Human-induced pluripotent stem cells pave the road for a better understanding of motor neuron disease

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While motor neuron diseases are currently incurable, induced pluripotent stem cell research has uncovered some disease-relevant phenotypes. We will discuss strategies to model different aspects of motor neuron disease and the specific neurons involved in the disease. We will then describe recent progress to investigate common forms of motor neuron disease: amyotrophic lateral sclerosis, hereditary spastic paraplegia and spinal muscular atrophy.

INTRODUCTION

In spite of intensive research, motor neuron diseases (MNDs) are still incurable. Some of the key factors in this failure to find a cure have been the lack of human cell-based disease models for molecular analysis and drug screening and the difficulty of obtaining homogeneous populations of specific cell types for clinical applications. Stem cell biology has the potential to transform our understanding of disease processes and to revolutionize our approach to develop novel therapies for MNDs. Specifically, new avenues of translational research are expected to develop from reprogramming adult somatic cells from patients and reverting them to disease-specific neurons. In this review, we will discuss recent advances in induced pluripotent stem cell (iPSC) research to model and investigate the most common forms of human MND: amyotrophic lateral sclerosis (ALS), hereditary spastic paraplegia (HSP) and spinal muscular atrophy (SMA).

Developmentally informed modeling of upper and lower motor neurons using hiPSC technology

Reliable and efficient differentiation of stem cells into disease-specific neurons is the prerequisites for modeling MND. The motor system consists of corticospinal motor neurons residing in the motor cortex (CSMNs, or upper motor neurons) and alpha-motor neurons (AMNs, or lower motor neurons) in the spinal cord. The CSMNs are glutamatergic pyramidal neurons of cortical layer 5 that form the corticospinal or pyramidal tract and extend their axons through the internal capsule to the spinal cord. These CSMN axons refine their axonal projections in a topographically specific manner and connect with AMNs in the spinal cord (see Fig. 1).

Important work came from the labs of Macklis and Arlotta, who identified numerous genes required for the specification of CSMNs and other cortical projection neurons and characterized the molecular mechanisms that control CSMN development by in vivo purification of CSMNs at different stages of development (1–4). One very important gene they identified is the transcription factor Forebrain Embryonic Zinc Finger 2 (Fezf2), which, when overexpressed, is necessary and sufficient to generate corticofugal neurons from non-committed progenitors in vitro and in vivo (5,6). Mouse embryonic stem cells (ESCs) could be directed into pyramidal neurons that were able to generate projections toward correct subcortical targets in mouse brains when co-cultured with MS5 stromal cells and by adding cyclopamine, an inducer of dorsal identities (7). In contrast to these findings, a recent study has shown that both human ESCs and induced pluripotent stem cells (hiPSCs) can recapitulate corticogenesis and sequential generation of functional pyramidal neurons in the absence of morphogenes (8).

The specific generation of CSMNs from human stem cells is still in progress. Important work for modeling cortex development using human stem cells was performed by the Livesey group, who established a chemically defined, multistep process for human cortical development from human iPSCs (9,10). This directed differentiation took advantage of dual SMAD
inhibition [inhibition of the BMP signaling pathway by addition of secreted molecules such as Noggin to induce neuronal fate and the TGF-b inhibitor SB431542 to inhibit Smad 2 and 3 in the nodal pathway (11)] and retinoid signaling to recapitulate aspects of in vivo development and generate different classes of cortical projection neurons in a temporally determined order (9,10). It is interesting to note that many of these neurons express chicken ovalbumin upstream promoter transcription factor-interacting protein 2 (Ctip2), an indicator of pyramidal neurons.

The use of FezF2 to model and/or sort (FezF2-GFP construct) upper motor neurons has been reported in hESCs, where a specific hFEzf2-YPF hESC reporter line has been generated (12); optimizing the distinct and pure generation of CSMN-specific cells will be a crucial next step in developing specific CSMN modeling in human models.

By comparison, AMN protocols for human ESCs and iPSCs have been elaborated for many years and are frequently used for disease modeling (13,14). Basically, most protocols use an

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**Figure 1.** (A) Schematic diagram of the cortical motor system, the corticospinal tract (CST) generated by CSMN, descending from the motor cortex, through the internal capsule. Most CST fibers (more than 80% in humans) decussate in the caudal medulla, before descending further through the spinal cord and connecting at the respective height to the AMN. The AMN then projects toward the muscles to form neuromuscular junctions. (B) Diffusion tensor imaging of the human CST indicating fibers from the motor cortex that descend through the internal capsule (courtesy of Prof. Dörfler, Dr. Engelhorn, University Hospital Erlangen).
embryoid body (EB) step by adding retinoic acid and sonic hedgehog. The different protocols vary in the length of EB cultures before dissociating them into adherent cultures. A very useful step within these protocols is the use of the homeobox gene Hb9::GFP reporter constructs to specify the AMNs (15).

MNDs (HSP, ALS, SMA)

MNDs are caused by selective degeneration of CSMN and/or AMN. Specifically, HSP is characterized by the specific degeneration in the CSMNs, whereas ALS involves CSMNs and AMNs and SMA causes selective degeneration of AMNs.

Degeneration of CSMNs causes paresis and spasticity; loss of AMNs causes amyotrophy and muscle wasting. However, disease onset and neurological phenotype vary a great deal between these diseases for yet unknown reasons.

HSP, also termed Strumpell-Lorrain disease, has a prevalence comparable with ALS, with 7 of 100,000 affected individuals in certain populations (16), and it represents the group of MNDs with the largest number of genetic mutations causative for HSP. Over the past 10 years, >71 spastic paraplegia (SPG) loci have been mapped and to date >40 genes have been identified (17). The identification of these genes has led to insights into genes that share common mechanisms. Dysfunction of axonal transport and membrane trafficking are among the most common speculated disease mechanisms. Clinically, all HSPs are characterized by spastic paraparesis, and in pure forms of HSP, other neural systems are classically spared from the disease. However, complicated HSPs are characterized by additional neurologic symptoms. So far, HSP research has focused on defining a large number of different genes involved in the disease but has suffered from the availability of valid disease models to mimic a spastic paraparesis of the lower limbs only.

The prevalence of ALS, also known as Lou Gehrig’s disease, is 4–6 of 100,000, and the disease is well known because it can be fatal within 5 years of diagnosis owing to severe muscle wasting and fasciculations, spasticity, dysarthria, dysphagia and dyspnea. An increasing number of loci, named ALS1-20, and 16 different genes associated with ALS have been identified within the last few years, shifting attention from the intensely studied mutations in superoxide dismutase 1 (SOD1, ALS1) to more frequently appearing mutations in other genes, including C9orf72, TARDBP (TDP-43) and FUS (18).

While familial forms of ALS were previously considered to occur infrequently (~2% of all cases), C9orf72 currently accounts for ~6% of all cases of ALS in Europeans (19). The low frequency of familial cases, in combination with differences in circuit complexity between species, diminishes the effectiveness of traditional transgenic mouse models of ALS.

 SMA is an autosomal recessive genetic disease caused by mutations in the survival motor neuron (SMN) 1 and 2 gene, resulting in a selective degeneration of AMNs owing to the reduced protein expression of SMN protein. The clinical symptoms are severe muscle wasting. SMN1 is crucial for survival of motor neurons, and the childhood onset of SMN1 results in a severe phenotype with limited life expectancy. Patients with mutations in the SMN2 gene, a nearly identical copy of SMN1, present with a less severe, adult-onset phenotype. SMN2 differs from SMN1 by a few nucleotides that lead to the skipping of exon 7 in SMN2 and a less stable, self-oligomerizing protein; only 10–20% of the transcripts produce a fully functional form of the protein [reviewed in (20)].

Spinal and bulbar muscular atrophy (SBMA, Kennedy’s disease) is an X-linked recessive inherited disease of motor nuclei of the cranial nerves and lower spinal motor neurons caused by a CAG/polyglutamine (polyQ) repeat expansion in the androgen receptor (AR) gene. SBMA is characterized by progressive bulbar and proximal limb weakness and atrophy, as well as signs of mild androgen insensitivity, such as gynecomastia, testicular atrophy and reduced fertility (21,22). Even though Drosophila and mouse AR overexpression models indicate that translocation of mutant AR to the nucleus in response to ligand binding is critical to pathogenesis, there is no direct biochemical evidence demonstrating mutant AR translocation in living human neurons derived from SBMA patients (23–26).

MODELING DIFFERENT MNDs USING iPSC TECHNOLOGY (ALSO REVIEWED IN TABLE 1)

HSP

All disease modeling using hiPSCs or other human cellular systems for HSP has focused on the most frequent cause of HSP, which are mutations in SPG4 encoding Spastin. These mutations account for ~40% of autosomal-dominant HSP cases. Many of the >60 different mutations are expected to cause loss of function owing to haploinsufficiency (27). Spastin is a member of the ATPase associated with diverse cellular activities (AAA) family of proteins. Spastin has at least four known isoforms, and their main function is to sever microtubules. The CNS-specific isoforms are involved in the endocytic pathway (Table 1).

Recently, we reported the generation of hiPSC lines from two patients of non-related families with an identical heterozygous nonsense mutation (p.R562X) that generated neurons with comparable efficiency to controls. The neurite complexity of the SPG4 neurons was severely impaired, because the number of primary neurites and their branching and length were significantly reduced. Moreover, these neurites displayed abundant neurite swellings, with loosely arranged, interrupted microtubules and an imbalance of axonal transport, with an increase in retrograde transport for mitochondria (28). Neurite swellings and altered axonal transport were also reported from neurons derived from one patient with a c683-1G > T mutation (29) and attributed to a loss of function of Spastin, suggesting there are common phenotypes in SPG4-related HSP. An important finding of our study was that elevation of Spastin levels by lentiviral expression of Spastin at low levels led to restoration of neurite complexity and reduction of neurite swellings in SPG4 neurons, whereas higher levels of Spastin expression were toxic to neurons (28). This implies that the generation of therapeutic strategies targeting an increase in Spastin expression levels to restore physiological Spastin levels will have a dose-dependent effect. It is interesting to note that, in this heterozygous human model, the neuritic disease phenotypes were more pronounced than in homozygous knockout mouse models (39,40).

Further, the levels of Spastin expression and its M1 and M87 isoforms were significantly decreased in SPG4 neurons. Interestingly, there was also a decrease in Spastin expression in the fibroblasts of these patients, indicating a potential role for patient-derived fibroblasts as a pharmacological screening tool in the...
### Table 1. Motor neuron disease modeling using iPSC

<table>
<thead>
<tr>
<th>Disease</th>
<th>Gene mutation or sporadic</th>
<th>Number of patients/controls</th>
<th>Type of neuron investigated</th>
<th>Neuronal phenotype observed in vitro</th>
<th>Correction in vitro</th>
</tr>
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<tbody>
<tr>
<td>References</td>
<td></td>
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</tr>
<tr>
<td>HSP</td>
<td>SPG4, Spastin</td>
<td>2/2 1/1</td>
<td>Forebrain neuron</td>
<td>Neurite swellings, altered axonal transport</td>
<td>Overexpression of Spastin rescues neurite length and reduces swellings (28,29)</td>
</tr>
<tr>
<td>ALS</td>
<td>ALS1 (SOD1) &lt;br&gt;ALS10 (TDP43) M337V</td>
<td>2/0 1/1</td>
<td>AMN</td>
<td>N/A</td>
<td>N/A (30)</td>
</tr>
<tr>
<td></td>
<td>ALS10 (TDP43) M337V</td>
<td>2/2</td>
<td>AMN and astrocytes</td>
<td>Glial cell autonomous pathological phenotype</td>
<td>N/A (31)</td>
</tr>
<tr>
<td></td>
<td>ALS10 (TDP43) Q343R, M337V G298S</td>
<td>3/6</td>
<td>AMN</td>
<td>Increased TDP-43 levels</td>
<td>Histone acetyltransferase inhibitor anacardic acid rescued Hb9::GFP-positive cells (32)</td>
</tr>
<tr>
<td>ALS/ALS with FTLD</td>
<td>ALS8 (VABP) C9ORF72 repeat</td>
<td>4/3 4/4</td>
<td>AMN AMN and CSMN/AMN</td>
<td>Reduced VAPB protein levels Toxic gain of function by C9ORF72 with potentially altered RNA metabolism</td>
<td>N/A Antisense oligonucleotide targeting C9ORF72 reversed gene expression alterations (34) (35)</td>
</tr>
<tr>
<td>ALS</td>
<td>ALS10 (TDP43) familial and sporadic</td>
<td>8 familial 16 sporadic 10 controls</td>
<td>AMN and CSMN</td>
<td>TDP-43 aggregation in some sporadic cases recapitulating postmortem pathology</td>
<td>Digoxin rescue (36)</td>
</tr>
<tr>
<td>SMA</td>
<td>SMN1</td>
<td></td>
<td>AMN</td>
<td>Lack of nuclear gems, motor neuron degeneration and abnormal neurite outgrowth</td>
<td>N/A (37,38)</td>
</tr>
<tr>
<td>SBMA</td>
<td>(polyQ) repeat expansion in the AR gene</td>
<td></td>
<td>AMN</td>
<td>Neuronal-specific upregulation and aggregation of AR in the presence of the AR ligand</td>
<td>Decreased levels of neuronal-specific aggregated AR after HSP90 inhibitor (26)</td>
</tr>
</tbody>
</table>
ALS

One of the first reports about modeling ALS in vitro from hiPSC-derived neurons involved two patients with the SOD1 mutation L144F [one of them was 82 years old (30)]. While these neurons did not have an obvious phenotype, subsequent studies demonstrated the efficiency of generating functional neurons in a larger number of hiPSC lines (42). These findings highlight the importance of not only studying the impact on neurons but also investigating the non-cell autonomous effects of certain mutations. In this regard, studies by Marchetto and DiGiorgio showed the importance of astrocytes for demonstrating disease-relevant phenotypes in hESC-derived models (13,14). In line with this, a recent study demonstrated that astrocytes, generated by direct conversion from one patient with an SOD1 mutation and three patients with the C9orf72 expansion repeat and three sporadic ALS patients, display toxicity toward AMNs (43). In the future, the impact of other cells present in the motor neuron niche, especially muscle and specifically the neuromuscular junctions and oligodendrocytes, will also be appreciated.

While a disease modeling study in ALS8 was able to show the expected decrease in protein expression of vamp-associated protein B/C (34), more recent studies have focused on modeling the more common genetic forms of ALS as well as sporadic ALS.

A cellular model of iPSCs derived from ALS patients carrying the C9orf72 repeat expansion demonstrated that transcription of the repeat was increased, leading to accumulation of GGGGCC repeat-containing RNA foci in C9orf72 hiPSC-derived motor neurons. These motor neurons showed altered expression of genes involved in membrane excitability, including DPP6, and demonstrated a diminished capacity to fire continuous spikes upon depolarization compared with control motor neurons. Anti-sense oligonucleotides targeting the C9orf72 transcript suppressed RNA foci formation and reversed gene expression alterations in C9orf72 motor neurons (35).

Recent advances in using iPSC-derived neuronal models from patients with TDP-43 mutations include (1) increased susceptibility to cellular stressors such as arsenite or LY295002, a selective inhibitor of PI3K (31), (2) deficits in RNA metabolism and cytoskeletal protein expression, resulting in motor neuron deficiency that can be rescued with histone acetyltransferase inhibitor (anacardic acid) (33) and (3) a role for mutant astrocytes in disease pathology in M337V mutations, indicative of glial cell homeostasis (43). Another promising approach for restoration of an MND phenotype that has been recently used is gene targeting. Specifically, Corti et al. used a targeted gene correction approach based on single-stranded oligonucleotides to convert the endogenous SMN2 gene into an SMN1-like gene in hiPSCs from patients with SMN1 mutations (44). The resulting cells gave rise to neurons that extended life span and ameliorated the disease phenotype in mouse models after transplantation. Similar to the gene dosage-dependent rescue in SPG4 hiPSC-derived neurons by Havlicek et al., these results suggest that genetic correction may be a promising approach for in vitro mechanistic studies and/or potentially in vivo gene/cellular therapy.

Polq diseases (SBMA)

The defining pathology of SBMA is intranuclear AR protein inclusions in the affected neuronal population, although misfolding and altered degradation of the mutant AR may also be involved in the pathogenesis. After deriving hiPSCs from a SBMA patient, Nihei et al. confirmed neuron-specific upregulation and aggregation of AR in the presence of the AR ligand dihydrotestosterone (26). In addition to confirming the biochemical phenotype, the group reported decreased levels of aggregated AR after HSP90 inhibitor treatment (17-allylamino-17α-testolactam). Their results demonstrate potential windows for drug discovery for PolyQ diseases.

Common characteristics and future perspectives

The power of human disease iPSC models for MND is their ability to recapitulate important aspects of the disease-relevant phenotype in a dish (see Fig. 2). One common theme for future studies will be to understand why these neurons are more vulnerable to traffic disruption within neurites than other cells. Another important aspect will be the significance of non-neuronal cells surrounding AMNs and CSMNs and their contribution to disease progression.

The crucial next step for profiling from hiPSC modeling for MND will be to exploit this technology for drug discovery. The advantage of stem cell technology in addition to the fact the cells are of human origin and derived from clinically and/or genetically well-defined patients is the ability to generate different cell types in the body, making possible additional studies of predictive safety and toxicology to avoid adverse effects for, i.e. the liver or heart. Furthermore, hiPSC-derived neuronal models offer the possibility of a targeted approach, as currently performed for testing kinase activity in LRRK2 mutations (46). Alternatively, phenotypic approaches investigating or reversing physiological changes can be performed [reviewed in (47)].
An important next step for the successful transition to the discovery of new drugs for clinical assessment will be the selection of relevant study cohorts. Therefore, the successful implementation of the new knowledge derived from stem cell technology will also depend on closely following up the patient cohorts used to generate hiPSC-derived disease models. As seen in complex neurodegenerative disorders such as Alzheimer’s and Parkinson’s disease, the ideal cohorts for testing novel compounds derived from hiPSC findings must be based on well-defined clinical and genetic characteristics. Despite all medico-economic pressure, a good starting point for clinical trials, therefore, may be monogenic forms of MNDs before immediately translating it to the much more frequent and diverse sporadic MNDs.

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