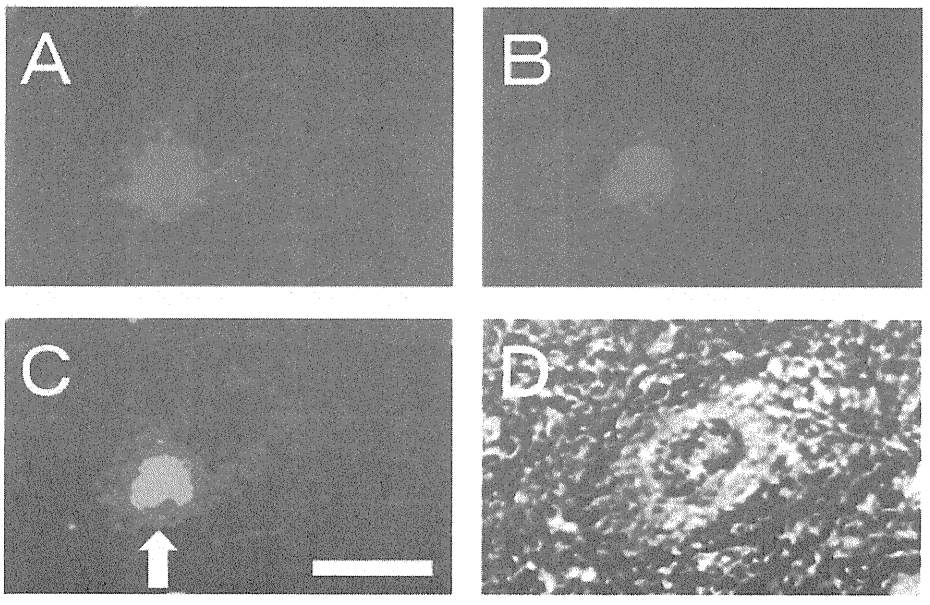

Figure 5
Double immunostaining of NCI from a patient with FALS (cervical spinal cord). SOD1 and PDI are co-localized in NCI (arrow). Green: anti-PDI antibody immunostaining (A). Red: anti-SOD1 antibody immunostaining (B). Yellow: merged immunostaning (C). Differential interference contrast (DIC): (D). Scale bar: 20 μm.
59x37mm (600 x 600 DPI)

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ORIGINAL PAPER

Clinicopathologic study on an ALS family with a heterozygous E478G *optineurin* mutation

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Abstract We investigated a family manifesting amyotrophic lateral sclerosis (ALS) with a heterozygous E478G mutation in the *optineurin* (*OPTN*) gene. Clinically, slow deterioration of motor function, mood and personality changes, temporal lobe atrophy on neuroimaging, and bizarre finger deformity were noted. Neuropathologically, TAR DNA-binding protein 43 (TDP-43)-positive neuronal intracytoplasmic inclusions were observed in the spinal and medullary motor neurons. In these cells, the immunoreactivity of nuclear TDP-43 was reduced. Consecutive sections revealed that the inclusions were also reactive with anti-ubiquitin and anti-p62 antibodies, but noticeably negative for OPTN. In addition, TDP-43/p62-positive glial cytoplasmic inclusions (GCIs) were scattered throughout the spinal cord and the medullary motor nuclei. Furthermore, Golgi fragmentation was identified in 70% of the anterior horn cells (AHCs). The presence of AHCs with preserved nuclear TDP-43 and a fragmented Golgi apparatus, which are unrecognizable in sporadic ALS, indicates that patients with the E4787G *OPTN* mutation would manifest Golgi fragmentation before loss of nuclear TDP-43. In the neocortex, GCIs were sparsely scattered among the primary motor and temporal cortices, but no neuronal TDP-43-positive inclusions were detected. In the amygdala and the ambient gyrus, argyrophilic grains and ballooned neurons were seen. The thorough neuropathologic investigations performed in this work demonstrated that OPTN-positive inclusion bodies, if any, were not prominent. We postulate that optineurinopathy is closely linked with TDP-proteinopathy and speculate that this heterozygous E478G mutation would cause ALS by acting through a dominant-negative mechanism.

Keywords Amyotrophic lateral sclerosis · Optineurin · TDP-43 · Golgi fragmentation

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Introduction

We recently reported that mutations in the gene encoding optineurin (*OPTN*) cause amyotrophic lateral sclerosis (ALS) [15]. *OPTN* had previously been identified as a causative gene of primary open-angle glaucoma (POAG) [18]. However, the sites of mutation in the *OPTN* gene found in ALS patients were distinct from those in cases with POAG. In addition, we demonstrated that OPTN is co-localized with TAR DNA-binding protein of 43 kDa (TDP-43) or Cu/Zn superoxide dismutase (SOD1) in the pathognomonic inclusion bodies of sporadic ALS (SALS) or familial ALS associated with *SOD1* mutation (SOD1-FALS), respectively [15]. The presence of OPTN immunoreactivity in TDP-43-positive inclusions of SALS patients was subsequently confirmed by other investigators [9, 17]. In addition, we recently demonstrated that OPTN is also co-localized with *fused* in *sarcoma* (FUS) within basophilic inclusions of ALS with the *FUS* mutation and in basophilic inclusion body disease [10]. Our findings thus indicate that OPTN associates with each of three major ALS-related proteins, i.e., TDP-43, SOD1, and FUS, suggesting that the underlying pathomechanism in ALS might be attributable to dysfunctional OPTN.

We identified eight ALS cases associated with three distinct types of *OPTN* mutation (OPTN-ALS) [15]: two siblings with a homozygous deletion of exon 5, two cases with a homozygous Q398X nonsense mutation, and four patients with a heterozygous E478G missense mutation within its ubiquitin-binding domain. Detailed clinicopathological features of patients with each mutation remain unknown. Moreover, whereas the pathomechanism causing the disease by the homozygous mutations is speculated to be a loss of function resulting from nonsense-mediated mRNA decay of the transcript, that of the heterozygous E478G mutation remains uncertain.

Here, we provide further clinicopathologic information about Family 4 [15] with the E478G mutation. Although their clinical features and our neuropathologic findings have previously been reported in brief [15], we obtained some new and novel information by examining the living patient and interviewing her daughters, and by investigating the autopsied material thoroughly.

Subjects and methods

Clinical features

Three siblings were affected in this family (Fig. 1a). Their mother died at age 32 from heart disease. Their father then married the mother's younger sister and had four more children (denoted by the diamond symbol). The father was over 80 years old at death, and all of his other four children are now over 60 years of age with no signs of ALS.

The demographic and clinical features of the three OPTN-ALS patients are summarized in Table 1.

Patient III-1 had noted right-hand weakness at age 58. Muscle weakness of all four limbs, dysarthria, and dysphagia followed. Her nieces noticed that she had become irritable and touchy. She was diagnosed as having ALS and died of pneumonia after artificial ventilation for several months at age 63.

Patient III-2 suffered from right-hand weakness at age 56. Flexion deformity of her fingers gradually developed four years later. Examinations at age 61 disclosed dysarthria, atrophy, fasciculation in the tongue, and exaggerated deep tendon reflexes and bilateral extensor plantar responses in all four extremities. She was depressed but not demented. A cranial MRI demonstrated mild atrophy of the medial temporal region (Fig. 1b). She died of CO_2 narcosis without respiratory support at age 66.

Patient III-3 suffered from right-hand weakness at age 64. Leg weakness, dysarthria, and dysphagia followed slowly afterward. She could communicate well with others until age 75, when she became taciturn and depressive. A cranial CT scan at age 76 showed pronounced temporal lobe atrophy (Fig. 1c). Examinations at age 78 revealed generalized atrophy and fasciculation of skeletal muscles, reduced deep tendon reflexes, and bilateral extensor plantar responses. Atrophy of the tongue was mild. Conspicuously, her fingers were bizarrely deformed, resulting in difficulty in passive movement of any finger joints (Fig. 1d). We observed 4-Hz rhythmic tremor of the fingers of her left hand. She was awake, and eye contact was preserved, but appeared expressionless and mute. She is alive after 14 years from the onset without respiratory support.

No patients developed decubitus, ophthalmoplegia, glaucoma, or cardiac or muscular abnormalities. Blood tests, including those on alkaline phosphatase and creatine phosphokinase, were normal. Chest and spine X-rays did not show any evidence of Paget's disease.

We had previously identified a heterozygous missense mutation (c.1743A>G, E478G, exon14) in the *OPTN* gene of Patients III-2 and III-3 [15]. Genetic analysis and cognitive testing were not performed on the other family members because of the lack of informed consent.

Neuropathological examinations

Formalin-fixed, paraffin-embedded 6-μm-thick sections were deparaffinized and stained with hematoxylin and eosin (H&E) or subjected to Gallyas–Braak silver impregnation. For immunohistochemistry, after antigen retrieval by heat/autoclaving (10 min at 121°C in 10 mM sodium citrate buffer, pH 6.0), the sections were incubated with a given

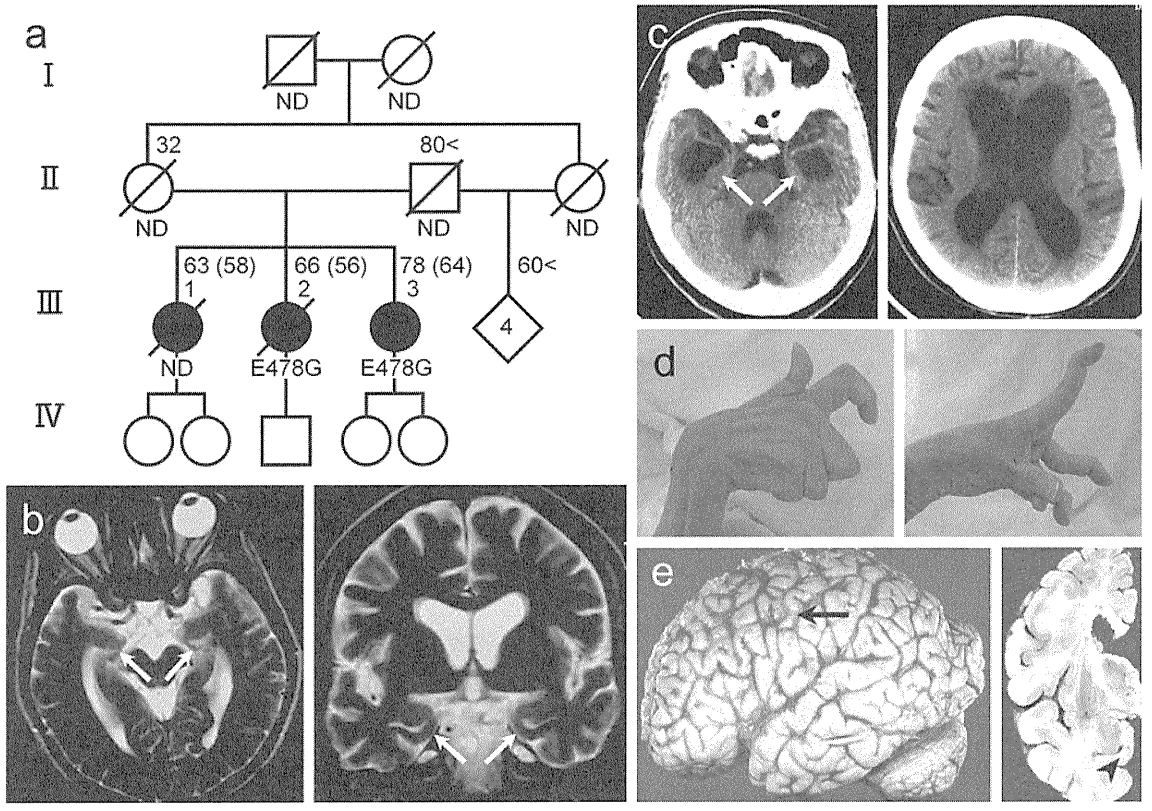
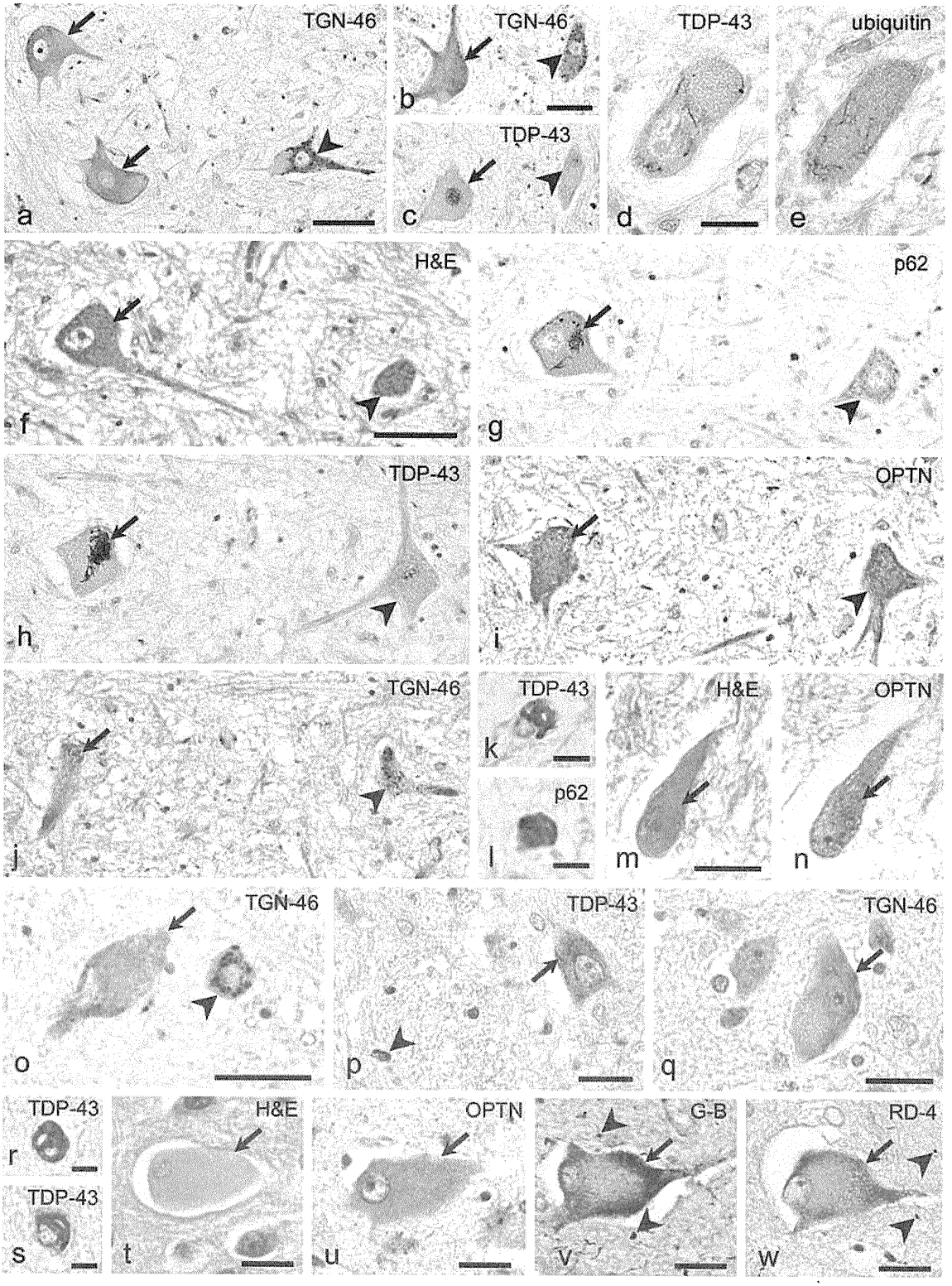


Fig. 1 Clinical and neuropathologic findings of the familial amyotrophic lateral sclerosis (FALS) patients with an *optineurin* (*OPTN*) mutation. The three patients in the family pedigree are indicated by the *solid circles* (**a**). A heterozygous E478G mutation in the *OPTN* gene was detected in Patients III-2 and III-3. *ND* not determined. Age at death or current age and age at disease onset are indicated *n (m)*. Deceased individuals are indicated by the *oblique line*. A cranial MRI of Patient III-2 at age 65 (**b**) reveals mild atrophy of the ambient gyri (*arrows*). A cranial CT scan of Patient III-3 at age 76 (**c**) reveals conspicuous atrophy of the medial temporal lobes (*arrows*) and mild atrophy of the frontal lobe. Gradually progressive bizarre deformity of the hands of Patient III-3 is striking (**d**). Photographs of the brain from Patient III-2 (**e**) reveal slight atrophy of the motor cortex (*arrow*) and of the ambient gyrus (*arrowhead*)

Table 1 Demographic and clinical features of patients with a heterozygous E478G *OPTN* mutation

Patient	III-1	III-2	III-3
Age at onset (years)	58	56	64
Gender	Female	Female	Female
Symptom at onset	Right-hand weakness	Right-hand weakness	Right-hand weakness
Upper motor neuron signs	Unknown	+	+
Lower motor neuron signs	+	+	+
Cognitive symptoms	Personality change	Depression	Depression
Other clinical features	–	Finger deformity	Finger deformity, Parkinsonian tremor
Neuroimaging	Unknown	Mild temporal lobe atrophy	Marked temporal lobe atrophy
Disease duration (years)	5	10	>14
Artificial ventilation	For several months	–	–
Cause of death	Pneumonia	CO_2 narcosis	Alive
Genetic analysis	Unavailable	E478G in *OPTN* gene	E478G in *OPTN* gene

primary antibody (listed in Online Resource 1) overnight at 4°C. Bound antibodies were detected with the appropriate Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA), with 3,3′-diaminobenzidine tetrahydrochloride used as the chromogen. All sections were counterstained with hematoxylin after immunohistochemistry. Some sections were stained with H&E, photographed, decolorized with 70% ethanol, and then immunostained for OPTN. The tissues from three age-matched neurologically normal subjects served as controls.



We assessed staining specificity by replacing the primary antibodies with an appropriate amount of non-immune rabbit serum or phosphate-buffered saline solution containing 3% bovine serum albumin. No deposits of reaction products were seen in the sections thus treated (data not shown).

Procedures involving use of human material were performed in accordance with ethical guidelines set by Shiga University of Medical Science and the Helsinki Declaration of 1983. No frozen tissue was available.

◀ **Fig. 2** Representative photomicrographs of the lumbar anterior horn (**a–n**), the facial nucleus (**o**, **p**), and the cerebral cortices (**q–w**) from Patient III-2. Immunostaining with anti-trans-Golgi-network 46 (*TGN-46*) antibody demonstrates evident fragmentation of the Golgi apparatus (GA) in some of the anterior horn cells (AHCs, *arrows*), in comparison with the preserved GA (*arrowhead*) in others (**a**). Consecutive sections stained with anti-TGN-46 (**b**) and anti-TDP-43 (**c**) antibodies indicate a neuron with normal nuclear TDP-43 immunoreactivity and obviously fragmented GA (*arrow*). The other neuron in these sections has a normal GA with preserved TDP-43 nuclear staining (*arrowhead*). A noticeable skein-like cytoplasmic inclusion immunoreactive for TDP-43 (**d**) and ubiquitin (**e**) is identifiable in consecutive sections. The physiological nuclear TDP-43 immunoreactivity is absent (**d**). Five consecutive sections stained with H&E (**f**) and immunostained for p62 (**g**), TDP-43 (**h**), optineurin (**i**), and GA (**j**) in this order reveal that a TDP-43-positive skein-like inclusion (**h**, *arrow*) is also reactive with anti-p62 antibody (**g**, *arrow*), which inclusion is indiscernible on the H&E-stained section (**f**, *arrow*). Note that the inclusion is devoid of optineurin (OPTN-I)-labeling (**i**, *arrow*). The GA is fragmented in this neuron (**j**, *arrow*) compared with the spared AHC with preserved TDP-43 nuclear staining (**f–j**, *arrowheads*). Glial cytoplasmic inclusions (GCIs) immunoreactive with anti-TDP-43 (**k**) and anti-p62 (**l**) antibodies are scattered throughout the spinal cord. The eosinophilic cytoplasmic hyaline region of this AHC (**m**) was decolorized and re-stained with the OPTN-C antibody (**n**), resulting in positive staining; however, prominent OPTN-positive inclusion bodies were not evident. GA fragmentation is apparent in this motor neuron of the facial nucleus immunostained with TGN-46 antibody (**o**, *arrow*), whereas another neuron has a preserved GA (*arrowhead*). By staining with anti-TDP-43 antibody, a skein-like inclusion (**p**, *arrow*) and a GCI (*arrowhead*) are clearly identifiable in the facial nucleus. A Betz cell within the primary motor cortex (**q**) shows reduced immunoreactivity with TGN-46 antibody (*arrow*). Only sparsely scattered TDP-43-positive GCIs are detectable in the frontal (**r**) and the temporal (**s**) cortices. Ballooned neurons in the ambient gyrus (**t–w**, *arrows*) are immunopositive in their entire cytoplasm for OPTN (**u**), stained at their periphery by Gallyas–Braak (*G–B*) silver staining (**v**, *arrow*), and are reactive with anti-4-repeat tau (*RD-4*) antibody (**w**, *arrow*). Argyrophilic grains (**v**, *arrowheads*), immuno-positive for 4-repeat tau (**w**, *arrowheads*), are also observed. *Scale bars* 50 μm (**a**, **b**, **f**, **o**), 20 μm (**d**, **m**, **p**, **q**, **t–w**), and 10 μm (**k**, **l**, **r**, **s**)

Results

The brain of Patient III-2 weighed 1,250 g. Macroscopically, the primary motor and medial temporal cortices appeared slightly atrophic (Fig. 1e).

Throughout the spinal cord, the anterior horns and the corticospinal tracts had degenerated. Additional immunohistochemical investigation revealed characteristic fragmentation of the Golgi apparatus (GA) in the anterior horn cells (AHCs; Fig. 2a). Quantitative analysis using a method described elsewhere [8] revealed that 72.8% (75/103) of the AHCs from eight distinct spinal cord segments had fragmented GAs. Analysis of consecutive sections immunostained for GA and TDP-43 revealed GA fragmentation not only in all the AHCs with reduced nuclear TDP-43 immunoreactivity but also in a substantial number of those with preserved nuclear TDP-43 (Fig. 2b, c). In contrast, a normal staining pattern for GAs was observed for non-motor neurons.

More importantly, we identified TDP-43/ubiquitin-positive skein-like inclusions in AHCs (Fig. 2d, e). The nucleus of these inclusion-bearing neurons was invariably immunonegative for TDP-43. Consecutive sections revealed that the TDP-43-positive inclusions were also reactive with anti-p62 antibody; they were difficult to recognize on H&E-stained sections and noticeably negative for OPTN on use of either the OPTN-C or OTPN-I antibodies (Fig. 2f–i). This finding was confirmed by double immunofluorescence investigation (Online Resource 2). The GA in AHCs with such inclusions was fragmented (Fig. 2j). We identified inclusions in 12.5% (19/152) of AHCs on 20 cervical and lumbar cord sections immunostained for TDP-43. In addition, TDP-43/p62-positive glial cytoplasmic inclusions (GCIs) were scattered throughout the spinal cord (Fig. 2k, l).

Careful examination of 265 AHCs on 30 H&E-stained sections revealed no Bunina bodies or round hyaline inclusions in these cells. Cystatin C immunohistochemistry failed to detect Bunina bodies in 182 AHCs examined. Eosinophilic intracytoplasmic regions were noted in several AHCs, which showed immunoreactivity when decolorized and then re-stained with each of the anti-OPTN antibodies (Fig. 2m, n). Occasionally, these eosinophilic retentions appeared to have formed inclusion-like structures; however, OPTN-positive prominent inclusion bodies delineated by a distinct margin were completely unrecognizable.

In the hypoglossal and facial nuclei, motoneurons were depleted in number, the GA was fragmented, and TDP-43-positive inclusions were identified (Fig. 2o, p). Betz cells were mildly depleted in number, and the remaining cells had reduced immunoreactivity for GA (Fig. 2q). TDP-43-immunoreactive GCIs were sparsely scattered among the medullary motor nuclei (Fig. 2p), primary motor and temporal cortices (Fig. 2r, s), putamen, and thalamus, but no neuronal intracytoplasmic inclusions were found other than in the spinal and medullary motor neurons. No intranuclear inclusions were identifiable throughout the central nervous system.

In the amygdala and the ambient gyrus, numerous argyrophilic, 4-repeat tau-positive grains, and several ballooned neurons were seen (Fig. 2t–w). The cytoplasm of these neurons was eosinophilic, and diffusely immunopositive for OPTN and phosphorylated neurofilaments; the cells were stained at their periphery by Gallyas–Braak silver impregnation and with anti-4-repeat tau antibody. There was faint, if any, immunoreactivity indicating ubiquitin, and the cells were negative for p62, α-synuclein, 3-repeat tau, TDP-43, FUS, SOD1, and ApoE. This III-2 case corresponded to argyrophilic grain disease (AGD), stage II [5, 20].

By amyloid β and AT8-immunohistochemistry this case was graded as amyloid stage A and NF stage II,

respectively [3]. Immunostaining for α-synuclein, FUS, and SOD1 revealed no pathologies. Additional genetic analysis of Patients III-2 and III-3 revealed no mutations in their *TARDBP*, *GRN* or *VCP* genes.

Discussion

Motor symptoms of our patients were indistinguishable from those of SALS. However, the rate of deterioration was noticeably slow in both of the genetically proven patients, suggesting that slow progression might be a feature of patients with a heterozygous E478G *OPTN* mutation. Progression was faster for their elder sister, whose DNA was unavailable, showed, implying intrafamilial variability. All the patients developed personality and mood changes, and neuroimaging showed medial temporal lobe atrophy. These features are consistent with those of AGD [5], which was confirmed neuropathologically for Patient III-2. In SALS, concomitant AGD is reported in 7.7–22% of cases [14, 20]. Whether FALS with mutated *OPTN* would be prone to coincide with AGD awaits clarification. Furthermore, finger deformity was observed in our patients. This feature might be a consequence either of dystonia or of chronic arthritis induced by disinhibited NF-κB because of the *OPTN* mutation [15]. The parkinsonian tremor observed in Patient III-3 could be a manifestation of the E478G mutation or simply coincidental.

Neuropathologically, neuronal intracytoplasmic inclusions immuno-positive for TDP-43, ubiquitin, and p62 were unequivocally identified in the spinal and medullary motoneurons. They were morphologically indistinguishable from those observed in SALS. However, OPTN was noticeably not co-localized within the inclusions, in contrast with those of SALS [15]. Although negative immunohistochemical results inherently warrant further investigation, this finding suggests that not only the mutated but also the wild-type OPTN would be impaired in its association with TDP-43. The molecular link between OPTN and TDP-43 is unknown. OPTN might function in TDP-43 transportation for degradation, and hence, dysfunctional OPTN could cause TDP-43 mislocalization, resulting in neurodegeneration.

TDP-43 pathology associated with FALS (ALS-TDP) and/or frontotemporal lobar degeneration (FTLD-TDP) has been reported [13] in patients with mutations in genes encoding TDP-43 (*TARDBP*), progranulin (*GRN*), and valosin-containing protein (*VCP*), and in one case with a mutation in *ANG* encoding angiogenin, who manifested atypical clinicopathological features [21]. TDP-43 pathology indistinguishable from that of SALS and/or FTLD was observed for mutations in *TARDBP* [13], through both gains and losses of function [22]. Patients with *GRN* mutations manifest FTLD, and TDP-pathology develops principally in the neocortex [12], through a haploinsufficiency mechanism [2, 4]. *VCP* mutations were originally identified in patients with inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia (IBMPFD) [23]. Identification of TDP-43, but not VCP, within ubiquitinated inclusions in these cases implies that *VCP* mutations lead to a dominant-negative loss of VCP function, with degradation of TDP-43 [16]. More recently, *VCP* mutations were also shown to cause autosomal dominantly inherited FALS [11]. One autopsy case revealed motor neuron degeneration with intracytoplasmic TDP-43-positive inclusions and Bunina bodies in the remaining cells. Our patients showed no mutation of their *TARDBP*, *GRN*, or *VCP* gene. However, the association of *OPTN* with Paget's disease, found by a recent genome-wide association study [1], and the similar biological function of OPTN and VCP warrant further investigation.

The presence of TDP-43 pathology has been reported in 60% of AGD cases [6]. In those cases, TDP-43-positive structures were mainly observed in the limbic regions and lateral occipitotemporal cortex [6]. The difference between the distribution of TDP-43 pathology of our patient and that of AGD cases implies that the pathomechanism of TDP-43 pathology in optineurinopathy would be distinct from that in AGD.

GA fragmentation in our Patient III-2 is plausible, because OPTN plays an important role in maintaining the GA [19]. The number of AHCs with GA fragmentation for our case (72.8%) was notably higher than that reported for SALS (8.3–52.6%, mean 29.6%) [8]. However, because this percentage varies markedly in SALS patients, it remains to be elucidated whether the ratio of GA fragmentation in AHCs of OPTN-FALS patients would be generally higher than that of SALS patients. Moreover, the presence of AHCs with preserved nuclear TDP-43 and showing fragmented GA, unrecognizable in SALS [7], indicates that patients with the E478G *OPTN* mutation would manifest GA fragmentation before loss of nuclear TDP-43. The relevance of GA fragmentation and TDP-43 nuclear staining to ALS awaits further clarification. In contrast, consistently preserved GA of non-motor cells implies that unrecognized GA-maintaining systems other than the OPTN system are operating in those neurons, affording them less vulnerability to dysfunctional OPTN.

The mutations of the *OPTN* gene causing FALS are unique in that recessive and dominant traits have similar symptoms. The mechanisms of neurodegeneration in the homozygous deletion of exon 5 and the homozygous Q398X nonsense mutation are conceivably speculated to be a loss function resulting from nonsense-mediated mRNA decay of the transcript. In contrast, the pathomechanisms operating in the case of the heterozygous E478G mutation remain unknown. The mechanism of dominant mutations causing a disease is assumed to be toxic gain-of-function, loss of function because of haploinsufficiency, or a dominant-

negative loss of OPTN function. Among these, a gain-of-function mechanism would be implausible because diseases caused by such a mechanism are usually associated with the presence of distinct inclusion bodies consisting of mutant proteins. However, the thorough neuropathologic investigations performed in this work demonstrated that OPTN-positive inclusion bodies, if any, were not prominent in our patient. A haploinsufficiency mechanism would be also unlikely, because individuals with the heterozygous exon 5 deletion or Q398X mutation, in whom half of the amount of OPTN is abolished by nonsense-mediated mRNA decay, manifest no motor neuron signs although the number of such subjects examined thus far is small. In contrast, a dominant-negative loss-of-function mechanism would be a possibility; being similar to that for patients with *VCP* mutations who manifest FTLD [16]; ubiquitinated inclusions identified in the AHCs of our patient demonstrated immunoreactivity for TDP-43, but not for OPTN. OPTN is reported to form homohexamers [24] and, thus, mutant OPTN could conceivably impair the formation of properly functioning hexamers, thus having a dominant-negative effect. This notion is consistent with the fact that the four patients with proved heterozygous E478G *OPTN* mutation [15] had later onset and longer disease duration (55.0 ± 6.7 years, longer than 7.6 ± 5.5 years (1 patient is still alive), respectively) than those with homozygous *OPTN* null mutations (41.3 ± 8.5 years and 4.0 ± 3.6 years, respectively).

For determination of the clinicopathologic features and pathomechanism of FALS with mutated *OPTN*, further studies with additional cases are needed.

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Conflict of interest The authors declare they have no conflict of interest.

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CORRESPONDENCE

Optineurin is co-localized with FUS in basophilic inclusions of ALS with *FUS* mutation and in basophilic inclusion body disease

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We recently reported that mutations in the gene encoding optineurin (OPTN) cause amyotrophic lateral sclerosis (ALS) [2]. In that report, we demonstrated the co-localization of OPTN with TAR DNA-binding protein of 43 kDa (TDP-43) or Cu/Zn superoxide dismutase (SOD1) in the pathognomonic inclusions of sporadic (SALS) or familial ALS (FALS) with mutated SOD1, respectively [2].

Fused in *sarcoma* (FUS) is another causative gene of ALS [1, 7]. FUS-immunoreactivity is identifiable in basophilic inclusions (BIs) from patients with sporadic basophilic inclusion body disease (BIBD) [4] and in those from 'FALS with FUS mutation' patients. The fact that both FUS and OPTN cause ALS when mutated prompted us to investigate the correlation between these proteins.

We analyzed postmortem material from three patients with sporadic BIBD and from three with FALS with FUS mutation. All the patients manifested upper and lower motor neuron signs, but no cognitive impairment was noted. Their demographic and clinical features are given in Online Resource 1. The 'FALS with FUS mutation' patients had missense mutations R514S, R521C, and P525L in their respective FUS gene. Genetic analysis of the sporadic BIBD patients for FUS and OPTN was unsuccessful, probably because of deterioration of the genomic DNA in the formalin-fixed material. No frozen tissue was available.

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Paraffin-embedded lumbar cord, frontal cortex, and brainstem were uniformly investigated immunohistochemically (Online Resource 2). The primary antibodies used are listed in Online Resource 3. To confirm the co-localization of OPTN and FUS, we employed a staining procedure on consecutive sections and double immunofluorescence staining (Online Resource 2).

In the sections from controls, the OPTN and myosin VI immunoreactivities were faintly recognizable in the neuronal cytoplasm; and the anti-FUS antibodies showed essentially no immunoreactivity when they were titrated in a way that did not recognize physiologic FUS (data not shown).

In the BIBD and 'FALS with FUS mutation' cases, H&E staining invariably demonstrated neuronal intracytoplasmic BIs in all the regions examined. Immunohistochemically, virtually all the BIs were positive for OPTN, FUS, and myosin VI (Fig. 1a–c, respectively). In contrast, the antibodies against TDP-43 and SOD1 did not react with the BIs (Fig. 1d, e, respectively). Noticeably, staining of the consecutive sections by H&E and immunohistochemistry for FUS and OPTN, as well as the double immunofluorescence staining for FUS and OPTN, evidently demonstrated that the distribution of OPTN immunoreactivity faithfully matched that of FUS within the BIs (Fig. 1f–l). OPTN immunoreactivity in FUS-positive glial inclusions was indiscernible. Further studies are warranted to clarify whether or not OPTN would co-localize with FUS within structures other than the BIs.

We recently showed that OPTN is co-localized with TDP-43 or SOD1 [2]. However, as shown here, the BIs in the above patients showed no immunoreactivity for TDP-43 or SOD1, but were positive for FUS as well as OPTN. Therefore, our present and earlier results provide evidence that OPTN associates with each of 3 major ALS-related proteins, i.e., TDP-43, SOD1, and FUS.

The pathomechanism of involvement of OPTN in the BIs and that of neurodegeneration in BIBD and 'FALS with FUS mutation' patients remain to be elucidated. Since OPTN and FUS share roles in intracellular trafficking in collaboration with myosin VI, it is likely, at least under pathologic conditions, that these proteins would encounter each other when delivering cargos, and could conceivably form a complex through myosin VI within the BIs. Thereby, OPTN and FUS would be sequestered from the cytoplasm.

FUS is known to act as a co-activator of NF-κB [6]. On the contrary, OPTN negatively regulates NF-κB activation [2]. Therefore, it is plausible that sequestration of both OPTN and FUS would induce dysregulation of NF-κB activation, leading to neurodegeneration.

Another promising hypothesis concerns a dysfunctional Golgi apparatus. OPTN and myosin VI play a role in the

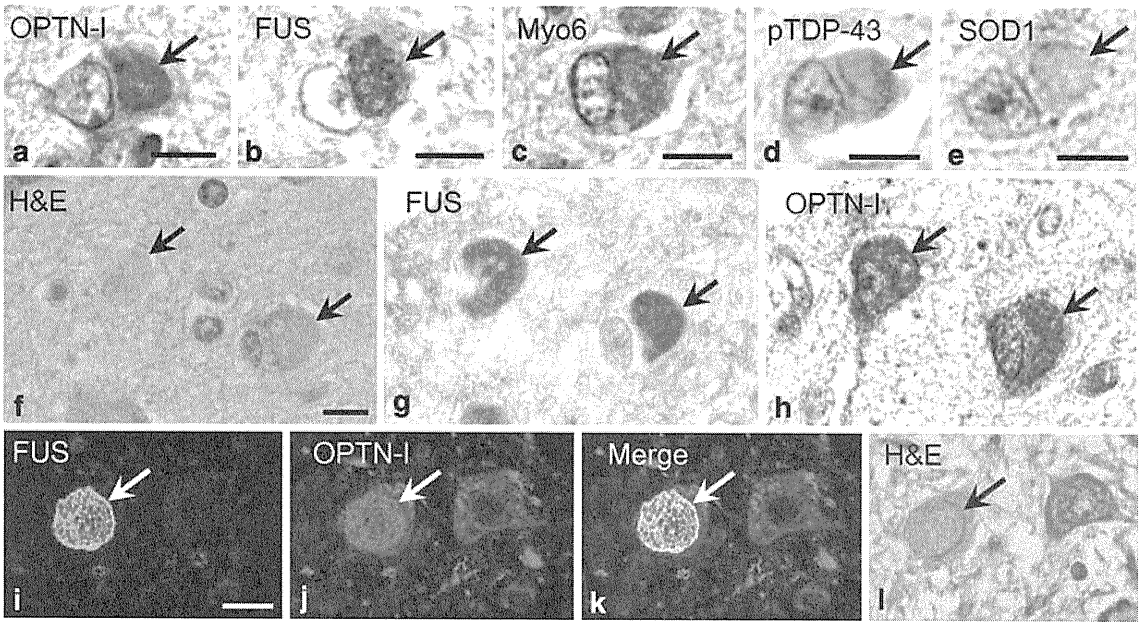

Fig. 1 Representative photomicrographs of basophilic inclusions (BIs). **a–e** BIs (*arrows*) within cortical neurons from BIBD patients are evidently immunopositive for OPTN (**a**), FUS (**b**), and myosin VI (**c**) throughout their entire structure. In contrast, no immunoreactivity indicating pTDP-43 (**d**) or SOD1 (**e**) is recognizable within the BIs (*arrows*). **f–h** Three consecutive sections from BIBD patient No. 1, stained with H&E (**f**) or subjected to immunohistochemistry for FUS (**g**) and OPTN (**h**), demonstrate that the 2 cortical BIs (*arrows*) seen are noticeably immunopositive for both FUS and OPTN. **i–l** Double immunofluorescence staining of neurons in the lateral cuneate nucleus in the medulla oblongata of 'FALS with FUS mutation' patient No. 1 evidently demonstrates that FUS (**i**; *green*) and OPTN (**j**; *red*) are faithfully co-localized (**k**; *merge*) in the BI (**l**; H&E). *Scale bars* 10 μm

maintenance of Golgi organization [5]. When OPTN is depleted from cells via RNA interference, the Golgi becomes fragmented [6]. This observation is noteworthy because Golgi fragmentation has been observed in the anterior horn cells in ALS [3]. Further investigations are warranted to determine whether dysfunctional OPTN could be essential for the underlying pathomechanism at play in ALS.

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Conflict of interest The authors report no conflicts of interest.

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再生医療と iPS 細胞

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はじめに

難治性の神経変性疾患である筋萎縮性側索硬化症(amyotrophic lateral sclerosis：ALS)に対して本邦で唯一認可されているリルゾールは，神経変性を遅延させることを目標として開発された．一方で，失われた神経細胞とその機能を補完する目的の細胞移植による再生医療研究は，後述する様々な課題を有している．

2006 年に人工多能性幹細胞(induced pluripotent stem cells：iPS 細胞)の作製技術が開発された．この革新的技術を用いることによって，患者自身の体細胞から多能性幹細胞を樹立し，さらに中枢神経系組織を含めた疾患標的細胞に分化させることが可能になり，これまでにない全く新たな医療開発が始まっている．

本稿では，既存の幹細胞を用いた ALS の再生医療研究と，近年始まりつつある神経疾患に対する胚性幹細胞(embryonic stem cells：ES 細胞)を用いた細胞移植臨床治療研究を紹介する．さらには，今後期待される iPS 細胞を用いた再生医療の展望と，予測される課題点を克服する試みについて述べる．

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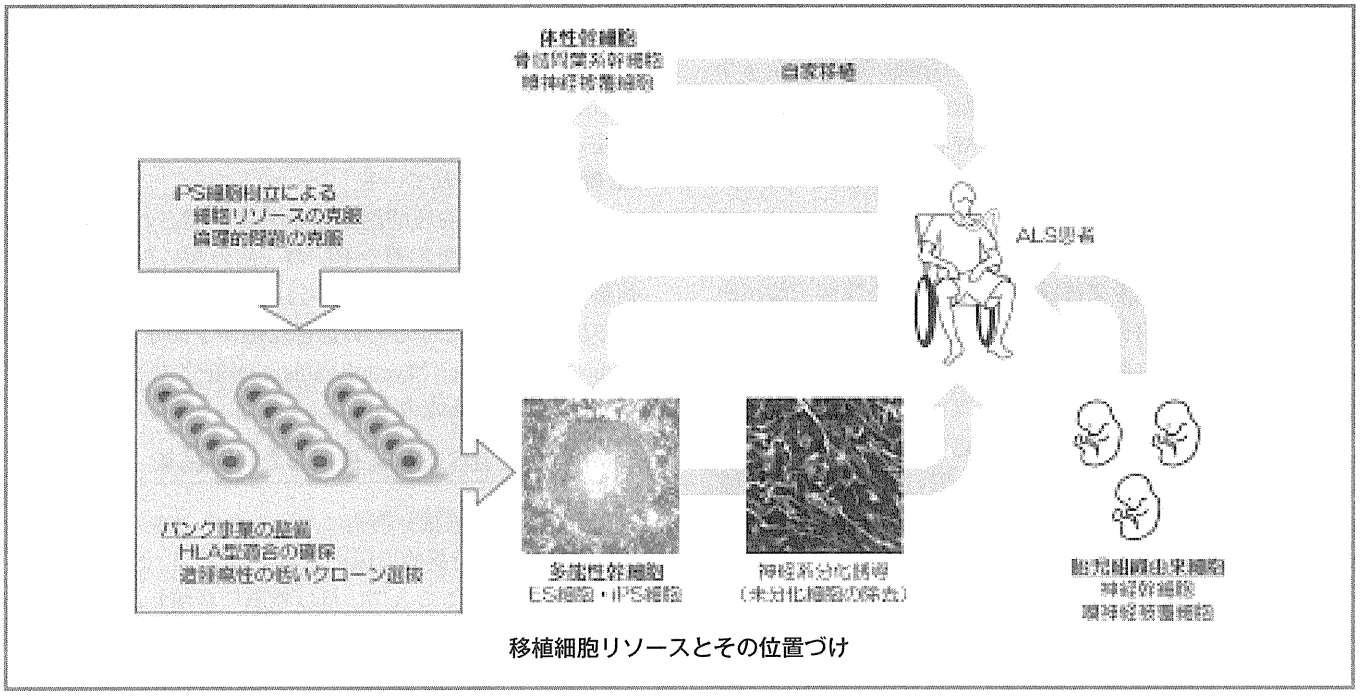
ALS における既存の移植治療細胞リソース

ALS における細胞移植治療研究は，ES 細胞や iPS 細胞といった多能性幹細胞の使用以前には，体性幹細胞を用いた治療研究が先行していた．この体性幹細胞リソースとしては，顆粒球コロニー刺激因子刺激により得られた末梢血幹細胞を用いた自家移植治療[1]や，造血幹細胞の他家移植治療が最も早くから行われたが，安全性が確保しやすい反面有効性も示されなかった[2]．

次に，骨髄由来間葉系幹細胞(mesenchymal stem cells：MSC)の応用が先行している．通常 MSC は，骨髄の中で造血系細胞の働きに適した環境を提供する役割が知られているが，Notch 遺伝子導入と線維芽細胞増殖因子・フォルスコリン(細胞内 cAMP 上昇作用)の併用により，胚葉分化を超えて 90～96％という高効率で神経前駆細胞に誘導が



移植細胞リソースとその位置づけ

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可能であることが示されている[3]．そしていくつかの研究でマウスALSモデルにヒトMSCを移植し，生存期間延長など有効性が示唆されている[4]．実際すでに，臨床治験としてMSCを脊髄内注入する自家移植がイタリア[5]・イスラエル[6]などから報告されており，少数例の第1相治験では安全性と，一部の症例でALS臨床スコア悪化の改善を認めている．しかし，継時的な脊髄MRIでは移植部の膨隆が認められるなど長期的な観察を必要としている．米国国立衛生研究所提供の登録型臨床試験データベース(http://clinicaltrials.gov/)では，スペイン(NCT01254539)とイスラエル(NCT01051882)でも同様の臨床試験が進行中である．

次に，汎用される細胞リソースとしては，嗅神経被覆細胞(olfactory ensheathing cells：OEC)があげられる．成体でも常に増殖再生している嗅神経粘膜から採取が可能であり，Schwann細胞のように神経の髄鞘化を行っている．OECの特徴として，末梢神経のみならず中枢神経系においても髄鞘形成・軸索再生に働くことがラット頸髄損傷モデルで示されている[7]．中国では，胎児嗅球由来のOECを両側放線冠に移植したオープンラベル試験[8]・二重盲検治験[9]で安全性と症状の改善が報告された．しかし，上記中国の施設でOEC移植治療を受けたオランダ人の追跡調査研究では，有効性に乏しく重篤な副作用例(急速な呼吸不全の進行と深部静脈血栓症)もあり，臨床的な有用性はないとしている[10]．この一連の臨床研究では，深部白質の局所にのみ注射するという移植細胞のデリバリー手法に問題があった可能性もある．ALSでは全脊髄が病変になりうるが，広範な病変に対する治療効果を有し侵襲性を低く抑える目的で，ALSマウスモデルに対するOECの第四脳室内投与が試みられ，限定的ながら有効性も示されており，今後の発展が期待される[11]．

最近になり，米国ではEmory大学とNeuralstem社が中心となり胎児脊髄由来神経幹細胞を用いた臨床治験が始まっている．この脊髄由来神経幹細胞はALSの中心的な障害部位である下位運動ニューロンを含むという点で，神経再生を最終目標とした場合優れているものの，細胞リソースを極めて限られる問題が残る．

MSCやOECなどの体性幹細胞を用いたALS細胞治療は，自家移植が可能であるという利点を持つが，神経組織の効率よい再生という観点からは治療効果が不十分となる限界も予測される．一方で，多能性幹細胞(ES細胞・iPS細胞など)からの，運動神経を含めた高効率の神経分化誘導法が急速に発展しており[12]，臨床応用への取り組みが活発化している．

ES細胞を用いた神経疾患治療

ヒト由来の代表的な多能性幹細胞であるヒトES細胞は1998年に初めて樹立され[13]，本邦では2003年に初めて樹立されて以来現在までに5クローンが樹立された．ヒトES細胞はその未分化状態を保ったまま，ほぼ無限に増殖を繰り返すことが可能であり，心筋細胞・肝細胞・神経細胞などへの *in vitro* における分化誘導法研究が進められてきた．

ES細胞が有する多能性は再生医療への応用が期待されていたが，倫理的側面・安全性の確保・異種動物由来因子除去を中心として超えるべきハードルが高かった．しかし，2010年に米国食品医薬品局(US Food and Drug Administration：FDA)が2社3案件の申請を承認，Geron社によるES細胞由来のオリゴデンドロ前駆細胞を用いた亜急性脊髄損傷治療[14]と，ACT社によるES細胞由来の網膜色素上皮細胞を用いたStargardt病・加齢性黄斑変性治療[15]が，それぞれ第1相臨床治験として開始された．また，California Stem Cell社が脊髄性筋萎縮症I型に対するES細胞由来の運動神経を用いた移植治療をFDA申請中だが，2011年4月現在保留中である．

ES細胞の臨床応用における問題点

ES細胞の樹立には体外受精に用いた余剰胚を用いるため，一個体の源を滅失する点において大きな倫理的問題が指摘されてきた．この点を克服するべく，single cell biopsyによるヒト胚を滅失しないES細胞樹立法も報告され[16]，前述のACT社臨床治験にはこの技術を用いて樹立されたES細胞が使用されている．

しかしながら倫理的問題は十分に配慮される必要があり，2011年時点で本邦やEUの一部の国においては，ES細胞の臨床応用が法的に認められていない．さらに，患者投与は他家移植となるため拒絶反応が予測され，Basiliximab・Tacrolimus・Mycophenolate mofetilなどの免疫抑制薬の投与が必須となりうる．

iPS細胞を使用した再生医療

ヒト成人皮膚線維芽細胞に，レトロウィルスベクターを用いてES細胞特異的遺伝子であるOct3/4・Sox2・Klf4・c-Mycを導入することで，ほぼ無限に増殖し，内・中・外胚葉それぞれへの分化能力を有するヒトiPS細胞が誕生した．その後も，より効率的なiPS細胞の樹立法研究の展開

により数多くのクローン数のヒトiPS細胞が樹立されていると予測され，幹細胞の未分化性維持・遺伝子発現特性の解析，さらには癌研究など広範な研究領域に大きなインパクトを与えている．

本邦においても，ヒト幹細胞を用いる臨床研究に関する指針が2010年に改訂され，ヒトiPS細胞の臨床研究が法的に認可された(ES細胞は2011年時点で禁止されている)．

神経疾患への応用を考えた場合，ヒトiPS細胞の神経分化誘導には，ヒトES細胞で培われた分化誘導法の適応が多くの場合応用可能である[17]点は大きな利点となりうる．そしてiPS細胞を用いた再生医療は，ES細胞樹立で議論される倫理的問題点が少なく，患者個々人から樹立したiPS細胞を用いることで拒絶反応を避けられる可能性もある．しかしながら，iPS細胞の安全性確認にかかる時間・コスト等についてはES細胞と同様に克服すべき課題を有する．

多能性幹細胞と造腫瘍性

さらに，iPS細胞・ES細胞に共通の課題として造腫瘍性がある．造腫瘍性には大きく分けて，未分化細胞から生じる奇形腫と，ある程度分化した細胞から生ずる腫瘍性増殖の可能性がある．

前者は，分化抵抗性のある未分化細胞が移植片に混入することが問題とされている[18]．未分化細胞に特異的な表面マーカーや遺伝子発現パターンを用いることで，この点は危険性を低減できると予測される．

一方で後者のある程度分化した細胞の腫瘍性増殖については，免疫不全動物における長期観察など前述のES細胞臨床治験を経た綿密な安全性確認が必要になるであろう．

今後の展望

これらの点を克服するため，本邦においてはHLA-A, B, DRの3遺伝子座についてホモ型であるドナー由来のiPS細胞株を50株作成し，バンク化しておくことで，将来的な移植治療を見据えたとき日本人の90.7%をカバーできることが報告されている[19]．

このバンク化されたiPS細胞の安全性をあらかじめ十分確認しておくことで，比較的病状進行の速いALSにおいても，安全性が高く拒絶反応の少ない細胞移植治療を速やかに実施できることが将来的に期待される．

むすび

体性幹細胞に端を発したALSの治療研究は，さらなる安全性の確認が必須であるが少しずつ進歩している．また多能性幹細胞を用いたALSの再生医療も，現時点では克服すべき課題が多い．しかしながら，ES細胞を用いた臨床研究が既に始まり，その課題を克服するべくiPS細胞の臨床応用にも道筋がつけられつつある現在，ALSの神経再生医療実現化に向けた着実な進歩を期待したい．

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Chapter V

Induced Pluripotent Stem Cell Technology for the Study of Neurodegenerative Diseases

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Abstract

Induced pluripotent stem (iPS) cell technology has paved new ways for disease modeling and drug discovery. Disease modeling with the differentiated neuronal cells from patient-specific iPS cells partially recapitulated the phenotypes of spinal muscular atrophy (SMA), familial dysautonomia (FD) and Rett syndrome. Furthermore, proof of the efficacy of candidate drugs by iPS cell-based assay on SMA and FD has been reported.

There are several obstacles for disease modeling using iPS cell-derived neuronal cells. First, differentiated neuronal cells from patient-specific iPS cells might not be provoked sufficiently toward senescence to manifest the phenotype of late-onset diseases such as Parkinson disease (PD) and amyotrophic lateral sclerosis (ALS). Second, there are heterogeneous populations in cultured cells differentiated from iPS cells that might affect the disease phenotype. The propensity of various differentiations among iPS cells leads to the heterogeneity (Figure 1).

In this review, we will describe recent literature concerning the application of iPS cell technology for the study of neurological diseases and also discuss some experimental requirements.

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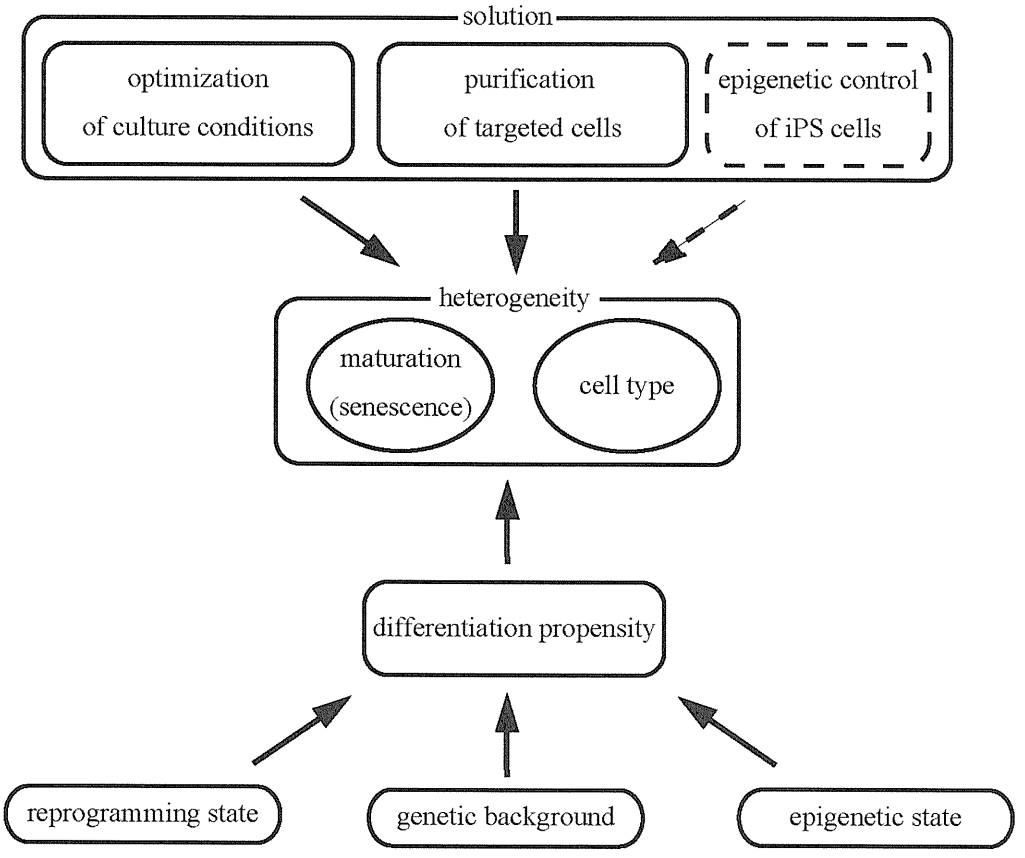

Figure 1. Heterogeneity, including maturation stage and cell population, is a result of the variety of differentiation propensities of iPS cells caused by reprogramming state, genetic background and epigenetic state. Purification of targeted cells and optimization of culture conditions will reduce the heterogeneity and lead to progresses in iPS cell technology.

Introduction

iPS cells are generated by transduction of transcription factors, which are expressed in embryonic stem (ES) cells, in somatic cells (Takahashi *et al.*, 2006; Takahashi *et al.*, 2007; Yu *et al.*, 2007). iPS cells are morphologically identical to ES cells and have the ability to self-renew and differentiate into cells of all germ layers. iPS cell technology makes it possible to analyze *in vitro* affected cell populations that are differentiated from disease-specific iPS cells. This then should enable us to determine the underlying mechanisms of disease, screen new drugs, and develop cell therapy.

Disease phenotypes were partially recapitulated in the differentiated cells from patient-specific iPS cells in some diseases such as spinal muscular atrophy (SMA) (Ebert *et al.*, 2009), familial dysautonomia (FD) (Lee *et al.*, 2009) and Rett syndrome (Marchetto *et al.*, 2010). Furthermore firm evidence of the efficacy of some candidate drugs for SMA (Hastings *et al.*, 2009) and FD (Lee *et al.*, 2009) has also been presented. Other disease phenotypes such as Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) have not yet been described in such terms, although midbrain dopaminergic neurons (Soldner *et al.*, 2009) and spinal motor neurons were successfully generated from patient-specific iPS cells (Dimos *et al.*, 2008).

Genetically corrected somatic cells from Fanconi anaemia patients can be reprogrammed to iPS cells (Raya *et al.*, 2009). Further, corrected patient-specific iPS cells can give rise to haematopoietic progenitors of myeloid and erythroid lineages whose phenotypes are normal. This demonstrates the potential value for cell therapy application.

In this review, we will describe recent literature featuring iPS cell technology for the study of neurological diseases, and we will discuss experimental requirements for overcoming several obstacles in disease modeling.

Main Document

iPS Cells Derived from Patients with Neurological Diseases

Since the start of the development of iPS cell technology, disease-specific iPS cell lines were generated from individuals with neurological diseases, as summarized in Table 1. Neurological diseases can be roughly divided into two categories. One includes early-onset, neurodevelopmental diseases such as Rett syndrome (Marchetto *et al.*, 2010), Prader-Willi/Angelman syndrome (Chamberlain *et al.*, 2010), Fragile X syndrome (Urbach *et al.*, 2010), SMA, FD, Friedreich's ataxia (Ku *et al.*, 2010) and Down syndrome (Park *et al.*, 2008). The other includes late-onset, neurodegenerative diseases like ALS, PD and Huntington's disease (Park *et al.*, 2008).

A few of the neurodevelopmental diseases caused by single gene abnormalities resulting in highly penetrant phenotypes were successfully recapitulated with iPS cell technology.

SMA is an autosomal recessive genetic disorder caused by mutations in the survival motor neuron 1 gene (SMN1). Mutations in SMN1 significantly reduce SMN protein expression and result in the selective degeneration of lower motor neurons. Ebert *et al.* (2009) showed that SMA patient-derived spinal motor neurons were reduced in number and cell body size at 6 weeks of differentiation compared to the patient's unaffected mother-derived motor neurons. SMN protein is localized in gems, one of the nuclear compartments, and the number of gems present is inversely correlated to disease severity. The authors detected an increased number of SMN gems in nuclei of both fibroblasts and iPS cells derived from the SMA patient. The homozygous loss of SMN1 is partially compensated for by the presence of another gene, SMN2, which also codes for SMN protein. However, the expression of full-length protein generated from SMN2 is substantially lower than that from SMN1. A single nucleotide replacement of SMN2 compared to SMN1 changes splicing, and that transcript encodes truncated protein. Hastings *et al.* (2009) reported that tetracyclines are able to increase the expression of full-length SMN protein from SMN2 by splicing modulation.

Table 1. Disease-specific iPS cell lines.

	Neurological disease	Primarily affected neuronal population	Control	*in vitro* phenotype	Mutated gene in disease-specific iPS cell
early-onset	Familial dysautonomia (FD)	sensory and autonomic neurons	unaffected	impaired migration	IKBKAP
	Fragile X syndrome	global			FMR1 CGG repeat truncation
	Friedreich's ataxia	sensory neurons	SMA-iPS cells, non-disease		FXN GAA·TTC repeat expansion

			unaffected		
	Angelman syndrome	global	unaffected		15q11-q13 paternal deletion
	Prader-Willi syndrome				15q11-q13 maternal deletion UBE3A
	Down syndrome	global			Trisomy 21
	Rett syndrome	global	unaffected	reduced number of synapses	MeCP2
	Spinal muscular atrophy (SMA)	spinal motor neurons	unaffected mother	motor neuron loss	SMN1
late-onset	Amyotrophic lateral sclerosis (ALS)	motor neurons			SOD1
late-onset	Huntington disease (HD)	striatal GABAergic neurons			Huntingtin CAG repeat expansion
late-onset	Parkinson disease (PD)	midbrain dopaminergic neurons			

FD is an autosomal recessive genetic disorder caused by mutations in IKBKAP gene involved in transcriptional elongation and characterized by the degeneration of sensory and autonomic neurons. Lee *et al.* (2009) showed marked defects in neurogenic differentiation and impaired migration of neural crest precursor cells derived from FD patients compared to those from non-affected control. Kinetin, one of the plant hormones, increased normal *IKBKAP* and ameliorated neural differentiation and migration.

Rett syndrome is a progressive neurological disorder caused by mutations in X-linked gene encoding MeCP2 protein. Marchetto *et al.* (2010) reported a reduced number of glutamatergic synapses and alteration of morphology in forebrain neurons derived from Rett syndrome patients compared to those from unaffected controls. At 6 weeks of differentiation, neurons from Rett syndrome patients have a significant decrease in frequency and amplitude of spontaneous postsynaptic currents when compared to those from unaffected individuals.

Obstacles of Modeling Late-Onset Neurodegenerative Diseases

A modeling by iPS technology of polygenic, late-onset neurodegenerative diseases has not been reported. Midbrain dopaminergic neurons and spinal cord motor neurons can be differentiated from PD and ALS patient-specific iPS cells, respectively. Perhaps the disease phenotype may never manifest itself under standard culture conditions because differentiated neurons might be too immature or an environment surrounding neurons might differ between *in vitro* (culture) and *in vivo* (brain or spinal cord). It might be revealed by challenging the neural cells with stressors such as oxygen reactive species, proinflammatory factors or co-culture with another type of cell (Marchetto *et al.*, 2008).

Recent investigations have shown that glial cells are affected by physiological brain aging regardless of the absence of any neurodegenerative pathology (Terry *et al.*, 1987). In astrocytes, advanced age initiates conditions similar to mild reactive gliosis. Astroglial cells from advanced-age brain have higher expressions of glial fibrillary acidic protein (GFAP) and glial calcium-bound protein S100 (Le Prince *et al.*, 1993). Additionally, the number of microglial cells is significantly increased (von Bernhardi *et al.*, 2010).

Moreover, the involvement of these non-neuronal cells in neurodegenerative diseases is increasingly being recognized. AD is characterized by profound neuronal loss and prominent reactive astrogliosis and activation of microglia. As Alois Alzheimer suggested, AD plaques are formed by Aβ deposits, degenerating neurites, astroglial processes and activated

microglial cells (Vehmas *et al.*, 2002). Activated astrocytes and microglia can release proinflammatory factors such as interleukin-1β and prostaglandin E_2 (Mhatre *et al.*, 2004).

Oxidative stress may play an important role in sporadic PD (Zhang *et al.*, 2000), as it is suggested that patient-derived midbrain dopaminergic neurons might be more vulnerable to oxidative stress.

Identification of new and more effective and relevant stressors or environments that mimic senescence or elicit neuronal phenotypes earlier in models of late-onset neurodegenerative diseases will therefore be a critical goal for future research.

Heterogeneous Population of Differentiated Cells

Although differentiation protocols have been reported for human ES cells and iPS cells in which neural stem cells and specific neuronal or glial cell types are enriched, it has in fact been revealed that heterogeneous cell populations exist under these conditions (Chambers *et al.*, 2009; Hu *et al.*, 2009; Lee *et al.*, 2010). Additionally, cells are not able to synchronize the developmental stage of cell populations to the extent seen in normal development *in vivo*, and consequently cells at different stages of maturation are present in such cultures. Naturally, this cellular heterogeneity impedes experimental and clinical utility.

The purification of specialized cells of interest is essential for recapitulating diseases and transplanting cells. Such procedures will rely on our technique of manipulating iPS cells genetically to express selectable markers under the control of cell type-specific promoters that would utilize fluorescent or magnetic cell sorting (Hockemeyer *et al.*, 2009; Placantonakis *et al.*, 2009).

Genomic insertion of reporter gene has been shown to alter gene function (Kustikova *et al.*, 2005). Therefore, identification of unique combinations of cell surface epitopes can facilitate cell therapy because cells of interest are purified without gene manipulation. Pruszak *et al.* (2007 & 2009) identified the combination of cluster of differentiation (CD) surface antigen code for neural lineage and Elkabetz *et al.* (2008) reported Forse 1 as being a marker for neural rosette cells. However, no markers for region-specific postmitotic neurons have so far been identified.

Inhibition of Notch activity by γ-secretase inhibitor resulted in a marked acceleration of differentiation, thereby shortening the time required for the generation of functionally active human ES cell-derived neurons (Borghese *et al.*, 2010). This kind of inhibitor can eliminate proliferating cells from differentiated neurons to reduce the risk of tumor formation after transplantation.

Resolution of this problem may assist in obtaining more robust phenotypes *in vitro*, finding more effective drugs, and supplying safe cells for cell therapy.

A Variety of Differentiation Propensities of iPS Cells

The generation of iPS cells can be accomplished by employing retroviral vectors to overexpress reprogramming factors. After infected somatic cells are fully reprogrammed into iPS cells, the vectors are silenced. Therefore, the reprogramming state may be evaluated by retroviral vector silencing. However, this viral system has been criticized for its permanent integration of exogenous genes into the genome and their possible involvement in differentiation propensity.

Alternatively, transfection of episomal plasmids or modified RNA was also successful in reprogramming somatic cells (Okita *et al.*, 2010; Yu *et al.*, 2009; Yue *et al.*, 2010; Warren *et al.*, 2010). Moreover, the direct delivery of recombinant protein was also reported to reprogram somatic cells (Zhou *et al.*, 2009). Although these methods eliminate the integrated

vector, the gene expression of iPS cells is reflected by that of donor cells (Ghosh *et al.*, 2010). Hence, it is considered that the epigenetic state, including DNA methylation, histone acetylation or histone methylation, may contribute to the differentiation propensities of the respective clones.

Direct conversion from differentiated cells to neurons has been reported (Vierbuchen *et al.*, 2010; Heinrich *et al.*, 2011). This conversion was used to generate mouse subtype-specific neurons from differentiated cells without the need for complete reprogramming to iPS cells. Although this direct conversion is independent from the differentiation propensity of iPS cells, the number of neurons available to study the disease and to be used in high-throughput analysis is limited, and the epigenetic states of these neurons would need to be further analyzed.

Human ES cells are widely variable with regard to epigenetic markers, expression profile and differentiation propensity (Osafune *et al.*, 2008; Rugg-Gunn *et al.*, 2007). And also there is significant intrinsic variance among the iPS cell lines generated to date, as pointed out by Hu *et al.* (2010) & Boulting *et al.* (2011). Pick *et al.* (2009) detected abnormal expression of imprinted genes in a significant number of iPS cell lines. Bock *et al.* (2011) reported that a small set of genes was hypermethylated in iPS cell lines compared to ES cell lines. iPS cells are morphologically similar to ES cells, but the epigenetic state is significantly different between them.

Since there are a variety of methods to generate iPS cells, it is critical for the study of neurodegenerative disease to choose a combination of reprogramming factors, a method of factor delivery, and a cell type to be reprogrammed. For instance, differentiation propensity is partially dependent on the cell type to be reprogrammed (Aoi *et al.*, 2008). For accurate comparison between independent experiments, all conditions should be the same. But there is as yet no standardized parameter for selecting safe and fully reprogrammed iPS cells.

The difficulty of disease modeling is partially attributed to the lack of uniform iPS lines. More robust phenotypes *in vitro* may be obtained in comparison with proper control iPS cell lines.

Conclusion

The development of iPS cell technology has provided both experimental and clinical applications. Once disease-specific phenotypes are identified, this can be translated into cell-based assays for drug screening. However, to accomplish these goals, further improvements are needed. Differentiation methods with higher efficiency will provide larger amounts of cells. The identification of cell surface markers on neuronal or non-neuronal cells will provide homogeneous populations. Highly purified, large amounts of cells of interest will facilitate high-throughput platforms for drug screening. Also crucial is the generation of uniform iPS cells and selection of appropriate iPS cells. This will result in patients having autologous transplantations after their own cells are genetically corrected and/or medicated.

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