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Nuclear TAR DNA-binding protein 43

A new target for amyotrophic lateral sclerosis treatment

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Research Highlights

(1) Chronic oxidative stress injury was induced by sodium malonate in cultured mouse cortical motor neurons, in a long-term *in vitro* model of amyotrophic lateral sclerosis. Pathological changes in TAR DNA-binding protein 43 (TDP-43) were observed during the later stages of the insult, but a transient increase in non-toxic nuclear TDP-43 occurred in the early stages.
(2) Wild type mouse TDP-43 was transfected into the cultures. Overexpression of nuclear TDP-43 in the cultured neurons exerted a neuroprotective effect against malonate injury.
(3) This is the first study to demonstrate the neuroprotective effect of TDP-43 overexpression and suggests a new strategy in the treatment of amyotrophic lateral sclerosis.

Abstract

Abnormal TAR DNA-binding protein 43 (TDP-43) inclusion bodies can be detected in the degenerative neurons of amyotrophic lateral sclerosis. In this study, we induced chronic oxidative stress injury by applying malonate to cultured mouse cortical motor neurons. In the later stages of the malonate insult, TDP-43 expression reduced in the nuclei and transferred to the cytoplasm. This was accompanied by neuronal death, mimicking the pathological changes in TDP-43 that are seen in patients with amyotrophic lateral sclerosis. Interestingly, in the early stages of the response to malonate treatment, nuclear TDP-43 expression increased, and neurons remained relatively intact, without inclusion bodies or fragmentation. Therefore, we hypothesized that the increase of nuclear TDP-43 expression might be a pro-survival factor against oxidative stress injury. This hypothesis was confirmed by an *in vitro* transgenic experiment, in which overexpression of wild type mouse TDP-43 in cultured cortical motor neurons significantly reduced malonate-induced neuronal death. Our findings suggest that the loss of function of TDP-43 is an important cause of neuronal degeneration, and upregulation of nuclear TDP-43 expression might be neuroprotective in amyotrophic lateral sclerosis.

Key Words

neural regeneration; neurodegenerative disease; amyotrophic lateral sclerosis; TAR DNA-binding protein 43; cortex; motor neurons; oxidative stress; sodium malonate; neuroprotection; grants-supported paper; neurodegeneration

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Author contributors: Zheng M performed western blot analysis, transfection, and immunocytochemical staining and wrote the paper. Shi YJ carried out cell culture and neuronal death analysis. Fan DS was responsible for subject design and closely reviewed the paper. All authors approved the final version of the manuscript.

Conflicts of interest: None declared.

INTRODUCTION

Amyotrophic lateral sclerosis is a late onset neurodegenerative disease characterized by a progressive loss of motor neurons in the motor cortex, brain stem and spinal cord[1-2]. The pathogenesis of amyotrophic lateral sclerosis remains elusive, but abnormal ubiquitinated inclusion bodies in the cytoplasm of the affected neurons have long been recognized as a prominent pathological feature of the disease[3-6]. The major protein component of the inclusion bodies is TAR DNA-binding protein 43 (TDP-43), a 414 amino acid protein of the heterogeneous nuclear ribonucleoprotein family[7-8]. TDP-43 has two RNA recognition motifs and a carboxy-terminal glycine-rich domain[9-10]. Its function is still unclear, but its most widely studied roles relate to the regulation of gene transcription, exon splicing, and exon inclusion through interactions with RNA, heterogeneous nuclear ribonucleoproteins, and nuclear bodies[9]. In addition, TDP-43 is the only human low molecular weight neurofilament mRNA-binding protein to alter its somatotopic localization[11], regulate retinoblastoma protein phosphorylation through the repression of cyclin-dependent kinase 6 expression, and play a key role in the maintenance of neuronal cell morphology and survival through protein geranylgeranylation of Rho family GTPases[12]. Furthermore, the most recent studies using cross-linking immunoprecipitation sequencing have shown that multiple RNAs interact with TDP-43[13-15]. As a transcription regulatory protein, TDP-43 is located in the nucleus under normal conditions, where it exerts its physiological actions.

In amyotrophic lateral sclerosis, TDP-43 accumulates in the cytoplasm and forms inclusion bodies, in which the protein is often ubiquitinated and hyperphosphorylated[7]. In addition, it is abnormally spliced to generate 26 kDa C-terminal fragments[7-8]. Interestingly, TDP-43 is cleared from nuclei in the affected neurons[7-8]. Presumably, the location of TDP-43 within a specific nucleoplasmic domain is required for its normal functions, therefore nuclear clearance of TDP-43 may cause neuronal degeneration. Recent studies have shown that knockdown or deletion of TDP-43 *in vitro* and *in vivo* induces cell apoptosis and embryonic death in mice[12, 16-19], suggesting that TDP-43 is a prosurvival protein in nuclei. Increasing expression of nuclear TDP-43 protein might promote neuronal survival; however, there is no direct evidence showing that enhanced expression of nuclear TDP-43 plays a protective role in degenerative neurons.

To study the role of TDP-43 in the pathogenesis of amyotrophic lateral sclerosis, we first set up a disease model. Transfection of human amyotrophic lateral sclerosis-linked TDP-43 mutants or TDP-43 C-terminal fragments can accurately replicate the histopathological findings of the disease, including cytoplasmic aggregation and fragments[20-25]. However, the fact that most cases of TDP-43 proteinopathies are sporadic rather than familial suggests that exogenous factors induce post-translational modifications of TDP-43 that manifest in the disease[26]. There is considerable evidence that reactive oxygen species and oxidative stress are associated with amyotrophic lateral sclerosis[27-30], and oxidative stress induced by paraquat or ethacrynic acid can reproduce pathological features of TDP-43 that occur in the disease[31-32]. However, the two agents induce acute oxidative stress injury, which differs greatly from the very slow process of amyotrophic lateral sclerosis pathology.

Malonate is an inhibitor of mitochondrial complex II. Mitochondria are an important link between cellular stress signals activated during long-term neuronal injury and the execution of apoptotic cell death[33-35]. Thus, malonate has been used to reproduce the histopathological manifestations of many neurodegenerative diseases, such as Huntington's, Alzheimer's, and Parkinson's diseases, and amyotrophic lateral sclerosis[35-42]. In previous studies including our own, malonate treatment caused chronic excitotoxicity by inducing the accumulation of intracellular reactive oxygen species, and has been used to create a model of amyotrophic lateral sclerosis in cultured organotypic brain or spinal slices[35-39].

Thus, in the present study, we established an *in vitro* cell model by adding malonate to cultures of mouse cortical motor neurons to investigate changes in TDP-43 expression and distribution during chronic oxidative stress. We further investigated how these changes affected cell survival, and whether overexpression of homologous TDP-43 had a neuroprotective effect.

RESULTS

Death of cortical motor neurons in mice after malonate treatment

We established an *in vitro* amyotrophic lateral sclerosis cell model by treating cultured cortical motor neurons with 3 mmol/L malonate for 9 days. To characterize the model, we performed a lactate dehydrogenase release assay and used propidium iodide staining to measure malonate-induced neuronal damage in cultured neurons.

The data showed that the rate of neuronal death was not significantly altered during the first 3 days of malonate treatment.

However, after 6 days, injured neurons began to show obvious apoptosis compared with control cells, and the rate of neuronal death then rapidly increased to almost 50% after 9 days of treatment (Figure 1A–C). Neuronal death only occurred in the later phase of malonate injury, closely mimicking the chronic neuronal degeneration observed in amyotrophic lateral sclerosis pathology.

Changes in nuclear TDP-43 protein expression levels in cortical motor neurons of mice after malonate treatment

To investigate the relationship between neuronal death and TDP-43 protein expression, we next performed western blot assays to measure the TDP-43 protein level in the specific injury paradigm. A reduction in nuclear TDP-43 is known to induce neuronal death[12, 16-19, 22, 43], so we hypothesized that the level of TDP-43 protein would gradually decrease during treatment with malonate until eventual neuronal death. However, surprisingly, TDP-43 protein increased in cultured motor neurons during the first 3 days of malonate treatment. Subsequently, expression returned to basal levels after 6 days of treatment, and was reduced as expected after 9 days (Figure 1D, E).

Location and pathological changes of TDP-43 in cortical motor neurons of mice after malonate treatment

To further validate the amyotrophic lateral sclerosis disease model, we performed immunocytochemical staining to determine the location and expression levels of TDP-43 in cultured neurons, using DAPI to label the nuclei.

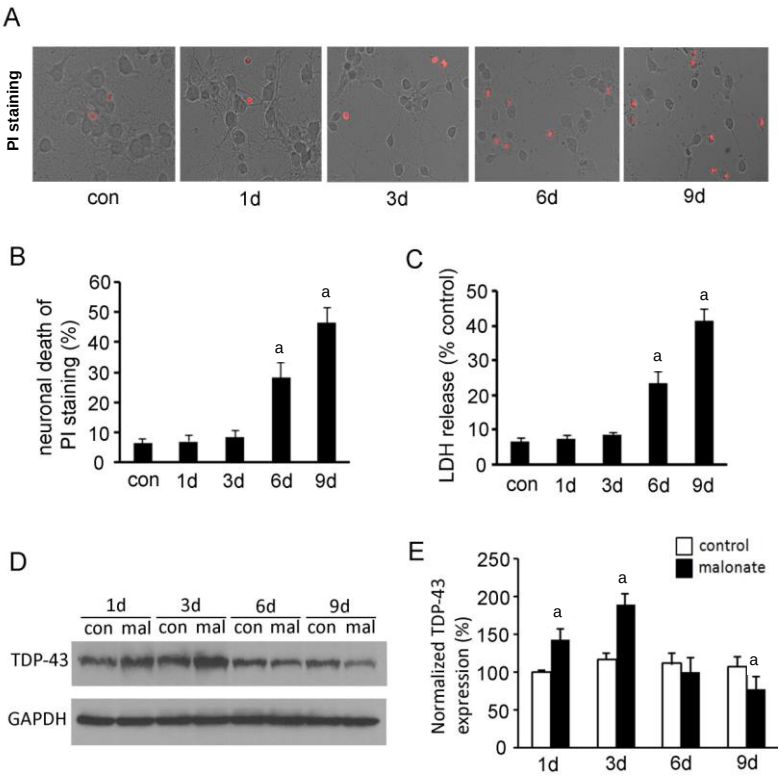

Figure 1 Neuronal death and protein expression level of nuclear TAR DNA-binding protein 43 (TDP-43) after malonate treatment.

Mouse cortical motor neurons were treated with 3 mmol/L malonate for 9 days. (A) Time course of malonate-induced neuronal damage detected by propidium iodide (PI) staining (red, × 400). The number of propidium iodide positive neurons increased between 6 and 9 days from the start of malonate insult. (B) Statistics of propidium iodide-positive neurons as a percentage of all green fluorescent neurons. (C) Neuronal death detected by lactate dehydrogenase release assay. (D) Western blot analysis of nuclear TDP-43 protein (TDP-43: 43 kDa; GAPDH: 37 kDa). (E) Quantitative analysis of nuclear TDP-43 protein in western blot (absorbance data are normalized to 1 day in con).

Data are expressed as mean ± SEM, there were six Petri dishes per group for propidium iodide staining and lactate dehydrogenase release assay, and eight Petri dishes per group for western blot analysis. The experiments were repeated three times. $^{a}P < 0.01$, *vs*. con; 1–9 d: 1–9 days after malonate treatment; con: control group; mal: malonate-treated group; LDH: lactate dehydrogenase.

After 9 days of malonate treatment, the protein level of TDP-43 in the nuclei was lower than that in control cells, and some of the TDP-43 had moved to the cytoplasm and formed aggregates (Figure 2A).

Separate nuclear and cytoplasmic TDP-43 protein levels were determined by subcellular fractionation assay. A small part of TDP-43 appeared in the cytoplasm at 9 days after malonate insult (Figure 2B); long exposure of immunoblots showed that a 26 kDa fragment of TDP-43 appeared at this stage (Figure 2C). Thus, malonate-induced chronic oxidative stress in cultured cortical motor neurons closely reproduced the typical TDP-43 pathology seen in amyotrophic lateral sclerosis. We also investigated the change in TDP-43 protein after 3 days of malonate treatment. Contrary to the pathological changes of TDP-43 after 9 days of treatment, the increased TDP-43 protein at 3 days was confined to nuclei without abnormal accumulation in the cytoplasm (Figure 2A, B), and was not truncated to 26 kDa fragments (Figure 2C).

Nuclear TDP-43 protein upregulation conferred protection against oxidative stress-induced neuronal death

The protein level of TDP-43 increased during the early stages of malonate treatment, and decreased during the later stages, displaying different phenotypes during both periods.

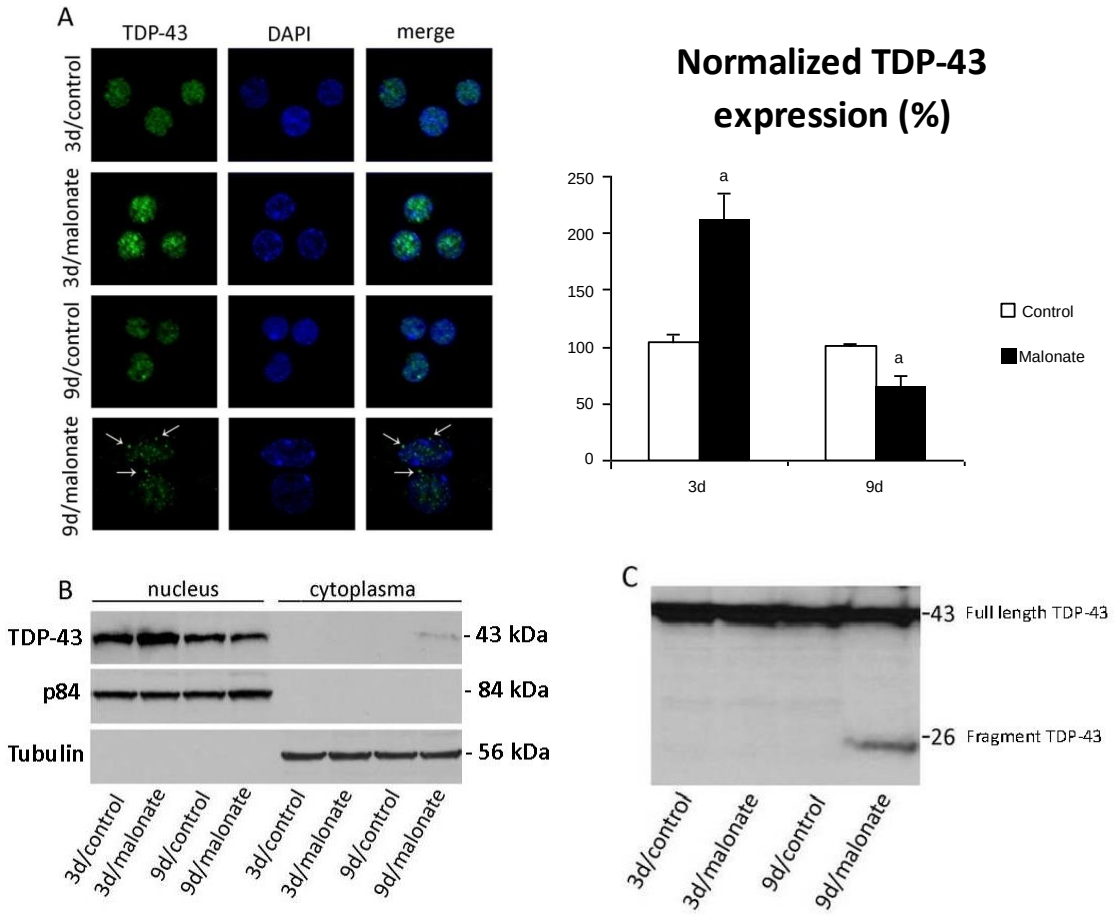

Figure 2 Location and pathological changes of nuclear TAR DNA-binding protein 43 (TDP-43) after malonate treatment.

(A) Distribution of TDP-43 protein after 3 mmol/L malonate treatment, detected by immunocytochemical staining (confocal microscope, × 630). Representative images (left) show that increased TDP-43 (green) is confined to nuclei (DAPI, blue) after 3 days of malonate insult; however, a small amount of TDP-43 protein is excluded from nuclei and forms condensed particles (white arrows) after 9 days of malonate treatment. Summarized data (right) show the quantitative analysis for the fluorescence intensity of TDP-43 protein. Data are expressed as mean ± SEM and normalized to 3 days in the control group, and there were 30 cells from three independent experiments for each group. $^{a}P < 0.01$, *vs*. control group.

(B) Location of TDP-43 within the nucleoplasmic domain detected by subcellular fractionation assay. After 9 days of malonate treatment, a small amount of TDP-43 is localized in the cytoplasm (p84 and tubulin are internal references for nuclei and cytoplasm respectively).

(C) TDP-43 fragment detected by long exposure of immunoblots. After 9 days of malonate treatment, a 26 kDa fragment of TDP-43 is present.

A reduction in nuclear TDP-43 has been linked to neuronal death[12, 16-19, 22, 43]. Therefore, we asked what the effect would be of enhancing nuclear TDP-43 expression on neuronal death or survival. To this end, we transfected the cultured cortical motor neurons with wild type mouse TDP-43 cDNA (Figure 3A). Control neurons were transfected with the non-targeting control, green fluorescent protein. TDP-43 protein expression was 1.93 times greater in the transfected neurons than in the control neurons (Figure 3A). We also investigated the localization and distribution of the overexpressed TDP-43. Immunocytochemical staining showed that overexpressed TDP-43 was confined to nuclei, without redistribution or aggregation (Figure 3A), similar to that seen in the early response of cultured neurons to the malonate insult.

To determine the effect of the increased nuclear TDP-43 protein level on neuronal death or survival, transgenic neurons and control cells were treated with malonate for 2 days. The mortality rate in neurons overexpressing TDP-43 was much lower than that in control cells (Figure 3B), indicating that the increased nuclear TDP-43 may protect normal neurons against malonate-induced oxidative stress. To further verify whether enhanced TDP-43 would still exert a protective effect in degenerating neurons, we transfected malonate-treated neurons with wild type mouse TDP-43 cDNA on day 7 of treatment. Indeed, overexpression of TDP-43 reduced neuronal death in damaged neurons (Figure 3B).

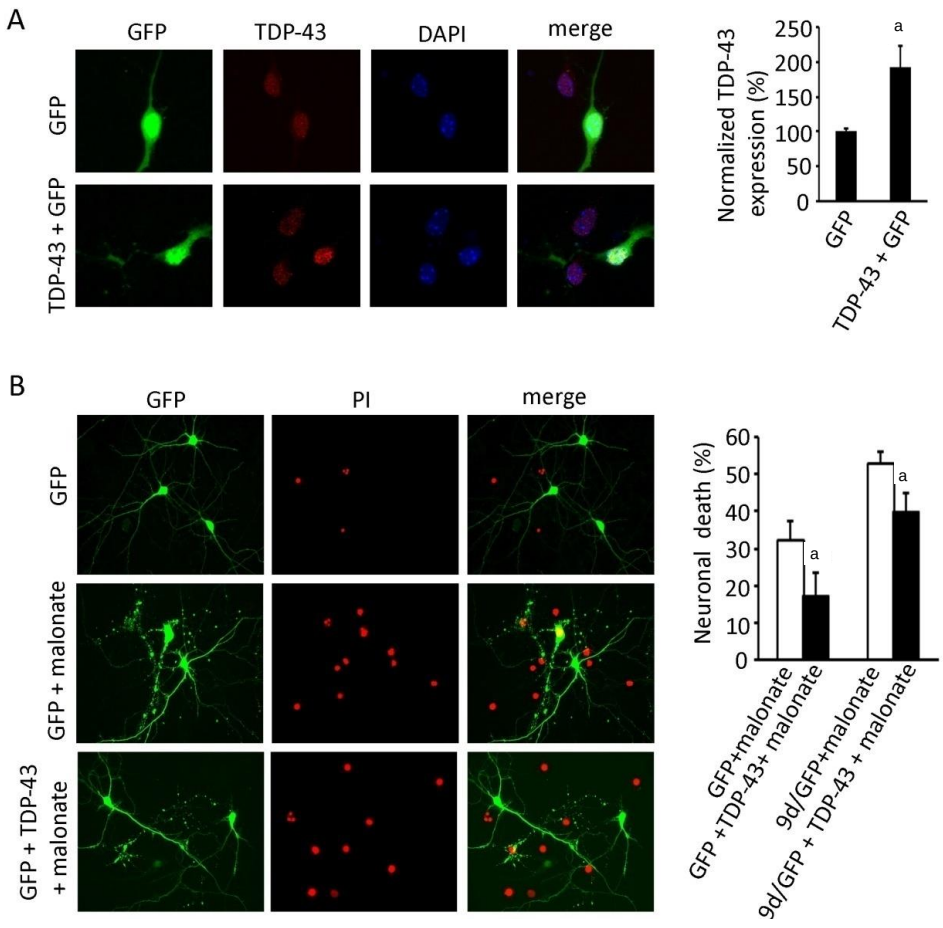

Figure 3 Effect of upregulation of nuclear TAR DNA-binding protein 43 (TDP-43) expression on oxidative stress-induced neuronal death.

(A) Representative confocal images (left; × 630) and summarized data (right) indicate that transient transfection with wild type mouse TDP-43 cDNA increases TDP-43 expression (data are normalized to GFP; there were 30 cells from three independent experiments for each group) in nuclei in normal cultured mouse cortical neurons.

(B) Representative confocal images (× 400; left) and summarized data (right) show that overexpression of TDP-43 in normal cultured neurons or neurons injured by malonate at 9 days. There were 50 cells from three independent experiments for each group. Data are expressed as mean ± SEM. [a]*P* < 0.01, *vs*. green fluorescent protein (GFP; A) or malonate-positive GFP (B) group.

DISCUSSION

In the present study, we successfully established an *in vitro* amyotrophic lateral sclerosis model by malonate-induced chronic oxidative stress. Since TDP-43 is the major protein component of abnormal inclusions in affected neurons in amyotrophic lateral sclerosis[7-8], models that mimic pathological changes in TDP-43 are very important in the study of the pathogenesis of the disease. In general, variation in a given protein is considered to be caused by a mutation of its encoding gene. At present, more than 40 kinds of TDP-43 gene mutations have been found in amyotrophic lateral sclerosis patients[44-47], most of them located in the C-terminal region[48]. This region is the location of protein-protein interaction, and is necessary for protein aggregation[49-50]. Transgenic studies indicate that expression of human amyotrophic lateral sclerosis-linked mutant TDP-43, or C-terminal fragments of the protein, can reproduce typical TDP-43 proteinopathies in mice or cultured cell lines[20-25]. Therefore, mutation in the TDP-43 gene would seem to be the cause of structural and functional abnormalities of the protein in amyotrophic lateral sclerosis. However, less than 3% of patients with this disease (including both familial and sporadic forms) have a mutation in the TDP-43 gene[26]. Environmental stresses, including ageing, oxidative stress, and inflammation, can modify the metabolism of TDP-43 through stress granules, caspases, and the ubiquitin proteasome system[31, 51-54]. As a degenerative disease, the progression of amyotrophic lateral sclerosis is closely associated with apoptosis and oxidative stress[27-30, 55]. Two recent studies used ethacrynic acid and paraquat, inducers of oxidative stress, to replicate TDP-43 proteinopathies in cell lines[31-32]. Both substances induced notable TDP-43 cytoplasmic aggregation and C-terminal fragmentation. However, pathologic TDP-43 changes occurred very quickly in both models. Ethacrynic acid, which increases cellular oxidative stress through glutathione depletion, induces TDP-43 redistribution, aggregation, and fragmentation in NSC34 cells within 5 hours[32]. Paraquat induces aggregation and fragmentation in SH-SY5Y and HeLa cells 24 hours after treatment[31]. A different inducer of oxidative stress is needed that can produce a chronic injury similar to the tardive pathological process of amyotrophic lateral sclerosis. Growing evidence indicates that malonate can induce oxidative stress over a very long time[36-38, 56]. In cultured organotypic brain or spinal slices, selective motor neuronal death occurs 14 days after malonate treatment. In the present study, we established a cell model of amyotrophic lateral sclerosis by treatment with malonate for 9 days in primary cultured cortical motor neurons. In our model, neuronal death occurred in the later stages of malonate insult and was accompanied by TDP-43 redistribution and aggregation. We established a cell model in primary cultured cortical motor neurons, which is closer to the *in vivo* situation than the ethacrynic acid and paraquat models in cell lines. In addition, histopathological studies in patients with amyotrophic lateral sclerosis have shown that there are more cytoplasmic TDP-43 inclusions in familial amyotrophic lateral sclerosis than in the sporadic form. Our chronic oxidative stress model displays the same pathological manifestation: there is a smaller number of aggregations in our model than in TDP-43 mutants or C-terminal fragments in other models. Therefore, our malonate-induced chronic oxidative stress model is suitable for studying the role of TDP-43 in the pathogenesis of amyotrophic lateral sclerosis.

Our data show that the protein level of TDP-43 in nuclei increases in the early stages of chronic oxidative stress and confirm that the enhanced nuclear expression of TDP-43 plays a protective role against oxidative stress-induced neurodegeneration. In our malonate-induced oxidative stress model, TDP-43 expression did not show a steady decrease as injury progressed; rather, a transient enhancement of TDP-43 expression occurred in the early stages of the malonate insult, with the increased protein being confined to nuclei without abnormal aggregation. This phenomenon is not seen in the ethacrynic acid or paraquat models[31-32]; the protein is cleared from nuclei 1 hour after ethacrynic acid treatment[32], whereas in the paraquat-induced model, cytoplasmic aggregation can be found 24 hours after treatment, but the protein level and morphology of nuclear TDP-43 show no detectable change after 30 hours[31]. Transfection of mice, C elegans, drosophilae, and zebrafish with human amyotrophic lateral sclerosis-linked mutant TDP-43 causes up to several-fold increases in TDP-43 expression, but the transfected protein forms abnormal inclusions in the cytoplasm, and sometimes in nuclei[20-25]. Endogenous TDP-43 in nuclei does not elevate in these transgenic animals[20-25]. Some previous studies indicated that cytoplasmic TDP-43 or abnormal TDP-43 inclusions were toxic. In a yeast model, when a panel of truncated TDP-43 constructs are transformed, the protein level of TDP-43 increases, but only the transformed TDP-43 aggregates in the cytoplasm produce toxicity[57]. In our study, TDP-43 expression was responsively upregulated during the early stages of malonate treatment, when the cultured neurons remained intact. Moreover, in contrast with the toxic theory, the increased TDP-43 protein did

not aggregate or redistribute. Therefore, we have reason to believe that the enhanced TDP-43 expression in the early stages of malonate-induced oxidative stress might play a protective role against neurodegeneration. We tested this theory by transfecting cultured mouse cortical motor neurons with TDP-43 cDNA and investigating the role of increased TDP-43 in neuronal survival. As with the transient increase in native TDP-43 expression, the overexpressed TDP-43 was also restricted to nuclei and did not show aggregation or redistribution, strongly suggesting an alleviation of malonate-induced neuronal death. Thus, enhanced TDP-43 expression in the early stages of malonate treatment plays a self-protective role against neurodegeneration mediated by malonate-induced oxidative stress.

The present data provide further evidence that a reduction in nuclear TDP-43 plays a key role in neurodegeneration. As TDP-43 proteinopathies are characterized by a loss of nuclear TDP-43 and an accumulation of abnormal protein inclusions in the cytoplasm, there are two different hypotheses about the pathogenesis of TDP-43 in neurodegeneration: loss of physiological function (mediated by nuclear clearance); or gain of toxic function (mediated by aggregates or abnormal cytoplasmic function)[58]. Both hypotheses are supported by numerous studies. Knockdown of TDP-43 in Neuro2a cells inhibits neurite growth and diminishes cell viability[18], and knockdown in HeLa cells or primary cultured mouse hippocampal neurons induces apoptosis[12]. Several groups have generated TDP-43 knockout mice, and in each instance all homozygous TDP-43 knockout mice (TDP-43$^{-/-}$) die during embryonic development owing to defective outgrowth of the inner cell mass[16, 59-60]. Even though heterozygous TDP-43 knockout mice (TDP-43$^{+/-}$) can survive for a long time, they have significantly decreased forelimb grip strength and display deficits in a standard inverted grid test, despite there being no evidence of pathologic changes in motor neurons[16]. Thus, TDP-43 is essential for viability, and a mild reduction in TDP-43 function is sufficient to cause motor deficits. On the other hand, aggregated TDP-43 may gain toxic function to induce neuronal degeneration[61], in the same way that increased β-amyloid production promotes senile plaque formation in Alzheimer's disease. A consensus from multiple reports of expression of human amyotrophic lateral sclerosis-linked TDP-43 mutations in worms, flies, and mice reveals abnormal aggregation of TDP-43 in the nucleus and cytoplasm accompanied by impaired locomotor activity, motor deficits, motor neuron loss, paralysis and reduced life span[20-25], implying that abnormal TDP-43 inclusions are toxic. Furthermore, a study in yeast testified that the toxicity induced by mutated TDP-43 is closely related to the number of aggregates in the cytoplasm[62]. Both loss of function and gain of toxicity have been cited as the cause of neurodegeneration, but a loss of nuclear TDP-43 is much more important than cytoplasmic TDP-43 aggregation. This is due to the fact that the number of inclusions in patients with amyotrophic lateral sclerosis without a mutation in TDP-43 is much lower than that in patients with the TDP-43 mutation[63], but both forms display the same neuronal degeneration, and extensive reduction or loss of nuclear TDP-43. As patients with TDP-43 mutations make up less than 3% of all patients with amyotrophic lateral sclerosis, we aimed to produce a non-mutated TDP-43 proteinopathy model. Our model shows obviously reduced nuclear TDP-43 and a relatively small number of TDP-43 aggregates in the later stages of the injury, closely mimicking non-mutated TDP-43 proteinopathy. To test whether a decrease in nuclear TDP-43 was the fundamental reason for neuronal death other than TDP-43 aggregation, we designed an experiment in which TDP-43 was overexpressed in cultured neurons with or without protein accumulation. If both could be protected against malonate-induced oxidative stress, it would indicate that they share the same mechanism, *i.e*. that decreased nuclear TDP-43 is the main reason for neuronal degeneration. Our data from malonate-treated TDP-43-overexpressing cells, which showed that increased nuclear TDP-43 reduces malonate-induced neuronal death, indicate that reduced nuclear TDP-43 expression is the cause of neuronal death. Furthermore, overexpression of TDP-43 in neurons treated chronically with malonate showed that even in neurons with cytoplasmic TDP-43 inclusions, an increase in nuclear TDP-43 protein can reduce neuronal death. This also indicates that TDP-43 aggregation is not the main cause of neuronal degeneration.

Our results revealed that overexpression of wild type mouse TDP-43 has a neuroprotective effect in cultured cortical motor neurons. In previous studies, overexpression of wild type full-length TDP-43 in transgenic animals achieved diverse results[20-22, 43, 64-67]. Some researchers concluded that the aggregations came from the excessive synthesized TDP-43 upon stimulation by the ubiquitin-proteasome system[21, 68-70]. Indeed, overexpression of wild type human TDP-43 in transgenic animals resulted in cytoplasmic aggregation similar to that seen with mutated TDP-43[21-22, 64-65, 67]. However, when a series of TDP-43 transgenic animals was analyzed[20-22, 43, 64-67], several characteristics were revealed: (1) overexpression of amyotrophic lateral sclerosis-linked TDP-43 mutants

produces more cytoplasmic aggregation than wild type TDP-43; (2) transgenic expression of wild type TDP-43 produces neurodegeneration in a dose-dependent fashion in mice; (3) overexpression of wild type TDP-43 to induce neurodegeneration appears to be a threshold effect. Transgenic animals usually begin to develop pathological and clinical features of amyotrophic lateral sclerosis when TDP-43 levels reach three times endogenous levels. Conversely, low levels of TDP-43 produce little or no amyotrophic lateral sclerosis phenotype. Our result is consistent with the above observations. In our transgenic study *in vitro*, we optimized experimental methods and obtained approximately twofold elevation of TDP-43 protein, therefore the increased TDP-43 was confined to nuclei without aggregation. Another interesting finding from the previous studies was that overexpression of wild type human TDP-43 and mutated TDP-43 in cultured cells and transgenic mice leads to a decrease in endogenous TDP-43 mRNA and protein[12, 69-70]. This phenomenon is explained by TDP-43 having an autoregulatory function[13-14]. As with many RNA regulatory proteins, TDP-43 can downregulate its own mRNA transcript levels *via* direct binding to its own 3' UTR sequence, and contributes to mRNA instability and degradation[71-72]. This autoregulatory feedback loop is crucial for maintaining appropriate levels of functional TDP-43 protein. In positive phenotype transgenic mice, TDP-43 protein is usually increased more than threefold, which may initiate autoregulation to reduce endogenous TDP-43 expression in nuclei. Mutated TDP-43 and environmental stressors can also disrupt such autoregulation, permitting abnormal TDP-43 expression and accumulation[73]. In our transgenic experiment, to avoid heterogenous TDP-43 inducing the autoregulation, we overexpressed homologous TDP-43 in cultured mouse cortical motor neurons. The result showed that when homogenous TDP-43 was overexpressed, the protein level increased approximately twofold, and it was this mild increase in TDP-43 that played a neuroprotective role.

In conclusion, the present study provides the first evidence that upregulated nuclear TDP-43 protein is a pro-survival factor in the early stages of malonate-induced chronic oxidative stress, and overexpression of homogenous TDP-43 confers neuroprotection. A series of previous studies disclosed that nuclear TDP-43 reduction or clearance was an important factor in neuronal degeneration in amyotrophic lateral sclerosis pathology[12, 16-19, 22, 43]. Therefore, an increase in nuclear TDP-43 using transgenic methods may rescue neurons that begin to degenerate. However, overexpression of wild type human TDP-43 in mice might result in further destruction. Our data show that overexpression of wild type mouse TDP-43 in cultured mouse cortical motor neurons results in a mild elevation of TDP-43 and plays a neuroprotective role. Thus, prolonged enhanced TDP-43 expression in nuclei or overexpression of homologous TDP-43 may represent novel strategies in the treatment of amyotrophic lateral sclerosis. As TDP-43 proteinopathies also occur in frontotemporal lobar degeneration with ubiquitinated inclusion bodies (Alzheimer's, Parkinson's and Huntington's diseases, and dementia with Lewy bodies)[31], it would be interesting in the future to test whether TDP-43 also protects from other forms of neuronal death or cytotoxicity in different cell types.

MATERIALS AND METHODS

Design

An *in vitro* molecular and genetic engineering experiment.

Time and setting

This study was performed at Peking University Third Hospital, China, from September 2010 to May 2012.

Materials

A total of 42 healthy pregnant C57BL/6 mice (specific pathogen free grade, aged over 6 months, weighing 24–30 g) were supplied by the Experimental Animals Center of Peking University Health Science Center, China (license No. SYXK (Beijing) 2006-0008). All procedures were carried out in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[74], and were approved by the Animal Ethics Committee of Peking University Health Science Center in China.

Methods

Culture of mouse cortical motor neurons and malonate treatment

Primary cultures of cortical motor neurons were prepared from embryonic motor cortex of C57BL/6 mice at embryonic day 17 using a modified protocol[19]. After removing meninges, cortices were placed in ice-cold plating medium (Neurobasal medium, 2% B-27 supplement, 0.5% fetal bovine serum, 0.5 μmol/L L-glutamax, and 25 μmol/L glutamic acid; Sigma, St. Louis, MO, USA). Dissociated motor neurons were prepared from precentral gyrus that was carefully dissected from the cortices. The neurons were suspended in plating medium and plated on 35 mm Petri dishes coated with poly-D-lysine. After 3 days in culture, half of the plating medium was

removed and replaced with maintenance medium (Neurobasal medium, 2% B-27 supplement, and 0.5 μmol/L L-glutamine). Thereafter, maintenance medium was changed in the same manner every 3 days. To induce chronic oxidative stress, the cultures were exposed to malonate (3 mmol/L; Sigma) on the 6th day after plating. The cultured neurons were collected for experiments at 1, 3, 6 and 9 days after malonate treatment.

Wild type mouse TDP-43 cDNA transfection of cortical motor neurons

The transfection experiments were performed in normal cultured mouse cortical motor neurons and damaged neurons on the 7th day after malonate treatment using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. The transfections were carried out by incubating cultures for 4 hours with 1 μg of DNA and 2 μL Lipofectamine per mL of culture medium. Cells were washed once with fresh culture medium and were maintained for 48 hours prior to experimentation. The transfection efficiency was approximately 5% and the transfection procedure was not toxic to the neurons. The cDNA encoding the longest mouse isoform of TDP-43 (wild type mice TDP-43 cDNA) (pEGFP-TDP-43-L; accession number AY 145556) was kindly provided by Academia Sinica[75]. The cDNA of green fluorescent protein (University of Nevada) was used as a marker of successful neuronal transfection[76]. The cells were fixed for immunocytochemical labeling 48 hours after transfection.

Neuronal death analysis

Lactate dehydrogenase release and propidium iodide staining were used to examine the death of cultured neurons. Lactate dehydrogenase release was measured using CytoTox 96 Cytotoxicity kit (Promega, Madison, WI, USA) based on the manufacturer's instructions[77]. Maximal neuronal death was measured by treating the cultures with 10 × lysis solution to completely lyse the cells. Absorbance data were obtained using a 96-well plate reader (Shenzhen Prokan Electronics Inc., Shenzhen, Guangdong Province, China) at 490 nm.

Propidium iodide labeling was performed to detect neuronal death. The culture medium was replaced by extracellular solution containing propidium iodide (Invitrogen Life Technologies) at a final concentration of 50 μg/mL. After 20 minutes of incubation in an ambient gas incubator at 37°C, cultures were washed with extracellular solution and then fixed with 4% paraformaldehyde. Neuronal death was determined by calculating the percentage of green fluorescent protein-expressing neurons that were labeled with propidium iodide [green neurons with propidium iodide labeling/(green neurons with propidium iodide labeling + green neurons without propidium iodide labeling)]. The investigator for the cell count was blinded to the experimental treatment.

Western blot analysis of TDP-43 protein expression

Cultured cortical neurons were lysed in 2% sodium dodecyl sulfate sample buffer (50 mmol/L Tris-HCl buffer, pH 7.5, 0.15 mol/L NaCl, 1.0 mol/L EDTA, 2% sodium dodecyl sulfate, 1% sodium deoxycholate, protein phosphatase inhibitors, and protease inhibitor cocktail). Lysates were sonicated and centrifuged at 100 000 × *g* for 15 minutes. Equal amounts of proteins were separated by 8–10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were electronically transferred to a polyvinylidene difluoride membrane and incubated with a blocking buffer for 1 hour at room temperature. The membranes were incubated with primary goat anti-TDP-43 polyclonal antibody (1:1 000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, and then with horseradish peroxidase-conjugated rabbit anti-goat IgG (1:2 000; Sigma) for 1 hour at room temperature. The protein bands were imaged using enhanced chemiluminescence. The membranes were reprobed with anti-GAPDH antibody as a loading control. Images were analyzed using ImageJ software (NIH, Bethesda, MD, USA).

Immunocytochemical staining of protein localization

Primary cultured mouse cortical motor neurons were grown on 15 mm diameter coverslips and treated as indicted above. Cells were fixed with 4% (w/v) paraformaldehyde in PBS for 30 minutes and blocked in 5% normal goat serum for 1 hour. The specimen was incubated with primary goat anti-TDP-43 polyclonal antibody (1:2 000) overnight at 4°C and then with Alexa Fluor 488 (green fluorescence)-conjugated secondary antibody (1:2 000; Invitrogen Life Technologies), or Alexa Fluor 568 (red fluorescence)-conjugated secondary antibody (1:2 000; Invitrogen Life Technologies) for 1 hour at room temperature. 4′,6′-diamidino-2-phenylindole (DAPI; Invitrogen Life Technologies) was used to label the nuclei. Fluorescent-labeled proteins were imaged using a 63 × or 40 × objective mounted on a Zeiss LSM 510 META confocal microscope (Oberkochen, Germany). Images were acquired using a Zeiss AxioCam digital camera in the linear range with constant settings and were analyzed using ImageJ software. The fluorescence intensity in each image was a z-series of 6–13 images, taken at 0.75 μm depth intervals. The resultant stack was "flattened" into a single image using a maximum projection.

All images were analyzed using identical acquisition parameters. During data acquisition and analysis, the investigator was blind to the treatment group.

Subcellular fractionation assay

The cultured neurons were harvested and washed in PBS by repeated centrifugation. The experiment was carried out at 4°C. The pellet was resuspended in five volumes of buffer N (15 mmol/L Tris-HCl pH 7.5, 60 mmol/L KCl, 15 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 1 mmol/L DTT, 2 mmol/L Na_3VO_4, 1 mmol/L phenylmethyl sulfonylfluoride, 0.25 mol/L sucrose, complete protease inhibitor cocktail). Cell lysis was obtained by adding an equal amount of buffer N plus 0.6% NP-40. Following 5 minutes of incubation, nuclei were pelleted and gently resuspended in 1 mL of buffer N. The nuclei were again pelleted and lysed using an equal volume of solution 2 (10 mmol/L HEPES pH 7.9, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.1 mmol/L EGTA , 0.1 mmol/L DTT, 0.5 mmol/L phenylmethyl sulfonylfluoride, 5% glycerol, 0.4 mol/L NaCl). The nuclear fraction was cleared by centrifugation following 30 minutes of incubation. Nuclear and cytoplasmic fractions were quantified and visualized by western blot staining with p84 (rabbit anti-mouse, 1:2 000, Abcam Biotechnology, Cambridge, MA, USA) and tubulin (goat anti- mouse, 1:2 000, Santa Cruz Biotechnology).

Statistical analysis

Results were analyzed using SPSS version 12.0 (SPSS, Chicago, IL, USA) and measurement data were expressed as mean ± SEM. Intergroup differences were compared by one-way analysis of variance. Least significant difference test was used in pairwise comparison. Differences between two groups were compared by Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

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