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RECOMBINANT COLLAGEN SCAFFOLDS AS SUBSTRATES FOR HUMAN NEURAL STEM/PROGENITOR CELLS

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Abstract

Adhesion to the microenvironment profoundly affects stem cell functions, including proliferation and differentiation, and understanding the interaction of stem cells with the microenvironment is important for controlling their behavior. In this study, we investigated the effects of the integrin binding epitopes GFOGER and IKVAV (natively present in collagen I and laminin, respectively) on human neural stem/progenitor cells (hNSPCs). To test the specificity of these epitopes, GFOGER or IKVAV were placed within the context of recombinant triple-helical collagen III engineered to be devoid of native integrin binding sites. HNSPCs adhered to collagen that presented GFOGER as the sole integrin-binding site, but not to IKVAV-containing collagen. For the GFOGER-containing collagens, antibodies against the β1 integrin subunit prevented cellular adhesion, antibodies against the α1 subunit reduced cell adhesion, and antibodies against α2 or α3 subunits had no significant effect. These results indicate that hNSPCs primarily interact with GFOGER through the α1β1 integrin heterodimer. These GFOGER-presenting collagen variants also supported differentiation of hNSPCs into neurons and astrocytes. Our findings show, for the first time, that hNSPCs can bind to the GFOGER sequence, and they provide motivation to develop hydrogels formed from recombinant collagen variants as a cell delivery scaffold.

Keywords

recombinant collagen; GFOGER; neural stem cells; integrin; IKVAV

INTRODUCTION

Great strides have been made in the field of regenerative medicine in identifying the importance of the cellular microenvironment for regulating basic cell functions such as adhesion, proliferation, and differentiation. The mechanical stiffness and epitopes displayed

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by the extracellular matrix (ECM) both significantly affect these basic cell processes. With this information, biomaterials can be tuned to optimize chemical and physical properties, polymerization and degradation rates, nanoscale architecture, and cell adhesion sites within the cellular microenvironment in order to modulate cell behavior[1-3]. The utilization of naturally-derived biomaterials as cell scaffold components is stymied by large-scale manufacturing bottlenecks and batch-to-batch variation of materials sourced from animals to be used in clinical settings[4,5]. One of the most commonly found naturally-derived ECM materials is collagen, comprising more than 90% of the natural ECM in mammals[6,7]. Collagen has been studied broadly as an ECM substrate for tissue engineering applications, but native collagen is typically acquired from animal sources, thereby permitting minimal control over cell-material interactions[8].

To combat these issues, we developed a modular recombinant human collagen III platform that allows for the introduction or removal of functional sites at desired locations and frequencies throughout the polymer[9,10]. By utilizing the degeneracy of the genetic code, the nucleotide sequence for collagen was optimized for assembly by PCR, allowing for complete control of the protein sequence at the nucleotide level. Collagen III, found in elastic tissues such as skin and vasculature[11,12], was chosen as it exists as a homotrimer, requiring only the expression of a single gene and removing complexity of separating various heterotrimer products. This recombinant human collagen III system yields stable triple-helical collagen that can be used as an effective biomaterial to support mammalian cells[13].

Collagen has been developed as a biomaterial for culturing neural stem/progenitor cells (NSPCs), and these cells can remain in a stem-like state or efficiently differentiate into neurons, astrocytes, and oligodendrocytes when cultured within 3-dimensional collagen scaffolds[14,15]. Collagen abundance within the mature nervous system is low, with collagen (likely collagen I) appearing in the connective tissue surrounding the central nervous system (CNS) as well as in the basement membranes (collagen IV)[16]. Despite their relatively low levels, collagen I and IV present in fractones may regulate adult neurogenesis (differentiation of neurons from neural stem cells)[16]. Furthermore, collagen used as a scaffold for NSPCs in a rotating wall vessel bioreactor culture system promotes their differentiation and supports formation of a two-layered structure mimicking cerebral cortical architecture of the embryonic brain[17,18]. Cells in the CNS bind to collagen primarily through the $\alpha 1\beta 1$, $\alpha 2\beta 1$, and $\alpha 3\beta 1$ integrin heterodimers[19]. Collagen has been used as a transplant scaffold for NSPCs in brain injury models, supporting both migration and differentiation of transplanted cells[20]. In this study, we investigated the effect of various recombinant collagen III scaffolds with inserted integrin-binding peptide sequences (GFOGER [Gly-Phe-Hyp-Gly-Glu-Arg] from collagen I and IKVAV [Ile-Lys-Val-Ala-Val] from laminin) on human NSPC (hNSPC) adhesion, proliferation, and differentiation. The ability of synthetic recombinant collagen to support hNSPC behavior would bode useful for translating NSPC/scaffold-based therapies into the clinic where the manipulability of collagen (e.g., size, sequence, number of cell interaction domains) would be of utmost importance.

MATERIALS AND METHODS

Collagen Variant Gene Synthesis

Recombinant collagen variants were fabricated using methods described previously[9]. Twelve modular, overlapping oligonucleotide fragments (primary fragments, PFs) encoding the entire triple-helical region of human collagen III were synthesized[9]. To introduce the desired functionalities, PFs were utilized as DNA templates for PCR-based site directed mutagenesis[21]. Mutagenesis primer pairs were amplified in a PCR reaction with the specified PF, digested with *Dpn*I, and transformed into *Escherichia coli*. Fragments containing the desired mutations were mixed-and-matched and assembled by PCR to produce the desired full-length variants as described in Table 1.

A variant (rCol-0G) which had the native integrin binding sites in collagen III (GROGER, GAOGER, GLOGEN, GLKGEN, and GMOGER) replaced with the non-binding sequence GSPGGK was produced, yielding a version of collagen that does not support cell adhesion[21]. This variant served as the template upon which to systematically introduce the binding motifs GFOGER or IKVAV, neither of which naturally exist in human collagen III. GFOGER is an α2β1 integrin binding motif found natively in collagen I[22]. As it has been shown that the density of the integrin binding motifs RGD, IKVAV, and YIGSR affected neural stem/progenitor cell differentiation[23], we designed variants to test whether the integrin binding motif GFOGER would produce similar results. Variants were produced with either 1, 2 or 4 GFOGER sequences introduced into rCol-0G to determine whether the amount of GFOGER presented had cellular effects[21]. GFOGER locations were chosen to minimize the number of amino acid changes and provide approximate even spacing between multiple sites. The pentapeptide IKVAV from laminin, reported to bind to α3β1 and α6β1 integrins [24] and enhance cell viability and neuronal differentiation[25], was also introduced into rCol-0G to test its function within the context of triple-helical collagen[26]. The location where IKVAV was introduced was chosen to minimize the number of amino acid changes and to replace only a single glycine (Gly) in the Gly-X-Y characteristic repeating sequence (to minimize disruption to structural stability).

Collagen Variant Expression, Purification, and Characterization

Recombinant collagens were expressed and purified utilizing previously described methods[21]. In summary, the full-length collagen variant genes were introduced into CEN/ARS plasmids and transformed into *S. cerevisiae* strain BYα2β2, which has 2 copies of genes encoding for human prolyl-4-hydroxylase (P4H) inserted into its chromosomes. The inclusion of P4H is required for the hydroxylation of proline, which enhances the thermostability of the triple helical structure of collagen. Expression of collagen was induced by culturing the yeast in galactose containing media (2% galactose, 5% casamino acids, 6.7 g/L yeast nitrogen base without amino acids, and 20 mg/L adenine sulfate) and the yeast was harvested at OD_{600} of 2.5 to 3.5. The resulting collagen variants were purified with two cycles of cellular disruption by French press at 25k psig, pepsin digestion (0.2 mg/mL) of the whole cell lysate, and serial salt precipitation (3M NaCl).

Expressed collagen variants were characterized by circular dichroism using published methods[10]. Triple-helical secondary structure was determined by scanning protein samples in 50 mM acetic acid using a spectropolarimeter equipped with a Peltier controller between 190-260 nm at 10°C with a scanning speed of 50 nm/min in a 0.1 cm path length quartz cell. Apparent melting temperature, defined as the temperature at which 50% of the protein is unfolded, was determined by monitoring ellipticity at 221 nm from 15°C to 65°C with a heating rate of 1°C/min. The thermal profile was fit to a thermodynamic model with multi-parameter, non-linear regression using the Levenberg-Marquardt algorithm within MATLAB[27,28].

Substrate Preparation

To prepare coated surfaces, 100 μL of 20 μg/mL of native and recombinant collagens (in MEM) were incubated per well in 96-well non-tissue culture treated polystyrene plates for 24 hours at 4°C. The solutions were removed prior to the seeding of the cells. Previous work quantifying surface protein densities have confirmed surface saturation of the collagen biopolymers at these concentrations and conditions[13]. As controls, surfaces were coated with 100 μL of laminin at 20 μg/mL and with 100 μL of fibronectin at 10 μg/mL. Table 1 details the different recombinant collagen variants used in these studies.

Cell Culture

Fetal-derived hNSPCs (SC27) were isolated from the cerebral cortices of brain by the National Human Neural Stem Cell Resource and were grown as adherent cultures on 6-well plates coated with 10 μg/mL human fibronectin[29]. HNSPC basal medium included DMEM/F12, 20% BIT 9500 (bovine serum albumin, insulin, and transferrin), and 1% antibiotic/antimycotic (penicillin/streptomycin/amphotericin). Proliferation media was prepared from basal media via addition of 40 ng/mL epidermal growth factor (EGF), 40 ng/mL basic-fibroblast growth factor (bFGF), and 40 ng/mL platelet-derived growth factor (PDGF-AB). Adhesion of SC27s to the substrate was measured at 1 day post cell-seeding and proliferation was measured at 3 days post seeding in proliferation media onto coated 96 well plates. Differentiation media consisted of 96% Neurobasal, 2% B-27, 1% L-glutamine (GlutaMAX, Thermo Fisher Scientific, Waltham, MA, USA), and 1% pen/strep (penicillin/streptomycin) with 20 ng/mL brain-derived neurotrophic factor (BDNF), 20 ng/mL glial-derived neurotrophic factor (GDNF), and 0.5 μM dibutyryl cyclic AMP (cAMP) (modified from Yuan et al.[30]) HNSPCs were differentiated for 14 days in this media formulation for assessment of neuron and astrocyte formation. Cells were routinely passaged 1:2 or 1:3 and seeded at 1×10^4 hNSPCs in 100 μL of media per well for experiments and equal numbers of viable cells (Trypan Blue staining) were used for each experimental group. Proliferation media was refreshed every day (50%) for passaging and every other day (100%) for proliferation assays to minimize mechanical disruption of the gels with daily media changes. Differentiation media was refreshed (100%) every other day.

Cell Adhesion and Integrin Blocking Assays

To assay cellular adhesion to the substrates, hNSPCs were dissociated and seeded in substrate-coated wells. Cells were imaged using phase contrast microscopy at 24 hours post-seeding. To quantify cellular adhesion to the surfaces, cells were visually counted on at least

three representative viewing frames per surface; cells that were rounded or spherical were considered not adhered, and percent cell adhesion was calculated.

To assess the role of integrins in cell adhesion, hNSPCs were dissociated and incubated for 30-45 minutes in 20 μg/mL function blocking (β1 (MAB2253Z), α1 (MAB1973Z), α2 (MAB1950Z), α3 (MAB1952Z)) or IgG1 isotype control antibodies (Millipore, Temecula, CA, USA). Cells were then plated and imaged using phase contrast microscopy at 24 hours post seeding. To quantify cellular adhesion to the surfaces after incubation with anti-β1 antibodies, cells were visually counted in at least three viewing frames per surface; cells that were rounded or spherical were considered not adhered, and percent cell. To determine how many living cells were adhered to each substrate, cells were washed once with MEM and subsequently incubated with 4 μM Calcein-AM in 0.1% bovine serum albumin (BSA)/PBS at 37°C for 1 hour. After a 5-minute wash in DPBS, solutions were removed and hNSPCs were lysed with detergent solution (CelLytic M, Sigma Aldrich, St. Louis, MO, USA) and fluorescence was measured on a plate reader at 494/517 nm (excitation/emission) and normalized to readout values for hCol I.

Immunostaining and Imaging

HNSPCs were fixed with 4% paraformaldehyde for 10 min as described previously[31]. Fixed cells were treated with 0.3% Triton X-100 in PBS for 5 minutes prior to blocking for 1 hour in 5% BSA in PBS. Cells were incubated in primary antibody in 1% BSA/PBS overnight at 4°C. For differentiation experiments, neurons were stained with 1:200 mouse anti-MAP2 and astrocytes with 1:200 rabbit anti-GFAP antibodies which have been used previously for identification of neurons and astrocytes; undifferentiated hNSPCs do not express these antigens[32]. For proliferation experiments, cells not in G_0 of the cell cycle were stained with 1:500 rabbit anti-Ki67. Following three 5-minute PBS washes, samples were incubated with secondary antibodies at a 1:200 dilution in 1% BSA/PBS at room temperature in the dark for 2 hours. The secondary antibodies used were donkey anti-mouse 488 and donkey anti-rabbit 555. Following three 5-minute PBS washes, cell nuclei were counterstained for 1 minute with Hoechst 33342 at 1:500 in PBS and wells were covered with 20 μL Antifade mounting medium (Vectashield, Vector Labs, Burlingame, CA, USA) and imaged using a confocal microscope with a 20X objective. ImageJ was used to quantitate positively stained cell nuclei for proliferation.

Statistical analysis

Statistical analysis was performed using GraphPad Prism. For the quantification of initial adhesion and of cell proliferation, a one-way ANOVA was performed followed by Tukey's HSD test. For the quantification of adhesion after integrin blocking, a two-way ANOVA was performed followed by Tukey's HSD test. Three independent biological replicates were used for experiments (N = 3), and data is reported as mean ± S.E.M. P-values less than 0.05 were considered significant.

RESULTS

Variants Display Characteristics Similar to those of Native Collagen

All recombinant collagen variants listed in Table 1 were successfully fabricated, and they displayed characteristics similar to those of recombinant collagen III (rCol), which has the native human collagen III amino acid sequence. The CD spectra for these variants confirmed the structure of triple-helical collagen with a positive peak at 221 nm and negative peak at 198 nm, and representative CD data for one variant (rCol-0G) is shown in Fig. 1. A complete list of spectra, apparent melting temperatures (T_m), and AFM images for the GFOGER variants and controls has been presented in Que et al.[21] T_m of variants were comparable to that for rCol (35.8 ± 0.5°C)[21].

HNSPCs Adhere to Recombinant Collagen-Coated Surfaces

The variants of collagen (Table 1) were investigated as substrates for hNSPCs. Human collagen I and III were used as controls for the mimetic collagens. A variant of rCol (rCol-0G) in which the non-binding GSPGGK peptide sequence replaced each of the reported α2β1 integrin binding sequences in recombinant collagen III (rCol) was previously shown not to support cell adhesion[21] and was used as the starting collagen template for the other recombinant variants. Three other recombinant collagens with 1, 2, or 4 inserted GFOGER peptide binding sequences were used as experimental surfaces (rCol-1G-8, rCol-2G-8,11, rCol-4G). The rCol substrates with incorporated GFOGER sites supported the adhesion and formation of focal adhesion complexes in HT-1080 cells, with more complexes formed on rCol-4G compared to both native and other recombinant collagens[21]. Fibronectin and laminin were used as positive controls in the study.

We observed that hNSPCs adhered to human collagen I and III, rCol, and all surfaces that contained at least 1 GFOGER site within the collagen variant (Fig. 2). Surfaces with rCol-4G (rCol-0G backbone with 4 GFOGERs) yielded similar cell adhesion and spreading as native human collagen I, collagen III, and laminin. There was a significant increase between rCol compared to rCol-4G, hCol I, and hCol III (Fig. 2j). While a general trend was observed between increasing number of GFOGER with adhesion, no statistically significant differences were detected between the GFOGER-containing variants rCol-1G-8, rCol-2G-8,11 and rCol-4G. Adhesion on the recombinant collagen (rCol) surface was not as robust compared to the native human collagen III (hCol III) control despite the same amino acid sequence. There was no cell spreading observed on rCol-0G, the substrate with the integrin-binding hexamers replaced with the non-binding GSPGGK sequence. This can be seen by the lack of cellular extension and the prevalence of clumped spheres as the cells preferred to adhere to one another instead of the protein-coated surface (Fig. 2h). Additionally, we tested hNSPC adhesion onto rCol substrates with the laminin-based integrin-binding sequence IKVAV (rCol-0G-1I). Interestingly, we did not see robust adhesion or spreading on this substrate (Fig. 2i).

Integrins α1 and β1 Play a Role in hNSPC Binding to Recombinant Collagen Substrates

Since there could be other mechanisms facilitating the adhesion of hNSPCs to the recombinant collagen substrates, we examined the major possible contributors. Non-specific

adhesion would be due to electrostatic interactions. To test for charged-based adhesion, we included the rCol-0G variant as a control, which would have similar charge to that of the GFOGER variants. Because the rCol-0G variant did not support robust cellular binding or spreading, adhesion of the cells to the other collagen variants is likely to be actively mediated rather than due to non-specific electrostatic interactions. Since we observed no binding on surfaces coated with rCol-0G, we omitted this variant from subsequent tests.

RNA sequencing (RNA-Seq) data shows that hNSPCs cultured on laminin or fibronectin express several α and β integrin subunits that mediate binding to the ECM at various expression levels[33], and they express collagen-binding integrins α1, α2, and α3 at moderate to high levels (Table 2). As we are interested in the initial binding of hNSPCS to the substrates and found the adhesion of the cells to be robust or impaired quite soon after plating, the RNA-Seq data should accurately reflect the state of the cells prior to encountering the surfaces.

Based on these data, we first studied whether the most promiscuous and highest-expressed integrin subunit in hNSPCs, β1, is involved in cell binding by using a blocking antibody against β1 to observe its effects on substrate adhesion. We found that blocking the β1 integrin fully disrupts binding to any of the collagen-based substrates, regardless of native or recombinant source (Fig. 3). In contrast, there was only a slight effect of blocking β1 integrin on hNSPC binding to laminin or fibronectin.

Since the β1 subunit is involved in mediating hNSPC adhesion to the collagen-based substrates, we tested whether its collagen-binding α subunit counterparts are also involved. We examined α3 because it is highly expressed in hNSPCs based on RNA-Seq data (Table 2), but found that blocking the α3 subunit did not have an effect on adhesion to any of the collagen surfaces (Fig. 4). There was also no effect of inhibiting α2 on hNSPC binding to collagen (Fig. 4). However, blocking the moderately-expressed α1 integrin in hNSPCs yielded a significant decrease in binding (Fig. 4; $p < 0.01$ and lower). Cells treated with a non-specific IgG1 control antibody demonstrated similar levels of adhesion as untreated cells. These findings suggest that hNSPCs bind our novel recombinant collagen substrates at least partially through the α1β1 integrin heterodimer.

HNSPCs Proliferate and Differentiate on Recombinant Collagen Substrates

Since hNSPCs can bind to recombinant collagen substrates through cell surface integrins, we assessed the proliferation and differentiation capacity of hNSPCs to gauge the ability of the substrates to support hNSPC function. We evaluated the proliferation of hNSPCs via Ki67 immunostaining. Ki67 is expressed in cycling cells that are not in the G_0-phase of the cell cycle. We observed similar percentages of hNSPC proliferation on laminin compared to hCol I, hCol III, rCol, rCol-1G-8, rCol-2G-8,11, and rCol-4G based on Ki67 immunostaining (Fig. 5). However, as seen in Fig. 2, the total number of cells is lower on collagen substrates compared to laminin controls. Although it is possible that the lower cell numbers observed on collagen substrates is due to lower cell survival on the substrates, the lack of statistically significant differences in proliferation levels between the surfaces over time (Day 1 and Day 3) suggests that differences in Fig. 2 are due to differences in adhesion to the substrate, rather than cell viability.

With proliferation on collagen substrates confirmed, we investigated the ability of hNSPCs to differentiate on these substrates. We evaluated differentiation via MAP2 (neurons) and GFAP (astrocytes) immunostaining. Differentiation of hNSPCs into neurons and astrocytes was observed after 14 days on all surfaces that supported cellular adhesion (Fig. 6). Cell clustering was observed on all collagen substrates but was not as apparent on rCol-4G. The cell clusters on the collagen substrates made assessment of neuronal and glial morphology difficult, although cells visibly expressed MAP2 and GFAP.

DISCUSSION

HNSPCs bound to varying degrees to the collagen variants expressing different adhesive domains. With no hNSPCs binding to the rCol-0G variant (which had native integrin binding sites replaced with the non-binding sequence GSPGGK), adhesion to the variants must be largely due to the introduced GFOGER sequences. To our knowledge, this observation of neural stem/progenitor cell binding to GFOGER peptide sequences has not been previously reported. The reduced adhesion on rCol compared to hCol III is likely due to the slightly lower thermostability of rCol compared to native hCol III[13] or other issues related to recombinant vs. native collagen expression. For example, native collagen undergo post-translational processing, (such as conversion from pro-collagen to mature collagen by metalloproteinases, or hydroxylysine cross-linking), which are not easily replicated in recombinant systems but can affect higher-order assembly. However, it is unlikely that reduced adhesion is due to reduced density of integrin binding sites, since both rCol and hCol III have the same amino acid sequence.

The lack of hNSPC binding to the collagen variant containing the integrin binding site IKVAV from laminin (rCol-0G-1I) was unexpected; one explanation could be that flanking amino acid sequences have typically been used with IKVAV to achieve binding of NSPCs and these sequences were lacking in our construct[23]. An alternative reason could be lower number of presented epitopes. Based on typical collagen surface densities[13], the number of IKVAV epitopes displayed on the surface within our system (10^{12} epitopes/cm^2) is approximately two orders of magnitude lower than previous studies that utilized IKVAV-containing peptides (10^{14} epitopes/cm^2)[34]. Finally, this sequence does not naturally occur within the context of triple-helical collagen and may not be in a conformation recognized by integrins.

To determine the mechanism of hNSPC adherence to collagen variants, we systematically blocked integrins with function blocking antibodies. Similar to our previous results[21] which utilized HT-1080 human fibrosarcoma cells, hNSPC adhesion to collagen substrates was abrogated when incubated with antibodies against β1 integrin. This clarified that collagen-binding integrins containing the β1 subunit (i.e., α1β1, α2β1, or α3β1[19]) facilitate hNSPC binding to the recombinant collagen substrates. However, when the cells were seeded in the presence of antibodies against α2 or α3 integrin, there was no detectable effect on adhesion. The lack of effect from blocking the α2 integrin subunit was somewhat unexpected, because we previously found that blocking the α2 integrin subunit impeded binding of HT-1080 cells to these collagen substrates[21]. However, RNA-Seq shows that SC27 cells have relatively low expression of the α2 integrin which could also explain the ineffectiveness of antibodies

against α2 integrin (Table 2). The reduced adhesion after blocking the α1 integrin subunit suggests that α1β1 integrin contributes to adhesion of hNSPCs to the GFOGER peptide sequence within collagen. One possible explanation for the incomplete inhibition when blocking the α1 integrin compared to the β1 integrin is that the blocking activity of the β1 antibody is more robust than that of the α1 antibody, solely due to differences in the antibodies which result in their ability to block adhesion. Since α2β1 and α3β1 are ruled out as integrins mediating binding of these cells, another possibility is that the cells bind to collagen via both α1β1 and α11β1, so blocking α1 removes only part of the binding. Other collagen-binding integrins that include subunits such as α10 may also play a role in hNSPC binding to our substrates; however, the relatively low expression of α10 decreases its probability of significantly affecting adhesion (Table 2).

Other non-β1 integrin heterodimers may explain the lack of effect of β1 integrin blocking antibodies on hNSPC adhesion to laminin and fibronectin. The laminin-binding integrin heterodimer α6β4 likely compensates when β1 is blocked and enables adhesion to laminin[35]. The integrin αVβ8 may mediate hNSPC adhesion to fibronectin when the prevalent fibronectin-binding β1-based integrins α3β1, α4β1, α5β1, and αVβ1 are blocked[36].

Supporting proliferation and differentiation are important attributes for a stem cell substrate. The similar level of proliferation on collagen as compared to laminin, indicates that these recombinant collagen substrates ably support hNSPC proliferation. Likewise, the recombinant collagen substrates permitted expression of neuronal and glial differentiation markers. These particular cells do not efficiently generate oligodendrocytes *in vitro*, so we did not pursue this lineage. Ability of hNSPC substrates to promote differentiation may stimulate repair in CNS transplant situations.

It was impractical to quantitatively analyze differentiated cells across samples due to the clustering of cells on collagen substrates over the 14 days of differentiation. The clustering of cells inhibits the ability to utilize the important criterion of cell morphology in determining cell type. Clustering was diminished on the rCol-4G substrate compared to rCol, hCol I, and hCol III, suggesting that the increased number of GFOGER sites may support greater cell adhesion to the surface. The initial binding of hNSPCs to the collagen substrates is not as strong as that on laminin controls (Fig. 2), and the strength of adhesion likely weakens over the course of 2 weeks of differentiation. Several possible explanations for the reduced adhesion are possible. The cells may secrete factors which affect binding to the collagen substrates. The collagen substrates may also denature over the course of the experiment, inhibiting integrin recognition[37]; at 37°C the triple helix of collagen denatures within days[38]. Integrin presentation may change as the cells differentiate, reducing the concentrations of the required integrins to adhere to collagen. However, it is evident from immunostaining that hNSPCs are able to express differentiated cell markers on both native and recombinant collagen substrates, suggesting that recombinant collagen substrates could provide a novel platform that supports hNSPC behavior. Previously, we showed the ability of the modular collagen platform to introduce non-native cysteines within the collagen polymer, which allowed for the formation of hydrogels with low stiffness[10] within the range that would be ideal for neurons[39,40]. By combining non-native cysteines with the GFOGER-

containing recombinant collagen, it could be possible to utilize these substrates for 3D scaffolds.

CONCLUSIONS

This work highlighted the development of novel recombinant collagen substrates for hNSPCs. HNSPCs adhered to collagen III-mimetic substrates that contained varying numbers of inserted GFOGER adhesion peptides. Since no group has previously demonstrated interaction of hNSPCs with GFOGER, this finding adds to the body of literature pertaining to short adhesion sequences that facilitate binding of NSPCs. Recombinant collagen surfaces enabled proliferation and differentiation of hNSPCs, and blocking of the α1 and β1 integrins perturbs binding to the substrate indicating cells interact with rCol variants through the α1β1 integrin heterodimer. These substrates serve as favorable biomaterials for hNSPC culture in 2D, and provide ample motivation to develop rCol-based substrates as 3D scaffolds. Analysis of hNSPC function within 3D rCol matrices would then set the stage for their use as cell delivery scaffolds for the treatment of neurological disease and injury.

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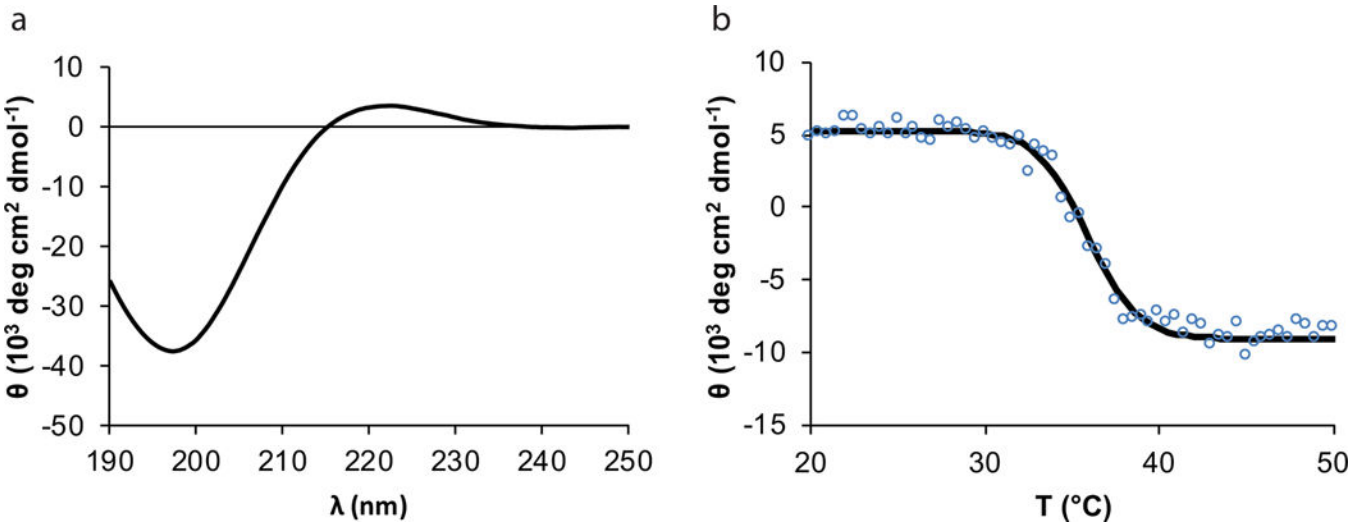

Figure 1. Representative CD wavelength and temperature scan of the non-binding collagen rCol-0G

(a) CD wavelength scans were representative of collagen-like triple-helical secondary structure with a positive peak at 221 nm and negative peak at 198 nm. (b) Thermostability scan measuring ellipticity at 221 nm vs. temperature for rCol-0G yields a melting temperature similar to rCol.

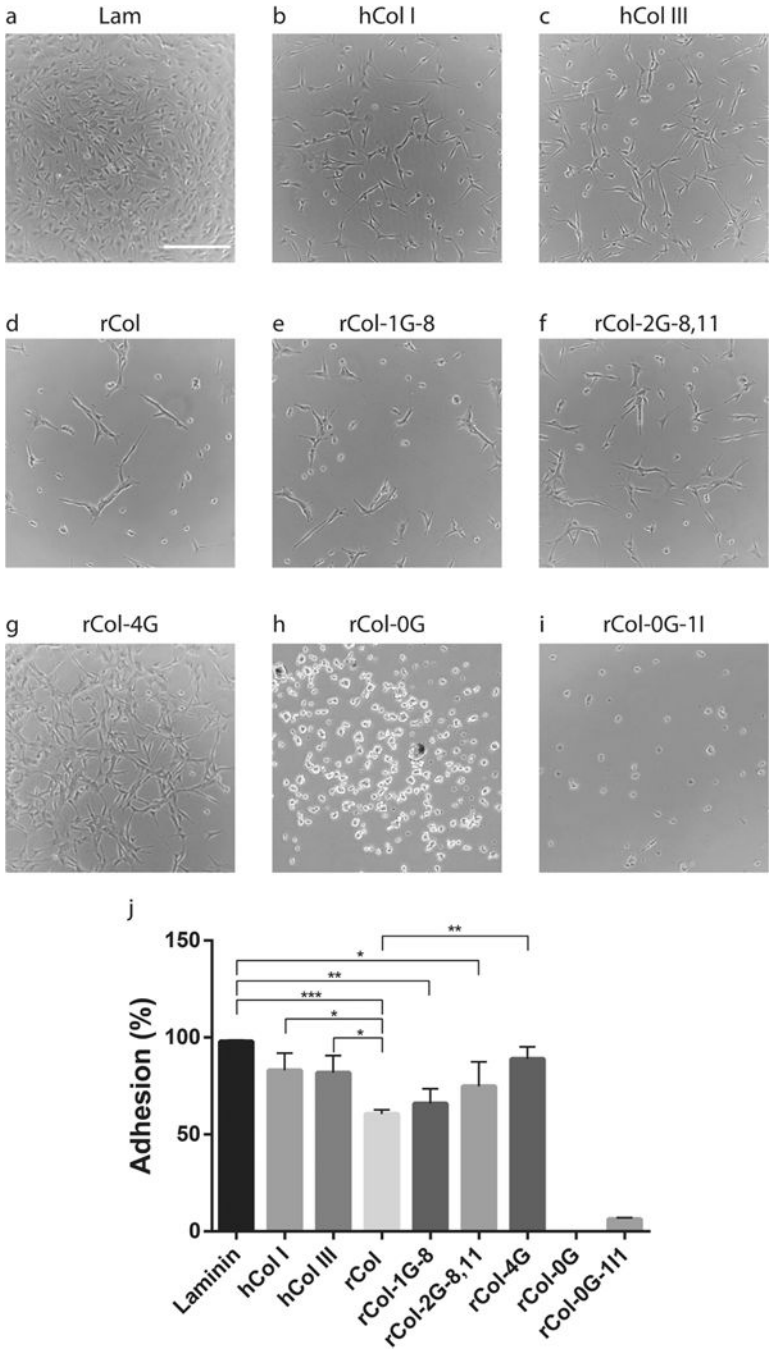

Figure 2. Adhesion of hNSPCs to recombinant collagen substrates

HNSPCs adhere and spread on (a) laminin (Lam), (b) hCol I, (c) hCol III, (d) rCol, (e) rCol-1G-8, (f) rCol-2G-8,11, and (g) rCol-4G. There was no cell binding and spreading on the recombinant collagen variants (h) rCol-0G and (i) rCol-0G-1I. (j) Quantification of adhesion (* denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$). Scale bar is 200 μm.

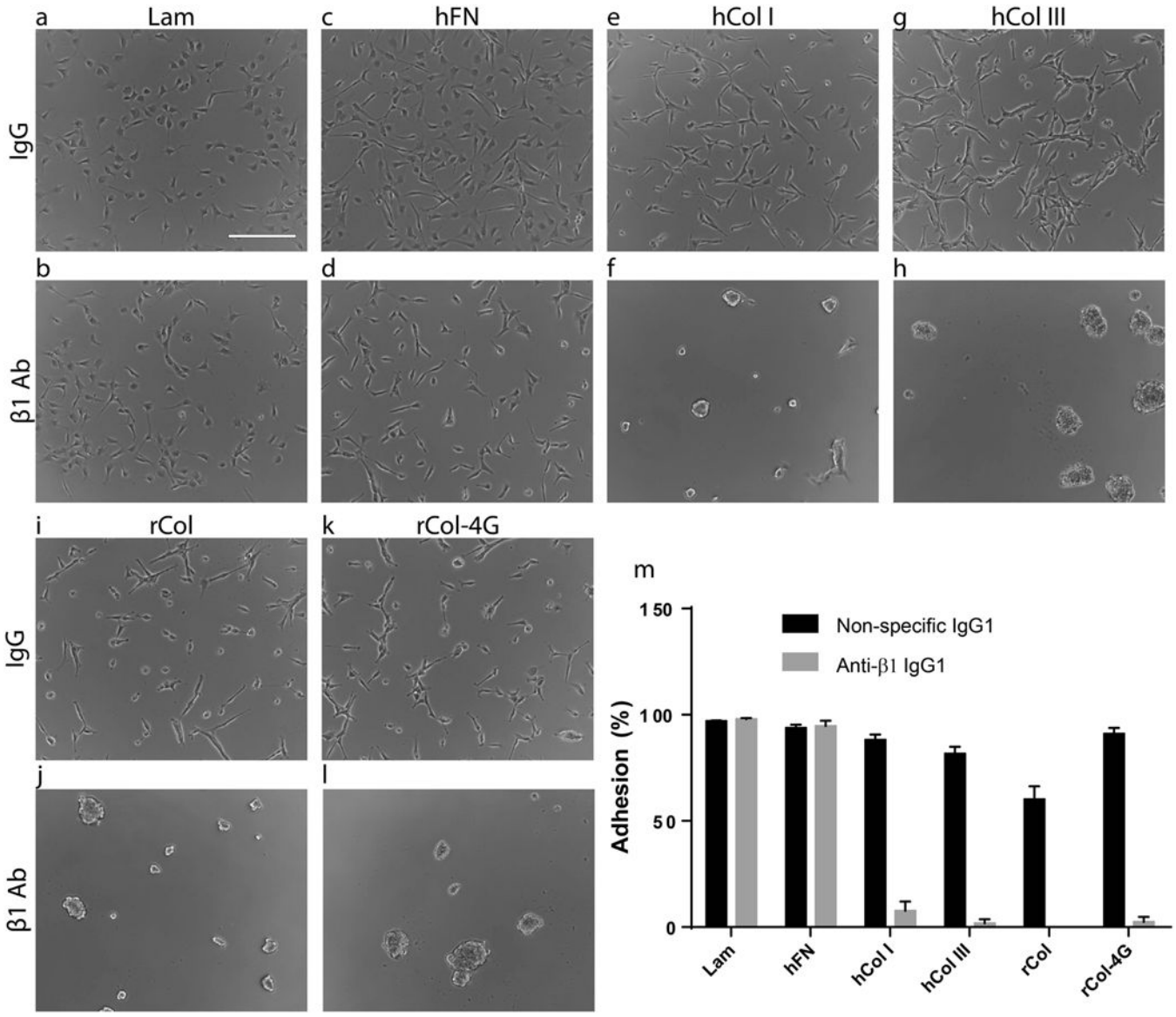

Figure 3. Blocking β1-integrin inhibits hNSPC binding to collagen substrates

HNSPCs were incubated with either a non-specific IgG1 control or function blocking β1 antibody prior to seeding onto (a-b) laminin (Lam), (c-d) human fibronectin (hFN), (e-f) hCol I, (g-h) hCol III, (i-j) rCol, and (k-l) rCol-4G substrates. (m) Quantification of adhesion. There was a minimal effect of the β1 antibody treatment on hNSPC adhesion to control hFN and Lam substrates, but binding was fully blocked on all collagen surfaces. Scale bar is 200 μm.

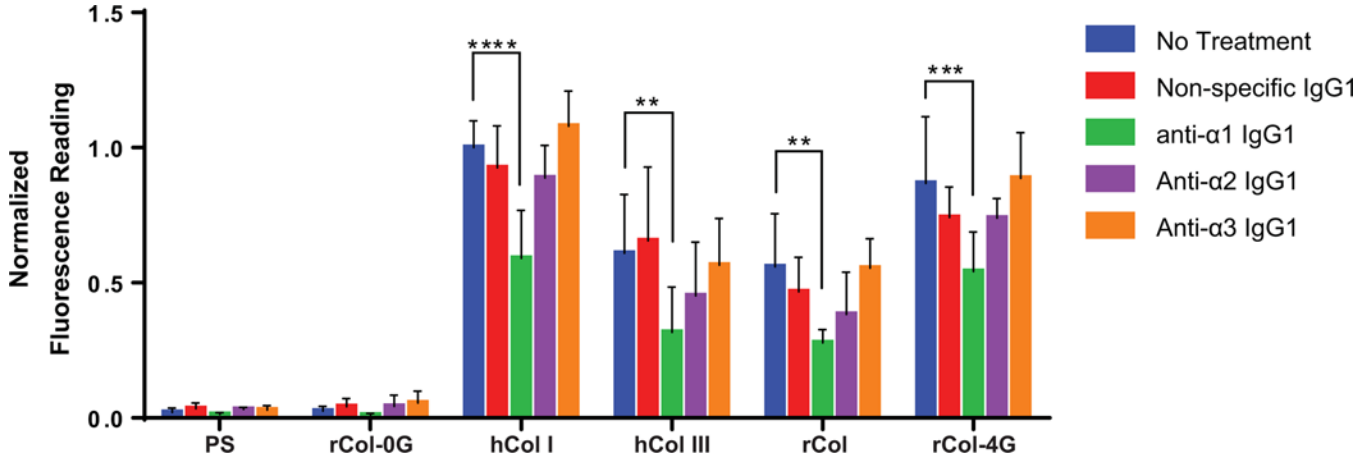

Figure 4. HNSPC adhesion after integrin blocking

HNSPCs were incubated with no antibody, non-specific IgG1 antibody, anti-α1, anti-α2 or anti-α3 antibody prior to seeding on various collagen substrates. The fluorescence readout quantifies the relative number of cells adhered to the substrate via calcein-AM assay. Fluorescence readings were normalized to the "No Treatment" hCol I group. PS = bare polystyrene surface. We note a significant decrease in hNSPC adhesion in α1 blocking antibody-treated groups compared to no treatment on hCol I, hCol III, rCol, and rCol-4G. A two-way ANOVA was performed followed by Tukey's HSD test. **P < 0.01. ***P < 0.001. ****P < 0.0001. Error bars represent S.E.M. N = 3 independent biological repeats.

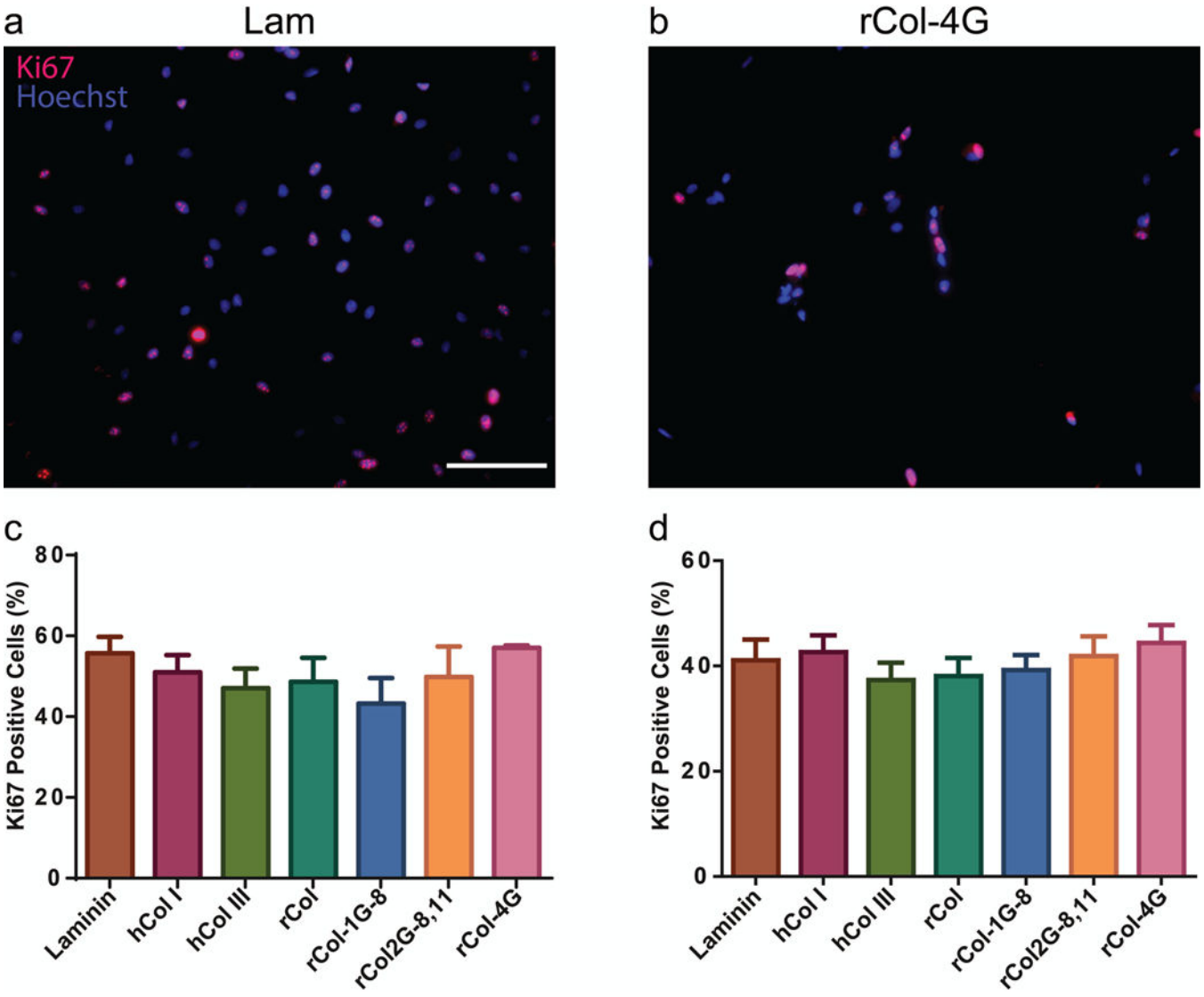

Figure 5. HNSPCs proliferate on collagen substrates

Proliferating hNSPCs not in G_0-phase of the cell cycle were detected by immunostaining for Ki67. Representative fields of cells on (a) laminin (Lam) and (b) rCol-4G are shown, with Ki67 stained in red and Hoeschst stain in blue. Ki67-positive nuclei were quantified after (c) 1 day and (d) 3 days in proliferating conditions. Similar percentages of cell proliferation were observed across all substrates at each timepoint. Scale bar is 100 μm. Mean ± S.E.M. N=3 independent biological repeats.

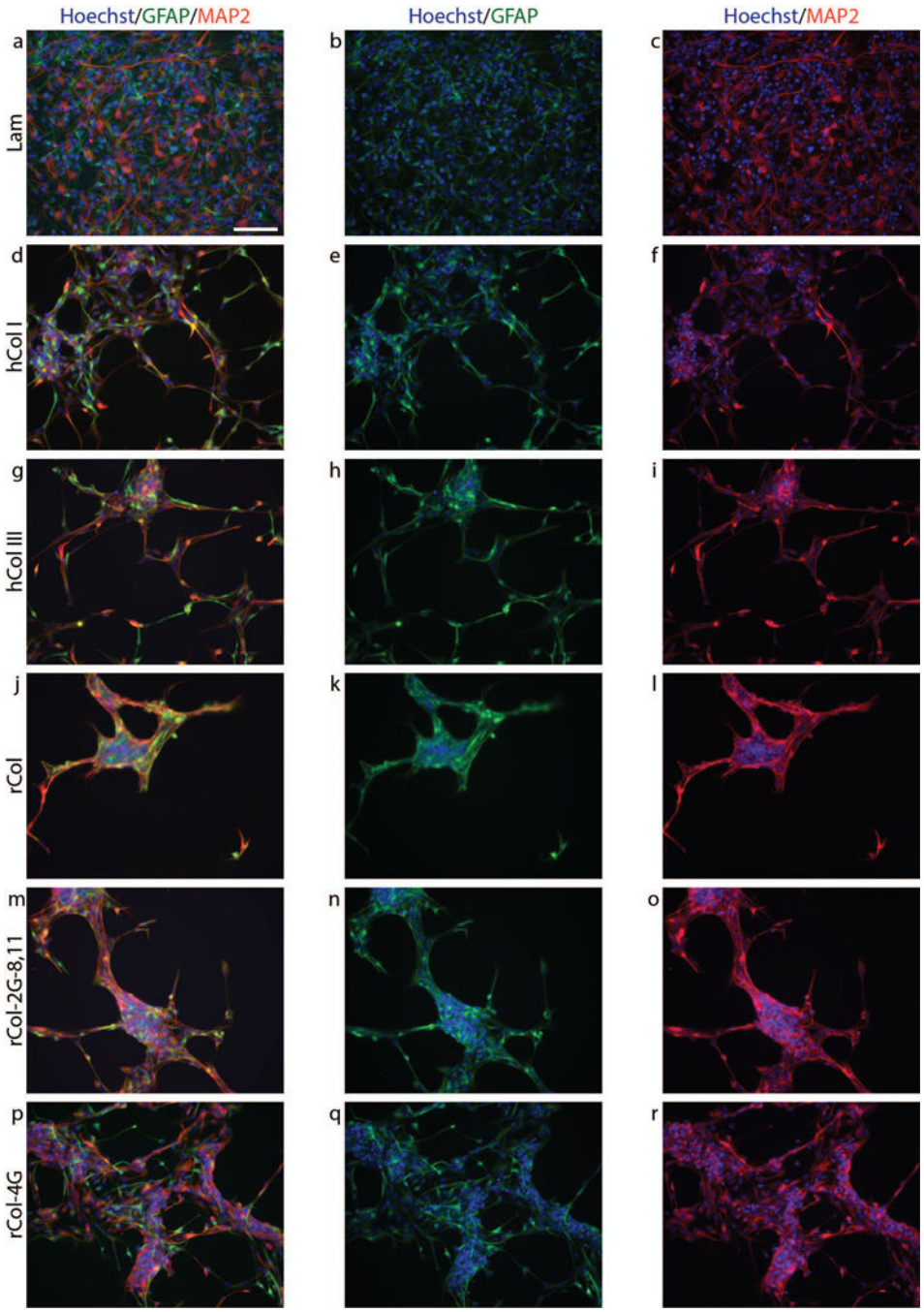

Figure 6. Neuronal and astrocyte differentiation of hNSPCs on collagen substrates

HNSPCs were differentiated for 14 days and differentiation was assessed using the markers GFAP (green; second column) for astrocytes and MAP2 (red; third column) for neurons. The overlay is shown in the first column. Cells express markers of both lineages on (a-c) laminin (Lam), (d-f) hCol I, (g-i) hCol III, (j-l) rCol, (m-o) rCol-2G-8,11, and (p-r) rCol-4G, although cells were more clumped together on the collagen surfaces than on laminin,

making the cell morphology difficult to ascertain. Nuclei were counterstained with Hoechst (blue). Scale bar is 100 μm.

Table 1
Summary of recombinant collagen variants and native human collagen controls

The abbreviated names of the variants, source of protein, and descriptions (with integrin-binding sites) are listed. PF = primary fragment; standard single amino-acid abbreviations are used.

Name	Source	Description
hCol I	Human	Human collagen I; 1 native GFOGER site
hCol III	Human	Human collagen III (no GFOGER, but contains GROGER, GAOGER, GLOGEN, GLKGEN, and GMOGER sites)
rCol	Recombinant	Human collagen III amino acid sequence (no GFOGER, but contains GROGER, GAOGER, GLOGEN, GLKGEN, and GMOGER sites)
rCol-0G	Recombinant	Human collagen III amino acid sequence with GROGER, GAOGER, GLOGEN, GLKGEN, and GMOGER integrin-binding hexamers replaced with non-binding GSPGGK
rCol-1G-8	Recombinant	rCol-0G with 1 GFOGER site inserted (in PF8)
rCol-2G-8,11	Recombinant	rCol-0G with 2 GFOGER sites inserted (in PF8 and PF11)
rCol-4G	Recombinant	rCol-0G with 4 GFOGER sites inserted (in PF1, PF4, PF8, and PF11)
rCol-0G-1I	Recombinant	rCol-0G with 1 IKVAV site inserted (in PF1)

Table 2

Expression levels of collagen-binding integrins in hNSPCs by RNA-Seq analysis

Expression levels are categorized as high (>10 RPKM), moderate (1-10 RPKM), or low (<1 RPKM)[33].

Integrin	RPKM (reads per kilobase per million mapped reads)	Relative Expression Level
α1	4.91	Moderate
α2	1.47	Moderate
α3	52.71	High
α10	0.35	Low
β1	420.28	High
β2	0.02	Low