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Review

Mislocalization, aggregation formation and defect in proteolysis in ALS

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Abstract: Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by motoneuron degeneration. The features observed in ALS are the mislocalization and aggregation of proteins in the affected motoneurons. The mutants of Cu/Zn superoxide dismutase (SOD1) or TAR DNA binding protein (TDP-43) that cause ALS have been reported to localize aberrantly. These aggregations contain the products of ALS causative genes, including SOD1 or TDP-43. Notably, TDP-43-positive aggregations have been identified even in sporadic ALS cases, indicating the importance of aggregate formation in the pathogenesis of ALS. Various proteins other than ALS causative gene products are also included in these aggregates. It is thought that the genetic mutation-induced conformational changes of proteins cause the aberrant redistribution and formation of aggregates, resulting in a loss of function or a gain of neuronal toxicity through the undesired interactions. Additionally, *valosin-containing protein (VCP)*, u*biquilin2 (UBQLN2)* and *optineurin (OPTN)*, which are related to the proteolysis system, have also been identified as causative genes in ALS. These facts suggest that the aberrant protein homeostasis mediated by mislocalization, aggregate formation, or defects in the proteolysis system are the underlying causes of neuronal toxicity in ALS. Here, we focus on the impaired protein homeostasis observed in ALS to discuss the potential for motoneuron toxicity.

Keywords: Amyotrophic lateral sclerosis (ALS); Cu/Zn superoxide dismutase (SOD1); TAR DNA binding protein (TDP-43); Fused in sarcoma (FUS); Chromosome 9 open reading frame 72 (*C9ORF72*); mislocalization; aggregation; proteolysis; endoplasmic reticulum (ER)

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by selective motoneuron cell death. Quadriplegia and respiratory disorders can occur during the disease progression, leading to death usually within 3–5 years after the onset of the disease. ALS is classified as either familial or sporadic, with familial ALS accounting for approximately 10% of all ALS cases. In 1993, *Cu/Zn superoxide dismutase (SOD1)* was first identified as a causal gene of familial ALS [1]. Since the discovery of *TAR DNA binding protein (TARDBP)* in 2008, which encodes TDP-43, another ALS causative gene [2], approximately 20 additional ALS causative genes have been reported due to the development of genetic techniques, such as the next generation sequencer. Notably, the distinguishing GC repeat expansion in the intronic region of the *Chromosome 9 open reading frame 72 (C9ORF72)* occurs in approximately 40% of all familial ALS cases [3-5]. In spite of these remarkable advances in inherited causal genes, the pathogenic mechanisms of familial and sporadic ALS still remain unclear. Therefore, there is a need to reveal the common motoneuron toxicity in ALS.

One of the hallmarks of ALS is the dysregulation of protein localization. The formation of aggregates was predicated to be involved in the pathogenesis of ALS. Several causative gene mutations, including *SOD1*, *TARDBP*, and *fused in sarcoma (FUS)*, have been reported to be mislocalized or form abnormal protein aggregates [1,6-8]. The mislocalization and aggregation of TDP-43 and FUS are observed even in sporadic ALS cases, suggesting that a defect in protein homeostasis also occurs in sporadic ALS cases. It is well known that these aggregations contain various ubiquitinated components [9]. In addition, u*biquilin2 (UBQLN2), valosin-containing protein (VCP),* and *optineurin (OPTN)*, which are associated with the proteolysis system, have also been identified in as causal genes of ALS [10-12]. Thus, protein mislocalization, aggregation formation, and defects in proteolysis could be the fundamental causes of ALS. However, there are many unresolved questions regarding these dysregulations of protein homeostasis (for example, their mechanisms, effects and significance in ALS pathology). Therefore, elucidating such questions would provide the possibility of developing ALS treatments.

The study of ALS has been steadily increasing, but the exact mechanisms of its pathology and effective treatments remain undiscovered. It is important to reveal whether the causal genes of familial ALS share the common motoneuron toxicity with sporadic cases. In this review, we will focus on recent studies of the aberrant distribution of proteins, aggregate formation, and the aberrant proteolysis in familial ALS. In addition, we discuss the possible involvement in sporadic cases.

2. SOD1

2.1. SOD1 mutation and ALS

SOD1 was the first ALS causal gene identified, and approximately 180 ALS-associated mutations have been reported to date [13]. SOD1 contains 153 amino acids and mainly exists in the cytosol, and it is abundant in neuronal cells, including motoneurons. SOD1 forms a homodimer, and each subunit contains one copper ion and one zinc ion. The main function of SOD1 is to eliminate reactive oxygen species, thus it was initially believed that changes in the enzymatic activity of SOD1 by ALS-related mutations caused motoneuron toxicity. However, the enzymatic activity of SOD1

mutants did not correlate with the severity of the ALS phenotype [14,15]. Actually, several mutants of SOD1 maintain their enzymatic activity compared with wild-type SOD1 [16]. Moreover, while *SOD1* mutant transgenic mice demonstrate ALS-like phenotypes, *SOD1* knockout mice do not [17]. These findings indicate that the motoneuron toxicity of SOD1 mutants was not caused by changes in enzymatic activity but by a gain of toxic functions. As one of the potential mechanisms, it was reported that misfolded SOD1 exerts toxicity through the formation of aggregates and/or the inhibition of cellular functions. Notably, several groups have generated antibodies specific for ALS-associated SOD1 mutants [18,19], and these antibodies recognized structurally abnormal SOD1 not only in *SOD1*-related but also in sporadic ALS patients [20]. Thus, the abnormal structural changes of SOD1 and subsequent motoneuron toxicity could be a common phenomenon in both familial and sporadic ALS (Figure 1).

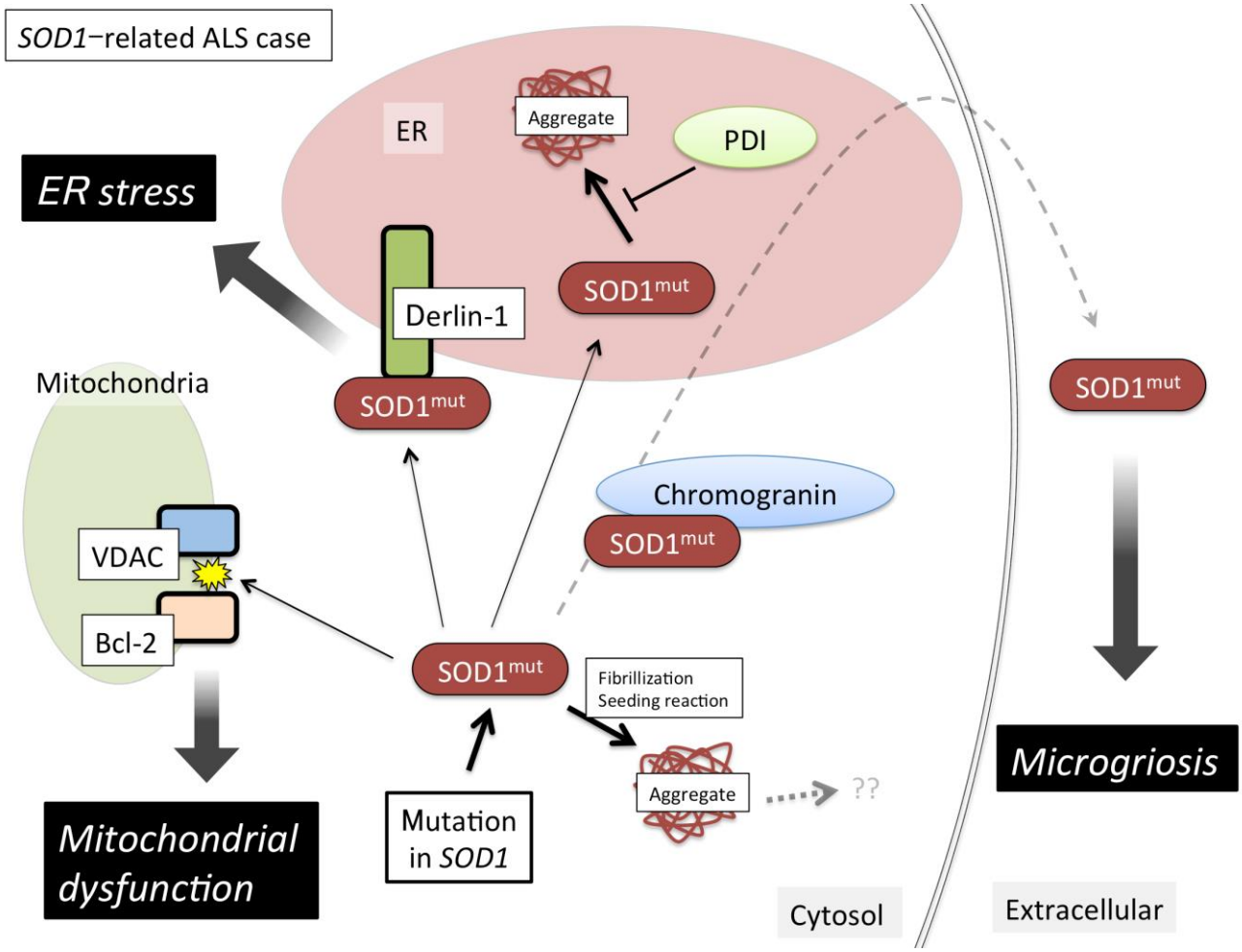

Figure 1. Overview of the potential pathogenic mechanisms of SOD1 toxicities in ALS. The proposed toxicities of SOD1 mutants in *SOD1*-related ALS. SOD1 mutants form aggregates, which could be prevented by the ER-localizing chaperon PDI in ER. The proper ER functions are inhibited by SOD1 mutants through interactions with Derlin-1 and BiP, resulting in ER stress. SOD1 mutants also abnormally localize in the mitochondrial outer membrane and inhibit the interaction between VDAC and Bcl-2. Chromogranin mediates the extracellular release of SOD1 mutants, triggering microgliosis.

2.2. SOD1 aggregation and post-translation modification

In histopathological studies, SOD1-positive Lewy body-like inclusions were detected in the ventral horn of the spinal cord in familial ALS patients [15]. Moreover, inclusions containing SOD1 were also observed in the spinal cord of *SOD1* mutant transgenic mice in the late stages of the disease [15,21]. It is known that some SOD1-positive inclusions are poly-ubiquitinated and fibrillized [22,23]. When an abnormal protein forms a fiber and eventually an aggregate, it functions as a 'seed'. The seed gradually gathers proteins that surround it in a process called 'seeding', and this process has been reported in SOD1 mutants. Interestingly, a SOD1 seed can induce seeding in cultured cells, and insoluble SOD1 inclusions have been observed [24]. SOD1 mutants associated with ALS have a low stability, and this lower stability causes an abnormal accumulation of SOD1 mutants [25]. Post-translational modifications, metallation and formation of disulfide bonds play a crucial role in maintaining the proper SOD1 conformation. In other words, the proper structure of SOD1 is obtained by the formation of disulfide bonds and metallation of copper and zinc atoms.

SOD1 contains four cysteine residues (Cys6, 57, 111 and 146), and the intramolecular disulfide bond between Cys57 and Cys146 contributes to the stabilization of SOD1 [26,27]. Indeed, the loss of an intramolecular disulfide bond has been shown to cause SOD1 misfolding [28]. Additionally, oxidative conditions in the ER were suggested to contribute to the formation of disulfide bonds with SOD1. There are over 20 enzymes in the ER that are involved in the oxidization and reduction of proteins. Ero1 is one of the enzymes engaged in the formation of disulfide bonds in the ER, and it strongly interacts with the protein disulfide isomerase (PDI). The Ero1-PDI complex forms a redox network [29] and facilitates the proper structure of proteins through the formation of disulfide bonds. Recently, it was reported that inhibition of PDI catalytic activity promotes SOD1 aggregation [30]. Moreover, a decrease in PDI catalytic activity by S-nitrosylation leads to the formation of SOD1 aggregates in ALS [31,32]. These reports indicate that PDI may help prevent the formation of SOD1 aggregates. This is supported by the finding that PDI co-localizes with SOD1 in familial ALS patients and with TDP-43 in sporadic ALS patients [33]. Thus, it is anticipated that some SOD1 mutants lose their disulfide bonds, which results in abnormal structures that form insoluble and fibrillated aggregates. However, cross-linking of SOD1 through irregular intermolecular disulfide bonds is also a suspected mechanism by which SOD1 forms inclusions [34] (Figure 1).

Although copper ions are required for substantial SOD1 enzymatic activity, zinc ions increase the stability of SOD1. Notably, mutations in the zinc binding site of SOD1 (H80R, D83G) were reported as causes of ALS. It is also known that the affinity to zinc is reduced in some SOD1 mutants that are prone to forming aggregations [35,36]. Furthermore, a zinc deficiency induces a conformational change of wild-type SOD1 and aberrant SDS-resistant disulfide-linked complex formation [37].

Considering these previous findings, the abnormal post-translation modifications of SOD1 lead to the formation of aggregates, and the aberrant metabolism of SOD1 can provide the mechanisms by which SOD1 mutants exert toxicity in ALS.

2.3. Aberrant localization of SOD1 mutants and its toxicities

SOD1 mutants have been reported to exert the motoneurons toxicities through the mislocalization to extracellular space, mitochondria and ER. The interaction with Chromogranin, a

neuronal secretory protein, causes the extracellular secretion of SOD1 mutants. Chromogranin was suggested to act as a chaperone that promotes the secretion of SOD1 mutants through the secretory pathway [38] (Figure 1). The extracellularly secreted SOD1 mutants trigger microgliosis and neurotoxicity.

Recently macrophage migration inhibitory factor (MIF) was identified as the chaperon that mediates the mislocalization of SOD1 mutants to the outer membrane of mitochondria [39]. SOD1 mutants in mitochondria induce the conformational change of Bcl-2 and inhibit the interaction between Bcl-2 and Voltage-dependent anion channel (VDAC), resulting in the cytotoxicity [40].

SOD1 mutants also accumulate in the ER and inhibit ER functions [41]. Induction of ER stress, which is an early event in ALS [42], was commonly observed in sporadic ALS patients and *SOD1*-related ALS animal models [30,41,43]. SOD1 mutants cause ER stress by disrupting protein folding in the ER and endoplasmic reticulum-associated degradation (ERAD), which degrades unfolded proteins in the ER. SOD1 mutants interact with BiP, an ER-resident chaperon, or Derlin-1, a component protein of the ERAD complex [41,44]. Because the induction of ER stress and conformational changes in SOD1 are also observed in sporadic ALS cases [22,45], SOD1-mediated ER stress could also be involved in other types of ALS in addition to *SOD1*-related ALS.

2.4. Recent studies of SOD1-mediated motoneurons toxicities using iPSC

For a long time, the studies of *SOD1*-related ALS have been heavily dependent on the analyses of overexpressed SOD1 mutants including ALS model animals. In recent years, induced pluripotent stem cells (iPSCs) derived from ALS patients have become a powerful tool. A *SOD1*-related ALS patient derived iPSCs enables us to analyze the toxicity of SOD1 mutants at the endogenous expression level in human motoneurons. At the beginning, it was reported that iPSCs generated from *SOD1*-related ALS patient could be differentiated into motoneurons and glial cells [46]. In motoneurons differentiated form *SOD1*-related ALS patient derived iPSCs, the activation of ER stress, the mitochondrial dysfunction and the accumulation of neurofilament were observed as well as in the *SOD1*-related ALS patients [47,48]. Furthermore, the recent advantage in genome editing clearly showed this abnormality depends on *SOD1* mutation, indicating the usability of iPSC-derived motoneurons as a new ALS model.

3. TDP-43

3.1. TDP-43 proteinopathy

In 2006, TDP-43 was identified in the abnormal protein aggregates that occur in ALS patients [8], and mutations of *TARDBP*, which encodes TDP-43, have been shown to cause ALS [2]. Notably, the TDP-43-positive aggregation and mutations of *TARDBP* were found in both familial and sporadic ALS [2]. TDP-43 is also a component of the ubiquitin-positive inclusion found in other neurodegenerative diseases, especially frontotemporal lobar degeneration (FTLD). FTLD presents with progressive neurodegeneration, progressive non-fluent aphasia and sematic dementia. We can classify FTLD pathologically into several subtypes, and one of these subtypes shows motoneuron degeneration similar to that in ALS and is characterized by the ubiquitin- and TDP-43-positive inclusion [49]. The aggregation of TDP-43 is also observed in patients suffering from dementia.

Co-localization of tau-protein and α-synuclein were reported in both Alzheimer's disease and Parkinson's disease [50]. Although these reports suggest that TDP-43 is widely associated with various neurodegenerative diseases, the detailed molecular mechanisms of TDP-43 pathology remain unclear.

3.2. TDP-43 function and mutants

TDP-43 was originally identified as a transcriptional repressor that binds to the TAR DNA of human immunodeficiency virus type 1 [51]; however, subsequent studies revealed that TDP-43 has multiple functions in RNA processing, and it is closely associated with hnRNP (e.g., transcription, splicing, transport, and localization). It was also reported that TDP-43 could stimulate dendritic growth and synapse formation in *Drosophila* [52], suggesting that TDP-43 is involved at least partially in the maintenance and growth of neurons. TDP-43 contains a nuclear localization signal (NLS), a nuclear export signal (NES), two RRM (RNA-recognition motifs, RRM1 and RRM2) and a glycine-rich domain [51]. Mutagenesis studies showed the presence of functional NLS and NES sequences in TDP-43, and the mutations of NLS and NES induced a restricted distribution of TDP-43 to the cytoplasm and nucleus, respectively [53]. RRM1 is necessary for binding to RNAs and usually recognizes the UG repeat region of RNAs. RRM2 is considered indispensable for interactions with RNAs and is involved in the formation of the TDP-43 dimer; however, its precise role remains unclear [54]. The C-terminal glycine-rich domain is essential for the interaction with some RNA binding proteins and the splicing activity of TDP-43 [55]. Interestingly, over 30 mutations of *TARDBP* reported in ALS are clustered in the glycine-rich domain [56], indicating the importance of the C-terminal region in ALS pathogenesis. An examination using domain deletion mutants revealed that a part of RRM1 and the C-terminal region were also essential to exerting toxicity in yeast [57].

3.3. Aberrant post-translational modification of TDP-43

As mentioned above, TDP-43 was identified as the main component of cytoplasmic inclusions, and various mutations of TDP-43 were found in ALS. Moreover, aberrant post-translational modifications of TDP-43 were found only in inclusions. In a biochemical analysis, the phosphorylation of S409/410 was identified as the major modification of TDP-43 aggregates [58,59]. These findings allowed the distinction between aggregate-forming and soluble TDP-43, but the mechanism by which phosphorylated TDP-43 accumulates in the cytoplasm is still unclear. At the same time, it was also revealed that TDP-43 is cleaved abnormally into a 17- to 25-kDa C-terminal fragment (CTF) (Figure 2). TDP-43 cleavage is mediated by caspase-3, which is activated in apoptotic conditions and in the late stage of the disease [60,61]. Most inclusions, including the pathological 25-kDa CTF, are recognized by the phosphorylated TDP-43-specific antibody [58,62]. However, phosphorylation is not necessary for the cleavage, accumulation and toxicity of TDP-43 [58,62], and only a correlation between the degree of phosphorylation and insolubility has been reported [60]. Although the relationship between the toxicity of the phosphorylated-CTF aggregation and its effect on disease progression is still unclear, the pathological characteristics of TDP-43 proteinopathy (e.g., cytoplasmic accumulation, insolubility, over-phosphorylation, poly-ubiquitination, and abnormal toxicity) are present in cells expressing the 25-kDa CTF [60]. Additionally, in transgenic mice expressing the wild-type TDP-43,

production of the 25-kDa CTF increased correspondingly with the disease progression, indicating a crucial role of CTF for motoneuron toxicity [64].

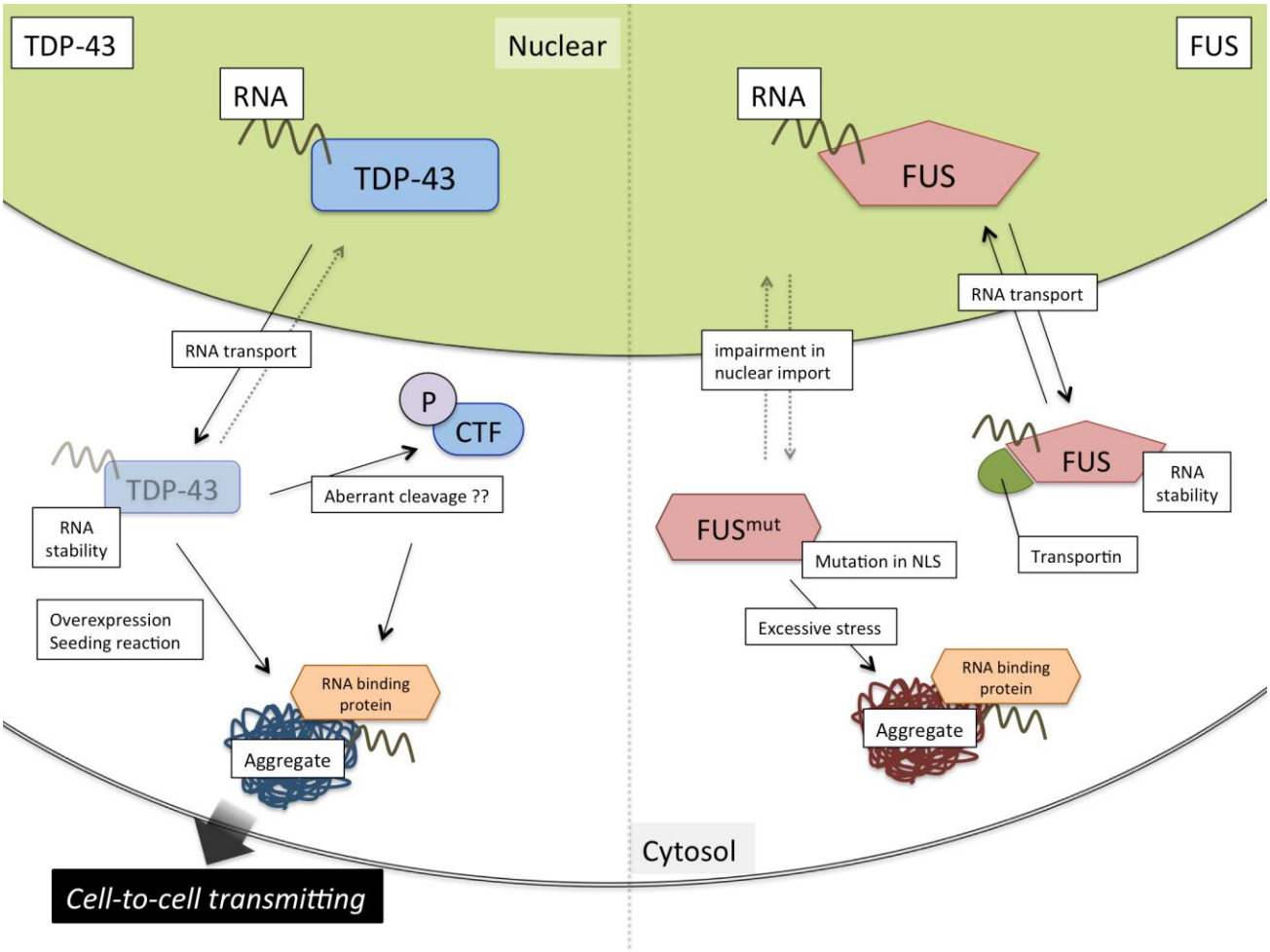

Figure 2. Overview of TDP-43 and FUS toxicity through aberrant localization and aggregation. (Left) TDP-43 mainly localizes in the nucleus, playing a role in RNA metabolism. TDP-43 shuttles between cytoplasm and nuclear, and mutation or dysregulation of the amount causes the aggregation formation of TDP-43 in cytoplasm. TDP-43-positive aggregations contain an abnormally cleaved and phosphorylated C-terminal fragment (CTF). Seeds and aggregates of TDP-43 have cell-to-cell transmitting ability. (Right) FUS with mutations in the NLS lost the ability to interact with transportin and is prone to localize in the cytoplasm. The excessive stresses facilitate the formation of the cytoplasmic aggregation. The aggregate formation of ALS causative gene products might cause the loss of functions or the gain of toxicity by sequestering the essential components for cell survival.

3.4. Aggregability of TDP-43 and the toxicity of aggregation

Although wild-type TDP-43 mainly localizes to the nucleus, ALS-related TDP-43 mutants typically translocate to the cytosol and form aggregates in the early stages of ALS [65]. The

C-terminal glycine-rich domain of TDP-43, where almost all disease-associated *TARDBP* gene mutations are clustered, plays an important role in mediating protein-protein interactions. Notably, this domain shows a similarity to yeast prion protein [66], and TDP-43 possesses a prion-like propagation ability. Insoluble TDP-43 isolated from patients with TDP-43 proteinopathy serves as the seed of the aggregation, accumulating the endogenous intact TDP-43 in abnormal aggregations. Furthermore, this seed has cell-to-cell transmitting ability and stability against heating, detergent and proteolysis [67] (Figure 2). ER stress is also suggested to be involved in the formation of TDP-43 aggregates [68]. As one possibility, weak ER stress in the early stage of neurodegenerative disease is reported to induce TDP-43 dysfunction and modulate its distribution. These impairments of TDP-43 then lead to greater ER stress through prion-like propagation and subsequent stabilization of aggregates. Thus, induction of ER stress could facilitate the accumulation of TDP-43 and its toxicity.

The overexpressed wild-type TDP-43 develops TDP-43 aggregates and cell toxicity, suggesting that dysregulation of the amount of TDP-43 in the cell causes TDP-43 toxicity [64] (Figure 2). It is possible that abnormal accumulation of TDP-43 results from a dysfunction in proteolysis. Indeed, *VCP* mutation causes the formation of TDP-43 aggregates [10], suggesting that an abnormality in the ubiquitin-proteasome system causes the aggregation. Additionally, it is known that TDP-43, which plays a role in RNA metabolism, autoregulates its expression level [69]. Moreover, it is noteworthy that ALS-related TDP-43 mutants have longer half-lives than the wild-type TDP-43, which correlates with the severity of ALS [70]. Therefore, it is possible that upregulation of TDP-43 through a dysfunction of the degradation system or its induction results in the propagation and subsequent stabilization of the TDP-43 aggregates, which leads to motoneuron death.

3.5. Studies of animal models and the possible toxicity of TDP-43

It is still unclear whether TDP-43-mediated neurotoxicity is caused by a loss or gain of function. Therefore, the toxicity of the aberrant localization and aggregation of TDP-43 in motoneurons has been a target of investigation, and several analyses of TDP-43 aggregation models have been attempted. The motoneuron-specific TDP-43 knockout mice exhibited selective and progressive ALS-like motoneuron degeneration, as well as morphological changes in the spinal cord [71]. However, these knockout mice showed different phenotypes than those of ALS, which included the absence of TDP-43 inclusions and survival of upper motoneurons. Because the precise function of TDP-43 in the brain is still unclear, these findings need further study and consideration. In contrast, transgenic mice carrying ALS-associated TDP-43 mutations have a shorter life span than non-transgenic mice and show ALS-like motoneuron degeneration [72]. In addition, transgenic mice overexpressing the wild-type human TDP-43 showed degeneration of cortical and spinal motoneurons, and the level of toxicity in these motoneurons correlated with the expression of TDP-43 [64] (Figure 2). These studies suggest that disturbances in the ability to regulate the amount of TDP-43 causes neurodegeneration, and both the loss of function and gain of toxic function could be mechanisms by which TDP-43 induces cell death.

The analysis of the possible toxicity of TDP-43 was also performed in smaller animal models such as yeast and *Drosophila*. In the *Drosophila* model, the loss of TDP-43 resulted in impairment of locomotive behaviors and life span [73]. Recently, it was also reported that the loss of TDP-43 impaired axonal transport of RNA granules [74] and TDP-43 mutant blocks autophagy-lysosome fusion in *Drosophila* [75]. In yeast, the modifiers of TDP-43 toxicity were searched through the

genome-wide loss of function screenings. The strongest suppression was achieved by the deletion of *DBR1*, which is a debranching enzyme of lariat introns [76]. This protective effect was confirmed in human neuronal cells or rat primary neuron, and the accumulated lariat introns in *DBR1* knocked down cells were suggested to sequester the toxic TDP-43. Another group performed expression screening to identify genes that modulate TDP-43 toxicity also in yeast. They identified *PBP-1*, an orthologue of human *ATXN2,* as a TDP-43 toxicity enhancer. This result was confirmed in *Drosophila* model expressing human wild-type TDP-43, resulting that unregulated *Drosophila* homologue Atx2 enhanced toxicity of TDP-43 [77]. These results suggested that the fundamental toxicity of TDP-43 would be conserved in small animals such as yeast and *Drosophila*. Thus, these models in which it is comparatively easy to perform the comprehensive analysis are valuable to reveal the molecular mechanisms and the modifiers of TDP-43 toxicity.

It is believed that several burdens (e.g., ER stress, genetic impairment of TDP-43, disruption of protein degradation) in cells facilitate the aggregation and stabilization of TDP-43. Because TDP-43 aggregations have been reported to include nonspecific RNAs and RNA binding proteins, the formation of aggregates could exhibit cell toxicity by isolating the essential factors for RNA metabolism. In contrast, a reduction in functional TDP-43 through mutations in *TARDBP* or other causal genes could suppress the original neuroprotective function of TDP-43. Although the functions of TDP-43 in the nuclear have been taken the notice, recently the importance of cytoplasmic TDP-43 was also highlighted. Cytoplasmic TDP-43 is usually co-localized with stress granules or RNA granules, suggesting that cytoplasmic TDP-43 also has a function in local RNA metabolism [74,78]. Moreover, TDP-43 has been shown to transport the RNA granules along with the axon in the *Drosophila* model, and the ALS-related mutants lost this ability [74]. These data indicate that the role of cytoplasmic TDP-43 in mRNA metabolisms could also be important in ALS pathogenesis. Further studies are needed to understand the precise mechanisms of TDP-43-mediated motoneuron toxicity.

4. FUS

4.1. FUS and neurodegenerative diseases

Fused in sarcoma (FUS), which is also known as *translocated in liposarcoma (TLS)*, was identified as a fusion oncogene in 1993 [79,80]. In human myxoid liposarcomas, the N-terminus of FUS fuses to a transcriptional factor, leading to an aberrant chromosomal translocation [79,80]. In 2009, *FUS* was reported as a causal gene of familial ALS, and the relationship between FUS and neurodegenerative diseases was suddenly noticed by researchers. At the same time, the pathological deposits of FUS was reported in *FUS*-related familial ALS and in FTLD without a *FUS* mutation [81-83].

FUS belongs to the FET family, which contains FUS, Ewing's sarcoma breakpoint region 1 (EWS) and TATA-binding protein-associated factor 15 (TAF-15). These three proteins have similar domains and regulate the expression of multiple genes [84]. It was reported that the mutation of *TAF15* is associated with ALS, and its mechanism resembles *FUS* [85]. FET family proteins are involved in not only gene expression but also RNA processing. Although the precise neuronal toxicity is unclear, it is possible that the mutation- or aggregation-mediated dysfunction of RNA maturation mediates neuronal degeneration.

4.2. Aberrant localization of FUS and aggregation

FUS contains an N-terminal SYGQ-rich domain, multiple nucleic acid domains and a C-terminal NLS. The NLS region may be the most important region for the physiological function of FUS. Indeed, many FUS mutations that exist in the NLS region have been associated with ALS. transportin, which is a nuclear transport receptor, recognizes the NLS region, and the NLS region is required for nuclear localization of FUS [86,87]. Mutations of FUS (P525L) in the NLS facilitated cytoplasmic redistribution, thus leading to the formation of FUS-positive aggregates [87] (Figure 2). Notably, a mutant's preference for the cytosol was reported to correlate with the onset of ALS [87]. Moreover, it is well known that the lack of arginine methylation in the RGG3 domain of FUS occurs in FTLD [88]. This defect in arginine methylation causes a tight interaction between FUS and transportin [86,87], and excessive interactions between FUS and transportin impairs the nuclear localization of FUS. Therefore, arginine methylation is required for nuclear translocation of FUS. These lines of evidence suggest that the disturbance in FUS nuclear localization is probably the key step for the formation of cytoplasmic inclusions and motoneuron cell death, which lead to ALS pathogenesis.

The necessity of a distinct mechanism that regulates the redistribution and aggregation of FUS was suggested. Cytoplasmic FUS is recruited to cytoplasmic deposits, and heat shock or oxidative stress then facilitates the formation of comparatively large inclusions [87] (Figure 2). It is thought that the formation of aggregations containing FUS requires two steps, which are the redistribution of FUS and an excessive amount of cell stress.

The FUS-positive inclusion was also observed in the brain of FTLD patients without *FUS* mutations [83], suggesting that *FUS* mutations are not the sole cause of the formation of FUS inclusions. There are some differences between the FUS-induced pathology in ALS and FTLD. In ALS, the *FUS* mutations cause impairment in nuclear import, whereas hypomethylation might be the most important step in FTLD. Consistent with these differences, the genetic defect in the *FUS* gene is always observed in FUS-inclusion-positive ALS, but in FTLD, FUS inclusions are detected even in the absence of a *FUS* mutation [83,89,90]. Additionally, FUS-specific granules are found in ALS, whereas FUS inclusions in FTLD contain other FET family proteins and transportin, which is required for nuclear import [91,92].

The toxicity of FUS likely results from the loss of functions in the nucleus and the gain of toxic functions through the aggregates in the cytoplasm. As with TDP-43, analyses involving animal models were performed with FUS to elucidate the source of its toxicity. *FUS* knockout mice do not show neuronal defects [93,94]. However, motoneuron defects caused by *FUS* depletion have been reported in zebrafish, which can be rescued by the expression of wild-type FUS [95]. In contrast, human wild-type *FUS* transgenic mice show a failure to gain weight and paralysis accompanied by impaired motoneuron function [96]. These animal model studies suggest that both the loss of protective functions in the nucleus and the gain of toxic functions through aggregates contribute to the *FUS*-mediated neurodegenerative pathology.

5. *C9ORF72* toxicity in ALS

5.1. C9ORF72 mutation in ALS

The GGGGCC (G_4C_2) hexanucleotide repeating expansion in the intron region of *chromosomal*

9 open reading frame 72 (C9ORF72) was reported as a cause of ALS in 2011. Additionally, the abnormal expansion in *C9ORF72* was also found in FTLD patients [3,4]. According to the epidemiological study, this abnormal repeating expansion was found in approximately 40% of familial ALS patients [5]. Remarkably, the *C9ORF72* mutation was also found in sporadic ALS cases, accounting for approximately 7% of sporadic ALS cases. In normal subjects, the G_4C_2 hexanucleotide is repeated two to three times, and this sequence is never repeated more than twenty times. However, it is believed that an abnormal expansion is repeated hundreds or even thousands of times in patients with *C9ORF72*-associated ALS.

5.2. Motoneuron toxicity resulting from the loss of function mechanism

Although the precise function of the *C9ORF72* encoding protein is still unknown, it was shown that *C9ORF72* is a homologue of differentially expressed in normal and neoplasia (DENN), a GDP/GTP exchange factor that activates Rab-GTPase [97], and it is involved in the endosomal trafficking [98]. Thus, mutations of *C9ORF72* are considered to disrupt vascular trafficking. *C9ORF72* gene is transcribed as three distinct variants and abnormal repeating expansion of variant 2 locates in the promoter region, decreasing its transcription [3]. The decrease of *C9ORF72* expression level is reported also in iPSC neurons [99]. To determine whether the loss of function of *C9ORF72* causes the motoneurons toxicities, several animal models were generated. While some of these model animals (*C. elegans* and zebrafish) showed neuronal deficit and dysfunctional behavior [100,101], neural-specific ablation of *C9ORF72* in mice does not result in the ALS-like phenotype [102]. Thus, it is still unclear whether the loss of function of *C9ORF72* causes motoneuron degeneration and further studies are required.

5.3. Motoneuron toxicity resulting from the gain of function mechanism

The abnormal RNA is considered to gain toxicity. The toxicity of abnormal RNA repeat expansions can be observed in several diseases. For example, in muscular dystrophy, which shows muscular necrosis and multiple organ failure, abnormal RNA repeats sequester essential proteins for RNA processing in aggregations such as RNA foci, leading to disruption of RNA metabolism and cell death [103]. In the case of *C9ORF72*-mediated ALS, RNA foci are observed in neurons derived from iPSC as well as in spinal and frontal cortex neurons of ALS patients [3,99]. It has been proposed that *C9ORF72* RNA containing a G_4C_2 repeat expansion also sequesters essential proteins and disturbs the RNA metabolism. The interactions with ribonucleoproteins like Pur-α and ADARB2 are considered to cause nucleolar stress [99,104,105].

A toxicity of abnormally expanded dipeptide repeats was also reported. Translation independent of the start codon (ATG) is induced in the presence of abnormal repeat expansions (repeat-associated non-ATG (RAN) translation) [106]. Theoretically, there are five expression patterns of the dipeptide repeat (DPR) protein generated through the RAN translation. Among them, the strong Gly-Ala DPR-positive inclusions, and to a lesser extent, the Gly-Pro and Gly-Arg DPRs-positive inclusions, were found in the brains of ALS and FTLD patients [107]. Notably, aggregations of DPRs were found together with TDP-43 negative/p62 positive inclusions, thus suggesting TDP-43-independent toxicity [3,107-109]. Several groups have demonstrated the toxicity of *C9ORF72* DPRs in various models, indicating the importance of DPRs in neurodegeneration [107,109]. The toxicity of each

DPRs are considered to differ from each other, and the analysis of the Gly-Arg DPR toxicity has been promoted. The expression of Gly-Ala DPR has been reported to evoke ER stress through activating PKR-like endoplasmic reticulum kinase (PERK) pathway [110]. Since other ER stress sensors were not activated by Gly-Ala DPR, the mere inhibition of ubiquitin proteasome system, one of proposed mechanism, was not likely and further analyses are needed to identify the specific target of Gly-Ala DPR.

To determine which of the abnormal RNA or DPR is the main cause of motoneuronal toxicity of *C9ORF72* mutation, two different fly models were generated. The one line has long repeat RNA with stop codons, which generates only RNA repeat expansion and not DPRs. The other line generates, besides RNA repeats, DPRs from RAN translation [111]. The line expressing both RNA repeats and DPRs showed toxicity, but the line expressing only RNA repeats had little effect. The other group generated fly model with a *C9ORF72* minigene containing G_4C_2 repeats [112]. This fly model showed RNA foci in nuclear but not significant toxicities. These reports utilizing small animals indicated that DPR is the major toxic species compared to abnormal RNA. Recently, several groups have also generated *C9ORF72*-ALS model mice with distinct techniques. One group generated mice carrying Bacterial artificial chromosome (BAC) harboring full-length human *C9ORF72* containing G_4C_2 repeat expansion. Despite RNA foci and DPR from RAN translation were observed in these mice, aberrant behavior and neurodegeneration were not [113]. The other group used adeno-associated virus (AAV) containing 66 G_4C_2 repeats and administered these viruses to postnatal day 0 mice. Six-month-old mice exhibit besides accumulation of RNA foci and DPRs, neuronal loss and gliosis [114]. Though several *C9ORF72* mutation models are evaluated, further studies are required to conclude the mechanism by which *C9ORF72* mutation causes toxicity. Notably, two recent studies suggested the involvement of a defect in nucleocytoplasmic transport in *C9ORF72* mutation-induced toxicity [115,116]. In *Drosophila* screening, RanGAP1 was identified as a suppressor of *C9ORF72* mutation-induced neuronal toxicity. They demonstrated the physical interaction of RanGAP1 with an RNA repeat expansion produced by G_4C_2 repeat. This interaction might cause inhibition of RanGAP1 and aberrant nucleocytoplasmic distribution [115]. Another report demonstrated that a wide variety of nuclear pore proteins could modulate *C9ORF72* mutation-induced toxicity, and the dysfunction of nucleocytoplasmic transport facilitated the accumulation of RNA in the nucleus. Moreover, it was reported that *C9ORF72* DPR toxicity is also modified by nucleocytoplasmic transport [117] (Figure 3).

It is still unclear how *C9ORF72* mutation causes toxicity through repeat expansion RNA or the production of DPRs, or both, the dysfunction of nucleocytoplasmic transport and subsequent aberrant distribution of proteins or RNAs could be the cause of motoneuron toxicity.

6. Other causal genes of ALS

6.1. OPTN

In 2010, *OPTN*, which is a known risk gene for primary open-angle glaucoma, was also identified as a causal gene for ALS [12,118]. OPTN contains 577 amino acids and has a coiled-coil domain, zinc-finger domain and ubiquitin-binding domain. OPTN has multiple functions, and its main function is negative regulation of NF-κB activation. It competes with NF-κB essential modulator (NEMO) for binding to ubiquitinated receptor interacting protein (RIP) to prevent NF-κB

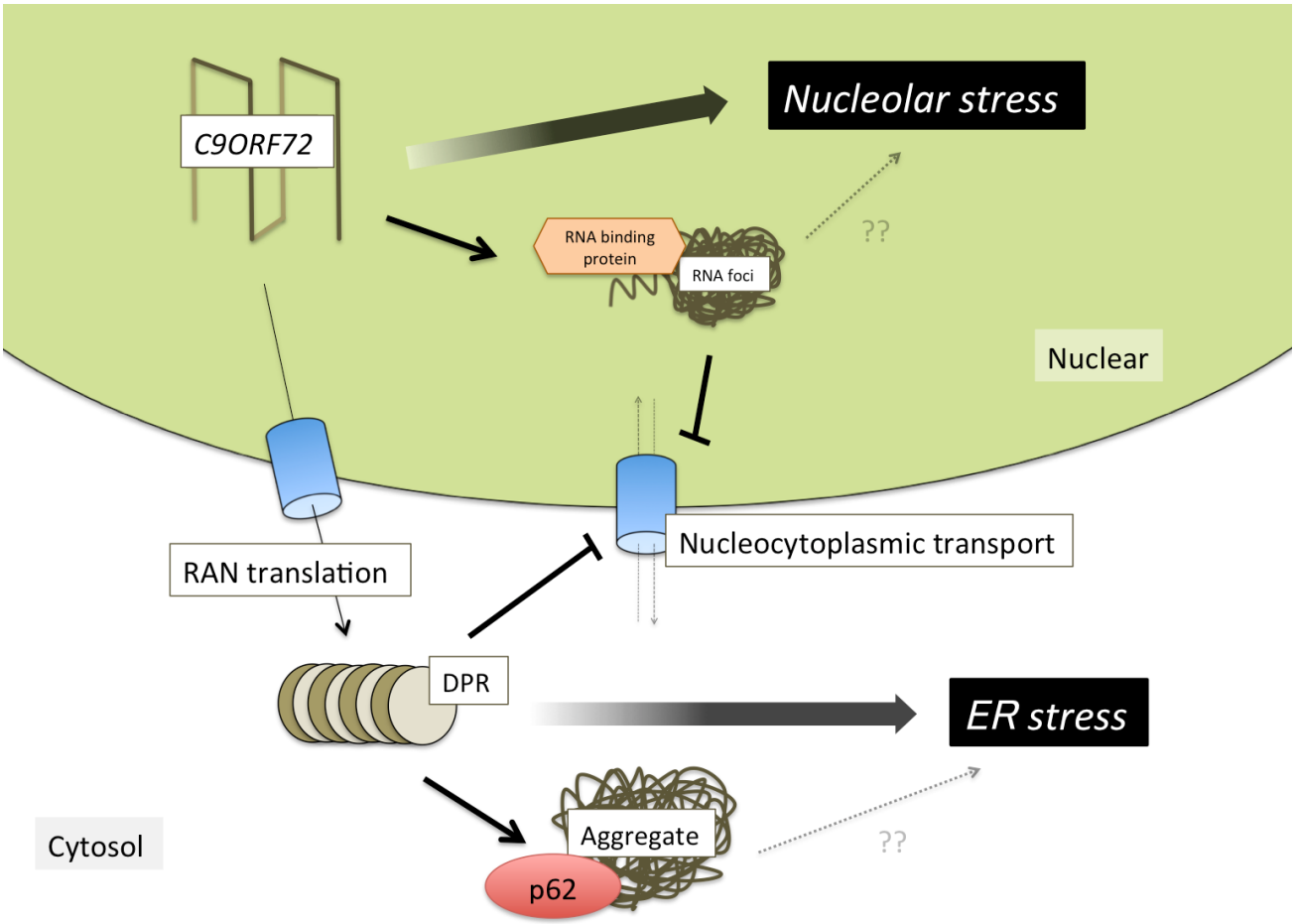

Figure 3. Overview of *C9ORF72* toxicity through repeat expansion of RNA and DPRs. The intronic hexanucleotide repeat expansion in *C9ORF72* causes the formation of RNA foci in the nuclear. RNA foci might sequester RNA binding proteins that are essential for cell survival. Dipeptide repeat proteins (DPRs) are generated from aberrant repeat expansion through RAN translation, evoking ER stress. DPR-positive aggregation is colocalized with p62 in the cytosol. The interaction of abnormal RNA repeat expansion with ribonucleoproteins is considered to cause nucleolar stress. A defect in nucleocytoplasmic transport has also been reported as the toxicity of the *C9ORF72* mutation.

activation [119]. The ALS-associated OPTN E487G mutant, which has a mutation in the ubiquitin-binding domain, has a loss of function for binding to ubiquitin that prevents inhibition of NF-κB activation [120]. It is likely that the functional disruption of OPTN induces an aberrant activation of NF-κB, which leads to neurodegeneration. OPTN E487G mutant shows aberrant cell localization and formation of cytosolic inclusions. In addition, OPTN has the functions of maintaining the Golgi body and transport from the Golgi body [121]. Moreover, OPTN also possesses the role of an autophagy receptor. OPTN localizes with the LC3 vesicle, and its ability to bind with ubiquitin is necessary for adapting to LC3. The OPTN E478G mutant loses the ability to bind to ubiquitin as well as to co-localize with LC3 [122]. OPTN is also detected in ubiquitin- and

TDP-43-positive inclusions in sporadic ALS, in SOD1-positive inclusions in familial *SOD1*-related ALS, and in FUS-positive inclusions in *FUS*-related ALS [119,123,124]. These results suggest that OPTN-positive inclusion is a common phenomenon in wide range of ALS types. Although the exact toxicity is unknown, the sequestration of OPTN to aggregations and its loss of function could be a common cause of ALS.

6.2. UBQLN2 and VCP

UBQLN2, which is associated with the ubiquitin-proteasome system, was also identified as a causal gene of ALS. In X-linked ALS-FTD, UBQLN2 had several mutations in the PXX repeat domain [11]. Recent studies also reported mutations in the vicinity of the PXX domain as a cause of ALS [125]. Although the precise function of the PXX domain is still unclear, UBQLN2 may play a role in ubiquitin-dependent proteolysis and the mutation might result in the dysfunction of protein degradation [11,126]. UBQLN2-positive inclusion was observed in ALS-FTD patients with or without *UBQLN2* mutations [11]. Skein-like inclusions are positive for UBQLN2 in the spinal cords of ALS patients with *SOD1*, *TARDBP* and *FUS* mutations [11,125].

The gene encoding VCP was also reported as a causal gene of ALS, and *VCP* mutation accounts for 1–2% of familial ALS cases [10]. It was shown that VCP interacted with TDP-43 and that disease-causing VCP mutants led to the redistribution of TDP-43 to the cytoplasm [127]. VCP forms a complex with its co-factors, Npl4 and Ufd1, and interacts with ubiquitinated proteins or ERAD complex, such as Hrd1 and Derlin-1 [128-131]. In ERAD, unfolded proteins in the ER are transported from the ER to the cytosol and subsequently degraded by the ubiquitin-proteasome system in the cytosol. It is suggested that VCP contributes to the retrograde-translocation of unfolded proteins through its ATPase activity [129,130]. Notably, UBQLN2 is also estimated to be involved in ERAD. UBQLN2 has a ubiquitin-like domain and a ubiquitin-associated domain and is considered to facilitate the transport of ubiquitinated substrates to proteasomes [126]. As mentioned before, SOD1 mutants also inhibit ERAD. Thus, it is possible that the dysfunction of ERAD, which leads to the defect in proteolysis, commonly plays an important role in ALS pathogenesis.

7. Conclusions

The pathological mechanism of ALS is still unclear, and there is no fundamental or effective therapy. Many causal genes of ALS have recently been identified. Numerous studies analyzing these genes have demonstrated multiple forms of motoneuron toxicity in each mutation-induced ALS. However, there is a need to reveal common mechanisms of familial ALS pathogenesis to elucidate the pathogenesis of sporadic ALS, which accounts for 90% of all cases. In order to approach the common toxicity in ALS motoneurons, some important questions below should be solved.

7.1. Whether the ALS model animals really represent the ALS pathology

Along with the discovery of ALS causative genes, there has been much effort to generate ALS model animals. Through the generation of knockout or transgenic animals of ALS-related genes, some animal models that show the motoneuronal degeneration and ALS-like symptoms have been established. While some of them possess high similarity to the ALS, the complete knockout or the

excessive expression of ALS-relate genes could cause artificial toxicities; but we should carefully interpret the results of these animal models. Especially, the pathological phenomenon that we focus on this review would be affected through the difference of expression levels. Since animal models give us significant information and are powerful tools to analyze *in vivo* effects, the confirmation in higher animals is desired. The generation of the knock-in animals of ALS-related mutation might also be useful. In addition, motoneurons differentiated from iPSC derived from ALS patients have been reported to reproduce the motoneurons toxicities [46,132,133]. In this model, the toxicity of each mutation could be assayed in the physiological conditions. Although there are several issues to be addressed (stability, clonal effect, lack of non-cell autonomous effects), iPSCs may also become prominent ALS model.

7.2. The problem of species difference

One of the big problems to develop the ALS treatment is that drugs effective in ALS model animals do not exert enough effect on ALS patients. The model animal could be unsuitable to evaluate the human ALS, which appears to be caused in part by the species difference. Although the animal models share the same toxicities with human ALS, most of the drugs target the motoneurons toxicity but not the fundamental mechanism of pathogenesis, which should be targeted to solve the problems of the species difference. Thus, the elucidation of the precise pathogenesis of ALS and discovery of molecular mechanism-based ALS treatment agent are required. Also, the iPSC-ALS model would be useful, in which we can evaluate the treatment efficiency in human.

7.3. Whether ALS cases share the common pathogenesis

An important question to deal with ALS (especially to approach sporadic ALS treatment) is whether ALS with different gene mutations is caused by the common mechanism. The symptoms observed in patients and the properties of the autopsy sometimes differ from the others. For example, in *SOD1*-related ALS the TDP-43 positive inclusion that is frequently observed in other type of ALS patients has not been found [134]. It would be possible that each genetic mutations of ALS exert its toxicity through different mechanisms. On the other hand, SOD1 has also been reported to be involved in the non-cell autonomous motoneurons toxicity in *SOD1* mutation-negative sporadic ALS, suggesting SOD1 as the common mediator of ALS toxicities [38,135]. Although the number of cases analyzed for the involvement of SOD1 in sporadic cases is still too small and SOD1 does not necessarily participate in all sporadic cases [134], the further large-scale analyses are desired. It is worth noting that the aberrant distribution of proteins/RNAs and the formation of aggregations were detected in a wide range of ALS types, even in the absence of their mutations. Thus, an analysis of the mislocalization, aggregation formation and defects in proteolysis could provide the insight into the common pathological events in ALS. In addition, the development of iPSC technology would enable us to investigate the molecular mechanisms of sporadic ALS cases. These attempts would reveal the common pathogenesis in wider variety of ALS cases and shed light on the fundamental ALS treatment.

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Conflict of interest

The authors declare that they have no conflict of interest.

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