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A unique mouse model for investigating the properties of amyotrophic lateral sclerosis-associated protein TDP-43, by *in utero* electroporation

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ABSTRACT

TDP-43 is a discriminative protein that is found as intracellular aggregations in the neurons of the cerebral cortex and spinal cord of patients with amyotrophic lateral sclerosis (ALS); however, the mechanisms of neuron loss and its relation to the aggregations are still unclear. In this study, we generated a useful model to produce TDP-43 aggregations in the motor cortex using *in utero* electroporation on mouse embryos. The plasmids used were full-length TDP-43 and C-terminal fragments of TDP-43 (wild-type or M337V mutant) tagged with GFP. For the full-length TDP-43, both wild-type and mutant, electroporated TDP-43 localized mostly in the nucleus, and though aggregations were detected in embryonic brains, they were very rarely observed at P7 and P21. In contrast, TDP-43 aggregations were generated in the brains electroporated with the C-terminal TDP-43 fragments as previously reported in *in vitro* experiments. TDP-43 protein was distributed diffusely—not only in the nucleus, but also in the cytoplasm—and the inclusion bodies were ubiquitinated and included phosphorylated TDP-43, which reflects the human pathology of ALS. This model using *in utero* electroporation of pathogenic genes into the brain of the mouse will likely become a useful model for studying ALS and also for evaluation of agents for therapeutic purpose, and may be applicable to other neurodegenerative diseases, as well.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a paralytic and fatal disorder caused by motor neuron degeneration in the brain and spinal cord. Familial ALS is represented in only 5–10% of all ALS patients, and most cases are sporadic. In familial ALS, mutations in superoxide dismutase 1 (*SOD1*), the TAR DNA-binding protein of 43 kDa (*TARDBP*, TDP-43), the fused in sarcoma protein (*FUS*, also known as the translocated in liposarcoma protein (*TLS*)), and *UBQLN2*, which encodes the ubiquitin-like protein ubiquilin 2, and the expansion of a hexanucleotide repeat in gene *C9orf72*, and in many other genes were reported (Deng et al., 2011; DeJesus-Hernandez et al., 2011). Although some mechanisms (Robberecht and Philips, 2013), such as RNA editing deficiency, are suggested (Takuma et al., 1999), the detailed pathogenic mechanisms of familial and sporadic ALS remain unknown.

TDP-43 is a nuclear factor functioning in RNA processing, and has a 414-amino-acid protein with two RNA recognition motifs (RRM1 and RRM2) and a carboxyl-terminal glycine-rich domain (Lagier-Tourenne et al., 2010; Lee et al., 2011). This protein is identified as the major deposited protein in inclusion bodies in the brains of both familial and sporadic ALS, as well as frontotemporal lobar degeneration (FTLD) (Arai et al., 2006; Giordana et al., 2010; Mori et al., 2008). There are many mutations in TDP-43 (*TARDBP*) even in the sporadic cases (Mackenzie et al., 2010). In neuropathological examinations of ALS patients' neurons, TDP-43 is found in neuronal cytoplasmic inclusions, dystrophic neurites, and neuronal intranuclear inclusions. Three types of inclusions are found in ALS, namely, skein-like inclusions, round inclusions, and dot-like inclusions (Arai et al., 2006; Giordana et al., 2010; Mori et al., 2008). The major disease-specific findings in ALS and FTLD include abnormal ubiquitination and phosphorylation of TDP-43, sarcosyl-insoluble TDP-43 inclusions, truncated 20–25 kDa TDP-43 C-terminal

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fragments (Arai et al., 2006, 2010; Hasegawa et al., 2008), and truncated N-terminal fragments (Yamashita et al., 2012). These abnormal features of TDP-43 and its fragments are purported to be associated with the mislocalization of the TDP-43 protein into the cytoplasm of motor neurons and loss of normal nuclear TDP-43 expression. Now TDP-43 aggregation, phosphorylation, truncation, and nuclear clearing of TDP-43 are considered hallmarks of ALS (Baloh, 2011). However, the mechanisms of inclusion body formation and the pathological function caused by the mislocalization of TDP-43 into cytoplasm are unknown.

To elucidate the problem, many researchers have established *in vitro* models. In mammalian cultured cells, TDP-43 remains predominantly soluble and localized in the nucleus, even though it is transfected by the full length TDP-43 protein (Baloh, 2011). Moreover the neuronal cell death and neuronal cell loss were not confirmed in *in vitro* experiments. However in some experiments, TDP-43 inclusion bodies were observed in *in vitro* (Nonaka et al., 2009b), and it was necessary to mutate the nuclear localization signal (NLS), mutate the RNA binding domain, or express truncation mutants containing C-terminal domain in order to make pathological inclusions (Baloh, 2011). In transfection experiments of C-terminal fragments or disruption of NLS of TDP-43 to primary neuron or cultured cells, aggregations and inclusion bodies of TDP-43 were observed, and some of them were phosphorylated and ubiquitinated (Nonaka et al., 2009a).

Many types of rodent models for TDP-43 were generated, such as those overexpressing wild type or ALS-related mutant TDP-43 with a neuronal specific promoter, ubiquitous promoter, or inducible promoter (Tsao et al., 2012; Janssens et al., 2013). Few, however, reported pathological changes similar to human ALS patients. An accumulation of ubiquitin is observed in the neurons of most TDP-43 transgenic mice, and contrary to the results from humans, phosphorylated TDP-43 immunopositive cytoplasmic inclusions are rarely observed (Gendron and Petrucelli, 2011). Only three transgenic mice (Wils et al., 2010; Xu et al., 2010; Igaz et al., 2011; Janssens et al., 2013) and one transgenic rat (Zhou et al., 2010) are reported to have shown phosphorylated TDP-43 inclusions in their neuronal cytoplasm.

In this study, we established an experimental model to express TDP-43 protein *in vivo* and to make pathological changes that are also detected in the human brain, such as inclusion bodies with ubiquitination and phosphorylation in the motor areas of the cerebral cortex by *in utero* electroporation of C-terminal fragments of TDP-43. Even though this method is much easier than making transgenic mice, it allows the targeted protein to be expressed *in vivo* and provides an analog to human pathology.

2. Materials and methods

2.1. Plasmids

To generate full-length TDP-43 expression constructs, we first amplified the human TDP-43 coding region from human HeLa cell line cDNA using the primers of TDP43UP (5′-AAAAAAACGCG-TGCCGCCATGTCTGAATATATTCGGGTAACCG-3′) and TDP43DW (5′-TATTACGCGTCTACATTCCCCAGCCAGAAGACTTAG-3′). After gel purification, PCR products were digested with *MluI* and inserted into the pCI vector (Promega, Madison, WI), then digested with the same restriction enzyme (pCI-hTDP-43). To construct N-terminally green fluorescent protein (GFP)-fused TDP-43 (pEGFP-C3-hTDP-43) and GFP-fused CTF-hTDP-43, a cDNA encoding full-length TDP-43 was amplified from pCI-hTDP-43, using the primers of GFP-C3-TDP-43UP (5′-AACCGCTCGAGATGTCTGAATATATTCGGG-TAACCGAA-3′) or GFP-C3-CTF-TDP-43UP (5′-AAAACTCGAGATGGT-CTTCATCCCCAAGCCATTC-3′) and GFP-C3-TDP-43DW (5′-AACGGGATCCCTACATTCCCCAGCCAGAAG-3′). The amplified fragments were digested with *XhoI*/*BamHI* cloned into the same cleavage sites of the pEGFP-C3 vector (Takara, Shiga, Japan). The pEGFP-C3-hTDP-43 and pEGFP-C3-CTF-hTDP-43 were digested with *NheI*/*BamHI*, and then a Klenow Fragment Kit (Takara) was used to unwind the fragments from the blunt-end duplexes. The pCX-EGFP (a gift from Prof. M. Okabe at Osaka University) was digested with *EcoRI*, and then unwind the fragments from the blunt-end duplexes. Either a GFP-hTDP-43 or a GFP-CTF-hTDP-43 fragment was cloned into the pCX vector. Among many mutations in the TDP-43 gene, we chose Met337 to Val (M337V), as we have dealt with a case that exhibited this mutation (Tamaoka et al., 2010). We used a KOD Plus mutagenesis kit (TOYOBO, Osaka, Japan) with the primers of TDP-43_M337VUP (5′-GTATGGTGGGCATGTTAGCCAGCCA-3′) and TDP43_M337VDW (5′-CCCAACTGCTCTGTAGTGCTGCCTG-3′) for site-directed mutagenesis of the full-length hTDP-43 and the CTF fragment hTDP-43 to substitute for Met337 to Val (M337V). All constructs were verified by DNA sequencing.

2.2. Animals

Timed-pregnant ICR mice were purchased from SLC (Shizuoka, Japan). For *in utero* electroporation, the pregnant mice were anesthetized by intraperitoneal injection of sodium pentobarbital (70 mg/kg body weight). For transcardial perfusion, the mice were deeply anesthetized by intraperitoneal injection of an excess of sodium pentobarbital (100 mg/kg body weight). All the animal experiments were approved by the animal care and use committee of the University of Tsukuba and performed under its guidelines.

2.3. In utero electroporation

Timed-pregnant mice on day 12.5–13.5 post-coitum were anesthetized and the uterus was pulled out of the abdominal cavity. A glass capillary was inserted into the lateral ventricle of the embryo through the uterus, and 2.5 μl of a plasmid solution, pCX-GFP-hTDP43-wt or pCX-GFP-hTDP43-M337V, pCX-GFP-hTDP-Ctf-wt, pCX-hTDP-Ctf-M337V (300 nM dissolved in 0.01% Fast Green FCF (Takara) in phosphate buffered saline (PBS)), or pCX-EGFP (150 nM), was injected by pressure (Okada et al., 2007; Saito, 2006). Around 5 min after injection, the head of the embryo was held with a forceps-type electrode positioned in front of the parietal region and behind the posterior part of the cerebellum (CUY650P5, Unique Medical Imada, Miyagi, Japan), with the anode on the forebrain side, and five cycles of square electric pulses (35 V, 50 ms) with 950 ms intervals were delivered through the uterine wall using an electroporator, CUY21 (Nepa Gene, Chiba, Japan). The uterus was then repositioned in the abdominal cavity, and the abdominal wall and skin were closed to allow the embryos to continue normal development.

2.4. Brain preparation and immunohistochemistry

The mouse embryos electroporated at embryonic day (E) 12.5 were removed from the pregnant mice at the stage of E15.5, and postnatal days (P) 7 or 21 mice were used. In the case of the E15.5 embryos, after their heads were cut off and immersion-fixed with 4% paraformaldehyde (PFA) in PBS at 4 °C overnight, the brains were removed. In the case of P7 and P21 mice, after transcardial perfusion with 4% PFA/PBS, the brains were removed from their head and immersed in the fixative at 4 °C overnight.

All the brains were incubated in 10%, 20%, and 30% sucrose/PBS at 4 °C overnight serially, and embedded in Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan); 20-μm-thickness sections were cut using a cryostat and mounted on slide glasses. The