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Review

Protein Misdirection Inside and Outside Motor Neurons in Amyotrophic Lateral Sclerosis (ALS): A Possible Clue for Therapeutic Strategies

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Abstract: Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease characterized by progressive muscle wasting and weakness with no effective cure. Emerging evidence supports the notion that the abnormal conformations of ALS-linked proteins play a central role in triggering the motor neuron degeneration. In particular, mutant types of superoxide dismutase 1 (SOD1) and TAR DNA binding protein 43kDa (TDP-43) are key molecules involved in the pathogenesis of familial and sporadic ALS, respectively. The commonalities of the two proteins include a propensity to aggregate and acquire detrimental conformations through oligomerization, fragmentation, or post-translational modification that may drive abnormal subcellular localizations. Although SOD1 is a major cytosolic protein, mutated SOD1 has been localized to mitochondria, endoplasmic reticulum, and even the extracellular space. The nuclear exclusion of TDP-43 is a pathological hallmark for ALS, although the pathogenic priority remains elusive. Nevertheless, these abnormal behaviors based on the protein misfolding are believed to induce diverse intracellular and extracellular events that may be tightly linked to non-cell-autonomous motor neuron death. The generation of mutant- or misfolded protein-specific antibodies would help to uncover the distribution and propagation of the ALS-linked proteins, and to

design a therapeutic strategy to clear such species. Herein we review the literature regarding the mislocalization of ALS-linked proteins, especially mutant SOD1 and TDP-43 species, and discuss the rationale of molecular targeting strategies including immunotherapy.

Keywords: seeding; subcellular localization; SOD1; TDP-43; non-cell-autonomous motor neuron death; antibody

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal paralytic disease with no effective cure. Although the etiology of ALS remains unclear, the current consensus implicates aberrant conformational changes in disease-associated proteins driving the diverse signaling cascades causing ALS [1–3]. The original pathological evidence came from case reports describing cellular inclusions either reminiscent of Lewy bodies in a familial ALS case [4] or resembling Lafora bodies in a sporadic ALS patient [5]. The Lewy body-like hyaline inclusions as well as the skein-like inclusions, are ubiquitinated [6,7], indicating that ALS pathogenesis involves protein mishandling.

Approximately 90–95% of ALS cases are sporadic with unknown etiology. However, intensive genetic approaches to familial ALS (FALS) patients have successfully identified diverse ALS-causative genes including SOD1, ALS2, SETX, SPG11, FUS, VAPB, ANG, and TDP-43, which are assigned as ALS1, 2, 4, 5, 6, 8, 9, and 10, respectively [8]. Moreover, an increasing number of new genes have been identified in familial ALS including optineurin [9], ubiquilin2 [10], and an expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 [11,12]. Strikingly, the wild type (WT) forms of SOD1, TDP-43, and FUS have been detected in pathological inclusions in sporadic ALS, justifying the research on familial ALS to understand sporadic cases. Indeed, many signaling mechanisms implicated in mutant SOD1-linked ALS may also underlie the pathogenesis of sporadic ALS, including mitochondrial damage, glutamate toxicity, proteasome impairment, ER stress, and axonal flow strangulation [13,14].

Physiological functions of the various ALS-associated proteins may vary widely despite the characteristic motor symptoms of ALS. This indicates huge diversities in ALS pathogenic mechanisms. However, aberrant gene products in ALS, and especially those involved in autosomal dominant inheritance such as SOD1, TDP-43, FUS, and, in several cases, optineurin, have been colocalized with ubiquitin-positive inclusions [9,15–17], indicating the disturbance of clearance pathways of misfolded proteins. Interestingly, the mutant products of these genes show abnormal subcellular distributions in cultured cells and affected tissues. For instance, mutant SOD1 protein, a major cytosolic protein, is mislocalized to mitochondria, endoplasmic reticulum, and the trans-Golgi network, and each such subcellular compartment could be a source of toxic signaling [13]. For example, the redistribution of TDP-43 to the cytosol induces aggregate formation and relocates nuclear TDP-43. Therefore, it is useful and practical to approach ALS pathogenesis from the standpoint of protein mislocalization driven by aberrant conformations.

Here, we review the roles of two major ALS-linked proteins, SOD1 and TDP-43, with a focus on their aberrant subcellular localizations.

2. Superoxide Dismutase 1 (SOD1)

2.1. Molecular Basis

In 1993, superoxide dismutase 1 (SOD1) was identified as the first genetic mutation in 20% of familial ALS patients [18]. SOD1 is an oxidation antagonist found in the cytosol that scavenges the superoxide to produce oxygen and hydrogen peroxide ($O_2^- + H_2O \rightarrow O_2 + H_2O_2$). It is one of the most abundant cellular proteins comprising about 2% of total cytosolic protein content. To date, more than 150 SOD1 mutations have been reported, which together cover most of the functional domains [19]. Moreover, the enzymatic activity of mutant SOD1 varies from null to "increased" despite similar symptoms in overall mutants. Moreover, SOD1 KO mice show no ALS phenotype [20]. These findings indicated that mutant SOD1-linked ALS is caused not by the loss of anti-oxidant function, but by the acquired toxic function resulting from protein conformational change.

Disulfide formation through this Cys57-Cys146 bond stabilizes the SOD1 structure and maintains proper dimerization [21], as shown by the study of Wang *et al*. [22] in which a substitution mutant of cysteine induced the misfolding of SOD1. On the other hand, Cys6 and Cys111 have been implicated in oligomer formation under oxidative conditions [22–24]. Considering that the cytosol is a reducing environment that would result in cleavage of disulfide bonds, this intramolecular Cys57-Cys146 bridge is protected from reduction by the proper dimer conformation. Therefore, it is reasonable to assume that a mutation-derived single amino acid replacement might affect the protein dimer structure, leading to misfolding, particularly as many mutant SOD1s are readily monomerized under mild reducing condition [25]. Rakhit *et al*. [26] reported that both WT and mutant SOD1 transiently form unnatural and partially folded monomers upon oxidation, before oligomerizing or aggregating. Based on these findings, the group then generated a rabbit polyclonal antibody against the potential dimer interface (SEDI), and demonstrated that this antibody specifically labels misfolded SOD1 [27]. We provided further evidence for the significant role of a transient hydrophobic surface by identifying Hsp70-SOD1 complex, which is present in the detergent-soluble, but not in the detergent-insoluble fraction. Moreover, demetallating SOD1 augmented the affinity of Hsp/Hsc 70 for SOD1 in both mutant and WT protein [28]. Mammalian cells precisely recognize misfolded SOD1 species and direct them for degradation through the ubiquitin-proteasome pathway [29]. Therefore, mutant SOD1 has a shorter half-life than the WT protein; however, the degradation capability of proteasome is gradually impaired under the continued expression of mutant SOD1, leading to the accumulation of ubiquitin-positive SOD1 species [29].

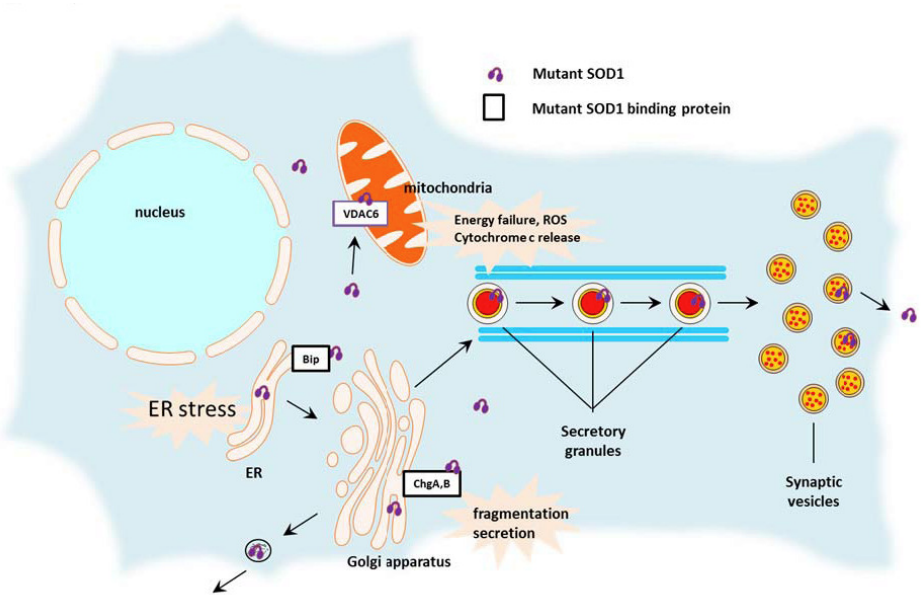
2.2. Toxic Cascades Caused by Mutant SOD1

SOD1 is a major cytosolic protein. Live-cell imaging of EGFP-fused SOD1 showed a homogenous distribution in the cytosol for both WT and mutant SOD1s. However, mild membrane perforation using digitonin in the presence of ATP to wash out soluble cytosolic protein unveiled punctate or vesicular locations of SOD1 including mitochondria, lysosomes, ER, and trans-Golgi network, and the effect was more apparent with the mutant SOD1 than with WT [30].

Although the molecular basis for these aberrant translocations of mutant SOD1 to various cellular organelles remains elusive, the hydrophobic nature of the mutant protein is a possible explanation.

Okado-Matsumoto and Fridovich [31] documented that the apo state of the SOD1 protein enables it to access mitochondria, but the heat shock chaperone prevents entry, indicating that the hydrophobicity of SOD1 is a key factor for its translocation to mitochondria [31]. Apo SOD1 can also be directly incorporated into microsomes in the presence of ATP *in vitro* [30]. Although WT SOD1 has also been localized at lysosomes, mitochondria, and nucleus as well as in the cytosol [32], it should be noted that only the mutant SOD1 causes undesirable detrimental effects on cell survival when redistributed to these other locations (Figure 1).

Figure 1. Aberrant subcellular localization of mutant SOD1 protein and the associated effect on ALS pathogenesis. Mutant SOD1 interacts with several "accompanying proteins", resulting in the abnormal subcellular localization.



2.2.1. Mitochondria

Mitochondria are indispensable suppliers of oxidative energy in addition to their role as calcium (Ca^{2+}) buffers. In neurons, mitochondria are transported to the distal axon, thus mitochondrial damage seriously affects the diverse functions of the growth cone and synaptic terminals. Indeed, blocking Ca^{2+} entry into mitochondria rescues cultured motor neurons from glutamate-induced cell death [33]. The role of mitochondria in mutant SOD1-linked ALS was first studied using a transgenic approach by Wong *et al*. [34], who demonstrated a prominent vacuolar change to mitochondria in G37R SOD1 Tg mice. Later work revealed mutant SOD1 inside mitochondria in mutant SOD1 Tg mice by immunofluorescence and immunoelectron microscopy [35]. Distinct from WT SOD1, mutant SOD1 induces morphological change and cytochrome c release in cultured neurons that resulted in apoptosis [36]. Two transgenic studies further indicated the involvement of mitochondria-mediated apoptosis in mutant SOD1-linked ALS. Inoue *et al*. [37] demonstrated that suppressing caspase-9 by overexpressing XIAP in motor neurons effectively slowed the progression of ALS in G93A SOD1 Tg mice, while Reyes *et al*. documented that neuron-specific deletion of BCL-associated X protein (BAX) or BCL2-homologous antagonist/killer (BAK), which are both proapoptotic BCL-2 family

proteins, delayed the onset and extended the longevity of disease in the same mice [38]. In addition, Vande Velde *et al*. [39] used sophisticated double transgenic mice co-expressing mutant SOD1 and a mitochondria-targeted fluorescent indicator to demonstrate aberrant morphology and distribution of mitochondria. Interestingly, these altered properties were influenced by the dismutase activity of SOD1, whereby dismutase-active G37R SOD1 induces mitochondrial clustering in the proximal site, whereas dismutase-inactive G85R SOD1 produces aberrantly elongated mitochondria in the axon, indicating that oxidative stress modifies the detrimental effect of mutant SOD1 [39]. One of the proposed molecules responsible for the entry of mutant SOD1 into mitochondria is a voltage-dependent anion channel (VDAC1), which resides in the mitochondrial outer membrane [40]. Mutant, but not WT SOD1, interacts with VDAC1, and the ablation of VDAC1 in G37R SOD1 Tg mice slowed the onset of paralysis. Mitochondria are the major source of reactive oxygen species (ROS) in cells, and these could contribute to the observed motor neuron death. However, conditional knockout of SOD2, a mitochondrial SOD, in the motor neurons produced no ALS phenotype [41], arguing against the hypothesis that mitochondrial ROS are a trigger for ALS. Surprisingly, a recent study by Zhu and Sheng [42] revealed that mutant SOD1 Tg mice with increased mitochondrial mobility in the motor neuron axons displayed a similar phenotype to those with impaired mobility. This result suggested that mislocalization of mitochondria alone is not enough to explain the detrimental events caused by the axonal transport impairment, and the malfunction of mitochondria themselves may be directly responsible for the ALS pathogenesis.

2.2.2. Endoplasmic Reticulum (ER)

Previously, the only luminal structures to which the SOD1 redistributed were regarded to be lysosomes and mitochondria [43]. Only one paper using hepatocytes had shown SOD1 in the ER [44]. However, Tobisawa *et al*. [45] showed that mutant SOD1, but not WT protein, colocalized with ER chaperone protein, GRP78/Bip, and induced ER stress in the transfected cells. Studies by our group [46] and by Kikuchi *et al*. [47] used immunoelectron microscopy to more precisely localize the mutant SOD1 in ER. Kikuchi *et al*. also demonstrated that mutant SOD1 interacts with GRP78/Bip, which mediates its entry into ER. We further confirmed the ER localization of SOD1 by sucrose density-gradient ultracentrifugation and floating ultracentrifugation using an iodixanol cushion and high-salt wash [30], while a recent paper demonstrated both WT and mutant SOD1 in microsome fractions and partial colocalization of these proteins with Derlin-1 in ER by immunocytochemistry [48].

ER stress is triggered by the accumulation of unfolded protein in the ER through the activation of three sensor proteins, IRE1, ATF6, and PERK, which downregulate protein synthesis and up-regulate BiP/GRP78 [49]. ER stress is thought to play a crucial role in both sporadic and familial ALS pathogenesis [50–54]. The ablation of BH3-only protein puma, which is necessary to induce ER stress, significantly delayed the disease onset and protected motor neurons [55]. However, the molecular mechanisms by which mutant SOD1 induces ER stress remains unclear. Notably, ER-targeted overexpression of mutant SOD1 did not injure Neuro2a cells [36], suggesting the existence of partner molecules inside or adjacent to ER that are involved in mutant SOD1-induced ER stress. To this end, Nishitoh *et al*. [56] reported that cytosolic mutant SOD1 induced ER stress through an interaction with Derlin-1, leading to an impaired unfolded stress response and the activation of apoptosis signal-regulating

kinase 1 (ASK1). In addition, protein disulfide isomerase, which is upregulated under ER stress, shows therapeutic potential based on its amelioration of cell toxicity induced by the unfolded stress response [57].

2.2.3. Golgi Apparatus, Secretory Pathway

Another noxious effect of ER stress caused by mutant SOD1 is fragmentation of the Golgi apparatus, which affects protein maturation and intracellular trafficking of membrane and secretory proteins [58]. Indeed, Gonatas and coworkers [59] clearly showed a fragmented Golgi apparatus in the motor neurons of ALS patients. This does not necessarily mean that mutant SOD1 is present in the Golgi apparatus, since Golgi morphology is affected by the structural dynamism of microtubules for axoplasmic transport. Indeed, SOD1 is synthesized in free ribosomes, and has no signal peptide that drives the entry of proteins into the ER-Golgi secretory pathway. Therefore, despite early findings of WT SOD1 secreted from cultured astrocytes and thymus-derived epithelial and fibroblast cells, secretion of SOD1 is thought to follow a non-classical pathway since brefeldin A, an ER-Golgi transport blocker, did not inhibit the secretion [60].

More recently, Turner *et al.* [61] presented compelling evidence that both WT and mutant SOD1 are trafficked via the classical secretory pathway. In addition to morphological evidence using immunoelectron microscopy, we confirmed the presence of mutant SOD1 in trans-Golgi network of mutant SOD1 Tg mice by immunoisolating the organelle using a Golgi-resident protein, TGN38 [46]. The molecular mechanism used by mutant SOD1 to enter the secretory pathway remains elusive. We identified chromogranin A (ChgA) and B (ChgB), which are neurosecretory proteins, as interacting partners with mutant SOD1, but not WT. Mutant SOD1 and chromogranins interact and colocalize in the G37R and G93A SOD1 transgenic mice, as verified by double immunoelectron microscopy and immunoprecipitation [46]. In addition, both ChgA and ChgB promote the secretion of mutant SOD1, but not WT protein, in cell culture experiments. The potential effects of this secreted and extracellular SOD1 on ALS pathogenesis are discussed below.

2.3. Cell-Autonomous and Non-Cell-Autonomous Motor Neuron Degeneration

The overexpression of mutant SOD1, but not WT, induces apoptotic cell death [36,62,63], with mutant SOD1 inducing diverse toxic signaling pathways inside cells, including those driving mitochondrial impairment, ER stress, oxidative stress, and proteasome suppression. However, an earlier study showed that transgenic mice expressing mutant SOD1 predominantly in the motor neuron do not develop ALS [64], while Lino and coworkers [65] showed that mutant SOD1 expression driven by the Thy-1 promoter neither produced an ALS phenotype nor affected the progression of mutant SOD1 Tg mice. On the other hand, transgenic mice harboring GFAP promoter-driven mutant SOD1 showed no palsy, but did show massive astrogliosis [66]. The interplay between motor neurons and glial cells has been intensively investigated by Cleveland's laboratory. First, Clement *et al.* [67] generated chimeric transgenic mice by randomly mixing morula from mutant SOD1 Tg and control mice (NF-L Tg mice or YFP mice). Their analysis revealed that the number of surrounding cells expressing mutant SOD1 regulates the survival of motor neurons. Specifically, mutant SOD1-expressing motor neurons were not injured if the surrounding cells were non-transgenic. Further studies on the role of motor neurons and glial cells in disease onset and progression, using Cre-loxP system showed

that cell-specific downregulation of G37R mutant SOD1 in motor neurons or microglia delays the development or progression, respectively [67]. Yamanaka *et al*. [68] further showed mutant SOD1 in astrocytes plays a crucial role in slowing the progression. This series of studies established the notion that mutant SOD1-induced ALS is mediated by non-cell-autonomous machinery. It should be noted that the type of mutation and promoter used in the experiments would influence these phenomena. Wang *et al*. then showed that depleting the G85R SOD1 mutant in astrocytes delayed the onset and slowed progression of the early symptomatic phase of ALS [69]. This group also documented that restricted expression of the G93A mutant in motor neurons and inter-neurons caused a mild clinical and pathological phenotype [70].

Two independent laboratories recently reported that mutant SOD1-expressing astrocytes secrete molecules that are toxic only to motor neurons [71,72]. One group identified several candidates for these molecules including prostaglandin D2 (PGD2) receptor, Mcp2, Cxcl7, and Rantes [73]. However, a PGD2 inhibitor only partial rescued the function of motor neurons cultured on monolayers of the mutant SOD1-expressing astrocytes; these cells also activate NOX2 to produce superoxide, which may be involved in motor neuron death [73]. Finally, mutant SOD1-expressing microglia were found to produce higher superoxide and nitrite, but lower IGF-1, than WT microglia, and these effects were augmented by LPS stimulation [74].

2.4. The Role of Extracellular SOD1 Mutants in the Non-Cell-Autonomous Pathology of ALS

The findings on this topic can be summarized as follows:

(1) The density of mutant SOD1-expressing cells in the anterior horns governs motor neuron survival;
(2) Mutant SOD1 in motor neurons or glial cells does indeed induce intracellular damage, and the motor neuron degeneration is accomplished by summation of these phenomena;
(3) Most of the chemical mediators identified so far are reactive oxygen species or proinflammatory molecules.

Based on our discovery of neurosecretory protein chromogranins as binding partners for mutant SOD1, we hypothesized that mutant SOD1 itself is a mediator of intercellular crosstalk in non-cell-autonomous motor neuron death. Indeed, mutant SOD1 secreted into the culture media, or externally applied recombinant mutant SOD1 protein, activates microglial cell lines and induces motor neuron death in culture [46]. We also documented that the toxicity of extracellular SOD1 mutant is mediated by CD14 on the microglia [75]. Moreover, overexpression of ChgA in G37R SOD1 Tg mice accelerated the disease onset and gliosis [76], while misfolded SOD1 was detected in the cerebrospinal fluid of patients with familial ALS using antibodies specifically recognizing misfolded SOD1 species [77,78].

2.5. WT SOD1

Interestingly, even WT SOD1 is misfolded by un- or de-metallation, and is recognized by Hsc70 *in vitro* [28]. Moreover, oxidation of WT SOD1 by H_2O_2 both *in vitro* and *in vivo* induces its misfolding and confers toxic effects on cultured motor neurons as well [79]. The role of WT SOD1 in

sporadic ALS is a matter of debate. Liu *et al*. [77] failed to detect misfolded SOD1 species in the lumbar spinal cord tissues of sporadic ALS patients using the SEDI antibody, while mutant SOD1 was immunoprecipitated by the same antibody from SOD1-linked familial ALS patients [77]. On the other hand, some work has suggested the involvement of SOD1 in the pathogenesis of sporadic cases as well. Gruzman *et al*. [80] showed that aberrantly dimerized SOD1 is a common finding in spinal cord lysates from sporadic and mutant SOD1-linked familial ALS patients, and recently, motor neurons of sporadic ALS patients were labeled by the monoclonal antibody (C4F6), which recognizes misfolded SOD1 protein, but not natively folded WT SOD1 [81]. Strikingly, Haidet-Phillips *et al*. [82] documented using iPS cells-derived astrocytes from ALS patients, that SOD1 is a target in the toxic machinery in astrocytes in familial and sporadic ALS. The misfolded SOD1 is indeed detected in the CSF of sporadic ALS patients using the antibody against misfolded SOD1, although its concentration is not high enough to induce motor neuron death [78]. However, posttranslational modifications, including oligomerization of a small fraction of SOD1 protein, would enhance the pathogenicity.

3. TAR DNA Binding Protein-43kDa (TDP-43)

In 2006, a groundbreaking discovery came from two independent research groups. A nuclear protein, TDP-43, was identified as a core protein of ubiquitinated inclusions of frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) and ALS [83,84]. Genetic mutations in TDP-43 have been detected in a fraction of familial ALS patients with or without FTLD, with several exceptions including pure FTLD [85], FTLD with progressive supranuclear palsy and chorea [86], and FTLD with Parkinsonism [87]. These lines of evidence indicate a primary role for TDP-43 in the development of ALS.

TDP-43 is a DNA/RNA binding protein, the physiological functions of which have been increasingly uncovered, including splicing or stabilization of RNA. The exon 9 skipping of the cystic fibrosis transmembrane receptor (CFTR) is a splicing example of TDP-43 [88]. In relevance to ALS, neurofilament light chain (NFL) mRNA was the first candidate target RNA to be recognized as stabilized by TDP-43 [89]. Recent attempts to grossly screen TDP-43-associated mRNAs using cross-linking and immunoprecipitation (CLIP) from brains of FTLD patients [90], mice brain with or without ablation of TDP-43 by siRNA [91], and human neuroblastoma cell line SHSY-5Y [92] revealed that huge amounts of RNA that bind TDP-43, which interacts with introns at UG/TG-rich repeat motifs of various mRNAs associated with neuronal development and neurological diseases, including TDP-43 itself and progranulin [90–92]. Knocking down TDP-43 in HeLa cells induced apoptosis via the upregulation of cyclin-dependent kinase 6 (cdk6) [93], while TDP-43 deletion in *Caenorhabditis elegans* downregulates histone deacetylase 6 (HDAC6), causing aggregate formation and promotion of the cytotoxicity caused by polyglutamine-expanded ataxin-3 [94]. TDP-43 knockdown in neuronal cultures also inactivated Rho family GTPases, including RhoA, Rac1, and Cdc42, leading to the inhibition of neurite outgrowth and cell death [95]. Based on these data, the current consensus on TDP-43-linked ALS pathogenesis argues for protein misfolding and defective RNA processing [96].

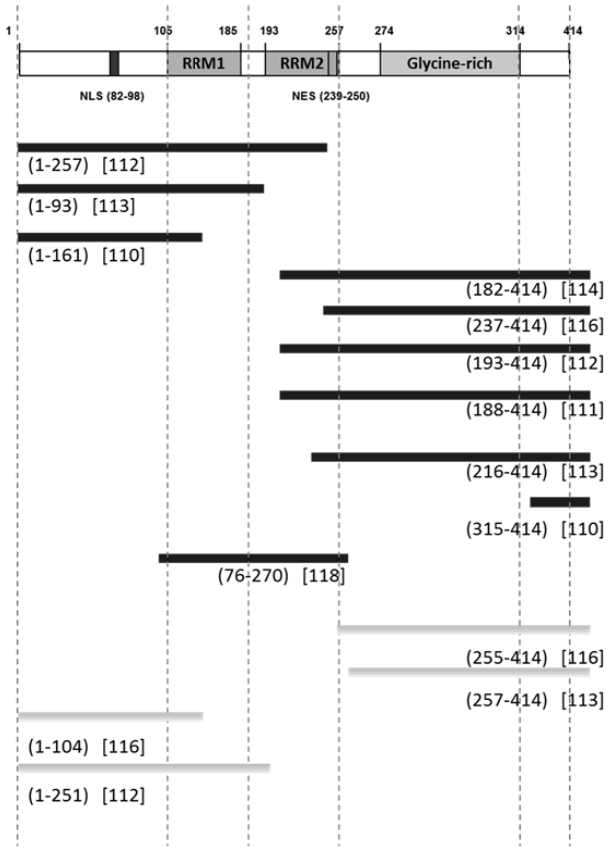
3.1. Cytosolic Redistribution of TDP-43 in ALS Pathogenesis

The original and consensus findings for TDP-43 pathologies include the aberrant cytosolic redistribution and the ubiquitinated and phosphorylated inclusions [83,84]. We have shown that both WT and mutant TDP-43 are constitutively polyubiquitinated, and are degraded in proteasomes and autophagosomes [97]. TDP-43 pathology is rarely detected in mutant SOD1-linked ALS, raising the question as to whether these two diseases are essentially different [98]. However, accumulating evidence indicates that long-lived mutant SOD1 Tg mice and a fraction of familial ALS patients with mutant SOD1 show TDP-43 pathology [99]. We also reported a familial ALS patient, with an I112T mutation in SOD1, presenting with massive expression of phosphorylated TDP-43 in the motor neurons of the brainstem and cervical cord [100]. Notably, TDP-43 redistribution has also been reported observed in several conditions not related to ALS or FTLD, including Perry syndrome [101], Lewy-body disease [102], Huntington disease [103], and inclusion body myositis [104]. Axonal injury also induces a transient redistribution of TDP-43 in rodent motor neurons [105,106].

3.2. Nuclear Localizing Signal (NLS) and the Responsible Domains for Cytosolic Redistribution

TDP-43 contains a bipartite lysine- or arginine-rich nuclear translocation signal (NLS) and leucine-rich nuclear export signal (NES), with nuclear-cytosol shuttling regulated by the importin system [105,107]. Substituting the NLS amino acids effectively altered the nuclear localization of TDP-43 [97,108]. In particular, modifying both NLS residues more efficiently induced cytosolic aggregates to be formed than a single amino acid alteration [108]. Studies by Arai *et al*. [83] and Neumann *et al*. [84] identified 35-kDa or 25-kDa fragments in the affected lesions of ALS or FTLD patients. TDP-43 possesses amino acid sequences that mimic caspase recognition sites just carboxyl to the NLS, and the cleavage of these also generated 25-kDa and 35-kDa fragments in the cytosol [109]. Importantly, Nishimoto *et al*. showed TDP-43 fragments of the same molecular sizes produced in the absence of caspase, and that these fragments are also generated as alternate isoforms [110]. This finding suggested that the fragmentation of TDP-43 could be a primary event in triggering ALS, although it remains unclear whether the generation of such aberrant isoforms is a regulated or random event. The cytosolic aggregates were predominantly carboxyl fragments, since they were labeled mostly by anti-*C*-terminal TDP-43, but much less frequently by the antibody recognizing *N*-terminus [111]. Accumulating evidence shows that the *C*-terminus of TDP-43 has a higher propensity for aggregate formation than other domains [112–114]. However, it should be noted that carboxyl fragments without the 2nd RNA binding domain, RRM2 do not form cytosolic aggregates [115]. In agreement with this, recent studies highlighted the importance of RRM2 domains in the cytosolic redistribution or aggregate formation of TDP-43 [114–116], although the molecular basis by which RRM2 would be involved in the TDP-43 misfolding remains unclear. In addition, RRM1, the 1st RNA binding domain, together with the amino terminal fragment of TDP-43 reportedly mediates dimer formation in transfected cells in culture [117]. The possible roles for the various TDP-43 fragments in cytosolic aggregate formation are summarized in Figure 2.

Figure 2. Fragments of TDP-43 and their effects on aggregate propensity or cytotoxicity. Black bars are reported to generate cytosolic aggregates or to induce cell death *in vitro* and *in vivo*. Grey bars were shown not to provide detrimental effects. Amino acid sequence and its source reference are indicated under each bar. References in square brackets are shown in the Reference section.



3.3. Pathogenic Role of Mislocalized TDP-43 in the Cytosol

The possible role of cytosolic TDP-43 as a trigger for ALS pathology remains unclear. In yeast, TDP-43 fragments without a functional NLS domain redistributed into the cytosol, although only those fragments containing the entire RRM2 and the carboxyl terminus induced toxicity [118]. Moreover, Tg mice expressing TDP-43 with an NLS substitution to alanine showed massive cytosolic aggregates and neuronal death [119]. On the other hand, a study in *C. elegans* revealed the motor phenotype only in the presence of both RRM1 and RRM2 together with the carboxyl terminal, while NLS-defective TDP-43 showed no motor impairment [120]. Aggregate formation of TDP-43 does not immediately follow its cytosolic redistribution, and using the elegant *in vivo* cleavage system with TEV protease, Pesiridis *et al*. [116] found that aggregates containing carboxyl fragments of TDP-43 were actually prompted by the disruption of dynein-mediated transport and RNA interaction. It should also be noted that the cytosolic redistribution of TDP-43 induces nuclear exclusion of the endogenous TDP-43 [108], which may link to the loss of function of nuclear TDP-43. On the other hand, the cytosolic redistribution of TDP-43 does not lead to neurodegeneration in either WT or mutant TDP-43 Tg mice [121,122]. Finally, we found that polyubiquitinated TDP-43 is present to a similar extent between

WT and NLS-defective TDP-43, arguing against the early notion that nuclear exclusion triggers the polyubiquitination of TDP-43 [97].

3.4. WT and Mutant TDP-43 in Motor Neuron Degeneration

Over 40 mutations involving amino acid replacement have been reported in TDP-43 [123], with most located in the carboxyl terminus (267-414), one mutation (D169G) in RRM1 (103-183), and two (Y214Y, P225P) in RRM2 (190-26) (Table 1). Biochemical studies suggested that mutant TDP-43 has a higher propensity for aggregate formation and carboxyl termini fragmentation of 25- or 35-kDa fragments [124–127]. Moreover, the overexpression of mutant TDP-43 induces cell death in chick embryonic spinal motor neurons [126], primary motor neuron cultures [124], *C. elegans* [128], and transgenic rodents [121,129,130]. Swarup *et al.* [131] found a moderate phenotype expressed by Tg mice driven by the native promoter of human TDP-43, including cognitive impairment and motor coordination with massive cytosolic redistribution of TDP-43, while ALS-linked mutant TDP-43 proteins more readily aggregate in stress granules (SG) than the WT protein [132].

Table 1. Previously identified genetic mutations in TDP-43. Mutations in the exon in regard with the cell toxicity are shown.

Domain	Mutation	Cell death	Reference
N-terminus	D65E		[133]
	A66A		[133]
	A90V		[126]
RRM1	D169G		[124]
RRM2	Y214Y		[134]
	P225P		[133]
	N267S		[135]
Glycine-rich	G287S		[136]
	G290A		[137]
	G290S		[136]
	G294A		[126]
	G294V		[136]
	G295R		[135]
	G295S		[135]
	G298S		[137]
	M311V		[136]
C-terminus	A315A		[133]
	A315T	Tg mice, Cultured cells, Zebrafish	[124,131]
	Q331K	Cultured cells	[126]
	S332N		[135]
	G335D		[136]
	M337V	Tg rat, Cultured cells, Zebrafish, chick embryo	[125,126,129]
	Q343R		[136]

Table 1. *Cont.*

Domain	Mutation	Cell death	Reference
	N345K		[136]
	G348C	Tg mice, Cultured cells, Zebrafish	[124,131]
	N352N		[133]
	N352S		[136]
	[144] 61S		[124]
	P363A		[136]
	Y374X		[136]
C-terminus	S379C		[136]
	S379P		[135]
	A382P		[136]
	A382T		[124]
	I383V		[136]
	N390D		[124]
	N390S		[136]
	S393L		[135]

Our review of the literature found limited types of mutations that induce cell death in cultured cells or Tg mice and all of these are located between the carboxyl terminus and the glycine-rich domain (Table 1). Further studies are needed to investigate the effect of mutations in other parts of the TDP-43 protein such as RRM1, RRM2, or the glycine-rich domains. WT TDP-43 transgenic mice have also showed motor neuron degeneration with paralysis [123,131], although several other studies observed no overt phenotype [129,130].

It remains a matter of debate whether TDP-43-induced ALS is caused by a loss or gain of protein function. A sciatic axotomy upregulates TDP-43 in rodents [106], and the expression level of TDP-43 in ALS patients was up to twice that in non-ALS control [131]. It should also be noted that the aberrant cytosolic TDP-43 brings endogenous TDP-43 to the cytosol, inducing loss of TDP-43 function in the nucleus. Further investigation comparing the temporal profiles of TDP-43 expression levels and the mislocalization of TDP-43, at least *in vivo*, would further our understanding of the TDP-43 proteinopathy.

4. Aberrant Localization and Therapeutic Hints

Non-cell autonomous neuronal cell death has been implicated in various neurodegenerative diseases [13]. The recent notion that disease-causative proteins can also affect neighboring cells via secretion is attracting significant attention as a possible mechanism underlying the temporal and spatial progression of disease [138,139]. Studies have shown that phosphorylated tau [140], α-synuclein [141], and Huntingtin [142] proteins can spread out and behave like a prion protein in cell culture models of Alzheimer's, Parkinson's, and Huntington's disease, respectively. Moreover, SOD1 aggregates in the culture media were recently detected inside cells, supporting a prion-like behavior of the misfolded SOD1 [143]. Aggregated SOD1 also acquires a seeding effect in which recombinant mutant SOD1 accelerates fibril formation of previously folded SOD1 species [144]. Furthermore, a recent paper has provided evidence that mutant or misfolded human SOD1 induces a species-specific conformational conversion of native WT SOD1 to misfolded species, which is surprisingly mediated by the tryptophan

at 32 amino acid residue [145]. We and others proved that extracellular SOD1 mutant can be a therapeutic target for vaccination or antibody therapy against misfolded SOD1 [146–148]. In the case of TDP-43, mounting data supports that TDP-43 aggregates have a seeding effect *in vitro* [114,149], although the possible spreading of TDP-43 is not proven. However, considering the clinical evidence that cerebrospinal fluid TDP-43 [150–152], it is possible that TDP-43 also behaves like a prion. In this sense, if mislocalized TDP-43 triggers loss of function of nuclear TDP-43, elimination of cytosolic TDP-43 is a challenging but attractive therapeutic strategy. For instance, the monoclonal antibody strategy could have extensive applications clinically including using intrabody or Fab molecules for molecular targeting of such species.

5. Conclusions

We present evidence in this review that the misdirection of the ALS-linked proteins can ultimately account for various pathological signaling. The efficacy of any molecular targeting strategy depends on our understanding of the aberrant subcellular localization of these proteins. Further investigation is required to uncover the molecular basis and the downstream cascades of the mislocalized, ALS-linked proteins to develop more innovative therapy.

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