Are iPSC-models the future of AD research?

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Abstract

Alzheimer's disease (AD) is an age-related neurodegenerative disease that affects a growing proportion of the human population. It is the main cause of dementia worldwide and, due to its increasing prevalence, it is estimated that the social and economic costs of caring for AD patients will have enormous impact on society. Thus, it is essential to understand the molecular basis of AD in order to develop more efficient therapies and reduce the burden of AD on society.

The lack of appropriate *in vitro* and *in vivo* models has hindered the progress for understanding AD mechanisms. However, the recent development of iPSC technology has enabled researchers to establish human neuron cultures that mimic the genetic and phenotypic profiles of AD patients. Despite that, obstacles such as variability and improper neuronal maturation are currently barriers for the use of iPSC-based models for AD research. In this essay, the contributions and limitations of iPSC-based models for AD research will be discussed. Finally, this essay also explores the future directions of iPSC-based research in AD research, namely the development of 2D and 3D neuronal-glial co-culture methods.

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Introduction

Neurodegenerative diseases are broadly defined as conditions where neuronal cells from the central and /or peripheral nervous system progressively lose their functions and die. It is the gradual death of neurons that gives rise to the neurological and psychological symptoms that are typical of each disease. The risk of developing neurodegenerative disorders often increases with age and, naturally, the prevalence of such diseases is expected to increase with the extension of the human lifespan. Indeed, between 1990 and 2016, the number of people suffering from dementia associated to Alzheimer's disease (AD) increased more than double to 43.8 million people worldwide[1]. This trend is also observed in other age-related neurodegenerative diseases[2]. In addition to the ageing of the population, while current treatments may help relieve physical and mental symptoms, there is no cure for these disorders. Altogether, these highlight the need for researchers to investigate the molecular basis of neurodegeneration in order to develop effective therapies. More effective therapies would not only have the obvious advantages for the patient but also cut the social-economic burden of caring for people suffering from a neurodegenerative disease. It is estimated that the total costs of care for people with AD or AD-like dementias could exceed 1 trillion dollars by 2050[3].

Despite the increasing research focus on AD, progress for understanding the molecular basis of AD has been hindered by a few obstacles. A major barrier is the reduced availability of brain tissue samples for study. Indeed, available brain tissue samples generally only display the final stages of disease, hence there is a lack of tissue presenting the early stages of AD pathology for study. This means that the mechanisms causatives of disease are still unknown. Most of what is known about AD progression and pathology comes from studies using animal models for AD. Nevertheless, the existing animal models do not often replicate human pathology, which is likely due to differences in species and genetic background[4]. Furthermore, animal models for AD are mostly based on genetic mutations linked to familial forms of AD[5]. Thus, many current models for AD do not mimic the etiology of sporadic AD, which is still unknown. Therefore, the scarcity of appropriate model systems has been crippling the progress in Alzheimer's research. Nevertheless, the recent development of induced pluripotent stem cells (iPSCs) may provide advantages and opportunities that typical animal and cellular models cannot provide[6,7].

iPSCs are pluripotent stem cells that can be generated from somatic cells via the introduction of specific transcription factors such as OCT4, C-MYC, KLF4 and SOX2[8]. It has been shown that these cells can proliferate and differentiate into the three germ layers *in vitro* and, therefore, can be differentiate into neuronal cells[8]. This provides benefits over using brain tissue samples as human neuron cultures can be engendered from somatic cells, such as

fibroblasts and, more importantly, generation of such models does not require the direct acquisition of neurons through biopsies. Additionally, iPSC-derived neurons made from patients' fibroblasts will have the same genetic makeup as the patient and, therefore, may replicate disease pathology more faithfully than animal models.

Thus, it seems that iPSC-derived neurons may present advantages over model systems previously used in neurodegeneration research and, consequently, may contribute to the understanding of the molecular basis of neurodegenerative disorders. Nevertheless, iPSC-based models may also have certain limitations. Major criticisms to the use of iPSCs include the restricted amount of comparison groups and the potential induction of cellular stress during iPSC generation and differentiation. Recent efforts are being conveyed to overcome these limitations. Genome editing techniques have been applied to the iPSC field to generate isogenic cell lines, this is, cell engineered from a parental cell line to model the genetics of a specific patient population. This way, the limited number of comparison groups and genetic variation between different patient-derived iPSC lines can be controlled[9]. Moreover, due to the differentiation capacities of iPSCs, 3D iPSC models are being developed to more accurately represent tissue-level disease pathology, which is lacking from more conventional 2D iPSC models[10].

This essay aims not only to evaluate the benefits and limitations of modelling AD using iPSCs but also explore the future directions of iPSC-based research, such as the use of isogenic cell lines, co-culture methods and 3D models.

Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disease characterized by the progressive death of neurons accompanied by the gradual worsening of cognitive functions. It is one of the most common neurodegenerative diseases and the main cause of dementia (responsible for between 60-70% of dementia cases)[11], which has enormous psychological, social and economic impacts on patients and their carers. It is estimated that around 50 million people worldwide suffer from dementia and 10 million new cases are diagnosed each year[11]. Despite being a great burden on society, the mechanisms that cause and promote AD progression are still not fully understood.

AD pathology in the brain is characterized by plaques composed of amyloid-β (Aβ) and neurofibrillary tangles (NFTs) of hyper-phosphorylated tau. According to the amyloid cascade hypothesis, disease progress is induced by the production of Aβ from the cleavage of amyloid precursor protein (APP) by the β- and γ-secretases. Aβ fragments then condense into plaques and promote the formation of NFTs, which eventually lead to toxicity and neuronal death[12,13]. This hypothesis is mainly supported by studies on models with mutations associated with familial forms of AD (fAD). Studies have shown that, in different animal models, mutations in either APP or in presenilin 1 (PSEN1) or presenilin 1 (PSEN2), which encode for proteins part of the γ-secretase complex, promote production of Aβ[14].

Over the years, the amyloid cascade hypothesis has been modified as it became clearer that accumulation of Aβ plaques does not linearly correlate with dementia and other cognitive impairments. Indeed, elderly nondemented individuals often show a substantial amount of plaques and neurofibrillary tangles, usually associated with AD pathology[15]. More recent evidence indicates that neurotoxicity may be caused by Aβ-derived diffusible ligands[16] and/or soluble toxic Aβ oligomers[17,18] (Figure 1). Nevertheless, the structure of such ligands and oligomers is still largely unknown.

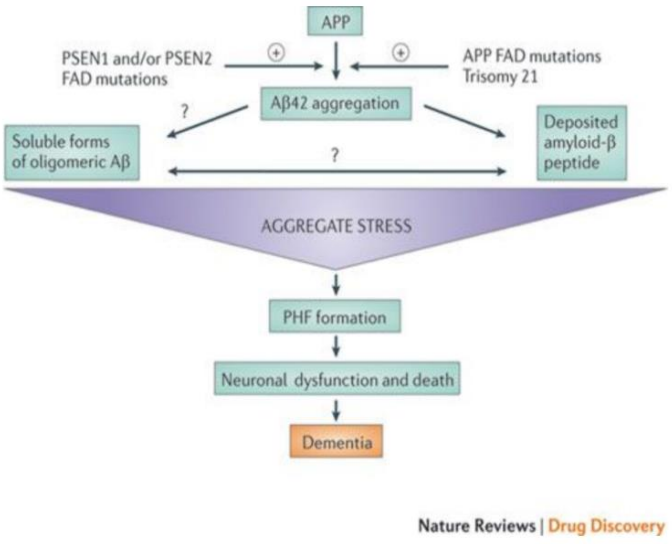

Figure 1 – The amyloid cascade hypothesis. Mutations in the APP, PSEN1 and PSEN2 can lead to accumulation and aggregation of Aβ peptides. This hypothesis has been modified over the years as it has become clearer that Aβ accumulation as plaques does not linearly correlate to dementia and cognitive impairment. It is proposed that intermediary forms of soluble oligomeric Aβ and/or Aβ plaques induce formation of paired helical fragments (PHF) of tau, which ultimately lead to neuronal dysfunction and death. Figure taken from: Karran, E., Mercken, M. & Strooper, B. De. The amyloid cascade hypothesis for Alzheimer's disease: An appraisal for the development of therapeutics. *Nature Reviews Drug Discovery* (2011). doi:10.1038/nrd3505

Mutations in the APP, PSEN1 and PSEN2 are a useful tool in understanding the molecular mechanisms underlying neurodegeneration in fAD however, it is becoming more clear that the mechanisms underlying sporadic forms of AD (sAD) are due to alterations in multiple pathways[19].

sAD accounts for over 95% of all AD cases and it is generally considered that the amyloid cascade hypothesis also occurs in sAD cases. Nevertheless, the triggers of Aβ accumulation in sAD are still unknown. GWAS studies revealed a number of genes that are implicated as risk factors for sAD, including the ε4 isoform of Apolipoprotein E (APOE) and loss of functions in triggering receptor expressed on myeloid cells 2 (TREM2)[20]. It was found that people heterozygous for the ε4 allele and four times more likely to develop sAD, while people homozygous for the same allele have around 12-fold increased risk of developing sAD. On the other hand, people with loss of function mutation in TREM2 are 2 to 3 times more likely to develop AD. Despite these findings, risk-associated genes encode for proteins with multiple functions thus, their contribution to the development of AD is still disputed.

It has been observed that chronic inflammation, characterized by increased proliferation and activation of astrocytes and microglia, as well as increased expression of cytokines and chemokines, accompanies the progress of AD. include the enhancement of proliferation and activation of astrocytes and microglia, activation of the complement system, and increased expression of cytokines or chemokines[21,22]. Thus, current therapies for AD are mostly inflammatory. Indeed, evidence suggests that retinoids and carotenoids have anti-inflammatory and neuroprotective roles as they may inhibit accumulation of Aβ accumulation, oxidative stress and secretion of pro-inflammatory cytokine secretion[23].

In spite of the development of better therapies, anti-inflammatory strategies do not terminate disease progression. Hence, it is essential researchers focus on understanding the molecular basis of AD and, in particular, sAD.

Contributions of iPSC models to understanding AD

The development of iPSC technology and the possibility to produce human neurons generated a new research field where iPSC-derived neurons are used to study disease mechanisms. Some studies comparing neurons differentiated from iPSCs of healthy donors and those of fAD and/or sAD patients have already been carried out.

iPSC-derived neurons from patients carrying mutations associated with fAD have been used to investigate the normal function of APP. Naturally, although much is known about the processing of APP into Aβ by secretases[12,13], the function of APP is still not fully understood. Indeed, it has been found that APP has various complex splice variants that can be cell specific, however, their roles, if any, in AD are yet to be discovered[24]. Recently, it has been found that while APP processing remained stable during cortical neuron differentiation, APP processing changes over time in iPSC-derived neurons[25]. Bergström and colleagues demonstrated that non-amyloidogenic soluble cleaved APP (sAPPα) is expressed in early differentiation, at the neural progenitor stage. Differently, amyloidogenic soluble cleaved APP (sAPPβ) only started to be secreted after formation of deep-layer neurons. Similarly, short Aβ peptides were secreted mostly early during differentiation while longer peptides, which are associated with formation of Aβ plaques, peaked when neurons were fully matured. Altogether, this study demonstrates that amyloidogenic APP processing is associated with mature neurons, thereby emphasizing the importance of using properly mature neuronal cultures to investigate AD disease pathways.

To research the function of the soluble fragment of APP (sAPP) and Aβ, Liao and colleagues measured the secretome of single neurons and astrocytes derived from iPSCs of healthy donors and patients with fAD-associated mutations[26]. In this study, it was observed that fAD mutations did not impact secretion of sAPP and Aβ, nevertheless, it was shown that deep layer GABAergic neurons tend to secrete high levels of Aβ. In addition, it was noted that astrocytes were able to secrete Aβ. Thus, Liao and colleagues propose the established method for detecting secreted proteins of iPSC-derived single neural cells could help answering important questions on APP and Aβ functions in healthy and disease conditions.

In other studies, iPSC-derived neurons have mostly been utilised to research cellular toxicity of Aβ oligomers[27–29]. Vazin and colleagues showed that pre-fibrillar forms of Aβ can bind to iPSC-derived glutamatergic and GABAergic neurons. Interestingly, it was noted that Aβ oligomers specifically induce death of glutamatergic but not GABAergic neurons[28]. These results support previous findings from postmortem human AD brain studies, which show selective degeneration of glutamatergic neurons. Differently, another research group demonstrated that iPSC-derived neurons had altered axonal vesicle clusters, disrupted postsynaptic AMPA signalling and increased levels of phosphorylated tau and endoplasmic

reticulum (ER) stress after being treated with Aβ oligomers for 8 days[29]. Although the concentrations of Aβ used to induce AD-like phenotypes were higher than normal physiological levels, these studies show that oligomeric Aβ induces synaptotoxicity and is particularly toxic to glutamatergic neurons. In 2017, Muratore and colleagues found that different types of neurons derived from iPSCs of patients with APP mutations show different degrees of AD phenotypes. Indeed, rostral neurons expressed higher levels of Aβ and phosphorylated TAU when compared to caudal neurons[30]. This is supported by the fact that rostral, cortical neurons are mostly affected during AD, while caudal neurons are often spared.

There have been less iPSC studies focusing on sAD when compared to fAD. Different studies have compared APP processing dynamics and levels of Aβ of iPSC-derived neurons from sAD and fAD patients with their respective control neurons. It was found that sAD iPSC-derived neurons have increased expression of APP and Aβ as well as altered ratios of Aβ peptides when compared to healthy iPSC-derived neurons[19,31,32]. Although these findings are consistent with what was observed in fAD neurons, it is important to note that results are highly variable in sAD iPSC-derived neurons. Indeed, Kondo and colleagues observed that iPSC-derived neuronal cells from two sAD patients responded differently to docosahxaenoic acid (DHA) treatment[19]. Furthermore, cells from the two patients showed different levels of Aβ aggregates and cellular stress (Figure 2). Similarly, the group of Mason Israel showed that iPSC-derived neurons from one sAD patient showed significantly higher hallmarks of AD than the other, including increased levels of Aβ, phosphorylated TAU and active glycogen synthase kinase 3β (GSK3β)[31]. Altogether, these studies show the significance of individual variability while studying disease mechanism and developing effective therapies. It would be expected that results are not consistent between neurons of sAD patients as it is known that disease pathways can be altered by a wide range of genetic and environmental risk factors.

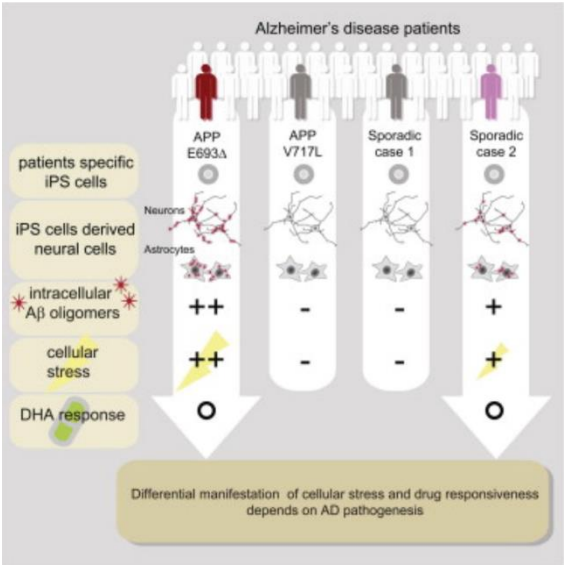

Figure 2 – Summary of the findings of Kondo and colleagues. iPSC-derived neuronal cells of fAD and sAD patients show different levels of AD pathological hallmarks. Neuronal cells from iPSCs of fAD patients with APPE693Δ mutation and of sAD case 2 displayed high levels of intracellular Aβ oligomers, cellular stress and responsiveness to DHA when compared to cells from the other patients. Figure taken from Kondo, T. et al. Modeling Alzheimer's disease with iPSCs reveals stress phenotypes associated with intracellular Aβ and differential drug responsiveness. Cell Stem Cell (2013). doi:10.1016/j.stem.2013.01.009

Limitations of iPSC models in AD research

A major limitation of iPSC-derived neuronal models is the relative developmental age of differentiated neuronal cells. Indeed, it has been reported that fibroblasts lose their ageing profile following reprogramming to iPSCs. A study on the utility of iPSC-derived neurons in modelling age-related diseases demonstrated that these neurons display an immature phenotype[33]. Thus, as differentiation protocols mirror developmental timing, cortical neurons generated by 100-day protocols model a foetal developmental stage. This is a significant obstacle for the use of iPSC models to investigate disease mechanisms of AD, where the main risk factor is age.

Another disadvantage of immature iPSC-derived neurons is the absence of tau isoform profile. The splicing of Tau and generation of different isoforms is regulated during development. Indeed, foetal tau expresses 3 microtubule binding region repeats while “adult” tau tends to express 4 microtubule binding region repeats, which is prone to aggregation. It has been observed that in disease conditions, there is an increase of 4-repeat tau expression. Nevertheless, iPSC-derived neurons mostly express 3-repeat tau, while 4-repeat tau was only found to be expressed when iPSC-derived neurons were cultured for longer periods of time (150-365 days)[34]. Similar to what was discussed above, this emphasizes the importance of using properly mature iPSC-derived neurons in investigating tauopathies, such as AD.

Although it is still disputed if the loss of ageing-profile is relevant for AD research, new protocols have been developed where fully mature, active neurons can be differentiated from iPSCs[35]. Another alternative for achieving a mature age-profile is direct reprogramming (transdifferentiation) of fibroblasts to neurons[36]. It has been demonstrated that transdifferentiation of fibroblasts to neurons maintains the ageing signature, as well as a mature tau expression profile[37]. Nevertheless, transdifferentiation protocols are less well-characterized and are less efficient than normal reprogramming protocols[38].

Genetic variability between iPSCs from different individuals and between iPSC clones from the same donor is also major limitation. Naturally, variability between cell lines can restrict chances of achieving statistical significance in AD research, as can be seen by the existence of studies with small number of cell lines. Indeed, a large-scale study in 2017 concluded that half of the variability found in over 300 iPSC lines from over 100 individuals is due to genetic differences[39]. This is an obstacle particularly for sAD research. A large number of genetic risk factors have been identified[20,31], suggesting that sAD could potentially be initiated by different molecular mechanisms. Thus, it is essential to select appropriate control cell lines to compare to iPSC cultures from patients.

One alternative to reducing variability between cell lines could be using iPSC cultures of close healthy family members. Few studies on neurodegeneration have been able to use

iPSC lines from discordant monozygotic twins, which is a strong indication that the genome between cell lines is identical except for the changes found in the diseased cells[40,41]. Another alternative to reduced genetic variability is the genetic stratification of iPSC cell lines through the generation of isogenic cell lines. As mentioned in the Introduction, isogenic cell lines are engineered from a parental cell line to model the genetics of a specific patient population, meaning that the genetic landscape of the cell lines is identical except for a mutation of interest. Most recent studies on iPSC field make use of genome editing techniques such as Transcription activator-like effector nucleases (TALENs) and the more recent technology CRISPR/Cas9 to generate isogenic cell lines[42]. Despite the accuracy of CRISPR/cas9, it has been demonstrated that this technology could induce cellular stress[43]. Hence, the use of CRISPR/Cas9 in iPSC research is somewhat disputed.

Role of glial cells in AD

In addition to neurons, glial cells are known to have important roles on the onset and progression of AD[44]. Microglia function as the innate immune cells of the central nervous system. In mice models of AD, increased levels of Aβ promote microglia accumulation for Aβ clearance thereby preventing formation of plaques[45]. Furthermore, few genetic risk factors for sAD have also been found in microglia. Guerreiro and colleagues observed that heterozygous mutations of TREM2, which plays a role in phagocytosis and cell signalling, in microglia are associated with increased risk of developing AD[46]. Astrocytes are another major cell type associated with AD. Various studies (reviewed by Dzamba and colleagues[44]) demonstrated that astrocytes become reactive in AD in response to increased levels of Aβ, which results in altered metabolism of Aβ, disrupted calcium homeostasis and synaptotoxicity due to defective clearance of glutamate. Similarly to microglia, genetic risk factors, including the APOEε4 isoform are often found enriched in astrocytes[6]. Taking the evidence for the roles of microglia and astrocytes in AD, it is becoming clearer that glial cells and their interactions with neurons could influence disease onset and progression.

To obtain a more realistic model of disease condition, efforts are being conveyed to develop efficient protocols for glial differentiation of iPSCs. Recently, an efficient protocol for microglia derivation from human pluripotent stem cells was developed[47]. Furthermore, microglia have been successfully co-cultured with cortical neurons, where it was demonstrated the these iPSC-derived microglia can mediate inflammatory responses[48]. Nevertheless, so far few studies have used iPSC-derived microglia in AD research, where it was observed that these cells could engulf fibrillar Aβ and brain-derived tau oligomers[49].

Various protocols to differentiate astrocytes from iPSCs have been developed, including transdifferentiation of astrocytes from fibroblasts[50,51]. It has been found that

astrocytes derived from iPSCs of patients with fAD mutations show increased Aβ levels, calcium dysregulation and oxidative stress[52], which are phenotypes often found in AD pathology. Similarly, to fAD cell lines, astrocytes have been differentiated from iPSCs of sAD patients homozygous for APOEε4 allele[53,54]. Contrary to neurons, it was observed that APOEε4 astrocytes showed morphological changes, dysregulated Aβ degradation and clearance[54], as well as disrupted differentiation from iPSCs[53]. Similar to microglial studies, efforts are being conveyed to build neuronal-astrocytic co-culture models. It has been reported that co-culture of human neurons with rodent astrocytes enhances growth and maturation of neurons[31] thus, it is plausible that co-culture of iPSC-derived neurons and astrocytes could provide benefits to neurons *in vitro*. Furthermore, considering the potential roles of glial cells in AD, including astrocytes into neuronal cultures could expose new disease phenotypes and venues for development of new therapies.

Studies have reported spontaneous differentiation of astrocytes in iPSC-derived neuron cultures at around 70 days post neural induction[35]. Hence, it is plausible that neurons co-exist with astrocytes in cultures that are maintained for long periods of time. Nevertheless, there are methods being developed to generate co-cultures more efficiently. One example is co-seeding, where two or more cell types are seeded onto a culture plate in relevant ratios however, it has not been well defined how the ratios of glial to neuronal cells vary between brain regions. It has been observed that co-seeding of iPSC-derived neurons and astrocytes improved neuronal maturation[55]. Other more complex co-culture systems, including culture on microfluidic chambers, enable the investigation of cell-cell interactions in a more detailed manner[56]. Indeed, it has been observed that synaptic interactions are stabilized when neurons are co-cultured with glial cells in microfluidic chambers, which indicates that neuron-glial communication play a role in the formation and maintenance of synapses[57].

Jumping to 3D

Despite the efforts for building better 2D *in vitro* models, it is proposed that 3D cultures could provide more advantages for AD research. Indeed, it is thought that these cultures can efficiently promote neuron maturation by better recapitulating the *in vivo* environment. Furthermore, due to regular replacement of media in 2D cultures, it has not been possible to observe certain AD phenotypes, including extracellular Aβ deposition and formation of NFTs. Nevertheless, few studies have tackled 3D cultures to study AD.

In vitro 3D cultures tend to be achieved by with culturing cells in a scaffold-like gel material or by self-organisation of cells into organoid structures. Recently, various research groups have developed protocols for generating neuronal organoid structures from iPSCs[27,58–60]. Raja and colleagues reported that 3D organoid cultures derived from iPSCs of fAD patients

with APP gene duplication or PSEN1 mutations recapitulate AD phenotypes such as Aβ aggregation, hyperphosphorylation of tau and aberrant endosome dynamics[58]. In addition, the group found that organoids treated with inhibitors of β- and γ-secretases, which cleaved the APP protein (discussed in Introduction) had significantly reduced levels of Aβ and phosphorylated tau[58]. Proteomic analysis of organoids derived from iPSCs of sAD patients revealed that key proteins involved in axonal injury and oxidative stress pathways are expressed in similar levels to ones found in post-mortem AD brain tissue[61]. Furthermore, a different study reported that efficacy of β- and γ-secretase inhibitors is reduced in organoids from iPSCs of sAD patients compared to 2D cultures and is varies between 5 AD cell lines[59]. Altogether, these findings suggest that 3D organoid structures can better mimic AD physiological conditions and may aid in the development of potential therapies.

Although organoid cultures have clear technical advantages, such as self-organization and are representative of *in vivo* models, they often display heterogeneity in cell numbers and types and, consequently variability between batches. This could become a source for inconsistent results due to variability, similar to what is found in the 2D iPSC field[27]. On the other hand, scaffold-based 3D cultures have a more limited range of variability as cell types are seeded onto the scaffold at ratios defined by the researcher. Few scaffold-based models are commercially available for AD research[62,63] nonetheless, the choice of hydrogel scaffold seems to be important for AD research. Indeed, hydrogels supplemented with heparan sulphate proteoglycans promote AD pathology[63], which could be advantageous in the study of sAD mechanisms. Differently, increased Aβ deposition and 4-repeat tau expression has been observed in APP and PSEN1 mutated neuronal cells cultured in a matrigel scaffold when compared to 2D cultures[63]. More organized and intricate hydrogel structures may also be generated by technologies such as 3D bioprinting to create more complex 3D models[64].

Conclusions and discussion

The progress for understanding the molecular basis of AD has been restrict by the lack of available tissue samples displaying the early stages of disease and animal/cellular models that mimic the etiology of AD. Nevertheless, the development of iPSC technology has opened new venues for the AD research field by providing a method for generating human neuronal cell cultures from healthy donors as well as AD patients. Despite this, the iPSC field is a relatively recent area of research thus, efforts are being conveyed to overcome current limitations of such models, including modulation of neuronal maturation and variability between cultures. In addition, new co-culture and 3D culture methods are being developed due to the increasing interest on the role of glial cells in AD onset and progression.

IPSC models provide the precise genome of the cell's donor. Hence, it is considered that IPSC cultures derived from AD patients are physiologically relevant *in vitro* models of AD has they display physiological expression of genes of interest. Indeed, certain AD phenotypes such as increased levels of Aβ and phosphorylated tau in rostral neurons compared to caudal neurons were observed in iPSC-derived neurons of patients with APP mutations[30]. This is consistent with what is observed in AD pathology, where rostral cortical neurons are more affected than more caudal neurons. Findings from other studies using iPSCs are also consistent with what is observed in physiological conditions. As discussed in "Contributions of iPSC models to understanding AD", Vazin and colleagues showed that pre-fibrillar forms of Aβ specifically induce death of glutamatergic but not GABAergic neurons[28]. These results support previous findings from postmortem human AD brain studies, which show selective degeneration of glutamatergic neurons.

Although evidence indicates that iPSC models faithfully mimic AD etiology, other studies have demonstrated that genetic variability between iPSC cultures can lead to heterogeneous results and, consequently, restrict the chances to achieve statistical significance[39]. This can be particularly disadvantageous for sAD research, to which a large number of genetic risks have been associated to[31]. In one study, it was reported that iPSC-derived neurons of two different sAD patients showed different levels of Aβ aggregation, cellular stress and response to drug treatment[19] (Figure 2). To reduce variability, genome editing techniques such as TALENs and CRISPR/Cas9 are being applied to generate isogenic cell lines[42], which model the genetics of a specific patient population, meaning that the genome is identical between cell lines except for a mutation of interest. Nevertheless, the application of these genomic editing techniques is still disputed as they may induce cellular stress[43].

Beside variability, iPSC-derived neuronal models often lack the presence of fully mature neurons. Naturally, following reprogramming to iPSCs, fibroblasts lose their ageing profile and, consequently, differentiated neurons display an immature phenotype[33]. Hence, the

utility of iPSC-derived neurons for studying age-related diseases, such as AD, is still disputed. Indeed, immature iPSC-derived neurons lack the presence of tau isoform profile found in AD. It has been reported that AD-like tau expression profile can only be observed when iPSC-derived neurons are cultured for long periods of time (150-365)[34]. Nonetheless, recent protocols have been developed where fully mature neurons can be derived from iPSCs[35]. In addition, it has been found that neurons transdifferentiated from fibroblasts maintain their ageing profile[36,37]. Therefore, establishing efficient transdifferentiation protocols may be an efficient method for generating mature neurons.

Establishing better protocols for generating iPSC-derived neuronal models of AD may help to better understand the molecular basis of AD. Nevertheless, as discussed in earlier chapters, glial cells may impact AD onset and progression. Naturally, AD is associated with chronic inflammation, which suggests that microglia, glial cells that regulate immune responses in the CNS, may play important roles in AD progression. Indeed, it was demonstrated that increased levels of Aβ induce microglia accumulation to prevent plaque formation in mice models of AD[45]. Moreover, it was found that TREM2 mutations in microglia are associated with increased risk of developing AD[46]. In addition to microglia, astrocytes are known to become reactive in response to increased levels of Aβ, which induces alterations in Aβ metabolism, calcium homeostasis and synaptotoxicity[44], which indicates that astrocytes may play key roles in AD progression.

To investigate the role of microglia and astrocytes in AD, various protocols for differentiating these cell types from iPSC have successfully been developed[47–50]. It is unknown if loss of ageing profile in glial cells is relevant to study of AD mechanisms in vitro. Despite that, astrocytes have successfully been transdifferentiated from fibroblasts, which, similarly to transdifferentiated neurons, should retain the donor's ageing profile[51]. iPSC-derived microglia and astrocytes can be co-cultured with iPSC-derived neurons and have been reported to improve neuronal maturation[55]. Furthermore, co-culture on microfluidic chambers revealed that glial cells stabilize neuronal synaptic interactions[57]. So, it seems that neuronal-glial co-culture methods could better mimic physiological conditions than neuron-only cultures. Therefore, it is suggested that AD researchers focus on building models with both neuronal and glial cells.

Notwithstanding, it is proposed that 3D culture methods may be better models than 2D co-culture methods. Certainly, it is considered that, because they better recapitulate the in vivo conditions, these cultures can promote neuronal maturation more efficiently than 2D cultures. In addition, particular AD phenotypes that have not been observed in 2D cultures may be observable in 3D conditions, such as extracellular Aβ deposition and formation of NFTs. Although few studies have tackled 3D culture for AD research, organoid cultures derived from fAD patients recapitulate certain AD pathological features and are responsive to current AD

drug treatments[58]. Moreover, such organoids express key axonal injury and oxidative stress proteins in similar levels to ones found in AD brain tissue[61], suggesting that 3D organoid structures mimic AD physiological conditions. Despite this, heterogeneity between organoid batches could become a source for inconsistent results and a barrier for achieving statistical significance. Hence, as proposed earlier, scaffold-based models could provide advantages such as better control of cell types present in the 3D culture. Moreover, modulation of the hydrogel scaffold could promote certain AD phenotypes, like increased Aβ deposition and AD-like tau isoform expression profile[63].

In conclusion, the use of iPSC-based models provides several benefits over using more traditional cell culture and *in vivo* models for AD research. Still, neuronal maturation, variability and lack of certain AD phenotypes are barriers that must be overcome in order to obtain accurate results from iPSC-derived neuronal models. One way of promoting neuronal maturation and better mimic AD physiological conditions is to include glial cells in iPSC-based culture methods, as they are known to have important roles in AD onset and progression. Furthermore, developing 3D culture methods could provide a better representation of AD *in vivo* conditions than 2D cultures.

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