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RESEARCH ARTICLE

The Effect of Culture Dimensionality and Brain Extracellular Matrix in Neuronal Differentiation

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ABSTRACT

Objective: Neuroblastoma cells are frequently used in neuroscience studies due to their human origin and ability of extensive propagation compared to animal-derived primary neuron cultures. Although they are tumor-derived, they exhibit neuronal differentiation capability in the presence of several agents including retinoid acid. Several studies have quested for successful differentiation protocols and faithful representation of neuronal characteristics. However, they predominantly pursued conventional two-dimensional (2D) cultures where the role of three-dimensional (3D) tissue microenvironment and cell-matrix interactions remained unknown. In this study, we investigated the effect of culture dimensionality and native brain extracellular matrix (ECM) on neuronal differentiation of neuroblastoma cells.

Materials and Methods: Decellularized brain ECM hydrogels offer a physiologically relevant *in vitro* 3D culture platform with the representation of key biochemical and biophysical aspects of the native tissue microenvironment for modeling cellular processes. We cultured SH-SY5Y cells on 2D or as encapsulated in 3D decellularized brain ECM hydrogels and assessed them for morphological shift, neurite extension, and expression of neuronal, synaptic, astrocytic, cholinergic, stemness, proto-oncogene and neuropathological markers.

Results: Our findings demonstrate that the 3D brain ECM microenvironment distinctly affects the differentiation process compared to conventional culturing. In 3D ECM, neuronal differentiation occurred as in 2D, with upregulation of neuronal markers, change in cell morphology, and promotion of neurite extension. However, during differentiation, maintenance of stemness was observed in a 3D-specific manner. Furthermore, 3D differentiation promoted significant upregulation of astrocytic and synaptic markers which was not observed in 2D.

Conclusion: This study highlights the importance of physio-mimetic 3D brain models.

Keywords: Neuronal differentiation, extracellular matrix, brain tissue engineering, decellularization, hydrogels

INTRODUCTION

The extracellular matrix (ECM) is the primary non-cellular component in all tissues.[1] Three-dimensional (3D) tissue models with the capability of representing the native ECM offer advantages over conventional, two-dimensional (2D) culturing and enable cell-matrix interactions. Decellularization is a widely used technique for the fabrication of 3D tissue models with the preservation of native ECM composition.[2] In the process, cellular content is efficiently removed and the remaining ECM is solubilized with enzymatic digestion which allows for temperature and pH-induced reconstitution into hydrogel form.[3,4] Decellularization of native brain tissues has been successfully demonstrated from sources including porcine and human for use in neuroscience and brain tissue engineering fields.[5,6] Lack of brain ECM ligands in 2D culture models has been emphasized for the insufficient responses in modeling processes such as neuronal differentiation, maturation, and synaptogenesis.[7,8] ECM-instructed cell signaling is known to modulate cell behavior in neurodevelopment, homeostasis, and neurodegeneration.[9–11] Therefore, understanding the role of cell-matrix interactions in neural cell behavior is still a viable aspiration in the field.

The human neuroblastoma cell line, SH-SH5Y, is a subcloned epithelial cell line from SK-N-SH, which originated in 1970 from a metastatic bone tumor retrieved from a 4-year-old female patient.[12] SH-SH5Y is frequently used in *in vitro* neu-

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roscience studies due to its human origin, ease of expansion, and neuronal differentiation capability.[13] Optimal neuronal differentiation requires obtaining a homogeneous cell population with moderate levels of neurotransmitter production.[5] Cellular proliferation is an important sign of immature phenotype and proliferation is expected to decrease significantly following differentiation.[14] For this purpose, one of the main approaches in differentiation protocols is the gradual decrease of serum in the growth medium from 10% to generally 2.5%.[15] On the other hand, starved cells are mostly supported with neurotrophic elements such as N2, B-27, retinoic acid (RA), brain-derived neurotrophic factor (BDNF), and potassium chloride (KCl).[16] RA, a form of vitamin A, has a key role in the differentiation of neuroblastoma cells into dopaminergic neurons by its interplay in the arrest of the cell cycle, elevated levels of cyclin-dependent kinase (CDK) inhibitors and anti-apoptotic proteins, and its promoting role in PI3K/AKT signaling cascade.[17] N2 supplement contains compounds such as insulin, transferrin, and selenium, which are formulated as 'Bottenstein's N-1 formulation' [18] and it promotes differential signaling at early stages. B-27 protects neuronal cells with the promotion of cell survival and inhibition of glycolysis.[19] The combination of these, including deprivation of serum and administration of neurotrophic factors, results in the negative selection of epithelial cells from the population, while differentiated mature neurons are expanded. According to the differentiation regime applied, different neuronal subtypes can be achieved including adrenergic, cholinergic, and dopaminergic neurons.[20] Differentiated neuroblastoma cells, which are phenotypically closer to primary neurons, have reduced proliferation rate, polarized cell structure, and long extended axons in connection with surrounding cells.[16]

Several molecular markers take an important role in the assessment of neuronal differentiation. Microtubule-associated protein family members play essential roles in both neuromorphogenic processes and neurodegenerative disease progression. Microtubule-associated protein 2 (MAP2) is responsible for stabilizing dendrites during neurogenesis, as well as guiding the coordination for the reconstruction of microtubules and F-actin proteins during neurite initiation.[21] While MAP2 is localized to the cell soma and dendrites, another microtubule-associated protein, TAU, is found in axonal regions, with both proteins having similar functions in promoting microtubule rigidity during dynamic cellular events within neuronal cells.[22] Class III beta-tubulin (TUBB3), also known as Tuj1, is another marker localized in the neuronal cytoskeleton and increased expression of TUBB3 is present in the early stages of neuronal differentiation with implications in axonal maturation. [23]

NEUN, a neuronal nuclei marker, is localized within the postmitotic neuronal nuclei and perinuclear cytoplasm beyond the previously mentioned cytoskeletal markers. Its expression is pronounced in neuronal nuclear areas, where low chromatin density with loosely packed DNA is present.[24] Synaptophysin (SYP), an integral membrane protein, was one of the first markers used to detect a neuronal cell. It regulates synaptic vesicle endocytosis and neurotransmitter release, which are crucial for neuronal cell communication within an organism.[25,26] The most prevalent neuronal differentiation marker retrieved from a stem cell perspective is SRY-box transcription factor 2 (SOX2). Studies have shown that the constitutive expression of SOX2 prevents terminal neuronal differentiation, resulting in intact progenitor features. Concordantly, downregulation of SOX2 expression is necessary during neuronal differentiation.[27] Ret proto-oncogene (RET) is an unusual marker of early neuronal differentiation and has an impact on neural crest development. Studies have shown that RET is upregulated during differentiation following RA treatment.[14,28,29]

Apart from the neuronal maturation markers, there are also genes and proteins which regulate the formation of neuronal subtypes, including dopaminergic, cholinergic, serotonergic, etc. neurons. Choline O-acetyltransferase (CHAT) is a gene required for the production of enzymes that synthesize the neurotransmitter acetylcholine, which completes the functionality of the cholinergic neurons.[30] In addition to neuronal markers, other markers regarding oligodendrocytes or glial cells are also important for the characterization of differentiated cells. Glial fibrillary acidic protein (GFAP) is an intermediate filament-III protein and is present in astrocytes in the central nervous system, non-myelinating Schwann cells in the peripheral nervous system, and enteric glial cells.[31]

Amyloid-beta precursor protein (APP) and presenilin 1 (PSEN1) are mostly studied for their pathological role in Alzheimer's disease and their physiological role is not completely understood. However, APP has been suggested to play an important role in synaptic plasticity and brain development. In particular, it guides the growth of axons, regulates dendritic morphology, and promotes early nervous system development.[32] PSEN1 is a component of the gamma-secretase enzyme responsible for the cleavage of APP with a possible role in calcium metabolism and signaling of Notch, and β-catenin along with APP.[33]

In this study, we aimed to investigate the role of culture dimensionality and brain ECM in neuronal differentiation of SH-SH5Y cells. To create a 3D brain tissue model, we fabricated decellularized brain ECM (db-ECM) hydrogels from bovine donors which we encapsulated with neuroblastoma cells. We compared the differentiation of cells in 3D db-ECM hydrogels to conventional 2D culturing regarding changes in morphology and expression of the abovementioned molecular markers.

MATERIALS AND METHODS

Decellularization

Fresh bovine brains removed from calves were delivered in a sealed container and placed on ice during transportation

from a local slaughterhouse. The brains were rinsed with 2% Penicillin-Streptomycin (P/S) containing distilled water (dH_2O) and the cerebellum was carefully disjoined. The cortex was meticulously sliced into small pieces ($1\times1\times1cm^3$) using a scalpel and scissors.

DeQuach et al.'s decellularization method was adapted for the decellularization of bovine brain tissues with minor modifications.[5] The dissected brain cortex tissues were treated with 0.1% (w/v) sodium dodecyl sulfate (SDS) in phosphate-buffered saline (PBS) solution containing 1% P/S placed in a beaker on a magnetic stirrer for 4 days. The solution was renewed each day. Followingly, 40 U/ml DNAse was applied for 2 h in 10 mM magnesium chloride ($MgCl_2$) buffer (pH 7.5). The viscous slurry was subdivided into falcon tubes and sequentially centrifuged at 10,000 rpm for 5 min before being rinsed with dH_2O in each centrifugation step. The leftover tissue pieces were kept at -80°C until lyophilization and a fragment of the decellularized tissue was saved for histological investigation. The tissue samples underwent lyophilization until they were dried entirely.

db-ECM Hydrogel Generation

Brain tissues that had been decellularized and lyophilized were cryo-milled into powder form. Then, 1 mg/ml pepsin in 0.1M hydrochloric acid (HCl) was used to digest powdered db-ECM for 24 h at room temperature at constant rotation. The total digest solution was then centrifuged at 13,000 rpm for 10 min to collect the solubilized form. The pH was then brought up to 7.4±0.2 by neutralizing the solubilized db-ECM on ice with sodium hydroxide. Hydrogel formation was achieved by incubating the neutralized and solubilized db-ECM at 37 °C for 40 min.

Histological Examination

The preserved wet tissue samples were used for histological and nuclear content examination. 3.7% formaldehyde solution was used to fix native and decellularized brain tissues overnight at 4°C. Before cryo-sectioning, the fixed samples were covered with OCT and frozen. Then, 10 µm cryo-sections were collected on glass slides. To indicate nuclear content, slides were first hydrated, then stained with 1 µg/ml Hoechst solution (Invitrogen) in PBS for 15 min. DNA content was visualized with fluorescence microscopy. To examine the structural changes between decellularized and native bovine brain tissues, Haematoxylin & Eosin staining was applied. First, slides were hydrated, then stained with Mayer's Haematoxylin for 3 min, and washed for 3 min with tap water. Slides were then treated with 95% ethanol for 45 sec and stained with Eosin alcoholic solution. All slides were dehydrated, mounted, and closed with a coverslip before being examined under a light microscope.

Cell Culture

The human neuroblastoma cell line SH-SY5Y (ATCC, CRL-2266TM) was grown in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L glucose, 10% heat-inactivated fetal bovine serum (FBS), and 1% P/S. Cells were incubated at 37°C and 5% CO_2, passaged when they reached sufficient confluency (80%), and the growth medium was replaced every other day.

Phalloidin/DAPI Staining of Neuroblastoma Cells Encapsulated in db-ECM

SH-SY5Y cells were harvested and embedded in decellularized brain hydrogels at a concentration of $5x10^5$ cells/ml, and after 5 days of incubation, fixation was done with 4% paraformaldehyde (Sigma). The gels were permeabilized by treating them with 0.1% Triton X-100 in PBS for 1 h at room temperature. After that, blocking was carried out with a 1% solution of bovine serum albumin (BSA) (Sigma, A2153) containing 10% goat serum for 2 h. The samples were stained with Phalloidin-FITC (Abcam) and 4',6-diamidino-2-phenylindole (DAPI, Sigma), and imaging was performed with Leica DMI8/SP8 laser scanning confocal microscope. Images were exported from the LAS X program (Leica, Wetzlar, Germany).

Neuronal Differentiation

For 2D experiments , SH-SY5Y cells were seeded onto a 6-well plate at a density of 30,000 cells/well. For 3D experiments, cells were encapsulated into db-ECM hydrogels at a density of 1.6 x 10^5 cells/ml. For this purpose, lyophilized db-ECM digest was thawed in DMEM high glucose medium with 1% PSA and 50 µg/ml Fungin. Cells were resuspended in a complete medium and mixed thoroughly with db-ECM by pipetting. Immediately, the pre-gel was plated on a 24-well plate and incubated at 37°C for 45 min to allow gelation. Then, 600 µl complete medium was carefully added into each well. During differentiation, FBS content was gradually decreased from 10% to 2.5%, and 10 µM RA (Sigma, #R2625) with 2 mM L-Glutamine (Biowest, #X0550-100) was added to the medium. After reaching day 9, the differentiation medium was switched to neurobasal medium (GibCo, #A35829-01) containing 2.5% FBS, 1% P/S, 10 µM RA, 2 mM L-Glutamine, 1X N-2 (Gibco, #17502), 1X B-27 Plus supplement (Gibco, #A35828-01) and 20 mM KCl (Sigma, P9333). At the end of the differentiation procedure, hydrogels were either collected for the extraction of RNA or fixed with 4% paraformaldehyde for immunostaining.

RNA Isolation and cDNA Synthesis

RNA extraction was applied following the manufacturer's instructions led by the NucleoSpin®-RNA isolation kit (Macherey-Nagel). For 3D samples, gels were homogenized in

RA1 buffer supplied by the kit and were centrifuged at 14,000 xg for 5 min to remove the debris. Ethanol was added to the supernatant and mixed well by vortexing followed by centrifugation at 14,000 xg for 10 min at 4°C. Supernatant was discarded and the pellet was air-dried. After resuspension of the pellet, the instructions were followed accordingly which include RNA binding, membrane desalting, DNA digestion, purification, and elution. The quantity and quality of the eluted RNA were evaluated with NanoDrop Spectrophotometer (Thermo Scientific 2000c, USA). The purified RNA was then reverse-transcribed into cDNA. First, random hexamer, 1 µg RNA, 10 mM dNTP-mix, and distilled water were mixed. After incubation at 65°C for 5 min, the second mixture including 5x First strand buffer, 100 mM DTT, and Ribonuclease Inhibitor (40 U/µl) were added to each sample. The samples were incubated at 37°C for 2 min and M-MLV RT enzyme (200 U) was added. The incubation steps for synthesis were as following: 25°C for 10 min, 37°C for 50 min, and 70°C for 15 min. The synthesized cDNA was then diluted 1:5 in RNase-free H_2O.

Gene Expression Analysis

Neuronal development-related gene expressions were quantified relatively by qRT-PCR. The primers were designed using Primer™ and sequences are shown in Table 1. QuantiNova SYBR Green PCR Master Mix (Qiagen, #172034345), forward and reverse primers, cDNA sample, and PCR grade H_2O were mixed for the reaction of each experimental group and transferred to the PCR plate. Reaction cycles were carried out by Roche's LightCycler 480 Instrument II. The mRNA expression levels (depicted as Cq values) of the related genes were normalized to a housekeeping control gene (GAPDH), and fold change was calculated using the $2^{-\Delta\Delta Ct}$ method.

Immunostaining with Neuronal Markers

SH-SY5Y cells encapsulated in db-ECM hydrogels at a concentration of $5x10^5$ cells/ml and 2D cultured cells for both undifferentiated control and the differentiated group were fixed with 4% paraformaldehyde (Sigma, 158127-500G) at day 15. Similarly, permeabilization with 0.1% Triton X-100 in PBS was done for 1 h at room temperature for 3D culture and 15 min for 2D culture. Then, blocking was carried out with 1% BSA solution including 22.52 mg/ml glycine in PBS-T for 2 h for 3D culture and 30 min for 2D culture. Primary antibodies anti-NEUN (Abcam, ab177487) and anti-Beta-Tubulin III (Biolegend, #801213) were used for immunostaining. After overnight incubation at 4°C in constant rotation, secondary antibodies Alexa Fluor 488 goat anti-mouse IgG (H+L) (Invitrogen, A11029) and Alexa Fluor 594 goat anti-rabbit (Invitrogen, R37117) were applied in dark. Nuclear counterstaining was done with DAPI and images were taken with Leica DMI8/SP8 laser scanning confocal microscope. Images were exported from the LAS X program (Leica, Wetzlar, Germany). Image analysis was achieved using ImageJ software (National Institutes of Health, USA).

Statistical Analysis

The quantitative data was stated as mean±S.D values resulting from minimum n=3 replicates. One sample t-test was applied to the experimental data using GraphPad Prism 8 software. P values smaller than 0.05 were considered statistically significant ($p<0.0001$=extremely significant (****), $0.0001<p<0.001$=extremely significant (***), $0.001<p<0.01$=very significant (**), and $0.01<p<0.05$=significant (*)).

RESULTS

Generation of Decellularized Brain ECM Hydrogels

The cortex of the bovine brain was cut into small pieces and treated with SDS and DNase to achieve decellularization. After decellularization, the wet tissue was lyophilized and cryomilled. Then, the powder form of the db-ECM was solubilized through digestion with the pepsin enzyme. The soluble part of the digest was neutralized, and gelation was achieved through temperature-induced crosslinking (Figure 1a). As shown in Figure 1b, the decellularization process resulted in changes in the tissue composition, and decellularized tissue represented fiber-like structures when compared to native tissue. Decellularization was validated with Hoechst staining, while nuclear elimination was completely achieved in the decellularized tissue. To encapsulate SH-SY5Y cells in db-ECM hydrogels, cells were blended with neutralized brain ECM digest at designated concentrations and incubated at 37°C (Figures 1c and d). Upon gelation, db-ECM hydrogels with SH-SY5Y cells were cultured for 5 days to assess the cytocompatibility of the reconstituted native matrix. Phalloidin/DAPI staining demonstrated optimal cell growth in 3D and typical morphology of SH-SY5Y cells (Figure 1c).

Differentiation of Neuroblastoma in 2D and 3D Conditions

Differentiation protocol was applied to neuroblastoma cells for 17 days and the procedure included two differentiation phases (Figures 2a and b). In the first phase, serum content was gradually decreased within the DMEM high glucose medium containing RA, while in the second phase, the cells were cultured in a neurobasal medium containing various indicated neurotrophic factors (Figure 2b). Cellular morphology was carefully monitored with brightfield microscopy during the differentiation protocol optimization. The brightfield images taken at the end of differentiation, on day 17, were shown in Figure 2c. Significant reduction in cell growth was observed in differentiated cells both in 2D and 3D conditions, a well-established indication for differentiated neuroblastoma.[14,34] Apart from reduced cell proliferation, the morphology of the cells remarkably changed in

Table 1. Gene-specific primers used in qRT-PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
APP	ATGGTAATGCTGGCCTGCTG	GAATCCCACTTCCCATTCTG
CHAT	GAGGAGCAGTTCAGGAAGAG	CCAGGAGTTTCTGCTGCAGG
GAPDH	CTGACTTCAACAGCGACACC	GTGGTCCAGGGGTCTTACTC
GFAP	CCAGTTATCAGGAGGCGCTG	TCCTGGTACTCCTGCAAGTG
MAP2	CTGTAGCAGTCCTGAAAGGTG	CTGTTCTGAGGCAGGTGATG
MAPI	TTAGCAACGTCCAGTCCAAG	TCAGGTCAACTGGTTTGTAG
PSENİ	AGTATCCTCGCTGGTGAAGAC	ACGAAACAGGCTATGGTTGTG
RET	ACCATGGGCGACCTCATCTC	CTGCCAAGTCCCGATGAACG
S0X2	CTTTTATGAGAGAGATCCTG	ACCGTACCACTAGAACTTT
SYP	GCTGTGTTCGCCTTCATGTG	AATGTTCTCTGGGTCTGTGG
TUBB3	GAGCGGATCAGCGTCTACTAC	GCGGACACTGTCCATGGTTC

2D and 3D conditions. In 2D, differentiated cells displayed elongated axons creating a synaptic network, while the cell body became smaller in size. In 3D culture, the encapsulated naïve cells in the control group were inclined to form round cell clump masses. On the other hand, differentiated cells in 3D tended to form neurites towards nearby cells across the hydrogel without forming cell clusters (Figure 2c).

Gene Expression in Differentiated Neuroblastoma

Next, we assessed the gene expression profile of differentiating neuroblastoma with qRT-PCR for 2D (Figure 3) and 3D (Figure 4) conditions to reveal the differences in molecular markers upon change in culture dimensionality. *TUBB3*, a mature neuron marker, is upregulated upon differentiation in SH-SY5Y cells encapsulated in both 3D db-ECM hydrogels and conventional 2D culturing. Expression of *CHAT*, a cholinergic neuron marker, was also significantly increased. Unexpectedly, *MAP2*, a gene responsible for neurogenesis, exhibited a small but significant decrease in gene expression upon differentiation in both conditions. Although the expression profile of neuronal markers was correlated for 2D and 3D, another set of genes showed different trends in response to differences in culture dimensionality during the differentiation of SH-SY5Y cells. Expression of *SOX2*, a stemness marker, was significantly decreased in SH-SY5Y cells differentiated on 2D in comparison to the non-differentiated control group (Figure 3). However, in 3D db-ECM hydrogels, cells maintained their stemness despite induction of differentiation and gene expression of *SOX2* remained unaltered (Figure 4). Similarly, the *MAPT* gene was significantly downregulated during differentiation in 2D whereas in 3D, its expression was unchanged. A proto-oncogene involved in neuronal differentiation, *RET*, exhibited a 2.64-fold increase in gene expression in cells following differentiation on 2D while in 3D, differentiation did not affect its expression. Interestingly, differentiation of SH-SY5Y cells in 3D db-ECM hydrogels significantly induced astrocytic (*GFAP*) and synaptogenesis (*SYP*) markers as opposed to 2D conditions in which expression of these genes did not demonstrate change. On the other hand, the differentiation protocol did not cause any change in the expression of neuropathological markers *APP* and *PSEN1* in either condition.

Evaluation of Neuronal Markers with Immunostaining in Differentiated Cells

To further evaluate the structural and morphological changes in differentiated SH-SY5Y cells in 2D and 3D cultures, protein expression of neuronal nuclei marker NEUN and mature neuron marker beta-tubulin III (TUBB3) was assessed with immunostaining. As shown in Figure 5, in 2D cultures, the number of cells in the differentiation group was reduced distinguishably concomitant with the emergence of elongated axons and TUBB3 expression. On the other hand, in 3D db-ECM hydrogels, a drastic change in morphology was observed (Figure 6). In non-differentiated cells in hydrogels, cell growth demonstrated clump formation, typical for cancerous cell lines. However, in the differentiated cells in 3D, a clear morphological shift was observed with the formation of a neuronal synaptic network alongside the expression of neuronal markers (Figure 6).

DISCUSSION

The neuroblastoma cell line is a preferred model for studying neurodegenerative diseases *in vitro* due to its human origin and ability for expansion and neuronal differentiation. However, the role of culture dimensionality and tissue-specific ECM in the differentiation of neuroblastoma cells has been unknown. A 3D microenvironment offers several advantages such as providing a physiologically relevant matrix that closely mimics the native tissue and enabling cell behavior and functionality observed *in vivo*. Furthermore, mechanical matrix parameters such as stiffness and viscoelasticity can be finely tuned.[5,35–37] In this study,

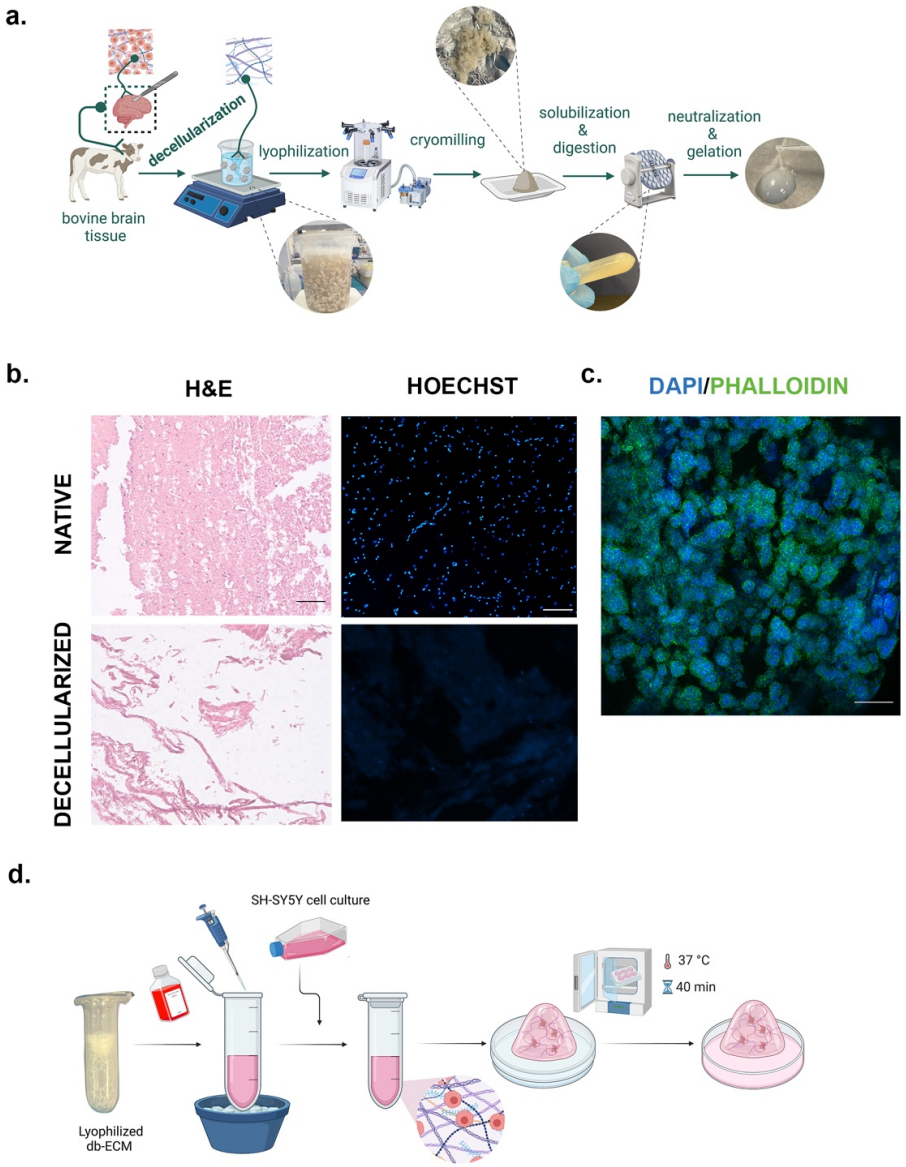

Figure 1. Decellularization of bovine brain tissue and hydrogel generation. a) Schematic description of hydrogel generation derived from decellularized bovine brain tissue (Created with BioRender.com). b) Histological examination of native bovine brain tissue and decellularized bovine brain tissue by H&E staining and Hoechst staining (scale bar: 100µm). c) Phalloidin and DAPI staining applied to SH-SY5Y cells in a 3D microenvironment at day 5 (scale bar: 75µm). d) Pipeline for hydrogel generation (Created with BioRender.com).

we investigated the role of culture dimensionality in neuronal differentiation of SH-SY5Y cells. As a 3D biomimetic model, we fabricated brain ECM hydrogels via decellularization of bovine brain tissues which allowed the culturing of cells within a reconstituted native brain matrix.

Neuroblastoma is a childhood cancer arising from the neural crest which holds the ability to differentiate into mature neuron phenotypes using certain agents.[38] In our work, SH-SY5Y differentiation is induced with a gradual decrease of serum content in the medium, the addition of RA, and neurotrophic factors. Serum deprivation allows for the elimination of epithelial cells to achieve a homogeneous neuronal cell population in the otherwise heterogeneous SH-SY5Y cells.[39] Until day 9, neuronal cells were selected after which the selected population was supported with neurotrophic factors including N2, B-27, KCl, and RA to induce neuronal maturation. RA plays a major role during neuronal differentiation by administrating complex signaling pathways, such as the protein kinase A-dependent pathway, transcription factors, and extracellular molecules, including Wnt signaling.[40] While N2 and B-27 are expected to

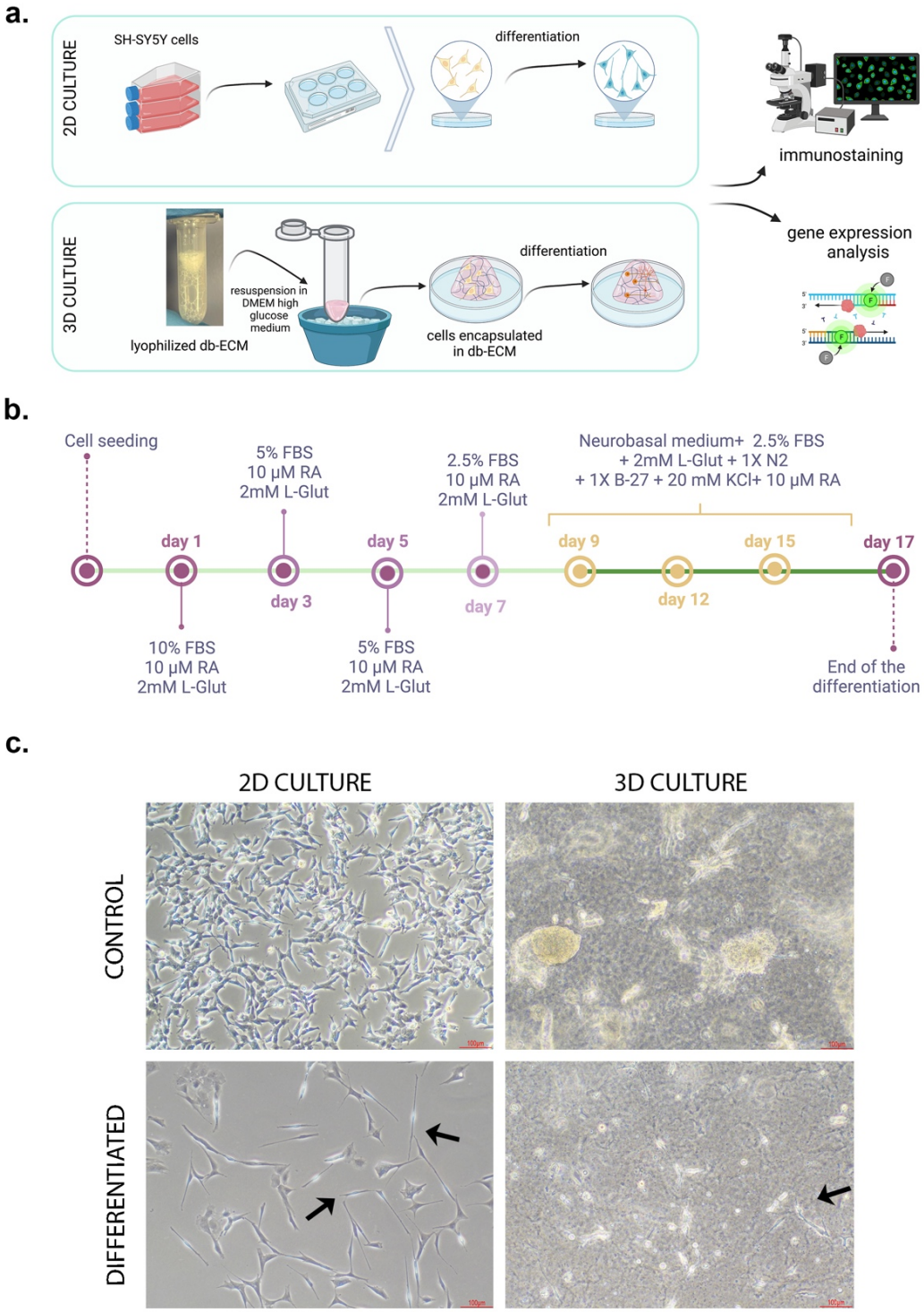

Figure 2. Neuronal differentiation of neuroblastoma cells in 2D and 3D culture. a) Schematic description of neuronal differentiation experiment setup (Created with BioRender.com). b) Neuronal differentiation treatment timeline for 2D and 3D application (Created with BioRender.com). c) Brightfield images of undifferentiated and differentiated neuroblastoma cells in 2D and 3D microenvironments at day 15 (scale bar: 100µm).

promote neurogenesis, KCl is known to induce depolarization of neurons by the activation of potassium channels.[41,42]

During neuronal differentiation, cell proliferation was reduced as expected, an important feature of differentiation, the expression of neuronal markers was distinctively changed, and a shift in cell morphology was observed in both 2D and 3D con-

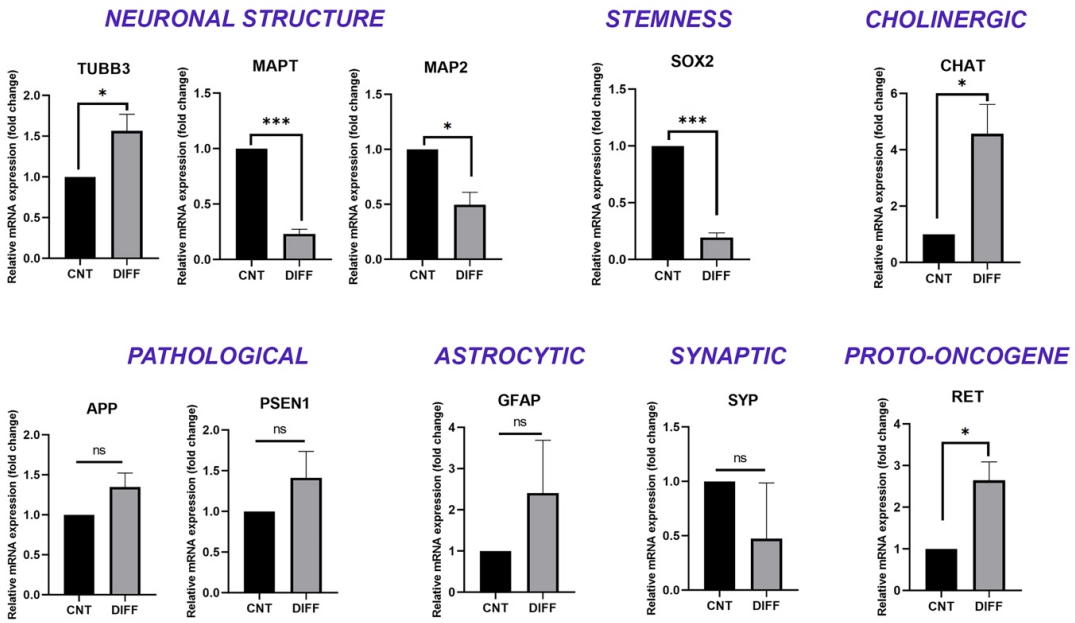

Figure 3. Gene expression analysis of 2D differentiated neuroblastoma cells; the results were normalized to GAPDH expression of the cells.

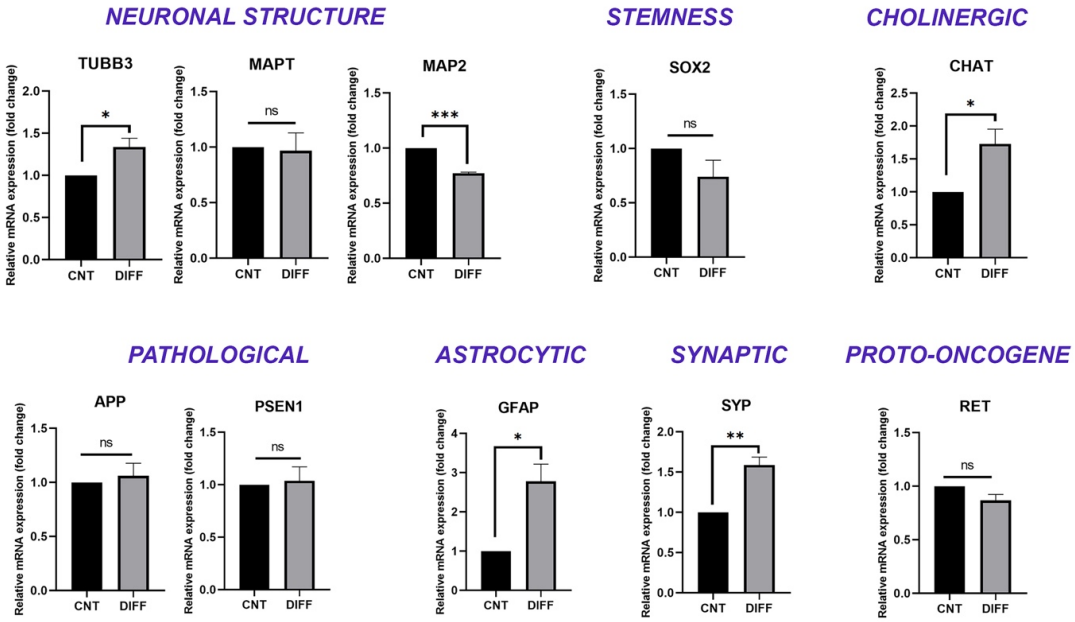

Figure 4. Gene expression analysis of 3D differentiated cells; the results were normalized to GAPDH expression of the cells.

ditions. In 2D culture, cells possessed long axons and formed a synaptic network with nearby cells upon differentiation (Figure 2c). Moreover, there was a significant increase in the mature neuron marker *TUBB3* gene expression and a significant decrease in the stemness marker *SOX2*, indicating neuronal maturation. On the other hand, the expression of the *CHAT* gene was upregulated which hints that cholinergic neuron differentiation was induced by the applied differentiation protocol. Unexpectedly, the expression of *MAP2* and *MAPT* was significantly reduced in differentiated cells. This might have been due to a lack of matching the timing of sample collection with the stage of differentiation in line with previous studies including Przyborski et al.'s, where it has been shown that upregulated levels of MAP2c mRNA transcripts in the early stages of differentiation

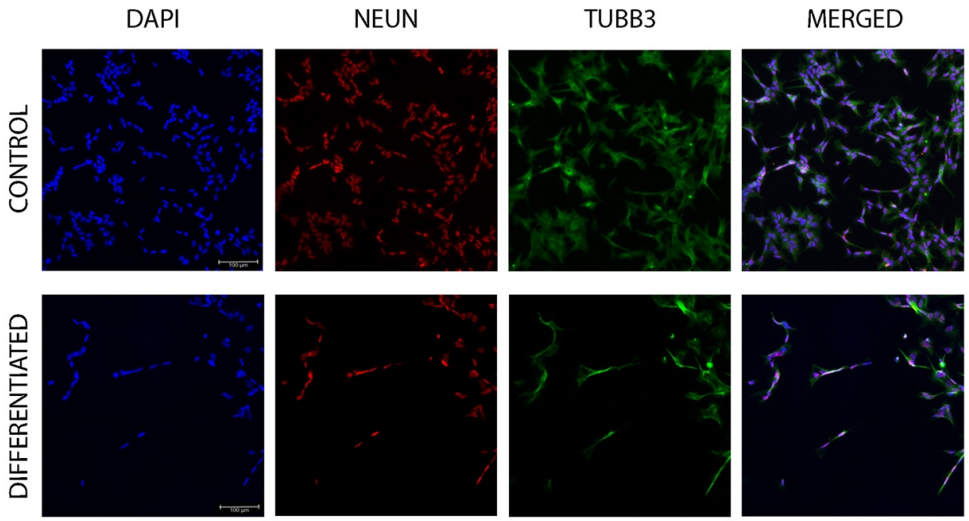

Figure 5. Immunostaining of undifferentiated and differentiated neuroblastoma cells in 2D culture at day 15. Neuronal markers, NEUN:red; Beta-tubulin III, TUBB3:green; Nuclear staining with DAPI:blue. Scale bar:100 µ m.

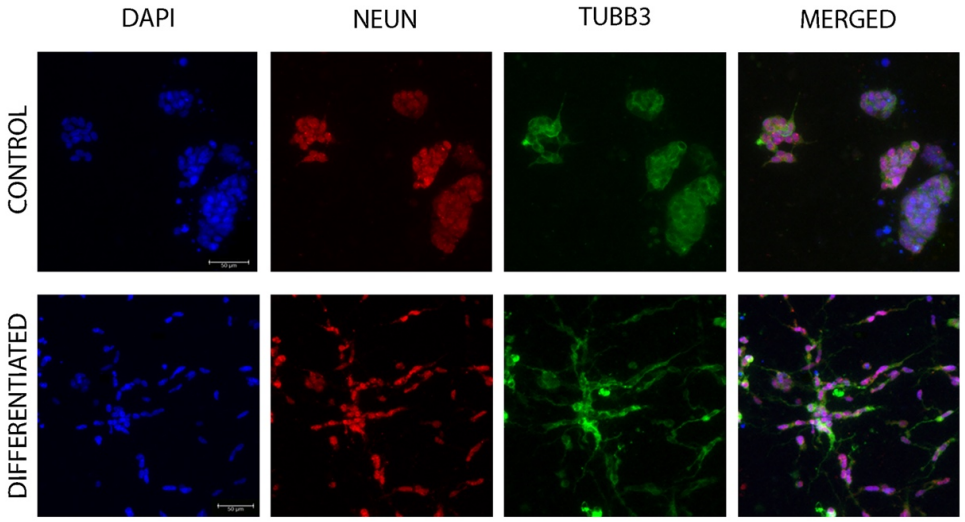

Figure 6. Immunostaining of undifferentiated and differentiated neuroblastoma cells in 3D culture at day 15. Neuronal markers, NEUN:red; Beta-tubulin III, TUBB3:green; Nuclear staining with DAPI:blue. Scale bar:50 µ m.

tended to decrease as developmental progression proceeded.[43] In our study, another gene that was affected by neuronal differentiation in 2D conditions was RET, a proto-oncogene marker. Studies have demonstrated an increased expression of RET starting from day 3 of RA treatment, which was hypothesized to stimulate neuronal differentiation.[29,44] RET expression is more common in dopaminergic neurons, and an increase in this gene is correlated with the promoted neurogenesis in dopaminergic

neurons. Moreover, the elevation of RET expression might be a sign of the completion of early differentiation phases.[14]

SH-SY5Y differentiation in 3D db-ECM hydrogels similarly revealed a morphological shift in cells and neuronal network formation in line with the expression of neuronal markers (Figure 6). On the other hand, non-differentiated neuroblastoma cells exhibited growth in spherical clumps, a behavior common for 3D cancer cell cultures.[45] In response to mechanical stimulus through hydrogels, cells tend to remodel their surroundings and change their cellular shape, behavior, and biological signaling.[46,47] Physical confinement and the presence of brain-specific ECM ligands provided by the 3D hydrogel model used in the study demonstrated distinct changes in gene expression during neuronal differentiation compared to 2D cultures (Figure 4). The expression of the *SYP* gene, a synaptic marker, was significantly increased in 3D matrices in line with neural network formation. Although the functionality of this gene has not been completely clarified, it has important roles in vesicular ion channel activity and endocytosis.[26] Expression profiles of neuronal markers *TUBB3*, *CHAT*, and *MAP2* were unaffected by culture dimensionality and similar in 2D and 3D conditions. On the other hand, SH-SY5Y cells that differentiated in 3D db-ECM hydrogels showed an upregulation of astrocytic marker *GFAP*. Although glial cell formation was not targeted during the differentiation procedure, due to the biomechanical characteristics of the hydrogel, glial cell formation was induced (Figure 4). Similarly, a study by Hu et al. demonstrated increased expression of GFAP in primary rat astrocytes in 3D soft matrices compared to stiff environments which was found to be mediated by the inhibition of Yes-associated protein (YAP), a key transcriptional coregulator of stiffness-induced cellular events.[48]

As opposed to the observed loss of stemness and reduction in *SOX2* gene expression in 2D conditions, neuroblastoma differentiation in 3D hydrogels showed preservation of stemness. This might be due to the presence of ECM components that help cells maintain their stemness characteristics thereby promoting their differentiation capability.[49] Furthermore, matrix properties such as stiffness, viscoelasticity, and degradability were defined as key parameters for regulating the stemness characteristic of stem cells.[50,51] Cells cultured in 3D microenvironments require matrix remodeling to migrate and expand.[52,53] Native tissue matrices are viscoelastic and support such remodeling where differentiation can be induced with instructive ECM ligands.[54]

The expression of neuropathological markers *PSEN1* or *APP* was investigated in different conditions since previous reports have shown that such markers could be upregulated upon differentiation. However, we did not encounter any significant changes in the expression of *PSEN1* or *APP* in either 2D or 3D hydrogels.[11]

CONCLUSION

In this study, we performed a comparative analysis of neuroblastoma cell differentiation within 3D hydrogels constructed from decellularized bovine brain tissue, in comparison to conventional 2D cell cultures. Our investigation focused on evaluating changes in cellular morphology and the expression of molecular markers. In conclusion, the findings of this study reveal that culture dimensionality distinctly affects the neuronal differentiation of neuroblastoma cells. 3D hydrogels with the ability to biomimick the brain microenvironment via reconstitution of native ECM show preservation of stemness, induction of synaptic markers, 3D network formation, and expression of glial markers as opposed to conventional 2D culturing. These hydrogel systems can be adapted to different pathophysiological states by modulating their biophysical and biochemical properties for developing faithful *in vitro* disease models in neuroscience.

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