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Peptide-modified, hyaluronic acid-based hydrogels as a 3D culture platform for neural stem/progenitor cell engineering

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Abstract

Neural stem/progenitor cell (NS/PC)-based therapies have shown exciting potential for regeneration of the central nervous system (CNS) and NS/PC cultures represent an important resource for disease modeling and drug screening. However, significant challenges limiting clinical translation remain, such as generating large numbers of cells required for model cultures or transplantation, maintaining physiologically representative phenotypes ex vivo and directing NS/PC differentiation into specific fates. Here, we report that culture of human NS/PCs in 3D, hyaluronic acid (HA)-rich biomaterial microenvironments increased differentiation toward oligodendrocytes and neurons over 2D cultures on laminin-coated glass. Moreover, NS/PCs in 3D culture exhibited a significant reduction in differentiation into reactive astrocytes. Many NS/PC-derived neurons in 3D, HA-based hydrogels expressed synaptophysin, indicating synapse formation, and displayed electrophysiological characteristics of immature neurons. While inclusion of integrin-binding, RGD peptides into hydrogels resulted in a modest increase in numbers of viable NS/PCs, no combination of laminin-derived, adhesive peptides affected differentiation outcomes. Notably, 3D cultures of differentiating NS/PCs were maintained for at least 70 days in medium with minimal growth factor supplementation. In sum, results demonstrate the use of 3D, HA-based biomaterials for long-term expansion and differentiation of NS/PCs toward oligodendroglial and neuronal fates, while inhibiting astroglial fates.

Keywords

hyaluronic acid; biomaterials; hydrogels; tissue engineering; neural stem/progenitor cells

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Additional Supporting Information may be found in the online version of this article.

INTRODUCTION

Although the central nervous system (CNS) has some innate ability to regenerate, this ability is insufficient to restore tissue function in people afflicted by injury or neurodegenerative conditions. In adults, limited regeneration is achieved by an endogenous pool of neural stem/progenitor cells (NS/PCs), which are actively recruited to damaged tissue where they have the capacity to become new neurons and glia.[1–3] Glia cells include oligodendrocytes and astrocytes and have distinct roles to support neuronal function. Oligodendrocytes form an insulating myelin sheath around neuronal axons to enable faster signal transmission and longrange conduction. Astrocytes have a variety of functions, including formation and maintenance of neuronal synapses and the blood–brain barrier. However, many NS/PCs present in damaged CNS tissue differentiate into reactive astrocytes, which create a glial scar and, when in excess, likely contribute to a chronic inflammatory environment thought to prevent regeneration.[4–10]

Over the past two decades, many studies have evaluated the therapeutic potential of transplanting human NS/PCs or oligodendrocyte progenitor cells (OPCs), the intermediary progenitors between NS/PCs and mature oligodendrocytes (Fig. 1), to regenerate CNS tissues after injury.[8,11–18] While NS/PCs can become neurons, astrocyte or oligodendrocytes, OPCs are restricted to the oligodendrocyte lineage. Differentiation of transplanted NS/PCs toward oligodendrocytes has led to significant gains in functional recovery in rodent models of spinal cord injury.[8,11–18] Furthermore, transplantation of OPCs has shown some promise for spinal cord injury repair in recent clinical trials.[17] Despite these positive results, differentiation of NS/PCs into OPCs is inefficient. The best *in vitro* yields of human OPCs remain below 30%[12,19–21] and *in vivo* below 25% of NS/PCs become oligodendrocytes after transplantation into the injured rodent spinal cord.[16,22,23] While relatively few transplanted cells differentiate and engraft into the host tissue, these few are able to induce some functional improvement.[14,17,24,25]

The potential of stem cell-based therapies to improve clinical outcomes after brain or spinal cord injury motivates development of more effective strategies to direct oligodendrocyte-lineage differentiation. Key factors in the tissue microenvironment include the extracellular matrix (ECM) and cell-secreted soluble factors.[26] Compared to neuron or astrocyte differentiation, oligodendrocyte differentiation of human NS/PCs has been more difficult to achieve.[21] The majority of protocols previously described for differentiation of NS/PCs toward oligodendrocytes rely on temporal application of formulations of soluble bioactive factors, a potent method of controlling differentiation.[20,21,27] However, as interactions between cells and the ECM are critical for oligodendrocyte differentiation,[28,29] this study aimed to evaluate whether provision of a defined, synthetic ECM could improve differentiation efficiency.

Biomaterials make excellent platforms for 3D cell culture that can provide ECM-mimetic cues in a highly modifiable, defined manner.[30] Biomaterial properties have been used to direct cell fate[31–33] through modulation of stiffness,[34] swelling ratio,[35] geometry,[36] and chemistry.[37] Hydrogel biomaterials are often used because of their similarities to native tissues, including high water content. They can be fabricated from biocompatible and

biodegradable polymers and their mechanical properties tuned to span those found in native brain and spinal cord.[38] Hydrogel scaffolds have the potential to enable large-scale production of therapeutic cells by providing control over their expansion and subsequent differentiation into defined phenotypes *ex vivo* prior to clinical transplantation.

Hydrogels were fabricated based on hyaluronic acid (HA), a nonsulfated glycosaminoglycan that is abundant in CNS tissue and particularly enriched during embryonic development and in adult NS/PC niches.[39] HA regulates proliferation and differentiation of $Sox2^+$ NS/PCs through the CD44 receptor.[40,41] In inflammatory environments, high molecular weight HA acts as a scavenger of damaging free radical species.[42] HA hydrogels can be modified to provide additional matrix-derived functionalities, such as integrin-binding and mechanical cues, in combination.[43–45] Use of aqueous, cytocompatible crosslinking chemistries enables 3D encapsulation of live cells within hydrogels.[46,47] HA hydrogels can be formulated with mechanical properties approximating native CNS tissues and are degraded by cell-produced hyaluronidases.[39] To enable cell adhesion, integrin-binding peptides derived from ECM proteins can be included.[31,39,43,47–51] Implantation of HA hydrogels into the brain or spinal cord after injury can reduce the local inflammatory response and glial scar deposition.[30,52] Furthermore, previous reports found that *in vitro* culture or *ex vivo* transplantation of NS/PCs within HA hydrogels with mechanical moduli mimicking healthy brain selectively increased differentiation of NS/PCs toward neurons or oligodendrocytes while decreasing astrocyte differentiation into reactive astrocytes.[53–58]

In the present studies, we evaluated the effects of combinatorial presentation of laminin-derived adhesion peptides on survival, proliferation and differentiation of human NS/PCs in 3D culture. Laminins are key mediators of NS/PC progression to mature, myelinating oligodendrocytes.[59] For example, when OPCs contact the laminin-rich ECM surrounding axons, engagement of integrin $\alpha_6\beta_1$ switches OPCs to a non-proliferative phenotype and initiates maturation.[60] Several regions of laminin I have been identified as mediators of cell adhesion, including RGD, YIGSR, and IKVAV amino acid sequences. RGD is a common integrin-binding site across several ECM proteins. YIGSR and IKVAV support neuron attachment, survival and neurite outgrowth in a synergistic manner.[61,62] Inclusion of peptides from these regions, rather than whole laminin protein, in biomaterial scaffolds increases local density of the bioactive sequence, and possibly biological potency.[63] As scaffolds are constructed from the “bottom-up”, adding individual bioactive regions of ECM proteins one at a time, they provide the opportunity to isolate the independent and combined effects of specific peptide-integrin interactions. Decoupling the effects of different ECM cues will enable creation of tailored, defined matrix environments containing the minimal elements necessary to efficiently direct cell fate.

Here, we characterized effects of ECM-mimetic features included in HA-based hydrogels on differentiation of 3D-cultured NS/PCs, with the ultimate goal of identifying matrix properties that can enrich differentiation toward oligodendrocytes that can support CNS repair. HA content and hydrogel modulus were chosen to best mimic properties of native CNS tissues. RGD, IKVAV and YIGSR peptides were included alone and in combination and their effects on viability and differentiation of NS/PCs were evaluated over 70 days in culture. Cultures in media formulated to maintain NS/PC “stemness” and to provide

minimally necessary support for oligodendrocyte differentiation were compared. Finally, we characterized the electrophysiological behavior of the differentiated neuronal cells in hydrogels.

MATERIALS AND METHODS

All reagents were purchased from Thermo Fisher Scientific, unless otherwise stated.

Hyaluronic acid preparation and hydrogel formation

Hydrogels were formed by Michael-type addition between 4-arm polyethylene glycol-maleimide (PEG-Mal, 20 kDa, Laysan Bio) and thiolated HA (700 kDa HA, Lifecore Biomedical).[64] In brief, carbodiimide chemistry (*N*-hydroxysuccinimide, NHS; 1-ethyl-3-(3-dimethylaminopropyl), EDC) was used to add a thiol from cystamine (Sigma-Aldrich) to the carboxylate group on the glucuronic acid moieties of HA, creating thiolated HA (HA-SH). Disulfide bridges were broken with dithiothreitol and newly synthesized HA-SH purified via dialysis (12–14 kDa molecular weight cut-off cellulose membrane, Spectrum Labs) in deionized water, adjusted to pH 4 with hydrochloric acid and changed at least twice daily for 3 days. Purified HA-SH was sterile-filtered and lyophilized for 2 days. A range of 4–6% thiolation, defined as thiol groups per HA disaccharide, was used as determined with Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid), Sigma-Aldrich).[65,66]

NS/PC culture

Neuralized H9 human embryonic stem cells (H9 hESCs) (Life Technologies) were expanded, and stored in liquid nitrogen vapors until use. NS/PCs were cultured in StemPro® hESC serum-free media (Life Technologies) made with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) containing 1× StemPro® neural supplement, 2 m*M* Glutamax™, 0.1× antibiotic/antimycotic, 20 ng/mL basic fibroblast growth factor (bFGF), and 20 ng/mL epidermal growth factor (EGF). Here, we will refer to this as "stem cell maintenance" medium. NS/PCs were cultured in a humidified 37° C incubator with 5% carbon dioxide. Half of the medium in each culture well was changed every 2 days. After 4 weeks in culture, NS/PCs were transitioned to pre-differentiation medium for 2 weeks, followed by an oligodendrocyte-promoting differentiation medium for 4 weeks. Compositions of pre-differentiation and differentiation media were adapted from Hu et al.[67] A timeline of the culture conditions is shown in Figure 1. Both pre-differentiation and differentiation media used the stem cell maintenance medium base without bFGF or EGF. Pre-differentiation medium was supplemented with 10 ng/mL platelet-derived growth factor AA (Peprotech) and 1 μ*M* purmorphamine (Sigma-Aldrich), a sonic hedgehog agonist to direct NS/PCs toward OPCs. Differentiation medium was supplemented with 1 μ*M* adenosine 3′,5′-cyclic monophosphate (cAMP; Sigma-Aldrich) and 60 ng/mL triiodothyronine (T3; Sigma-Aldrich) to direct OPCs toward oligodendrocytes. Minimal growth factor supplementation was used for these studies to obtain information about how insoluble cues provided by 3D culture matrices affected differentiation.

Prior to seeding 2D cultures for assessments of viability and mRNA expression, tissue culture-treated polystyrene wells were coated with CELLstart™ (Life Technologies) diluted

in phosphate buffered saline (PBS) in a humidified, 37°C incubator for at least 2 h. After aspirating the CELLstart™ solution, 25,000 NS/PCs per cm^2 were seeded. TrypLE™ Express (Life Technologies) was used to dissociate adherent NS/PCs during passaging. For immunostaining experiments, cells were seeded onto laminin-coated, 12 mm-diameter glass coverslips. Prior to coating, glass coverslips were cleaned by submerging in sulfuric acid for 20 min to remove residues, rinsed three times with distilled, deionized water (ddH_2O), and autoclaved. Coverslips were first coated with a 50 μg/mL solution of poly-D-lysine (Sigma-Aldrich) in sterile ddH_2O in a humidified, 37°C incubator for 1 h, rinsed three times with sterile PBS (without calcium or magnesium), and then incubated with 10 μg/mL mouse laminin I in sterile ddH_2O for 2 h in a humidified, 37°C incubator. Coverslips were rinsed a final three times in PBS before seeding with 25,000 NS/PCs per cm^2.

Prior to gelation and NS/PC encapsulation, PEG-Mal was dissolved in PBS and sterile-filtered. Peptides (Genescript, Supplementary Table 1) containing thiols (i.e., cysteines) near the *N*-termini were conjugated via Michael-type addition to an average of one arm of PEG-Mal prior to hydrogel formation for a total peptide concentration of 270 μ*M* in the final hydrogels.[64,68] PEG-Mal/peptide solutions were adjusted to 10 mg/mL with PBS and kept on ice. HA-SH was dissolved in DMEM/F12 (15 mg/mL). Once dissolved, the HA-SH solution was adjusted to approximately pH 6 with sterile 0.1 *M* NaOH. Next, 2.5 million NS/PCs per mL of HA-SH (around 25,000 NS/PCs per a 20 μL hydrogel) were added. The HA-SH solution with suspended NS/PCs was adjusted to 10 mg/mL with DMEM/F12 and kept on ice. To form cell-laden hydrogels, HA-SH with NS/PCs and PEG-Mal/peptide solutions (10 μL of each) were mixed in sterile, press-to-seal silicone isolators (12 × 4.5 mm diameter by 2 mm deep, Grace BioLabs) adhered to the bottom of a polystyrene Petri dish. Hydrogels became solid rapidly (within 30 s). Hydrogel cultures were placed in a humidified 37°C incubator for 15 min to complete gelation before they were removed from the molds, and transferred to 48-well tissue culture plates (400 μL media per well, 1 hydrogel culture per well).

Rheology

Hydrogel samples were tested for rheological stiffness using a Discovery Hybrid Rheometer (DHR2) from TA Instruments. Samples were formulated as described in the previous section, but with a final volume of 150 μL in 8 × 9 mm diameter by 2 mm deep, press-to-seal silicone isolators (Grace BioLabs). After gelation, hydrogels were allowed to equilibrate in PBS overnight at 4°C. The next morning, hydrogels were brought to room temperature and mounted onto the rheometer for testing. A 9 mm, parallel-plate geometry was used to perform an oscillation-frequency sweep with a 1% strain logarithmic sweep at a frequency range of 0.1–10 Hz. The storage and loss moduli were recorded and used to determine the complex modulus. Rheological properties of hydrogel are reported as average ± standard deviation of 15 independent experiments, each with at least three technical replicates.

Immunocytofluorescence

Neural stem/progenitor cells grown on 2D laminin-coated, coverslips or in 3D hydrogels were fixed by removing half of their medium and replacing it with fresh, 4% paraformaldehyde in PBS. After 20 min, the fixative was removed and coverslips rinsed

three consecutive times in PBS. Fixed coverslips were stored at 4°C in PBS until use. Fixed hydrogel cultures were then transitioned in to a solution containing 20% sucrose in Optimal Cutting Temperature compound (OCT, Tissue-Tek) for sectioning samples using the following successive steps: 5% sucrose in PBS (1 h), 20% sucrose in PBS (2 × 30 min), 20% sucrose in PBS (overnight), 20% sucrose in OCT (2 h), 20% sucrose in OCT. In the final sucrose OCT solution, samples were frozen in a slurry of dry ice and 2-methylbutane, embedded in plastic molds, and cryo-sectioned (18 μm thick sections).

Sections were permeabilized with 0.1% Triton-X 100 and blocked using 4% normal donkey serum (Sigma-Aldrich) with 2% bovine serum albumin in PBS with 0.05% Tween-20. Triton-X 100 and Tween-20 were not used with antibodies labeling cell surface antigens. Samples were incubated with primary antibodies (Supplementary Table 2) overnight, rinsed three times in PBS, and incubated for 2 h with dye-conjugated, secondary antibodies raised in donkey (1:500; Life Technologies) and Hoechst 33342 (1:1000; ThermoFisher) to visualize nuclei. Samples were rinsed three times in PBS and mounted with Fluoromount-G® (SouthernBiotech). Widefield fluorescence images were acquired using an Axio Observer Z1 (Zeiss), Zen software (Zeiss), and an ORCA-Flash4.0V.2 CMOS (Hamamatsu). Confocal fluorescence images were acquired using a Leica TCS-SP5. Maximum projections of confocal image stacks were produced using Leica Application Suite X software.

mRNA expression

Prior to lysis, cells were isolated from hydrogels by incubating in a 1 mg/mL hyaluronidase (Sigma-Aldrich) solution in 0.01 *M* citrate buffer (pH 5.5) for 20 min at 37°C in a humidified incubator and then rinsed once in PBS. mRNA was isolated from lysed samples using the RNeasy and Qiashdredder kits (Qiagen) following instructions provided by the manufacturer. Real-time PCR (RT-PCR) was performed using a PCR master mix from Qiagen and an iQ5 qPCR machine (Bio-Rad). All primer/probe mixtures were purchased from Applied Biosystems (Supplementary Table 3). Samples were diluted to equivalent RNA concentrations before running RT-PCR and were normalized to GAPDH mRNA expression levels from NS/PCs at day 0.

Viability measurement

Cell viability was measured with CellTiter-Glo® 3D Cell Viability Assay (Promega) to quantify ATP present. Medium was removed from hydrogels, which were then homogenized in CellTiter-Glo® reagent and incubated for 30 min at room temperature. Hydrogel viability was determined by measuring luminescence on a plate reader (BioRad Synergy II) and comparing raw values to luminescence of a standard curve constructed from known numbers of NS/PC measured at the same time.

Electrophysiology

Electrophysiology was performed at room temperature with an Olympus BX51W1 upright microscope with optics for differential interference contrast. All recordings were performed at room temperature in an electrophysiology microscope chamber containing regular artificial cerebral spinal fluid (ACSF) composed of 130 m*M* NaCl, 26 m*M* $NaHCO_3$, 10 m*M* glucose, 3 m*M* KCl,2 m*M* $MgCl_2$, 2 mM $CaCl_2$, and 1.25 m*M* NaH_2PO_4. Samples

are oxygenated with 95% O_2 and 5% CO_2, maintained with a pH between 7.2 and 7.4 and osmolality between 290 and 310 mOsm/L. Whole-cell patch clamp recordings in current-clamp mode were performed using a MultiClamp 700B amplifier (Molecular Devices) and pCLAMP 10.3 acquisition software. The patch pipette (3–5 MΩ resistance) contained a solution comprised of 112.5 m*M* potassium-gluconate, 17.5 m*M* KCl, 10 m*M* HEPES buffer, 5 m*M* ATP potassium salt, 5 m*M* ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 4 m*M* NaCl, 1 m*M* $MgCl_2$, 1 m*M* NaGTP, 0.5m*M* $CaCl_2$, and pH 7.2 with 270–280 mOsm/L. Capacitance, input resistance, decay time constant and resting membrane potential were recorded while holding the membrane potential at −70 mV and electrode access resistances were maintained at <30 MΩ.

Statistics

Statistical significance for viability differences between peptide groups was determined by evaluation with a Student's *t*-test and a Holm–Šidák method for multiple comparison correction. A $p < 0.05$ was considered statistically significant after Holm–Šidák's correction. Standard deviations for gene expression data were determined by propagating error from threshold cycle measurement for gene of interest and control gene (GAPDH) as well as control RNA (NS/PCs from day 0) following guidelines from Applied Biosystems.[69] A two-way ANOVA was performed on gene expression levels and statistically significant differences in means were determined with a Hochberg method for multiple comparison correction (Supplementary Table 4). The false-discovery rate was set to 0.05. A $p < 0.05$ was considered statistically significant after Hochberg's correction.

RESULTS

Mechanical properties

The average complex shear modulus of HA hydrogels was found to be 188 ± 42 Pa with a range of 134–282 Pa, which is consistent with native CNS tissue.[55,70,71] Hydrogel storage modulus was not significantly affected by the addition of up to 500 μ*M* peptide (Supplementary Fig. 1).

Cell viability and proliferation

There were no significant differences among groups day 7 after encapsulation (Supplementary Fig. 2). At day 28, more viable cells were detected in all conditions. Multivariable ANOVA (peptide condition, time) indicated significant differences in cell viability (assessed by ATP content) at days 28 and 70 for all conditions ($p < 0.02$), but not day 42, for both the stem cell maintenance and pre-oligodendrocyte medium conditions (Fig. 2A). Within these timepoints, a Holm–Šidák comparison test was used to evaluate statistical significance between peptide groups. At day 28, viability of NS/PCs cultured in hydrogels with RGD in maintenance medium was significantly higher than in hydrogels with YIGSR ($p < 0.05$) or CYS ($p < 0.005$). This difference may be attributed to some combination of improved cell survival and higher levels of proliferation being present in the RGD conditions. No significant differences in NS/PC viability among hydrogels with RGD, IKVAV, YIGSR+IKVAV, or YIGSR+IKVAV +RGD were observed. At day 70 of culture in differentiation medium, NS/PCs in hydrogels containing RGD (i.e., RGD or

YIGSR+IKVAV+RGD) had significantly more viable cells than those in hydrogels without RGD (i.e., YIGSR, IKVAV, YIGSR +IKVAV, or CYS) ($p < 0.05$). For all peptide conditions, NS/PCs cultured in pre-differentiation or differentiation medium had decreased ATP activity compared to those in stem cell maintenance medium.

Immunofluorescent images of cleaved poly (ADP-ribose) polymerase (cPARP) and Ki67 were used to indicate NS/PC apoptosis and proliferation, respectively (Fig. 2B). There were no observable differences in numbers of cPARP+ cells among any conditions. However, there was a clear, qualitative decrease in the numbers of proliferative cells (i.e., $Ki67^+$ nuclei) when cultured in differentiation medium (days 42 and 70), compared to stem cell maintenance medium (day 28), in agreement with results from ATP assays (Fig. 2A). Cells in stem cell maintenance medium continued to proliferate throughout their culture period. By day 42, these cultures became overconfluent within scaffolds. Thus, cultures in stem cell maintenance medium were discontinued at day 42. As cells cultured in differentiation medium had slowed proliferation, they were continued to day 70.

Stem cell maintenance.—Effects of peptides on NS/PC fate were assessed based on mRNA (RT-PCR) and protein (immunostaining) expression of lineage-specific markers. *SOX2*, a transcription factor indicative of multipotent NS/PCs, was not significantly altered by 3D culture as measured by mRNA (Fig. 3A). Protein levels of SOX2 and nestin, another commonly used marker for NS/PCs,[72] were evaluated using immunofluorescence (Fig. 3B). Qualitatively, expression of SOX2, in particular nuclear SOX2, and nestin protein appeared to decrease after culture in differentiation medium. Together, results suggest that only a few NS/PCs maintain stemness throughout the 70-day culture period.

Levels of CD44, a marker of NS/PCs and receptor for HA, were evaluated. Compared to 2D culture, NS/PCs in 3D culture HA hydrogels displayed a trend toward reduced levels of *cd44* mRNA in all peptide and media conditions at all time points (Fig. 4A). However, this difference was only statistically significant at day 28 in stem cell maintenance medium and day 42 in pre-differentiation medium (for all peptide conditions). Immunofluorescent labeling in 3D HA hydrogels indicated sporadic CD44 expression in few cells (Fig. 4B).

Oligodendrocyte-lineage differentiation.—In all 3D cultures independently of peptide or media conditions, mRNA levels of *olig2*, a transcription factor present in CNS glial and motor neuron progenitors,[73,74] significantly increased (Fig. 5A).[75] Furthermore, increased levels of OLIG2 protein was observed in 3D hydrogel cultures in all media conditions (Fig. 5B). Qualitatively, more NS/PCs appeared positive for nuclear OLIG2 protein after culture in differentiation medium in both 2D and in 3D conditions. This shift from cytoplasmic to nuclear localization of OLIG2 protein indicates functional differentiation away from glial fibrillary acidic protein (GFAP)-positive astrocytes and toward progenitors of either oligodendrocytes, motor neurons, or fibrous astrocytes.[58,76–78] Oligodendrocyte-lineage differentiation was characterized at days 42 (stem cell maintenance medium) and 70 (differentiation medium) using immunofluorescence staining for platelet-derived growth factor-α (PDGFR-α, OPCs), O4 (pre-oligodendrocytes), and 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase, pre-oligodendrocytes, and mature oligodendrocytes).[67,79,80](Fig. 5C–E). In 2D, NS/PCs in differentiation medium tended to

form rosette-like clusters positive for OPC markers, similar to previous reports.[81] Cells cultured in 3D, HA hydrogels consistently showed robust labeling of oligodendrocyte-lineage markers, which was qualitatively more abundant when compared to culture in stem cell maintenance medium in 2D or 3D and on laminin-coated, 2D substrates coverslips in any medium. However, no immunolabeling of myelin basic protein (MBP) was observed, indicated that oligodendrocyte-lineage cells did not produce myelin (*data not shown*).

Astrocyte-lineage differentiation.—Glial fibrillary acidic protein is an intermediate filament protein whose expression is associated with activated astrocytes. GFAP is upregulated in response to inflammation and in the glial scar that forms to isolate injured tissue from surrounding healthy tissue in the CNS.[21,82,83] Levels of *GFAP* mRNA were significantly decreased in NS/PCs cultured in 3D, compared to 2D, in pre-differentiation and differentiation media conditions (Fig. 6A). In 2D cultures, immunofluorescent staining confirmed a substantial increase in *GFAP* levels in differentiation medium, compared to minimal expression in stem cell maintenance medium. In 3D cultures, NS/PCS in all conditions had no detectable *GFAP* (Fig. 6B).

While GFAP expression indicates activated astrocytes, it is possible that NS/PCs in 3D culture differentiated toward non-activated astrocytes. Thus, we assessed levels of S100β, a marker of astroglial differentiation that is not specific to activated or reactive astrocytes (Supplementary Fig. 3).[21,82,84]

While immunofluorescence staining revealed the presence of a few $S100\beta^+$ cells in both 2D and 3D cells in all conditions, overall low levels of *S100β* mRNA expression, which is often present even on fibrous astrocytes derived from $olig2^+$ progenitors,[58] in 3D culture provides evidence that the $OLIG2^+$ cells observed at days 42 and 70 in 3D, differentiation culture are likely OPCs (Fig. 5A, B). NS/PC culture in HA hydrogels with CYS peptides in stem cell maintenance medium at day 28 was the only condition with a statistically significant difference in *S100β* mRNA levels compared to 2D cultures at the same time point.

Neuron-lineage differentiation.—Next, we evaluated expression of βIII-tubulin (TUBB3) and doublecortin (DCX), which both indicate a neuronal or neuronal precursor phenotype (Fig. 7).[21,85] A decrease in *TUBB3* mRNA was observed for 2D-cultured NS/PCs in maintenance medium at day 28 compared to those in pre-differentiation medium at day 42 (Fig. 7A). No other statistically significant differences in levels of *TUBB3* mRNA were observed. Immunofluorescence staining for βIII-tubulin protein showed no obvious differences in expression, in agreement with mRNA results (Fig. 7B). Immunofluorescence staining for DCX indicated the presence of more DCX^+ cells in 3D culture and an additional increase when cultured in differentiation medium (Fig. 7B).

To assess differentiation into more mature neurons, we evaluated expression of neurofilament 200 (NF200), nuclear antigen (NeuN), and synaptophysin (SYN). Presence of NF200, an intermediate filament protein associated with mature axons, increased with both 3D culture and differentiation medium at day 70[86] (Fig. 7C). NeuN is a transcription factor active in the nucleus of many mature neurons. Nuclear NeuN was observed only in 3D cultures in differentiation medium, indicative of active transcriptional regulation of genes

associated with mature neurons[86] (Fig. 7D). SYN is a protein found in pre-synaptic vesicles associated with early stages of synapse maturation.[87] In 2D cultures, SYN was only present when cultured in differentiation medium at day 70 (Fig. 7E). In 3D cultures, SYN was present in all conditions and increased with differentiation medium at day 70, suggesting that 3D HA hydrogels are conducive to maturation of functional neurons. However, markers for more specific mature neuronal subtypes, including serotonin, tyrosine hydroxylase, and choline acetyltransferase, were not present (*data not shown*).

Electrophysiology

Previous reports have demonstrated that the ECM provided to NS/PCs in culture can alter their electrophysiological characteristics.[88,89] Thus, we performed electrophysiology on NS/PCs cultured in 3D HA hydrogels with YIGSR+IKVAV+RGD for the full 70-day differentiation period (Fig. 8). Recorded NS/PCs had membrane channels that conducted potassium and sodium, but were too immature to determine an electrophysiological phenotype associated with any mature neuronal subtype. Measured values for capacitance, input resistance, and resting membrane potential were similar to values measured in embryonic stem cells[89] and induced pluripotent stem cells[90] undergoing neuronal differentiation but not yet fully matured. While this data is insufficient to definitively characterize the differentiation of NS/PCs, the observed electrophysiological characteristics suggest that NS/PCs were in an intermediate stage of maturation.

DISCUSSION

Hydrogel biomaterials have immense potential to enable successful stem cell-based therapies for CNS regeneration. This study investigated whether 3D culture in an HA-rich environment incorporating laminin-derived, adhesive peptides could direct NS/PC differentiation more efficiently and in a more controlled manner than is currently possible with 2D cultures. Results demonstrated that HA-based hydrogels support viable, 3D cultures of human NS/PCs for at least 70 days and that culture conditions can be used to promote NS/PC expansion or differentiation, as desired. In addition, culture in 3D, HA hydrogels increased differentiation toward both neurons and oligodendrocytes. Very few, if any, astrocytes expressing S100β or GFAP were present in 3D cultures. In contrast, both astrocyte markers were widely observed in 2D culture. As GFAP expression is typically associated with inflammatory astrocytes *in vivo*, this result agrees with previous reports that HA hydrogels can reduce the presence of GFAP+ astrocytes in the spinal cord after injury.[52,91]

The ECM acts through integrins to regulate NS/PC phenotype, including maintenance of a stem-like phenotype and differentiation toward specific mature cell types.[92,93] Pinkstaff and colleagues identified a specific set of integrins are expressed in the rat brain.[94] Of these, integrin subunits α_1, α_3, α_6, α_V, and β_1 are abundant and are known to interact with laminin and other ECM proteins bearing the RGD sequence. Integrin dimers $\alpha_V \beta_1$ and $\alpha_V \beta_5$ have been linked to proliferation of NS/PCs.[95] For OPCs, various integrin dimers with the β_1 subunit have been found to promote survival,[96] regulate a switch from proliferation to differentiation[29] and mediate myelination by mature oligodendrocytes.[97,98]

Several studies have found that inclusion of IKVAV peptides in hydrogel-type biomaterials increased neuronal differentiation of transplanted NS/PCs after traumatic brain injury.[99] Inclusion of IKVAV has also been reported to reduce astrogliosis after injury.[100,101] *In vitro*, IKVAV-bearing hydrogels are reported to promote differentiation of neurons while reducing differentiation of astrocytes.[102,103] YIGSR has also been reported to support survival and neurogenesis of transplanted NS/PCs[104] and has been widely found to promote neurite extension.[105,106] YIGSR and IKVAV are known to synergistically improve neurite attachment and outgrowth.[61,62] Their combination has been reported to maximize neurogenesis of NS/PCs cultured in 3D HA hydrogels.[44] Collectively, these results and prior studies confirm that RGD, YIGSR, and IKVAV are supportive of NS/PC survival and differentiation into oligodendrocytes and neurons.

Despite progress in our understanding, integrin regulation of NS/PC differentiation is understudied and has potential to be a valuable tool for developing and implementing stem cell-based therapies. While this study demonstrated that RGD peptides likely increases cell proliferation in HA hydrogels, no other peptides or combinations investigated significantly altered NS/PC maturation compared to non-bioactive cysteine. However, higher cell viability was observed in 3D hydrogels including RGD peptide, either exclusively or in combination with YIGSR and IKVAV, after 70 days in culture. Given that NS/PCs cultured in hydrogels with IKVAV and/or YIGSR without RGD had equivalent viabilities to those with cysteine, which served as a negative control as it does not bind integrins, higher viability with RGD may be because of the abilities of RGD to interact with many different integrins and bind more strongly to integrins, thereby improving cell adhesion. The decrease in numbers of viable NS/PCs between days 28 and 42 likely can be attributed to the cells filling, or overgrowing, hydrogel cultures by the later time point. After day 28, NS/PC spheroids continued to grow but often breached the edges of the hydrogel and were released into the surrounding medium. These spheroids were not included in analysis of cell numbers and were rinsed away before viability and phenotype experiments.

As anticipated, removal of the mitogenic factors bFGF and EGF from culture medium after day 28 in the PREDIFF and DIFF media reduced proliferation and initiated differentiation of NS/PCs.[107,108] A subset of NS/PCs in a population are expected to undergo apoptosis in response to the differentiation stimuli,[108–110] which helps to explain the reduced numbers of viable cells in differentiation medium at day 70. However, many NS/PCs were still viable at day 70. Notably, apoptotic cells were identified throughout the 3D culture volume, indicating that cell death was likely not caused by inadequate diffusion through the hydrogels.

Although CD44 has been reported widely as a marker for NS/PCs,[41,111,112] immunofluorescence labeling in 3D HA hydrogels indicated sporadic CD44 presence in only a few cells in all 2D and 3D conditions. Moreover, there was a trend toward less CD44 expression in 3D compared to 2D cultures. While the implications of this result remain unclear, it is possible that the increased HA binding of CD44 may lead to reduced CD44 expression. Given that CD44 is associated with stemness[41] and our data showing that NS/PCs cultured in 3D, HA-based hydrogels are more mature than those in 2D, differentiation in 3D cultures may lead to reductions in CD44 expression.[41] Another

study reported that during differentiation, CD44 remains high in astrocyte progenitors, but is substantially reduced in OPCs.[112] CD44 expression is also seen in developing and mature, branched fibrous astrocytes.[112] In this study, we observed no astrocyte-lineage differentiation, but significant oligodendrocyte-lineage differentiation. Thus, the reduction in CD44 expression in 3D cultures may correlate with an increased oligodendrocyte-lineage differentiation. However, further investigation is warranted in future studies.

Overall, results from this study demonstrate the potential utility of HA-based hydrogels for biomanufacturing of stem cell-based therapies for CNS regeneration. First, NS/PCs could be expanded for 42 days when cultured in hydrogels in the presence of mitogens. 3D cultures provide a larger space for culture expansion than 2D cultures. Thus, more new cells are generated without the need for passaging, which may lead to significant phenotypic drift affecting therapeutic potential. Furthermore, as relatively high numbers of NS/PCs are required for clinical use, a 3D culture method for cell expansion could address this significant challenge to translation. HA hydrogels maintained high levels of *SOX2* mRNA and immunostaining consistent with observations that high expression of *SOX2* is conserved throughout embryogenesis in vertebrate NS/PCs.[113]

Second, with mitogen withdrawal and progressive addition of soluble factors intended to promote oligodendrocyte-lineage differentiation, 3D culture in HA-based hydrogels generally increased differentiation toward OPCs over monolayer culture on CELLstart™, evidenced by increased olig2 expression and nuclear localization coinciding with an increase in expression of PDGFR-α and followed by increased expression of O4 and CNPase, early markers of differentiating oligodendrocytes. The goal of this study was to establish a baseline for how these 3D scaffolds affect differentiation. Thus, minimal soluble growth factor support was provided. However, there have been several reports detailing how progression addition of soluble factor cocktails can drive oligodendrocyte differentiation.[20,21,27] In future work, we expect that combining 3D scaffolds with these soluble factor protocols will likely increase numbers of oligodendrocytes generated.

While many NS/PCs in 3D culture progressed down the oligodendrocyte lineage, others expressed the neuronal lineage markers βIII-tubulin and DCX. After 70 days, many cells in differentiation medium exhibited signs of more mature neurons, including nuclear NeuN and synaptophysin expression. However, electrophysiological measurements and immunostaining for markers of mature synapses showed that these cells did not differentiate completely. As the differentiation protocol used here was designed to promote differentiation into oligodendrocytes, and not neurons, use of a neuron-promoting protocol may further synaptic maturation in 3D cultures.

Notably, NS/PC differentiation into astrocytes (assessed via GFAP or S100β expression) was only observed in 2D and not in 3D cultures. As GFAP expression is associated astrocyte activation, NS/PC culture on substrates with non-physiological stiffness, like glass, may promote an inflammatory response from differentiating astrocytes.[114–116] High molecular weight HA, as used here for hydrogel fabrication, has also been shown to reduce astrocyte proliferation and activation, both of which may contribute to inhibition of axon regeneration by the glial scar after injury.[91,117]

Schwann cells also express S100β and can promote regeneration of damaged CNS tissue.[118,119] Schwann cells normally myelinate peripheral nerve axons, but have been observed to remyelinate CNS axons after injury. However, Schwann cell myelination reduces axon density to a pattern more similar to peripheral nerves than CNS, limiting the overall effectiveness of Schwann cells in CNS repair.[119,120] As NS/PCs are unlikely to differentiate into Schwann cells due to differences in their developmental origins,[118,121] it is likely $S100\beta^+$ cells in these studies, even those not expressing GFAP, were astroglial cells.

Hyaluronic acid hydrogel stiffness and degradability are areas of particular importance for future studies. Hydrogel stiffness affects differentiation of cultured NS/PCs, with peak differentiation toward neurons when stiffness is closest to that of normal brain tissue.[122] Previous work found that NS/PCs respond to stiffness in a time-dependent manner, typically on a time scale of seconds to minutes.[123] This finding suggests that NS/PCs may "remember" a previous mechanical stimulus that primes them toward a particular lineage. Thus, it is possible that stiffness of a culture material could be transiently altered to create an environment selecting for differentiation to a specific phenotypic fate.

In general, HA found in the normal CNS tissues is high molecular weight (>500 kDa), where it is more thought to help maintain NS/PC stemness[124,125] and quiescence.[39,126] However, low molecular weight HA (<30 kDa) may promote differentiation,[127] while mid-sized HA (~65 kDa) has been reported to inhibit OPC maturation and myelination.[128] Thus, the presence of high molecular weight HA (~700 kDa) in the 3D cultures may prevent complete maturation. While HA may be a beneficial material to initiate NS/PC proliferation and early OPC differentiation, it may also create an inhibitory "valley" that prevents full maturation. In the future, it will be beneficial to evaluate effects of lower molecular weight HA in hydrogel cultures, either by using smaller HA to form hydrogels or by digesting larger HA molecules with hyaluronidase after cell encapsulation. In future studies, incorporating additional regions that can be enzymatically degraded by cells cultured 3D hydrogels may provide an extra parameter to direct NS/PC fate.[129]

Hyaluronic acid hydrogels are a useful scaffolding material for 3D culture of NS/PCs and as bioactive carriers of therapeutic cells for CNS regeneration. HA hydrogels have beneficial effects on recovery after brain or spinal cord injury in rodents as they decrease inflammation[52,101] and improve NS/PC cell survival following transplantion.[130,131] The tunable hydrogel platforms reported here can be used to characterize how stiffness, peptide concentration, ECM content, degradability, and soluble factors interact to dynamically coordinate NS/PC differentiation into specific fates, helping to inform the optimal design of regenerative scaffolds. As the hydrogel chemistry used in this study is compatible with injection and gelation *in situ*, which is an advantage to avoid excessive tissue damage when delivered to the CNS, the same scaffolds found to direct NS/PC fate *in vitro* can be easily translated *in vivo* to evaluate their therapeutic effects.

CONCLUSIONS

This study demonstrates that 3D culture in HA-based hydrogels can be used to expand or differentiate human NS/PCs with improved efficiency over standard 2D cultures. Inclusion

of the RGD adhesive peptide increased cell viability in the early growth phase and through the 70-day differentiation period. Differentiation toward neurons and oligodendrocytes was increased significantly in 3D over 2D cultures, while differentiation into reactive astrocytes was reduced. However, differentiation into fully mature oligodendrocytes or neurons was not observed. The laminin I-derived adhesive peptides included had no significant effects on NS/PC fate. Taken together, these results establish that 3D, HA-based culture platforms can improve viability, expansion and differentiation of human NS/PCs and underscore the exciting potential of 3D, HA-based culture system for biomanufacturing of therapeutic NS/PCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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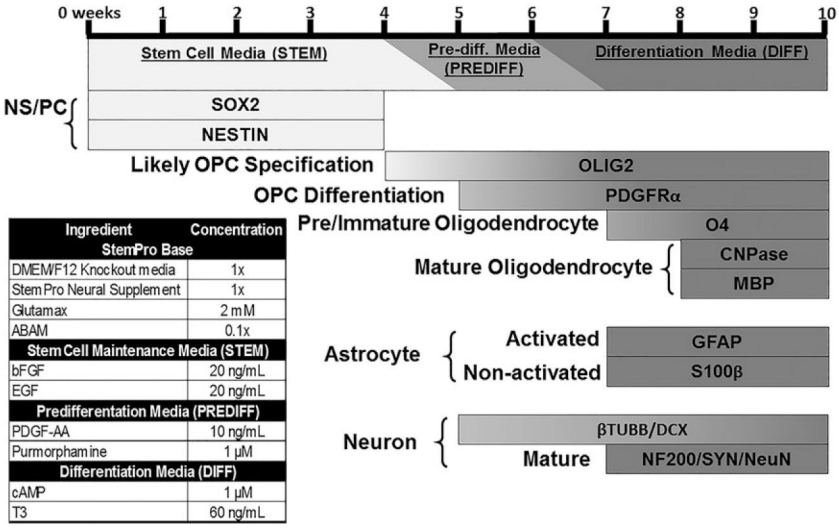

Ingredient	Concentration
StemPro Base	
DMEM/F12 Knockout media	1x
StemPro Neural Supplement	1x
Glutamax	2 mM
ABAM	0.1x
Stem Cell Maintenance Media (STEM)	
bFGF	20 ng/mL
EGF	20 ng/mL
Predifferentation Media (PREDIFF)	
PDGF-AA	10 ng/mL
Purmorphamine	1 μM
Differentiation Media (DIFF)	
cAMP	1 μM
T3	60 ng/mL

FIGURE 1.

Culture conditions and timeline. A timeline of media conditions is depicted above. The media composition is detailed in the inset table, while the expected observation timelines for markers of NS/PC, OPC, pre-oligodendrocyte, mature oligodendrocyte, astrocyte, and neuron are included below the media timeline. Differentiation marker timelines are from Miron et al.132

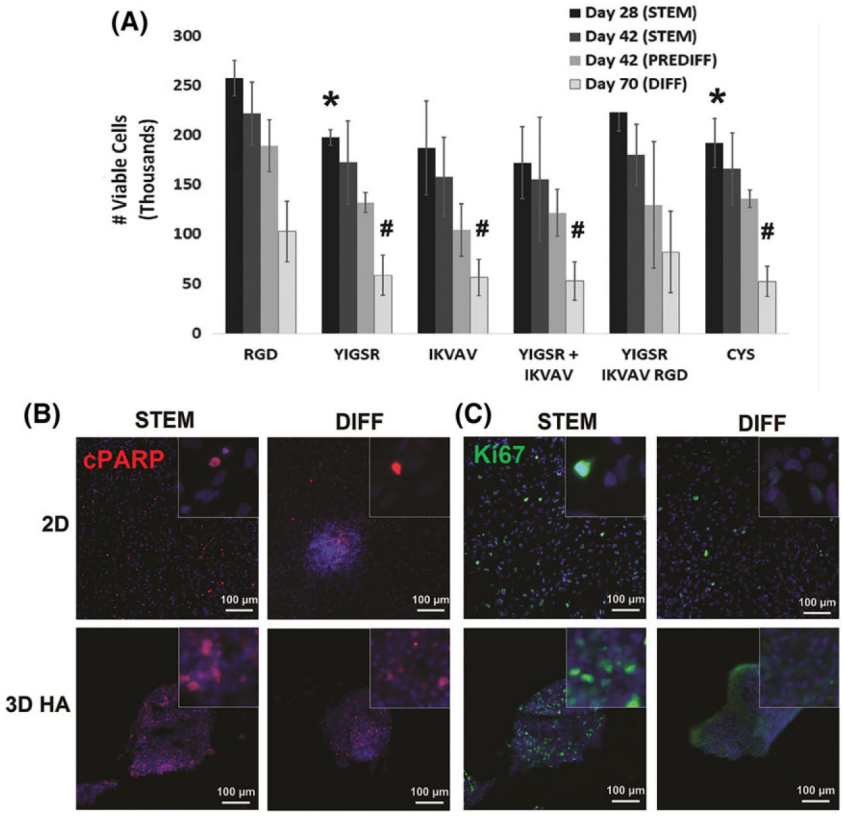

FIGURE 2.

Viability in NS/PCs in 3D hydrogels. (A) The number of viable cells was quantified in each hydrogel condition using CellTiter-Glo®. Statistically significant differences were found between hydrogels with and without RGD at day 28 in STEM is represented with (*) and day 70 in DIFF is represented by (#). Statistical significance was evaluated using Student's t-test with a Holm–Šidák sequential correction for multiple comparisons. Error bars represent standard deviation ($N \geq 4$). (B, C) Immunofluorescence imaging was used to visualize apoptotic (B, cPARP, red) and proliferating (C, Ki67, green) cells, along with nuclear staining (B and C, Hoechst, blue), in 2D and 3D hydrogels with YIGSR, IKVAV, and RGD peptides at day 28 in culture. Images are magnified 200×; inset images are magnified 1000×. Media conditions were also varied: stem cell maintenance (STEM), oligodendrocyte-lineage conditioning (PRE-DIFF), and oligodendrocyte differentiation (DIFF).

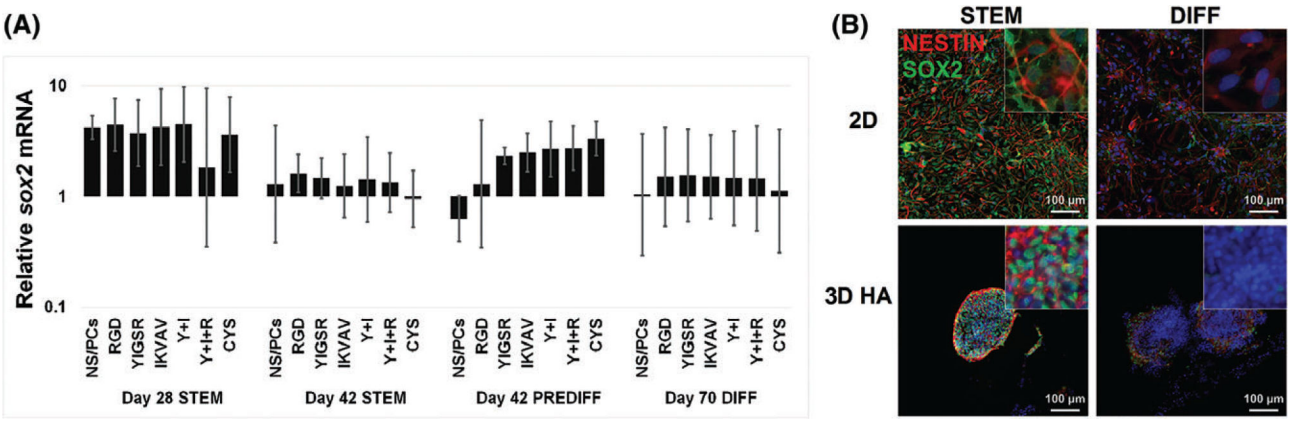

FIGURE 3.

Maintenance of "stemness" in cultured NS/PCs. (A) Levels of *SOX2* mRNA were evaluated using RT-PCR in 2D and 3D cultures under varying media conditions: stem cell maintenance (STEM), oligodendrocyte-lineage conditioning (PRE-DIFF), and oligodendrocyte differentiation (DIFF). Error bars show the standard deviation ($N \geq 4$). (B) NS/PCs cultured in 2D (top) or 3D with YIGSR, IKVAV, and RGD peptides (bottom) were immunofluorescently labeled for nestin (red) and SOX2 (green). Cells cultured in STEM (42 days, left) or in differentiation conditions (PREDIFF, days 28–42 + DIFF, days 43–70; right) both showed expression of nestin and SOX2. Images are 200×; inset images are 1000×.

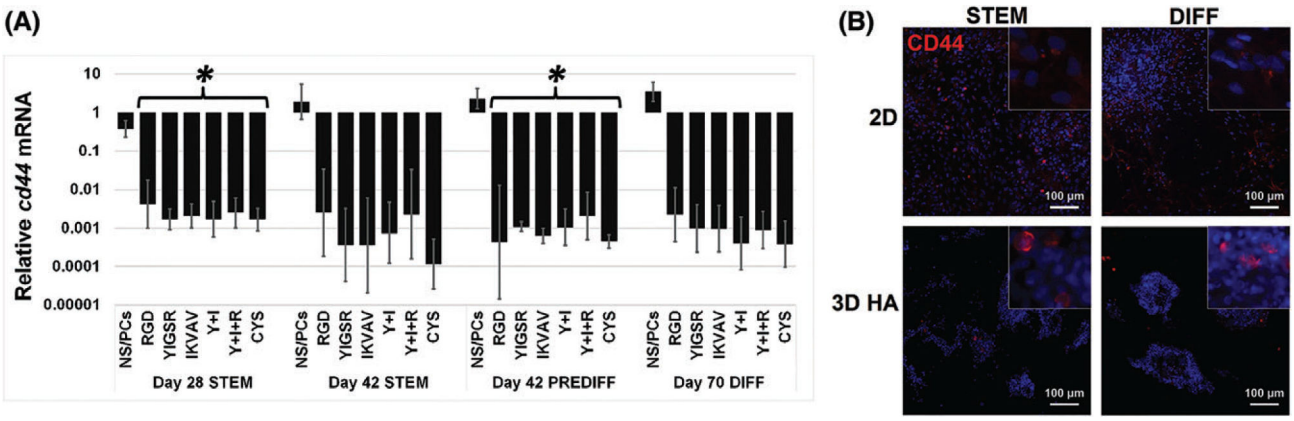

FIGURE 4.

CD44 expression by NS/PCs decreases when cultured in 3D, HA-based hydrogels. (A) *CD44* mRNA was evaluated with RT-PCR in 2D and 3D cultures with varying peptides under varying media conditions: stem cell maintenance (STEM), oligodendrocyte-lineage conditioning (PRE-DIFF), and oligodendrocyte differentiation (DIFF). All 3D culture conditions showed statistically significant decreases in *CD44* expression for day 28 STEM and day 42 PREDIFF compared to 2D controls (*). Error bars show the standard deviation ($N \geq 4$). (B) Cells cultured in either 2D (top) or 3D with YIGSR, IKVAV, and RGD peptides (bottom) were immunofluorescently labeled for CD44. NS/PCs showed very little discernable CD44 expression in all conditions Images are 200×; inset images are 1000×.

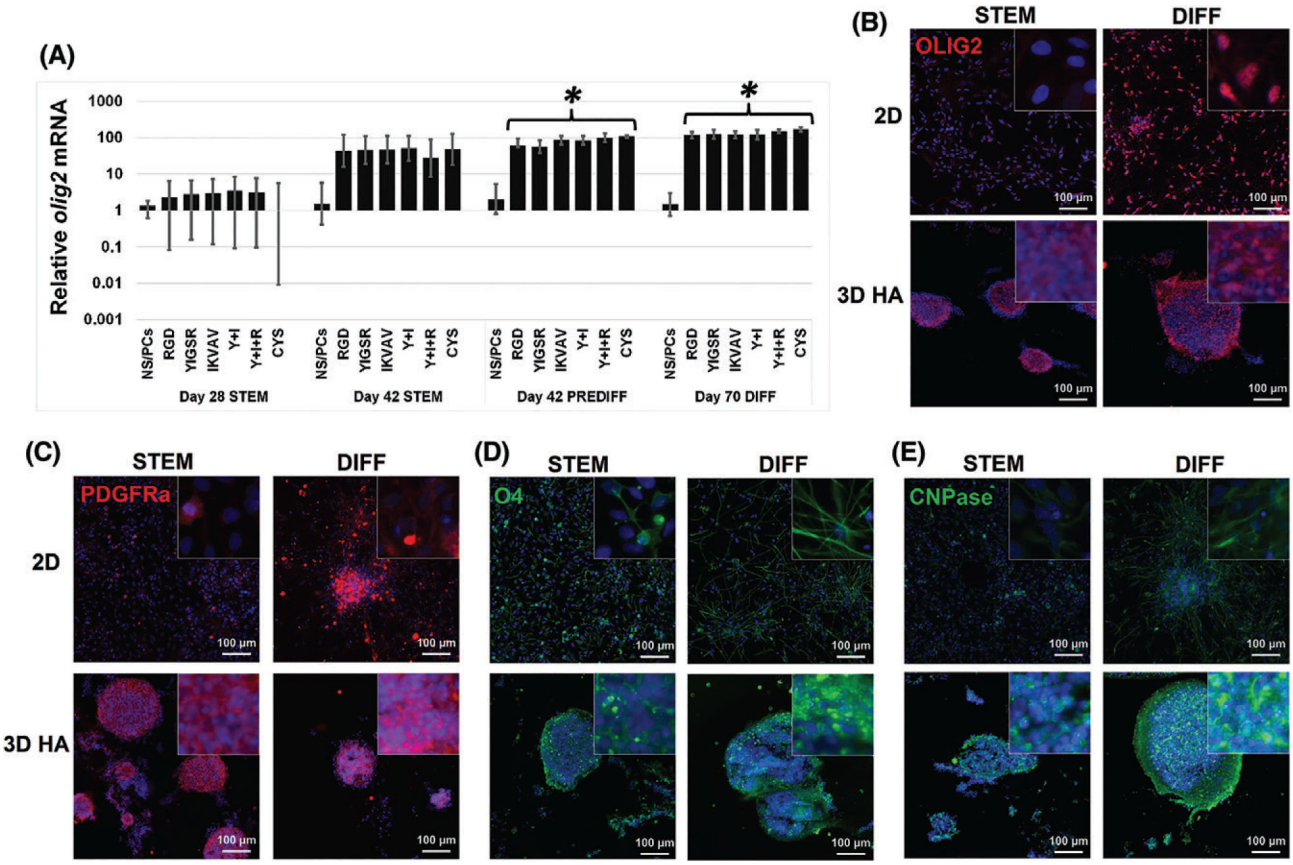

FIGURE 5.

Oligodendrocyte-lineage differentiation increases when NS/PC are cultured in 3D under differentiation conditions. (A) *OLIG2* mRNA was evaluated with RT-PCR in NS/PCs cultured in 2D and 3D with various peptides exposed to different media conditions: stem cell maintenance (STEM), oligodendrocyte-lineage conditioning (PRE-DIFF), and oligodendrocyte differentiation (DIFF). NS/PCs grown in 3D HA with PREDIFF (day 42) and with full differentiation conditions (PREDIFF, days 28–42 + DIFF, days 43–70) showed a statistically significant increase in *OLIG2* expression compared to 2D NS/PCs treated with the same media (*). Error bars show the standard deviation ($N \geq 4$). (B) NS/PCs cultured in either 2D (top) or 3D with YIGSR, IKVAV, and RGD peptides (bottom) were immunofluorescently labeled for OLIG2 expression (red). Fewer NS/PCs expressed OLIG2 in 2D, compared to 3D, when cultured in STEM for 42 days. In 2D, OLIG2 expression OLIG2 increased when cultured in differentiation conditions (day 70) compared to STEM medium (42 days). (C–E) NS/PCs showed an increase in expression of the OPC marker PDGFRα (C), pre-oligodendrocyte marker O4 (D), and oligodendrocyte marker CNPase (E) when cultured in 3D, HA hydrogels with YIGSR, IKVAV, and RGD peptides (bottom) compared to NS/PCs cultured in 2D (top) in both STEM and DIFF media conditions. Images are 200×; inset images are 1000×.

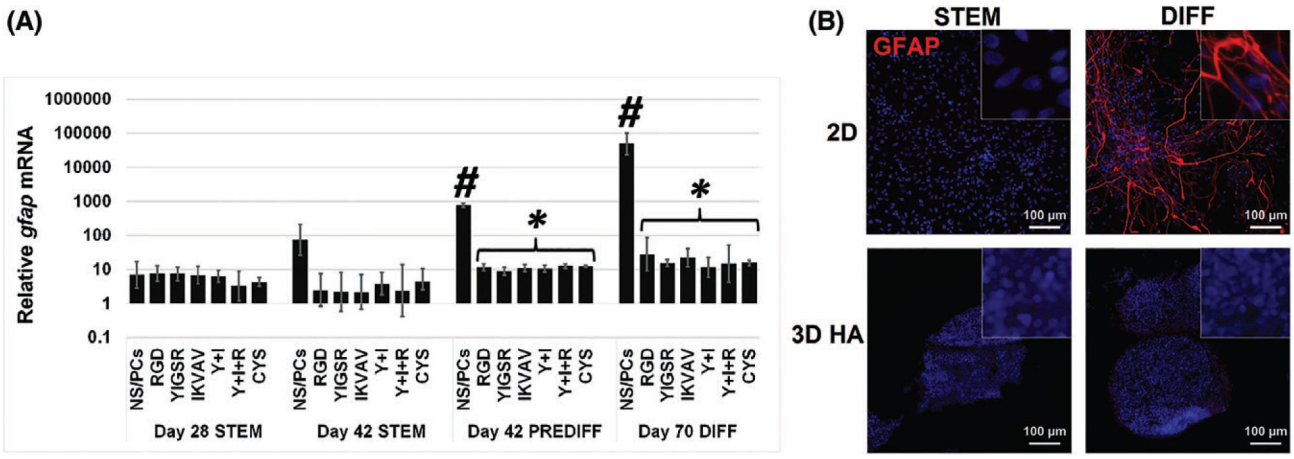

FIGURE 6.

GFAP expression by NS/PCs increases with 2D culture and differentiation conditions. (A) *GFAP* mRNA was evaluated with RT-PCR in 2D and 3D hydrogels. In 2D, both PREDIFF (day 42) and full differentiation (PREDIFF, days 28–42 + DIFF, days 43–70) conditions significantly increased *GFAP* expression compared to 2D STEMRPO (day 28) (#). Both PREDIFF (day 42) and differentiation (day 70) conditions in 3D cultures showed a statistically significant decrease in *GFAP* expression compared to NS/PCs cultured in the same media conditions in 2D (*). Error bars show the standard deviation of multiple experiments. Error bars show the standard deviation ($N \geq 4$). (B) NS/PCs cultured in either 2D (top) or 3D with YIGSR, IKVAV, and RGD peptides (bottom) were immunofluorescently labeled for GFAP (red), a marker of activated astrocytes. Images are 200×; inset images are 1000×. Media conditions: stem cell maintenance (STEM), oligodendrocyte-lineage conditioning (PRE-DIFF), and oligodendrocyte differentiation (DIFF).

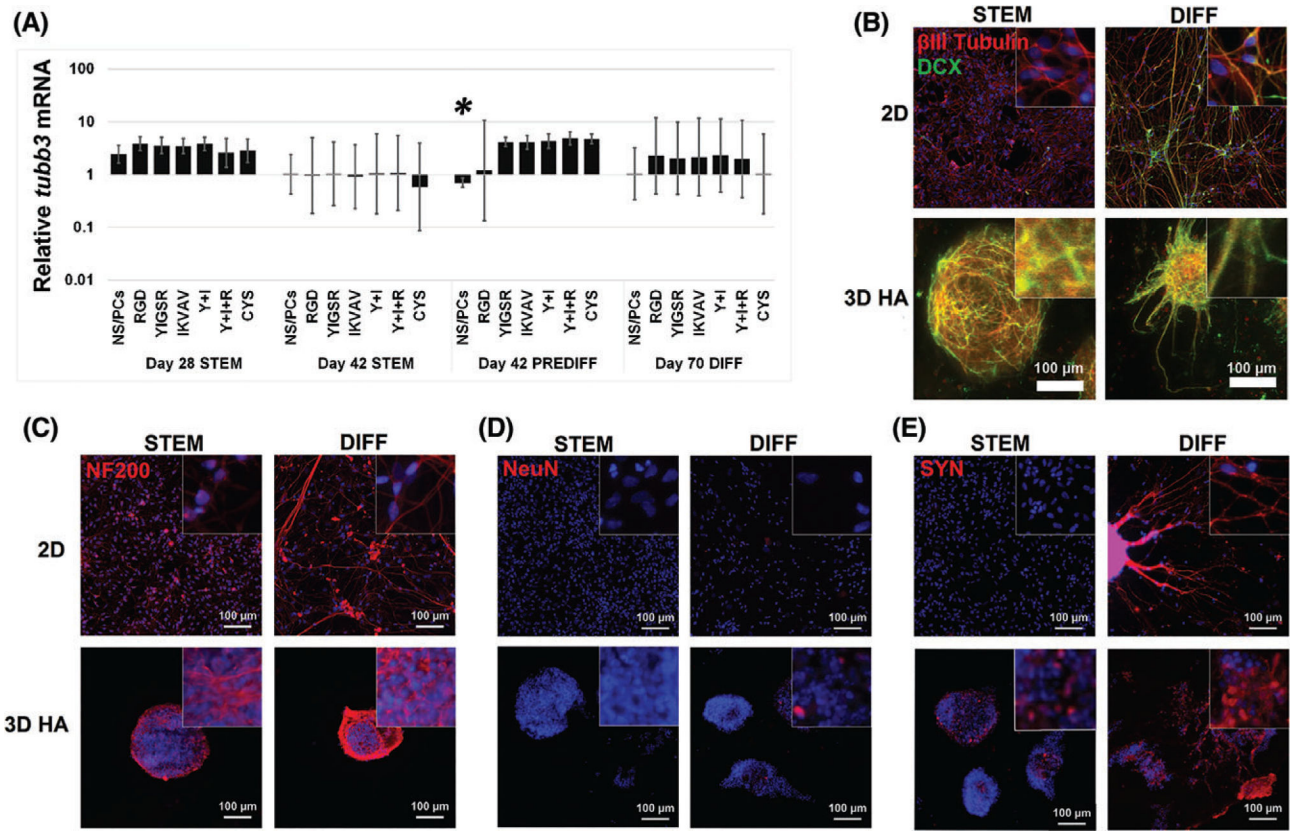

FIGURE 7.

3D culture increased neuronal differentiation of NS/PCs. (A) *βIII-tubulin* (*TUBB3*) mRNA was evaluated with RT-PCR in 2D and 3D HA with varying adhesive peptides. In STEM (day 42), all 3D conditions showed a statistically significant decrease in *TUBB3* compared to 2D NSPC at day 28 in STEM (*). Error bars show the standard deviation ($N \geq 4$). (B–E) NS/PCs cultured in either 2D (top) or 3D with YIGSR, IKVAV, and RGD peptides (bottom) were immunofluorescently labeled for neuronal markers. Neuronal differentiation of NS/PCs in 3D cultures increases under oligodendrocyte-promoting differentiation conditions. (B) Expression of TUBB3 (red) and doublecortin (DCX, green) were observed in all conditions. DCX expression appeared to increase after undergoing the full, 70-day differentiation protocol (PREDIFF, days 28–42 + DIFF, days 43–70) and in 3D cultures. For 3D cultures, maximum projections of stacked images acquired using confocal microscopy are shown. (C–E) NS/PCs showed an increase in expression of mature neuronal markers neurafilament 200 (NF200, C), NeuN (D) synaptophysin (SYN, E) after undergoing the full, 70-day differentiation protocol. NS/PCs grown in 3D hydrogels with YIGSR, IKVAV, and RGD peptides (bottom) showed increased NF200 compared to those cultured in 2D in both STEM (day 42) and DIFF (day 70) conditions. (D) NS/PCs grown in 3D in DIFF conditions (day 70) exhibited more cells with nuclear NeuN than 3D cultures in STEM (day 42) or 2D cultures. (E) In 2D, SYN expression increased between STEM (day 42) and DIFF (day 70). In 3D cultures, SYN was observed in both STEM (day 42) and DIFF (day 70) conditions. Images are 200×; inset images are 1000×. Media conditions:

stem cell maintenance (STEM), oligodendrocyte-lineage conditioning (PRE-DIFF), and oligodendrocyte differentiation (DIFF).

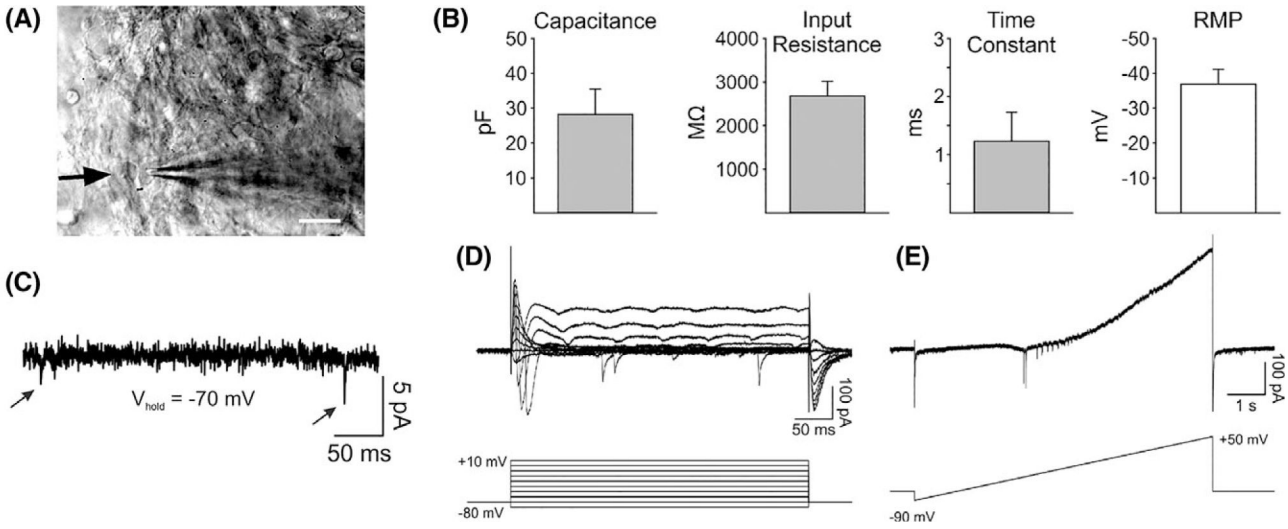

FIGURE 8.

NS/PCs differentiated in 3D HA hydrogels exhibit electrophysiological properties of immature neurons. (A) Infrared differential interference contrast microscopy (IR-DIC) image taken from a recorded cell (arrow). Scale bar = 20 μm. (B) Membrane properties of recorded NS/PCs ($N = 5$). (C) Whole-cell patch clamp recording at a holding potential of −70 mV shows the presence of occasional synaptic inputs (see arrows) in a recorded NS/PC. (D) Sample recording from an NS/PC at different voltage steps (−80 mV to +10 mV, 10 mV increments) shows the presence of transient voltage-gated Na+ inward currents K+ outward currents. (E) Recording of voltage-gated currents from the same NS/PC as shown in D that are activated during a slowly depolarizing ramp (8 s).