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Review

Using induced pluripotent stem cell neuronal models to study neurodegenerative diseases

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ABSTRACT

Current application of human induced pluripotent stem cells (hiPSCs) technology in patient-specific models of neurodegenerative disorders recapitulate some of key phenotypes of diseases, representing disease-specific cellular modeling and providing a unique platform for therapeutics development. We review recent efforts toward advancing hiPSCs-derived neuronal cell types and highlight their potential use for the development of more complex *in vitro* models of neurodegenerative diseases by focusing on Alzheimer's disease, Parkinson's disease, Huntington's disease and Amyotrophic lateral sclerosis. We present evidence from previous works on the important phenotypic changes of various neuronal types in these neurological diseases. We also summarize efforts on conducting low- and high-throughput screening experiments with hiPSCs toward developing potential therapeutics for treatment of neurodegenerative diseases. Lastly, we discuss the limitations of hiPSCs culture system in studying neurodegenerative diseases and alternative strategies to overcome these hurdles.

1. Introduction

Neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS), are featured by progressive loss of neuronal structure and function which leads to movement disorder and cognitive impairment. Despite great progress in understanding the etiology of neurodegenerative diseases, the pathogenesis underlying these disorders remains elusive and there is no effective treatment for these devastating diseases. While transgenic and knock-in animal models of neurodegenerative diseases are extensively used and have yielded important insights into pathogenesis of these diseases, the animal models may be insufficient to capture the complexity of human disease, given that large differences in neuroanatomy and distribution of neuronal cell types within the brain exist between rodent and human. Moreover, animal models are difficult to be used for a large scale of drug screening. In addition, the lack of access to well-characterized living and postmortem patient brain tissue has made the study of neurodegenerative diseases progressed slowly.

Human induced pluripotent stem cells (hiPSCs) now overcome some limitations of rodent animal models and provide a unique human-based culture system to permit disease-in-a-dish modelling and analysis of the phenotypic characteristics of neurological pathologies. Moreover, hiPSCs have great self-renewal and differentiation capacity and can produce a large number of patient-specific hiPSCs harboring genetic variations implicated in disease, which make it possible for low- and high-throughput screening to discover and evaluate the efficacy and safety of former drugs and novel therapeutics. In this review, we discuss previous findings from hiPSCs-based cellular models of neurodegenerative diseases by focusing on AD, PD, HD and ALS. We also present evidence on therapeutics development by utilizing the cellular cultures of patient iPSCs. Finally, the experimental challenges and limitations of using hiPSC-based models for studying neurodegenerative diseases are also discussed.

2. Induced pluripotent stem cell model

2.1. Generation and development of iPSCs

In 2006, Takahashi and Yamanaka reported the conversion of adult mouse fibroblasts to iPSCs by expression of four transcriptional factors including c-Myc, Sox2, Klf4 and Oct3/4 [1]. In the following year, Yamanaka and his colleagues successfully derived iPSCs from human fibroblasts [2], which was the first time that human somatic cells were

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converted to the embryonic stem cell state. The basic features of iPSCs are similar with human embryonic stem cells (hESCs). They both express pluripotency markers, are self-renewable and can differentiate into cells of all three germ layers [2,3]. Importantly, the iPSCs have advantages over hESCs: 1) they do not raise any ethical concerns which limit the practical application of hESCs; 2) they also do not have immunologic incompatibility between donors and recipients, and can be derived from patients specifically [4]. Subsequently, the technology of generation of iPSCs was rapidly used in various fields of research. To date, researchers have developed methods to establish iPSCs from numerous somatic cell sources including dermal fibroblasts, adipocyte, hematopoietic stem cells and peripheral blood mononuclear cells [5–10]. In addition to viral transduction of reprogramming factors to generate iPSCs, RNA viruses [11], virus-free DNA [12,13], RNAs [14], proteins [15] and a cocktail of chemical compounds [16] have been used to replace integrative virus to enhance the genetic stability and safety of iPSCs [17–19]. In addition, modified messenger RNA (mRNA) encoding reprogramming factors has been explored [14,20,21] to improve the efficiency of iPSCs generation. These strategies strengthen the application of hiPSCs in studying human diseases and developing therapeutics.

2.2. Induction of brain cells from iPSCs

Differentiation of iPSCs into candidate cellular lineages is the key step to recapitulate disease phenotypes. Neuronal induction was first achieved by overexpressing three transcriptional factors (*Brn2*, *Ascl1* and *Myt11*) in mouse fibroblasts [22]. The same group later showed that the three factors, when combined with the basic helix-loop-helix transcriptional factor *NeuroD*1, could generate functional neurons from hiPSCs [23]. Moreover, the neurons differentiated from hiPSCs were able to generate action potentials and matured to receive synaptic contacts when co-cultured with primary mouse cortical neurons [23]. In a parallel study, Zhang et al. showed that hESCs and hiPSCs can be converted into functional neuronal cells with nearly 100% yield and purity in < 2 weeks by forced expression of a single transcription factor *neurogenin 2* (*NGN2*) [24]. The resulting neuronal cells exhibited quantitatively reproducible properties independent of the cell line of origin, formed mature pre- and post-synaptic specializations, and integrated into existing synaptic networks when transplanted into mouse brain [24]. To study neurodegenerative diseases, specific neuronal subtypes have been induced through the addition of cell type specific transcriptional factors, growth factors, and even chemical cocktails that suppress Wnt signaling [25]. More recently, human astrocytes, microglia and oligodendrocytes have also been induced *in vitro* and applied to modeling the diseased cells related neurological disorders. The methods and research progress have been summarized by a number of other reviews [26–29].

2.3. Brain organoid in a dish

Single cell culture or 2-dementioanl (2D) neuronal culture have rapidly deepened our understanding of neuronal development and function in diseases. However, the 2D *in vitro* culture may not be sufficient to reproduce complex of the nervous system; lack of the ability to model neuronal architecture and network connectivity of human brain is an issue. Within the past few years, 3-dimentioal (3D) neuronal culture systems or brain organoid culture systems have been generated and provided a new platform to investigate human brain development. The first neuronal organoid-like structure was derived from telencephalic precursors of mouse embryonic stem (ES) cells using optimized serum-free suspension culture (SFEB culture) [30]. Importantly, the authors showed that the organoid-like structure could self-organize into polarized neuro-epithelial structures and acquire sub-regional identities by responding to extracellular patterning signals [30]. Lancaster et al. generated large and complex cerebral organoids by seeding neuro-ectodermal aggregates embedded in Matrigel droplets in a spinning bioreactor to enhance nutrient absorption [31]. Moreover, cerebral organoids were shown to recapitulate features of human cortical development [31]. In order to reduce the large volumes and space placing the spinning bioreactor approach, Qian et al. further developed a miniaturized spinning bioreactor (SpinΩ) to generate forebrain-specific organoids from human iPSCs. These organoids recapitulated key features of human cortical development, including progenitor zone organization, neurogenesis, gene expression, and, notably, a distinct human-specific outer radial glia cell layer [32]. The brain-region-specific organoids and SpinΩ thus provide an accessible and versatile platform for modeling human brain development and diseases [32]. Subsequently, a number of groups have generated organoids of cortical brain [33], midbrain [34], cerebellum [35] and neural tube [36], which have been reviewed by others [37–39]. However, whether these organoids can be really applied to study neuronal development and to model disease remains to be investigated.

3. Modeling neurodegenerative disease *in vitro* with patient-specific iPSCs

Neurons derived from hiPSCs have been used to model various forms of neurodegenerative disorders, with Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and Amyotrophic lateral sclerosis (ALS) among the most extensively studied. These *in vitro* patient-related culture models deepen our understanding on the pathological mechanisms underlying neurodegenerative diseases and provide insights for new therapeutic strategies. Below we summarize the reported phenotypic changes of iPSCs modeling of neurodegenerative diseases (see Table 1).

3.1. Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disorder and is pathologically characterized by neuronal death, neurofibrillary tangles and β-amyloid plaques in cerebral cortex and hippocampus. AD includes familial AD (FAD) and sporadic AD (SAD). FAD and SAD are similar clinically, both of which are characterized by progressive cognitive decline and memory loss. Accumulation of amyloid β peptide (Aβ) is proposed to initiate the pathogenic cascade that ultimately leads to AD. Aβ is produced by β- and γ-secretase-mediated sequential proteolysis of amyloid precursor protein (APP) and plays a central role in AD pathogenesis. FAD has an early onset of disease and accounts for < 5% of all AD cases. It is primarily due to rare autosomal dominant mutations in the amyloid precursor protein (*APP*) gene and in the presenilin genes, *PSEN1* and *PSEN2*. Both PSEN1 and PSEN2 are required in the formation of a γ-secretase complex which functions to cleave APP, leading to Aβ formation [40].

AD patient-derived iPSCs were firstly generated from patients carrying mutations in *PS1 (A246E)* and *PS2 (N141I)*. Neurons differentiated from these iPSCs with *PS1* and *PS2* mutations have increased amyloid-β42 (Aβ42) secretion which recapitulates the molecular pathogenesis of FAD. Moreover, secretion of Aβ42 from the neurons derived patient iPSCs responded to the treatment of compound E, a γ-secretase inhibitor [41]. Further, Sproul et al. generated iPSCs from 6 patients carrying *PS1* mutations and found that in addition to producing higher ratio of Aβ42/Aβ40 in neurons derived from these patient iPSCs, there was a significant change in gene expression pattern in the neurons derived iPSCs of *PS1* mutant patients, when compared to that from control subjects [42]. These findings indicate the potential application of the iPSCs in FAD modeling. Further, Israel et al. compared neurons derived from two patients with FAD caused by a duplication of the APP gene (APP^{Dp}), two patients with SAD and two non-demented control subjects [43]. The purified cultures contained > 90% neurons that all exhibited normal electrophysiological activity. Compared with controls, iPSC-derived neurons from the two FAD patients and one SAD

Table 1
Neuronal phenotypes of patient iPSCs of neurodegenerative diseases and therapeutic options.

Model	Genetic mutation	Neuronal phenotypes	Therapeutic options
Alzheimer's disease	*PS1 (A246E)* and *PS2 (N141I)*	Increased Aβ42 secretion and Aβ42/40 ratio [41,42]	Compound E, an γ-secretase inhibitor [41]
	PS1 mutations	Increased production of endogenous Aβ40 and increased Aβ42/40 ratio [141], APP processing, phosphor-tau, and activated GSK-3β [53,159]; diminished autophagy degradation, lysosomal abnormalities, impaired mitophagy [160]; increased content of BRCA1(Ser1524) [161]	
	$PSEN2^{N141I}$ mutation	Increased Aβ42, enhanced ratio of Aβ42/40, elevated phosphor-Tau and GSK3β, impaired insulin signaling [162]	
	APP^{Dp}	Increased Aβ40, phosphor-tau, and activated GSK-3β, increased RAB5-positive endosomes [43]	β-Secretase inhibitors (βSi-II and OM99-2) [43]
	APP-E693Δ	ER stress and oxidative stress, increased apoptotic markers [45]	Docosahexaenoic acid (DHA) [45]
	APPV717I	Increased Aβ42, APP processing, enhanced tau phosphorylation [163]	Aβ antibody [163]
	ApoE4	Increased tau phosphorylation, GABAergic neuron degeneration [50]; increased synapse number and elevated Aβ42 secretion in neurons whereas impaired Aβ uptake and cholesterol accumulation in astrocytes [164,165]	PH002, an ApoE structure corrector [50]; HDAC3 inhibitor [165]
	Sporadic	Increased Aβ42, elevated ratio of Aβ42/40, oxidative stress [45]; hyper-phosphor Tau and activated GSK3β [43,54]	Cdk2 inhibitor [142]; an anti-Aβ cocktail (bromocriptine, cromolyn, and topiramate) [143];
Parkinson's disease	LRRK2 mutation (G2019S)	Oxidative stress and increased amount of α-synuclein, sensitive to caspase-3 activation and cellular stressors [58,66]; reduced numbers of neurites and neurite arborization, and impaired autophagy [63]; impairment of mitochondrial respiration [59], mtDNA [61] and trafficking [62]; excessive mitochondrial fragmentation, enhanced autophagy and neurite shortening of DA neurons [60].	Coenzyme Q(10), rapamycin, or the LRRK2 kinase inhibitor GW5074 [59]; P110, a peptide inhibitor of Drp1-Fis1 [60]
	α-Synuclein A53T	Nitrosative stress and ER stress [70,71], and mitochondrial damage and impaired mitophagy [73]; α-synuclein aggregation [76], and phosphorylation [85]	Isoxazole [125]
	α-Synuclein triplication	Oxidative stress [79]; lower lysosomal degradation capacity [80]; reduced capacity to differentiate into DA or GABAergic neurons, decreased neurite outgrowth and lower neuronal activity [81]; ER stress [82] and α-synuclein phosphorylation [85]	
	PINK1	Impaired recruitment of Parkin to mitochondria, increased mitochondrial copy number, and upregulated PGC-1α [87]. Decreased mitochondrial membrane potential [87,88], mitochondrial complex I activity [89,90], and deficient mitochondrial trafficking [62]	Rapamycin and GW5074 [59]
	Parkin	Decreased DA uptake and increased spontaneous DA release [91]; Oxidative stress, α-synuclein accumulation [92]; abnormalities in endosomal processes and trafficking [93]; disrupted calcium shuttling between mitochondria and ER [94]; enhanced sensitivity to metal toxins [95,96]; abnormal neurite outgrowth and complexity [97]	
	GBA	Elevated α-synuclein protein levels, reduced capacity to synthesize and release dopamine, increased monoamine oxidase B [166,167]; ER stress and abnormal cellular lipid profile, impaired autophagy and lysosome activity [167]	
Huntington's disease	mtHtt	Mitochondrial dysfunction and enhanced caspase activity upon growth factor deprivation [102–104]; mitochondrial fragmentation and neurite shortening of medium spiny neurons [145,146]; DNA damage [148]; Increased vulnerability to stress/toxicity [104]; lysosomal dysregulation and impaired cholesterol biosynthesis pathway [106,107]; oxidative stress and reduced cytoskeleton-associated proteins [108].	P110, a peptide inhibitor of Drp1-Fis1 interaction [145]; HV-3, a peptide blocker of mtHtt-VCP interaction [146]; bexarotene, a potent retinoid X receptor agonist [147]; KU55933, an ATM protein inhibitor [148]; CGS-21680 and APEC, adenosine receptor 2A agonists [149].
ASL	SOD1	Reduced soma size and altered dendrite length of motor neurons, and dysregulated neurofilaments [119]; impaired mitochondrial function and structure [120]; ER stress [120] and neuronal hyperexcitability [121];	Retigabine, a clinically approved anticonvulsant [121]
	TDP-43	Cytosolic aggregates of TDP-43 and shorter neurites of motor neurons [133–135]; increased vulnerability in a variety of stressors [134,135], mitochondrial fragmentation and mitochondrial bioenergetics deficiency [136].	PM1, a peptide inhibitor of TDP-43 mitochondrial localization [136]; Digoxin [137]; anacardic acid, a histone acetyltransferase inhibitor [44]
	C9ORF72	Increased transcription of C9ORF72, accumulation of GGGGCC repeat-containing RNA foci, susceptibility to excitotoxicity [124–127]; impaired endosomal trafficking and autophagy [129–131]	Antisense oligonucleotide (ASO) to the C9ORF72 [124,126,150]

patient exhibited significantly higher levels of the pathological markers Aβ40, phosphor-tau, and activated glycogen synthase kinase-3b (GSK-3β). These neurons were sensitive to treatment with β-secretase inhibitors, which caused significant reduction of phosphor-tau and GSK-3β levels. This study also extended phenotypic characterization by looking at endosomal and synaptic markers since AD severity is known to be associated with synaptic loss. Neurons derived from FAD patient-iPSCs showed increased RAB5-positive early endosomes. However,

there were no differences in the synaptic marker synapsin-1 in mutants, which is contrast with other study showing the reduction of synapsin-1 in AD patient brains [44]. Further, Kondo et al. compared 7 types of *APP* mutations including *APP* E693 deletion and *APP* V717L mutation [45]. However, these cell lines did not consistently replicate the same phenotypes with differential manifestation of ER stress, oxidative stress and Aβ oligomer accumulation. Moreover, one candidate AD compound, docosahexaenoic acid (DHA), was shown to only rescue some types of AD neurons and showed no effect on others. This raises concerns regarding the inherent variability of iPSCs and highlights the need for further refining of AD modeling using iPSCs.

Adults with Down syndrome (caused by trisomy of chromosome 21) develop early-onset Alzheimer's disease, probably due to increased expression of *APP* encoded by a gene on chromosome 21. Thus, Down syndrome patients have a predilection to develop AD [46]. The cortical neurons generated from iPSCs of patients with Down syndrome exhibited neuronal Aβ secretion, insoluble intracellular and extracellular amyloid aggregates, and tau hyper-phosphorylation and altered localization [47], the phenotypes of which recapitulate later stages of AD pathogenic process. Interestingly, these phenotypes could be observed within months instead of years and were free from spontaneous mutations introduced by cellular reprogramming [47]. In contrast, another study has recently showed that, though cortical neurons derived from iPSCs of Down syndrome exhibited increased Aβ42 production, altered Aβ42/40 ratio and plague formation due to an increased APP gene expression, tau-related AD phenotypes and apoptotic markers were lack [48]. These findings challenge the idea that increased APP level is required for tau pathology and enhanced neuronal cell death in Down syndrome-associated AD pathogenesis.

Apolipoprotein (Apo) E4 is a strong genetic risk factor for aging-related cognitive decline as well as late-onset AD [49]. Neurons derived from iPSCs of ApoE4 carriers had higher levels of tau phosphorylation, unrelated to their increased production of Aβ peptides, and they also displayed GABAergic neuron degeneration [50]. Treatment of ApoE4-expressing neurons with a small-molecule structure corrector ameliorated the detrimental effects, suggesting that correcting the pathogenic conformation of ApoE4 is a valuable therapeutic approach for ApoE4-related AD [50]. In addition to neurons, iPSC-derived astrocytes have been generated from carriers of *APOE* ε4, given the natural function of ApoE4 in astrocyte [51]. These astrocytes showed an increased ApoE lipoprotein secretion, and impaired neurotrophic support when co-cultured with iPSC-derived neurons, as compared to astrocytes derived from *APOE* ε3 carriers [52].

To date, only few of studies has utilized iPSCs from patients with SAD. Two early studies have compared the levels of Aβ between neurons derived from SAD and APP mutation FAD patient lines [43,45]. The cellular phenotypes in SAD patient iPSCs-derived neurons appeared similar to those in FAD patients derived neurons; cells exhibited increased Aβ levels [43,45], altered Aβ42/40 ratios and increased APP expression [53]. However, the authors also reported that these changes are not consistent in all SAD patients [43,45], which is likely owing to the complex disease pathogenesis of the SAD. Further, using mixed neuronal culture derived from iPSCs of an 82-year old SAD patient, Hossini et al. characterized an AD-related protein interaction network composed of APP and GSK3β among others [54]. Moreover, transcriptome analysis of the SAD-iPSCs derived neuronal cells revealed significant changes in the expression of genes associated with AD and with the constitutive as well as the inducible subunits of the proteasome complex [54]. However, the study only used iPSCs derived from one patient. Whether the reported changes are consistent in other SAD patient iPSCs remains to be validated.

3.2. Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease. Patients experience progressive motor dysfunction, such as tremors, bradykinesia, rigidity and gait disturbance. Pathological changes of PD are the loss of dopaminergic (DA) neurons in the pars compacta region of the Substantia Nigra (SN) and the presence of cytoplasmic inclusion (Lewy bodies, LB), containing α-synuclein [55]. A great advantage of the iPSCs model system for studying PD is to generate middle brain DA neurons from PD patients with defined genetic backgrounds, which allows to characterize morphological and functional deficiencies of the vulnerable neuronal type in PD. Familial (including *LRRK2*, *SNCA*, *PINK1*, and *PARK2* mutation) and idiopathic PD iPSCs have been generated and differentiated into DA neurons.

Mutations in Leucine-rich repeat kinase 2 (LRRK2) are associated with sporadic and familial PD [56]. While over 50 variants have been identified throughout the different LRRK2 domains in PD patients, the mutation G2019S (Gly2019 to Ser) that takes place in the MAPKKK domain has been recognized as the most common cause of dominant familial PD and accounts for up to 2% of sporadic PD cases [57]. Nguyen et al. first generated iPSCs from a patient carrying LRRK2 G2019S mutation and showed that DA neurons differentiated from the LRRK2 G2019S patient iPSCs displayed abnormal accumulation of α-synuclein, increased oxidative stress-related genes and highly susceptibility to chemical stressors [58]. Moreover, neurons derived from the same LRRK2 mutation or R1441C mutation exhibited mitochondrial dysfunction [59,60], mitochondrial DNA (mtDNA) damage [61], abnormal retention of Miro [62], enhanced autophagy and abnormal neurite outgrowth [59,61,63]. In addition, neurons generated from patient with LRRK2 mutation had DA neuron-specific hypermethylation and altered expression of transcriptional factors relevant to PD [64], and exhibited altered NF-kB signaling and inflammatory response [65]. The cellular vulnerability associated with mitochondrial dysfunction and autophagy in LRRK2 mutant iPSC-derived DA neuronal cells could be rescued with coenzyme Q(10), rapamycin, or the LRRK2 kinase inhibitor GW5074 [59]. We previously reported that DA neurons derived from LRRK2 G2019S patient iPSCs displayed excessive mitochondrial fragmentation and impaired autophagy. Importantly, inhibition of excessive mitochondrial fission by a peptide inhibitor P110 can attenuate mitochondrial fragmentation, excessive autophagy and neuronal morphological abnormality [60], suggesting that mitochondrial damage might be a key step in initiation of DA neuronal degeneration in the context of LRRK2 G2019S mutation. In addition to pharmacological regulation, gene correction of LRRK2 G2019S mutation has been reported to result in phenotypic rescue in differentiated DA neurons [66]. Moreover, LRRK2 G2019S-induced neuronal degeneration might be mediated by increased extracellular-signal-regulated kinase 1/2 (ERK) phosphorylation which in turn led to transcriptional dysregulation of CADPS2, CPNE8, and UHRF2 [66]. Beyond the G2019S mutation, iPSCs lines have been generated from patients with other LRRK2 mutations, including R1398H [67], R1628P [68] and I2012T [69].

α-Synuclein is the key component of Lewy body (LB) and aberrant α-synuclein aggregation has long been implicated in the PD pathogenesis, which is also a pathological hallmark of PD. iPSC lines with point mutant (A53T) in α-synuclein, isogenic control and triplicate SNCA have been generated to study cellular and molecular mechanisms disturbed by α-synuclein. Cortical neurons from iPSCs of patients harboring α-synuclein A53T mutation exhibited nitrosative stress, accumulation of endoplasmic reticulum (ER)-associated degradation substrates, and ER stress [70]. In another study, Ryan et al. reported that DA neurons derived from α-synuclein A53T patient iPSCs displayed increased nitrosative/oxidative stress which resulted in S-nitrosylation of transcription factor MEF2C in A53T DA neurons compared to isogenic controls. This redox reaction inhibited the MEF2C-PGC1α transcriptional network, contributing to mitochondrial bioenergetic defects and apoptotic cell death [71]. Accumulation of α-synuclein in DA neurons derived from A53T patient iPSCs has also been associated with impaired mitochondrial trafficking by retaining Miro on the mitochondria [72], fragmented mitochondria and autophagic impairment due to interaction with cardiolipin [73]. α-Synuclein physiologically

presents as a helically folded nontoxic tetramer (around 55 kDa) that resists aggregation [74,75]. Destabilization of the helically folded tetramer precedes α-synuclein misfolding and aggregation in PD and other human synucleinopathies [75]. DA neurons derived from α-synuclein A53T patient iPSCs consistently altered the ratios of tetramers to monomers which decreased α-synuclein solubility and induced neurotoxicity [76], suggesting a consistency in pathological change of α-synuclein in neurons derived from patient iPSCs and human PD.

Triplication of *SNCA*, encoding α-synuclein, causes a fully penetrant and aggressive form of PD with dementia [77]. DA neurons derived from a PD patient with *SNCA* triplication mutation produced double amount of α-synuclein protein relative to neurons from the unaffected relative, recapitulating the cause of PD in these 10 individuals [78]. These DA neurons were sensitive to peroxide-induced oxidative stress [79] and exhibited lower lysosomal degradation capacity [80]. In addition, these iPSCs exhibited a reduced capacity to differentiate into DA or GABAergic neurons, decreased neurite outgrowth and lower neuronal activity compared with control cultures [81]. Neurons derived from iPSCs of patient bearing SNCA triplication also revealed an ER stress phenotype, marked by induction of the IRE1α/XBP1 axis of the unfolded protein response (UPR) and UPR activation [82].

Given that α-synuclein aggregates and LB formation start with abnormal accumulation and phosphorylation of α-synuclein in the neurons, one of major focuses in the PD iPSCs modeling is to recapitulate α-synuclein accumulation in iPSCs-derived neurons to understand the potential toxic properties of α-synuclein aggregation. Normal levels of α-synuclein have been observed in α-synuclein A53T and sporadic patient iPSC-derived DA neurons [71,76,83], whereas an enhanced protein level of α-synuclein was observed in DA neurons derived from iPSCs of *SNCA triplication* [78,79]. Phosphorylated α-synuclein at serine 129 (pS129) is the most abundant form found in patient LBs [84], making this post-translationally modified protein important to identify in patient iPSC-derived neurons *in vitro*. Increased levels of phosphorylated α-synuclein were identified in *SNCA triplication* patient iPSC-derived DA neurons and iPSC-derived cortical neurons from *SNCA A53T* mutation carriers, and neurons derived from sporadic PD patient iPSCs [85], indicating that this abnormal protein expression is common across different PD genotypes.

Mutations in both PTEN-induced putative kinase 1 (PINK1) and Parkin are implicated in autosomal recessive forms of familiar PD. In response to mitochondrial depolarization, PINK1 accumulates on the mitochondria where it recruits Parkin to the damaged mitochondria. Parkin subsequently ubiquitinates mitochondrial proteins, directing them for mitophagy [86]. Siebler et al. generated iPSCs from skin fibroblasts taken from three PD patients with nonsense (c.1366C > T; p.Q456X) or missense (c.509T > G; p.V170G) mutations in the PINK1 gene [87]. DA neurons derived from these iPSCs showed impaired recruitment of lentivirally expressed Parkin to mitochondria, increased mitochondrial copy number, and upregulation of PGC-1α, an important regulator of mitochondrial biogenesis upon mitochondrial depolarization. Lentiviral expression of wild-type PINK1 in the DA neurons derived from mutant PINK1 iPSCs can correct these mitochondrial phenotypes [87]. In addition, neurons from PINK1 mutant iPSCs showed a decreased mitochondrial membrane potential [87,88], mitochondrial complex I activity [89,90], and deficient mitochondrial trafficking [62]. Besides a concerted role with PINK1 in mitophagy and oxidative stress, Parkin is also associated with dopamine homoeostasis. Jiang et al. showed that iPSC-derived DA neurons from patients with Parkin mutation had decreased DA uptake and increased spontaneous DA release, whereas lentiviral expression of Parkin, but not its PD-linked mutant, rescued these phenotypes [91]. The results suggest that Parkin controls dopamine utilization in human midbrain DA neurons by enhancing the precision of DA neurotransmission and suppressing dopamine oxidation. Besides, increased oxidative stress, α-synuclein accumulation [92], abnormalities in endosomal processes and trafficking [93], disrupted calcium shuttling between mitochondria and ER [94] and enhanced sensitivity to metal toxins [95,96] were all observed in the neurons derived from Parkin mutant iPSCs. In addition, Parkin mutant-iPSCs derived DA neurons exhibited abnormal neurite outgrowth and complexity, including neurite shortening, less number of terminals and branch points, which were due to destabilization of microtubule. These phenotypes could be reduced by overexpression of Parkin [97], suggesting that Parkin maintains the morphological complexity of human neurons by stabilizing microtubules.

In DA neurons derived from idiopathic PD patient iPSCs, mitochondria deficiency, autophagy dysregulation and neurite length shortening have been repeatedly reported [62,63,98], suggesting that these aberrant events might be common mechanisms implicated in both familial and idiopathic PD. However, the expression of specific early cytopathies such as a-synuclein accumulations was restricted to familial PD with appointed genetic mutations.

3.3. Huntington's disease

Huntington's disease (HD) is a hereditary autosomal dominant neurodegenerative disorder. HD is caused by an expansion of CAG repeats within the huntingtin (Htt) gene [99]. This mutant protein (mHtt) leads to progressive and prominent degeneration of the GABAergic projection neurons in the striatum and ultimately more widespread loss of other brain regions. People who carry the HD mutation progressively develop involuntary movement, psychiatric disturbance, personality changes and weight loss and eventually death within 10–15 years of disease onset [100]. Though the disease mutant, mtHtt, was discovered twenty years ago, the mechanism underlying HD-associated neurodegeneration remains elusive and no treatment is currently available. Because of monogenic mutation and a strong correlation between the CAG length and disease onset, HD is considered as an ideal disorder to utilize iPSCs for modeling.

Shortly after the discovery of human iPSCs, Park et al. generated a number of iPSCs from patients with genetic diseases, including HD [101], demonstrating the feasibility of reprogramming HD patient fibroblasts into iPSCs. Further, Zhang et al. reported that HD patient iPSCs can be differentiated into GABAergic and medium spiny neurons, the neuronal populations most susceptible in HD [102]. These HD-iPSCs derived striatal neurons contained the same CAG expansion as the mutation in the HD patients from whom the iPSC lines were established, and showed mitochondrial dysfunction and enhanced caspase activity upon growth factor deprivation compared to neurons from normal subjects [102,103]. In 2012, the HD Consortium generated fourteen iPSC lines derived from fibroblasts originating from seven individuals, healthy or affected by HD, representing cell models ranging from asymptomatic controls to HD models with varying CAG repeat numbers and disease severity [104]. Neurons were differentiated from these iPSC lines with CAG repeat numbers 21, 33, 60, 109, and 180, and exhibited decreased cell adhesion and adenosine triphosphate production, increased caspase-3 activation, increased cell death after prolonged culture or BDNF withdrawal, and increased vulnerability to stress/toxicity. Importantly, these disease-associated phenotypes correlated to CAG repeat number: more severe phenotypes were found in HD cells with longer CAG repeats [104,105]. In addition, other phenotypes in HD patient iPSCs-derived neurons have been reported to associate with lysosomal dysregulation and altered genes involved in cholesterol biosynthesis pathway [106,107], and enhanced oxidative stress and reduced cytoskeleton-associated proteins [108]. Multiple molecular pathways that are characteristically dysregulated in HD were also present in undifferentiated pluripotent HD-iPSCs, including dysregulation of the MAPK and Wnt signaling pathways and altered expression of p53 [109]. In a more recently study, Victor et al. generated medium spiny neurons from HD patient fibroblasts through microRNA-based direct neuronal conversion [110]. This new strategy bypasses the induction of pluripotency and retains age-associated marks of the original fibroblasts. The medium spiny neurons directly derived from HD

patient fibroblasts exhibited mtHtt aggregates, mitochondrial dysfunction and spontaneous degeneration over time [110]. Intriguingly, the authors showed that cellular age was an essential component underlying the manifestation of these HD phenotypes, highlighting the importance of age in modeling late-onset neurological disorders. In addition to GABAergic neurons, astrocytes derived from iPSCs of HD patients carrying mtHtt with 50 and 109 CAG repeats displayed a CAG-repeat dependent increase in cytoplasmic vacuolization and alteration in autophagy [111], and a TNF-α inhibitors, XPro-1595, lowers cytokine (TNF-α and IL-β) induced iNOS production in astrocytes derived from iPSCs with 43 CAGs [112]. Further exploration of HD iPSC derived glia, along with co-culture of iPSC derived neurons and glia, may provide new insights into sources of non-cell autonomous toxicity in HD pathogenesis.

To overcome genetic background differences in individual iPSCs lines, especially between HD and normal control, isogenic iPSCs lines in which disease-causing mutations are genetically corrected to produce a wild type allele, have been generated to produce genetically identical control iPSC lines. An et al. reported the successful correction of the mtHtt in HD patient iPSCs. They generated two human HD isogenic iPSC lines using a homologous recombination based genetic correction method in which a 72 CAG repeat was replaced with a normal 21 CAG repeat in the gene *HTT* [103]. They showed that genetic correction of the HD mutation could reverse disease-associated phenotypes such as elevated cell death and caspase-3/7 activity as well as lower BDNF levels and energy metabolism [103]. Further, using CRISPR/Cas9 gene editing approach, Xu et al. reported that both HD and corrected isogenic hiPSCs can be differentiated into excitable, synaptically active forebrain neurons and that the phenotypic abnormalities in HD hiPSC-derived neural cells, including impaired neural rosette formation, increased susceptibility to growth factor withdrawal, and deficits in mitochondrial respiration, were rescued in isogenic controls [113]. Therefore, the phenotypes identified in these isogenic iPSCs can be solely attributed to the disease-causing mutation. These cell lines should be particularly suitable for screening of drugs or identifying mechanisms that target phenotypes caused by the disease mutation.

3.4. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset neurodegenerative disorder characterized by the progressive loss of motor neurons in the brain, brainstem, and spinal cord, which culminates in paralysis and death within a few years of diagnosis [114,115]. While mostly sporadic, a small population of patients is associated with genetic mutations, among which 20% are caused by mutations in the Cu/Zn superoxide dismutase (*SOD1*) gene [114–116]. In addition, other mutations have been identified in genes coding for *C9ORF72*, *TAR DNA-Binding Protein 43* (TDP-43, encoded by TARDBP) and *Fused in sarcoma* (FUS gene), which are considered as key causative factors in familial ALS [114,115].

Mutations in gene encoding SOD1 are most studied related to ALS. These mutations are found in the exon regions, suggesting that their toxic effects are the consequence of protein dysfunction with an increase of oxidative stress [114,117]. Dimos et al. first reported the generation of disease-derived iPSCs from an 82-year-old women diagnosed with a familial form of ALS carrying SOD1 mutation, and the success in differentiation of these iPSCs into motor neurons [118]. Subsequently, two groups back-to-back reported that motor neurons derived from SOD1 mutations patients recapitulated the spontaneous and progressive decrease in cell viability observed in humans, when compared to those derived from isogenic controls [119,120]. Chen et al. reported a reduction in soma size and an altered dendrite length of motor neurons, and the dysregulation and aggregation of neurofilaments (NF), an event that preceded the occurrence of neuronal apoptosis, in the motor neurons derived from ALS SOD1 mutation iPSCs [119]. Importantly, conditional expression of NF-L in the SOD1 iPSC-derived motor neurons corrected the NF subunit proportion, mitigated NF aggregation and neurite degeneration [119]. Thus, NF misregulation underlies mutant SOD1-mediated NF aggregation and axonal degeneration in ALS. In parallel, Kiskinis et al. showed that several genes related to cytoskeletal organization, mitochondrial function and structure, and protein translation were dysregulated in the patient-derived motor neurons in comparison to isogenic controls [120]. Moreover, these motor neurons derived from SOD1 mutations iPSCs exhibited ER stress [120] and hyperexcitability [121]. Significantly, in a following study, Wainger et al. found that Retigabine, a clinically approved anticonvulsant, blocked hyperexcitability in these patient-derived motor neurons by activating subthreshold Kv7 currents and increased survival of motor neurons [121].

Expansions of a hexanucleotide repeat (GGGGCC) in the noncoding region of the C9ORF72 gene are the most common cause of the familial form of ALS (C9-ALS), as well as frontotemporal lobar degeneration and other neurological diseases [122,123]. Motor neurons differentiated from iPSCs of ALS patients carrying the C9ORF72 repeat expansion have been reported to recapitulate major pathological signatures of the disease, 15 including an increase in transcription of the repeat of C9ORF72, accumulation of GGGGCC repeat-containing RNA foci, gene expression alteration and susceptibility to excitotoxicity [124–127]. Several other studies further showed that in the motor neurons derived from patient iPSCs, *C9ORF72* protein was found to colocalize with Rab proteins and to be involved in endosomal trafficking and autophagy [128], and played an important role in the induction of autophagy [129–131]. In addition, Sivadasan et al. recently profiled the interactome of *C9ORF72* in motor neurons derived from patient iPSCs and found that *C9ORF72* was present in a complex with cofilin and other actin binding proteins. They showed that *C9ORF72* modulated the activity of the small GTPases Arf6 and Rac1, resulting in enhanced activity of LIM-kinases 1 and 2 (LIMK1/2) and axonal outgrowth deficits [132].

Tar DNA binding protein-43 (TDP-43) is found in cytoplasmic inclusions in 95% of ALS and about 4% of familial ALS is caused by mutations in TDP-43 [115]. There are > 30 mutations in the TDP43 are involved in both familial and sporadic ALS cases. Motor neurons from iPSCs of familial ALS patients, who carry mutations in TDP-43, formed cytosolic aggregates of TDP-43, similar to those seen in ALS patients, and exhibited shorter neurites [133–135]. Moreover, the motor neurons derived from ALS patients with TDP-43 mutations exhibited increased vulnerability in response to a variety of stressors [134,135], mitochondrial fragmentation and bioenergetics deficiency [136].

In addition, Burkhardt et al. generated iPSCs of three sporadic ALS patients [137]. The authors reported that motor neurons derived from these sporadic ALS patients showed *de novo* TDP-43 aggregation. Moreover, the aggregates recapitulated pathology in postmortem tissue from one of the same patients from which the iPSC were derived. Sun et al. further showed that the surviving motor neurons derived from both sporadic and familial ALS iPSCs exhibited TDP-43 aggregates and higher neurofilament (NF) inclusion [138]. The neurite mitochondria density was significantly lower in ALS motor neurons than that in the control neurons. Thus, changes in TDP43 protein, NF inclusion, and impairment of mitochondrial distribution seem to be common early pathologies both in familial and sporadic ALS.

4. Implementation of iPSCs for therapeutic development

One of major reasons for the failure of therapeutics development for neurodegenerative diseases is the poor prediction of preclinical experimental models. *Bona fide* disease-relevant model or proper drug-targeted tissue would thus be required for an improved pharmacological *in vitro* profiling. In addition to enable understanding pathological mechanism and progression, disease-specific iPSCs have the potential to revolutionize drug development, because 1) iPSCs disease models offer unique platforms where patient biology and physiologically relevant

assays can be used for preclinical drug discovery, and 2) iPSCs originate from the patients and thus have great potentials for the development of personalized medicine, allowing drug discovery and testing based on a patient's genetic background and specific disease characteristics [139,140]. Below we summarized a number of therapeutic approaches that have been applied to neurons derived from iPSCs of patients with AD, PD, HD or ALS (also see Table 1).

The accessibility of iPSCs allows many compounds to be tested simultaneously. Studies have shown that treatment of AD iPSC-derived neurons with β-secretase inhibitors, but not γ-secretase inhibitors, caused significant reductions in phosphorylated Tau expression and GSK-3β levels [41,43,141]. In 2013, Xu et al. observed that in neurons differentiated from AD patient iPSCs, Aβ-induced toxicity correlated with cell cycle re-entry which was inhibited by pharmacological inhibitors or shRNAs against Cyclin-dependent kinase 2 (Cdk2). They then screened a chemical library containing several hundred compounds and discovered several small molecules as effective blockers against Aβ1–42 toxicity, including a Cdk2 inhibitor [142]. This study provides an excellent example of how hiPSCs can be used for disease modeling and high throughput screening for neurodegenerative diseases. In a more recently study, Kondo et al. utilized AD patient neurons purified from iPSCs to screen a pharmaceutical compound library with the aim of reducing amyloid amount and toxicity [143]. They prioritized hits by chemical structure-based clustering, and selected 6 leading compounds. To maximize the anti-Aβ effect, they selected a synergistic combination of bromocriptine, cromolyn, and topiramate as an anti-Aβ cocktail, and showed a significant and potent anti-Aβ effect on patient cells [143]. In addition to reduction of Aβ toxicity, lowering total tau level is an attractive therapeutic strategy for AD and other tauopathies. Wang et al. engineered an isogenic iPSC line that harbored an inducible *neurogenin 2* transgene, a transcription factor that rapidly converts iPSCs to neurons. Using a simplified two-step protocol, they differentiated these iPSCs into cortical glutamatergic neurons with minimal well-to-well variability. They further utilized high throughput screening assay to identify tau-lowering compounds and identified adrenergic receptors agonists as a class of compounds that reduce endogenous human tau [144]. The technique enables the use of human neurons for high throughput screening of drugs to treat AD with less variability.

iPSC-derived PD models have also been used for screening therapeutic compounds. In DA neuronal cells generated from iPSCs derived from PD patients carrying mutations in the PINK1 or LRRK2 genes, Cooper et al. screened a number of small molecules and found that coenzyme Q10, rapamycin and the LRRK2 kinase inhibitor GW5074, can rescue cytotoxicity caused by valinomycin or concanamycin A. Moreover, they showed that rapamycin and GW5074 selectively reduced reactive oxygen species production in iPSC-derived neurons with PINK1 mutation but not in neural cells from healthy subjects, highlighting the difference in susceptibility to pharmaceutical compounds between diseased neurons and artificial disease models [59]. More recently, nitrosative and oxidative stress were found to cause mitochondrial dysfunction and apoptotic cell death in A9 DA neurons with α-synuclein A53T mutation through S-nitrosylation of transcription factor MEF2C. Ryan et al. screened a chemical library of compounds for their ability to activate MEF2C transcription in the context of human neurons. They showed that small molecule isoxazole effectively drove expression of both *MEF2C* and *PGC1α* in A53T human neurons, and protected neurons from apoptosis induced by the mitochondrial toxins rotenone or PQ/MB [125]. Their findings suggest the target potential of MEF2C pathway in PD and possible clinical implications for the repurposing of known drugs, such as isoxazone, to treat PD. Therefore, iPSC-based technology may facilitate identification of therapeutic compounds by elucidating authentic signaling pathways in diseased human neurons.

Major phenotypic readouts used to test the effectiveness of novel therapies in HD iPSCs derived neuronal cells include mitochondrial dysfunction, cell death induced by a stressor such as growth factor withdrawal, DNA damage, or oxidative toxicity. We have recently found that neurons derived from HD patient iPSCs exhibited extensive mitochondrial fragmentation, increased mitochondrial oxidative stress, decreased mitochondrial membrane potential and neurite shortening [145,146]. Importantly, either blocking mitochondrial fission protein dynamin-related protein 1 (Drp1) by a peptide P110 or inhibition of the interaction between Valosine-containing protein (VCP) and mtHtt by a peptide HV-3, abolished these aberrant mitochondrial events and improved neuronal morphology and survival in medium spiny neurons derived from HD patient iPSCs [145,146]. These findings suggest the possibility that correcting mitochondrial defects in neurons derived from HD patient iPSCs might provide a unique approach for developing HD therapeutics. In another study, researchers showed that FDA-approved drug bexarotene, a potent retinoid X receptor (RXR) agonist, reduced cell death in medium spiny-like neurons from HD patient iPSCs, which was likely a result of synergistically activating the peroxisome proliferator-activated receptors (PPARs), the ligand-activated transcription factors that promote mitochondrial biogenesis and oxidative metabolism [147]. An ATM (ataxia-telangiectasia mutated) protein inhibitor KU55933 reversed both neocarzinostatin (a DNA damaging agent) induced increases in phosphorylation of p53, CHK2 and γH2AX, and Mn^{2+} decreases in p53 phosphorylation in 70 and 180 CAG “striatal-like” neurons [148]. In addition, adenosine receptor 2A agonists CGS-21680 and APEC produced a dose dependent reduction of oxidative stress toxicity induced by exposure to H2O2 in 43 CAG neuronal cultures, as measured by decreased γH2AX induction and caspase3 cleavage [149]. These findings reveal multiple targets that are implicated in the pathogenesis of HD. Further optimization for high throughput screening of these targets would be very beneficial not only for HD but for many other neurodegenerative diseases.

In motor neurons derived from *C9ORF72* ALS patients, antisense oligonucleotide (ASO) therapeutics to the *C9ORF72* transcript or repeat expansion have been found to mitigate intranuclear RNA foci formation, dysregulated gene expression, impaired nuclear import and excitotoxicity [124,126,150]. Moreover, iPSC-derived motor neurons from ALS patients were found to be hyperexcitable compared to controls, and Retigabine, an approved drug for epilepsy, could rescue this hyperexcitability phenotype in motor neurons derived from patients with different ALS-associated mutations [121]. Burkhardt et al. used the TDP-43 aggregation phenotype as readout in a high-content chemical screen in lower and upper motor neuron-like cells, and identified FDA-approved small molecule modulators including Digoxin that could modulate TDP-43 aggregation [137]. Further, in motor neurons from TDP43 mutation iPSCs, Egawa et al. examined four chemical compounds and found that a histone acetyltransferase inhibitor called anacardic acid rescued the abnormal ALS motor neuron phenotype including TDP43 aggregations and neurite shortening [133]. These findings suggest that motor neurons generated from ALS patient-derived iPSCs may provide a useful tool for screening drug candidates.

In addition to drug screening, patient hiPSCs have advantages on revealing new targets and pathways that are druggable for neurodegenerative diseases, given that iPSCs-derived cellular lineages have patient's specific disease characteristics. By a phenotypic screen for pTau accumulation in AD-patient iPSC-derived neurons, Kant R et al. identified cholesteryl esters (CE), the storage product of excess cholesterol, as upstream regulators of Tau early during AD development, and that the CE regulated Aβ secretion and Tau by independent pathways. Importantly, they found that allosteric activation of CYP46A1, a key enzyme to eliminate brain cholesterol accumulation, lowered CE specifically in neurons [151]. These findings identify a role of CYP46A1-CE-Tau axis in AD, which sets a good example on utilizing iPSCs culture system to reveal druggable pathway in AD. To determine pathways altered in ALS, Tank et al. recently performed unbiased proteomic analysis of ALS *C9orf72* iPSCs and identified reductions in mitochondrial components and compensatory increases in protein

synthesis in ALS iPSCs [152], suggesting previously unidentified mechanisms that cause cell death in ALS by disrupting energy production and protein synthesis pathways. Kishinevsky et al. reported that PD-related genetic or toxic stimuli altered the neuronal proteome of iPSCs-derived DA neurons, thereby altering the stress-specific chaperome networks, which produced changes detected by chemical sensors [153]. They identified STAT3 and NF-κB signaling activation as examples of genetic stress, and phospho-tyrosine hydroxylase (TH) activation as an example of toxic stress-induced pathways in PD neurons. Interestingly, they showed that pharmacological inhibition of the stress chaperome network reversed abnormal phospho-STAT3 signaling and phospho-TH-related dopamine levels and rescued PD neuron viability [153]. Using chemical sensors on hiPSC-derived lineages may present a useful strategy to identify molecular events associated with neurodegenerative diseases.

5. Future perspectives

HiPSCs are generated directly from affected patients, representing a genetically accurate *in vitro* model of the disease. Therefore, using iPSC lines for disease modeling may fill in the gaps between animal models and human neural cells, helping to elucidate the molecular basis of disease and revealing therapeutic targets directly associated with disease progression. Moreover, iPSC technology could be used for the low- and high-throughput screening that provides efficacious platforms to assess a number of former and novel drug candidates. However, several issues regarding the application of iPSC-derived cells remain unresolved.

First, variability between cell lines needs to be optimized. Marked differences in differentiation propensity between pluripotent stem cell lines, even between iPSC lines generated from the same individual, have been documented [154]. Optimizing and standardizing protocols across laboratories for iPSCs characterization and differentiation are thus necessary to permit the generation of accurate and reproducible data. To solve this issue, several large-scale hiPSCs initiatives, such as HD, PD and ALS iPSC consortium and repository, Kyoto University Center for iPSC research and application, National Institute of Health hiPSCs biobank have been established to create biorepositories of well-characterized hiPSC lines from patients, control subjects and isogenic controls. These large-scale initiatives will help researchers to minimize the variation of cell lines and to improve statistical power of hiPSC-related studies. In addition, researchers have recently applied the genome editing techniques to generate isogenic controls or isogenic mutated cell lines. RNA-guided-engineered-nuclease (RGENs, derived from clustered-regularly-interspaced-short-palindromic-repeat (CRISPR)-Cas), zincfinger-nuclease (ZFN), and transcription-activator-like-effector nuclease (TALEN)-based disease modeling are widely utilized. These strategies enable to 1) investigate disease mechanisms resulting from causative mutations rather than the individual's genetic background and 2) compare the cellular phenotypes between control and diseased lines under the same stages of cells (*e.g.* epigenetic states and differentiation capacities). Thus, using isogenic controls or isogenic mutated lines may help to reduce variability among different lines and simplify analyses of interactions between genotype and phenotype.

Second, identification of correlation between early abnormal phenotypes observed from neurons of patient iPSCs and real neuronal degeneration in the patient brains. Neurodegenerative diseases are featured with the aggregations of toxic proteins, such as Aβ, TDP-43, mtHtt and α-synuclein. The accumulation of these aberrant protein aggregates has been used as readouts in neurons of hiPSCs to assess the success of disease modeling. However, pathological protein aggregates are hard to reproduce in many of iPSCs culture systems. Recently, Victor et al. reported a direct conversion of medium spiny neurons from HD patient fibroblasts by using microRNAs [110]. They showed that mtHtt formed aggregates in the medium spiny neurons, and neurons exhibited age-associated marks of the original fibroblasts and spontaneous degeneration over time [110]. This new strategy may be applicable to other types of neurodegenerative diseases in which protein aggregates manifest, overcoming the limitation using iPSCs culture system. In addition, it remains difficult to understand how these cytopathies occurring during the later stages of neurodegeneration correlate with early phenotypes of signaling, subcellular organelle dysfunctions and neuronal damages in neurons derived from patient iPSCs. Therefore, identification and application of early markers to reflect disease development may be the next step to overcome. Uncovering detailed mechanisms that connect the early neuronal phenotypes and later neuronal loss in culture would also help to maximize the use of patient iPSCs.

Third, aging is an unsolved issue for the utilization of iPSCs-based diseased model. The majority of neurodegenerative diseases are age-related neurological disorders. When reprogramming, most aging-related genes are turned off and the cells derived from hiPSCs are generated as young cells, compared to the cells in patient brains. Such different stages of neurons less accurately mimic real pathological stages of the diseases. To accelerate disease phenotypes, researchers utilized various stimuli to enhance mitochondrial stress, nutrition depletion or oxidative stress. However, whether these factors reflect nature process of aging remains to be investigated. Brain organoids mimic brain structures, exhibiting similar developmental stages to endogenous neural development and to generate multiple brain tissue types and regions [38]. Brain organoids can survive long periods in culture, thus providing a relative “aged” model with more mature and functional neurons, which could be another alternative to mimic aged cells in diseased brains. This system has already been utilized to model microcephaly, and organoids generated from microcephaly patient iPSCs exhibited premature neuronal differentiation [31,155,156]. Due to the difficulty of obtaining human living brain tissue samples, cerebral organoids currently also offers one of comprehensive model conditions to represent *in-vivo* brain structure. For example, cerebral organoids (COs) produced from iPSCs of familial AD or Down syndrome have been reported to spontaneously develop over time with pathological features of AD, including accumulation of structures highly reminiscent to amyloid plaques and neurofibrillary tangles. These pathological abnormalities were not observed in COs generated from various controls [157,158]. Further development and optimization of this technology might be beneficial for many neurodegenerative diseases, in addition to neuronal developmental disorders.

In conclusion, hiPSCs are powerful tool for the study of neurodegenerative diseases. The application of new technology, such as CRIPSR/Cas9 gene editing and 3D culture system, may help to yield *in vitro* diseased model that more faithfully reflect the complex network of human brains and disease phenotypes. These will also pave the solid foundation for pharmacological drug screening and validation. Therefore, the iPSCs-derived neuronal culture system provides an invaluable platform and resource for patients suffering from neurodegenerative diseases.

Conflict of interests

The authors claim no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

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