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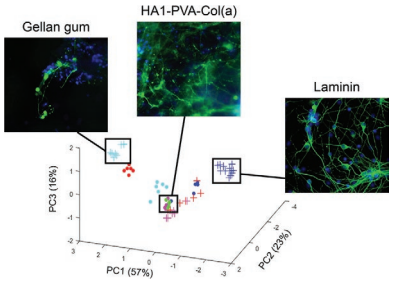

This work presents a wide comparison of 3D scaffolds for human cell–based neuronal tissue engineering. As an outcome, this study gives an interpenetrated network hydrogel, which supports and enables adhesion of the cells by its collagen component. Moreover, it is revealed that integrin $\alpha 6\beta 4$ is a neuronal cell–specific adhesion marker in both 2D and 3D cultures.

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Screening of Hydrogels Reveals that a Hyaluronan-Polyvinyl Alcohol-Collagen-Based Interpenetrating Polymer Network Hydrogel Provides an Improved Scaffold for Human Pluripotent Stem Cell–Derived Neural Cells

Laura Ylä-Outinen,* Venla Harju, Tiina Joki, Janne T. Koivisto, Jennika Karvinen, Minna Kellomäki, and Susanna Narkilahti

There is a clear need for novel in vitro models, especially for neuronal applications. Development of in vitro models is a multiparameter task consisting of cell-, biomaterial-, and environment-related parameters. Here, three different human origin neuronal cell sources are studied and cultured in various hydrogel 3D scaffolds. For the efficient evaluation of complex results, an indexing method for data is developed and used in principal component analysis. It is found that no single hydrogel is superior to other hydrogels, and collagen I (Col1) and hyaluronan–poly(vinyl alcohol) (HA1-PVA) gels are combined into an interpenetrating network (IPN) hydrogel. The IPN gel combines cell supportiveness of the collagen gel and stability of the HA1-PVA gel. Moreover, cell adhesion is studied in particular and it is found that adhesion of neurons differs from that observed for fibroblasts. In conclusion, the HA1-PVA-col1 hydrogel is a suitable scaffold for neuronal cells and supports adhesion formation in 3D.

1. Introduction

Much interest has recently been focused on in vitro models of human organ development and diseases.[1] Especially for studying human central nervous system (CNS) development, function, and dysfunctions, in vitro models could offer new insights because the human CNS is very complex and challenging to study in vivo in detail.[2–4] To meet that goal, neurons of human origin should be cultured in a reproducible and reliable manner. Importantly, to better mimic in vivo–like growth and maturation of human neuronal cells in vitro, 3D cultures with appropriate scaffolds are needed.[3] Various materials have been tested as scaffolds for human pluripotent stem cell (hPSC)-derived neuronal cell–based models. The crucial aspect of the material selected for these scaffolds is that it allows an interaction between cells and the material that is similar to that in vivo between cells and the extracellular matrix (ECM). Different material properties, including mechanical properties, such as stiffness, material chemistry, availability for binding sites, and porosity, affect this interaction.[1,5,6]

Hyaluronan (HA), collagen, and synthetic peptides, such as RADA-16 (PuraMatrix, PM), are all found to have these suitable properties when used as scaffolds for CNS cells in vitro and in vivo.[7–9] Interactions between neuronal cells and ECM modify not only cell movement and adhesion but also neuronal cell differentiation and maturation.[10] Substrate stiffness is well known to modulate cell behavior in both in vitro and in vivo conditions,[11] and more specifically, the 3D environment of ECM or scaffold affects neuronal cell differentiation.[12] In addition, neuronal differentiation can be supported by, for example, electrical conductivity, topography (macro-, micro-, or nano-scale), and chemical composition of the scaffold.[10] These physical and chemical features modify cell signaling via adhesion pathways and mechanotransduction. Integrins are cell membrane–bound proteins mediating these cell–ECM interactions and thus play an important role in cell attachment and behavior.[13] Some integrins are especially associated with neurons such as integrin $\alpha 6\beta 4$, which is found to act as a laminin receptor.[14] Moreover, neuronal cells have various specific ECM receptors, such as 40S ribosomal protein SA (RPSA), that have important roles in growth cones.[15] Kinases, enzymes that add phosphate groups to other molecules, are very important in these signaling events. ECM receptors are linked to kinase activity that transduces the cellular responses to ECM binding. Some of the best-known kinases are focal adhesion kinase (FAK, also known as protein tyrosine kinase 2,

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PTK2), mitogen-activated protein kinase/extracellular signal–regulated kinase (MAPK/ERK), and Rho-associated protein kinase (ROCK). FAK is involved in regulating neuronal cell migration and is associated with axon pathfinding both in vivo and in vitro.[16] In neurons, FAK is localized close to nucleus-attaching microtubules, unlike its localization in other cell types.[17] MAPK is crucial in early neuronal cell development and migration and is linked to doublecortin and microtubule protein expression.[18] ROCK also plays an important role in cellular organization during development, and its dysfunctions have been associated with neurodevelopmental disorders.[19] ROCK also limits neurite extension and downregulates transcriptional regulator yes-associated protein 1 (YAP) expression, thus limiting neuronal differentiation and spreading.[20] Therefore, YAP inhibition plays an important role in neuronal maturation and neurite extension.[21] The active form of YAP in developing neuronal cells maintains proliferation, thus limiting maturation.[22] The stiff substrate (≈10 kPa) promotes YAP localization into the nucleus and keeps cells in the self-renewal stage, whereas the soft substrate (≈0.7 kPa) results in cytoplasmic YAP localization leading to neuronal differentiation.[23]

In summary, several adhesion pathways are associated with neuronal cell growth and maturation. In **Figure 1**, their relation trends to neurite spreading are shown in a simplified schematic drawing.[20,23,24] Understanding the mechanisms of these regulatory factors in cell differentiation is important when reliable and efficient in vitro models and novel therapeutic solutions are wanted.[25] Today, only little is known about the effects of ECM mimicking scaffolds on the cell maturation via adhesion pathways. Natural biomaterials like HA or collagen have cell-responsive binding sites that activate adhesion pathways, like FAK-pathway.[26] Moreover, synthetic biomaterials, like RADA-16, have added binding sites potentially activating adhesion pathways, making all these hydrogels interesting for in vitro adhesion studies.[27,28] In 3D neuronal cultures, a lack of tissue mimicry associated with the 3D architecture and degrees of freedom and the immature stage of hPSC-derived neurons are two main challenges that more careful studies on cell adhesion may help overcome.[29–31] There are several studies in which biomaterials for human neuronal cells have been screened;[5] however, the best is yet to be discovered. Even though many natural and synthetic hydrogels seem to provide a suitable 3D environment for human neurons, more research is needed to study their relevant properties for neuronal cell cultures. One strategy is to create a multicomponent hydrogel because ECM is a complex mixture of polymers with many structural subnetworks. These types of hydrogels are called interpenetrating network (IPN) hydrogels.[32] To date, IPN hydrogels consisting of hydrazone cross-linked hyaluronan (HA) combined with collagen have been used to create human mesenchymal stem cell scaffolds.[32]

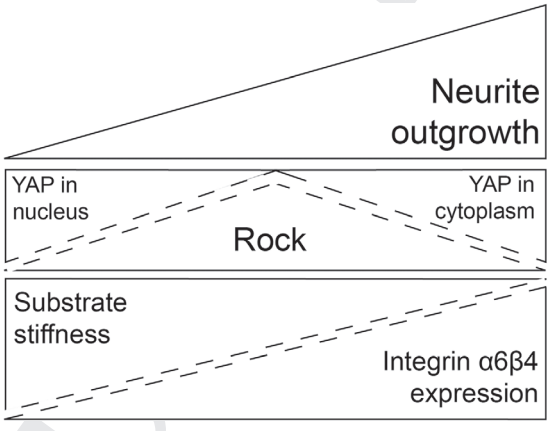

Figure 1. Relationship trends between neurite outgrowth and adhesion-related phenomena based on in vitro studies.

Here, we cultured human neurons derived from three pluripotent stem cell lines in several ECM-mimicking hydrogels, including an IPN hydrogel, and evaluated their performance as a 3D scaffold for human neurons. Neuronal network formation in various hydrogels and hydrogel performance as long-term scaffolds were evaluated with several parameters and analyzed with principal component analysis (PCA). In addition, cell adhesion was studied at the gene and protein levels and was associated with neuronal network formation.

2. Experimental Section

2.1. Cells and Differentiation

The following cells/cell lines were used: human neural progenitor cells hNP1 (hNP1; ArunA Biomedicals; Athens, GA, USA), Regea 08/023 human embryonic stem cell (hESC) line (08023; in-house derived); 10212.EURCCs human induced pluripotent stem cell (hiPSC) line (10212; in-house derived); and a primary human foreskin fibroblast cell line ATCC-CRL_2429 (hFF; ATCC; Manassas, Virginia, USA). All experiments were performed under approval from the Finnish Medicines Agency (FIMEA) to perform research using human embryos (Dnro 1426/32/300/05), and supportive statements were obtained from the Regional Ethics Committee of Pirkanmaa Hospital District for the derivation, characterization, culture, and differentiation of hESCs (R05116) and the use of hiPSCs in studies (R14023). Both used in-house-derived hPSC lines (08023 and 10212) and were under quality control with frequent gene and protein expression analysis, karyotype, and mycoplasma assays. Cultures were maintained at +37 °C in a 5% CO_2 atmosphere and 95% humidity.

Both in-house-derived hPSC lines (08023 and 10212) were cultured with or without a feeder cell layer of human foreskin fibroblasts (Supporting Information 1)[33,34] and differentiated to neurons using the neurosphere method, as described previously.[35] Neurospheres were cultured in neural differentiation media (NDM). The NDM composition was 1:2 DMEM/F:12 and 1:2 Neurobasal, supplemented with GlutaMax (2 mM), B27 supplement (20 µL mL^{-1}), N2 supplement (10 µL mL^{-1}) (all purchased from Thermo Fisher Scientific, Waltham, MA, USA), penicillin/streptomycin (25 U mL^{-1}; Lonza Group Ltd., Basel, Switzerland), and basic fibroblast growth factor (FGF; 8 µL mL^{-1}; R&D Systems Inc., Minneapolis, MN, USA). LDN193189 (1 mM; STEMCELL Technologies, Cambridge, UK) was used as a promoter of neural differentiation. One-third of the medium was changed three times a week, and neurospheres were cut mechanically to a size of 500 µm two times a week. The cells

Table 1. Modified polymer components of HA1-PVA- and HA2-PVA-based hydrogels.

Polymer	Supplier	Molecular weight [g mol^{-1}]	Modified polymer	DS%	Reference
HA	Sigma-Aldrich (St. Louis, MO, USA)	$1.5–1.8 \times 10^6$	HA1 (HAALD1H)[a)]	5	[39]
HA	Lifecore (Chaska, MN, USA)	1.5×10^5	HA2 (HALD1)[b)]	15	[38]
PVA	Sigma-Aldrich (St. Louis, MO, USA)	2.7×10^4	PVA (PVAHY)[a),b)]	13	[39]

[a)]Name according to ref. [39]; [b)]Name according to ref. [38]; DS%, degree of substitution.

were cultured in suspension culture for 8 weeks to induce neural differentiation prior to the experiments.

HNP1 cells were cultured and expanded according to the manufacturer's instructions, except the medium used, which was made from Neurobasal supplemented with GlutaMax (10 mM; Thermo Fisher Scientific), B27 supplement without vitamin A (20 µL mL^{-1}; Thermo Fisher Scientific), penicillin/streptomycin (25 U mL^{-1}; Lonza Group Ltd), and FGF (8 µL mL^{-1}; R&D Systems Inc.). Briefly, hNP1 cells were plated onto Matrigel-coated (1:200; Corning Incorporated, Kennebunk ME, USA) dishes, passaged, and divided 1:2 or 1:3 when they reached 95–100% confluence. Twenty-four hours after each passage, the medium was changed and thereafter every other day until confluence. Frozen vials from passages 7–9 of hNP1 cells were thawed rapidly according to the manufacturer's instructions and used in experiments after 1–3 passages, thus in passages 9 or 10.

HFF cells from passage 8 were cultured adherently on T75 bottles in hFF–fetal bovine serum (FBS) medium containing 1 × 1MDM (Thermo Fisher Scientific), Pen/Strep (5 µL mL^{-1}; Lonza), and FBS (100 µL mL^{-1}; Sigma-Aldrich, St. Louis, MO, USA).

2.2. Hydrogel Preparation

A total of seven different hydrogels were prepared and used in 3D culture experiments: PuraMatrix (PM; BD Biosciences, San Jose, CA, USA [Catalog No. 354250]/3DM Inc., Cambridge, MA, USA[27]), gellan gum (GG; Gelzan, Sigma-Aldrich;[36]), collagen hydrogel (Col1), hyaluronan–polyvinyl alcohol based hydrogels (HA1-PVA, HA2-PVA, HA1-PVA-Col), and hyaluronan–collagen type I–poly(ethylene glycol) ether tetrasuccinimidyl glutarate hydrogel (HA-Col1-4SPEG).

2.2.1. Controls

Based on previous studies and in-house laboratory standards, both positive and negative 3D hydrogel controls were used. The controls were as follows: positive control PM[37] and negative control nonfunctionalized GG.[36] In addition, in-house 2D laboratory standard coating control mouse laminin (Sigma-Aldrich) was used.[14] For PM, cells were mixed with 0.25% PM diluted in 10% sucrose in phosphate-buffered saline (PBS). GG solution (5 mg mL^{-1}) was prepared as previously described,[36] and 1.25% spermidine trihydrochloride (SPD; Sigma-Aldrich) was used as a cross-linker, both dissolved in 10% sucrose.

2.2.2. First-Generation Hydrogels

Aldehyde groups were introduced to HA either by using periodate oxidation (HA1) or selective oxidation of diol-modified HA (HA2). The syntheses and determinations of the degree of substitution (DS%) of modified HA1 and HA2 components were carried out according to previously reported procedures.[38,39] Hydrazide groups were introduced to PVA using glycine ethyl ester and hydrazine as a source of the hydrazide unit. The synthesis and determination of the DS% of the modified PVA component were carried out according to the previously reported procedure.[39] The modified components are presented in **Table 1**.

Two types of HA-PVA hydrogels (HA1-PVA and HA2-PVA) were prepared as previously described.[39] Freeze-dried HA1 or HA2 were dissolved in 10% sucrose to a concentration of 20 mg mL^{-1}, and PVA was dissolved to a final concentration of 10 mg mL^{-1}. To make a 200 µL hydrazone cross-linked hydrogel, 100 µL of both HA1 or HA2 and PVA were used. HA-Col1-4SPEG hydrogel gelation was performed as described previously[40] using following components: rat tail collagen type I (Cultrex, Trevigen, Gaithersburg, MD, USA), HA (Contipro group, Dolni Dobrouc, Czech Republic), and 4SPEG (1000 MW, JenKem Technology, Allen, TX, USA). Collagen type I (Col1) hydrogel (0.5 mg mL^{-1}) was prepared by mixing rat tail collagen type I (5 mg mL^{-1}), 10× PBS, sterile H_2O, and $NaHCO_3$. For example, to make a 200 µL collagen solution, 20 µL collagen stock was mixed with 20 µL 10× PBS, 157.5 µL sterile H_2O, and 2.5 µL $NaHCO_3$.

2.2.3. Second-Generation Hydrogel

IPN hydrogel HA1-PVA-Col was prepared by mixing HA1, PVA, and neutralized collagen solution in a volume ratio of 2:2:1. The final concentration of collagen was 0.5 mg mL^{-1}. To make 200 µL HA1-PVA-Col gel, HA1 was pipetted in the wells. Then, neutralized and diluted collagen solution was first mixed with PVA, and then the cell suspension was added and mixed. Finally, PVA-Col-cell solution was added and mixed in the well with HA1. Hydrogel formation was confirmed by incubating at 37 °C for 15 min, and then the media was added on top of it.

2.3. Mechanical Testing of Hydrogels

Compression testing was performed as previously described,[36,41] using a BOSE Electroforce Biodynamic 5100 machine equipped with a 225 N load sensor and Wintest 4.1 software (Bose Corporation, Eden Prairie, Minnesota, USA). Briefly, HA1-PVA-Col and

HA2-PVA hydrogel samples were cast into a homemade cylindrical mold with an approximate height of 6 mm and a diameter of 12 mm and stored overnight before compression testing to ensure complete gelation. At least five parallel samples of each hydrogel were tested. Unconfined compression was performed with a constant 10 mm min^{-1} strain rate, and samples were compressed until 65–75% strain was reached from the original height, depending on the fracture point of the material. PM and collagen hydrogels could not, however, be measured because they were too soft to hold their shape.

After compression, the data were analyzed with MS Excel. The data obtained from the stress–strain curve were used to estimate the so-called stiffness of the hydrogels. Because the stress–strain curve of hydrogels (or tissues) was nonlinear in the elastic portion (even at low strains), a polynomial fit was used for the data, and the stiffness of hydrogels was determined according to the previously described method.[41]

2.4. Cell Culture

Neurospheres derived from hPSC lines 08023 and 10212 were enzymatically dissociated with 1 × TrypLE Select (Thermo Fisher Scientific) into single cells or small aggregates for the hydrogel experiments. Adherently cultured hNP1 cells were detached mechanically according to the manufacturer's instructions into single cells. Additionally, adherently cultured hFF cells were detached with trypsin for the experiments.

In all 3D hydrogel samples, neural cells were encapsulated at a final concentration of 5×10^6 cells mL^{-1}, while in 2D samples on laminin or on top of the hydrogels, neural cells were plated at a density of 50 000 cells cm^{-2}. Fibroblasts were plated in 2D samples at a density of 10 000 cells cm^{-2}. All the platings were done into 48-well plates Nunc, Nunclon, flat bottom (Thermo Fisher Scientific) or MatTek, glass bottom, (MatTek Corporation, Ashland, MA, USA).

Further maturation of neural cells in hydrogels was induced by withdrawing FGF and, in the case of 08023 and 10212 cells, LDN, from the medium. Medium was changed three times a week during the hydrogel experiments. Every cell–hydrogel combination was repeated in two separate experiments, and in every experiment, there were at least three parallel sample wells.

2.5. Immunocytochemistry and Imaging

The primary antibodies used targeted β-tubulin III (mouse, 1:1500, Sigma-Aldrich or chicken, 1:4000; Abcam, Cambridge, UK), microtubule-associated protein 2 (MAP2; chicken; 1:4000; Novus Biologicals, Littleton, CO, USA), yes-associated protein (YAP, 63.7, mouse, 1:100; Santa Cruz Biotechnology, Dallas, Texas, USA), CD56 (rabbit, neural cell adhesion molecule (NCAM), 1:800; Merck KGaA, Darmstadt, Germany), vinculin (rabbit, 1:500, Thermo Fisher Scientific), and CD104 (mouse, integrin $\alpha6\beta4$, 1:500; Thermo Fisher Scientific). Secondary antibodies conjugated with Alexa 488, 568, or 647 (Thermo Fisher Scientific) were used at a 1:400 dilution, and phalloidin TRITC 568 (Sigma-Aldrich) was used at a 1:800 dilution.

A 3D hydrogel staining protocol optimized previously[36] was used after 2 or 4 weeks of cell culture, and the same protocol was also used for 2D controls. Briefly, cells were fixed in 4% paraformaldehyde for 30 min, and unspecific staining was blocked by 10% normal donkey serum, 0.1% Triton-X 100, and 1% bovine serum albumin (all from Sigma-Aldrich) for 1 h at room temperature. An exception for this was staining for integrin $\alpha6\beta4$; blocking, washes, and primary antibody solutions were used without Triton-X. Primary antibodies were incubated on cells for 48 h and secondary antibodies for 24 h, both in +4 °C. Finally, after washes, the cells were mounted with VECTASHIELD containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA, USA).

The hydrogels were imaged with an Olympus IX51 fluorescence microscope (Olympus Corporation, Japan, Tokyo). The representative samples were imaged with a Zeiss LSM 780 LSCM confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

2.6. Quantitative Polymerase Chain Reaction

Hydrogels were lysed with mechanical disruption by Qiagen PowerLyser with ceramic 2.8 mm PowerBead Tubes (both Qiagen, Hilden, Germany) before obtaining total RNA with a NucleoSpin RNA kit (MACHEREY-NAGEL GmbH & Co, Düren, Germany). To prepare complementary DNA (cDNA), 50 ng of total RNA was reverse-transcribed using a high-capacity reverse transcription kit. Polymerase chain reaction (PCR) was conducted in reaction mixtures (15 µL) containing 2.5 ng of cDNA, 0.75 µL PCR primers (TaqMan Gene Expression Assay), and 7.5 µL Taq DNA polymerase in PCR buffer on a thermal cycler (7300 Real-Time PCR System). Thermal cycling conditions were as follows: initial incubation at 95 °C for 10 min and 40 cycles of 95 °C for 15 s (annealing) and 60 °C for 60 s (extension). The PCR primers used (TaqMan Gene Expression Assay, Thermo Fisher Scientific) were glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Assay ID: Hs02786624_g1), MAPK1 (Hs01046830_m1), ROCK1 (Hs01127701_m1), PTK2 (Hs01056457_m1), RPSA (Hs00347791_s1), TUBB3 (Hs00801390_s1), and ITGB4 (Hs00236216_m1).

2.7. Data Processing and Analyses

2.7.1. Imaging Data

The images taken with an Olympus IX51 microscope were processed with Adobe Photoshop CS4 (Adobe Systems Inc., USA, San Jose, CA), and z-stack images acquired with a Zeiss LSM 780 microscope were managed with ZEN microscope software (Carl Zeiss AG). Figures were composed and modified with Adobe InDesign CC (Adobe Systems Inc.).

2.7.2. Indexing of the Neurite Spreading, Cell Adhesion, and Gel Performance

To develop a systemic neurite spreading index, all hydrogel samples stained with neuronal markers (β-tubulin III or

β-tubulin III + MAP2) were semiquantitatively evaluated throughout imaging with an Olympus IX51 microscope. Evaluations were done non-blindly directly at the microscope from at least five different areas for each well. Values from 0 to 3 were given for every sample, where 0 represents cells with no neurite spreading, and 3 represents long and branched neurite structures throughout the hydrogel (Figure S1, Supporting Information). For every cell line, there were at least two separate experimental repeats, including 1–3 parallel samples for every hydrogel, from which the total neurite spreading index was averaged.

For indexing of adhesion, staining for vinculin, NCAM, and integrin α6β4 were semiquantitatively evaluated, and indexing was performed as for neurite spreading (Figure S1, Supporting Information).

To develop an index for gel performance, multiple properties of the gel behavior during the cell culture period were semiquantitatively evaluated (Table S1, Supporting Information). These properties were usability of the gelation procedure, performance of the gelation procedure, usability of cell culturing, and performance of the gel after 2 and 4 weeks of cell culturing. Values from 0 to 3 were given accordingly.

2.7.3. Principal Component Analysis

Indexes for neurite spreading, cell adhesion, and gel performance as well as results of mechanical testing were further used to classify different hydrogels using PCA. A more detailed methodology is found in Supporting Information 2. Analysis was performed using MATLAB (2017b, MathWorks, Kista, Sweden), and the results were expressed according to the three most explanatory principal components.

2.7.4. Quantitative PCR Data

qPCR data were analyzed with relative quantification using the comparative quantitation method and are presented as fold differences of ΔCt values. Fold differences were calculated as in Equation (1), where GOI = gene of interest, normalizer = endogenous control/housekeeping gene GAPDH. In Figure 7B, values of relative gene expression are represented for integrin α6β4, which are calculated with the ΔΔCt method as in Equation (2), where calibrator = fibroblasts. The efficiency of endogenous control amplification was approximately equal to the amplification of target genes.

$$\mathrm{Ct_{GOI}} - \mathrm{Ct_{normalizer}} = \Delta\mathrm{Ct}, \cdot \text{Relative Quantity} = 2^{(-\Delta\mathrm{Ct})} \quad (1)$$

$$\Delta\mathrm{Ct_{sample}} - \Delta\mathrm{Ct_{calibrator}} = \Delta\Delta\mathrm{Ct}, \cdot \text{Relative Quantity} = 2^{(-\Delta\Delta\mathrm{Ct})} \quad (2)$$

2.7.5. Statistical analysis

All quantitative results were formed and statistics were calculated with GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Statistics for the qPCR results were calculated either with unpaired *t*-test (two sample types comparison) or one-way ANOVA and Tukey's multiple comparison test (three or more sample type comparisons). All quantitative results were reported as the mean ± standard deviation (SD). In all qPCR results, $n = 3$, and significance in Figures are shown as *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$.

3. Results and Discussion

3.1. The Adhesion of Neurons Differs from the Adhesion of Fibroblasts in 2D Culture

In most cell adhesion–related studies, fibroblasts have been used as a model cell type,[42] whereas the adhesion of neurons has not been widely addressed. Here, cell adhesion of neurons was studied and compared to that of fibroblasts in two dimensions at the gene and protein expression levels. There was a clear difference in the expression of genes related to cell adhesion between neuronal cells and fibroblasts at 1 and 28 days of culture (**Figure 2**). In fibroblasts, the expression of all studied genes (MAPK, ROCK, PTK2, RPSA, ITGB4, TUBB3) was already lower than the expression in neurons after 1 day in vitro and was even lower after 28 days, and the difference was very significant ($p < 0.001$ in all comparisons; Figure 2A, d28). This difference in expression might be because fibroblasts stabilize their migration much quicker and possess contact inhibition after cells have formed enough contacts with each other,[43] whereas the neuronal cells used here do not have strong contact inhibition and thus can continue their migration much longer. There are also differences in microtubule organization in fibroblasts and neurons that can affect the organization and expression of microtubule-associated adhesion pathways.[44]

Here, we clearly showed that β-tubulin III-positive neuronal cells did not express YAP in their nucleus after 28 days of culture on laminin, whereas hFF cells cultured on the same substrate expressed YAP mainly in the nucleus (Figure 2B). This finding is supported by earlier literature.[23]

In conclusion, cell adhesion of neurons seems to differ remarkably from the adhesion of fibroblasts. The expression of adhesion markers in these cell types differed by their expression levels both temporally and spatially. Importantly, cells behaved very differently on rigid substrates, indicating that their most important adhesion-related pathways differ in those conditions; thus, neuronal adhesion must be studied more specifically.

3.2. The Cell Source Affects Neuronal Cell Adhesion and Neurite Spreading in Two Dimensions

Neuronal adhesion has not been widely studied, and the existing results are derived from neuronal cells from various sources. In this study, we utilized three sources for human neurons: hESC- and hiPSC-derived neurons differentiated with the same protocol and commercial hESC-derived NPCs. Interestingly, we found that hiPSC-derived neurons had the highest expression of all studied genes (MAPK, ROCK, PTK2, RPSA, and TUBB3), which significantly differed from the expression in 08023- and hNP1-derived neurons at 28 days of culture on

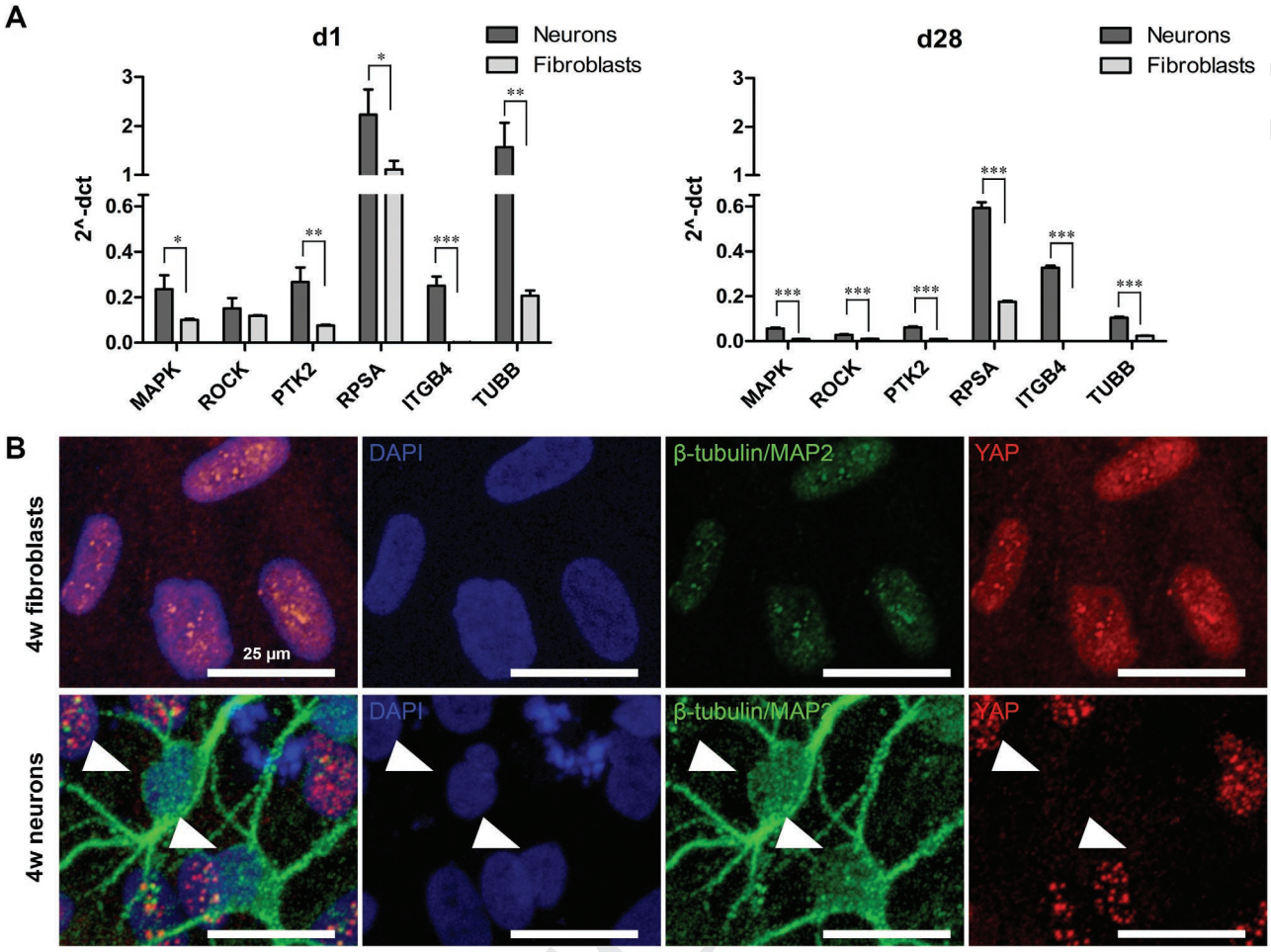

Figure 2. The expression and localization of adhesion-related markers differed between neurons and fibroblasts. A) After d1, the expression of adhesion-related genes MAPK, PTK2, and RPSA was significantly higher in neurons than in fibroblasts (shown here are values of 2^{-dCt}). The expression of all studied adhesion-related genes MAPK, ROCK, PTK2, and RPSA was significantly higher in neurons than in fibroblasts at d28 when cultured on laminin. Moreover, the expression of the ITGB4 gene was significantly higher in neurons both at d1 and d28. Value one represents the level of the housekeeping gene GAPDH for that cell type. Neuronal cells were differentiated from the 08023 cell line. Stars indicate: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. B) The adhesion-related marker YAP (red) was localized in the fibroblast nucleus but was not observed in the neuronal nucleus (marked with white arrows) when cells were cultured for 4 weeks on laminin. Staining for β-tubulin III + MAP2 (green) is shown for neurons and for DAPI (nuclear stain, blue) for both neurons and fibroblasts. Scale bars are 25 µm.

the 2D laminin surface (**Figure 3**A). There were no significant differences between 08023- and hNP1-derived neurons, which were derived from hESCs but produced with different differentiation protocols. However, TUBB3 expression was significantly higher in 10212-derived neurons. This difference might indicate a less mature stage of neuronal cells because immature neurons are known to actively remodel their cytoskeleton via a process called the "dynamic instability" of the microtubule network, resulting in a greater need for the growth of neurites and upregulation of β-tubulin III.[45] These dynamically instable neurons might expressed of adhesion-related genes more robustly, which was supported by the results shown in Figure 3. 10212-derived neurons were seemingly actively finding ways to migrate, as both RPSA (growth cone gene) and ROCK (inhibitor of axonal growth) were upregulated at the same time.

In addition to the differential expression of adhesion markers, neurite spreading and neuronal network formation varied among the neuronal populations derived from different hPSC lines. The 08023-derived neurons had the best neurite and neuronal network forming capacity, while hNP1 and 10212 neurons were less efficient (Figure 3B). Previously, we showed that similarly differentiated hESC and hiPSC neurons do not express ECM- and adhesion-related molecules at the same levels, nor do they form neurites or neuronal networks similarly in different laminin formats.[14] Even though hPSC cell lines have differential initial differentiation capacity despite their origin,[46] the differentiation method used does not necessarily make them more similar in their cell type–specific behavior.

In general, both hESC- and hiPSC-derived neuronal cells are valid for building both 2D and 3D in vitro models for particular brain-related diseases.[1] Upcoming studies should examine how similar results can be achieved, for example, in disease modeling between multiple patient-derived cell lines in CNS-related disorders.

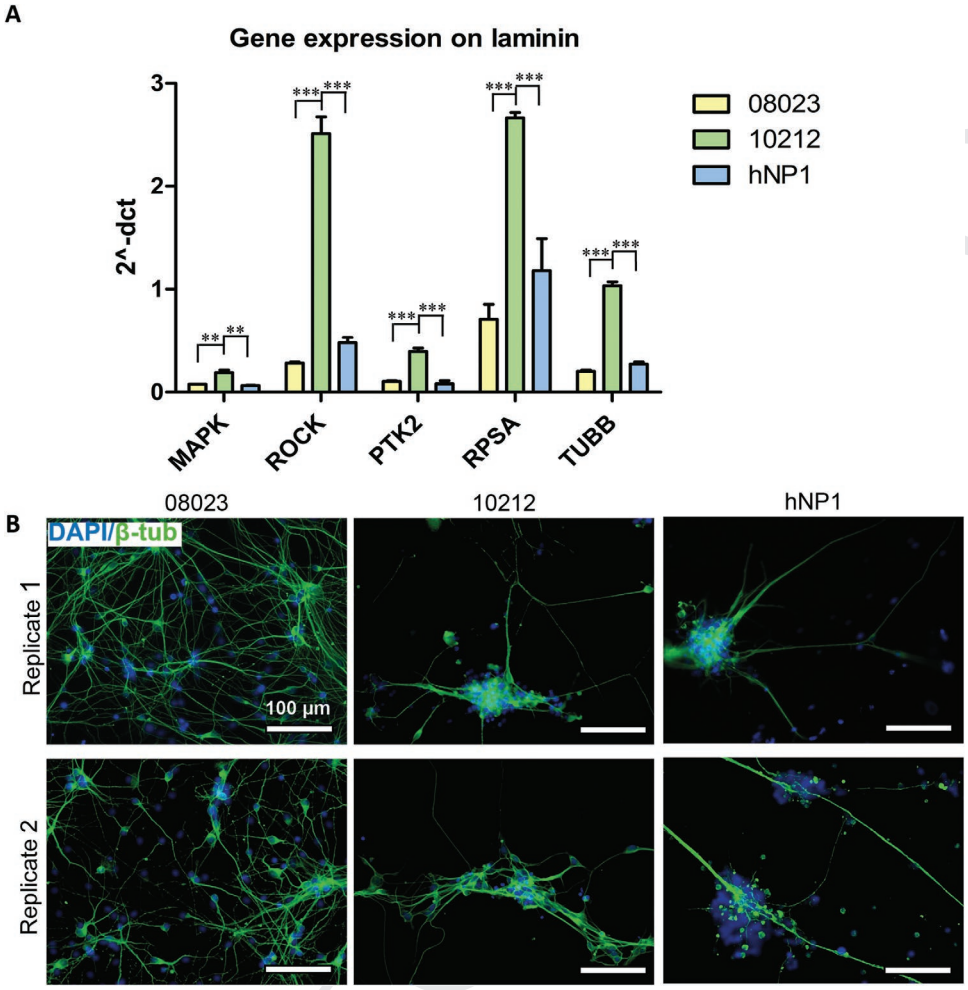

Figure 3. The expression of adhesion-related genes differed between neurons derived from three different hPSC lines cultured on laminin in 2D. A) The expression of all studied genes (MAPK, ROCK, PTK2, RPSA, and TUBB3) was significantly higher in 10212 neurons than in either 08023 or hNP1 neurons at the d28 timepoint. Stars indicate: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. B) Neurite spreading and neuronal network formation in neurons derived from three different hPSC lines. The neurite spreading and network formation were repeatable between experiments as shown here at d14 for two replicates from different experiments. Staining is for DAPI (blue) and β-tubulin III (green). Scale bars are 100 µm.

As further suggested by our results, one should consider the rather massive variation in the behavior of differentiated cells, whether these differences are cell line- or differentiation protocol–dependent, as the variation can influence the observed results. The most robust conclusions in CNS disorder–related in vitro studies can be made using several relevant human origin cell lines in the same study.

3.3. Evaluation of Hydrogel Performance using Multiple Parameter Analysis

Here, we evaluated the performance of various hydrogels in supporting 3D neuronal cultures. First, we measured 1) the mechanical properties of hydrogels; then, we used immunostaining to create indexes (explained in Section 2.7.2) for 2) hydrogel performance during gel preparation and culturing, 3) cell adhesion in 3D, and 4) neurite spreading in 3D. These four parameter groups were combined in PCA analysis to gain an overview of hydrogel performance.

As the stiffness of the 3D scaffold affects neuronal differentiation and maturation, using scaffolds with stiffness values near those of natural brain tissue is reasonable. Mechanical tests performed with the compression method showed that all the studied hydrogels had compression moduli under 10 kPa, which is in the range of brain tissue.[36,39,41] The highest measured modulus of the hydrogels used in PCA analysis, shown in **Figure 4**, was the modulus of GG (1.25% SPD) at 9.4 ± 2.7 kPa. For others, the modulus was 6.8 ± 2.1 kPa for HA2-PVA and 2.8 ± 0.8 kPa for HA1-PVA. More informative stiffness–strain curves are presented in Figure S2, Supporting Information. The modulus for PM, HA-Col1-4SPEG, and Col1 could not be measured with the compression testing method because they were too soft and did not maintain their shape during testing.

Among the gels tested, Col1 best supported neurite spreading (Figure 4A), but did not perform well in gel performance, as it

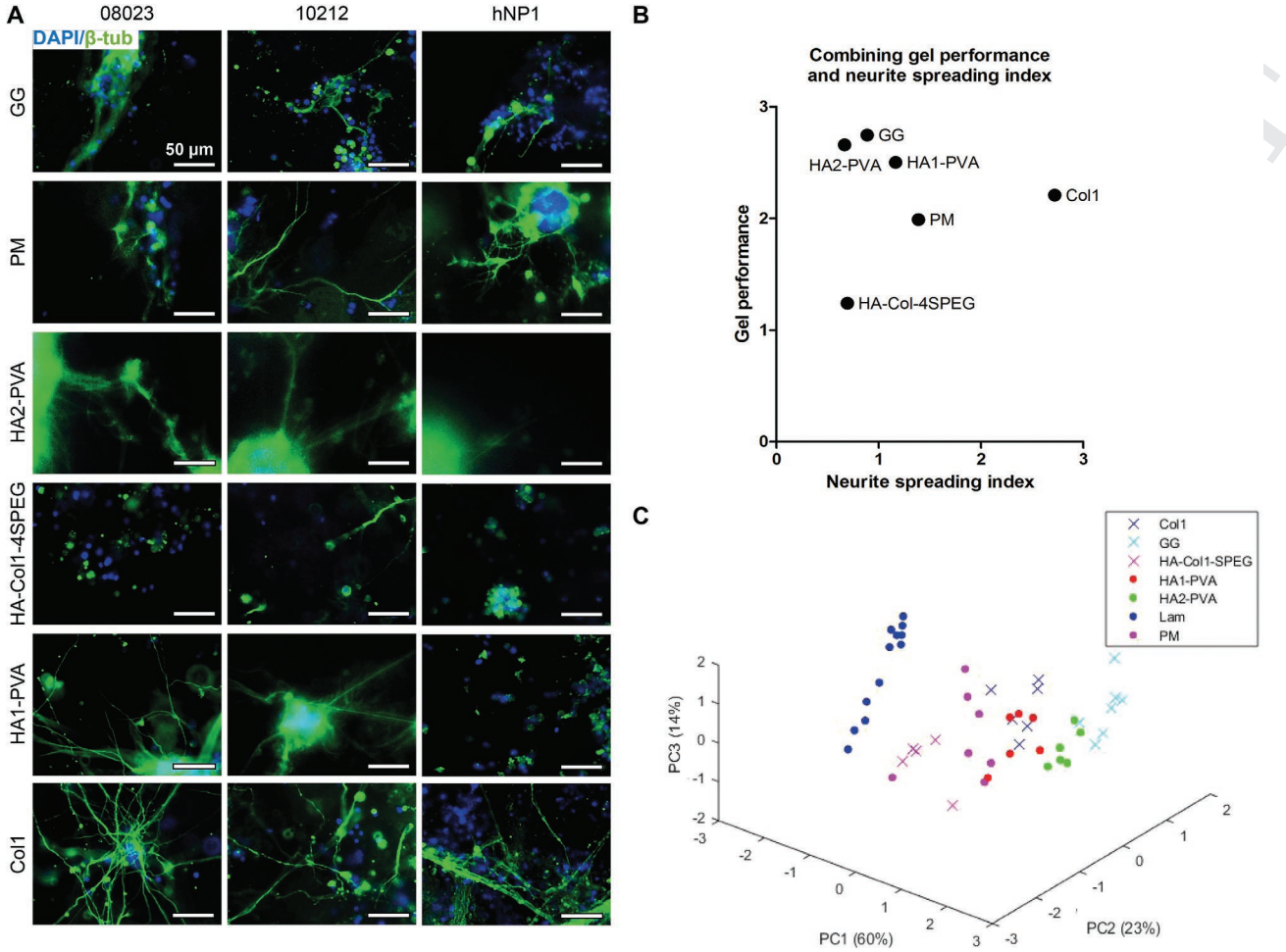

Figure 4. Neuronal network formation varied between hydrogels in 3D at day 28. A) The most prominent expression of β-tubulin III–positive neurites (green) was found in Col1 hydrogels with neurons derived from all three cell lines. DAPI (blue) was used to stain nuclei. The scale bar is 50 µm in all images. B) The combination of two parameters, the gel performance index and neurite spreading index, showed that Col1 had the best influence on neurite spreading, while GG, HA2-PVA, and HA1-PVA were better in terms of gel performance. C) Principal component analysis (PCA), including gel performance, adhesion index, neurite spreading index, and mechanical testing, clearly classified different culturing conditions into separate clusters. The most important principal component is shown as PC1, the second important as PC2, and the third as PC3.

was reduced in size at 28 days of culture and was difficult to handle (Figure 4B). On the other hand, GG, HA1-PVA, and HA2-PVA achieved the best scores in gel performance but did not provide good neurite spreading support (Figure 4B). Details of the hydrogel performance indexes for 28 days are shown in Table S3, Supporting Information. According to the literature, the modulus of collagen type I at low concentrations (1–3 mg mL^{-1}) is under 1 kPa,[47,48] being the lowest of all hydrogels studied here. The modulus of HA-Col1-4SPEG could not be measured either, but contrary to Col1, it did not support neurite spreading. These results highlight that the mechanical properties of the hydrogel are not necessarily the most important parameters for predicting neural cell growth and neuronal network formation in 3D.

The cell adhesion indexes were combined with ICC staining of vinculin, NCAM, and integrin $\alpha 6\beta 4$ and are shown in Table S2, Supporting Information. NCAM is not only a widely used marker for neuroectodermal and immature neuronal cells[35,49] but also related to neuronal synapse formation.[50] Vinculin is a link protein between cell adhesion receptors and the actin cytoskeleton, and its expression is increased on focal adhesion points.[51] As mentioned earlier, integrin $\alpha 6\beta 4$ is related to neural cell laminin adhesion.[14]

Combining information from different indexes reveals results from a wider perspective, as shown recently.[6] According to the evaluated indexes during the experiments, gel behavior differed between hydrogels both in the case of gel performance properties (gel performance index, Table S3, Supporting Information) and supportiveness of neurite spreading (neurite spreading index, Table S2, Supporting Information) (Figure 4B). To combine these indexes with other important parameters, mechanical testing and the neural adhesion index, PCA was performed, and the results clearly separated different culturing conditions into different classes (Figure 4C). PM was used here as positive control, and it is known to have binding sites for the cells and its supportiveness to hPSC derived neuronal cells has been shown earlier.[27,28] PCA analysis classified well-performing gels, PM, Col1, and HA1-PVA, closely together. Moreover, GG and HA2-PVA were clustered closely,

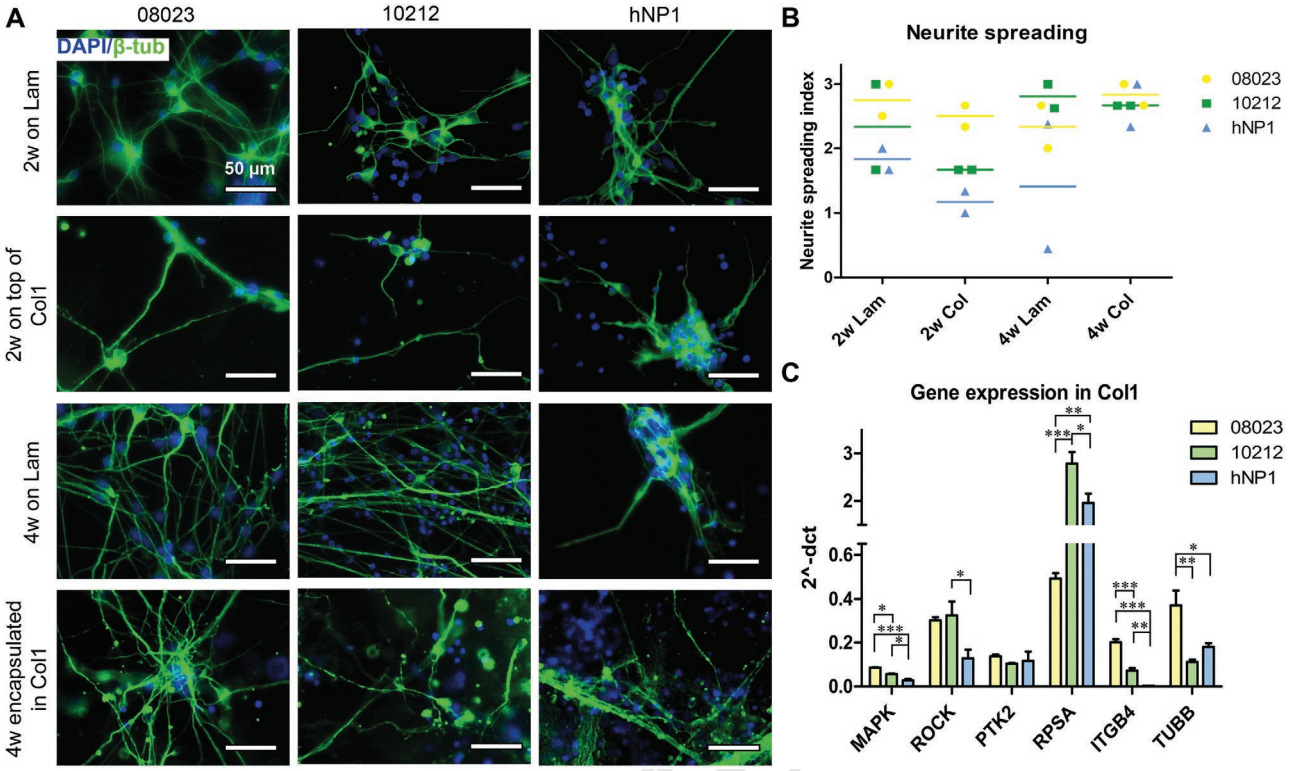

Figure 5. The expression of adhesion-related genes and proteins varied between the neurons derived from three different hPSC lines cultured in 3D and between those cultured in 2D and 3D. A) Immunostaining of β-tubulin III (green) is shown in all three cell lines cultured for 2 weeks on laminin or on top of Col1 and 4 weeks on laminin or encapsulated in Col1. Counterstaining is for DAPI (blue). Scale bars are 50 µm. B) Neurite spreading indexes from laminin and collagen cultured cells at 2 and 4 weeks. Neurite spreading was widest and most stable for different cell sources with the neurons encapsulated and cultured for 4 weeks in Col1 C. Gene expression of adhesion markers differed between neuronal cells derived from different cell lines in Col1 3D culture at d28. For example, the expression of RPSA was significantly lower in 08023 cells than in the other two cell lines. Stars indicate: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

both of which were demonstrated as non-supportive gels. GG does not have binding sites for the cells and has been earlier shown to act as non-supportive gel for hPSC-derived neuronal cells.[36] HA-Col1-4SPEG formed its own cluster, although a scattered one. Laminin 2D controls clearly separated from gel samples, indicating that the 2D surface acts differently from the 3D hydrogels.

We conclude that the use of more complex analysis methods, such as multiparameter analysis and PCA, can allow more relevant information to be obtained with sufficient time, in line with a recent study.[6] Here, Col1 alone was the most supportive scaffold, although it was mechanically unstable.

3.4. Collagen Supports Neurite Spreading of All Studied Neuronal Cells, Particularly in Three Dimensions

Col1 was found to be the most supportive hydrogel for neurite spreading in 3D. It has been shown that Col1 interactions are important regulators in neural stem cell development and maturation.[52] The used rat collagen is a good option for in vitro models due to its stable quality, availability, and inexpensiveness. However, for transplantation therapies and studies related to clinical aim, the collagen needs to be changed to human derivative.[53] Thus, we wanted to study how Col1 influences neuronal cell adhesion both on stiff and soft cultures and whether neuronal cells from different cell lines behave differently in those conditions. We selected culturing time of 2 weeks for 2D cultures and 4 weeks for 3D encapsulated cultures because hPSC derived neuronal cells have different maturation speed on these different environments as shown in previous study.[27] Here, the neurite spreading of cells cultured on stiff 2D (laminin), soft 2D (Col1), or soft 3D (Col1) cultures was compared (**Figure 5**). There were no significant differences in neurite spreading indexes between the neurons derived from different hPSC lines or stiff versus soft culture types, even though neurite spreading of hNP1-derived neurons seemed slightly weaker in 2D cultures (Figure 5B). The immunostaining results showed the same trend (Figure 5A). Additionally, neurite spreading was more robust and less variable in 3D soft Col1 than in 2D soft Col1 (Figure 5A,B). Overall, the neurite spreading of neurons derived from different hPSC lines was good in Col1.

Neuronal cells cultured in 3D in Col1 expressed all studied adhesion markers despite the original cell line used. Of those, the expression of the adhesion marker RPSA was highest in neurons derived from all different hPSC lines and was significantly higher in 10212 neurons than in 08023- ($p < 0.001$) and hNP1 ($p < 0.05$)-derived neurons. Interestingly, 10212 neurons also had the highest RPSA expression in the 2D laminin substrate (Figure 3A). The neurite spreading index of

10212 neurons compared to 08023 neurons may indicate that 10212 neurons are still in an active phase of migration and thus in a less mature stage. Cells that express lesser amounts of β-tubulin III might be more mature with already stabilized neurites.[45] Altogether, neurons derived from different hPSC lines are most likely undergoing temporally varying neuronal maturation steps in 3D. Additionally, neuron-specific adhesion marker ITGB4 expression varied among cell lines, being highest in 08023 neurons and lowest in hNP1 neurons.

We conclude that Col1 is a supportive substrate for human neuronal cells in 3D even though it is unstable in long-term (up to 4 weeks) culturing. Longer culturing periods are needed in more sophisticated in vitro models where, for example, electrical properties of neurons are studied because the

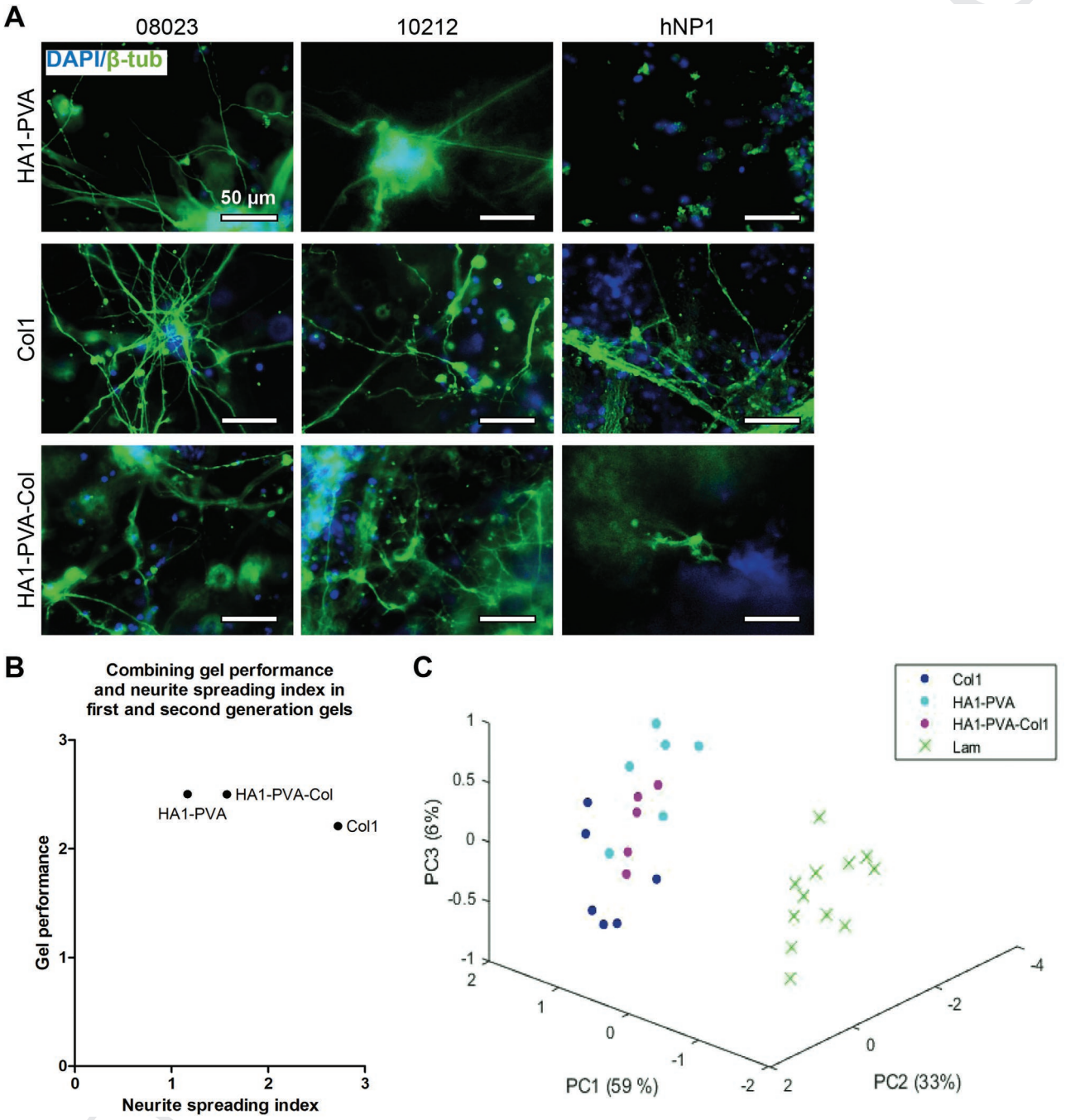

Figure 6. The best properties of Col1 and HA1-PVA could be combined by mixing these two hydrogels and preparing an IPN hydrogel of HA1-PVA-Col. A) Immunostainings showing neurite network formation in these three hydrogels in 4 weeks timepoint. Shown are β-tubulin III (green) and DAPI (blue), and scale bars are 50 µm. B) The combination of two parameters, the gel performance index and neurite spreading index, shows that HA1-PVA-Col had good gel performance and better neurite spreading than HA1-PVA. C) PCA revealed how 2D laminin clearly differs from the three tested hydrogels and how HA1-PVA-Col is localized between HA1-PVA and Col1.

functional maturation of human neurons is longer than that of rodent neurons in vitro.[54]

3.5. Creation of an IPN Hydrogel for Successful 3D Culturing of Neurons

As stated, Col1 was supportive for neurite spreading but an unstable hydrogel for long-term culturing. In contrast, HA1-PVA was stable for long-term culture with moderate support for neurite spreading (Figure 4B). To optimize the 3D scaffold, we generated an IPN gel from HA1-PVA and Col1. IPN gels have previously been used as 3D scaffolds but not with neuronal cells.[32] Our aim was to combine the supportiveness of Col1 with the stability of HA1-PVA. According to mechanical tests, the compression modulus of HA1-PVA-Col was 1.0 ± 0.3 kPa, which was lower than that of HA1-PVA (2.8 ± 0.8 kPa) but higher than that reported for Col1 (under 1 kPa) in the literature.[47,48] The IPN hydrogel maintained its shape almost fully for 4 weeks of cell culture (**Figure 6**B). Immunostainings showed that HA1-PVA-Col supported neurite spreading at least as well as Col1 (Figure 6A). Thus, our IPN hydrogel successfully combined the good properties of the gels used. Moreover, we compared the HA1-PVA-Col1 gel with the HA1-PVA gel, Col1 gel, and stiff laminin coated surface with multiparameter PCA analysis combining neurite spreading, gel performance, mechanical testing, and adhesion indexes. According to the PCA results, laminin as a 2D control separated clearly from 3D hydrogels (Figure 6C). Importantly, HA1-PVA-Col was localized very closely together with Col1 and HA1-PVA.

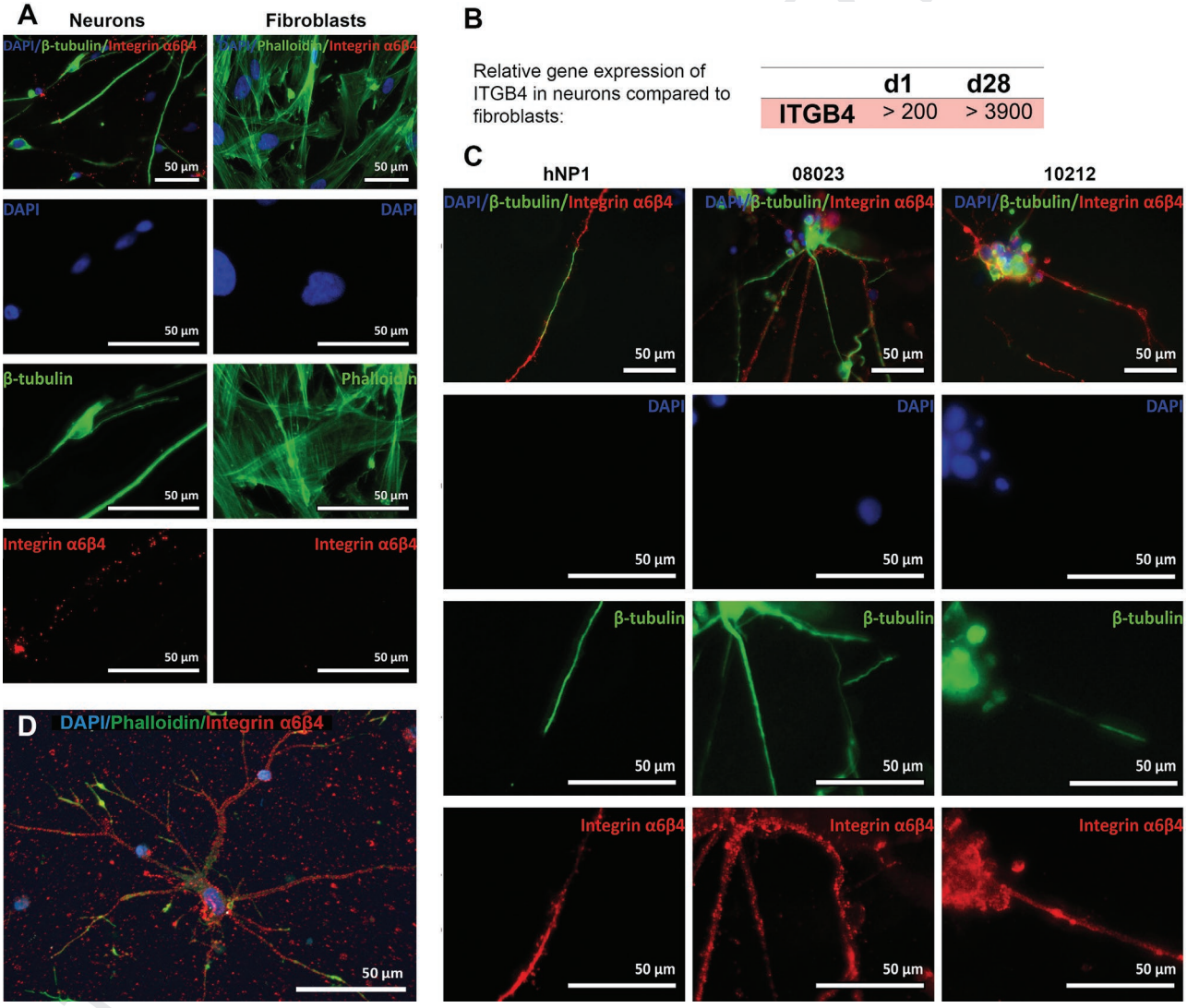

Figure 7. Integrin $\alpha6\beta4$ is widely expressed in neurons. A) At the protein level, integrin $\alpha6\beta4$ was not detectable in 2D cultured fibroblasts (d28). Staining is shown for DAPI (blue), β-tubulin III on neurons (green) or phalloidin on fibroblasts (green), and integrin $\alpha6\beta4$ (red). B) Relative expression of integrin $\alpha6\beta4$ was over 200 times higher in neurons than in fibroblasts at d1 and over 3900 times higher at d28 when cultured on 2D laminin. C) When cultured on top of Col1 for 14 days, integrin $\alpha6\beta4$ was expressed in neurons differentiated from all three cell lines. Staining is shown for DAPI (blue), β-tubulin III (green), and integrin $\alpha6\beta4$ (red). D) Additionally, when cultured in three dimensions in a HA1-PVA-Col gel for 28 days, integrin $\alpha6\beta4$ was widely expressed in neurons. Staining is shown for DAPI (blue), integrin $\alpha6\beta4$ (green), and phalloidin (red).

In conclusion, the HA1-PVA-Col1 IPN gel acted as a supportive scaffold material for all used neuronal cell sources and was stable for long-term culturing up to 28 days.

3.6. Integrin $\alpha6\beta4$ Is an Important Adhesion Marker in Neurons

The relationship between the cell adhesion pathways of the neuronal network development process and the effect of 3D hydrogel culture on development are combined in this study. In addition to other adhesion-related molecules, one molecule identified in our previous work was integrin $\alpha6\beta4$.[14] In the neural field, integrin $\alpha6\beta4$ (also known as CD104) has been previously associated mostly with Schwann cells,[55] neural stem cell differentiation[24,56,57] and pathways regulating cell adhesion, survival, and maturation.[24] Integrin $\alpha6\beta4$ mediates cell–ECM interactions involving laminin, the protein that is enriched in neural basal lamina.[14,57] Here, these earlier findings were strengthened, and in addition, integrin $\alpha6\beta4$ was shown to be highly expressed in more mature-appearing neuronal cells. In contrast, we could hardly detect any integrin $\alpha6\beta4$ gene or protein expression in human fibroblasts (**Figure 7**). Adhesion can vary greatly between cell types, so the expression of integrin $\alpha6\beta4$ was validated with neurons derived from three different hPSC lines, all of which showed prominent integrin $\alpha6\beta4$ expression at the protein level (Figure 7B). The 3D environment, which has previously been associated with a more mature phenotype of neuronal cells,[1,37] also promoted the expression of integrin $\alpha6\beta4$ remarkably (Figure 7D).

These findings prove that the adhesion-related regulatory system for neurons is highly different compared with that for fibroblasts. Thus, more intensive research about the specific adhesion mechanisms of neuronal cells is needed.

4. Conclusions

This study highlights the importance of proper study design for in vitro model studies, including choosing relevant cell sources and hydrogel scaffolds and selecting relevant adhesion markers. The findings of this study showed that: 1) to build a reliable in vitro model, hPSC cell sources need to be selected carefully and that the use of multiple cell sources is preferable; 2) IPN hydrogels can combine the good properties of used components and thus are a relevant strategy to build more in vivo-like scaffolds for neuronal cells; 3) cell adhesion of neurons differs from that of fibroblasts, and integrin $\alpha6\beta4$ is a neuronal cell–specific adhesion marker in both 2D and 3D cultures.

Here, we present a method with which the materials can be valued based on multiple parameters: mechanical testing, neurite spreading, adhesion, and gel performance properties. PCA formed from these parameters revealed how different hydrogel scaffolds separate from each other and which scaffold materials resemble each other the most. When summarizing different viewpoints, the HA1-PVA-Col hydrogel was found to be the best for 3D neuronal cell cultures derived from three different cell lines. In the future, this hydrogel can be used in various 3D in vitro studies to better mimic the in vivo growth and maturation of human neurons.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

adhesion, human pluripotent stem cells, hydrogel, neurons, tissue engineering

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