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RESEARCH ARTICLE

The influence of sustained dual-factor presentation on the expansion and differentiation of neural progenitors in affinity-binding alginate scaffolds

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Abstract

Biomaterials capable of controlling the release of multiple growth factors (GFs) could potentially promote the integration of co-transplanted neural progenitor cells (NPCs) and stimulate the plasticity and regenerability of the lesioned spinal cord. As a first step towards the employment of such a vehicle for cell therapy, this study examined the capability of an alginate–sulphate/alginate scaffold, able to capture and rigorously control the release of GFs, to promote the expansion and lineage differentiation of NPCs *in vitro*. Epidermal growth factor (EGF) and fibroblast growth factor-2 (bFGF) were affinity-bound to alginate–sulphate (200 ng/scaffold) and the bioconjugates were mixed with partially calcium-crosslinked alginate. NPCs isolated from 18 day-old rat embryo brains and seeded into the scaffold during preparation were found to proliferate and differentiate within the vehicle. A continuous release of both bFGF and EGF was noted for a period of 21 days. The concentrations of released GFs were sufficient to promote extensive NPC proliferation at initial cultivation times; the number of neurospheres in the scaffold was twice the number found in the 2D cultures supplemented with 20 ng/ml each factor every 3 days. Between days 10–14, when the GF concentrations had substantially declined, extensive cell migration from the neurospheres as well as lineage differentiation were noted in the scaffold; immunocytochemical analyses confirmed the presence of neurons, astrocytes and oligodendrocytes.The scaffold has a potential to serve as cell delivery vehicle, with proven capability to promote cell retention and expansion, while enabling NPC lineage differentiation *in situ*. **Copyright © 2013 John Wiley & Sons, Ltd.**

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Keywords **alginate–sulphate; alginate scaffold; epidermal growth factor; fibroblast growth factor-2; neural progenitor cells; neurospheres**

1. Introduction

Spinal cord injury (SCI) in humans produces devastating irreversible neurodegenerative changes associated with progressive inflammation, demyelization, loss of neurons and glial cells. These injuries often lead to permanent disability, with clinical signs of partial or complete paralysis and spasticity (Elbasiouny *et al*., 2010). Multiple post-traumatic factors, including increase of axonal inhibitory molecules, lack of neurotrophic factor support and scar tissue formation, are responsible for the poor spontaneous neural repair after SCI (Fawcett and Asher, 1999). Yet, by addressing each of these factors, such as neutralization of growth inhibitors, delivery of neurotrophic factors or utilizing stem cells/progenitors, considerable progress has been achieved in enhancing the growth of injured adult axons in various SCI preclinical animal models (Fawcett, 2006; Schwab, 2004).

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In recent years, much attention has been focused on the transplantation of neural progenitor cells (NPCs) following spinal cord injury, leading to various levels of functional recovery (Cummings *et al*., 2006; Nakamura *et al*., 2005; Marsala *et al*., 2004; Webber *et al*., 2007). The transplantation process typically involves the injection of a cell suspension into the central cyst site to replace lost neurons or remyelinate impaired spinal axons caused by SCI (Salazar *et al*., 2010). However, the lack of chemical signals, including cytokines and neuropeptides such as trophic/growth factors, and physical determinants such as extracellular matrix (ECM) proteins and supporting matrix at the lesion site, have led to the limited survival, retention and differentiation of the implanted NPCs. In addition, the hostile environment in the lesion site immediately after the damaging event does not provide a suitable place for transplanted cells (Karimi-Abdolrezaee *et al*., 2006).

In an attempt to enhance endogenous plasticity and integration of transplanted NPCs into functional neural circuitry, various cell-delivery vehicles have been developed in the form of natural/synthetic scaffolds, releasing growth factors (Shanbhag *et al*., 2010; Jain *et al*., 2006; Perale *et al*., 2011; Baumann *et al*., 2010). In most of these systems, the growth factors are attached to the scaffolds via covalent binding or are physically entrapped in the matrix. Such factor presentation is considered unnatural, since growth factors, especially those of the heparin-binding protein family, bind the proteoglycans heparin and heparan sulphate in the ECM via high-affinity, specific electrostatic interactions (Taylor *et al*., 2006). To mimic such interactions and create an appropriate cell-delivery vehicle, our group has developed a bio-inspired scaffold made of alginate–sulphate/alginate, wherein heparin-binding proteins are bound to alginate–sulphate with an affinity similar to that realized upon their binding to heparin (Freeman *et al*., 2008). We demonstrated that multiple GFs can be attached to a single affinity-binding scaffold and that their release can be controlled by adjusting the initial concentration of attached GFs and according to their equilibrium binding constant to the matrix (Freeman and Cohen, 2009; Dvir *et al*., 2009).The use of alginate–sulphate-containing hydrogel as a delivery system has advantages compared to heparin-crosslinked biomaterials. Heparin is a multifaceted molecule with several biological functions other than binding growth factors, such as anticoagulation, and its delivery may induce distinguishable blood-thinning effects (Park *et al*., 2008) and other biological responses (Oschatz *et al*., 2011).

In the present study, we aimed to investigate the appropriateness of the affinity-binding alginate scaffold to support the survival and expansion of neural progenitor cells (NPCs) and enable their lineage differentiation into neurons, astrocytes and oligodendrocytes. We hypothesized that the presentation of epidermal growth factor (EGF) and fibroblast growth factor-2 (bFGF) as affinity-bound to the alginate–sulphate/alginate scaffold would provide the desired initial neurotrophic support for their survival and expansion and, after GFs expenditure, the environment would enable the differentiation of the seeded NPCs. The GFs selected have important roles in regulating NPC proliferation and differentiation; bFGF is a known mitogen for stem cell self-renewal, while EGF induces both proliferation and differentiation in many mammalian stem cells (Lam *et al*., 2010; Wilson and Stice, 2006), including NPCs. The study reported herein constitutes a preliminary feasibility evaluation of the appropriateness of the affinity-binding scaffold to serve as a cell-delivery vehicle *in vivo*, with a capability to support the viability, integration and function of transplanted cells and their lineage differentiation.

2. Material and methods

2.1. Harvesting and culture of NPCs from embryonic brain

This study was performed with the approval of, and according to the guidelines of, the Institutional Animal Care and Use Committee of the Slovak Academy of Sciences and with the European Communities Council Directive (86/906/EEC) regarding the use of animals in research, Slovak Law for Animal Protection No. 115/1995.

Neural progenitor cells (NPCs) were derived from the telencephalon of 18 day-old embryos ($n = 27$) isolated from four Wistar pregnant rat females. Isolation of NPCs was performed according to a multistep dissociation protocol, as described previously (Marsala *et al*., 2004). Briefly, the entire forebrains were excised, washed in cooled (4 °C) phosphate-buffered saline (PBS) with antibiotics, after which the meningeal membrane and blood vessels were removed, the dissected tissue was cut into small pieces (< 2 mm), mechanically disaggregated and incubated in an enzyme solution, consisting of 0.01% papain and 0.01% DNase1 (Worthington Biochemical Corp., Lakewood, NJ, USA) at 37 °C for 30 min. Afterwards, the enzymatic reaction was stopped with ovomucoid protease inhibitor and the final cell suspension was centrifuged for 5 min at $300 \times g$, using a density gradient to remove cell membrane fragments. Finally, cell counts and viability were determined using the standard trypan blue method.

Neural progenitor cells (24×10^6), defined in our previous studies by specific cell surface markers (Cizkova *et al*., 2009; Marsala *et al*., 2004), were plated in Nunc T75 culture flasks at a density of 6×10^5 cells/10 ml and grown in complete culture medium (CM) composed of Dulbecco's modified Eagle's medium (DMEM; D 6429) and Ham's F12 (1:1 v/v) with 3 mM glucose, 5 mM HEPES, pH 7.2 (all from Sigma-Aldrich Corp., Seelze, Germany), 5 mg/ml streptomycin and 5 IU/ml penicillin, supplemented with B27 (10 ng/ml), N2 (10 ng/ml), recombinant human bFGF (20 ng/ml) and human EGF (20 ng/ml; all from Gibco/Invitrogen, Grand Island, NY, USA). The cells were incubated in a humidified incubator supplied with 5% CO_2. The initially formed primary neurospheres were

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dissociated on day 3 by mechanical trituration; half of the suspended cells (neurospheres from 12×10^6) were used for seeding into the bFGF/EGF affinity-bound scaffold, while the remaining half were replated in 24-well plates in medium supplemented with human bFGF (20 ng/ml) and human EGF (20 ng/ml) for an additional week. Cell differentiation was induced on pre-poly(D-lysine)-coated 24-well plates (PDL, 100 μg/ml; Sigma), or a Lab-Tek chamber slide system (Nalge Nunc International, Roskilde, Denmark) at a density of 2×10^5 viable cells/chamber in differentiation medium (DM), free of mitogenic factors, containing fetal bovine serum (FBS) 5–10% and 10 μM retionic acid (RA), for 7–21 days.

2.2. Preparation of alginate scaffold with affinity-bound factors

Fabrication of the scaffold with the affinity-bound dual growth factors (ALG/ALG-S + GFs) involved preparing bioconjugates of bFGF and EGF with alginate–sulphate and then mixing both bioconjugate solutions with the solution of a partially calcium-crosslinked alginate. The bioconjugates were prepared by mixing bFGF or EGF with alginate–sulphate solution (1%, w/v) and incubating for 1.5 h at 37 °C, to allow equilibrium binding. The partially calcium-crosslinked alginate solution was prepared as previously described (Tsur-Gang *et al.*, 2009). Briefly, stock solutions of sodium alginate (VLVG, 30–50 kDa, > 65% guluronic acid content; NovaMatrix FMC Biopolymers, Drammen, Norway) and D-gluconic acid/hemi calcium salt were prepared by dissolving the materials in double-distilled water (DDW) and stirring at room temperature. Each solution was filtered separately through a sterile 0.2 μm filter membrane into a sterile container in a laminar flow cabinet. Equal volumes from each stock solution (2.08% and 0.68% w/v for VLVG alginate and D-gluconic acid, respectively) were combined by extensive homogenization for several minutes to facilitate homogeneous distribution of the calcium ions and crosslinking of alginate chains. Finally, the bFGF and EGF alginate–sulphate bioconjugates were mixed with the partially crosslinked alginate to yield an bFGF/EGF-containing, affinity-binding alginate scaffold (0.1% alginate–sulphate, 0.9% alginate, 0.3% D-gluconic acid w/v) (ALG/ALG-S + GFs). For the control system lacking GFs, the scaffold was prepared with no affinity-bound factors (ALG/ALG-S).

2.3. NPC cultivation in scaffold and study design

The cells were mixed with the alginate biomaterial solutions to yield alginate/alginate–sulphate scaffolds harbouring NPCs, either without (group A, Figure 1) or with (group B) the two GFs. The amount of each GF loaded onto each scaffold was 200 ng. The cell suspension in biomaterial solution (500 μl) was placed into a well in a 24-well plate and was supplemented with 500 μl DMEM (Sigma–Aldrich) with 5 mg/ml streptomycin and 5 IU/ml penicillin (no GFs added to the external medium). Upon addition of the medium, which contains calcium ions, the partially crosslinked alginate underwent phase transition to form a scaffold containing 2×10^5 NPCs/500 μl scaffold/well. The cell constructs were cultivated for up to 21 days under standard cultivation mode; the medium was replaced with fresh DMEM supplemented with antibiotics (500 μl) on days 4, 7, 14 and 21 ($n = 6$ wells/period).

An additional control group (group C) was a two-dimensional (2D) culture of NPCs supplemented with GFs into the external medium. Here, 2×10^5 NPCs were plated on the surface of a well in a 24-well plate (no alginate scaffold) with 500 μl culture medium (CM) composed of DMEM supplemented with 5 mg/ml streptomycin, 5 IU/ml penicillin, 20 ng/ml bFGF and 20 ng/ml EGF. This group was cultivated for 10 days to allow for the formation of numerous neurospheres. The medium containing the GFs was replaced every 3 days. After the 10 day cultivation period, the growth factors were replaced with 5–10% v/v FBS to initiate disaggregation of the neurospheres and achieve final cell differentiation.The cells of all three experimental groups were cultured under same conditions in a humidified atmosphere of 5% CO_2 at 37 °C.

2.4. Release study and ELISA analysis

The release pattern of bFGF and EGF from the affinity-binding scaffolds, in the presence or absence of seeded NPCs, was examined using enzyme-linked immunosorbent assay (ELISA; Quantikine, R&D Systems, Minneapolis, MN, USA). On days 4, 7, 14 and 21 the medium was replaced and the releasing medium was analysed in triplicate by ELISA. For blank controls, alginate/alginate–sulphate scaffolds supplemented with GFs but with no cells (ALG/ALG-S + GFs) were used.

2.5. Neurosphere formation and migration activity of NPCs

The proliferation, migration and differentiation capabilities of NPCs seeded in the bFGF/EGF affinity-bound cell construct or plated in 2D cultures and supplemented with bFGF/EGF in the medium were evaluated and quantified on days 4, 7, 14 and 21. Neurosphere formation, as indication for proliferation, was quantified by counting the number of neurospheres formed and measuring their diameter in 10 random fields and calculated per 1 mm^2. NPC migration activity (as an indicator of initial differentiation) was analysed according to the formula: radius of the furthest migrated cell distance out from the neurosphere (RMC) divided by the radius of the neurosphere (RM); 10–40 neurospheres/time point were analysed in a blinded manner.

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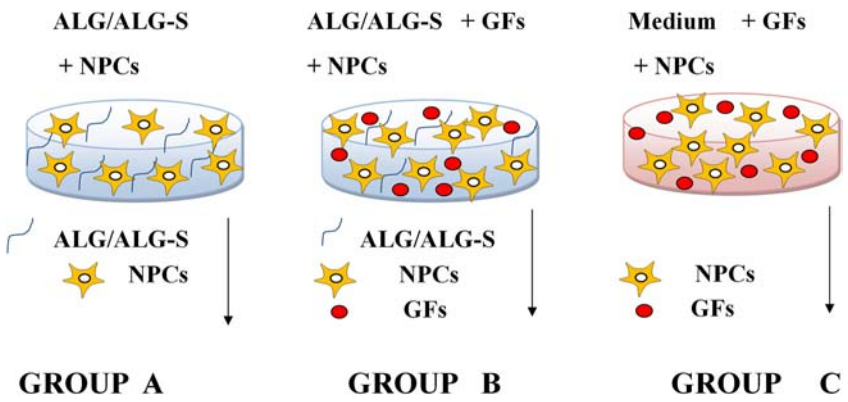

Figure 1. A schematic illustration of the experimental groups in the study: (A) NPCs seeded in ALG/ALG-S scaffold lacking growth factors (GFs); (B) NPCs seeded in ALG/ALG-S scaffold with affinity-bound GFs; and (C) NPCs cultured in 2D cultivation mode with GFs supplemented into the medium. All experimental groups were cultured under standard *in vitro* conditions. ALG/ALG-S, alginate/alginate–sulphate; NPCs, neural progenitor cells; GFs (bFGF, EGF)

2.6. Immunocytochemistry

The cell construct (ALG/ALG-S + GFs + NPCs; loaded with 200 ng of each GF) and the cells in the 2D culture supplemented with GFs were fixed with 4% paraformaldehyde, washed in PBS and incubated with the following primary antibodies: rabbit anti–MAP2 (1:1000); mouse anti-NeuN (1:500); rabbit anti-NF-200 (1:1000); mouse or rabbit anti-GFAP (1:1000); rabbit anti-NG2 (1:1000); and mouse anti-receptor-interacting protein RIP (1:300) (all from Millipore, Temecula, CA, USA), followed by fluorescent-labelled secondary antibodies AlexaFluor 488 anti-mouse IgG (1:200) or AlexaFluor 594 anti-rabbit IgG (1:200) (all from Invitrogen/Molecular Probes, Eugene, OR, USA). After nuclear staining with DAPI, the cells were viewed under an Eclipse Ti-Nikon fluorescence or a Leica confocal microscope (Nikon, Amstelveen, The Netherlands; Leica Microsystems CMS, Mannheim, Germany). To calculate the percentage of neuronal or glial progeny, immunopositive cells for each antibody were counted in 10 random fields (600 × 600 μm) scanned for each treatment.

2.7. Statistical analysis

For all data, comparison was performed by one-way analysis of variance (ANOVA) followed by a Tukey–Kramer test using Graphpad Prism software. $p < 0.05$ was considered statistically significant ($*p < 0.05$; $**p$ 0.01; $***p < 0.001$). Data from neurosphere analyses and immunocytochemistry were collected and analysed using Microsoft Office Excel (Microsoft, Denver, CO, USA) and Ellipse 3D software (Vidito, Košice, SR) and are presented as mean ± standard deviation (SD).

3. Results

3.1. Release pattern of affinity-bound bFGF and EGF from the scaffold

The amount of bFGF released into the external medium from affinity-binding alginate scaffolds (initially loaded with 200 ng bFGF and 200 ng EGF) with no seeded NPCs peaked at a value of 21.9 ± 1.1 ng/ml on day 7, then gradually declined to 7.6 ± 0.6 ng/ml on day 21 (Figure 2A). In scaffolds seeded with NPCs, significantly lower levels of released bFGF were detected on day 7 (17.4 ± 0.7 ng/ml) and day 14 (9.6 ± 1.2 ng/ml; $p < 0.001$), while on day 21, a similar level of 7.8 ± 0.2 ng/ml was measured for both the cellular and acellular constructs (Figure 2A). In scaffolds loaded with 100 ng bFGF/scaffold, with or without seeded NPCs, the concentration of released bFGF into medium was below the amount (20 ng/ml) used in standard 2D cultures at all time-points (Figure 2A). Loading with 100 ng each factor/scaffold resulted in medium concentrations of bFGF that were lower than those supplemented to the medium in standard 2D cultures (data not shown). Based on these results, an affinity-binding alginate scaffold loaded with 200 ng bFGF was used in further studies. The EGF release profile exhibited a similar trend to that found for bFGF. On day 7, 128.5 ± 31.0 ng/ml released EGF was measured in the medium of the affinity-binding alginate scaffold with no seeded cells, initially loaded with 200 ng EGF/scaffold (Figure 2B). Later, the amount of released EGF gradually declined to 29.1 ± 4.9 ng/ml on day 21. A significantly lower concentration of released EGF was detected on day 7 (75.7 ± 6.4 ng/ml) from the NPC-seeded scaffolds, in comparison to the acellular scaffold ($p < 0.001$), while at later time points the release levels from cellular and acellular scaffolds were similar (Figure 2B). Loading with 100 ng each factor/scaffold resulted in low medium concentrations of EGF; the release levels were similar for the cellular and acellular constructs (data not shown).

3.2. Neurosphere formation in scaffold and migration activity of NPCs

In group A, in which NPCs were seeded onto the alginate/alginate–sulphate scaffolds with no addition of GFs, only scattered cells occurred, neurospheres were not formed and only a minority of cells (15–20%) survived after 4 days in culture (Figure 3C). In group B, in which NPCs

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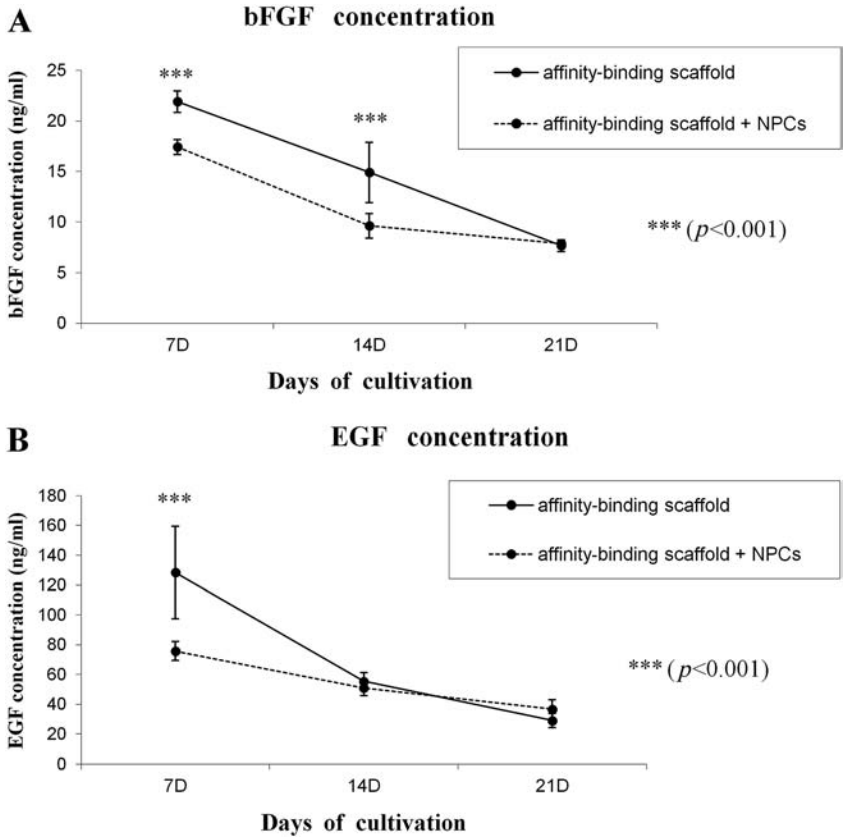

Figure 2. Release patterns of (A) bFGF and (B) EGF from the affinity-binding alginate scaffold; the scaffold was seeded with (dashed lines) or without (solid lines) NPCs. Each scaffold was loaded with 200 ng each factor, bFGF and EGF. Error bars represent SD; ***$p < 0.001$

were seeded onto alginate/alginate–sulphate scaffolds with affinity-attached bFGF and EGF, the NPCs survived in culture and had already formed the typical spherical neurospheres at day 4 (Figure 3), which by day 21 spontaneously dissociated into a single-cell population exhibiting a neural (neurogenic and gliogenic) phenotype (Figure 4). The transition from compact neurospheres to single differentiated cells is demonstrated in the day 14 cell constructs (Figure 4A, B). These constructs contain a confluent population of fully differentiated cells of various types, as well as occasional neurospheres. Some of the neurospheres are small and compact (Figure 4B), others are dissociating (Figure 4A) and both are linked throughout a rich network of extending processes with the surrounding cells. By day 21, all neurospheres had disintegrated and single differentiated cells prevailed in the culture, although the presence of a few small oval bright-like cells indicated ongoing differentiation (Figure 4C, D).

The number of neurospheres in the GF-supplemented scaffolds was higher than that found in the 2D culture at all time-points (Figure 5A). The numbers of spheres in the scaffolds on days 4, 7, 14 and 21 in culture were 23.3 ± 8.9, 11.6 ± 4.9, 22.9 ± 9.1 and $8.3 \pm 2.2/mm^2$, respectively ($p < 0.05$ at days 4 and 14), was twice that found under the 2D cultivation mode.

Another indicator for NPCs proliferation in culture is the increase in neurosphere size with time. In the alginate scaffolds with affinity-bound bFGF/EGF, the neurospheres increased in size until a peak diameter of $157.2 \pm 38.8\,\mu m$ on day 7 in culture. Of note, the average size of the neurospheres formed in the scaffolds at all time points was smaller in comparison with the 2D standard cultivation, which exhibited a maximum diameter of $232.3 \pm 69.4\,\mu m$ on day 7 and $221.3 \pm 30.0\,\mu m$ on day 14 (Figure 5B). This is probably due to the confinement conferred by the environment within the scaffold.

The migratory activity of NPCs, as an indication of initial cell differentiation, was evaluated on days 4, 7 and 14 (Figure 5C). The measured distances were normalized using the ratio of the cell migration distance out of the neurosphere and the radius of the corresponding neurosphere, as shown in Figure 3D. On day 4, the migratory activity of NPCs from the newly formed neurospheres was minimal; the average ratio migration distances:original colony radius measured for the affinity-binding hydrogel system was 1.2 ± 0.2, which is shorter than that measured for the 2D cultivation system, 2.2 ± 0.1. On days 7 and 14, the migratory activity in the two cultures was more extensive, with similar average ratios of migration of 2.5 ± 0.7 and 5.8 ± 0.4, respectively (Figure 5C).

3.3. Lineage differentiation of NPCs

NPCs isolated from embryonic brains and 2D-cultured on poly(D-lysine)-coated dishes and in medium supplemented with EGF and bFGF proliferated and formed neurospheres within 4 days in culture. Some of the cells within the

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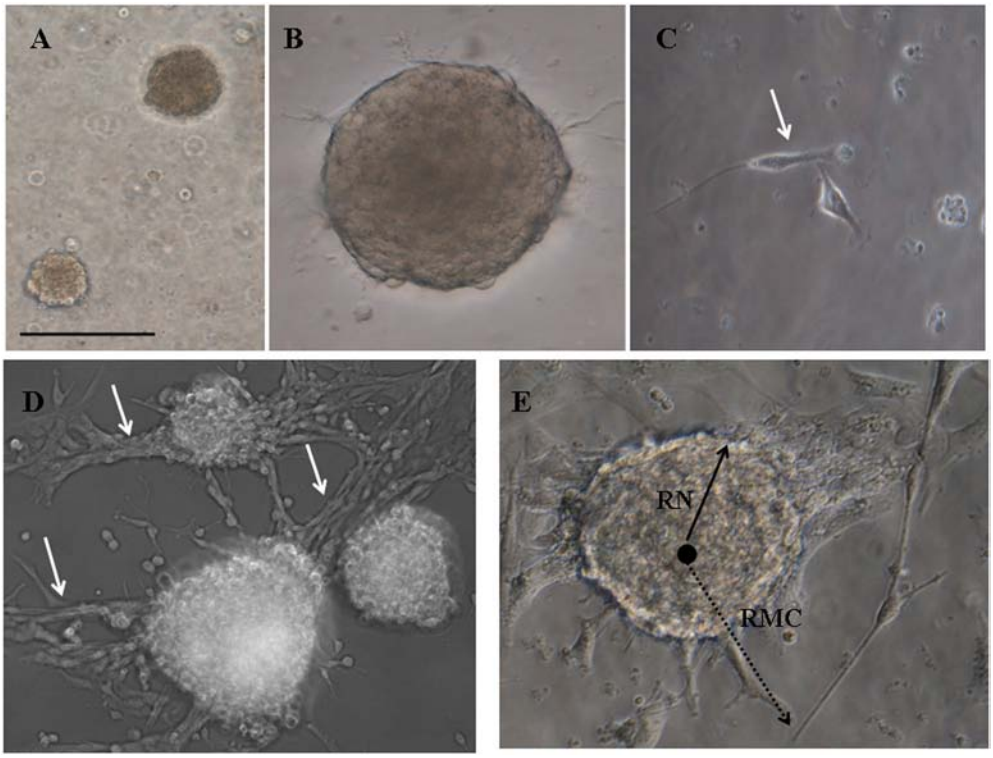

Figure 3. Representative phase-contrast images of neurospheres formed in the bFGF/EGF affinity-binding alginate scaffold (B) group (the amount loaded was 200 ng/scaffold for each factor). Typical small, round-shaped primary neurospheres are noted on days 4 (A) and 7 (B), but not in alginate scaffold lacking growth factors (A group), where only a few scattered cells occurred (C). Initial differentiation is seen in cells migrating (white arrows) out from neurospheres on day 10 (D). (E) Calculation of cell migration distance from a neurosphere, as a ratio of the radius of the farthest migrated cell distance out from the neurosphere (RMC) to the radius of the neurosphere (RN) (RMC:RN). Scale bars (A–D) = 100 μm

neurospheres were already positive for neurogenic as well as gliogenic markers on day 7, as detected by immunostaining. Groups of neurons which stained positive for both MAP2 and NeuN were found in distinct parts of the neurospheres (Figure 6A). Double-label immunofluorescence and confocal microscopy revealed that NeuN outlined the nuclei, while MAP2 was expressed in the cytoskeleton and in extended bipolar processes mostly orientated parallel to cell bodies, indicating an initial state of ramification (Figure 6A, A′). Cells with a glycogenic phenotype, expressing markers such as GFAP and NG2, were also found within the neurospheres (Figure 6D, E, G). Withdrawal of the mitotic factors from the medium led to full differentiation of the cells, yielding postmitotic neurons (MAP2-positive cells 21 ± 5.6% and NF-200-positive cells 18 ± 7.3%; Figure 6B, C, E′′), astrocytes (GFAP-positive 51 ± 6.1%; Figure 6E′, F) and oligodendrocytes (RIP-positive 16 ± 3.8%; Figure 6H, I).

NPCs cultivated in the bFGF/EGF affinity-binding alginate scaffold maintained multipotency, as judged by their differentiation into neurons, astrocytes and oligodendrocytes. Immunocytochemical analyses and quantification of the different cell markers revealed that in general the percentages of the different differentiated cells were less than those measured in the 2D cultures at corresponding time points. Staining for mature neuronal phenotype showed that MAP2-positive neurons (14 ± 7.2% of seeded NPCs) occurred either in residual neurospheres or within the final confluent cell population on day 21 (Figure 7A–C). MAP2-positive cells showed unambiguous neuronal morphology with intensely labelled cell bodies and long thin processes. However, more profound MAP2 staining occurred within residual neurospheres (Figure 7A), rather than in the single-cell population (Figure 7B, C). Staining with NF-200 for the more advanced neuronal maturation phenotype exhibited a substantially lower number of mature neurons (9 ± 5.2% of seeded NPCs) as compared to that measured in the 2D cultures (Figure 7D–F). The NF-200-positive neurons revealed poor branching but with extended long processes.

Glial cell-derived astrocytes (GFAP-positive) were found within and around the scattered residual clusters of neurospheres in the scaffold on day 21 (Figure 8A). The astrocytes comprised the largest cell population differentiating from the scaffold-seeded NPCs (32 ± 5.8%). Distinct astrocytic subtypes appeared as stellate and flat cells with rather short thick projections (Figure 8D) or spindle-shaped with long, thin and foot-like processes (Figure 8E–H). The astrocytes appeared to be relatively similar to those found in the 2D cultivation mode, whereas within the scaffold more fibrous-like astrocytes could be detected.

Terminally differentiated oligodendrocytes represented 6 ± 2.9% of the total differentiating cell population in the

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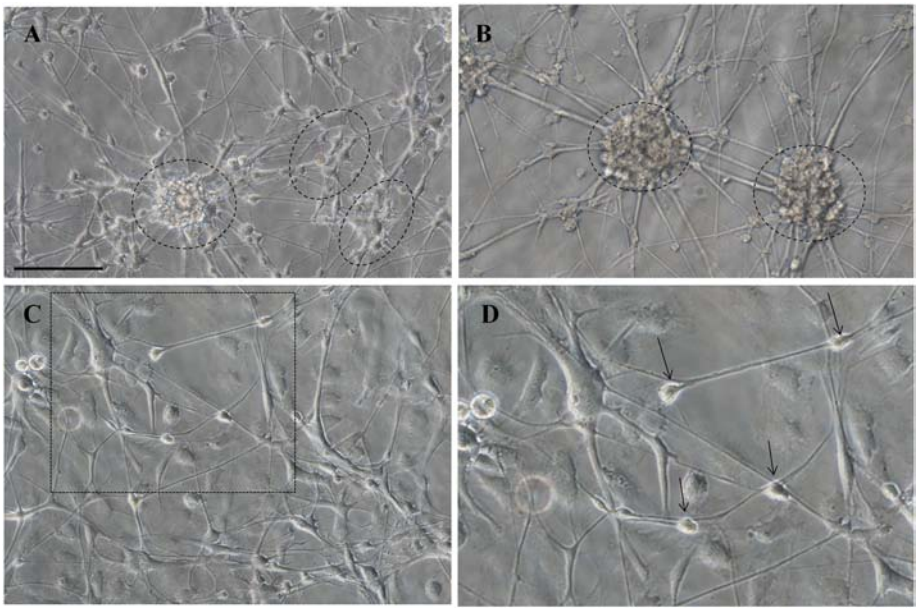

Figure 4. Differentiation of NPCs seeded in the bFGF/EGF affinity-binding alginate scaffold. On day 14 in culture, a few small compact neurospheres (dashed circle) (A, B) or dissociated-like neurospheres (dashed oval) (A), interconnected through a rich network of fully differentiated cells with long processes, are seen. On day 21, the differentiated NPCs form a confluent single-cell population, containing variously shaped and cross-connected cells (C). A few small ovoid phase-bright cells (arrows) with long and thin processes are seen in culture on day 21. (D) Higher magnification of boxed area from (C). Scale bars = (A–C) 100 μm; (D) 50 μm

affinity-binding scaffold (Figure 9). The RIP-positive mature oligodendrocytes had large somata, either with few short processes (Figure 9B) or with excessive branching (Figure 9A–C).

4. Discussion

We have previously shown that heparin-binding proteins can specifically interact with alginate–sulphate, with equilibrium binding constants closely mimicking those observed upon their interaction with heparin; among these proteins were bFGF and EGF (Freeman *et al*., 2008). The present study was designed to investigate the capability of a tailor-made alginate/alginate–sulphate scaffold with affinity-bound bFGF and EGF to: (a) sustain the presentation and release of both GFs; and (b) preserve the viability, appropriate expansion, migration and neural lineage differentiation of the scaffold-seeded NPCs as compared to a standard 2D culture system.

Our results demonstrate that an affinity-binding alginate scaffold with a sustained release of both EGF and bFGF is capable of supporting the viability, extensive proliferation and lineage differentiation of scaffold-seeded NPCs. The affinity binding of the dual GFs to alginate–sulphate/alginate scaffold enables their presentation to the seeded NPCs in a more natural and localized way, while eliminating the need for multiple supplementations of GFs to the external medium during cultivation. Such a scaffold with its affinity-bound GFs could potentially be used as a cell delivery vehicle *in vivo*, having an appropriate microenvironment for promoting the survival of transplanted NPCs and enabling their differentiation *in situ* (Lee *et al*., 2011).

In the present study, the combination of EGF and bFGF was selected due to their known mitotic effect on NPCs and their routine application in standard 2D cultures performed to obtain the desired amount of cells prior to transplantation. We hypothesized that the strong but reversible binding of both factors to alginate–sulphate [K_A (bFGF) = 2.6×10^6/M; K_A (EGF) = 9.9×10^6/M] as previously shown (Freeman *et al*., 2008), would enable their prolonged and sustained local presentation to the scaffold-seeded NPCs, thus eliminating the need for continuous factor supplementation to the external medium during cultivation. To decide upon the GF concentration within the fabricated scaffolds, we considered the bFGF/EGF concentrations used in a routine 2D cultivation mode (about 10–20 ng /ml for each factor, every 3 days) and their respective equilibrium binding constants to alginate–sulphate.

The affinity-binding scaffold loaded with the dual factors bFGF and EGF (200 ng each) exhibited a continuous release of the factor for over 21 days, yielding medium concentrations of 8–22 ng bFGF/ml, and slightly lower concentrations, 8–17 ng /ml, when NPCs were seeded in the scaffold. The lower bFGF concentrations in the external medium of the cellular construct possibly indicates factor binding to the seeded cells and its consumption.

A prolonged release profile from the affinity-binding scaffold was also noted for EGF. Here, greater concentrations of EGF were measured in the external medium, 29–129 ng /ml, compared to bFGF release levels from the same scaffold. The three- to four-fold higher release levels of EGF compared to bFGF can be attributed to the higher initial molar concentration of EGF as compared to bFGF in the affinity-binding scaffold (MW of EGF is 6 kDa and that of bFGF is 20–24 kDa; for a loading

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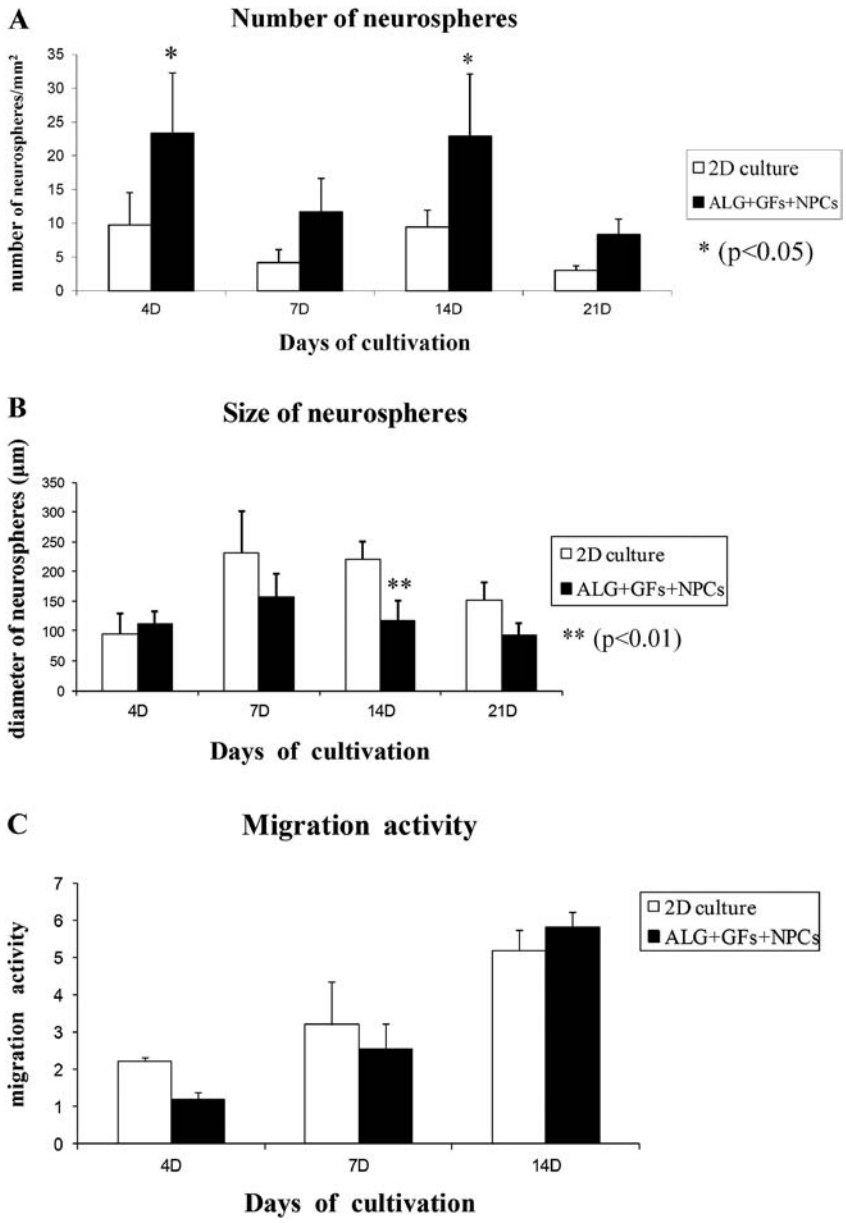

Figure 5. Quantification of the number (A) and size (B) of neurospheres as well as cell migration from neurospheres (C) in the bFGF/EGF affinity-binding alginate scaffold (ALG) compared to 2D cultivation mode. The numbers of neurospheres formed and their diameters in 10 random fields were determined, as well as their migration (see legend to Figure 3D). Error bars represent SD; *$p < 0.05$, **$p < 0.01$

amount of 200 ng/factor, the molar concentration of EGF is 3.5- to four-fold greater than that of bFGF). The presence of NPCs in the affinity-binding scaffold led to a lower concentration of EGF in the medium on day 7, but at later days the factor concentrations released from the acellular and cellular constructs were similar. Cell binding of EGF could account for the early time differences in EGF release pattern. Of note, the difference between EGF release levels from the cellular and acellular constructs was not as prominent as that found for bFGF. This may be due to the different extents of cell receptor expression for the two factors on NPCs. Previously, it was shown that bFGF and fibroblast growth factor receptor 1 (FGFR-1) are expressed during neurogenesis (Weise *et al*., 1993) and that telencephalic progenitor cells express FGFR-1, with only a minority of them expressing EGF or EGF receptor (EGFR) (Maric *et al*., 2003). Thus, the marked difference in EGF release level compared with bFGF from the cellular constructs may well reflect the fact that few of these cells actually express the EGF receptor, whereas most do express the receptor for bFGF.

The release pattern of bFGF/EGF noted from the affinity-binding scaffolds, viz. high factor levels at initial cultivation times and lower levels at later cultivation times, mimics to some extent the frequently used protocol of factor supplementation and withdrawal during NPCs cultivation. According to this protocol, NPCs seeded in 2D cultures are supplemented with the mitogenic factors bFGF/EGF (20 ng/ml of each factor) at initial cultivation times to promote their proliferation, while after approximately 10–14 days of cultivation these factors are withdrawn from the medium to allow NPC complete differentiation into

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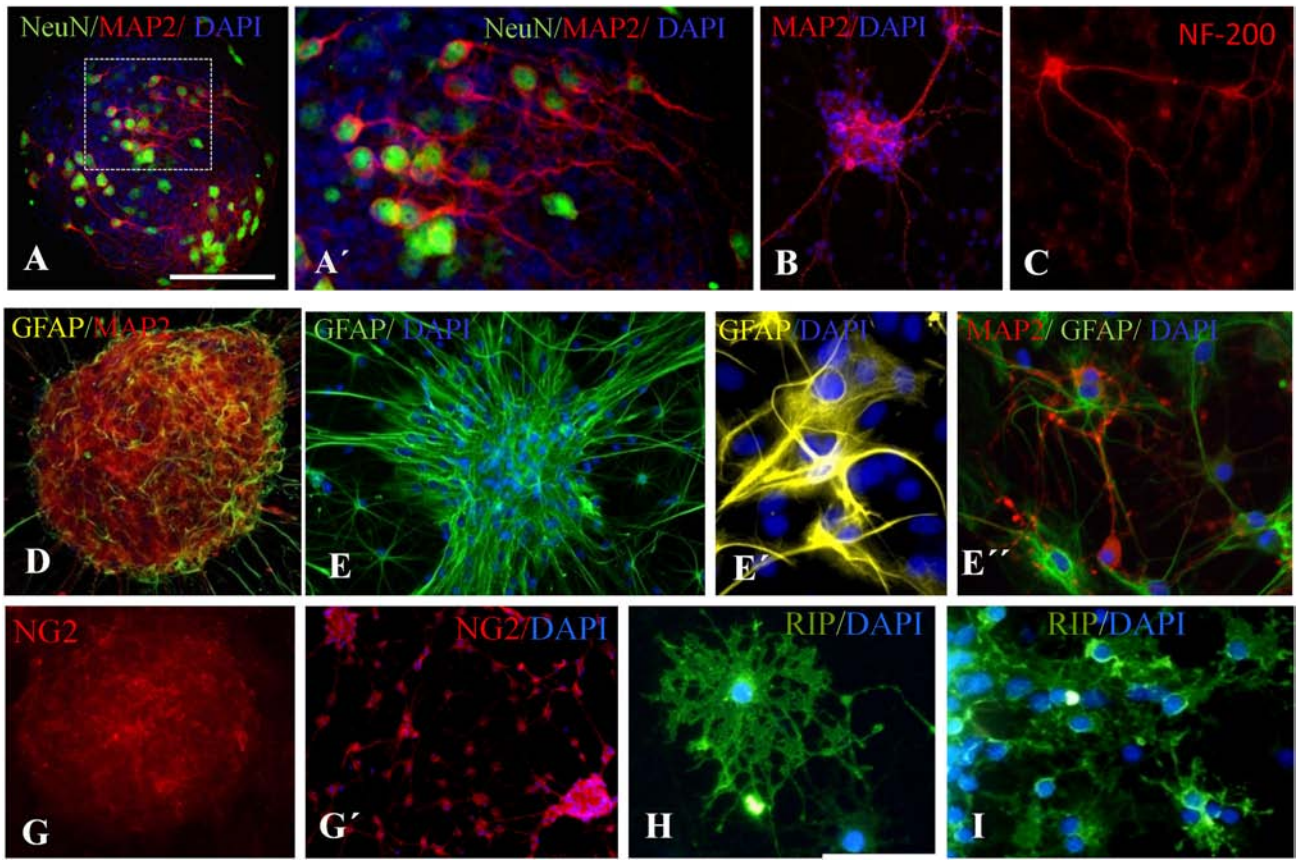

Figure 6. Immunocytochemical analyses of NPCs differentiation in 2D cultivation mode. Occasional clusters of neurons expressing MAP2 (involving cytoskeleton and processes, red) and NeuN (within the nuclei, green) are seen in neurospheres (A, A′). Similarly, compact (D, G) or dissociated (E, G′) neurospheres containing GFAP-positive (D, E) and NG2-positive (G, G′) cells were found on days 7–10. Further cultivation led to a final differentiation of MAP2-positive and NF-200-positive postmitotic neurons (B, C), GFAP-positive astrocytes (E′) and RIP-positive oligodendrocytes (H, I) on day 21. Note, in the double-staining image (E′′), that the MAP2 antibody outlines a few branching neurons (red) and the GFAP antibody labels intermediate filaments of adult astrocytes (green), counterstained with DAPI for nuclei (blue). Images taken with Leica confocal microscope = A, A′, D, E, E′. Scale bars = (A–E, G, G′) 100 μm; (A′, E′, E′′, F, H, I) 50 μm

neurons, astrocytes and oligodendrocytes. In a similar manner, the high concentrations of bFGF/EGF released from the affinity-binding scaffold, at initial cultivation times, enable the extensive proliferation of NPCs. Our results show that the process is more efficient in the affinity-binding scaffold than in 2D cultivation mode, as reflected by the greater numbers of neurospheres formed in the scaffold. This is probably due to the localized effect of the mitogenic factors on the scaffold-seeded NPCs. At later cultivation times, the low concentrations of bFGF/EGF in the scaffold cultivation system are suboptimal for inducing extensive cell proliferation, thus allowing the multilineage differentiation of NPCs to proceed.

Indeed, we found that NPCs in the affinity-binding scaffolds differentiated to neurons, astrocytes and oligodendrocytes. Yet, when quantitatively comparing the percentages of differentiated cells, it appears that at day 21 the profile of cell phenotypes is slightly different between the two cultivation modes. While in the 2D cultivation mode the percentage of mature neuron (NF-200-positive cells) was 18 ± 7.3% of total cells, in the scaffold their percentage was two-fold less (9 ± 5.2%). The percentages of glial cell-derived astrocytes (GFAP-positive) and terminally differentiated oligodendrocytes were also lower in the scaffold. These differences may be explained by: (a) the effect of residual bFGF/EGF in the scaffold, which promotes proliferation and maintenance of the neurosphere for a longer time; and (b) the slower kinetics of cell migration from the neurosphere outside, due to the confinement boundary of the scaffold, which eventually affects the kinetics of NPC differentiation. Such a phenomenon of delayed migration in scaffolds has been also noted by other groups (Shanbhag *et al.*, 2010; Li *et al.*, 2006; Purcell *et al.*, 2009a, 2009b). In future studies, we plan to design scaffolds with a more rapid degradation/erosion rate, to allow better cell migration and to characterize neural progenitors. Thus, there is a need to develop a biomaterial that supplies appropriate amount of growth factors to preserve the undifferentiated population for a sufficient period of time when transplantation experiments are performed (Subramanian *et al.*, 2009).

The affinity-binding alginate scaffold appears to be an appropriate system for the cultivation of NPCs as well as a potential delivery vehicle for cell transplantation into the central lesion of injured spinal cord. The affinity-binding strategy offers technological as well as scientific advantages

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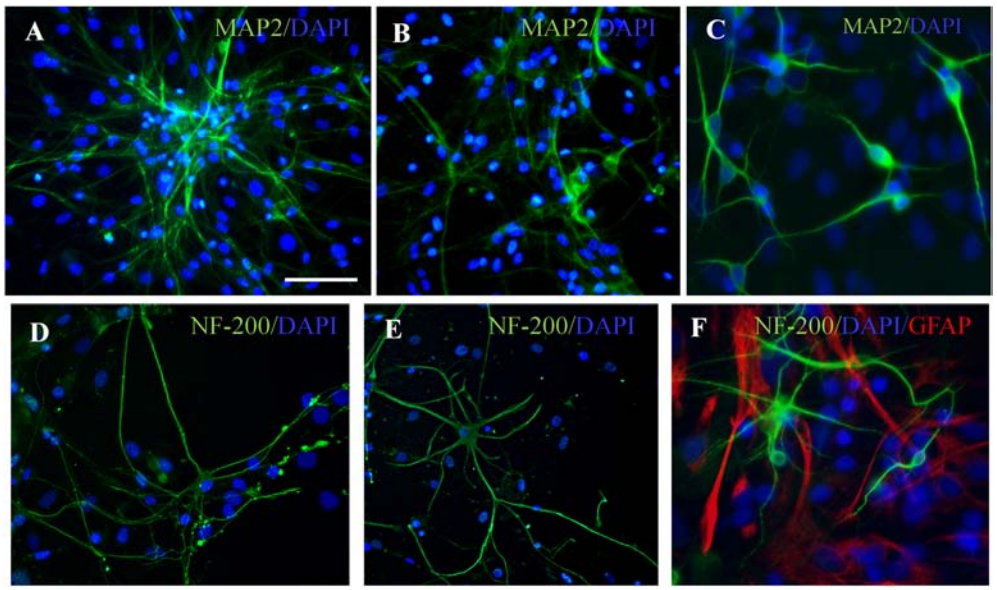

Figure 7. Neuronal differentiation of NPCs in the GF affinity-binding scaffold. MAP2-positive neurons are observed within the partially dissociated neurospheres (A) or throughout the confluent cell population on day 21 (B, C). Note a lower occurrence of more advanced NF-200-positive neurons with fine long interconnected processes (D, E). Double-labelled images with NF-200 (green) and GFAP (red) counterstained with DAPI show a predominance of astrocytes in comparison to neurons (F). Scale bars = (A, B, D, E) 100 μm; (C, F) = 50 μm

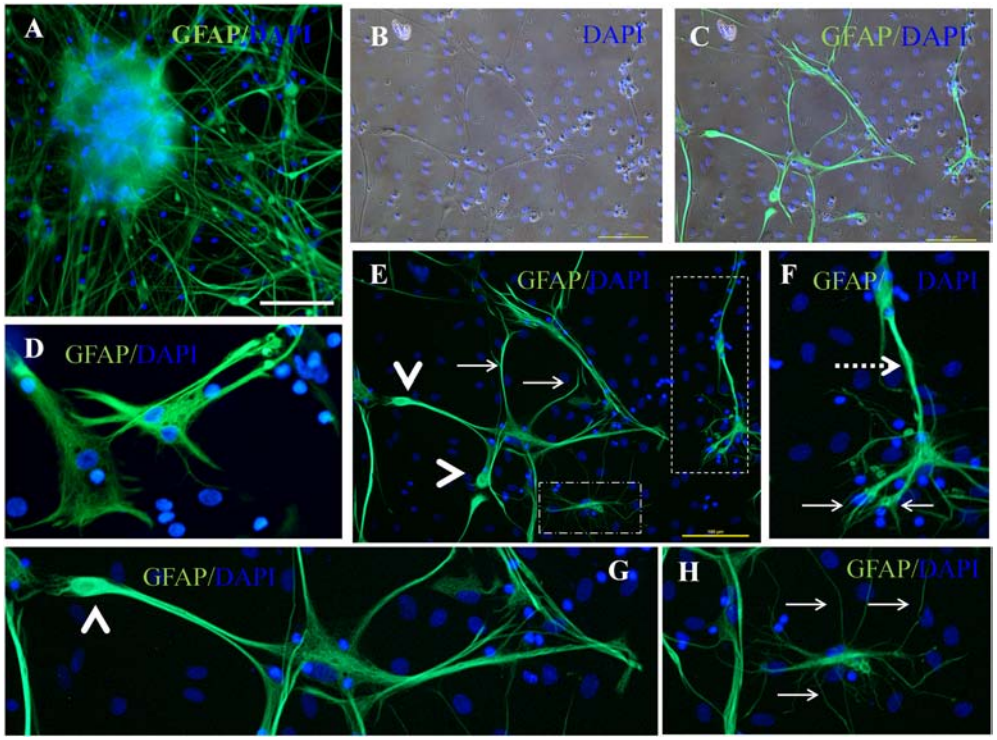

Figure 8. Astrocytic differentiation in the GF affinity-binding scaffold. GFAP-positive neurospheres surrounded by numerous astrocytic figures are found on day 21 (A). A combination of brightfield with DAPI (B) or GFAP and DAPI (C) fluorescence microscopy was used in analysis. Various stellate and flat astrocytes with large bodies and short, thick projections (D), or spindle-shaped astrocytes with long thin (thin arrows, E–H) and foot-like (arrowheads, E, G) processes were seen in the scaffold. Furthermore, a triangular astrocyte having thick apical projection (arrow dotted) and lateral cytoplasmic processes branching into fine collateral fibres (arrows, (F), or a spider-like astrocyte with fine processes coursing in all directions (arrows, H) was visualized at higher magnification, taken from two boxed areas of (E). Scale bars = (A, B, C, E) 100 μm; (D, G, F, H) 50 μm

over other existing approaches to obtain local and sustained delivery of GFs. From a technological point of view, our process of factor incorporation into the scaffold via affinity interactions with alginate–sulphate is simple, scalable and does not require the chemistry of covalent binding of the factor. In addition, multiple growth factors can be

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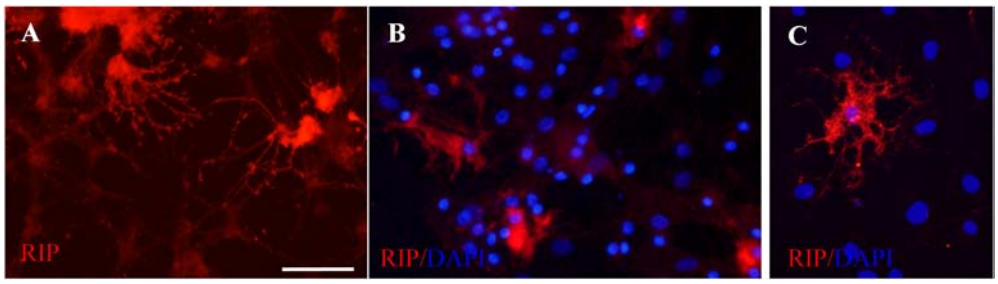

Figure 9. Differentiation of NPCs into oligodendrocytes in the GF affinity-binding scaffold on day 21. RIP-positive mature oligodendrocytes reveal large somata with short processes (B) or with rich branching (A, C). Scale bars = (A, B) 100 μm; (C) 50 μm

incorporated into one device. Furthermore, the possible effects of covalent binding on factor conformation, activity and spatio-temporal presentation manner are irrelevant in our approach.

As a potential cell delivery vehicle, the scaffold described herein can be injected into irregular cavities, adjust itself into the cavity shape and, in the presence of calcium ions, can undergo gelation *in situ*, thus filling the cavity. Such a non-invasive technique for vehicle administration is potentially advantageous, particularly when considering the fragility of the spinal cord site. In future studies, we plan to transplant the affinity-binding alginate scaffold seeded with NPCs and with EGF and bFGF into a rat model of SCI injury, and to test cell retention, lineage differentiation and therapeutic efficacy in terms of functional recovery and nerve tissue repair.

5. Conclusions

We describe herein the features of an affinity-binding alginate scaffold, which enables the capture and sustained presentation of bFGF/EGF to seeded NPCs in a manner mimicking their presentation by the ECM. The prolonged presentation, as confirmed by the ELISA analysis for released GFs into the medium, supported the extensive proliferation of NPCs. At late cultivation time-points, when GFs concentrations are suboptimal for inducing proliferation, the cells underwent differentiation into neurons, astrocytes and terminally differentiated oligodendrocytes. The scaffold whose development is described herein has the potential to serve as a useful cell delivery vehicle in a model of SCI damage, with proven capability to promote cell retention and expansion, while enabling NPC lineage differentiation *in situ*.

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Conflict of interest

The authors have declared that there is no conflict of interest.

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