

Original Article

Effects of mammalian target of rapamycin on proliferation, apoptosis and differentiation of myoblasts undergoing mechanical stress

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Abstract: Myoblasts characterize by the potential to transform into skeletal muscle, and involve in processes of proliferation, differentiation and apoptosis. Mammalian target of rapamycin (mTOR) is an important protein of PI3K signaling pathway in muscle metabolism and physiology. This study aimed to investigate effects of mTOR on proliferation, apoptosis and differentiation of myoblasts undergoing mechanical stress. We paid much attention on mTOR function undergoing mechanical stress in myoblasts. C2C12 myoblasts were cultured and mTOR gene was knocked down by using Crisper/Cas9 method. Western blot assay and quantitative polymerase chain reaction (Q-PCR) were used to test 4E-binding protein 1 (4EBP1) and p70 ribosomal protein S6 kinase (p70S6k) expression. Cell counting kit 8 (CCK-8) was used to measure cell proliferation, and flow cytometry to used to detect cell apoptosis. Differentiation was counted by using immunofluorescence staining. Results showed that the knockdown of mTOR reduced the phosphorylation of 4EBP1 and p70S6k levels undergoing mechanical stress and decreased PI3K signaling pathway proteins synthesis. In addition, proliferation of myoblasts was decelerated by the mTOR knockdown. However, when mTOR knocked down cells treated with mechanical stress, apoptosis rate increased significantly and the differentiation speed was slow down. In conclusion, our study revealed the mTOR function on regulating myoblast proliferation, apoptosis and differentiation undergoing mechanical stress.

Keywords: Myoblast, mTOR, proliferation, mechanical stress

Introduction

Skeletal muscle is one of three muscle types, and as a form of striated muscle tissue, which is under control of somatic nervous system [1]. Muscle cells are formed from the fusion of developmental myoblasts in a process known as myogenesis. In skeletal muscle regeneration processes, myoblasts are derived from activated satellite cells and start the proliferation, differentiation and apoptosis process, and finally become myofibers [2-5]. The satellite cells can be activated by mechanical stress to provide additional myonuclei for muscle growth and repair. According to our previous research, mechanical stress activates the myoblasts apoptotic processes by the NF- κ B signaling pathway [6]. We also paid much attention on PI3K/mTOR pathway, which regulates the classical proliferation of myoblasts. Moreover, the

mTOR has been considered as an important factor that need to be fully addressed.

The mammalian target of rapamycin (mTOR), is a kinase which as a member of the phosphatidylinositol 3-kinase-related kinase family of protein kinases [7]. The mTOR signaling pathway is a central regulator of mammalian metabolism and physiology. mTOR plays important roles in some tissues, including liver, muscle, white and brown adipose tissue. Meanwhile, the mTOR is always dys-regulated in a few human diseases, such as diabetes, obesity, depression and certain cancers [8, 9]. The increasing of mTOR signaling leads to the cell proliferation and activates the synthesis of proteins, such as 4E-binding protein 1 (4EBP1) and p70 ribosomal protein S6 kinase (p70S6k). Both of 4EBP1 and p70 are the downstream proteins for the PI3K/mTOR signaling pathway.

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Table 1. Primers for the quantitative PCR

Genes		Primers
4EBP1	Forwards	5'-GATGTCCGGGGCAGCAGCTG-3'
	Reverse	5'-AATGTCCATCTCAAAGTGTGACTC-3'
p70S6K	Forwards	5'-GATGAGGCGACGAAGGAGGGG-3'
	Reverse	5'-TAGATTCATACGCAGGTGCTCTG-3'
mTOR	Forwards	5'-AAGAAGTACCCCATCGAGCAC-3'
	Reverse	5'-CATCAGAGTCAAGTGGTCATAGTCCG-3'
β -actin	Forwards	5'-CTGAAGTACCCCATCGAGCAC-3'
	Reverse	5'-ATAGCACAGCCTGGATAGCAAC-3'

The phosphorylation of P70S6k always be activated by the mTOR and leads to the increase of protein synthesis and up-regulation of the cell proliferation. Meanwhile, the 4EBP1 is phosphorylated in response to the mRNA translation. Zhang et al. [10] reported that mTOR was an essential factor for the satellite cell function and skeletal muscle regeneration through controlling the expression of myogenic genes.

It is evident that mechanical stress affects skeletal muscle developments and function. A recent study [11] revealed that diagram of the molecular signaling cascades are involved in the myofibrillar muscle protein synthesis response to the physical exercise. Phillips et al. [12] also found that the resistance training stimulated the muscle protein synthesis for a period of up to 48 h following exercise. Physical exercise activates the protein synthesis via phosphorylation of p70S6k in a pathway that depends on mTOR signaling. In a previous published study [13], the increased 4EBP1 phosphorylation was consistent with the regular exercise and rest. Bond et al. [14] found that the mTORC1 was regulated by the mechanical stimuli. However, the functional role of mTOR in skeletal muscle undergoing mechanical stress is also unclear. In order to reveal the mechanism, we build up mTOR knockdown cells to prove the potential functional roles of mTOR in regulating the myoblast proliferation, differentiation and apoptosis.

Materials and methods

Cell culture

C2C12 myoblasts were cultured in growing medium (Gibco BRL, Co. Ltd., Grand Island, New York, USA), supplementing with 10% fetal

bovine serum (FBS), 100 U/ml penicillin (Sigma-Aldrich, St. Louis, Missouri, USA) and 100 μ g/ml streptomycin (Sigma-Aldrich, St. Louis, Missouri, USA) at 37°C and 5% CO₂. Media were changed every two days. For mTOR knockdown by Crispr/Cas9, cells were grown in trypsin (Biyotime Biotech., Shanghai, China) digestion and switched into sorting buffer (Biyotime Biotech., Shanghai, China). Fluorescent cells sorting was used for sorting positive transfected cells.

Crispr/Cas9 knockdown mTOR gene expression

We detected mTOR gene by National Center for Biotechnology Information (NCBI) reference sequence, and designed target by crisper. Then, the carrier was built up and separated into 3 types, including px458-mTOR-T1, px458-mTOR-T2 and px458-mTOR-T3. After sequencing to screen the positive expression and extracting the plasmid by endotoxin transfection, we selected target-1 reached higher active and knocked down mTOR-T1 to single the cell clone.

Western blot assay

Muscle cells were separated in radioimmunoprecipitation assay (RIPA) buffer (Biyotime Biotech., Shanghai, China) containing rabbit inhibitor and inhibitor sheep IgG (Sigma-Aldrich, St. Louis, Missouri, USA). Cells were washed by using phosphate buffered saline (PBS, Biyotime Biotech., Shanghai, China) adding with 0.1 ml RIPA buffer, and then the cells were subjected to standard Western Blot protocol. The antibodies (Abcam Biotech., Cambridge, Massachusetts, USA) including anti-4EBP1 antibody, anti-p-S6 antibody and anti-beta-actin antibody were used to detect the signals. We disposed bovine serum albumin (BSA) standard solution to test low concentration protein and diluted BSA to test samples twice. Then, we detected protein depending on standard curve. Then, fluorescent signals of blots were detected by using the GDS8000 scan system (UVP corporation, Sacramento, CA, USA) and analyzed by using the Labworks TM Analysis Software (Labworks, Upland, CA, USA) to analysis the results.

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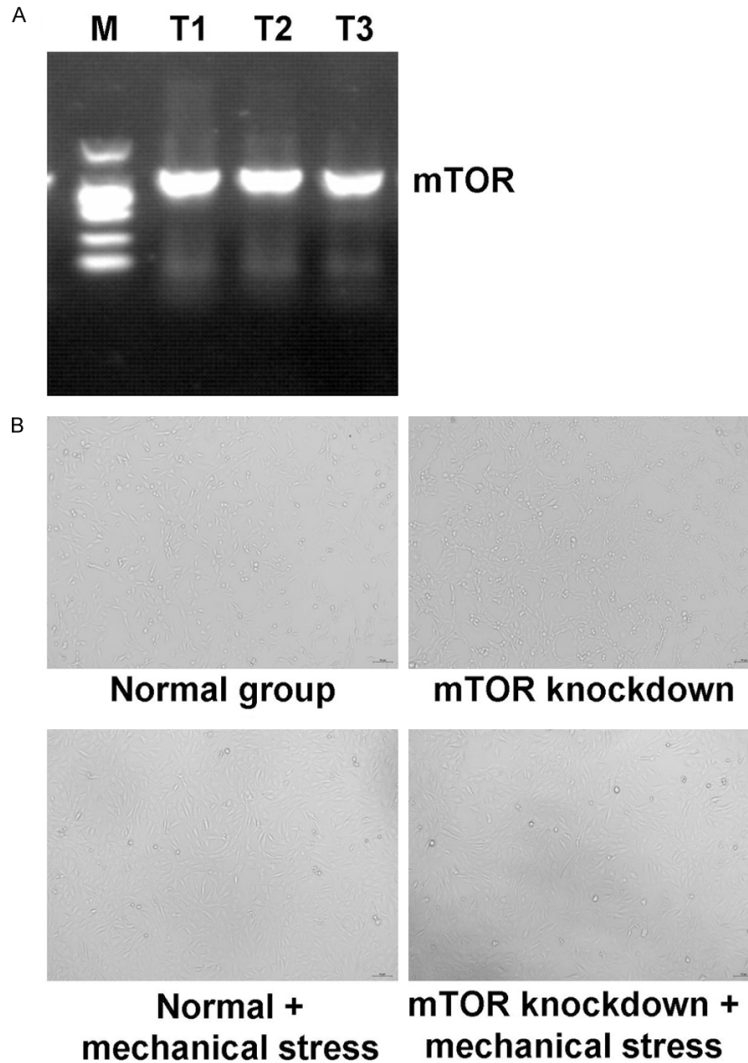


Figure 1. mTOR gene clone and morphology of C2C12 cells undergoing mTOR knockdown. A. Target-1 gets higher activity than T2 and T3. B. The number of C2C12 cells changed by mechanical stress. The microscope showed mTOR knockdown groups get lower level compared with the normal groups. Under the mechanical stress, the cells number reversed while mTOR knockdown groups decreased. And the stress led the cells to the force direction.

Quantitative-polymerase chain reaction

We used quantitative polymerase chain reaction (Q-PCR) to detect the gene expressions of 4EBP1, p70S6k and mTOR. Cells were separated into 4 groups and tested twice. A groups were normal cells comparing with B groups mTOR knockdown cells. While C groups were loading stress on normal cells related with the D groups, which were the mTOR knockdown cells undergoing stress. Primer 5 was used to design 4EBP1, s6K and mTOR primers (Table

1). After reverse transcription, we calculated mRNA or gene expression according to the $2^{-\Delta C_t}$ method. Quantitative PCR conditions were conducted as 94°C and 30 s as one cycle and repeated for 35 times, which included in 4 min then tested signals in 72°C.

Cell proliferation by testing cell counting kit 8 (CCK-8)

Cell counting kit was used to detect cell proliferation, and Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL. Co. Ltd., Grand Island, New York, USA) culture was made with 10% FBS (Gibco BRL. Co. Ltd.), 100 U/ml penicillin (Sigma-Aldrich, St. Louis, Missouri, USA) and 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, Missouri, USA). After antibiotic solution dilution in 100 times, C2C12 cells and mTOR knockdown cells were cultured in penicillin streptomycin DMEM, and inoculated in 96-well plates after 6 h, 12 h, 24 h, 36 h and 72 h cultured, we added 10 µl CCK-8 solution with 1-4 h incubation. Finally, we detected cell vitality to figure out which time point reached proliferation high level and which group stayed at proliferation high level.

Flow cytometry was used to detect cell apoptosis

C2C12 cells were cultured on 6 well plate in DMEM and treated with mTOR knockdown, as described above. At the end of treatments, cells were washed with PBS and fixed with 7-AAD for 5-15 min avoiding light. For apoptosis, we added 450 µl Binding Buffer to cells, and mixed with the Annexin V-PE for 5-15 min. For the flow cytometry tests, the excitation wavelength was assigned as 488 nm and emission wavelength was assigned as 578 nm. Meanwhile, the FL2 tunnel was suggested by the Annexin V-PE. For 7-AAD, which excitation wave-

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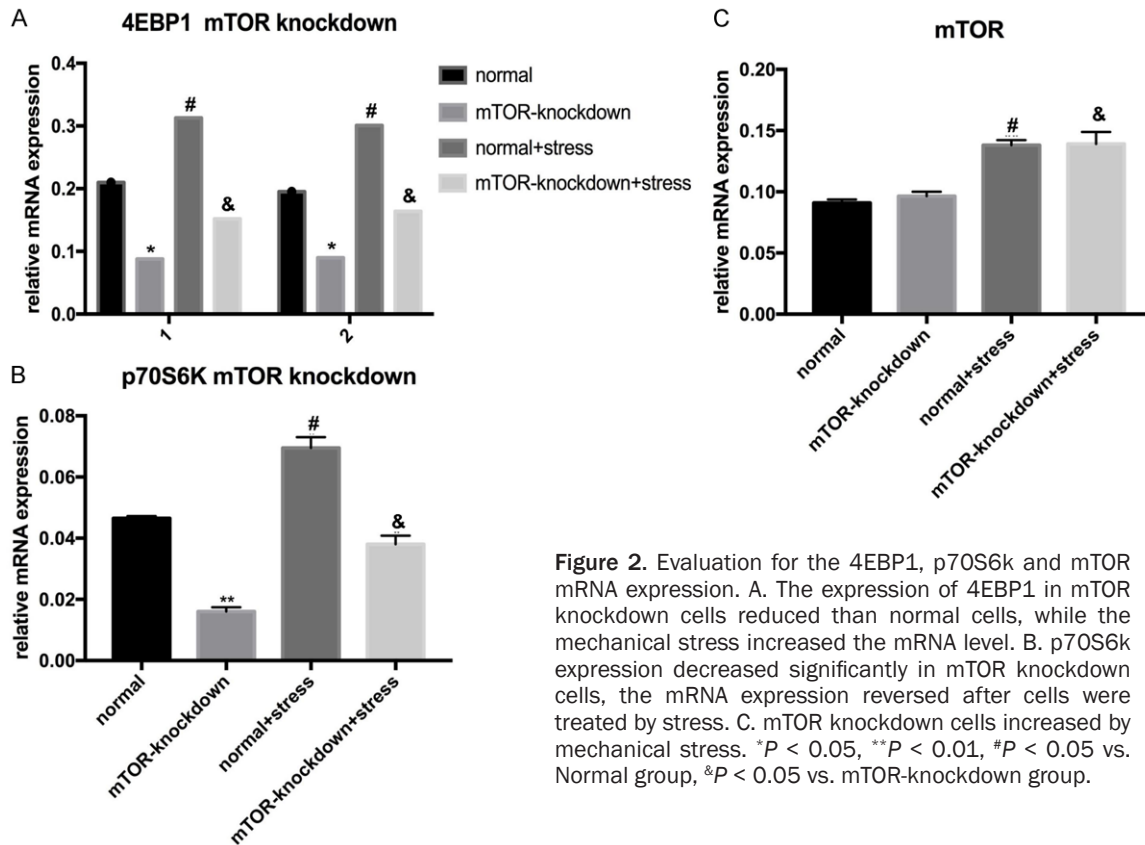


Figure 2. Evaluation for the 4EBP1, p70S6k and mTOR mRNA expression. A. The expression of 4EBP1 in mTOR knockdown cells reduced than normal cells, while the mechanical stress increased the mRNA level. B. p70S6k expression decreased significantly in mTOR knockdown cells, the mRNA expression reversed after cells were treated by stress. C. mTOR knockdown cells increased by mechanical stress. * $P < 0.05$, ** $P < 0.01$, # $P < 0.05$ vs. Normal group, & $P < 0.05$ vs. mTOR-knockdown group.

length was assigned as 546 nm and emission wavelength was assigned as 647 nm, and FL3 tunnel was suggested. Cells were separated into 4 groups as previous, and we choose P3-Q2 added P3-Q3 to calculate the apoptosis rate.

Differentiation of cells were counted by immunofluorescence staining: DAPI + F-actin double staining

Cultured cells were washed 3 times with PBS, and fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, Missouri, USA). After treating with 0.5% Triton X-100 (Sigma-Aldrich) for 15 min, the cells were incubated with 6% goat serum and sealed for 30 min at room temperature. Then, the cells were incubated with primary antibodies overnight at 4°C. The primary antibodies from rabbit were diluted. After washed with PBS, cells were incubated with secondary antibodies conjugated with Alexa Fluor 647 for 30 min under dark room. After washed, cells were counted on a glass slide and observed with laser scanning microscope.

Statistical analysis

Data were analyzed by using statistical package SPSS Version 18.0 for Windows (SPSS, Inc., Chicago, IL, USA). Data were represented as mean \pm standard deviation (SD). Student's t test was utilized for the statistical analysis between two groups. Tukey's post hoc test was used to validate the ANOVA for comparing measurement data between groups. A statistical significance was defined when $P < 0.05$. Statistical significances were defined as p value less than 0.05.

Results

Build up knockdown of mTOR model of C2C12 myoblast

In order to synthesize three study target gene mTOR, we designed three targets gene plasmids or vectors. The issuing individual mTOR was cloned and tested for mRNA/DNA quality, while the paired sets were tested as px458-MTOR-T1, px458-mTOR-T2 and px458-mTOR-

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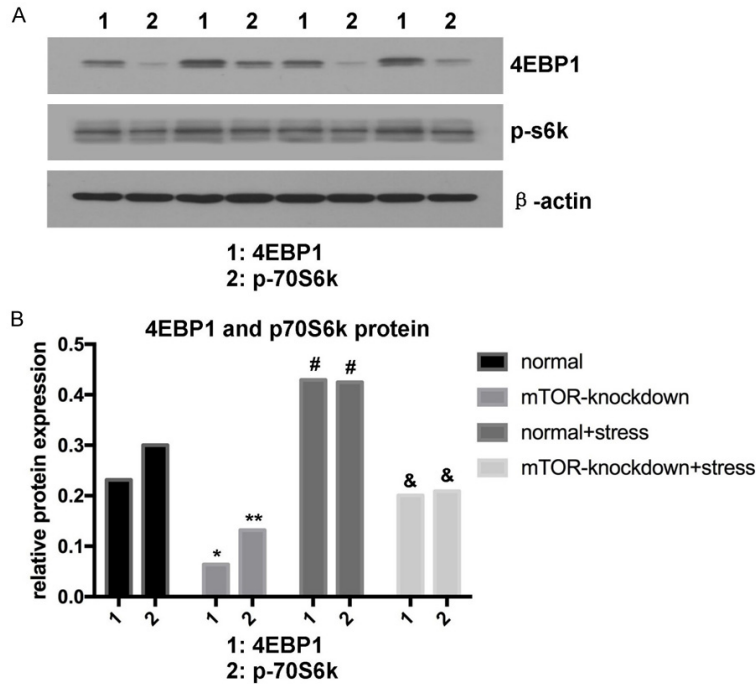


Figure 3. Evaluation for the effects of mTOR on the PI3K pathway associated proteins by using western blot assay. A. Western blot assay for 4EBP1 and p70S6k expression. B. Statistical analysis for western blot data. mTOR knockdown reduced the PI3K pathway protein synthetic, because 4EBP1 and p70S6k as the important downstream protein of PI3K pathway level declined obviously. * $P < 0.05$, ** $P < 0.01$, # $P < 0.05$ vs. Normal group, & $P < 0.05$ vs. mTOR-knockdown group.

T3. Although all three pairs demonstrated > 50% relative positive control activity, the first pair or set 1 (px458-mTOR-T1) offered the most promising efficient target mutagenesis *in vitro*. This work was done by way of endotoxin transfected cells in culture.

CRISPER/Cas9 and px458-mTOR-T1 plasmids were selected by flow cytometer for positive test. In order to test the activity, the quality of each plasmid vector was analyzed by PCR (Figure 1A). Figure 1 shows the T2 and T3 CRISPER gRNA get the lower activity compared with T1. Therefore, we choose target-1 knockdown to culture the monoclonal antibody. After cultured knockdown cells colonies, we used PCR to amplified mTOR target-1 and analyzed by sequencing and sequence alignment.

mTOR knockdown decreased the number of C2C12 cells but the mechanical stress reversed this effect

In mouse myoblast C2C12 cells, we firstly observed that mTOR the level was relatively

high in mechanical stress groups compared to that of normal groups (Figure 1B). As cells were switched into knock-down groups, the number began to decline as we expected. Meanwhile, when the knockdown cells were exposed under the mechanical stress, C2C12 cells number was continuously increased. We also observed that the cells were separated by stress force (Figure 1B). The change of mTOR knockdown cells number suggests that mechanical stress may be functionally associated with these processes.

mTOR was a positive factor for PI3K/mTOR pathway under mechanical stress

The number increased under mechanical stress in mTOR knockdown groups to the without stress ones as we noticed in microscope. To test this, we used CRISPER/Cas9 effectively knocking down mTOR in C2C12 myoblast cells. 4EBP1 and p70S6K are the downstream proteins in PI3K/mTOR pathway. We marked the target protein to test whether this signal pathway involved under mechanical stress. RT-PCR, which detects mRNA expression, indicated that mTOR knockdown significantly reduced 4EBP1 synthesis as compared with normal one. Under mechanical stress, the mTOR knockdown groups negatively regulates 4EBP1 mRNA. As we expected, the mechanical stress enhanced mTOR knockdown cells 4EBP1 synthetic (Figure 2A).

In order to confirm the PI3K signaling pathway participates in the mechanical stress process, we detected the mRNA level of p70S6k. There was no significant differences between p70S6k and 4EBP1. The mTOR knockdown significantly decreased ($P < 0.01$) the stress on the normal cell groups ($P < 0.05$). These results indicate that p70S6k, as a downstream protein, is at least influence by the mechanical stress (Figure 2B).

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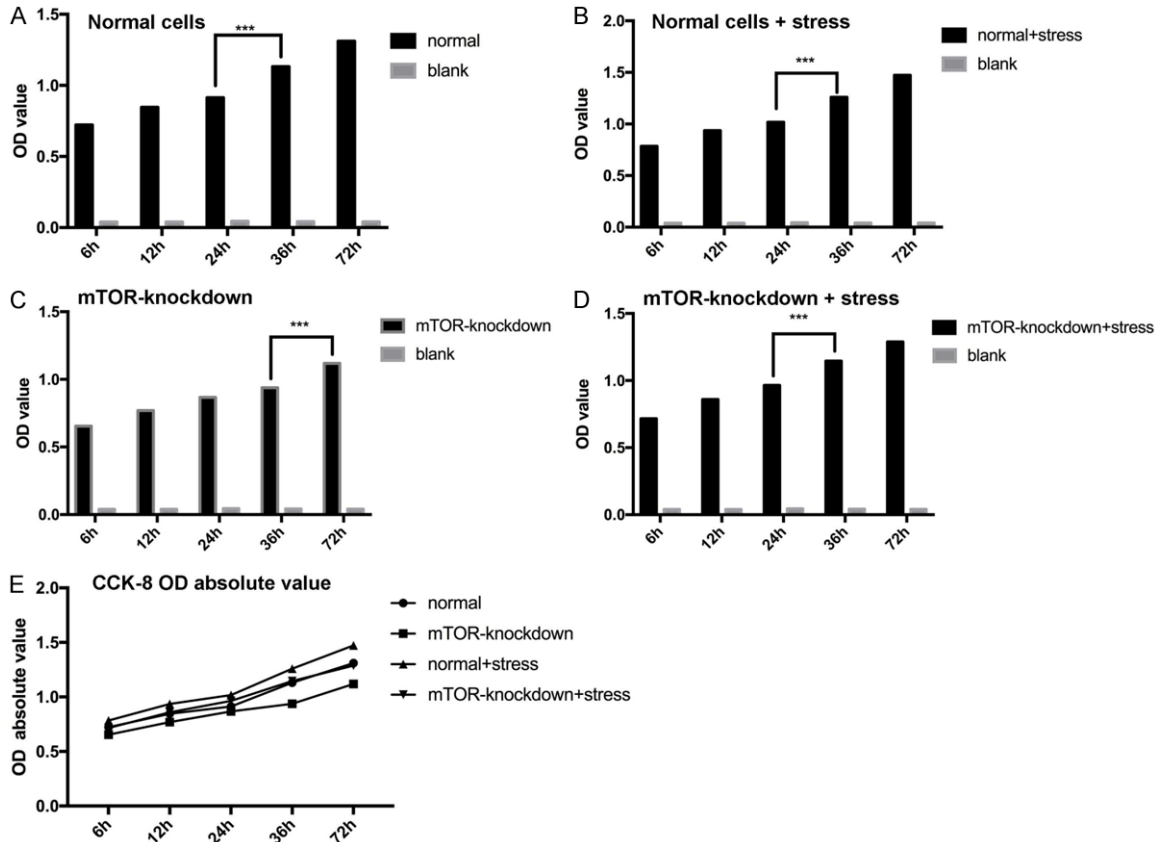


Figure 4. Examination for the myoblast cells proliferation in different groups. (A) Proliferation for Normal cells. (B) Proliferation for Normal cells + stress. (C) Proliferation for mTOR-knockdown. (D) Proliferation for mTOR-knockdown + stress. (E) Statistical analysis for CCK-8 data in (A-D). mTOR knockdown myoblast cells proliferation rate accelerated at 36 h compared with the normal cells at 24 h, while normal cells treated by stress proliferation speed up at 24 h, and proliferation rate of mTOR knockdown cells under stress went up rapidly at 24 h. *** $P < 0.001$.

As the upstream protein of PI3K signaling pathway, we found that mTOR mRNA levels were significantly higher post the stress compared to the normal groups ($P < 0.01$). Interestingly, we detected the mTOR knockdown groups treated with stress did not significantly enhance the mTOR expression (Figure 2C, $P < 0.05$). These data suggest that mTOR has a positive effect for PI3K signaling pathway under the mechanical stress.

mTOR knockdown decreased PI3K pathway protein synthesis under mechanical stress

Given this change of C2C12 myoblast following the mTOR knockdown, we further hypothesized that the mTOR knockdown was the key protein affecting the 4EBP1 and p70S6k (Figure 3A). Western blot results showed that 4EBP1 and p70S6k phosphorylation levels were declined post the mTOR knockdown distinctly. Mean-

while, compared with the normal stress groups, mTOR knock down groups were also declined apparently (Figure 3B, $P < 0.05$). We also observed that stress-treated myoblasts still stimulated 4EBP1 and p70S6k phosphorylation (Figure 3B). Our results showed that mTOR knockdown down-regulated the PI3K signaling pathway but mechanical stress altered the protein synthesis. All of the above changes were represented by the changed phosphorylation of PI3K signaling pathway downstream protein, 4EBP1 and p70S6k.

mTOR accelerated C2C12 proliferation under mechanical stress

Because PI3K signaling pathway is regarded as a classical proliferation pathway, we then further examined the effectively knocking down mTOR in C2C12 cells under stress. Cells staining by stress were separated into 4 groups

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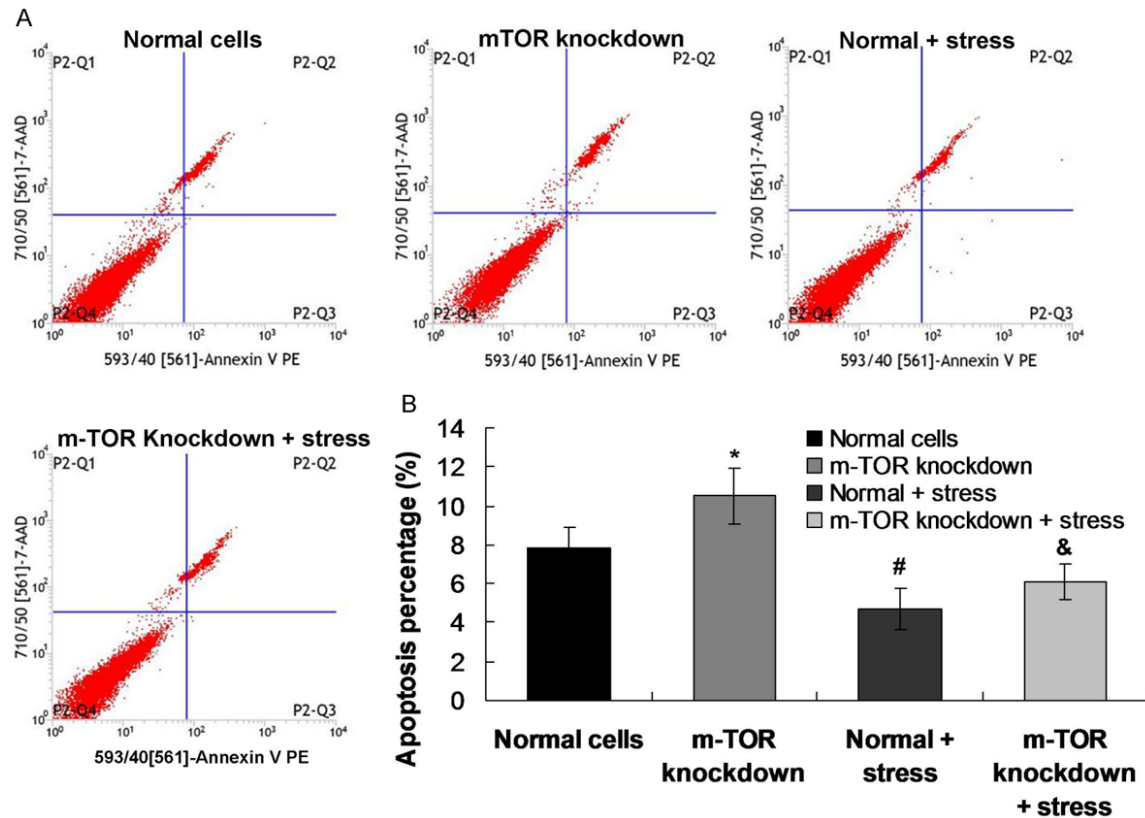


Figure 5. Analysis for the apoptosis in different groups. A. Flow cytometry images for different groups. B. Statistical analysis for the flow cytometry data. The apoptosis level increased in mTOR knockdown groups than the normal cells, after mechanical stress loading on cells, the apoptosis rate in mTOR knockdown cells decreased. * $P < 0.05$, # $P < 0.05$ vs. Normal group, & $P < 0.05$ vs. mTOR-knockdown group.

comparing with the blank group. CCK-8, which detects cell proliferation, showed that mTOR knockdown significantly reduced C2C12 proliferation at 36 h compared with normal cells proliferation with high level at 24 h (Figure 4A, 4B, $P < 0.05$). When the normal cells treated with stress (Figure 4C), the proliferation rate began at 24 h, which was higher than the mTOR knockdown cells with stress at 24 h (Figure 4D). Meanwhile, we also found the proliferation rate was another rising trend by time depended (Figure 4E). These results suggest that mTOR up-regulates the myoblast proliferation undergoing mechanical stress.

mTOR knockdown increased myoblasts apoptosis under stress

In C2C12 myoblasts, we hypothesized whether mTOR could affect cell apoptosis undergoing mechanical stress and used the flow cytometry to evaluate the apoptosis (Figure 5A). Meanwhile, during the apoptosis, the levels of mTOR

knockdown groups were increased compared to that of the control groups. In stress groups, stress on the normal cells apoptosis rate was significantly lower compared to that of the mTOR knockdown cells treated by stress (Figure 5B, $P < 0.05$). But when stress loading on mTOR knockdown cells, the apoptosis levels began to decrease (Figure 5B). Overall, these results indicated that the C2C12 apoptosis by mTOR knockdown was increased under stress. Meanwhile, the mTOR might partially play a negative role in C2C12 apoptosis.

mTOR knockdown reduced C2C12 differentiation after mechanical stress

We tested the differentiation by immunofluorescence staining to confirm whether mTOR involved in the myoblast differentiation undergoing mechanical stress. Immunofluorescent staining results showed that mTOR knockdown groups illustrated the differentiation rate at low level. Moreover, we found that myoblast levels

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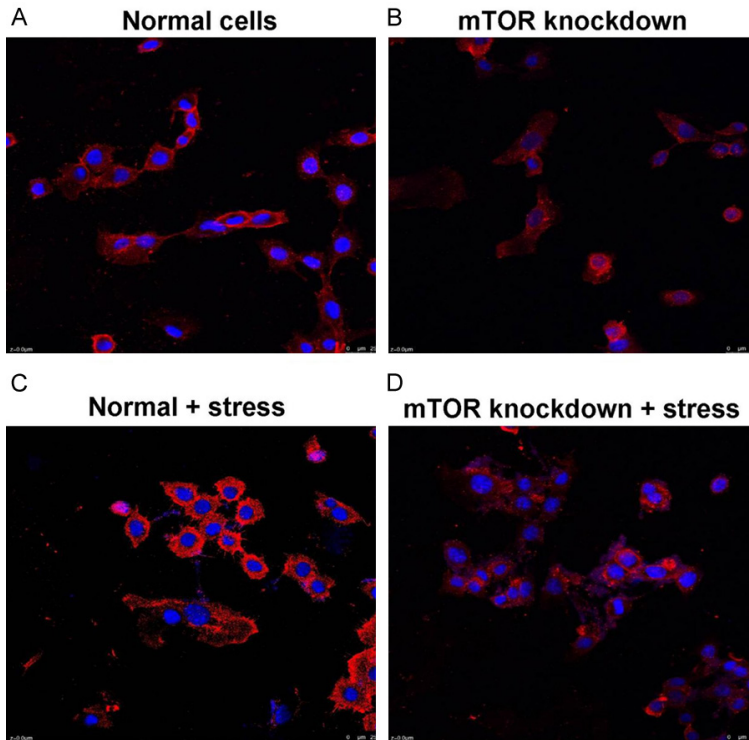


Figure 6. Evaluation for the cell differentiation in different groups. A. Normal cells group. B. mTOR knockdown group. C. Normal + stress group. D. mTOR knockdown + stress group. mTOR knockdown cells differentiation rate reached lower level than normal cells. Compared with the normal cells, the stress treated cells began differentiation. When the stress loading on mTOR knockdown cells, which the differentiation slower than the stress treated normal cells, comparing with the mTOR knockdown cells, the stress reversed the differentiation rate. Magnification, 400 \times .

were also significantly rose up under the stress (**Figure 6A, 6B**). As an evidence of mTOR knockdown, stress reversed the low differentiation levels (**Figure 6B-D**). When normal cells treated with stress, C2C12 stayed at high level, but mTOR knockdown decreased the differentiation process (**Figure 6C, 6D**). These data suggest that mTOR plays a role in regulation of myoblast activation and differentiation during the muscle processes.

Discussion

PI3K/Akt/mTOR signaling pathway is regarded as a classical proliferation pathway in some organs, which regulates human's life activities. mTOR, as a core component, regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, autophagy, and transcription [15, 16]. The mTOR pathway plays an important role in the functional tissues, such as muscle tissues. Furthermore, the mTORC1 activation is required for the muscle protein

synthesis and skeletal muscle hypertrophy in humans in response to the physical exercise [17, 18]. When the inhibition of mTOR signaling pathway persistently, the skeletal muscle begin to loss muscle mass and strength during muscle wasting in aging and muscle atrophy from physical inactivity [17-19].

Our previous study showed that the mechanical stress affects PI3K/mTOR signaling pathway in myoblasts. Moreover, mTOR is regarded as a critical factor for the PI3K signaling pathway, which is the efficient proliferation pathway for the muscle satellite cells. Therefore, we assumed that mTOR is involved in the regulation of myoblast proliferation and participates in the differentiation and apoptosis undergoing the mechanical stress. In order to confirm our hypothesis, we used Crisper/Cas9 system to build up knockdown mTOR model. We observed that mTOR knockdown reduced the C2C12 number, which means that mTOR promoted the myoblast proliferation and mechanical stress regulated myoblasts by involving the mTOR signaling pathway. Overall, our data suggest that mTOR has important effects on promoting myoblast growth and mechanical stress may participate in this processes.

As the downstream proteins of PI3K/mTOR signaling pathway, 4EBP1 and p70S6k were marked positively in PI3K signaling pathway. Our results also revealed that 4EBP1 and p70S6k protein were decreased post the mTOR knockdown, which suggests that the 4EBP1 and p70S6k can promote the proliferation of myoblast that newly derived from activated mTOR protein. Indeed, we observed that in C2C12 cells 4EBP1 and p70S6k levels began to rise when cells treated under stress. Therefore, mTOR plays important roles in PI3K signaling pathway in C2C12 myoblasts by mechanical stress.

Many recent studies reported that PI3K/mTOR signaling pathway was overactive, therefore, reducing the apoptosis and allowing the proliferation in cancer. This pathway is significant to promote the growth and proliferation in adult stem cells [20]. Moreover, it is difficult to find an appropriate amount to balance the proliferation and differentiation and researchers are trying to utilize various therapies to conquer diseases [20]. This signaling activated the PI3K/Akt signaling pathway which works to promote proliferation. Our data support their conclusion and provide these observations. According to our data, mTOR knockdown prevented the myoblast proliferation and decelerated the proliferation speed. Furthermore, mechanical stress promoted the proliferation and speeded up the mTOR knockdown proliferation. Therefore, inhibition the mTOR in PI3K signaling pathway illustrated a negative effect on the myoblast proliferation and the mechanical stress consequently accelerated the proliferation rate.

Many researches reported that many families of proteins act as negative regulators, such as pro-survival factors like Akt and NF- κ B [21, 22]. A cell initiates intracellular apoptosis signaling in response to the stress, which may bring about cell suicide. Mechanical stress may damage cells by the trigger the intracellular apoptosis signals, and may help regulate apoptosis. According to our flow cytometry data, we observed that in C2C12 cells with mTOR knockdown began apoptosis process. This result revealed that the mTOR might have a negative effect on myoblast apoptosis. Indeed, down-regulation of mTOR could promote apoptosis rate and is essential for preventing proliferation. Even when the cells treated with stress, mTOR knockdown resulted in significant decrease apoptosis rate. These results showed that stress increased myoblast apoptosis might be involved in the mTOR activation. It would be interesting to prove the functions of mTOR in balancing myoblast proliferation and apoptosis, which would point to positive directions in myoblast reconstruction.

In conclusion, our data supported our conclusion and provide further details to understand and explain our observations. Meanwhile, we tested the mTOR knockdown differentiation to complete mTOR biological function. Furthermore, we used immunofluorescence to prove

our hypothesis, and the results showed that the mTOR knockdown cells reached low level and mechanical stress promoted differentiation in C2C12 cells. As suggested by our figures, mTOR knockdown resulted in significant decrease in myoblast differentiation, suggesting that the mTOR may contribute to accelerate myoblast differentiation.

The role of mTOR in regulation of myoblasts in C2C12 cells under mechanical stress is significant, but still remains further study. Our study showed that mTOR decreased myoblasts proliferation and inhibited PI3K signaling pathway by down-regulating the p70S6K and 4EBP1 factors. Moreover, mechanical stress accelerated PI3K signaling pathway and reversed mTOR knockdown cells proliferation. Secondly, our data also showed that mTOR up-regulated the myoblast apoptosis, which suggests that mTOR may play a negative role in myoblast apoptosis. It is possible that mTOR was involved in myoblast differentiation but not as an essential factor for stimulating differentiation. As a conclusion, our study revealed a function of mTOR regulating myoblasts cell proliferation, apoptosis and differentiation. This result helped us to understand how mTOR regulates skeletal muscle regeneration.

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Disclosure of conflict of interest

None.

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References

- [1] Birbrair A, Zhang T, Wang ZM, Messi ML, Enikolopov GN, Mintz A, Delbono O. Role of pericytes in skeletal muscle regeneration and fat accumulation. *Stem Cells Dev* 2013; 22: 2298-2314.

Effects of mTOR on myoblasts undergoing mechanical stress

- [2] Wang J, Zhang F, Yang H, Wu H, Cui R, Zhao Y, Jiao C, Wang X, Liu X, Wu L, Li G, Wu X. Effect of TEAD4 on multilineage differentiation of muscle-derived stem cells. *Am J Transl Res* 2018; 10: 998-1011.
- [3] Bentzinger CF, Wang YX, Rudnicki MA. Building muscle: molecular regulation of myogenesis. *Cold Spring Harb Perspect Biol* 2012; 4: a008342.
- [4] Comai G, Tajbakhsh S. Molecular and cellular regulation of skeletal myogenesis. *Curr Top Dev Biol* 2014; 110: 1-73.
- [5] Tajbakhsh S. Skeletal muscle stem cells in developmental versus regenerative myogenesis. *J Intern Med* 2009; 266: 372-389.
- [6] Tan J, Kuang W, Jin Z, Jin F, Xu L, Yu Q, Kong L, Zeng G, Yuan X, Duan Y. Inhibition of NF- κ B by activated c-Jun NH2 terminal kinase 1 acts as a switch for C2C12 cell death under excessive stretch. *Apoptosis* 2009; 14: 764-770.
- [7] Mitra A, Luna JI, Marusina AI, Merleev A, Kundu-Raychaudhuri S, Fiorentino D, Raychaudhuri SP, Maverakis E. Dual mTOR inhibition is required to prevent TGF- β -mediated fibrosis: implications for scleroderma. *J Invest Dermatol* 2015; 135: 2873-2876.
- [8] Kennedy BK, Lamming DW. The mechanistic target of rapamycin: the grand conductor of metabolism and aging. *Cell Metab* 2016; 23: 990-1003.
- [9] Beevers CS, Li F, Liu L, Huang S. Curcumin inhibits the mammalian target of rapamycin-mediated signaling pathways in cancer cells. *Int J Cancer* 2006; 119: 757-764.
- [10] Zhang P, Liang X. mTOR is necessary for proper satellite cell activity and skeletal muscle regeneration. *Biochem Biophys Res Commun* 2015; 463: 102-108.
- [11] Brook MS, Wilkinson DJ, Phillips BE, Perez-Schindler J, Philp A, Smith K, Atherton PJ. Skeletal muscle homeostasis and plasticity in youth and ageing: impact of nutrition and exercise. *Acta Physiologica* 2016; 216: 15-41.
- [12] Phillips SM. A brief review of critical processes in exercise-induced muscular hypertrophy. *Sports Med* 2014; 44: S71-S77.
- [13] Abou Sawan S, van Vliet S, Parel JT, Beals JW, Mazzulla M, West DWD, Philp A, Li Z, Paluska SA, Burd NA, Moore DR. Translocation and protein complex co-localization of mTOR is associated with postprandial myofibrillar protein synthesis at rest and after endurance exercise. *Physiol Rep* 2018; 6.
- [14] Bond P. Regulation of mTORC1 by growth factors, energy status, amino acids and mechanical stimuli at a glance. *J Int Soc Sports Nutr* 2016; 13: 8.
- [15] Lipton JO, Sahin M. The neurology of mTOR. *Neuron* 2014; 84: 275-291.
- [16] Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes Dev* 2004; 18: 1926-1945.
- [17] Brook MS, Wilkinson DJ, Phillips BE, Perez-Schindler J, Philp A, Smith K, Atherton PJ. Skeletal muscle homeostasis and plasticity in youth and ageing: impact of nutrition and exercise. *Acta Physiologica* 2016; 216: 15-41.
- [18] Brioché T, Pagano PF, Py G, Chopard A. Muscle wasting and aging: experimental models, fatty infiltrations, and prevention. *Mol Aspects Med* 2016; 50: 56-87.
- [19] Drummond MJ, Dreyer HC, Fry CS, Glynn EL, Rasmussen BB. Nutritional and contractile regulation of human skeletal muscle protein synthesis and mTORC1 signaling. *J Appl Physiol* 2009; 106: 1374-1384.
- [20] Peltier J, O'Neill A, Schaffer DV. PI3K/Akt and CREB regulate adult neural hippocampal progenitor proliferation and differentiation. *Dev Neurobiol* 2007; 67: 1348-1361.
- [21] Razaghi A, Heimann K, Schaeffer PM, Gibson SB. Negative regulators of cell death pathways in cancer: perspective on biomarkers and targeted therapies. *Apoptosis* 2018; 23: 93-112.
- [22] Cheng L, Zhou S, Zhao Y, Sun Y, Xu Z, Yuan B, Chen X. Tanshinone IIA attenuates osteoclastogenesis in ovariectomized mice by inactivating NF- κ B and Akt signaling pathways. *Am J Transl Res* 2018; 10: 1457-1468.