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Invited Mini Review

Patient-specific pluripotent stem cell-based Parkinson's disease models showing endogenous alpha-synuclein aggregation

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After the first research declaring the generation of human induced pluripotent stem cells (hiPSCs) in 2007, several attempts have been made to model neurodegenerative disease *in vitro* during the past decade. Parkinson's disease (PD) is the second most common neurodegenerative disorder, which is mainly characterized by motor dysfunction. The formation of unique and filamentous inclusion bodies called Lewy bodies (LBs) is the hallmark of both PD and dementia with LBs. The key pathology in PD is generally considered to be the alpha-synuclein (α-syn) accumulation, although it is still controversial whether this protein aggregation is a cause or consequence of neurodegeneration. In the present work, the recently published researches which recapitulated the α-syn aggregation phenomena in sporadic and familial PD hiPSC models were reviewed. Furthermore, the advantages and potentials of using patient-derived PD hiPSC with focus on α-syn aggregation have been discussed. [BMB Reports 2019; 52(6): 349-359]

INTRODUCTION

Parkinson's disease (PD) is a progressive, age-related neurodegenerative disease with noteworthy motor impairments, and is the second most common neurodegenerative disease after Alzheimer's disease (AD). PD is primarily linked with the explicit loss of midbrain dopaminergic (mDA) neurons in the substantia nigra pars compacta (SNpc), and physically displays as weakened movements in affected individuals (1, 2). The formation of unique and filamentous inclusion bodies called Lewy bodies (LBs), comprised mostly of alpha-synuclein (α-syn, *SNCA* gene product), is considered as the hallmark of PD or dementia with LBs (DLB) (1-6). Although the key pathology in PD or DLB is commonly known to be the accumulation of misfolded- and aggregated-α-syn (2-7), the formation of pathological α-syn aggregates is not typically displayed in general neurotoxin-based PD animal models (8, 9). In addition, several trials for PD drugs continue to fail; this leads to a significant socioeconomic burden on our healthcare system and emphasizes the need for a new approach to model PD pathogenesis.

Human pluripotent stem cells (hPSCs), including embryonic stem or induced pluripotent stem cells (hESCs/hiPSCs), differentiated into specific types of neurons have emerged as a promising model for studying human neural diseases (10-14) and have the potential to be used as cell sources for transplantation (15, 16). Particularly, disease-specific hiPSCs provide us with an exceptional opportunity to recapitulate human disease phenotypes *in vitro*, thereby enabling disease investigation and drug development; although there are several challenges which need to be addressed [reviewed in (17)]. Using PD patients' hiPSCs, many researchers have tested whether PD-relevant phenotypes are reproduced in the patient-derived mDA neurons *in vitro* to reveal PD pathology and to identify therapeutic targets; however, abnormally phosphorylated detergent-insoluble α-syn aggregates are rarely recapitulated in these model systems [reviewed in (18, 19)].

In this review, it was investigated whether the α-syn aggregation phenomena in PD or DLB can be reproduced in hiPSC-based models, the present extent of development, and the type of further researches necessitated in future.

α-SYNUCLEIN AGGREGATION AND α-SYNUCLEINOPATHY

At the time of initial cloning, α-syn was called as the 'precursor of non-Aβ component of AD amyloid' (precursor of NAC, NACP) because the NAC was first detected in and isolated from AD amyloid plaques (20). α-Syn protein is a soluble protein and exists in the form of an unfolded monomer (21). Furthermore, it has been considered that α-syn undergoes a conformational change to the α-helical structure only upon binding to lipid vesicles (22). As unstructured α-syn monomers tend to eagerly undergo a conformational change to β-sheet structure and aggregate together, it has been hypothesized that

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binding of lipid vesicles with the α-helical conformation of α-syn monomers is a crucial intrinsic mechanism for sequestering unfolded cytosolic α-syn to prevent spontaneous α-syn aggregation. However, recent studies indicate that α-syn exists in the form of an α-helically folded tetramer in the physiological conditions and not as a natively unfolded monomer, and rarely this tetramer is converted into the pathological aggregates (23, 24). The PD missense mutations located in the lipid-binding motif of α-syn increase the transition from tetramer to monomer (25), leading to the formation of β-sheet oligomers and eventually to insoluble aggregates in pathological conditions. A growing number of evidence indicates a causative role of α-syn misfolding and aggregation in the pathogenesis of PD (2-7, 26, 27). α-Synucleinopathies (also called synucleinopathies) are neurodegenerative diseases representing the abnormal accumulation of intracellular aggregates of α-syn in neurons (LB and Lewy neurite) or glial cells (28). Among α-synucleinopathies, PD, DLB, and multiple system atrophy (MSA) are of the most common occurrence. The incident rate of α-synucleinopathies related to parkinsonism was 21.0 per 100,000 person-years (PD, 68% of α-synucleinopathies; DLB, 28%; MSA, 4%), based on the investigation of the medical records in Olmsted County, Minnesota, USA, 1991-2005 (29). Classical neurotoxin-based animal models do not model the molecular pathology of α-synucleinopathies (8, 9). However, many attempts have been made to recapitulate α-synucleinopathies in the mammalian system using α-syn transgenic mouse models, viral vector models of α-syn overexpression, and α-syn transmission models [reviewed in (9)].

PHOSPHORYLATION OF α-SYNUCLEIN

Phosphorylation is the most widely and deeply studied posttranslational modification of α-syn. This modification may affect the ability of aggregate formation as well as the subcellular localization and function of α-syn. In recent years, an increasing number of studies have reported that α-syn within LBs is subjected to phosphorylation at serine 129 (S129), and it may have serious implications for α-syn-induced neurodegeneration (7, 30-34). Especially, S129-phosphorylated α-syn (pS129-α-syn) is an excellent marker for α-synucleinopathies because ~90% of α-syn in LBs is phosphorylated at S129, compared with only ~4% of α-syn under physiological conditions (7). However, the molecular and cellular mechanisms of α-syn aggregation controlled by phosphorylation and other effects of α-syn phosphorylation at S129, remain to be elucidated (35, 36), probably due to a lack of a pathophysiologically relevant model system for investigating α-syn aggregation of α-synucleinopathies. Therefore, it should be clearly addressed whether α-syn phosphorylation is a cause or a consequence of aggregation, or whether phosphorylation is neurotoxic or neuroprotective in α-synucleinopathies including PD and DLB.

HUMAN PLURIPOTENT STEM CELL-DERIVED MIDBRAIN DOPAMINERGIC NEURONS

Developing the most ideal protocol for human mDA neuronal differentiation from hPSCs for applications in PD modeling and/or transplantation therapy has been an intense area of research during the past decade. Arenas *et al*. have reviewed the molecular mechanisms underlying mDA neuronal development *in vivo* and their applications for *in vitro* generation of human mDA neurons differentiated from hPSCs or directly induced from somatic cells [reviewed in (37)]. In this section, popular and major protocols for the human mDA neuronal differentiation from hPSCs are briefly introduced (Fig. 1).

Stromal feeder-induced midbrain dopaminergic neurons

The classical approach to developing a protocol for the human mDA neuronal differentiation from hPSCs was based on adaptations of mouse neural stem cell (mNSC) and mESC protocols, which required co-culture with feeder cells (37). Three groups have published the initial protocols to derive mDA neurons from hESCs co-cultured with the murine stromal cell lines (38-40). In 2007, Sonntag *et al*. reported an enhanced protocol to generate neural rosette-derived mDA neurons from hESCs co-cultured with the MS-5 cells as a feeder, appropriate for cell therapy in PD (41) (Fig. 1A).

Embryonic body-derived midbrain dopaminergic neurons

For initiating spontaneous differentiation from hPSC towards specific cell types, embryonic body (EB)-formation is often considered as a basic starting method. After inducing EB-formation, putative mDA neurons are generated from hESCs (42, 43). In 2009, Swistowski *et al*. published a protocol to derive expandable mDA progenitors and mDA neurons from hESCs *via* EB-formation and neural rosette-isolation (44) (Fig. 1B).

Floor plate-derived midbrain dopaminergic neurons

Studer group introduced a "dual-SMAD inhibition" method for differentiating hPSCs into neural cells in an exceedingly efficient manner to eliminate the influence of undefined factors, including unknown secreted molecules and unidentified effects of co-culturing with murine stromal cell lines or astrocytes, as well as to increase the efficiency and to reduce heterogenous nature on neuronal differentiation (45). Combined blockage of SMAD signaling at the beginning of the monolayer differentiation protocol using Noggin (to inhibit BMP-mediated SMAD signaling) and SB431542 (to inhibit TGF-β/nodal/activin-mediated SMAD signaling), synergistically facilitated neural induction of hPSCs and eliminated the need for feeder layers (45).

The developing midbrain co-expresses the roof plate marker LMX1A and the floor plate (FP) marker FOXA2. Administration

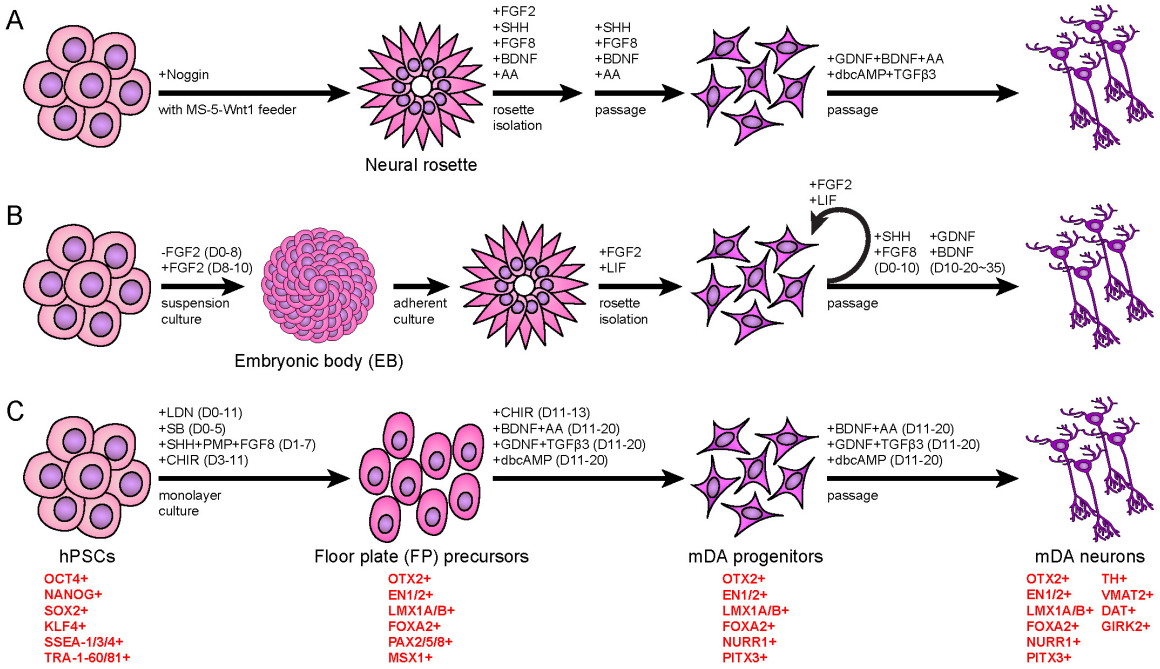

Fig. 1. Derivation of human midbrain dopaminergic neurons from hPSC. (A) A protocol developed by Sonntag *et al*. for the generation of human mDA neurons *via* the neural rosette-isolation, from hESCs co-cultured with the MS-5 cells as a feeder (41). (B) A protocol developed by Swistowski *et al*. for the generation of expandable mDA progenitors and mDA neurons from hESCs *via* the embryonic body (EB)-formation and neural rosette-isolation (44). (C) A protocol developed by Kriks *et al*. for the generation of hPSC-derived mDA neurons *via* the floor plate (FP)-induction (47). AA, ascorbic acid. CHIR, CHIR99021. dbcAMP, dibutyryl-cyclic AMP. LDN, LDN193189. MS-5-Wnt1, Wnt1 transgenic MS-5 cells. PMP, purmorphamine. SB, SB431542.

of high levels of SHH along with the dual-SMAD inhibition during neural induction has been considered as essential for FP specification (46). By synthesizing the existing knowledge of midbrain development, Studer group succeeded in generating correctly specified hPSC-derived mDA neurons in a reliable and efficient manner (47). A follow-up study has described the use of a small molecule, LDN193189 (to inhibit BMP-mediated SMAD signaling), that can replace Noggin for neural induction of hPSCs (48). Modified dual SMAD inhibition (termed ''LSB'' for two inhibitors LDN193189 and SB431542) along with activation of SHH and WNT signaling, enhances the efficiency and reproducibility of the monolayer differentiation of hPSC-derived mDA neurons *via* FP-induction.

GENETIC MODELS USING PATIENT-DERIVED HUMAN INDUCED PLURIPOTENT STEM CELL

Jaenisch group reported the first example of PD modeling using PD patient-derived hiPSC-based DA neurons in 2009, but the focus of the study did not cover the phenotypical differences between patient's cells and healthy controls (49). After the pioneer study, many groups attempted to model PD *in vitro* using patient hiPSC-derived neuronal cells and reported various *in vitro* phenotypes consistent with PD pathophysiology [reviewed in (18, 19)]. While tons of studies failed to repeat the aggregation of α-syn in PD hiPSC-based models, a handful of researches recapitulated α-syn aggregation in PD patient-derived hiPSC-based neuronal models by employing unique conditions (Table 1). It is necessary to state that cases showing simple augmentation of α-syn expression were intentionally omitted in the present review.

Sporadic PD hiPSC-based model

The causes of sporadic PD and DLB are largely unknown; however, the aggregation of α-syn is heavily implicated in the degeneration of neurons in sporadic PD and DLB (50). In 2016, Krainc group reported the detection of the thioflavin S-positive α-syn aggregates in the FP-derived 110 days old mDA neurons, which were differentiated from a sporadic PD hiPSC (51). The authors also showed that the level of pathogenic α-syn species detected by Syn303 antibody (52) was significantly increased in the 1% Triton X-100-insoluble-and-2% SDS-soluble fraction of the sporadic PD mDA neurons, as compared to healthy controls (51). Another report described the increased level of pS129-α-syn in the FP-derived 28-49 days old mDA neurons differentiated from a

Table 1. Selected reports showing the endogenous α-synuclein aggregation using PD hiPSC-derived cell models

Genetic mutation	Tested model	Differentiation protocol (ref.)	Differentiation marker	Stimulus	Aggregate		Cell death	Note	Ref.
					WB	ICC			
Sporadic									
Unknown	mDA	2D-based (45, 47)	n.d.	No	Yes	Yes	n.d.	Increased detergent-insoluble α-syn aggregates at day 110; thioflavin S-positive α-syn aggregates formation at day 110; lysosomal defects at day 90	(51)
SNCA									
p.[Ala53Thr];[=]	mDA GABAergic GLUergic	EB-based (43, 45, 49) ~20% TH^+; ~25% $GABA^+$; ~20% $VGLUT1^+$	*FOXA2*, *NURR1*, *PITX3*, *TH*, *MAP2*, *GAD67*, *VGLUT1* (qRT-PCR); TH, GABA, VGLUT1 (ICC)	No	n.d.	Yes	Yes	Detection of thioflavin S-positive and proteinase K-resistant α-syn aggregates at day 50; increased pS129-α-synat day 50; decreased neurite length at day 50	(54)
p.[Ala53Thr];[=]	mDA	2D-based (45, 47) ~73% TH^+/$GIRK2^+$	OTX2, LMX1A, FOXA2, NURR1, TH, GIRK2 (ICC)	No	n.d.	Yes	No	Increased thioflavin T-positive or S129-phosphorylated α-syn aggregates formation and ROS production than genetically corrected controls at day 35	(55)
				paraquat	n.d.	n.d.	Yes	Increased apoptosis than genetically corrected controls at day 35	
				maneb	n.d.	n.d.	Yes	Increased apoptosis than genetically corrected controls at day 35	
				rotenone	n.d.	n.d.	Yes	Increased apoptosis than genetically corrected controls at day 35	
p.[Ala53Thr];[=]	mDA	2D-based (45, 47)	n.d.	No	n.d.	Yes	n.d.	Detection of thioflavin S-positive α-syn aggregates at day 90; reduced aggregates formation by 758 treatment at day 90	(56)
Duplication	Neuron	EB-based (82)	n.d.	No	Yes	n.d.	n.d.	Neural rosette-derived neurons co-cultured with astrocytes; increased detergent-insoluble α-syn aggregates; increased presence of α-syn aggregation intermediates	(57)
Triplication	Cortical	2D-based (45) ~70% neurons activated by glutamate	Glutamate response (Ca^{2+} imaging)	No	n.d.	Yes	Yes	Increased filamentous α-syn aggregates formation and cell death than genetically corrected controls at day 70-90; increased NADH redox index than control at day 70-90	(58)
Triplication	mDA	EB-based (53) ~28% TH^+/$TUBB3^+$ (83)	*FOXA2*, *TH* (qRT-PCR); FOXA2, TH (ICC)	No	n.d.	Yes	n.d.	Neural rosette-derived mDA; increased number and size of punctate α-syn aggregates at day > 71 than at day 45-50	(59)
	BFCN	EB-based (84, 85) ~36% $VCHT^+$/$TUBB3^+$ (83)	*CHAT* (qRT-PCR); CHAT (ICC)	No	n.d.	Yes	n.d.	Neural rosette-derived BFCN; increased number and size of diffused α-syn aggregates at day > 71 than at day 45-50	

Table 1. Continued 1

Genetic mutation	Tested model	Differentiation protocol (ref.)	Differentiation marker	Stimulus	Aggregate		Cell death	Note	Ref.
					WB	ICC			
Triplication	mDA	2D-based (45, 47) ~70% TH^+/ $FOXA2^+$	LMX1A, FOXA2, TH (ICC)	No	Yes	Yes	n.d.	Increased detergent-insoluble α-syn aggregates at day 55-330; thioflavin S-positive α-syn aggregates formation at day 100 or 120; increased low expressing TH cells at day 330; lysosomal defects at day 180 or 330; reduced aggregates formation by 758 treatment at day 120	(51, 56)
LRRK2									
p.[Gly2019Ser];[=]	Astrocyte	3D-based (86) ~95% $GFAP^+$	*AQP4*, *GFAP* (qRT-PCR); GFAP, S100β, GLT1 (ICC)	No	n.d.	Yes	n.d.	Increased α-syn puncta area at day 28; transmitting α-syn to mDA neurons during 28 days co-culture	(61)
PRKN									
p.[Arg42Pro];[=]	mDA	EB-based (44) ~7% TH^+	FOXA2, TH (ICC)	No	u.c.	u.c.	n.d.	Decreased mDA neuronal differentiation than healthy control (~22% TH^+) at day 28	(62)
p.[Arg275Trp];[=]	mDA	EB-based (44) ~15% TH^+	FOXA2, TH (ICC)	No	u.c.	u.c.	n.d.	Decreased mDA neuronal differentiation than healthy control (~22% TH^+) at day 28	(62)
p.[Arg42Pro];[EX3del]	mDA	EB-based (44) ~7% TH^+	FOXA2, TH (ICC)	No	u.c.	u.c.	n.d.	Decreased mDA neuronal differentiation than healthy control (~22% TH^+) at day 28	(62)
p.[Asn52fs];[EX3_4del]	mDA	EB-based (44) ~7% TH^+	FOXA2, TH (ICC)	No	u.c.	u.c.	n.d.	Decreased mDA neuronal differentiation than healthy control (~22% TH^+) at day 28	(62)
p.[Val324fs];[Val324fs]	mDA	2D-based (45, 47) ~70% TH^+	LMX1A, FOXA2, NURR1, TH (ICC)	No	Yes	u.c.	n.d.	Increased Triton X-100-insoluble α-syn aggregates at day 60; increased intracellular dopamine level; increased susceptibility to mitochondria toxin at day 60	(63)
PINK1									
p.[Gln456Ter];[Gln456Ter]	mDA	2D-based (45, 47) ~75% TH^+	LMX1A, FOXA2, NURR1, TH (ICC)	No	Yes	u.c.	n.d.	Increased Triton X-100-insoluble α-syn aggregates at day 60; increased susceptibility to mitochondria toxin at day 60	(63)
ATP13A2									
p.[Leu1059Arg];[Leu1085fs]	mDA	2D-based (45, 47) ~60% TH^+/ $FOXA2^+$	FOXA2, TH (ICC)	No	Yes	n.d.	n.d.	Increased detergent-insoluble α-syn aggregates at day 90; lysosomal defects at day 90	(56)

sporadic PD hiPSC, although the authors did not assess the formation of α-syn aggregates in these neurons (53).

Familial PD hiPSC-based model: *SCNA* mutations

Mutations in *SCNA* (also known as *PARK1* and *PARK4*) gene, which encodes α-syn, lead to autosomal dominant PD (1). The thioflavin S-positive and proteinase K-resistant α-syn

Table 1. Continued 2

Genetic mutation	Tested model	Differentiation protocol (ref.)	Differentiation marker	Stimulus	Aggregate		Cell death	Note	Ref.
					WB	ICC			
GBA									
p.[Asn409Ser];[Leu29fs]	mDA	2D-based (45, 47) ~60% $TH^+/FOXA2^+$	LMX1A, FOXA2, TH (ICC)	No	Yes	Yes	n.d.	Increased detergent-insoluble α-syn aggregates at day 90; thioflavin S-positive α-syn aggregates formation at day 100 or 120; increased low expressing TH cells at day 330; lysosomal defects at day 110, 120 or 180; reduced aggregates formation by 758 treatment at day 120	(51, 56)
p.[Asn409Ser];[=]	mDA	2D-based (45, 47) ~75% TH^+	*FOXA2*, *NURR1*, *TH* (qRT-PCR); FOXA2, PITX3, TH (ICC)	No	u.c.	n.d.	No	Reduced ratio of tetramers and related multimers, which resist aggregation, to monomers at day 60-65	(71)
				α-PFF	Yes	n.d.	Yes	Increased detergent-insoluble α-syn aggregates at day 73; increased pS129-α-syn at day 73; increased cell death at day 73 or 79	

2D, monolayer differentiation. 3D, neurosphere differentiation. 758, NCGC00188758. α-PFF, α-syn pre-formed fibril (72). BFCN, basal forebrain cholinergic neuron. cortical, cortical neuron. del, deletion. EB, embryonic body differentiation. EX, exon. fs, frameshift. GABAergic, GABAergic neuron. GLUergic, glutamatergic neuron. ICC, confirmed by immunocytochemistry. mDA, midbrain dopaminergic neuron. n.d., not determined. NPC, neuronal precursor cell. p., protein sequence. qRT-PCR, quantitative real-time PCR. u.c., unclear. WB, confirmed by western blotting.

aggregates were detected in the EB-derived 50 days old neurons differentiated from a familial PD hiPSC with a point mutation in *SNCA* gene (c.[157G>A];[=]/p.[Ala53Thr];[=], also known as heterozygous A53T) (54). Moreover, the level of pS129-α-syn was increased in the A53T neurons (54). Ryan *et al*. also reported an increase in thioflavin T-positive or Ser129-phosphorylated α-syn aggregates formation and ROS production in the FP-derived 35 days old A53T mDA neurons, as compared to genetically corrected controls (55). The authors have further revealed the susceptibility of A53T mDA neurons to apoptosis induced by environmental pesticides, such as paraquat, maneb, and rotenone, compared to the controls (55). The presence of thioflavin S-positive α-syn aggregates in the FP-derived 90 days old A53T mDA neurons has been demonstrated along with the reduction in aggregate formation upon treatment with β-glucocerebrosidase (GCase) activator, NCGC00188758 (56).

Copy number variations in *SNCA* locus are also linked to familial PD [duplication (5), triplication (4)]. Using a familial PD hiPSC with *SNCA* locus duplication, Prots *et al*. reported a significant increase in the level of 1% Triton X-100/1% NP-40/1% SDS-insoluble-and-8 M urea/5% SDS-soluble α-syn aggregates in the EB-derived PD neurons co-cultured with primary human cerebellar astrocytes, as compared to healthy controls (57). The authors performed sucrose density gradient centrifugation analysis to assess the composition of α-syn aggregates and revealed an increase in the presence of α-syn aggregation intermediates in the PD neurons (57). Other report exhibited the increased formation of filamentous α-syn aggregates and cell death in the 70-90 days old cortical neurons differentiated from a familial PD hiPSC with *SNCA* locus triplication, as compared to genetically corrected controls (58). In addition, Tagliafierro *et al*. described an increase in the number of punctate α-syn aggregates in the EB-derived 45-50 days old mDA neurons differentiated from a familial PD hiPSC with *SNCA* locus triplication compared to the controls (59). Moreover, the increment and the size of punctate α-syn aggregates were more augmented in > 70 days old PD mDA neurons, as compared to 45-50 days old neurons (59). The authors have additionally shown an increase in the formation and size of diffused α-syn aggregates in > 70 days old PD basal forebrain cholinergic neurons (BFCNs), which are primarily affected in DLB, compared to 45-50 days old neurons (59). Mazzulli *et al*. reported the detection of thioflavin S-positive α-syn aggregates in the FP-derived 100 or 120 days old mDA neurons differentiated from a familial PD hiPSC with *SNCA* locus triplication (51, 56). In addition, the amount of pathogenic α-syn detected by Syn303 antibody was increased in the 1% Triton X-100-insoluble-and-2% SDS-soluble fraction of 55-330 days old PD mDA neurons carrying an *SNCA* locus triplication than healthy hiPSC-derived mDA neurons (51). Another report described the increased level of

pS129-α-syn in the FP-derived 44-46 days old mDA neurons and revealed the increased susceptibility to neurotoxins in 34 days old mDA neurons, differentiated from a familial PD hiPSC with *SNCA* locus triplication compared to healthy hiPSCs (53).

Familial PD hiPSC-based model: *LRRK2* mutations

Mutations in *LRRK2* (also known as *PARK8*) gene, which encodes leucine-rich repeat serine/threonine-protein kinase 2, lead to autosomal dominant PD (60). Even though an attempt was made to analyze numerous articles which attempted to recapitulate PD phenotypes using PD hiPSCs with *LRRK2* mutations, none of the reports provided solid evidence showing α-syn aggregation in patient-derived mDA neurons. Instead, one recently published paper reported an increase in α-syn puncta in 28 days old PD astrocytes with a point mutation in the *LRRK2* gene (c.[6055G>A];[=]/p.[Gly2019Ser];[=], also known as heterozygous G2019S), as compared to healthy astrocytes (61).

Familial PD hiPSC-based model: *PRKN* mutations

Mutations in *PRKN* (also known as *PARK2*) gene, which encodes E3 ubiquitin-protein ligase parkin, lead to autosomal recessive PD (60). Shaltouki *et al*. demonstrated the formation of tentative α-syn aggregates in the EB-derived 28 days old mDA neurons differentiated from familial PD hiPSCs with various mutations in *PRKN* gene; i.e., p.[Arg42Pro];[=], p.[Arg275Trp];[=], p.[Arg42Pro];[*EX3del*], and p.[Asn52fs];[*EX3_4del*] (62). On the other hand, Chung *et al*. reported a significant increase in the level of 1% Triton X-100-insoluble-and-2% SDS-soluble α-syn aggregates in the FP-derived 60 days old mDA neurons differentiated from a PD hiPSC with same point mutations in both the *PRKN* alleles (c.[971delT];[971delT]/p.[Val324Alafs*111];[Val324Alafs*111], also known as homozygous V324fs), as compared to healthy controls (63). Furthermore, the researchers provided evidence of the dependency on the type of differentiation protocol utilized; the FP-derived PD mDA neurons recapitulated PD phenotypes *in vitro* while the neural rosette-derived PD mDA neurons (40) did not recapitulate these phenotypes (63).

Familial PD hiPSC-based model: *PINK1* mutations

Mutations in *PINK1* (also known as *PARK6*) gene, which encodes serine/threonine-protein kinase PINK1 mitochondrial, lead to autosomal recessive PD (60). In the FP-derived 60 days old mDA neurons differentiated from a PD hiPSC with point mutations in the *PINK1* gene (c.[1366C>T];[1366C>T]/p.[Gln456Ter];[Gln456Ter], also known as homozygous Q456X), the level of 1% Triton X-100-insoluble-and-2% SDS-soluble α-syn aggregates was significantly increased compared to healthy controls; however, it was not observed in the neural rosette-derived PD mDA neurons (63).

Familial PD hiPSC-based model: *ATP13A2* mutations

Mutations in *ATP13A2* (also known as *PARK9*) gene, which encodes cation-transporting ATPase 13A2, lead to autosomal recessive PD (60). Mazzulli *et al*. reported that the amount of 1% Triton X-100-insoluble-and-2% SDS-soluble α-syn was increased in the FP-derived 90 days old mDA neurons differentiated from a familial PD hiPSC with point mutations in *ATP13A2* gene (c.[3176T>C];[3253delC]/p.[Leu1059Arg];[Leu1085Trpfs*4], also known as heterozygous L1059R;L1085fs) compared to healthy control (56).

PD-related hiPSC-based model: *GBA* mutations

GBA (also known as *GBA1*) gene, which encodes lysosomal acid glucosylceramidase (also known as β-glucocerebrosidase or GCase), has a critical role in glycolipid metabolism. Loss-of-function mutations in the *GBA* gene cause lysosomal defects *via* accumulation of lipid substrates in the lysosome that results in autosomal recessive Gaucher disease (GD) (64). Moreover, mutations in the *GBA* gene are well-known to increase the risk of developing PD and DLB (65-70). Using a GD hiPSC with mutations in the *GBA* gene (c.[1226A>G];[84dupG]/p.[Asn409Ser];[Leu29Alafs*18], also known as heterozygous N409S;L29fs), Mazzulli *et al*. reported the detection of thioflavin S-positive α-syn aggregates in the FP-derived 100 or 120 days old GD mDA neurons (51, 56). In addition, the authors demonstrated that the amount of pathogenic α-syn detected by Syn303 antibody was increased in the 1% Triton X-100-insoluble-and-2% SDS-soluble fraction of the 90 days old GD mDA neurons compared to healthy hiPSC-derived mDA neurons (51).

Groundbreaking researches suggested that α-syn exists as a helically folded tetramer in the physiological conditions and not in the form of natively unfolded monomer; the tetramer is rarely gets converted into the pathological aggregates (23, 24). A recent study has reported that the ratio of α-syn tetramers and related multimers, which resist aggregation, to monomers was reduced in the FP-derived 60-65 days old mDA neurons differentiated from a PD hiPSC with a point mutation in *GBA* gene (c.[1226A>G];[=]/p.[Asn409Ser];[=], also known as heterozygous N409S) compared to healthy iPSCs; indirectly revealing that the level of α-syn aggregates may be increased in the cells of the patient (71). The authors also revealed an increase in the levels of 1% Triton X-100-insoluble-and-2% SDS-soluble α-syn aggregates and pS129-α-syn in the α-syn pre-formed fibril [α-PFFs (72)]-treated FP-derived 73 days old PD mDA neurons compared to healthy controls (71).

PERSPECTIVES

It is feasible to reproduce the molecular pathology of α-synucleinopathies without using any exogenous factors and by employing only hiPSC-based PD modeling system along with the patient's genetic background. However, only a handful of papers demonstrated success in recapitulating α-syn

aggregation *in vitro*, among a large number of PD hiPSC-based models (Table 1). It is hypothesized that there might be three potential underlying causes of this problem: i) insufficient aging, ii) incomplete differentiation protocol, and iii) lack of environmental cues.

Even though hiPSCs were generated by reprogramming of somatic cells from aged PD patients, their ages seemed to be reset to embryonic ages during the reprogramming process (73-76). Moreover, hiPSC-derived neurons, such as human mDA neurons, are largely regarded as embryonic stage neurons (73-76). Therefore, resolving an aging issue and/or a maturation issue is necessary to model late-onset diseases, such as PD and DLB. Apparently, the progerin-induced aging method could be used as one of the possible solutions (77).

We are constantly attempting to improve mDA neuronal differentiation protocols for modeling PD more complete, beyond EB-derived, feeder-dependent, neural rosette-derived, and FP-derived methods (Fig. 1). Very recently, the protocols to differentiate hPSCs or neuroepithelial stem cells into human midbrain organoids have been developed for the generation of more ideal human mDA neuronal models (78-80). These midbrain organoids will serve as a completer and more important biologically relevant cell sources for PD modeling.

Environmental factors may cause PD along with genetic factors. Peng *et al*. demonstrated that environmental factors, such as neurotoxins, together with genetic mutations in PD in the same animal, synergistically accelerate age-related neurodegeneration (81). Similarly, only after treatment of α-PFFs, PD-relevant phenotypes including increased levels of detergent-insoluble α-syn aggregates and pS129-α-syn were detected in mDA neurons differentiated from PD hiPSC with a *GBA* mutation (p.N409S) (71). These results suggest that the addition of environmental factors to the PD iPSC models might be the key to reveal the hidden phenotypes.

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CONFLICTS OF INTEREST

The author has no conflicting interests.

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