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The effect of immobilized platelet derived growth factor AA on neural stem/progenitor cell differentiation on cell-adhesive hydrogels

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ABSTRACT

Neural stem/progenitor cells (NSPCs) hold great promise in regenerative medicine; however, controlling their differentiation to a desired phenotype within a defined matrix is challenging. To guide the differentiation of NSPCs, we first created a cell-adhesive matrix of agarose modified with glycine–arginine–glycine–aspartic acid–serine (GRGDS) and then demonstrated the multipotentiality of NSPCs to differentiate to the three primary cell types of the central nervous system on this matrix: neurons, oligodendrocytes and astrocytes. We then examined whether immobilized platelet derived growth factor AA (PDGF-AA) would promote differentiation similarly to the same soluble factor and found similar percentages of NSPCs differentiated to oligodendrocytes as determined by immunohistochemistry (IHC) and quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Interestingly, the gene expression of the differentiated oligodendrocytes was similar for 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) but different for myelin oligodendrocyte glycoprotein (MOG) in the presence of soluble PDGF-AA vs. immobilized PDGF-AA. These results demonstrate for the first time, that it is possible to control the differentiation of NSPCs, and specifically to oligodendrocytes, in cell-adhesive matrices with immobilized PDGF-AA.

1. Introduction

There is great interest in adult stem cells and their resulting progenitors because of their inherent capacity to self-renew and differentiate into multiple cell types, providing promising cell-based therapeutic strategies after tissue loss due to disease or injury [1–5]. For example, neural stem/progenitor cells (NSPCs) can self-renew or differentiate into neurons, astrocytes, and oligodendrocytes and can be transplanted to promote recovery of functions lost due to neurological diseases or disorders of the brain and spinal cord [3–6].

A better understanding of the factors controlling NSPC differentiation is required for their effective and safe application in potential cell-based therapies. To date, most studies have focused on the optimization of culture methods for the maintenance and differentiation of NSPCs, as well as the effect of soluble factors on differentiation [7–10]. Results from these studies have revealed that rodent NSPCs derived from adult brain tissues are expanded *in vitro* in floating colonies called "neurospheres" in the presence of epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and heparin [7–9]. The withdrawal of these mitogens and the addition of certain growth factors and cytokines promote the expansion and differentiation of NSPCs into specific neuronal cell lineages [11,12]. Several *in vitro* differentiation assays have shown that platelet derived growth factor AA (PDGF-AA) is a key signaling molecule that specifically regulates the differentiation of NSPCs to oligodendrocytes [13–15].

NSPC differentiation is influenced by the cellular microenvironment, including the matrix on which the NSPCs are cultured [9]. The chemical, physical and mechanical properties of the matrix have been shown to influence stem cell fate [5]. Incorporating extrinsic factors, such as growth factors, into a matrix, instead of adding these factors exogenously to the growth media, may better mimic the way these factors are presented to cells *in vivo* [16,17]. PDGF-AA has been shown to promote differentiation of NSPCs to oligodendrocytes, which have been shown to limit axonal degeneration after spinal cord injury [15]. Agarose is a naturally derived polysaccharide that has been specifically modified with cell-adhesive peptides, such as arginine–glycine–aspartic acid (RGD), to guide cell adhesion and growth [9,18]. The tripeptide RGD sequence is found in basement membrane proteins, including fibronectin and

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laminin, and is considered to be a ubiquitous cell adhesion peptide. It has been shown to promote cell adhesion to normally non-adhesive materials, such as agarose, via integrin receptors, $\alpha_v\beta_1$, on cell membranes [19]. By integrating both cell adhesive and cell differentiating stimuli into the hydrogel design, it will more closely mimic the stem cell microenvironment, or 'niche', which is required for regenerative medicine strategies.

To immobilize bioactive PDGF-AA to agarose hydrogels, agarose was first modified with 2-nitrobenzyl cysteine which was then photocleaved to yield agarose-SH. Importantly, 2-nitrobenzyl groups are stable in aqueous environments, making this protecting group particularly useful for hydrogel modification [18]. The use of photochemistry is particularly attractive for matrix modification since coupling reactions are fast and biomolecules can be stably immobilized in non-cytotoxic reactions [20,21].

In this study, we compared the NSPC fate in the presence of soluble vs. immobilized PDGF-AA, first in a model system and then on an agarose matrix. Agarose was chemically modified with the glycine–arginine–glycine–aspartic acid–serine (GRGDS) peptide to enhance cell adhesion and then the differentiation to oligodendrocytes was investigated on this matrix in the presence of either soluble or immobilized PDGF-AA. Both IHC using neural cell type-specific antibodies and qRT-PCR based expression profiling of NSPCs for oligodendrogenic, neurogenic and astrogenic markers were used to characterize the differentiated cells.

2. Materials and methods

2.1. Cell isolation and culture

Neural stem/progenitors cells (NSPCs) were isolated from the subependymal region of the lateral ventricles in the forebrain of adult male Wistar rats as previously described [10,22]. Cells were grown in complete medium containing neurobasal media (Gibco-Invitrogen, Burlington, ON, Canada), B27 neural supplement (Gibco-Invitrogen), 2 mM L-glutamine (Sigma–Aldrich, Oakville, ON), 100 μg/ml penicillin–streptomycin (Sigma–Aldrich), 20 ng/ml EGF (recombinant human EGF; Gibco-Invitrogen, Carlsbad, CA), 20 ng/ml bFGF (recombinant human bFGF; Gibco-Invitrogen) and 2 ng/ml heparin (Sigma–Aldrich). Cell numbers and viability were determined with a haemocytometer and the trypan blue exclusion test. Dissociated cells were plated in complete media and incubated at 37 °C with 5% CO_2. Neurospheres were observed within 2–3 weeks, after which cells were passaged weekly. All experiments were conducted using cells at passage 4.

2.2. Synthesis and characterization of the modified PDGF-AA

Recombinant rat PDGF-AA was purchased from R&D Systems (Minneapolis, MN, USA) and dissolved in 4 mM HCl with 10% 1,2-propanediol at a concentration of 400 μg/ml (40 μg/100 μl). The water-soluble carbodiimide, 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 50 μl; Sigma–Aldrich) and *N*-hydroxysulfosuccinimide (sulfo-NHS, 50 μl; Pierce, ON) in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, 1 M NaCl, pH 6 (reaction buffer) were added to the PDGF-AA solution to obtain a final concentration of 11 mM EDC and 12 mM sulfo-NHS. After mixing and reacting the above reagents at room temperature for 15 min, Alexa Fluor 350 hydrazide sodium salt (5 mM) in dimethyl sulfoxide (DMSO) and 4-(4-*N*-maleimidophenyl) butyric acid hydrazide (MPBH, 7 mM) in 0.1 M sodium acetate pH 5.5 (200 μl) were added to the PDGF-AA for 75 min at room temperature. After adding 20 mM HCl (80 μl), the maleimide- and Alexa Fluor-modified PDGF-AA (MI-PDGF-AA-f) was purified with AKTA-FPLC (Amersham Pharmacia, Piscataway, NJ) using Sephadex G-25 (Sigma–Aldrich) column (10 × 200 mm, Amersham Pharmacia) equilibrated in 4 mM HCl pH 2.3, 150 mM NaCl, 5% propanediol. Elution of MI-PDGF-AA-f was monitored at 280 nm by UV–Vis (UPC-900 Amersham Pharmacia) on the FPLC. Fluorescently tagged MI-PDGF-AA-f was confirmed by measuring fluorescence emission at 445 nm on a fluorescence plate reader (345 nm excitation, Molecular Device, Sunnyvale, CA). The concentration of MI-PDGF-AA-f was determined using the PDGF-AA enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN). The yield of MI-PDGF-AA-f was determined to be 30.4 μg (7.6 μg/ml), resulting in 76% recovery. To confirm the conjugation of MPBH to PDGF-AA, the molecular weight of conjugated PDGF-AA-MPBH (without Alexa Fluor) was determined using MALDI-TOF (MDS Sciex API QSTAR XL Pulsar MALDI QTOF, Applied Biosystems, Foster City, CA).

2.3. Bioactivity of the modified PDGF-AA

The bioactivity of MI-PDGF-AA-f was confirmed by comparison to unmodified PDGF-AA in terms of cell differentiation. NSPCs were dissociated into a single cell suspension and seeded onto glass coverslips coated with poly-D-lysine (Sigma–Aldrich) and mouse laminin (Gibco-Invitrogen) in complete medium as described in Section 2.1, at a cell density of 10^4 cells/cm^2. Cells were incubated at 37 °C, 5% CO_2 and allowed to attach overnight. The complete medium was removed the next day, and differentiation medium containing either unmodified soluble PDGF-AA or soluble MI-PDGF-AA-f and neurobasal media, B27 neural supplement, 2 mM L-glutamine, 100 μ/ml penicillin–streptomycin were added and incubated at 37 °C, 5% CO_2. Preliminary studies comparing 20 and 140 ng/ml of PDGF-AA showed no difference, suggesting that the saturation concentration of PDGF-AA is less than 20 ng/ml. Results comparing 140 ng/ml soluble PDGF-AA vs. MI-PDGF-AA-f samples are shown. The medium was changed every 3 d for up to 7 d. NSPCs were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS) for 20–30 min for immunohistochemical studies. Differentiated oligodendrocytes were stained with a monoclonal antibody against mouse- receptor-interacting protein (RIP, 1:5 dilution, in blocking solution; Development Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa City, IA). Immunostaining was carried out as described in Section 2.10.

2.4. Photolabile hydrogel substrate preparation

Photolabile agarose was synthesized by reaction with S-2-nitrobenzyl cysteine (S-NBC) in DMSO as described by Luo and Shoichet [18]. The substitution level of S-NBC in the resulting agarose was determined by measuring its absorbance at 266 nm using a UV spectrophotometer (Ultrospec 4000, Amersham Pharmacia). The S-NBC agarose solution was dissolved at 1 wt% in PBS by heating. After cooling to room temperature, the solution was sterilized by filtration (0.22 μm) before forming hydrogels at 4 °C.

2.5. Immobilization of the modified PDGF-AA to S-NBC agarose hydrogel

Conjugation of MI-PDGF-AA-f to S-NBC agarose hydrogel was investigated using 1 wt% agarose gelled in a 96-well plate. The fluorescent intensity of immobilized PDGF-AA in the hydrogel was calibrated using a set of standard solutions of 0 ng, 76 ng, 228 ng, 380 ng in 120 μl hydrogel, which corresponded to 0, 0.63 μg/ml, 1.89 μg/ml, 3.15 μg/ml of fluorescently-modified PDGF-AA. For covalent immobilization of the MI-PDGF-AA-f, S-NBC agarose hydrogel samples were irradiated for 90 s with a UV lamp (EFO X-Cite XC60000, Santa Clara, CA) to photo-release nitrobenzyl groups, leaving reactive sulfhydryl groups. Then, 50 μl of 7.6 μg/ml MI-PDGF-AA-f in 4 mM HCl, 150 mM NaCl and 5% propanediol (neutralized by 1 M Tris–HCl pH 7.6, 10 μl) was added onto the hydrogel and kept for 4 h at 4 °C in the dark. Controls (for PDGF-AA adsorption) were treated identically without irradiation of S-NBC agarose. Non-covalently bound MI-PDGF-AA-f was removed by successively washing the hydrogels in 0.01 M PBS over 36 h. The amount of MI-PDGF-AA-f covalently coupled to hydrogels was determined by measuring fluorescence intensity as described above.

2.6. Bioactivity of the immobilized PDGF-AA

The bioactivity of the immobilized PDGF-AA was assessed by the percentage of NSPCs that differentiated to oligodendrocytes compared to controls of soluble unmodified PDGF-AA, adsorbed PDGF-AA or no PDGF-AA. Because differentiation was difficult to quantify when cells were cultured on agarose hydrogels, 10^4 NSPCs (passage 4) were cultured as single cells on laminin/poly-D-lysine-coated glass coverslips (1 cm^2) and then covered with an agarose hydrogel (120 μl), that was preformed in a 1 cm^2 round mold equilibrated in the differentiation medium. Fig. 1 summarizes the methodology where NSPCs on laminin/poly-D-lysine glass coverslips were cultured under agarose hydrogels with one of the following: soluble unmodified PDGF-AA, immobilized PDGF-AA, adsorbed PDGF-AA, or no PDGF-AA. The differentiation medium, as described at Section 2.1, was changed every 3 d for up to 7 d. In order to make a fair comparison between soluble and immobilized PDGF-AA, the same amount of PDGF-AA immobilized to agarose hydrogel, as calculated in Section 2.5, was added to each well in the soluble PDGF-AA control (i.e., 210 ng). After 7 d of culture, agarose hydrogels were carefully removed and then NSPCs were fixed in 4% PFA as before and IHC was performed, as described in Sections 2.3 and 2.10.

2.7. Neural stem cell differentiation analysis on GRGDS-agarose hydrogels

In order to culture NSPCs on agarose, it was modified with maleimide-GRGDS (MI-GRGDS) (Ana Spec, San Jose, CA), which reacts by Michael addition with thiol-reactive agarose, (as previously described by Luo and Shoichet [18]), and resulting in 60–70 nmol/ml of immobilized peptide on the agarose hydrogels [18]. NSPCs were dissociated into a single cell suspension and seeded onto GRGDS-agarose hydrogels in complete medium as described in Section 2.1 at a cell density of 2 × 10^4 cells/cm^2, incubated at 37 °C, 5% CO_2 and allowed to attach for 2 h. After removing the complete medium, differentiation medium (see Section 2.3) containing 5% fetal bovine serum (FBS, Gibco-Invitrogen) was added and cells were incubated at 37 °C, 5% CO_2 for 5 d. After gently removing the differentiation media, NSPCs on the hydrogels were fixed in 4% PFA in 100 mM PBS for 2 h to ensure that PFA diffused throughout the hydrogel for IHC analysis, as described in Section 2.10.

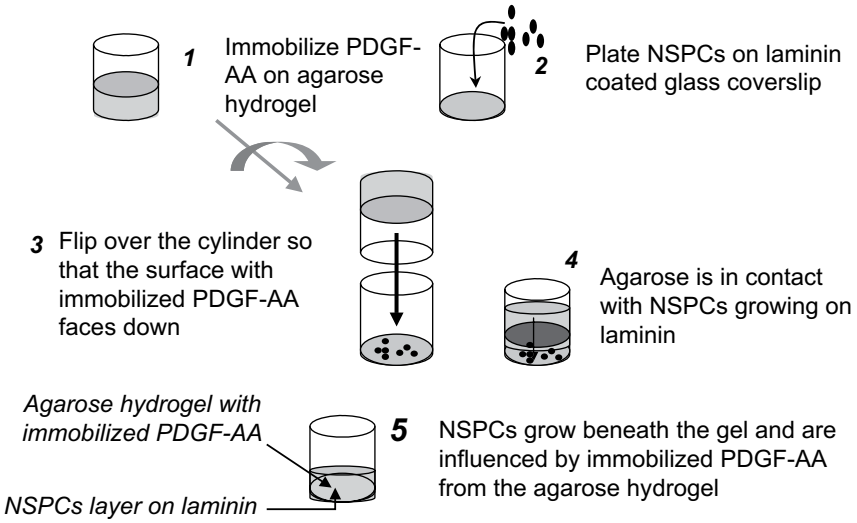

Fig. 1. Experimental method to test the bioactivity of immobilized PDGF-AA.

2.8. Effects of immobilized PDGF-AA on neural stem cell differentiation on GRGDS-agarose hydrogels

NSPC differentiation was studied directly on GRGDS-agarose hydrogels with immobilized PDGF-AA (210 ng) vs. controls with either soluble unmodified PDGF-AA (210 ng) or no PDGF-AA. The hydrogel was formed in a round 1 cm^2 mold and UV-irradiated for 90 s and then modified with 50 μl MI-PDGF-AA-f (7.6 μg/ml) for 2 h as described in Section 2.5, followed by 50 μl of MI-GRGDS (1 mg/ml) in PBS for 2 h. Unreacted MI-PDGF-AA-f and MI-GRGDS were rinsed off by successive washes with PBS as described in Section 2.5. NSPCs were dissociated into a single cell suspension and seeded onto the above hydrogels in the complete medium at a cell density of 2×10^4 cells/cm^2, incubated at 37 °C, 5% CO_2 for 2 h to allow NSPCs to attach. After removing the complete medium, differentiation medium was added and incubated at 37 °C, 5% CO_2 for 5 d. For GRGDS-agarose samples with soluble PDGF-AA, the same amount of unmodified PDGF-AA was added to the differentiation medium as had been immobilized, as calculated in Section 2.5. The differentiation media were carefully removed after 5 d, and the NSPCs on the hydrogels were fixed in 4% PFA for IHC as described in Section 2.10.

2.9. Quantitative real-time RT-PCR for neural stem cell differentiation analysis

The effects of immobilized vs. soluble vs. no PDGF-AA on NSPC differentiation were compared quantitatively by qRT-PCR. Samples, prepared as described in Section 2.8, were lysed after 5 d and their total RNA isolated using the RNAqueous micro kit (Ambion, Austin, TX). Total RNA concentration and purity were measured by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), which allowed standardization by total RNA into the reverse transcription (RT) reaction. Before the RT reaction, RNA was subjected to DNase I treatment. For the RT reaction, 15.7 μl of RNA was incubated with oligo(dT) primers and random hexamers at 65 °C for 5 min. After cooling to room temperature, a 20 μl reaction was incubated with the RNA-oligo(dT)/hexamer mix, buffer, dNTPs, RNase inhibitor, Stratagene AffinityScript RT enzyme (La Jolla, CA) for 60 min at 42 °C. The reaction was terminated by 70 °C for 15 min. After cDNA synthesis, real-time RT-PCR amplification was performed for hypoxanthine phosphoribosy transferase (HPRT) as a housekeeping gene, and 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNPase), myelin oligodendrocyte glycoprotein (MOG), β-III tubulin and glial fibrillary acidic protein (GFAP) using an Applied Biosystems 7900HT. A forward primer, reverse primer, with SYBR Green I (Molecular Probes, Eugene, OR) detection chemistry was used. The 5′ to 3′ sequences for the forward primers and reverse primers were designed from rat mRNA sequences from the National Center for Biotechnology Information (NCBI): for HPRT, the oligonucleotides used were CTCATGGACTGATTATGGACAGGAC (sense primer) and GCAGGTCAGCAAAGAACTTATAGCC (antisense primer); for CNPase CAACAGGATGTGGTGAGGA (sense primer) and CTGTCTTGGGTGTCACAAAG (antisense primer); for MOG CTCATTGCCCTTGTGCCTA (sense primer) and GCACGGAGTT TTCCTCTCAG (antisense primer); for β-III tubulin ACTTTATCTTCGGTCAGAGTG (sense primer) and CTCACGACATCCAGGACTGA (antisense primer); for GFAP GAGA GAGATTCGCACTCAGTA (sense primer) and TGAGGTCTGCAAACTTGGAC (antisense primer). For real-time RT-PCR analysis of each sample, the following were prepared in a 11 μl reaction buffer: 1 μl of DNA sample, buffer, 3–4 mM $MgCl_2$, 0.2 mM DNTPs, 200–400 nM of each forward and reverse primer, 1X SYBR Green I, 1X ROX reference dye, and 0.055 U HotStarTaq (Qiagen, Valencia, CA). The real-time analysis involved a 15 min activation step at 95 °C, followed by 50 cycles of 15 s at 94 °C, 30 s at 60 °C, 60 s at 72 °C and a fluorescence measurement. All qRT-PCR reactions were performed in triplicate. A standard curve (cycle threshold value vs. template concentration) was prepared for each target gene and for the endogenous reference (HPRT) in each sample. The expression for each target gene was compared to controls (no PDGF-AA treatment), and relative fold change was calculated by normalizing against HPRT gene expression according to the relative fold change (ΔΔCt) analysis [23], following the equation:

$$\Delta\Delta Ct = 2\exp\Big[-\Big[\Big(Ct_{(\text{gene of interest})\text{sample}} - Ct_{(\text{gene of interest})\text{control}}\Big) - \Big(Ct_{(\text{HPRT})\text{sample}} - Ct_{(\text{HPRT})\text{control}}\Big)\Big]\Big]$$

2.10. Immunohistochemistry

The following primary antibodies were used for the immunocytochemical studies: monoclonal mouse anti-β-III tubulin (β-III tubulin; 1:1000, Chemicon, Temecula, ON, Canada) for neurons; monoclonal mouse anti-glial fibrillary acidic protein (GFAP; 1:100, Chemicon) for astrocytes; and monoclonal anti-RIP for oligodendrocytes. For all immunohistochemical procedures, appropriate controls were obtained by omission of the relevant primary antibody.

Cells on the substrates were fixed with PBS solution containing 4% PFA for 20 min (on glass) or 1 h (on the hydrogel) at room temperature and then washed with PBS. After cell membrane permeation and blocking by treating with 1.5% BSA and 0.2% Triton X-100 solution at room temperature for 1 h, each specific primary antibody solution as described above, was added for 2 h (on glass) or 5 h (on hydrogel) at room temperature. After washing with PBS, samples were exposed to rabbit Cy3 anti-mouse IgG (1:1000 Jackson Immunoresearch, West Grove, PA) for 1 h (on glass) or 5 h (on hydrogel) at room temperature and then washed with PBS buffer. Finally, cell nuclei were counterstained with 1 μg/ml DAPI for 1 h and washed with PBS buffer.

2.11. Statistical analysis

All statistical analyses were performed using JMP IN 5.1 (SAS Institution Inc., Cary, NC). Differences among groups were assessed by one-way ANOVA with the Tukey's *post hoc* analysis to identify statistical differences among three or more treatments. A student's *t*-test was used to statistically compare unmodified and MI-PDGF-AA-f responses. All data are presented as mean ± SD.

3. Results

3.1. Characterization of modified PDGF-AA

Recombinant rat PDGF-AA contains 14 carboxyl groups – two aspartic acids and 12 glutamic acids – which were activated with EDC/sulfo-NHS chemistry and then modified with the hetero-bifunctional cross-linker, MPBH [24], yielding maleimide-modified PDGF-AA. Using MALDI-TOF mass spectrometry, the peak molar mass of PDGF-AA was measured at 25,200 g/mol. After chemical modification, the peak molar mass of PDGF-AA-MPBH was

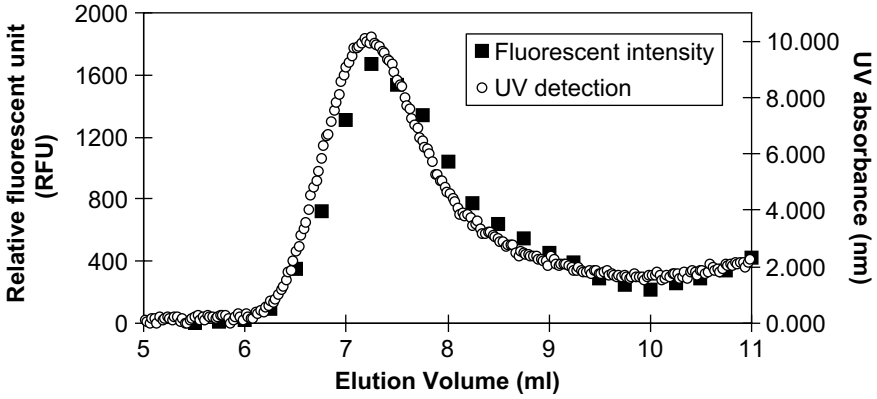

Fig. 2. The overlapping peaks of (■) fluorescent intensity measurement and (○) UV measurement of modified PDGF-AA. The *X*-axis values are the elution volumes from the FPLC. The UV absorbance was measured at 280 nm and the fluorescence emission at 445 nm with 345 nm excitation.

26,251 g/mol, which was estimated at three MPBH groups per PDGF-AA. When PDGF-AA was modified with both Alexa-350 and MPBH (MI-PDGF-AA-f) and eluted on the FPLC, the UV absorbance peak at 280 nm of PDGF-AA overlapped with the fluorescence peak at 345 nm of Alexa-350, confirming the successful reaction of PDGF-AA with Alexa-350, as shown in Fig. 2.

In order to compare the bioactivity of soluble MI-PDGF-AA-f to unmodified soluble PDGF-AA, each was added at 140 ng/ml to NSPC cultures and evaluated in terms of the NSPC differentiation to oligodendrocytes. After 7 d of culture, $58 \pm 8.8\%$ and $61 \pm 4.7\%$ of NSPCs differentiated into RIP-positive cells in the presence of unmodified PDGF-AA and MI-PDGF-AA-f, respectively ($n = 3$). These RIP-positive cells exhibited oligodendrocytic morphologies having slender processes radiating out from their cell bodies. These data demonstrate that MI-PDGF-AA-f remained bioactive.

3.2. Conjugation of modified PDGF-AA to agarose hydrogel matrices

Having demonstrated the bioactivity of soluble MI-PDGF-AA-f, we next focused on testing its bioactivity after immobilization. Agarose was modified with S-NBC which yielded agarose-thiol after exposure to UV light, as previously described [18]. MI-PDGF-AA-f was added to agarose-thiol where a Michael-type addition occurred between the agarose-thiol and PDGF-AA maleimide, yielding agarose-PDGF-AA-f. After thorough washing and by comparison to a standard curve of fluorescence intensity, 210 ± 8 ng of MI-PDGF-AA-f was immobilized to agarose-SH. When MI-PDGF-AA-f was added to agarose-S-NBC (without deprotection), 30 ± 10 ng of physically adsorbed MI-PDGF-AA-f was detected. Thus approximately 86% of MI-PDGF-AA-f was covalently bound to agarose and ~14% was physically adsorbed. Since the PDGF-AA could not be completely removed from the agarose gel, despite thorough washing for several days, it is likely that the PDGF-AA was stably adsorbed and unlikely to diffuse from the gel.

3.3. Bioactivity of covalently immobilized PDGF-AA

To test the ability of immobilized PDGF-AA to promote differentiation of NSPCs to RIP-positive cells, agarose hydrogels with 210 ng of immobilized PDGF-AA-f (as was quantified in Section 3.2) were compared to: agarose hydrogels with 210 ng of soluble unmodified PDGF-AA; agarose hydrogels with 30 ng of adsorbed MI-PDGF-AA-f (to account for the concentration of adsorbed PDGF-AA that could not be removed after thorough washing, as quantified in Section 3.2); and agarose hydrogels with no PDGF-AA. Since practically it was difficult to quantify differentiation of cells cultured on agarose, NSPCs were cultured on laminin-coated glass coverslips which were then covered with agarose hydrogels (see Fig. 1). The percentage of RIP-positive cells was compared between the different groups as shown in Fig. 3. Agarose-immobilized PDGF-AA showed a similar percentage of RIP-positive cells ($57 \pm 1.5\%$) to soluble unmodified PDGF-AA controls ($59 \pm 3.8\%$), which was not significantly different ($p > 0.05$, $n = 3$). Thus, chemically immobilized PDGF-AA maintained bioactivity and resulted in a similar percentage of RIP-positive cells as soluble unmodified PDGF-AA. NSPCs cultured in the presence of physically adsorbed MI-PDGF-AA-f agarose showed a similar differentiation profile to RIP-positive cells ($27 \pm 0.7\%$) as control cells cultured in the absence of PDGF-AA ($28 \pm 2.3\%$). Thus, physically adsorbed PDGF-AA did not stimulate NSPC differentiation above that of controls. Interestingly, different oligodendrocyte morphologies were observed for NSPCs cultured in the presence of immobilized PDGF-AA vs. soluble unmodified PDGF-AA (Fig. 4), which is discussed more fully below.

3.4. Neural stem cell differentiation analysis on GRGDS-agarose hydrogels

With the goal of culturing NSPCs directly on agarose hydrogel matrices, agarose was modified with the ubiquitous cell-adhesive

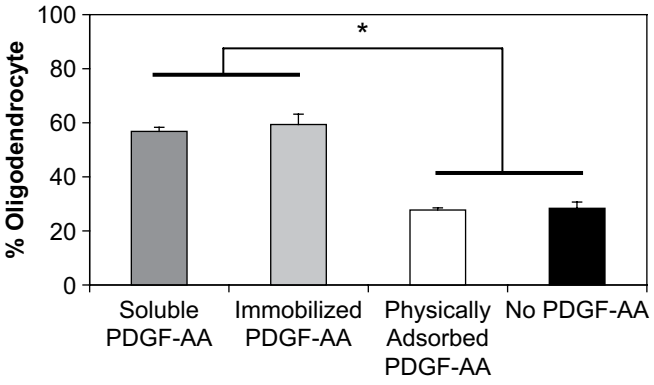

Fig. 3. Comparison of % RIP-positive cells among soluble unmodified PDGF-AA, immobilized PDGF-AA, physically adsorbed PDGF-AA, and no PDGF-AA treatments. (An asterisk (*) shows the statistical significance measured by ANOVA, $p < 0.0001$). All sections were examined and photographed using a BX 61-microscope (Olympus) using a 20× objective. Quantitative immunohistochemistry was performed by determining differentiation within a population of at least 50 cells in four randomly selected fields. DAPI was used to measure total cell numbers. Each determination was performed in triplicate ($n = 3$), and each experiment was repeated at least three times. The total cell number between soluble PDGF-AA vs. immobilized PDGF-AA was not significantly different.

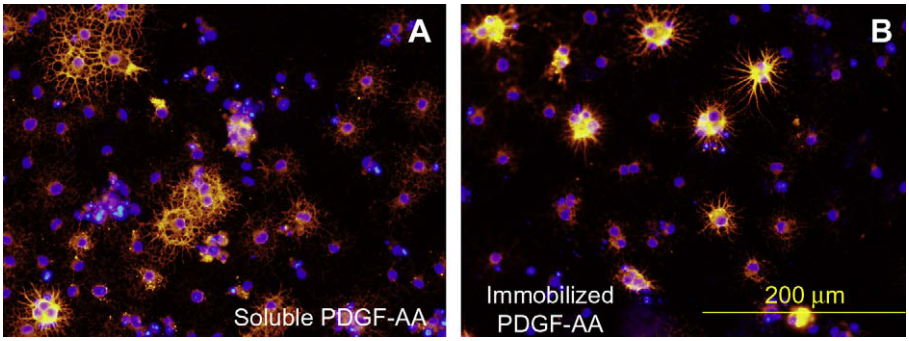

Fig. 4. Comparison of the morphology of oligodendrocytes between (A) soluble unmodified PDGF-AA and (B) immobilized PDGF-AA as assessed by immunohistochemistry (blue, DAPI and red, RIP). After 7 d, the cells on PDL/laminin-coated glass coverslips under the agarose hydrogel with immobilized PDGF-AA demonstrate a different morphology compared with the cells under the agarose hydrogel with soluble PDGF-AA.

ligand, GRGDS (as previously described [18], binding agarose-thiol with maleimide-GRGDS). NSPCs were cultured on agarose-GRGDS and tested for multipotentiality in the presence of 5% FBS after 5 d in culture. Cells were immunostained with β-III tubulin (a marker for neurons), GFAP (for astrocytes) and RIP (for oligodendrocytes). These data, shown in Fig. 5, demonstrate the multipotentiality of these NSPCs cultured on agarose-GRGDS where cells immunostained positively for β-III tubulin, GFAP and RIP. Interestingly, when NSPCs were cultured on agarose hydrogels that lacked immobilized GRGDS, the NSPCs survived yet formed large aggregates in suspension, confirming the importance of GRGDS-modified agarose for cell adhesion and subsequent differentiation.

3.5. Neural stem/progenitor cell differentiation on GRGDS-agarose hydrogel with immobilized PDGF-AA

The differentiation of NSPCs was investigated on agarose hydrogels with either immobilized or soluble unmodified PDGF-AA by IHC and real-time RT-PCR. NSPCs that were plated on GRGDS-agarose hydrogels with immobilized PDGF-AA preferentially differentiated to RIP-positive cells with few β-III tubulin positive cells and no GFAP-positive cells (Fig. 6A–C). A similar differentiation profile was observed for NSPCs cultured on GRGDS-agarose hydrogel in the presence of soluble unmodified PDGF-AA (Fig. 6D–F) where NSPCs preferentially differentiated to RIP-positive cells. To determine the level of gene expression in the differentiated cells, the following were quantified using qRT-PCR: oligodendrogenic specific mRNA markers, CNPase [25] and MOG; the neuronal marker, β-III tubulin; and the astrogenic marker, GFAP. To facilitate comparison between soluble and immobilized PDGF-AA, data were normalized relative to the no PDGF-AA controls. GFAP expression was not detected in any of the samples (data not shown). NSPCs cultured on GRGDS-agarose with either immobilized or soluble unmodified PDGF-AA showed a similar 2.5-fold increase in CNPase mRNA levels compared to controls having no PDGF-AA (Fig. 7A). NSPCs cultured on GRGDS-agarose in the presence of both soluble and immobilized PDGF-AA did not express significantly different amounts of β-III tubulin mRNA

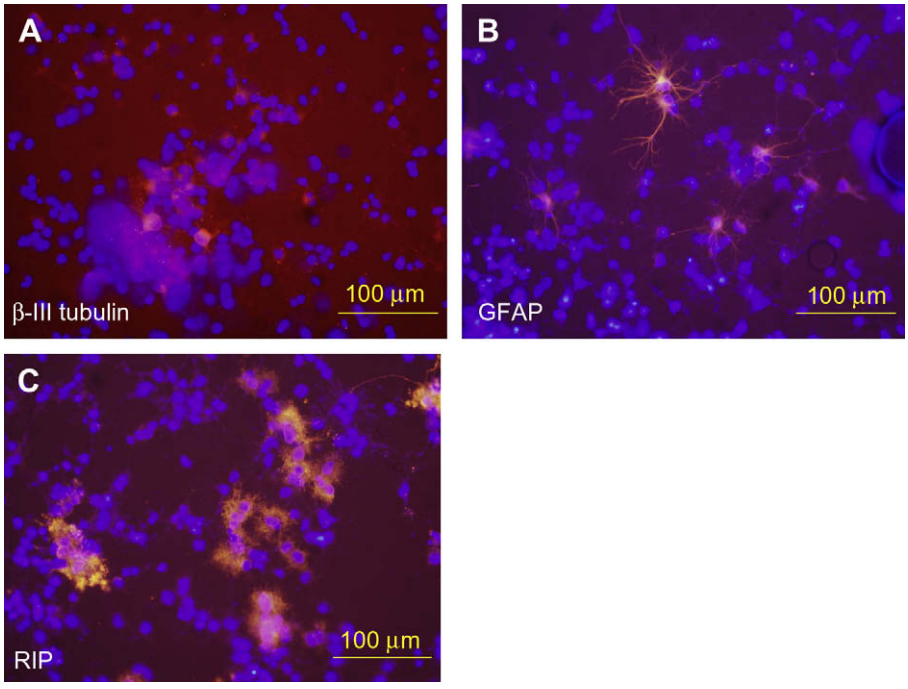

Fig. 5. Expression of various markers of neural cell differentiation on GRGDS-agarose hydrogels as assessed by immunocytochemistry. By 5 d, the cells showed morphology consistent with and expressed antigenic markers of (A) neurons (blue, DAPI and red β-III tubulin), (B) astrocytes (blue, DAPI and red, GFAP), and (C) oligodendrocytes (blue, DAPI and red, RIP).

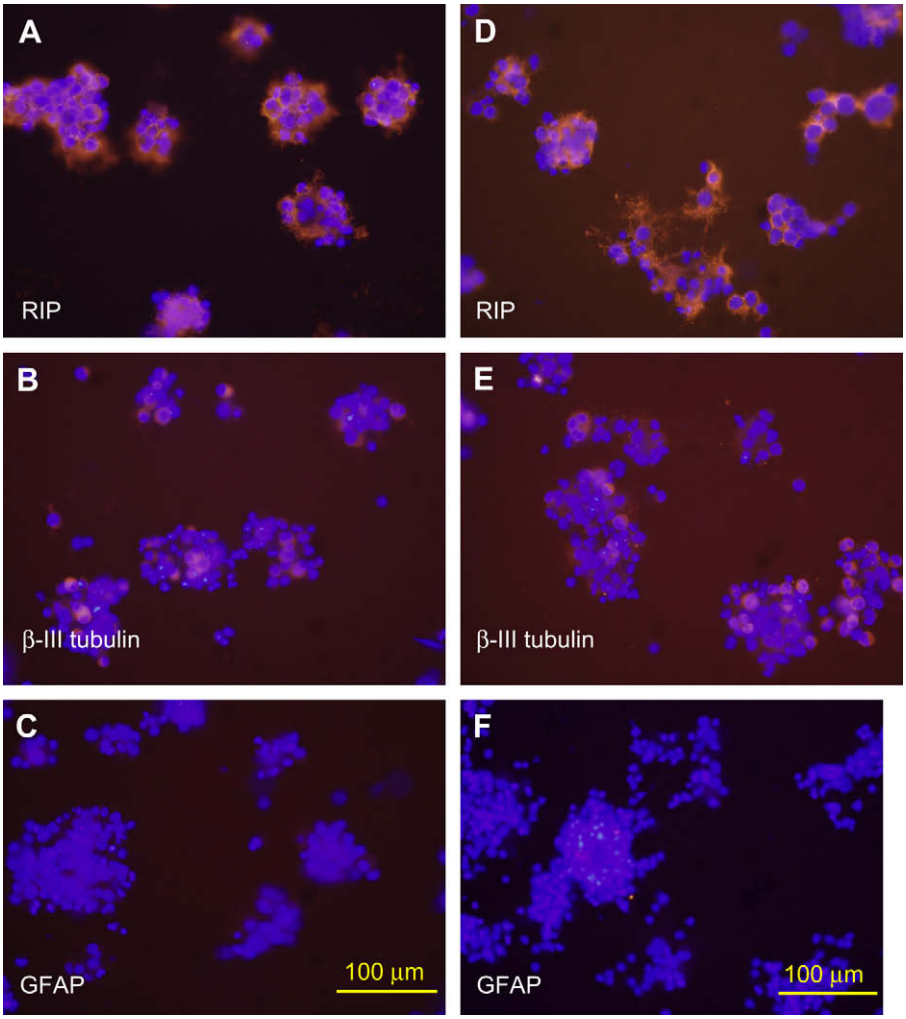

Fig. 6. Expression of various markers of neural cell differentiation as assessed by immunohistochemistry. By 5 d, the cells on GRGDS-agarose hydrogel with immobilized PDGF-AA exhibited morphology consistent with and expressed antigenic markers of (A) oligodendrocytes (blue, DAPI and red, RIP). (B) In contrast, there were few β-III tubulin-expressing cells (blue, DAPI and red, β-III tubulin) and (C) no GFAP expressing cells were detected (blue, DAPI and no GFAP-positive cells). A similar differentiation profile was observed in the presence of soluble unmodified PDGF-AA: (D) RIP, (E) β-III tubulin, (F) GFAP.

relative to controls having no PDGF-AA (Fig. 7B). Interestingly, the MOG mRNA level of NSPCs cultured in the presence of soluble unmodified PDGF-AA was greater (although not significantly) than that of NSPCs cultured on immobilized PDGF-AA and significantly greater ($p < 0.05$) than controls without PDGF-AA (Fig. 7C). Taken together, the mRNA and immunohistochemical markers demonstrate that immobilized PDGF-AA on GRGDS-agarose hydrogel promotes oligodendrogenic differentiation, notwithstanding some differences, relative to soluble unmodified PDGF-AA, in terms of morphology and MOG-expression.

4. Discussion

Many protein conjugation chemistries have been described in the literature [26]. One of the most common methods is activation on the protein carboxylic acid groups with EDC/NHS followed by reaction with amine groups to form stable amide bonds [27]. However, such conjugation lacks selectivity and may alter protein function. As a result, more chemically selective approaches have been developed and include the reaction of protein sulfhydryl groups (such as free cysteines) with acryloyl or maleimide functionalized materials [28]. Since PDGF-AA has no free sulfhydryl groups and the primary amine groups at residues 157–162 (valine–arginine–lysine–lysine–phenylalanine–lysine) are involved in activating receptor homo-dimerization with PDGF-R α/α [29], the carboxylic acid groups in PDGF-AA were activated with EDC/sulfo-NHS chemistry to introduce MPBH. The maleimide functionalized PDGF-AA was immobilized to sulfhydryl functional groups on agarose hydrogels by Michael-type addition. Importantly, MPBH is stable under the acidic (pH 2–3) conditions that are optimal for PDGF-AA stability, unlike more common cross-linkers, such as polyethylene glycol (PEG) which are not stable at pH 2–3 [30]. MALDI-TOF indicated that PDGF-AA was successfully modified with MPBH and the immobilization chemistry confirmed the presence of maleimide (relative to controls). Since soluble MI-PDGF-AA-f triggered NSPC differentiation into oligodendrocytes similar to that of unmodified PDGF-AA, the fluorophores and maleimide attached to PDGF-AA did not disrupt its binding to receptors.

Immobilized agarose-PDGF-AA induced differentiation of NSPCs to oligodendrocytes which was similar to results obtained with soluble unmodified PDGF-AA at the same concentration. While a high amount of PDGF-AA was immobilized, it is significant that similar concentrations of immobilized and soluble PDGF-AA elicited a similar differentiation pattern. Importantly, 30 ng of physically adsorbed PDGF-AA to agarose hydrogels did not lead to preferential differentiation over controls with no PDGF-AA added. Thus the differentiation observed from immobilized PDGF-AA was not affected by PDGF-AA that may have desorbed from the matrix

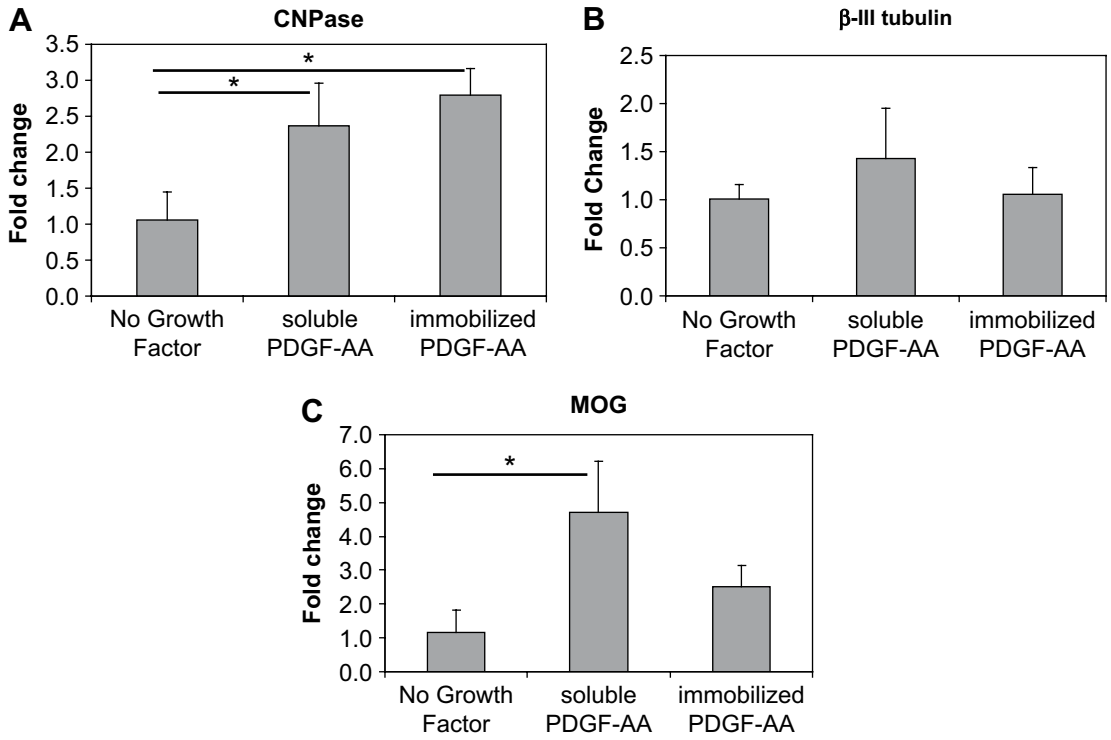

Fig. 7. Quantitative analysis of expression of neural cell fate determinants in NSPCs. NSPCs were plated on GRGDS-agarose hydrogel with immobilized PDGF-AA, soluble unmodified PDGF-AA, or no PDGF-AA for 5 d. mRNA was prepared and quantitative reverse transcription-polymerase chain reaction was performed. Data are expressed as relative mRNA concentration (ΔΔCt) with respect to the control (no PDGF-AA treatment) for (A) CNPase, (B) β-III tubulin, and (C) MOG. (An asterisk (*) shows the statistical significance measured by ANOVA, CNPase $p < 0.0004$, MOG $p < 0.05$, mean ± SD for triplicate samples).

during cell culture. Adsorbed growth factors may be denatured and/or inaccessible by cellular receptors. Unlike the similar cell differentiation profile observed in this study between soluble and immobilized factors, high concentrations of immobilized growth factors have often been required to elicit cellular responses. For example, when nerve growth factor (NGF) was immobilized in poly(2-hydroxyethyl methacrylate) (PHEMA) hydrogels, a threefold increased concentration gradient of NGF was required to guide primary dorsal root ganglia neurites compared with soluble NGF gradients [31,32].

Intriguingly, the morphology of the RIP-positive oligodendrocytes was different when exposed to soluble vs. immobilized PDGF-AA (Fig. 4). The inability of the NSPCs to endocytose the immobilized PDGF-AA may explain the different oligodendrocyte morphologies observed between immobilized and soluble PDGF-AA and possibly the maturation of the oligodendrocytes.

The multipotentiality of NSPCs cultured on GRGDS-agarose hydrogels was demonstrated by the cells expressing markers of β-III tubulin (neurons), GFAP (astrocytes), and RIP (oligodendrocytes) in medium containing 5% FBS (Fig. 5). This result laid the foundation to probe whether immobilized PDGF-AA (and soluble unmodified PDGF-AA) would preferentially promote differentiation to oligodendrocytes on GRGDS-agarose as had been observed on laminin-coated glass coverslips. Our results demonstrate that growth factors immobilized in matrices play a major role in regulating the differentiation of NSPCs. Immobilized PDGF-AA on GRGDS-agarose hydrogels specifically directed the differentiation of NSPCs primarily to oligodendrocytes.

NSPCs exposed to soluble PDGF-AA expressed one of the more mature oligodendrogenic genes, MOG, than NSPCs exposed to immobilized PDGF-AA (Fig. 7C). The inability of the NSPCs to endocytose PDGF-AA when it was immobilized may explain the different gene expression. While we have shown that endocytosis of PDGF-AA is not required to preferentially differentiate NSPCs to oligodendrocytes, the endocytic process may be necessary to promote oligodendrocyte maturity.

To our knowledge, this is the first time that PDGF-AA has been immobilized and shown to both retain its bioactivity and influence NSPC differentiation to oligodendrocytes. Since PDGF-AA is also known to be a major mitogen for fibroblasts [33], smooth muscle cells [34], and other glial cells [35], our process of modification and immobilization may be useful for regenerative strategies involving these cells as well.

5. Conclusions

Adult NSPCs hold great promise for regenerative medicine strategies of the central nervous system. Using maleimide-modified PDGF-AA and photocleaved agarose-thiols, we demonstrated that MI-PDGF-AA-f retains its bioactivity when immobilized to agarose hydrogels. Moreover, cell-adhesive agarose, GRGDS-agarose, supported NSPC adhesion and preferential differentiation to oligodendrocytes in the presence of both immobilized and soluble PDGF-AA. The percentage of differentiated oligodendrocytes was neither affected by PDGF-AA modification nor immobilization. This study is the first to demonstrate that differentiation of NSPCs to oligodendrocytes in the presence of immobilized PDGF-AA is possible and demonstrates the potential of immobilized PDGF-AA for spatial control of differentiation within 3D matrices for use in regenerative medicine.

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References

[1] Gage FH. Mammalian neural stem cells. Science 2000;287:1433–8.
[2] Gobbel GT, Choi SJ, Beier S, Niranjan A. Long-term cultivation of multipotential neural stem cells from adult rat subependyma. Brain Res 2003;980:221–32.
[3] Rivera FJ, Couillard-Despres S, Pedre X, Ploetz S, Caioni M, Lois C, et al. Mesenchymal stem cells instruct oligodendrogenic fate decision on adult neural stem cells. Stem Cells 2006;24:2209–19.
[4] Li L, Xie T. Stem cell niche: structure and function. Annu Rev Cell Dev Biol 2005;21:605–31.
[5] Mimeault M, Batra SK. Concise review: recent advances on the significance of stem cells in tissue regeneration and cancer therapies. Stem Cells 2006;24:2319–45.
[6] Wang JH, Hung CH, Young TH. Proliferation and differentiation of neural stem cells on lysine–alanine sequential polymer substrates. Biomaterials 2006;27:3441–50.
[7] Temple S. The development of neural stem cells. Nature 2001;414:112–7.
[8] Armstrong RJ, Svendsen CN. Neural stem cells: from cell biology to cell replacement. Cell Transplant 2000;9:139–52.
[9] Nakajima M, Ishimuro T, Kato K, Ko IK, Hirata I, Arima Y, et al. Combinatorial protein display for the cell-based screening of biomaterials that direct neural stem cell differentiation. Biomaterials 2007;28:1048–60.
[10] Zahir T, Nomura H, Guo XD, Kim H, Tator CH, Morshead CM, et al. Bioengineering neural stem/progenitor cell-coated tubes for spinal cord injury repair. Cell Transplant 2007;17:245–54.
[11] Recknor JB, Sakaguchi DS, Mallapragada SK. Directed growth and selective differentiation of neural progenitor cells on micropatterned polymer substrates. Biomaterials 2006;27:4098–108.
[12] Nakaji-Hirabayashi T, Kato K, Arima Y, Iwata H. Oriented immobilization of epidermal growth factor onto culture substrates for the selective expansion of neural stem cells. Biomaterials 2007;28:3517–29.
[13] Hu JG, Fu SL, Wang YX, Li Y, Jiang XY, Wang XF, et al. Platelet-derived growth factor-AA mediates oligodendrocyte lineage differentiation through activation of extracellular signal-regulated kinase signaling pathway. Neuroscience 2008;151:138–47.
[14] Jackson EL, Garcia-Verdugo JM, Gil-Perotin S, Roy M, Quinones-Hinojosa A, VandenBerg S, et al. PDGFR alpha-positive B cells are neural stem cells in the adult SVZ that form glioma-like growths in response to increased PDGF signaling. Neuron 2006;51:187–99.
[15] Baumann N, Pham-Dinh D. Biology of oligodendrocyte and myelin in the mammalian central nervous system. Physiol Rev 2001;81:871–927.
[16] Sakiyama-Elbert SE, Hubbell JA. Functional biomaterials: design of novel biomaterials. Annu Rev Mater Res 2001;31:183–201.
[17] Park KI, Teng YD, Snyder EY. The injured brain interacts reciprocally with neural stem cells supported by scaffolds to reconstitute lost tissue. Nat Biotechnol 2002;20:1111–7.
[18] Luo Y, Shoichet MS. A photolabile hydrogel for guided three-dimensional cell growth and migration. Nat Mater 2004;3:249–53.
[19] Petrie TA, Capadona JR, Reyes CD, Garcia AJ. Integrin specificity and enhanced cellular activities associated with surfaces presenting a recombinant fibronectin fragment compared to RGD supports. Biomaterials 2006;27:5459–70.
[20] Wosnick JH, Shoichet MS. Three-dimensional chemical patterning of transparent hydrogels. Chem Mater 2008;20:55–60.
[21] Wieboldt R, Gee KR, Niu L, Ramesh D, Carpenter BK, Hess GP. Photolabile precursors of glutamate: synthesis, photochemical properties, and activation of glutamate receptors on a microsecond time scale. Proc Natl Acad Sci U S A 1994;91:8752–6.
[22] Morshead CM, Reynolds BA, Craig CG, McBurney MW, Staines WA, Morassutti D, et al. Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. Neuron 1994;13:1071–82.
[23] Pfaffl M. Relative quantification. In: Dorak MT, editor. Real-time PCR. New York: Taylor & Francis; 2006. p. 66–8.
[24] Chamow SM, Kogan TP, Peers DH, Hastings RC, Byrn RA, Ashkenazi A. Conjugation of soluble CD4 without loss of biological activity via a novel carbohydrate-directed cross-linking reagent. J Biol Chem 1992; 267:15916–22.
[25] Watanabe M, Sakurai Y, Ichinose T, Aikawa Y, Kotani M, Itoh K. Monoclonal antibody Rip specifically recognizes 2′3′-cyclic nucleotide 3′-phosphodiesterase in oligodendrocytes. J Neurosci Res 2006;84:525–33.
[26] Blawas AS, Reichert WM. Protein patterning. Biomaterials 1998;19:595–609.
[27] Rowley JA, Madlambayan G, Mooney DJ. Alginate hydrogels as synthetic extracellular matrix materials. Biomaterials 1999;20:45–53.
[28] Lutolf MP, Lauer-Fields JL, Schmoekel HG, Metters AT, Weber FE, Fields GB, et al. Synthetic matrix metalloproteinase-sensitive hydrogels for the conduction of tissue regeneration: engineering cell-invasion characteristics. Proc Natl Acad Sci U S A 2003;100:5413–8.
[29] Szabo P, Weksler D, Whittington E, Weksler BB. The age-dependent proliferation of rat aortic smooth muscle cells is independent of differential splicing of PDGF A-chain mRNA. Mech Ageing Dev 1993;67:79–89.
[30] DeLong SA, Moon JJ, West JL. Covalently immobilized gradients of bFGF on hydrogel scaffolds for directed cell migration. Biomaterials 2005;26: 3227–34.
[31] Cao X, Shoichet MS. Defining the concentration gradient of nerve growth factor for guided neurite outgrowth. Neuroscience 2001;103:831–40.
[32] Moore K, MacSween M, Shoichet M. Immobilized concentration gradients of neurotrophic factors guide neurite outgrowth of primary neurons in macroporous scaffolds. Tissue Eng 2006;12:267–78.
[33] Dereka XE, Markopoulou CE, Vrotsos IA. Role of growth factors on periodontal repair. Growth Factors 2006;24:260–7.
[34] Raines EW. PDGF and cardiovascular disease. Cytokine Growth Factor Rev 2004;15:237–54.
[35] Valenzuela CF, Kazlauskas A, Weiner JL. Roles of platelet-derived growth factor in the developing and mature nervous systems. Brain Res Brain Res Rev 1997;24:77–89.