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Human Induced Pluripotent Stem Cells and their Derivatives for Disease Modeling and Therapeutic Applications in Alzheimer's Disease

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ABSTRACT

Human induced pluripotent stem cells (hiPSCs) have recently been generated for various inherited diseases. These hiPSC have the capacity to differentiate into any given cell type with the help of small compounds and growth factors aiding the process. In Alzheimer's disease (AD) several specific neural subpopulations in the brain are more susceptible to degeneration and apoptosis and hiPSCs can be used in order to generate these subpopulations in cell culture dishes via directed differentiation. Subsequently these cells can be used to optimize small compound screens to identify novel drug targets and to study AD pathology on a cellular level. Recently, it has also become possible to repair the genetic defect found in familiar forms of AD (FAD) through the application of CRISPR Cas9 mediated gene editing. Both hiPSC from FAD patients and isogenic controls generated via gene editing provide an excellent basis for investigation of cellular pathologies of AD and a screening platform to develop novel drugs for treatment. Furthermore, the current efforts to optimize neural 3D differentiation methods provide an even more natural

platform compared to 2D differentiation approaches. These human cellular platforms complete, but also in some cases contradict the already gathered knowledge obtained from AD transgenic animals. This further underlines the importance of human based cellular models to fully understand AD pathology. In this chapter we will first summarize the current status of hiPSC for AD, followed by a description of the methods used to generate isogenic controls. We will also discuss the possibilities and limitations of current neural differentiation protocols for AD to obtain relevant neuronal subtypes. In the end we will elaborate on the possibilities and current issues of hiPSC for cell replacement therapies in AD.

INTRODUCTION

AD is the most prevalent age-related neurodegenerative disorder, which is characterized in its final stages by severe memory loss, personality changes and cortical brain atrophy. The pathological hallmarks of AD are amyloid plaques and neurofibrillary tangles (NFTs) [1]. Most of our current knowledge of the disease phenotype and progression stems from patients with FAD, with mutations in specific genes such as *Amyloid Precursor Protein* (*APP*), *Presenilin 1* (*PSEN1*) and *Presenilin 2* (*PSEN2*) [2], and mouse models generated with the human mutations of these genes [3]. Mutations in APP, PSEN1 or PSEN2 affect the processing of APP and have been implicated in the production of toxic Abeta forms and formation of amyloid plaques [4]. Another pathological hallmark of AD are neurofibrillary tangles (NFTs), which arise from instable hyperphosphorylated tau proteins [5]. The amount of NFTs is correlates well with the severity of AD, but mutations in the *MAPT* gene encoding the tau protein are strictly associated with frontotemporal dementia (FTD) and mutations in *MAPT* have so far not been directly correlated with AD. Despite of these rare FAD cases the majority of AD cases are classified as sporadic AD (SAD) cases with a later age of onset and absence of a family history or specific mutation. The only other well-described risk factor for AD is *apolipoprotein E* (*APOE*). Mutations in this gene can result in the expression of a certain isoform of APOE namely APOE4, which has been linked to increased susceptibility to develop SAD [6-8]. Besides *APOE* several other genes have been identified via GWAS to be potential risk factors for SAD, including *ABCA7, BIN1, CD33, CLU, CR1, CD2AP, EPHA1, MS4A6A-MS4A4E, PICALM, HLA-DRB5-DRB1, SORL1, FERMT2, CASS4, PTK2B* [9-13]. Despite these risk factors, at least 50% of the SAD cases are of unknown aetiology [14]. From these findings it is clear that AD has a very complex pathology and a cell-based approach could provide certain benefits over animal models in order to elucidate underlying mechanisms of the disease, which might be human-specific.

The development of human iPSC in 2007 [15] has opened up the possibility to generate patient-specific iPSC, which can be amplified and propagated easily as well as differentiated into any given cell type. Originally these iPSC were generated via integrative methods using retroviral or lentiviral delivery of the reprogramming genes [15-17]. Nowadays the methods of choice are footprint-free reprogramming approaches using for example Sendai virus [18] or episomal plasmids [19]. These approaches guarantee to a large extend that the observed pathological

phenotype is truly caused by the mutation and not a side effect of a gene expression perturbation caused by the integration of the lentivirus or adenovirus. If iPSC can be used in clinical applications, such as transplantation of progenitor cells derived from iPSC, a complete absence of integration of the reprogramming factors is obligatory. This is crucial in order to prevent knock-out effects caused by integration or inappropriate reactivation, which harbors a potential risk of cancerous growth due to inappropriate activation of pluripotency-related genes [20]. Another important development within the iPSC field is the generation of isogenic controls via gene editing. Originally gene editing was performed using Zinc finger nucleases (ZFN) and Transcription activator-like effector nucleases (TALENs) technology, but these have recently been replaced by a much more time efficient method named Clustered regularly interspaced short palindromic repeats (CRISPRs). Several groups have generated iPSC from AD patients and investigated their cellular pathology after differentiation of these into relevant neuronal subtypes. To this date all studies have used neurons derived from iPSC of age- and sex-matched controls, which has been proven to be problematic. With the advent of easy and time efficient targeted gene corrections using the CRISPR-Cas9 system it is foreseeable that these isogenic patient lines will replace the use of age- and sex-matched controls. These isogenic patient control lines are especially important for subtle, but disease relevant, phenotype detection, which can be missed in the noise of patient to patient and line to line variation.

The subsequent step in the process towards patient-specific disease cell models is differentiation of the iPSCs (as well as their isogenic controls) into the affected neuronal subtypes. In AD, neurons of the hippocampus and entorhinal cortex are affected early in the disease, whilst with its progression wider areas of the frontal and temporo-parietal cortex are affected. Numerous protocols for differentiation of neuronal subtypes of importance for AD modeling have been published ranging from unspecific neurons to cortical neurons and basal forebrain cholinergic neurons. These differentiation protocols enrich for certain neural cell populations, but with all currently used protocols there is also a tendency of other neural subtypes to emerge including glial cells and sometimes even non-neuronal cells. Maturation of the neurons into adult-like cell types, which are capable of recapitulating an AD-associated phenotype, is not trivial and poses several challenges. Hence, it may be necessary to stress the neurons by e.g. oxidative stress in order to provoke the disease phenotype.

The differentiated patient-specific neural cell cultures can, together with their isogenic controls, be used for modeling of AD and allow for both in depth studies of molecular disease mechanisms and, in the future, for implementation in drug discovery. A number of different AD-related phenotypes have so far been discovered in iPSC-derived models from patients including increased Abeta 42:40 ratios, increased Tau phosphorylation and enlargement of endosomes [21].

Due to the fact that so far all medications in clinical trials failed to substantially halt the progression of AD, several groups are pursuing the possibility of cellular replacement therapies. This form of therapy though is still in its infancy due to several obstacles, which still need to be sufficiently addressed before it can become reality in AD treatment. AD is a slowly progressing disease and by the time patients develop the symptoms, which attracts the attention of clinicians, significant damage to the brain has already occurred. The challenges of performing cell transplantations are multifold. Firstly, the cells need to be able to migrate out from the injection site, maintain their identity and functionally integrate. The functional integration can be especially challenging since the cells enter a diseased and toxic environment and the damage within the neural circuits they are supposed to integrate might be too advanced to efficiently integrate. Another aspect is the graft rejection. Even though the brain is considered to be immune privileged at a minimum the implanted cells need to be matched in regards to the human leukocyte antigen (HLA) haplotype and immunosuppression needs to be provided. Therefore, the ideal cell type for such transplantation attempts would be patient-specific isogenic genome modified iPSCs in which the disease causing mutation has been repaired through gene editing. Since these cells stem from the patients there will be no risk of a rejection response. The other important consideration is the maturation state of the injected cells. These need to be specified in order to develop into the desired neuronal subpopulation, but they should also not be developmentally too advanced. If the cells are too immature, inappropriate differentiation into unwanted cell types is possible and even tumorigenic overgrowth can occur. If the cells are too differentiated, then they might have issues with survival and integration.

In summary, iPSC have been derived from AD patients and isogenic controls are currently being developed to generate optimal matched controls for the cellular assessment of disease phenotypes. Several groups are developing differentiation protocols, which can be used to obtain disease-specific neural subtypes and efforts to further develop 3D differentiation might lead to a more natural mimicking of disease processes in the dish. Moreover, these iPSC and especially the isogenic controls might at some point be utilized for cell replacement/transplantation therapies.

MATERIAL AND METHODS

Generation of hiPSCs

Multiple scientific advances have led to improved reprogramming methods resulting in increased efficiency, safety and shorter duration of the overall procedure. This also includes in some cases a variation of the original transcription factor cocktail [22] (is reviewed in detail in [23]).

Several different approaches to generate iPSC as well as diverse primary cell sources have been used as elaborated below. In general this diversity of procedures to obtain iPSC is based on the fact that this field is still relatively young and researchers are still optimizing procedures.

This currently causes currently problems with Good Manufacturing Practices (GMPs) in regards to safety, reliability and reproducibility, which are crucial in order to embark on personalized medicine using cell replacement therapy [24].

Reprogramming Methods

Integrating reprogramming systems (viruses)

The first reprogramming studies were reported in 2006 and 2007 and used a retroviral-delivery method to deliver exogenous pluripotency factors [15,22]. With this system, hiPSC colonies could be observed after ~25-30 days post-transduction and the efficiency was around 0.01–0.02% for human cells [15]. However, retroviruses can only transduce proliferating cells as opposed to lentiviruses, which can target both dividing and non-dividing cells with acceptable efficiency. Subsequently the use of lentiviruses became more popular. The first report of a lentiviral-based strategy was in 2007 where Yu et al. delivered a different transgene combination (OCT4, SOX2, NANOG and LIN28) with ~0.02% efficiency, obtaining hiPSC colonies 20 days after infection [25].

Viral-dependent delivery methodologies are the most efficient, but these lead to random integration of the provirus in the host genome conveying a high risk of insertional mutagenesis and tumorigenicity. Subsequently iPSC generated via integrative viral-mediated approaches are not safe to be used in clinical trials and regenerative medicine [26]. Moreover, there are expression problems between the different reprogramming vectors, which can deviate from a 1:1:1:1 stochiometry and hamper the reprogramming process [27]. As a consequence, the reprogramming vectors were merged into one single cassette with a self-cleaving peptide signal separating the factors in a polycistronic lentiviral-based system [28,29]. In order to avoid reactivation issues of integrated pluripotency factors, lentiviral vectors were designed containing loxP sites, with makes it possible to excise any transgene integrated sequences by Cre-mediated excision [29,30]. This technique was already reported to work in Parkinson's-hiPSCs via expression of a Cre-puromycin episomal plasmid [31].

Nonintegrating reprogramming systems

In order to generate iPSC, which are potentially applicable in regenerative medicine, several footprint-free methods have been successfully developed and reported, such as Sendai virus, episomal plasmids, direct mRNA, proteins and small molecules [25,32-34]. However, the reprogramming efficiencies vary a lot amongst methods.

Sendai virus is a RNA virus-based approach commonly used in research and quite attractive for reprogramming of translational-grade iPSCs. It has two major advantages – it is replicated within the cytoplasm, cannot enter the nucleus and is lost around 10 passages after targeting. Secondly, it has a high protein expression of the transgenes from the vector [35]. Moreover, it is able to reprogram adult fibroblasts with ~1% efficiency in less than 30 days. Sendai virus-based reprogramming works also with neonatal fibroblasts and blood cells [36-38]. Sendai viruses

containing the Yamanaka factors are commercially available and ready to use but it is technically much more challenging to work with these virus when compared to lenti- or retrovirus [38].

Transient expression of the exogenous transcription factors as episomal plasmids can generate integration-free iPSCs, however the genomic reprogramming process is relatively slow, meaning the expression of the transgenes has to be maintained for around 10–20 days [39]. Unless transfections are performed every day, a standard vector cannot be expressed long enough for the cells to be reprogrammed, and the efficiency is also extremely low [40]. Consequently, Epstein–Barr nuclear antigen (OriP/EBNA)-based plasmids were constructed and coupled with *OCT4*, *SOX2*, *NANOG*, *KLF4* + *OCT4*, *SOX2*, *SV40 Large T antigen* + *C-MYC* and *LIN28* vectors, allowing for longer expression periods and successfully generating iPSCs, but still with low efficacy [41]. New oriP/EBNA plasmids were then designed containing the Yamanaka cocktail and *LIN28* together in one single reprogramming cassette and an aditional oriP/EBNA vector with *SV40 Large T antigen* [42]. With one transfection, the iPSCs gene expression, pluripotency studies and *OCT4*/*NANOG* methylation resembled ESCs. Moreover, the exogenous transgenes were lost at passage ~12 and no footprint was detected in whole genomic sequencing [43]. Later, these episomal-based protocols were subjected to changes (i.e. inclusion of nontransforming *L-MYC* and suppression of p53) in order to increase efficiency, and xeno- and feeder-free systems were adapted [34,44,45]. The latter is highly relevant for reliable, reproducible and therapeutic-grade iPSCs. Episome-mediated systems still show low reprogramming efficiency of fibroblasts but this method is becoming more attractive since it allows for footprint-free and clinically-approved iPSCs that can be used in translational studies.

Using reprogramming cocktails directly as proteins is extremely appealing for the field as it allows for integration-free systems and translational-grade iPSCs. However, it is quite challenging to produce sufficient amounts of proteins that are able to enter through the plasma membrane while maintaining their biological activity. Some labs were already able to derive bioactive proteins in *E. coli* and successfully reprogrammed mouse and human fibroblasts, although with relatively low efficiency [33,46]. This system might be viable in the future but it is still struggling with several technical challenges like infeasible efficiency, lengthy timeline and problems when reprogramming cells that are not fibroblasts.

mRNA transfection is another integration-free system that consists of expressing exogenous transgenes by providing mRNA. This was firstly achieved by Warren et al., who reported reprogramming factors being successfully transcribed as mRNAs in human fibroblasts [47]. The strong immunogenic response upon synthetic mRNA transfection leads to high cell death but this was solved with several modifications such as substitution of 5-methylcytidine RNA bases for cytidine and pseudouridine for uridine, plus addition of interferon inhibitor B18R to the culture media [47]. Using the Yamanaka cocktail paired with *LIN28*, valproic acid and 5% O_2 in culture, it still took 20 days to generate iPSCs, but the efficiency was greatly improved to ~4.5% efficiency

compared to the initial 1.5% [47]. Like the protein-based system, and despite being commercially available, reprogramming mRNAs are costly and labor intensive and still haven't been reported in other cells besides fibroblasts.

Other popular reprogramming methods are adenovirus, miRNA transfection, PiggyBac and minicircle vector-based systems. Adenovirus-delivery technology is promising since these do not integrate into the host genome; nevertheless the reprogramming efficiencies are very low [32,48]. It has previously been reported that ESCs express many miRNA clusters, which are important to establish and maintain pluripotency. Therefore several groups are now adding mature miR-372 and/or miR-302b together with the lentiviral-delivery of the Yamanaka factors to reprogram fibroblasts [49]. This approach showed a significant increase in efficiency when compared to only Yamanaka factors in lentiviral constructs [49]. Recently it has even been possible to use specific miRNAs by themselves, without the Yamanaka cocktail, to generate iPSCs with decent efficiency [50,51]. Both expression of seed sequences from miR302/367 as lentivirus particles and transfection of mir-200c, mir-302s and mir-369s generated iPSCs [50,51]. However, none of these studies was yet successfully replicated, making it difficult to assess the efficiency and reliability of the mentioned system. Another reprogramming technology is based on cloning the Yamanaka factors into a piggyBac vector. PiggyBac is a transposon than can be integrated in and cleanly excised from TTAA chromosomal sites if the enzyme transposase enzyme is expressed. Therefore this method can also be considered foot print-free. If the Yamanaka-piggyBac vector is transfected into mouse embryonic fibroblasts (MEFs), reprogramming can be achieved within 14-25 days at ~0.05% efficiency [52,53]. Despite the inefficiency (0.02%), when a similar set-up (including sodium butyrate) was tried in human mesenchymal stem cells (MSCs), these were also successfully reprogrammed [54].

Finally, reprogramming was also achieved using the minicircle method - small vectors consisting only of the cDNA that is expressed and a eukaryotic promoter. Narsinh et al. (2011) published the first human adipose stromal cells reprogrammed using a minimal vector containing *OCT4*, SOX2, *NANOG*, *LIN28* and *GFP*, also with low efficacy (~0.005%). Later, neonatal fibroblasts were also reprogrammed but with an even smaller efficiency rate [55]. For robust results, the method needs further and extensive validation.

Validation of iPSC lines

After reprogramming, confirmation of the pluripotency status of the generated iPSC is necessary. In the past, the gold standard for assessment of hiPSCs pluripotency were *in vivo* teratoma assays [56,57]. This method is costly, time and labor demanding and requires the use of laboratory animals [57]. Consequently, a shift towards *in vitro* assays has been made during the past years. *In vitro* assays can be more easily streamlined and are better suited for high-throughput and standardization. Most popular are gene expression analysis with qPCR-based methods, such as PluriTest [58], to directly compare the generated hiPSCs with hESCs. Another

common method is embryoid body (EBs) formation to assess trilineage potential [59], since truly pluripotent cells should be able to differentiate into all three germ layers namely mesoderm, endoderm and ectoderm.

Gene Editing in hiPSC Models

Generation of AD isogenic controls for potential gene/cell therapy

The genome editing field has been revolutionized in a particular short time period, allowing research to reach new frontiers by correcting diseased cells obtained from patients and therefore the generation of patient-specific cell therapeutics. Pluripotent stem cells (PSCs) – iPSCs, ESCs and adult stem cells - are quite responsive to gene editing since they can maintain their genome integrity and pluripotent state even after lengthy culture and manipulations, drug selection and clonal expansion. This opens up possibilities for gene corrections, which are ideal control cells for *in vitro* assays of the patient cell lines and excel age- and sex-matched controls. Furthermore, if GMP standards, reproducibility and safety of iPSC derived progenitors are achieved, cell replacement therapies can be envisioned with such gene edited patient cell lines.

In general, targeted synthetic nucleases technology is based on engineered DNA-binding sequences fused to a non-specific FokI endonuclease catalytic domain that requires dimerization to cleave at a specific site, activated by sequence-specific binding. The binding leads to a double-strand break (DSB), which then triggers DNA repair primarily via non-homologous end joining (NHEJ) mechanism or less frequently by homologous recombination (HR) [60]. Zinc finger nucleases (ZFNs) belong to first generation of engineered nucleases and are modular proteins with the FokI catalytic unit fused to several Cys_2-His_2 zinc finger (ZF) DNA-binding motifs [61]. These ZF domains recognize 3-4 bps, and are linked in tandem. Two ZFNs are designed to have both FokI units oriented towards each other and bind in opposite sides of the specific sequence to be cleaved [62], which associated with the inclusion of ~4-6 motifs increase specificity [63]. ZFNs are commercially available (Sigma) and have been successfully used to target different genomes, including human cells [61,64]. Unfortunately, this system is quite expensive, its design and construction are difficult, laborious and time consuming, and most importantly, off-target cleavage can occur and cause undesired mutations, cytotoxicity and hampers the analysis of the programmed genetic modifications (reviewed in [62]).

New techniques such as Transcription activator-like effector nucleases (TALENs) have emerged and allow for the repair of specific disease-linked mutation(s) in a relatively simple manner. TALENs resemble ZFNs since they consist of a FokI unit fused to DNA-binding domain that target contiguous DNA sites; these have 35 tandem repeats and each contains two amino acids that are specific for a single basepair of DNA sequence [65]. This means the design is easier than for ZFNs, and it is also not necessary to re-design the spacer/linker sequences when changing the target site, which is necessary when working with ZFNs. Therefore, TALENs can be designed and produced much faster, they show higher specificity, allow multiplexing and are more cost

efficient. Moreover, openly available repositories (www.addgene.org) contain most plasmids sets necessary for the construction of these nucleases [66-68].

Another recent method emerged and is reported to be even easier and quicker than TALENs and ZFNs. This system is known as Clustered regularly interspaced short palindromic repeats (CRISPRs) and it consists of a guide RNA (sgRNA) sequence that is designed to specifically recognize the genomic loci target [69,70]. Right after the target sequence comes the protospacer adjacent motif (PAM), which together with a seed sequence contained within the sgRNA, direct the Cas9 endonuclease to the target site, which originates a DSB in the DNA sequence [69,70]. This method is quick, versatile, simple and relatively cheap since sgRNAs (~18-20 nts) are easily designed and produced; like TALENs, most plasmids needed are also available in free repositories. The CRISPR-Cas9 system was already used successfully in iPSCs and it was shown that this technology has higher efficacy when compared to the previously mentioned systems, also allowing for an easy re-design so that other genomic sequences can be targeted [71]. There are, however, some constrains in the target design due to the PAM sites that, in most cases, must contain specific sequences.

If homologous DNA fragments containing the correct (healthy) target sequences are included in the transfections, the cells where HR happens can use these as a template to replace the host sequence, therefore correcting the specific mutation. This system allows the generation of isogenic control lines, which resemble exactly the disease cell line except for the disease-linked mutation that was corrected. Several disease-causing mutations have been corrected in specific cell types and even when targeting mouse oocytes, the progeny was corrected for the mutation and disease [72-78]. An important advantage of TALENs and CRISPRs is that these are able to "act" without leaving any traces at the target site.

A particularly problematic caveat with CRISPR-Cas9 strategy is the shorter target RNA sequence (when compared to ZFNs and TALENs), which makes it more complicated to control genomic off-target changes. This means that genetic changes in other loci that have either a similar or the same sequence as the target might occur. These off-target disruptions may lead to alterations in other genes throughout the genome (being loci-dependent) and it has been previously reported [73,76,78]. Some variations of this system have already been developed (e.g., CRISPR nickases) to reduce the off-target activity [79]. Still, improvements on the design and activity of TALENs and CRISPRs are necessary, especially ones that allow only the desired target site to be recognized. These advances may even be necessary before the next step to clinical transplantation can be envisioned. It is also possible to adapt the set-up according to the specific needs using more than one methodology and recently some combination systems such as iCRISPR (TALEN-CRISPR/Cas) have been developed, reporting an increase in efficiency and multiplex/high-throughput genetic analysis ability [80], which was also reported to work in hPSCs [81].

A successful correction of an AD phenotype with these gene editing systems has not yet been published, but this research field will surely be further developed and improved with future research. Importantly, it will aid the generation of realistic control lines (i.e. isogenic controls) by reverting the specific mutation back to a healthy state. This allows for disease and control cells to only differ by the disease-associated mutation, therefore annulling any other line-specific variations (as it happens when using age/sex-matched controls) and allowing for an accurate interpretation of phenotypes in diseased lines. Moreover, the generation of healthy patient-specific cells will be facilitated and these might potentially be allowed for autologous transplants, therefore escaping immunological rejection.

Direct lineage conversion

Interestingly, although with low efficiency, it is also possible to generate functional neuronal cells through direct reprogramming [82]; its use is unfortunately limited since the cells generated cannot proliferate. This is a limiting factor in regards to large scale compound screens for drug development and cell replacement therapies. In contrast, neuronal progenitor cells are capable of proliferation and expansion and recent reports on induced neural progenitors cells (iNPCs) showed that these cells are capable to differentiate into all types of neuronal lineages, suggesting these directly-reprogrammed lines might be a relevant alternative to iPSC-derived neuronal cells for future screening platforms or cell replacement [83-85]. Direct conversion of somatic cells into iNPCs via ectopic expression of specific transcription factors was already achieved and these cells resembled dividing primary neural progenitors with self-renewal and differentiation capacities [86,87]. The same group is also using specific transcription factors ectopically to generate directly-reprogrammed region- and subtype-specific iNPCs [88]. Moreover, iNPCs derived from MEFs with hypoxic conditions and chemical cocktails (ciNPCs) were also reported to share cell properties and gene expression similarities with murine brain-derived NPCs. These cells are generated without any kind of transgene, therefore surpassing the critical issues of insertional mutagenesis, tumorigenicity and ethical criteria mentioned earlier [89,90].

RESULTS

So far, the majority of reprogramming methods used in AD-hiPSCs models were based on retroviral-based delivery and used the traditional Yamanaka cocktail or a slight variation substituting *c-MYC* for *L-MYC*, including *LIN28* and short-hairpin for *p53*. There are also studies that chose lentiviral- [91] and episomal-based systems [92,93], although the exogenous factors used remain the same as mentioned before (Table 1). At this point it makes sense to strive for the highest efficiency possible to generate AD iPSC since the current focus is based on *in vitro* studies of the disease phenotype or compound screens for drug development. Obviously, once robust *in vitro* disease models have been generated, and if the AD-iPSC-derived progenitor cells should be used in cell based replacement therapies, non-integrative and footprint-free reprogramming methods are needed.

Table 1: Reprogramming and differentiation strategies for induction of induced pluripotent stem cells and their neural progeny.

Reprogramming Strategy	Differentiation Protocol	Cell Type Formed	Reference
Retrovirus *OCT4, SOX2, KLF4, LIN28, NANOG* Human dermal fibroblasts	EB induction w/o bFGF 8d EBs plated gelatin w/o bFGF 8d Neuron induction w/o growth factors 2wks Added compound E or compound W 48h	Neurons βIII-Tubulin +MAP2+	[99]
Retrovirus *OCT4, SOX2, KLF4, cMYC* Human dermal fibroblasts	Neuronal rosette induction on PA6 stromal cells 11d NPCs isolated by FACS CD184+CD15+CD44-CD271- NPC cultured 4 wks Neuron induction - BDNF/GDNF/cAMP 3 wks CD24+CD184-CD44- neurons selected by FACS. Cultured in BDNF/GDNF/cAMP 5d	Neurons βIII-Tubulin+MAP2+ >90% VGluT1+15% GABA+8% Expressed tetrodotoxin-sensitive voltage-activated currents GABA+AMPA receptors Spontaneous inhibitory/excitatory synaptic currents	[21]
hESC (I3) transduced with lentivirus containing mutations in PS1 iPSC – Retrovirus Human dermal fibroblasts (PKa)	It-NES induction with bFGF+EGF+B27 Neuron induction - Matrigel w/o factors, +N2+B27+cAMP 4 wks	Neurons βIII-Tubulin+ 80% Astrocytes 6%	[116]
Trisomy 21 Retrovirus *OCT4, SOX2, KLF4, cMYC* Human dermal fibroblasts	Matrigel+N2+B27+Noggin+SB431542 Dissociated and cultured with 3N+bFGF 100d	Cortical neurons; Early born, TBR1+βIII-Tubulin + CTIP2+βIII-Tubulin+ 30% Late born, BM2+βIII-Tubulin+ SATB2+βIII-Tubulin+ 20-25% Functional synapses Glutamatergic+ PSD95+	[102]
Episomal vectors *SOX2, KLF4, OCT4, L-MYC, LIN28*, shRNA p53 Human dermal fibroblasts	EB induction DMEM/HamsF12+5%KSR+SB431542 8d Neural induction – plated on Matrigel +N2+SB431542 16d Cortical neuron induction – dissociated and cultured in NB media + B27+BDNF+GDNF+NT3 48d As above, but on day 58 cortical neuron induction, cells passaged Repeated passages on day 96, 126, 156, 176	Cortical neurons SATB2+TBR1+ Astrocytes	[117]
Retroviral vector *Klf4, Oct4, Sox2, cMyc* Human fibroblasts	RA + bFGF 7d Neurosphere formation w/o bFGF 7d Neurospheres cultured with bFGF+EGF 4d Neurospheres cultured with SHH+FGF8 3d Dissociated and transfected with Lhx8/Gbx1-IRES-EGFP 2d Lhx8+/Gbx1+ cells selected by FACS and cultured in NB media + bFGF+NGF 2wks (+ arabinoside from day 5-10 of NB culture step)	Basal forebrain cholinergic neurons 95% MAP2 66% ChAT VaChT+P75R+, NKX2.1+HB9- Expessed tetrodotoxin-sensitive voltage-activated currents, voltage-gated calcium channels	[96]
Reprogramming Strategy	**Differentiation Protocol**	**Cell Type Formed**	**Reference**
Lentivirus *OCT4 SOX2, cMYC, KLF4* Human dermal fibroblasts	Aggregates with iPS cell media 4d + neural media+N2 2d Aggregates plated on matrigel, Neural media+N2 10d Suspension culture, neural media+B27+N2+cAMP+IGF1 7d Neural rosettes selected manually or Neural Rosette selection agent Dissociated + plated on Matrigel+NBmedia+N2+B27+ cAMP+ BDNF+GDNF+IGF1 35d-76d	Neurons 90% MAP2 Tau+, βIII-Tubulin+Cux1+ Tbr1+PSD95+VGLUT1+ Spontaneous activity from microelectrode array	[91]

MMLV retrovirus *Oct4, Sox2, Klf4, cMyc* Human dermal fibroblasts	Neuronal rosette induction on PA6 stromal cells + Noggin and SB431542 6d -Noggin and SB431542 8d CD24+/CD184+/CD271-/CD44- cells selected by FACS Cultured in neural media (DMEM:F12+N2+B27+BDNF+GDNF+dcAMP) for 3 wks w/o bFGF CD24+/CD184-/CD44- neurons selected by FACS	Neurons βIII-Tubulin+MAP2+	[100]
Retrovirus *OCT4, KLF4, SOX2, cMYC* Human dermal fibroblasts	Neuronal progenitor induction using dual-SMAD inhibition 9d NB media 26-46d	Neural progenitors 79% NESTIN+, small pop'nβIII-Tubulin+ Neurons Active Na^+ channels K^+ channels Produce action potentials 40% neurons Ca^{2+} spikes	[101]
Episomal vectors *OCT4, SOX2, NANOG, KLF4, MYC, LIN28, SV40 LT* Human dermal fibroblasts	1. EB induction 4d Neural Induction: DMEM:F12+N2+NEAA+heparin+bFGF for 3d. Cells then plated only polyornithine/laminin-coated dishes in either neural induction medium for 8-10d forming rosettes 2. EB induction 10d Neural Induction: Cells plated on matrigel-coated dishes in complete NB medium (NB+BSA+NEAA+B27+N2+noggin+SB31542) for 5 d and w/o SB31542 thereafter. Rosettes isolated and cultured either as monolayers in NB complete medium+bFGF+EGFor as suspension in DMEM:F12+B27+bFGF+EGF+heparin Neuronal differentiation: cells plated as monolayers in NB complete medium or DMEM:F12+N2+B27+cAMP+AA+BDNF+GDNF for 1-2months	Cortical neurons (MAP2+, SYNAPSIN1+, TBR1+, SATB2+) Electrophyisologicareadings show sodium and potassium currents and spontaneous postsynaptic currents	[93]
Retrovirus *OCT4, SOX2, KLF4, cMYC* Human dermal fibroblasts	Neuronal rosette induction on PA6 stromal cells 11d NPCs isolated by FACS CD184+CD15+CD44-CD271- NPC cultured 4 wks Neuron induction - BDNF/GDNF/cAMP 3 wks CD24+CD184-CD44- neurons selected by FACS. Cultured in BDNF/GDNF/cAMP 5d	NPCs (NESTIN+) Neurons (MAP2+)	118
Retrovirus *OCT4, SOX2, KLF4, cMYC* Human dermal fibroblasts	Neuronal induction: MEF conditioned medium+SB431542+PD0325901 minus bFGF for 4 weeks.	Immature neurons (PAX6+, NESTIN+, βIII-Tubulin +)	[95]

AA-Ascorbic Acid; D-Days; B27-B27 Supplement; BDNF-Brain-Derived Neurotrophic Factor; bFGF-Basic Fibroblast Growth Factor; BSA-Bovine Serum Albumin; cAMP-cyclic adenosine monophosphate AMP; dcAMP -Dibutrylcyclic AMP; EB-Embryoid body; EGF-Epidermal Growth Factor; EGFP-Enhanced Green Fluorescent Protein; FACS-Fluorescence Activated Cell Sorting; GDNF-Glialcell-Derived Neurotrophic Factor; IGF-Insulin Growth Factor; IPS cell-Induced Pluripotent Stem Cells; It-NES- Neuroepithelial Stem Cells; KSR-Knockout Serum Replacement; N2-N2 supplement; NB-Neural Basal Media; NGF-Nerve Growth Factor; NPC-Neural Progenitor Cell; RA-Retinoic Acid; SHH- Sonic hedgehog; wks-weeks; w/o-without; 3N-Modified bold 3N medium.

As mentioned before, in general AD can be divided into two groups. Early onset AD with known mutations also referred to as familiar forms of Alzheimer's disease (FAD) and late onset with unknown genetic background and usually no family history of AD also referred to as sporadic forms of Alzheimer's disease (SAD).

Sporadic AD

Sporadic AD with no known genetic mutations

The main neuropathological lesions of sporadic AD are considered to be the accumulation of Abeta and tau, however other proteins also accumulate, including a-synuclein, TDP-43 and ACTIN which result in Lewy-like bodies and Hirano bodies [94]. Depression, agitation, apathy, social withdrawal, insomnia, delusion and emotional/physical outbursts are all typical symptoms associated with AD. In the case of sporadic AD, the symptoms occur later than the familial forms of AD [94]. However, the complexity and heterogeneity of both symptoms and pathology are widespread in sporadic SAD, which may be due to multiple risk factors including mutations in varying genes and effects of environmental factors [94].

Three studies investigating neurons produced from sporadic AD patient iPSCs without any known risk factor mutations have been performed. Little investigation of APP processing has been done performed, although one study reported no change in Abeta 40 [21]. Increased hyperphosphorylated tau (p-tau) was also reported in neurons from one patient [21]. Interestingly, enlarged endosomes were also observed in the same patient [21]. One other study found altered WNT signaling and glutamate metabolism in mixed cell culture's from one patient and gene alterations related to proteosome function, ROS and cell death [95] (Table 2).

Table 2: Phenotypes of neural cells analyzed from differentiated patient-specific induced pluripotent stem cells for studying Alzheimer's disease.

Mutation	Cell Type analyzed	Analyses performed	Phenotype	Reference
Familial PSEN1 (A246E) 2 clones Familial PSEN2 (N141I) 2 clones	Neurons (βIIITubulin+ MAP2+)	Extracellular A Tau accumulation (HT7 antibody) Tangle formation Treatment with γ-secretase inhibitor and modulator of γ-secretase-mediated APP cleavage	Increased Aβ42:Aβ40 No Tau accumulation No tangle formation Decreased Aβ40+Aβ42 with γ-secretase inhibitor and modulator of APP cleavage Increased Aβ42:Aβ40 No Tau accumulation No tangle formation Decreased Aβ40+Aβ42 with γ-secretase inhibitor and modulator of APP cleavage	[99]
Familial APP dup'n $APP^{Dp}1$ 3 clones Familial APP dup'n $APP^{Dp}2$ 3 clones Sporadic sAD1 3 clones Sporadic sAD2 3 clones	Neurons (βIII-Tubulin+ MAP2+) >90%	Genome-wide mRNA seq. Extracellular Aβ p-tau (Thr231) GSK-3β activity Treatment with γ-secretase and β-secretase inhibitors Endosome markers Synaptic markers	Increased Aβ40 Increased p-tau (Thr231) Increased aGSK-3β γ-/β-secretase inhibitors reduced Aβ40 Increased Aβ40 Increased p-tau (Thr231) Increased aGSK-3β γ-/β-secretase inhibitors reduced Aβ40 β-secretase inhibitor reduced aGSK-3β+p-tau had large/very large Rab5+ early endosomes No change in synapsin I+ puncta on dendrites No change in Aβ40 No increase of p-tau (Thr231) No increase aGSK-3β γ-/β-secretase inhibitors reduced Ab40 No change in Aβ40 Increased p-tau (Thr^{231}) and aGSK-3β γ-/β-secretase inhibitors reduced Aβ40 β-secretase inhibitor reduced aGSK-3β+p-tau had large/very large Rab5+ early endosomes No change in synapsin I+ puncta on dendrites	[21]
Familial PSEN1 (D385N) Familial PSEN1 (L166P)	It-NES progenitor cells (NESTIN+ SOX2+) Neurons (βIII-Tubulin+ MAP2ab+ GABA+) + <10%Astrocyte (GFAP+) It-NES cells (NESTIN+ SOX2+) Neurons (βIII-Tubulin + MAP2ab+ GABA+) <10%Astrocyte(GFAP+)	Expression APP + γ-secretase components Extracellular Aβ Aβ length qPCR	Dominant-negative effect on S3 cleavage of Notch in progenitors, decreased HES5 Increased full-length APP Decreased Aβ40 Dominant-negative effect on S3 cleavage of Notch in progenitors, decreased HES5 Decreased Aβ40	116

Mutation	Cell Type analyzed	Analyses performed	Phenotype	Reference
Trisomy 21 DS1-iPS4	Cortical neurons; Early born, (TBR1+ βIII-Tubulin+ /CTIP2+ βIII-Tubulin+) 30% Late born, (BM2+ βIII-Tubulin+ /SATB2+ βIII-Tubulin+) 20-25% Functional synapses Glutamatergic(PSD95+)	Extracellular Aβ Aggregation of Aβ Treatment with γ-secretase inhibitor p-tau expression Cell death	Increased Aβ40 Increased Aβ42 (>70d cultures) Increased Aβ42:Aβ40 Intracellular and extracellular Aβ42 aggregates Decreased Aβ40+Aβ42 with γ-secretase inhibitor p-tau localized in cell bodies and dendrites Increased secretion of total tau and p-tau Increased cell death (2 fold)	[102]
Familial APP(E693Δ) 3 clones Familial APP(V717L) 2 clones Sporadic AD3E211 1 clone Sporadic AD8K213 1 clone	Cortical neurons (SATB2+ TBR1+) Astrocytes Cortical neurons (SATB2+ TBR1+) Cortical neurons (SATB2+ TBR1+) Cortical neurons (SATB2+ TBR1+) Astrocytes	Extracellular Aβ Intracellular Aβ Aβ Oligomers Gene expression profiling ROS expression Aβ Oligomers ROS expression	Decreased Aβ40 and Aβ42 Elevated Aβ oligomers in neural cells Elevated levels of oxidative stress-related genes Elevated ROS Elevated Aβ oligomers Elevated ROS Increased Aβ42, increased Aβ42:Aβ40 Elevated levels of oxidative stress-related genes No change in Aβ40 or Aβ42 Elevated levels of oxidative stress-related genes No change in Aβ40 or Aβ42 Elevated Aβ oligomers in neural cells Elevated levels of oxidative stress-related genes and ROS Elevated Aβ oligomers Elevated ROS	117
Sporadic Early ApoE3/E4 AG04402 (2 clones) Sporadic Early APOE3/E4 AG11414 Sporadic Late APOE3/E4 AG05810 Familial AG07872 Familial PSEN1 (A246E) AG066848	Basal forebrain cholinergic neurons (MAP2+ChAT+ VaChT+P75R+ NKX2.1+HB9-) Expessed tetrodotoxin-sensitive voltage-activated currents and voltage-gated calcium channels	Extracellular Aβ Treatment with γ-secretase inhibitors Treatment with ionomycin+glutamate Fura-2 calcium imaging	Elevated Aβ42 Increased Aβ40 with γ-secretase inhibitor Increased susceptibility to glutamate-induced excitotoxic death Increased calcium transient Elevated Aβ42 Increased Aβ40 with γ-secretase inhibitor Susceptibility to cell death following calcium influx. No elevated Aβ42 Increased susceptibility to glutamate-induced excitotoxic death Increased calcium transient Elevated Aβ42 Reduced Ab40 with γ-secretase inhibitor No elevated Aβ42 Reduced Aβ40 with γ-secretase inhibitor	[96]
Familial APP (V717I) (fAD1) (2 clones) (fAD2) asymptomatic (2 clones)	Forebrain neurons (MAP2+Tau+ βIII-Tubulin +Cux1+ TBR1+PSD95+ VGLUT1+)	Extracellular Aβ APP cleavage product expression Treatment with γ-secretase inhibitor Expression of tau Treatment with Aβ antibodies	APP holoprotein 1.4x increased Increased Aβ42:Aβ40 Increased Aβ42 Increased Aβ38 Decreased APPsα:APPsβ (Increased APPsβ) γ-secretase inhibitor blocked APPsβ cleavage Increased total tau Increased p-tau (Ser^{262}) d100 Aβ antibodies blocked increased total tau (early differentiated neurons only)	[91]

Familial PSEN1 (A246E) (2 patients) PSEN1 (H163R) asymptomatic PSEN1 (M146L)	Neurons (βIII-Tubulin+ MAP2+)	Extracellular Aβ Treatment with γ-secretase inhibitors	Increased Aβ42:Aβ40 Increased Aβ42 γ-secretase inhibitor lowered total Aβ, Aβ40, Aβ42, A38 Increased Aβ42:Aβ40 Increased Aβ42 γ-secretase inhibitor lowered Aβ42 Increased Aβ42:Aβ40 Increased Aβ42 γ-secretase inhibitor lowered Aβ42	[100]
Familial PSEN1 (A246E) (2 clones) 7671C/7768C PSEN1 (M146L) (2 clones) 8446B/8446D	D14 immature neurons (79% NESTIN+ small pop'nβIII-Tubulin +) Neurons Electrical signaling properties D14 immature neurons (79% NESTIN+ small pop'nβIII-Tubulin +) Neurons Electrical signaling properties	Extracellular Aβ Total Aβ	Increased Aβ42:Aβ40 Increased *NLRP2, ASB9, NDP* Increased Aβ42:Aβ40 Increased Aβ42:Aβ40 Increased *NLRP2, ASB9, NDP* Increased Aβ42:Aβ40	[101]
Familial PSEN1 (A246E) (2 patients) iPSCAD1/6/ 10/36/38/40/41	Cortical neurons (MAP2+, SYNAPSIN1+, TBR1+, SATB2+) Electrophyiso logicareadings show sodium and potassium currents and spontaneous postsynaptic currents	Extracellular Aβ	Increased Aβ42 Increased Aβ42:Aβ40	[93]
Sporadic (13 patients) (1 clone of each)	NSCs Mix of glutamatergic, GABAergic, cholinergic neurons	SORL1 haplotypes SORL1 expression Extracellular Aβ	No difference in SORL1 expression compared to SORL1 haplotypes No difference in SORL1 expression compared to SORL1 haplotypes Increased SORL1 expression following high treatment of BDNF in lines containing protective alleles in SORL1 Decreased Aβ40 following high treatment of BDNF in lines containing protective alleles in SORL1	[39]
Sporadic (1 patient) (2 clones) AD-iPS5/AD-iPS26B	Immature neurons (PAX6+, NESTIN+, βIII-Tubulin +)	Treatment with γ-secretase inhibitor Tau expression Gene expression profiling	Treatment with γ-secretase inhibitor did not alter expression of tau or p-tau WNT signaling and alanine, aspartate and glutamate metabolism altered in AD-iPS26B Proteosome genes, ROS genes, AD-related genes and cell death-related genes altered	[95]

(NSCs-Neural Stem Cells)

Sporadic cases with mutations in APOE

A polymorphism within the *APOE* gene is a risk factor for the disease and results in the presence of the e4 allele (APOE4), which has been strongly linked to development of AD [94]. One copy of this allele increases the risk factor of AD by two fold, whereas two copies increase the risk to develop AD by 12 fold [96]. The e4 allele has been shown to be less efficient at transporting cholesterol from neurons [94]. One iPSC study has been performed on patients with an APOe3/e4 genotype and found elevated Abeta 42 in neurons derived from two of the three patients studied. Two of the patients also showed increased sensitivity of neurons to cell death following glutamate-

induced excitation [96]. No investigation of cholesterol transport was performed. There are also no iPSC studies on patients with the Apoe3/e4 genotype (Table 1).

Familial AD

Pathology common to all familial mutations includes increased Abeta plaque formation compared to sporadic AD in most, but not all studies [97]. However, plaques containing Abeta 42 show often no increase in Abeta 40 compared to sporadic AD [97].

PSEN1 mutations

Patients with mutations in *PSEN1* have the earliest age of onset and are often accompanied by seizures, myoclonus, paraparesis and cerebeller signs [98]. All patients, who carry *PSEN1* mutations present with classic hallmark pathologies, including neuritic plaques, NFTs, tissue atrophy, neuronal loss and inflammation [97]. However, some differences do exist depending on the gene affected, and even the location of the mutation. Pathological mechanisms that are general for both PSEN1 and PSEN2 are the effects on Abeta peptide metabolism induced by y-secretase cleavage, which results in increased Abeta 42 production. Curiously, in some *PSEN1* mutations severe neurodegeneration has been shown without any Abeta pathology. Contrasts in pathological findings are also described, such as the discovery that Abeta 40 positive plaques are the predominant type in the cortex of some PSEN1 mutation patients [97]. Studies have also highlighted that soluble and insoluble levels of Abeta 42 are higher in familial AD brain tissue compared to sporadic AD [97].

Given these specific pathological findings in patients, it is interesting to know whether iPSC-derived neurons share the same reported pathology. To date, the most commonly evaluated mutation so far for patient-specific iPSC studies has been on *PSEN1*. These studies have revealed that most of the neurons generated from *PSEN1* AD patients also have increased Abeta 42 levels [93,99-101]. However, little other pathology has been described so far. In one study, no tau accumulation or tangle formation was observed [99]. One other study has reported small changes in gene function in *PSEN1* mutant-derived neurons including increased *NLRP2, ASB9* and *NDP* [101].

PSEN2 mutations

Patients with mutations in *PSEN2* have a delayed age of onset, suffer from disorientation and endure a long duration of the disease [98]. Pathologically, similar to PSEN1, mutations affect Abeta peptide metabolism by y-secretase cleavage, which results in increased Abeta 42 production. To date, only one study has investigated the pathology from neurons derived from a patient carrying a mutation in *PSEN2*. This study reported increased Abeta 42:Abeta 40 ratio, but no tau accumulation or tangle formation [99]. Clearly, more research is required to investigate Abeta metabolism, y-secretase function and whether neural atrophy, inflammation and tau and NFT pathology can be observed *in-vitro* from patient cells carrying this mutation.

APP mutations

Patients with *APP* mutations frequently have more aggression and those presenting with APP duplication frequently have apraxia [98]. Different *APP* mutations induce neural death by different mechanisms, for example, some mutations increase production of AICD and other C-terminal *APP* fragments, which regulate apoptosis [97]. Other mutations have been observed to trigger intracellular mechanisms, which increase oxidative stress and death [97]. Studies have also highlighted that soluble and insoluble levels of Abeta 42 are increased in cases with *APP* mutation compared to sporadic AD cases.

To date three studies have investigated pathology in neurons derived from patients carrying mutations in *APP*. These studies have conflicting results in relation to *APP* processing. One study found elevated Abeta 40 [21], another study found one patient had increased Abeta 42, whilst a second patient had decreased Abeta 40 [92] and a third study revealed a patient with both increase Abeta 42 and Abeta 38 [91]. One study also reported an increase in Abeta oligomers in the analyzed neural cells and astrocytes [92]. Increased p-tau has been observed in patients from two different studies [21,91] and increase in total tau has also been reported [91]. Increased aGSK-3b has been reported as well [21]. No studies have looked at AICD function; however oxidative stress has been partially investigated in one study, which revealed elevated ROS and oxidative stress-related genes in the cortical neurons and also elevated ROS in astrocytes [92] (Table 1).

Other Models of AD

Trisomy 21

Trisomy 21 otherwise known as Down's syndrome results in duplication of the *APP* gene, which leads to symptoms and pathology very similar to AD [102]. In addition, duplication of Dyrk1A kinase that phoshorylates tau may also contribute to the pathology and symptoms [102]. Increased Abeta peptides can be observed in early childhood, which leads to early onset dementia [102]. Therefore Trisomy 21 has been used to model AD in one iPSC study. In this case, perturbed Abeta processing was observed including increased Abeta 40, increased Abeta 42 in long-term cultured neurons as well as intracellular and extracellular aggregates of Abeta 42 [102]. Furthermore, this study also reported increased p-tau and total tau, and increased cell death [102]. This is the only study to date, which reports cell death, which suggests this may be a relevant and worthy model of *APP* duplication and study of AD-like dementia (Table 1).

DISCUSSION

The *in vitro* models for FAD and SAD are still very limited and the disease phenotype is so far not sufficiently replicated. Nevertheless, as illustrated here a few models for FAD and SAD have been generated, which have used mainly retroviral, to a lesser extent lentiviral reprogramming strategies and only a couple of groups published iPSC derivation via episomal vectors. All of these studies have used age- and sex-matched controls, which could make it more challenging to identify

subtle disease phenotypes. Most studies report the elevated ratio between Abeta 40 and Abeta 42 as the only significant phenotype and none of the studies could detect hyperphosphorylated tau or tau tangles. Therefore, most of the preliminary insights have been gained from Abeta related phenotypes and toxicity.

There are currently at least three main problems, which make the use of AD-iPSC-derived neurons lag behind its potential.

The first one is the use of age- and sex-matched controls. Clones derived from the same parental fibroblast line can already vary significantly in regards to gene expression and differentiation potential [103]. This line-to-line variation is very challenging in regards to distinguish between individual line characteristics and a true cellular disease phenotype. Due to the recent advances with genome editing, using the CRISPR-Cas9 system, it will be easier to compare these isogenic controls with the original patient iPSC and the random line-depended differences should be neglectable. A recent example is the study of dopaminergic neurons derived from Parkinson's patient iPSC and comparison to their genome edited isogenic controls of the same patients. This study revealed several differentially expressed genes in the patient line vs. isogenic control [104]. These differences in gene expression would have been more difficult to observe using age- and sex-matched controls. Obviously, these isogenic controls could also be relevant for regenerative medicine via cell replacement therapy. The only issue remaining is the relative uncertainty regarding off-targets (as described in the materials and methods section) and methods for off-target predictions are only emerging recently [105], which makes this approach not yet suitable for gene therapy with subsequent cell replacement therapy.

The second challenge for AD iPSC derived cellular models is the diversity of neural differentiation protocols. There are several protocols for 2D differentiation, which can result in enrichment of different subtypes of neurons. Neurons, which are of specific interest due to their sensitivity towards the AD pathology, are cortical and pyramidal neurons, entorhinal neurons and hippocampal neurons. Hippocampal neurons have been successfully generated from human pluripotent cells [106] as well as pyramidal cortical neurons [107], cortical projection neurons [108] and cortical interneurons [109]. These subtypes of neurons are all pathology relevant, susceptible neuron types, and located in affected brain regions. All protocols vary in regards to their efficiencies, but it is clear that these differentiation protocols only enrich for certain neuronal subpopulations and pure populations cannot be obtained. Pure populations of neurons might be advantageous in order to study the specific effect of mutations and disease phenotype in a particular neuron type, but they do not reflect the natural environment of the affected neurons in the brain. 3D differentiation protocols result in a three dimensional, more natural assembly of a mixed population of neurons and this approach to model disease phenotypes might be more suitable to obtain mature neurons. Nevertheless, current 3D AD models are based on genetically modified human NPCs overexpressing human FAD genes and are therefore very similar to the

mouse models in AD. Even though overexpression is achieved now in relevant human neuronal subpopulations the system is not reflecting the situation in AD patients [110,111]. These 3D systems with overexpression of FAD mutations are very relevant though in compound screens for drug development, since compounds, which have been identified in mice, have not yet resulted in development of drugs, which can ameliorate AD.

Finally, it has also been widely accepted now that neurons derived from iPSC are relatively immature and represent neonatal neurons [112,113]. This is an obvious challenge to overcome if the research is focused on age-related neurodegeneration such as AD. Several attempts have been explored to artificially age neurons derived from iPSC and one prominent example is the expression of progerin to induce premature ageing [114]. On the other hand the previously mentioned direct induction of neurons enables the cells to retain their specific aging-associated gene expression signature [115]. These cells could be much more suitable in regards to exploration of late disease phenotypes, whilst iPSC-derived neurons give the opportunity to investigate into early pre-symptomatic cellular changes.

A lot has been achieved in regards to generation of AD models using patient fibroblasts, or other cellular sources, to generate iPSC. Combined with the novel methods to generate isogenic controls, using the CRISPR-Cas9 system, these human cellular systems are very valuable for basic research and as screening platforms. Obviously, non-integrative reprogramming methods are necessary in order to avoid reactivation of transgenes or inappropriate gene inactivation due to insertional events. This is even more important if these gene-edited cells should ever be applied in personal medicine. Several crucial hurdles need to be overcome before such cells could be implanted into AD patients. They need to be footprint free and a reliable screen for off-targets need to be established. These cells should also be free of any tumor potential and in such a progenitor state that they can differentiate into the desired cell types, migrate from the injection site towards the injury site and withstand the already toxic and apoptotic environment in the patients brain. If ESCs instead of iPSC will be used they will need to be at least human leukocyte (HLA) matched. Even though there is still a lot to be solved cell replacement therapies for AD are becoming a potential alternative treatment and in the case of Parkinson's disease (PD) this form of treatment is already more advanced, which gives hope for other neurodegenerative diseases such as AD.

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