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Alginate microcapsule as a 3D platform for the efficient differentiation of human embryonic stem cells to dopamine neurons

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Abstract Human embryonic stem cells (hESCs) are emerging as an attractive alternative source for cell replacement therapy since the cells can be expanded in culture indefinitely and differentiated into any cell types in the body. In order to optimize cell-to-cell interaction, cell proliferation and differentiation into specific lineages as well as tissue organization, it is important to provide a microenvironment for the hESCs which mimics the stem cell niche. One approach is to provide a three-dimensional (3D) environment such as encapsulation. We present an approach to culture and differentiate hESCs into midbrain dopamine (mdDA) neurons in a 3D microenvironment using alginate microcapsules for the first time. A detailed gene and protein expression analysis during neuronal differentiation showed an increased gene and protein expression of various specific DA neuronal markers, particularly tyrosine hydroxylase (TH) by >100 folds after 2 weeks and at least 50% higher expression after 4 weeks respectively, compared to cells differentiated under conventional two-dimensional (2D) platform. The encapsulated TH^+ cells co-expressed mdDA neuronal markers, forkhead box protein A-2 (FOXA2) and pituitary homeobox-3 (PITX3) after 4 weeks and secreted approximately 60 pg/ml/10^6 cells higher DA level when induced. We propose that the 3D platform facilitated an early onset of DA neuronal generation compared to that with conventional 2D system which also secretes more DA under potassium-induction. It is a very useful model to study the proliferation and directed differentiation of hESCs to various lineages, particularly to mdDA neurons. This 3D system also allows the separation of feeder cells from hESCs during the process of differentiation and also has potential for immune-isolation during transplantation studies.

Introduction

One of the foremost causes of human ailment late in life is neurodegenerative disease. PD is a prominent type of such disease, which presents with various motor and mental disabilities, and affects 1% of the human population above the age of 65 (Chiba et al., 2008). The main pathological cause of PD is the progressive degeneration of DA neurons which reside in the midbrain substantia nigra (SN) and project their axons to the basal ganglia striatum (Ma et al., 2011; Jiang et al., 2012; Hardy, 2010). Although many pharmacological and surgical treatments for PD are currently available, none provides a cure and all have risks of side effects (Cai et al., 2009; Snyder and Olanow, 2005). Hence, there has been extensive research in the field of DA neuron regenerative medicine as an alternative treatment for PD, with the focus being on stem cells (Cai et al., 2009; Jaderstad et al., 2010; Zhou et al., 2010).

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Stem cells, particularly, hESCs which are pluripotent and can proliferate indefinitely in an undifferentiated state *in vitro*, are arguably the most suitable cell source for developing cell replacement therapy for PD. Many studies have demonstrated differentiation of hESCs to DA neurons *in vitro* and *in vivo via* various strategies in 2D monolayer cultures either on extracellular matrices or stromal cell lines such as PA6 cells (Cho et al., 2008, 2011; Kawasaki et al., 2000; Schwartz et al., 2008). Some of the protocols include application of soluble factors into the culture condition such as sonic hedgehog (SHH), fibroblasts growth factor 8a (FGF8a), SPIE factors (combination of four factors; stromal cell-derived factor 1 (SDF-1), ephrin B1 (EFNB1); insulin-like growth factor 2 (IGF2); pleiotrophin (PTN)) (Vazin et al., 2009; Yamazoe et al., 2005) and compound C (C) (Zhou et al., 2010).

Although many protocols have been published for differentiating hESCs to the DA neurons, the research on scalable and controlled culture systems is still needed before the clinical application of hESCs becomes feasible (Kriks et al., 2011). The limitations of existing protocols include low mdDA neuronal generation efficiency and risk of contamination with other cell types which may cause undesirable side effects during transplantation (Wernig et al., 2008). Previous studies have demonstrated that hESCs can be propagated and differentiated under 3D environment involving alginate microcapsules (Chayosumrit et al., 2010; Dean et al., 2006). The entrapment of cells within alginate microcapsules allows high-density cell culture and exchange of nutrients, oxygen and stimuli across the membrane, whereas the cells are protected from external threats such as antibodies from the host (Dawson et al., 2008; Serra et al., 2011). It has been shown that the alginate encapsulation technique promotes better growth, differentiation, maturation or protein secretion of various cell types including mesenchymal stem cells, mouse ESCs and hESCs (Chayosumrit et al., 2010; Dean et al., 2006; Serra et al., 2011; Addae et al., 2012). Despite the success in many cell types, hESC differentiation studies using alginate encapsulation are limited (Chayosumrit et al., 2010; Addae et al., 2012; Bidarra et al., 2010; Raof et al., 2011; Fang et al., 2007).

In this study, we present an approach to differentiate hESCs to mdDA neurons in a 3D microenvironment using alginate microcapsules on PA6 cell co-culture, along with SHH and FGF8a as compared to that under 2D environment. To further optimize the differentiation, the influence of supplementation with SPIE factors and C to direct pluripotent hESCs to mdDA neurons was also investigated. We have demonstrated a proof-of-principle that the mdDA neurons can be obtained early from hESCs under a 3D system compared to that under a 2D environment and secrete more DA under potassium-induction.

Materials and methods

Cell culture

The hESC lines, Endeavor-1, 2 (Sidhu and Tuch, 2006) and MEL-1 (a gift from Monash University, Melbourne) were cultured and maintained in a pluripotent state on matrigel-coated (BD Sciences, California, US) culture plates with mTeSR®1 media (STEMCELL Technologies, Vancouver, CA). The culture medium was changed daily, with routine passaging of hESCs every 5 to 6 days by using 1 mg/ml dispase (STEMCELL Technologies, Vancouver, CA).

The mouse stromal cell line, PA6, was purchased from Riken BioResource Center Cell Bank (Tsukuba, Japan, http://www.brc.riken.jp/inf/en) and cultured in α-minimum essential media supplemented with 10% fetal bovine serum. The medium was changed every other day, with routine passaging of every 4 to 6 days by using trypsin (all from Invitrogen, Camarillo, CA).

Encapsulation of hESCs

Encapsulation of hESCs was carried out as described earlier from our laboratory (Chayosumrit et al., 2010; Dean et al., 2006). Prior to encapsulation, hESCs were washed with phosphate buffered saline (PBS; Invitrogen, Camarillo, CA) twice and treated with 10 μM of Rho-associated protein kinase inhibitor, also known as Y-27632 (RI; Merck, Darmstadt, Germany), supplemented in mTeSR®1 media for 2 h. hESCs were dissociated with accutase (Millipore, Temecula, CA) for 10 min and filtered through 40 μm cell strainers, followed by washing with 0.9% saline. A mixture of 1.25×10^6 cells/ml in 1.1% purified sodium alginate (high glucuronic acid content $\geq$60%, viscosity $>$200 mPa s and endotoxin $\leq$100 EU/g) (Pronova UP MVG, Novamatrix, Sandvika, Norway) was passed through an air-driven droplet generator (Nisco Engineering Inc., Zurich, Switzerland) at an air flow rate of 8 l/ml and a pressure of 100 kPa as described (Chayosumrit et al., 2010). The capsules were incubated in sterile 100 mM $CaCl_2$/10 mM HEPES (pH 7.4) for 7 min and washed twice with 0.9% saline solution, followed by further treatment with 10 μM of RI in mTeSR®1 media for 3 days prior to the differentiation.

DA neuronal differentiation under 3D environment (encapsulation)

PA6 cells were seeded at 1.0×10^5 cells per cm^2 in 0.1% gelatin-coated T75 flask and the ratio between PA6 cells and hESCs was 1.0×10^5 PA6 cells per 1.2×10^6 hESCs. After 24 h, PA6 culture medium was replaced with differentiation medium which consists of Glasgow minimum essential medium supplemented with 10% knockout serum replacement, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 0.1 mM β-mercaptoethanol (all from Invitrogen, Camarillo, CA). The RI-treated encapsulated hESCs were transferred to the PA6 cell culture flask to initiate differentiation on the next day. The medium was changed on day 4 and every other day thereafter. The hESCs were allowed to differentiate for 21 days, followed by supplementation with FGF8a (100 ng/ml) and SHH (100 ng/ml) for additional 7 days (all from R&D systems, Minneapolis, MN) (Fig. 1).

DA neuronal differentiation under 2D environment

To observe the difference of generating mdDA neurons between 3D and conventional 2D differentiation systems, PA6 cell co-culturing with cell culture insert differentiation protocol was followed (Vazin et al., 2008). After pre-treatment with RI for 2 h, the accutase treated single cells were filtered through 40 μm cell strainers. The cells were seeded at 3.0×10^6 cells per well onto matrigel-coated 12-well cell culture plate,

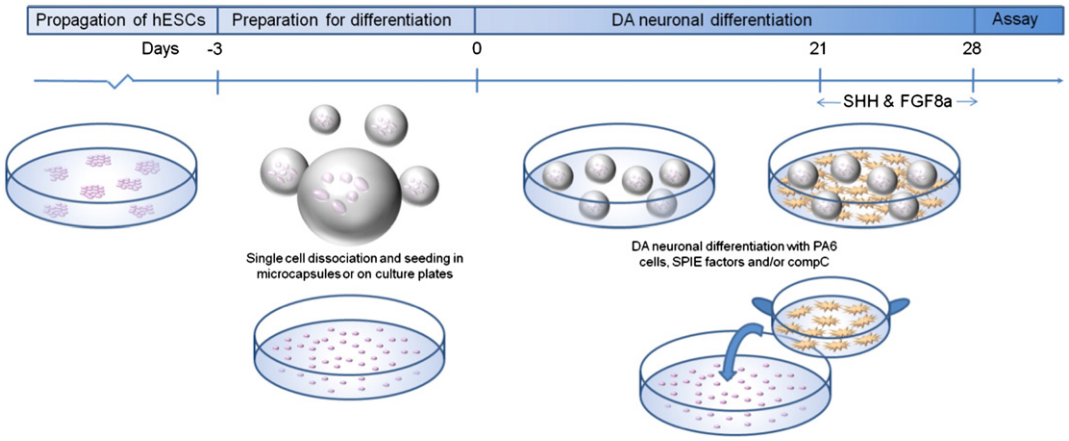

Figure 1 Overview of DA neuronal differentiation. The dissociated RI-treated cells were either encapsulated or seeded onto matrigel-coated dish and treated with RI for 3 days prior to differentiation. The prepared hESC were differentiated on PA6 cells or with SPIE factors and/or C for 28 days.

followed by further treatment with RI for 3 days. During the period of RI treatment, PA6 cells were seeded at 4.0×10^4 cells per 12-well cell culture insert (Millipore, Temecula, CA). After 24 h, PA6 culture medium was replaced with differentiation medium as described above. The medium of RI-treated hESCs was replaced with PA6 cell conditioned-differentiation medium along with the PA6 cells seeded cell culture inserts. The differentiation was carried out for 21 days, followed by supplementation of FGF8a (100 ng/ml) and SHH (100 ng/ml) for additional 7 days in the same manner as in 3D differentiation system (Fig. 1).

DA differentiation with SPIE factors and C

To further optimize the DA differentiation under 3D platform protocol by increasing the rate of DA neuron production and reducing the usage of xenogens, the effects of SPIE factors (SDF-1; 100 ng/ml, PTN; 100 ng/ml, IGF2; 100 ng/ml, insulin-like growth factor binding protein-4 (IGFBP4); 500 ng/ml, and EFNB1; 200 ng/ml; all from R&D systems, Minneapolis, MN) (Vazin et al., 2009) and C (10 μM; Merck Chemicals, Darmstadt, Germany) (Zhou et al., 2010) were investigated. After the encapsulation and post-treatment with RI as described from above, the cells were cultured in differentiation media supplemented with heparin (100 μg/ml; Sigma-Aldrich, St. Louis, MO), and SPIE factors with or without C for 21 days. The two morphogens FGF8a (100 ng/ml) and SHH (100 ng/ml) were supplemented in the differentiation media for additional 7 days in the same manner as in the 3D differentiation system with the change of media every third day (Fig. 1).

Decapsulation of cells for further analysis

The alginate microcapsules can be depolymerized (decapsulated) in the presence of a chelating agent ethylenediaminetetraacetic acid (EDTA) (Chayosumrit et al., 2010). Cells were retrieved from the capsules by incubating in decapsulating solution (50 mM EDTA and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in PBS) for 4–5 min at room temperature. Decapsulated cells were collected and analyzed for gene and protein expression or cultured onto a matrigel-coated culture plate with a density of approximately $1.0–2.0 \times 10^5$ cells per 10 cm^2 for functional and immunofluorescent staining analyses.

Immunofluorescent staining

Expression of stem cell and neuronal markers was examined by immunofluorescent staining. The cells were fixed with 4% paraformaldehyde for 20 min, permeablized with 0.2% Triton X-100 (all from Sigma-Aldrich, St. Louis, MO) in PBS for 10 min and blocked with 10% donkey serum (Millipore, Temecula, CA) in PBS for 1 h. The cells were then incubated with primary antibodies overnight at 4 °C, followed by incubation with appropriate secondary antibodies for 30–45 min at room temperature. Nuclei were then stained using Prolong gold antifade reagent with DAPI (Invitrogen, Camarillo, CA).

The primary antibodies used were as follows: rat anti-octamer-4 (OCT4) (1:300, R&D systems, Minneapolis, MN), rabbit anti-paired box gene-6 (PAX6) (1:50, Santa Cruz Biotechnologies, Santa Cruz, CA), mouse anti-neurogenin-2 (NGN2) (1:100, R&D systems, Minneapolis, MN), mouse anti-NESTIN (1:1000, Millipore, Temecula, CA), rabbit anti-tubulin βIII (TUBB3) (1:1000, Covance, New Jersey, US), mouse anti-nuclear related receptor-1 (NURR1) (1:100, Abnova, Taipei, Taiwan), sheep anti-TH (1:100, Abcam, Cambridge, England), goat anti-LIM LIM homeobox transcription factor 1, α (LMX1A) (1:50, Santa Cruz Biotechnologies, Santa Cruz, CA), mouse anti-FOXA2 (1:100, Abnova,Taipei, Taiwan) and mouse anti-PITX3 (1:100, AbD Serotec, Kidlington, UK). Secondary antibodies (1:500) used were: Alexa fluor® 488 donkey anti-rabbit IgG (H + L) antibody, Alexa fluor® 594 donkey anti-rabbit IgG (H + L) antibody, Alexa fluor® 488 donkey anti-goat IgG (H + L) antibody, Alexa fluor® 488 donkey anti-mouse IgG (H + L) antibody, Alexa fluor® 594 donkey anti-mouse IgG (H + L) antibody and Alexa fluor® 594 donkey anti-sheep IgG (H + L) (all from Invitrogen, Camarillo, CA).

Quantitative polymerase chain reaction (PCR) analysis

Total RNA was extracted from differentiated, undifferentiated hESCs and human fetal brain using Illustra RNAspin Mini Kit (GE Healthcare, Piscataway, NJ). Standard reverse transcription was performed using SuperScript III First-Strand Synthesis System and Oligo (dT) primers (Invitrogen, Camarillo, CA). Real-time PCR analysis was carried out on the CFX96 Real-Time PCR system using the SsoFast EvaGreen Supermix (all from Biorad, Benecia, CA). The PCR reaction consisted of 5 μl of SsoFast EvaGreen Supermix, 0.5 μl of 500 nM forward and reverse primers, 2.5 μl of milliQ water, and 2 μl of diluted template cDNA (5 ng/reaction) in a total volume of 10 μl. Initial enzyme activation was performed at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, and primer annealing/extension at 60 °C for 10 s. Melting curve analysis was performed at 65 °C for 5 s and 95 °C for plate read. Relative fold changes were calculated using Pffafl's calculations based on the ΔΔCt method (Pfaffl, 2001). All genes of interest were normalized to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and samples were in general, relatively compared to a zero time point or to control cell line, which was always set as 1 arbitrary unit, unless, otherwise indicated.

Western blot analysis

The differentiated and non-differentiated hESCs were lysed with radio immune precipitation assay (RIPA) buffer supplemented with cocktail of protease inhibitor (all from Sigma-Aldrich, St. Louis, MO). The proteins were purified by centrifugal filter units (Millipore, Temecula, CA) and quantified by bicinchoninic acid (BCA) protein assay (Thermo Fisher, Beverly, MA). 40 μg of protein was separated on a 10% precast polyacrylamide gel (Biorad, Benecia, CA) at 90 V for 2 h and transferred to a nitrocellulose membrane (Amersham Biosciences, Sunnyvale, CA) at 300 mA for 45 min. The membrane was blocked for 1 h and probed with primary antibodies for overnight at 4 °C. This was followed by incubation with appropriate secondary antibodies for 1 h at room temperature. Western blotting detection reagents (Millipore, Temecula, CA) were added to the membrane for 1 min. The membrane was then exposed to the x-ray film (Radincon, Dee Why, Australia), developed and fixed in the dark.

The primary antibodies used were rabbit anti-β-ACTIN (1:5000, Santa Cruz Biotechnologies, Santa Cruz, CA), rabbit anti-PAX6 (1:2000, Abcam, Cambridge, England) and rabbit anti-TH (1:1000, Millipore, Temecula, CA). The secondary antibody used was goat anti-rabbit IgG, (H + L) HRP conjugate (1:3000, Millipore, Temecula, CA). The relative expression of each protein was normalized against the housekeeping protein, β-ACTIN. After normalization, samples were in general, relatively compared to a zero time point or to control cell line, which was always set as 1 arbitrary unit, unless, otherwise indicated.

Enzyme-linked immunosorbent assay (ELISA) analysis

The decapsulated cells after seeding were analyzed for DA secretion. The differentiating hESCs under 3D platform were decapsulated at days 14 and 28. Similarly, the cells that were differentiated in conventional 2D environment were seeded at days 14 and 28. After 3–4 days, the medium was removed and replaced with Ca^{2+} containing Hank's balanced salt solution (Ca^{2+}/HBSS; Invitrogen, Camarillo, CA) and incubated at 37 °C for 10 min, followed by incubation with 56 mM KCl supplemented in Ca^{2+}/HBSS at 37 °C for 30 min. The cell culture supernatant was collected and stabilized at a final concentration of 150 mM H_3PO_4 and 50 mM diethylene–triamine–pentaacetate (DTPA). The sample was immediately frozen in liquid nitrogen and stored at −80 °C until assay. Prior to assay, the sample was thawed and centrifuged at 16,700 ×*g* at 4 °C for 12 min. The supernatant was collected and DA level was analyzed with high-sensitive DA ELISA kit (Labor Diagnostika Nord, Nordhorn, Germany). DA concentration was quantified by comparison to known standard dilutions and well to well variation was adjusted by counting the total number of cells per well.

Statistical analyses

All differentiation experiments were performed in 3 independent biological replicates for each hESC line, Endeavor-1, 2 and MEL-1. The data were obtained from technical duplicates for each sample and significance was calculated from the 3 biological replicates using Student's *t*-test in Microsoft Excel, unless otherwise indicated; P-values were <0.05 (*), 0.005 (**), 0.0005 (***), and 0.00005 (****). All graph values were plotted as mean ± SEM using GraphPad Prism 5 software.

Results

Encapsulation of hESCs as a 3D culture model

The diameter of alginate microcapsules remained 500 ± 100 μm with cell viability of >80 ± 5% when encapsulated (Sidhu et al., 2012). The number of cells within the capsule was estimated by calculating the total number of cells divided by the total number of capsules per run, resulting in approximately 5.0×10^4 cells per capsule (Sidhu et al., 2012). To sustain the cell viability of encapsulated hESCs prior to DA neuronal differentiation, the RI treatment of hESCs before and briefly after encapsulation was employed and found to be essential (Chayosumrit et al., 2010; Sidhu et al., 2012). During the process of differentiation, the single cell suspension within the capsules formed clusters or EBs with gradual increase of its size (Fig. 2). A minimum number of hESCs required for encapsulation were optimized to prevent bursting of capsules.

Differentiation of encapsulated hESCs by co-culture with PA6 cells

The microcapsules *via* encapsulation acted as a physical barrier preventing direct contact between the differentiating hESCs and PA6 cells. To verify whether the spherical shaped EBs express neuronal morphology and mdDA neuronal markers, the EBs were decapsulated each week and seeded

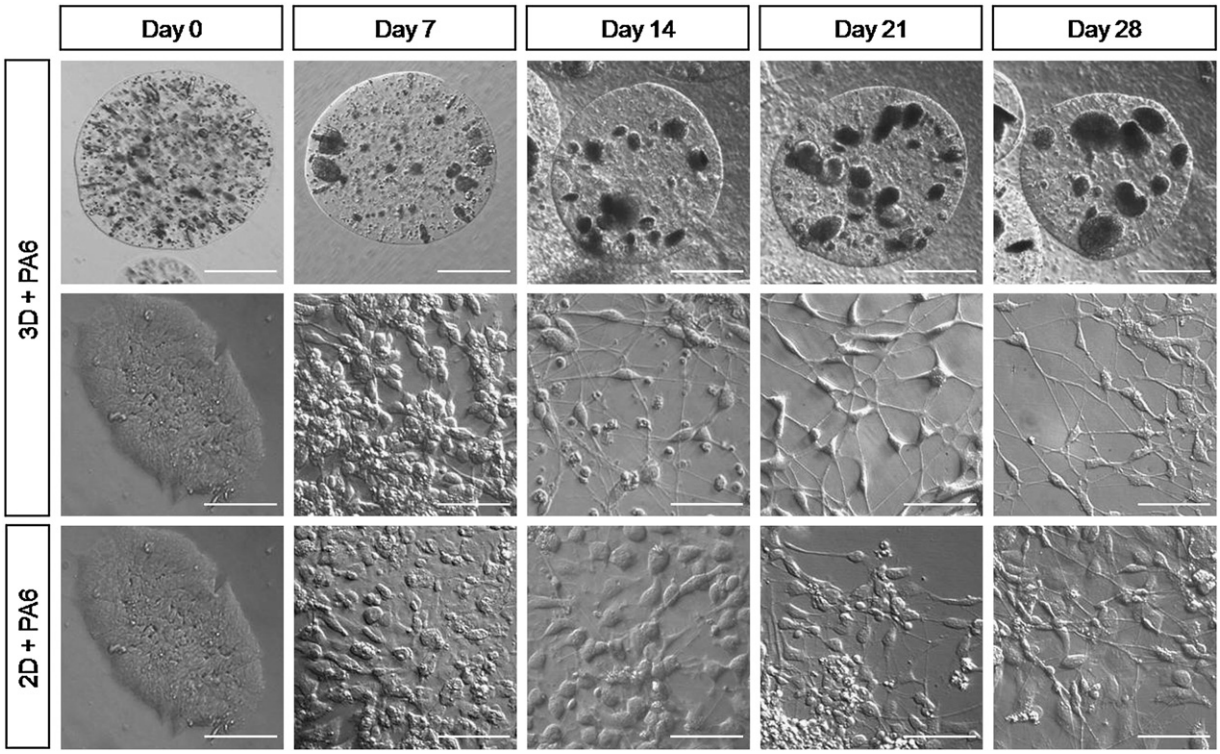

Figure 2 Morphology of 3D and 2D differentiated hESCs with PA6 cells. Single-cell dissociated hESCs were encapsulated and differentiated in 3D manner for 28 days (top panel). The differentiated cells under 3D system were decapsulated at each week, seeded and cultured for 4 days prior to observation (middle panel). Similarly, the differentiated hESCs under 2D system were collected, seeded and cultured on matrigel-coated culture plates for 4 days (bottom panel). Top panel scale bar = 200 μm. Middle and bottom panel scale bar = 50 μm.

onto matrigel-coated culture plates for 3–4 days. The seeded cells showed progressive neuronal differentiation with extensive neurite formations (Fig. 3A). After 7 days of differentiation, the cells were negative for pluripotent markers; OCT4 and NANOG (data not shown) but positive for NP marker; PAX6, and early neuronal marker; NESTIN (Fig. 3A). At day 14, cells began to show more defined neuron-like cells which expressed early mdDA neuronal marker; NGN2 and mature neuronal marker; TUBB3 (Figs. 2 and 3A). After 21 days, the cells were positive for mature DA neuronal marker; TH which co-expressed early DA neuronal markers; NURR1 and LMX1A. After 28 days of differentiation, TH^+ cells co-expressed late mdDA neuronal markers; FOXA2 and PITX3 (Fig. 3A). The percentage of TH^+ cells after 28 days of differentiation was estimated to be approximately 93 ± 3% (Fig. 5B).

To study differential gene expressions during differentiation, RNA samples were taken at certain time points and analyzed by qPCR. The analysis showed down-regulation of pluripotent marker; *OCT4* after 7 days, while neuronal markers; *PAX6* (>1000 fold) and *NGN2* (>1000 fold), *TH* (>1000 fold) and *LMX1B* (>100 fold) were up-regulated after 7 days (Fig. 4A). However, 3D differentiated hESCs after 28 days showed lower *TH* and *LMX1B* gene expression levels when compared to human fetal brain. Corresponding to these data, western blot analysis showed up-regulation of PAX6 (>100 fold) at day 7, followed by down-regulation after day 14 while TH (>100 fold) expression steadily up-regulated after day 7 (Fig. 4B).

Comparison of derived DA neurons under 3D *vs* 2D systems

To maintain the variability of indirect contact with PA6 cells during differentiation, cell culture inserts were used. In comparison to the 3D system, hESCs differentiated under the 2D system showed a slower progression towards neuronal differentiation with less extensive neurite formations (Fig. 2). The cells that were differentiated under the 2D environment showed negative expressions for pluripotent markers; OCT4 and NANOG (data not shown). However, the neuronal markers; PAX6, NESTIN, NGN2 and TUBB3 at day 7 were also negative but only showed the expressions after day 14 (Fig. 3). After 28 days, the cells were positive for TH (approximately 52 ± 5%) and co-expressed NURR1 and LMX1A (Fig. 3B). Interestingly, the TH^+ cells co-expressed FOXA2 and PITX3 after extended 35 days of differentiation (Fig. 3B).

In correspondence to the data, qPCR analysis showed lower level of expressions in all of the neuronal markers above in comparison to differentiated cells under 3D platform and human fetal brain, particularly *TH* level, which showed significant ($P < 0.05$) difference between the two systems

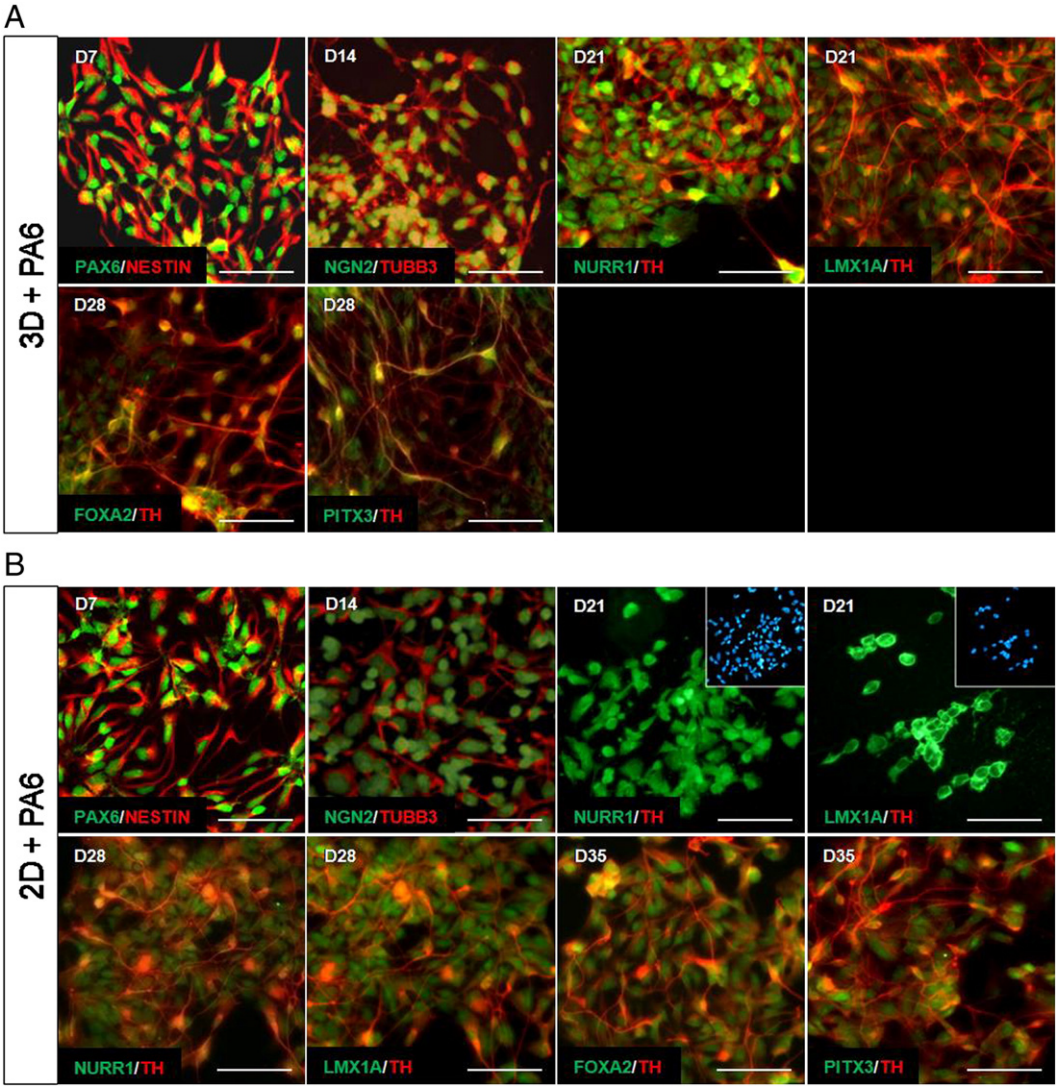

Figure 3 Immunofluorescent analysis of 3D + PA6 *vs* 2D + PA6. The cells that were differentiated under 3D platform with PA6 cells were decapsulated, seeded and cultured on matrigel-coated culture plates for 4 days prior to analysis (A). Similarly, hESC under 2D manner with PA6 cells were collected, seeded and cultured on matrigel-coated cultured plates for 4 days prior to analysis (B). The images represent the onset of DA neuronal markers. Scale bar = 50 μm.

(Fig. 4A). Western blot data analysis also revealed that TH expression level was significantly ($P < 0.05$) much lower than that observed under the 3D platform while maintaining a significantly ($P < 0.05$) higher level of PAX6 expression from days 14 to 28 (Fig. 4B).

The direct comparison of gene and protein expression analyses revealed that the differentiated hESCs under the 3D environment expressed the mature DA neuronal markers earlier than the cells that were cultured under the 2D system (Figs. 4A and B). In addition to this, the 3D system promoted down-regulation trend of PAX6 after 7 days, while the hESCs under the 2D environment maintained high expression level after 14 days (Fig. 4B). This indicates that the 3D differentiation system has the capacity to drive the pluripotent hESCs to mature neuronal lineage faster than the cells under the 2D environment that has a slower rate of differentiating the cells into mature neurons. Concurrent with these data, TH marker was highly expressed in hESCs under the 3D environment than the 2D system throughout the differentiation process (>100 fold after 2 weeks) (Fig. 4A). Also, we have shown that the 3D differentiated cells depicted higher expression of early and late mdDA neuronal markers; *NGN2*, *LMX1B*, in comparison to the 2D environment (Fig. 4A).

Further optimization of 3D system for future therapeutics under feeder-free environment

To further optimize the 3D differentiation protocol by increasing the production of DA neurons and reducing the usage of xenogens in the culture system, PA6 cells were replaced with SPIE factors and with or without C. After 7 days of differentiation, the decapsulated and seeded cells showed distinctive neuronal morphology which is an early stage of differentiation. Similarly with hESCs differentiated under 3D platform with PA6 cells, both samples were

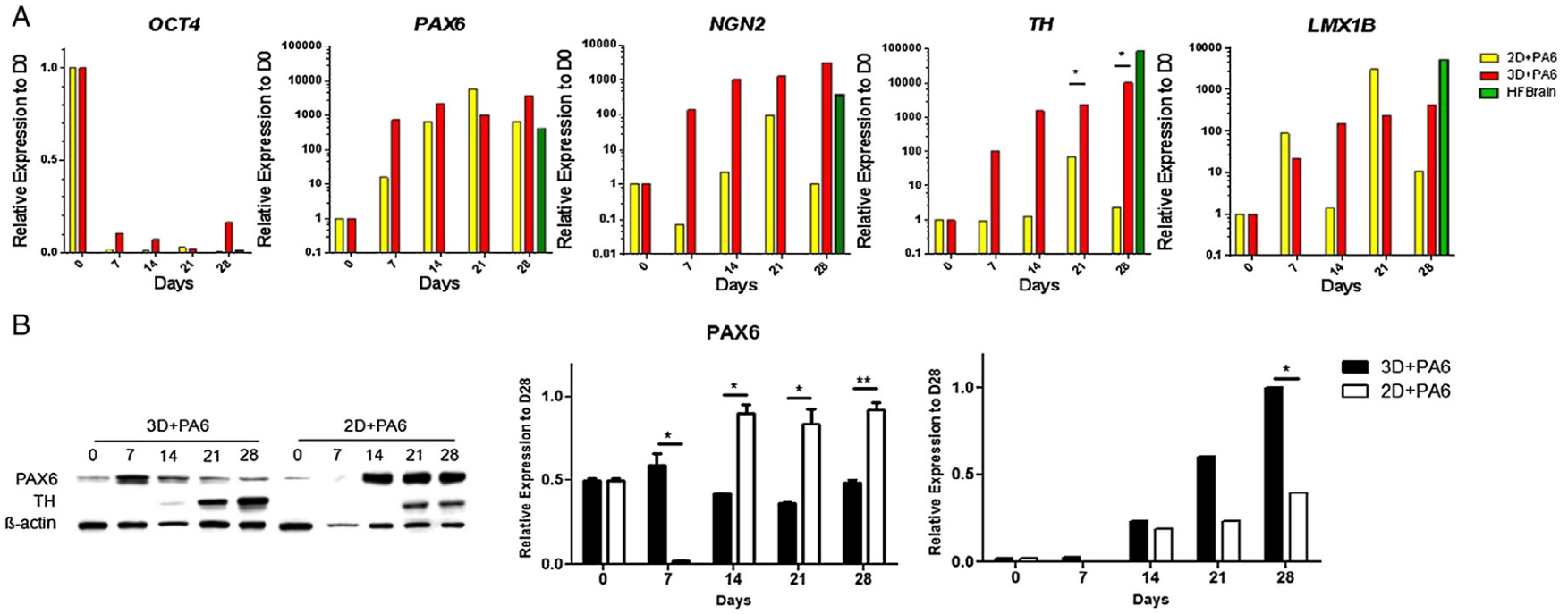

Figure 4 Gene and protein expression analysis of 3D + PA6 *vs* 2D + PA6. RNA samples of 3D + PA6 *vs* 2D + PA6 (A) were isolated at different time points for qPCR analysis (including RNA sample from human fetal brain as a positive control). Protein samples of 3D + PA6 *vs* 2D + PA6 (B) were isolated at different time points for western blot analysis, showing immunoblot bands and their representative graphs. Data presented as mean ± S.D. (biological replicates = 3 of each hESC line), Student's *t*-test, unpaired, two-tailed. The results were considered significant when P-values were <0.05 (*), 0.005 (**), 0.0005 (***), and 0.00005 (****). *Abbreviation* — HFBrain: human fetal brain.

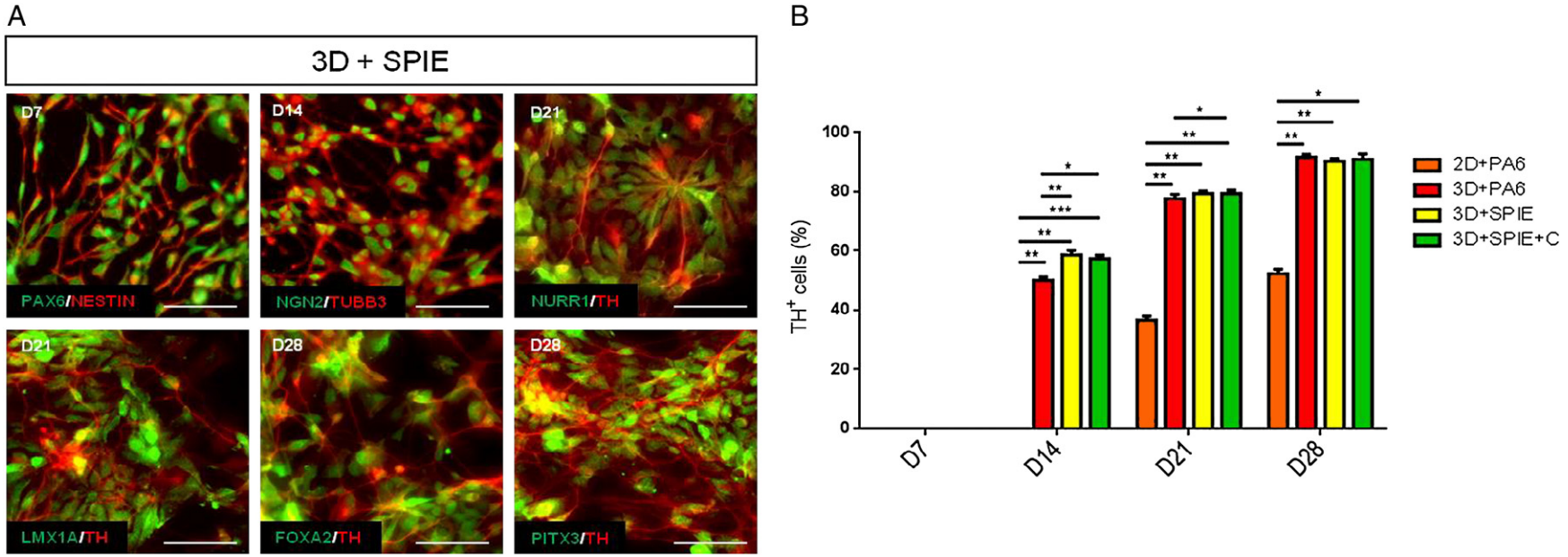

Figure 5 Immunofluorescent analysis of 3D + SPIE *vs* 3D + SPIE + C and estimated TH + cells of differentiated hESCs. The cells that were differentiated under 3D platform with SPIE with/without C were decapsulated, seeded and cultured on matrigel-coated culture plates for 4 days prior to analysis (A). Total of 300 cells of each differently differentiated hESC were counted at weekly points, estimated the average percentage of TH^+ cells (B). The images represent the onset of DA neuronal markers. Scale bar = 50 μm. Data presented as mean ± S.D. (biological replicates = 3 of each hESC line), Student's *t*-test, unpaired, two-tailed. The results were considered significant when P-values were <0.05 (*), 0.005 (**), 0.0005 (***), and 0.00005 (****).

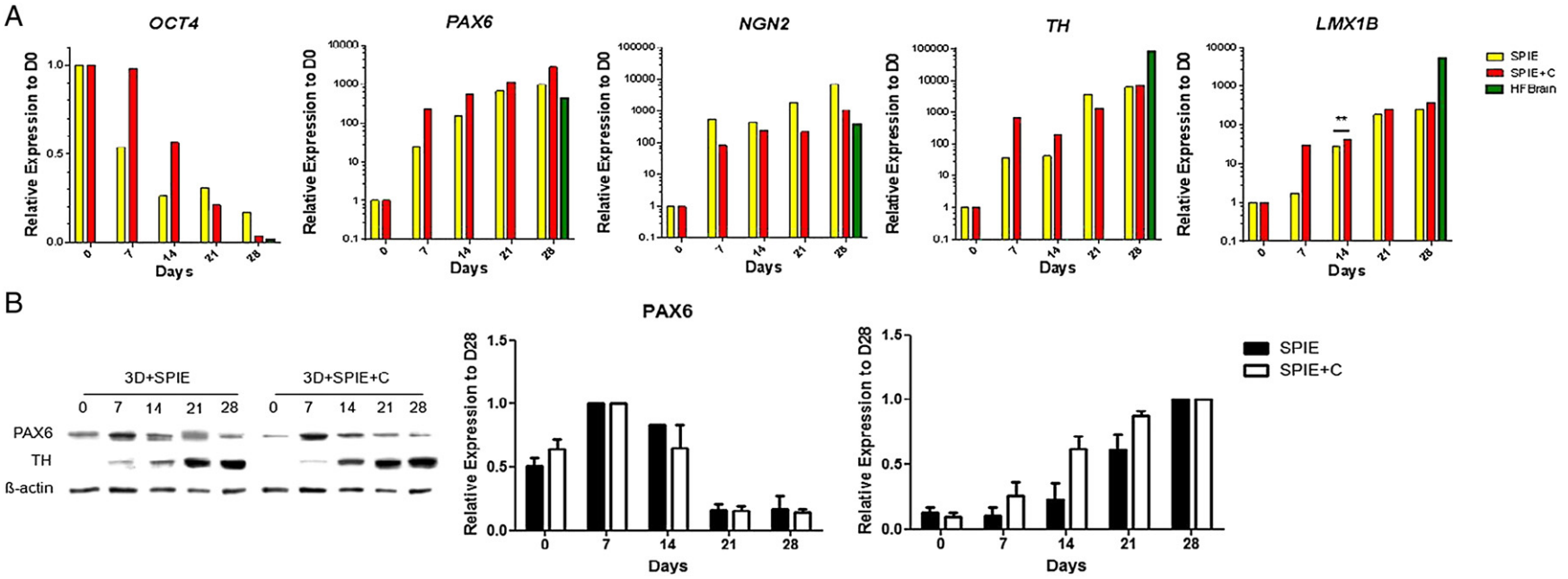

Figure 6 Gene and protein expression analysis of 3D + SPIE *vs* 3D + SPIE + C. RNA samples of 3D + SPIE *vs* 3D + SPIE + C (A) were isolated at different time points for qPCR analysis (including RNA sample from human fetal brain as a positive control). Protein samples of 3D + SPIE *vs* 3D + SPIE + C (B) were isolated at different time points for western blot analysis, showing immunoblot bands and their representative graphs. Data presented as mean ± S.D. (biological replicates = 3 of each hESC line), Student's *t*-test, unpaired, two-tailed. The results were considered significant when P-values were <0.05 (*), 0.005 (**), 0.0005 (***), and 0.00005 (****). *Abbreviation* — HFBrain: human fetal brain.

negative for OCT4 and NANOG (data not shown) but positive for PAX6 and NESTIN at day 7, followed by co-expression of NGN2 and TUBB3 after day 14. After 21 days of differentiation, TH^+ cells co-expressed NURR1 and LMX1A, followed by co-expression of FOXA2 and PITX3 after 28 days (Fig. 5A). After 28 days of differentiation, both SPIE and SPIE + C samples showed approximately 91 ± 3% and 92 ± 4% TH positive expression, respectively (Fig. 5B).

To investigate whether the gene expression analysis will show any differences, qPCR was conducted. The data showed similar expression levels of neuronal markers with no significant difference between the two treatments and lower *TH* and *LMX1B* gene expression levels than human fetal brain (Fig. 6A). Western blot analysis showed down-regulation of PAX6 expression after day 7 while TH expression showed steady up-regulation throughout the differentiation (Fig. 6B). However, there were no significant differences between the two treatments.

To observe the rate of DA neuronal differentiation from the four different systems, the expression levels of neuronal markers at each end point were directly compared. The hESCs that were differentiated in a conventional 2D protocol showed significantly higher PAX6 expression after 28 days (Fig. 7). However, the hESCs under the 3D platform, SPIE and SPIE + C differentiated hESCs showed higher expression of other mature neuronal markers compared to the 2D differentiation system (Fig. 7). The data suggested that the combination of co-culturing with PA6 cells *via* microencapsulation allows the pluripotent hESCs to mature mdDA neurons faster than the other systems within 28 days of differentiation.

Functional analysis of DA neurons obtained under 3D and 2D platforms

The differentiated cells under 3D and 2D PA6 co-culturing systems were analyzed for DA secretion under KCl-induction *in vitro* using high-sensitive DA ELISA assay. Overall, the cells that were not induced with KCl had much lower DA level in the supernatant than the samples with KCl-induced (Fig. 8). The direct comparison of DA secretion levels between KCl-induced samples showed significantly ($P < 0.05$) higher DA level in cells under the 3D platform in both days 14 and 28, particularly day 28 samples of cells under the 3D system resulted in >4 fold higher DA level than cells differentiated for 28 days in the 2D platform (Fig. 8). The data from ELISA correlate with the above gene and protein expression data indicating there are higher numbers of functionally active DA neurons when differentiated under the 3D environment than the conventional 2D system.

Discussion

Microcapsules and microcarriers have been used in many studies as 3D culture systems to grow and differentiate stem cells despite their potential application for neuronal differentiation (Dawson et al., 2008; Fang et al., 2007; Sidhu et al., 2012). In this study, a novel protocol has been established that involves 3D differentiation strategies using alginate microcapsules. The advantage of this method is that it is a single step process and physically separates the differentiating

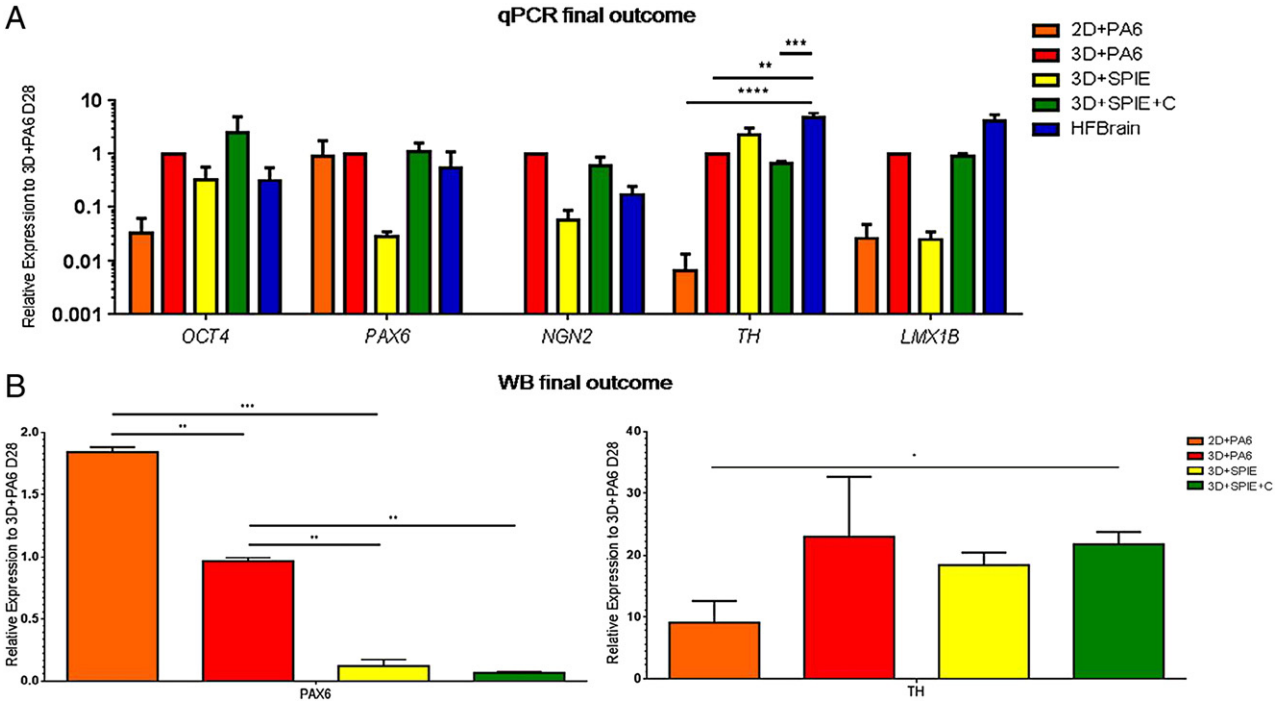

Figure 7 Expression level comparison at each end point. Graph of qPCR analysis at day 28 of differently treated hESCs (including human fetal brain as a positive control) (A). Graph of western blot analysis at day 28 of differentiated hESCs under different conditions (B). Data presented as mean ± S.D. (biological replicates = 3 of each hESC line), Student's *t*-test, unpaired, two-tailed. The results were considered significant when P-values were <0.05 (*), 0.005 (**), 0.0005 (***), and 0.00005 (****). *Abbreviation* — HFBrain: human fetal brain.

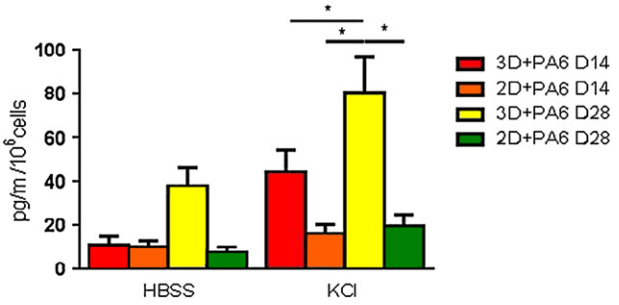

Figure 8 DA secretion analysis using ELISA. After 14 and 28 days of differentiation, the cells that were differentiated under 3D or 2D environment with PA6 cells were collected and seeded onto matrigel-coated culture dish. After 4 days, the cell culture supernatants were collected which were incubated in Ca^{2+}/HBSS and followed by 56 mM KCl supplemented in Ca^{2+}/HBSS. Data presented as mean ± S.D. (biological replicates = 3 of each hESC line), Student's *t*-test, unpaired, two-tailed. The results were considered significant when P-values were <0.05 (*), 0.005 (**), 0.0005 (***), and 0.00005 (****).

hESCs, thus avoiding the necessary enzymatic digestion of PA6 cells to isolate the differentiated cells. Furthermore, the 3D strategy to derive DA neurons has shown to produce faster expression of TH^+ neurons which also secretes higher level of DA when induced compared to the conventional 2D protocol after 28 days of differentiation. However, it must be noted that the percentage data of TH^+ neurons from this study are still estimation and require further verification by FACS.

The optimized encapsulation protocol from above allows for single hESC to survive, proliferate and form aggregates within the microcapsules in the presence of RI. The chemical compound has been shown to inhibit cell apoptosis, resulting in >80 ± 5% hESC viability inside a capsule diameter size of 500 ± 100 μm as reported previously (Chayosumrit et al., 2010; Sidhu et al., 2012). The encapsulated cells can be released *via* decapsulation process and cultured as a monolayer on matrigel-coated culture plates. The cells were seeded at a density of approximately 1.0–2.0 × 10^5 cells per 10 cm^2 which had the viability of 95 ± 1% (Sidhu et al., 2012).

The data further demonstrated that the encapsulated hESCs can be differentiated into mdDA neurons after 28 days as a 3D model environment. The starting number of hESCs that can be encapsulated with minimum risk of EB overgrowth and capsule breakage were optimized prior to differentiation. It is crucial to note that throughout the differentiation process, the number and size of EB increases (approximately 10–20 EB per capsule with 50–200 μm in diameter per EB) which may limit the diffusion of growth factors and gas substrates within aggregates (Yi et al., 2010). However, the low oxygen/hypoxic environment within the EB may have increased the rate of DA neuronal expressions which has been previously reported that the cells in hypoxic culture condition enhance the DA neuronal differentiation (Francis and Wei, 2010). In addition, it has been shown that cells cultured in a 3D environment can freely interact with neighboring cells in a 3D environment and respond more readily to each other, which significantly affects stem cell fate decisions such as apoptosis, self-renewal and differentiation (Yi et al., 2010). In comparison to the cell–cell interaction and the hypoxic environment of EB, the conventional 2D differentiation system has limited cell–cell contact with its neighbor cells and higher exposure to oxygen may have led to slower mdDA neuronal expressions than differentiated hESCs under 3D environment. However, the comparison of DA neuronal gene expression levels of 3D differentiated hESCs after 28 days against human fetal brain suggests that the cells differentiated in 3D manner are early DA neurons and may require to be differentiated for longer period of time to become fully mature DA neurons.

In order to allow the transition of hESCs to clinical application, it is essential that the culture system is xeno-free to eliminate any harmful effect it may cause to patients. Therefore, the 3D differentiation platform with PA6 cells is needed to be modified as the culture system contains several xenogens such as mouse-derived PA6 cells, FBS supplementation and xeno-derived trypsin. The application of human-derived SPIE and a chemical compound, C helps to limit the usage of xenogens. The data demonstrated that the differentiation in the 3D environment with SPIE factors can produce approximately >90% TH^+ neurons after 28 days, showing faster rate of TH^+ neuronal expression than the conventional 2D differentiation with SPIE factors which produces approximately 50% DA neurons after 28 days (Vazin et al., 2009). However, it is suspected that there are still necessary factors other than SPIE yet to be investigated which assist in mdDA neuronal differentiation (Vazin et al., 2009). The inhibitor C showed no significant further improvement on differentiating hESCs to DA neuronal lineage when used in conjunction with SPIE. However, the effectiveness of using C alone in a 3D environment to derive DA neurons could be an interesting approach which could potentially be investigated in the future. Although the presence of xenogens in the culture is limited, the necessary supplementation of RI to maintain the viability of encapsulated hESCs or the C for DA neuronal differentiation may cause concerns as the long-term effect of the chemical compounds to human is still unknown.

The functional *in vitro* data using high-sensitive ELISA suggested that the 3D model-derived TH^+ neurons secrete DA at a higher level than hESCs derived from the conventional 2D platform upon K^+ induction. However, it is recommended that the ELISA data be validated *via* high performance liquid chromatography (HPLC) assay in the future. Also, further functional analysis is required such as electrophysiology using whole cell patch clamp in order to fully characterize the differentiated cells.

Conclusions

In conclusion, the experiments herein demonstrated and provided as a proof-of-principle that 3D differentiation *via* microencapsulation is a powerful tool for DA neuronal differentiation. However, further analyses by differentiating the neurons under 3D and 2D platforms for longer period, followed by characterization of these cells using electrophysiology and transplantation assay in Parkinsonian rat model are required. Generating robust functional DA neurons efficiently is an essential requirement if cell therapy for PD is to become a reality. The study's proposed 3D platform is an effort in that direction.

Author contribution

Jaemin Kim: Conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing

Perminder Sachdev: Financial support, administrative support, provision of study material or patients, final approval of manuscript

Kuldip Sidhu: Conception and design, administrative support, provision of study material or patients, data analysis and interpretation, manuscript writing, final approval of manuscript

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