Journal of Integrative Agriculture 2026, 25(3): 1125–1136

Available online at ScienceDirect

Journal of Integrative Agriculture



RESEARCH ARTICLE

Myostatin promotes proliferation of bovine muscle satellite cells through activating TRPC4/Ca^{2+}/calcineurin/NFATc3 pathway

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Highlights

- Ca^{2+} content in muscle tissues and muscle satellite cells of MSTN mutated cattle was detected.
- Ca^{2+} signaling pathway was used to explain the phenomenon that MSTN mutation activates cell proliferation and induces animal muscle overgrowth.
- The molecular mechanism by which MSTN mutation activates the TRPC4/Ca^{2+}/calcineurin/NFATc3 pathway to promote muscle development was revealed.

Abstract

Myostatin (MSTN) is principally expressed in skeletal muscle and negatively regulates muscle growth and development. MSTN mutation can induce muscle overgrowth in cattle by activating cell proliferation, presenting a “double-muscle” phenotype. However, the specific regulatory mechanism is still unclear. Here, we found that Ca^{2+} content in muscle tissue and muscle satellite cells of MSTN mutated ($MSTN^{-/-}$) cattle were significantly increased compared to wild-type (WT). Furthermore, transcriptome analysis of muscle satellite cells revealed that TRPC4 was significantly increased in $MSTN^{-/-}$ cattle. And the expression of TRPC4 in muscle tissue of $MSTN^{-/-}$ cattle was detected by RT-qPCR and Western blot, which was significantly higher than that of WT. These results suggested that MSTN mutation promoted muscle satellite cells proliferation through activation of TRPC4 channel. To further verify, ML204, a specific inhibitor of TRPC4, was used to treat $MSTN^{-/-}$ muscle satellite cells. We found that cell proliferation was inhibited, calcineurin expression was downregulated, and the entry of NFATc3 into nuclei was reduced, which was similar to WT group. Thus, MSTN mutation leads to the activation of TRPC4 channel, which increases intracellular Ca^{2+} content, further activates calcineurin/NFATc3 pathway, and ultimately promotes the proliferation of muscle satellite cells.

Keywords: myostatin, cell proliferation, Ca^{2+}, TRPC4, NFATc3

1. Introduction

Myostatin (MSTN), a member of TGF-beta (transforming growth factor beta) family involved in a variety of metabolic processes (McPherron and Lee 1997), which is mainly expressed in skeletal muscle (Kollias and Mcdermott 2008). MSTN negatively regulates skeletal muscle growth (Liu *et al.* 2020; Lee 2021; Wang *et al.* 2021; Zhang G *et al.* 2021; Han *et al.* 2022). The effect of MSTN on the number of muscle fibers is due to its participation in the proliferation and differentiation of myoblasts during muscle development (Thomas *et al.* 2000), while the effect on the size of muscle fibers is regulated by muscle satellite cells (Figeac *et al.* 2007). It is precisely because of the negative regulatory

Received 31 October 2023; Received in revised form 5 March 2024; Accepted 13 March 2024; Available online 13 April 2024
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doi: 10.1016/j.jia.2024.04.020

effect of MSTN on muscle growth (McPherron and Lee 1997).

Calcium ion (Ca^{2+}) is a kind of important intracellular second messenger and participates in the regulation of biological processes such as cell growth, proliferation, differentiation, and apoptosis (Puri 2020; Matikainen *et al.* 2021; Sukumaran *et al.* 2021). A typical transient receptor potential canonical (TRPC) channel family consists of seven members (TRPC1-TRPC7) (Nelson *et al.* 2011), which transport Ca^{2+} through the formation of homologous or heterologous tetramers (Hofmann *et al.* 2002). TRPC channels regulate intracellular calcium levels, participate in regulating calcium homeostasis, and play an important role in cell proliferation (Yu *et al.* 2004; Zhan and Shi 2017; Farfariello *et al.* 2022). Activation of TRPC channels leads to a continuous increase in intracellular Ca^{2+} levels, which in turn activates calcineurin and promotes dephosphorylation of NFAT into the nucleus (Li *et al.* 2016; Han *et al.* 2019).

Calcineurin, a Ca^{2+}/calmodulin-dependent serine/threonine phosphatase, is a downstream target of intracellular calcium (Rusnak and Mertz 2000) and plays a central role in calcium-dependent signaling pathways (Li *et al.* 2011; Fu *et al.* 2018; Hendrikx *et al.* 2019). The most characteristic substrate of calcineurin is the nuclear factor of activated T cells transcription factor family (NFATc1-c4) activated by intracytoplasmic calcium elevation dynamically (Masaki and Shimada 2022). In the nuclei, NFAT binds to DNA motifs which activate transcription to regulate the expression of transcription factors, signaling proteins, cytokines, and cell surface receptors (Rao *et al.* 1997; Horsley and Pavlath 2002; Mojsa *et al.* 2015; Park *et al.* 2020; Ren *et al.* 2021).

Under natural or artificial conditions, MSTN gene mutation can lead to a significant increase in muscle mass and induce double-muscle phenotype (McPherron and Lee *et al.* 1997). MSTN and its effect on meat yield have been studied extensively in the field of animal breeding. Understanding the molecular mechanism of MSTN has great application potential in animal breeding. This study explored the molecular mechanism of MSTN deletion mutation promoting proliferation of muscle satellite cells from the perspective of Ca^{2+} channels and their regulatory pathways.

2. Materials and methods

2.1. Animals

The wild type cattle (WT) and MSTN gene-edited cattle ($MSTN^{-/-}$) used in our study were Luxi cattle bred by our laboratory and raised in the experimental base of Hohhot (Zhao *et al.* 2022). WT cattle (4 male) and $MSTN^{-/-}$ cattle (6 male) aged 24–26 mon were selected for routinely slaughtered. Skeletal muscle was collected for tissue sectioning and molecular biology studies. Semitendinosus was collected for primary muscle satellite cell culture.

2.2. Real-time PCR

Total RNA was extracted from skeletal muscle and muscle satellite cells using RNAiso Plus Kit (TaKaRa, Japan). cDNA was synthesized by using a PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Japan). Roche LightCycler 480 real-time PCR (Roche, Switzerland) and ChamQ SYRB qPCR Master Mix (Vazyme, China) were used to amplify the cDNA. Primer sequences were listed in Appendix A. PCR amplification was performed as follows: one cycle at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 60°C for 34 s. To ensure normalization, housekeeping gene *GAPDH* was used as an internal reference, and fold-changes in differential gene expression were determined using comparative threshold cycle ($2^{-\Delta\Delta C_T}$) method.

2.3. Western blot

Total proteins were extracted from skeletal muscle and muscle satellite cells of the cattle and lysed in precooled radio immunoprecipitation (RIPA) buffer containing protease inhibitors. After maintaining on ice for 30 min, the lysis solution was centrifuged at 12,000×g for 5 min at 4°C, and the supernatant was taken for western blotting. Proteins were separated on 10% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membranes by electric blotting. Sealed with 5% skim milk containing Tris-buffered saline with 0.1% Tween-20 (TBST) at room temperature for 1 h. And then, the membranes were incubated with the primary antibodies in TBST containing 0.5% skim milk at 4°C overnight. The primary antibodies are as following: anti-MSTN (Abcam, USA, ab201954), anti-TRPC4 (Alomone labs, Israe, ACC-018), anti-calcineurin A (Abcam, USA, ab282104), anti-calcineurin B (Abcam, USA, ab342082), anti-NFATc3 (Proteintech, USA, 18222-1-AP), anti-α-tubulin (Proteintech, USA, 66031-1-Ig), anti-GAPDH (Proteintech, USA, 60004-1-Ig), and anti-lamin B1 (Proteintech, USA, 12987-1-AP). According to the resistance of primary antibody, goat anti-mouse (Proteintech, USA, SA00001-1) or goat anti-rabbit secondary antibodies (Proteintech, USA, SA00001-2) conjugated with horseradish peroxidase were used to incubate the membranes at room temperature for 1 h respectively, followed by detection using chemiluminescent labeling reagent ECL Plus (Thermo, USA). Quantification analysis of blots was performed with the ImageJ software (1.52a).

2.4. Cell culture and treatment

Muscle satellite cells were isolated and cultured from bovine semitendinosus muscle in this study. Cells were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F-12; Gibco, USA) containing 10% inactivated fetal bovine serum (FBS; Gibco, USA) at 37°C in a humidified incubator with 5% CO_2. The cells were cultured to the 3rd generation for cells experiments. After 4 hours of cultivation, the bovine muscle satellite cells were seeded and treated with 1.0 μmo L^{-1} ML204 hydrochloride (MCE, USA) for 24 h to determine the effect of ML204 or TRPC4 on cell proliferation.

2.5. Immunofluorescence

Muscle satellite cells were cultured on cell slides to 80% confluence. The cells were fixed with 4% paraformaldehyde in PBS (pH=7.4), permeabilized by incubating in PBS containing 0.1% Triton X-100, and then blocked with 1% bovine serum albumin (BSA). After the following primary antibodies: anti-Calreticulin (Abcam, USA, ab92516), anti-

TRPC4 (Alomone, Israel, ACC-018) and anti-NFATc3 (Proteintech, USA, 18222-1-AP) were incubated overnight at 4°C, the appropriate secondary antibody (Abcam, USA, ab150081) was incubated at room temperature for 30 min. All cells were restained with DAPI (Thermo, USA). Specific fluorescence was imaged by laser-scanning confocal microscopy (Nikon, Japan).

2.6. Cellular proliferation assays

Two methods were used to detect muscle satellite cells proliferation: Live cell count using trypan blue exclusion and EdU (5-ethynyl-2′-deoxyuridine) fluorescent labeling assay. For the trypan blue exclusion method, cells of the same generation were seeded into 24-well culture plates at a same density. Cells were stained with trypan blue and live cells were counted using a hemocytometer every 12 h. Three wells for each treatment group were counted. For EdU assay, cells were seeded in 24-well plates at a same density. The cultured cells were EdU labeled, fixed, washed and permeated according to the instructions of *BeyoClick*™ EdU Cell Proliferation Kit with Alexa Fluor 488 (Beyotime, China). After click reaction, Hoechst 33342 stained the nuclei. EdU detection was performed using a laser-scanning confocal microscopy (Nikon, Japan).

2.7. Inductively coupled plasma-mass spectrometry (ICP-MS)

The gastrocnemius, semitendinosus, longissimus, soleus, and myocardium of WT cattle and MSTN$^{-/-}$ cattle were processed using microwave digestion. The obtained samples were filtered through a 0.45 μm water-based MCE (NAVIGATOR, China) and stored at 4°C. Inductively coupled plasma-mass spectrometry (ICP-MS) (AnalytikJENA, Germany) was used to analyze Ca^{2+} standard samples and test samples separately, to plot the standard curve and detect the Ca^{2+} concentration in different muscle tissues of WT cattle and MSTN$^{-/-}$ cattle.

2.8. Transcriptomic analysis

RNA-Seq and analysis of muscle satellite cells from WT and MSTN$^{-/-}$ group were completed by Lianchuan Biotechnology Co., Ltd. The mapped fragments were normalized using the FPKM method. DEGs between WT and MSTN$^{-/-}$ cattle were identified by the DEG-seq software package applying the MA-plot-based method with random sampling (MARS) model methods. *P*-value<0.05 and absolute value of fold change>2 was considered to have significant expression abundance. All DEGs were mapped to terms in KEGG database. The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA012945) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa.

2.9. Statistical analysis

All data were expressed as means±SE (*n*=3). The significance of difference was analyzed by student's paired *t*-test (two-group comparison) or one-way ANOVA (multiple group comparison). *P*-value<0.05 was considered as statistically significant. And plot the histogram with GraphPad Prism 8.0.

3. Results

3.1. MSTN mutation promotes muscle satellite cells proliferation

MSTN is a key factor in regulating the development of skeletal muscle (Chen *et al.* 2021). The fusion of activated muscle satellite cells with existing muscle fibers is the main cause of muscle growth after birth, and the inhibition of muscle fiber thickening by MSTN is partly due to the inhibition of activation, proliferation and renewal of muscle satellite cells (Figeac *et al.* 2007). The MSTN gene-edited cattle (MSTN$^{-/-}$) bred in our laboratory presented a "double-muscle" phenotype is caused by the deletion mutation of MSTN gene (Appendix B). Similar to WT, after isolation and culture of MSTN$^{-/-}$ bovine muscle satellite cells, it was observed that the cells were spindle shape (Fig. 1-A). MSTN expression was detected at mRNA and protein levels in muscle satellite cells, indicating that MSTN mutation resulted in a significant decrease in MSTN expression (Fig. 1-B–D). The MSTN$^{-/-}$ group cell proliferation and DNA synthesis were significantly increased, which were detected by live cell count and EdU assay (Fig. 1-E–G). We detected the expression levels of cell cycle-related genes in bovine muscle satellite cells, including Cyclin-dependent kinases (CDKs; CDK1, CDK2, CDK4, CDK6), cyclins (Cyclin A2, Cyclin D2, Cyclin E2), and Cyclin-dependent kinase inhibitors (CKIs; CDKN1A, CDKN1B, CDKN1C, CDKN2A, CDKN2B, CDKN2C, CDKN2D). The expression level of cell cycle-related genes showed that CDKs and Cyclins were up-regulated and CKIs were down-regulated in MSTN$^{-/-}$ group (Fig. 1-H and I), which promoted cell cycle and made cells enter S phase. These results all suggested that MSTN mutation enhanced cell proliferation.

3.2. MSTN mutation increases the Ca^{2+} content

Ca^{2+} is an important intracellular second messenger, which is involved in the regulation of cell growth, survival, proliferation, differentiation, and apoptosis (Matikainen *et al.* 2021). Ca^{2+} and calcium signaling play an important role in development, growth, regeneration and function of skeletal muscle. The Ca^{2+} content in muscle tissue and muscle satellite cells of WT and MSTN$^{-/-}$ cattle were detected by ICP-MS and Calreticulin specific immunofluorescence respectively (Fig. 2). The results showed that the Ca^{2+} content in MSTN$^{-/-}$ cattle was significantly increased.

3.3. MSTN mutation leads to changes in calcium signaling pathway

In order to explore the molecular mechanism of MSTN mutation enhancing cell proliferation, transcriptomic analysis was performed on muscle satellite cells in WT and MSTN$^{-/-}$ groups to screen for differentially expressed genes (DEGs). After statistical analyses and filtering, there were 2864 differentially expressed genes (FC>2, *P*<0.05) (Fig. 3-A; Appendix C-a). Among them, 1,313 were up-regulated and

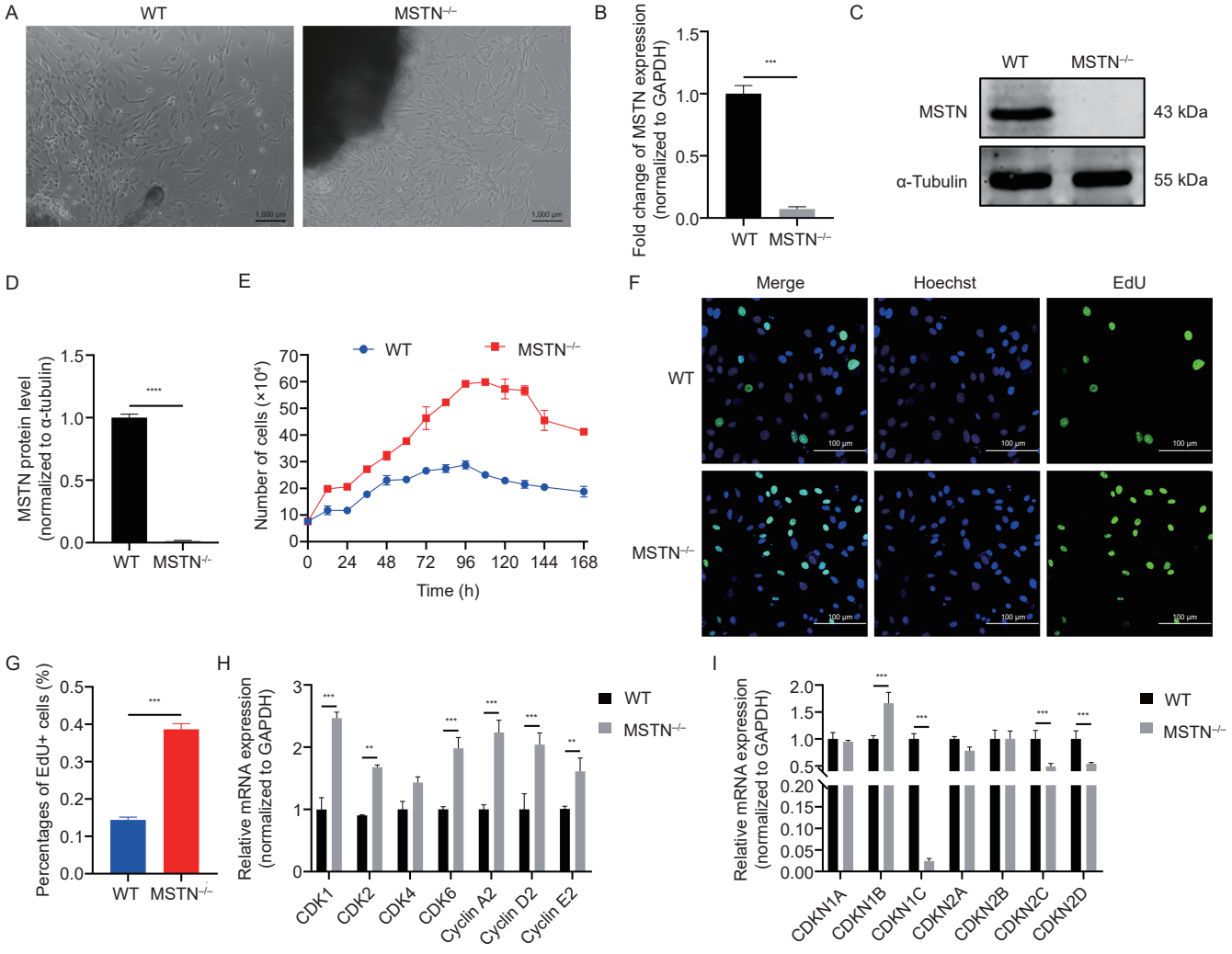


Fig. 1 MSTN (myostatin) mutation promotes muscle satellite cells proliferation. A, the primary bovine muscle satellite cells were isolated and cultured, and cells were spindle-shaped, and there was no apparent morphological change compared with WT. B–D, the mRNA and protein levels of MSTN were significantly decreased in muscle satellite cells. E–G, live cell count and EdU showed significantly increased cell proliferation and accelerated DNA synthesis in the MSTN$^{-/-}$ (MSTN mutated) group (green, EdU+; blue, nuclei). H–I, the expression of cell cycle-related genes showed that the expression of CDKs and Cyclins were up-regulated in MSTN$^{-/-}$ group, while CKIs was down-regulated, thus promoting cell cycle. CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor. Data are mean±SEM. *t*-test was used to generate *P*-value. Compared with WT group, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, ****, $P<0.0001$.

1,551 were down-regulated in MSTN$^{-/-}$ group (Appendix C-b). KEGG analysis of the DEGs revealed that the enrichment occurred in calcium signaling pathway and others (Fig. 3-B). DEGs in calcium signaling pathway were mainly related to Ca^{2+} transport and regulation and were involved in calcium homeostasis of cells. Among the 31 genes with significant differences, 21 genes were up-regulated and 10 genes were down-regulated (Fig. 3-C).

There are thousands of channels on the surface of each cell that precisely control the flow of Ca^{2+} in and out. TRPC channels regulate intracellular Ca^{2+} levels, participate in the regulation of calcium homeostasis (Yu *et al.* 2004), and play an important role in cell proliferation (Zhan and Shi *et al.* 2017). Analysis of differentially expressed genes of calcium channels on cell membrane showed that TRPC4 is the only up-regulated gene in TRPC family in MSTN$^{-/-}$ muscle satellite cells (Fig. 3-D). The expression of TRPC4 in skeletal muscle and muscle satellite cells were detected by RT-qPCR and Western blot, and the expression level of MSTN$^{-/-}$ cattle was significantly increased (Fig. 3-E–G and Appendix C-e–g). It was preliminarily concluded that MSTN mutation increased intracellular Ca^{2+} content, of which TRPC4 was the key calcium channel.

3.4. MSTN mutation activates TRPC4 channel to promote cell proliferation

To further verify whether MSTN mutation regulates cell proliferation by affecting calcium signaling pathway, ML204, a TRPC4 specific inhibitor, was used to treat MSTN$^{-/-}$ muscle satellite cells to detect the effect of TRPC4 on cell proliferation. The minimum inhibitory concentration of ML204 on proliferation of MSTN$^{-/-}$ muscle satellite cells was 1.0 μmo L^{-1}

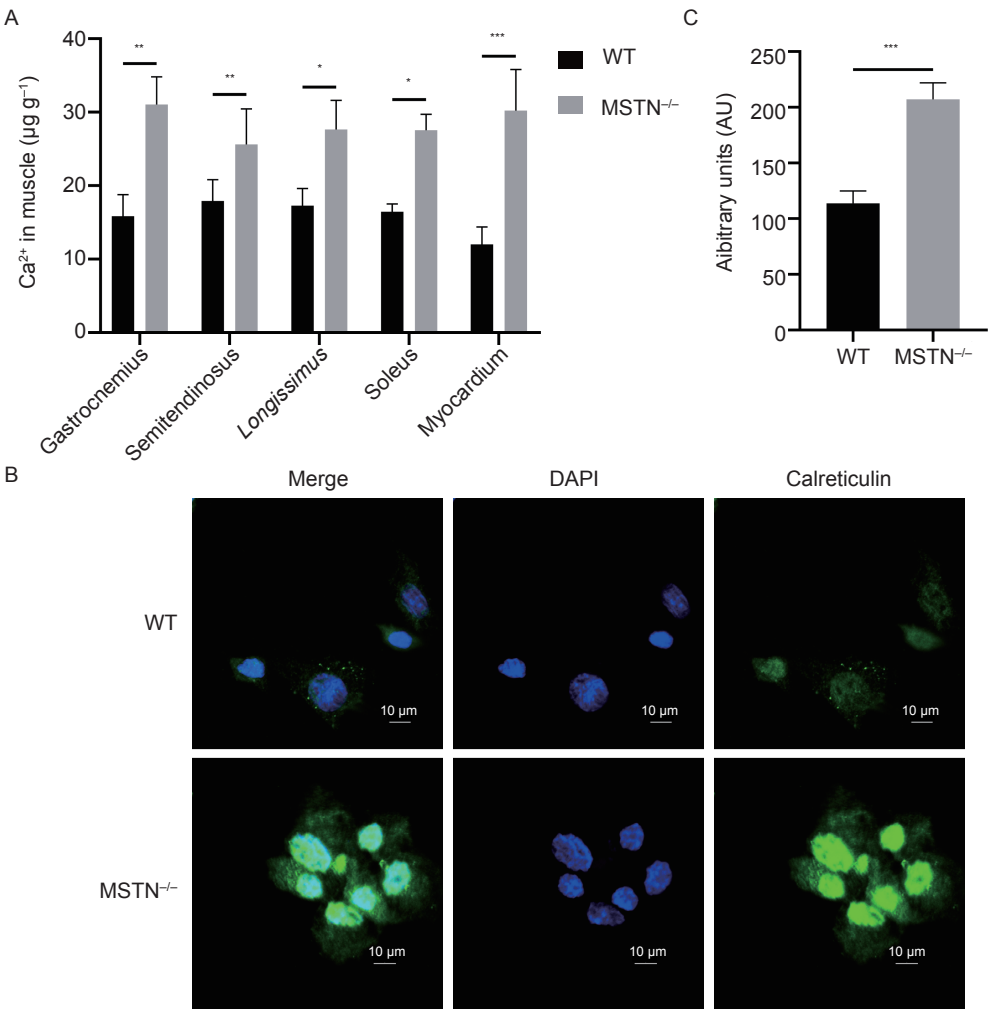


Fig. 2 Ca^{2+} in $MSTN^{-/-}$ (MSTN mutated) bovine muscle tissue and muscle satellite cells was significantly increased. A, determination of Ca^{2+} content in muscle by ICP-MS. B and C, the characterization of Ca^{2+} content in muscle satellite cells by specific immunofluorescence staining of Calreticulin (green, calreticulin; blue, nuclei). Data are mean±SEM. *t*-test was used to generate *P*-value. Compared with WT group, * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

(Appendix C-h). TRPC4 specific immunofluorescence of WT, $MSTN^{-/-}$ and $MSTN^{-/-}$+ML204 cells showed that 1.0 μmol L^{-1} ML204 could inhibit the expression of TRPC4 in cells (Appendix C-i). In addition, after the treatment with ML204, there was a prominent decrease of Ca^{2+} in $MSTN^{-/-}$ muscle satellite cells, similar to that in WT (Fig. 4-A). The results of live cell count, EdU assay and the expression of cell cycle-related genes showed that ML204 significantly inhibited cell proliferation, and $MSTN^{-/-}$ cells treated with ML204 had no significant difference compared with WT cells (Fig. 4-B–E). These results suggested that MSTN mutation activated the TRPC4 channel and increased extracellular Ca^{2+} influx, thereby promoting cell proliferation. And ML204 had the opposite effect by inhibiting TRPC4 channel activity.

3.5. MSTN mutation regulates cell proliferation through calcineurin/NFATc3 pathway

To further explore how MSTN mutation regulates cell proliferation through calcium content, the calcium signaling pathway should be paid more attention. As a key downstream calcium signaling pathway, the calcineurin/NFATc3 pathway has been reported to regulate the expression of genes associated with cell proliferation (Li *et al.* 2011; Zhang *et al.* 2020). Calcineurin is a calcium- and calmodulin-dependent serine/threonine protein phosphatase (Zhang J *et al.* 2021). It is a heterodimeric protein consisting of a catalytic subunit calcineurin A and a regulatory subunit calcineurin B, which is tightly associated with calcineurin A (Rusnak and Mertz 2000). Therefore, we chose to represent the expression of calcineurin by measuring the abundance of calcineurin A and calcineurin B. With the treatment of 1.0 μmo L^{-1} ML204, the expression of calcineurin and the amount of NFATc3 transferred into the nuclei were decreased in $MSTN^{-/-}$ muscle satellite cells, which were similar to those of WT (Fig. 5-A–D). NFATc3 specific immunofluorescence showed similar fluorescence intensity in three groups, WT, $MSTN^{-/-}$ and $MSTN^{-/-}$+ML204 cells (Fig. 5-E and F). These results indicated that MSTN mutation did not affect the total amount of NFATc3, but 1.0 μmo L^{-1} ML204 could

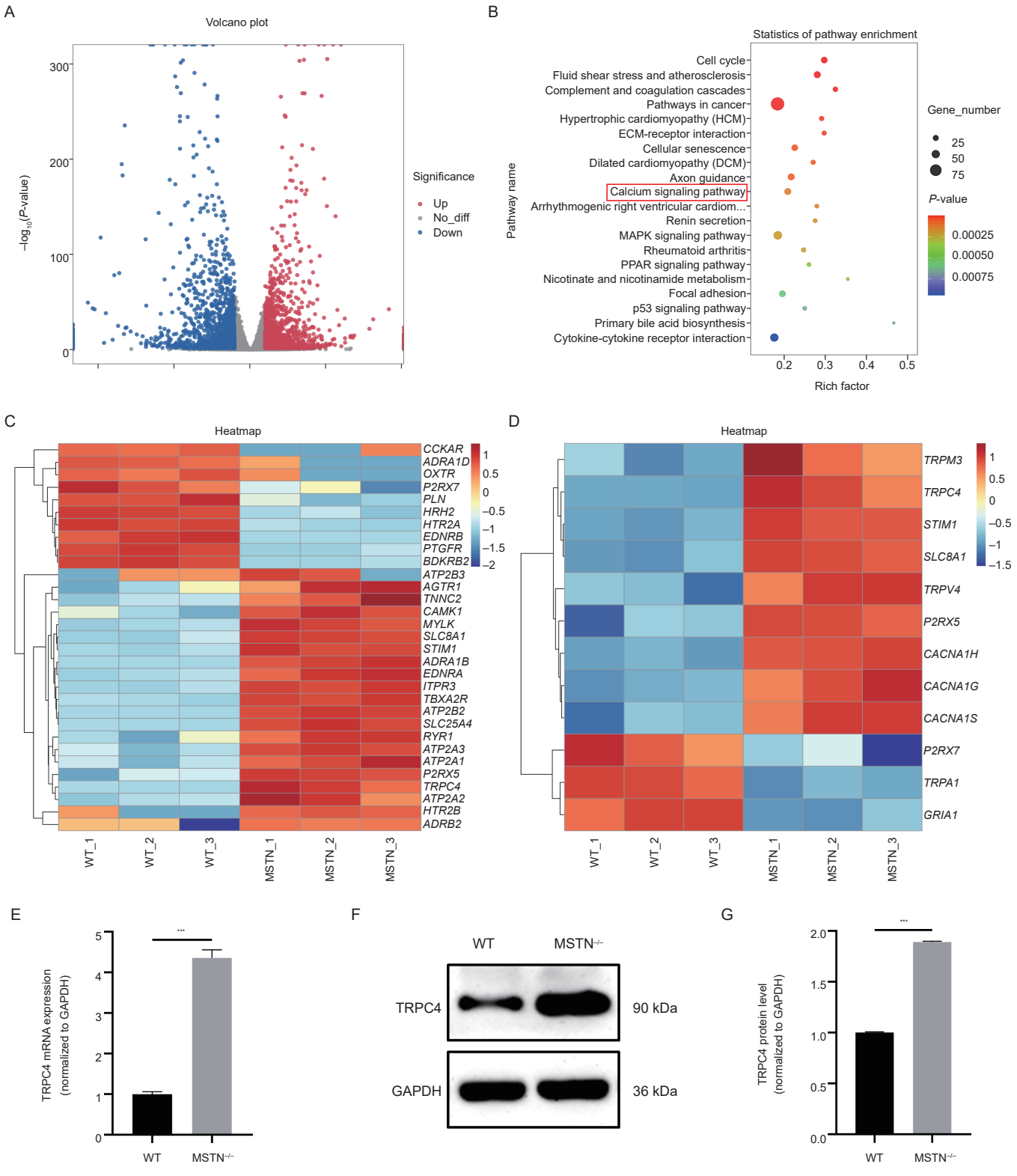


Fig. 3 MSTN (myostatin) activates calcium signaling pathway by activating calcium channel TRPC4. A, transcriptome analysis of bovine muscle satellite cells was performed in WT and MSTN$^{-/-}$ (MSTN mutated) groups showed significant differences between them. B, KEGG analysis of differentially expressed genes was enriched to calcium signaling pathway. C, differentially expressed genes in calcium signaling pathway were mainly related to calcium transport and regulation. Among the 31 genes differentially expressed genes, 21 genes were up-regulated and 10 genes were down-regulated. D, there were calcium channel genes on the cell membrane with significant differences, and only TRPC4 in the TRPC family was up-regulated in MSTN$^{-/-}$ muscle satellite cells. Red indicates up-regulated genes, blue indicates down-regulated genes, and gray indicates genes with no significant difference. E–G, and the mRNA and protein levels of TRPC4 in MSTN$^{-/-}$ muscle satellite cells were significantly increased. Data are mean±SEM. *t*-test was used to generate *P*-value. Compared with WT group, * $P<0.05$, **,$P<0.01$, *** $P<0.001$.

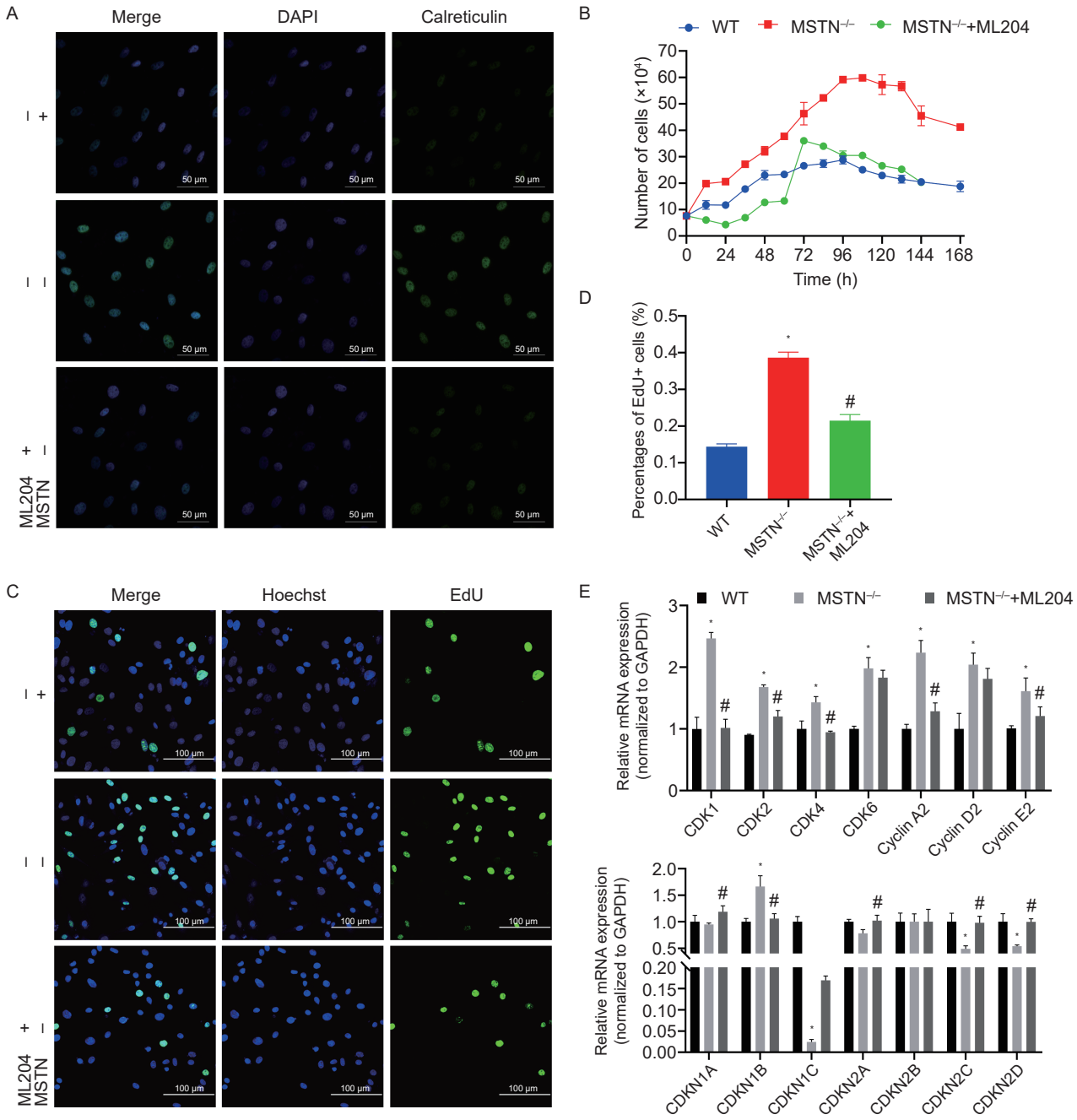


Fig. 4 MSTN (myostatin) mutation activated TRPC4 channel and increased extracellular Ca^{2+} influx, thereby promoting cell proliferation, while ML204 played an opposite role by inhibiting TRPC4 channel activity. A, the specific immunofluorescence staining of calreticulin indicated that Ca^{2+} decreased significantly in MSTN$^{-/-}$ (MSTN mutated) muscle satellite cells after treatment with ML204, similar to that in WT (green, calreticulin; blue, nuclei). B–E, cell count, EdU and the expression of cell cycle-related genes suggested that the treatment with 1.0 μmol L^{-1} ML204 had obvious inhibitory effect on cell proliferation, and cells treated with ML204 showed no significant difference compared with WT group (green, EdU+; blue, nuclei). The cells in A, B–E were treated with 1.0 μmol L^{-1} ML204 for 24 h. Data are mean±SEM. *t*-test was used to generate *P*-value. $^{*}P<0.05$, *vs.* WT group; $^{\#}P<0.05$, *vs.* MSTN$^{-/-}$ group.

effectively inhibit the NFATc3 entry in MSTN$^{-/-}$ muscle satellite cells caused by MSTN deletion.

4. Discussion

4.1. MSTN mutation promotes the proliferation of muscle satellite cells and skeletal muscle development

MSTN is mainly synthesized and expressed in skeletal muscle, which negatively regulates the growth and development of skeletal muscle (McPherron *et al.* 1997; Chen *et al.* 2021). The deletion of MSTN leads to the thickening of muscle fibers, and the animals show obvious "double-muscle" phenotype. Muscle hypertrophy caused by MSTN mutation is presented in a variety of mammals, including cattle (Kambadur *et al.* 1997), rat (McPherron *et al.* 1997), sheep (Boman and Våge 2009), dog (Mosher *et al.* 2007), and human (Elliott *et al.* 2012), and etc. The MSTN mutation

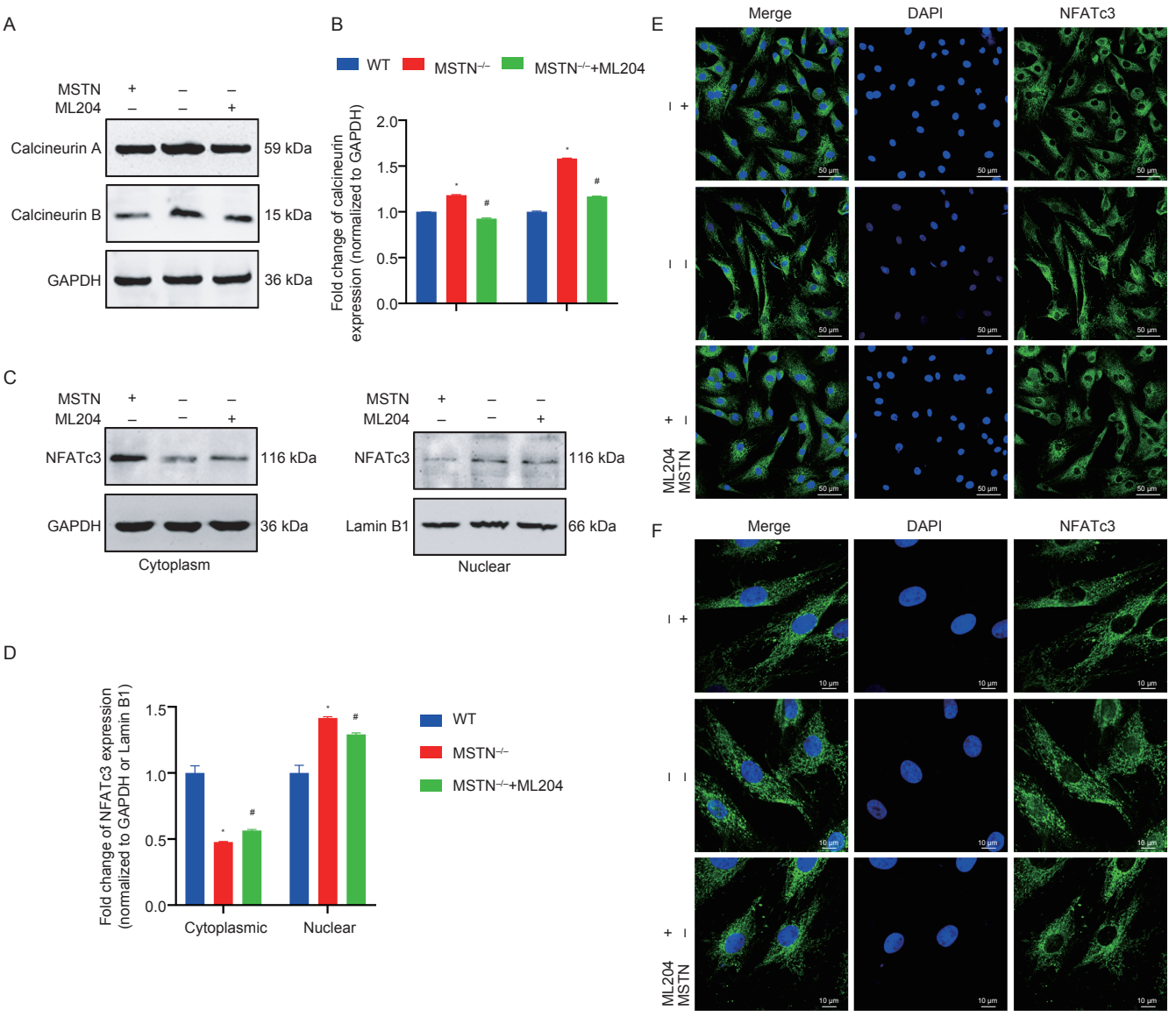


Fig. 5 After MSTN (myostatin) mutation, cell proliferation is regulated through calcineurin/NFATc3 signaling pathway downstream of Ca^{2+}. A–D, with the treatment of 1.0 μmol L^{-1} ML204, the expression of calcineurin and the amount of NFATc3 transferred into the nuclei were decreased in MSTN$^{-/-}$ (MSTN mutated) muscle satellite cells, which were similar to those of WT. E and F, NFATc3 specific immunofluorescence staining showed similar fluorescence intensity in three groups. WT, MSTN$^{-/-}$ and MSTN$^{-/-}$+ML204 cells (green, NFATc3; blue, nuclei). Data are mean±SEM. *t*-test was used to generate *P*-value. $^{*}P<0.05$, *vs*. WT group; $^{\#}P<0.05$, *vs*. MSTN$^{-/-}$ group.

resulted in enhanced muscle development, manifested as a distinct "double-muscle" phenotype. The knockout of MSTN promotes the increase of muscle mass, which is caused by the stimulation of the increase in the number and volume of muscle satellite cells. Ge continuously edited four based at the 3′-UTR of MSTN, thereby reducing the translation level of MSTN and significantly promoting the proliferation and differentiation of C2C12 myoblasts (Ge *et al.* 2020). The research by McCroskery found that compared with the wild type, MSTN gene knockout mice possessed more muscle satellite cells, which proliferated faster *in vitro* (McCroskery *et al.* 2003). In our study, cultured MSTN$^{-/-}$ muscle satellite cells proliferated faster than WT, and showed a significant increase in cell count. Cells in MSTN$^{-/-}$ group had shorter doubling time and were able to reach the plateau stage more quickly. The expression of cell cycle-related genes was detected. The expression levels of CDKs and cyclins in MSTN$^{-/-}$ group were higher than those in WT, while the expression of CKIs were decreased. All the results have indicated that MSTN mutation promotes the proliferation of muscle satellite cells and the development of skeletal muscle.

4.2. Ca^{2+} plays an important role in skeletal muscle of MSTN mutant animals

Ca^{2+} plays a key role in excitation-contraction coupling of all types of muscle (Sweeney and Hammers 2018; Allen 2019). The rise of intracellular Ca^{2+} will activate the contraction device of muscle fibers (Sweeney and Hammers 2018). The Ca^{2+} content in fast switch fibers is higher and is used for rapid muscle contraction (Schiaffino and Reggiani 2011). In this

study, Ca^{2+} was detected in four skeletal muscles, and Ca^{2+} in gastrocnemius (fast switch fiber) was increased by 95.73%, significantly higher than that in semitendinosus (both fast and slow switch fiber), longissimusdorsi (both fast and slow switch fiber) and soleus (slow switch fiber). The expression of MyHC-I was down-regulated in the skeletal muscle of MSTN gene knockout mice, while that of MyHC-IIB was up-regulated, resulting in a decreased proportion of type I muscle fibers and an increased proportion of type II muscle fibers (Girgenrath *et al.* 2005). The increased proportion of fast switch fibers was the main cause of muscle hypertrophy in mice (Hennebry *et al.* 2009). Ca^{2+} signaling pathway played an important regulatory role in the transformation of muscle fibers (Mu *et al.* 2007; Luo *et al.* 2019). Compared with wild type, the number of type IIb muscle fibers in $MSTN^{-/-}$ was significantly increased (Bodnár *et al.* 2014). MSTN mutation promoted the conversion of muscle fibers from slow switch fibers to fast switch fibers.

Calcium signaling was involved in a variety of cellular processes in a temporal and spatial dynamic manner, regulating cell secretion, molecular transport, chemotaxis, proliferation, differentiation, apoptosis, and gene expression (Snoeck 2020). Increased cytoplasmic Ca^{2+} concentration activated the signal transduction proteins and transcription factors necessary for cell proliferation (Hardingham *et al.* 1997; Hasegawa *et al.* 2021). Intracellular Ca^{2+} can be up-regulated in two ways. One was Ca^{2+} influx through Ca^{2+} channels, and the other was released from intracellular calcium pool (Pinto *et al.* 2015). Detection of Ca^{2+} content in cytoplasm of WT and $MSTN^{-/-}$ muscle satellite cells showed that MSTN mutation increased Ca^{2+} content in the cytoplasm and activated signal transduction proteins and transcription factors essential for cell proliferation, thereby promoting cell proliferation (Pinto *et al.* 2015). Therefore, it was believed that the proliferation of muscle satellite cells caused by MSTN mutation was mediated by Ca^{2+} and its downstream signals.

4.3. MSTN mutation activates TRPC4 channel and promotes an increase in intracellular Ca^{2+}

TRPC family plays an important role in Ca^{2+} regulation and cell proliferation (Zhan and Shi *et al.* 2017). Studies have shown that overexpression of TRPC in mammalian cells leads to an increase in intracellular Ca^{2+} (Yu *et al.* 2004). Makarewich and Zhang (Makarewich *et al.* 2014) found that the increased expression of TRPC3 promoted myocardial cell hypertrophy in some animal models of myocardial hypertrophy. The TRPC/calcineurin/NFATc3 pathway was involved in the regulation of PASMC proliferation (Junli *et al.* 2018). And the expression of TRPC6 was positively correlated with S and G2/M phases of cell cycle, and related to the expression of Ki67. TRPC6 participated in the transition of PASMCs from quiescence to mitosis, and regulated the proliferation of human PASMCs (Yu *et al.* 2004). In this study, transcriptomic analysis was performed on muscle satellite cells, and the DEGs were enriched to calcium signaling pathway. DEGs in calcium signaling pathway were mainly genes related to Ca^{2+} transport and regulation, and they participated in the homeostasis of Ca^{2+} in cells. Differential gene expression analysis of Ca^{2+} channels in cell membrane revealed that only TRPC4 gene of TRPC family was significantly increased in $MSTN^{-/-}$ group. In addition, among the 12 DEGs, TRPC4 was next to TRPM3 in significance, but the relative expression level and differential multiple of TRPM3 were lower than those of TRPC4 (TRPM3: 0.44, 0.36, 0.39, 0.95, 0.75, 0.69; TRPC4: 48.51, 48.02, 47.47, 142.46, 130.91, 110.88). Thus, it was considered that MSTN mutation significantly affected TRPC4 channel. The expression of TRPC4 in skeletal muscle and muscle satellite cells were significantly increased in $MSTN^{-/-}$ cattle, indicating that MSTN mutation affected Ca^{2+} content by up-regulating TRPC4 expression. Though, the possibility of Ca^{2+} entering cells through other Ca^{2+} channels is not ruled out. However, $MSTN^{-/-}$ muscle satellite cells were treated with ML204, a TRPC4-specific inhibitor, and the cell proliferation was significantly reduced, suggesting that TRPC4 was a major Ca^{2+} channel for the regulation of cytoplasmic Ca^{2+} and the cell proliferation in $MSTN^{-/-}$ cattle.

4.4. Ca^{2+} promotes cell proliferation *via* TRPC4/calcineurin/NFATc3 pathway

Regulation of gene expression is achieved through a variety of signaling pathways. Ca^{2+} controls cell fate by regulating all kinds of calcium-dependent pathways and transcription factors (Puri 2020). There are many signaling cascades involved in the regulation of hypertrophy response, including calcineurin/NFATc3 and calcium-calmodulin-dependent kinase II (CaMKII)/histone deacetylase 4 (HDAC4) (Tham *et al.* 2015; Chen *et al.* 2017; Lin *et al.* 2021). Calcineurin directly binds to its main downstream effector factor NFATc3 during activation, making it dephosphorylated and transferred into the nucleus to promote the activation and expression of hypertrophy-related genes (Zhang *et al.* 2018; Hong *et al.* 2021). CaMKII is a serine/threonine kinase that phosphorylates various substrates including NFATc3 (Hook and Means 2001). Similarly, CaMKII has been reported to phosphorylate HDAC4 with the activation of hypertrophy-related genes (Backs *et al.* 2006; Kreusser and Backs 2014).

As a Ca^{2+}-dependent phosphatase, calcineurin is activated upon the stimulation of TRPC channels (Nakayama *et al.* 2006), and initiates a variety of intracellular responses through its downstream transcriptional effector, NFAT (Abramowitz and Birnbaumer 2009; Gao *et al.* 2012; Zhao *et al.* 2014). Several studies have shown that TRPC channels contribute to the activation of calcineurin-NFAT signaling and hypertrophic response (Eder and Molkentin 2011; Gao *et al.* 2012). Catherine supported this and suggested that in adult feline myocytes, the organization of TRPC4 channels in caveolae membrane microdomains influenced their ability to coordinate calcineurin-NFAT signaling (Makarewich *et al.* 2014). TRPC4 contributes to excessive SOCE, aberrant proliferation, exacerbated migratory, and apoptosis resistance of PHA-hPASMCs (Masson *et al.* 2023). Cooley demonstrated that TRPC4α, a splice variant of TRPC4, selectively participated in the downstream of PLCβ1b, providing Ca^{2+} to activate the responses of calcineurin (Cooley *et al.* 2014). Kirschmer *et al.* (2016) also indicated that upregulation of TRPC4α

and TRPC4β in both human and mice hearts induced the hypertrophic growth of cardiomyocytes, which is associated with the elevated Ca^{2+} transient peaks and an enhanced calcineurin/NFTA activity, thereby activating hypertrophy signaling pathway. In a SAH rat model, Wang (Wang *et al.* 2016) concluded that overexpression of TRPC1/4 could increase calcineurin activity, which in turn promoted the dephosphorylation of N-methyl-D-aspartate receptor (NMDAR) and the nuclear translocation of NFAT, thus inhibiting SAH-induced EBI. Since NFAT is a transcription factor promoting the expression of TRPC1/4 (Serrano-Pérez *et al.* 2015), it also suggests that a positive feedback regulatory loop between increased TRPC expression and enhanced calcineurin activity. Consisting with Jiang (Jiang *et al.* 2016), the treatment of LPS promotes PASMCs proliferation through enhancement of TRPC mediated Ca^{2+} entry. Further research is needed to verify whether such positive feedback regulation exists in our study.

NFAT is a ubiquitous regulator of cell differentiation and adaptation, affecting the development and adaptation of a variety of mammalian cells (Horsley and Pavlath 2002). Interestingly, Ca^{2+} influx through TRPC channel is an important determinant of the regulation of NFAT activity in skeletal muscle (Rosenberg *et al.* 2004). During the continuous increase of Ca^{2+}, calcineurin dephosphorylates NFATc1-c4, which is transferred to nucleus and binds to the corresponding DNA sites to regulate transcription and activate the target genes related to cell proliferation (Buchholz and Ellenrieder 2007). Once intracellular Ca^{2+} concentration was restored to basic level, NFATs was phosphorylated by resident protein kinases such as GSK-3, NFATs exposed nuclear exporting sequence and translocated to cytoplasm, where NFAT signal was terminated (Graef *et al.* 2001; Gwack *et al.* 2006). NFATc1 and NFATc3 were major subtypes expressed in human skeletal muscle. The expression of NFATc3 can be activated by stimulation of increased intracellular Ca^{2+} level (Hoey *et al.* 1995). Up to now, there have been limited studies on NFATc3 in $MSTN^{-/-}$ cattle. In study, we found that, the treatment of muscle satellite cells in $MSTN^{-/-}$ group with ML204 revealed decreased expression of calcineurin and decreased nuclear influx of NFATc3, with levels similar to those in WT group. Therefore, ML204 was effective in inhibiting the increase of alcineurin and the entry of NFATc3 in $MSTN^{-/-}$ cells due to MSTN mutation. It can be said that, the increase of Ca^{2+} and the accelerated proliferation of muscle satellite cells caused by the deletion mutation of MSTN were mainly attributed to the Ca^{2+} channel TRPC4. As MSTN is a key gene regulating muscle growth and development, intensive and meticulous research on it is of great significance, whether in improving human health or promoting economic development in animals husbandry. This study is the first to explain the effect of MSTN on proliferation of muscle satellite cells from the perspective of Ca^{2+} regulation in large animals such as cattle, providing a new research direction. The limitation of our study is that we did not specify the specific way in which MSTN regulates TRPC4. And we will conduct further and more thorough research on this aspect in subsequent studies.

5. Conclusion

In summary, this study suggested that MSTN deletion mutation promoted cell proliferation of muscle satellite cells by activating the TRPC4/Ca^{2+}/calcineurin/NFATc3 pathway. According to the schematic diagram, MSTN deletion mutation activated the expression of calcium channel TRPC4, resulting in an increase in intracellular Ca^{2+} content, thus activating the calcineurin/ NFATc3 pathway. Cytoplasmic Ca^{2+} activates the activity of calcineurin, which making NFATc3 dephosphorylated and transferred into the nuclei, where it activates the expression of proliferation-related genes and promotes cell proliferation.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (32360837 and 32341052), the STI2030 Major Projects, China (2023ZW0404803), the Inner Mongolia Open Competition Projects, China (2022JBGS0025), the Inner Mongolia Science and Technology Leading Team, China (2022LJRC0006), the Inner Mongolia Science and Technology Major Projects, China (2021ZD0009, 2021ZD0008, 2022ZD0008, 2023KJHZ0028), the Inner Mongolia Young Talents Projects, China (NJYT23138), the Inner Mongolia Natural Science Foundation, China (2023MS03004), the Central Government Guides Development, China (2022ZY0212), the National Agricultural Science and Technology Project of China (NK2022130203), the Collaborative Innovation among Universities in Hohhot, China (XTCX2023-06), and the Ministry of Education Engineering Centre Project, China (JYBGCSYS2022).

Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Ethical approval

The animal study was reviewed and approved by the Committee on the Ethics of Animal Experiments at Inner Mongolia University, China. Written informed consent was obtained from the owners for the participation of their animals in this study. All treatments are performed in strict accordance with relevant guidelines and regulations.

Appendices associated with this study are available at https://doi.org/10.1016/j.jia.2024.04.020

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