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Role of Mutant TBP in Regulation of Myogenesis on Muscle Satellite Cells*

Dong-ming ZHAO[1], Sui-qiang ZHU[2], Fu-rong WANG[2], Shan-shan HUANG[2#]
[1]*Department of Orthopedics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*
[2]*Department of Neurology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*

Summary: In polyglutamine (PolyQ) diseases, mutant proteins cause not only neurological problems but also peripheral tissue abnormalities. Among all systemic damages, skeletal muscle dystrophy is the severest. Previously by studying knock-in (KI) mouse models of spinal cerebellar ataxia 17 (SCA17), it was found that mutant TATA box binding protein (TBP) decreases its interaction with myogenic differentiation antigen, thus reducing the expression of skeletal muscle structural proteins and resulting in muscle degeneration. In this paper, the role of mutant TBP in myogenesis was investigated. Single myofibers were isolated from tibialis anterior muscles of wild type (WT) and SCA17KI mice. The 1TBP18 staining confirmed the expression of mutant TBP in muscle satellite cells in SCA17KI mice. In the $BaCl_2$-induced TA muscle injury, H&E cross-section staining showed no significant change in myofibril size before and after $BaCl_2$ treatment, and there was no significant difference in centralized nuclei between WT and SCA17KI mice, suggesting that mutant TBP had no significant effect on muscle regeneration. In the cultured primary myoblasts from WT and SCA17KI mice *in vitro*, representative BrdU immunostaining showed no significant difference in proliferation of muscle satellite cells. The primary myoblasts were then induced to differentiate and immunostained for eMyHC, and the staining showed there was no significant difference in differentiation of primary myoblasts between WT and SCA1KI mice. Our findings confirmed that mutant TBP had no significant effect on myogenesis.
Key words: TATA box binding protein; spinocerebellar ataxia 17; myoblast; myogenesis

Polyglutamine (PolyQ) diseases are a family of hereditary neurodegenerative disorders, including Huntington's disease (HD), spinocerebellar ataxia (SCA) 1, 2, 3, 6, 7, and 17, spinobulbar muscular atrophy (SBMA), and dentate-rubral-pallidoluysian atrophy (DRPLA)[1–3]. To date, how mutant proteins cause pathological changes of each disease remains unknown, and there are no curable therapeutic strategies and disease modifying treatment. Although previous researches have mostly focused on neurodegeneration, the abnormalities in peripheral tissues mediated by PolyQ-expanded proteins however inevitably draw attention recently[4–6]. These emerging features can be explained as firstly, all PolyQ proteins are not selected but ubiquitously expressed and involved in a variety of cellular functions; Secondly, the aberrant expansion of PolyQ proteins indeed causes pathogenic effect on each protein so called gain-of function and loss-of function[7]. The peripheral phenotypes of PolyQ diseases include weight loss, skeletal muscle atrophy, cardiac failure, testicular atrophy and even dysfunction of blood-derived cells, etc[5]. Among all these peripheral phenotypes, skeletal muscle atrophy seemed is another hallmark of most PolyQ diseases[8].

Spinocerebellar ataxia type 17 (SCA17) is one of nine PolyQ diseases caused by a CAG repeat expansion in the TATA-box binding protein gene (TBP)[9]. Because TBP is a well-documented transcriptional factor which accounts for almost 30% transcriptions of eukaryotic genes, the clinical presentation for SCA17 is more sever and distinct from other PolyQ diseases[10]. The disease has a varied age of onset and heterogeneous clinical spectrum. Symptoms of SCA17 patients range from ataxia, which is manifested as gait instability, and slurred speech, dementia, psychiatric symptoms, chorea to systemic symptoms like body weight loss.

Dong-ming ZHAO, E-mail: 57800184@qq.com
#Corresponding author, E-mail: shanahuang3@gmail.com
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The normal range of CAG/CAA repeats of TBP is 30 to 42[9, 10]. If PolyQ tract reaches more than 42, obvious symptoms start to be shown. The authors have previously discovered preferential skeletal muscle degeneration in SCA17 by generating SCA17 knock-in (KI) mice that express mutant TBP with 105 PolyQ[11]. The authors further found muscle degeneration was resulted from reduced expression of muscle-specific structural genes. Mutant TBP mediated less association with MyoD, a muscle specific transcription factor, attributed to the cascade event[11].

Skeletal satellite cells, helping skeletal muscle to operate adaptive changes during postnatal development and injury, are considered the most promising source of muscle stem cells possessing a high regenerative potential[12]. Activated muscle satellite cells undergo myogenic differentiation or self-renewal to maintain stable skeletal muscle metabolism[12]. In SCA17KI mice, whether mutant TBP was expressed in muscle satellite cells and affects functions of satellite cells such as regeneration, proliferation or differentiation remain unknown.

Using SCA17KI mouse skeletal muscles, we first isolated single myofibers and confirmed the expression of mutant TBP in muscle satellite cells, we then challenged muscles with $BaCl_2$ and found that expression of mutant TBP in skeletal muscle satellite cells does not affect the regeneration of muscle satellite cells. We cultured primary myoblasts from SCA17KI muscle and discovered that expression of mutant TBP has no effect on the proliferation and differentiation of muscle satellite cells. Overall, we conclude that the myogenic activities of muscle satellite cells are not impaired by aberrant expansion of CAG/CAA repeats in TBP.

1 MATERIALS AND METHODS

1.1 Mouse Models

The TBP105Q SCA17KI mice were generated as described previously[13]. All mice were bred and maintained in the animal facility at Medical School, Emory University under specific pathogen-free conditions in accordance with institutional guidelines of The Animal Care and Use Committee at Emory University. Primers used for genotyping are listed below: for mutant TBP (forward: 5′-CCA CAG CCT ATT CAG AAC ACC-3′; reverse: 5′-AGA AGC TGG TGT GGC AGG AGT GAT-3′); for Cre (forward 5′-GCG GTC TGG CAG TAA AAA CTA TC-3′; reverse: 5′-TGT TTC ACT ATC CAG GTT ACG G-3′).

1.2 Antibodies and Immunohistochemistry

Primary antibodies from commercial sources used in this study included: 1TBP18 (QED Bioscience, 70102, USA), Sydecan4 (Abcam, ab24511, USA), and eMyHC (F1.652, neat hybridoma supernatant; Developmental Studies Hybridoma Bank, USA). All secondary antibodies were purchased from Jackson Immunoresearch (USA).

Immunofluorescence and immunohistochemistry were performed as described previously[14]. When the eMyHC antibody was used for immunocytochemistry, fixed myoblasts were incubated with eMyHC at 1:100 dilution. For immnofluorescence, primary antibodies 1TBP18 (1:1000) and Sydecan4 (1:500) were used. Light micrographs and fluorescent images were acquired on a Zeiss microscope (Axiovert 200 MOT; Carl Zeiss Imaging, Germany) equipped with a digital camera (Hamamatsu Orca-100, Japan) and Openlab software (Improvision Inc, GK).

1.3 Isolation of Single Myofibers

Single myofibers were isolated from tibialis anterior (TA) muscles as described previously[15]. Briefly, the TA was dissected and digested in DMEM containing 25 mmol/L HEPES and 0.1% collagenase (type Ⅰ, Worthington) for 90 min with gentle agitation. Single myofibers were extracted individually into fresh plates, then transferred to 15 mL conical tubes and washed with media 3 times to remove contaminants. Washed myofibers were returned to a 100 mm dish prior to plating. Individual myofibers were transferred to 24-well plates pre-coated with 10% Matrigel (BD Biosciences, USA), fixed immediately upon plating with 3.75% formaldehyde, and immunostained with Syndecan4 and 1TBP18 antibodies.

1.4 Collection of Muscles and Morphometric Measurements

Mice were sacrificed and TA muscles were removed, embedded in OCT mounting medium, and frozen in 2-methybutane cooled in liquid nitrogen. Serial cross-sections were collected onto gelatin-subbed slides at 400- to 500-μm intervals along the entire length of the muscle and analyzed histologically by H&E staining. To analyze muscle growth during regeneration, injury was induced in the TA muscles of 1.5 months old WT and SCA17KI mice (n=4) by injection of 40 μL of 1.2% $BaCl_2$[16]. Muscles were collected 14 days after injury using standard dissection techniques and frozen. Serial 15-μm-thick sections were collected along the entire length of the muscle and stained with H&E. Photography was performed and analyzed using a Zeiss Axioplan microscope equipped with a video camera and Axiovision Rel. 4.8 software (Germany). The imaging software ImageJ (NIH) was used to quantitatively analyze the cross-sectional area. The cross-sectional area is determined in the muscle belly, and anatomical landmarks of each muscle were used to find the same region in different samples.

1.5 Culture of Primary Muscle Myoblasts, Proliferation Assay and Cell Differentiation Assay

Primary myoblasts were derived from the hindlimbs of 2 months old WT or SCA17-KI mice

and cultured to 99% purity as previously described[17]. Briefly, the hindlimbs were removed from mice and the bones were dissected away. The remaining muscle was minced into a coarse slurry. Cells were enzymatically dissociated by the addition of a solution of dispase (grade Ⅱ, 2.4 U/mL, Boehringer Mannheim Corp., USA) and collagenase (class Ⅱ, 1%; Boehringer Mannheim Corp., USA), supplemented with $CaCl_2$ to a final concentration of 2.5 mmol/L. The slurry, maintained at 37°C for 30–45 min and then passed through 80 μm nylon mesh (Nitex; Tetko, Inc., USA). The filtrate was re-suspended in growth medium, and the suspension was plated on collagen-coated dishes. Myoblasts were maintained in growth media (GM: Ham's F10, 20% FBS, 5 ng/mL bFGF, 100 U/mL penicillin G, 100 mg/mL streptomycin) in a humidified 5% CO_2 incubator at 37°C on collagen coated dishes.

The cell proliferation was assayed as described before[17]. Briefly, myoblasts were incubated in GM with 10 mmol/L bromodeoxyuridine (BrdU) (Sigma, USA) starting 24 h after plating. Following 1 h incubation with BrdU, cells were fixed and analyzed by Click-iT BrdU Alexa Fluor 594 Imaging Kit (Invitrogen, C10339, USA). The percentage of BrdU positive nuclei in 6 random fields (1000 total nuclei) was determined for each condition. At least three independent experiments were performed.

To induce differentiation, cells were plated on dishes coated with Entactin-Collagen Ⅳ-Laminin (ECL; Upstate Biotechnology, USA) in GM and shortly switched to differentiation media[16] [DM: DMEM, 1% insulin-transferrin-selenium-A supplement (Invitrogen, USA), 100 U/mL penicillin G and 100 mg/mL streptomycin]. After 24 or 48 h in DM, cells were fixed in 3.7% formaldehyde for 10 min and nonspecific binding was blocked with TNB buffer (NEN Life Science Products, USA) for 1 h at room temperature. The cells were incubated with eMyHC (F1.652, neat hybridoma supernatant; Developmental Studies Hybridoma Bank, USA) antibody for 1 h at room temperature. Cells were washed in PBS with 0.1% Tween, and then incubated in biotinylated goat anti-mouse IgG (1:200, Jackson ImmunoResearch Laboratories, USA). Antibody binding was detected using Vectastain Elite ABC reagent (Vector Laboratories, USA). To analyze differentiation, the number of nuclei in eMyHC-positive cells was counted and expressed as a percentage of the total number of nuclei analyzed (250). The fusion index was determined by dividing the number of nuclei within myotubes (two or more nuclei) by the total number of nuclei analyzed (100–250).

1.6 Statistical Analysis

Each experiment was repeated three or more times, and the results were expressed as the mean±standard deviation (SD). Statistical significance was calculated based on *t* test, and a P value <0.05 was considered as significant.

2 RESULTS

2.1 Expression of Mutant TBP in Skeletal Muscle Satellite Cells in SCA17KI Mice

To verify the expression of mutant TBP in skeletal muscle satellite cells in SCA17KI mice, we first isolated single myofibers from TA muscles of WT and SCA17KI mice and performed immunofluorescent analysis with two antibodies: anti-TBP (1TBP18), which can preferentially recognize expanded PolyQ TBP (fig. 1, red), and Sydecan-4, which is the marker of muscle satellite cells (fig. 1, green). Mutant TBP was strongly expressed in myobifers isolated from SCA17KI mice (fig. 1) and well co-localized with muscle satellite cells (fig. 1, arrows); 1TBP18 staining was absent in myofibers from WT mice. These results confirmed that mutant TBP was expressed in muscle satellite cells of SCA17KI muscles.

2.2 No Impairment of Muscle Regeneration Post-injury by Mutant TBP in Satellite Cells

To detect whether mutant TBP affects regeneration of muscles after injury in SCA17KI mice, we induced

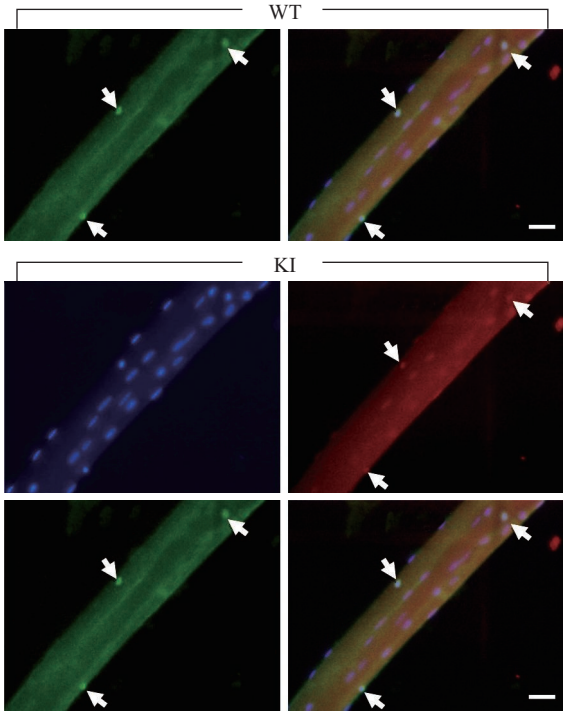

Fig. 1 Expression of mutant TBP in muscle satellite cells of WT and SCA17KI mice
Immunofluorescent images show the co-localization of 1TBP18 (red) and Syndecan-4 (green, marker for muscle satellite cells) in isolated single myofibers from tibialis anterior muscle in KI mice. Single myofibers isolated from WT mice were not stained with 1TBP18 (upright). The merged image displays nuclear staining (blue). Arrows indicate muscle satellite cells. Scale bar=20 μm

muscle injury using $BaCl_2$ injection. $BaCl_2$ induces myofiber degeneration but leaves satellite cells sufficiently unharmed to allow regeneration[18]. The TA muscles in both WT and SCA17KI mice were efficiently regenerated following $BaCl_2$ induced injury, as judged by histological analysis of HE-stained tissue sections (fig. 2A). Quantification of the fiber diameter before and after injury confirmed the above observations (fig. 2B). Quantification of the centralized muscle nuclei, which is another parameter to indicate muscle regeneration after injury, revealed no significant difference in the rate of centralized nuclei between WT and SCA17KI mice (fig. 2B). Overall, it suggests that the regeneration of muscles post-injury is not impaired by mutant TBP in muscle satellite cells of SCA17KI mice.

2.3 Proliferation of Primary Myoblasts in WT and SCA17KI Mice

In adult myogenesis, muscle satellite cells can form myoblasts and proliferate. To investigate whether mutant TBP can impair proliferation of muscle satellite cells, primary myoblasts were derived from the hind-limb of 2 months WT or SCA17-KI mice and cultured to 99% purity. The proliferation rate of primary myoblasts was revealed by BrdU immnunofluorescent staining (fig. 3A, red). Notably, there was no significant difference in the staining of BrdU between WT and SCA17KI mice (fig. 3A). No significant difference was seen in the ratio of positive cells/total cells in primary myoblasts between WT and SCA17KI mice (fig. 3B). Taken together, the proliferation of primary myoblasts was not impaired by mutant TBP in SCA17KI mice.

2.4 Differentiation of Primary Myoblasts in WT and SCA17KI Mice

To determine the role of mutant TBP in differentiation of primary myoblasts, cultured primary myoblasts were incubated in differentiation media for 24 to 48 h. Embryonic myosin heavy chain (eMyHC), a later differentiation marker of myoblasts was used to stain the cells (fig. 4A, brown). As expected, eMyHC positive myotubes were observed in both primary myoblasts from WT and SCA17KI mice after 24 h and 48 h (fig. 4A). The undifferentiated myoblasts were not stained for eMyHC (fig. 4A, arrows). The ratio of nuclei in positive cells/total nuclei was counted, and there was no significant difference in differentiation rate between WT and SCA17KI mice (fig. 4B). Quantification of the ratio of nuclei in myotubes/total nuclei revealed there was no significant in fusion rate between WT and SCA17KI mice (fig. 4C). The differentiation of primary myoblasts was not affected by mutant TBP in SCA17KI mice.

3 DISCUSSION

Growing evidence has shown in addition to the classic neurological symptoms, peripheral symptoms in PolyQ diseases are rather independent than directly associated with changes in brain functions[19, 20]. Among all peripheral pathologies, skeletal muscle atrophy is one of the most striking changes, and can cause dysregulation of other organs and exacerbate progress of PolyQ diseases. The authors have generated SCA17KI mouse model that expressed endogenous mutant TBP *in vivo*. Using this mouse model, the authors found large PolyQ repeats in TBP cause severe muscle atrophy, presented as dramatic shrink in skeletal muscle mass. The discovery was then further confirmed by selective expression of mutant TBP in skeletal muscle *in vivo*. Indeed, reduced skeletal muscle mass was frequently

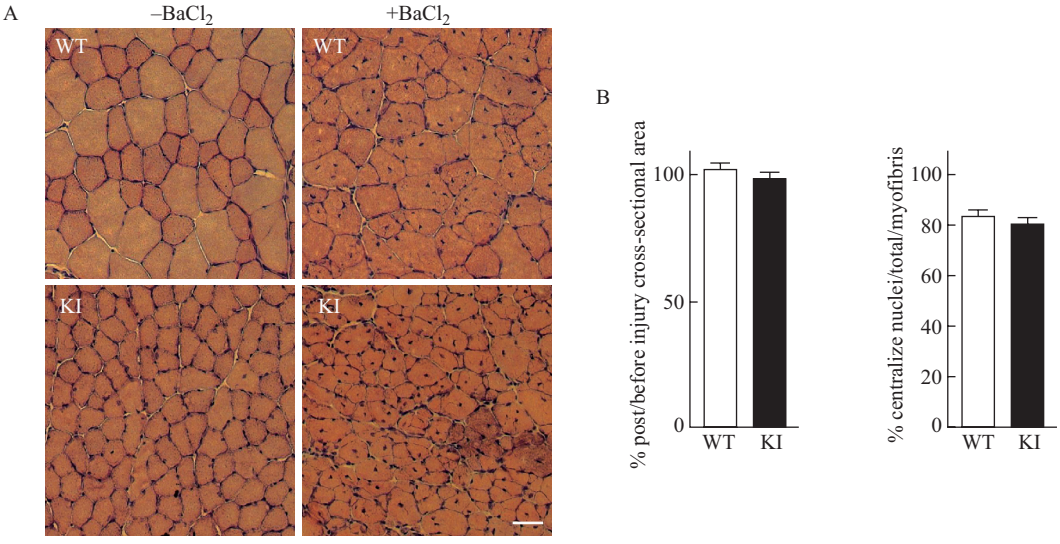

Fig. 2 Effect of mutant TBP on regeneration of skeletal muscle after injury

A: Haematoxylin and eosin (H&E) cross-section staining of tibialis anterior (TA) muscles from 1.5-month-old WT and SCA17KI mice showing myofibril size before and after 14 days of $BaCl_2$-induced muscle injury; B: Quantification of cross-sectional area of TA myofibrils (left) and centralized nuclei (right) before and after 14 days of $BaCl_2$-induced muscle injury. Scale bar=50 μm

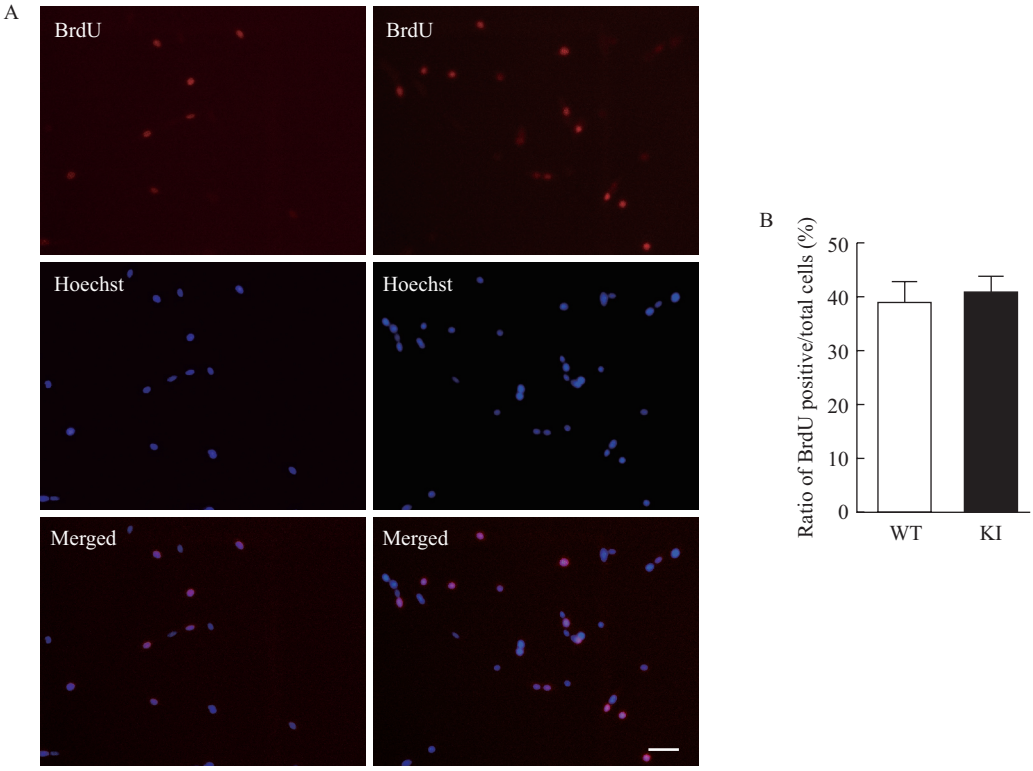

Fig. 3 Effect of mutant TBP on proliferation of primary myoblasts
A: The myoblasts were isolated from the tibialis anterior muscles of WT and SCA17KI skeletal muscles and cultured, and nuclei of proliferative myoblasts were positive for BrdU (red) and nuclei of total myoblasts were positive for Hoechst (blue); B: The relative percentage of BrdU positive nuclei of myoblasts in WT and KI mice indicates no significant change. Scale bar=10 μm

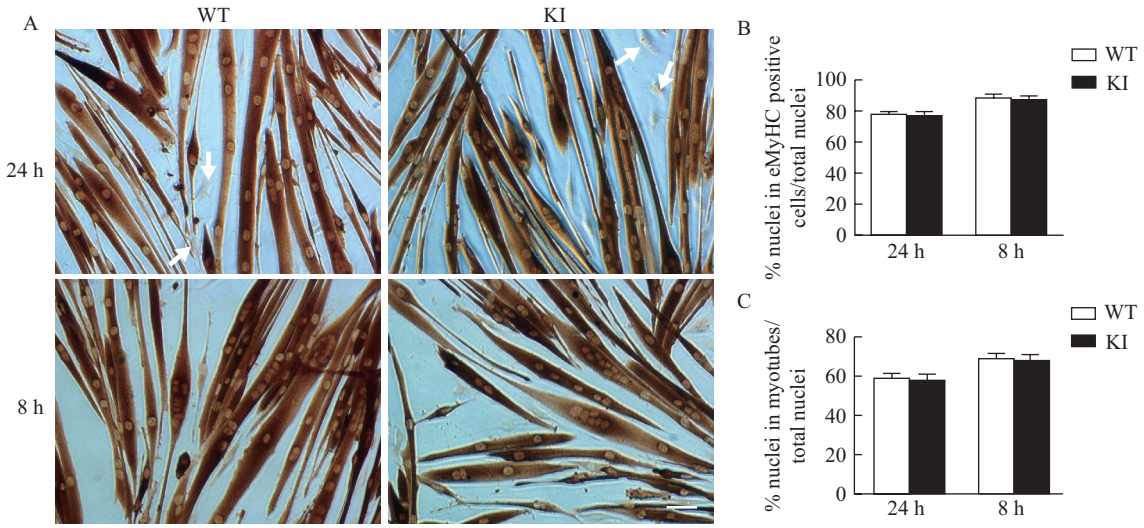

Fig. 4 Effect of mutant TBP on differentiation of primary myoblasts
A: The myoblasts were isolated from WT or SCA17KI skeletal muscles and cultured. DAB stained eMyHC positive cells (brown) indicate differentiated myoblasts after 24 h and 48 h in differentiation media, and the undifferentiated myoblasts were not stained with eMyHC (arrow); B: For the differentiation rate of myoblasts, the nuclei of eMyHC myoblasts/nuclei of total myoblasts indicates no significant change of differentiation between WT and KI TA myoblasts; C: For the fusion rate of myoblasts, the nuclei of eMyHC myoblasts/nuclei of total myoblasts indicates no significant change of differentiation between WT and KI TA myoblasts. Scale bar=50 μm

seen in almost all polyQ diseases[19–21]. However, how mutant PolyQ proteins attribute to severe skeletal muscle atrophy remains largely unknown.

Similar to neurons, adult skeletal muscle fibers are terminal differentiated cells and hardly to achieve self-renew upon injury or other stress[22]. The regulation of adult skeletal muscle mass has been traditionally considered as a problem of protein turnover, namely as

the result of the balance between protein synthesis and protein degradation[22]. Our previous research revealed that the expression of structural proteins of skeletal muscle was generally reduced in SCA17KI mice. The reduced synthesis of muscle structural proteins was resulted from the decreased association of mutant TBP with MyoD, a muscle specific transcription factor. The study confirmed the imbalance of protein synthesis and degradation in adult skeletal muscle could cause muscle atrophy in PolyQ diseases. However, emerging evidence indicates that muscle satellite cells mediated cell turnover is also involved in muscle growth and maintenance of muscle mass. Muscle satellite cells retain the capability to both maintain the quiescence in uninjured muscles and can be promptly activated thus undergoing myogenic differentiation for muscle-renewal. It brought up the question whether functions of muscle satellite cells were impaired by mutant TBP in SCA17.

Our study had shown that although mutant TBP is expressed in muscle satellite cells, the ability of regeneration after injury in muscle satellite cells was not impaired. BrdU test suggested proliferation of primary myoblasts, myogenic progenitor cells, was not impaired by expression of mutant TBP either. Primary myoblasts can be activated, differentiated and fused to form myotubes. Our study found neither differentiation nor fusion was impaired by mutant TBP in SCA17. Overall, there was no significant difference in satellite cell mediated myogenesis upon mutant TBP expression. We conclude that the myopathy in SCA17 is a result of imbalanced protein metabolism on matured muscle fibers; Muscle satellite cells seemed not involved in pathogenesis of SCA17. Given the fact that the stem cells reduce its number with age, the SCA17 skeletal muscle gradually induces dystrophy phenotype as the mice grow older.

skeletal muscle is distinct from the central nevous system by means of its accessibility. The peculiar ability of skeletal muscle tissue to operate adaptive changes during adulthood brought the concept that amelioration of muscle dysfunction is a very optimistic strategy to mitigate a deterioration of PolyQ disease symptoms[23]. The stem cell feature of satellite cells makes it a great candidate for exploring therapeutic strategies for PolyQ diseases. Since the functions of muscle satellite cells were not impaired by mutant TBP, it's feasible to self-transplant molecular and chemical compound modified muscle satellite cells to disease affected muscles. Our study shed light on the role of mutant PolyQ protein in the function of stem cells. It's urgent to figure out whether PolyQ proteins have pathogenic effect on other type of cells, particularly neuronal stem cells. The type of investigation will bring tremendous newly targets for treating PolyQ diseases.

Conflict of Interest Statement

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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