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Cellular models of alpha–synuclein toxicity and aggregation

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Abstract

Misfolding and aggregation of alpha-synuclein (α-synuclein) with concomitant cytotoxicity is a hallmark of Lewy body related disorders such as Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy. Although it plays a pivotal role in pathogenesis and disease progression, the function of α-synuclein and the molecular mechanisms underlying α-synuclein-induced neurotoxicity in these diseases are still elusive. Many in vitro and *in vivo* experimental models mimicking α-synuclein pathology such as oligomerization, toxicity and more recently neuronal propagation have been generated over the years. In particular, cellular models have been crucial for our comprehension of the pathogenic process of the disease and are beneficial for screening of molecules capable of modulating α-synuclein toxicity. Here, we review α-synuclein based cell culture models that reproduce some features of the neuronal populations affected in patients, from basic unicellular organisms to mammalian cell lines and primary neurons, to the cutting edge models of patient-specific cell lines. These reprogrammed cells known as induced pluripotent stem cells (iPSCs) have garnered attention because they closely reproduce the characteristics of neurons found in patients and provide a valuable tool for mechanistic studies. We also discuss how different cell models may constitute powerful tools for high-throughput screening of molecules capable of modulating α-synuclein toxicity and prevention of its propagation.

Keywords

cellular model; dopaminergic neurons; oligomers; Parkinson's disease; α-Synuclein

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It is well-established that mutations in, and multiplications of, the *SNCA* gene encoding alphaαasynuclein (α-synuclein) cause Parkinson's disease (PD) (Stefanis 2012), a neurodegenerative disorder that presents clinically with a collection of motor impairments referred to as *parkinsonism* as well as non-motor symptoms such as sleep disorder, depression, gastrointestinal disturbances, and often dementia (Chaudhuri *et al.* 2006; Langston 2006). Related neurological disorders with symptoms of parkinsonism include Parkinson's disease dementia (PDD), dementia with Lewy bodies, and multiple system atrophy (Jellinger 2003). These disorders, also known as alpha-synuclein opathies, are neuropathologically characterized by the accumulation of α-synuclein in cytoplasmic inclusions known as Lewy bodies (LBs) in vulnerable neuronal and glial populations. The first identification of a genetic cause for PD was made more than 20 years ago, when Polymeropoulos and colleagues identified a mutation in the *SNCA* gene in an Italian family with an autosomal dominant form of PD (Polymeropoulos *et al.* 1997; Nussbaum 2017). Soon after, histological studies in post mortem brains of idiopathic PD and dementia with Lewy bodies patients revealed that α-synuclein is a major constituent of LBs, strengthening the case for α-synuclein's pivotal role in the pathogenesis of PD and related disorders (Spillantini *et al.* 1997; Goedert *et al.* 2017). Since then, various mutations in and multiplications of *SNCA* have been discovered and have been directly linked to disease progression and severity (Devine *et al.* 2011). Subsequently, experimental in vitro and *in vivo* models involving overexpression of wild-type or mutant α-synuclein have been developed in an effort to model the disease pathology of α-synuclein aggregation and toxicity (Kirik *et al.* 2002; Lo Bianco *et al.* 2002; Giasson *et al.* 2002).

Cumulative evidence implicates a causative role of α-synuclein aggregation in neurodegeneration (Irizarry *et al.* 1998; Chartier-Harlin *et al.* 2004; Winner *et al.* 2011). However, the molecular mechanisms underlying cytotoxicity in PD are still elusive, hampering the development of disease modifying therapies. α-synuclein is a soluble presynaptic protein that may exist as a natively unfolded monomer (Fauvet *et al.* 2012; Binolfi *et al.* 2012; Burre *et al.* 2013; Theillet *et al.* 2016) or a functional tetramer (Bartels *et al.* 2011; Gurry *et al.* 2013; Selkoe *et al.* 2014; Dettmer *et al.* 2015). Under pathological circumstances, α-synuclein forms aggregates via the assembly of soluble oligomeric intermediates that mature into the insoluble amyloid fibrils found in LBs. Whether such cytoplasmic inclusions contribute to neuronal death or protect cells from the toxic effects of misfolded proteins remains controversial. The current hypothesis in the field suggests that α-synuclein pre-fibrillar forms represent the toxic species, making them the subject of intense investigations (Conway *et al.* 2000; Outeiro *et al.* 2008; Villar-Pique *et al.* 2016). Furthermore, it is now widely recognized that α-synuclein aggregates can spread throughout the central nervous system via cell-to-cell propagation, possibly in a prion-like manner (Kordower *et al.* 2008; Masuda-Suzukake *et al.* 2013; Recasens and Dehay 2014) driving disease progression. Taken together, it appears that lowering α-synuclein levels and/or eliminating toxic α-synuclein species in cells, will be an attractive target for therapeutics to halt disease progression (Nasstrom *et al.* 2011; Brundin *et al.* 2017). Above all, the availability of reliable experimental models is essential to garner a deeper understanding of the mechanisms associated with α-synuclein-mediated toxicity and, most importantly, to aid development and validation of future pharmacological interventions.

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Mechanistic aspects of a disease often emerge from studies at the cellular and subcellular level. Mammalian cell lines and unicellular organisms such as yeast have already provided valuable insights into the pathophysiology of synucleinopathies and are key translational approaches prior to validation in preclinical animal models and in human specimens (Alberio *et al.* 2012). Cellular models have tremendous advantages over *in vivo* approaches as they are fast and reproducible and importantly, are a cost-effective tool. They are also readily amenable to genetic modifications and pharmacological manipulations, making the direct targeting of specific cellular processes involved in disease feasible. With a central role for α-synuclein in disease, several cellular models that mimic important aspects of α-synuclein biology, such as aggregation and toxicity, have been developed over the years and have contributed to many advances in our comprehension of the pathogenesis of PD and other synucleinopathies (Lazaro *et al.* 2017). Although cell models are simplified systems which do not fully reproduce neuronal networks or recapitulate the complexity of the diseases, they are powerful tools to unravel pathophysiological mechanisms at play in neurodegeneration as well as being useful for high-throughput screening of new therapeutic compounds (Schule *et al.* 2009).

Herein, we review α-synuclein cell-based models that are currently available and discuss their contribution to the understanding of molecular neurodegeneration and how these models may shed light on new drug discovery in synucleinopathies.

Lessons learned from the baker's yeast Saccharomyces cerevisiae

The baker's yeast *Saccharomyces cerevisiae* has been used for thousands of years for industrial applications, including baking bread. Therefore, our understanding of the biology and genetics of this organism is vast, and many of its characteristics can be exploited for the study of basic biological processes that are conserved among all eukaryotes. In particular, the detailed understanding of molecular machines involved in protein folding and degradation, and the identification of yeast proteins with *prion* behavior led to the use of *S. cerevisiae* (herewith referred to as yeast) as a living test tube with which to investigate the molecular underpinnings of neurodegenerative diseases associated with protein misfolding and aggregation. At around the same time that multiplications of the *SNCA* gene were linked with familial forms of PD, heterologous expression of human α-synuclein in yeast resulted in two key aspects of synucleinopathies, dose-dependent cytotoxicity and inclusion formation (Outeiro and Lindquist 2003). Strikingly, the toxic effects of α-synuclein resulted from an impairment in intracellular trafficking, altered lipid metabolism, and increased levels of oxidative stress (Outeiro and Lindquist 2003). Several other groups have exploited the yeast toolbox to further dissect the molecular mechanisms underlying α-synuclein toxicity and aggregation (Zabrocki *et al.* 2005). Importantly, using powerful genetic screens, modifiers of toxicity and aggregation have been identified (Willingham *et al.* 2003; Cooper *et al.* 2006; Liang *et al.* 2008; Su *et al.* 2010), and further validated, in primary neuronal cultures and in vivo, in multicellular organisms such as *Caenorhabditis elegans*, drosophila, and mice (Tardiff *et al.* 2014). From the genetic screens, endoplasmic reticulum (ER) to Golgi trafficking and membrane and lipid metabolism alterations have emerged as highly conserved pathways affected by α-synuclein in the cell (Zabrocki *et al.* 2008). Despite initial skepticism, these basic studies in yeast have provided tremendous insight into the biology

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and pathobiology of α-synuclein and other PD-associated genes (Buttner *et al.* 2008; Sampaio-Marques *et al.* 2012; Tardiff *et al.* 2014; Dhungel *et al.* 2015), and have enabled the identification of several small molecules that are capable of alleviating α-synuclein -induced cytotoxicity (Fleming *et al.* 2008; Su *et al.* 2010).

More recently, yeast has proven a useful tool to study the effect of PD-associated mutations in α-synuclein (Lazaro *et al.* 2014; Lazaro *et al.* 2016) and of post-translational modifications, such as phosphorylation (Tenreiro *et al.* 2014; Mbefo *et al.* 2015; Kleinknecht *et al.* 2016; Tenreiro *et al.* 2017; Bras *et al.* 2018), sumoylation (Shahpasandzadeh *et al.* 2014), or glycation (Vicente Miranda *et al.* 2017). The development of effective therapies for synucleinopathies has been a tremendous challenge, and continues to be an awesome task. Yeast alone will not be the solution, but together with more complex models, it has already proven to be an invaluable tool/model organism in which to investigate the basic biology and pathobiology of α-synuclein (Tenreiro and Outeiro 2010; Menezes *et al.* 2015).

Non-neuronal cell models of α-synuclein overexpression

Since mutations in and multiplications of *SNCA* are considered causative for some cases of PD, numerous cellular models based on overexpression have been generated in the last two decades aimed at understanding the contribution of α-synuclein to the disruption of cellular processes (Table 1). Indeed, several biochemical pathways known to be affected in synucleinopathies including mitochondrial dysfunction, increased apoptosis, and oxidative stress, as well as defects in protein degradation machinery, were all discovered in cellular overexpression models (Outeiro *et al.* 2008; Klucken *et al.* 2012; Lazaro *et al.* 2014). In patients with *SNCA* multiplications, increased expression of wild-type α-synuclein is sufficient to cause *parkinsonism*, and rare point mutations seem to increase α-synuclein aggregation leading to neurodegeneration (Devine *et al.* 2011). Consequently, generating cellular models where α-synuclein accumulates and forms oligomers has been considered a useful strategy. Two of the most commonly used immortalized human non-neuronal cell lines in this context are human embryonic kidney 293 (HEK293) and human H4 neuroglioma (H4) lines. These cells are easily transfected with transient and constitutive (stable) overexpression of human wild-type or mutated α-synuclein widely reported in both cases (Tabrizi *et al.* 2000; McLean *et al.* 2001; Outeiro *et al.* 2008; Lazaro *et al.* 2016). Increased expression of wild-type α-synuclein can be detected in a short period of time and most importantly, α-synuclein positive inclusions are often formed, depending on the specific paradigm. Interestingly, time-lapse imaging performed in an HEK293 model illustrated how cells formed and accumulated aggregated forms of α-synuclein (Opazo *et al.* 2008). These cell lines are also suitable to study the effect of α-synuclein mutations. Among them, the A53T or A30P point mutations are known to cause familial early onset PD with A53T being the most highly penetrant and widely studied of the mutations. Interestingly, these two mutations show greater toxicity in cellular models than wild-type α-synuclein. Thus it seems plausible that these mutations render α-synuclein more susceptible to aggregation (Lazaro *et al.* 2014).

Even though overexpression of wild-type or mutated α-synuclein in cells seems to recapitulate aspects of synucleinopathies, one major drawback in these models is the

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low level of toxicity associated with α-synuclein overexpression. A second limitation is the absence of abundant α-synuclein aggregates to mimic the major pathologic features observed in diseased post mortem brains. Many of these cell models exhibit small inclusions that are Thioflavin S positive or resistant to protease digestion, but only a few will actually develop larger aggregates which share properties of LBs found in patients. Thus, additional insults have commonly been used to challenge the cells and create a toxic environment. For example, the frequency of intracytoplasmic inclusions increases when toxins such as 1- methyl-4- phenylpyridinium (MPP+), rotenone, or proteasome inhibitor are applied to α-synuclein overexpressing cells (McLean *et al.* 2001; Lee *et al.* 2002). Importantly, co-expression with specific proteins facilitates the formation of more mature aggregates. Co-expression of synphilin-1, an α-synuclein interacting protein, can induce the formation of inclusions in H4 cells (McLean *et al.* 2001) as well as HEK293 cells (Engelender *et al.* 1999; O'Farrell *et al.* 2001; Tanaka *et al.* 2004). Lastly, the brain specific protein, p25a, has also been identified as a stimulator of α-synuclein aggregation in vitro (Lindersson *et al.* 2005; Ejlerskov *et al.* 2013).

Tracking α-synuclein oligomers in vitro

A large body of evidence indicates that the assembly of toxic oligomeric species of α-synuclein may be one of the key processes underlying the induction of pathology and spread of synucleinopathies. Understanding how these aggregates form and assessing their impact on neuronal function will contribute to the development of therapeutic targets to prevent disease progression. It is well known that in vitro induced α-synuclein oligomers ectopically applied to cell cultures are formed because of overexpression of α-synuclein, induce cell death and toxicity (Chen *et al.* 2007; Danzer *et al.* 2007; Tetzlaff *et al.* 2008). However, the lack of sensitive in situ detection methods has hindered the study of oligomeric α-synuclein species. Therefore tremendous efforts have been made to generate cell lines that allow tracking and visualization of aggregation in living cells (Fig. 1). Innovative technologies using biosensors from fluorescence labeling to protein complementation assays (PCA) have been developed to monitor α-synuclein/α-synuclein interaction. Intracellular α-synuclein oligomerization was visualized for the first time in H4 cells using a highly sensitive molecular fluorescence lifetime imaging microscopy by fusing a small epitope tag to α-synuclein (Klucken *et al.* 2006). A similar approach was used in HEK293 cells when α-synuclein was tagged with a six amino acid PDZ binding motif and co-expressed with the corresponding PDZ domain fused with enhanced green fluorescent protein (Opazo *et al.* 2008) and in contrast to traditional approaches with fusion proteins, provided higher sensitivity. Lastly, fluorescent-based biosensor cell lines have allowed the detection and the quantification of α-synuclein seeding activity. α-synuclein 'seeds' isolated from post mortem brain tissue with pathologically confirmed synucleinopathy, were found to trigger aggregation in HEK293T cells stably expressing α-synuclein-yellow fluorescent protein (YFP) resulting in a fluorescent readout that can be visualized and quantified using regular confocal microscopy (Prusiner *et al.* 2015). Likewise, Holmes and colleagues developed a HEK-based biosensor cell line that takes advantage of a Förster resonance energy transfer readout for seeding that can be detected and quantified using flow cytometry (Holmes and Diamond 2017).

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Another method to study α-synuclein/α-synuclein interactions that has been developed is based on the principle of bimolecular protein-fragment complementation (Remy and Michnick 1999; Kerppola 2008). PCA have been adapted to enable rapid and non-destructive reporting of α-synuclein oligomerization in living cells by the fusion of α-synuclein molecules with the inactive C-terminal or N-terminal fragments of a fluorescent (ie, YFP) (fPCA) or luminescent (ie. humanized *Gaussia* luciferase) reporter bioluminescent PCA [bioluminescent PCA (bPCA)] (Outeiro *et al.* 2008). The functional fluorophore or bioluminescent protein is reconstituted upon α-synuclein/α-synuclein interactions and acts as a surrogate reporter for the formation of oligomers that can be detected with readouts such as fluorescence microscopy, flow cytometry, or photometric measurement (Fig. 1c). α-synuclein -fPCA and bPCA have been widely used to confirm a major role for multimeric species in cytotoxicity and disease propagation (Outeiro *et al.* 2008; Danzer *et al.* 2012; Lazaro *et al.* 2014; Jiang *et al.* 2017). Moreover, this technology allows the investigation of early stages of α-synuclein aggregation, at a time before larger oligomers formation occured. Recently, stable cell lines co-expressing α-synuclein PCA using bioluminescent and fluorescence reporters, were developed and used in high-throughput screening (HTS) to identify inhibitors of α-synuclein oligomerization (Moussaud *et al.* 2015). Lastly, because the spread of α-synuclein from neuron to neuron is now considered an important step in PD pathogenesis, PCA can be applied to develop model systems where cell-to-cell transmission can be quantitatively analyzed. Using co-cultures, cells expressing α-synuclein tagged with either the N-or C-terminal fragment of a reporter allow the identification of cells where α-synuclein has been transferred from cell to cell by monitoring the signal of the reconstituted complete reporter (Bae *et al.* 2014). Using α-synuclein-bPCA, Danzer and colleagues found that α-synuclein oligomers are present in secreted extracellular vesicles that are taken up by recipient cells inducing toxicity (Danzer *et al.* 2012).

Although a powerful tool, PCA requires the use of tagged-proteins and does not discriminate between dimers and larger multimeric species of aggregates. To overcome these issues, the use of the newly developed proximity ligation assay (PLA) represents an alternative strategy (Fig. 1d). PLA has attracted a lot of attention because it allows the investigation of protein interactions at the endogenous level without the need for tagging or overexpression of proteins (Soderberg *et al.* 2006; Dettmer and Bartels 2015). A pair of oligonucleotide-labeled secondary antibodies (PLA probes) generate a signal only when the two PLA probes are bound in close proximity. The signal from each detected pair of PLA probes is then amplified and visualized as an individual fluorescent spot. In an elegant study, Roberts and colleagues (Roberts *et al.* 2015) developed the α-synuclein -PLA and demonstrated the sensitivity of the technique to detect α-synuclein oligomers with minimal recognition of monomeric and higher molecular weight species.

Primary neurons

An alternative to immortalized cell lines is the use of primary neurons prepared from embryonic or early post-natal mouse or rat pups. These cultures closely simulate a neuronal environment and may yield more physiologically significant results. They also offer the possibility to isolate neurons from specific brain regions allowing enrichment of specific neuronal populations. In synucleinopathies, and in particular PD, neuronal cell

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loss is observed in dopamine (DA) producing neurons and to lesser extent in cholinergic neurons. Therefore preparation of primary DAergic neurons from the ventral mesencephalon of mice has been extensively used to closely model this pathological hallmark of PD (Dryanovski *et al.* 2013; Gaven *et al.* 2014). Overexpression of wild-type or mutated forms of α-synuclein can be easily achieved in these models using transfection techniques (Tonges *et al.* 2014; Hassink *et al.* 2018) or neurons overexpressing α-synuclein can be directly cultured by preparing primary neurons from α-synuclein transgenic animals (Li *et al.* 2013). Recently recombinant α-synuclein has been utilized to generate small seeds of pre-formed α-synuclein fibrils (PFFs) and induce aggregates with characteristics of those found in diseased brains (Volpicelli-Daley *et al.* 2014). The addition of PFFs to primary neurons leads to the recruitment of endogenous α-synuclein into LB-like aggregates that are insoluble in detergent, hyperphosphory-lated, ubiquitinated, and have a filamentous ultrastructure when examined using electron microscopy (Volpicelli-Daley *et al.* 2014). This model system provides researchers with an opportunity to examine aggregation of α-synuclein from early formation to spread throughout the neuron, and ultimately neuronal death. In addition, intercellular trafficking of internalized α-synuclein seeds in primary neurons has been characterized using microfluidic devices that allow fluidic separation of neuronal soma from axonal projections. Freund *et al.* used live cell imaging to show the transfer of fluorescent α-synuclein PFFs to a second-order neuron (Freundt *et al.* 2012) and fluorescently labeled PFFs have also recently been utilized to image the internalization of α-synuclein seeds (Karpowicz *et al.* 2017; Jiang *et al.* 2017). Recently, mechanistic studies using PFFs revealed that the immune receptor Lag3 is a receptor for PFF α-synuclein in neurons initiating α-synuclein fibrils endocytosis, transmission and toxicity (Mao *et al.*, 2016). While other receptors for α-synuclein have been proposed based on proteomics studies, none have been validated functionally to date (Shrivastava *et al.* 2015; Bieri *et al.* 2018).

Differentiated dopaminergic cell-model

Many of the concepts discussed above are common to the multiple neurodegenerative diseases classified as synucleinopathies. Although cell death is a common key pathologic feature, the selective loss of DAergic neurons from the substantia nigra is specific to PD. DA deficit observed in PD patients underlies the three cardinal motor symptoms tremor, rigidity and akinesia which can be substantially improved by DA replacement therapy. Over the years, efforts to develop DAergic cell lines have expanded as an alternative strategy to obtain faithful cellular models of PD. The SH-SY5Y neuroblastoma and the PC12 pheochromocytoma cell lines bear many similarities to the neuronal populations affected in PD and are widely used to unravel neurodegenerative mechanisms. Indeed, PC12 and SH-SY5Y cells have the ability to differentiate into neurons after sequential exposure to retinoic acid or brain derived neurotrophic factor respectively. Upon differentiation, changes in morphology and function are observed with the extension of neuron-like processes and production and release of catecholamines. These DAergic-like neuronal cell lines are very similar to mesencephalon-derived primary neurons with the advantage that they can be continuously expanded and are less labor intensive (Westerink and Ewing 2008; Xicoy *et al.* 2017). Transient and constitutive (stable) overexpression of wildtype or mutant α-synuclein in PC12 and SH-SY5Y shows cytotoxicity and can affect cell survival (Stefanis *et al.*

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2001; Matsuzaki *et al.* 2004; Vekrellis *et al.* 2009). Remarkably, in a stable PC12 cell line expressing non-toxic levels of wild-type human α-synuclein or A30P mutant, impaired DA release is observed (Larsen *et al.* 2006). Addition of extracellular α-synuclein from PD brain lysates in differentiated SH-SY5Y also triggers the formation of α-synuclein aggregates (Xin *et al.* 2015). Finally, Desplats and colleagues demonstrated that α-synuclein is transmitted to neighboring cells via endocytosis and forms Lewy body–like inclusions that displayed ubiquitin immunoreactivity and were thioflavin S positive in a differentiated SH-SY5Y model (Desplats *et al.* 2009). Collectively, these studies demonstrate the usefulness of DAergic neuron-like cell lines as a predictive tool to mimic a PD-like phenotype in vitro.

Alternatively, the Lund human mesencephalic cells are a well-established model of human DAergic neurons (Lotharius *et al.* 2002; Lotharius *et al.* 2005; Scholz *et al.* 2011). The cells can be differentiated within a few days into a DAergic phenotype when cultured in dibutyryl cyclic adenosinemonophosphate and glial cell-derived neurotrophic factor. They are post-mitotic neurons with electrical properties similar to those of DAergic neurons (Scholz *et al.* 2011) and produce DA and wild-type human α-synuclein (Lotharius *et al.* 2002). With these characteristics they represent a good model to mimic a PD-like phenotype and have been used to study neurodegeneration caused by α-synuclein-induced toxicity (Lotharius *et al.* 2005; Paiva *et al.* 2017). Recently they were used as a model for high-throughput screening where 1600 FDA approved drugs were screened in differentiated Lund human mesencephalic cells overexpressing α-synuclein to identify neuroprotective compounds (Höllerhage *et al.*2017). Also worthy of a mention in the present review is the immortalized human neural stem cell (NSC) line, ReNcell VM. Overexpression of the myc family of transcription factors in human primary cells from developing mesencephalon was used to produce a stable multipotential NSC line that can be continuously expanded in monolayer culture and presents with neuronal activity (Hoffrogge *et al.* 2006; Donato *et al.* 2007). This line can also be differentiated to exhibit a DAergic phenotype as identified using immunocy-tochemical markers, however further characterization is still necessary. Regardless, immortalized mesencephalic NSCs may well provide a promising model to studying disease mechanisms related to DAergic neurons.

Patient-derived cell lines modeling disease in a dish

As described throughout this review, numerous α-synuclein based-cellular models have been established and are available to the scientific community for studies of pathophysiological mechanisms, toxicity or for high-throughput screening of future therapeutics (Table 1). Nonetheless they represent simplified models that may not accurately recapitulate the complexity of synucleinopathies and discoveries from these models may not precisely reflect the pathogenesis seen in patients. Human skin fibroblasts are an alternative to study disease mechanisms and can be used to develop patient-specific cell lines. However this approach is limited by the fact that fibroblasts in long-term culture become senescent and undergo clonal selection. Interestingly, transduction of specific pluripotency regulators has proven to be sufficient to convert skin fibroblasts into induced pluripotency stem cells (iPSCs). The emergence of patient-derived iPSCs has opened up new possibilities to create physiologically relevant disease models in a culture dish (Piper *et al.* 2018). Induced pluripotent stem cells offer unprecedented and exciting opportunities to understand disease

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mechanisms and have potential for high-throughput drug screening to test personalized therapies. Importantly, these new models facilitate the study of patient-specific risk factors or disease-specific mutations (e.g., A53T) in relevant cell types (i.e., DAergic neurons) while comparing the function and phenotype to iPSC lines derived from healthy control individuals (Piper *et al.* 2018). Lastly in combination with cutting edge genome-editing technologies such as CRISPR-Cas9, disease-causing mutations can be corrected in patient-derived iPSCs or mutations can be introduced into control iPSCs to further elucidate the role of disease-causing mutations (Calatayud *et al.*, 2017; Cobb *et al.* 2018).

To date several human iPSC models with mutations and genetic variations in the *SNCA* gene have been engineered to model specific molecular and cellular phenotypes. The generation of iPSC-derived midbrain DAergic neurons from patients with a *SNCA* triplication produces twice the amount of α-synuclein protein compared to healthy controls (Byers *et al.* 2011; Devine *et al.* 2011) and, importantly, the cell lines exhibit disease-related phenotypes such as increased expression of oxidative stress and protein aggregation-related genes. Several iPSCs lines also show lysosomal dysfunction induced by α-synuclein accumulation (Mazzulli *et al.* 2016). In SNCA triplication iPSC-derived DA neurons, α-synuclein aggregates were shown to physically interact with ATP synthase and lead to premature mitochondrial permeability transition pore (PTP) opening, making the neurons more vulnerable to cell death (Ludtmann *et al.* 2018). Interestingly, increases in reactive oxygen species following exogenous addition of α-synuclein oligomers were observed in *SNCA* triplication iPSCs (Deas *et al.* 2016), while another study demonstrated that the presence of *SNCA* triplication in iPSC-derived neural precursor cells reduces their capacity to differentiate into DA neurons, decreases neurite outgrowth, and lowers neuronal activity compared to control neurons (Oliveira *et al.* 2015) Additionally, microglia differentiated from *SNCA* triplication iPSCs had impaired phagocytosis compared to isogenic controls, suggesting that impaired microglial clearance of extracellular α-synuclein, contributes to α-synuclein accumulation and aggregation phenotype (Haenseler *et al.* 2017).

Patient-derived iPSCs differentiated into midbrain DA neurons carrying the A53T mutation, have been found to contain higher concentrations of α-synuclein monomers relative to tetramers when compared to the corresponding isogenic controls supporting a possible neuroprotective role of the α-synuclein conformation (Dettmer *et al.* 2015). Also in A53T iPSC-neurons, α-synuclein nitrosylation was found to be increased compared to isogenic control lines, while correction of the A53T mutation reversed nitrosative stress and ER stress suggesting that the mutation contributes to the aberrant phenotype (Chung *et al.* 2013). Increased sensitivity to environmental mitochondrial toxins with consequent nitrosative stress-induced neuronal loss has also been observed in A53T SNCA iPSC-DA neurons (Stykel *et al.* 2018).

In summary iPSCs represent a means to derive physiologically relevant cell types from patients and healthy controls, offering promise for the testing of individualized medicine approaches. Genome-editing techniques to correct genetic variants provide optimal isogenic controls and enable investigations into mechanistic links between genotype and phenotype. As well as utilizing cells from carriers of *SNCA* multiplications and point mutations, sporadic PD iPSC models can be generated which appear to recapitulate most cellular

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phenotypes of corresponding monogenic models. Since sporadic cases account for 90% of all PD, these iPSCs models may be considered more relevant. IPSC lines can be utilized in the high-throughput screening of individuals with both genetic and sporadic forms of PD to attempt to identify novel biomarkers or therapeutic approaches.

Although it can be argued that iPSCs are the cellular model with the most translatable relevance, there are some important caveats to be considered. Firstly the epigenetic profile of iPSC may not accurately resemble that of mature neurons. Potentially related to this, α-synuclein accumulation and aggregation is not so far a common feature in iPSC-derived models unless they are maintained in culture for extremely long periods of time. Therefore, more studies must be performed where aging of iPSC-DA neurons is provoked pharmacologically or another method (Vera *et al.* 2016; Tagliafierro *et al.* 2019). Alternatively, technological advances in cell differentiation may improve our ability to directly convert patient somatic cells into specific neuronal populations (iNeurons) without altering the epigenetic profile.

Concluding remarks

The central role of α-synuclein in the neurodegenerative process of synucleinopathies has led to the generation of cellular models aiming to elucidate its contribution to the dysregulation of various cellular processes and cellular toxicity (Table 1). As reviewed here, α-synuclein cellular models offer myriad opportunities for studying pathogenic mechanisms and aid development and validation of future pharmacological interventions. Each model has the potential to be a powerful tool provided the limitations and disadvantages of such simplified models are taken into account. Finally, the availability of newer cell culture systems, such as deriving iPSCs from patient somatic cells, offers hope that we will soon be able to closely mirror the disease in a petri dish which will pave the way towards personalized medicine and thus enhance the probability of success of future clinical trials.

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Abbreviations used:

DA	dopamine
DLB	dementia with Lewy bodies
FLIM	fluorescence lifetime imaging microscopy
H4	human H4 neuroglioma
HEK293	human embryonic kidney 293
IPSCs	induced pluripotent stem cells

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LB	Lewy bodies
LUHMES	Lund human mesencephalic cells
MSA	multiple system atrophy
NSC	neural stem cell
PCA	protein complementation assay
PD	Parkinson's disease
PFFs	pre-formed fibrils
PLA	proximity ligation assay

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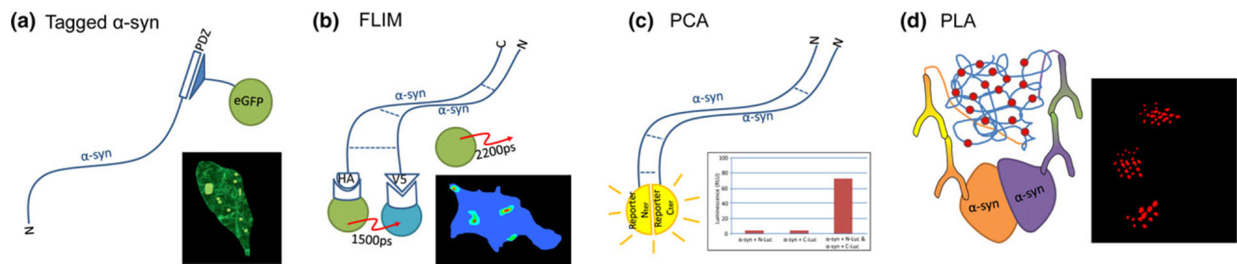
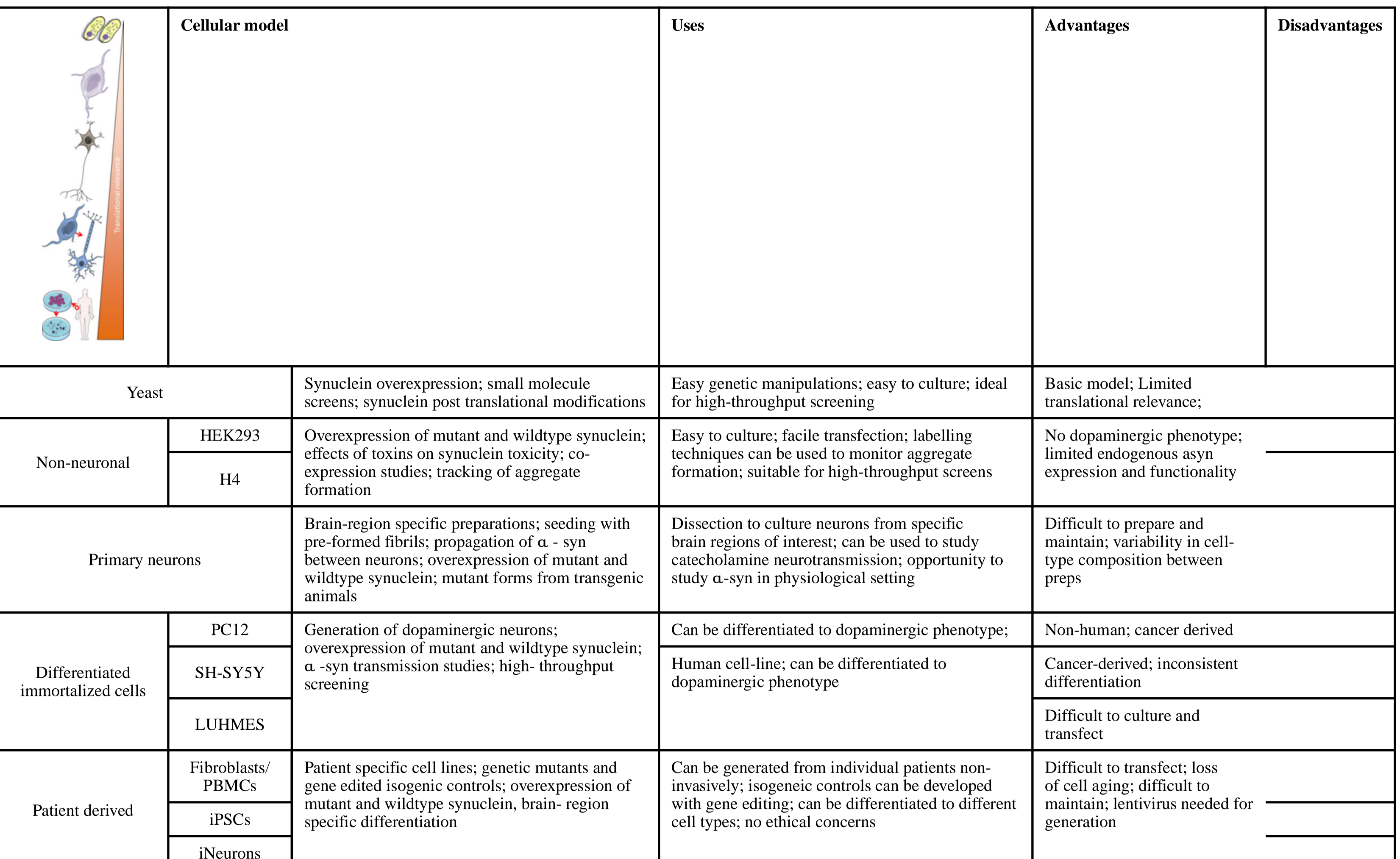


Fig. 1.

A number of methods have been developed to visualize aggregating α-synuclein in cellular models. These methods include the indirect (a) where synuclein containing a PDZ binding motif epitope is co-expressed with the corresponding PDZ domain fused to eGFP, sensitively labeling α-synuclein, with aggregates presenting as bright puncta (Opazo et al, 2008). Multimeric conformations of α-synuclein are more directly visualized using fluorescence lifetime imaging microscopy (FLIM) (b) by expressing α-synuclein containing small epitope tags. These can be targeted with FRET donor and acceptor secondary antibodies where the proximity of the interaction between synuclein molecules determined as the fluorescence lifetime of the donor fluorophore (Klucken *et al.* 2006). More recently (c) protein complementation assay (PCA) approaches have been used where synuclein tagged with either N- or C- terminal portions of a split reporter (fluorescent or bioluminescent) is expressed and the reporter signal used as a proxy for dimeric or higher order oligomeric species (Moussaud et al, 2015). In order to label endogenous α-synuclein aggregates, PLA can be employed (d) where proximity-dependent rolling circle amplification of oligonucleotide-labeled antibodies generates a signal to mark α-synuclein aggregates (Roberts et al, 2015)

Table 1

Cell lines available to study α-synuclein toxicity and aggregation with their associated advantages and disadvantages

	Cellular model	Uses	Advantages	Disadvantages
Yeast		Synuclein overexpression; small molecule screens; synuclein post translational modifications	Easy genetic manipulations; easy to culture; ideal for high-throughput screening	Basic model; Limited translational relevance;
Non-neuronal	HEK293	Overexpression of mutant and wildtype synuclein; effects of toxins on synuclein toxicity; co-expression studies; tracking of aggregate formation	Easy to culture; facile transfection; labelling techniques can be used to monitor aggregate formation; suitable for high-throughput screens	No dopaminergic phenotype; limited endogenous asyn expression and functionality
Non-neuronal	H4	Overexpression of mutant and wildtype synuclein; effects of toxins on synuclein toxicity; co-expression studies; tracking of aggregate formation	Easy to culture; facile transfection; labelling techniques can be used to monitor aggregate formation; suitable for high-throughput screens	No dopaminergic phenotype; limited endogenous asyn expression and functionality
Primary neurons		Brain-region specific preparations; seeding with pre-formed fibrils; propagation of α - syn between neurons; overexpression of mutant and wildtype synuclein; mutant forms from transgenic animals	Dissection to culture neurons from specific brain regions of interest; can be used to study catecholamine neurotransmission; opportunity to study α-syn in physiological setting	Difficult to prepare and maintain; variability in cell-type composition between preps
Differentiated immortalized cells	PC12	Generation of dopaminergic neurons; overexpression of mutant and wildtype synuclein; α -syn transmission studies; high- throughput screening	Can be differentiated to dopaminergic phenotype;	Non-human; cancer derived
Differentiated immortalized cells	SH-SY5Y	Generation of dopaminergic neurons; overexpression of mutant and wildtype synuclein; α -syn transmission studies; high- throughput screening	Human cell-line; can be differentiated to dopaminergic phenotype	Cancer-derived; inconsistent differentiation
Differentiated immortalized cells	LUHMES	Generation of dopaminergic neurons; overexpression of mutant and wildtype synuclein; α -syn transmission studies; high- throughput screening	Human cell-line; can be differentiated to dopaminergic phenotype	Difficult to culture and transfect
Patient derived	Fibroblasts/ PBMCs	Patient specific cell lines; genetic mutants and gene edited isogenic controls; overexpression of mutant and wildtype synuclein, brain- region specific differentiation	Can be generated from individual patients non-invasively; isogeneic controls can be developed with gene editing; can be differentiated to different cell types; no ethical concerns	Difficult to transfect; loss of cell aging; difficult to maintain; lentivirus needed for generation
Patient derived	iPSCs	Patient specific cell lines; genetic mutants and gene edited isogenic controls; overexpression of mutant and wildtype synuclein, brain- region specific differentiation	Can be generated from individual patients non-invasively; isogeneic controls can be developed with gene editing; can be differentiated to different cell types; no ethical concerns	Difficult to transfect; loss of cell aging; difficult to maintain; lentivirus needed for generation
Patient derived	iNeurons	Patient specific cell lines; genetic mutants and gene edited isogenic controls; overexpression of mutant and wildtype synuclein, brain- region specific differentiation	Can be generated from individual patients non-invasively; isogeneic controls can be developed with gene editing; can be differentiated to different cell types; no ethical concerns	Difficult to transfect; loss of cell aging; difficult to maintain; lentivirus needed for generation

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