MSTN mutant promoted bovine muscle satellite cell proliferation by regulating the binding of SMAD2/SMAD3 with *CDKN1C*

Li Gao
Baotou Teachers College
Miao-Miao Yang
State Key Laboratory of Reproductive Regulation & Breeding of Grassland Livestock, Inner Mongolia University
Ming-Juan Gu
State Key Laboratory of Reproductive Regulation & Breeding of Grassland Livestock, Inner Mongolia University
Yun-Peng Liu
Baotou Teachers College
Cai-Hong Bu
Baotou Teachers College
Peng Cheng
Baotou Teachers College
Chunling Bai (chunling1980_0@163.com)
State Key Laboratory of Reproductive Regulation & Breeding of Grassland Livestock, Inner Mongolia University https://orcid.org/0000-0003-0531-3973

Research

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Abstract

Background: Myostatin (*MSTN*), also known as growth/differentiation factor 8, mostly expressed in skeletal muscle and plays negative roles in regulation of muscle development. Previous studies had proved that *MSTN* have important effect on cell proliferation. Therefore we aimed to investigate the mechanism of *MSTN* in regulating the proliferation of bovine muscle satellite cells (MSCs).

Methods: Bovine MSCs of *MSTN* mutant (MT) and wild type (WT) were obtained, we detected the cell proliferation and cell cycle by EdU proliferation assay and Flow cytometry. Then we detected the expression of genes associated with cell cycle by Real-time PCR and Western blotting . RNA-seq and Chromatin immunoprecipitation (ChIP)assay were performed to research the mechanism of *MSTN* in regulating the cell proliferation.

Results: In this study, we found that *MSTN* mutant promoted the proliferation of MSCs. The expression of *CyclinA*, *CyclinD* and *CyclinE* were all increased after MSTN mutant, while the expression of *CDKN1C* (*P57*), *CDKN2A*, *CDKN2C* and *CDKN2D* were down-regulated, which were consistent with the promotion of cell proliferation. Among these genes, *CDKN1C*(*P57*) down-regulated most significantly. RNA-seq results showed that *MSTN* mutant affected the SMAD binding, so we performed ChIP-qPCR and demonstrated that the SMAD2/SMAD3 transcription factor combined with the promoter of *CDKN1C* thus to increase the expression of *CDKN1C*, this demonstrating that *MSTN* regulated the expression of *CDKN1C* through SMAD2/SMAD3 complex. Finally, overexpression of *SMAD3* in wild type cells increased the expression of *CDKN1C*, further suggested that *SMAD3* regulated the expression of *CDKN1C*.

Conclusion: *MSTN* mutant down-regulated the expression of SMAD2/SMAD3, then reduced the promotion of SMAD2/SMAD3 to the expression of *CDKN1C*, thus to inhibit the expression of *CDKN1C*, then promoting the cell cycle.

Background

Myostatin (*MSTN*), is a member of the transforming growth factor β (TGF-β) family, also known as growth/differentiation factor-8; it is expressed in many tissues but mostly expressed in skeletal muscle, played important roles in the regulation of muscle development[1]. Many studies had proved that *MSTN* mutant leads to muscle hypertrophy due to both increased myofiber numbers and increased myofiber sizes, and muscle mass is the most important trait in livestock production, so *MSTN* has received a lot of attention. Up to now, many species of *MSTN* deficiency animals have been obtained, including cattle[2], humans[3], sheep[4], dogs[5], pigs[6],goats[7] and birds[8] without causing severe adverse consequences.

Previous studies had proved that *MSTN* have important effect on cell proliferation. Transcriptome analysis of muscle tissue of *MSTN* knockout chickens found that differentially expressed genes were enriched in the process of cell proliferation and differentiation[9].Knockout of *MSTN* promote cell proliferation, while overexpression of *MSTN* inhibit cell proliferation and DNA synthesis[10,11]. McCroskery *et al*. (2003) showed that overexpression of *MSTN* up-regulates *P21* and downregulates

CDK2 expression[12], *MSTN* negatively regulated the progression of G1/S phase, thus to inhibit the cell cycle and maintain the resting state of satellite cells. Inhibition of *MSTN* expression by RNAi in C2C12 cells promoted the cell cycle, cells of G0/G1 phase decreased, while S phase cells increased[13,14]. Inhibition and knockout of *MSTN* expression by RNAi and zinc finger synthase in goat and sheep myoblasts promoted cell proliferation, decreased the expression of *P21* while increased *CDK2* expression[14,15]. But the further mechanism of *MSTN* regulating cell cycles were not well known. In this study, we used *MSTN* mutant bovine muscle satellite cells (MSCs) described in the previous study[16], detected the effect of *MSTN* mutant to cell cycles and the regulating pathway in the *MSTN* mutant bovine muscle satellite cells we obtained.

Methods

Cell culture

Muscle satellite cells (MSCs) cultured from cattle of *MSTN* mutant (MT) and wild type (WT) were obtained and identified as our previously reported [16]. MSCs of both MT and WT were cultured in DMEM supplemented with 10% horse serum (HS) and 20% fetal bovine serum (FBS) with 5% CO_2 at 38.5°C. When the confluence of cells reached 100%, subculture was performed.

Ethynyl-2'-deoxyuridine (EdU) assays

The proliferation of MT and WT MSCs were detected by using EdU proliferation kit(RuiBo, Guangzhou, China), the operation method is carried out according to the manufacturer's protocol. Both MT and WT bovine muscle satellite cells were incubated with EdU for 12 h and washed twice with PBS for 5min each time; then fixed the cells by 4% paraformaldehyde for 30min, permeabilized the cells with 0.5% Triton X-100 for 20min, labeled the cells with Apollo® fluorescent dye (RuiBo, Guangzhou, China) for 30min in dark. Finally infiltrated the cells with PBS. Images were captured using a confocal microscope (Nikon, Tokyo, Japan) and calculated the percentage of EdU-positive cells.

Myogenic cells differentiation

Myogenic differentiation was induced when the confluence of the cultured cells reached 80-90% by using low serum medium of which 2% horse serum was supplemented to DMEM. Myogenic differentiation induction lasts for 3d.

Flow cytometry assay

Cell cycles were detected by the Cell Cycle and Apoptosis Analysis Kit (Beyotime, China).MSCs and differentiated MSCs were collected and washed with PBS. Next, cells were fixed for over 24h in 4°C with 70% ethanol, then the fixed cells were washed with pre-cooled PBS and then labeled with PI solutions avoiding light for 30min in 37°C solid bath, then the cells were detected using a flow cytometer(BD Biosciences). Cell cycles were determined by FlowJo 7.6 software.

Real-time PCR

Expression of genes associated with cell cycle in mRNA level was detected by Real-time PCR. RNA of the myogenic cells and myotubes of MT and WT cattle were extracted by RNAiso Plus kit (9108; Takara), then the RNA was reverse-transcribed into cDNA by reverse transcription reagent (RR047A; Takara). The genes associated with cell cycle were amplified using ABI7500 real-time PCR (Applied Biosystems, America). The primers used for PCR are listed in Table S1. DNA amplification was performed as the previously described program: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 60°C for 34 s, and a final melting curve stage [16]. The cycle threshold (Ct) values of housekeeping gene GAPDH were used to normalized the targeted genes using the $2^{-\Delta\Delta Ct}$ method [17].

Western blotting

Western blotting was performed according to reference of our previously reported[16]. Briefly, the protein of cell samples were extracted using Radio Immunoprecipitation Assay (RIPA) buffer, then boiled and mixed with the loading buffer in proportion, electrophoresed in a 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane by electroblotting. Then blocked the membrane, incubated with anti-CyclinA (sc-271682; Santa Cruz, USA), anti-CyclinD (sc-376676; Santa Cruz, USA), anti- P21 (sc-6246; Santa Cruz, USA), anti-P57 (sc-56341; Santa Cruz, USA) and anti-SMAD3 (ab40854; Abcam, USA) monoclonal antibodies at 1:500 in 4°C for overnight. Then washed and incubated for 1h with secondary antibodies, finally captured the images using Tanon Gel Imaging System (Tanon 4800).

RNA-seq analysis

Total RNA of wild type (WT) and *MSTN* mutant (MT) bovine muscle tissues were extracted using TRIzol® reagent (Invitrogen). RNA sequencing was operated in Sangon Biotech company. RNA Sequencing data are shown in Table S3. Clean reads were aligned to Bos Taurus genome (NCBI:ID82) as described in reference [18]. KEGG pathway analysis and GO functional enrichment were conducted by Omicshare tools and David online database.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using Pierce™ Magnetic ChIP Kit (26157; Thermo Fisher Scientific) as previously described [19]. anti-SMAD2+SMAD3 (ab202445; Abcam, USA) monoclonal antibodies was used to settle the bound DNA, the settled DNA was analyzed by quantitative PCR. Primers used for ChIP-qPCR were provided in Table S2.

Construction and transfection of SMAD3 overexpression plasmid

cDNA of *SMAD3* was cloned from cattle muscle tissue. The primers were as follows: 5'-ATGTCGTCCATCCTGCCTTTC-3' (forward) and 5'-CTAAGACACGCTGGAGCAG-3' (reverse). The obtained *SMAD3* cDNA and the pCAG-IRES2-AcGFP vector were digested by restriction endonucleases of *EcoR*I and *BamH*I. Nextly, the *SMAD3* fragment and the linearized pCAG-IRES2-EGFP vector were ligated. Then the

SMAD3 overexpression vector was transfected using Lipofectamine LTX Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Transfection was conducted when the MSCs reaching 85% confluence. RNA was isolated 48 h after transfection.

Statistical analysis

T-tests were used to analysis the significance difference between *MSTN* mutant and wild type MSCs. p-values of less than 0.05 were considered statistically significant.

Results

MSTN mutant promoted the proliferation of muscle satellite cells (MSCs)

The muscle satellite cells (MSCs) derived from both MT and WT cattle were shown in Fig 1A, and identified in our previously reported reference[16]. We analyzed the proliferation of the MSCs isolated from MT and WT to detect the effect of *MSTN* mutant to cell proliferation. EdU proliferation assay results showed that *MSTN* mutant promoted the proliferation of MSCs(Figure 1B, 1C). Then we analyzed the cell cycle of these two cells by Flow cytometry. Results showed that cell numbers of G0/G1 and G2/M phase decreased while the cell numbers of S phase increased compared with WT(Figure 1D, 1E). This results indicated that MSTN mutant promoted the cell cycle mainly by promoting the DNA synthesis of cells.

MSTN mutant promoted the myogenic diffefentiation by exiting the cell cycle earlier

We then induced the MSCs of MT and WT to myogenic differentiation and the cell morphology was observed. Results indicated that myotubes were formed in MT cells after 1 day of myogenic differentiation, while this occurred after 2 days of myogenic differentiation. After 3 day' myogenic differentiation, there were more myotubes formed in MT cells than that in WT cells (Figure 2A). Then we analyzed the cell cycle of MSCs after 1, 2 and 3 day's myogenic differentiation in MT and WT cells. As shown in Figure 2B,2C and 2D, for MT MSCs, Cell number of G1 phase increased immediately after differentiated, while S phase decreased immediately after 1 day of myogenesis. For WT MSCs, Cell number of G1 phase increased significantly after 2 days of myogenesis, while the S phase increased on day 1, but decreased after 1 day of myogenesis. These results showed that MT MSCs exited the cell cycle and began to differentiate immediately when induced to myogenic differentiation, but WT MSCs occurred these after 2 days of myogenesis induction. The cell cycles results were consistent with the cell morphology change results observed by microscope.

MSTN mutant promoted cell proliferation mainly by down-regulating *CDKN1C*

We then detected the expression of cell cycle related genes. Among the Cyclins we detected, the expression of *CyclinA*, *CyclinD* and *CyclinE* were all increased after *MSTN* mutant, and the most significantly increased is *CyclinA* (Figure 3A, 3C and 3D). The expression of *CDK1*, *CDK2* and *CDK6* were all increased(Fig 3A). The expression of Cyclin dependent kinase inhibitors were not the same after *MSTN* mutant, *CDKN1C* (*P57*), *CDKN2A*, *CDKN2C* and *CDKN2D* were down-regulated, which were consistent with

the promotion of cell proliferation. Among these genes, *CDKN1C*(*P57*) down-regulated most significantly (Figure 3B, 3C, 3D). These results indicated that *MSTN* mutant promoted MSCs proliferation mainly by down-regulating *CDKN1C*, then promoting the CyclinA-CDK2 expression, thus to promote the synthesis of DNA and cell cycle.

RNA-seq analysis

To further analysis the mechanism of *MSTN* mutant to muscle cell proliferation and cell differentiation, we compared transcripts of *MSTN* mutant (MT) bovine and the controls. Skeletal muscle of six cattle were performed RNA-seq analysis consist of 3 MT cattle and 3 WT controls. DEGs were identified by P-value < 0.05 at the same time |fold change (FC)| > 1.5. Notably, expressions of 721 genes were found significantly changed has occurred in the MT cattle. Of these, the expressions of 74 genes were up-regulated, while 647 genes were down-regulated in the MT cattle (Figure 4A). We then performed Real-time quantitative PCR to verify the expression of the most significant DEGs (Figure 4B), results were consistent with the RNA-seq results (Figure 4C), proving that the transcriptome data is usable. Succeeding Gene Ontology (GO) analysis indicated that the significantly differently expressed genes were with regard to the biological processes of muscle cell differentiation regulation, muscle cell proliferation, especially G1/S transition, and TGF-β pathway (Figure 4D). At the same time, the DEGs were associated with SMAD binding (Figure 4D), this indicated that *MSTN* mutant may affect the binding of SMAD with downstream genes, thus to regulate cell proliferation.

MSTN regulated the expression of *CDKN1C* by SMAD2/SMAD3 pathway

Since SMAD2/SMAD3 complex were downstream transcription factors of the *MSTN* type II receptors, *MSTN* mutant increased the expression of *CDKN1C*, so we speculated that SMAD2/SMAD3 complex may combine with *CDKN1C* promoter and regulate the expression of *CDKN1C*. This was proved by the transcription factor binding prediction analysis using the JASPAR database, results showed that SMAD2/SMAD3 complex bound with the promoter region of *CDKN1C* (NC_037355.1) from −804 to −792 (Figure 5B). Furthermore, we also confirmed the binding of SMAD2/SMAD3 complex with promoter of *CDKN1C* by performing a ChIP-qPCR assessment. We used an anti-SMAD2/SMAD3 monoclonal antibody to settle the DNA bound to SMAD2/SMAD3 complex, then amplified the *CDKN1C* promoter region from the deposited DNA. Region detected was from −926 to −757 on the *CDKN1C* promoter (Figure 5C).

Overexpression of *SMAD3* promoted the expression of *CDKN1C*

To further discuss the influence of *SMAD3* to *CDKN1C* expression, we then constructed the overexpression vector of *SMAD3* (Figure6A, 6B), then transfected the WT MSCs with this overexpression vector. Results indicated that the expression of SMAD3 in the transgenic cells increased both in mRNA and protein levels as displayed in Figure 6C, 6D, and 6E. Then we detected the expression of CDKN1C, results showed that the expression of CDKN1C both in mRNA and protein levels increased significantly after overexpression of *SMAD3*(Figure 6F, 6G, 6H). These results indicated that SMAD3 promoted the expression of CDKN1C. So MSTN mutant decreased the expression of SMAD3, then

suppressed the expression of CDKN1C, thus to promote the CyclinA-CDK2 complex expression, promoted DNA synthesis and cell proliferation.

Discussion

Myostatin (*MSTN*), is mainly expressed in skeletal muscle, and has a negative regulatory effect on the development of muscle mass[1]. Studies in many species have shown that MSTN gene mutation can cause abnormal muscle development, including an increase in the number of muscle fibers and an increase in the cross-sectional area of muscle fibers without causing severe adverse consequences, as previously reported in cattle[2], humans[3], sheep[4], dogs[5], pigs[6],goats[7] and birds[8]. Muscle mass is an important indicator in animal production, so many effective strategies have been made to product animals with increased muscle masses by preventing the expression of MSTN[7,29-23].

Previous studies had proved that *MSTN* have important effect on cell proliferation. Knockout of *MSTN* promote cell proliferation, while overexpression of *MSTN* inhibit cell proliferation and DNA synthesis[10,11,14]. In the present study, we studied the proliferation of MSTN mutant (MT) and wild-type (WT) bovine muscle satellite cells.

The *MSTN* mutant (MT) satellite cells were found to have a accelerated cell cycle than the control cells. Then we analyzed the cell cycle of these two cells by Flow cytometry. Results showed that cell number of G0/G1 phase reduced while the cell number of S phase increased compared with WT, these results indicated that *MSTN* mutant promoted the cell cycle mainly by promoting DNA synthesis. This was consist with the previous studies[10,12]. RNA-seq results also showed that *MSTN* mutant regulated the cell proliferation and cell cycle, especially G1/S transition. This further demonstrated that *MSTN* mutant promoted the cell cycle mainly by promoting DNA synthesis.

During the process of myogenic differentiation, myoblasts will withdraw from the cell cycle and obtain the apoptotic phenotype to fuse into myotubes. In the present study, when the MT and WT MSCs were induced to myogenic differentiation, MT cells quit from the cell cycle and began to differentiation earlier than the WT controls. This was consist with the previous study[11,24,25]. Then we detected the cell cycle during myogenic differentiation, results showed that MT MSCs withdraw from the cell cycle and began to differentiate immediately when induced to myogenic differentiation, but WT MSCs occurred these after two days of myogenesis induction. So the promotion of myogenic differentiation due to *MSTN* mutant had two reasons: on one hand, *MSTN* mutant promoted the expression of myogenic related genes such as *MyoD* and *MyoG*[26,27], then to promote myogenic differentiation; on the other hand, *MSTN* mutant promoted the cells withdraw from the cell cycle and began to differentiation earlier than the control, thus to promote myogenic differentiation[24].

Cell cycle progression was regulated by a series of related genes, so we detected the expression of cell cycle related genes. The expression of Cyclins and CDKs were all up-regulated, while the Cyclin dependent kinase inhibitors, such as CDKN1C (*P57*), *CDKN2A*, *CDKN2C* and *CDKN2D* were down-regulated, which were consistent with the promotion of cell proliferation. Among which the expression of *CyclinA* up-

regulated most significantly and CDKN1C down-regulated most significantly, these results indicated that *MSTN* mutant promoted MSCs proliferation mainly by down-regulating *CDKN1C*, then promoting the *CyclinA-CDK2* expression, thus to promote the synthesis of DNA and cell cycle.

SMAD were key transcription factors in TGF-β pathway. In this study, we obtained DEGs from MT and WT cattle muscle tissue focused on SMAD binding, this indicated that *MSTN* may regulate cell cycle by affecting the binding of SMAD transcription factors with the downstream genes. At the same time, previous studies have shown that up-regulation of TGF-β will increase the expression of CDK inhibitor p15, p16, p21 and p27 by increasing the binding of pSMAD3 to the their promoters[28]. So we intend to investigate that if *CDKN1C* (*P57*) can also bind with SMAD2/SMAD3. We predicted the binding of SMAD2/SMAD3 complex to the region of *CDKN1C* promoter by JASPAR database, subsequently a ChIP assay were performed using an anti-SMAD2/SMAD3 monoclonal antibody. Results of ChIP-qPCR confirmed the directly bound of SMAD2/SMAD3 complex with the *CDKN1C* promoter region. Moreover, overexpression of *SMAD3* promoted the expression of CDKN1C. Since MSTN mutations have been found to reduce the activity of SMAD2/SMAD3 complex[29,30], so in the present study, *MSTN* mutant decreased the promotion of SMAD2/SMAD3 to the expression of CDKN1C, so the expression of CDKN1C was decreased, and then promoted the CyclinA-CDK2 expression, thus to promote the synthesis of DNA and cell cycle.

Conclusion

In summary, the *MSTN* mutation leads to decreased activity of SMAD2/SMAD3, which bound directly to the promoter region of *CDKN1C*, *SMAD3* overexpression promoted the expression of *CDKN1C*, so *MSTN* mutant down-regulated the expression of *CDKN1C* by SMAD2/SMAD3 pathway. Down-regulation of *CDKN1C* then promoted the CyclinA-CDK2 complex, then promoted the cell proliferation.

Abbreviations

MSTN: Myostatin; MSCs:smuscle satellite cells; MT: *MSTN* mutant; WT: wild type; ChIP: Chromatin immunoprecipitation; KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: Gene Ontology; DEGs: Differentially Expressed Genes.

Declarations

Acknowledgments

Not applicable.

Authors' contributions

The authors' contributions are as follows: Chunling Bai and Li Gao conceived and designed the study; Li Gao , Mingjuan Gu , Yunpeng Liu and Caihong Bu conducted the research, Miaomiao Yang and Peng

Cheng analyzed and interpreted the data; L. G. wrote the manuscript. All authors read and approved the final version of the manuscript.

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Declarations

Ethics statement

All animal operating procedures were authorized by the Inner Mongolia University and Baotou Teacher's College Committee on the Ethics of Animal Experiments. All methods were performed compliance with the relevant policies and guidelines.

Competing interests

The authors declare that there is no conflict of interest.

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Figures

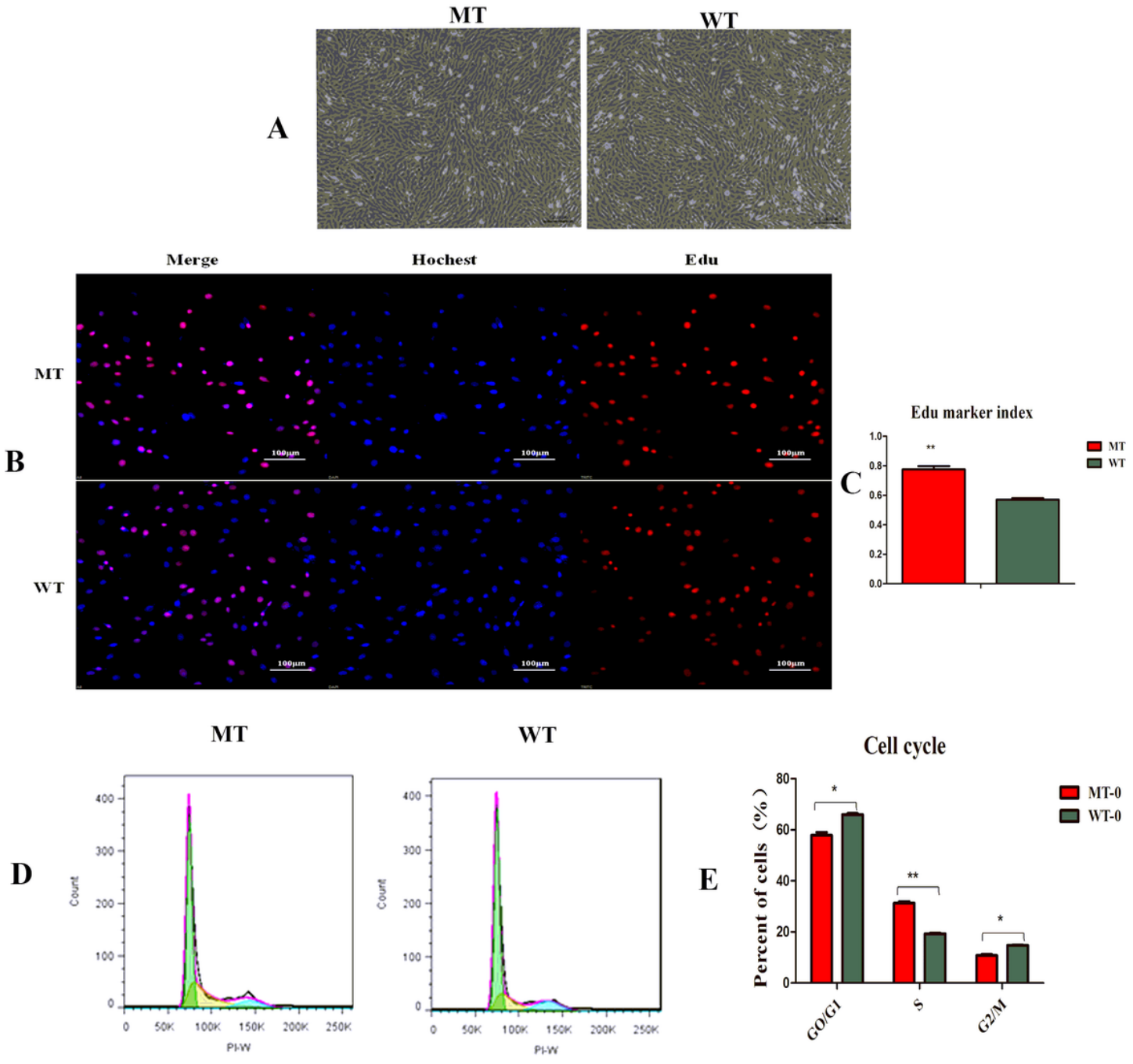

Figure 1

MSTN mutant promoted the proliferation of muscle satellite cells (MSCs). A, Morphology of the WT and MSTN mutant (MT) bovine muscle cells. Magnification of microscope is 50×. B, Proliferation of MSCs was analyzed by EdU assays. Blue, cell nuclei stained with Hoechst 33342. Red, EdU staining; C, Edu marker index (the number of new synthesized nuclei (red)/the number of all nuclei (blue), n=5).D,Cell cycle of MT and WT MSCs analyzed by Flow cytometry. E, cell Cycles (n=3). T-test was used to generate p-value. *p<0.05,**p<0.01,***p<0.001.

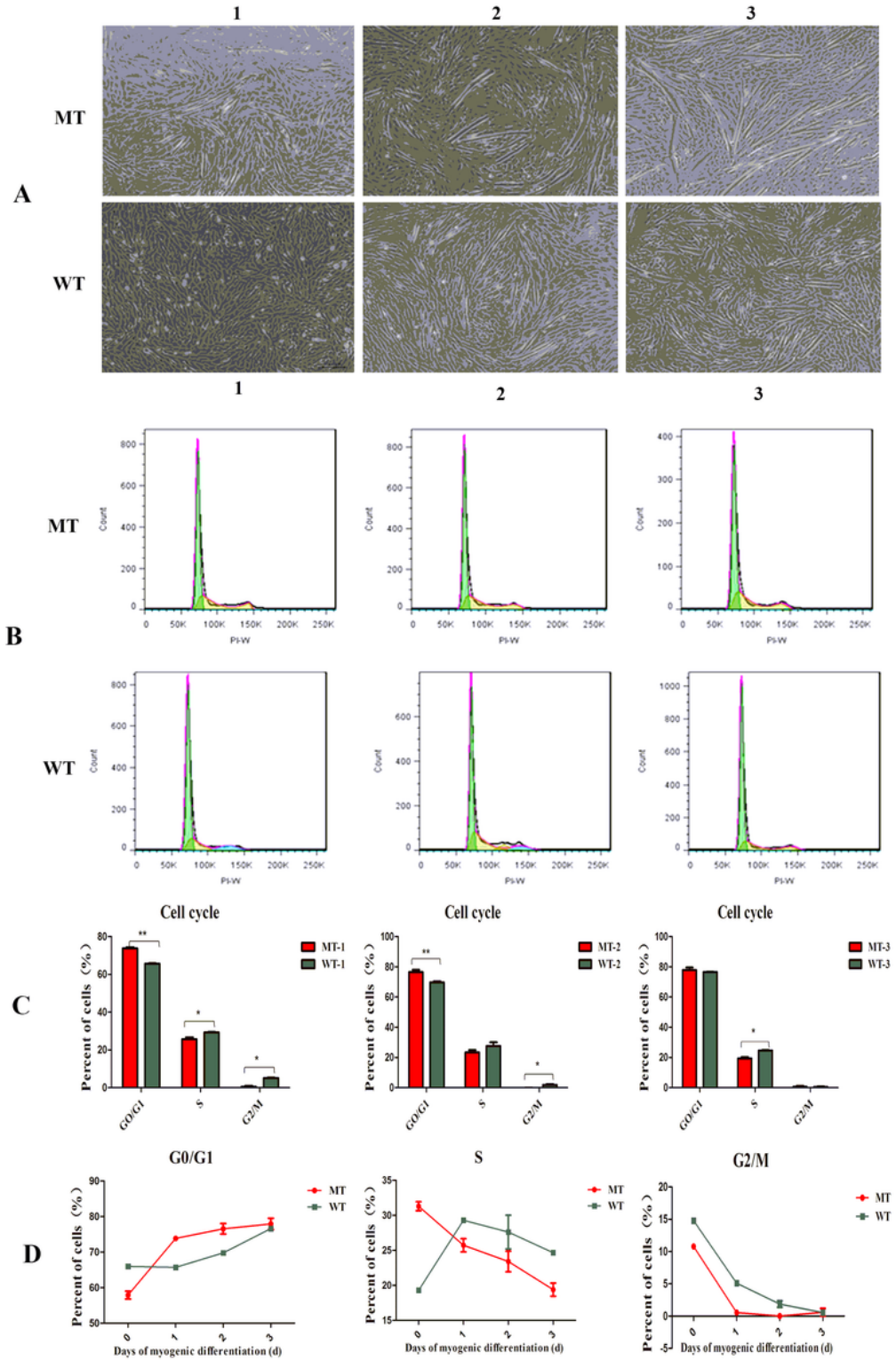

Figure 2

MSTN mutant promoted the myogenic diffefentiation by exiting the cell cycle earlier. A,The differentiated MT and WT MSCs of 1, 2 and 3 days. MT MSCs began to differentiate on day 1 when induced to myogenic differentiation, while WT MSCs began to differentiate on day 2 when induced to myogenic differentiation. B, Cell cycle of differentiated cells analyzed by Flow cytometry. C, Counted cell cycles

(n=3). D, Changes in the proportion of cells in each stage of MT and WT cells after induced to myogenesis. T-test was used to generate p-value. *p<0.05,**p<0.01,***p<0.001.

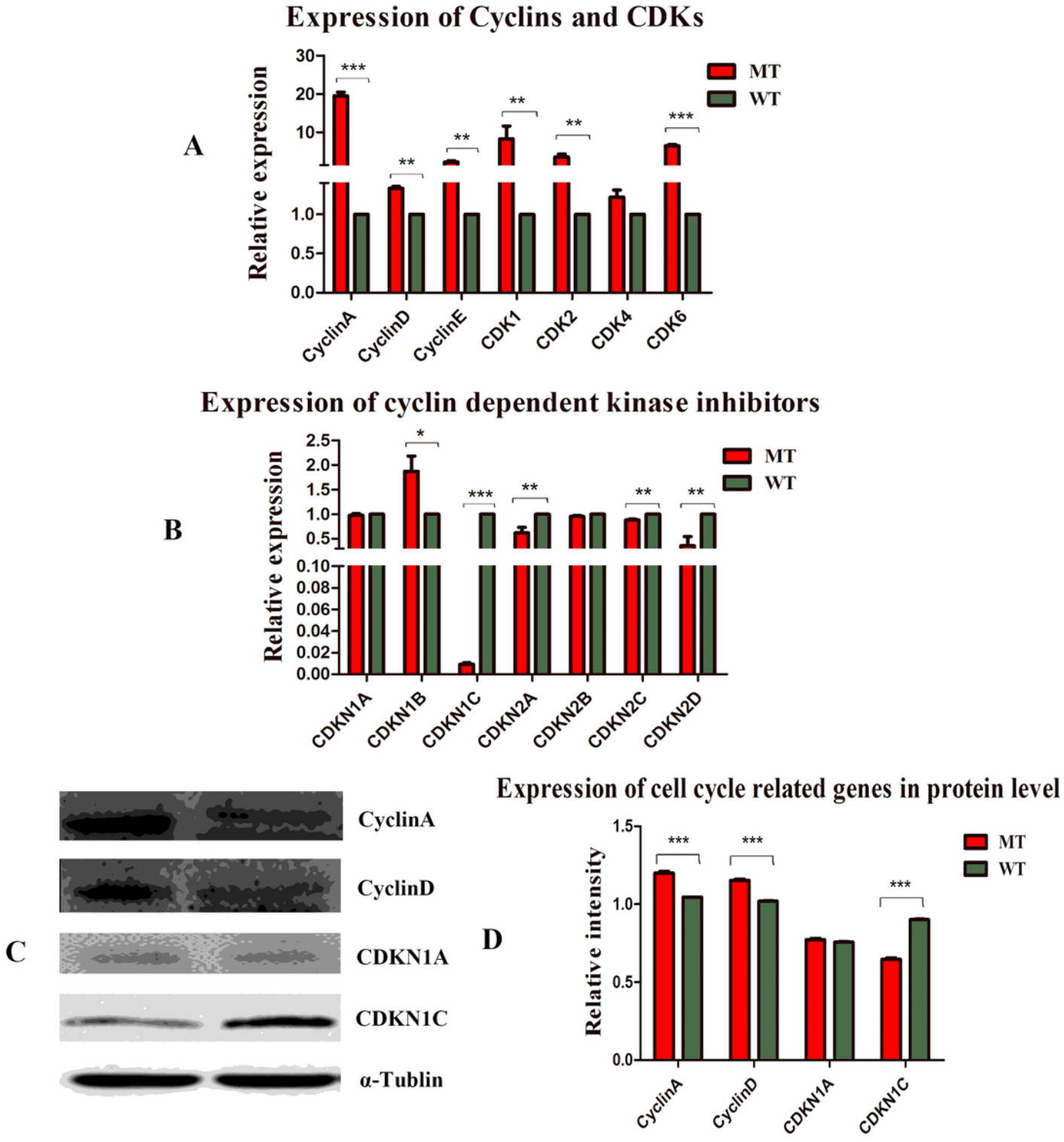

Figure 3

MSTN mutant promoted cell proliferation mainly by down-regulating CDKN1C. A, Expression of CDKs and Cyclins in mRNA level. B, Expression of cyclin dependent kinase inhibitors in mRNA level. C, Expression of

Cyclins and cyclin dependent kinase inhibitors in protein level detected by western blot and quantified in D. T-test was used to generate p-value.*p<0.05,**p<0.01,***p<0.001.

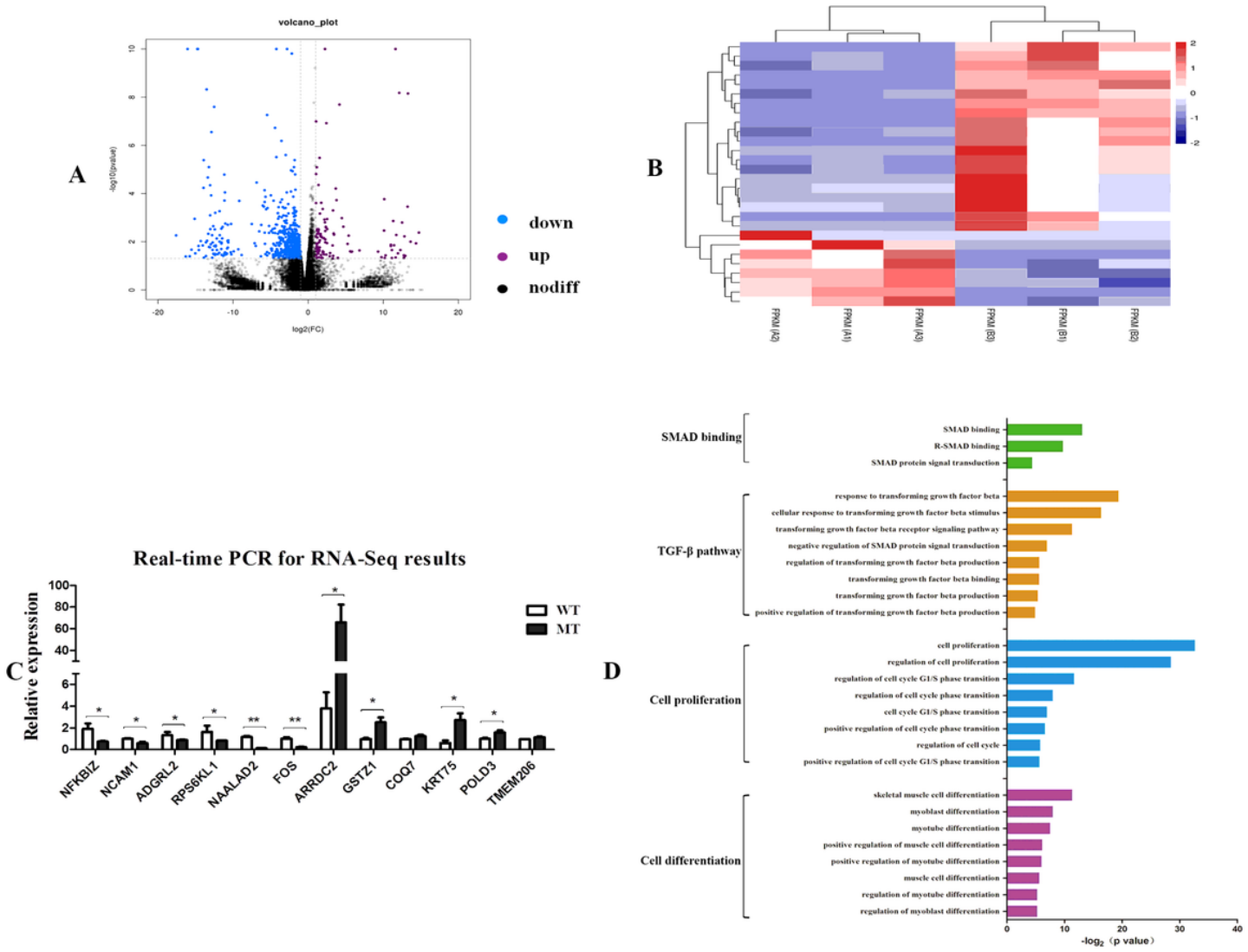

Figure 4

RNA-seq analysis. A, The differential expression genes (DEGs) between MSTN gene edited and control cattle muscle tissues. B, Heatmap of the most 30 significant DEGs between MSTN gene edited and control cattle muscle tissues. Red represents the up-regulated genes, blue represents the down-regulated genes. C, qPCR data for differential expression genes. D, GO enrichment analysis of DEGs. The DEGs were enriched on GO terms of cell differentiation, cell proliferation, TGF-β pathway and SMAD binding.

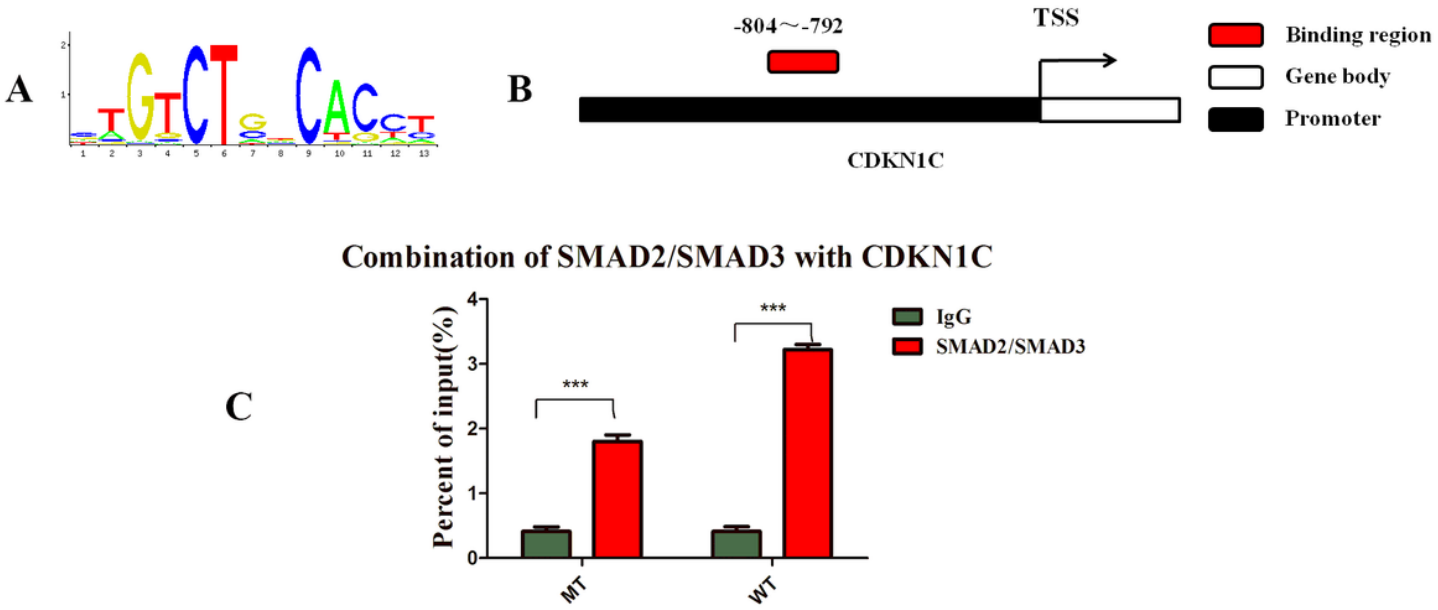

Figure 5

MSTN regulated CDKN1C expression by SMAD2/SMAD3 pathway. A, The motif of SMAD2/SMAD3 transcription factor. B, Binding region of SMAD2/SMAD3 complex with CDKN1C promoter. C, The binding of CDKN1C promoter with SMAD2/SMAD3 complex detected by ChIP-qPCR. The binding region on the promoter was from -804 to -792 of CKDN1C. T-test was used to generate p-value.*p<0.05,**p<0.01,***p<0.001.

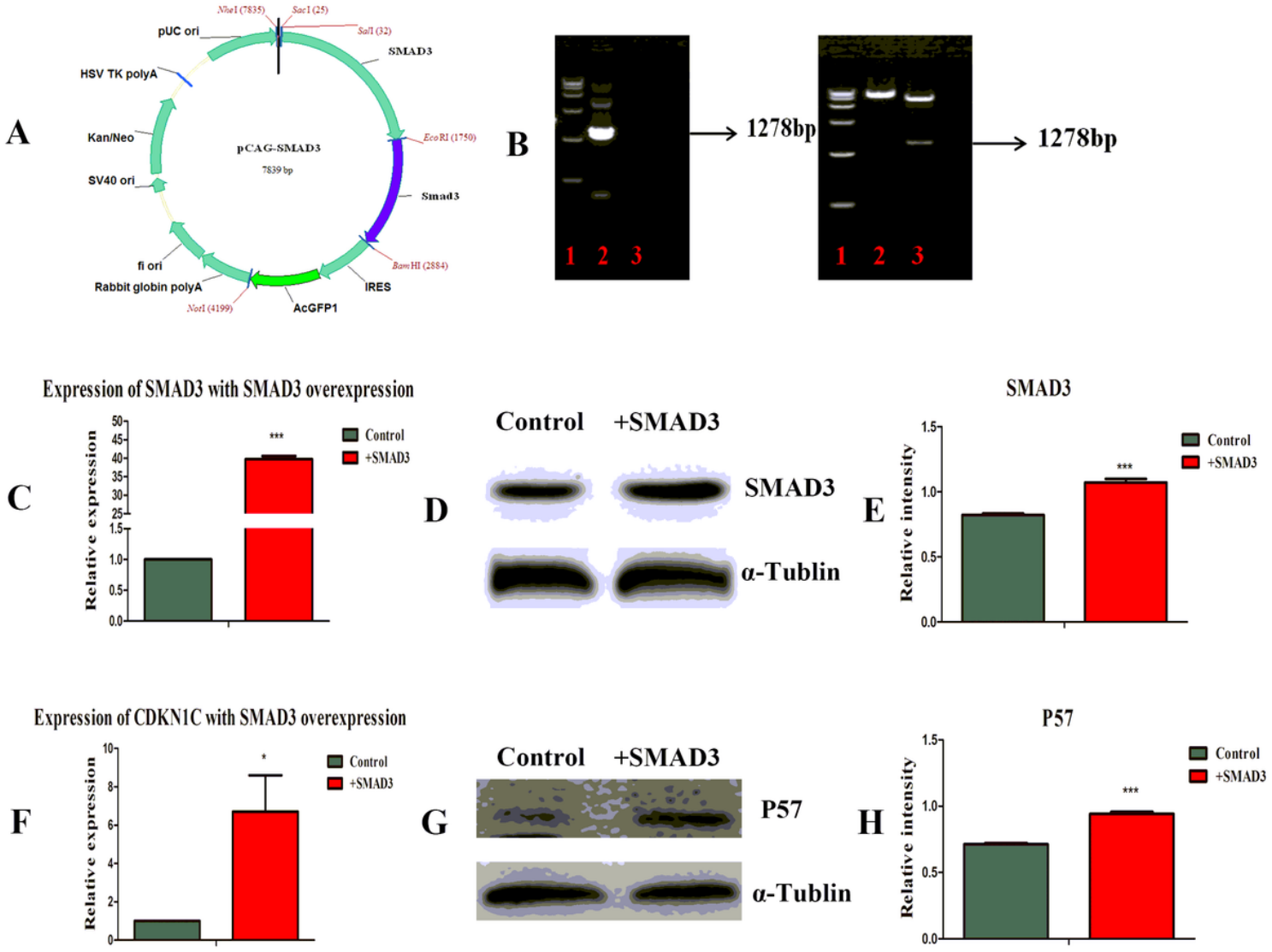

Figure 6

Overexpression of SMAD3 promoted the expression of CDKN1C. A, The overexpression vector of SMAD3. B, left: identification of the pCAG-SMAD3-IRES-AcGFP plasmid by PCR, 1,DL 5000 marker; 2, plasmid; 3, ddH2O; right: identification of the pCAG-SMAD3-IRES-AcGFP plasmid by restriction enzyme digestion. 1, DL 5000 marker; 2, Single digestion by EcoRI; 3, Double digestion by EcoRI and BamHI. C, Expression of SMAD3 in mRNA level after overexpression of SMAD3 to muscle satellite cells of wild type; D and E, Expression of SMAD3 in protein level. F, Expression of CDKN1C in mRNA level after overexpression of SMAD3 to wild type muscle satellite cells. G and H, Expression of CDKN1C in protein level. T-test was used to generate p-value.*p<0.05,**p<0.01,***p<0.001.

Supplementary Files

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- SupplementaryFiles.pdf