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The ALS/FTLD-related RNA-binding proteins TDP-43 and FUS have common downstream RNA targets in cortical neurons☆

Daiyu Honda[a], Shinsuke Ishigaki[a,*], Yohei Iguchi[a], Yusuke Fujioka[a], Tsuyoshi Udagawa[a], Akio Masuda[b], Kinji Ohno[b], Masahisa Katsuno[a], Gen Sobue[a,*]

[a]Department of Neurology, Nagoya University Graduate School of Medicine, Nagoya, Japan
[b]Division of Neurogenetics, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine, Nagoya, Japan

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ABSTRACT

TDP-43 and FUS are linked to amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD), and loss of function of either protein contributes to these neurodegenerative conditions. To elucidate the TDP-43- and FUS-regulated pathophysiological RNA metabolism cascades, we assessed the differential gene expression and alternative splicing profiles related to regulation by either TDP-43 or FUS in primary cortical neurons. These profiles overlapped by >25% with respect to gene expression and >9% with respect to alternative splicing. The shared downstream RNA targets of TDP-43 and FUS may form a common pathway in the neurodegenerative processes of ALS/FTLD.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by the death of motor neurons in the spinal cord, brainstem, and motor cortex [1]. Frontotemporal lobar degeneration (FTLD) is a dementia syndrome characterized by progressive changes in behavior, personality, and/or language resulting from the gradual deterioration of the frontal and temporal lobes [2,3]. Transactive response (TAR) DNA-binding protein 43 (TDP-43) and fused in sarcoma (FUS) have been genetically and pathologically linked to ALS and FTLD; however, the underlying mechanisms by which TDP-43 and FUS induce ALS and FTLD pathologies are unknown [2,3].

TDP-43 was identified as a major component of cytoplasmic neuronal inclusions in sporadic ALS and FTLD patients [4,5], and missense mutations in *TARDBP*, the gene encoding TDP-43, are a known cause of familial ALS and FTLD [6–8]. Familial cases of ALS and FTLD involving TDP-43 mutations and sporadic cases of these diseases exhibit highly similar clinical and pathological characteristics [9], suggesting that TDP-43 plays an important role in the pathogenesis of sporadic ALS and FTLD. Similarly, FUS is also a causative gene for familial ALS and FTLD; in these diseases, redistribution to the cytoplasm and the formation of cytoplasmic aggregates occur for both the TDP-43 and FUS proteins [10,11]. TDP-43 and FUS also share many common pathophysiological characteristics. In particular, these proteins are structurally similar heterogeneous ribonucleoproteins (hnRNPs), as both TDP-43 and FUS are RNA-binding proteins with RNA recognition motifs (RRMs); they are typically predominantly found in the nucleus; their pathological forms are located mainly in the cytosol; and they are involved in transcription, alternative splicing, translation, and RNA transport [12–14].

Although it remains unclear whether a loss of function or gain of toxicity of TDP-43 or FUS is a major cause of ALS/FTLD, the loss of these RNA-binding proteins in the nucleus is a plausible trigger of neurodegeneration. This hypothesis has been supported by several lines of evidence, including the fact that TDP-43 or FUS nuclear staining is lost in the nuclei of neurons in both human ALS/FTLD tissue [15,16] and TDP-43 overexpressing mice [17,18]. In addition, animal models involving the loss of either TDP-43 or FUS mimic the pathology of ALS/FTLD [19–22]. Recently, analyses of TDP-43 using fly models revealed

Abbreviations: ALS, amyotrophic lateral sclerosis; Cugbp1, CUG triplet repeat, RNA-binding protein 1; DAVID, Database for Annotation, Visualization and Integrated Discovery; FTLD, frontotemporal lobar degeneration; FUS, fused in sarcoma; GFAP, glial fibrillary acidic protein; GO, Gene Ontology; hnRNAPs, heterogeneous ribonucleoproteins; LTP, long-term potentiation; RIN, RNA integrity numbers; RMA, robust multichip average; RRMs, RNA recognition motifs; SBMA, spinal and bulbar muscular atrophy; shCont, shRNA/control; shCugbp1, shRNA/Cugbp1; shFUS, shRNA/FUS; shTDP, shRNA/TDP-43; TDP-43, transactive response (TAR) DNA-binding protein 43; TGF, transforming growth factor.

* Corresponding authors. Address: Department of Neurology, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-8550, Japan. Tel.: +81 52 744 2391; fax: +81 52 744 2785. Tel.: +81 52 744 2385; fax: +81 52 744 2785.
E-mail addresses: ishigaki-ns@umin.net (S. Ishigaki) sobueg@med.nagoya-u.ac.jp (G. Sobue).

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that the up- and down-regulation of TDP-43 produced highly similar transcriptome alterations [23]. Cross-rescue analysis in *Drosophila* demonstrated that FUS acted together with and downstream of TDP-43 in a common genetic pathway [21]. Thus, it is intriguing to compare the transcriptome profiles from neurons with silenced TDP-43 or FUS. This experiment could clarify the common molecular mechanisms of ALS/FTLD that are associated with TDP-43 and FUS.

Recently, we investigated the transcriptome profiles of FUS regulation in different cell lineages of the central nervous system and determined that FUS regulates both gene expression and alternative splicing events in a cell-specific manner that is associated with ALS/FTLD [24]. In the current study, we investigated the transcriptome profiles of TDP-43-silenced primary cortical neurons and compared these profiles with the transcriptome profiles of FUS-silenced neurons. The gene expression and alternative splicing event profiles related to regulation by TDP-43 and by FUS were rather similar, suggesting that TDP-43 and FUS may regulate common downstream RNA targets and molecular cascades that could potentially be associated with the pathomechanisms of ALS/FTLD.

2. Methods

2.1. Lentivirus

We designed two different shRNAs against mouse *Tardbp* (*Tdp-43*), *Fus*, and a control shRNA. The targeted sequences were 5′-CGATGAACCCATTGAAATA-3′ for shRNA/TDP-43–1 (shTDP1); 5′-GAGTGGAGGTTATGGTCAA-3′ for shRNA/TDP-43–2 (shTDP2); 5′-GCAACAAAGCTACGGACAA-3′ for shRNA/FUS1 (shFUS1); 5′-GAGTGGAGGTTATGGTCAA-3′ for shRNA/FUS2 (shFUS2); 5′-GGCTTAAAGTGCAGCTCAA-3′ for shRNA/Cugbp1 (shCugbp1); and 5′-AAGCAAAGATGTCTGAATA-3′ for shRNA/control (shCont). The shRNAs were cloned into a lentiviral shRNA vector (pLenti-RNAi-X2 puro DEST, w16–1, which was a kind gift from Dr. Eric Campeau at Resverlogix Corp.). Lentivirus was prepared in accordance with the protocols detailed by Campeau et al. [25].

2.2. Primary cortical neuron culture and the depletion of TDP-43 and FUS

Primary cortical neurons were obtained from the fetal brains of C57BL/6 mouse embryos on embryonic day 15 (E15). The detailed procedure for acquiring these neurons was described in previously published reports [26]. On day 5, neurons were infected with 2×10^{10} copies/well (1.5×10^{7} copies/μl) of lentivirus expressing shRNA against mouse *Tdp-43* (shTDP1 or shTDP2), mouse Cugbp1 (CUG triplet repeat, RNA-binding protein 1) (shCugbp1), or scrambled control (shCont). The virus-containing media was removed at 4 h after infection. The neurons were then cultured for 6 additional days and harvested on day 11 for RNA extraction and cDNA synthesis. Each knockdown experiment was performed in triplicate for each microarray analysis. Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health and with the approval of the Nagoya University Animal Experiment Committee (Nagoya, Japan). The experiments on FUS-silenced primary cortical neurons were performed in the manner described above and have been detailed in a previously published report [26].

For immunohistochemical analyses, we used an anti-β-tubulin antibody (TU20, Santa Cruz, Santa Cruz, CA), an anti-glial fibrillary acidic protein (GFAP) antibody (EB4, Enzo Life Sciences, Plymouth Meeting, PA), and 4′,6-diamidino-2-phenylindole (DAPI) staining.

For immunoblot analyses, cells were lysed in TNE buffer containing protease inhibitors for 15 min on ice. The lysates were then cleared by centrifuging the cells at 13,000*g* for 15 min at 4 °C. Lysates were normalized for total protein (10 μg per lane), separated using a 4–20% linear gradient SDS–PAGE and electroblotted. For immunoblot, we used anti-FUS antibodies (A300–293A, Bethyl Laboratories, Montgomery, TX), anti-TDP-43 antibody (Proteintech, Chicago, IL), and anti-actin antibody (Sigma, St. Louis, MO).

2.3. Microarray analysis

Total RNA was extracted from primary cortical neurons using the RNeasy Mini Kit (Qiagen, Hilden, Germany). We confirmed that the RNA integrity numbers (RIN) for the extracted samples were all greater than 7.0. We synthesized and labeled cDNA fragments from 100 ng of total RNA using the GeneChip WT cDNA Synthesis Kit (Ambion, Austin, TX). Hybridization and signal acquisition for the GeneChip Mouse Exon 1.0 ST Array (Affymetrix, Santa Clara, CA) were performed according to the manufacturer's instructions. Each array experiment was performed in triplicate. The robust multichip average (RMA) and iterative probe logarithmic intensity error (iterPLIER) methods were employed to normalize exon-level and gene-level signal intensities, respectively, using Expression Console 1.1.2 (Affymetrix). We utilized the gene annotation provided by Ensembl version e!61, which is based on the National Center for Biotechnology Information (NCBI) Build 37.1/mm9 of the mouse genome assembly. All microarray data were registered in the Gene Expression Omnibus with accession numbers of GSE36153 (shFUS) and GSE46148 (shTDP-43 and shCugbp1).

Using Student's *t*-test, we compared the gene-level signal intensities from three controls treated with shCont with the gene-level signal intensities of three samples treated with either shTDP1 or shTDP2. We also analyzed alternative splicing profiles by filtering the exon-level signal intensities, using a *t*-test with a threshold of p-value ≤ 0.1. Gene expression and alternative splicing profiles related to FUS regulation in primary cortical neurons were also obtained by comparing gene-level and exon-level signal intensities from three controls treated with shCont with the corresponding signal intensities from three samples treated with either shFUS1 or shFUS2, as previously described [26]. As a control for the RNA-binding protein-silencing model, we analyzed the gene-level and exon-level signal intensities of three samples treated with wither shCugbp1or shCont.

2.4. RT-PCR for alternative splicing analyses

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen). The extracted RNA was then treated with DNase I (Qiagen). cDNA was synthesized from 1 μg of total RNA using oligo(dT) primers (Promega, Madison, WI). Primers for each candidate exon were designed using the Primer3 software program (http://frodo.wi.mit.edu/primer3/input.htm). The primer sequences are provided in Supplementary Table 1. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using Ex Taq (Takara Bio Inc., Otsu, Japan), with the following amplification conditions: 25–30 cycles of 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 min. The PCR products were electrophoresed on a 15% acrylamide gel and stained with ethidium bromide. The intensity of each band was measured using the Multi Gauge software program (Fujifilm, Tokyo, Japan).

2.5. Real-time qPCR for gene expression analysis

The RNeasy Mini Kit (Qiagen) was used to isolate total RNA from cells; 1 μg of total RNA was then reverse transcribed, using oligo-dT primers. This transcription utilized the CFX96 system (BioRad, Hercules, CA) and thermocycler conditions of 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s and 55 °C for 30 s.