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Original Article

The influence of human TDP-43-M337V on ROCK/PTEN signaling pathway in NSC-34 cells

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Abstract: The mechanism underlying the pathology of amyotrophic lateral sclerosis (ALS) is unclear. Thus, we sought to study the effect of TDP-43-M337V overexpression on the ROCK/PTEN signaling pathway in mouse motor neuron (NSC-34) cells. The hTDP-43-M337V gene reduced cellular protection against oxidative damage, increased intracellular lipid peroxidation, and MTT assay confirmed inhibition of growth in NSC-34 cells transfected with an hTDP-43-M337V overexpression plasmid. Western blot confirmed increased p-PTEN protein and decreased p-Akt protein. PTEN and Akt protein were not different in ALS transfected cells. ROCK activity indicated that Rho-kinase did not change among TDP-43-M337V mutant cells, compared with other cell groups. Transfected hTDP-43-M337V reduced NSC-34 cell activity and oxidative damage resistance, and increased vulnerability of motor neurons to stress-induced injury. Human TDP-43-M337V on NSC-34 cells did not influence ROCK activity or protein expression but modified PTEN and Akt phosphorylation. Thus, a transfected TDP-43 mutant gene may activates apoptosis signaling pathways and induce apoptosis via different mechanisms. These results suggest that hTDP-43-M337V overexpression augments oxidative stress and damages the ROCK/PTEN signaling pathway and suggests a novel mechanism of toxicity that can be used to increase our understanding of signaling during ALS.

Keywords: Amyotrophic lateral sclerosis, NSC-34 cells, TDP-43, ROCK/PTEN signaling pathways

Introduction

Amyotrophic lateral sclerosis (ALS) is a gradually fatal nervous system degenerative disease. As the most common adult-onset motor neuron disease, it is characterized by selective loss of motor neurons in the motor cortex, brainstem, and spinal cord, with a progressive development. ALS or Lou Gehrig's disease (US) or motor neuron disease (UK), has an annual incidence of 2-3 cases per 100,000. Clinical manifestations of ALS include muscle weakness, atrophy, tremors and tendon hyperreflexia. Approximately 10% of ALS cases are inherited, and the remaining 90% have no known cause. At present, the pathogenesis of ALS is unclear and no effective treatments are available. Patients die from ALS due to respiratory muscle denervation more than 40 months after the initial clinical onset [1, 2].

Rho kinase (ROCK) signaling pathways are key to reconstruction of the cytoskeleton, cell differentiation and migration, and apoptosis. Research suggests that rho kinase activates apoptosis signaling cascades and promotes apoptosis and cell death. Phosphatase and tensin homolog (PTEN) has been confirmed to be a ROCK substrate, and phosphatase activity is elevated after ROCK phosphorylation [3]. PTEN has a negative regulatory role in the PI3K/Akt signaling pathway, and an important role in apoptosis: ROCK/PTEN signaling has been implicated in apoptosis [4-6].

Recently activated ROCK was reported to suppress phosphorylation of Akt via activation of PTEN. Also, in a *SOD1* mutant mouse model, ROCK signaling pathways are involved in apoptosis induced by *SOD1-G93A* mutations [7]. ROCK activation by *SOD1-G93A* elevates PTEN

phosphorylation and reduces Akt phosphorylation, inducing neuronal death. Because some ROCK inhibitors are protective against cell death, new target treatments of neurodegenerative disease focused on the ROCK/PTEN signaling pathway have been proposed [8, 9].

Studies indicate that the onset and progress of cardiovascular diseases are associated with elevated ROCK activity [10-12] and ROCK pathways are involved in the occurrence and progress of various diseases [12-16]. Growing concern about ROCK signaling pathway roles in nervous system diseases have stimulated research into ROCK signaling pathways and ALS, and inhibitors of ROCK are expected to provide a new strategy for treatment of ALS.

Numerous studies verified that mutant *TDP-43* may could be involved in 2.9% of sporadic ALS cases and 3% of familial ALS [17, 18]. Also, inclusions caused by *TDP-43* mutations may initiate neurodegenerative diseases such as ALS [17]. Although *TDP-43* inclusions are now recognized as a common characteristic of ALS patients, how this affects ALS is uncertain.

At present, multiple *TDP-43* gene mutations have been identified in different populations of sporadic and familial ALS patients [19, 20]. The human *TDP-43* mutation expressed in transgenic mice and human ALS have similar clinical manifestations and pathological features, and the human *TDP-43* mutant transgenic mouse models is a relatively common for studying ALS (hTDP-43-Q331K and *hTDP-43-M337V* are typical. Data suggest that TARDBP genetic abnormalities may be tied to the onset and progression of ALS [21]. *TDP-43* mutant and *SOD1-G93A* transgenic mouse models are well established for researching pathogenesis and potential therapies. Here, we studied the effect of the mutant *hTDP-43-M337V* gene on the ROCK/PTEN signaling pathway in NSC-34 cells transfected with *hTDP-43-M337V* to better understand ALS and how to treat it.

Materials and methods

Chemicals

An antibody recognizing anti-β-actin was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other antibodies, including anti-ROCK1, anti-ROCK2, anti-PTEN, anti-phospho-PTEN, anti-Akt and anti-phospho-Akt were purchased from Abcam Biotechnology, Inc. (Abcam, Cambridge, MA). Malondialdehyde (MDA) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). MTT Cell Proliferation and Cytotoxicity Assay Kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Total protein extraction kit were purchased from Pulitzer gene technology co., LTD (Beijing, China). A CycLex Rho-kinase Assay Kit was purchased from MBL (CycLex Co. Ltd, Nagano, Japan).

Cell lines and cell cultures

NSC-34 is a hybrid cell line, produced by fusion of motor neuron enriched, embryonic mouse spinal cord cells with mouse neuroblastoma, which possesses several unique morphological and physiological characteristics of motor neurons. NSC-34 cells stably transfected with the pDEST30-EGFP plasmid, pDEST30-EGFP-TDP-43-WT plasmid and the pDEST30-EGFP-TDP-43-M337V plasmid had been successfully established in our laboratory. Cell lines were removed from liquid nitrogen and thawed quickly in a 37°C water bath. Then, cells were diluted 5-fold with complete medium (containing 90% Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 100 IU/ml penicillin and 0.1 mg/ml streptomycin) and seeded to 25-cm^2 glass culture bottles. Cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere. After attachment of cells to culture bottles (4-6 h), the medium was refreshed to remove DMSO. Medium was changed every 2-3 days, depending on growth rate. Once cells reached 90% confluence, they were used for experiments and divided into three groups: empty plasmid transfected with pDEST30-EGFP, TDP-43-WT transfected with pDEST30-EGFP-TDP-43-WT plasmid and TDP-43-M337V transfected with pDEST30-EGFP-TDP-43-M337V plasmid.

Measurement of MDA

MDA is a degradation product of lipid peroxidation that reacts with thiobarbituric acid (TBA) in a colorimetric assay. We measured reacted TBA (532 nm) in confluent cells at room temperature after cell disruption. Then, cells were incubated in a 95°C water bath for 40 min, the reaction products were rapidly cooled with running water. Product absorbance was measured and a BCA method was used to quantify protein

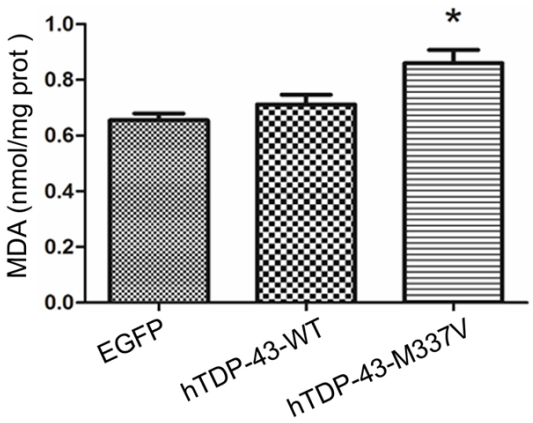

Figure 1. Cells were harvested after incubating for 48 h and MDA was measured in three cell lines (mean ± SD, n = 3). Note: EGFP: NSC-34 cell lines transfected with empty pDEST30-EGFP plasmid; hTDP-43-WT: NSC-34 cell lines transfected with pDEST30-EGFP-TDP-43-WT plasmid; hTDP-43-M337V: NSC-34 cell lines transfected with pDEST30-EGFP-TDP-43-M337V plasmid. *Statistically different from EGFP, hTDP-43-WT cell lines (*P<0.05).

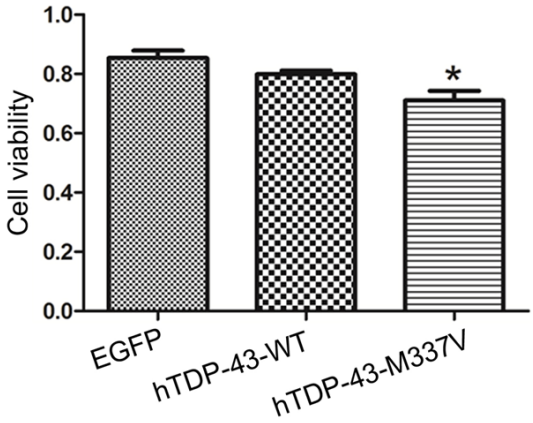

Figure 2. Cells were harvested after incubating for 48 h and an MTT assay was used to measure cell viability in three cell lines (mean ± SD, n = 4). *Statistically different from EGFP, hTDP-43-WT cell lines (*P<0.05).

using BSA as a standard. Data were expressed as nmol/mg prot.

Measurement of MTT

The three groups of transfected cells were seeded in 96-well sterile culture plates (2×10^3/ml), with three duplications for each group. Cells with no plasmid were blank controls. Once cells were 90% confluent, they were incubated for 4 h in the dark and 20 μl of 5 mg/mL MTT was added. Culture medium was discarded and 150 μl DMSO was added to wells. Cells were vortexed to mix solutions for 10 min. Then, activity was measured by monitoring the change in absorbance at 570 nm.

Western blot

NSC-34 cells were lysed with RIPA buffer and proteins were extracted using a total protein extraction kit. Protein was quantified by comparison with a known concentration of BSA using a BCA Protein Assay kit. A total of 60 μg of extracted protein was resolved on 10% SDS-PAGE and resolved proteins were transferred to polyvinylidene difluoride membranes. Membranes were incubated overnight at 4°C with the following specific primary antibodies: rabbit monoclonal anti-ROCK1 (1:500), rabbit monoclonal anti-ROCK2 (1:5,000), rabbit monoclonal anti-PTEN (1:500), rabbit monoclonal anti-Akt (1:5,000), rabbit monoclonal anti-phospho-PTEN (1:1,000), rabbit monoclonal anti-phospho-Akt (1:5,000), and mouse monoclonal anti-β-actin (1:500). Subsequently, the membranes were incubated with corresponding secondary antibody (the dilution of β-actin secondary antibody was 1:10,000 and the others were 1:5,000) for 1 h at room temperature and immunodetection was performed with an enhanced chemiluminescent substrate. Data were calculated by comparing the density of target protein bands to the density of corresponding β-actin bands.

ROCK activity assay

ROCK activity was assayed using a CycLex Rho-kinase assay kit, a single-site, semi-quantitative immunoassay, according to the manufacture's recommendations. Stably transfected cells were immediately prepared in extraction buffer (25 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 5% glycerol, 10 mM β-mercaptoethanol, and protease and phosphatase inhibitor cocktails) at 4°C, then centrifuged at 12,000×g to obtain lysates. Lysates were added to precoated plates with myosin-binding subunit of myosin phosphate (MSB), including a threonine residue that is phosphorylated by Rho kinase, for 60 min at room temperature. After washing, horseradish peroxidase-conjugated anti-phospho-specific MSB threonine-697 specific antibody was applied into wells and incubated for 1 h at room temperature. Products were devel-

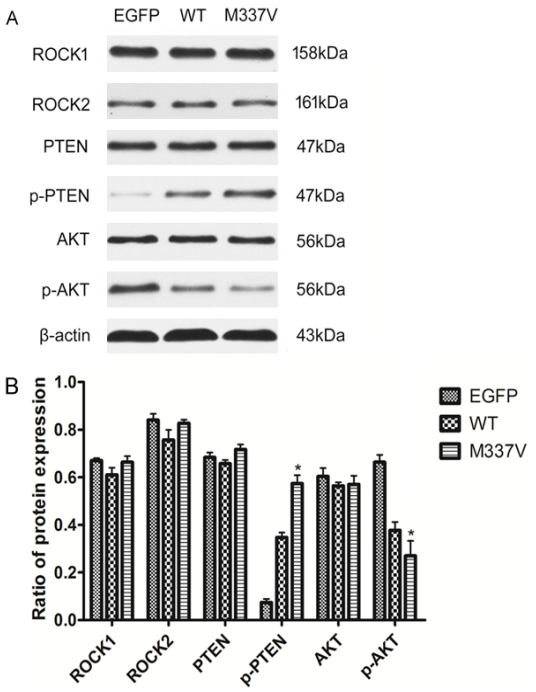

Figure 3. Western blot of ROCK (ROCK1, ROCK2), PTEN, p-PTEN, Akt and p-Akt protein in three cell lines. Protein was separated on SDS-PAGE as indicated and probed with specific primary antibodies and β-actin as a control. Protein band densities were measured and their ratio to β-actin was calculated (mean ± SD, n = 3) (EGFP: empty plasmid, WT: *hTDP-43-WT*, M337V: *hTDP-43-M337V*). *Statistically different from EGFP, *hTDP-43-WT* cell lines (*P<0.05).

oped by incubation with the horseradish peroxidase substrate tetramethylbenzidine at room temperature for 10 min. The reaction was stopped by adding stop solution containing 0.5 M H_2SO_4. Colored products were quantified with spectrophotometry (450 nm). Purified Rho kinase (CycLex) was used as a positive control.

Statistical analysis

Data are expressed as means ± standard deviation. Statistical analyses were performed using one-way ANOVA (P<0.05 statistically significant differences).

Results

Lipid peroxidation

MDA measurement is a proxy for lipid peroxidation so we measured this in NSC-34 cells stably transfected with the *hTDP-43-M337V* gene. MDA content is depicted in **Figure 1**. MDA in NSC-34 cells transfected with the *hTDP-43-M337V* gene was significantly greater than the other two treatment groups and MDA was not statistically significantly different between the other two cell groups.

The hTDP-43-M337V gene and NSC-34 cell viability

An MTT assay was used to measure cell viability (**Figure 2**). Data show that MTT in NSC-34 cells transfected with the *hTDP-43-M337V* gene was less than in the other two treatment groups but the other two cell groups were not different from each other. Thus, the *hTDP-43-M337V* gene did not increase cell viability compared to cells transfected with empty and *hTDP-43-WT* genes.

The hTDP-43-M337V gene and ROCK protein: downstream factors of the ROCK/PTEN signaling pathway in NSC-34 cells

We measured the effect of *hTDP-43-M337V* on the ROCK/PTEN signaling pathway in NSC-34 cells, by assessing ROCK protein and the downstream ROCK/PTEN pathway factors using Western blot. p-PTEN protein in NSC-34 cells transfected with the *hTDP-43-M337V* gene increased, and p-Akt protein decreased compared with the other two cell lines, which showed the opposite effect (**Figure 3A** and **3B**, P<0.05). There was no difference between empty and *TDP-43-WT*-expressing cells with respect to p-PTEN or p-Akt protein (**Figure 3A** and **3B**, P>0.05). There was also no significant difference between ROCK1, ROCK2, PTEN and Akt protein in these three cell lines (**Figure 3A** and **3B**, P>0.05). Thus, the *hTDP-43-M337V* gene increased p-PTEN protein expression and inhibited p-Akt protein expression. Therefore, the ROCK/PTEN signaling pathway is likely affected by *hTDP-43-M337V*.

Effect of the hTDP-43-M337V gene on ROCK activity in NSC-34 cells

Previous studies and our work indicate that the *hTDP-43-M337V* gene modifies downstream factors in the ROCK/PTEN signaling pathway. Next, we measured differences in ROCK activity to determine if this was the mechanism by which the ROCK/PTEN signaling pathway was manipulated. Data show that ROCK activity was not different in cells transfected with pDEST30-EGFP-TDP-43-WT and pDEST30-EGFP-TDP-43-

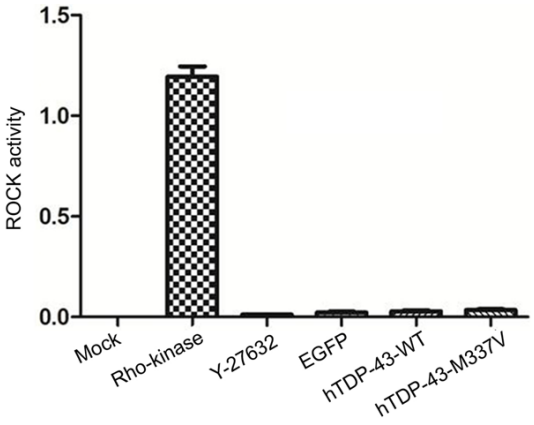

Figure 4. Cells were harvested after incubating for 48 h and ROCK activity was measured in three cell lines (mean ± SD, n = 3) (Mock: blank control, Rho-kinase: positive control, Y-27632: negative control). No difference was noted among EGFP, *hTDP-43-WT* and *hTDP-43-M337V* cell lines (P>0.05).

M337V compared with empty NSC-34 cells (**Figure 4**, P>0.05).

Discussion

Mechanisms underlying ALS pathology are unknown but several hypotheses suggest that ALS onset may be promoted by a series of complex interactions such as oxidative stress [22, 23], gene mutation, apoptosis, excitatory amino acid toxicity, iron metabolism [24-26], mitochondrial damage [27, 28], axonal transport barriers [29], environmental factors, and abnormal protein accumulation [30]. Of these ideas, oxidative stress may be the dominant contributor to chronic motor neuron degeneration as well as a mechanism contributing to selective motor neuron and corticospinal tract degeneration. Studies with spinal cord slices and cerebrospinal fluid of ALS patients suggest that oxidative stress may explain the pathogenesis of ALS [23], and oxidative damage of nucleic acids, proteins, and lipids was noted in *SOD1*-mutant animal models [31, 32].

Presently, 17 mutant genes associated with ALS have been reported, and the four most common genes are *SOD1*, *FUS sarcoma fusion*, *C9orf72*, and *trans activation reaction-DNA binding protein* (*TARDBP*) genes [33]. An ubiquitin abnormal protein aggregation (43 kDa comprised of the TAR DNA-binding protein, TDP-43) was found in the nucleus and cytoplasm of motor neurons and glial cells among ALS patients. *TDP-43* contributes to ALS pathogenesis and this gene has been mentioned in this context by another group [19]. To date, *TDP-43* gene mutations have been confirmed in different ALS populations-from sporadic to familial ALS, and these mutations are diverse and include missense mutations [19, 20]. *TDP-43* mutations can destroy proteostasis and contribute to protein dysfunction [34] and abnormal phosphorylation.

Studies using ALS disease models caused by a *TDP-43* mutation suggest that 2.9% of ALS cases are sporadic and 3% are familial [17]. *TDP-43* mutations in ALS patients are common but how they function is unclear. Huang's group reported that mutant *TDP-43* expression in rat motor neurons triggered the onset and progression of ALS [35] but how the *TDP-43* gene mutations degenerates motor neurons is not well understood.

To investigate the toxic effects of *TDP-43-M337V* on motor neurons, NSC-34 cell lines transfected with *hTDP-43-M337V* and the ROCK/PTEN pathway was observed. *hTDP-43-M337V* reduced cellular protection against oxidative damage and increased intracellular lipid peroxidation and cellular damage. Oxidative damage is documented to occur according to biochemical and histopathological studies of postmortem tissue of ALS patients, and this was correlated to disease severity. These studies verified that nucleic acid oxidation, lipid peroxidation, protein nitration and protein glycosylation in ALS patients was significantly elevated [36, 37]. Oxidative damage also has been reported to be present in the cerebral cortex and CSF of ALS patients. We used empty, *hTDP-43-WT* and *hTDP-43-M337V* transfected cells to measure oxidative damage, and MDA in *hTDP-43-M337V* cells was significantly increased compared with the other two cell treatments (**Figure 1**). Thus, antioxidant detoxification in *hTDP-43-M337V* gene transfected cells decreased, which may be a potential mechanism by which mutant NSC-34 cells are vulnerable to external stimuli and subsequent injury.

To observe the influence of *hTDP-43-M337V* on NSC-34 cell vitality, an MTT assay was used to measure viability and *hTDP-43-M337V*-treated cells had fewer viable cells compared to the other two cell groups (**Figure 2**). Thus, in NSC-34 cells, *hTDP-43-M337V* gene overexpression inhibited motor neuron growth so the *hTDP-43-*

M337V gene could inhibit motor neuron cells viability.

To understand the role of *TDP-43-M337V* on ROCK/PTEN signaling pathways, we compared empty- or *hTDP-43-WT*-expressing, and *TDP-43-M337V*-expressing NSC-34 cells and noted upregulation of phosphorylated PTEN and downregulation of phosphorylated Akt. However, total protein expression of PTEN or Akt was not different in either group of cells (**Figure 3A** and **3B**). Moreover, there was no significant difference among empty-, *hTDP-43-WT*- and *hTDP-43-M337V*-expressing NSC-34 cells on ROCK1 or ROCK2 protein (**Figure 3A** and **3B**). This is consistent with results obtained by Tanaka's group [7] in motor neuron cells of human *SOD1* mutant transgenic mouse models.

ROCK activity in *hTDP-43-M337V* cells was not different than in the other cell groups (**Figure 4**), but these data differed from published results [7]. It is suggested that elevated ROCK activity suppressed phosphorylation of protein kinase B (Akt) though activation of phosphatase and tension homolog deleted on chromosome 10 (PTEN) [4], which was activated by *SOD1*-G93A, *in vivo* and *in vitro*. In contrast, when cells were exposed to oxidative stress, the mutant *TDP-43* gene transformed phosphorylation of downstream effectors PTEN and Akt, but not via activating ROCK. Thus, a *TDP-43* gene mutation may participate in the pathogenesis of ALS using different means or mechanisms. ROCK appears to be implicated in positive and negative regulation of PI3K/Akt signaling pathway, and outcomes may depend on cell types or stimuli [6]. Previous research indicates that the ROCK/PTEN/Akt pathway regulates cell death in neurons [38] and the PTEN/Akt pathway accounts for motor neuron survival in human *SOD1*-related ALS [39]. In a *SOD1-G93A* transgenic mouse model, ROCK activated by *SOD1-G93A* downregulates phosphorylated Akt by enhancing phosphorylated PTEN, causing apoptosis. Thus, *TDP-43* mutant transgenic mouse models might undergo different pathogenesis from that of *SOD1*, inducing apoptotic signaling pathways and contributing to apoptosis in ways that are currently unclear.

In conclusion, oxidative stress was elevated during ALS and the *hTDP-43-M337V* gene damaged the ROCK/PTEN signaling pathway, thereby decreasing cell viability and reducing cell defenses against oxidative damage. This caused increased vulnerability of injury induced by stress in motor neurons. Transfection of the *hTDP-43-M337V* gene in NSC-34 cells activated apoptosis signaling pathways and induced apoptosis but this was not via elevated ROCK activity.

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Disclosure of conflict of interest

None.

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References

[1] Mitchell JD, Callagher P, Gardham J, Mitchell C, Dixon M, Addison-Jones R, Bennett W and O'Brien MR. Timelines in the diagnostic evaluation of people with suspected amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND)--a 20-year review: can we do better? Amyotroph Lateral Scler 2010; 11: 537-541.

[2] Traub R, Mitsumoto H and Rowland LP. Research advances in amyotrophic lateral sclerosis, 2009 to 2010. Curr Neurol Neurosci Rep 2011; 11: 67-77.

[3] Fusella F, Ferretti R, Recupero D, Rocca S, Di Savino A, Tornillo G, Silengo L, Turco E, Cabodi S, Provero P, Pandolfi PP, Sapino A, Tarone G and Brancaccio M. Morgana acts as a proto-oncogene through inhibition of a ROCK-PTEN pathway. J Pathol 2014; 234: 152-163.

[4] Wu J, Li J, Hu H, Liu P, Fang Y and Wu D. Rho-kinase inhibitor, fasudil, prevents neuronal apoptosis via the Akt activation and PTEN inactivation in the ischemic penumbra of rat brain. Cell Mol Neurobiol 2012; 32: 1187-1197.

[5] Li R, Wang X, Zhang XH, Chen HH and Liu YD. Ursolic acid promotes apoptosis of SGC-7901 gastric cancer cells through ROCK/PTEN mediated mitochondrial translocation of cofilin-1. Asian Pac J Cancer Prev 2014; 15: 9593-9597.

[6] Li G, Liu L, Shan C, Cheng Q, Budhraja A, Zhou T, Cui H and Gao N. RhoA/ROCK/PTEN signaling is involved in AT-101-mediated apoptosis in human leukemia cells in vitro and in vivo. Cell Death Dis 2014; 5: e998.

[7] Takata M, Tanaka H, Kimura M, Nagahara Y, Tanaka K, Kawasaki K, Seto M, Tsuruma K, Shimazawa M and Hara H. Fasudil, a rho kinase inhibitor, limits motor neuron loss in experimental models of amyotrophic lateral sclerosis. Br J Pharmacol 2013; 170: 341-351.

[8] Hensel N, Rademacher S and Claus P. Chatting with the neighbors: crosstalk between Rho-kinase (ROCK) and other signaling pathways for treatment of neurological disorders. Front Neurosci 2015; 9: 198.

[9] Labandeira-Garcia JL, Rodriguez-Perez AI, Villar-Cheda B, Borrajo A, Dominguez-Meijide A and Guerra MJ. Rho kinase and dopaminergic degeneration: a promising therapeutic target for Parkinson's disease. Neuroscientist 2015; 21: 616-629.

[10] Na W, Peng G, Jianping Z, Yanzhong C, Shengjiang G and Li C. RhoA/ROCK may involve in cardiac hypertrophy induced by experimental hyperthyroidism. Toxicol Ind Health 2012; 28: 831-839.

[11] Hartmann S, Ridley AJ and Lutz S. The function of Rho-associated kinases ROCK1 and ROCK2 in the pathogenesis of cardiovascular disease. Front Pharmacol 2015; 6: 276.

[12] Shimizu T and Liao JK. Rho kinases and cardiac remodeling. Circ J 2016; 80: 1491-1498.

[13] Soga J, Noma K, Hata T, Hidaka T, Fujii Y, Idei N, Fujimura N, Mikami S, Maruhashi T, Kihara Y, Chayama K, Kato H, Liao JK and Higashi Y. Rho-associated kinase activity, endothelial function, and cardiovascular risk factors. Arterioscler Thromb Vasc Biol 2011; 31: 2353-2359.

[14] Al-Shboul O. The role of the RhoA/ROCK pathway in gender-dependent differences in gastric smooth muscle contraction. J Physiol Sci 2016; 66: 85-92.

[15] Banerjee S and McGee DW. ROCK activity affects IL-1-induced signaling possibly through MKK4 and p38 MAPK in Caco-2 cells. In Vitro Cell Dev Biol Anim 2016; 52: 878-884.

[16] Bei Y, Hua-Huy T, Nicco C, Duong-Quy S, Le-Dong NN, Tiev KP, Chereau C, Batteux F and Dinh-Xuan AT. RhoA/Rho-kinase activation promotes lung fibrosis in an animal model of systemic sclerosis. Exp Lung Res 2016; 42: 44-55.

[17] Lagier-Tourenne C and Cleveland DW. Rethinking ALS: the FUS about TDP-43. Cell 2009; 136: 1001-1004.

[18] Brown JA, Min J, Staropoli JF, Collin E, Bi S, Feng X, Barone R, Cao Y, O'Malley L, Xin W, Mullen TE and Sims KB. SOD1, ANG, TARDBP and FUS mutations in amyotrophic lateral sclerosis: a United States clinical testing lab experience. Amyotroph Lateral Scler 2012; 13: 217-222.

[19] Daoud H, Valdmanis PN, Kabashi E, Dion P, Dupre N, Camu W, Meininger V and Rouleau GA. Contribution of TARDBP mutations to sporadic amyotrophic lateral sclerosis. J Med Genet 2009; 46: 112-114.

[20] Lattante S, Rouleau GA and Kabashi E. TARDBP and FUS mutations associated with amyotrophic lateral sclerosis: summary and update. Hum Mutat 2013; 34: 812-826.

[21] Corcia P, Valdmanis P, Millecamps S, Lionnet C, Blasco H, Mouzat K, Daoud H, Belzil V, Morales R, Pageot N, Danel-Brunaud V, Vandenberghe N, Pradat PF, Couratier P, Salachas F, Lumbroso S, Rouleau GA, Meininger V and Camu W. Phenotype and genotype analysis in amyotrophic lateral sclerosis with TARDBP gene mutations. Neurology 2012; 78: 1519-1526.

[22] Wang F, Lu Y, Qi F, Su Q, Wang L, You C, Che F and Yu J. Effect of the human SOD1-G93A gene on the Nrf2/ARE signaling pathway in NSC-34 cells. Mol Med Rep 2014; 9: 2453-2458.

[23] Barber SC and Shaw PJ. Oxidative stress in ALS: key role in motor neuron injury and therapeutic target. Free Radic Biol Med 2010; 48: 629-641.

[24] Hadzhieva M, Kirches E, Wilisch-Neumann A, Pachow D, Wallesch M, Schoenfeld P, Paege I, Vielhaber S, Petri S, Keilhoff G and Mawrin C. Dysregulation of iron protein expression in the G93A model of amyotrophic lateral sclerosis. Neuroscience 2013; 230: 94-101.

[25] Oshiro S, Morioka MS and Kikuchi M. Dysregulation of iron metabolism in Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. Adv Pharmacol Sci 2011; 2011: 378278.

[26] Yu J, Qi F, Wang N, Gao P, Dai S, Lu Y, Su Q, Du Y and Che F. Increased iron level in motor cortex of amyotrophic lateral sclerosis patients: an in vivo MR study. Amyotroph Lateral Scler Frontotemporal Degener 2014; 15: 357-361.

[27] De Vos KJ, Morotz GM, Stoica R, Tudor EL, Lau KF, Ackerley S, Warley A, Shaw CE and Miller CC. VAPB interacts with the mitochondrial protein PTPIP51 to regulate calcium homeostasis. Hum Mol Genet 2012; 21: 1299-1311.

[28] Jaiswal MK and Keller BU. Cu/Zn superoxide dismutase typical for familial amyotrophic lateral sclerosis increases the vulnerability of mitochondria and perturbs Ca2+ homeostasis in SOD1G93A mice. Mol Pharmacol 2009; 75: 478-489.

[29] De Vos KJ, Chapman AL, Tennant ME, Manser C, Tudor EL, Lau KF, Brownlees J, Ackerley S, Shaw PJ, McLoughlin DM, Shaw CE, Leigh PN, Miller CC and Grierson AJ. Familial amyotrophic lateral sclerosis-linked SOD1 mutants perturb fast axonal transport to reduce axonal mitochondria content. Hum Mol Genet 2007; 16: 2720-2728.

[30] Boillee S, Vande Velde C and Cleveland DW. ALS: a disease of motor neurons and their nonneuronal neighbors. Neuron 2006; 52: 39-59.

[31] Bozzo F, Mirra A and Carri MT. Oxidative stress and mitochondrial damage in the pathogenesis of ALS: new perspectives. Neurosci Lett 2017; 636: 3-8.

[32] Hitchler MJ and Domann FE. Regulation of CuZnSOD and its redox signaling potential: implications for amyotrophic lateral sclerosis. Antioxid Redox Signal 2014; 20: 1590-1598.

[33] Siddique T and Ajroud-Driss S. Familial amyotrophic lateral sclerosis, a historical perspective. Acta Myol 2011; 30: 117-120.

[34] Yerbury JJ, Ooi L, Dillin A, Saunders DN, Hatters DM, Beart PM, Cashman NR, Wilson MR and Ecroyd H. Walking the tightrope: proteostasis and neurodegenerative disease. J Neurochem 2016; 137: 489-505.

[35] Huang C, Tong J, Bi F, Zhou H and Xia XG. Mutant TDP-43 in motor neurons promotes the onset and progression of ALS in rats. J Clin Invest 2012; 122: 107-118.

[36] Mariani E, Polidori MC, Cherubini A and Mecocci P. Oxidative stress in brain aging, neurodegenerative and vascular diseases: an overview. J Chromatogr B Analyt Technol Biomed Life Sci 2005; 827: 65-75.

[37] Rulten SL, Rotheray A, Green RL, Grundy GJ, Moore DA, Gomez-Herreros F, Hafezparast M and Caldecott KW. PARP-1 dependent recruitment of the amyotrophic lateral sclerosis-associated protein FUS/TLS to sites of oxidative DNA damage. Nucleic Acids Res 2014; 42: 307-314.

[38] Yang S and Kim HM. The RhoA-ROCK-PTEN pathway as a molecular switch for anchorage dependent cell behavior. Biomaterials 2012; 33: 2902-2915.

[39] Kirby J, Ning K, Ferraiuolo L, Heath PR, Ismail A, Kuo SW, Valori CF, Cox L, Sharrack B, Wharton SB, Ince PG, Shaw PJ and Azzouz M. Phosphatase and tensin homologue/protein kinase B pathway linked to motor neuron survival in human superoxide dismutase 1-related amyotrophic lateral sclerosis. Brain 2011; 134: 506-517.