A Matrigel-based 3D construct of SH-SY5Y cells models the α-synuclein pathologies of Parkinson’s disease

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Abstract

Parkinson’s disease (PD) is featured with α-synuclein-based Lewy body pathology, which however was difficult to observe in conventional two-dimensional (2D) cell culture and even in animal models. We herein aimed to develop a three-dimensional (3D) cellular model of PD to recapitulate the α-synuclein pathologies. All-trans-retinoic acid-differentiated human SH-SY5Y cells and Matrigel were optimized for 3D construction. The 3D cultured cells displayed higher tyrosine
hydroxylase expression and improved dopaminergic-like phenotypes than 2D cells as suggested by RNA-sequencing analyses. Multiple forms of α-synuclein, including monomer, low and high molecular weight oligomers, were differentially present in the 2D and 3D cells, but mostly remained unchanged upon the MPP⁺ or rotenone treatment. Phosphorylated α-synuclein was accumulated and detergent-insoluble α-synuclein fraction was observed in the neurotoxin-treated 3D cells. Importantly, Lewy body-like inclusions were captured in the 3D system, including proteinase K-resistant α-synuclein aggregates, ubiquitin aggregation, β-amyloid and β-sheet protein deposition. The study provides a unique and convenient 3D model of PD which recapitulates critical α-synuclein pathologies and should be useful in multiple PD-associated applications.

**Keywords:** Parkinson’s disease, 3D modeling, α-synuclein, Lewy body, dopaminergic neurons

**INTRODUCTION**

Parkinson’s disease (PD) is the second most common neurodegenerative disorder after Alzheimer’s disease (AD). The prevalence of PD increases dramatically with aging, and is about 1.1% in people over 60 years old (Cui et al., 2020). Clinical manifestations of PD include resting tremor, bradykinesia, rigidity, and postural instability. Its principal pathophysiology is determined by the degeneration of dopaminergic neurons in the substantia nigra pars compacta, leading to impaired neurotransmission in the dorsolateral striatum. A number of studies have shown that PD develops from a complicated interplay between genetics and environment (Kalia and Lang, 2015).
Lewy bodies are recognized as the pathological hallmark of PD and used in Braak staging to define the disease temporal and spatial progression (Kalia and Lang, 2015). The Lewy body mainly comprises α-synuclein aggregates and other proteins such as ubiquitin, β-amyloid protein, and neurofilament proteins, but its form is not monolithic. For example, it appears in a particulate form in dopaminergic neuronal bodies of the substantia nigra, an acidophilic and argyrophilic core and a pale-staining halo in the brainstem, and spindle- or thread-like in axons and dendrites of affected neurons (Goedert et al., 2013, Braak et al., 1994, Kuusisto et al., 2003). The α-synuclein, encoded by the gene SNCA, is an abundant 140-residue neuronal protein. This protein is in a dynamic equilibrium in forms of monomer, oligomer, and fibril (Dehay et al., 2015). Oligomers define α-synuclein in a wide range of molecular weight, and are usually classified into low molecular weight (LMW) and high molecular weight (HMW) (Cullen et al., 2009, Gu et al., 2010, Lehri-Boufala et al., 2015), or single size molecular weight such as aS100 (α-synuclein at 100 kDa) (Dettmer et al., 2013). Mutations in the SNCA gene and abnormal post-translational modification of α-synuclein can break the assembly equilibrium (Paleologou and El-Agnaf, 2012), such that A53T mutation leads to the formation of the annular and tubular structures (Lashuel et al., 2002, Sardi et al., 2013), and phosphorylation at Ser-129 promotes insoluble fibril formation in vitro (Fujiwara et al., 2002). Misfolded α-synuclein and its toxic aggregates, but not loss of its function, are involved in the pathogenesis and progression of PD (Makin, 2016). In recent years, a prion theory of α-synuclein has gained mounting support over a controversy, with piling evidence showing that α-synuclein propagates through the brain as well as between inter-neurons and neurons-glial cells (Desplats et al., 2009, Hansen et al., 2011, Loria et al., 2017, Makin, 2016).
The capture of the multifactorial nature of PD is often difficult in cellular or animal models. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned non-human primate remains to be the gold-standard animal model of PD and is frequently used to evaluate the effectiveness of molecules on parkinsonism and psychosis (Veyres et al., 2018). Higher MPTP dose can lead to acute dopaminergic cell loss within 1 month, while low dose usually takes 8-12 months to achieve a stable parkinsonism (Bezard et al., 2001, Seo et al., 2019, Barth et al., 2020). Intraneuronal inclusions reminiscent of Lewy bodies have been described in the primate MPTP model (Dauer and Przedborski, 2003, Tieu, 2011, Forno et al., 1986). However, other animal models induced by neurotoxins or by genetic manipulations cannot consistently recapitulate this important neuropathological feature (Chia et al., 2020, Giraldez-Perez et al., 2014, Cicchetti et al., 2009), not to even mention the conventional two-dimensional (2D) cell culture system (Zhuang et al., 2018).

Three-dimensional (3D) models of human-derived cells have recently been extensively developed to study disease mechanisms and to screen drugs (Duval et al., 2017). Compared to the 2D cultures, these models contain mechanical structural cues and the extracellular microenvironment that bring them closer to physiological conditions (Baker and Chen, 2012). Matrigel is the ideal skeleton of 3D model, which contains rich extracellular matrix molecules such as laminin, collagen IV, and entactin. A Matrigel-based 3D model of human neural cells overexpressing familial AD mutants of β-amyloid precursor protein and/or presenilin 1 was recently reported to successfully recapitulate all key known neuronal hallmarks of AD (Choi et al., 2014). By using SH-SY5Y cells, an immortalized cell line often used for PD study and with the advantage of fast reproduction, simple culture, and low cost for use in 3D cultivation, we herein for the first time provided a neurotoxin-induced, Matrigel-based 3D model of PD that recapitulated unique features of Lewy body-like aggregations.
MATERIALS AND METHODS

Cells and reagents

SH-SY5Y cells (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; 086-150, Wisent) and 1% penicillin/streptomycin (Gibco) and maintained in a humidified incubator at 37°C with 5% CO₂. All-trans-retinoic acid (RA; PHR1187), N-methyl-4-phenyl pyridine (MPP⁺; M0896), rotenone (R8875) and 12-O-tetradecanoylphorbol-13-acetate (TPA; P8139) were purchased from Sigma. Primary antibodies used in this study were α-synuclein (6210789, BD Biosciences), pS129-α-synuclein (phosphorylated α-synuclein at serine 129; ab59264, Abcam), LB509-α-synuclein (180215, Thermo Fisher), β-amyloid (803004, Biolegend), β-actin (4970, Cell Signaling Technology), dopa decarboxylase (DDC; 10166-1, Proteintech), microtubule-associated protein 2 (MAP2; 4542, Cell Signaling Technology), tyrosine hydroxylase (TH; 22941, ImmunoStar), ubiquitin (ab7780, Abcam), and vesicular monoamine transporter 2 (VMAT2; 20873-1-AP, Proteintech).

3D model construction

SH-SY5Y cells were differentiated in a differentiation medium as optimized in the Results section. The cells were embedded in the Matrigel matrix containing 60% laminin, 30% collagen IV, and 8% entactin (356237, Corning) to generate 3D constructs. In detail, Matrigel was taken out from -80 °C, and placed in a 4 °C refrigerator 2 h before use. SH-SY5Y cells were collected at a confluency of 70-80%, resuspended in DMEM with 1% FBS and placed on ice. Cold Matrigel was added into the cell suspension to obtain final concentrations of Matrigel and cells at 4.5 mg/mL and 6-7×10⁶/ml, respectively. The mixture was prepared on ice and vortexed for 30
For thin-layer 3D blocks (used for 3D reconstruction of confocal imaging), 400 μL of the mixture was dispensed into a glass-bottom dish (D35-20-1-N, Cellvis) using prechilled pipettes. For thick-layer 3D blocks (used for all other experiments), 300 μL of the mixture was dispensed into a cell culture insert (MCEP24H48, Millipore) in 24-well plates. The constructs were incubated in a CO₂ incubator at 37 °C for 2 h, followed by the addition of prewarmed differentiation medium to the dishes or inserts, that is, 3 mL for thin-layer 3D cultures, and 0.5 mL to the insert and 1 mL to the surrounding well for thick-layer 3D cultures. The constructs were then cultured for 6 days before use. The differentiation medium was refreshed every 3 days.

**RNA-sequencing**

Total RNA of SH-SY5Y cells in the RA-treated 2D and 3D cultures was extracted using RNAiso Plus (9108, Takara) according to the manufacturer’s protocol. RNA sequencing was performed on an Illumina X10 at LC Sciences (Hangzhou, China). Prior to assembly, the low-quality reads that contained sequencing adaptors, sequencing primer, and nucleotide with q quality score lower than 20 were removed. HISAT package was used to map reads of samples to the homo sapiens reference genome at http://genome.ucsc.edu/. The mapped reads of each sample were assembled using StringTie and merged to reconstruct a comprehensive transcriptome using Perl scripts. After that, StringTie was used to calculate expression levels of the mRNAs by calculating fragments per kilobase per million mapped reads (FPKM). The differentially expressed mRNAs and genes were selected by Ballgown with fold change > 2 and with statistical significance $p < 0.05$. Bioinformatic analyses were performed using the OmicStudio tools at https://www.omicstudio.cn/tool. The dopaminergic synaptic pathway diagram was mapped using KEGG mapping tools at Disease Models & Mechanisms • DMM • Accepted manuscript
RNA-sequencing data were deposited in the Sequence Read Archive database (https://www.ncbi.nlm.nih.gov/sra/) under the accession number PRJNA761139.

**Cell lysate preparation and Western blot**

Cells in thick-layer 3D blocks were recovered following incubation with Cell Recovery Solution (354253, Corning) at 4 °C for 1-2 h, and then collected at 330 g for 5 min at 4 °C. For total cell lysates, cells from either 2D or 3D cultures were lysed in a buffer containing 60 mM Tris-HCl, pH 6.8, 5% glycerol, and 2% SDS. For insoluble fraction preparation, cells were first lysed in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.5) supplemented with 1% Triton X-100, protease inhibitor cocktail (7012L, Cell Signaling Technology), and 1 mM phenylmethane sulfonyl fluoride (PMSF; ST506-2, Beyotime). After sonication using a fine probe (0.5 sec pulse at an amplitude of 20% for 10 times), cell lysates were incubated on ice for 30 min and centrifuged at 100,000 g for 30 min at 4°C. The pellet was washed with the lysis buffer, sonicated as above again, and centrifuged for another 30 min at 100,000 g. After removing the supernatant, the pellet was resuspended in TBS supplemented with 2% SDS, protease inhibitor cocktail and 1 mM PMSF, and sonicated as above for 15 times. Cell lysates were then boiled for 10 min. Total protein concentration was measured using a BCA kit (ST023, Beyotime) after centrifugation. Western blot was performed as previously described (Liu et al., 2014). As a note, for α-synuclein analysis, the membrane was pre-fixed with 0.4% paraformaldehyde (PFA) for 30 min before blocked with 5% milk. Membranes were probed with primary antibodies against α-synuclein (1:1000), pS129-α-synuclein (1:1000), β-amyloid (1:1000), VMAT2 (1:1000), DDC (1:1000), TH (1:1000) or β-actin (1:2000). Anti-mouse (7076)
and anti-rabbit (7074) secondary antibodies, and LumiGLO Reagent and Peroxide chemiluminescence detection kit (7003) were purchased from Cell Signaling Technology.

**Immunofluorescence staining**

For 2D cultures, cells were fixed in 4% PFA for 30 min, followed by permeabilization in 0.2% Triton X-100 for 15 min. Samples were then blocked with 5% bovine serum albumin (BSA; P0010, Beyotime) for 1 h at room temperature. The washing buffer is phosphate-buffered saline (PBS). For thin-layer 3D blocks, the procedure was slightly modified from Kim et al. (Kim et al., 2015). In brief, cells were fixed in 4% PFA containing 0.1% glutaraldehyde for 1 h, followed by permeabilization in 0.5% Triton X-100 for 1 h at room temperature. Samples were then blocked with 5% BSA overnight at 4°C. The washing buffer is Tris-buffered saline containing 0.2% (v/v) Tween-20. Thereafter, samples were incubated with primary antibodies against α-synuclein (1:500), MAP2 (1:200) and/or TH (1:500) at 4°C overnight, followed by incubation with Alexa Fluor 555 anti-rabbit (A21428, Thermo Fisher) and/or Alexa Fluor 488 anti-mouse (A11001, Thermo Fisher) secondary antibodies at room temperature for 2 h. Cells were then counterstained with Hoechst 33342 (H3570, Thermo Fisher) for 5 min at room temperature and mounted with an anti-fade mounting medium (P0126, Beyotime). The fluorescence images were captured and reconstituted using a Nikon confocal microscope (C2si).

**Sectioning of thick-layer 3D blocks**

The thick-layer 3D blocks were fixed in 4% PFA containing 0.1% glutaraldehyde for 2 h, dehydrated in 30% sucrose for 24 h, and then subjected to optimal cutting
temperature (OCT) compound (80202-0001, CellPath) embedding and frozen sectioning at 15 μm thickness using a Leica microtome (CM1860) following the manufacturer’s procedure. Next, sections were incubated with PBS to remove the OCT compound at room temperature, followed by incubation with 3% (v/v) H₂O₂ for 20 min to block endogenous peroxidase activities.

Immunohistochemical (IHC) staining

The 3D sections were permeabilized in 0.5% Triton X-100 for 15 min and blocked with 5% BSA for 1 h at room temperature. The washing buffer was PBS. The sections were then incubated with primary antibodies against LB509-α-synuclein (1:500), pS129-α-synuclein (1:200), β-amyloid (1:500) or ubiquitin (1:500) at 4°C overnight, followed by incubation with anti-mouse or anti-rabbit Ig HRP polymer conjugates (PV-6002 and PV-6001, respectively, Zhongshan Golden Bridge) at room temperature for 1 h. Signals were developed using 3,3′-diaminobenzidine peroxidase substrate kits (ZLI-9018, Zhongshan Golden Bridge). Nuclei were counterstained with hematoxylin (C0107, Beyotime).

Proteinase K digestion

To detect proteinase K-resistant α-synuclein aggregates, the 3D sections were treated with 50 μg/mL proteinase K prepared in a buffer containing 10 mM Tris-HCl (pH 7.4) and 100 mM NaCl at 37 °C for 30 min. The sections were then subjected to IHC processing as described above using the LB509-α-synuclein antibody.
**Thioflavin-S staining**

Thioflavin-S indicates β-sheets and is often used in β-amyloid and Lewy body pathologies (Urbanc et al., 2002, Mclellan et al., 2003, Li et al., 2010). The 3D sections were incubated in PBS to remove the OCT compound, then permeabilized in 0.2% Triton X-100 for 15 min and blocked with 5% BSA for 1 h at room temperature. The sections were then incubated with 0.05% Thioflavin-S for 5 min at room temperature, followed by cleaning with 50% anhydrous ethanol 3 times for 5 min each.

**Statistical Analysis**

Statistical difference was evaluated using Student’s *t*-test, factorial analysis of variance (ANOVA), or one-way ANOVA followed by Tukey’s post hoc test. Data were expressed as means ± SE from at least three independent experiments. Differences were considered statistically significant when *p* was < 0.05.

**RESULTS**

**Development of Matrigel- and SH-SY5Y cell-based 3D construct**

RA is often used to induce dopaminergic differentiation of SH-SY5Y cells as indicated by the TH expression (Xicoy et al., 2017). Different medium FBS concentrations (1, 2, 3, 5 and 10%) had no impact on the RA (10 μM)-induced TH expression in regular 2D cultures (Fig. S1A; all statistical results listed in Table S1). Then 1% FBS was selected for subsequent cultures to avoid over-confluency and cell aggregations. Cells used for the 3D constructs were at 6-7×10^6 per mL based on a previous study by Choi et al (Choi et al., 2014), and cultured in different concentrations of Matrigel (3.5, 4.0, 4.5 and 6.0 mg/mL). Results showed that 4.5
mg/mL of Matrigel conferred on cells the most appropriate spacing and the least cell aggregations (Fig. S1B) and was then selected for subsequent experiments. It was previously reported that SH-SY5Y cells treated with RA followed by the addition of TPA [RA/(RA+TPA)] would improve dopaminergic differentiation compared to the RA alone in the conventional 2D cultures (Presgraves et al., 2004). However, in the Matrigel-based 3D constructs, the treatment of RA/(RA+TPA) at 10 μM/(10 μM+80 nM) resulted in a severe aggregation of SH-SY5Y cells (Fig. S1C), suggesting that this type of induction is not appropriate for the 3D cultures.

As a result, 6-7×10^6 per mL of SH-SY5Y cells, 4.5 mg/mL of Matrigel, and a differentiation medium containing 1% FBS and 10 μM RA were used to establish the 3D culture with a culture time of 6 days. The 2D counterpart showed a high expression of TH during 2-4 days in the differentiation medium (Fig. S1D), and thus the culture time of 3 days was selected for comparison. The 2D and 3D preparation and modeling procedures were illustrated in Fig. 1A and detailed in the Methods section. An axonal network of SH-SY5Y cells in the 3D construct was reconstituted using confocal images immunostained with TH and the neuronal marker MAP2 (Fig. 1B). The TH expression was compared between the 2D and 3D cultures with or without RA differentiation. Results showed that the 3D culture per se led to a massive increase of TH expression compared to the 2D culture without RA treatment (by 17.8-fold; Fig. 1C). In contrast, the RA treatment induced a similar amount of increase in TH expression in the 2D and 3D cultures (Fig. 1C).
Transcriptomic analysis of gene expression in the RA-treated 2D and 3D cultures

RNA-sequencing was used to analyze transcriptomic expression of the 2D and 3D cultures differentiated with RA as above for differentially expressed genes (DEGs). Compared to the 2D cultured cells, a total of 1127 up-regulated and 692 down-regulated genes were disclosed in the 3D cultured cells (Fig. 2A; full list in Table S2). Consistent with the above change in protein expression, the mRNA expression of TH was massively elevated in the 3D culture (by 81.8-fold; Fig. 2A). We could also validate the elevated expression of two additional DEGs, VMAT2 and DDC, by Western blot (Fig. 2B). The DEGs were enriched in extracellular matrix, glycolytic process, canonical glycolysis, plasma membrane, response to hypoxia, etc., as suggested by Gene ontology (GO) biological function analysis (Fig. 2C). Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis suggested that the DEGs were enriched in cellular community, membrane transport, replication and repair, neurodegenerative disease, energy metabolism, nervous system, etc. (Fig. 2D). Besides, gene set enrichment analysis (GSEA) revealed the enrichment of DEGs in dopaminergic neuron differentiation, dopaminergic synaptic transmission, extracellular matrix receptor interaction, and neuroactive ligand-receptor interaction (Fig. 2E). Results of the dopaminergic synaptic signal pathway diagram of DEGs showed altered expressions in TH (elevated), DDC (elevated), VMAT1 and VMAT2 (elevated), dopamine receptor D5 (DRD5; reduced), and electric potential and signal transduction associated genes including calcium voltage-gated channel subunit alpha1 (CACNA1D; reduced) and protein kinase C gamma (PRKCG; elevated). Products of the first four DEGs are involved in presynaptic dopamine production and transport, while the others function in postsynaptic neurons (Fig. S2).
MPP⁺- and rotenone-induced α-synuclein pathologies in the 2D and 3D cultures

Cells of the 2D and 3D cultures were treated with classical neurotoxins, MPP⁺ at 10 μM and rotenone at 0.5 μM, for 24 h. Western blot results of the 2D cells showed that the MPP⁺ treatment induced significant increase in levels of α-synuclein monomer and LMW oligomer (< 75 kDa), but not in HMW oligomer (> 75 kDa). The rotenone treatment induced no change in the α-synuclein forms. In contrast, neither MPP⁺ nor rotenone treatment changed the α-synuclein forms in the 3D culture (Fig 3A). Western blot results of pS129-α-synuclein appeared to be different. While the MPP⁺ treatment caused no changes, the rotenone treatment increased pS129-α-synuclein HMW oligomer, but not monomer/LMW oligomer, in the 2D cultures. In contrast, MPP⁺ and rotenone increased both pS129-α-synuclein forms in the 3D cultures (Fig 3B). Comparing at the baseline level to that in the 2D cells, we observed that the proportion of monomer to total α-synuclein was elevated in the 3D cells (Fig. 3C). The pS129-α-synuclein was largely present as HMW oligomer in the 2D cells, while the forms existed roughly in similar abundance in the 3D cells (Fig. 3D).

Immunofluorescence analysis of α-synuclein in the 2D cultures showed that the protein was significantly elevated by the MPP⁺ treatment, but not by rotenone (Fig. 4A), which is consistent with the above Western blot results. However, immunofluorescence of α-synuclein in the 3D constructs showed a high background staining, probably resulting from the Matrigel nonspecific binding (Fig. S3). We thus used the IHC staining for the 3D constructs, by which there were much less background signals. Results showed an elevated aggregation area of α-synuclein immunopositivity upon treatment of either MPP⁺ or rotenone (Fig 4B). We also prepared detergent-insoluble fractions and found that the insoluble α-synuclein level
was increased by both treatments, mainly in the form of HMW oligomer (Fig. 4C). However, β-amyloid protein was not detectable in the fractions (data not shown). Similar to α-synuclein (Fig. 3A), β-amyloid protein levels were not altered by the neurotoxin treatments in 3D cultures (Fig. S4A).

Intraneuronal aggregation of Lewy body α-synuclein in the 3D cultures administered with MPP⁺ and rotenone

Lewy pathology was detected by a standard histological methodology, including the application of LB509-α-synuclein, an antibody raised against Lewy body α-synuclein, and proteinase K digestion (Ip et al., 2017, Goedert et al., 2013). The IHC staining of LB509-α-synuclein showed a large and significant increase of intracytoplasmic aggregation dots in the SH-SY5Y cells of the 3D constructs following treatment of 10 μM MPP⁺ or 0.5 μM rotenone for 24 h (Fig. 5A). The intracytoplasmic aggregation appeared to be of different degrees of severity, from light, medium, high to condense around the nuclei (Fig 5B). To demonstrate that these aggregates are degradation-resistant, the sections were pretreated with proteinase K. IHC results showed that the MPP⁺- and rotenone-induced aggregation dots remained in significant amount after the treatment. (Fig. 5C). However, Western blot analysis did not detect any bands of proteinase K resistant α-synuclein (Fig. S4B). The 3D constructs were then analyzed for phosphorylated α-synuclein, ubiquitin and β-amyloid protein by IHC staining, and β sheet protein by thioflavin-S staining. Like results of the LB509-α-synuclein staining, treatment of MPP⁺ or rotenone in the 3D constructs led to significant increase in the number of cells accumulated with phosphorylated α-synuclein (Fig. 6A), ubiquitin aggregation (Fig. 6B), β-amyloid
protein (Fig. 6C) and β-sheet protein (Fig. 6D), which together suggest a Lewy body-like formation.

**Discussion**

An appropriate human 3D cellular model of disease may provide a unique and convenient platform for studying molecular mechanisms and screening therapeutic compounds. As noted earlier, neurofibrillary tangles, a hallmark pathology of AD, have been recently recapitulated in the 3D cellular model overexpressing familial AD mutants (Choi et al., 2014). Herein, we provide a convenient 3D model of PD based on Matrigel, differentiated SH-SY5Y cells and neurotoxins which remarkably recapitulates α-synuclein pathologies, including elevated α-synuclein phosphorylation and Lewy body-like inclusions.

2D cell culture is arguably too simple and overlooks many parameters which are important for reflecting cell and tissue physiology or pathology. These include interactions between cells and matrix, communication between adjacent cells, and mechanical connections. On the other hand, in vivo animal models are time-consuming, such as for pharmaceutical compound screening, and may have low predictability for human diseases (Van Der Worp et al., 2010, Leist and Hartung, 2013, Warren et al., 2015). For instance, a binding mapping of four conserved transcription factors in human and mouse hepatocytes suggests that 41-89% of their binding events are species-specific (Odom et al., 2007). Moreover, genomic responses in mouse models correlate poorly with the human conditions of inflammatory diseases such as trauma, burns, and sepsis (Seok et al., 2013). The poor predictability may also result from bias in animal studies such as randomization, blinded outcome assessment, and sample size calculation (Van Der Worp et al., 2010). A 3D-culture system using
human cells of wildtype or mutants may thus act as a bridge in between, where cells are subjected to mechanical, structural and surrounding cues and may better reproduce physiological conditions and/or pathological changes of diseases (Baker and Chen, 2012).

Indeed, the 3D culture brings SH-SY5Y cells a few good indicators to be relatively closer to a dopaminergic neuron like state. The 3D culture per se leads to a high-fold increase in TH expression, which is usually not achievable by the existing cell differentiation protocols, including RA, RA/TPA, or RA/brain-derived neurotrophic factor (BDNF) (Kovalevich and Langford, 2013, Presgraves et al., 2004, Xicoy et al., 2017). RNA-sequencing-based DEGs further suggest that cells in the 3D culture better resemble the in vivo dopaminergic phenotype as manifested by results of the GO, KEGG and GSEA analyses. Indeed, extracellular matrix proteins are enriched in the scaffold Matrigel, which together with mechanostructural cues may be crucial for induction of proper synaptic structures, functional axonal vesicle transport, and sustainable neuronal morphology (Agholme et al., 2010, Baker and Chen, 2012, Hughes et al., 2010). The DEGs also provide molecular insights for further exploring how this transition occurs. In addition, human embryonic stem cells and inducible pluripotent stem cells have also been used for 3D model construction (Choi et al., 2014, Duval et al., 2017, Jo et al., 2016). Stem cells have the advantage of differentiating to desired cell types and being closely like primary cells but are relatively more expensive and time-consuming in culturing. In contrast, immortalized cells are cost-effective, usually fast-growing, and easier to manipulate. Both cell types are worth 3D modeling with if in vivo pathological or physiological key features can be recapitulated.
When comparing at the normal state (i.e., untreated), the proportion of total or phosphorylated α-synuclein shifts more to the monomer/LMW oligomer state in the 3D model than in the 2D culture. It remains unclear how the shifts occur but suggests that the 3D model per se is closer to the in vivo physiological state based upon the premise that greater levels of aggregation are closer to the disease state (which may be in debate). After that, the neurotoxin treatments lead to a building of the disease state. The 3D model also appears superior to the 2D culture with a better and more sophisticated manifestation of the disease state. The most exciting and meaningful finding is the recapitulation of α-synuclein pathologies of PD in the 3D model upon neurotoxin induction, including accumulation of the phosphorylated α-synuclein and detergent-insoluble α-synuclein fraction, and the Lewy body-like inclusions as manifested by the intracytoplasmic LB509-stained α-synuclein aggregates, the resistance to proteinase K, the ubiquitin-stained aggregates, the β-amyloid, and thioflavin-S-stained β sheet protein deposits.

As a note, not like the α-synuclein phosphorylation, the neurotoxin-induced α-synuclein resistance to proteinase K appears to be solely observable by IHC, but not by Western blot. Theoretically, the availability of α-synuclein for digestion by proteinase K under conditions used for IHC is limited, hence digesting lysates and analyzing by Western blot provide a more sensitive assay for protease resistance. By searching literatures and to the best of our knowledge, it seems that Western blot analysis of proteinase K resistance are mostly against exogenous α-synuclein preformed fibrils (Guo et al., 2013, Pujols et al., 2018), or requires large amount of sample such as approximately 1 g of human brain extracts (Neumann et al., 2002). Instead, IHC is more widely used for detection of endogenous resistant α-synuclein (Spencer et al., 2016, Ip et al., 2017, Svarcbahs et al., 2018). Indeed, even limited
aggregation dots from IHC are observable under microscope, but the amount may not be enough to reach Western blot detection limit. Nonetheless, IHC has its own limitations, such that how specific the antibody is and how fully proteinase K can penetrate the tissue slides for aggregates digestion.

Abnormally phosphorylated α-synuclein has been observed in multiple cellular and animal models (Lou et al., 2010, Sugeno et al., 2008, Gorbatyuk et al., 2008, Kim et al., 2006), which promotes insoluble fibril formation (Fujiwara et al., 2002), and is associated with pathological α-synuclein deposits contained within Lewy bodies and Lewy neurites (Lee et al., 2014, Spillantini et al., 1997). In contrast, the Lewy body-like inclusions are mostly observed in 2D, 3D or in vivo models induced by exogenously added preformed α-synuclein fibrils (Mahul-Mellier et al., 2020, Uemura et al., 2018, Volpicelli-Daley et al., 2014, Taylor-Whiteley et al., 2019), but are hardly observed in neurotoxin-based models aside from monkeys as noted earlier (Tieu, 2011, Dauer and Przedborski, 2003, Forno et al., 1986, Chia et al., 2020, Giraldez-Perez et al., 2014, Cicchetti et al., 2009, Zhuang et al., 2018). A 3D organoid system derived from stem cells containing a G2019S mutation in Leucine-rich repeat kinase 2 (LRRK2) displayed an accumulation of pS129-α-synuclein with no change in total α-synuclein, which is consistent with our observation. However, the Lewy body pathology was not reported to be recaptured in this organoid (Kim et al., 2019). In addition, our model uses neurotoxins as a variable to induce α-synuclein dysfunction. Given potential disconnection between toxin models and idiopathic PD, using either SNCA overexpression (modelling the duplication and triplication mutations observed in human populations) or point mutations would be an alternative disease modeling approach.
In conclusion, the current study provides a novel neurotoxin-based 3D human cellular model of PD exhibiting accumulation of α-synuclein phosphorylation and Lewy body-like inclusions. The model is convenient to construct, manipulate and observe, and should be decently useful in investigating PD mechanisms and screening drugs targeting α-synuclein pathologies.

CONFLICT OF INTEREST STATEMENT
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS
JHZ and XZ designed and supervised the study. ZFL and LC performed experiments and data analysis. MMJ, DYH and XGH contributed to partial experiments. SSL assisted statistical analysis. ZFL and JHZ wrote the manuscript. All authors have read and approved the final manuscript.

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**Figures**

**A**

Figure 1. Matrigel- and SH-SY5Y cell-based 3D constructs. (A) Preparation and processing scheme of the 2D and 3D cultures. (B) Reconstituted 3D images of SH-SY5Y cells in a thin-layer 3D construct after 6 days in the differentiation medium.
Z-sections were captured in a 0.85-μm and 0.4-μm interval for 36 and 58 consecutive times respectively for the ×200 and ×600 magnifications. Green, TH; red, MAP2; blue, nuclei; bar size, 50 μm. (C) TH expression in the 2D and 3D cultures with or without RA induction. Protein levels were quantified and normalized to their respective β-actin levels. Values were expressed relative to the 2D cells without RA treatment, which was set as 1. Data are means ± SE, n = 3. Statistical analyses were performed using factorial ANOVA. Different letters in the same casing and * between the indicated groups represent $p < 0.05$. MAP2, microtubule-associated protein 2; RA, all-trans-retinoic acid; TH, tyrosine hydroxylase.
Figure 2. RNA-sequencing analyses of SH-SY5Y cells in the RA-treated 2D and 3D cultures. The data represent 3 replicates. (A) Volcano plot showing DEGs in the 3D cultures compared to the 2D. The threshold was set at fold change > 2 and \( p < 0.05 \). (B) Western blot validation of the differential expression of VMAT2 and DDC in the RA-treated 2D and 3D cultures. Protein levels were quantified and normalized to their respective β-actin levels. Values were expressed relative to the 2D cells, which was set as 1. Data are means ± SE, \( n = 3 \). Statistical analyses were performed using Student’s \( t \)-test. *, \( p < 0.05 \). (C) GO analysis of the DEGs. (D) KEGG analysis of the DEGs. (E) GSEA analyses of the RNA-sequencing data in dopaminergic neuron differentiation, dopaminergic synaptic transmission, ECM receptor interaction, and neuroactive ligand-receptor interaction. DDC, dopa decarboxylase; DEGs, differentially expressed genes; ECM, extracellular matrix; RA, all-trans-retinoic acid; VMAT2, vesicular monoamine transporter 2.
Figure 3. Western blot analyses of α-synuclein and pS129-α-synuclein expression. (A-B) Analysis of α-synuclein (A) and pS129-α-synuclein (B) in the RA-induced 2D and 3D cultures treated with 10 μM MPP+ or 0.5 μM rotenone for 24 h. Protein levels were quantified and normalized to their respective β-actin levels. Values were expressed relative to the control, which was set as 1. Data are means ± SE, n = 3. Statistical analyses were performed using Student’s t-test. *, p < 0.05. (C-D) Baseline comparison of α-synuclein (C) and pS129-α-synuclein (D) in the RA-treated 2D and 3D cultures. As a note, we observed that α-synuclein aggregation state may vary a bit among different cell batches. Thus, 2D and 3D direct comparison should be more appropriate using cells of the same batch.
Figure 4. Analyses of α-synuclein in the 2D and 3D cultures treated with 10 μM MPP⁺ or 0.5 μM rotenone for 24 h. (A) Immunofluorescence staining of the 2D cultures. Green, α-synuclein; red, MAP2; bar size, 25 μm. (B) Immunohistochemical staining of sections of the 3D cultures. The images were contrasted for quantitative analysis of α-synuclein aggregates using ImageJ software. Bar size, 100 μm. (C) Western blot analyses of insoluble α-synuclein fraction in the 3D cultures. Protein levels were quantified and normalized to β-actin. Values were expressed relative to the control, which was set as 1. Data are means ± SE, n = 3. Statistical analyses were performed using Student’s t-test. *, p < 0.05.
Figure 5. Analyses of α-synuclein aggregation in the 3D cultures treated with 10 μM MPP⁺ or 0.5 μM rotenone for 24 h. (A) Immunohistochemical staining of LB509-α-synuclein. (B) Representative images of LB509 positive cells with different severity degrees. (C) Immunohistochemical staining of LB509-α-synuclein after digestion with 50 μg/mL proteinase K. Bar size, 50 μm. LB509 positive cells and total cells were counted from 3-4 random areas. n = 3. Data are means ± SE, n = 3. Statistical analyses were performed using Student’s t-test. *, p < 0.05.
Figure 6. Aggregation and deposition analyses in the 3D cultures treated with 10 μM MPP⁺ or 0.5 μM rotenone for 24 h. (A-C) Immunohistochemical staining of pS129-α-synuclein (A), ubiquitin (B) and β-amyloid (C). Bar size, 50 μm. (D) Thioflavin-S staining for β-sheet protein. Bar size, 25 μm. The positive cells and total cells were counted from 3 random areas. n = 3. Data are means ± SE, n = 3. Statistical analyses were performed using Student’s t-test. *, p < 0.05.
**Fig. S1.** Optimization of SH-SY5Y cell cultures. (A) SH-SY5Y cells were 2D cultured and treated with 10 μM RA for 3 days in media containing different concentrations of FBS. (B) Bright-field images of SH-SY5Y cells in 3D constructs with different concentrations of Matrigel. The constructs were cultured for 6 days in a differentiation medium containing 10 μM RA and 1% FBS. Bar size, 200 μm. (C) Bright-field images of cells in 3D constructs with 4.5 mg/mL of Matrigel. The constructs were cultured in a medium containing 1% FBS with two different differentiation inducers, that is, 10 μM RA for 6 days (RA) or 10 μM RA for 3 days followed by 10 μM RA and 80 nM TPA for 3 days [RA/(RA+TPA)]. Bar size, 100 μm. (D) Cells were 2D cultured and treated with 10 μM RA for different days in a medium containing 1% FBS. TH levels were quantified and normalized to their respective β-actin levels. Values were expressed relative to the one without RA treatment, which was set as 1. Data are means ± SE, n = 3. Statistical analyses were performed using one-way ANOVA, followed by Tukey’s post hoc test. Different letters indicate p < 0.05. FBS, fetal bovine serum; RA, all-trans-retinoic acid; TH, tyrosine hydroxylase; TPA, 12-O-tetradecanoylphorbol-13-acetate.
**Fig. S2.** Dopaminergic synaptic signal pathway analysis. (A) The differentially expressed genes in cells of 3D versus those of 2D in the dopaminergic synaptic signal pathway. (B) Dopaminergic synaptic signal pathway diagram by KEGG analysis.
Fig. S3. Matrigel and α-synuclein. (A) Immunofluorescence staining of α-synuclein using the 3D constructs. Green, α-synuclein; red, MAP2. Bar size, 20 μm. (B) Western blot analysis of α-synuclein using SH-SY5Y cells and Matrigel, respectively.
Fig. S4. Western blot analyses of β-amyloid (A) and proteinase K resistant α-synuclein (B) expression in the RA-treated 3D cultures. Cells were treated with 10 μM MPP⁺ or 0.5 μM rotenone for 24 h. For (B), total cell lysates were treated with 5 μg/mL proteinase K at 37 °C for 30 min. APP, amyloid precursor protein; β-amyloid monomer is at about 4 kDa.
### Table S1. Results of all statistical analyses undertaken

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Table S2. The differentially expressed genes in the 3D cultures compared to the 2D cultures by RNA-sequencing analyses (fold change > 2 and p < 0.05)

Click here to download Table S2