CRISPR/Cas9-Mediated Modulation of mTOR Signaling: A Multi-Dimensional Approach for Muscle Disease Intervention, Metabolic Regulation, and Mechanical Stress

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ABSTRACT

BACKGROUND

Myoblasts (satellite cells) possess the characteristics to distinguish between skeletal muscles and play crucial roles in the proliferation, differentiation, and growth of skeletal muscles. The mammalian target of rapamycin (mTOR), a key regulatory protein in the mTOR signaling pathway, is crucial to muscle metabolism and physiology. Understanding the modulation of mTOR signaling under mechanical stress provides insights into muscle-related diseases, metabolic regulation, and mechanical stress.

OBJECTIVE

This article is set to elaborate on the effects of mTOR knockdown on the growth, mechanical stress, and differentiation of C2C12 myoblasts under mechanical stress using CRISPR/Cas9 technology.

METHODS

C2C12 satellite cells were grown on media, and mTOR expression was demolished through CRISPR/Cas9. The expression levels of 4E-binding protein 1 (4EBP1) and p70 ribosomal protein S6 kinase (p70S6k) were examined using quantitative polymerase chain reaction. Cell growth/proliferation was evaluated by the Cell Counting Kit-8.

RESULTS

mTOR knockdown significantly reduced the phosphorylation of 4EBP1 and p70S6k, leading to decreased protein synthesis in the mTOR signaling pathway under mechanical stress. The proliferation of myoblasts was notably slowed by mTOR knockdown. Furthermore, mechanical stress applied to mTOR-deficient cells resulted in a prominent decrease in differentiation speed.

CONCLUSION

The study demonstrates that mTOR performs a critical role in regulating myoblast proliferation and distinction under mechanical stress. These findings highlight the potential of CRISPR/Cas9-mediated mTOR modulation as a strategy for disease intervention, longevity enhancement, metabolic regulation, and mitigating mechanical stress effects.

Keywords: mTOR signaling, CRISPR-Cas9, Myoblast differentiation, Mechanical stress, Metabolic regulation

Introduction

Skeletal muscle growth is a highly regulated process involving the proliferation, differentiation, and apoptosis of myoblasts—precursor cells capable of fusing to form mature muscle fibers. Skeletal muscle basically performs the role of a unit for movement, and various studies have proven that it is a critical regulator of complete body catabolism and anabolism.[1,2] Several conditions involving maturing, nerve disruption, misuse, and blazes reflect the dependence on skeletal muscle mass[3] and regulates disability and high threat of mortality.[4] Therefore, the stability of muscle cells has been revolutionized as a causal factor that steadily affects the characteristics of life. Considering the molecular processes governing these mechanisms is critical for inducing therapeutic techniques for muscle-related diseases, enhancing muscle regeneration, and addressing metabolic disorders. Among the key regulators of muscle physiology is the mammalian target of rapamycin (mTOR), a key component of the mTOR pathway, which incorporates signals from nutrients, growth hormones, and mechanical stimuli to modulate protein synthesis, cell growth, and metabolism.[1]

The mTOR is known as the mechanistic/mammalian target of rapamycin. Rapamycin was invented on the island of Rapa Nui (Easter Island) in 1994. Mainly, it exhibits immunosuppressive and anti-tumor properties, showing regulated growth, proliferation, and survival.

Its two pathways (mTORC1 and mTORC2) play a critical role in muscle metabolism, particularly due to the effect of mechanical stress, which is known to stimulate muscle hypertrophy and adaptation. C2C12 myoblasts, a widely used murine cell line, serve as an excellent model for studying skeletal muscle biology due to their characteristic to distinguish into myotubes under suitable conditions. C2C12 myoblasts are a commonly used cell line derived from mouse skeletal muscle, and they are widely studied to understand muscle development, differentiation, and metabolism. The mTOR signaling pathway is highly relevant in C2C12 cells because it regulates key cellular processes like proliferation, differentiation, protein synthesis, and metabolism.[5] In these cells, mTOR regulates downstream effectors such as 4E-binding protein 1 (4EBP1) and p70 ribosomal protein S6 kinase (p70S6K), which are critical for protein translation and muscle fiber formation.[2] Dysregulation of mTOR signaling has been applied in various pathological conditions, including muscle atrophy, metabolic diseases, and even mechanical stress responses.

Recent improvements in gene-editing techniques, mainly the CRISPR/Cas9 system, have highlighted the ability to precisely deregulate gene expression, proposing new contributions to dissect the fundamental roles of key signaling pathways. By employing CRISPR/Cas9-mediated knockdown of mTOR in C2C12 myoblasts, researchers can gain deeper insights into how

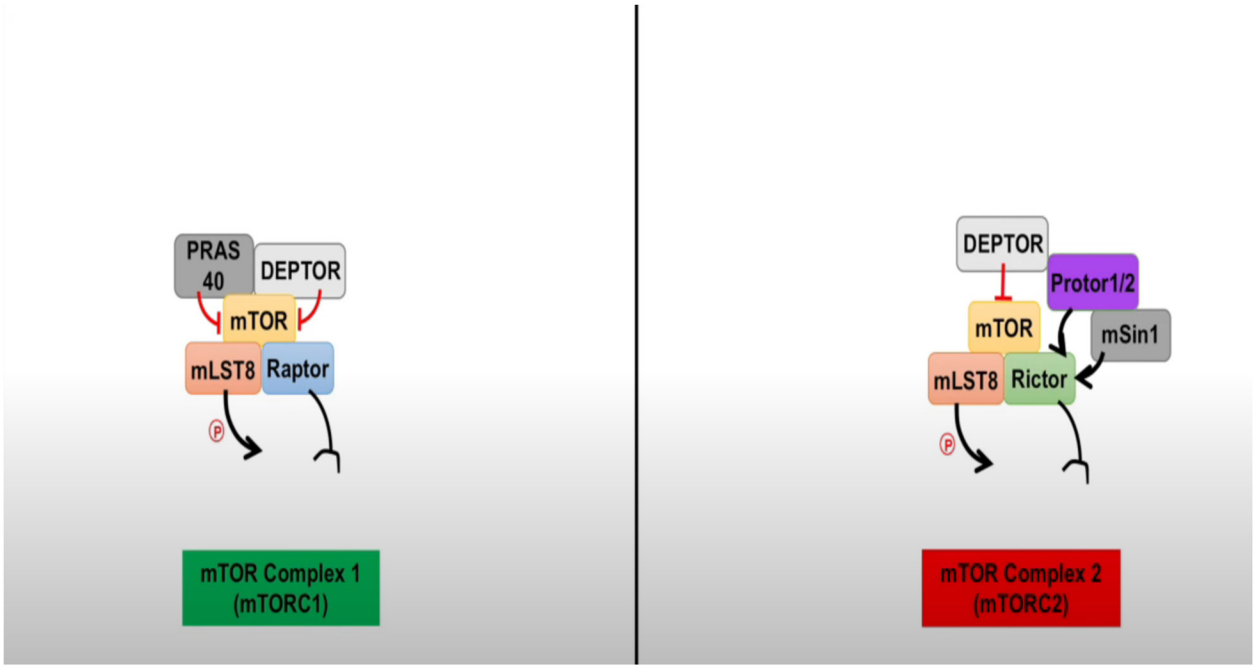

Fig 1 | Biochemical and structural presentation of mTOR C1 (on the left side) and mTOR C2 (on the right side)

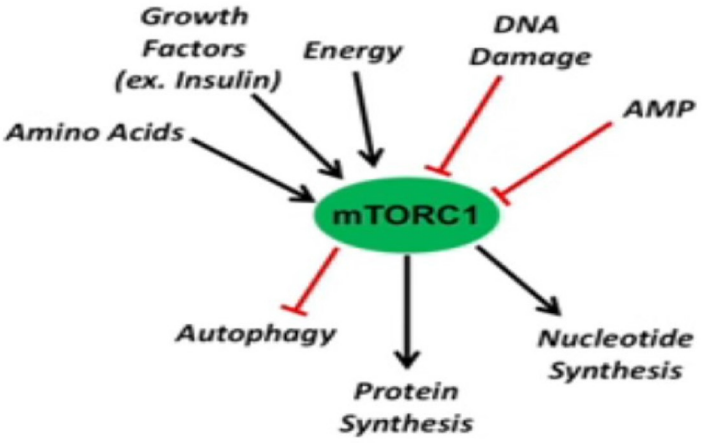

Fig 2 | Effects of mTORC1

mTOR influences muscle cell behavior under different physiological conditions, including mechanical stress.[6]

This study evaluates the role of mTOR in regulating the differentiation of C2C12 myoblasts subjected to mechanical stress.[3] Through targeted mTOR knockdown and subsequent analysis of downstream signaling components, we seek to integrate the multi-dimensional effects of mTOR modulation. The findings have potential implications not only for muscle disease intervention and metabolic regulation but also for understanding how mTOR affects mechanical stress. Bond et al.[5] proved that the mTORC1 was governed by mechanical stimuli. Nonetheless, the active character of mTOR in skeletal muscle under pressure (mechanical stress) is also not clear. We create mTOR knockdown cells to demonstrate the functional functions of mTOR in promoting myoblast differentiation and proliferation in order to unravel the mechanism.

Research Statement

How does CRISPR/Cas9-mediated mTOR knockdown affect the growth, differentiation, and mechanical stress response of C2C12 myoblasts?

The hypothesis for this study can be formulated as follows:

CRISPR/Cas9-mediated knockdown of mTOR in C2C12 myoblasts will lead to reduced proliferation, impaired differentiation, and altered response to mechanical stress due to disrupted mTOR signaling, affecting key downstream targets such as 4EBP1 and p70S6k.

This hypothesis aligns with the study's focus on how mTOR influences muscle cell behavior under mechanical stress and its potential role in muscle disease intervention and metabolic regulation.

mTOR Complexes: mTORC1 and mTORC2

Two functionally and structurally different mTOR complexes, mTORC1 and mTORC2, exist.[7] Although each structure has unique components, mTOR is the fundamental functional component of both. The DEP domain-containing mTOR-interacting protein (DEPTOR), the mammalian SEC13 protein 8 (mLST8), and the associated protein of mTOR (raptor) make up mTORC1. The rapamycin-insensitive companion of mTOR and the mammalian stress-activated map kinase-interacting protein 1 are two of the components of the second mTOR complex, mTORC2, which shares mLST8 and DEPTOR with mTORC1 (Figure 1).

By considering its genetic and therapeutical evidence and restricting the present evaluation of the molecular mechanism of mTOR in the upgradation of skeletal muscle mass, this work combines the critical role of mTOR complexes and their signaling in the functioning of skeletal muscle (Figures 2 and 3).

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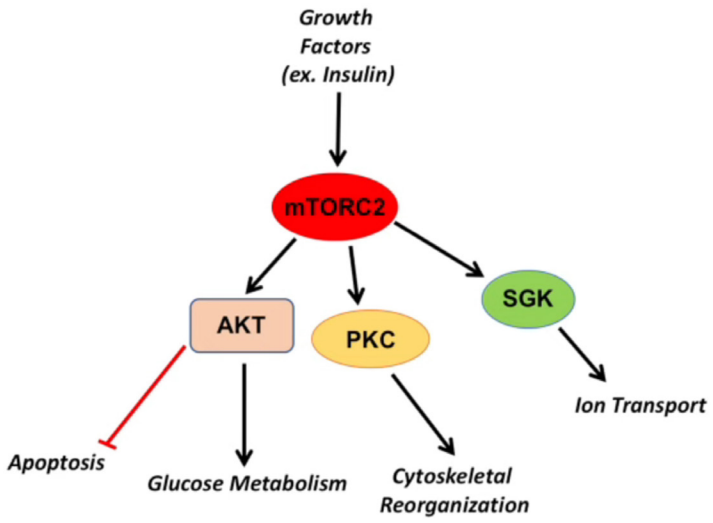

Fig 3 | Effects of mTORC2

The complex mTORC1 processes multiple signals, such as amino acid availability, energy status, growth factors, and oxygen levels. mTORC1 stimulates protein synthesis by controlling S6 kinase 1 (S6K1) and inhibiting 4EBP1.[8] In contrast, mTORC2 phosphorylates AGC kinases, glucocorticoid-regulated kinase 1, AKT, and protein kinase C control cell stability and metabolism.[9]

Materials and Methods

Cell Culture

Skeletal muscle myoblasts C2C12 were procured from an approved cell culture bank. The cells were kept in Dulbecco's Modified Eagle Medium (DMEM), which was enhanced with 90 U/ml of penicillin, 90 µg/ml of streptomycin, and 10% fetal bovine serum (FBS). To provide ideal conditions for cell development, the culture media was changed every 48 hours. To replicate physiological circumstances, cells were incubated at 37°C in a humidified environment with 5% CO_2. The cells underwent two rounds of washing in phosphate-buffered saline (pH 7.4) before being exposed to 0.25% trypsin-EDTA for three minutes at 37°C until they separated. After DMEM supplemented with 10% FBS was added to halt trypsinization, cells were centrifuged at 300 × g for 5 minutes before being resuspended in new culture media.

CRISPR/Cas9 Knockdown of mTOR Gene Expression

Guide RNA (gRNA) Design and Plasmid Construction

The National Center for Biotechnology Information (NCBI) database provided the mTOR gene sequence. Using the CRISPR Guide RNA Design Tool (Benchling), gRNA sequences that target mTOR's exonic regions were created. To assess transfection efficacy, three different gRNA sequences (mTOR-T1, mTOR-T2, and mTOR-T3) were cloned into the pSpCas9(BB)-2A-GFP (PX458) vector, which expresses both Cas9 and a GFP reporter.

Transfection and Selection of Knockdown Cells

Following the manufacturer's instructions, Lipofectamine 3000 (Thermo Fisher Scientific, USA) was used to transfect C2C12 cells at 70% confluency. Basically, Opti-MEM Reduced Serum Medium (Gibco, USA) was used to dilute 2.5 µg of the CRISPR/Cas9 plasmid, which included gRNA and Cas9. It was then incubated with Lipofectamine reagent for 15 minutes before being added to the cells. A BD FACSAria III Cell Sorter was used to sort GFP-positive cells using Fluorescence-Activated Cell Sorting (FACS) after 48 hours. Sanger sequencing and Western blot analysis verified that mTOR-T1 was the most efficient of the three gRNA variations. As a result, it was chosen for single-cell cloning in order to create a stable knockdown line.

Quantitative Polymerase Chain Reaction (Q-PCR)

RNA Extraction and cDNA Synthesis

As directed by the manufacturer, total RNA was isolated from cells using the TRIzol Reagent (Invitrogen, USA). A Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA) was used to measure the concentration and integrity of the RNA. DNase I (Promega, USA) was used to treat RNA samples in order to remove genomic DNA contamination. The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was used to create first-strand cDNA from 1 µg of total RNA using the following protocol: 25°C for 10 minutes, 42°C for 60 minutes, and 70°C for 10 minutes.

Q-PCR Protocol

The SYBR Green Master Mix (Applied Biosystems, USA) was used in a StepOnePlus Real-Time PCR System to conduct Q-PCR. Ten microliters of SYBR Green Mix, one microliter of each primer (10 µM), one microliter of cDNA template, and seven microliters of nuclease-free water were used in each reaction. The following were the cycling conditions: Initial Denaturation: 30 seconds at 94°C 35 cycles of amplification: 15 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C Final Extension: 4 minutes at 72°C Primer5 software was used to construct the primer sequences for mTOR, 4EBP1, and p70S6K, and BLAST (NCBI) was used for validation (see Table 1). Gene expression levels were normalized to GAPDH as an internal control, and each reaction was carried out twice. Relative gene expression was analyzed using the ΔΔCt technique.

Experimental Design

Cells were divided into four experimental groups:

Group A: Untreated control cells (no transfection, no stress)

Group B: Cells with mTOR knockdown (CRISPR/Cas9 transfection, no stress)

Group C: Normal cells subjected to mechanical stress

Group D: mTOR knockdown cells under mechanical stress

Mechanical Stress Application

The Flexcell Tension System FX-5000 (Flexcell International, USA) was used to apply mechanical tension to cells. In order to replicate physiological mechanical loading conditions, cells were seeded on collagen-coated

BioFlex plates and exposed to cyclic stretch (10% strain, 1 Hz frequency) for 6 hours every day for 3 days.

Table 1 | Primers for the Q-PCR

Genes		Primers
4EBP1	Forwards	5′-GATGTCCGGGGGCAGCAGCTG-3′
	Reverse	5′-AATGTCCATCTCAAACTGTGACTC-3′
p70S6K	Forwards	5′-GATGAGGCGACGAAGGAGGGG-3′
	Reverse	5′-TAGATTCATACGCAGGTGCTCTG-3′
mTOR	Forwards	5′-AAGAAGTACCCCATCGAGCAC-3′
	Reverse	5′-CATCAGAGTCAAGTGGTCATAGTCCG-3′

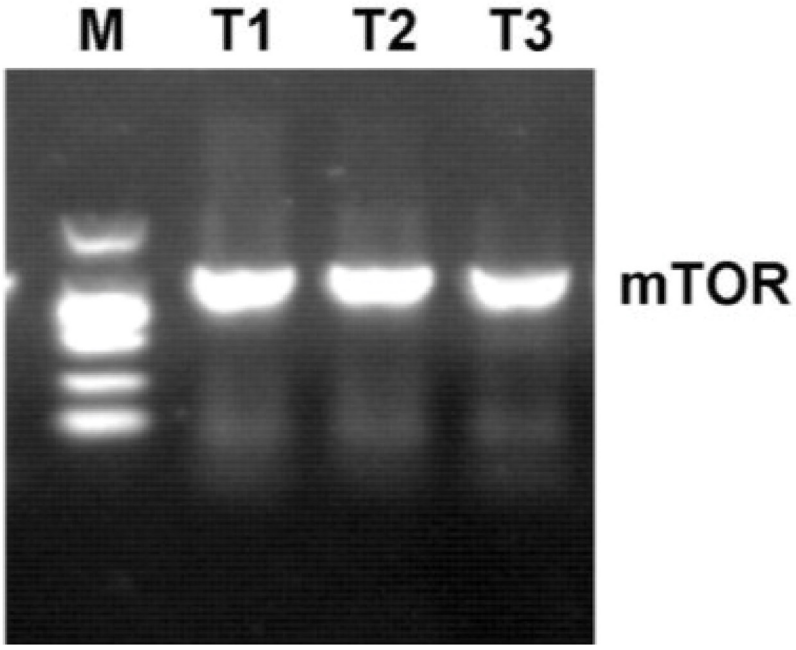

Fig 4 | Target-1 performs greater activity than T2 and T3

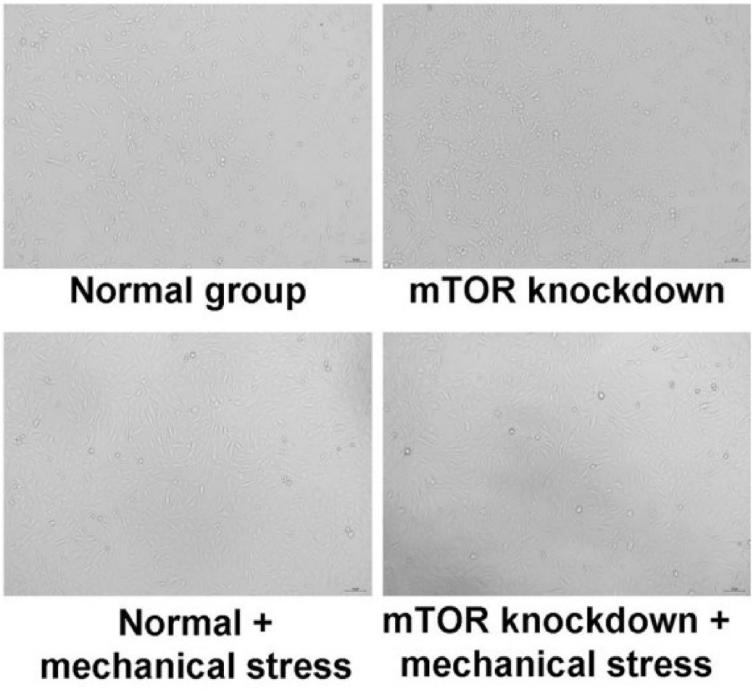

Fig 5 | The number of C2C12 cells altered by mechanical stress

Assay for Cell Proliferation

Cell Counting Kit-8 (CCK-8) test (Dojindo, Japan) was used to measure cell proliferation. After seeding 5000 cells per well in 96-well plates, the cells were treated for two hours at 37°C with 10 µl of CCK-8 reagent in each well. Utilizing a microplate reader (BioTek, USA), absorbance was determined at 450 nm.

Western Blot Analysis

Western blot analysis was used to examine the protein expression of mTOR, 4EBP1, p70S6K, and their phosphorylated variants (p-4EBP1, p-p70S6K). In short: RIPA lysis solution (Thermo Fisher, USA) containing protease and phosphatase inhibitors was used to extract the proteins. After being separated on a 10% SDS-PAGE gel, 30 µg of total protein was put onto PVDF membranes (Millipore, USA). After blocking the membranes with 5% non-fat milk in TBST, primary antibodies (1:1000 dilution) were incubated at 4°C for the whole night. Membranes were cleaned and then incubated for one hour at room temperature with HRP-conjugated secondary antibodies (1:5000 dilution). ECL substrate (Thermo Fisher, USA) was used to create the blots, and a ChemiDoc XRS+ Imaging System (Bio-Rad, USA) was used to visualize them.

Statistical Analysis

GraphPad Prism 9 (GraphPad Software, USA) was used to analyze the data. The mean ± standard deviation is used to display the results. Tukey's post hoc test was used after a one-way ANOVA for statistical comparisons. P-values less than 0.05 were regarded as statistically significant.

Results

Establishment of mTOR Knockdown Model in C2C12 Myoblasts

To target the mTOR gene in this study, three specific plasmid vectors were designed. Each vector, corresponding to a unique mTOR target site, was cloned and assessed for mRNA/DNA integrity. These vectors were labeled as mTOR-T1, mTOR-T2, and mTOR-T3. While all three constructs exhibited over 50% relative activity compared to the positive control, px458-mTOR-T1 demonstrated the highest efficiency in inducing target mutagenesis in vitro. The transfection was performed using endotoxin-free protocols in cultured cells.

For further selection, CRISPR/Cas9, along with the px458-mTOR-T1 plasmid was introduced into the cells, and positive transfection, was confirmed using flow cytometry. The activity and quality of each plasmid were then evaluated through PCR analysis (Figure 4). Results indicated that the gRNAs for T2 and T3 showed lower activity relative to T1, as depicted in Figure 1. Consequently, target-1 was selected for knockdown, and monoclonal cell lines were cultured. Following colony formation, PCR amplification of the mTOR target-1 region was conducted, and the results were validated through sequencing and sequence alignment.

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Effect of Mechanical Stress on C2C12 Myoblast Cell Proliferation and Regulation

Mechanical stress influenced the proliferation of C2C12 cells, with noticeable differences observed under the microscope. The mTOR knockdown groups exhibited a reduced cell count compared to the control groups. However, when subjected to mechanical stress, this trend was reversed, and the cell numbers in the mTOR knockdown levels increased. Additionally, the mechanical stress caused the cell units to align in the direction of the applied force.[9]

Mechanical Stress Counteracts mTOR Knockdown-Induced Reduction in C2C12 Cell Numbers

In murine C2C12 myoblasts, we initially observed elevated mTOR expression levels in cells exposed to mechanical stress compared to unstressed controls (Figure 5). Upon mTOR knockdown, cell numbers declined as anticipated. Interestingly, when these knockdown cells were subjected to mechanical stress, their proliferation increased significantly. Microscopic examination revealed that mechanical stress also influenced cell orientation, causing them to align along the force vector (Figure 5). These results proposed that mechanical stress may perform a compensatory character in modulating cellular responses following mTOR knockdown.

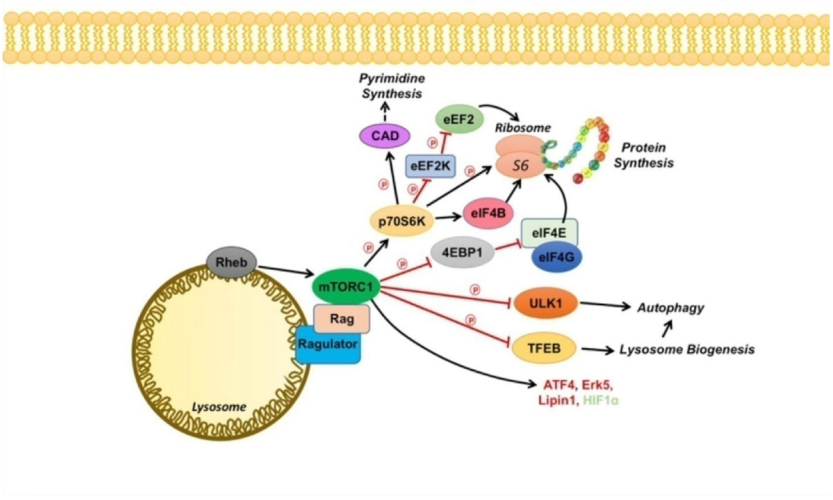

Fig 6 | mTOR signaling alters muscle protein synthesis

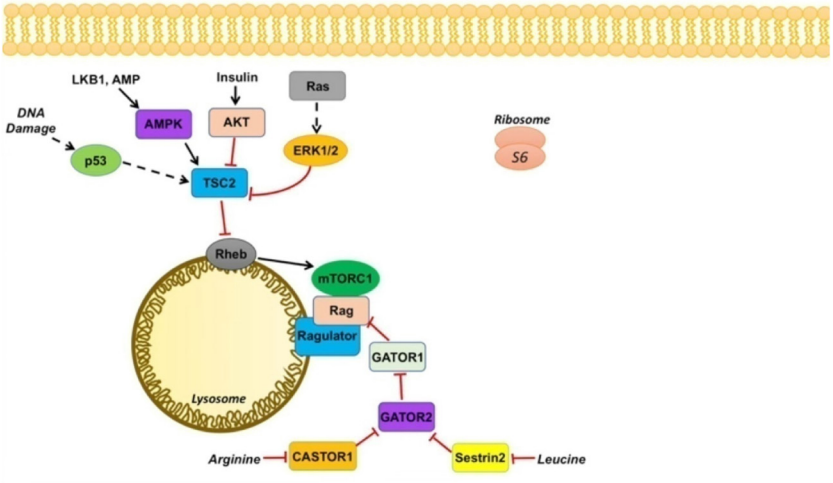

Fig 7 | Regulation of mTOR pathway

mTOR as a Positive Regulator in the PI3K/mTOR Pathway Under Mechanical Stress

Microscopic analysis revealed that mechanical-stress-enhanced cell proliferation in mTOR knockdown groups compared to unstressed conditions. To explore this further, we utilized CRISPR/Cas9 to effectively knock down mTOR in myoblasts. Since p70S6K and 4EBP1 are significant downstream regulators in the PI3K/mTOR pathway, we examined their expression to assess pathway involvement under mechanical stress. RT-PCR analysis revealed that mTOR knockdown significantly reduced 4EBP1 mRNA levels compared to controls. However, when mechanical stress was applied, there was an upregulation of 4EBP1 expression in the mTOR knockdown groups, indicating that mechanical stress can partially restore pathway activity (Figures 4 and 6).

mTOR's Role in IGF1-Dependent Pathway of Muscle Cells

Various studies reveal that IGF1 is a crucial factor for skeletal muscle development and resurgence,[10–15] as well as a well-known upstream activator of mTOR in skeletal muscle. IGF1 gets links to the IGF1 receptor (IGFR) and subsequently activates insulin receptor substrate-1 (IRS-1). The role of the IRS-1 pathway in skeletal muscle is ambiguous.[16] Rather, the Akt/mTOR signaling pathway by IGF1 proves to be crucial in activating muscle hypertrophy.[1] Akt phosphorylates TSC1/2, which blocks the GTPase-activating protein (GAP) activity of TSC1/2 toward small G protein Rheb. Then, GTP-bound Rheb activates mTORC1, resulting in phosphorylation of S6K1and 4EBP1, which stimulate the production of proteins by releasing the translation initiation factor eIF-4E and activating the ribosomal protein S6, respectively. In tissue culture, IGF1 causes skeletal myofiber hypertrophy in accordance with IGF1-Akt-mTORC1 regulation.[11] Transgenic mice with muscle-specific IGF1 expression exhibit at least a twofold increase in muscular growth,[12,13] indicating that muscle hypertrophy depends on the IGF1/Akt/mTORC1 pathway. Furthermore, GSK β1 is phosphorylated and deactivated by Akt, which then inhibits the eukaryotic translation initiation factor 2B (eIF2B) in a GSK β1-dependent manner to control muscle mass (Figure 7).[17,18]

Despite this fact, a current study revealed that IGF1 and its receptors were not crucial to the activation of mTOR in mechanical signaling.[19] The utterance of dominance negative (DN)-IGFR-I, mainly in skeletal muscles, produced myocyte hypertrophy with the help of an active overload model produced by interactive excision.[19] Interestingly, DN-IGFR-I showing muscle showed an equivalent amount of expression of Akt

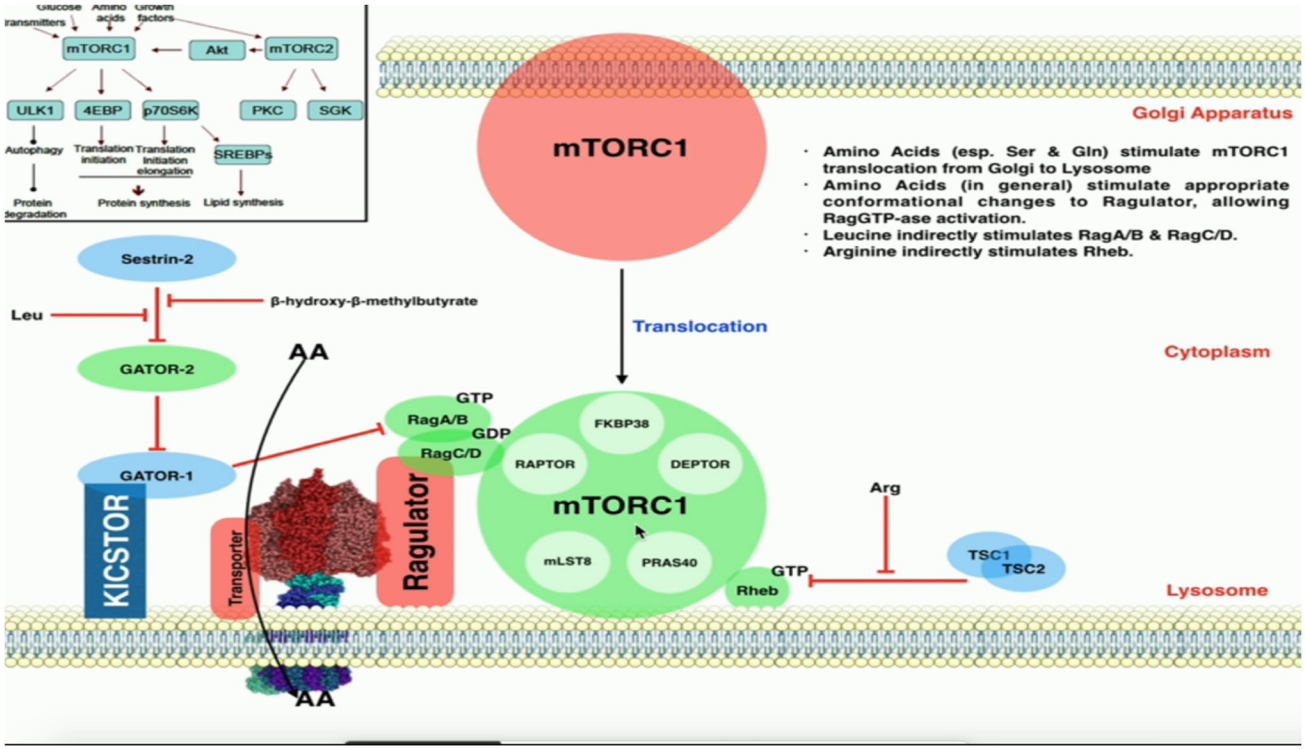

Fig 8 | The role of mTORC1 in amino acid stimulation

and p70S6K1. These findings indicate that an unidentified upstream mediator besides IGFR may activate Akt/mTOR signaling in skeletal muscle hypertrophy (Figure 8).

The Role of mTOR in IGF1-Independent Pathway of Skeletal Muscle; Phosphatidic Acid (PA)-Induced mTOR Activation in Mechanical Stimulus

IGFR-independent mTOR's one of the strongest activators in muscle cells is PA. IGF1-involved involuntary stress enhances PAs, as revealed by Hornberger et al., governed through mTOR regulation.[4] PA is strictly linked to the FKBP12 unit in relation to rapamycin and regulates signaling in mTOR.[20] PA is produced through a number of pathways: by phosphatidylcholine by phospholipase D (PLD) and through diacylglycerol (DAG) by the use of kinases.[21,22] Through the different enzymes used in PA synthesis, PLD function was accelerated by mechanical strain and accompanied by mTOR regulation.[23] Moreover, treating with 1-butanol, a PLD inhibitor, prevented the rise in mTOR functioning and strengthened the character of PLD in mechanical stains.[23] Nevertheless, the PA number used to retain a high number after the regulated PLD activation went back to the primary level 15–16 minutes after stretch using [3H] arachidonic acid, indicating that other enzymes synthesized PA through mechanical stretch. Hornberger et al. revealed that DGK synthesized PA with the help of mechanical activation, which is governed by mTOR activation.[24] Mechanical indication does not induce PLD activation under the influence of [3H] myristic acid, indicating that labels PC and a PLD inhibitor did not block mechanical activation. Besides, both DAG and DGK outer layer activity, crucial for mTOR regulation and activation, has been enhanced through mechanical stimulation. However, the past data reported that PLD1-produced Pas displace DEPTOR, governing the activation of mTORC1.[22] Therefore, interrogation into whether PA synthesized by DGK during muscle extension combines with the FRB or regulates mTOR with the help of an FRB-independent process is justified.

The Function of mTOR in the Skeletal Muscle IGF1-Independent Pathway, the Process by Which Mechanical Stimulus Activates mTORC1

It is being reported that mTORC1 moves to the lysosome by the activation of the Rag regulator in amino acid signaling.[25,26] Lysosomal migration of mTOR does not regulate mTOR specifically; instead, it gives a close location to Rheb, a crucial activator of mTOR.[27] mTORC1 is regulated by straight linkage with the GTP-linked form of Rheb,[25,26] which is activated by the TSC complex TSC1, TSC2, and Tre2-Bub2-Cdc16-1 domain family member 7 (TBC1D7),[28] a GAP of Rheb.[29] The occurrence of Rheb, shown to be on the lysosomal units, was not altered by either insulin or amino acid. In spite of that, the TSC regulates the GTPase functioning of Rheb on the outer 30 layers of the lysosome and moves to the lysosome, at least half by its linkedness with Rheb-GDP when the growth components are not present. Insulin regulates Akt, which gradually activates the TSC complex by phosphorylation, forming the breakout of the TSC complex from Rheb before the mTORC1 activation.[30]

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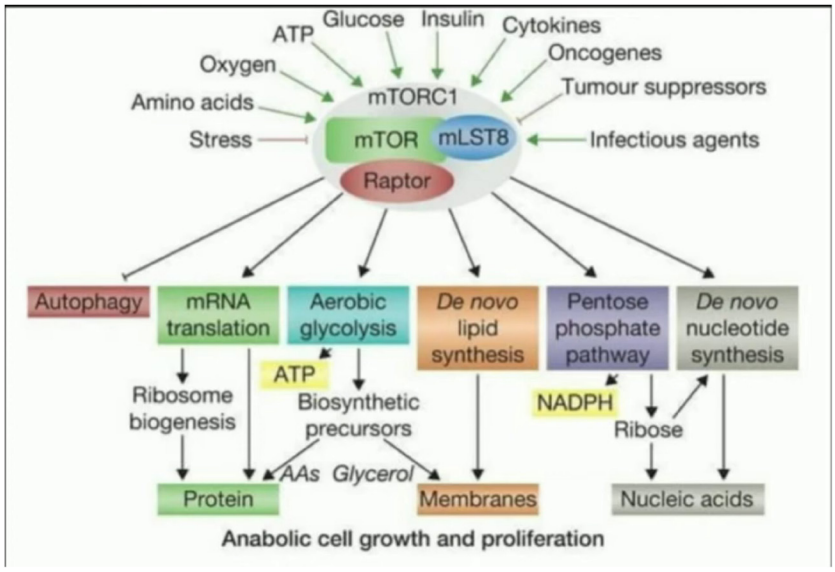

Fig 9 | Summary of overall mTOR effects

Significance of mTOR Pathway

mTOR is a significant serine/threonine protein kinase that manages homeostasis in cells and organisms by processing anabolic and catabolic processes in response to nutrient, energy, and oxygen availability and growth factor signaling.

Cells and living creatures encounter various stresses that disrupt mTOR-governed homeostatic systems. Stress can arise from imbalances in upstream signals or genetic disruptions in upstream pathway effectors, influencing mTOR's role.

mTOR forms two large protein complexes that are crucial in buffering cells from stress and are linked to stress-related conditions such as aging, cancer, and diabetes (Figure 9).

Discussion

mTOR signaling pathway is considered a long-established regulation pathway in various parts of the body that signals life functions. mTOR, as a basic unit, alters cell development, cell proliferation, movement, survival, protein formation, autophagy, and RNA formation.[31,32] The mTOR signaling plays a crucial part in the active cells, such as muscle cells. Moreover, mTORC1 functioning is needed for muscle protein formation and skeletal muscle hyperplasia in the human body in correspondence to the physical workout.[33,34] Meanwhile, the blockage of mTOR resists the skeletal mass, starts to decrease muscle volume and stability during muscle breakout in old age, and muscle shrivels from physical indolence.[35]

Previous studies revealed that the mechanical stress regulates mTOR signaling in muscle cells. In addition, mTOR is reported as a crucial unit for the PI3K pathway, being the most effective regulation pathway for the muscle cells. Hence, we thought that mTOR is concerned with the activation of skeletal muscle regulation and is involved in the disparity and cell death following mechanical stress. To validate our considerations, we applied the CRISPR/Cas9 system to make up the knockdown mTOR system. We analyzed that mTOR knockdown lessens the C2C12 cells, which implies that mTOR accelerates the myoblast growth and mechanical stress activation of myoblasts by connecting the mTOR signaling.[36] To sum it up, our study suggests that mTOR has crucial consequences on regulating myoblast proliferation, and mechanical stress may be involved in this response.

As the downstream regulators of mTOR signaling pathway, 4EBP1 and p70S6k were considered positively in mTOR signaling pathway. Our findings depicted that the above-mentioned proteins declined followed by the mTOR knockdown, which emphasizes that the p70S6k and 4EBP1 can increase the growth of myoblast acquired from activated mTOR protein. Certainly, we have seen that in C2C12 cells, levels of these two proteins start to increase when cells are prone to stress. Hence, mTOR performs vital roles in PI3K signaling in C2C12 myoblasts due to mechanical stress. Microscopic analysis revealed that mechanical-stress-enhanced cell proliferation in mTOR knockdown groups compared to unstressed conditions. To explore this further, we utilized CRISPR/Cas9 to effectively knock down mTOR in myoblasts.[37]

Many recent researches described that the mTOR signaling pathway was overactive, hence lessening the apoptosis and giving permission for growth of cancer. This signaling is important to accelerate the proliferation of mature stem cells.[38] In addition, it is not easy to find a suitable figure to equalize the growth, and scientists are working to use several techniques to combat dysfunction.[38] This signaling regulated the PI3K/Akt pathway, which was utilized to enhance proliferation. Our data on mTOR might possess a negative control on muscle cell apoptosis. In fact, down-regulation of mTOR might accelerate apoptosis and is crucial for blocking proliferation. Even if the cells are served with stress, mTOR knockdown turns out to be a considerably low apoptosis rate. These findings strengthen the results and gather these findings. With respect to our study, mTOR knockdown lessened the myoblast growth and decreased the proliferation rate. Moreover, mechanical stress increased the growth rate and accelerated the mTOR knockdown proliferation. Hence, preventing the mTOR in the PI3K pathway demonstrated a downstream impact on the muscle cell growth rate, and the mechanical stress increased the proliferation rate.

Limitations

A number of limitations should be taken into account, even if our work offers valuable insights into mTOR signaling and its function in myoblast proliferation under mechanical stress. The results may not be entirely applicable to muscle physiology in vivo because the study was carried out in vitro using C2C12 myoblasts. Second, although we examined important downstream regulators like p70S6k and 4EBP1, we did not investigate other elements that affect mTOR signaling, such as the AMPK and FoxO pathways. Third, our study's mechanical stress settings might not accurately represent physiological muscle loading. A more thorough knowledge of mTOR's function in muscle metabolism

and stress responses might be possible with future research that includes animal models and other signaling pathways.

Conclusions and Perspectives

In conclusion, our study supported our results and gave more details to consider and demonstrate our findings. At the same time, we examined the mTOR knockdown differentiation to understand mTOR biological activation. In addition, we utilized immunofluorescence to analyze our considerations, and the findings revealed that the mTOR knockdown cells crossed low limits and mechanical-stress-enhanced differentiation in C2C12 cells. As declared by our figures, mTOR knockdown resulted in low levels of muscle cell differentiation, pointing out that the mTOR may participate in increased muscle cell differentiation. Delving deeper into mTOR signaling has led to useful strategies for inducing muscle atrophy and hypertrophy, as current studies highlight mTOR as a main activator of muscle protein synthesis.

The role of mTOR in the activation of myoblasts in C2C12 units under the influence of mechanical stress is important, even though it leaves some gaps. Our research showed that mTOR lowered myoblasts' growth and proliferation and prevented PI3K signaling by down-regulating the p70S6K and 4EBP1 units using CRISPR/Cas9. In addition, mechanical stress speeded up the PI3K signaling pathway and remained intact in mTOR knockdown cell proliferation. It might be due to the fact that mTOR was found in myoblast differentiation but not as a crucial unit for inducing differentiation. In conclusion, our research exhibited the role of mTOR in activating myoblast cell proliferation, apoptosis, and differentiation. These findings worked for us to reach the point of how mTOR regulates and activates skeletal muscle re-synthesis.

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