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Neural progenitor cell-derived extracellular matrix as a new platform for neural differentiation of human induced pluripotent stem cells

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ABSTRACT

The culture microenvironment has been demonstrated to regulate stem cell fate and to be a crucial aspect for quality-controlled stem cell maintenance and differentiation to a specific lineage. In this context, extracellular matrix (ECM) proteins are particularly important to mediate the interactions between the cells and the culture substrate. Human induced pluripotent stem cells (hiPSCs) are usually cultured as anchorage-dependent cells and require adhesion to an ECM substrate to support their survival and proliferation *in vitro*. Matrigel, a common substrate for hiPSC culture is a complex and undefined mixture of ECM proteins which are expensive and not well suited to clinical application. Decellularized cell-derived ECM has been shown to be a promising alternative to the common protein coatings used in stem cell culture. However, very few studies have used this approach as a niche for neural differentiation of hiPSCs.

Here, we developed a new stem cell culture system based on decellularized cell-derived ECM from neural progenitor cells (NPCs) for expansion and neural differentiation of hiPSCs, as an alternative to Matrigel and poly-L-ornithine/laminin-coated well plates. Interestingly, hiPSCs were able to grow and maintain their pluripotency when cultured on decellularized ECM from NPCs (NPC ECM). Furthermore, NPC ECM enhanced the neural differentiation of hiPSCs compared to poly-L-ornithine/laminin-coated wells, which are used in most neural differentiation protocols, presenting a statistically significant enhancement of neural gene expression markers, such as *βIII-Tubulin and MAP2*.

Taken together, our results demonstrate that NPC ECM provides a functional microenvironment, mimicking the neural niche, which may have interesting future applications for the development of new strategies in neural stem cell research.

1. Introduction

Human induced pluripotent stem cells (hiPSCs) can be derived by reprogramming adult somatic cells [1,2] and have the ability to self-renew indefinitely and to differentiate into cells from all the three embryonic germ layers (endoderm, mesoderm and ectoderm), when cultured *in vitro* under appropriate conditions [3]. These properties allow to generate large numbers of hiPSCs, and their derivatives, with great potential for applications in cell therapies, drug discovery and disease modeling. In fact, these cells constitute a less ethically prohibitive alternative to other human pluripotent stem cells, such as human embryonic stem cells (hESCs) [4] and, importantly, patient-specific hiPSC lines can be derived which may lead to personalized therapies.

Engineering the microenvironment in which these cells are cultured is a common strategy for quality-controlled hiPSC maintenance and to direct differentiation to a specific lineage [5]. Currently, hiPSC culture conditions are complex, expensive and not well suited to clinical application [5]. Undifferentiated hiPSCs can be maintained on feeder cells (*e.g.* fibroblasts) but, for most applications, feeder-free culture is preferred [5,6]. Although hiPSCs can be cultured in suspension [7], these cells are usually cultured as a 2D monolayer, requiring an appropriate culture substrate to support their adhesion, survival and proliferation *in vitro* [5,6]. hiPSCs are usually cultured in serum-free media and, under these conditions, do not adhere efficiently to uncoated glass or tissue culture-treated plastic. Instead, hiPSCs require a pre-coating with specific extracellular matrix (ECM) proteins, peptides or synthetic polymers as culture substrates [6]. Basement membrane complex matrices, such as Matrigel (Corning), or different individual ECM proteins, have been used to enhance hiPSC adhesion, while maintaining the pluripotency and a normal karyotype [5,8,9]. Matrigel, the most widely used feeder-free substrate, is extracted from the Engelbreth-Holm-Swarm mouse tumor and contains a mixture of laminin, collagen, entactin, heparin sulfate proteoglycan and growth factors that varies from lot to lot [8,10]. The ill-defined composition and animal origin of Matrigel lead to variability in cell culture outcomes and limit clinical applicability [11,12]. Thus,

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efforts have been made to identify the minimum number of required components to develop a suitable matrix for hiPSC culture without compromising their quality. Synthetic polymers have been developed as alternative to Matrigel, supporting hiPSC proliferation and maintenance [12]. For instance, the polymer poly(2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide) (PMEDSAH) has been used as a coating to culture hiPSCs and sustain their maintenance [13]. A poly(OEGMA-co-HEMA) film decorated with a vitronectin-derived peptide has also demonstrated the capacity to support long-term hiPSC culture and maintenance [14]. Synthemax II, a synthetic peptide containing the RGD sequence of human vitronectin, has been also shown to support hiPSC growth [15]. However, these synthetic alternatives to Matrigel do not recreate the native microenvironment, especially the architecture, topography or biochemical composition and thus a better alternative is needed for mimicking the *in vivo* niche. Moreover, since hiPSCs can differentiate into different cell lineages, specific culture substrates should be selected to promote efficient and robust differentiation methods [6]. In particular, neural stem cells (NSCs) and neural progenitor cells (NPCs) derived from hiPSCs may be used for disease modeling and, potentially, for treating a range of neurological diseases [16]. Different culture substrates for neural differentiation of hiPSCs have been investigated [17] but the majority of the reported studies uses poly-L-ornithine/laminin-coated plates [6,16–18. Laminin has been widely used for NPC/NSC culture, promoting adhesion and proliferation of NSCs [17,19] and also enhancing neural differentiation [20]. Poly-L-ornithine has been shown to promote differentiation of NPCs/NSCs via ERK signaling pathway [18] and is often used in combination with other ECM proteins to support their function, such as laminin and fibronectin [21]. However, despite these advantages, these substrates present some features that make them less favorable in some culture conditions. Although laminin efficiently supports neural differentiation, this molecule is often collected from mouse sarcomas, not being suitable for clinical application. Alternatively, recombinant laminin or human laminin are available but extremely expensive [17]. Poly-L-ornithine, in contrast, is cheaper than laminin since it can be easily obtained from polycondensation reactions. However, it is not commonly used alone but in combination with other proteins and some studies have reported that poly-L-ornithine can be toxic to cells, being important to find the optimal concentration for coating [22]. Moreover, most of these culture substrates consist in a single or a few ECM proteins, used as protein coatings, not mimicking the complex native ECM composition and architecture. Interestingly, besides biochemical composition, substrate stiffness and topography deeply influence cell behavior, such as proliferation and differentiation responses [23]. Therefore, new cell substrates are needed to optimize hiPSC culture and, in particular, differentiation into a neural lineage in order to better mimic the *in vivo* neural niche and establish a robust and cost-effective platform to obtain NPCs/NSCs for biomedical applications.

Decellularized extracellular matrix (dECM) has gained increasing interest as an adhesion substrate for cell culture, providing mechanical and structural support and signaling cues to cells, regulating cell survival, proliferation and differentiation [24]. dECM is composed of fibrillar proteins (*e.g.* collagens, fibronectin, laminin), proteoglycans (*e.g.* decorin, versican, aggrecan), glycosaminoglycans (*e.g.* heparin sulfate, chondroitin sulfate, hyaluronan), and matricellular proteins (*e.g.* osteopontin, thrombospondin) that assemble into a complex and highly organized structure [25]. dECM can be obtained from *in vivo* tissue ECM (tissue-derived ECM) or from ECM fabricated by cells cultured *in vitro* (cell-derived ECM) [26]. In the field of neural tissue engineering, tissue-derived ECM, in particular from decellularized brain sections, has been used to design innovative scaffolds [27–29] where NSCs were able to attach, proliferate, retain their stemness and differentiate into neural cells [27,28]. Furthermore, Baiguera and colleagues reported that mesenchymal stem/stromal cells differentiated into neural cells on electrospun gelatin scaffolds containing decellularized brain ECM [30]. Recently, functionalization of culture substrates using decellularized brain ECM enhanced the differentiation of hiPSCs into oligodendrocytes, providing brain-specific biochemical, biophysical and structural signals [31]. Decellularized cell-derived ECM has also been used as an *in vitro* ECM model to regulate stem cell differentiation, modeling a specific stem cell niche and recreating the ECM at different maturational stages [32]. Cell-derived ECM can be obtained by culturing cells *in vitro* until confluence, allowing them to secrete ECM, followed by a decellularization treatment to remove the cellular components while retaining the ECM structure [33]. However, although decellularized cell-derived ECM has been shown to induce cell proliferation and differentiation [26,33], to date there are scarce reports describing the use of cell-derived ECM as a culture substrate to recreate the niche of anchorage-dependent cells that need ECM to attach, such as hiPSCs, hESCs or NPCs. Sart and colleagues demonstrated that dECM derived from ESCs (monolayers or aggregates) promoted ESC proliferation and differentiation [34]. Furthermore, mouse embryonic fibroblasts were decellularized to obtain a cell-derived ECM model to culture NPCs, facilitating NPC attachment, proliferation and neuronal differentiation [35]. However, recent studies have reported that different cell types generate cell-derived ECM with different compositions and this may impact cellular behavior and differentiation [32,33]. In this study, we developed a new culture system based in dECM derived from human NPCs and evaluated the feasibility of expanding hiPSCs and differentiating them into neural cells on NPC ECM, mimicking the neural niche. This work will allow the development of hiPSC differentiation platforms that incorporate signals from the ECM, closely recapitulating the *in vivo* microenvironment and resulting in improved cell maturation of neural cells and providing an alternative to standard substrates, such as Matrigel and poly-L-ornithine/laminin.

2. Materials and methods

2.1. Human induced pluripotent stem cell culture and maintenance

Two different hiPSC lines were used in this work. The F002.1A.13 cell line (TCLab – Tecnologias Celulares para Aplicação Médica, Portugal) was reprogrammed from human healthy fibroblasts (46, XX) through retroviral transduction of human genes OCT4, SOX2, C-MYC and KLF4. The Gibco® episomal hiPSC line (Thermo Fisher Scientific, USA) was derived from $CD34^+$ cord blood through EBNA-based episomal transfection of factors SOX2, OCT4, KLF4, C-MYC, NANOG, LIN28 and SV40 T antigen. hiPSCs were thawed and plated in tissue culture plates coated with Matrigel (1:100; Corning, USA) using mTeSR1 culture medium (STEMCELL Technologies, Canada). Cells were kept at 37 °C and 5% CO_2 in a humidified atmosphere. Medium was changed everyday and, after reaching 80% confluence, cells were passaged using EDTA (Thermo Fisher Scientific) at a split ratio of 1:3. Briefly, cells were washed twice and incubated with EDTA solution (0.5 mmol/L in Dulbecco's phosphate-buffered saline, DPBS, Thermo Fisher Scientific) for 5 min. After that, EDTA was removed and cells were collected with mTeSR1 and plated in new tissue culture plate pre-coated with Matrigel or with dECM substrate.

2.2. Neural commitment of hiPSCs

Following expansion of hiPSCs, neural induction was performed by culturing cells in N2B27 medium (50% N2 medium+50% B27 medium) [16,36]. N2 medium was composed of DMEM-F12 with GlutaMAX™ (Thermo Fisher Scientific) supplemented with 1% N2 (Thermo Fisher Scientific), 1.6 g/L glucose (Sigma-Aldrich, USA), 20 μg/mL insulin (Sigma-Aldrich) and 1% penicillin/streptomycin (P/S, Thermo Fisher Scientific). B27 medium was composed of Neurobasal medium supplemented with 2% B27 supplement, 2 mM L-glutamine and 1% P/S (all reagents from Thermo Fisher Scientific). During 12 days of neural commitment, cells were cultured with N2B27 medium supplemented with 10 μM SB431542 (SB, Sigma-Aldrich) and 100 nM LDN193189 (LDN, StemGent, USA). The medium was renewed daily.

2.3. Decellularized cell-derived ECM preparation and characterization

ECM was derived from decellularized NPCs after neural commitment of hiPSCs through 12 days, according to Section 2.2. Therefore, after 12 days of neural commitment of hiPSCs (N2B27 medium+LDN+SB), medium was discarded and cells were washed with DPBS [16,36]. Based on previously reported methods [26,33], ECM decellularization was performed using a 20 mM ammonium hydroxide (NH_4OH, Sigma-Aldrich) and 0.5% Triton X-100 (Sigma-Aldrich) solution in DPBS containing 150 U/mL DNase. NPCs were incubated with this solution for 3 min at room temperature. After microscopic confirmation of complete cell lysis and presence of intact ECM on the surface of the wells, NPC ECM was gently washed three times with distilled water. In all experiments, NPC ECM was used freshly after decellularization treatment. ECM protein components and distribution pattern of NPC ECM were evaluated by immunofluorescence staining of human collagen I, collagen IV, fibronectin and laminin. Thus, NPC ECM was fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) for 20 min at room temperature. After washing three times with 1% bovine serum albumin (BSA, Sigma-Aldrich) in DPBS, samples were blocked with a solution of 0.3% Triton X-100, 1% BSA and 10% fetal bovine serum (FBS, Thermo Fisher Scientific) in DPBS for 45 min at room temperature. Primary antibodies including collagen I, collagen IV, fibronectin and laminin (R&D Systems, USA) were added into the samples and incubated overnight at 4 °C (10 μg/mL in a solution of 0.3% Triton X-100, 1% BSA, 10% FBS in DPBS). After washing with DPBS, goat anti-mouse IgG-AlexaFluor 546 (1:500, Thermo Fisher Scientific) and goat anti-rabbit IgG-AlexaFluor 488 (1:500, Thermo Fisher Scientific) were added into the samples and incubated for 1 h at room temperature in the dark. Afterwards, samples were washed twice with DPBS and cells nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 1.5 μg/mL, Sigma-Aldrich) for 5 min at room temperature in the dark. The staining was imaged by fluorescence microscope (Leica DMI 3000B) and recorded by an attached digital camera (Nikon DXM 1200).

For further quantification of DNA, collagens and sulfated glycosaminoglycans (sGAGs), NPC ECM and cells were lyophilized and stored at −80 °C for further use. Briefly, the quantification of double-stranded DNA (dsDNA) was measured using a Quant-iT^{TM} PicoGreen^{TM} dsDNA assay kit according to the manufacturer's instructions (Thermo Fisher Scientific). sGAGs were determined using a 1,9 dimethylmethylene blue (DMMB) assay. A papain buffer consisting on 50 mM Na_2HPO_4, 2 mM EDTA, 2 mM N-acetyl cysteine and 100 μg/mL papain was used to digest dECM and cells overnight at 60 °C. 50 μL of samples were mixed with 200 μL of DMMB solution and incubated at room temperature for 5 min. The standard curve ranged from 0 to 40 μg/mL chondroitin-6-sulfate. Absorbance was measured at 525 nm. Collagen content was evaluated using a hydroxyproline assay kit according to the manufacturer's instruction (Sigma-Aldrich). All quantifications were performed per mg of dried cells/ECM.

2.4. hiPSC culture and neural differentiation on decellularized NPC ECM

hiPSCs were cultured on decellularized NPC ECM using mTeSR1 medium at a density of 1×10^5 cells/cm^2 and medium was renewed daily. As a control, hiPSCs were cultured on Matrigel-coated tissue culture plates using mTeSR1 medium at a density of 1×10^5 cells/cm^2. Supplementary Figure S1 shows the experimental protocol used in this study for neural differentiation. After reaching confluence, cells were cultured in 1:1 N2 and B27 medium for 12 days supplemented with 10 μM SB431542 and 100 nM LDN193189, according to Section 2.2. Medium was replaced daily and after 12 days human neural progenitor cultures were passaged using EDTA dissociation buffer (0.5 mM in DPBS) for 5 min at room temperature. NPCs cultured on NPC ECM were replated to a new NPC ECM plate at a density of 1×10^5 cells/cm^2. On the other hand, NPCs cultured on Matrigel-coated tissue culture plates were replated to poly-L-ornithine-treated (15 μg/mL, Sigma-Aldrich) and laminin-coated (20 μg/mL, Sigma-Aldrich) plates at a density of 1×10^5 cells/cm^2. At day 14, when neural rosette-like structures were observed, N2B27 medium was supplemented with basic fibroblast growth factor (bFGF, 20 ng/mL, Peprotech) for 48 h and then withdrawn for both conditions (NPC ECM and poly-L-ornithine/laminin-coated wells). At day 17, rosette cells were split 1:3 using EDTA and replated to NPC ECM substrate or to poly-L-ornithine/laminin-coated plates and cultured in N2B27 medium without supplementation. The medium was replaced every other day. At day 27, neurons were split 1:3 with Accutase (Sigma-Aldrich) and plated into NPC ECM substrate or into poly-L-ornithine/laminin-coated plates at a density of 1×10^5 cells/cm^2. N2B27 medium without supplementation was replaced every two days until day 40.

2.5. Flow cytometry

After several passages, cells were analyzed for the expression of pluripotency markers. For intracellular staining, a minimum of 5×10^5 cells was washed with DPBS and fixed with 2% PFA for 20 min. Cells were centrifuged at 1000 rpm for 3 min and washed with DPBS. Cells were then resuspended in 3% normal goat serum (NGS, Sigma-Aldrich) and permeabilized using a solution 1:1 of 3% NGS and 1% saponin (Sigma-Aldrich) for 15 min at room temperature. After washing with 3% NGS, cells were incubated with primary antibody solution (in 3% NGS) for 1.5 h at room temperature. Afterwards, cells were washed twice with 1% NGS and incubated with the secondary antibody solution (in 3% NGS) for 45 min in the dark at room temperature. Finally, cells were washed with 1% NGS and resuspended in DPBS prior to flow cytometry analysis. For intracellular staining, antibodies for OCT4 (1:300, Millipore, USA) and SOX2 (1:150, R&D Systems) were used and goat anti-mouse IgG-AlexaFluor 488 (1:300, Thermo Fisher Scientific) was used both as a secondary antibody and as a negative control. For surface marker staining, antibodies for SSEA-4 (1:10, PE-conjugated, Miltenyi Biotec, Germany) and TRA-1–60 (1:10, PE-conjugated, Miltenyi Biotec) were used. Approximately 1×10^5 cells were resuspended in 100 μL of FACS buffer (3% FBS in DPBS) with the primary antibody and incubated for 15 min at room temperature in the dark. Cells were washed twice with DPBS and resuspended in DPBS prior to flow cytometry analysis. All flow cytometry analyses were performed in FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Acquisition of the data was performed with the CellQuest software (Becton Dickinson) and a minimum of 10,000 events was collected for each sample. For data analysis, FCSalyzer Software (https://sourceforge.net/projects/fcsalyzer/) was used.

2.6. Immunocytochemistry

For intracellular staining, cells were fixed with 4% PFA for 20 min and blocked with 10% FBS and 0.1% Triton X-100 (Sigma-Aldrich) in DPBS at room temperature for 1 h. Cells were incubated with primary antibodies in staining solution (5% FBS and 0.1% Triton X-100 in DPBS) for 3 h at room temperature. Then, cells were washed twice with DPBS and incubated with secondary antibodies diluted in staining solution for 1 h at room temperature in the dark. For intracellular staining of hiPSCs, antibodies for OCT4 (1:150, Millipore) and SOX2 (1:200, R&D Systems) were used and goat anti-mouse IgG-AlexaFluor 546 (1:500, Thermo Fisher Scientific) was used as a secondary antibody. For neural differentiation, staining was performed with PAX6 (1:400, Covance), NESTIN (1:400, R&D Systems), Zo-1(1:100, Novex), SOX2 (1:200, R&D Systems), TUJ1 (1:1000, Covance), MAP2 (1:250, Sigma-Aldrich) and GFAP (1:200, Abcam). Secondary staining was performed with goat anti-mouse IgG-AlexaFluor 546 (1:500, Thermo Fisher Scientific), goat anti-rabbit IgG-AlexaFluor 546 (1:500, Thermo Fisher Scientific), goat anti-mouse IgG-AlexaFluor 488 (1:500, Thermo Fisher Scientific), goat anti-rabbit IgG-AlexaFluor 488 (1:500, Thermo Fisher Scientific) and goat anti-rat IgG-AlexaFluor 546 (1:500, Thermo Fisher Scientific). For

surface staining of hiPSCs, cells were incubated with primary antibodies in 3% BSA solution for 3 h at room temperature. Cells were washed twice with DPBS and incubated with secondary antibodies diluted in 3% BSA solution for 1 h at room temperature in the dark. For surface staining, cells were labelled with antibodies for TRA-1–60 (1:135, StemGent) and SSEA-4 (1:135, StemGent) with the secondary antibody goat anti-mouse IgM-AlexaFluor 546 (1:500, Thermo Fisher Scientific) or goat anti-mouse IgG-AlexaFluor 546 (1:500; Thermo Fisher Scientific), respectively. Afterwards, samples were washed twice with DPBS and cells nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 1.5 μg/mL, Sigma-Aldrich) for 5 min at room temperature in the dark. Finally, cells were washed three times with DPBS and observed under the fluorescence optical microscope (Leica DMI 3000B) and images were taken using a digital camera (Nikon DXM 1200).

2.7. RNA extraction and quantitative reverse transcription-polymerase chain

Total RNA was extracted using the High Pure RNA Isolation Kit (Roche, Switzerland). cDNA was synthesized from 1 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using the StepOne Plus real-time PCR system (Thermo Fisher Scientific). Taqman^{TM} Gene Expression Assays (Thermo Fisher Scientific) were selected for *OCT4, Nanog, SOX1, SOX17, T/BRACHYURY, FOXG1* and *GAPDH. PAX6, βIII-Tubulin, MAP2, TBR1, VGAT, VGLUT* and *GAPDH* analysis were performed using NZYSpeedy qPCR Green Master Mix, ROX plus (NZYTech, Portugal) (Supplementary Table S1). Reactions were run in triplicate and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control to normalize differences in total RNA levels in each sample. A threshold cycle (Ct) was observed in the exponential phase of amplification and quantification of relative expression levels was performed with the use of standard curves for target genes and endogenous control. Geometric means were used to calculate the ΔΔCt values and expressed as $2^{-\Delta\Delta Ct}$. The mean values from triplicate analysis were compared. The values obtained for "Matrigel group" or "Poly-L-ornithine/laminin group" were set as 1 and were used to calculate the fold difference in the target gene.

2.8. Differentiation potential assays

To confirm the pluripotency capacity of hiPSCs cultured on NPC ECM, directed differentiation of hiPSCs into cardiomyocytes, neural progenitors and endoderm progenitors were performed. Cardiac differentiation was performed through temporal modulation of the WNT signaling pathway. Therefore, hiPSCs previously cultured on NPC ECM and Matrigel substrates were plated on a 12-well Matrigel-coated tissue culture plate at a density of 1×10^5 cells/cm^2 using mTeSR1 medium. After reaching confluence, the medium was changed to RPMI/B27-ins (RPMI 1640 medium (Thermo Fisher Scientific), supplemented with 1x B-27 minus insulin (Thermo Fisher Scientific)) with 6 μmol/L of CHIR99021 (StemGent). After 24 h, the medium was changed to RPMI/B27-ins. At day 3, half-medium was changed to RPMI/B27-ins supplemented with 5 μmol/L of IWP-4 (StemGent). At day 5, the medium was changed to RPMI/B27-ins. At day 7, the medium was changed every two days to RPMI/B27 (RPMI 1640 medium supplemented with 1x B27 (Thermo Fisher Scientific)) until day 15. Cells were then fixed with 4% PFA and stained for cTNT (1:200, Abcam).

Neural induction was performed through the dual-SMAD inhibition protocol according to Section 2.2. After neural induction, cells were fixed and stained for PAX6 (1:400, Covance), NESTIN (1:400, R&D Systems) and TUJ1 (1:1000, Covance).

For endoderm induction, hiPSCs were plated on a 12-well Matrigel-coated tissue culture plate at a density of 1×10^5 cells/cm^2 using mTeSr1 medium. After reaching confluence, the medium was changed to RPMI/B27-ins medium supplemented with 6 μmol/L of CHIR99021 and 100 ng/mL of Activin-A (Peprotech). After 24 h, medium was changed to RPMI/B27-ins medium supplemented with Activin-A and was renewed everyday until day 3. Cells were then fixed and stained for SOX17 (1:500, Abcam).

2.8. Statistical analysis

Each experiment was conducted in triplicate. Data are expressed as the mean ± standard error of the mean (SEM). Statistical analysis of the data was performed using GraphPad Prism version 7 (GraphPad Software, USA). Statistical significance was determined by Student's *t*-test, comparing each condition with the control condition ("Matrigel group" or "Poly-L-ornithine/laminin group"). Differences were considered statistically significant at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1. Characterization of decellularized ECM derived from neural progenitor cells

The detailed process of the experimental protocol is presented in Fig. 1. Decellularized ECM was obtained from NPCs derived from hiPSCs upon 12 days under neural induction [16,36]. Confluent cultures of NPCs expressing PAX6 and Nestin were decellularized adapting protocols from similar reports [26,33,37,38] (Fig. 2A). Fig. 2A revealed that NPC culture was completely confluent before decellularization. After decellularization treatment, the ECM secreted by NPCs remained as a substrate, presenting a fibrillar architecture (Fig. 2A, Supplementary Figure S2). Before decellularization treatment, DAPI stained cultures showed well-defined cell nuclei while after decellularization the absence of DAPI staining confirmed that most of the cellular nuclei were disrupted (Fig. 2B, C). To confirm an efficient decellularization, DNA quantification was evaluated after the decellularization treatment. Results showed that NPC ECM contained 38.2 ± 2.4 ng DNA/mg dry weight and NPCs contained 846.7 ± 22.7 ng DNA/ mg dry weight (Fig. 2D). The DNA content of the dECM was significantly reduced after the decellularization process, as expected. Thus, the adapted decellularization protocol was efficient to obtain dECM derived from NPCs. Furthermore, NPC culture, before and after decellularization, was characterized for common ECM proteins. The results demonstrated that, after decellularization, the substrates were coated with a fibrillar network of ECM containing collagen type I and IV, laminin and fibronectin (Fig. 2C, Supplementary Figure S2). In fact, expression of these proteins was detected before and after decellularization, indicating that ECM protein composition is mostly preserved during decellularization treatment (Fig. 2B, C). Furthermore, the sGAGs and collagen contents present in the NPC ECM after decellularization were relatively high when compared to native cells, since the weight of the ECM was more diluted because there was no cellular material (Fig. 2F, E). These results indicated that the decellularization process did not cause severe compositional damages to the NPC ECM. Interestingly, we did not observe significant variability in sGAGs and collagen composition between NPC ECM derived from both cell lines used in this study (F002.1A.13 cell line from TCLab and Gibco® episomal hiPSC line) (Supplementary Figure S3).

3.2. NPC ECM as a new substrate for hiPSC culture and maintenance of pluripotency

To investigate if NPC ECM is able to promote hiPSC adhesion, these cells were seeded on two different substrates: Matrigel and NPC ECM (Fig. 1). hiPSCs were able to adhere on both substrates, forming colonies which grew throughout time (Fig. 3A). Fig. 3A showed how hiPSCs adhered and spread on the surface of NPC ECM. Interestingly, no obvious difference in hiPSC colonies morphology was observed between cells cultured on both substrates (Matrigel and NPC ECM). Moreover, after 5 days, hiPSCs were characterized according to their pluripotency

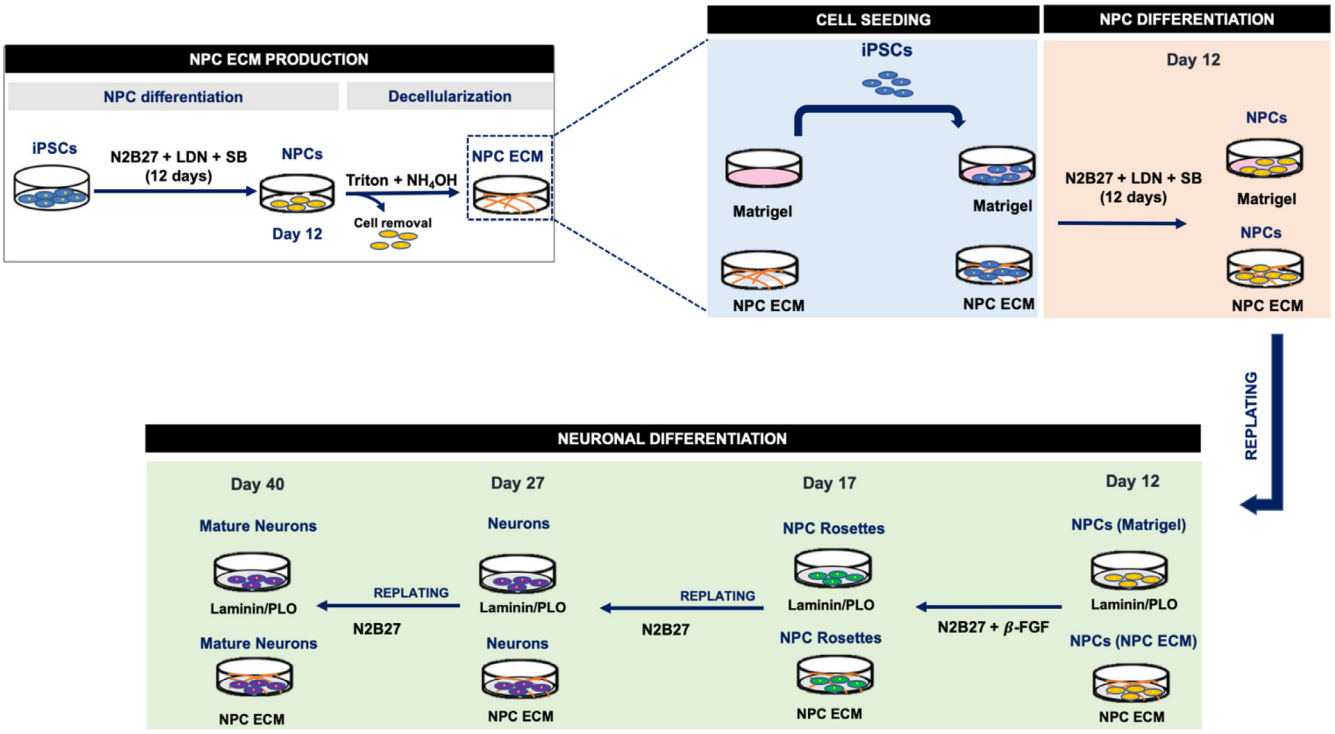

Fig. 1. A scheme of the experimental procedure used for decellularized NPC ECM production, human iPSC seeding and neural differentiation on the different substrates (Matrigel/Laminin/poly-L-ornithine (PLO) and NPC ECM) to obtain NPCs (after 12 days) and neurons (after 40 days of differentiation). N2B27 medium was used to differentiate iPSCs into NPCs and neurons. b-FGF: basic fibroblast growth factor; ECM: extracellular matrix; iPSCs: induced pluripotent stem cells; LDN: LDN193189; NPCs: neural progenitor cells; PLO: poly-L-ornithine; SB: SB431542.

markers (Fig. 3). Quantification of pluripotency marker expression was performed through flow cytometry and qRT-PCR analysis. Flow cytometry analysis confirmed that, after 5 days of expansion, expression of pluripotency markers OCT4, SOX2, TRA-1–60 and SSEA-4 was above 90% for hiPSCs cultured on both substrates: Matrigel and NPC ECM (Fig. 3B). Furthermore, immunostaining results demonstrated that hiPSCs cultured on both substrates (Matrigel and NPC ECM) were expressing pluripotency markers, such as OCT4, SOX2, TRA-1–60 and SSEA-4 (Fig. 3C). Before and after hiPSC expansion on both substrates, total RNA was extracted and the expression of pluripotency (*OCT4* and *NANOG*) and neural differentiation genes (*PAX6* and *SOX1*) was assessed (Fig. 3D). Expression of pluripotency and differentiation genes was not significantly different before and after expansion on both substrates. These results overall demonstrated that hiPSCs are able to adhere on dECM derived from NPCs and that, during expansion, NPC ECM did not compromise the pluripotency of the cells. Thus, NPC ECM may be used as an alternative substrate to Matrigel basement matrix, since similar results were obtained for NPC ECM and Matrigel substrate. Importantly, since NPC ECM is derived from neural committed cells, this decellularized cell-derived ECM did not induce hiPSCs to spontaneously differentiate into a neural lineage, since gene expression of *PAX6* and *SOX1* markers was not enhanced after 5 days of culture (Fig. 3D). In order to evaluate if the capacity of NPC ECM to support hiPSCs is maintained in long-term cultures, hiPSCs were consecutively replated into new NPC ECM for five passages (Fig. 4A). Flow cytometry for pluripotency markers and gene expression analysis of pluripotency and differentiation markers were evaluated. Results showed that, after five consecutive passages on NPC ECM substrate, expression of pluripotency markers OCT4, SOX2, TRA-160 and SSEA-4 was always above 90% (Fig. 4B, C). Furthermore, gene expression analysis revealed that no significant differences were observed when hiPSCs were replated into new NPC ECM substrate. Cells were tested for pluripotency (*OCT4* and *NANOG*) (Fig. 4D) markers and markers of cells from the three germ layers (*SOX1, T/BRACHYURY* and *SOX17*, representing ectoderm, mesoderm and endoderm, respectively) (Fig. 4E, F) and no significant differences were observed. Besides, assessment of the differentiation potential of the hiPSCs cultured on NPC ECM after five consecutive passages was evaluated by directed differentiation assays (Supplementary Figure S4). Overall, hiPSCs cultured on both substrates were able to differentiate in the three germ layers, staining for PAX6 and Nestin (ectoderm), cTNT (mesoderm) and SOX17 (endoderm), demonstrating that after five consecutive passages hiPSCs cultured on NPC ECM maintained their pluripotency (Supplementary Figure S4).

3.3. NPC ECM as a new platform for neural differentiation of hiPSCs

After demonstrating the suitability of NPC ECM as a substrate for hiPSC culture, we evaluated if it could be used as well as a substrate for neural differentiation of hiPSCs. Supplementary Figure S1 shows the experimental protocol used in this study for neural differentiation. hiPSCs were first seeded on Matrigel or NPC ECM and then differentiated into NPCs during 12 days. After this step, NPCs cultured on Matrigel were replated to poly-L-ornithine/laminin-coated well plates while NPCs previously cultured on NPC ECM substrate were replated to a new NPC ECM substrate for further neuronal differentiation (Supplementary Figure S1).

After 12 days of neural commitment, NPCs differentiated from hiPSCs cultured on both substrates (Matrigel and NPC ECM) showed the presence of the neural markers Nestin and Pax6 (Fig. 5A). As expected, NPCs downregulated the gene expression levels of pluripotency markers, such as *OCT4* and *Nanog* (Fig. 5B). In addition, differentiated NPCs on both substrates upregulated the gene expression levels of *PAX6, SOX1, FOXG1, βIII-Tubulin, MAP2* and *TBR1* (Fig. 5C, D). Interestingly, when hiPSCs were differentiated on NPC ECM for 12 days, gene expression levels of *PAX6, SOX1, βIII-Tubulin* and *TBR1* presented a statistically significant improvement compared to hiPSCs differentiated on Matrigel,

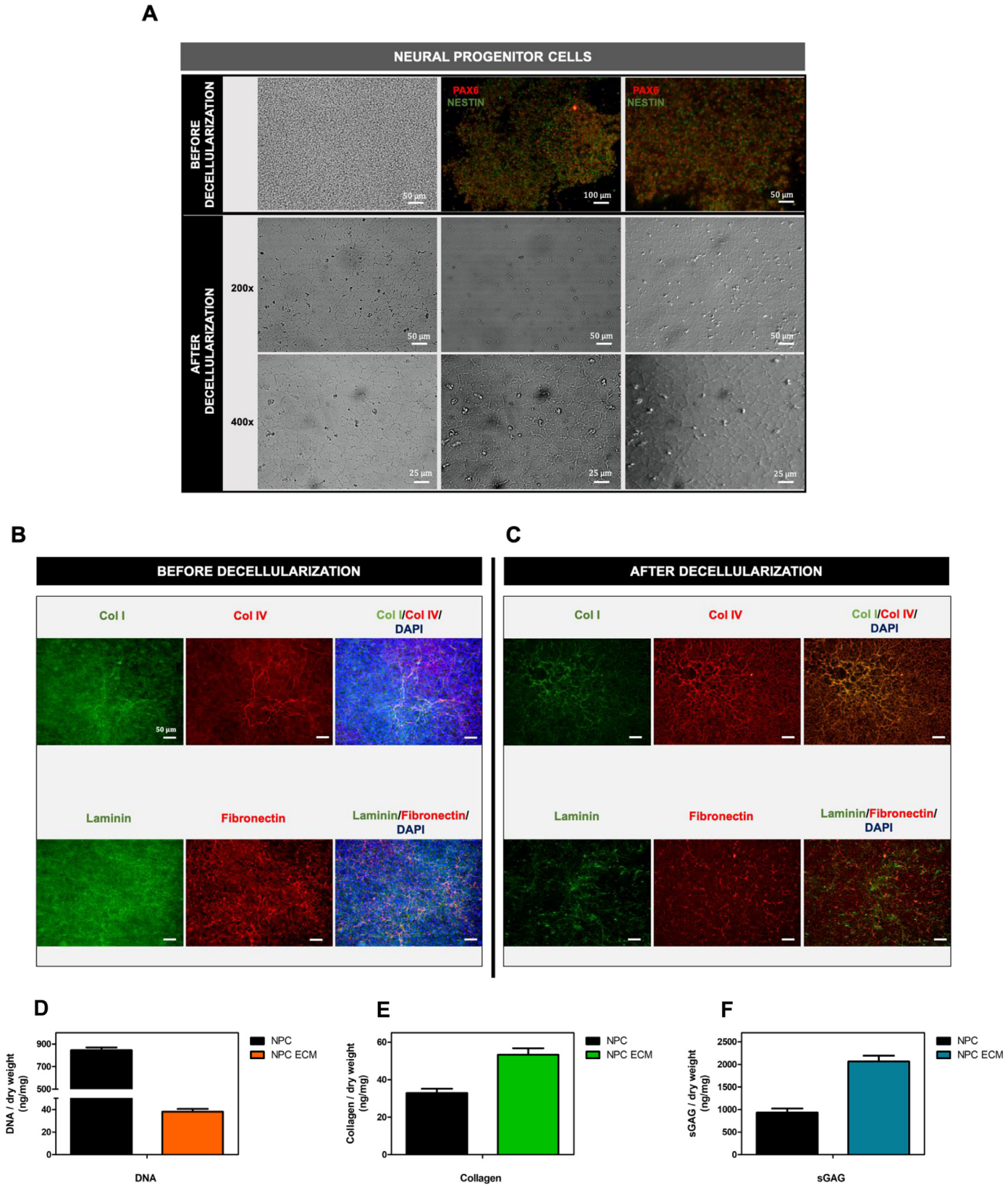

Fig. 2. Characterization of decellularized ECM derived from neural progenitor cells. **A)** Characterization of NPCs before and after decellularization. Before decellularization, NPCs derived from hiPSCs expressed PAX6 and Nestin. Decellularization treatment revealed the deposition of ECM with a fibrillar architecture. **B,C)** Immunofluorescence staining images of collagen I (Col I), collagen IV (Col IV), laminin and fibronectin before **(B)** and after **(C)** the decellularization protocol. DAPI staining revealed the absence of nuclei after decellularization. Scale bars, 50 μm. **D)** DNA content present in NPCs and decellularized ECM (NPC ECM). **E)** Content of collagen present in NPCs and NPC ECM. **F)** Quantification of sGAGs in NPCs and NPC ECM. Quantification was performed per mg of dry weight. DAPI: 4′,6-diamidino-2-phenylindole; ECM: extracellular matrix; hiPSCs: human induced pluripotent stem cells; NPCs: neural progenitor cells; sGAGs: sulfated glycosaminoglycans.

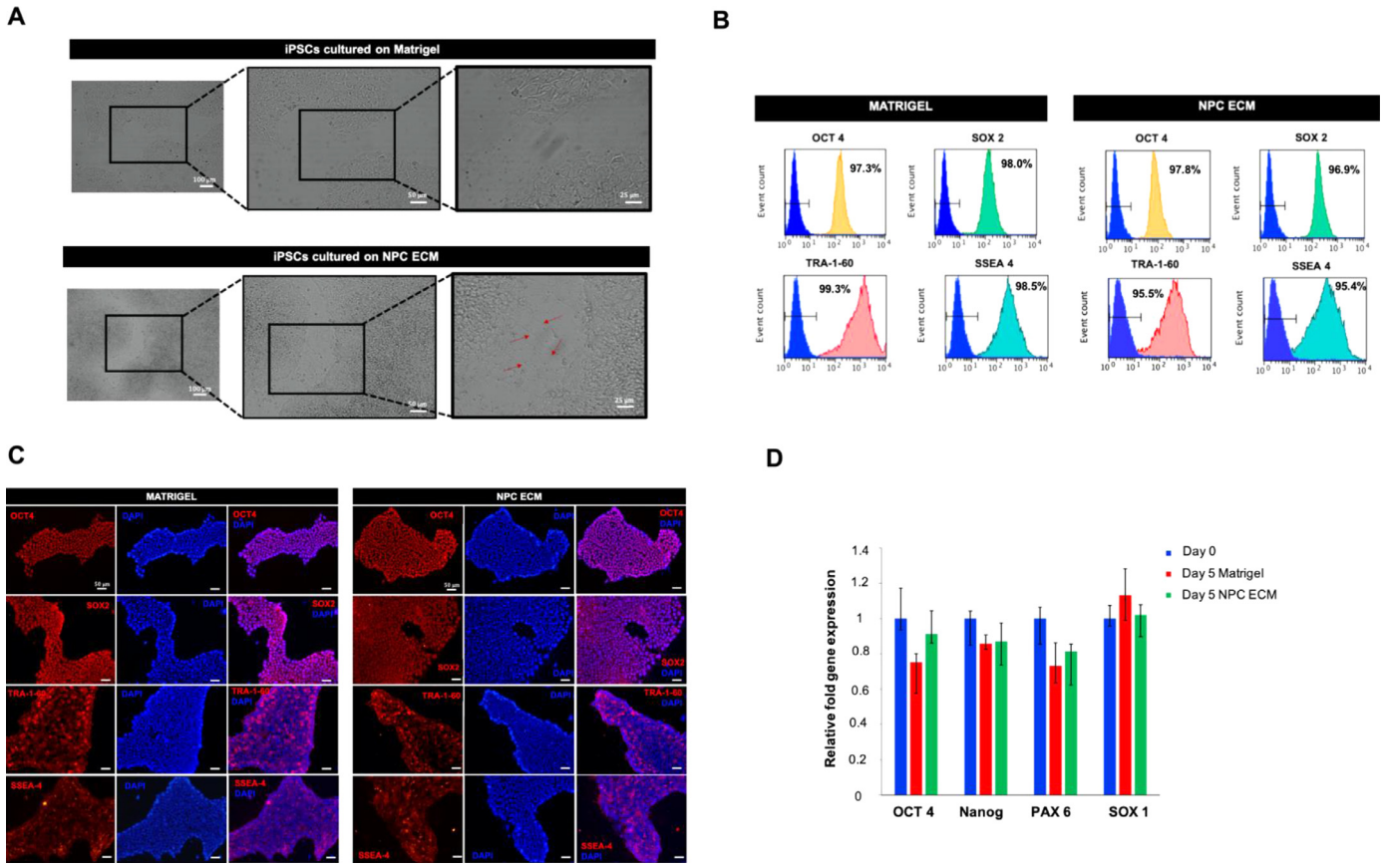

Fig. 3. Characterization of hiPSCs after expansion in NPC ECM substrate. **A)** hiPSCs colonies formed on Matrigel and NPC ECM substrate after 5 days. Red arrows show the presence of the ECM. **B)** Flow cytometry analysis of hiPSCs after 5 days of culture on Matrigel and NPC ECM. Cells were labelled for pluripotency markers, such as OCT4, SOX2, TRA-1–60 and SSEA-4. **C)** Immunocytochemistry staining for pluripotency markers OCT4, SOX2, TRA-1–60 and SSEA-4 of hiPSCs cultured on Matrigel and NPC ECM at day 5 of expansion. Scale bars, 50 μm. **D)** qRT-PCR analysis of hiPSCs at day 0 and after 5 days of expansion on Matrigel substrate and NPC ECM. Expression of pluripotency (OCT4 and NANOG) and neural (PAX6 and SOX1) markers was tested. ECM: extracellular matrix; hiPSCs: human induced pluripotent stem cells; NPCs: neural progenitor cells; qRT-PCR: quantitative reverse transcription-polymerase chain reaction.

demonstrating that NPC ECM enhanced the neural commitment of hiPSCs at this stage (Fig. 5C, D). Afterwards, NPCs cultured both on Matrigel and NPC ECM were replated to tissue culture plates coated with poly-L-ornithine/laminin or NPC ECM, respectively. After 2 days (at day 14), NPCs started to form rosette-like structures in both substrates and bFGF was added to promote proliferation (Supplementary Figure S1). At day 17, immunocytochemistry analysis revealed that NPCs differentiated onto NPC ECM were organized as neural rosette-like structures, containing progenitor cells expressing SOX2 and polarized expression of the tight junction marker ZO-1 in the apical side of the rosettes (Fig. 5E). After 17 days, rosette-like cells cultured on the different substrates (NPC ECM and poly-L-ornithine/laminin) were replated into new substrates: NPC ECM and poly-L-ornithine/laminin, respectively, for 10 additional days. At day 27 of neural differentiation, cells differentiated on both substrates started to express βIII-tubulin (TUJ1), an early neuronal marker (Fig. 5E). Furthermore, qRT-PCR analysis revealed that cells upregulated neuronal gene expression markers such as *βIII-Tubulin, MAP2* and *TBR1* (Fig. 5F). Interestingly, after 27 days of differentiation, results demonstrated a statistically significant improvement on neuronal gene expression levels, such as *βIII-Tubulin, MAP2* and *TBR1*, when cells were differentiated on NPC ECM instead of a poly-L-ornithine/laminin-coated substrate, as commonly performed in most neuronal differentiation protocols (Fig. 5F). Afterwards, we demonstrated that NPCs derived from hiPSCs were able to further differentiate into mature neurons when cultured on NPC ECM as a substrate. After 40 days of differentiation, the replated cells expressed the neuronal markers TUJ1 and MAP2 (Fig. 5G). Moreover, the presence of glial cells, namely astrocytes, was also detected by staining with glial fibrillary acidic protein (GFAP) (Fig. 5G). qRT-PCR analysis demonstrated that the expression of markers of NPC/NSC and early neurons (*βIII-Tubulin*), mature neurons (*MAP2*), Glutamatergic neurons (vesicular glutamate transporter, *VGLUT*) and GABAergic neurons (vesicular GABA transporter, *VGAT*) were upregulated when neurons were differentiated on both substrates. Remarkably, gene expression level of *MAP2* was statistically significantly higher on neurons differentiated on NPC ECM compared to neurons differentiated on poly-L-ornithine/laminin-coated plates (Fig. 5H). Additionally, the gene expression levels of *MAP2* increased after 40 days of neural differentiation, when compared to day 27, demonstrating the generation of a more mature neuronal population. Thus, these results demonstrate that NPC ECM is able to efficiently generate NPCs from hiPSCs and to differentiate them into mature neurons, presenting a statistically significant increase in the gene expression of *MAP2* compared to cells differentiated on poly-L-ornithine/laminin pre-coated plates.

4. Discussion

ECM provides different signals to cells, such as growth factors, bioactive molecules and biophysical cues, controlling cell behavior and response to microenvironment [39], being an essential element to control stem cell fate *in vitro*. Indeed, biophysical properties of ECM, such as elasticity, matrix pore size, architecture and topography, have been shown to play an important role in neural development and regener-

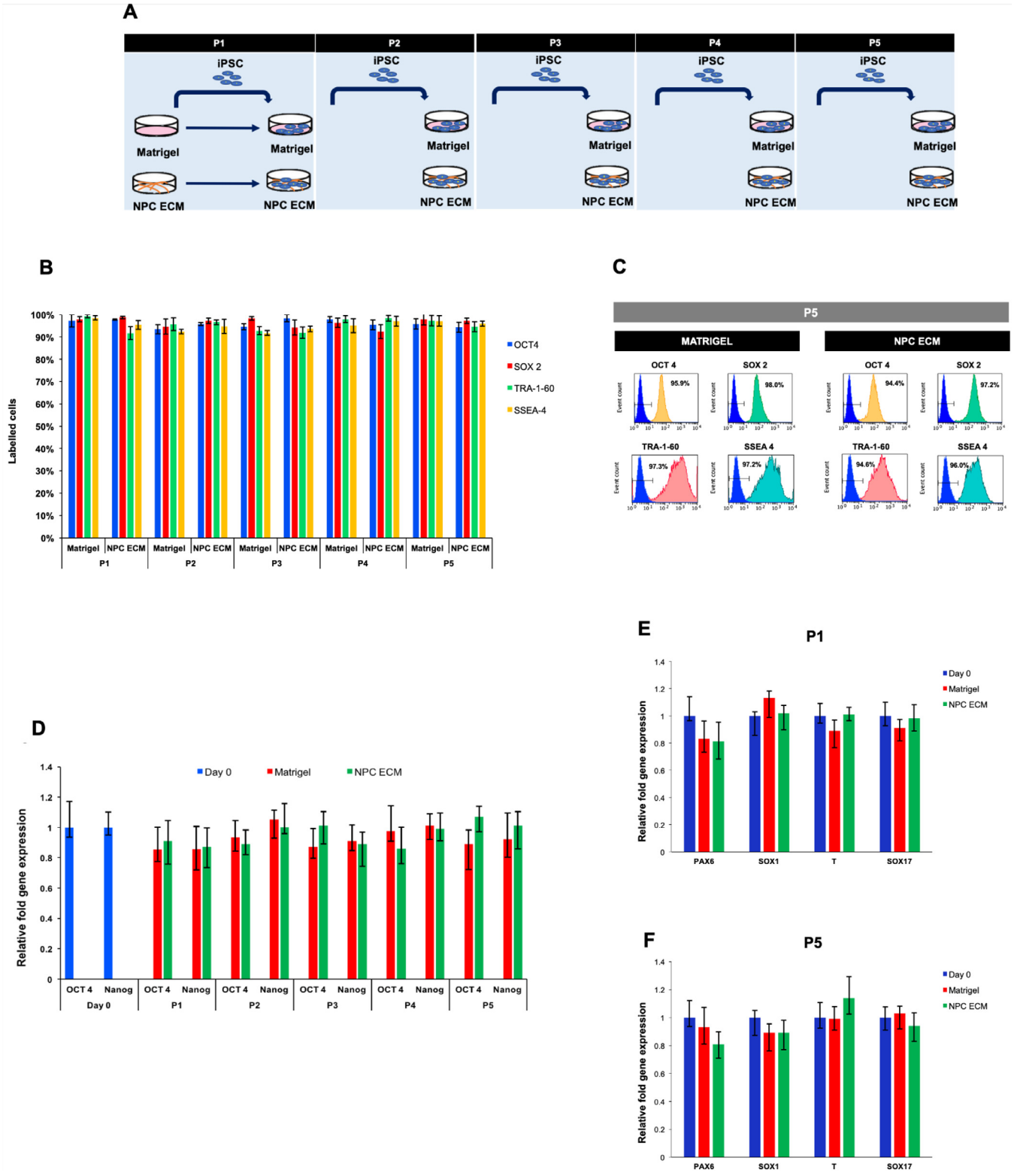

Fig. 4. Long-term culture of hiPSCs culture on two different substrates, Matrigel and NPC ECM, for five consecutive passages. **A)** Schematics of consecutive replating of hiPSCs cultured on Matrigel and NPC ECM during five passages. **B)** Flow cytometry analysis of hiPSCs expanded on Matrigel and NPC ECM after each passage (P1-P5). Cells were labelled for pluripotency markers, such as OCT4, SOX2, TRA-1–60 and SSEA-4. **C)** Immunophenotypic profiles of hiPSCs cultured on Matrigel and NPC ECM at passage 5. Flow cytometry histograms showed the expression of pluripotency markers, such as OCT4, SOX2, TRA-1–60 and SSEA-4. **D)** Analysis of pluripotency gene expression in hiPSCs cultured on Matrigel and NPC ECM after each passage (P1-P5). **E, F)** Gene expression analysis of markers from the three germ layers in hiPSCs cultured on Matrigel and NPC ECM after **(E)** passage 1 and **(F)** passage 5 (SOX1, T/BRACHYURY and SOX17, representing ectoderm, mesoderm and endoderm, respectively). ECM: extracellular matrix; hiPSCs: human induced pluripotent stem cells; NPCs: neural progenitor cells; P1: passage 1; P5: passage 5.

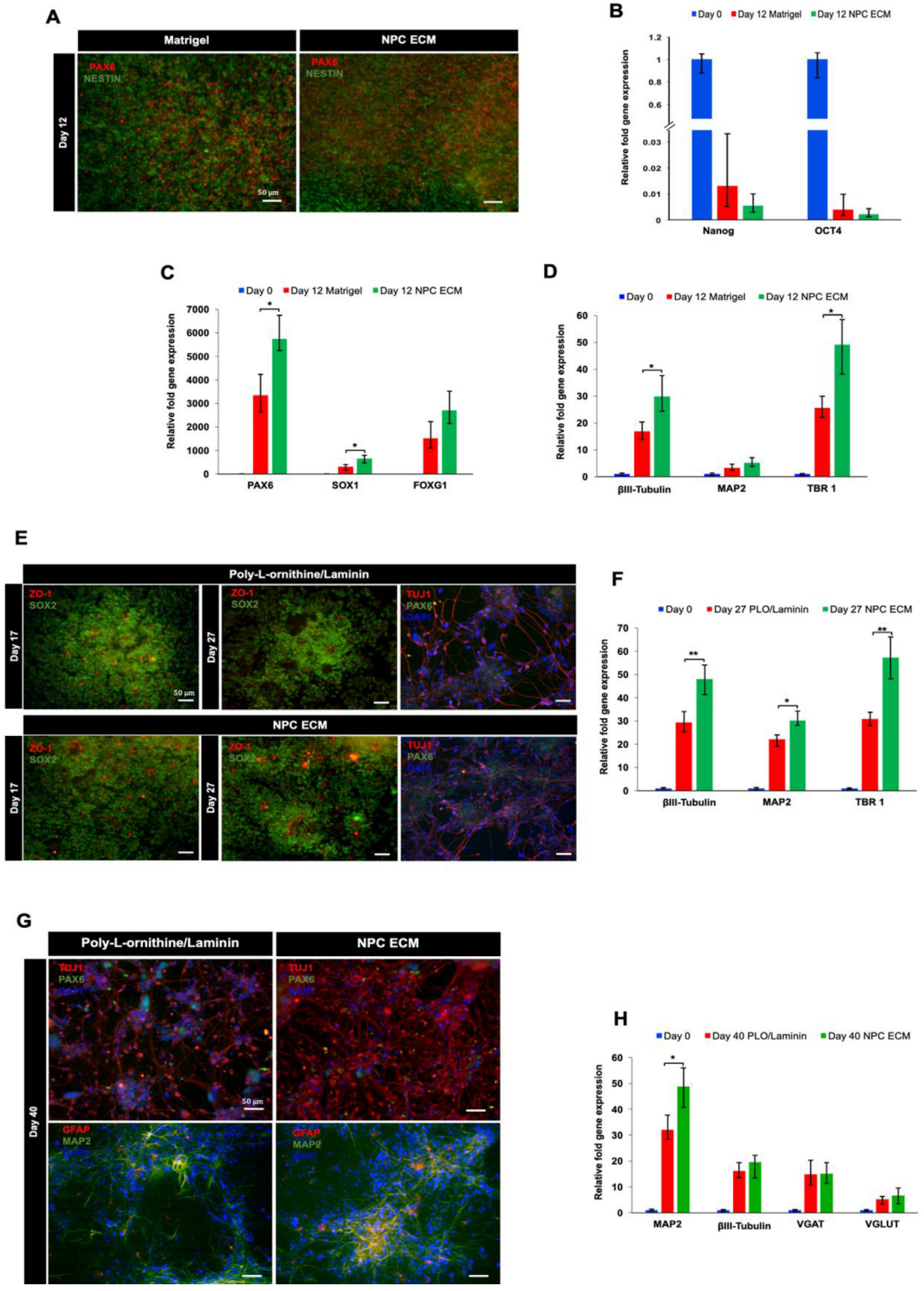

Fig. 5. Neural differentiation of hiPSCs in NPC ECM substrate. **A)** Immunocytochemistry staining of NPCs differentiated on Matrigel and NPC ECM after 12 days. PAX6 and Nestin are neural progenitor markers. Scale bars, 50 μm. **B)** Pluripotency gene expression analysis of NPCs after 12 days of neural commitment on Matrigel and NPC ECM. **C, D)** Neural gene expression analysis of NPCs after 12 days of neural commitment on Matrigel and NPC ECM. **E)** Immunocytochemistry straining of NPCs differentiated on PLO/laminin-coated wells and NPC ECM after 17 and 27 days of neural differentiation. Scale bars, 50 μm. **F)** qRT-PCR analysis of cells after 27 days of neural differentiation on PLO/laminin-coated wells and NPC ECM. **G)** Immunocytochemistry straining of cells differentiated on PLO/laminin-coated wells and NPC ECM after 40 days of neural differentiation. Scale bars, 50 μm. **F)** qRT-PCR analysis of cells after 40 days of neural differentiation PLO/laminin-coated wells and NPC ECM. ECM: extracellular matrix; hiPSCs: human induced pluripotent stem cells; NPCs: neural progenitor cells; PLO: poly-L-ornithine; qRT-PCR: quantitative reverse transcription-polymerase chain reaction.

ation, such as neurogenesis, neuronal cell migration and nerve repair [40,41,42].

The *in vitro* culture of hiPSCs has been regarded as a valuable model to study neural development as well as to understand disease mechanisms and, ultimately, to provide new therapies for neurologic diseases [43,44]. Matrigel, poly-L-ornithine and laminin have been used as substrates to culture and differentiate hiPSCs into the neural lineage but these substrates present some limitations. Matrigel is derived from murine tumors (Engelbreth-Holm-Swarm (EHS) mouse sarcoma), therefore its composition is not well-defined and thus can generate a source of variability. Additionally, the animal-derived nature of Matrigel also limits its clinical applicability, due to safety concerns. Other substrates typically used for hiPSC culture and differentiation, such as poly-L-ornithine, laminin or synthetic coatings, do not mimic the composition and architecture of native ECM [12,17], employing only individual proteins or polypeptides to provide cell binding motifs for cells [45,46]. From this perspective, decellularized cell-derived ECM appears as a promising alternative for hiPSC culture, since it provides a more physiologically relevant microenvironment which closely mimics the complexity of native ECM composition and biophysical cues [47,48]. Recently, the effect of decellularized cell-derived ECM has also been explored as a different avenue for cell differentiation and can be potentially used as a tool to modulate hiPSC differentiation. Different cell types have been used to generate decellularized cell-derived ECM [26,33,34,38,49]. We hypothesized that dECM derived from NPCs may offer a flexible approach not only acting as a new substrate to expand and differentiate hiPSCs, since it allows the deposition of molecules and growth factors, but also recreating a microenvironment closer to the neural niche, through its fibrillar architecture and composition.

In this work, we assessed the potential of decellularized NPC ECM substrate as a new platform for hiPSC expansion and neural differentiation, as an alternative substrate to Matrigel and poly-L-ornithine/laminin coatings. Therefore, Matrigel-coated wells were used as the control group for hiPSC expansion and laminin/poly-L-ornithine-coated wells were used as the control group for neural differentiation. hiPSCs were first differentiated into NPCs using a previously established protocol [16,36]. After 12 days of neural commitment, NPCs were embedded in secreted ECM deposition. To produce the decellularized NPC-derived ECM, cells were lysed and the resulting dECM was composed of a large number of interconnected filaments distributed into a network. Immunofluorescence staining and collagen/sGAGs quantification revealed that NPC-derived ECM was abundant with structural proteins, such as collagen I, collagen IV, laminin and fibronectin before and after decellularization, demonstrating that the decellularization protocol did not affect the presence of these ECM molecules. In the case of DNA quantification, the DNA content of the NPC-derived ECM was significantly reduced after the decellularization treatment, as expected. In fact, some criteria must be met to confirm the successful decellularization of tissues and cells, such as: decellularized tissues/cells must contain less than 50 ng dsDNA per mg ECM dry weight and no visible nuclear material should be present in the ECM analyzed by DAPI staining [50]. Results obtained showed that NPC ECM contained 38.2 ± 2.4 ng DNA/mg dry weight, which is according with the prerequisites [50]. Moreover, DAPI/Phalloidin staining also showed no evidence of cell nuclei.

ECM proteins play a crucial role in stem cell survival, proliferation and differentiation, being collagen the major structural protein in the ECM and responsible for mechanical properties of native tissues [35]. Regarding the neural niche, collagen and laminin are important components for cell anchorage, thus, influencing cell adhesion, migration, viability and cell-cell signaling [51]. Moreover, proteoglycans and laminins have been shown to regulate proliferation of neural progenitors/stem cells [19, 52] by modulating growth factors, such as fibroblast growth factor (FGF) signaling [53].

Interestingly, as observed by immunofluorescence stainings, flow cytometry and qRT-PCR analysis, NPC-derived ECM was able to maintain the pluripotency properties of hiPSCs after 5 days of culture. Long-term expansion of hiPSCs on NPC ECM was also demonstrated. After five consecutive passages on NPC ECM, the pluripotency capacity of these cells was not lost, as assessed by flow cytometry, immunocytochemistry assays, qRT-PCR analysis and directed differentiation assays. Hence, our results demonstrated that NPC ECM did not affect the pluripotency of hiPSCs and could be used as a substrate to expand these cells.

After observing that hiPSCs were able to grow on NPC ECM, while maintaining their pluripotent potential, neural differentiation of hiPSCs cultured on NPC ECM was evaluated. hiPSCs were firstly differentiated into NPCs, which were afterwards differentiated into neurons, culturing the cells on NPC ECM for up to 40 days under the conditions outlined in Supplementary Figure S1. After 12 days of neural commitment, NPCs were obtained from hiPSCs differentiated on both substrates: Matrigel and NPC-derived ECM. It was possible to observe that NPC ECM significantly increased mRNA expression levels of *PAX6, SOX1, βIII-Tubulin* and *TBR1* when NPCs were cultured on the cell-derived ECM in relation to the Matrigel control. *PAX6* has been reported to be expressed in an early phase of hESC neural differentiation and human embryonic brain development [54,55]. Moreover, the expression levels of *PAX6* and *SOX1* have been shown to be crucial for promoting neural differentiation, controlling the balance between cell self-renewal and neurogenesis [56,57], with increased *PAX6* and *SOX1* gene expression levels driving the system towards neurogenesis [56,57,58]. Thus the enhanced levels observed on cells cultured on NPC ECM compared to Matrigel suggest a more efficient neural differentiation of hiPSCs. Moreover, microtubules composed of tubulin proteins are essential for the generation, migration and differentiation of neurons [59] and therefore we believe that the statistically significant improvement of *βIII-Tubulin* gene expression levels on cells differentiated on NPC ECM substrate may also confirm the potential of this specific neural ECM to regulate stem cell fate. Afterwards, at day 27 of neural differentiation, cells differentiated on NPC ECM substrates presented a statistically significant upregulation of *MAP2, βIII-Tubulin* and *TBR1* gene expression levels compared to cells differentiated on poly-L-ornithine/laminin-coated wells. In fact, *MAP2* is a marker of neuronal differentiation and its upregulation on cells differentiated on NPC ECM may indicate the presence of a more mature cell population. Moreover, the statistically significant increase of gene expression levels of *βIII-Tubulin* and *TBR1* (intermediate neuronal markers) may also explain the generation of a more mature population of neurons. After 40 days of neuronal differentiation, neurons cultured on NPC ECM also presented significantly increased mRNA expression levels of *MAP2*, a mature neuronal marker, suggesting improved neural maturation compared to neurons differentiated on poly-L-ornithine/laminin-coated wells, the traditionally used substrate. In addition, the presence of glial cells was also detected by staining with GFAP when cells were differentiated on NPC ECM, demonstrating the potential of the cells for differentiation into other neural lineages.

In this study, we used two different cell lines and did not observe significant variability regarding NPC ECM production, such as sGAGs and collagen content. Moreover, we have observed the same benefit effect of NPC ECM derived from both cell lines on neural differentiation of hiPSCs.

The underlying mechanism responsible for the enhancement of neural differentiation on NPC ECM is not yet known but will be the subject of future investigations. Future studies should focus on further characterization of ECM, including proteomic analysis, such as mass-spectrometry-based strategies to evaluate the ECM derived from NPCs. However, we believe that ECM elements can slowly release bioactive factors into the microenvironment. We hypothesize that the enhanced neural differentiation of cells cultured on NPC ECM was due to the chemical and physical properties of the ECM and due to the complex interaction between cells and ECM, namely the rich variety of growth factors and proteins, such as proteoglycans and glycoproteins, present in its composition. For instance, some reports have shown that heparin sulfate proteoglycans present in cell-derived ECM can drive the differentiation of neural cells by promoting FGF and bone morphogenetic protein (BMP) signaling

[60]. Furthermore, immunocytochemistry analysis revealed that NPC ECM presented a collagen I fibrillar network as opposed to Matrigel that showed a different architecture with collagen I being organized dispersedly. Thus, we believe that the fibrillar architecture of the ECM can also influence cell behavior, facilitating cell adhesion and differentiation and closely mimicking the native niche. Besides, the topography of the ECM surface may also lead to the adsorption of more proteins and growth factors present in the differentiation medium and/or released by cells in the proximity, providing more bioactive factors important for neural differentiation.

Although the influence of the microenvironment on neural stem cell differentiation has been evaluated, very few studies have explored the potential of decellularized tissues or cells for neural differentiation of hiPSCs [47,61]. Aiming to recreate the neural niche, decellularized tissue-derived ECM from porcine brain has been used to culture neurons derived from hiPSCs and, compared to Matrigel, neurons cultured on dECM exhibited increased dendritic branching, suggesting improved neural maturation [62]. Regarding the effect of decellularized cell-derived ECM on neural differentiation, Yang and colleagues have reported that dECM derived from mouse embryonic fibroblasts promoted adhesion and differentiation of NPCs [35]. However, no studies were performed using decellularized cell-derived ECM derived from neural stem/progenitor cells, which may more closely mimic the neural niche. Interestingly, our results demonstrated that NPC ECM can be effective not only for neural differentiation but may facilitate the attachment and expansion of hiPSCs. Thus, in this study, we developed a new platform that allows to perform the complete workflow of hiPSC differentiation into neurons, including the previous expansion of undifferentiated hiPSCs and the whole differentiation into $MAP2^+$ neurons, by using decellularized cell-derived ECM derived from NPCs as a substrate. To our knowledge, these results describe the first study suggesting that NPC ECM can be used as a biomimetic material for neural differentiation, as an alternative to Matrigel and protein-coated wells, and could constitute an advantageous substrate to improve clinical outcomes in neural regeneration. Further studies are still required to unveil the complete composition of NPC ECM and to understand its mechanism on the modulation of the culture microenvironment. Nevertheless, we envision a potential use of NPC-derived ECM for several regenerative purposes, including stem cell expansion, pluripotency maintenance, neural differentiation and neural tissue engineering.

5. Conclusion

ECM has been reported to regulate stem cells fate but very few studies have used decellularized cell-derived ECM as a niche for neural differentiation of hiPSCs. Moreover, the mechanism of action of ECM in hiPSC self-renewal and differentiation is not fully understood. In this work, we have successfully demonstrated that decellularized cell-derived ECM produced by NPCs supports not only hiPSC expansion and maintenance of their pluripotency but also enhances neural differentiation. We propose that NPC ECM can be used as a new platform to culture hiPSCs *in vitro,* as an alternative to Matrigel, and as a new strategy for neural differentiation of hiPSCs. We believe that NPC ECM architecture and its diverse composition recreate closely the neural niche and promote neural differentiation. To our knowledge, these results describe the first study suggesting that NPC ECM can be used as a biomimetic substrate for neural differentiation and could be further used to develop new biomaterials for regenerative medicine applications that better mimic the *in vivo* niche.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.bbiosy.2022.100070.

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