Research Report

Compatibility of human fetal neural stem cells with hydrogel biomaterials in vitro

Jason R. Thonhoff, Dianne I. Lou, Paivi M. Jordan, Xu Zhao, Ping Wu*

Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, TX 77555-0620, USA

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ABSTRACT

Stroke and spinal cord or brain injury often result in cavity formation. Stem cell transplantation in combination with tissue engineering has the potential to fill such a cavity and replace lost neurons. Several hydrogels containing unique features particularly suitable for the delicate nervous system were tested by determining whether these materials were compatible with fetal human neural stem cells (hNSCs) in terms of toxicity and ability to support stem cell differentiation in vitro. The hydrogels examined were pluronic F127 (PF127), Matrigel and PuraMatrix. We found that PF127, in a gelated (30%) form, was toxic to hNSCs, and Matrigel, in a gelated (1–50%) form, prevented hNSCs' normal capacity for neuronal differentiation. In contrast, PuraMatrix was the most optimal hydrogel for hNSCs, since it showed low toxicity when gelated (0.25%) and retained several crucial properties of hNSCs, including migration and neuronal differentiation. Further optimization and characterization of PuraMatrix is warranted to explore its full potential in assisting neural regeneration in vivo.

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1. Introduction

An impediment to neural regeneration after injury is that a cavity often forms that lacks any structure to aid cellular replacement and differentiation. Thus, potential regeneration of neurons after transplantation of stem cells would undoubt-
edly be aided by being combined with appropriate biomater-
ials. The properties of an ideal biomaterial would include that it is biodegradable, non-toxic, provides a three-dimensional scaffold for developing tissue, can be vascularized due to its porous structure and elicits minimal immune responses (Holmes et al., 2000). Previously, rodent stem/progenitor cells have been seeded on scaffolds that meet some of these criteria and applied in cell-based therapies in animal models of neurotrauma (Vacanti et al., 2001; Lavik et al., 2002; Tate et al., 2002; Teng et al., 2002; Park et al., 2002; Bhang et al., 2007). However, hydrogel polymer scaffolds possess a further characteristic that may be advantageous in neural grafting in that they can be injected as a fluid into spinal cord or brain cavities and then polymerized in situ, thus conforming to the shape of the cavity caused by the injury. The present study focuses on three hydrogels; pluronic F127 (PF127), Matrigel and PuraMatrix.

PF127 is a nonionic surfactant composed of a block co-
polymer of propylene oxide and ethylene oxide. It gels at room temperature and has mostly been used in controlled-release drug delivery (Barichello et al., 1999; Desai and Blanchard, 2000; El Kamel, 2002; Wenzel et al., 2002). However, hydrogel polymer scaffolds possess a further characteristic that may be advantageous in neural grafting in that they can be injected as a fluid into spinal cord or brain cavities and then polymerized in situ, thus conforming to the shape of the cavity caused by the injury. The present study focuses on three hydrogels; pluronic F127 (PF127), Matrigel and PuraMatrix.
and as a construct to support the growth and differentiation of lung progenitor cells (Cortiella et al., 2006).

Matrigel is composed of basal membrane extract derived from Engelbreth–Holm–Swarm mouse sarcoma cells. It solidifies at room temperature forming a hydrogel. The extracellular matrix components in this hydrogel include laminin, collagen IV, entactin, nidogen and heparan sulfate proteoglycans. Growth factors such as TGF-β, basic fibroblast growth factor, insulin-like growth factor-1 and tissue plasminogen activator are also present (Novikova et al., 2003; Kleinman and Martin, 2005). Matrigel has previously been used in neuronal culture and has been shown to stimulate neurite outgrowth in vitro (Tisay and Key, 1999; Novikova et al., 2006).

PuraMatrix is a synthetic peptide hydrogel composed of ionic self-complementary oligopeptides similar to the EAK16 tandem repeats found in the yeast protein, zuotin. It is a 16-mer peptide with a repeating sequence of arginine, alanine, aspartate and alanine (RADARADARADARADA or RAD16) and has a composition of more than 99% water. The amphiphilic nature of PuraMatrix gives rise to the spontaneous assembly of a water-soluble β-sheet structure in the presence of monovalent cations (Zhang et al., 1993, 1995). This hydrogel

Fig. 1 – Cytotoxicity and morphological characteristics of human neural stem cells in hydrogels. Phase-contrast images of human neural stem cells primed for 5 days and then differentiated for 2 days in the absence of a hydrogel (control) (a), 10% PF127 (b), 20% PF127 (c), 30% PF127 (d), 1% Matrigel (e), 10% Matrigel (f), 50% Matrigel (g), 0.25% PuraMatrix (h), 0.5% PuraMatrix (i) and 1% PuraMatrix (j). Scale bar=20 μm.
has been shown to support cell attachment and proliferation (Zhang et al., 1995), formation of neurite outgrowth and active synapses (Holmes et al., 2000), and cell entrapment (Semino et al., 2004) and differentiation (Semino et al., 2003).

None of these hydrogels have been assessed for biocompatibility with human fetal neural stem cells (hNSCs), a type of stem cell with self-renewing and multipotential differentiation properties as well as an origin most suitable for human transplantation. These cells proliferate in vitro in neurospheres, which consist of a heterogeneous population of neural stem cells and neural progenitors (Suslov et al., 2002). We have previously shown that mitogen-expanded and primed (Wu et al., 2002; Tarasenko et al., 2004) hNSCs become cholinergic motoneurons when grafted into acutely or chronically injured rat spinal cords (Gao et al., 2005; Tarasenko et al., 2007). However, grafted hNSCs showed limited survival rates and migrational capabilities within the injured cavity indicating the need for a scaffold support. In this study, we screened several scaffolding biomaterials and determined the optimal hydrogel based on its support of hNSC survival, differentiation and migration in vitro.

2. Results

2.1. Survival and morphological characteristics of primed human neural stem cells after 2 days differentiation beneath hydrogels

Cell survival is one of the critical parameters to determine whether a given material is biocompatible and suitable for hNSC tissue engineering. Therefore, we first assessed the survival of primed hNSCs through both morphological analyses and measurements of overall activity of mitochondrial dehydrogenases in live cells. After priming, hNSCs were exposed to hydrogels at different concentrations: PF127 (10–30%), Matrigel (1–50%) and PuraMatrix (0.25–1%).

Primed and differentiated hNSCs without hydrogel treatment showed many presumptive neurons based on morphology that appeared bright in phase-contrast microscopy and emitted processes (Fig. 1a). Further immunostaining studies confirmed their neuronal phenotype (Fig. 3). Similarly, 10% PF127 supported many phase-bright presumptive neurons (Fig. 1b) whereas more cells with a presumptive glial morphology were present in 20% PF127 (Fig. 1c). The presumptive glial cells exhibited large cell bodies and hypertrophied nuclei. 30% PF127 was toxic as fewer cells and more cellular debris were observed (Fig. 1d). A mix of presumptive neurons and glia were seen in 1% Matrigel (Fig. 1e). As the concentration of Matrigel increased, the number of presumptive glial cells increased (Figs. 1f, g). Little cellular debris was observed indicating that hNSCs survived well in Matrigel. A mix of presumptive neurons and glia were also present in 0.25% and 0.5% PuraMatrix (Figs. 1h, i). However, the concentrated PuraMatrix (1%) seemed to exert a toxic effect on the hNSCs as the cells seemed shrunken and degenerated (Fig. 1).

Cytotoxicity of these hydrogels was determined quantitatively using the WST-1 assay to evaluate mitochondrial enzymatic activities in viable cells. The viability of hNSCs decreased steadily as the concentration of PF127 and PuraMatrix increased. Surprisingly, however, Matrigel significantly decreased the viability of hNSCs at a 10% concentration (Fig. 2).

2.2. Differentiation capacity of primed human neural stem cells beneath Matrigel and PuraMatrix

Another parameter of an optimal hydrogel is whether the material allows hNSCs to retain their natural differentiation capacity into both neurons and glial cells. To test the differentiation capacity, hNSCs were primed and allowed to differentiate in B27 alone as a control or in contact with 20% Matrigel or 0.25% PuraMatrix. The particular concentrations of Matrigel and PuraMatrix were selected based on their gelation capacity to support hNSCs after potential transplantation into the injured brain or spinal cord. PF127 was eliminated from this study, since it was too toxic to hNSCs at concentrations necessary for gelation. Differentiation of primed hNSCs in B27 medium, without hydrogel, showed a mix of both Tuji+ neuronal cells (17.2±5.2%) and GFAP+ astroglial cells (27.6±9.5%) (Figs. 3a–d). Primed hNSCs differentiated in the presence of 20% Matrigel for 7 days showed primarily GFAP+ glial cells (95.3±3.6%) (Figs. 3e–h) with larger nuclei and cell bodies. Very few Tuji+ neurons (4.7±3.6%) were present. The normal differentiation pattern seen in primed hNSCs (Figs. 3a–d) was retained in primed hNSCs that were differentiated in the presence of 0.25% PuraMatrix for 7 days (Tuji: 17.3±4.2% and GFAP: 25.8±5.3%) (Figs. 3i–l).

![Fig. 2 - Viability of human neural stem cells beneath hydrogels. WST-1 assay on human neural stem cells primed for 5 days and then differentiated for 2 days in the absence of a hydrogel (control), PF127 (10–30%), Matrigel (1–50%) and PuraMatrix (0.25–1%). PF=PF127, MG=Matrigel and PM=PuraMatrix. A reference absorbance at 630 nm was taken and subtracted from the absorbance at 450 nm for each sample. *p<0.05 compared to the control group.](image-url)
2.3. Migration patterns and differentiation capacity of hNSC neurospheres embedded within PuraMatrix

Since PuraMatrix appeared to be the most optimal among the three hydrogels that were tested in terms of supporting the survival and differentiation capacity of hNSCs, we then asked whether hNSCs retained their migration capacity within PuraMatrix. We chose neurospheres rather than primed cells to address the migration issue, since the extensive migration of hNSCs that occurs during priming could prevent a full assessment of the cells' migration capacity in PuraMatrix. Spheres, 4 days post-passage, were embedded within 0.25% PuraMatrix and allowed to differentiate for 7 days. Images were taken using a light microscope at 3.5 and 7 days to determine the extent of hNSC migration. NIS-Elements imaging software (Nikon Instruments, Inc., Melville, NY) was

Fig. 3 – Differentiation capacity of human neural stem cells beneath Matrigel and PuraMatrix. Immunofluorescent images of human neural stem cells primed for 5 days and then differentiated for 7 days in the absence of a hydrogel (control, a–d); in contact with 20% Matrigel (e–h); and in contact with 0.25% PuraMatrix (i–l). DAPI (a, e and i), GFAP (b, f and j), Tuj1 (c, g and k) and overlay (d, h and l). Scale bar=20 μm.
used to measure the migration distance of the cell that traveled furthest from the edge of each control sphere and sphere embedded within the PuraMatrix hydrogel. Some hNSCs were seen migrating from spheres 3.5 days post-embedding with an average furthest distance of $108.8 \pm 32.3 \mu m$ ($n=9$) (Fig. 4c). After 7 days, a more extensive network of migrating cells was observed with an average furthest distance of $245.6 \pm 49.5 \mu m$ ($n=13$) (Fig. 4d), although the distance of cell migration was much less than that observed in medium without a hydrogel after only 3.5 days ($670.8 \pm 17.5 \mu m$, $n=8$) (Fig. 4a). After 7 days in medium alone (Fig. 4b), a much higher proportion of migrating cells was observed from each sphere and many of these cells migrated further than the field of view at the lowest magnification in just a few days.

We then asked whether hNSC neurospheres without priming retained their neuronal differentiation capacity in PuraMatrix, and compared the cell phenotypes to undifferentiated neurospheres. Immunostaining of sectioned spheres, which were cultured for 5 days in growth media and not seeded in PuraMatrix, showed heterogeneous populations with GFAP$^+$ cells distributed evenly throughout the spheres and some Tuj1$^+$ cells mainly in the center of spheres (Figs. 5a–e). Interestingly, spheres that were seeded in PuraMatrix and differentiated for 7 days in B27 revealed a concentration of GFAP$^+$ cells on the outside regions of the spheres and an even distribution of Tuj1$^+$ cells throughout (Figs. 5f–j).

3. Discussion

The major thesis of the present study was to determine an optimal hydrogel that was biocompatible with hNSCs and thus, could potentially be used in combination with stem cells to facilitate neural regeneration after stroke or traumatic injury. The data collected from the in vitro experiments revealed that PuraMatrix hydrogel was most compatible with hNSCs as it supported hNSC survival, differentiation and migration.

Previously, poly($\alpha$-hydroxy acid) polymers including poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and their copolymers (PLGA) have been seeded with rodent stem/progenitor cells (Vacanti et al., 2001; Teng et al., 2002; Bhang et al., 2007; Gelain et al., 2007). It was unknown, though, whether these types of polymers were compatible with hNSCs. In an initial trial experiment, we found that hNSCs seeded and primed on PGA attached to and migrated along the PGA fibers (unpublished observation). However, PGA seemed to be particularly rigid and thus not suitable for transplantation into neural tissue as the rigid PGA fibers would need to be pressed, rather than injected into the cavity resulting from most neural injuries. An ideal biomaterial for neural transplantation would have the ability to be mixed with stem cells and injected into the cavity in a fluid form. Once in vivo, the biomaterial would solidify forming a three-dimensional matrix, in which the

Fig. 4 – Migration capacity of human neural stem cells embedded as neurospheres after 7 days within PuraMatrix. Phase-contrast images of human neural stem cell neurospheres seeded without a hydrogel for 3.5 days (a) and 7 days (b) and within 0.25% PuraMatrix for 3.5 days (c) and 7 days (d). A much higher proportion of migrating cells was observed from each sphere after 7 days in medium alone compared to 3.5 days. Scale bar=20 μm.
Fig. 5 – Differentiation capacity of human neural stem cell neurospheres embedded within PuraMatrix. Immunofluorescent images of human neural stem cell neurospheres 5 days after passage (control) (a–e); and neurospheres differentiated within 0.25% PuraMatrix for 7 days (f–j). DAPI (a and f), GFAP (b and g), Tuj1 (c and h) and overlay in a low magnification (d and i). Insets in panels d and i are shown in a high magnification in panels e and j, respectively. Scale bars=20 μm.
stem cells could migrate and form connections across the cavity. PF127, Matrigel and PuraMatrix hydrogels possessed this important characteristic. The biocompatibilities of PuraMatrix and Matrigel with rodent neural precursor cells were recently compared to other commonly used scaffolds including poly(α-hydroxy acid) polymers (Gelain et al., 2007). Although some differences were observed in cell viability and differentiation capacity, the compatibilities of the hydrogel materials and other scaffolds were comparable.

However, in our study, PF127 showed some toxicity to hNSCs at concentrations needed for in vivo gelation. Also, although more viable cells were found in the case of 10% PF127 hydrogel as compared to the control, this increase was not significant and may be attributed to cell plating variation. Furthermore, cell viability in 10% Matrigel was significantly decreased compared to the control whereas cells in 50% Matrigel had a much higher viability. This phenomenon observed under conditions of increasing concentrations of Matrigel likely involves a complex dynamic between cell toxicity (or inhibition of cell proliferation) and growth factor stimulation. The fact that hNSCs cultured with Matrigel become mainly astrocytes indicates a toxic effect of Matrigel on hNSC-derived neurons. This initial phase of neuronal death may then be followed by the proliferation of astrocytes that is stimulated by growth factors in the increasing concentration of Matrigel. Alternatively, Matrigel, a mixed basement membrane extract from mouse sarcoma cells containing various growth factors and molecules in addition to collagen and laminin, may inhibit the neuronal differentiation of hNSCs. In any case, this observation is inconsistent with previous reports that showed neuronal differentiation and migration of neural stem/progenitor cells when placed on top of a surface coated with Matrigel (Katakowski et al., 2005; Flanagan et al., 2006). An apparent difference is that we placed hNSCs underneath the Matrigel. However, toxicity due to insufficient nutrient access is unlikely as both Matrigel and PuraMatrix at the concentrations we used have adequate pore sizes (approximately 2 µm in 50% Matrigel and 0.05–0.2 µm in 0.5% PuraMatrix) to allow medium infusion (Semino et al., 2003; Zaman et al., 2006). Whether toxicity in some conditions was due to the release of harmful or acidic byproducts during degradation is yet to be determined. Taken as a whole, Matrigel and PuraMatrix at gelating concentrations showed a lower toxicity than PF127 to hNSCs.

Besides low cytotoxicity, an ideal biomaterial scaffold should allow the full differentiation capacity of hNSCs. Along this line, primed hNSCs mixed with Matrigel and PuraMatrix revealed different differentiation patterns. Almost every cell in Matrigel stained positively for GFAP, indicating either an astroglial or undifferentiated phenotype. In contrast, PuraMatrix supported both astroglial and neuronal differentiation in a similar pattern as the primed hNSC control group without a hydrogel. Interestingly, as the concentration of Matrigel was decreased, more TuJ1+ staining was observed, but the vast majority of cells were still GFAP+ (unpublished observation). This indicated that one or more components incorporated into the Matrigel prevented neuronal differentiation or survival. Another observation was that hNSCs mixed with Matrigel had larger nuclei. Hypertrophy of astrocytic nuclei has previously been described in activated astrocytes (Albrecht et al., 2002). Since Matrigel incorporates several growth factors and other extracellular matrix components, it is perceivable that one or more of these factors may be inducing the morphological change in the hNSC-derived astroglia. Our observation seems contradictory to the previous reports of a supporting role of Matrigel for neuronal differentiation and migration of human and rodent neural progenitor cells (Katakowski et al., 2005; Flanagan et al., 2006). The discrepancy may be due to the different concentrations of Matrigel used in these studies as we observed the most inhibition on neuronal formation from hNSCs when 50% Matrigel was used (total protein concentration of 10 mg/ml), which is 60 and 18 times more concentrated than what were used in the previous neuronal differentiation (Flanagan et al., 2006) and migration (Katakowski et al., 2005) studies, respectively.

While it is important to test markers for all three lineages when evaluating the differentiation capacity of hNSCs, oligodendrocytes expressing the O4 marker are undetectable in vitro until approximately 2–3 weeks post-differentiation using the priming and differentiation procedure described in these experiments (unpublished observation). Although the slow biodegradation of hydrogels is a necessity after transplantation in vivo, just a 7-day differentiation was chosen for this in vitro experiment to minimize hydrogel degradation due to a diluting effect caused by medium changes. A short-term differentiation allowed for the evaluation of differentiation during the critical period in which hNSCs were in direct contact with the hydrogel materials. Consequently, immunostaining for oligodendrocytes may be performed post-transplantation in vivo. Previously, we have shown that primed hNSCs have the capacity to differentiate into oligodendrocytes at least 3 months after transplantation (Tarasenko et al., 2007). Furthermore, the self-renewal property of hNSCs was not determined in this short-term in vitro study. With the purpose of replacing functional neural cells (neurons and glia), further in vivo studies are warranted to confirm that hNSCs mixed with PuraMatrix hydrogel do not form tumors after long-term transplantation.

For transplantation, we routinely prime cells on a poly-D-lysine- and laminin-coated surface first and then collect cells for transplantation. This allows us to obtain neurons when the primed hNSCs are grafted into non-neurogenic areas in adult rats (Wu et al., 2002; Tarasenko et al., 2007). Collecting primed cells and embedding them within the hydrogels would most appropriately mimic the environment that the cells would experience post-transplantation. However, when this particular experimental paradigm was attempted, too many cells were lost during the immunostaining process. Thus, in order to mimic the procedure of priming and grafting hNSCs to some extent while maintaining the capability to test differentiation through immunostaining, we added the hydrogels on top of the primed hNSCs, which were attached to poly-D-lysine- and laminin-coated glass coverslips. This method still allowed the hydrogels to interact with hNSCs during the differentiation process and mimicked the in vivo situation in that nutrients had to penetrate through the hydrogels to reach hNSCs. However, a limitation of this in vitro model was that the hNSCs were receiving cues from the coated coverslips as well as the hydrogels rather than solely the hydrogels. The effect of the extra cues in vitro compared to the condition post-transplantation is yet to be determined.
Since a low concentration of PuraMatrix hydrogel showed sufficient gelling capability, low toxicity and supported a normal differentiation pattern, we next sought to determine the migration capability of hNSCs embedded as spheres within PuraMatrix. We found that hNSCs were able to migrate from spheres in gelled PuraMatrix. In vitro, hydrogels would be expected to physically impede migration to some extent (e.g., physically block the extension of neurites of a cell) when compared to controls without hydrogels. However, hydrogels may enhance migration capability across the injured cavity in vivo due to their ability to provide a three-dimensional matrix scaffold suitable for cellular adhesion, migration and support. This migration capability of hNSCs through a PuraMatrix-formed scaffold will be important in terms of enhancing cell potentials to fill the cavity resulting from brain or spinal cord injuries.

4. Conclusion

The ultimate goal of the present study was to determine an optimal hydrogel that can form scaffolding for hNSC differentiation when placed in cavities in the damaged brain or spinal cord. Such a hydrogel should show low toxicity to hNSCs and the scaffold should permit hNSC adhesion, migration and differentiation. Among the three hydrogels tested in this study, PuraMatrix was most suitable for hNSC transplantation for the following reasons: 1) PuraMatrix showed low toxicity and suitable gelling capability at a low concentration, 2) Unlike PF127 and Matrigel, PuraMatrix did not require freezing temperatures to maintain a fluid form, but rather gelled upon salt incorporation, 3) PuraMatrix allowed a similar differentiation pattern to hNSCs primed in the absence of a hydrogel material and 4) PuraMatrix allowed hNSC migration.

5. Experimental procedures

5.1. Hydrogels

Hydrogels tested were pluronic F127 (PF127) (Sigma, St. Louis, MO), Matrigel™ (High Concentration, total protein concentrations of 18–22 mg/ml, BD Biosciences, Bedford, MA) and PuraMatrix (1% w/v amino acids, BD Biosciences). In vitro, hydrogels would be expected to physically impede migration to some extent (e.g., physically block the extension of neurites of a cell) when compared to controls without hydrogels. However, hydrogels may enhance migration capability across the injured cavity in vivo due to their ability to provide a three-dimensional matrix scaffold suitable for cellular adhesion, migration and support. This migration capability of hNSCs through a PuraMatrix-formed scaffold will be important in terms of enhancing cell potentials to fill the cavity resulting from brain or spinal cord injuries.

5.2. Cell culture

Human fetal NSCs, line K048, were isolated from an 8-week fetal forebrain (Svendsen et al., 1998). Cells were cultured as neurospheres and passaged every 10–11 days according to our previous description (Tarasenko et al., 2004). Growth media contained a basic medium supplemented with 20 ng/ml recombinant human epidermal growth factor (EGF) (R&D Systems, Minneapolis, MN), 20 ng/ml recombinant human basic fibroblast growth factor (bFGF) (R&D Systems), 5 μg/ml heparin (Sigma), and 10 ng/ml recombinant human leukemia inhibitory factor (LIF) (Chemicon, Temecula, CA). The basic medium is serum-free defined medium containing DMEM (high glucose, L-glutamine)/Ham’s F12 (3:1) (Invitrogen/Gibco, Grand Island, NY), 15 mM HEPES (Sigma), 1.5% D-glucose (Sigma), 67 IU/ml/67 μg/ml penicillin/streptomycin (Cellgro, Herndon, VA), 25 μg/ml bovine insulin (Sigma), 100 μg/ml human transferrin (Sigma), 100 μM putrescine (Sigma), 20 nM progesterone (Sigma), 30 nM sodium selenite (Sigma) and 2 mM L-glutamine (Sigma). Cells were incubated at 37 °C with 8.5% CO2 to maintain pH 7.4–7.5.

5.3. Cell priming and differentiation

Priming was performed by plating hNSCs (passage # 15–32) either in 96-well plates for the WST-1 assay or onto German glass coverslips (Carlosina Biological Supply, Burlington, NC) in 24-well plates for immunostaining. The plates and coverslips were pre-coated with 0.01% poly-D-lysine (PDL) (Sigma) in calcium and magnesium free Dulbecco’s phosphate-buffered saline (dPBS) (Cellgro) for at least 1 h at 37 °C and then 0.5 μg/cm² of laminin (LMN) (Invitrogen/Gibco) in dPBS overnight at 37 °C. Spheres, 3–4 days post-passage, were plated onto PDL/LMN-coated areas at 1×10⁶ cells/0.28 cm² for the WST-1 assay or 2×10⁶ cells/1.8 cm² for immunostaining in the basic medium supplemented with 10 ng/ml bFGF, 2.5 μg/ml heparin and 1 μg/ml LMN for 5 days. Half of the medium was changed after 2 days. After 5 days in priming medium, 80% of the medium was changed to a B27 medium containing DMEM/Ham’s F12 (3:1), 15 mM HEPES, 1.5% d-glucose, 67 IU/ml/67 μg/ml penicillin/streptomycin and B27 (Invitrogen/Gibco) to allow for differentiation. When cells were mixed with PF127 or Matrigel, the hydrogels were diluted with B27 medium to the appropriate concentration and then added on top of the primed hNSCs after the priming medium was removed. After solidification of these hydrogels, additional B27 medium was added to avoid cellular desiccation. PuraMatrix was first diluted to the appropriate concentration with 10% sucrose and then added on top of the primed hNSCs after removal of the priming medium. B27 medium was added immediately to initiate solidification of the PuraMatrix and to avoid cellular desiccation. Cells in B27 medium were maintained at 37 °C with 5% CO2 to maintain pH 7.4–7.5. When cells were allowed to differentiate longer than 2 days, half of the B27 medium was changed every 3–4 days.

5.4. WST-1 assay

Cell viability was assessed using WST-1 (Roche, Indianapolis, IN). Mitochondrial dehydrogenases in live cells cleave WST-1, a tetrazolium salt, into a colorimetric product, formazan, which was assayed to determine the amount of viable cells. Spheres were plated in a 96-well plate at approximately 1×10⁴ cells/0.28 cm² and primed for 5 days. Priming medium was then removed and PF127 (10%, 20%, 30%), Matrigel (1%, 10%, 50%) and PuraMatrix (0.25%, 0.5%, and 1%) (n=7/group) were added on top of the primed hNSCs for 2 days. Another group of primed hNSCs was changed to just B27 medium for 2 days to serve as a priming control. After 2 days, the WST-1 reagent was incubated at a dilution of 1:10 for 1.5 h. The absorbance of the formazan produced by the metabolically active cells in each sample was measured at a wavelength of 450 nm with a 630 nm reference using an ELx808uv Universal Microplate Reader (Biotek Instruments, Inc., Winoski, VT). Absorbance readings were normalized against control wells with medium alone. A one-way ANOVA and Dunnett multiple-comparisons...
test was used to compare each hydrogel group to the priming control group. A \( p \) value less than 0.05 was considered statistically significant. Data were expressed as means±S.E.M. Statistical analyses were done using GraphPad Prism Version 4 software (GraphPad Software, Inc., San Diego, CA).

5.5. Immunofluorescent staining

Four-day spheres that were allowed to differentiate in PuraMatrix for 7 days and 5-day spheres were embedded in Optical Cutting Temperature (O.C.T.) medium (Tissue-Tek, Tokyo, Japan) and sectioned on a Leica CM1900 cryostat (Meyer Instruments, Houston, TX) into 15 μm sections. These sections along with cells that were primed and then differentiated in Matrigel, PuraMatrix or B27 medium alone for 7 days were fixed for 20 min with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature and rinsed three times with 0.1 M PBS pH 7.4. Cells were then permeabilized for 1 h at room temperature with 0.25% Triton X-100 (Sigma) and treated with 0.3% bovine serum albumin (BSA) (Sigma) with 5% normal goat serum (NGS) in Tris-buffered saline (TBS) pH 7.4 to block nonspecific bindings. Primary antibodies were diluted in 0.1% Triton X-0.3% BSA/TBS to the following working concentrations: neuronal class III β-tubulin (TuJ1) 1:5000 (Babco, Berkeley, CA) and glial fibrillary acidic protein (GFAP) 1:1000 (Chemicon). Cells were incubated with primary antibodies overnight at 4 °C and rinsed with TBS prior to the addition of the secondary antibody. Alexafluor 488-conjugated secondary goat anti-mouse and 568-conjugated goat anti-rabbit antibodies (Molecular Probes, Eugene, OR) were both diluted 1:300 in 0.1% Triton X-0.3% BSA/TBS and added to cells for 3 h at room temperature in the dark. Cells were then washed with PBS and cell nuclei were counterstained with 1 μg/ml DAPI (Sigma) in TBS for 5 min at room temperature in the dark. Glass coverslips with cells were mounted onto glass slides and microscope coverglass (Fisher Scientific, Pittsburgh, PA) was mounted onto sectioned neurospheres with Fluoromount-G (Fisher, Fair Lawn, NJ). Fluorescent images were taken on a Nikon Eclipse 80i epifluorescent microscope.

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