles of Laboratory Animal Science : a Contribution to the Humane Use and Care of Animals and to the Quality of Experimental Results. Rev. Ed, Elsevier, Amsterdam ; London, 2001.
[29] N.A. Silva, R.A. Sousa, A.O. Pires, N. Sousa, A.J. Salgado, R.L. Reis, Interactions between Schwann and olfactory ensheathing cells with a starch/polycaprolactone scaffold aimed at spinal cord injury repair, J. Biomed. Mater. Res. A 100 (2012) 470–476.
[30] A. Torres-Espin, D. Santos, F. Gonzalez-Perez, J. del Valle, X. Navarro, Neurite-J: an image-J plug-in for axonal growth analysis in organotypic cultures, J. Neurosci. Methods 236 (2014) 26–39.
[31] N.A. Silva, R.A. Sousa, J.S. Fraga, M. Fontes, H. Leite-Almeida, R. Cerqueira, A. Almeida, N. Sousa, R.L. Reis, A.J. Salgado, Benefits of spine stabilization with biodegradable scaffolds in spinal cord injured rats, Tissue Eng. Part C Methods 19 (2013) 101–108.
[32] D.M. Basso, M.S. Beattie, J.C. Bresnahan, A sensitive and reliable locomotor rating scale for open field testing in rats, J. Neurotrauma 12 (1995) 1–21.
[33] N. Sousa, O.F. Almeida, C.T. Wotjak, A hitchhiker's guide to behavioral analysis in laboratory rodents, Genes Brain Behav. 5 (Suppl. 2) (2006) 5–24.
[34] R.K. Chen, A.J. Shih, Multi-modality gellan gum-based tissue-mimicking phantom with targeted mechanical, electrical, and thermal properties, Phys. Med. Biol. 58 (2013) 5511–5525.
[35] R.C. Assuncao-Silva, C.C. Oliveira, O. Ziv-Polat, E.D. Gomes, A. Sahar, N. Sousa, N.A. Silva, A.J. Salgado, Induction of neurite outgrowth in 3D hydrogel-based environments, Biomed. Mater. 10 (2015) 051001.
[36] I. Allodi, M.S. Guzman-Lenis, J. Hernandez, X. Navarro, E. Udina, In vitro comparison of motor and sensory neuron outgrowth in a 3D collagen matrix, J. Neurosci. Methods 198 (2011) 53–61.
[37] A. Ould-Yahoui, O. Sbai, K. Baranger, A. Bernard, Y. Gueye, E. Charrat, B. Clement, D. Gigmes, V. Dive, S.D. Girard, F. Feron, M. Khrestchatisky, S. Rivera, Role of matrix metalloproteinases in migration and neurotrophic properties of nasal olfactory stem and ensheathing cells, Cell Transpl. 22

(2013) 993–1010.
[38] E.D. Gomes, S.S. Mendes, R.C. Assuncao-Silva, F.G. Teixeira, J.M. Gimble, N. Sousa, N.A. Silva, A.J. Salgado, Transplantation of adipose tissue stem cells and olfactory ensheathing cells: a combinatorial approach for spinal cord injury regenerative medicine, Tissue Eng. Part A. 21 (2015). S387–S387.
[39] O. Kjaerulff, O. Kiehn, Distribution of networks generating and coordinating locomotor activity in the neonatal rat spinal cord in vitro: a lesion study, J. Neurosci. 16 (1996) 5777–5794.
[40] O. Kiehn, S.J. Butt, Physiological, anatomical and genetic identification of CPG neurons in the developing mammalian spinal cord, Prog. Neurobiol. 70 (2003) 347–361.
[41] A. Ferrero-Gutierrez, Y. Menendez-Menendez, M. Alvarez-Viejo, A. Meana, J. Otero, New serum-derived albumin scaffold seeded with adipose-derived stem cells and olfactory ensheathing cells used to treat spinal cord injured rats, Histol. Histopathol. 28 (2013) 89–100.
[42] H. Ren, M. Han, J. Zhou, Z.F. Zheng, P. Lu, J.J. Wang, J.Q. Wang, Q.J. Mao, J.Q. Gao, H.W. Ouyang, Repair of spinal cord injury by inhibition of astrocyte growth and inflammatory factor synthesis through local delivery of flavopiridol in PLGA nanoparticles, Biomaterials 35 (2014) 6585–6594.
[43] R. Gonzalez, J. Glaser, M.T. Liu, T.E. Lane, H.S. Keirstead, Reducing inflammation decreases secondary degeneration and functional deficit after spinal cord injury, Exp. Neurol. 184 (2003) 456–463.
[44] S.X. Zhang, F. Huang, M. Gates, E.G. Holmberg, Role of endogenous Schwann cells in tissue repair after spinal cord injury, Neural Regen. Res. 8 (2013) 177–185.
[45] V.M. Tysseling, V. Sahni, E.T. Pashuck, D. Birch, A. Hebert, C. Czeisler, S.I. Stupp, J.A. Kessler, Self-assembling peptide amphiphile promotes plasticity of serotonergic fibers following spinal cord injury, J. Neurosci. Res. 88 (2010) 3161–3170.
[46] E. Ansorena, P. De Berdt, B. Ucakar, T. Simon-Yarza, D. Jacobs, O. Schakman, A. Jankovski, R. Deumens, M.J. Blanco-Prieto, V. Preat, A. des Rieux, Injectable alginate hydrogel loaded with GDNF promotes functional recovery in a hemisection model of spinal cord injury, Int. J. Pharm. 455 (2013) 148–158.
[47] M.M. Pakulska, K. Vulic, M.S. Shoichet, Affinity-based release of chondroitinase ABC from a modified methylcellulose hydrogel, J. Control Release 171 (2013) 11–16.