Induced pluripotent stem cell models of frontotemporal dementia

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Abstract

The increasing prevalence of dementia in the ageing population combined with the lack of treatments and the burden on national healthcare systems globally, make dementia a public health priority. Despite the plethora of important research findings published over the past two decades, the mechanisms underlying dementia are still poorly understood and the progress in pharmacological interventions is limited. Recent advances in cellular reprogramming and genome engineering technologies offer an unprecedented new paradigm in disease modeling. Induced pluripotent stem cells (iPSCs) have enabled the study of patient-derived neurons *in vitro*, a significant progress in the field of dementia research. The first studies using iPSCs to model dementia have recently emerged, holding promise for elucidating disease pathogenic mechanisms and accelerating drug discovery. In this review, we summarise the major findings of iPSC-based studies in Frontotemporal Dementia (FTD) and FTD overlapping with Amyotrophic Lateral Sclerosis (FTD/ALS). We also discuss some of the main challenges in the use of iPSCs to model complex, late-onset neurodegenerative diseases such as dementias.

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Introduction

It is estimated that over 44 million people are currently affected by dementia globally, a figure that is predicted to double every 20 years [1]. The devastating nature of the syndrome, as well as its immense socio-economic impact, highlight the urgency for reliable pre-clinical biomarkers and effective treatments.

Our current understanding of dementia has been greatly shaped by genetic studies that have led to the identification of numerous dementia-causing genes and genetic risk factors. Such genetic data have allowed the development of animal and cell models for functional studies which have proven crucial in elucidating certain aspects of disease biology. There are however some limitations in their use to model complex human diseases such as dementias as well as their fidelity as candidate drug screening platforms. Transgenic mouse models of overexpressed mutant proteins have been dominant in dementia modeling and drug development, however, most of these models have not faithfully reproduced the full spectrum of pathology or dementia symptoms resembling the ones seen in humans. Most significantly, pre-clinical screens in such systems have pointed towards series of candidate drugs that failed in subsequent human clinical trials. Despite any similarities between human and mouse brains and conserved sequences of genes and proteins, there are profound differences in physiology, genome, transcriptome, proteome and connectome that highlight the importance of relevant human *in vitro* systems for (i) faithful and fully translational disease modeling and (ii) additional preclinical evaluation of candidate drugs. Human immortalised cell lines are also widely used in dementia research, however their suitability as modeling systems is questioned as most of these lines are non-neuronal cancer lines characterised by dysregulated signaling pathways, wide genomic alterations and karyotypic abnormalities relying on non-physiological protein overexpression. Studies of port-mortem human brain tissues have been fundamental so far in elucidating neuropathological hallmarks of dementias, however, this only serves as an end-point snapshot into the pathology of a severely degenerated brain following years of disease underlying processes.

The lack of relevant human cellular models in dementia research has been recently overcome by advances in reprogramming of adult somatic cells into induced pluripotent stem cells (iPSCs) [2]. This technology has revolutionised the field of neurological disease modeling and allowed the generation of patient-derived iPSCs, by overexpression of four transcription factors OCT3/4, SOX2, KLF4 and c-MYC in patient's cells such as fibroblasts [2] (**Figure 1**). IPSCs are in principle indistinguishable from embryonic stem cells (ESCs) in their ability to self-propagate and differentiate into any type of somatic cell. Differentiation of these iPSCs into neurons and glia is possible, offering a highly relevant system to study dementia pathogenic mechanisms or use as drug screening platforms. Ultimately, the ability to generate patient-derived neurons *in* vitro has raised hopes for the development of potential cell replacement therapies for several neurodegenerative diseases [3].

In this review, we discuss the major findings of iPSC studies in Frontotemporal Dementia (FTD) and FTD overlapping with Amyotrophic Lateral Sclerosis (FTD/ALS) to date, as well as the main challenges of using iPSCs to model complex, neurodegenerative diseases such as dementias.
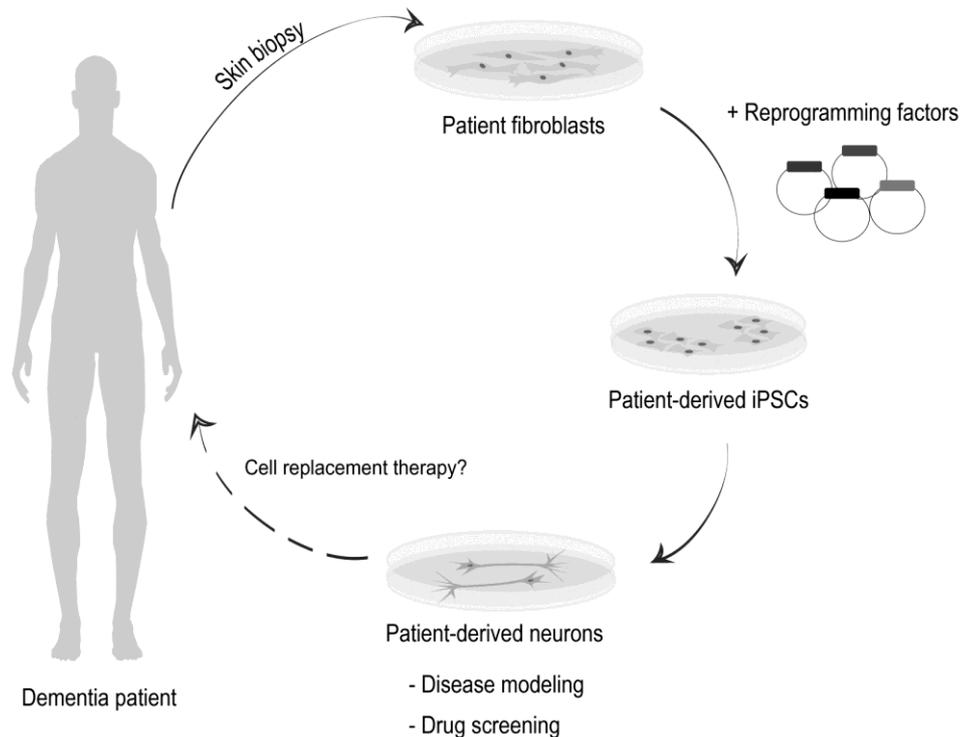
Figure 1. Induced pluripotent stem cells (iPSCs) have allowed for the first time the study of patient-derived neurons in dementia. Patient somatic cells such as fibroblasts can be reprogrammed to iPSCs by overexpression of four transcription factors. iPSCs can subsequently differentiated to neurons or glia for modeling dementia mechanisms or as drug screening platforms or potentially used in the future as cell replacement therapy.

Frontotemporal dementia

Frontotemporal dementia (FTD) is the second most common form of young-onset dementia, characterised by progressive loss of neurons in the frontal and temporal lobes of the brain and subsequent gradual deterioration in the functions controlled by these regions such as personality, behaviour, language as well as certain types of movement [4-6]. FTD is a clinical syndrome characterised by clinical, genetic and neuropathological heterogeneity [7, 8]. The clinical phenotypes of FTD include behavioural variant FTD (bvFTD) and two variants of primary progressive aphasia (PPA), the semantic and nonfluent/agrammatic. FTD can also overlap with other movement disorders such as progressive supranuclear palsy (PSP), corticobasal syndrome (CBS) and amyotrophic lateral sclerosis (FTD/ALS) [9]. Neuropathologically, FTD is often described as frontotemporal lobar degeneration (FTLD). Analysis of FTLD post-mortem tissue has revealed characteristic patterns of abnormal deposition of tau, TDP-43, FUS and ubiquitin proteins in neurons and/or glia of cases and based on these findings, FTLD is classified into four main subgroups; FTLD-tau, FTLD-TDP43, FTLD-FUS and FTLD-UPS [10].

Mutations in several genes have been described that can cause FTD with or without amyotrophic lateral sclerosis (ALS). A common cause of familial FTD are mutations in *MAPT* and *GRN* genes, both located on chromosome 17 [11-14], while rare mutations in *CHMP2B* have also been linked to FTD [15]. Mutations in *VCP* [16, 17], *SQSTM1* [18-20], *TBK1 [21, 22]* and *UBQLN2* [23, 24] genes have been described that cause FTD with or without ALS. In recent years, an intronic expansion in *C9orf72* has been reported as the most common genetic cause of both FTD and familial ALS [25, 26]. Mutations in *TARDBP* [27] and *FUS* [28] genes usually lead to pure ALS, however these proteins are found mislocalised and aggregated into characteristic neuropathological inclusions in FTD [29]. All this clinical, genetic and neuropathologic overlap suggests FTD and ALS are part of a spectrum of neurodegeneration with overlapping molecular aetiology. Here we review the findings of iPSC studies in FTD and FTD/ALS which are also summarised in **Table 1**.

MAPT

Hyperphosphorylated, insoluble aggregates of the microtubule-associated protein tau are a pathological hallmark of a range of disease, collectively termed the tauopathies, including Alzheimer's disease (AD) and FTD. Evidence for a causative link between tau and disease was established by the discovery of mutation in *MAPT* linked to FTD with tau pathology. Mutations in *MAPT* either alter the coding sequence of tau or are located in intronic regions leading to an alteration in tau splicing [11, 30].

Tau splicing is complex and subjected to developmental regulation and dysregulation in disease. The alternative splicing of exons 2, 3 and 10 of *MAPT* generates six protein isoforms in the adult human CNS, differing by the presence of 0, 1 or 2 N-terminal repeats (0N, 1N, 2N) and 3 or 4 microtubule-binding repeats (3R or 4R) at the C-terminus of the protein [31-33]. The balance of tau isoform expression is tightly controlled, and only the smallest tau isoform (0N3R) is expressed during fetal stages but all six isoforms are expressed post-natally. The precise balance of 3R and 4R tau appears to be critical for neuronal function, the 3R/4R ratio is approximately 1 under normal conditions, but mutations in *MAPT* that disrupt this ratio (generally increasing the relative amount of 4R tau) are sufficient to cause disease [11, 34].

This splicing of *MAPT* is not recapitulated in tumour cell lines or most model organisms, and therefore iPSC-neurons represent a potentially viable way to generate physiologically-relevant models, expressing the appropriate complement of tau isoforms. Overcoming the developmental regulation of tau splicing has proved challenging in this regard, with several reports demonstrating that the regulation of tau splicing is conserved in iPSC-neurons, where the full complement of tau isoforms is only apparent at extended *in vitro* time points [35, 36]. This is an important observation given that many clinically-aggressive mutations in *MAPT* are located within exon 10, and therefore expression of 4R tau isoforms is necessary in order to have mutant protein present in the model. Interestingly, iPSC-neurons generated from patients with splice-site mutations in *MAPT* demonstrated an early increase in 4R tau expression, suggesting these mutations are able to override the developmental regulation of tau splicing [35-38].

In spite of these challenges several reports have described neuronal phenotypes linked to mutation in *MAPT*. Iovino and colleagues demonstrated that neurons with the *MAPT* N279K (splicing) and P301L (coding) mutations displayed a premature maturation phenotype, with earlier electrophysiological maturity than controls [36]. Tau's role as a microtubule associated protein means that tau dysfunction could result in disrupted axonal transport, and indeed vesicle trafficking and altered distribution of mitochondria has been observed in patient neurons [36, 37]. Another study reported increased oxidative stress and activation of the unfolded protein response in the presence of *MAPT* mutations [38].

Tau pathology spreads throughout the brain in a trans-synaptic manner, and abnormal tau is able to seed pathology in recipient neurons. This has been modeled effectively in vivo and in non-neuronal human cell lines, but a human, iPSC-neuron model for the spread of tau pathology would be a step forward in modeling this aspect of disease. Tau fragments are released by iPSC-neurons and tau oligomers, but not monomer, can be internalized by iPSC-neurons, resulting in pathogenic tau accumulation and neurotoxicity [39, 40]. Thus, iPSC-neurons represent a good system for the study of both coding and splice-site *MAPT* mutations and findings yielded from these models will have broad implications for the tauopathies.

The A152T variant in MAPT is a risk factor associated with increased risk for FTD, AD and tauopathies [41, 42]. Fong and colleagues generated iPSC from patients with the A152T variant in *MAPT*, and also used genome engineering to generate a homozygous 152T/T line and an isogenic control 152A/A line [43]. A152T neurons had a significant increase in cleaved fragments of tau, an effect that was further exacerbated in homozygous 152T/T neurons. Immunostaining with specific antibodies revealed these to be caspase-cleaved tau fragments, which accumulated in the axon. This was accompanied by abnormal neuronal morphologies such as neurite fragmentation in certain neuronal subtypes (GABA and glutamatergic). All of these findings were eliminated in the isogenic control line, suggesting a central role of the A152T variant in degeneration of these cells.

GRN

Progranulin (PGRN) and its proteolytic cleavage products, granulins, are a family of secreted proteins with important roles in cell growth regulation, development, wound healing and tumorigenesis. Mutations in the *GRN* gene account for ~25% of familial FTD cases and their pathogenicity is consistent with a loss-of-function mechanism [13, 14]. In the first study using human iPSCs to model PGRN-deficient FTD, Almeida and colleagues generated iPSC lines from one unaffected individual, one sporadic FTD patient and one FTD patient with the S116X *GRN* mutation [44]. The differentiated neurons and microglia from the S116X *GRN* lines showed ~50% decrease in levels of both secreted and intracellular PGRN, recapitulating the PGRN haploinsufficiency disease phenotype. Induction of cellular stress revealed compromised PI3K/Akt and MEK/MAPK signaling pathways in S116X neurons, phenotypes which were rescued by overexpression of wild-type PGRN. The patient-derived neurons of the above study and also neurons derived from *C9orf72* GGGGCC expansion carriers, were used in a study by Gascon and colleagues, to evaluate findings from their *in vivo* mouse study of behavioural

alterations on FTD [45]. They confirmed an age-dependent reduction in miR-124 levels and upregulation of Gria2 and Gria4 AMPAR subunits in 8 week old patient-derived neurons compared to controls. Their findings linked for the first time the behavioural deficits observed in bvFTD with altered expression of AMPA receptor (AMPAR) subunits opening another possible therapeutic avenue for behavioural variant FTD [45].

PGRN-deficient FTD offers the most promising clinical subtype for therapeutic intervention, where restoration of PGRN levels could modify pathogenic pathways andalleviate the disease symptoms. However, such therapeutic approaches should be taken cautiously as little is known about the pleiotropic functions of progranulin and granulins and their anti- and pro-inflammatory properties respectively. Two recent studies in PGRN-deficient iPSC-neurons have provided further evidence supporting modulation of PGRN levels as a potential therapeutic intervention. In the first, Lee and colleagues restored PGRN levels in S116X *GRN* neurons by reducing SORT1-mediated endocytosis of PGRN using a SORT1 suppressor bioactive compound MPEP [46]. Suppression of SORT1 expression via MPEP was specific and led to increased extracellular levels of PGRN without affecting intracellular PGRN levels or other sortilin-related family members. In the other study, Raitano and colleagues used iPSCs with a different *GRN* mutation, the GRNIVS1+5G>C, which they differentiated into cortical neurons, the most physiologically relevant neuronal subtype to model FTD. IPSCs with the GRN mutation had reduced ability to generate cortical neurons compared to controls, a phenotype that was rescued after introducing *GRN* cDNA by homologous recombination with zinc finger nucleases (ZFNs) in the safe-harbour AAVS1 locus [47].

C9orf72

Much research has focussed recently on *C9orf72*, an expanded repeat in which is responsible for the majority of both FALS and FTD cases. The hexanucleotide repeat is located in an intronic region of the gene, between two non-coding exons. The disease-causing mechanism of the repeat expansion remains largely unknown and initial findings support the existence of both gain and loss of function pathogenic mechanisms. Consistent with a toxic gain of function of the expanded repeat, preliminary studies in patient fibroblasts and post-mortem tissue have shown that the expanded repeats form RNA aggregates in the nuclei of patient cortex and motor neurons [25]. In addition, the expanded repeat has been found to be translated via a repeat-associated non-ATG (RAN) translation mechanism, producing long dipeptide repeat proteins (DRPs) from either sense: Gly-Ala (GA), Gly-Arg (GR), Gly-Pro (GP) or anti-sense: Pro-Arg (PR), Pro-Ala (PA), Pro-Gly (PG) transcripts that are also found in cytoplasmic aggregates in patient tissue [48, 49]. On the other hand, loss of normal C9orf72 normal function is another possible underlying disease mechanism. *C9orf72* has three pre-mRNA transcript variants, some of which have been found to be ~50% reduced in patients carrying the repeat [25, 50, 51] and epigenetic studies show that repeat-associated hypermethylation near *C9orf72* can also be responsible for reduced C9orf72 expression [52, 53]. C9orf72 is a distant homologue of the differentially expressed in normal and neoplastic cells (DENN) family of GDP–GTP exchange factors (GEFs) that activate Rab GTPases [54]. Although its biological function remains

largely unknown, some initial evidence suggest C9orf72 regulates endosomal trafficking [55]. A more recent study has revealed that C9orf72 is required for the normal function of macrophages and microglia, therefore linking toxicity and loss of function mechanisms in a dual-effect pathogenic mechanism [56].

In the first C9-FTD iPSC study by Almeida and colleagues, patient-derived neurons recapitulated certain aspects of *C9orf72* expansion-related pathology in FTD [57]. Interestingly, the GGGGCC expanded repeats showed instability during reprogramming to iPSCs and differentiation into neurons. Moreover, *C9orf72* expression was found to be reduced in patient-derived neurons compared to controls. Patient-derived neurons exhibited RNA foci and RAN GP dipeptide pathology as well as significantly high levels of p62 protein and compromised autophagy mechanisms.

Additional evidence supporting a toxic gain of function disease mechanism came from two recent studies on C9-ALS iPSC models using antisense oligonucleotide (ASO) treatment to rescue the disease phenotypes [58, 59]. In terms of *C9orf72* repeat stability, Sareen and colleagues observed instability of the repeat in iPSCs and motor neurons, whereas Donnelly and colleagues found iPSCs and neurons maintained a stable number of repeats which was attributed to clonal selection of fibroblasts. Moreover, in one study the levels of two *C9orf72* variant transcripts were found reduced in patient-derived neurons compared to controls [58], recapitulating *C9orf72* downregulation seen in patient tissue, whereas in the other no changes in *C9orf72* expression were observed in patient-derived motor neurons [59]. Knockdown of *C9orf72* transcript was not toxic to cultured motor neurons, arguing against a loss of function mechanism as the drive of neurodegeneration in this *in vitro* model [59]. Both groups were able to detect intranuclear RNA foci in their neuronal models, whereas dipeptide RAN pathology was only observed by Donnelly and colleagues. Patient-derived neurons of both studies displayed toxicity phenotypes of dysregulated gene expression and sequestration of RNA binding proteins like ADARB2, hnRNPA1 and Pur-a by the expanded RNA repeat [58, 59] in contrast to previous reports from Almeida and colleagues on lack of sequestration of major RNA binding proteins by the RNA foci [57]. In addition, one group reported susceptibility to glutamate excitotoxicity, whilst patient-derived motor neurons of Sareen and colleagues showed decreased electrical excitability compared to control motor neurons. Antisense oligonucleotide (ASO) treatment in both studies reversed toxicity phenotypes in patient-derived neurons supporting a GGGGCC repeat-associated RNA toxic gain-of-function as a primary mechanism for neurodegeneration.

Non-cell-autonomous toxicity mechanisms in ALS were investigated in the study of Meyer and colleagues who showed that directly converted astrocytes from either skin or spinal cord of familial and sporadic ALS, including *C9orf72* mutation carriers, were toxic to co-cultured motor neurons [61]. Satoh and colleagues analysed transcriptome data performing molecular network analysis showing reduction in the expression of extracellular matrix proteins and matrix metalloproteinases in patient-derived motor neurons [60]. Investigation of electrophysiological properties of *C9orf72* and *TARDBP* (M337V) patient-derived motor neurons by Devlin and colleagues revealed abnormal patterns of initial hyperexcitability followed by diminished firing and synaptic activity [62]. Interestingly, the loss of excitability phenotype is in line with previous reports from Sareen and colleagues [59] and appears at a similar time point during motor neuron maturation. Yang and colleagues identified

Notch pathway to be affected by RAN GR toxicity and found reduced levels of some Notch target genes in patient-derived cortical neurons. Moreover, they suggest RAN GA dipeptides act as suppressors of RAN GR toxicity by recruiting GR into cytoplasmic inclusions in patient-derived neurons [63].

Three recent *in vivo* studies in fly and yeast models of the repeat expansion have revealed impaired nucleocytoplasmic transport as a novel phenotype in FTD/ALS and confirmed findings *in vitro* in patient-derived neurons [64-66]. RanGAP was identified by Zhang and colleagues as a suppressor of neurodegeneration in Drosophila expressing 30 hexanucleotide repeats and was shown to bind the sense RNA G-quadruplex of the repeat expansion. The human orthologue RanGAP1 was found in puncta in *C9orf72* patient-derived neurons that also co-localised with the expanded repeat RNA foci. Impaired RanGAP1 function was also linked to disrupted nuclear-cytoplasmic pattern of Ran, leading to higher cytoplasmic levels of the protein in patient-derived neurons, subsequently rescued by overexpression of RanGAP1. Impaired nucleocytoplasmic transport was responsible for the abnormal nuclear/cytoplasmic ratios of Ran and TDP-43 which were restored after ASO treatment in *C9orf72* patient-derived neurons. In the other study, Freibaum and colleagues also observed impaired nucleocytoplasmic transport phenotypes due to the hexanucleotide repeat toxicity. Investigation of total RNA distribution revealed a 35% increase in the nuclear/cytoplasmic ratio of RNAs in 60 days-old patient-derived cortical neurons compared to controls. Finally, in the third study, directly converted patient neurons showed decreased nuclear localisation of the Ran- GEF RCC1 compared to controls [66]. Taken together, the above findings suggest nucleocytoplasmic transport as a novel target for therapeutic intervention in *C9orf72*-linked FTD and ALS.

TARDBP

Mutations in the TAR DNA-binding protein 43 (*TARDBP*) gene are a rare cause of ALS while a few have also been linked to FTD [27]. Transactive response DNA binding protein 43 (TDP-43) is a DNA and RNA binding protein with important roles in regulation of gene expression, RNA stability and splicing. It is mainly localised in the cell nucleus but can shuttle to the cytoplasm to exert its RNA regulatory functions. However, in some neurodegenerative diseases such as ALS and a subtype of FTD (FTD-TDP), TDP-43 has been found mislocalised to the cytoplasm as a major component of ubiquitin-positive aggregates. This has been a key finding for ALS and FTD research, highlighting how two so phenotypically different clinical syndromes can share the same molecular pathology. Moreover, this has allowed the classification of several neurodegenerative diseases that share the same TDP-43 pathology into a group of TDP-43 proteinopathies.

In the first study of a human neuronal model of FTD linked to TDP-43 mutations, Zhang and colleagues generated neurons from an FTD/ALS patient carrying the *TARDBP* A90V mutation. Patient neurons were found to be sensitive to staurosporine-induced stress [67]. When stressed, patient-derived neurons exhibited mislocalised TDP-43 in the cytoplasm, lower total TDP-43 as well as decreased levels of microRNA-9 (miR-9) and its precursor pre-miR-9-2 compared to controls. MiR-9

downregulation was also observed in neurons derived from patients carrying the M337V mutation suggesting miR-9 downregulation may be a common downstream event for FTD and ALS.

The majority of TDP-43 iPSC modeling studies focused on ALS assessing cell-autonomous phenotypes in differentiated motor neurons or glia. In one of them, Bilican and colleagues generated iPSCs with the M337V mutation and differentiated them into motor neurons. They observed cell-autonomous phenotypes of increased levels of soluble and insoluble TDP-43, decreased survival in longitudinal studies and increased vulnerability to antagonism of the PI3K pathway in patient-derived neurons compared to controls [68]. Cell-autonomous phenotypes were also observed in another study of iPSC-derived M337V astrocytes which showed elevated levels and mislocalisation of TDP-43 as well as and decreased cell survival compared to controls [69]. In contrast to the observed toxicity of astrocytes from another ALS mutation in *SOD1*, TDP-43 patient astrocytes did not display any toxic effects longitudinally in co-cultured control and patient motor neurons in this study.

A number of iPSC modeling studies in TDP-43-ALS have used patient-derived neurons in drug screening assays, showing some interesting candidate compounds that were able to rescue ALS-related phenotypes. In one of them, Egawa and colleagues performed a small chemical screen on iPSC-derived motor neurons from patients with the Q343R, M337V and G298S *TARDBP* mutations [70]. The differentiated motor neurons displayed mislocalised, aggregated TDP-43, elevated insoluble TDP-43 bound to SNRPB2 spliceosomal factor and shorter neurites compared to control motor neurons. Moreover, gene expression profiling revealed upregulation of RNA metabolism genes and downregulation of cytoskeletal genes. They found that anacardic acid, an inhibitor of histone acetyltransferases, was able to rescue ALS disease phenotype in patient motor neurons. A larger high content chemical screen in upper and lower motor neurons from a large cohort of control, familial and sporadic ALS cases identified four classes of compounds that reduced TDP-43 aggregation phenotype in sALS; cyclin-dependent kinase inhibitors, c-Jun N-terminal kinase inhibitors (JNK), Triptolide and FDA-approved cardiac glycosides, Digoxin, Lanatoside C, and Proscillaridin A [71]. Finally, a drug screening in human motor neurons from fALS patients showed that kenpaullone, a multikinase inhibitor, strongly improved motor neuron survival [72]. More importantly, the effect of kenpaullone on motor neuron survival was found to be much stronger than the corresponding effect of olesoxime and dexpramipexole, two compounds that have failed in ALS clinical trials, highlighting the importance of iPSC-derived human neurons as relevant drug screening platforms.

Challenges in iPSC-dementia modeling

Although it is still early days in dementia iPSC modeling, it is becoming apparent that this technology has the potential to accelerate dementia research and identify new therapeutic avenues. As iPSC studies become more prevalent in dementia research, it is therefore crucial to consider some of the challenges in the use of iPSCs to model complex, late-onset dementias. Here we focus on two main

general challenges, heterogeneity and cellular phenotyping, and discuss ways they can be addressed in order to develop robust models and most importantly reproducible data for the field.

Heterogeneity

The first and significant challenge to generate robust iPSC models is heterogeneity in all its forms, namely genetic, epigenetic and cellular. Genetic and epigenetic heterogeneity can be attributed to reprogramming methods, human genetic variation among study lines or can be a result of selective pressure due to culture conditions. Cellular heterogeneity reflects differences in expression, function or morphology between cells or cell subpopulations within the study system. Such sources of heterogeneity in iPSC cultures are illustrated in **figure 2**.
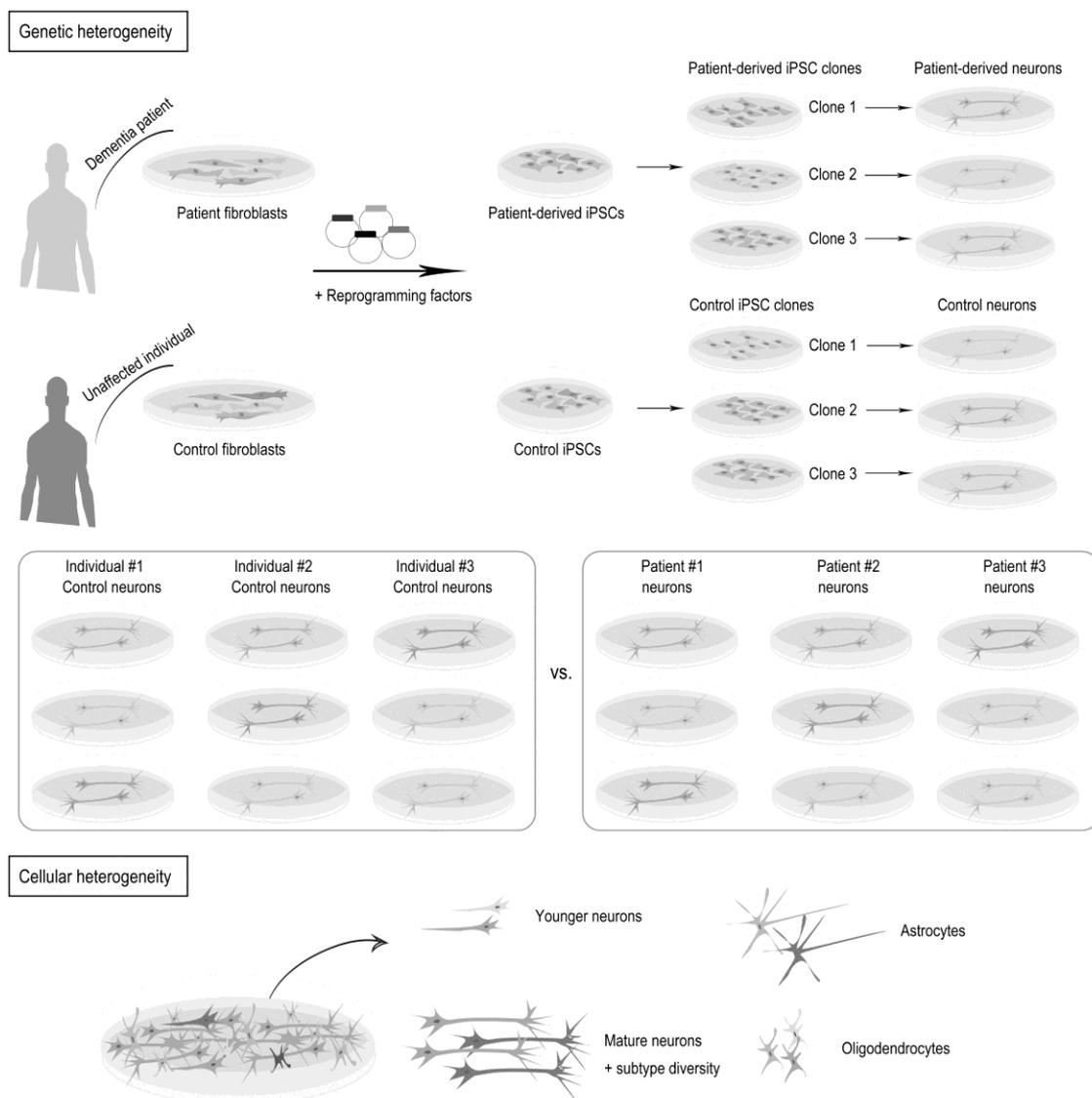
Figure 2. Types of heterogeneity in iPSC-derived neuronal cultures. Genetic or epigenetic heterogeneity may already exist in fibroblasts but are also induced by the reprogramming process. That leads to the generation of heterogeneous iPSC clones that need to be isolated and characterised individually. Karyotype analysis is a necessary step in the establishment of iPSC clones. Ideally, several clones are used per iPSC line to account for variability. That leads to large and labour-intensive study cohorts of patient and control-derived neurons. Cellular heterogeneity in neuronal cultures reflects cell-to-cell variation and also variation between different cellular types in culture. Different neuronal subtypes, for instance neurons from all layers of the cortex, with a wide range of *in vitro* ages, as well as different glial subtypes lead to a diverse cellular population in culture.

Traditionally, the generation of iPSCs has relied on delivery of the reprogramming transgenes via lentiviral or retroviral vectors which randomly integrate in the genome [73, 74]. This random virus integration in the genome has unknown effects and can limit the differentiation potential of the generated iPSCs, whereas excision of reprogramming transgenes has been found to significantly improve their differentiation capacity [75]. Therefore, several integration-free strategies have been developed based on the use of excisable [75, 76] or episomal vectors [77, 78], non-integrating Sendai virus [79, 80], as well as direct protein [81] or mRNA [82] to minimise random mutagenesis. Although in theory integration-free methods are less mutagenic, comparative studies have shown no

difference in the frequency of somatic coding mutations [83] or karyotypic abnormalities [84] between integrating and non-integrating methods. Regardless of the method though, reprogramming is a mutagenic procedure, leading to increased frequency of CNVs [85, 86] or somatic coding mutations [83, 87, 88] in iPSCs compared to parental cells. Reprogramming-induced benign variants have also been detected by a recent study in isogenic iPSCs derived with three different reprogramming methods, namely integrating retroviral vectors, non-integrating Sendai virus and synthetic mRNAs [89]. A large scale karyotype analysis on more than 1,700 human ESC and iPSC cultures from independent laboratories has revealed similar types and frequency of karyotypic abnormalities, with trisomies 8 and 12 the most common, suggesting that these are independent of reprogramming procedures [84]. Karyotypic alterations and CNVs are closely dependent on culture conditions and especially prolonged cultures, reflecting adaptation of the iPSC and ESC cultures to positive selection pressure [85, 90-92]. Other factors that may influence culture-induced variability are the passage number, passaging method, substrates and culture media. Generally, choice of integration-free reprogramming methods, the use of same reprogramming method across all study lines, selection of multiple clones per generated line and careful characterisation are the best ways to control reprogramming-derived heterogeneity. Moreover, a way to control culture-induced variability is maintenance of iPSCs under feeder-free conditions, in defined serum-free media, using non-enzymatic passaging with frequent karyotype analysis checkpoints. All the above highlight the necessity for standardised and controlled procedures in iPSC line generation and differentiation, a fact also reflected by the creation of consortia and stem cell banks for the reprogramming, storage and distribution of iPSCs to research centres. Validated and reproducible protocols are critical to the success of iPSC derived dementia models.

Human genetic variation among the different iPSC lines in a study is the main source of genetic heterogeneity that needs to be considered early on during study design. Frequently that problem has been somewhat circumvented by increasing the power of iPSC modeling studies, including several patient and control lines. However, increasing the power may reduce the robustness of the study model due to genomic background noise, result in large labour-intensive iPSC cohorts and finally may not always be possible, especially in the case of studying rare mutations, such as in *PSEN1* or *PSEN2* genes in AD. Recent advances in genetic engineering technologies such as TAL-effector nucleases (TALENs) and the most recent CRISPR/Cas9 systems, have allowed the cost-effective generation of isogenic iPSC lines that share the same genetic background (Figure 3). Although some initial studies linked the use of such "designer" nucleases with the generation of "off-target" mutations in a range of additional genomic sites apart from the intended ones [93], novel strategies such as obligate-dimer nucleases and nickase versions offer improved, highly specific and "scarless" genome editing [94]. Effectively, with the use of these tools, one can study the effect of a mutation by inserting the mutation in a control iPSC line or rescue a disease phenotype by replacing the mutation with the wild-type sequence in a patient iPSC line. The generation of such isogenic lines eliminates the genetic heterogeneity between the study lines and allows the study of the net effect of a disease mutation in a robust and reproducible way. Isogenic lines can also accelerate research as they do not rely on patient material, therefore can bypass ethical requirements or enable the study of extremely rare or even hypothetical mutations.
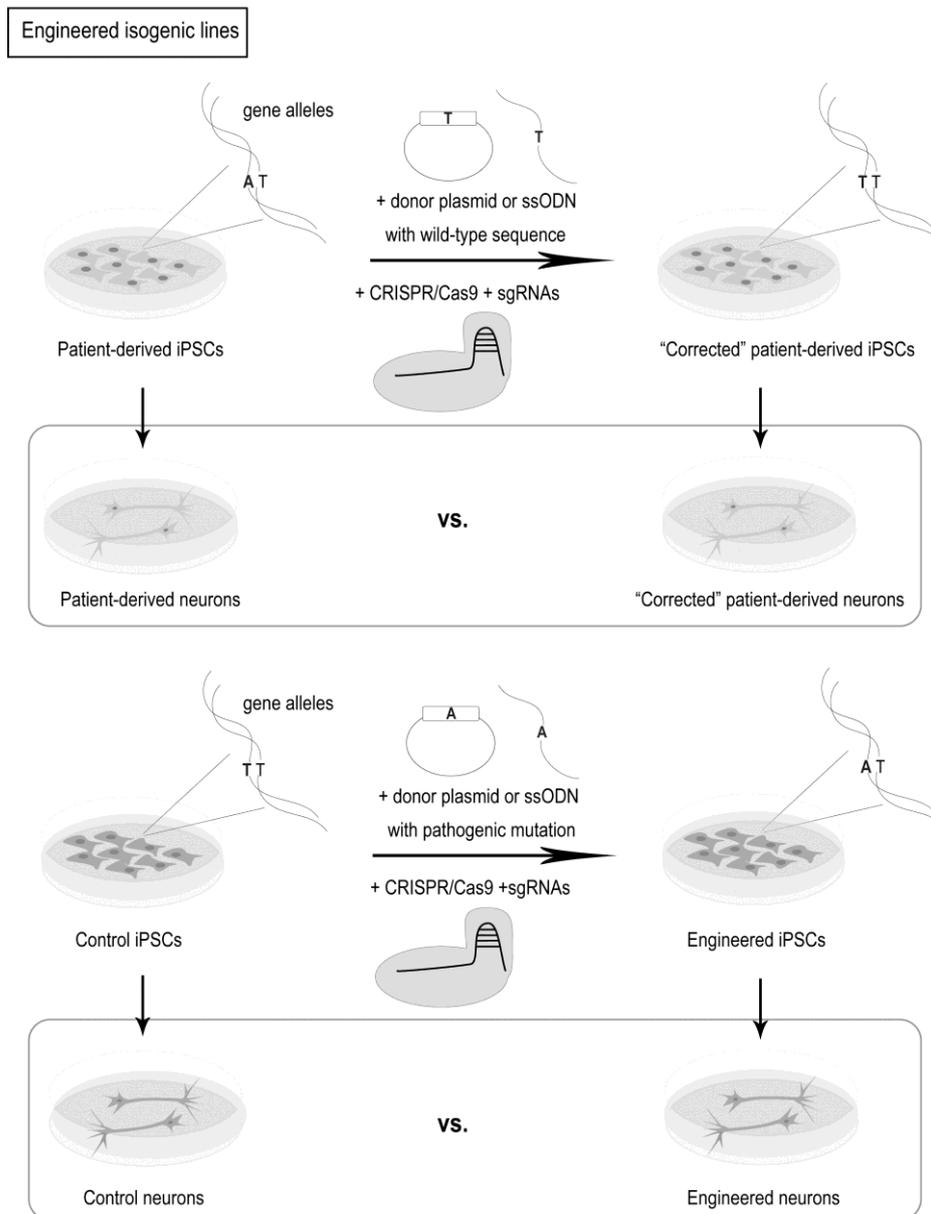
Figure 3. The generation of isogenic iPSC lines with the use of CRISPR/Cas9 nucleases can solve the problem of iPSC line heterogeneity and lead to robust systems for cellular phenotyping. Isogenic lines can be applied to correct a disease mutation by replacing the mutation with the wild-type sequence in patient-derived iPSCs, or to study the effect of a mutation by inserting the mutation in a control iPSC line. In both systems, the result is robust and smaller-scale studies.

Interclonal variation in iPSCs is very much dependent on cell culture procedures as already discussed, all these are sources of selective pressure and subsequent genetic drift in the cultured highly-proliferative iPSCs. Such cellular heterogeneity is not limited to proliferating cells but can also be found in post-mitotic neurons which are susceptible to DNA damage during their lifespan. Single-cell whole genome sequencing of individual neurons from the human prefrontal cortex has revealed thousands of somatic single-nucleotide variants (SNVs) that cluster into distinct lineages [95]. Cellular heterogeneity of human brain has also been investigated by single-cell RNA sequencing [96]. *In vitro*, cellular heterogeneity becomes a challenge after differentiation of iPSCs to neurons, as cultures become enriched in several different neuronal and glial subtypes of variable *in vitro* ages. All

this cellular variability has major implications in cellular phenotyping as we will discuss in the next section.

Finally epigenetic heterogeneity exists in iPSC model systems in the same way as genetic that we already described; it can be heterogeneity between the lines or a result of reprogramming. In the latter case, it is generally accepted that the forced expression of the reprogramming factors in somatic cells leads to a genome-wide change in the epigenetic landscape where chromatin is re-organised to an ESC-like state. Chromatin architecture in ESCs is in an "open" state with abundant euchromatin and less heterochromatin modifications, whereas lineage-committed somatic cells are characterised by highly condensed heterochromatin. Despite the epigenetic similarities between iPSCs and ESCs, several lines of evidence suggest that iPSCs retain some epigenetic "memory" from the somatic cell type of origin and aberrant methylation patterns [97].

Cellular phenotyping

Identifying disease-specific phenotypes is particularly challenging in late-onset dementias in which ageing is the main risk factor. IPSC modeling is tied to developmental procedures, therefore, manifestation of phenotypes may not happen early on *in vitro*, unless there is an underlying developmental phenotype, or it may require the application of appropriate stressors (figure 4).

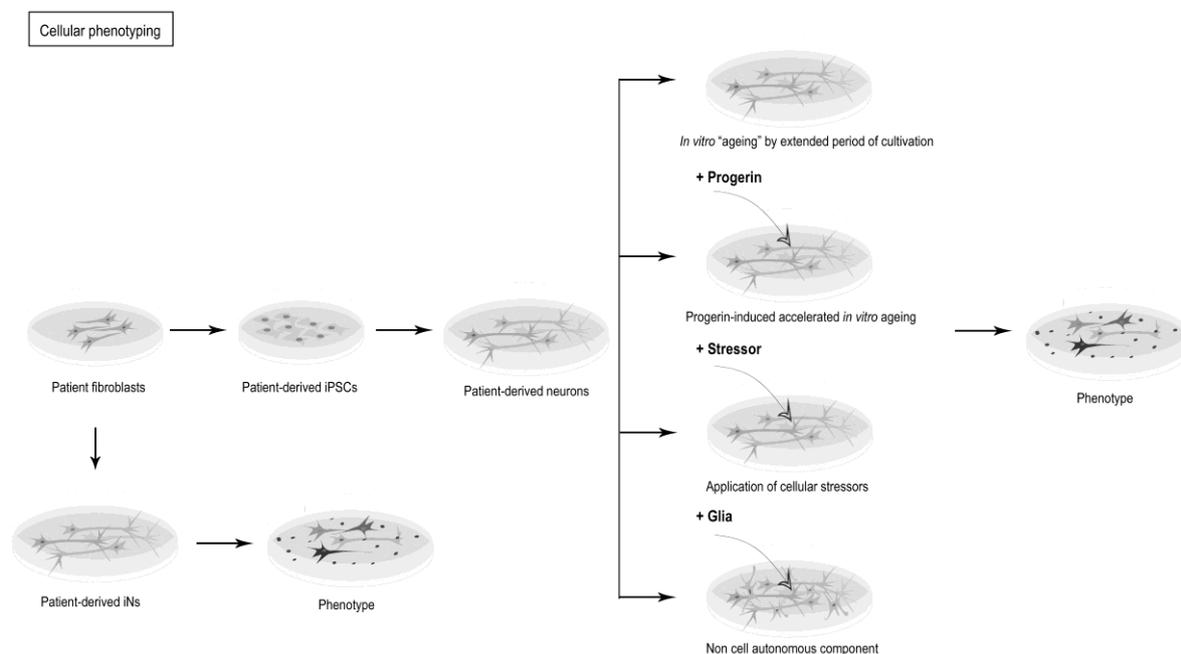**Figure 4.** Manifestation of dementia-related phenotypes in iPSC-neuronal cultures may require longer *in vitro* cultures, the application of several stressors, co-cultures or other ways to accelerate ageing phenotypes such as expression of progerin. Transdifferentiation of fibroblasts to induced neurons (iNs) has also been described as a way to potentially accelerate phenotypes as iNs retain the "age memory" of the fibroblasts.

This developmental component and its implications in FTD modeling is highlighted in our study of a 10+16 splice-site mutation MAPT patient-derived neuronal model, where using tau splicing as a surrogate marker of neuronal maturation *in vitro*, we showed that control neurons expressed the fetal isoform of tau (0N3R) up to 100 days *in vitro* and only started expressing the full range of tau isoforms in extended culture time-points of one year *in vitro* [35]. The 10+16 mutation was able to override this developmental regulation of exon 10 splicing early on in cultures. However, neuronal maturation and proper tau splicing are important considerations for studies of mutations located in exon 10 of *MAPT*, which may be absent from neurons at conventionally assessed timepoints (day 80 or day 100 *in vitro*) leading to major implications in phenotyping.

Accelerated neuronal maturation and aging are important factors for optimisation in iPSC studies, as they can lead to shorter and cost-effective time-frames that are more suitable for disease modeling. In an effort to generate an iPSC model of late-onset Parkinson's disease (PD), Miller and colleagues expressed progerin, to induce premature ageing in their cultures of dopaminergic neurons [98]. Progerin-induced ageing successfully recapitulated phenotypes associated with both ageing and genetic composition, suggesting it is a useful system in late-onset disease modeling.

The concept of biological age in iPSC-derived neurons, as reflected by epigenetic modifications and transcriptome signatures, still remains unclear. In the study of Miller and colleagues, age memory was shown to be lost after reprogramming and could not be re-established upon differentiation but required rather long *in vitro* maturation in culture [98]. In a different study, induced neurons (iNs) directly converted from fibroblasts retained the age-specific transcriptional profile of the donor fibroblasts, in contrast to the generated iPSCs and iPSC-neurons, suggesting iNs may be useful in late-onset disease modeling [99].

In the context of cellular phenotyping, another important consideration is the autonomous versus non-autonomous mechanisms. Cell-autonomous phenotypes can be observed in the affected neuronal type that may recapitulate certain aspects of dementias. However, the complexity of brain networks as well as the apparent spread of pathology across brain regions may be an indication of non-cell autonomous co-existing mechanisms. This can either be due to direct toxicity of one cell to the other or simply a gradual loss of neuronal networks, as a result of loss of synaptic connections or glial support. It is a great challenge to model human brain in a dish, however, progress in co-culture systems such as microfluidics and 3D cultures offer a good way of investigating the spread of pathology or network interactions and employing more physiological, brain-mimicking conditions respectively. Indeed, most of the studies use two-dimensional cultures, a non-physiological spatial conformation of neurons *in vivo*. Such a 3D cell culture system has been described for Alzheimer's disease, recapitulating key pathogenic events, such as increase levels of hyperphosphorylated tau and extracellular aggregation of amyloid-β (Aβ) [100]. A limitation in the current model systems, 2D or 3D, is that they are isolated, lacking blood supply and interactions with the vascular system. Significant developments have recently been reported on 3D neural constructs where different cellular precursors combined on chemically defined hydrogels self-assembled into 3D neural constructs comprised of diverse populations of neurons and glia, including ramified microglia, and interconnected vascular networks [101].

As we already discussed in the previous section, heterogeneity in iPSC cultures directly affects the robustness of cellular phenotypes. Genetically engineered isogenic lines offer the best solution to this problem by eliminating background genomic noise. Moreover, cellular heterogeneity in neuronal cultures, combined with selective vulnerability of specific neuronal subtypes is making the detection of phenotypes even harder. Dissecting real data from background noise from pooled cells is very hard and perhaps adds an unnecessary level of complexity. Single-cell transcriptomics is a powerful way to extract informative data from single neurons rather than profiling the bulk population of cells that may lead to noisy data masking any potential phenotypes [102]. Finally, another hurdle to overcome is the limited ability to model certain aspects of dementia due to unavailability of robust differentiation protocols for specific and topographically defined neuronal subpopulations.

Conclusions

Like with any other model system, the use of iPSCs to model dementia is challenging. Nevertheless, iPSCs have bridged the gap between modeling systems and human neurodegenerative diseases, enabling studies of patient-derived neurons *in vitro*. Moreover, isogenic iPSC lines offer an unprecedented way of modeling the effect of mutations by eliminating genomic noise and producing robust data. They also offer a great tool to address the major issue of reproducibility in the field of cell modeling research. Some of the first studies in FTD and ALS that we have reviewed here show interesting results, recapitulating some major disease phenotypes and even elucidating novel pathogenic mechanisms [64]. It is however too early to conclude on mechanisms and more studies are required in order to have a complete picture of the pathogenic processes involved in FTD.

Study	Gene (mutation)	iPSC generation method	Differentiated cell type	Disease	Phenotypes observed	Phenotype rescue
Fong et al. 2013	MAPT (A152T)	Retroviral OCT3/4, SOX2, KLF4, and c-MYC	Mixed neurons	Tauopathy	↑ tau fragmentation and phosphorylation Axonal degeneration Dose-dependent effect of A152T mutation on neurodegeneration Vulnerability of DA neurons to A152T neurotoxicity	Genetic correction of the mutation with Zing-Finger nucleases (ZFNs) Elimination of tauopathy phenotypes
Ehrlich et al. 2015	MAPT (N279K, V337M)	Lentiviral OCT4, SOX2, KLF4, and c-MYC	Mixed neurons	FTD	tau pathology; ↑ tau fragmentation and phosphorylation ↓ neurite extension ↑ oxidative stress response to inhibition of mitochondrial respiration (reversible) Unfolded protein response (UPR) activation Distinct disease-associated gene expression profiles	N/A
Sposito et al. 2015	MAPT (10+16)	Retroviral OCT3/4, SOX2, KLF4, and c-MYC	Cortical neurons	FTD	Mutation overrode developmental regulation of exon 10 splicing Patient-derived neurons expressed 0N3R and 0N4R tau At 365 days in culture, neurons expressed all 6 tau isoforms with ↑ levers of 4R in patient neurons Faithful recapitulation of human developmental tau expression	N/A
Wren et al. 2015	MAPT (N279K)	Episomal OCT4, SOX2, KLF4, L-MYC, LIN28, and p53 shRNA	Neural stem cells (NSCs)	FTD	Mutation dramatically affected neuronal viability and differentiation of NSCs to mature neurons ↑ ratio of 4-repeat to 3-repeat tau Accumulation of stress granules; ↑ cellular stress Impaired endocytic trafficking	N/A
Iovino et al. 2015	MAPT (P301L, N279K)	Retroviral OCT3/4, SOX2, KLF4, and c-MYC	Cortical neurons (+ DAPT Notch inhibitor for neuronal maturation)	FTD	Earlier electrophysiological maturation Altered mitochondrial transport N279K neurons; premature developmental 4R tau, 3R:4R isoform ratio changes, AT100-hyperphosphorylated tau aggregates P301L neurons; contorted processes with varicosity-like structures, some containing both alpha-synuclein and 4R tau	N/A
Almeida et al. 2012	GRN (S116X)	Retroviral OCT3/4, SOX2, KLF4, and c-MYC	Mixed neurons and microglia	FTD	↓ intracellular and secreted PGRN Compromised PI3K/Akt and MEK/MAPK signaling pathways	PGRN expression
Gascon et al. 2014*	GRN (S116X) Same line as Almeida et al. 2012 C9ORF72 (GGGGCC expansion)	Retroviral OCT3/4, SOX2, KLF4, and c-MYC	Mixed neurons	bvFTD	Age-dependent (8-week-old neurons) ↓ miR-124 levels ↑ Gria2 and Gria4 AMPAR subunits mRNA	N/A
Lee et al. 2014	GRN (S116X) Same line as Almeida et al. 2012	Retroviral OCT3/4, SOX2, KLF4, and c-MYC	Mixed neurons (2 week-old)	FTD	PGRN haploinsufficiency	Restoration of PGRN levels by reduction of SORT1-mediated endocytosis via blocking with MPEP compound
Raitano et al. 2015	GRN (GRNIVS1+5G>C)	Retroviral OCT3/4, SOX2, KLF4, and c-MYC	Cortical neurons	FTD	↓ generation of cortical neurons due to PGRN haploinsufficiency Altered gene expression Wnt signalling pathway dysregulated	ZFN-mediated introduction of GRN wild-type cDNA in the AAVS1 locus corrected cortical neurogenesis defects and restored altered gene expression and Wnt

					pathway dysregulation	
Almeida *et al.* 2013	*C9ORF72* (GGGGCC expansion)	Retroviral OCT3/4, SOX2, KLF4, and c-MYC	Mixed neurons	FTD	RNA foci detected in fibroblasts, iPSCs and neurons RAN dipeptides detected in neurons ↑ p62 levels Compromised autophagy mechanisms	N/A
Donelly *et al.* 2013	*C9ORF72* (GGGGCC expansion)	Retroviral OCT3/4, SOX2, KLF4, and c-MYC	Mixed neurons	ALS	↓ C9orf72 RNA levels Detection of intranuclear RNA foci and cytoplasmic poly-(Gly-Pro) RAN dipeptides Sequestration of RNA binding proteins by the expanded GGGGCC RNA, validation of ADARB2 interaction Aberrant gene expression Neurons susceptible to glutamate excitotoxicity	Antisense oligonucleotides (ASOs) targeting C9orf72 the repeat expansion or transcript rescue toxicity phenotypes
Sareen *et al.* 2013	*C9ORF72* (GGGGCC expansion)	Episomal OCT4, SOX2, KLF4, L-MYC, LIN28, and p53 shRNA	Motor neurons	ALS ALS/FTD	No reduction of C9orf72 RNA Detection of RNA foci but absence of RAN dipeptide products Co-localisation of RNA foci with hnRNPA1 and Pur-α Aberrant gene expression Reduced excitability of C9orf72 motor neurons	Antisense oligonucleotides (ASOs) targeting C9orf72 transcript suppressed RNA foci formation and rescued gene expression profile
Meyer *et al.* 2014	*C9ORF72* (GGGGCC expansion) *SOD1* (A4V)	Direct conversion of fibroblasts to NPCs	Astrocytes (i-astrocytes)	ALS	Non-cell autonomous toxicity of patient induced astrocytes to co-cultured motor neurons	N/A
Satoh *et al.* 2014	*C9ORF72* (GGGGCC expansion) Molecular network analysis of Sareen *et al.* 2014 RNA-seq dataset	N/A	N/A	ALS	↓ expression of a wide range of extracellular matrix proteins and matrix metalloproteinases in patient-derived motor neurons No reduction of C9orf72 mRNA in patient motor neurons compared to controls	N/A
Devlin *et al.* 2015	*C9ORF72* (GGGGCC expansion) *TARDBP* (M337V)	Lentiviral or Sendai OCT3/4, SOX2, KLF4, and c-MYC	Motor neurons	ALS	Initial hyperexcitability followed by progressive loss of action potential output and synaptic activity No changes in cell viability	N/A
Yang *et al.* 2015	*C9ORF72* (GGGGCC expansion)	Retroviral OCT3/4, SOX2, KLF4, and c-MYC and Episomal OCT4, SOX2, KLF4, L-MYC, LIN28, and p53 shRNA	Mixed neurons	FTD/ALS	↓ expression of some Notch target genes Co-expression of $(GA)_{80}$ partially suppressed $(GR)_{80}$ toxicity by recruitment of $(GR)_{80}$ into cytoplasmic inclusions	N/A
Zhang *et al.* 2015	*C9ORF72* (GGGGCC expansion	Retroviral OCT3/4, SOX2, KLF4, and c-MYC	Mixed neurons	ALS	Mislocalisation of RanGAP1 and interaction with expanded repeat RNA Disrupted nuclear-cytoplasmic pattern of Ran; ↑ cytoplasmic levels of Ran in patient-derived neurons - rescued by RanGAP1 overexpression Abnormal nuclear/cytoplasmic ratio of TDP-43 Impaired nucleocytoplasmic transport	Small molecules and antisense oligonucleotides targeting the repeat expansion G-quadruplexes rescued nucleocytoplasmic transport deficits
Freibaum *et al.* 2015	*C9ORF72* (GGGGCC expansion)	Episomal OCT4, SOX2, KLF4, L-MYC, LIN28, and p53 shRNA	Mixed neurons	FTD/ALS	RNA nuclear export defect, retention of RNA in nuclei ↑ nuclear to cytoplasmic ratio of RNA in patient neurons vs controls ~ 35% ↑ in the nuclear:cytoplasmic ratio of RNA density in patient neurons	N/A
Jovicic *et al.* 2015	*C9ORF72* (GGGGCC expansion)	Direct conversion of fibroblasts to neurons (iNs) via NGN2 and ASCL1 expression	Induced neurons (iNs)	FTD/ALS	↓ nuclear localization of RCC1 in patient iNs	N/A

Study	Mutation	Reprogramming factors	Cell type	Disease	Phenotype	Drug/Treatment
Bilican et al. 2012	*TARDBP* (M337V)	Retroviral OCT3/4, SOX2, KLF4, and c-MYC	Motor neurons	ALS	↑ soluble and insoluble TDP-43 ↓ survival in longitudinal studies increased vulnerability to antagonism of the PI3K pathway	N/A
Serio et al. 2013	*TARDBP* (M337V)	Retroviral OCT3/4, SOX2, KLF4, and c-MYC	Astrocytes	ALS	↑ levels and subcellular mislocalisation of TDP-43 ↓ cell survival Absence of non-cell-autonomous component; patient astrocytes not toxic to neurons in co-culture systems	N/A
Zhang et al. 2013	*TARDBP* (A90V, M337V)	Retroviral OCT3/4, SOX2, KLF4, and c-MYC	Mixed neurons	FTD/ALS	Neurons sensitive to staurosporine-induced stress Stress-induced phenotypes; TDP-43 mislocalisation in the cytoplasm ↓ total TDP-43 ↓ levels of microRNA-9 (miR-9) and pre-miR-9-2 ↓ miR-9 expression also in M337V neurons	N/A
Egawa et al. 2012	*TARDBP* (Q343R, M337V, G298S)	Retroviral OCT3/4, SOX2, KLF4, and c-MYC or episomal OCT4, SOX2, KLF4, L-MYC, LIN28, and p53 shRNA	Motor neurons	ALS	Mislocalised, aggregated TDP-43 ↑ insoluble TDP-43 bound to SNRPB2 spliceosomal factor Shorter neurites compared to control motor neurons Upregulation of RNA metabolism genes and downregulation of cytoskeletal genes in patient neurons compared to controls	Anacardic acid, an inhibitor of histone acetyltransferases, rescued ALS phenotype in patient motor neurons
Burkhardt et al. 2013	8 familial ALS lines from which known mutations in; *TARDBP* (A315T), *SOD1* (N139K, A4V), *FUS* (G1566A) and 16 sporadic ALS	Retroviral OCT3/4, SOX2, KLF4, and c-MYC	Motor neurons (upper and lower)	ALS	TDP-43 aggregates Aggregates recapitulate pathology in postmortem tissue of patients	High-content chemical screen revealed four classes of compounds that reduced TDP-43 aggregation phenotype in sALS neurons; cyclin-dependent kinase inhibitors, c-Jun N-terminal kinase inhibitors (JNK), Triptolide and FDA-approved cardiac glycosides, Digoxin, Lanatoside C, and Proscillaridin A
Yang et al. 2013	*TARDBP* (M337V) *SOD1* (L144F)	Retroviral OCT3/4, SOX2, KLF4, and c-MYC	Motor neurons	ALS	Reduced survival of patient motor neurons compared to controls	Kenpaullone, a multikinase inhibitor, strongly improved patient motor neuron survival

Table 1. Summary of iPSC studies in FTD and FTD/ALS.

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Author contributions

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