

Dysfunction of autophagy as the pathological mechanism of motor neuron disease based on a patient-specific disease model

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Autophagy is the main catabolic pathway in cells for the degradation of impaired proteins and organelles. Accumulating evidence supports the hypothesis that dysfunction of autophagy, leading to an imbalance of proteostasis and the accumulation of toxic proteins in neurons, is a central player in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS). The clinical pathology of ALS is complex and many genes associated with autophagy and RNA processing are mutated in patients with the familial form. But a causal relationship between autophagic dysfunction and ALS has not been fully established. More importantly, studies on the pathological mechanism of ALS are mainly based on animal models that may not precisely recapitulate the disease itself in human beings. The development of human iPSC techniques allows us to address these issues directly in human cell models that may profoundly influence drug discovery for ALS.

Keywords: motor neuron disease; iPSC; autophagy; amyotrophic lateral sclerosis; spinal muscular atrophy

General Introduction to Motor Neuron Diseases

Motor neuron (MN) diseases are a heterogeneous group of sporadic or familial disorders of the nervous system that mostly lead to a progressive loss of MNs and the subsequent impairment of neuromuscular function, such as spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis (ALS). To date, there is no effective treatment for these diseases^[1]. SMA is an autosomal recessive disease caused by loss or mutation of the SMN1 gene and retention of the SMN2 gene, which results in reduced levels of survival motor neuron (SMN) protein. With a casualty rate of 0.01%, it is the number one inherited killer of children below the age of two^[2]. Currently, it is still largely unclear why low levels of SMN protein result specifically in an MN disease, although it has been proposed that loss of SMN may result in alteration of the splicing of a specific gene or

the disruption of mRNA transport, which eventually leads to SMA^[2, 3]. ALS is another devastating neurodegenerative disorder affecting upper and lower MNs that leads to death within 2–3 years from diagnosis. Between ~90% and 95% of cases are sporadic in origin, whereas the remaining 5%–10% are familial and usually of autosomal dominant inheritance. Of these, the first ALS gene, superoxide dismutase 1 enzyme (SOD1), accounts for ~20%. TAR DNA-binding protein (TDP-43/TADBP) has been identified as a major component of ubiquitinated inclusions in ALS and other neurodegenerative diseases. Hexanucleotide GGGGCC intronic expansions in the newly-identified C9ORF72 gene (chromosome 9 open reading frame 72) are the most common cause of both familial and sporadic ALS, and are responsible for up to 50% of the familial and ~10% of the sporadic form^[4]. Other genes, such as FUS/TLS, angiogenin, SQSTM1, valosin-containing protein

(VCP), UBQLN2, optineurin (OPTN)^[6], and very recently Matrin3, have also been found to be linked to familial ALS^[6, 7]. The pathophysiology of ALS is complex and various mechanisms have been proposed for MN injury including protein misfolding and aggregation, mitochondrial dysfunction, oxidative stress, defective axonal transport, excitotoxicity, defects in RNA processing and protein degradation by autophagy, dysregulated transcription and RNA processing, endoplasmic reticulum stress, and apoptosis, as well as toxicity caused by non-neuronal cells^[8]. Of these mechanisms, autophagic dysfunction is considered to play a key role^[6, 8, 9].

Defective Autophagy May Be a Central Player in MN Diseases and Other Neurodegenerative Diseases

Autophagy, an intracellular degradation pathway for the clearance of damaged organelles and aggregation-prone proteins by lysosomes, is essential for the maintenance of protein homeostasis^[9, 10]. Generally, there are three distinctive types: macroautophagy, microautophagy, and chaperone-mediated autophagy. Of these, macroautophagy (here referred to as autophagy) is the main catabolic pathway for maintaining protein homeostasis and sustaining neuronal functions^[11, 12]. Recent studies have revealed that autophagy is involved in many essential biological functions including cell survival, cell death, cellular metabolism, development, aging, antigen-presentation, and anti-infection^[13]. Autophagic degradation can be divided into several steps: induction, autophagosome formation, cargo recognition/sequestration, and autophagosome clearance. Failure at each of these steps can lead to neurodegenerative diseases^[12, 14]. The common pathological hallmark of several major neurodegenerative diseases is the accumulation of misfolded proteins in both sporadic and genetic cases. In Alzheimer's disease (AD), the misfolded protein inclusions are mainly plaques of amyloid β peptide and intracellular neurofibrillary tangles comprising hyperphosphorylated tau. Parkinson's disease (PD) is characterized by the presence of Lewy bodies composed of aggregated α -synuclein and polyubiquitinated proteins. In Huntington's disease (HD), mutant huntingtin forms protein aggregates in the cytoplasm^[15]. Similarly, in ALS, TDP43 aggregates have

been identified in almost all sporadic and genetic cases^[9, 10, 15-20]. Although it is still not conclusive that these cytoplasmic inclusions themselves are toxic or merely serve to precipitate toxic cellular components and minimize their detrimental consequences, accumulating evidence reveals that the perturbation of autophagy primarily contributes to their existence and the pathogenesis of these diseases^[21].

The very first link between the deregulation of autophagy and neurodegeneration came from studies showing that genetic inactivation of autophagy-related protein 5 (Atg5) or Atg7, which are key regulators of autophagosome formation, leads to massive neuronal death and eventually animal lethality accompanied by evident protein aggregation^[22, 23]. Recent studies have established a tight connection between the pathogenesis of AD and the deregulation of autophagy. For example, Beclin1-deficiency can cause the aggregation of $A\beta$ and amyloid precursor protein and subsequent neuronal loss attributable to autophagic dysfunction^[19]. Under normal conditions, $A\beta$ stimulates autophagy and facilitates its own degradation, which is important for maintaining a relatively low level of $A\beta$. Under pathological conditions, the cellular $A\beta$ aggregates can block autophagosome clearance and lysosomal degradation, which results in exacerbation of the pathogenetic condition of AD^[24]. Presenilin 1 (PS-1) is known to be an essential regulator for maintaining the acidic environment in lysosomes. PS-1 mutation interferes with the acidification of lysosomes and leads to impaired cargo clearance by autophagolysosomes. Similarly, many PD-associated genes, such as PINK1, Parkin, and LRRK2, have also been found to interact with Beclin1, and their mutant forms can alter the induction of PD and may contribute to its progress^[25-27]. As a matter of fact, treatment with rapamycin, which enhances autophagic activity, can improve the symptoms of several neurodegenerative diseases including AD, PD, and HD^[20, 28].

With regard to the link between proteinopathy and the development of MN diseases, autophagic dysfunction is considered to be one of the major factors in MN degeneration. Typical pathological features of ALS include protein inclusions enriched in ubiquitin, TDP-43, FUS, and SOD1. Especially, TDP-43-positive inclusions have been shown to be common to 97% of ALS cases, both sporadic and familial^[7]. Growing evidence suggests that many ALS-

linked genetic mutations actually affect autophagy receptor proteins (ubiquilin-2, OPTN, SQSTM1/p62) and regulators (VCP) and eventually result in alteration of the autophagic process^[29]. Such mutations may impair cargo recognition and the clearance of autophagy substrates, with severe consequences. TDP-43 proteostasis is normally maintained by the coordinated action of the ubiquitin–proteasome system and autophagy, which is particularly important for clearing TDP-43 oligomers and aggregates. The linkage of SQSTM1, VCP, UBQLN2, and OPTN with ALS suggests the impaired turnover of TDP-43 by autophagy may have profound impact on the pathogenesis of ALS^[6, 7]. Similarly, deposition of TDP-43 is also the major feature of tau-negative frontotemporal dementia. In addition, the most common ALS-linked mutation is an intronic GGGGCC repeat expansion in C9ORF72, the pathology of which is also characterized by classical TDP-43 inclusions in the motor cortex and spinal cord, which are decorated by ubiquitin, p62, and/ or ubiquilin 2^[16, 30]. Currently, it is not clear whether mutant TDP43 and C9ORF72 affect MN degeneration through similar pathways by modulating autophagy. Another common form of familial ALS is correlated with mutant SOD1. It has been demonstrated that mSOD1 is capable of unmasking the inhibition of Beclin1 by BCL-XL through association with this complex, further suggesting that perturbation of autophagy induction profoundly affects the fate of MNs in ALS^[31]. However, in contrast to other neurodegenerative diseases, the pathological mechanism of MN diseases seems to be more complex given the facts that rapamycin treatment causes diverse responses in various ALS models with detrimental consequences in mSOD1 VCP transgenic mice but beneficial results for mutant TDP-43 mice^[16, 32, 33]. Nassif and colleagues also reported that autophagic activity compromised by reducing the level of Beclin1 in the mSOD1 ALS model is potentially protective for the progress of ALS^[31]. Coincidentally, in a recent study, upregulation of autophagy and the accumulation of autophagosomes have been thought to be pathogenic in a cellular model of SMA^[18]. It is conceivable that in some forms of MN disease autophagy has become malfunctioning, and a further boost of autophagy under this circumstance can only lead to overproduction of defective autophagosomes and exhaust the capacity to maintain protein homeostasis, which may

have detrimental consequences and result in eventual cell loss^[34]. Therefore, understanding the exact nature of autophagic dysfunction in different forms of human ALS as well as SMA is critical for finding proper therapeutic approaches.

Potential Link between Autophagy and the Pathogenesis of MN Diseases and Other Neurodegenerative Diseases Based on Patient-Specific iPSC Models

A major challenge in studying the molecular pathological mechanisms underlying MN diseases is the limited access to disease-affected human tissue from the central nervous system. Over the years, several cell and transgenic animal models of ALS/SMA have been developed in yeast, zebrafish, *Drosophila*, mice, and rats. Although these models have made contributions of inestimable importance for molecular studies and drug testing, or for gene therapy, in terms of genetic background they often have limitations in replicating human ALS/SMA-like phenotypes. It has been reported that results obtained in transgenic animals cannot always be directly transferred to humans^[35].

Human induced pluripotent stem cells (iPSCs) are a source of great hope, because they provide access to virtually unlimited numbers of patient-specific cells for modeling MN diseases *in vitro*. Several groups have generated iPSCs from patients with various MN disease-specific mutations. Importantly, the pathology of ALS/SMA has been partially recapitulated in MNs derived from patient-specific iPSCs. Dimos *et al.* obtained fibroblast lines from two elderly siblings with early and late manifestations of ALS, including weakness of the arms and legs, caused by a disease-associated mutation in the SOD1 gene^[36]. These cells were then used to generate iPSCs after infection with retroviruses containing human OCT3/4, SOX2, C-MYC, and KLF4. The resulting iPSC lines appeared to be pluripotent *in vitro*, as they spontaneously differentiated into representative phenotypes of the three embryonic germ-cell layers. Furthermore, they were driven to a spinal MN-like phenotype and expressed key MN markers, including HB9 and ISLET1/2. These first showed the possibility of modeling disease using iPSC techniques, although they were not functionally characterized for basic

neuronal properties and disease phenotypes^[36].

Egawa *et al.* obtained MNs carrying disease-causing mutations in the gene encoding TDP-43^[37]. The ALS MNs in culture recapitulated cellular and molecular abnormalities associated with ALS. Of particular interest, cytosolic aggregates were found in ALS patient-specific iPSC-derived MNs similar to the postmortem tissue from ALS patients, suggesting that deregulation of autophagy plays a role in its pathogenesis. Mutant TDP-43 in the ALS MNs was bound to the spliceosomal factor SNRPB2 and formed aggregates in the nucleus, resulting in perturbed RNA metabolism. It is likely that disturbances in autophagy and RNA processes are intrinsically connected and jointly lead to the disease phenotypes. It will be very interesting to explore whether the dynamics of stress granule formation is altered in this cellular disease model. Consequently, the ALS iPSC-derived MNs were more vulnerable to cellular stressors such as arsenite. The researchers then used the ALS MNs in a drug-screening assay and identified a compound called anacardic acid, a histone acetyltransferase inhibitor that reversed some of the ALS phenotypes in MNs. The new work provides an encouraging step toward using MNs generated from iPSCs derived from ALS patients to learn more about what triggers the death of MNs in this disease and to identify new candidate drugs that may be able to slow or reverse the devastating loss of MNs^[37].

In addition, very recent studies with ALS C9ORF72 iPSC lines provide compelling evidence that the human cellular model can have utility that exceeds prior disease models^[38]. In their new study, Sareen *et al.* reported a cellular model of C9ORF72-ALS with MNs differentiated from iPSCs derived from ALS patients carrying the C9ORF72 repeat expansion. Transcription of the repeat was increased, leading to the accumulation of GGGGCC repeat-containing RNA foci selectively in C9ORF-ALS iPSC-derived MNs^[38]. Repeat-containing RNA foci co-localized with hnRNPA1 and Pur-a, suggesting that they may be able to alter RNA metabolism. Consistently, other groups have also reported that C9ORF72 interacts with both hnRNPA1 and hnRNA2B1 and may regulate the formation of stress granules, which in turn profoundly influence RNA processing^[17, 39]. C9ORF72-ALS MNs show altered expression of genes involved in membrane excitability including dipeptidyl-peptidase 6, and demonstrate a

diminished capacity to fire continuous spike trains upon depolarization compared to control MNs. Antisense oligonucleotides targeting the C9ORF72 transcript suppress the formation of RNA foci and reverse the gene expression alterations in C9-ALS MNs. These data show that patient-derived MNs can be used to delineate pathogenic events in ALS. Donnelly *et al.* demonstrated that fibroblasts and iPSCs from patients contain intranuclear GGGGCC RNA foci similar to those found *in vivo*, and which are toxic due to the sequestration of RNA-binding proteins such as ADARB2^[34]. Simultaneously, cytoplasmic GGGGCC foci have been found in C9ORF72-ALS MNs and in C9ORF72 ALS postmortem motor cortex, suggesting that the deregulation of autophagy is likely to be the next important issue to be addressed regarding the pathogenesis of the disease.

The study by Ebert *et al.* took a similar approach to address the major spinal MN degenerative disorder SMA, typically caused by a genetic mutation in the SMN1 gene^[40, 41]. The iPSCs obtained could in turn differentiate into a variety of cell types, including cells with a spinal MN phenotype that harbor the SMN1 mutation. Ebert *et al.* reported a specific reduction in the accumulation and size of these cells in patient cultures *versus* normal controls. In addition, the patient iPSCs displayed a predicted deficiency in nuclear SMN protein aggregates. Treatment of the cells with inducers of SMN protein expression—valproic acid or tobramycin—suppressed this phenotype. It will be of interest to determine whether patient iPSC-derived MN reduction can be similarly suppressed by these compounds or by novel SMN inducers.

In parallel, iPSC techniques have been implemented to generate human AD models for mechanistic studies. Yagi *et al.* recently discovered that the iPSCs derived from fibroblasts from AD patients carrying mutations in PS1 and PS2 can differentiate into neurons that show increased A β 42 secretion compared to healthy controls^[42]. Similarly, neurons derived from iPSCs carrying a duplication of the amyloid precursor protein express higher amounts of both A β 40 and A β 42 proteins, and form insoluble intracellular and extracellular amyloid aggregates with augmented phosphorylated tau protein, recapitulating the progress of AD^[43]. However, it remains to be determined in these patient-specific AD models whether autophagy is defective

and correlated to the pathophysiology. More importantly, it would be of great value to test the efficacy of the compounds such as rapamycin that have been shown to be beneficial for treating the disease through the experiments mainly carried out in animal models.

With regard to PD, iPSC-derived dopaminergic neurons from patients carrying an LRRK2 mutation have increased expression of oxidative stress response genes and α -synuclein. The PD iPSC-derived neurons are also more susceptible to cell death when exposed to hydrogen peroxide, MG-132, and 6-hydroxydopamine than healthy controls^[44], mimicking the pathology of PD, so they can be potentially used as an alternative model to explore the molecular mechanisms of neurodegeneration. Moreover, neurons derived from PD iPSCs carrying LRRK2, G2010S, and PINK1 mutations have been shown to exhibit higher mitochondrial superoxide formation and mitochondrial DNA lesions that may explain their susceptibility to cell death in responses to oxidative stress^[45]. Increased expression of α -synuclein protein and augmented sensitivity to oxidative stress have also been reported in the dopaminergic neurons derived from iPSCs bearing a triplication of SNCA genes^[46]. Most importantly, dopaminergic neurons derived from both sporadic-PD and LRRK2-PD iPSCs manifest impaired maturation of autophagosomes and defective autophagosome clearance compared to healthy controls, directly linking the pathophysiology of PD to autophagic dysfunction^[47].

Conclusions

Taken together, recent advances in iPSC techniques provide a good opportunity for researchers to directly explore the pathological mechanisms underlying neurodegenerative diseases, especially MN diseases, in human cell-based models with almost unlimited sources. However, several major obstacles remain to be overcome for better understanding the kind of role autophagic dysfunction plays in the clinical pathology of these diseases. First, efforts need to be made to optimize the methods to obtain purer populations of MNs either by increasing the differentiation efficiency or through a better sorting strategy so that detailed molecular studies can be implemented using newly-developed methods such as genome-wide sequencing techniques. Second,

a large consortium of patient-specific iPSC lines carrying all kinds of mutated genes should be generated, allowing comprehensive examination of the pathogenesis of each individual gene. As noted above, despite direct links between autophagic malfunction and pathophysiology in the PD-iPSC model^[47], a clear correlation between a disturbance of autophagy and the pathogenesis of MN diseases has not been established, not to mention exploration of the molecular mechanisms in great detail. This is a major challenge in the field of MN diseases, given the complexity of mutant genes and the diversity of the potential mechanisms as well as the potential involvement of multiple steps of autophagy. In spite of these difficulties, the preliminary studies based on iPSC disease models in the past few years have already shed light on the potential involvement of proteostasis and RNA metabolism as central players in the pathogenesis of MN diseases. Elucidation of the disease mechanism based on these human disease models will strengthen our understanding of the roots of the diseases, how they progress, and eventually provide an ideal system to evaluate drugs that may provide new therapeutic solutions.

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