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Reverse Engineering Human Neurodegenerative Disease Using Pluripotent Stem Cell Technology

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Abstract

With the technology of reprogramming somatic cells by introducing defined transcription factors that enables the generation of “induced pluripotent stem cells (iPSCs)” with pluripotency comparable to that of embryonic stem cells (ESCs), it has become possible to use this technology to produce various cells and tissues that have been difficult to obtain from living bodies. This advancement is bringing forth rapid progress in iPSC-based disease modeling, drug screening, and regenerative medicine. More and more studies have demonstrated that phenotypes of adult-onset neurodegenerative disorders could be rather faithfully recapitulated in iPSC-derived neural cell cultures. Moreover, despite the adult-onset nature of the diseases, pathogenic phenotypes and cellular abnormalities often exist in early developmental stages, providing new “windows of opportunity” for understanding mechanisms underlying neurodegenerative disorders and for discovering new medicines. The cell reprogramming technology enables a reverse engineering approach for modeling the cellular degenerative phenotypes of a wide range of human disorders. An excellent example is the study of the human neurodegenerative disease amyotrophic lateral sclerosis (ALS) using iPSCs. ALS is a progressive neurodegenerative disease characterized by the loss of upper and lower motor neurons (MNs), culminating in muscle wasting and death from respiratory failure. The iPSC approach provides innovative cell culture platforms to serve as ALS patient-derived model systems. Researchers have converted iPSCs derived from ALS patients into MNs and various types of glial cells, all of which are involved in ALS, to study the disease. The iPSC technology could be used to determine the role of specific genetic factors to track down

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what's wrong in the neurodegenerative disease process in the “disease-in-a-dish” model. Meanwhile, parallel experiments of targeting the same specific genes in human ESCs could also be performed to control and to complement the iPSC-based approach for ALS disease modeling studies. Much knowledge has been generated from the study of both ALS iPSCs and ESCs. As these methods have advantages and disadvantages that should be balanced on experimental design in order for them to complement one another, combining the diverse methods would help build an expanded knowledge of ALS pathophysiology. The goals are to reverse engineer the human disease using ESCs and iPSCs, generate lineage reporter lines and in vitro disease models, target disease related genes, in order to better understand the molecular and cellular mechanisms of differentiation regulation along neural (neuronal versus glial) lineages, to unravel the pathogenesis of the neurodegenerative disease, and to provide appropriate cell sources for replacement therapy.

Keywords

Motor neurons; Glia; Lou Gehrig disease; Induced pluripotent stem cells; CRISPR

1. Introduction

There has been tremendous interest to apply pluripotent stem cell technologies for disease modeling, drug screening and regenerative medicine, as well as to determine genetic factors contributing to disease onset and treatment response as a means of improving health outcomes. The focus of this review article is amyotrophic lateral sclerosis (ALS) (Boillee et al., 2006a; Brown, 1997; Cole and Siddique, 1999), a devastating neurodegenerative disease with a worldwide prevalence of 4–6 per 100,000 people. ALS affects lower motor neurons (MNs) in brainstem and spinal cord, upper MNs in motor cortex, and the corticospinal tract, resulting in progressive weakness and atrophy of skeletal muscles. Death results from respiratory failure within three years on average of initial diagnosis. Despite the selective functional deficiency due to MN loss, recent evidence has implicated glial cells (astrocytes and oligodendrocytes) and microglia as contributors to MN death (Beers et al., 2006; Frakes et al., 2014; Ilieva et al., 2009; Kang et al., 2010; Maragakis and Rothstein, 2006). Although several mechanisms have been proposed to likely contribute to sporadic disease pathogenesis, the etiology of selective MN death in this disease remains elusive. As a result, there exists no effective treatment for ALS.

The groundbreaking development of a cellular reprogramming technology, through which “induced pluripotent stem cells (iPSCs)” could be derived from easily accessible somatic cells such as dermal fibroblasts by forced expression of defined pluripotency-inducing “reprogramming” factors, has provided an unprecedented approach that enables generation of patient-specific cells for cell-specific pathogenesis studies and for cell-based therapeutic developments (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007). Patient-derived iPSCs have been used to investigate the key pathogenic processes of ALS, using the reprogramming technology to “de-differentiate” patient-specific skin fibroblasts back to stem cells, and then “re-differentiate” them into specific neural lineages to create appropriate in vitro models of “disease in a dish” (Bilican et al., 2012; Donnelly et al., 2013; Kiskinis et al., 2014; Wainger et al., 2014). Such a “de- and re-differentiation” approach is to

make a “cellular U-turn” and is ideal to track down what’s wrong in the neurodegenerative disease process. The iPSC technology has proven useful for the generation of individual cell lines from different patients to study the nature of the disease. To complement the iPSC-based approach, researchers have also performed gene targeting to knock-in disease-relevant mutations in human embryonic stem cells (ESCs) for disease modeling and for comparative mechanistic studies. By taking advantage of the ESC/iPSC-based platform to determine the key pathogenic events in disease progression and pathogenic development, researchers are investigating how the patients’ iPSCs take various lineages (neuronal/glial versus non-neuronal), monitor the development of the neurodegenerative disease phenotypes, and determine their regulatory role in ALS development, in hope to gain new insights into the pathogenesis and treatment of the neurodegenerative disease. This approach thus combines a reverse engineering concept with molecular studies in order to identify key mechanisms in the neuronal versus glial causes of ALS.

2. Disease modeling for ALS using human pluripotent stem cells (PSCs)

Both the molecular mechanisms of neurodegeneration in ALS and the reasons accounting for the selective vulnerability of MNs in ALS remain poorly understood. Several pathogenic mechanisms have been taken into consideration, including impaired RNA metabolism, aberrant protein mis-folding, mitochondrial alterations, defective axonal transport, excitotoxicity and local inflammation (Beers et al., 2006; Beers et al., 2008; Cozzolino et al., 2013; Fischer et al., 2011; Israelson et al., 2015; Ling et al., 2013; Magrane and Manfredi, 2009; Maragakis and Rothstein, 2006; Wong et al., 1995). The discovery of causative genes has given new inputs to the field. After the first report of ALS causative mutations in the gene encoding the Cu/Zn-dependent antioxidant enzyme superoxide dismutase-1 (SOD1), researchers have started to investigate non-cell-autonomous mechanisms linked to the development of ALS disease. The identification of C9ORF72 repeat expansion as the major factor responsible for ALS onset (DeJesus-Hernandez et al., 2011; Renton et al., 2011) in the familial forms has focused attention on the causative role of alterations in RNA metabolism, a line of research supported also by the involvement of mutations in TARDBP and FUS genes that encode DNA/RNA binding proteins in ALS development (Arai et al., 2006; Lagier-Tourenne et al., 2012; Neumann et al., 2006).

Although much work needs to be done with ALS iPSCs, this particular disease is probably the most heavily investigated neurological disorder with human iPSC modeling. The establishment of human cell platforms has allowed to test in vitro some of these pathogenic hypotheses and to model and investigate early disease mechanisms. Neural stem cells or MNs derived from ALS patients or bearing ALS mutations could be used in long-term cell cultures or co-culture with glial cells to examine pathophysiological hallmarks of the disease, including analysis of protein aggregates, altered pathways in the neurodegenerative process, in particular the imbalance of mitochondrial dynamics, electrophysiological properties, gene expression analyses, and examination of patterns of alternative pre-mRNA splicing. These studies might provide important insights into ALS, and provide cell-based assays for drug screening (Bilican et al., 2012; de Boer et al., 2014; Di Giorgio et al., 2008; Dimos et al., 2008; Donnelly et al., 2013; Kiskinis et al., 2014; Li et al., 2015b; Marchetto et al., 2008; Sareen et al., 2013; Wainger et al., 2014).

2.1. Modeling ALS with human PSCs

As a proof of principle for generating disease models using human ESCs, well characterized common human ESC lines were used to mimic the genetic changes in familial ALS patients by overexpressing mutant SOD1 (Karumbayaram et al., 2009). Alternatively, one could introduce mutations of known genes into normal human ESC lines. For instance, creation of SOD1 mutation at the original genomic SOD1 locus would recapitulate the genetic defects in a wide range of familial ALS patients (with A4V being the most lethal form, Figure 1). This approach could be extended to additional SOD1 mutants and other gene mutations identified in ALS. Another strategy makes use of safe-harbor or pre-engineered docking platform human ESC lines. These human ESC (or iPSC) lines usually harbor Cre-lox cassette or R4 integrase binding site at constitutive (such as the human ROSA, HPRT1, and the chromosome 19 adeno-associated virus AAV integration) loci, or lineage specific genes (such as the neural specific transcription factor Olig2) loci (Cerbini et al., 2015; Irion et al., 2007; Liu et al., 2011; Macarthur et al., 2012; Pei et al., 2015; Xue et al., 2009). This strategy ensures the re-targeting of mutation at predetermined and validated genomic loci, facilitating the subsequent cell differentiation and purification, as well as phenotype identification and comparison.

Using genetic modification to generate disease models with human ESCs is limited to known gene mutations, which only cover a small percentage of ALS patients (Boillee et al., 2006a; Brown, 1997; Cole and Siddique, 1999). In order to model a wider range of ALS cases, and to provide the opportunity to examine disease complexity, researchers have generated disease models using ALS patient-derived iPSCs, which open a new avenue to the generation of human neurons and glia that carry the genomes of individuals with familial or sporadic ALS. Differentiation of such stem cells to human MNs and glia is already offering new insights into ALS and molecular pathways that might provide new targets for effective therapy (Bilican et al., 2012; Donnelly et al., 2013; Haeusler et al., 2014; Kiskinis et al., 2014; Sareen et al., 2013; Wainger et al., 2014). There is a need for standardization of models so that isogenic lines differing only in the familial ALS mutation could be compared. There are exciting opportunities for more complex culture systems in either 3 dimensions, organoids culture (Lancaster et al., 2013), or with the addition of other defined cell types, such as astrocytes, oligodendrocytes, and microglia. This system opens many new doors for testing mechanisms and pathways, as well as for discovery of therapeutic targets and agents.

A unique opportunity of the human iPSC system is the ability to probe how complex human genomic architectures, i.e, the complexity of the genome content (single nucleotide polymorphism or SNPs, repeat sequences, duplications, copy number variations or CNVs, etc.), epigenetic composition, as well as the organization of the genome (Alkan et al., 2009; Dixon et al., 2015; Guo et al., 2015), predispose patients to ALS and how they influence the behavior of the various participating cell types. There is enormous promise in the utility of a human cellular system to predict how individual genetic and cellular phenotypic variation contributes to response to pharmacological intervention at clinically relevant levels. Moreover, the genetic technologies available with human iPSCs might allow the deciphering of how complex genomic architectures found in individual humans act together to generate

susceptibility and variation in response to the environmental factors that might also contribute to or pharmacologically modify ALS phenotypes in patients.

Because non-cell autonomous effects have been observed in SOD1G93A mice (Boillee et al., 2006b; Nagai et al., 2007; Wichterle et al., 2002), the co-culturing of human iPSC-derived MNs with glial cells would prove very useful for the establishment of a robust in vitro system that could serve as the basis for detailed mechanistic studies of the interactions between SOD1 mutant MNs and glia; these studies also provide an assay for diffusible factors toxic to MNs. They might also provide a cell based assay for small molecules that promote survival of mutant SOD1 MNs. In addition, such studies validate the use of stem cells carrying disease-causing genes to study disease mechanisms. Thus, it is necessary and important to carry out such an approach using fibroblasts from both familial and sporadic ALS patients, converting them to iPSCs and then to MNs and glia for detailed mechanistic studies. The goal is to understand the mechanisms that underlie familial and sporadic forms of ALS, and to ultimately identify approaches for the development of new therapies. This is clearly of great importance, considering the devastating effects of the disease, the lack of mechanistic understanding, and the fact that neither a cure nor treatment is available. A robust in vitro model system in which the pathological events leading to ALS could be studied could lead to the identification of the factors that influence MN survival. The capacity of pluripotent stem cells to self-renew in culture, while retaining their pluripotent potential, provides the opportunity to produce virtually unlimited numbers of differentiated cell types to replenish those lost as a consequence of disease processes. An alternative, but equally important potential of iPSCs is to provide insights into disease mechanisms. In this case, iPSCs carrying the genes responsible for a particular disease could be induced to differentiate into the cell types affected in that disease. Studies of the differentiated cells in culture could provide important information regarding the molecular and cellular nature of events leading to pathology.

An objective of this line of research is to characterize the molecular and physiological nature of the events leading to MN degeneration in ALS. This objective could be approached by detailed comparisons of the mRNAs and proteins produced in normal and patient derived MNs, and by physiological studies. An important goal is to identify gene products that track with progressive MN degeneration. Since patient derived MNs likely behave indistinguishably from primary MNs and the patients' iPSCs could be readily expanded, this work would neither be limited by availability of material, nor are the observations likely to be confounded by possible heterogeneity in tissue samples. To validate results obtained using the in vitro system, one could employ microarray and RNA sequencing (RNAseq) analyses to profile patterns of gene expression in patient-derived as well as normal MNs obtained from healthy control individuals. These studies would also include detailed studies of the changes in pattern of alternative pre-mRNA splicing.

2.2. Limitations and potential mitigation strategies

Human ESCs are not always available in substantial amount for large scale in vitro studies. Furthermore, a great advantage of iPSCs is that they carry the genetic combination of an individual, thus permitting the study of patient-specific mutations. Issues concerning the

heterogeneity of iPSC lines, which could provide misleading results, might be overcome by a careful experimental setting. This should be based on rigorous statistical analysis and standardized protocols. Moreover, the use of cutting-edge molecular methods allows correcting the mutation in patient-derived iPSCs, thus obtaining isogenic controls. Genome editing of human cells aims to repair or eliminate a mutation that could cause disease. The premise is that corrective changes to a sufficient number of cells carrying the mutation, in which the genetic fixes would last the lifetimes of the modified cells and their progeny, could provide isogenic controls for rigorous research or even a “one and done” curative treatment for patients. The newest addition to the genome-editing arsenal is CRISPR/Cas9, a bacteria-derived system that uses RNA molecules that recognize specific human DNA sequences. The RNAs act as guides, matching the nuclease to corresponding locations in the human genome. CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated) system is the simplest genome-editing tool to work with because it relies on RNA-DNA base pairing, rather than the engineering of proteins that bind particular DNA sequences. The CRISPR technique has dramatically expanded research on genome editing in human stem cells (Cong et al., 2013; Fu et al., 2014; Jinek et al., 2012; Li et al., 2015a; Xue et al., 2014; Yang et al., 2013a).

Another important advancement is that reliable results could be obtained with the use of non-integrative reprogramming methods. Advances in reprogramming methods have also led to the possibility of directly reprogramming mature cells (i.e., fibroblasts or astrocytes) into relevant cell types (i.e., induced MNs). These trans-differentiation methods could be assessed for ALS disease modeling. In addition to the well-studied role of MNs in ALS pathology, attention has recently been paid on glial cells in ALS pathogenesis. Indeed, astrocytes, oligodendrocytes and microglia have all proved to contribute to MN dysfunction and play key roles in neurodegeneration in ALS. It is crucial to pay attention to the cellular subtypes obtained in culture with varying differentiation protocols in order to avoid misleading results. Well-established cellular markers need to be used to assess both the cell phenotype and the maturation state. In addition, several studies have highlighted the possibility that reprogrammed cells might maintain an epigenetic memory of the cell of origin, which could interfere with the expression of membrane markers. It also needs to be pointed out that pathogenesis studies benefit from long-term observation, from the cell pluripotent state to the mature phenotype, in order to speculate on the time-dependent disease alteration. Nonetheless, patient-derived iPSCs have truly proven as invaluable tools for disease modeling, as they provide a direct way to test the possibility that a key molecule or pathway or a specific defect is responsible for a particular neurodegenerative disorder in humans. This approach involves the production of iPSCs from adult cells derived from individuals with the disorder and then inducing these iPSCs to form neurons and glial cells. Such an approach using patient-derived iPSCs has been applied to model ALS, revealing key pathogenic events in this disease.

3. Investigating key pathogenic events in ALS using PSCs

The development and optimization of iPSC-based platforms has allowed elucidating disease-specific mechanisms, which are exquisitely human. Further advances might eventually lead to the path to a full understanding of key pathogenic events, leading to the development of

effective treatments for ALS. The majority of ALS cases are sporadic with only 5–10% of patients having a family history of the disease. Studies of familial ALS patients have identified a number of genes, mutation of which likely accounts for the onset of the disease. However, twin studies have estimated ALS heritability at greater than 60%, strongly suggesting that a majority of sporadic ALS cases also have a genetic basis. Genomic sequencing studies of sporadic ALS patients and their parents demonstrate that patients often carry compound heterozygous mutations that potentially inactivate genes whose loss leads to ALS onset. Examining the functions of these genes in iPSC-based models and systems to assess their likely association with ALS would provide a format for tailoring treatment options on the basis of the genetic underpinning of the disease.

3.1. Identification of genetic defects

While mutations of the SOD1 gene in an autosomal dominant trait have been identified in a small percentage of familial patients, no consistent genetic alterations have been found in sporadic subjects, who are the majority of ALS cases. Currently, transgenic rodents are the disease models available for pathogenesis study and drug testing. As no sporadic ALS disease models exist, the majority of preclinical trials are based on animal models of familial ALS that do not recapitulate sporadic ALS. In addition, rodent models, although do mimic disease phenotypes, often are not satisfying tools for studying pathogenesis in humans. These needs prompt us to model ALS using a reverse engineering approach, allowing for a developmental perspective for the neurodegenerative disease and providing a platform to determine key pathogenic events in disease development. ALS iPSCs could be used to determine how developmental dysfunction and pathogenic signaling might critically contribute to the development of MN and glial pathology in human ALS.

3.2. Investigation of mitochondria dysfunction

Mitochondrial dysfunction has been implicated in many human neurodegenerative diseases including ALS. Understanding the molecular basis of mitochondrial function and dysfunction in human cells would transform our understanding of the role of this critical organelle in human health and disease. It would be interesting to investigate mitochondrial function in the human ESC/iPSC-based models, i.e. to study how mitochondrial function dictates the fate of ALS human ESC/iPSCs and their differentiation toward MNs and glia, in hope to gain new insights into pathogenesis and treatment of the neurodegenerative disease. For example, it would be interesting to examine mitochondrial dysfunction in human ESCs/iPSCs with ALS SOD1 mutations. In addition, iPSC technology could be used as an innovative tool for human mitochondrial research since we could generate iPSCs with normal or dysfunctional mitochondria from healthy people and ALS patients, and then force the cells to differentiate into various cell lineages to evaluate how the state of mitochondrial function dictates the ability of the iPSCs to provide distinct lineages. Elucidating the complexity of human mitochondrial function through the use of ALS patient-derived iPSCs that are reprogrammed from a disease state allows for investigation into mechanisms that regulate mitochondrial mechanisms in ALS-specific iPSCs. Comparison of mitochondrial morphology and function in a variety of human ESCs and iPSCs would allow evaluation of basic mitochondrial function and structure to gain an appreciation for the variability in these human ESC/iPSCs and for the global effects on human ESC/iPSC function. It would also be

by misregulation of TDP43-dependent splicing, then a genome-wide analysis of its targets could provide major advances in our understanding of the disease (Bilican et al., 2012).

Given the technological feasibility in genomic sequencing-based technologies (RNASeq, ChIPSeq and CLIPSeq), it would be interesting to investigate the role of TDP43 in regulating alternative pre-mRNA splicing in MNs (Johnson et al., 2007; Ule et al., 2003; Ule et al., 2005a; Ule et al., 2005b; Ule et al., 2006), and to sequence RNA from stem cell-derived MNs treated with control siRNA or siRNA targeting TDP43 for knock down. Analysis of these data could identify genes dependent on TDP43 not only for their transcriptional regulation but also for different stages of the processing of their encoded RNAs such as alternative promoter usage, splicing, poly-adenylation and editing. It would also be interesting to then sequence biochemically purified DNA (ChIPSeq) or RNA (CLIPSeq) cross-linked to TDP43 using a TDP43 antibody. Comparison of these sequence tags with the RNASeq data might lead to testable hypotheses about the implications of TDP43 binding such as its preferred binding sites and the effect of TDP43 binding on transcription and RNA processing. Sequence analysis of the tags could also identify potential binding partners of TDP43. These data could be examined for genes and RNA processing events already known to be perturbed in ALS to uncover the role TDP43 pathology might play in these deleterious processes.

Hexanucleotide (GGGGCC)_n repeats, located at the noncoding exon of the C9orf72 (C9) gene, has been identified to be the most common causative genetic changes in amyotrophic lateral sclerosis (ALS) patients with or without frontotemporal dementia (FTD) (Almeida et al., 2013; Donnelly et al., 2013; Haeusler et al., 2014; Sareen et al., 2013). ALS research has therefore focused more on possible RNA toxicity caused neurodegeneration. Aberrant expansion of GGGGCC repeats might act through forming RNA foci of G-quadruplexes and RNA-DNA hybrids, binding and sequestering RNA-binding proteins, and subsequently disrupting the normal process of transcription, promoter methylation/demethylation, or translation, resulting in dipeptide formation, cytoplasmic inclusion, and RAN (repeat associated non-ATG) translation (Almeida et al., 2013; Donnelly et al., 2013; Gijssels et al., 2012; Haeusler et al., 2014; Sareen et al., 2013; Todd and Paulson, 2013; Xi et al., 2013; Zu et al., 2011). However, the size of repeats and their exact function in disease initiation and progression have yet to be determined. The relationship of the size of the GGGGCC repeat expansion across different neural cell types or non-neural cell types that might correlate disease pathogenesis, disease onset and progress has not been fully understood (Nordin et al., 2015). One of the limitations is the lack of availability of matched patient isogenic samples. iPSCs provide a renewable source of patient autologous cells that not only retain identical genetic information but also give rise to all neural cell types. Meanwhile, the rapid advancement of genome editing tools including the CRISPR/Cas9 system would allow for the generation of a collection of isogenic iPSCs with desired (GGGGCC)_n length from patient iPSCs. These advancements combined have provided unprecedented opportunities to whittle down the versatile symptoms in ALS/FTD (Figure 2). They may also facilitate in the research on other diseases involving GGGGCC or trinucleotide repeat expansion including Parkinson's disease, Huntington's disease, Fragile X syndrome, and myotonic dystrophy.

4. Drug screening for ALS

Cellular reprogramming offers a unique approach to modeling human disorders, as it captures a patient's genome in relevant cell types that could be propagated in vitro. This kind of "disease-in-a-dish" approach allows progressive time course analyses of target cells, offering a window of opportunity to reveal molecular or pathway alterations before symptomatic onset. It would be interesting to integrate neurotransmitter/calcium imaging probes to patient-specific iPSC-derived neurons and glial cells to create a platform for modeling "brain disease in a dish". Such cultured neural networks would enable us to visualize how the precise, guided communication between neurons and glia develops, and how it breaks down in disease. With this system, we could test a library of drugs to identify ones that could correct communication defects in a patient- and disease-specific manner (Bilican et al., 2012; Di Giorgio et al., 2008; Drawnel et al., 2014; Egawa et al., 2012; Mercola et al., 2013; Yang et al., 2013b). Such drug screening would not be possible in living patients. Working with large patient cohorts is important to understand how brain cells derived from the diverse human genetic background respond to specific drugs. As many neurodegenerative disorders share similar neurological symptoms, comparison of in-vitro-derived patient-specific neural cells provides insights into common trends and unique aspects of each neurological disease.

A distinctive new approach to CNS drug discovery is multi-scale network analysis based systems drug screening on the human iPSC-based technology platform of "brain in a dish" in which neural network or brain connectome is derived from neural differentiation of patient-derived iPSCs. This network neuropharmacology (Csermely et al., 2005; Hopkins, 2008) approach involves application of network analysis to determine the set of proteins within the brain network or the wiring of neural circuits that are most critical in the disease pathway, and then chemical biology to identify molecules capable of targeting that set of proteins. By addressing the complexity of disease and by seeking to harness the ability of drugs to influence many different proteins, network pharmacology differs from conventional drug discovery approaches, which have generally been based on highly specific targeting of a single protein. Network pharmacology has the potential to provide new treatments for complex diseases such as ALS where conventional approaches have failed to deliver satisfactory therapies.

In all of life that is relevant to human diseases, there are hundreds of thousands of proteins in the dysfunctional brain network. Every drug compound binds in varying degrees to many different proteins, and could affect very many proteins in other ways, such as through changing their expression, or phosphorylation state. The huge variety of possible protein targets and the large variety of patterns of possible effects on proteins create an enormous range of possible interactions between a drug molecule and the underlying biological system, i.e. the dysfunctional brain network. For this fundamental reason, the biological effects of a molecule's interactions with pathogenic, pathological and normal cells have been difficult to predict. Existing pharmacotherapies are limited and carry substantial risk of adverse effects. The iPSC-based technology platform of network pharmacology enables analysis of networks associated with the dysfunctional neural network underlying a particular CNS disease. In each case it identifies a disease signature, a set of points in the

network at which intervention would have maximum impact. Then the goal is to seek drug candidates whose footprint, the set of proteins impacted by the presence of the drug, matches the relevant disease signature. Drug candidates discovered from this platform of iPSC-based systems pharmacology for disorders of neuro-connectome would more rapidly progress to development pipelines. Widespread integration of iPSC platform with genome-wide association studies and -omics would ultimately allow implementation of this technology in a personalized medicine context. In the case of ALS, iPSC technology provides unique advantages for the discovery of factors that cause ALS and how these factors respond to genetic or pharmacologic manipulation. The generation of human neurons and glia carrying various familial ALS mutations or sporadic ALS genomes would provide a uniquely human cellular system for the evaluation of potential therapeutic interventions. Probing the human genetic and biochemical pathways in MNs and glial cells provides unique advantages owing to the likely differences between humans and other model organisms such as mice. Future work along this direction is warranted.

5. Cell therapy for ALS

Studies on the treatment of ALS are roughly divided into two strategies. One is to reproduce the pathology of ALS and search for a therapeutic drug to improve it. The other is the complementation and regeneration of MNs with ALS, which have lost their function to appropriately stimulate muscle, by cell transplantation. Recently, much attention has been placed on stem cell-based regeneration strategies as a promising new treatment for ALS (Lepore et al., 2008; Papadeas et al., 2011). Cell therapy for ALS aims to regenerate MNs and additionally provide a favorable microenvironment by replacing astrocytes and microglia. MNs have been generated in vitro from several stem cell sources. However, to be clinically successful in ALS, transplanted MN precursors should extend axons across long distances and integrate with the existing neural circuitries and innervate target muscle fibers. Preclinical studies in rat models showed that spinal transplantation of MN progenitors could extend axons to ventral roots and innervate host muscles resulting in partial recovery from paralysis. Although this sounds promising, several finer characteristics need to be defined before clinical translation of this technology. For example, MN progenitors need to be delivered at multiple sites along the spinal cord and they should be directed to cervical, thoracic or lumbar phenotypes. It should also be evaluated whether corticospinal neurons that degenerate in ALS could be effectively replaced. Perhaps the most important hurdle in MN regeneration would be to ensure that the hostile microenvironment for this cell type in the spinal cord is also favorably modified.

Replacing lost MNs using human ESCs or iPSCs remains a potential therapy for ALS, but relies on transplanting MNs expected to survive and form long-distance projections (from brain to spinal cord, and/or spinal cord to muscle) with functional connections. This is a very challenging proposition given the long distances required for axonal outgrowth in the adult compared to development (when the MNs originally make contact with the muscle). In addition, the new MNs are placed in a very toxic environment where all those around them are dying. Alternatively, transplanting cells that have the ability to support the survival of existing MNs or potentially “detoxify” the environment is a far more practical idea. Support cell types could be generated from other tissues such as mesenchymal stem cells (MSCs)

isolated from the bone marrow and neural stem cells (NSCs) isolated from fetal brain. These cells are not as likely to make primitive tumors as those derived from pluripotent cells. They are a safer potential product and have provided a much faster path to the clinic, although pluripotent stem cell research is developing rapidly and clearly this field would expand enormously in the coming years.

There have been several advancements using MSCs and NSCs, all of which rely on using the stem cells to stimulate the survival of existing MNs rather than MN replacement. MSCs and NSCs have been used to generate immunomodulatory cells, growth factor-releasing cells, functional support cells such as glia, or GABAergic interneurons to modify MN survival and activity. Several clinical studies (Choi et al., 2010; Connick et al., 2011; Karussis et al., 2010; Mazzini et al., 2012; Xiao et al., 2015) have examined the effect of transplanting MSCs to alter the inflammatory microenvironment in the spinal cord of ALS patients. Intravenous allo-transplantation of MSCs showed migration of the cells to the pathologic lesions. Intra-spinal injections of autologous MSCs reduced inflammation and MN loss and led to significant improvement in ALS patients. Although these studies show that ALS patients could benefit from MSC transplantation, continued fundamental research to understand the specific effects mediated by these transplanted cells would be necessary to improve therapeutic directions. This understanding would also facilitate optimizing human ESCs or patient specific iPSCs protocols for ALS treatment.

Transplantation of human VEGF-overexpressing NSCs conferred protection on MNs and delayed disease progression (Vande Velde and Cleveland, 2005). Based on these principles, NeuralStem Inc., a company based in the US has received FDA approval for ALS clinical trials by transplantation of human fetal-derived NSCs into the spinal cord (<https://clinicaltrials.gov/ct2/show/NCT01348451?term=neuralstem&rank=2>). At the present time, fetal NSC transplantation is considered safer than using of human ESC-derived NSCs due to risk of teratoma formation. NSCs differentiated from human iPSCs were administered by intravenous injections in the ALS mice which exhibited improved neuromuscular function and motor unit pathology and significantly increased life span (Boulis and Federici, 2011; Boulis et al., 2011; Glass, 2010; Glass et al., 2012; Lunn et al., 2011; Riley et al., 2012). The therapeutic effects of these cells were partially attributed to the production of neurotrophic factors (Krakora et al., 2013).

In addition, during early signs of ALS, counteracting MN loss by transplantation of supporting glial cell types to release neurotrophic molecules or by modifying the inflammatory milieu is a more realistic clinical goal for ALS treatment. Several lines of preclinical data have provided the rationale for this approach. For example, in a rodent model for ALS, glial restricted precursors (GRPs) extensively differentiated into mature astrocytes in grafts, and prevented MN loss and reduced microgliosis (Lepore et al., 2008; Papadeas et al., 2011).

A recent study showed that transplantation of human iPSC-derived glial-rich neural progenitor cells into ALS model mice extends their lifespans. The iPSC-derived glial-rich neural progenitor cells were transplanted them into the lumbar cord of the ALS model mice. The transplanted cells differentiated into astrocytes, and the survival time of mice with the

graft was extended compared to that of those without the graft. It was also suggested that the transplanted cells improve the spinal cord environment by increasing neurotrophic factors. Neurotrophic factor is important in the pathology of ALS, and it is being investigated as a target of treatment. Cells of the glial lineage have a function to prepare the environment around MNs. In the future, it would be interesting to test whether the transplantation of healthy iPSC-derived MNs or combination of MNs and glia might more efficiently recover the function. This iPSC-based approach would help to realize the promise of autologous cell-based stem cell therapy as well as avoid many ethical concerns associated with human ESCs, but many issues remain to be overcome, and a lot of time is necessary before reaching the stage of investigating the therapeutic effects in humans.

6. Summary

We reviewed the current progress of disease modeling of ALS. A reverse engineering approach of using patient specific iPSCs allows for modeling the cellular degenerative phenotypes ALS. The iPSC technology together with advanced genome editing also provides an excellent platform for detailed investigation of ALS pathogenesis, as well as drug screening and testing. Such a platform combined with animal studies will offer useful clues for preclinical studies and clinical trials for the treatment of ALS.

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Glossary

ALS	amyotrophic lateral sclerosis
C9ORF72	chromosome 9 open reading frame 72
ChIPSeq	chromatin immunoprecipitation with high throughput sequencing
CLIPSeq	cross-linking immunoprecipitation with high throughput sequencing
CNV	copy number variation
ESCs	embryonic stem cells
FTD	frontotemporal dementia
GRP	glial restricted precursors
PSCs	pluripotent stem cells (ESCs and iPSCs are collectively called PSCs)
MNs	motor neurons
MSCs	mesenchymal stem cells
NSCs	neural stem cells
RNAseq	High throughput RNA sequencing

SNP	single nucleotide polymorphism
SOD1	superoxide dismutase-1
VEGF	Vascular endothelial growth factor

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Highlights

- Patient-derived iPSCs can be used to investigate the key pathogenic processes of ALS, using the reprogramming technology to “de-differentiate” patient-specific skin fibroblasts back to stem cells, and then “re-differentiate” them into specific neural lineages to create appropriate in vitro models of “disease in a dish”.
- The ESC/iPSC-based platform can be used to determine the key pathogenic events in ALS disease progression and pathogenic development by a reverse engineering approach to identify key mechanisms in the neuronal versus glial causes of ALS.
- The iPSC-based technology platform of network pharmacology enables analysis of networks associated with the dysfunctional neural network underlying ALS and other CNS diseases.
- iPSCs provide promising cell source for cell therapy in ALS.

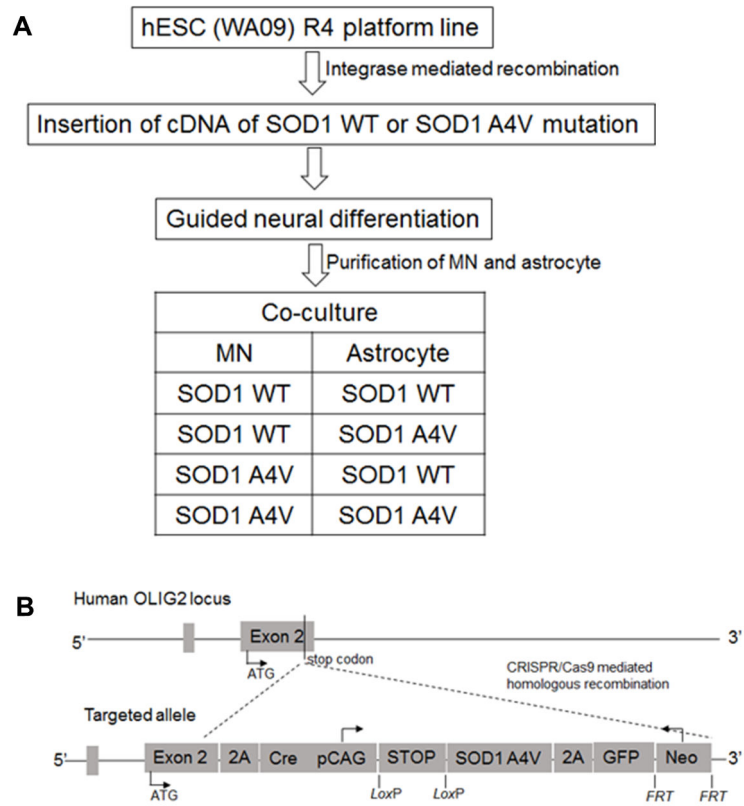


Figure 1.
An example for using human ESC and genetic engineering tools to study ALS. (A) Work flow. (B) Vector design for creating SOD1 A4V mutation in human ESC.

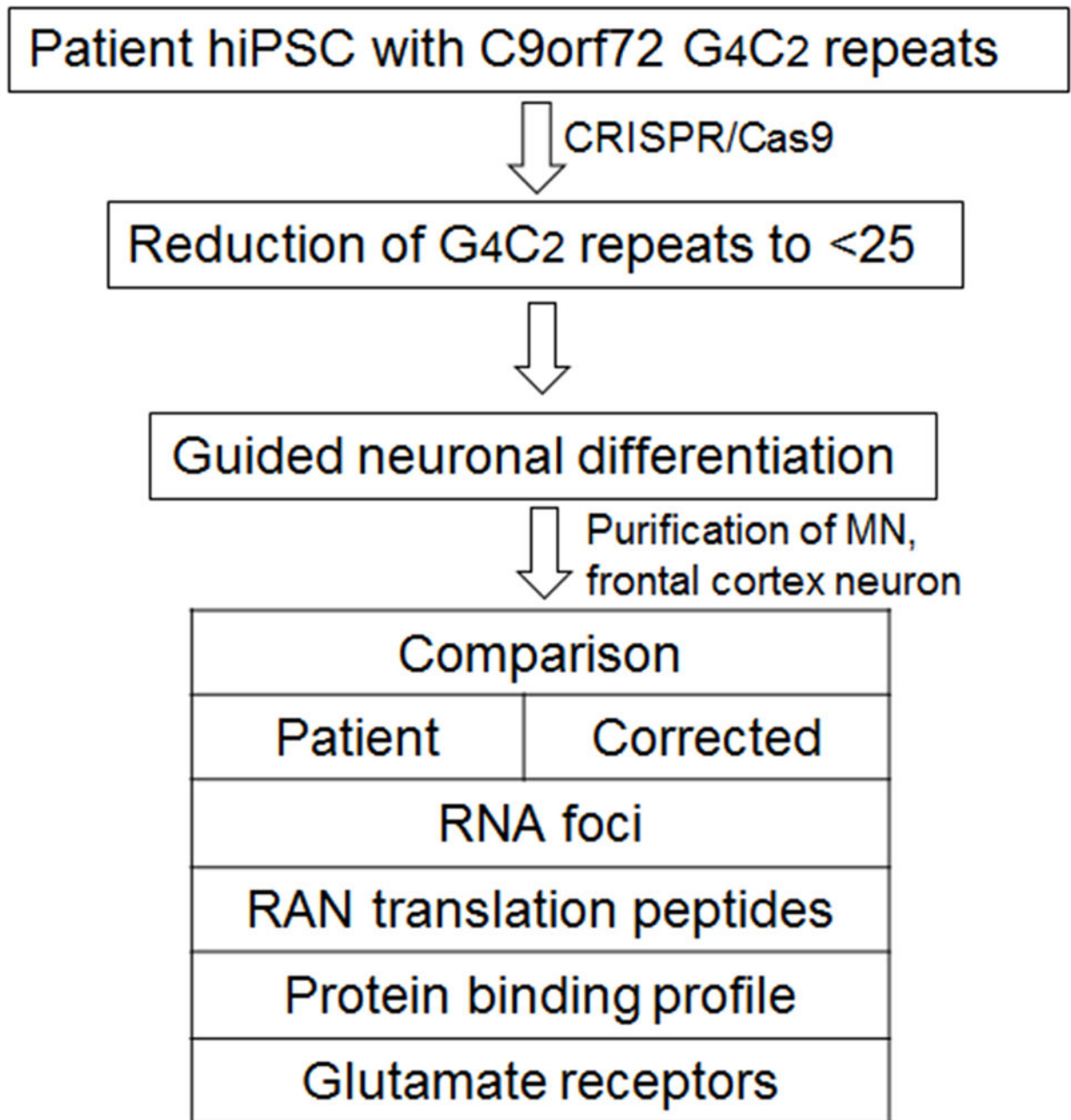


Figure 2. An example for using patient derived human iPSC and CRISPR/Cas9 system for genetic correction in ALS. RAN foci: repeat-associated non-ATG translation.