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The effects of peptide modified gellan gum and olfactory ensheathing glia cells on neural stem/progenitor cell fate

Nuno A. Silva [a,b,c,d,e], Michael J. Cooke [d,e], Roger Y. Tam [d,e], Nuno Sousa [b,c], António J. Salgado [b,c], Rui L. Reis [a,b], Molly S. Shoichet [d,e,f,*]

[a] 3B's Research Group – Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, Guimarães, Portugal
[b] ICVS/3B's – PT Government Associated Laboratory, Braga/Guimarães, Portugal
[c] Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal
[d] Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, ON M5S 3E5, Canada
[e] Institute of Biomaterials and Biomedical Engineering, 164 College Street, Room 407, Toronto, ON M5S 3G9, Canada
[f] Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, ON M5S 3H6, Canada

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ABSTRACT

The regenerative capacity of injured adult central nervous system (CNS) tissue is very limited. Specifically, traumatic spinal cord injury (SCI) leads to permanent loss of motor and sensory functions below the site of injury, as well as other detrimental complications. A potential regenerative strategy is stem cell transplantation; however, cell survival is typically less than 1%. To improve cell survival, stem cells can be delivered in a biomaterial matrix that provides an environment conducive to survival after transplantation. One major challenge in this approach is to define the biomaterial and cell strategies *in vitro*. To this end, we investigated both peptide-modification of gellan gum and olfactory ensheathing glia (OEG) on neural stem/progenitor cell (NSPC) fate. To enhance cell adhesion, the gellan gum (GG) was modified using Diels–Alder click chemistry with a fibronectin-derived synthetic peptide (GRGDS). Amino acid analysis demonstrated that approximately 300 nmol of GRGDS was immobilized to each mg of GG. The GG–GRGDS had a profound effect on NSPC morphology and proliferation, distinct from that of NSPCs in GG alone, demonstrating the importance of GRGDS for cell-GG interaction. To further enhance NSPC survival and outgrowth, they were cultured with OEG. Here NSPCs interacted extensively with OEG, demonstrating significantly greater survival and proliferation relative to monocultures of NSPCs. These results suggest that this co-culture strategy of NSPCs with OEG may have therapeutic benefit for SCI repair.

1. Introduction

Traumatic spinal cord injury (SCI) usually leads to significant neurological deficits and disabilities that result in loss of sensory and motor function, termed paraplegia or tetraplegia depending on the site of injury. This can subsequently result in other related problems such as infections of the bladder and kidneys and dysfunction of the bowel, as well as heart and respiratory system. All of these problems have a negative effect on the physiological, psychological and social behavior of SCI patients. Current clinical approaches are limited and mainly based on the use of anti-inflammatory agents, such as methylprednisolone [1]; however, its use is controversial as recent studies failed to reveal conclusive beneficial outcomes [2]. Some of the other strategies that have been tested clinically include: minocycline [3], anti-NogoA [4] and transplantation of oligodendrocyte precursor cells [5]. The latter trial was recently canceled, underlying the urgent need to develop therapeutic strategies that can promote regeneration after SCI.

One approach currently under investigation for regeneration following SCI is the transplantation of cells into the spinal cord. Several groups have reported that the injection of cells, such as olfactory ensheathing glia cells (OEG or OECs) [6,7], neural stem/progenitor cells (NSPCs) [8] or mesenchymal stem cells [9,10], leads to motor improvements and/or tissue repair in SCI animal models. OEG are compelling because they support and guide olfactory axons [11], are able to grow through the glial scar [12] and secrete several

* Corresponding author. University of Toronto, Donnelly Centre for Cellular & Biomolecular Research, 160 College Street, Room 514, Toronto, Ontario M5S 3E1, Canada. Tel.: +1 416 978 1460; fax: +1 416 978 4317.
E-mail address: molly.shoichet@utoronto.ca (M.S. Shoichet).

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neurotrophic factors [13]. NSPC transplants have shown some functional repair, taking advantage of their ability to differentiate to neurons, oligodendrocytes and astrocytes [14]. However, to date no single repair strategy has successfully induced full functional recovery following SCI. Extensive cell death of the transplanted cells after injection, due to inflammation and/or the absence of matrix support [15,16], has limited the therapeutic benefit. To enhance cell survival, we investigated the co-delivery of two cell types in an engineered matrix where the second cell type provides trophic support and the matrix contributes to overcoming cell death through anoikis [17].

Biomaterials have been designed as vehicles for cell transplantation in order to enhance cell survival after transplantation. Hydrogels are particularly appealing for soft tissue applications because they can be designed to match the mechanical properties and water content of these tissues. For example, delivering NSPCs in chitosan tubular scaffolds demonstrated NSPC survival and promising results for tissue and functional repair [8]. The gellan gum hydrogel is compelling because it can be injected in a minimally-invasive way to form a gel *in situ*. Gellan gum (GG) is a linear anionic microbial polysaccharide composed of repeating units of glucose, glucuronic acid and rhamnose [18] and thus has multiple hydroxyl groups available for chemical modification. It has been studied for drug delivery and cartilage regeneration [19–21] and is approved by the FDA as a food additive.

In order to better mimic the extracellular matrix (ECM), biomaterials have been modified with several peptide sequences [22–24] to influence biological processes, such as cell adhesion, growth and development [25,26]. Cells transplanted in a material that mimics both the mechanical and chemical properties of native tissue have been shown to be more efficacious after transplantation [27,28].

Here we aim to synthesize GG with enhanced cell-ECM and cell–cell interactions for ultimate use in cell transplantation. To promote cell survival and interaction with gellan gum, we chemically conjugated the fibronectin-derived peptide sequence GRGDS via Diels–Alder click chemistry and studied the impact of GRGDS-modified gellan gum (GG–GRGDS) on cell fate. To enhance cell survival beyond that provided by the ECM-mimetic peptide sequence, cell–cell interactions are investigated. Since OEG have been shown to provide guidance pathways to axons and NSPCs have been shown to promote some functional recovery, we investigated the co-culture of these two cell types to determine whether OEG affected NSPC survival and/or differentiation. To gain a greater appreciation of the OEG–NSPC interaction, the two cell types were cultured in direct contact, in a transwell system or in the GG–GRGDS. Importantly, both OEG and NSPCs can be isolated from human patients [29,30], which enhance the eventual feasibility of this therapeutic strategy for SCI.

2. Materials and methods

2.1. Synthesis of furan-modified gellan gum hydrogel

Gellan gum (Sigma, USA) was first dissolved in 2-(N-morpholino)ethanesulfonic acid (MES) buffer (100 mM, pH 5.5) 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 (M_w cutoff 12–14 kDa, Spectrum Labs, USA) alternately against distilled water and PBS (0.1 M, pH 7.2) for 5 days. Finally, water was removed by lyophilization to obtain furan-modified gellan gum (furan–GG) as a white powder. ^{1}H NMR spectra were used to analyze the degree of furan substitution. ^{1}H NMR was recorded in D_2O on a Varian Mercury-400MHz NMR spectrometer (Palo Alto, USA). As a negative control, GG was incubated with furfurylamine in the absence of DMT-MM.

2.2. Synthesis of maleimide-modified GRGDS peptide

Maleimide-modified GRGDS (mal-GRGDS) peptide was prepared by linear solid-phase synthesis (SPS) using standard Fmoc chemistry [31]. Fmoc-serine Wang resin (Anaspec, USA) with a loading capacity of 0.48 mmol/g was swollen in DMF (Sigma, USA) for 30 min. The solution was then drained and a solution of 20% piperidine (Caledon, Canada) in DMF was added and mixed for 30 min. The resin was then washed with DMF and tested for free amines using 1% trinitrobenzenesulfonate, TNBS (TCI America, USA), in DMF. The first amino acidbuilding block (Fmoc–Asp–OH, 3.0 equivalents, NovaBiochem) was pre-mixed with 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate, HCTU (3.0 equivalents, Anaspec, USA) in DMF for 15 min, then transferred to the SPS flask. 1.0 M diisopropylethylamine, (DIPEA, 4.0 equivalents, Sigma, USA) in DMF was then added and the flask was stirred until the coupling reaction was complete, which was determined by the TNBS test (negative test outcome). The Fmoc deprotection and coupling steps were repeated until five amino acid residues (GRGDS) were coupled to the resin. To conjugate the maleimide linker to the deprotected N-terminus of the peptide, 4 equivalents of maleimidopropionic acid (TCI America, USA) and 12 equivalents of diisopropylcarbodiimide (Sigma, USA) were pre-mixed in dichloromethane for 45 min and then added to the SPS flask and stirred for 24 h. The maleimide-modified GRGDS (mal-GRGDS) sequence was then cleaved from the resin using 95% trifluoroacetic acid (Caledon, Canada) in water. The peptide was allowed to precipitate in cold diethyl ether for 30 min. Then, the precipitate was recovered by centrifugation. Finally, the product was purified by HPLC (Shimadzu, Japan) in a C18, 250 × 10 mm, 5 um, 100 Å column. A mobile phase gradient from 5% to 20% (Acetonitrile (with 0.1% TFA):ddH_2O (with 0.1% TFA)) over 30 min was performed. A 90% yield was obtained and the product was confirmed by ^{1}H NMR and mass spectrometry (MS).

2.3. Immobilization of mal-GRGDS peptide on furan–GG hydrogel by Diels–Alder chemistry

Immobilization of maleimide-containing GRGDS (mal-GRGDS) to furan-modified gellan gum was performed via Diels–Alder chemistry between the maleimide functional group of the peptide with the furan group of the gellan gum. Furan–GG was first dissolved in MES buffer (100 mM, pH 5.5) at 37 °C (4 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 (M_w cutoff 12–14 kDa) alternately against distilled water and PBS (0.1 M, pH 7.2) for 5 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 negative control, mal-GRGDS was incubated with unmodified gellam gum.

2.4. Cell isolation and culture

All animal work was carried out in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care) and approved by the Animal Care Committee at the University of Toronto. Neural stem/progenitors cells were isolated from the subependymal region of the lateral ventricles in the forebrain of 6–8 weeks old male Wistar rats as previously described [32]. Cells were grown in neurobasal medium (Invitrogen, Canada), with 2% of B27 neural supplement (Invitrogen), 1% of L-glutamine (Sigma), 1% of penicillin–streptomycin (Sigma), 20 ng/ml EGF (recombinant human EGF; Invitrogen), 20 ng/ml bFGF (recombinant human bFGF; Invitrogen) and 2 ng/ml heparin (Sigma). Cell number and viability were determined with a hemocytometer using the trypan blue exclusion test. Dissociated cells were plated in complete media and incubated in a humidified atmosphere at 37 °C with 5% of CO_2. Neurospheres were observed within 1–2 weeks, after which cells were passaged weekly.

Olfactory ensheathing cells were isolated from adult male Wistar rats as previously described [33]. Briefly, upon the olfactory bulb dissection all meninges were removed and the tissue was digested with 0.125% collagenase type I (Sigma) for 20 min at 37 °C. The digested tissue was mechanically dissociated with a pipette and then filtered through a 40 μm cell strainer (BD Falcon, USA). After centrifugation at 1000 rpm for 10 min, cells were resuspended and plated in uncoated plates for 18 h. A posterior change to new uncoated plates for 36 h was made, as it is expected that most of the fibroblasts and astrocytes will attach in the first and second period, respectively. Finally, the cell suspensions were transferred to fibronectin treated flasks (coated overnight with 1 μg/ml fibronectin solution, (Sigma, USA) and cultured in DMEM/F12 (Gibco) with 10% of FBS (Gibco) and 1% of antibiotic–antimycotic solution (Sigma) at 37 °C and 5% CO_2. OEG were enriched by the supplementation with Bovine Pituitary Extract (5 μg/ml, Gibco) and Forskolin (2 μg/ml, Sigma).

2.5. Neural stem/progenitors cell culture on GRGDS-modified gellan gum

Bioactivity of GG–GRGDS was assessed by the analysis of NSPC growth, morphology and differentiation when cultured on the hydrogel for 2 and 7 days. NSPCs were mechanically dissociated into a single cell suspension and either seeded on the surface or encapsulated into GG–GRGDS hydrogel in complete medium (as described in Section 2.1). The cell density was 2×10^4 cells/cm^2 and hydrogels were

incubated in a humidified atmosphere at 37 °C and 5% of CO_2. Furan-modified GG was used as a control. Cell growth and morphology was evaluated by phalloidin/DAPI staining and differentiation was evaluated using immunocytochemistry (ICC). Analysis was carried out using a Zeiss Observer Z1 microscope with a Yokogawa confocal scan unit; images were captured and processed using Volocity 4.3.2 software.

2.6. Co-cultures between OEG and NSPCs

To evaluate the potential synergistic or antagonistic effects between OEG and NSPCs, direct and transwell co-culture experiments were performed. OEG (1×10^5 cells/cm^2) and NSPCs (4×10^4 cells/cm^2), obtained as described in section 2.4, were seeded either simultaneously onto the same fibronectin-coated cover glass, or in indirect contact by using a transwell (with OEG cultured on the fibronectin-coated cover glass and NSPCs on the transwell). Cells were allowed to grow in complete NSPC culture medium (see section 2.4) for 24 h and then cultured in the absence of the growth factors, FGF2 and EGF. In the direct co-culture experiment, in order to clearly identify one cell population from another, OEG were labeled (according to manufacturer instructions) with a cell tracing reagent (C34554, Invitrogen) before seeding. After 7 days of incubation, cell growth and NSPC differentiation was assessed by ICC. Analysis was performed using an Olympus BX61fluorescence microscope. OEG and NSPCs cultured alone were used as controls.

After these initial experiments, OEG and NSPCs were then encapsulated together in the GG–GRGDS hydrogel. Both cells were pre-labeled before encapsulation. A green tracer was used for OEG labeling (C34554, Invitrogen) and NSPCs were labeled with a far red tracer (C34553, Invitrogen). Both labeling protocols were performed according to the manufacturer instructions. Cell interactions and growth were then analyzed by confocal analyses after 7 days of culture.

2.7. Immunocytochemistry and phalloidin/DAPI staining

The following primary antibodies were used for the immunocytochemical studies: monoclonal rabbit anti-β-III tubulin (1:500, Chemicon, Canada) for neurons; monoclonal mouse anti-GFAP (1:100, Chemicon) for astrocytes; monoclonal mouse anti-O4 (1:200, R&D Systems, Canada) for oligodendrocytes; monoclonal mouse anti-Nestin (1:100, Millipore, USA) for progenitor cells; and polyclonal rabbit anti-p75 (1:100, Millipore) for OEG. For all immunocytochemical procedures, the appropriate controls were obtained by omission of the relevant primary antibody. Cells on the substrates were fixed with PBS solution containing 4% paraformaldehyde PFA for 20 min (on glass) or 1 h (in the hydrogel) at room temperature and then washed with PBS. Next, cell membrane permeation (except for p75 and O4 antibodies) and blocking by treating cells with 0.3% TritonX-100 (Sigma, USA) and 10% of FBS solution at room temperature for 1 h, each specific primary antibody solution was added for 1 h (on glass) or 12 h (on hydrogel). After washing with 0.5% of FBS in PBS, the samples were exposed to the specific secondary antibody (1:500 dilution of Alexa Fluor 488 anti-rabbit and 1:500 dilution of Alexa Fluor 594 anti-mouse, Invitrogen) for 1 h (on glass) or 5 h (on hydrogel) and then washed with 0.5% FBS. Finally, cell nuclei were counterstained with 1 μg/ml DAPI (Invitrogen) for 1 h.

For phalloidin/DAPI staining, cells were fixed with 4% of PFA for 30 min at room temperature and then treated with 0.3% TritonX-100. After washing several times with PBS, 0.1 μg/ml of phalloidin (Sigma) was added to the cells for 30 min. Finally, cell nuclei were counterstained with DAPI (1 μg/ml, Invitrogen) for 10 min.

2.8. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, USA). Differences among groups were assessed by two-way ANOVA followed by a Bonferroni post-hoc test (proliferation and differentiation analyses of NSPCs) and by t-student test (proliferation analyses of NSPCs and OEG in the co-cultures experiments). A p-value of ≤ 0.05 (95% confidence level) was set as the criteria for statistical significance. All data are presented as mean ± standard deviation.

3. Results

3.1. Synthesis and characterization of GG–GRGDS hydrogel

Immobilization of the synthetic peptide (mal-GRGDS) to the gellan gum (GG) hydrogel was achieved in two synthetic steps (Fig. 1A). First, the carboxylic acid groups of the glucuronic acid monosaccharide of GG were activated with DMT-MM, and then conjugated to furfurylamine to functionalize the GG with a furan. By ^{1}H NMR analysis, the degree of furan substitution to GG was calculated to be 27% (Fig. 1B). This was calculated by comparing the ratio of the areas under the furan peaks at 6.26, 6.46 and 7.65 ppm to the methyl peak at 1.2 ppm (of the rhamnose monosaccharide of GG). To confirm that the furfurylamine was covalently bound to GG, and not simply adsorbed, a control reaction (in the absence of the coupling agent, DMT-MM) was similarly characterized, and analysis of this ^{1}H NMR spectrum did not show any furan peaks (Fig. 1C). Second, the furan–GG hydrogel was reacted with maleimide-modified GRGDS peptide to yield GG–GRGDS hydrogel. After excessive dialysis to remove unbound peptide, quantification by amino acid analysis of the immobilized peptide was calculated to be 304.0 nmol of GRGDS peptide per mg of GG (Fig. 1D). A control reaction (using unmodified GG) was also performed to determine the amount of unbound peptide adsorbed on the hydrogel. In the absence of furan substitution to GG, only 5.3 nmol of GRGDS/mg of GG (Fig. 1E) was detected. Thus, approximately 300 nmol/mg of GRGDS peptide was covalently bound to the GG by the Diels–Alder [4 + 2] cycloaddition.

3.2. Biological effect of GG–GRGDS on NSPCs

NSPCs were either seeded on the surface or encapsulated within GG–GRGDS and compared to NSPCs cultured with unmodified GG hydrogels. Cell proliferation, morphology and differentiation were assessed after 2 and 7 days of culture. The results revealed pronounced differences in NSPC behavior when cultured in the peptide-modified hydrogel relative to the unmodified hydrogel. In the presence of immobilized GRGDS, the cells were able to migrate and successfully expand throughout the hydrogel whereas in the absence of GRGDS, NSPCs interacted preferentially with each other, forming cell aggregates or neurospheres (Fig. 2). Visible cytoplasmatic extensions were observed in the GG–GRGDS, both on the surface (Fig. 2A) and inside the gel (Fig. 2B), but not in the unmodified GG. Moreover, proliferation (number of singles cells between days 2 and 7) and morphological analyses revealed that in the absence of peptides, the cells were only able to proliferate as neurospheres whereas in the GG–GRGDS hydrogel, cells proliferated as single cells. This observation reflects the interaction of NSPCs with GRGDS peptides and their lack of interaction with the GG hydrogel. To quantify this observation, the number of single cells found either encapsulated within or on the gel surface was quantified (Fig. 3A and B): after 7 days of culture, we observed an average of $11.8 \times 10^4 \pm 5.1 \times 10^4$ single cells/cm^2 on the GG–GRGDS gel surface whereas only $7.8 \times 10^3 \pm 4.2 \times 10^3$ single cells/cm^2 on the GG surface. A similar trend was observed with encapsulated cells. The number of single cells in the GG–GRGDS hydrogel of $8.1 \times 10^4 \pm 2.3 \times 10^4$ single cells/cm^2 was significantly higher than that in the GG hydrogel of $8.2 \times 10^3 \pm 7.3 \times 10^3$. Interestingly, while GRGDS modification influenced the number of single cells present, it did not affect the differentiation profile, which was not significantly different (Fig. 3C). After 7 days, the majority of NSPCs on GG–GRGDS and GG hydrogels were O4 positive oligodendrocytes (72 ± 11% and 64 ± 30%, respectively) with relatively little differentiation into astrocytes 7 ± 12% vs. 0 ± 0% and no differentiation into neurons. Moreover, as judged by the nestin expression, approximately one third of the NSPCs remained as progenitors (32 ± 16% vs. 36 ± 19%).

3.3. Co-culture of OEG and NSPCs

The interaction of OEG and NSPCs was analyzed after 7 days in culture for cell proliferation, and differentiation as a function of direct contact or indirect contact (i.e. same media, but no contact through use of a transwell). Interestingly, the differentiation profile of NSPCs was not altered by the presence of OEG (Fig. 4A); however, NSPCs proliferated significantly more when co-cultured with OEG vs. alone. There was a 2.3 fold increase in the number of NSPCs

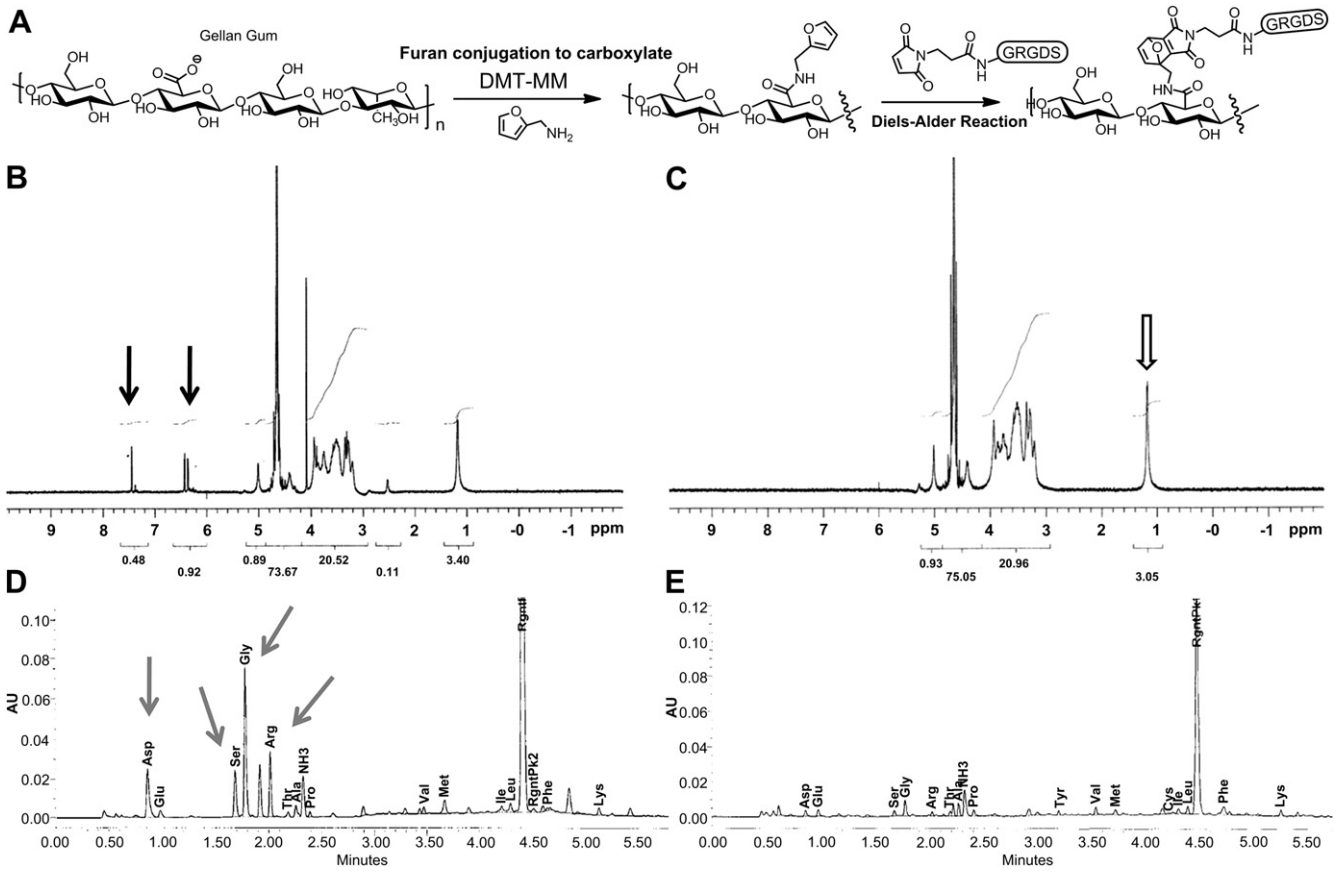

Fig. 1. Immobilization of the GRGDS peptide into the gellan gum hydrogel. (A) Schematic representation of the Diels–Alder reaction (furan–maleimide conjugation) used for the GG modification. When DMT-MM and furfurylamine were incubated with the GG, a furan-modified gel with 27% substitution was synthesized. (B) ^{1}H NMR spectrum of furan–GG. The degree of substitution was calculated by comparing the ratio of the areas under the proton peaks at 6.26, 6.46 and 7.65 ppm (black arrows point to furan protons) to the peak at 1.2 ppm (white arrow point to methyl group of GG). (C) ^{1}H NMR spectrum of GG, after incubation with furfurylamine in the absence of the coupling reagent DMT-MM. Furan peaks were not detected in the GG spectrum (D) By reverse phase HPLC, the amino acid analyses revealed that when furan–GG and mal-GRGDS were incubated together it was possible to obtain approximately 300 nmol GRGDS/mg of GG (gray arrows point to each amino acid). (E) In contrast, only 5.3 nmol of GRGDS peptide were detected in the control reaction between maleimide-modified GRGDS and unmodified GG.

when co-cultured in direct contact with OEG vs. NSPCs alone (Fig. 4B) and a 1.8-fold increase in the number of NSPCs when co-cultured indirectly in transwells with OEG vs. NSPC monocultures (Fig. 4C). These results suggest that OEG secrete factors that enhance NSPC proliferation and that the cells do not need to be in contact in order for this affect to be observed. Unlike the proliferative effect that the OEG had on the NSPCs, the reverse was not true: the total number of OEG was not significantly affected by co-culture with NSPCs either in direct contact (Fig. 4D) or indirect, transwell (Fig. 4E) contact. Notwithstanding that OEG do not have to be in direct contact with NSPCs to influence their proliferation, immunocytochemistry illustrates that OEG are closely associated with NSPCs when co-cultured together (Fig. 5). By pre-labeling OEG green with CFSE34554 and counterstaining NSPCs with either nestin (red for progenitors) or O4 (red for oligodendrocytes), the two cell types appeared to be closely associated with each other. These data suggest that OEG may be able to provide a guidance pathway for NSPC growth.

3.4. Co-culture of OEG and NSPCs in the GG–GRGDS hydrogel

To gain greater insight into the interactions of OEG and NSPCs, they were co-cultured together in the 3D gellan gum hydrogel modified with the cell-adhesive peptide, GRGDS (GG–GRGDS). We initially seeded 10^5 OEG and 4×10^4 NSPCs. Different cellular densities were used because NSPCs have a higher proliferative rate than OEG. After 7 days we estimated that both should be near confluence. To facilitate identification, the OEG were pre-labeled green with CFSE34554 and the NSPCs red with DDAO-SE 34553 prior to encapsulation in the GG–GRGDS hydrogel. As was observed in the 2D co-culture study, the total number of NSPCs was significantly higher when co-cultured with OEG than when cultured alone (Fig. 6A). Similar to the 2D data presented in Fig. 4, the number of OEG was unaffected by the presence of NSPCs (Fig. 6B).

4. Discussion

Gellan gum (GG) is a natural biomaterial that has shown promise for tissue regeneration. It is a thermo-reversible gel that was previously shown to be cytocompatible with adipose stem cells, rabbit articular chondrocytes and immortalized oligodendrocytes [20,34]. Until recently, most of the research on GG modification was focused on controlling its mechanical properties through gel network formation [21,35]. To the best of our knowledge, there is no literature describing the chemical modification of GG with ECM-derived peptides, even though the cellular microenvironment is defined, in part, by its chemical nature. In order to create a hydrogel which mimics the chemical nature of the ECM, bioactive peptides were conjugated to GG, thereby providing a more suitable environment for encapsulated cells to survive and migrate.

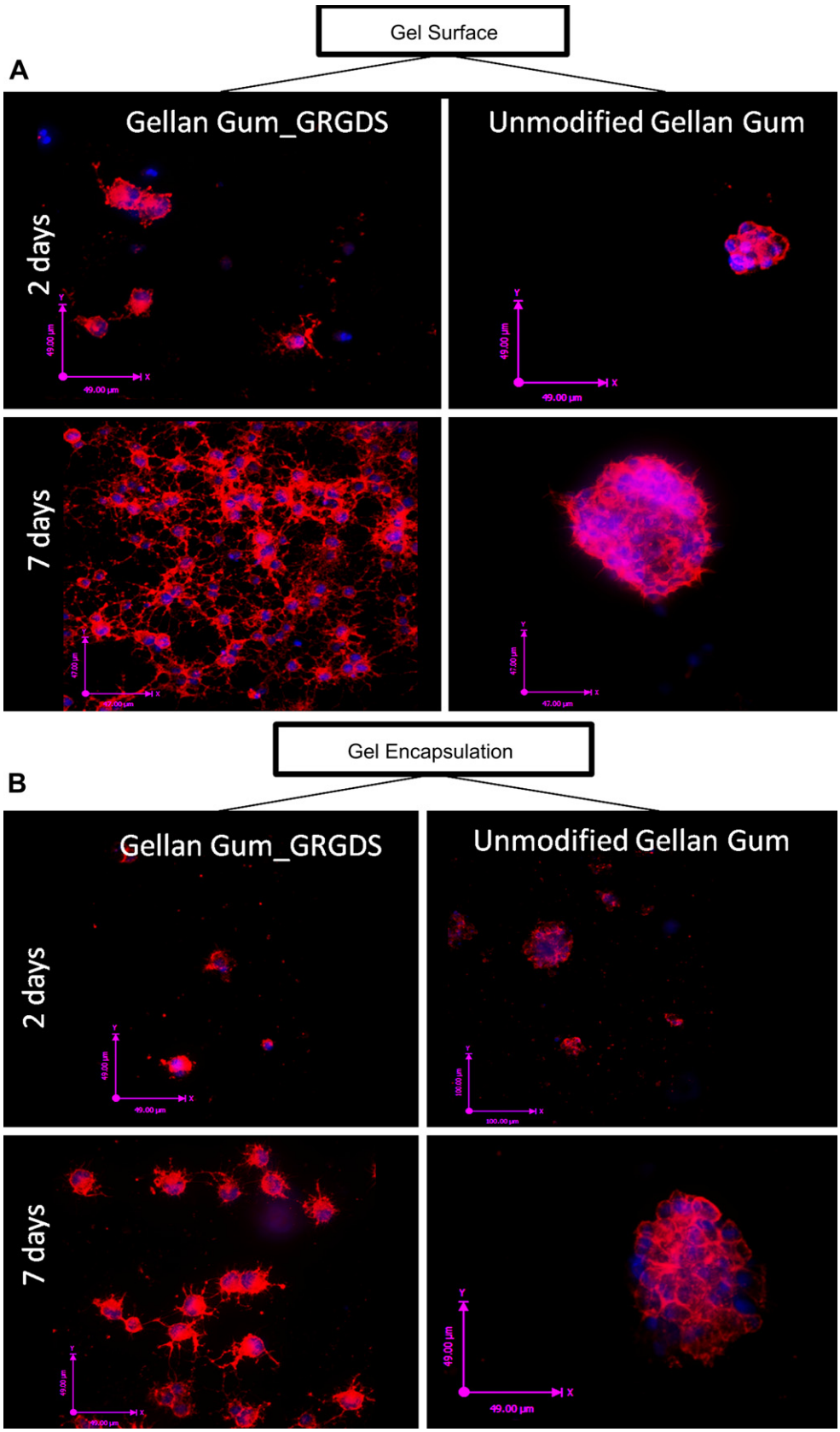

Fig. 2. Morphology and dispersion of NSPCs on the GG–GRGDS. Confocal analyses revealed substantial differences in NSPC morphology when cultured either (A) on the surface or (B) encapsulated within the GG–GRGDS vs. unmodified GG gel. Cell spreading and visible cytoplasmatic extensions were only observed in the GG–GRGDS. In the unmodified GG, NSPCs proliferated as neurospheres. The cytoplasm was stained with the anti-F-actin/phalloidin (red) and nuclei counterstained with DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

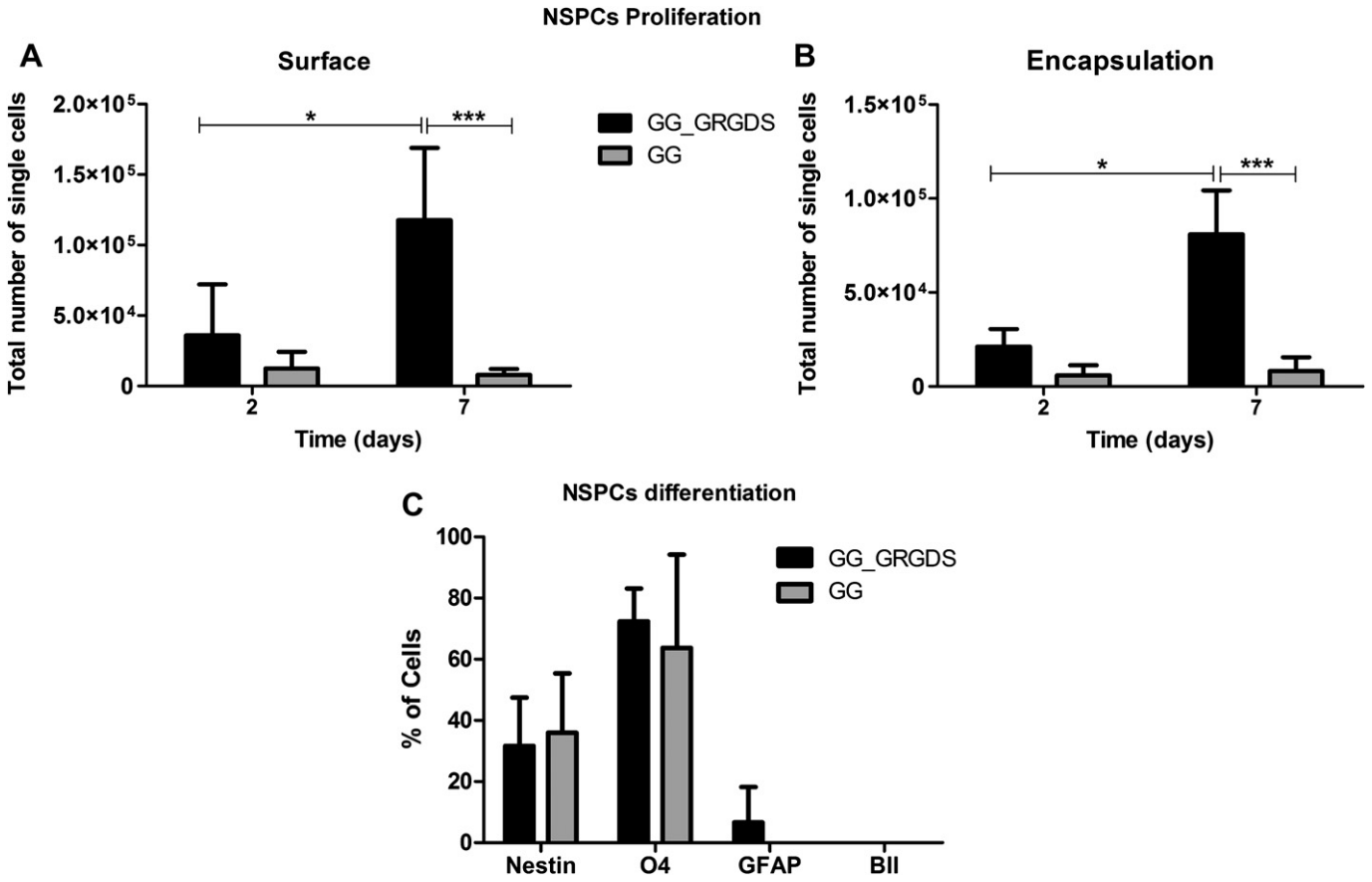

Fig. 3. Bioactivity of the GG–GRGDS hydrogel. Proliferation analyses of NSPCs cultured either (A) on the surface of or (B) in the gel of the GG–GRGDS (black bars) vs. unmodified GG (gray bars) showed that on the seventh day a significantly higher number of single cells were found in the GG–GRGDS. Moreover, only in the GG–GRGDS was a significant increase in cell number observed from day 2 to day 7. (C) Immunocytochemistry revealed that the GRGDS sequence did not significantly influence the differentiation profile of the NSPCs. Values are shown as mean ± standard deviation ($n = 3$ samples of 2×10^4 cells/sample, $^*p < 0.05$; $^{***}p < 0.001$).

The peptide sequence GRGDS is a ubiquitous cell-adhesive peptide, which has been shown to enhance cell–biomaterial interactions, support cell survival and influence cell morphology [22,36]. In this work we show that it is possible to immobilize the GRGDS peptide to GG. By taking advantage of the Diels–Alder click cycloaddition reaction between chemically modified GG–furan and maleimide-GRGDS, GG–GRGDS was synthesized in aqueous conditions.

Immobilization of GRGDS to GG had a profound effect on NSPC morphology, distinct from that observed on NSPCs in unmodified GG alone, demonstrating the importance of GRGDS for cell–GG interaction. Importantly, these morphological differences were observed both within the 3D hydrogels and on their surfaces. Unlike most studies that focus only on cells cultured on surfaces, herein we cultured cells both on the hydrogel surface and encapsulated within [36,37]. Given that the ultimate goal involves cell transplantation via an injectable hydrogel, it was reassuring that the cell morphology in 3D mirrored that in 2D. The NSPCs were able to adhere to and extend processes within the GG–GRGDS hydrogel, yet their differentiation profile was not affected by the presence of GRGDS. This reflects the cell-adhesive property ascribed to GRGDS and not a differentiation property. To achieve preferential differentiation to a given phenotype, GG would typically require further modification with growth factors, such as PDGF-AA for oligodendrocytes [38] or interferon-gamma for neurons [39].

The co-culture experiments of NSPCs and OEG, in direct contact or in the transwell, suggested that diffusible factors are responsible for the effects observed. OEG have been shown to secrete several neurotrophic factors including: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF) [13], basic fibroblast growth factor (FGF2) and neurotrophin-3 (NT-3) [40]. This rich profile of secreted neurotrophins from OEG may explain the proliferation of NSPCs that we observed in our co-culture studies. For example, NGF and FGF promote NSPC proliferation [41,42]; and NT-3 enhances their survival [43]. The combination of both mechanisms, survival and proliferation, may explain the increased number of NSPCs in all of the co-culture studies.

Other than this study, very little is known about the effects of OEG on the behavior of NSPCs. Interestingly, Cao *et al.* [33], also observed that OEG promoted NSPC proliferation, yet they also observed increased neurogenesis and oligodendrogenesis of NSPCs cultured with OEG. Differences in the co-culture experiments may account for this discrepancy. Cao *et al.* harvested the NSPCs from newborn mice, whereas we obtained NSPCs from adult rats; moreover, they co-cultured the cells for only 3 days in DMEM with F12 and N2 supplements whereas we co-cultured the cells for 7 days in neurobasal medium with B27 supplements.

The immunocytochemistry figures suggest that OEG and NSPCs interact with each other. This interaction may be attributed to OEG expressing cell-adhesive molecules, such as N-CAM and L1 [44], and NSPCs expressing the corresponding receptors [45]. The cellular interactions may suggest a role for co-transplantation of OEG and NSPCs.

Cell transplantation has been pursued for several years for SCI repair. Both OEG and NSPCs have been investigated independently, each with some success. Notably, biomaterial scaffolds have been

shown to enhance cell survival after transplantation. For example, Cummings *et al.* [46] showed that transplanting human NSPCs in SCI mice led to some locomotor recovery and remyelination. Johnson *et al.* [47] demonstrated that implantation of fibrin hydrogels containing neural progenitor cells resulted in some functional recovery in SCI rats. Ballios *et al.* demonstrated greater cell survival after transplantation in a hyaluronan/methylcellulose hydrogel compared to saline [48]. OEG support and guide axon elongation in their native olfactory system and have shown promise after spinal cord injury as well [49,50]. OEG transplanted immediately or up to 2 months after SCI in rats resulted in functional recovery and/or tissue regeneration [51,52]. Significantly,

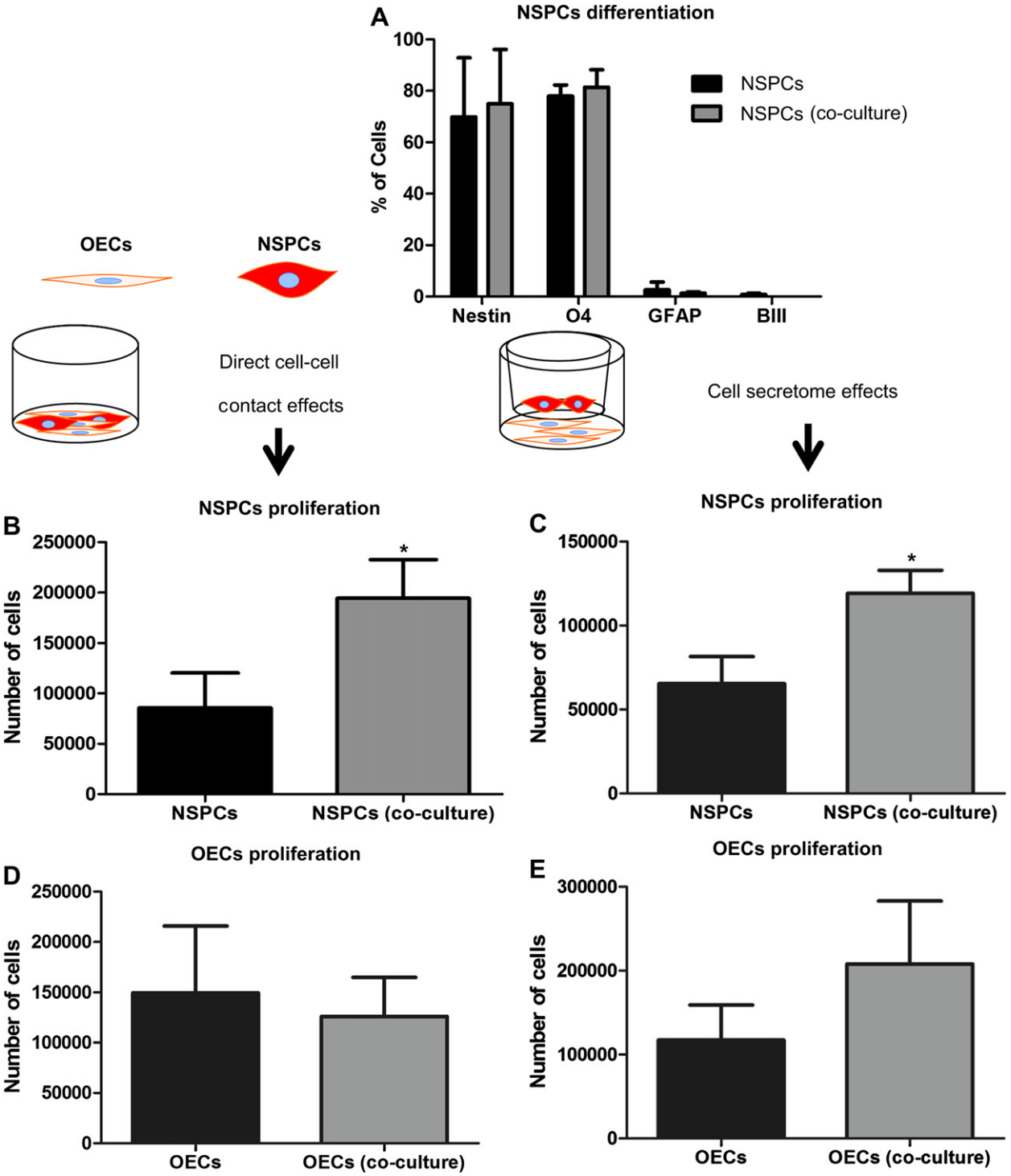

Fig. 4. OECs and NSPCs proliferation in transwells and direct contact co-culture. (A) The differentiation profile of NSPCs was not affected by the presence of OECs. Proliferation analysis showed that the total number of NSPCs is significantly higher when in culture with OECs than when cultured alone. This occurred both in the (B) direct cell–cell contact co-cultures and in the (C) indirect transwell co-cultures. OECs proliferation was not significantly affected by the presence of NSPCs, either in (D) direct contact or in the (E) indirect transwell co-culture. Values are shown as mean ± standard deviation ($n = 3$ independent studies of a minimum of 4000 cells counted per study, $^{*}p < 0.05$).

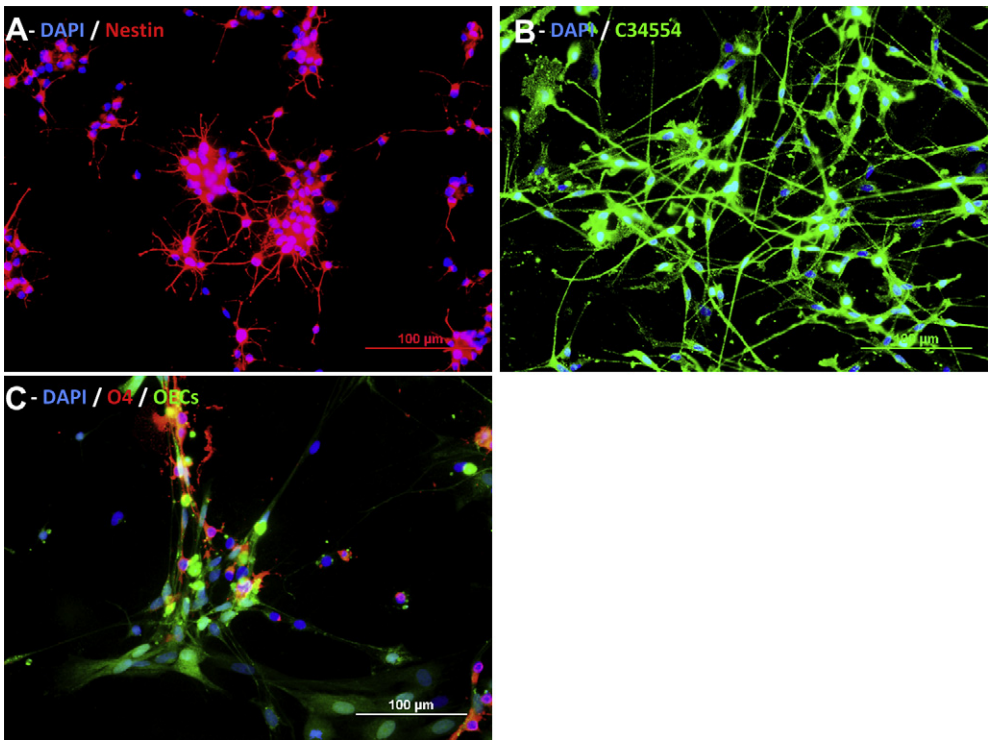

Fig. 5. OECs and NSPCs interact with each other during co-culture. (A) Monocultures of NSPCs and (B) Monocultures OECs served as controls. (C) OECs (green, CFSE 34554) and NSPCs (red, O4) appear to be closely associated when the cells are cultured together in direct contact. NSPCs were identified by immunocytochemistry and nuclei are counterstained with DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

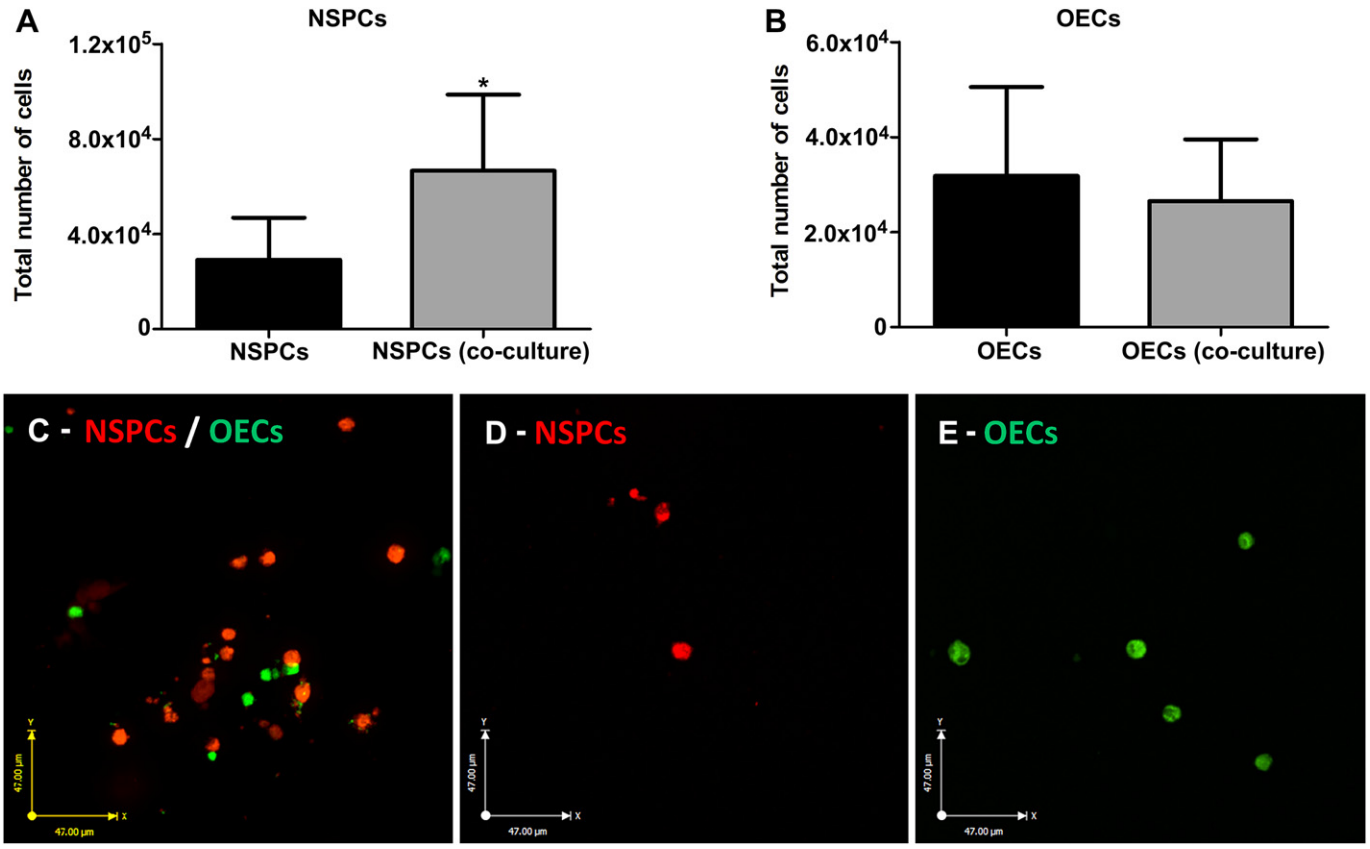

Fig. 6. Co-culture between OECs and NSPCs in the GG–GRGDS hydrogel. (A) After counting the cells, it was possible to observe that the number of NSPCs was significantly higher when co-cultured with OECs than when cultured alone. (B) In contrast, OEC proliferation was unaffected by the presence of NSPCs in culture. (C) Co-cultures of NSPCs (red, DDAO-SE 34553) and OECs (green, CFSE 34554) suggested some interaction between the two cell types as observed by confocal microscopy. (D) NSPCs and (E) OECs were cultured alone to serve as controls. Values are shown as mean ± standard deviation ($n = 3$ independent experiments of 200 cells counted per experiment, $^{*}p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

OEG have been tested in three separate clinical trials [30,53,54]; however, only very modest (if any) motor improvements were observed. Despite the promise for cell therapy, with OEG or NSPCs, the functional recovery has been modest, underlining the need for innovative strategies, such as the combined transplantation of OEG and NSPCs in a GG–GRGDS hydrogels. The simplicity and broad applicability of the Diels–Alder click chemistry can be easily extended to other biomolecules to further promote NSPC differentiation for greater integration with the host tissue. This combination strategy could be powerful, with OEG and gellan gum providing a cellular pathway on which NSPCs could differentiate, thereby replacing the damaged tissue and achieving greater functional repair.

5. Conclusions

Using well-established Diels–Alder click chemistry, we immobilized the GRGDS fibronectin-derived peptide to gellan gum hydrogels, which promoted greater adhesion and proliferation of neural stem/progenitor cells than gellan gum controls. Moreover, NSPCs co-cultured with olfactory ensheathing glia showed greater survival and outgrowth than NSPCs cultured alone. These results suggest that the combined use of NSPCs and OEG with bioengineered GG–GRGDS hydrogels may be beneficial in regenerative medicine cell transplantation strategies to promote repair after spinal cord injury.

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