Porous chitosan scaffold and NGF promote neuronal differentiation of neural stem cells *in vitro*

Xin Yi, Guohua Jin, Meiling Tian, Weifeng Mao, Jianbing Qin

Department of Anatomy and Neurobiology, The Key Laboratory of Neuroregeneration of Jiangsu Province, Nantong University, Nantong, China

Correspondence to: Guohua Jin
Department of Anatomy and Neurobiology,
The Key Laboratory of Neuroregeneration of Jiangsu Province, Nantong University
19 Qixiu Road, Nantong 226001, China.
TEL: +86 513 85051718; FAX: +86 513 85051718; E-MAIL: jguohua@ntu.edu.cn

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Abstract

OBJECTIVE: Successful neural stem cells (NSCs) therapies require the controlled differentiation of NSCs into neurons. Porous chitosan scaffold was explored if it promoted neuronal differentiation of NSCs in the presence of nerve growth factor (NGF) in 3-dimensional (3-D) culture.

METHODS: Chitosan scaffold was made by the freeze-drying technique. NSCs were cultured under four different conditions: on flat cover slips (2-D structure) in media with or without NGF, and on chitosan scaffold (3-D structure) in media with or without NGF. Immunohistochemical staining was used to observe multi-directional differentiation of cultured NSCs. Photomicrographs were taken and analyzed for cell number, soma size, and neuronal process length.

RESULTS: The porosity index of chitosan scaffold was around 90% and the diameter of pores was 50–350 μm. NSCs could differentiate into neurons, astrocytes, and oligodendrocytes under all culture conditions. The rank efficacy for neuronal differentiation was 3-D culture with NGF group > 3-D culture without NGF group > 2-D culture with NGF group > 2-D culture without NGF group.

CONCLUSION: The results suggest that the combination of chitosan scaffold and NGF exerts a synergistic effect on neuronal differentiation of NSCs, a requirement for successful integration into the damaged central nervous system.

INTRODUCTION

Neural stem cells (NSCs) have the potential for proliferation, migration, and differentiation into different neuronal lineages, including neurons, astrocytes, and oligodendrocytes. However, most transplanted NSCs differentiate into astrocytes, which weakens the functional impact of implantation (Han *et al.* 2002).

Tissue engineering utilizes 3-dimensional (3-D) biomaterial scaffolds to support the proliferation, migration, and differentiation of progenitor cells. Scaffolds ideally guide progenitor cell migration and stimulate expansion and differentiation (Andersson & van den Berg 2004). The properties that determine scaffold efficacy include biocompatibility, porosity, pore size, surface properties, pH, surface charge, biodegradability, and ideally, the ability to recruit progenitor cells (Wiesmann *et al.* 2004). Chitosan is a biodegradable polyglucosamine chitin derivative that has low toxicity, is inexpensive, and has characteristics that assist in nerve repair (Cheng *et al.* 2007).

NSCs derived from various sources release a myriad of neurotrophic factors, including nerve growth factor (NGF), brain derived neurotrophic

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factor (BDNF), and glial-derived neurotrophic factor (GDNF) (Llado *et al.* 2004; Lu *et al.* 2003). Growth factors that stimulate progenitor cell signaling cascades enhance proliferation and differentiation (Muschler *et al.* 2004). NGF can enhance the survival of NSCs and promote differentiation into neurons (Longhi *et al.* 2004). In the present study, we fabricated porous chitosan scaffolds by the freezing-drying technique (Whang *et al.* 1999). NSCs derived from the cortices of fetal rats were cultured on scaffolds with NGF to investigate whether this combination can enhance the differentiation of NSCs into neurons.

MATERIALS AND METHODS

Preparation of porous chitosan scaffold

Porous chitosan scaffolds were fabricated by the freeze-drying technique. In brief, chitosan (Sigma) was dissolved in acetic acid (1%, v/v) at 2% (w/v) and pipetted into 24-well culture plates at 1 ml/well. Treated plates were maintained in a refrigerator at 4 °C for 6 h. The plates were then frozen at –20 °C for 12 h and lyophilized in a freeze-dryer at –60 °C for 24 h. After neutralization with NaOH solution (0.1 M), scaffolds were rinsed with distilled water, dried at room temperature, sterilized with alcohol (70%, v/v), rinsed with physiological saline solution, and irradiated by a viltalight lamp for 20 min before culturing NSCs.

Detecting the characterization of porous chitosan scaffold

Detecting the porosity of porous chitosan scaffold

The porosity of chitosan scaffold was detected by anhydrous ethanol substitution method (Hsu *et al.* 1997). A sample of freeze-dried chitosan was immersed in a graduated cylinder containing a known volume (V1) of anhydrous ethanol. Air was removed from the cylinder by a series of brief evacuation-repressurization cycles to force liquid into the void volume of the scaffold. Cycling was continued until no air bubbles were seen emerging from the chitosan. The volume difference recorded (V2 – V1) represented the volume of chitosan only. The liquid impregnated chitosan was then removed from the cylinder and the new volume (V3) recorded. The quantity (V1 – V3) or the volume of liquid held by the scaffold was accepted as the void volume. Thus, the chitosan scaffold porosity (p_s) can be expressed as $p_{s\,=}$ (V1 – V3)/(V2 – V3).

Detecting the pore size of porous chitosan scaffold

Fifteen micrometer sections were obtained from chitosan samples using a cryostat (Leica CM1900, Germany) and thaw-mounted onto gelatin covered slides. Sections were observed by microscopy and the pore average diameter was quantified by JD-801 morphology software (Nanking, China).

Isolation and culture of NSCs

Cerebral cortical NSCs were prepared from pregnant Sprague-Dawley rat embryos on E14–15 as described previously (Zhang *et al.* 2007). Briefly, rat embryonic cerebral tissue was dissociated into a cell suspension by 0.25% trypsin digestion. Cell number and viability were determined by staining a small volume of cell suspension with 0.4% trypan blue. The cell suspension was then maintained at a density of 4×10^4 cells/ml in Dulbecco' Modified Eagle' Medium (DMEM) plus Ham's F12 medium (1:1, v/v) and supplemented with 2% B27, 10 ng/ml EGF, and 10 ng/ml basic fibroblast growth factor (bFGF). Cells were incubated at 37 °C in a 95% air /5% CO_2 humidified atmosphere and split every 7–8 d by dissociation of bulk neurospheres with 0.05% trypsin. Neurospheres were characterized by immunocytochemistry against anti-BrdU antibody (1:400, Roche, Germany) or anti-nestin antibody (1:100, Chemicon) as described previously.

After four passages, NSCs (1×10^5 cells/ml) from these neurospheres were cultured for 14 d on chitosan scaffolds (3-D structure) in 24-well plates containing serum-free medium or serum-free medium supplemented with 5% NGF (Roche, Germany). As the control, NSCs were plated onto poly-D-lysine coated cover slips (2-D structure) in 24-well plates containing serum-free medium or serum-free medium supplemented with 5% NGF.

Immunocytochemistry

After 14 d in culture, cells cultured on poly-D-lysine coated cover slips were fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) for 1 h. Cells cultured on chitosan scaffolds were fixed with 4% PFA in 0.1 M PB for 1 h, then 15-μm sections were obtained using a cryostat and thaw-mounted onto gelatin covered slides. Cells or sections were blocked in 5% goat serum for 30 min at room temperature and then incubated for 12 h at room temperature with primary antibodies. After washing three times in phosphate buffered saline (PBS), the cells or sections were incubated in secondary antibodies for 6 h at room temperature. Antibodies were as follows: the primary monoclonal antibodies were against mouse microtubule associated proteins-2 (anti-MAP-2, 1:200, Chemicon), mouse glial fibrillary acidic protein (anti-GFAP, 1:200, Sigma), and mouse 2',3'-cyclic nucleotide 3'-phosphodiesterase (anti-CNP, 1:200, Sigma). Secondary antibodies were Alexa Fluor 568-conjugated (red) goat anti-mouse IgG (1:500, Invitrogen) or FITC-conjugated (green) goat anti-mouse IgG (1:200, Millipore). Cell nuclei were counter-stained with Hoechst33342 for 30 min at room temperature. Immunopositive cells were observed using a fluorescent microscope. Photomicrographs were taken and analyzed for cell phenotype, number, soma size, and neuronal process length.

Statistical analysis

Images were analyzed by Leica Qwin image processing and analysis software (Leica imaging System, Cambridge, UK). Statistical analysis was performed using statistics package for social science 16.0 (SPSS 16.0). Data were presented as mean ± standard deviation (M ± SD). Statistical comparisons were performed using one-way analysis of variance (ANOVA) and differences at $p<0.05$ were considered statistically significant.

RESULTS

Characterization of porous chitosan scaffold

Porous chitosan scaffold is a circular cylinder, height with 0.08 cm and area of undersurface with 0.02 cm^2. The porosity of freeze-dried chitosan was about 90% as determined by anhydrous ethanol substitution, and most pores had a diameter ranging from 50–350 μm (Figure 1). Thus, freeze-dried chitosan formed a porous scaffold structure with pores large enough for cell migration, growth, and axonal pathfinding.

Multi-directional differentiation of cultured NSCs

NSCs derived from cerebral cortical of E14–15 rat embryos were nestin and BrdU positive (Figure 2A). NSCs were cultured under the four conditions outlined in materials and methods. After 14 d in culture, NSCs exhibited multi-directional differentiation (Figure 2B). MAP-2 positive neurons, GFAP positive astrocytes, and CNP positive oligodendrocytes were observed under the four conditions.

Neuronal differentiation of NSCs cultured in different media

In the present study, we observed neuronal differentiation of NSCs cultured in four different conditions. NSCs that were plated onto poly-D-lysine-coated cover slips (2-D structure) in serum-free medium without NGF differentiated a small number of MAP-2 positive neurons with small bodies and very short processes. When NSCs were plated onto poly-D-lysine coated

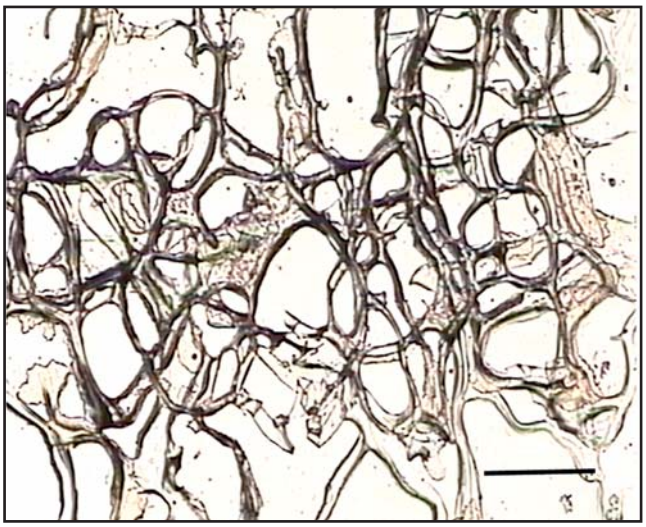

Figure 1. A thin slice of freeze-dried chitosan scaffold. Chitosan formed a sponge-like 3-dimensional structure that was highly porous. Pores ranged from 50-350 μm wide. Scale bar, 200 μm.

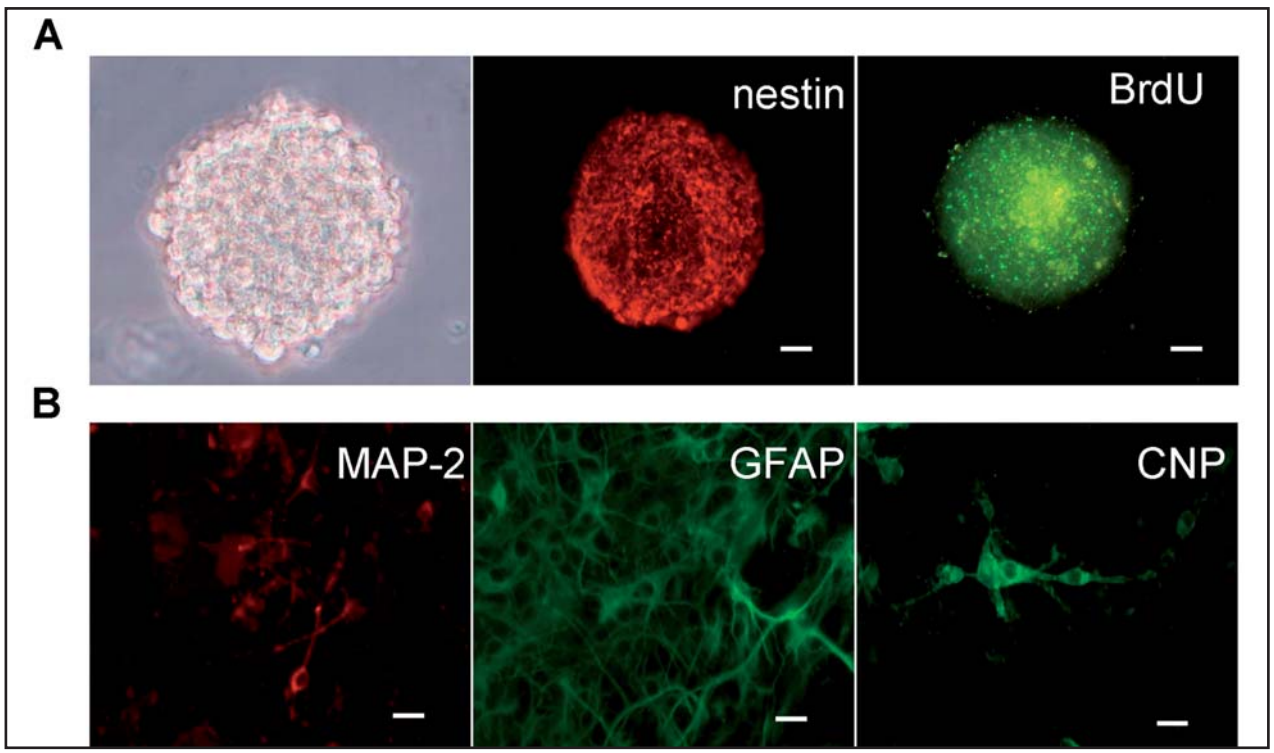

Figure 2. Multi-directional differentiation of NSCs derived from cerebral cortical of E14-15 rat embryos. (A) NSCs (left, bright field) were detected by nestin (middle) and BrdU antibody (right). Scale bar, 100 μm. (B) NSCs differentiated into MAP-2 positive neurons, GFAP positive astrocytes, and CNP positive oligodendrocytes. Scale bar, 20 μm.

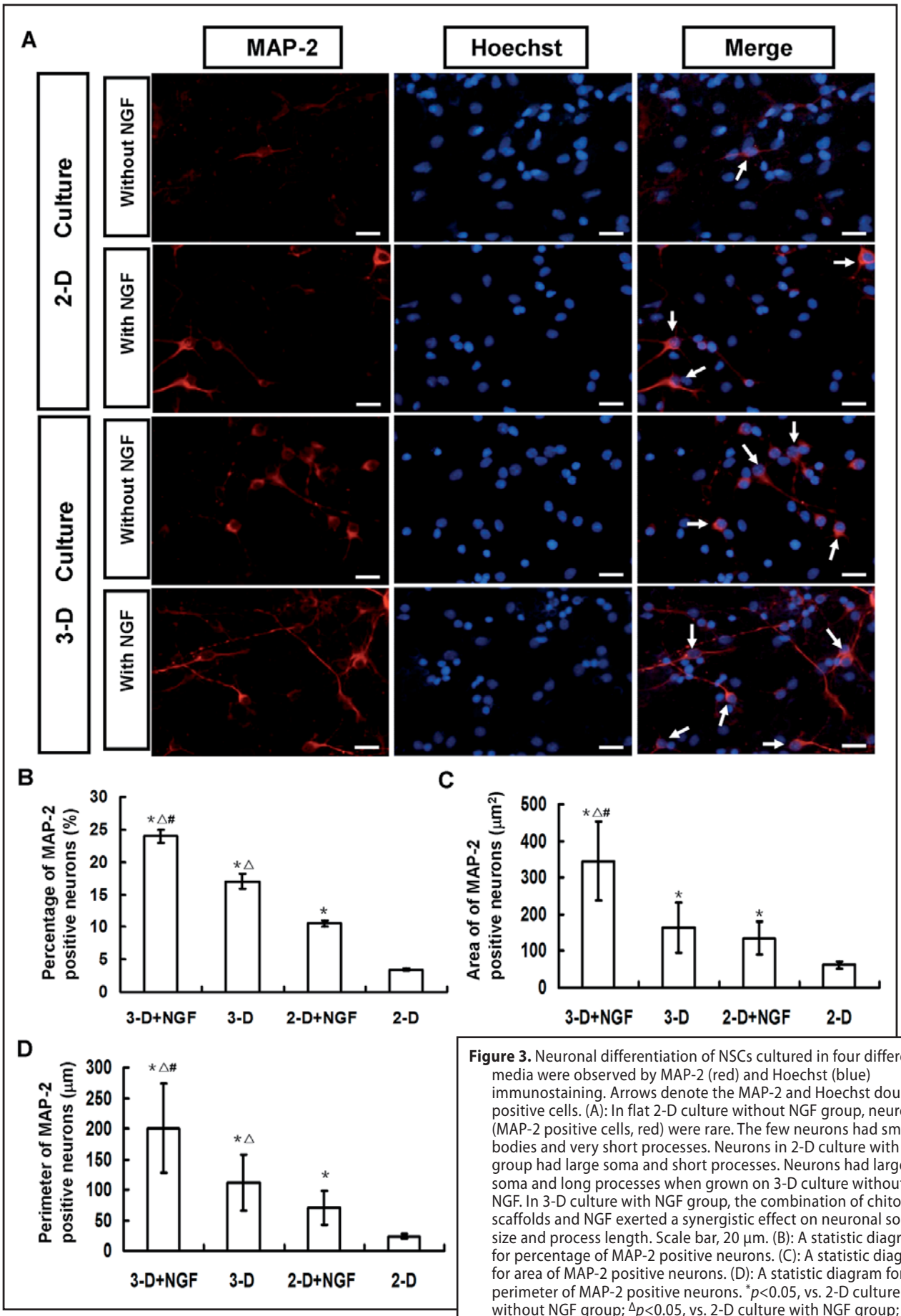

Figure 3. Neuronal differentiation of NSCs cultured in four different media were observed by MAP-2 (red) and Hoechst (blue) immunostaining. Arrows denote the MAP-2 and Hoechst double positive cells. (A): In flat 2-D culture without NGF group, neurons (MAP-2 positive cells, red) were rare. The few neurons had small bodies and very short processes. Neurons in 2-D culture with NGF group had large soma and short processes. Neurons had large soma and long processes when grown on 3-D culture without NGF. In 3-D culture with NGF group, the combination of chitosan scaffolds and NGF exerted a synergistic effect on neuronal soma size and process length. Scale bar, 20 μm. (B): A statistic diagram for percentage of MAP-2 positive neurons. (C): A statistic diagram for area of MAP-2 positive neurons. (D): A statistic diagram for perimeter of MAP-2 positive neurons. $^{*}p<0.05$, vs. 2-D culture without NGF group; $^{\Delta}p<0.05$, vs. 2-D culture with NGF group; $^{\#}p<0.05$ vs. 3-D culture without NGF group.

cover slips in serum-free medium supplemented with 5% NGF, MAP-2 positive cells were more numerous and had larger soma. Neuronal processes, however, were still under development. In contrast, many more MAP-2 positive cells with large soma and extensive processes were found in the two chitosan scaffold (3-D structure) culture groups (Figure 3A). The percentage of MAP-2 positive cells cultured on scaffolds with NGF was significantly higher than on scaffold culture without NGF. The average neuronal cell body area and cell perimeter in the scaffold with NGF culture were significantly higher than in scaffold culture without NGF. Neurons under both scaffold cultures had far larger cell bodies and longer processes than those observed in 2-D cultures with or without NGF (Figure 3B,C,D). In sum, these results showed that porous chitosan scaffold significantly enhanced neuronal differentiation compared to conventional 2-D cultures, and porous chitosan scaffold with exogenous NGF synergistically enhanced neuronal differentiation.

DISCUSSION

Most central nervous system (CNS) diseases are caused by the progressive loss of function and eventual death of neurons. Stem cell therapy provides a potential method for replacing neurons lost by injury or disease. To realize efficient stem cell therapy, the transplanted NSCs must differentiate into the desired phenotypes (Caldwell *et al.* 2001). However, the poor survival, migration, and the partial differentiation of transplanted NSCs limits the therapeutic effect (Cao *et al.* 2002). The factors responsible for the proliferation, migration, and differentiation of NSCs have been the focus of intense investigation to determine optimal conditions for deriving functionally integrated neurons after stem cell therapy (Cameron *et al.* 1998). In our study, the rank efficacy for neuronal differentiation of NSCs was 3-D culture with NGF group > 3-D culture without NGF group > 2-D culture with NGF group > 2-D culture without NGF group. Our results indicate that porous chitosan scaffolds have good NSCs biocompatibility, and the chitosan scaffolds with NGF exert a synergistic effect on the differentiation of NSCs into neurons with a morphologically mature phenotype.

This study is part of a larger program to evaluate the utility of various chitosan scaffold configurations with NGF for the effective differentiation of NSCs. Surprisingly, more neurons were observed in cultures of NSCs grown on chitosan scaffolds without NGF than on flat substrate with NGF, underscoring the growth-permissive properties of chitosan. The microenvironment is a major influence on the differentiation of NSCs. Axonal targets and surrounding supporting cells release neurotrophic factors and cytokines that modulate differentiation by activating a number of signaling cascades (Dodla *et al.* 2010). The extracellular matrix is composed of a variety of permissive and non-permissive substrates that can promote differentiation, process formation, and guide axons to their targets (Young *et al.* 2000). Chitosan is a linear polyglucosamine that forms porous scaffolds after freeze-drying. Chitosan is derived from natural chitin and possesses good biocompatibility, biodegradability, and low toxicity. In addition, chitosan can be fabricated in films (Cheng *et al.* 2007), porous scaffolds (Bithell & Williams 2005), and tubes (Li *et al.* 2009) to guide tissue architecture. In fact, chitosan is a widely used biomaterial in nerve tissue engineering. The porosity and pore size of porous chitosan scaffold can be controlled by chitosan concentration and temperature of freeze-drying. The freeze-drying method employed here yielded a sponge-like scaffold with a porosity of about 90% and pore diameters ranging 50–350 μm. The somal diameter of NSCs derived from fetal rat cortex range from 6–12 μm and grow to less than 70 μm in diameter, so the pore size is compatible with cell migration, axonal growth, and elaboration of dendrites. Chitosan scaffolds provide an environment that support the survival, migration, and differentiation of NSCs in 3-D structure.

Cell-cell interactions might play an important role in controlling NSCs differentiation when seeded onto 2-D surfaces (Wang *et al.* 2010; Young *et al.* 2000). Indeed, cell survival and differentiation are greatly influenced by cell density in culture, possibly because adjacent cells can maintain local elevations of growth factors (Young *et al.* 2000). In addition to growth factors, the topology of chitosan (film, tube, or porous) determines the phenotype of differentiated NSCs. However, particular combinations of chitosan forms and growth factors exert effects not readily predicted by each condition separatel. In our study, porous chitosan scaffolds without NGF was superior to 2-D culture with NGF. The reason for this has not been determined, but may relate to cell density. When NSCs were seeded on 3-D scaffolds, cells could enter into the pores, possibly through capillary action, and adhered on the scaffold walls. The complex 3-D structure provided more space and surface area for cell proliferation, migration, and differentiation, while still maintaining relatively in close proximity and high cell density.

BDNF (Zhao *et al.* 2004), NGF (Longhi *et al.* 2004) and neurotrophin-3 (NT-3) (Park *et al.* 2006) all increased the survival of NSCs and promoted neuronal differentiation. Chitosan conjugated to a variety of growth factors could dynamically regulate and fine tune the cellular composition of the tissue (Reimers *et al.* 2008). In the current study, combining NGF with porous chitosan scaffold exerted a synergistic effect on neuronal differentiation, possibly because cells have access to more NGF in 3-D culture. These results suggest that NGF combined with porous chitosan scaffold may be suited to induce neuronal differentiation of NSCs on a large scale, providing a promising method for neural repair following CNS injury.

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REFERENCES

1 Andersson H & van den Berg A (2004). Microfabrication and microfluidics for tissue engineering: state of the art and future opportunities. Lab Chip. **4**: 98–103.
2 Bithell A & Williams BP (2005). Neural stem cells and cell replacement therapy: making the right cells. Clin Sci (Lond). **108**: 13–22.
3 Caldwell MA, He X, Wilkie N, Pollack S, Marshall G, Wafford KA, et al (2001). Growth factors regulate the survival and fate of cells derived from human neurospheres. Nat Biotechnol. **19**: 475–479.
4 Cameron HA, Hazel TG & McKay RD (1998). Regulation of neurogenesis by growth factors and neurotransmitters. J Neurobiol. **36**: 287–306.
5 Cao QL, Howard RM, Dennison JB & Whittemore SR (2002). Differentiation of engrafted neuronal-restricted precursor cells is inhibited in the traumatically injured spinal cord. Exp Neurol. **177**: 349–359.
6 Cheng H, Huang YC, Chang PT & Huang YY (2007). Laminin-incorporated nerve conduits made by plasma treatment for repairing spinal cord injury. Biochem Biophys Res Commun. **357**: 938–944.
7 Dodla MC, Mumaw J & Stice SL (2010). Role of astrocytes, soluble factors, cells adhesion molecules and neurotrophins in functional synapse formation: implications for human embryonic stem cell derived neurons. Curr Stem Cell Res Ther. **5**: 251–260.
8 Han SS, Kang DY, Mujtaba T, Rao MS & Fischer I (2002). Grafted lineage-restricted precursors differentiate exclusively into neurons in the adult spinal cord. Exp Neurol. **177**: 360–375.
9 Hsu YY, Gresser JD, Trantolo DJ, Lyons CM, Gangadharam PR & Wise DL (1997). Effect of polymer foam morphology and density on kinetics of in vitro controlled release of isoniazid from compressed foam matrices. J Biomed Mater Res. **35**: 107–116.
10 Li X, Yang Z, Zhang A, Wang T & Chen W (2009). Repair of thoracic spinal cord injury by chitosan tube implantation in adult rats. Biomaterials. **30**: 1121–1132.
11 Llado J, Haenggeli C, Maragakis NJ, Snyder EY & Rothstein JD (2004). Neural stem cells protect against glutamate-induced excitotoxicity and promote survival of injured motor neurons through the secretion of neurotrophic factors. Mol Cell Neurosci. **27**: 322–331.
12 Longhi L, Watson DJ, Saatman KE, Thompson HJ, Zhang C, Fujimoto S, et al (2004). Ex vivo gene therapy using targeted engraftment of NGF-expressing human NT2N neurons attenuates cognitive deficits following traumatic brain injury in mice. J Neurotrauma. **21**: 1723–1736.
13 Lu P, Jones LL, Snyder EY & Tuszynski MH (2003). Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. Exp Neurol. **181**: 115–129.
14 Muschler GF, Nakamoto C & Griffith LG (2004). Engineering principles of clinical cell-based tissue engineering. J Bone Joint Surg Am. **86-A**: 1541–1558.
15 Park KI, Himes BT, Stieg PE, Tessler A, Fischer I & Snyder EY (2006). Neural stem cells may be uniquely suited for combined gene therapy and cell replacement: Evidence from engraftment of Neurotrophin-3-expressing stem cells in hypoxic-ischemic brain injury. Exp Neurol. **199**: 179–190.
16 Reimers D, Gonzalo-Gobernado R, Herranz AS, Osuna C, Asensio MJ, Baena S, et al (2008). Driving neural stem cells towards a desired phenotype. Curr Stem Cell Res Ther. **3**: 247–253.
17 Wang G, Ao Q, Gong K, Wang A, Zheng L, Gong Y, et al (2010). The effect of topology of chitosan biomaterials on the differentiation and proliferation of neural stem cells. Acta Biomater. **6**: 3630–3639.
18 Whang K, Healy KE, Elenz DR, Nam EK, Tsai DC, Thomas CH, et al (1999). Engineering bone regeneration with bioabsorbable scaffolds with novel microarchitecture. Tissue Eng. **5**: 35–51.
19 Wiesmann H, Joos U & Meyer U (2004). Biological and biophysical principles in extracorporeal bone tissue engineering: part 1. Int J Oral Maxillofac Surg: 325–332.
20 Young TH, Huang JH, Hung SH & Hsu JP (2000). The role of cell density in the survival of cultured cerebellar granule neurons. J Biomed Mater Res. **52**: 748–753.
21 Zhang X, Jin G, Tian M, Qin J & Huang Z (2007). The denervated hippocampus provides proper microenvironment for the survival and differentiation of neural progenitors. Neurosci Lett. **414**: 115–120.
22 Zhao LX, Zhang J, Cao F, Meng L, Wang DM, Li YH, et al (2004). Modification of the brain-derived neurotrophic factor gene: a portal to transform mesenchymal stem cells into advantageous engineering cells for neuroregeneration and neuroprotection. Exp Neurol. **190**: 396–406.