Control of Neural Cell Composition in Poly(Ethylene Glycol) Hydrogel Culture with Soluble Factors

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Poly(ethylene glycol) (PEG) hydrogels are being developed as cell delivery vehicles that have great potential to improve neuronal replacement therapies. Current research priorities include (1) characterizing neural cell growth within PEG hydrogels relative to standard culture systems and (2) generating neuronal-enriched populations within the PEG hydrogel environment. This study compares the percentage of neural precursor cells (NPCs), neurons, and glia present when dissociated neural cells are seeded within PEG hydrogels relative to standard monolayer culture. Results demonstrate that PEG hydrogels enriched the initial cell population for NPCs, which subsequently gave rise to neurons, then to glia. Relative to monolayer culture, PEG hydrogels maintained an increased percentage of NPCs and a decreased percentage of glia. This neurogenic advantage of PEG hydrogels is accentuated in the presence of basic fibroblast growth factor and epidermal growth factor, which more potently increase NPC and neuronal expression markers when applied to cells cultured within PEG hydrogels. Finally, this work demonstrates that glial differentiation can be selectively eliminated upon supplementation with a γ -secretase inhibitor. Together, this study furthers our understanding of how the PEG hydrogel environment influences neural cell composition and also describes select soluble factors that are useful in generating neuronal-enriched populations within the PEG hydrogel environment.

Introduction

CELLS ISOLATED FROM the rat forebrain at the peak of neurogenesis (E14-15) contain two major cell populations: neurons and multipotent neural precursor cells (NPCs).¹ The NPCs are important because they function as a model system for neural lineage development and lay the foundation for neural cell therapies.¹⁻⁴ When grown *in vitro*, NPCs proliferate for a period of time before some undergo neuronal differentiation, and the remainder gradually restricts their potential until they generate only glia.⁵⁻⁸ Increasing neuronal yields from NPC populations are a priority for treating neurodegenerative diseases. Toward that end, researchers have used this cell population to identify soluble factors such as basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) that can increase overall cell supplies by maintaining multipotent NPCs in a proliferative state.^{8,9} Still other soluble factors such as the γ -secretase inhibitor, N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT), can increase the percentage of NPCs that undergo neuronal rather than glial differentiation.^{10,11}

Much of this research has been performed in monolayer culture; however, characterizing and controlling NPCs

within three-dimensional (3D) tissue engineering matrices is of increasing importance given the potential of these matrices to support integration of transplanted cells.¹²⁻¹⁶ Poly(ethylene glycol) (PEG)-based hydrogels are a particularly useful matrix for studying the effects of soluble factors on NPC behavior in 3D because, like monolayer cultures, they provide a minimally instructive environment devoid of exogenous peptides. The overall objective of this work is to assess if neural cells seeded within PEG hydrogels are as responsive to bFGF, EGF, and DAPT as are cells in monolayer culture. However, further characterization is first needed in the absence of soluble factors to define how the baseline cell composition present throughout PEG hydrogel culture compared to standard monolayers. To date, all PEG hydrogel studies containing quantitative cell composition data included bFGF in the culture media, which influences NPC specification.^{17,18} Further, cell composition shortly after seeding within PEG hydrogels has not yet been examined.

Seeding dissociated tissue within PEG hydrogel rather than monolayer culture exposes cells to a unique environment that may alter the composition of surviving cells. For example, PEG reduces adsorption of cell-derived proteins compared to tissue culture polystyrene (TCPS),^{19,20} so

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adherent-dependant cells seeded within PEG hydrogels may be more vulnerable to anoikis-mediated death.^{21,22} Also, the photopolymerization process requires acute exposure of encapsulated cells to free radicals. Given that proliferative cells are more resistant to anoikis²³ and free-radical oxidation,²⁴ PEG hydrogels might select against neurons and enrich the surviving population for NPCs, even when using PEG hydrogel conditions that best preserve cell viability (100% degradable macromer²⁵ and 7.5–10 wt%²⁶). Also, single NPCs proliferate in PEG hydrogels to form immobilized spherical clusters²⁷ that create a long-term microenvironment more reminiscent of clonal neurospheres than monolayers. Finally, the low compressive modulus of 7.5 wt% PEG hydrogels resembles native brain tissue²⁶ but differs greatly from stiff TCPS, which eventually permits glial over growth.^{12,26}

Here, biochemical and immunological assays are used to identify differences in cell composition when neural cells are seeded within PEG hydrogel compared to monolayer cultures. To postulate which environmental attribute of PEG hydrogel culture might cause observed differences, results are also compared against numerous control cultures as follows: (1) to assess the impact of anoikis, cells are encapsulated into PEG hydrogels containing extracellular matrix (ECM) proteins or growth factors that can inhibit anoikis by, respectively, activating or cross-activating integrin receptors^{21,22,28,29}; (2) to assess the impact of photopolymerization, cells are encapsulated within temperature-curable, inert agarose hydrogels; (3) to assess the impact of growth within cell-dense clusters, cells are grown as suspended neurospheres in the absence of PEG hydrogels; and (4) to assess impact of substrate stiffness, cells are seeded as monolayers onto the surface of stiffness-matched PEG hydrogels.

After characterizing cell composition in the absence of soluble factors, the effectiveness of bFGF, EGF, and DAPT is assessed when applied to PEG hydrogel cultures as compared to monolayers. The extent to which bFGF and EGF can maintain NPCs in each culture system may vary if significant differences are observed between the baseline cell compositions. The expected reduction in glial presence within PEG hydrogels may preserve growth factor action for NPCs. Previous studies supplementing with bFGF have not assessed nestin or glial fibrillary acidic protein (GFAP) expression levels to indicate whether NPCs and/or glia, respectively, were the proliferative cell population within PEG hydrogels.^{26,27,30,31} EGF has not yet been applied to PEG hydrogel cultures and may be more useful than bFGF for maintaining NPCs that are competent for neurogenesis.^{8,32} In fact, growth factor concentrations present in the bulk media may not even equal the levels present within NPC-laden hydrogels because NPCs express cell-surface syndecan-1,³³ which might immobilize and concentrate growth factors^{34,35} within the hydrogel. This is particularly true within the PEG hydrogel environment because the small hydrogel mesh size (~ 15 nm)²⁷ can restrict the diffusion of exogenously applied or cell-derived proteins.^{26,36}

Finally, the ability of DAPT to achieve selective neuronal differentiation within PEG hydrogel cultures is assessed as compared to monolayers. Efficiently inducing NPCs to undergo neuronal rather than glial differentiation within PEG hydrogel cultures has not yet been achieved. DAPT supplementation may be an effective approach given that, even in

cell dense cultures, DAPT can inhibit the ligand-dependent, γ -secretase-mediated cleavage and activation of notch receptors that is required for the onset of gliogenesis.^{10,11,37,38}

Materials and Methods

Neural cell isolation and culture

Neural tissue was obtained from E14–15 rat forebrains (Charles River) and dissociated as described.³¹ The single cell suspension was seeded into each culture system as described (Preparation of PEG hydrogel, monolayer, and control cultures section) and cultured at 37°C and 5% CO₂ in DMEM:F12 (MediaTech) supplemented with 1 $\times$ N2 (Invitrogen), 100 U/mL penicillin–streptomycin (Hyclone), and 1 mM L-glutamine (Invitrogen). Select experiments included 20 ng/mL bFGF (Sigma) and/or EGF (Sigma), or 0.25 μ M DAPT (Chemicon). The medium was refreshed every other day.

Preparation of PEG hydrogel, monolayer, and control cultures

PEG hydrogel. Degradable, methacrylated PEG macromers (4600 g/mol) were synthesized, purified, and characterized as described previously.³¹ Macromers were verified to be free of impurities, contained an average of 2.5 lactide units on each end of the PEG monomer, and possessed methacrylation efficiencies greater than 80%. Cells were encapsulated within PEG hydrogels by combining PEG macromer (7.5% [w/w]), photoinitiator (0.025% [w/w]) (Irgacure 2959; Ciba), and cells (1×10^7 cells/mL) to make a prepolymerization solution. The solution was polymerized within an optically transparent mold (0.5 mL syringe with the tip removed) upon exposure to long-wavelength, low-intensity ultraviolet light (365 nm, ~ 4 mW/cm²) (UVP) for 10 min (unless otherwise indicated). Resulting hydrogels were sectioned into 3-mm thick discs.

Two-dimensional monolayer. Cells (2×10^5 cells/cm²) were seeded upon poly(ornithine)-coated (Sigma) TCPS or 7.5% (w/w) PEG hydrogels as described.²⁶ Cells seeded as two-dimensional (2D) monolayers upon PEG hydrogels are termed “2D-PEG.”

Neurosphere. Cells (2×10^5 cells/cm²) were seeded in untreated wells upon a rotating shaker. Periodic trituration was performed for 24 h to prevent cell attachment. After 2–3 days, suspended spherical neurospheres formed.

Agarose. Cells (1×10^7 cells/mL) were encapsulated within 1% low melting-point agarose (Sigma) gels upon exposure to 4°C for 10 min using molds described above then sectioned into 3-mm discs. Agarose concentration was chosen to match the day 1 compressive modulus of PEG hydrogels (data not shown).

ECM protein-loaded PEG hydrogel. Purified fibronectin (FN), collagen-4 (Col4), and collagen-1 (Col1) (all from Invitrogen) were PEGylated as described in³⁹ and Supplementary Methods, and incorporated at 250 μ g/mL. Resulting hydrogels are termed “PEG-FN,” “PEG-Col1,” and “PEG-Col4.” ECM protein mass accounted for <0.025% of the gel mass and did not significantly alter hydrogel compressive

modulus or mass swelling ratios (Supplementary Fig. S1a; Supplementary Data and Methods are available online at www.liebertonline.com/tea). Even ECM distribution was verified immunologically, and covalent ECM incorporation was confirmed by bicinchoninic acid assessment (Pierce) of protein concentration over time (Supplementary Fig. S1c, b).

Cell viability assessments

Measurement of ATP, DNA, and caspase in cell cultures. Total caspase activity, and ATP and DNA content was, respectively, measured using the Apo-one (Promega), Cell-glow (Promega), and Quant-iT PicoGreen dsDNA assay kit (Invitrogen) as described.²⁶ At discrete time points, samples were collected from monolayer wells ($n=18$), PEG hydrogels ($n=18$), agarose hydrogels ($n=12$), neurosphere wells ($n=12$), 2D-PEG gels ($n=6$), and ECM-loaded PEG hydrogels ($n=15$) obtained from three different experiments.

Visualization of live and dead cells. Live and dead cells were labeled using calcein-AM (Invitrogen) and ethidium bromide (Invitrogen). At discrete time points, samples were collected from monolayer wells ($n=10$), PEG hydrogels ($n=10$), agarose hydrogels ($n=10$), neurosphere wells ($n=10$), 2D-PEG gels ($n=6$), and ECM-loaded PEG hydrogels ($n=15$) obtained from three different experiments. Samples were imaged as described in Image collection and analysis section.

Assessment of cell composition

qRT-PCR. qRT-PCR was performed as described.²⁶ Purified RNA was accurately quantified using RiboGreen Quantification Reagent (Invitrogen), and 100 ng was included in each reverse transcriptase reaction, thus normalizing reactions for cell number differences and alleviating the need for house-keeping genes.⁴⁰ Primers (Invitrogen) are listed in Table 1 and efficiencies were confirmed to be <90%. Melt curves and DNA electrophoresis was used to confirm single product amplification of the correct size. At discrete time points, samples were collected from monolayer wells ($n=12$), PEG hydrogels ($n=16$), agarose hydrogels ($n=6$), neurosphere wells ($n=9$), 2D-PEG gels ($n=6$), and ECM-loaded PEG hydrogels ($n=9$) obtained from two to three different experiments.

Immunocytochemistry. At discrete time points, samples were collected from monolayer wells ($n=5$) and PEG hydrogels ($n=5$) obtained from two to three different experiments. Samples were fixed in 4% paraformaldehyde (Sigma). Hydrogels were cryoprotected using 15% sucrose (Sigma), and then cyrosectioned into 40 μ m slices. Standard immunological

techniques²⁶ were employed to stain samples with primary antibodies (Chemicon) directed against nestin (1:1000, MAB353), β -tubulin III (1:1000, MAB380), and GFAP (1:1000, AB5804); secondary antibodies (Invitrogen; 1:400 dilution) Alexafluor 546 conjugated goat-anti-mouse or goat-anti-rabbit; and 40,6-diamidino-2-phenylindole (DAPI) (1 μ g/mL; Invitrogen) for nuclear counterstaining. Samples were imaged as described in the Image collection and analysis section.

Image collection and analysis

Images ($n=10$ from two to three experiments) were acquired using a confocal microscope (Zeiss) equipped with a 10 $\times$ water immersion objective. Each image represents a z-stack compiled from 10 optical slices acquired at equal intervals spanning the entire depth of monolayer and 100 μ m into hydrogels. The number of live/dead cells and negative/positive nuclei per image were manually counted by two independent researchers. Cells were considered positive for an antigen when cell processes displayed the immunocytochemical signal. Pyknotic nuclei of apoptotic cells were also quantified to account for total cell numbers (data not shown).

Growth factor sequestration

Purified bFGF (Sigma) was fluorescently labeled with Alexafluor 546 microscale protein labeling kit (Invitrogen). Cells were encapsulated within PEG hydrogels as described (Preparation of PEG hydrogel, monolayer, and control cultures section) at increasing concentrations: 0 $\times$, 0.5 $\times$, 1 $\times$, 2 $\times$, and 4 $\times$ ($x=1 \times 10^7$ cells/mL). On day 7, cell-loaded control samples were collected. The culture medium was then replaced with the medium containing fluorescent bFGF. After a 4 h incubation, $t=0$ samples were collected. Remaining samples were placed in the medium containing no fluorescent bFGF, and samples were collected at select time intervals to monitor the release of fluorescent bFGF from the hydrogels. At each interval, $n=12$ hydrogels from three different experiments were collected and individually homogenized in 200 μ L of PBS using disposable pestles, and fluorescence intensity was quantified using a fluorometer (Fluostar Optima). To account for cellular autofluorescence, data are presented as the average fluorescence intensity measured when cell-loaded hydrogels were incubated with fluorescent bFGF minus the values obtained from control samples. Data are normalized with respect to 1 $\times$ (Fig. 3e) or $t=0$ (Fig. 3f).

Statistical analysis

Statistical significance was determined using two-tailed Student's t -test. The standard significance threshold $\alpha=0.05$

TABLE 1. qRT-PCR PRIMER SEQUENCES

Cell type	Gene	Forward primer (5' $\rightarrow$ 3')	Reverse primer (5' $\rightarrow$ 3')
NPC	<i>Nestin</i>	gtggcctctgggatgatg	ttgaccttctccccctc
Neuron	<i>Syndecan-1</i>	acacgcatagcacttaccc	ctgctggggctctaaaacag
Glia	<i>β-tubulin III</i>	gtttgtgatgggtgtgaacc	tcttctgagtggcagtgatg
N/A	<i>GFAP</i>	aggacaaatctcacacagg	gactcaaccttctctcca
	<i>Hairy and enhancer of split-1 (Hes-1)</i>	cccacctctctctctgacg	aggcgcaatccaatatgaac

NPC, neural precursor cells; N/A, not applicable; GFAP, glial fibrillary acidic protein.

is used unless otherwise stated. In select experiments, Bonferroni correction was applied when multiple pair-wise testing required more stringent significance thresholds. Data are presented as mean $\pm$ SEM.

Results

Baseline cell survival in the absence of soluble factors

The extent to which PEG hydrogel and monolayer culture contain apoptotic or metabolically active, live cells was assessed over time. Results show that by day 1 of PEG hydrogel culture, caspase activity increased 3.2 ± 0.4 -fold (Fig. 1a), ATP levels decreased to 0.45 ± 0.10 of day 0 levels (Fig. 1b), and only $43\% \pm 6\%$ of cells were alive (Fig. 1c). In monolayer culture, no significant changes in day 1 caspase activity or ATP levels were detected and $66 \pm 5\%$ of cells were alive (Fig. 1a–c). Viability values observed in PEG hydrogels recovered by day 3 and did not significantly differ from time-matched monolayers for the remainder of the culture period (Fig. 1a–c). Despite the initial loss of

viable cells, DNA levels steadily increased over time, suggesting that cells survive and proliferate in both culture systems (Fig. 1d).

Day 1 viability in PEG hydrogels was not improved upon growth factor supplementation (Fig. 1e); while ECM-loaded hydrogels resulted in a reduction of day 1 caspase activity (0.65–0.69 of caspase levels in control PEG hydrogels), ATP levels and the percentage of live cells did not significantly improve (Fig. 1e) even after increasing ECM concentrations to 1 mg/mL (data not shown). Day 1 viability was higher in the three control culture conditions that do not require free-radical polymerization (3D agarose, 2D-PEG, and neurosphere), as they exhibited decreased caspase activity (0.3–0.4-fold), increased ATP levels (1.7–2.1-fold), and a higher percentage of live cells (1.5–1.7-fold) relative to day 1 PEG hydrogel culture (Fig. 1e). As further evidence that photopolymerization is responsible for the day 1 cell death in PEG hydrogels, decreasing day 1 ATP levels were observed as the length of time that cells were exposed to free-radical polymerization increased (Fig. 1f).

FIG. 1. Cell viability in poly(ethylene glycol) (PEG) hydrogel culture. **(a)** Cellular apoptosis was assessed by quantifying caspase activity. **(b)** Cellular metabolic activity was assessed by quantifying ATP concentration normalized with respect to DNA concentration. **(c)** The percent of live cells was determined after direct visualization then manual quantification of live and dead cells. **(d)** Cell number was assessed by quantifying total DNA concentration. For **(a)**, **(b)**, and **(d)**, data acquired on days 0, 1, 3, 7, and 14 are normalized relative to day 0 for both PEG hydrogel and monolayer cultures. For **(a–d)**, asterisks indicate statistically significant differences in PEG hydrogel relative to time-matched monolayer cultures. **(e)** Day 1 caspase activity (white bars), ATP/DNA (gray bars), and the % of live cells (black bars) were determined when cells were cultured in PEG hydrogels that were either loaded with extracellular matrix proteins (left plot) or cultured in the presence of growth factors (middle plot). In addition, these parameters were determined in control agarose, neurosphere, two-dimensional PEG hydrogel, and monolayer conditions (right plot). Data are normalized relative to values observed in day 1 PEG hydrogel culture (represented by dotted line). Asterisks indicate statistically significant differences relative to day 1 PEG hydrogel culture. **(f)** Day 1 metabolic activity was determined after photopolymerization was performed using increasing ultraviolet exposure times. Asterisks indicate statistically significant differences relative to the standard 10 min polymerization time that results in day 1 values displayed in **(b)**. Significance thresholds are as follows: **(a, c, e)** (caspase and % live): $p < 0.005$; **(b, e)** (ATP/DNA); **(f)** $p < 0.0035$; **(d)** $p < 0.05$.

Baseline cell composition in the absence of soluble factors

qRT-PCR and immunocytochemistry were utilized to characterize cell composition changes over time in PEG hydrogel compared to monolayer culture. Freshly isolated cells contained 50%±3% nestin-positive NPCs, 57%±4% β -tubulin-positive neurons, and 0±0% GFAP-positive glia (Fig. 2g). After 1 day of growth within PEG hydrogels, the percentage of nestin-positive NPCs (45%±9% positive) and mRNA levels (1.0 ± 0.1) were maintained at day 0 levels ($p>0.05$) (Fig. 2a, b, h), whereas the percentage of β -tubulin-positive neurons (8%±4%) and mRNA levels (0.24 ± 0.05) significantly decreased (Fig. 2c, d, h). In comparison, after 1 day of growth within monolayer culture, a significant decrease in nestin-positive NPCs (26%±4%) and mRNA levels (0.30 ± 0.10) (Fig. 2a, b) was observed, but there was only a slight decrease in β -tubulin-positive neurons (45%±5%) and mRNA levels (0.80 ± 0.09) (Fig. 2c, d, h). No GFAP-positive glia were present in either culture system (0%±0%) (Fig. 2f, h), and pyknotic nuclei accounted for the remaining marker-negative nuclei (data not shown). These results suggest that dissociated neurons are selectively damaged early in PEG hydrogel culture. Consistent with the survival

data, day 1 β -tubulin mRNA expression was not rescued upon ECM protein incorporation, but was significantly higher in control cultures that do not require photopolymerization (Table 2).

After day 1, the percentage of β -tubulin-positive neurons and mRNA expression in PEG hydrogels recovered to levels observed in time-matched monolayer cultures (Fig. 2c, d, i). Meanwhile, the percentage of nestin-positive NPCs and mRNA expression decreased, but were maintained at higher levels in PEG hydrogel compared to monolayer culture (Fig. 2a, b, i). The percentage of GFAP-positive glia and mRNA expression rose over time in both systems, though significant suppression of glial growth was observed in PEG hydrogels (3%±1% on day 14) relative to monolayer culture (19%±4% on day 14) (Fig. 2e, f, i). The high GFAP-mRNA levels observed in monolayer culture were reduced to levels observed in time-matched PEG hydrogel cultures when cells were grown as monolayers upon stiffness-matched hydrogel surfaces (Table 2, 2D-PEG).

bFGF and EGF supplementation

Given the opposing bias of PEG hydrogels for higher NPC percentages and monolayer cultures for increased glia, it

FIG. 2. Cell composition in the absence of soluble factors. The expression of nestin, β -tubulin III, and glial fibrillary acidic protein (GFAP) was assessed on days 0, 1, 7, and 14 of culture in both monolayer and PEG hydrogel culture using qRT-PCR [left column (a, c, e)] and immunocytochemistry [right column (b, d, f)]. Asterisks indicate statistically significant differences in hydrogel values relative to time-matched monolayer values. Representative immunocytochemistry images observed 4 h after dissociation (g), and after culturing in PEG hydrogel or monolayer culture for 1 day (h) or 14 days (i). DAPI is used as a nuclear counterstain. Scale bar=30 μ m and applies to all images. (i) Inset shows rare GFAP-positive cell cluster within PEG hydrogel culture on day 14, though most clusters exhibited very diffuse staining. Significance threshold is $p<0.0055$. qRT-PCR, quantitative reverse-transcription polymerase chain reaction. Color images available online at www.liebertonline.com/tea

TABLE 2. qRT-PCR RESULTS IN CONTROL CULTURES

Culture condition	Gene	Time in culture (days)			
		0	1	7	14
Neurosphere	<i>Nestin</i>	1.0±0.2	0.7±0.1	0.3±0.1	0.2±0.0
	<i>β-tubulin III</i>	1.0±0.2	0.6±0.0 ^a	0.6±0.0	0.6±0.1
	<i>GFAP</i>	1.0±0.4	0.8±0.2	104.3±14.1	5601.4±912.3 ^a
2D-PEG hydrogel	<i>Nestin</i>	1.0±0.1	0.2±0.1 ^a	0.2±0.0 ^a	0.1±0.0 ^a
	<i>β-tubulin III</i>	1.0±0.1	0.7±0.1 ^a	0.6±0.1	0.7±0.1
	<i>GFAP</i>	1.0±0.4	1.2±0.6	92.8±13.6	1082.9±209.3
FN-PEG hydrogel	<i>Nestin</i>	1.0±0.1	0.9±0.1	0.9±0.3	0.6±0.3
	<i>β-tubulin III</i>	1.0±0.1	0.2±0.4	1.1±0.5	0.9±0.2
	<i>GFAP</i>	1.0±0.3	1.5±0.8	94.0±16.0	1079.0±217.0
Agarose hydrogel	<i>Nestin</i>	1.0±0.0	0.4±0.1 ^a	0.1±0.0 ^a	0.1±0.0 ^a
	<i>β-tubulin III</i>	1.0±0.2	0.6±0.2 ^a	0.1±0.1 ^a	0.1±0.0 ^a
	<i>GFAP</i>	1.0±0.4	3.2±0.6	444.8±31.4 ^a	3952.2±1089.6 ^a

qRT-PCR was employed to assess the expression of *nestin*, *β-tubulin III*, and *GFAP* on days 0, 1, 7, and 14 when cells were cultured in neurosphere, as 2D-PEG gels, in FN-loaded PEG hydrogels, and in agarose hydrogel control conditions.

^aStatistically significant differences in gene expression relative to time-matched PEG hydrogel cultures. Significance threshold is $p < 0.0055$. 2D, two-dimensional; FN, fibronectin; PEG, poly(ethylene glycol).

was interesting to compare growth factor-mediated NPC maintenance in each culture system. The comparison was made on day 7 of culture because growth factor supplemented monolayer cultures became over-confluent by day 14. Growth factor supplementation improved *nestin* maintenance in both culture systems whether bFGF and EGF were applied separately or in combination (Fig. 3a, b). More potent

increases in *nestin* expression were observed in PEG hydrogel (5–6-fold, Fig. 3a) compared to monolayer culture (2–3-fold, Fig. 3b). Slight growth factor-induced increases in *β-tubulin III* expression (<2-fold) were restricted to cells in PEG hydrogels (Fig. 3a). In contrast, growth factor-induced increases in *GFAP* expression were largely restricted to cells in monolayer culture (4–13-fold increase, Fig. 3b), though a

FIG. 3. Growth factor-mediated neural precursor cell maintenance in PEG hydrogel and monolayer culture. (a, b) qRT-PCR was employed to assess the expression of *nestin*, *β-tubulin III*, and *GFAP* on day 7 when cells were grown in both (a) PEG hydrogel and (b) monolayer culture in the presence of basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), or both bFGF & EGF. (c) qRT-PCR was employed to assess the expression of *syndecan-1* on day 7. (a–c) Asterisks indicate statistically significant differences in mitogen-supplemented conditions relative to no growth factor controls. (d, e) Growth factor sequestration was assessed by monitoring the concentration of fluorescently labeled bFGF in PEG hydrogels that contain increasing cell concentrations both (d) before and (e) after withdrawing fluorescent bFGF from culture media. Asterisks indicate statistically significant differences relative to 1× cell concentration. Significance thresholds are as follows: (a, b) is $p < 0.0055$, (c) $p < 0.05$, (d, e) $p < 0.0125$.

6-fold increase in *GFAP*-mRNA was observed in bFGF-supplemented PEG hydrogels (Fig. 3a).

Consistent with the increased NPC percentages observed in PEG hydrogel cultures, *syndecan-1* mRNA levels were observed to be 3.4 ± 0.4 -fold greater in PEG hydrogels than in monolayer cultures (Fig. 3c). A previous study demonstrated that *syndecan-1* levels are also reduced in 2D-PEG cultures.²⁶ Syndecan-1 sequesters bFGF,³⁵ so it was interesting to assess if bFGF was sequestered within cell-laden PEG hydrogels. Results show that increased cell density was correlated with increased bFGF concentrations (Fig. 3d) and slower bFGF release rates (Fig. 3e). No evidence of EGF sequestration was observed (data not shown).

DAPT supplementation

Finally, the ability of DAPT to achieve selective neuronal differentiation within both culture systems was assessed.

Consistent with reports demonstrating that DAPT interrupts notch signaling in monolayer culture,^{37,38} a 20-fold DAPT-induced decrease in the notch-dependent mRNA transcript, *Hes-1*, was confirmed in PEG hydrogel cultures (Supplementary Fig. S2). Cell composition results show that DAPT-treated cultures eliminated any increase in GFAP-positive glia and mRNA expression in both PEG hydrogel (Fig. 4a, b) and monolayer (Fig. 4c, d) cultures. DAPT supplementation also resulted in a faster decline of nestin-positive NPCs and mRNA, and a significant increase in β -tubulin-positive neurons and mRNA expression relative to untreated cultures by days 7 and 14 (Fig. 4a, c). The DAPT-induced decrease in both proliferative cell types correlated with a $\sim 50\%$ decrease in day 7 DNA content in both culture systems (data not shown), though cosupplementation of bFGF, EGF, and DAPT was an effective approach to increase total cell number without evidence of glial presence (Supplementary Fig. S2).

FIG. 4. N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT)-mediated neuronal differentiation in PEG hydrogel and monolayer culture. qRT-PCR (left column) and immunocytochemistry (right column) were employed to assess the expression of GFAP, β -tubulin III, and nestin on days 0, 1, 7, and 14 when cells were cultured in both PEG hydrogel (upper row) and monolayer culture (lower row) in the presence of DAPT. (a, c) The mRNA expression levels normalized relative to day 0 are displayed for DAPT-treated (thick lines) and untreated control cultures (thin lines). Untreated control data are replicated from Figure 2 to facilitate direct comparisons. Statistically significant differences in DAPT-treated culture relative to time-matched untreated cultures are indicated by offset asterisks. (b, d) The percentage of live cells positive for each cell-type-specific marker in both PEG hydrogel (upper row) and monolayer culture (lower row) are shown. Unlabeled cells with pyknotic nuclei are excluded from analysis. (c) Asterisks indicate statistically significant differences in DAPT-treated cultures relative to untreated control cultures. DAPI is used as a nuclear counterstain. Representative images of cell-type-specific immunolabeling observed in DAPT-treated cultures on day 14 are also displayed. Scale bar = 30 μ m and applies to all images. Significance threshold is $p < 0.0055$. Color images available online at www.liebertonline.com/tea

Discussion

Cell survival and composition in the absence of soluble factors

PEG hydrogels are being developed as cell delivery vehicles that have great potential to improve neuronal replacement therapies. This study characterized a therapeutically relevant cell population during *in vitro* PEG hydrogel culture. Consistent with previous reports,^{1,41} cell composition before encapsulation was comprised of roughly half NPCs, half neurons, and no glia. Enzymatic dissociation typically results in a 20%–30% loss of viable cells after 24 h in monolayer culture,⁴² similar to the $34\% \pm 5\%$ loss observed in monolayer cultures here. However, the cell loss observed in PEG hydrogels was more severe ($55\% \pm 7\%$ loss), suggesting that the PEG hydrogel environment itself may damage cells. Other studies have provided evidence of cell death early in PEG hydrogel culture, including images of ethidium-bromide-stained dead cells and decreases in day 1 metabolic activity to levels within range of those observed here.^{26,27}

PEG hydrogels deplete dissociated neurons. The cells that survive until day 1 of PEG hydrogel culture were depleted of neurons and enriched for NPCs. The initial neuronal death could be due to environmental factors such as a lack of ECM contacts or the presence of free radicals during photopolymerization. The rationale for incorporating growth factors and ECM proteins into the hydrogel environment was to test if anoikis is responsible for the observed neuronal death. We report here that neither growth factors nor specific ECM proteins known to support neuronal survival^{28,29,43–46} and prevent caspase activation in dissociated cells^{21,22} are able to rescue cells encapsulated into PEG hydrogels. We further demonstrate that neuronal survival is unimpaired within other culture systems that do not require free-radical polymerization despite their lack of ECM contacts and growth factors (i.e., agarose hydrogels and 2D culture upon PEG hydrogels). Finally, we demonstrate that improved cell viability is inversely correlated with photopolymerization exposure times. Together, these results suggest that free-radical polymerization rather than anoikis is predominantly responsible for the early neuronal death within PEG hydrogels. While the mechanism of free-radical damage was not explored, a previous study proposed that Igacure 2959-generated free radicals may cause direct peroxidation of plasma membrane lipids.^{47,48} Neurons may be particularly susceptible to free-radical damage given their lipid rich cellular membranes.⁴⁹

While maximizing the number of neurons present in PEG hydrogel culture is a priority for neuronal replacement therapies, efforts to rescue the fixed number of dissociated neurons may not be the most efficient approach to increase neuronal yields. Another attractive approach is to utilize soluble factors such as bFGF and EGF, which can stimulate NPC proliferation to generate increased cell numbers, and DAPT, which can efficiently promote NPCs, to undergo neuronal rather than glial differentiation. In this study, efforts were focused in the latter direction, so NPC maintenance and differentiation within PEG hydrogels was analyzed both in the absence and the presence of these soluble factors. For reference, results were compared to those observed in standard monolayer culture.

PEG hydrogels suppress glial growth. In the absence of soluble factors, surviving NPCs give rise to new neurons, and then to glia in both culture systems. After 1 week, no difference was observed between the percentages of neurons present in PEG hydrogel compared to monolayer culture. However, PEG hydrogels did suppress the percentage of glial cells and overall cell proliferation relative to monolayer cultures. Reduced glial growth in PEG hydrogels may be attributable to reduced substrate stiffness given that decreased GFAP expression was also observed in 2D-PEG cultures, and that previous reports have shown decreasing GFAP expression and neural cell proliferation on substrates of decreasing stiffness.²⁶ While a recent study reported decreases in GFAP expression on poly(dimethylsiloxane) substrates of increasing stiffness,¹¹ the results observed here are substantiated by a mechanistic study demonstrating reduced actin polymerization within glia and suppressed glial proliferation upon soft substrates.¹² Additional studies are needed to determine the mechanism by which neural cells sense substrate stiffness, possibly through traditional mechanotransduction after cell-secreted ECM proteins adsorb to the polymer surface.⁵⁰

PEG hydrogels enrich for NPCs. Despite the initial neuronal loss, PEG hydrogels enrich the initial cell population for NPCs and maintain higher NPC percentages throughout the culture period relative to monolayers. The increased *nestin* levels in PEG hydrogels were similar to those observed in neurosphere culture, suggesting that any potentially mitogenic factors within the PEG hydrogel environment itself, such as reactive oxygen species⁵¹ or lactic acid degradation products,⁴⁷ do not substantially contribute to *nestin* maintenance. It was also determined that aggregated growth is insufficient for increased *nestin* expression given the decreased *nestin* observed in agarose cultures. Instead, the prolonged increase in *nestin* expression is likely a residual effect of the NPC enrichment that occurs immediately in PEG hydrogels and gradually over a few days in neurosphere culture⁵² (data not shown). To what extent the original NPCs present upon dissociation are truly maintained within each culture system still remains open for investigation because no distinction was made in this study between multipotent NPCs, or transient amplifying neuronal or glial progenitors which may all express *nestin*. Nonetheless, the ability of the PEG hydrogel culture system to select for NPCs from a mixed cell population may be both scientifically and clinically useful, particularly given reports that neurosphere-forming NPCs need to be immobilized for unambiguous clonal analyses⁵³ and that NPCs undergoing terminal neuronal differentiation after transplantation *in vivo* integrate better with host neurons when compared to predifferentiated neurons.⁵⁴

bFGF sequestration within PEG hydrogels. The increased percentage of NPCs is accompanied by increased *syndecan-1* expression in PEG hydrogels relative to monolayer culture. *Syndecan-1* codes for an NPC surface protein known to sequester bFGF.^{35,55–57} While mechanistic studies are still needed to confirm direct interaction between *syndecan-1* and bFGF within the hydrogel, this study provides the first evidence of bFGF sequestration within neural PEG hydrogel cultures. Without sequestration, bFGF concentrations within

the hydrogel would be the same independent of cell density, or perhaps lower in high cell density cultures given more opportunity for receptor–ligand internalization.⁵⁸ In contrast, the results show a cell dose-dependent increase in bFGF concentration. In addition, about 30 min are required for proteins the size of bFGF (molecular weight=17.43 kDa, hydrodynamic radius= ~3–4 nm⁵⁹) to passively diffuse from PEG hydrogels,⁶⁰ but the results shown here demonstrate that the actual time required for complete release of bFGF from hydrogels increases in a cell dose-dependent manner.

Our data do not take into account how the fluorochrome-label impacts bFGF sequestration. While the theoretical diffusion coefficients of labeled and unlabeled bFGF are similar given the slight (3%) increase in molecular weight of labeled bFGF (18.01 kDa), a previous study demonstrated reduced binding affinity and sequestration of labeled compared to unlabeled bFGF within heparin-sulfate containing hydrogels that have very similar properties (97% water content, 2.51 kPa compressive modulus, 11.7 nm mesh size) to the PEG hydrogels used here.⁶¹ The tetramethylrhodamine label (430 g/mol) used in the previous study is smaller than the Alexafluor 546 label (1079 g/mol) used here, so it is possible that our study underestimates the extent of bFGF sequestration that occurs within PEG hydrogels. Nonetheless, this is the first study to our knowledge demonstrating increased bFGF concentrations within neural PEG hydrogel cultures relative to levels present in bulk media.

Cell composition in the presence of bFGF, EGF, and DAPT

The opposing bias of PEG hydrogels for higher NPC percentages and monolayer cultures for increased glia was accentuated upon supplementation with bFGF and EGF. Growth factors improved *nestin* maintenance in both culture systems as expected,^{8,9} but the increase observed in PEG hydrogels was more potent. Although mechanistic studies were not performed, we speculate that the intrinsically reduced glial presence within PEG hydrogels may preserve growth factor activity for NPCs. The slight growth factor-induced increase in β -tubulin III expression observed only in PEG hydrogels may translate to an insignificant increase in β -tubulin III protein levels.³⁰ The growth factor-induced increases in *GFAP* expression were more robust and largely restricted to monolayer culture. While bFGF also increased *GFAP* expression in PEG hydrogel culture, EGF supplementation increased *nestin* without increasing *GFAP* expression. This result is consistent with reports showing EGF supplementation results in glial-free NPC cultures either with or without bFGF provided that cultures are kept either at low cell densities⁸ or upon soft substrates.³²

Finally, DAPT supplementation significantly reduces notch-dependant transcription within PEG hydrogels, and efficiently prevents glial differentiation within both PEG hydrogel and monolayer cultures. This result is consistent with previous studies showing that constitutive notch activation promotes gliogenesis in peripheral and central nervous system progenitors⁶² and that notch inhibition induces a severe neurogenic phenotype in zebrafish,³⁷ increases neuronal differentiation in neuralized embryoid bodies,³⁸ and accelerates neuronal differentiation in monolayer cultures of human embryonic stem cell-derived neural stem cells.¹⁰ Interruption

of notch signaling is thought to be the mechanism by which DAPT supplementation prevents glial differentiation; however, further mechanistic studies will be needed to confirm that other γ -secretase substrates such as E-cadherin and ErbB-4 do not also play a role.^{63,64} Further, the ability of DAPT to inhibit glial differentiation may not be effective for neural progenitors isolated before they have undergone differentiation induction and express the transcription factors Mash1 and Prox1,⁶⁵ as notch inhibition before induction accentuates both neurogenesis and gliogenesis.^{10,66}

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Disclosure Statement

No competing financial interests exist.

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