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Roles of Mitogen-Activating Protein Kinase Kinase Kinase Kinase-3 (MAP4K3) in Preterm Skeletal Muscle Satellite Cell Myogenesis and Mammalian Target of Rapamycin Complex 1 (mTORC1) Activation Regulation

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Statistical Analysis C
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Background: Preterm skeletal muscle genesis is a paradigm for myogenesis. The role of mitogen-activating protein kinase kinase kinase kinase-3 (MAP4K3) in preterm skeletal muscle satellite cells myogenesis or its relationship to mammalian target of rapamycin complex 1 (mTORC1) activity have not been previously elaborated.

Material/Methods: Small interfering RNA (siRNA) interference technology was used to inhibit MAP4K3 expression. Leucine stimulation experiments were performed following MAP4K3-siRNA interference. The differentiation of primary preterm skeletal muscle satellite cells was observed after siRNA-MAP4K3 interference. Western blot analysis was used to determine the expression of MAP4K3, MyHC, MyoD, myogenin, p-mTOR, and p-S6K1. The immunofluorescence fusion index of MyHC and myogenin were detected. MAP4K3 effects on preterm rat satellite cells differentiation and its relationship to mTORC1 activity are reported.

Results: MAP4K3 siRNA knockdown inhibited myotube formation and both MyoD and myogenin expression in primary preterm rat skeletal muscle satellite cells, but MAP4K3 siRNA had no effect on the activity of mTORC1. In primary preterm rat skeletal muscle satellite cells, MAP4K3 knockdown resulted in significantly weaker, but not entirely blunted, leucine-induced mTORC1 signaling.

Conclusions: MAP4K3 positively regulates preterm skeletal muscle satellite cell myogenesis, but may not regulate mTORC1 activity. MAP4K3 may play a role in mTORC1 full activation in response to leucine.

MeSH Keywords: **MAP Kinase Kinase Kinase 4 • Satellite Cells, Skeletal Muscle • TOR Serine-Threonine Kinases**

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Background

Neonatal period growth rate is greater than at any other postnatal development stage. Skeletal muscle constitutes most of this growth, making it a significant determinant of amino acid and energy requirements. Skeletal muscle is 30% of neonatal body mass and is the most rapidly growing part. The muscle protein pool largely determines overall body protein metabolism and amino acid requirements [1]. Skeletal muscle satellite cells are a heterogeneous population of stem and progenitor cells necessary for embryonic skeletal muscle development [2]. Satellite cells are a significant proportion of the total muscle nuclei in newborns. The proportion decreases as myonuclei-per-fiber numbers increase. In rats, satellite cells constitute approximately 35% of all muscle nuclei at birth, decreasing to 10% at 4 weeks. A large number of satellite cells support neonatal muscle growth [3]. Preterm skeletal muscle genesis is a paradigm for myogenesis [4].

Amino acids and proteins play pivotal roles in preterm infant growth and development [5]. Leucine, a branched-chain amino acid, is critical for muscle growth and acts, in part, by triggering mammalian target of rapamycin complex 1 (mTORC1) [6]. mTORC1 has recently been determined to be a key regulator of skeletal myogenesis. Sun et al. [7] showed that the mTOR-miR-1-HDAC4-follistatin pathway regulates, *in vitro*, myocyte fusion during myoblast differentiation, and, *in vivo*, skeletal muscle regeneration. Our recent study suggests that leucine promotes skeletal muscle satellite cell differentiation during myotube formation, in part, via the mTORC1-MyoD signal pathway [8].

How amino acids activate mTORC1 in preterm skeletal muscle satellite cell myogenesis remains unclear. Mitogen-activating protein kinase kinase kinase kinase-3 (MAP4K3) may be involved in mediating the effects of amino acids on mTORC1 signaling. MAP4K3, an MAP4K family member belonging to a subfamily of the sterile 20 protein-like serine/threonine kinases [9], may regulate gene transcription, apoptosis, and immune inflammation in response to extracellular signals [10,11]. Cell-growth regulation studies suggest that MAP4K3 is upstream from mTORC1 and regulates mTORC1 in response to amino acids [12].

However, no MAP4K3 role in preterm skeletal muscle satellite cell myogenesis or in mTORC1 activation regulation has been previously reported. This study used small interfering RNA (siRNA) interference technology to inhibit MAP4K3 expression, and perform post-siRNA MAP4K3 interference leucine simulation experiments. MAP4K3 effects on preterm skeletal muscle satellite cells differentiation and its relationship to mTORC1 activity were observed.

Material and Methods

Materials

MAP4K3 siRNA was designed and synthesized at Shanghai GenePharma. Anti-MAP4K3, anti-mammalian target of rapamycin (mTOR) and anti-p-mTOR (Ser2448) antibodies were purchased from Cell Signaling Technology. Anti-MyoD and anti-Myogenin antibodies were supplied by BD Biosciences. Anti-myosin heavy chain antibody (MyHC), anti-ribosomal protein S6 kinase, polypeptide 1 (S6K1), and anti-p-S6K1 (Thr389) antibodies were purchased from Abcam. Lipofectamine 2000 was supplied by Life Technologies. Anti-myosin heavy chain antibody (MyHC) for immunofluorescence was purchased from Proteintech. Fluor 594 anti-mouse immunoglobulin G (IgG) and 488 anti-rabbit IgG were from Invitrogen Life Technologies. HRP-conjugated anti-mouse, and anti-rabbit, IgG antibodies were obtained from EarthOx and Abmart, respectively. Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/F12ham) (D9785), Leucine Type I Collagenase, and Trypsin were from Sigma-Aldrich. Fetal bovine serum (FBS) was obtained from Gibco Life Technologies. Basic Fibroblast Growth Factor (b-FGF) was purchased from BBI Solutions. Antifade solution was obtained from Life Technologies.

Cell culture and treatments

This study was approved by the Ethics Committee of the First Affiliated Hospital, Sun Yat-sen University. The SD rats were obtained from the Animal Experiment Center of the First Affiliated Hospital, Sun Yat-sen University. Cell culture was carried out as previously described [8]. The preterm rats were delivered by caesarean on gestation day 18. The limb skeletal muscles were isolated from preterm rats under a surgical microscope, disassociated with type I collagenase and trypsin by 2-step digestion method, and purified via differential adhesion. Growth medium contained 70% DMEM/F12ham, 20% FBS, 10% HS, and 5 ng/ml bFGF. Differentiation medium (DM) contained 98% DMEM/F12ham and 2% HS. CCK-8 assay was used to record preterm rat skeletal muscle satellite cells growth curves. The expression of desmin was identified by immunocytochemistry staining. The differentiation of preterm rat skeletal muscle satellite cells was observed by inverted microscopy. These 2 steps of pre-plating resulted in satellite cell purity of greater than 90%. The cells were then cultured to approximately 80% confluence in DMEM/F12ham with 2% horse serum for 3 days to initiate differentiation. The medium was changed each day. Myotubes were photographed using an inverted fluorescence microscope.

The primary skeletal muscle satellite cells were starved for leucine and serum in DMEM/F12ham for 2 h prior to the leucine stimulation experiments. Cells were stimulated with 0.5 nM leucine for 30 min after starvation.

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siRNA transfection

Transfection of MAP4K3 with siRNA was performed using lipofectamine 2000 transfection reagent according to the manufacture's protocols. Primary skeletal muscle satellite cells were plated in 5×10^4 cells per well of growth medium and cultured until reaching 30–40% confluence after 24 h. Then, the cells were transfected with 20 nM-100 nM siRNA of MAP4K3 plus 5–10 ul lipofectamine 2000 for 6 h in 10%FBS DMEM/F12 and sequentially cultured in growth medium for 48 h in a 37°C 5% CO_2 humidified incubator. The silencing efficiency was determined by immunoblotting. The sequence (r-300) corresponding to MAP4K3 siRNA was: 5'-CGG UUG UAC AGC AAG AAA UTT-3'. The negative control sequence was: 5'-UUC UCC GAA CGU GUC ACG UTT-3'.

Western blot analysis

Western blot analysis was performed to assay the protein expression levels of MAP4K3, MyHC, MyoD, myogenin, mTOR, p-mTOR, S6K1, p-S6K1, and GAPDH by standard methods. Briefly, protein from cells was extracted using cell lysis buffer. Protein levels of the cell lysate were quantified using a BCA protein assay kit (Beyotime Biotechnology Co. Ltd., Shanghai, China), according to the manufacturer's instructions. The protein extracts were then loaded on SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). After blocking in blocking buffer containing 5% BSA for 1 h at room temperature, we incubated the membranes with primary antibody against MAP4K3 (1: 1000), MyHC (1: 5000), myogenin (1: 2000), MyoD (1: 500), mTOR (1: 1000), p-mTOR (1: 1000), p-S6K1(1: 500), S6K1 (1: 2000), and GAPDH (1: 10000) at 4°C for overnight. After washing 3 times, the membranes were then incubated with appropriate horseradish peroxidase-linked secondary antibodies at 25°C for 1 h. After using enhanced chemiluminescence, the protein blots were visualized. The abundance of protein bands was quantified using Image J software (National Institutes of Health, Bethesda, Maryland, USA). Target protein expression level was normalized to individual GADPH levels. The relative phosphorylated protein expression level was calculated according to the ratio of phosphorylated protein and total protein expression.

Immunofluorescence microscopy

To assess the protein expression of MyHC and myogenin and observe the forms and structures of myotubes, the cells differentiated in 6-well plates were fixed after all treatments. The fixed cells were washed with ice-cold PBS (pH 7.4) and fixed with 4% paraformaldehyde for 30 min, followed by permeabilization (0.3% Triton-X) for 15 min and blocking with 5% goat serum albumin for 1 h at room temperature. Primary antibody dilutions (1: 500) were prepared in 5% goat serum albumin in PBS using anti-MyHC and anti-myogenin, then incubated overnight at 4°C. Following incubation, cells were washed 3 times by PBS for 5 min and incubated with fluorescence-labeled (488 nm) anti-rabbit antibody and fluorescence-labeled (594 nm) anti-mouse antibody for 1 h followed by washing 3 times with PBS and counterstaining with the nuclear stain 4',6-diamidino-2-phenylindol (DAPI) for 5 min. Slides were dried and mounted using antifade solution. The stained cells were captured and examined with an Olympus BX63 fluorescence microscope, and the fluorescence images were analyzed with Q-capture Pro51 software.

Statistical analysis

The statistical analyses were performed using SPSS13.0 software. The results are presented as mean ± standard deviation (SD). Differences between groups were analyzed by one-way analyses of variance (ANOVA). $P<0.05$ was considered significant.

Results

Transfection efficiency of the MAP4K3 siRNA

In the results of Western blot analysis, no significant differences with MAP4K3 protein were seen between the mock control and negative siRNA control. MAP4K3 protein in the siRNA sequence 2 and 3 groups decreased significantly compared to the negative control (NC) group ($p<0.05$). The siRNA sequence 1 (r-300) was decreased more significantly ($P<0.01$) (Figure 1). The transfection efficiency of r-300 achieved over 74% in cells. Thus, we used the sequence 1 (r-300) approach to knock down MAP4K3 expression and assess its effects.

MAP4K3 siRNA knockdown inhibits primary preterm skeletal muscle satellite cell differentiation

Primary preterm rat skeletal satellite cells reached a confluence of approximately 80% and then were induced to differentiate in DM. Cells were lysed every 24 h and the lysates were subjected to Western blot analysis and immunofluorescence microscopy. Western blot analysis was performed to determine MAP4K3 and MyHC levels. The immunofluorescence fusion index of MyHC was detected. MAP4K3 and MyHC expression increased gradually during primary preterm rat skeletal muscle satellite cell differentiation (Figure 2).

After siRNA-MAP4K3 interference, when primary preterm rat skeletal satellite cells reached approximately 80% confluence, they were cultured in a differentiation medium for 3 days, followed by Western blot analysis and immunofluorescence microscopy. MAP4K3 siRNA knockdown resulted in

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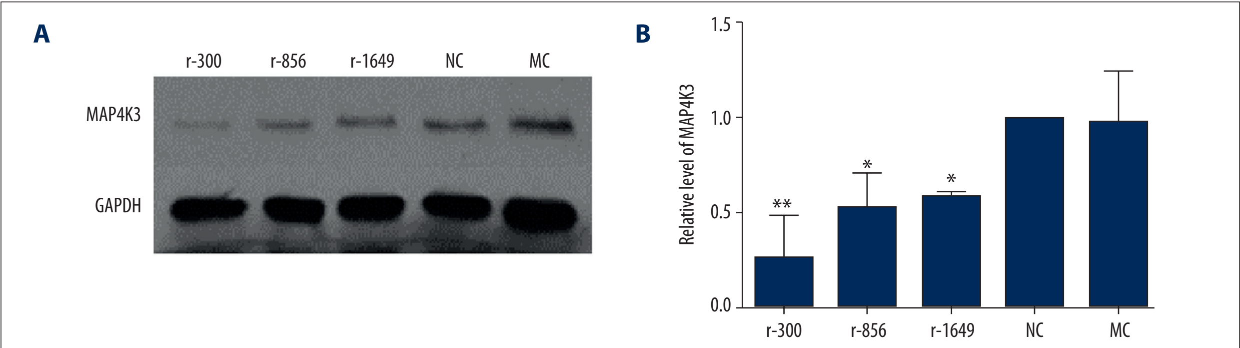

Figure 1. Effects of siRNA interference on MAP4K3 protein expression in preterm skeletal muscle satellite cells. (**A**) Western blot of MAP4K3 protein: 1. siRNA sequence 1 group: r-300; 2. siRNA sequence 2 group: r-856; 3. siRNA sequence 3 group: r-1649; 4. negative control (NC) group; 5. mock control (MC) group; (**B**) A histogram of relative level of MAP4K3 protein expression in all groups. The data were presented as the mean ±standard deviation (SD) (n=3 in each group). * P<0.05 versus NC group. ** P<0.01 versus NC group.

A

d0 d1 d2 d3

MAP4K3

MyHC

GAPDH

Relative level of MAP4K3

3 2 1 0

d0 d1 d2 d3

Relative level of MyHC

20 15 10 5 0

d0 d1 d2 d3

B

d0 d1 d2 d3

Fusion index of MyHC

2.5 2.0 1.5 1.0 0.5 0.0

d0 d1 d2 d3

Figure 2. MAP4K3 and MyHC expression in primary preterm rat skeletal muscle satellite cell differentiation. (**A**) MAP4K3 and MyHC protein expression increased gradually during primary preterm rat skeletal muscle satellite cell differentiation. (**B**) Cells were stained for MyHC (red) and DAPI (blue) and calculated the quantification for fusion index. The fusion index of MyHC, forms and structures of myotubes increased gradually during primary preterm rat skeletal muscle satellite cell differentiation. The data were presented as the mean ±SD (n=3 in each group). * P<0.05 versus control (d0) group, ** P<0.01 versus control (d0) group, *** P<0.001 versus control (d0) group (original magnification ×200).

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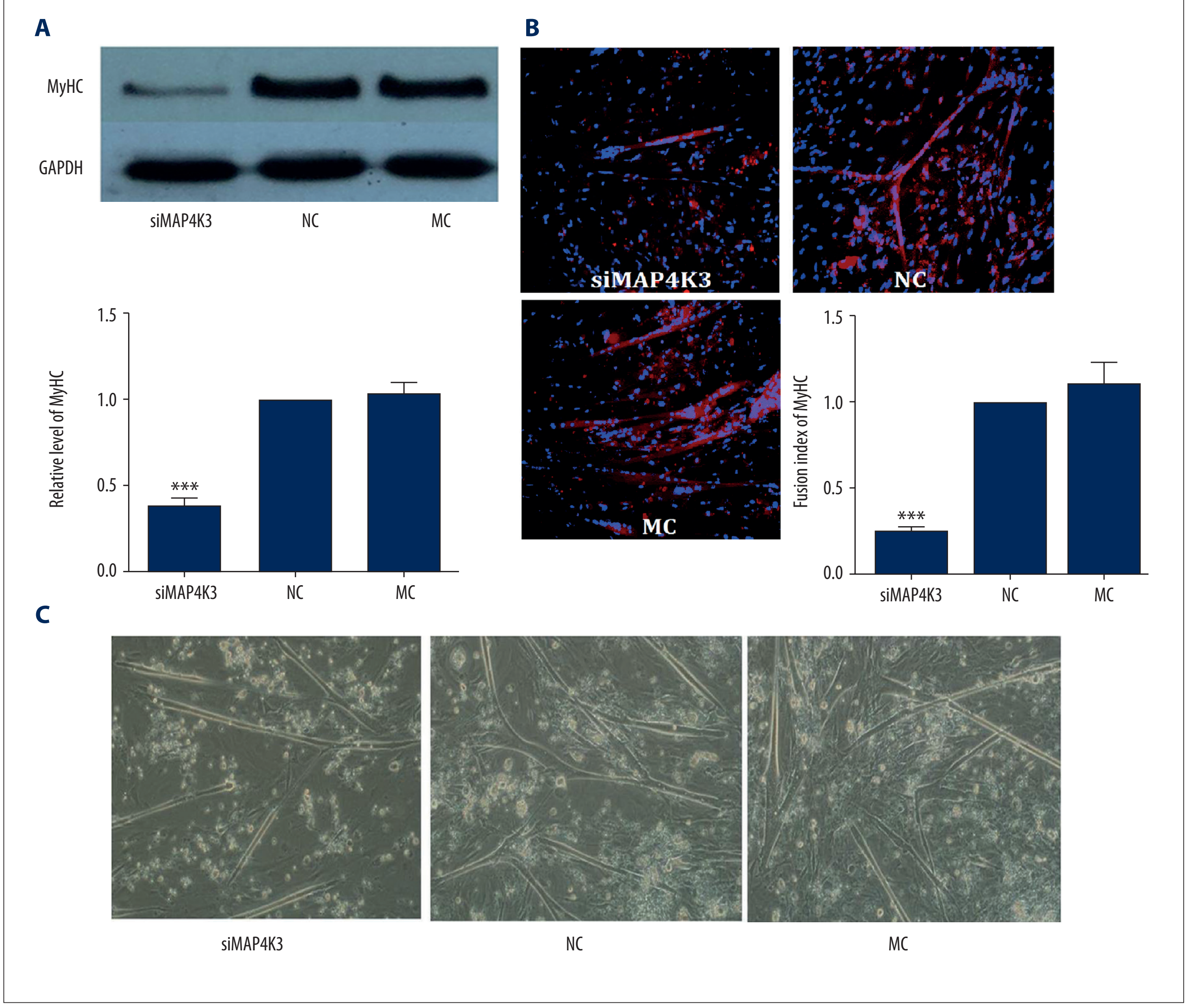

Figure 3. Effect of MAP4K3 siRNA on MyHC expression and myotubes formation. (**A**) MyHC levels decreased after siRNA-MAP4K3 interference. (**B**) After siRNA-MAP4K3 interference, the cells were stained for MyHC (red) and DAPI (blue) and calculated the quantification for fusion index. The fusion index of MyHC was lower in siMAP4K3 group. The data are presented as the mean ±SD (n=3 in each group). *** P<0.001 versus negative control (NC) group. (**C**). After siRNA-MAP4K3 interference, the forms and structures of myotubes were smaller and thinner in the siMAP4K3 group and visibly larger in the NC and MC groups (original magnification ×200).

MyHC expression decrease in primary preterm rat skeletal muscle satellite cells. After siRNA-MAP4K3 interference, the forms and structures of myotubes were smaller and thinner in the siMAP4K3 group and were visibly larger in the NC and MC groups (Figure 3).

MAP4K3 siRNA knockdown inhibits MyoD and myogenin expression in primary preterm rat skeletal muscle satellite cells

After 48 h of siRNA-MAP4K3 interference, relative MyoD levels were determined using Western blot analysis. siRNA-MAP4K3 knockdown caused MyoD expression decrease in primary preterm rat skeletal muscle satellite cells (Figure 4).

After siRNA-MAP4K3 interference, primary preterm rat skeletal satellite cells, having a confluence of approximately 80%, were cultured for 3 days in DM, followed by Western blot analysis and immunofluorescence microscopy of myogenin relative levels. siRNA-MAP4K3 knockdown caused decreased myogenin expression in primary preterm rat skeletal muscle satellite cells (Figure 5).

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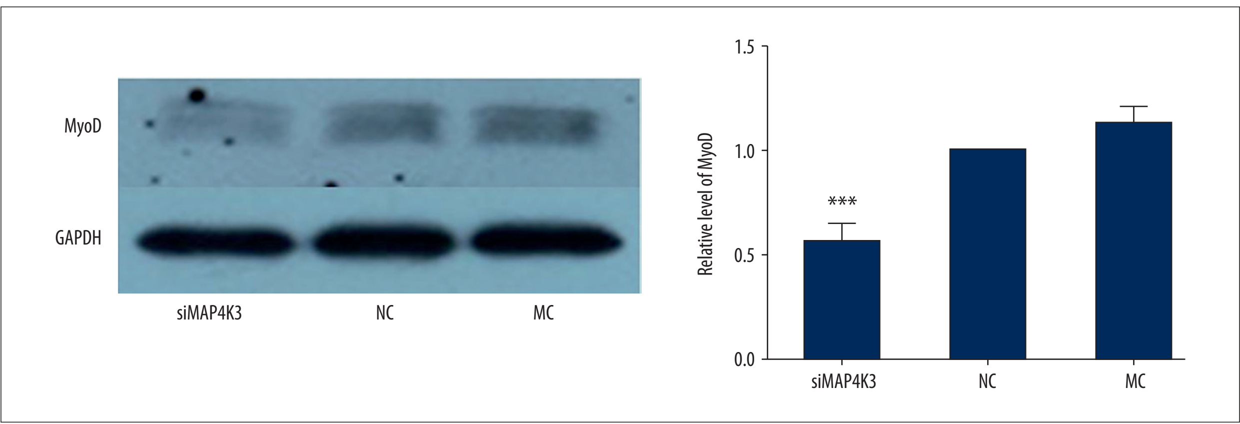

Figure 4. Effect of MAP4K3 siRNA on MyoD expression. After siRNA-MAP4K3 interference, MyoD relative levels decreased. The data are presented as mean ±SD (n=3 in each group). *** P<0.001 versus NC group.

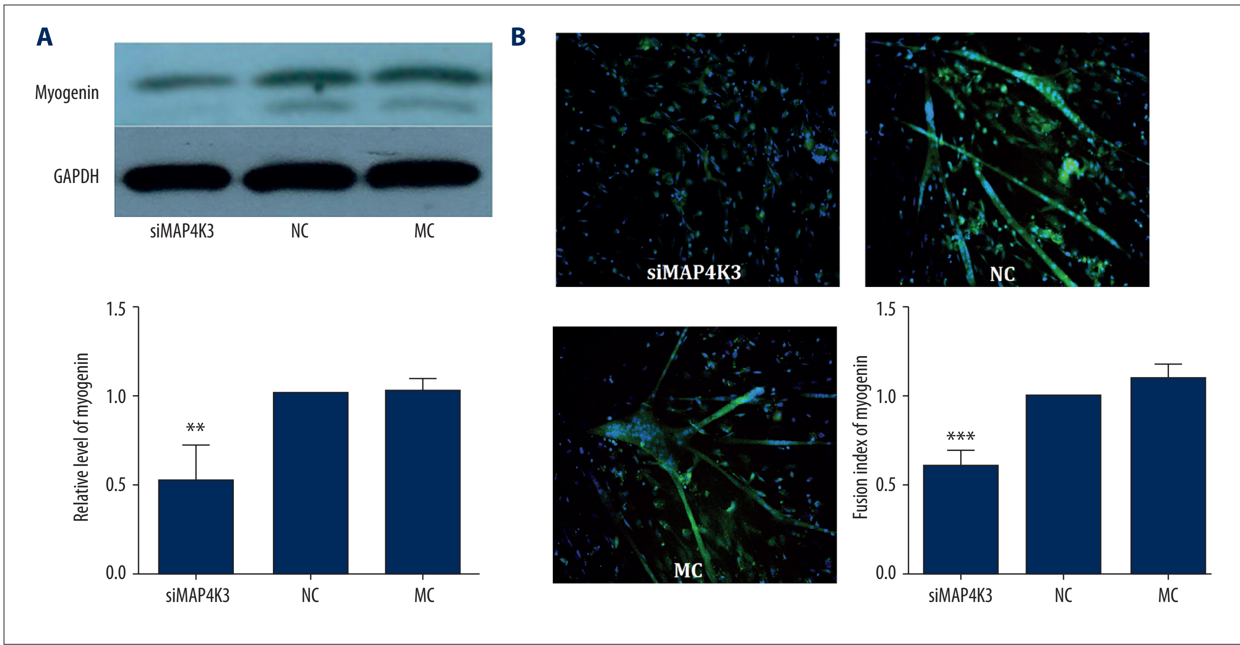

Figure 5. Effect of MAP4K3 siRNA on myogenin expression. (**A**) After siRNA-MAP4K3 interference, myogenin relative levels decreased. (**B**) After siRNA-MAP4K3 interference, the cells were stained for myogenin (green) and DAPI (blue) and quantified for fusion index. The fusion index of myogenin was lower in the siMAP4K3 group. The data are presented as mean ±SD (n=3 in each group). ** P<0.01 versus NC group (Original magnification ×200).

siRNA-MAP4K3 does not regulate mTORC1 activity in primary preterm skeletal muscle satellite cell differentiation

Post-siRNA-MAP4K3 interference with primary preterm rat skeletal satellite cells that had reached a confluence of approximately 80% were cultured for 3 days in DM, followed by Western blot analysis of the relative levels of p-mTOR and p-S6K1. As depicted in Figure 6, MAP4K3 knockdown by siRNA did not affect either p-mTOR or p-S6K1 expression in primary preterm rat skeletal muscle satellite cells.

Leucine-induced p-mTOR expression significantly increases in non-specific siRNA cells compared with siRNA-MAP4K3 interference cells

As shown in Figure 7, After 72 h of siRNA-MAP4K3 interference, compared to the non-specific siRNA group, p-mTOR relative levels in the siRNA-MAP4K3 interference group showed no significant changes. Following leucine starvation, cells were stimulated with 0.5 nM leucine for 30 min. Leucine-induced p-mTOR expression increased in both the siRNA-MAP4K3 interference group and the non-specific siRNA group. Leucine-induced p-mTOR expression was significantly increased in the

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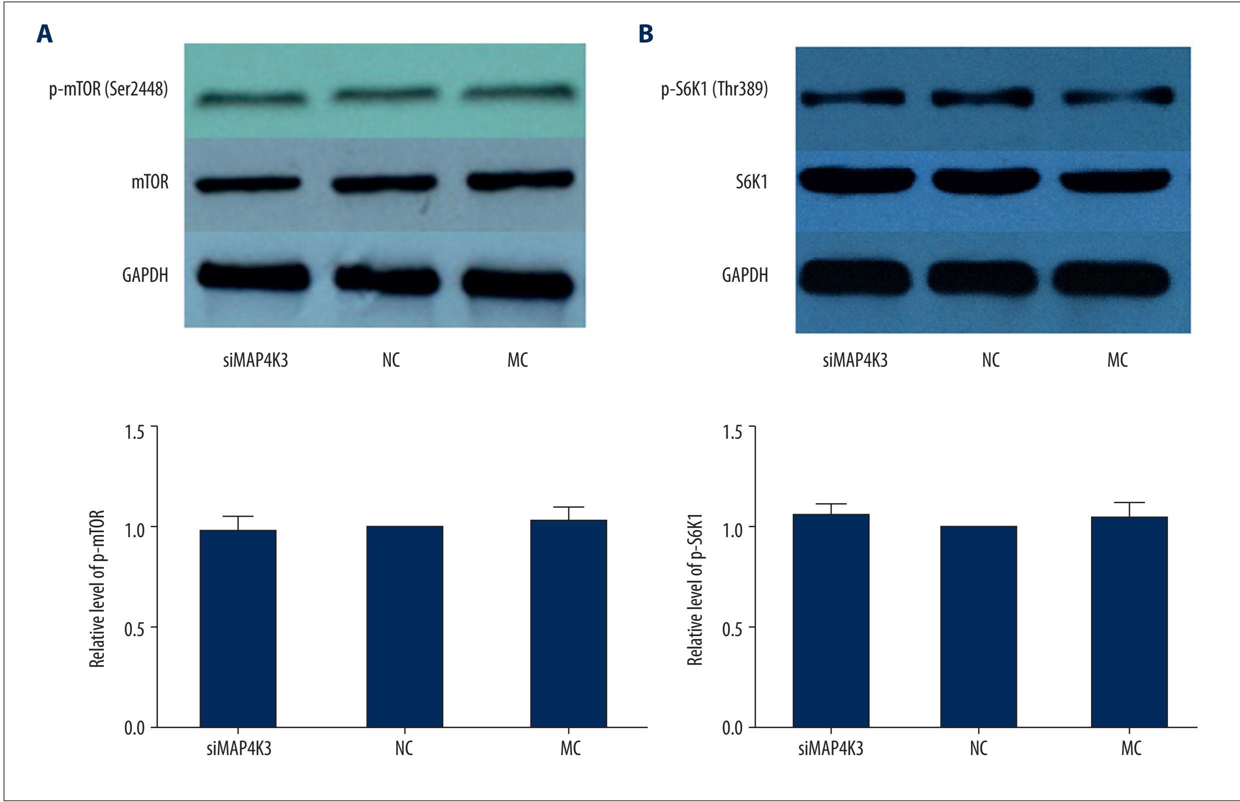

Figure 6. Effect of MAP4K3 siRNA on p-mTOR and p-S6K1 expression. (**A**) Compared to the NC group, p-mTOR relative levels in the siRNA-MAP4K3 interference group and MC group showed no significant difference (P>0.05). (**B**) Compared to the NC group, p-S6K1 relative levels in the siRNA-MAP4K3 interference group and MC group showed no significant difference (P>0.05). The data are presented as mean ±SD (n=3 in each group).

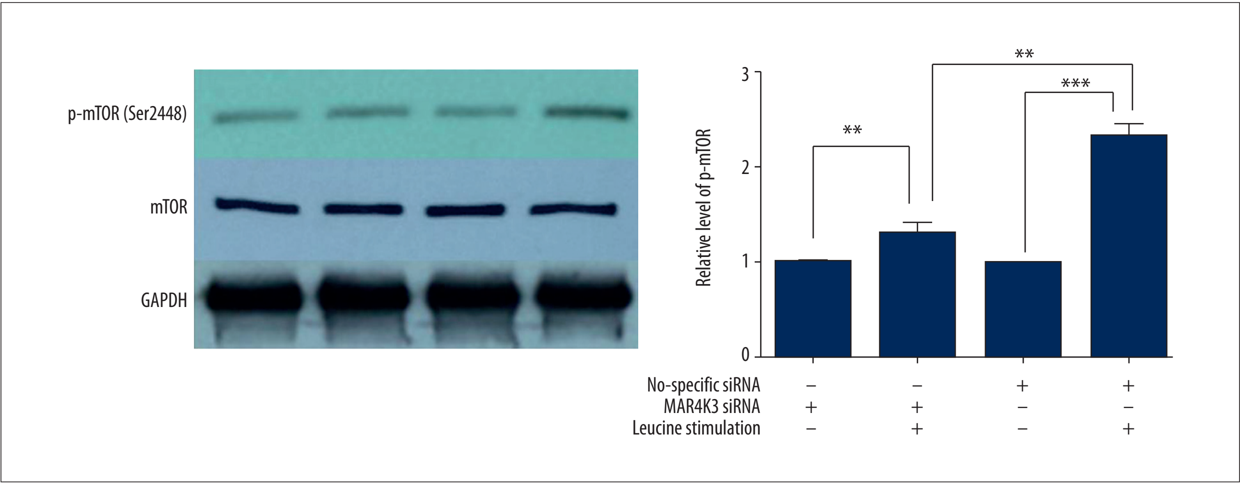

Figure 7. Leucine-induced p-mTOR expression in the siRNA-MAP4K3 interference group and the non-specific siRNA group. Compared to the non-specific siRNA group, p-mTOR relative levels in the siRNA-MAP4K3 interference group showed no significant changes in their basic station (P>0.05). After leucine stimulation, leucine-induced p-mTOR expression increased in both the siRNA-MAP4K3 interference group and the non-specific siRNA group. Leucine-induced p-mTOR expression was significantly increased in the non-specific siRNA group compared with the siRNA-MAP4K3 interference group. The data are presented as mean ±SD (n=3 in each group). ** P<0.01, *** P<0.001.

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non-specific siRNA group compared with the siRNA-MAP4K3 interference group.

Discussion

Leucine is required to activate mTORC1, which has pivotal roles in controlling protein synthesis, cell growth, and cell proliferation [13]. Rag GTPase, Vps34, and MAP4K3 have been identified as leucine-signaling mediators in mTORC1 activation [14]. It has been reported that both Rag GTPase and Vps34 mediate mTORC1 amino acid activation in C2C12 myoblasts. They have opposing functions during myogenic differentiation [15]. Our study presents evidence that MAP4K3 positively regulates primary skeletal muscle satellite cell differentiation and that MAP4K3 might not regulate mTORC1 activity during myogenic differentiation.

Myogenic regulatory factors (MRFs) control muscle lineage specification and differentiation. MyoD expresses during the terminal differentiation of myogenic precursor cells into myotubes and myofibers. MyoD, a primary MRF, appears to be required for myogenic determination [16]. Myogenin, the secondary MRF, acts later, during myogenesis, to allow myotube formation and maturation [17]. MyHC accounts for 25–30% of all muscle protein, making it an important skeletal muscle growth factor [18]. Increased MAP4K3 and MyHC expression during primary preterm rat skeletal muscle satellite cell differentiation was found. After siRNA-MAP4K3 interference, there was decreased MyoD expression in the early differentiation stage, and myogenin expression decreased in a later differentiation stage. This suggests that MAP4K3 has a positive role in myogenic differentiation.

We further showed that siRNA-MAP4K3 interference did not affect either p-mTOR or p-S6K1 relative levels. Our results describe a process of myogenic differentiation, indicating that MAP4K3 may not be involved in mTORC1 activity, in contrast to regulating cell growth. Findlay et al. [12] reported that in Drosophila and mammalian cells, MAP4K3 suppression inhibited mTORC1 signaling pathways. mTORC1 positively regulates protein synthesis and cell growth by phosphorylating 2 translational regulatory proteins: S6K1 and 4E-binding protein 1 (4E-BP1) [14]. However, mTORC1 substrate S6K1 and 4E-BP1 appear to play no role in myogenesis, despite their documented involvement in cell growth [19, 20]. How MAP4K3 activates MyoD or myogenesis in the process of preterm rat primary skeletal muscle satellite cells differentiation needs further investigation.

MAP4K3 may function to only partially modulate targeting of rapamycin complex 1 (TORC1) signaling. Bryk et al. [21] showed that MAP4K3 mutant flies displayed reduced, but not completely inhibited, TORC1 activity. This suggests that MAP4K3 might play a more pivotal role in TORC1 activity when nutrients are plentiful. In agreement with a recent study which found that in human fibroblasts, MAP4K3 is required for mTORC1 full activation in response to leucine [22], we demonstrated that in primary preterm rat skeletal muscle satellite cells, MAP4K3 was needed for full mTORC1 activation in response to leucine stimulation. Similarly, Yan et al. [23] found that during amino acid stimulation, amino acids phosphorylate MAP4K3 in Ser170 and activate both MAP4K3 and mTORC1 in HEK293 cells.

Conclusions

Our data indicates that in primary preterm rat satellite cells, MAP4K3 may play a role in mTORC1 full activation in response to leucine. MAP4K3 is a positive regulator of preterm skeletal muscle satellite cell myogenesis, but may not be involved in mTORC1 activity during myogenesis.

Conflicts of interest

There are no conflicts of interest.

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