Article

MSTN Regulatory Network in Mongolian Horse Muscle Satellite Cells Revealed with miRNA Interference Technologies

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Abstract: Myostatin (MSTN), a member of the transforming growth factor-β superfamily, inhibits the activation of muscle satellite cells. However, the role and regulatory network of MSTN in equine muscle cells are not well understood yet. We discovered that MSTN knockdown significantly reduces the proliferation rate of equine muscle satellite cells. In addition, after the RNA sequencing of equine satellite cells transfected with MSTN-interference plasmid and control plasmid, an analysis of the differentially expressed genes was carried out. It was revealed that MSTN regulatory networks mainly involve genes related to muscle function and cell-cycle regulation, and signaling pathways, such as Notch, MAPK, and WNT. Subsequent real-time PCR in equine satellite cells and immunohis- tochemistry on newborn and adult muscle also verified the MSTN regulatory network found in RNA sequencing analysis. The results of this study provide new insight into the regulatory mechanism of equine MSTN.

Keywords: horse; satellite cell; MSTN gene; RNA sequencing; signaling pathway; cell proliferation

1. Introduction

MSTN knockout increases mice skeletal muscle mass as a result of both hyperplasia (increase in number) and hypertrophy (enlargement) of the muscle fibers [1], and the mutations in the MSTN gene could result in the “double muscling” phenotype in cattle [2,3], sheep [4], dogs [5], and humans [6]. MSTN, often known as the “speed gene” in horses [7], has been acknowledged as a significant genetic factor influencing race distance aptitude [8,9]. Numerous sequence variations have been found in the MSTN gene’s upstream and downstream regions, and research has examined their relationships to Thoroughbred horses’ race performance [10–12]. Researchers have used CRISPR/Cas9 editing technologies to make MSTN-null horse embryos with the goal of artificially enhancing equine sports performance [13,14]. The equine’s general health is significantly impacted by skeletal muscle metabolism. The majority of research on equine skeletal muscle has concentrated on a small number of specific metabolites produced during acute exercise or training [15]. After suffering a muscle-fiber injury, skeletal muscles in particular have an extraordinary ability for regeneration, allowing for the complete restoration of their structure and function within a few weeks [16]. Direct trauma and excessive exercise can harm a horse’s myofibers over the course of a lifetime [17], and the activation [18], proliferation [19], and terminal...
differentiation of muscle quiescent satellite cells (SCs) [20] are crucial to their recovery. At present, little is known about the MSTN regulation network in equine muscle SCs, despite the fact that the impact of MSTN knockdown and MSTN-related signaling pathways in other mammalian skeletal muscle SCs have been examined [20–25]. In this study, we used miRNA interference approaches to expose the MSTN regulation network in equine muscle SCs because MSTN is an important gene for comprehending the mechanisms underpinning equine muscle development and regeneration [26].

2. Materials and Methods

2.1. In Vitro Culture of Horse Muscle SCs

Semitendinosus muscle samples were collected from healthy horses at the local slaughter house. When the muscle sample arrived at the lab, it was immediately sanitized with 70% ethanol and washed three to four times with 4-fold volume cold DPBS. After the visible adipose and connective tissues on the muscle mass were removed with a knife, all the muscles were excised and chopped into little pieces with scissors. With collagenase type IV (Sigma-Aldrich, St. Louis, MO, USA) and trypsin (0.25%, Sigma-Aldrich) solutions, fractionated enzymatic digestion was carried out for 2–30 min at 37 °C while stirring in a water bath. The cell suspension was then progressively filtered through 70 µm and 40 µm cell strainers. The cell pellet was then extracted from the filtrates using a centrifuge. The cells pellet was then transferred to culture disks and resuspended in media (20% FBS/DMEM/AB), which were then incubated at 37 °C with 5% CO₂. In total, 1.5 h of preplating were utilized to reduce any potential fibroblast contamination. Satellite-cell-containing supernatant was then added to culture disks. Every two days, the growing media were changed. The cells were passaged at a ratio of 1:3 after reaching 80% confluence.

2.2. Immunofluorescence Assay

After being fixed with 4% paraformaldehyde for 30 min, the cells were rinsed with PBS containing BSA and Triton X-100, blocked for 1 h at 37 °C with PBS containing Triton X-100, and then incubated with the primary Pax7 (AB-528428, DSHBY) and desmin (LSB3122, LSBio) antibodies overnight at 4 °C. Following three cycles of washing, samples were incubated with the secondary antibody for 1 h at room temperature. The cells were then washed three times, exposed to Hoechst (1 mg/mL, 10 min at RT), incubated, rinsed once more, and mounted.

2.3. Plasmid Construction and Transfection Analysis

The miRNAs targeting MSTN (miRNA267 and miRNA364) were designed with BLOCKit RNA DESIGN (https://www.thermofisher.cn/cn/zh/home/life-science/rnai.html (accessed on 7 October 2022)) based on the sequence of Mongolian horse MSTN CDS.

In order to create the MSTN knockdown plasmid, these two miRNAs with BglII and EcoRI restriction sites were separately ligated with Td Tomato-C1 vector and then transformed into the competent cells DH5α. The plasmid was verified by restriction digestion and sequencing, and finally the correct interference plasmid was purified and named Td Tomato-mirRNA267(364)-eMSTN.

SCs were transfected with the Td Tomato-miRNA plasmid using Lipofectamine 2000 (Life Science) at a plasmid density of 60,000 cells/cm². At 36 h post transfection, the fluorescence signal was observed with a fluorescence microscope to verify the transfection efficiency.

2.4. RNA Sequencing (RNA-seq)

At 36 h, the cell culture fluid was removed and the cells were washed with PBS three times. Finally, each well was collected with 1 mL Trizol reagent (Invitrogen, Waltham, MA, USA) to collect the cell sample; then, the total RNA could be extracted immediately, or it could be stored in an ultra-low temperature refrigerator at −80 °C for later use. The RNA
Library Prep Kit for Illumina (NEB, Ipswich, MA, USA) was used to create a library, and the Illumina HiseqTM2500 was used to perform the sequencing.

2.5. Screening of Differentially Expressed Genes (DEGs)

DEGs in MSTN knockdown group compared with controls of equine SCs were identified using the DESeq R package from www.bioinfo.au.tsinghua.edu.cn/software/degseq, accessed on 7 October 2022. Differential gene screening mainly refers to the difference fold (Fold change value) and q value (padj value, corrected p value) as related indicators; the genes with expression \(|\log_2 \text{fold change}| \geq 1\) and \(q < 0.05\) were selected as DEGs.

2.6. Gene Ontology (GO) and KEGG Pathway Enrichment Analysis

DEGs were annotated using the GO enrichment analysis, in which gene length bias was corrected. GO terms with the corrected value \(p < 0.05\) were considered significantly enriched. Signaling pathways were investigated using KEGG (Kyoto Encyclopedia of Genes and Genomes), and KOBAS version 2.0 software. \(q < 0.05\) was considered a significant value.

2.7. Quantitative PCR (qPCR) Assay

Trizol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to separate the RNA from the MSTN knockdown and control groups, and a reverse transcription kit was used to convert the RNA samples into cDNA (Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). Using \(\beta\)-actin as a control, 14 genes were chosen for validation of the RNA sequencing gene profiling results. In total, 10 \(\mu\)L of SYBR Green PCR Master Mix, 2 \(\mu\)L of cDNA, 1.2 \(\mu\)L of each primer (10 \(\mu\)M), and 6.8 \(\mu\)L of RNase-free water made up the SYBR Green PCR experiment. The cycling schedules were as follows: 95 °C for 1 min, 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Using the \(2^{-\Delta\Delta CT}\) approach, the levels of gene expression were calculated. The parallel experiment has at least three replicates, and the data’s mean value of Mean ± S.D. is shown. In this study, a significance level of \(p < 0.05\) is accepted. Table S1 contains a list of the primers utilized for this study.

2.8. Immunohistochemistry

Equine muscle cryosections of 8 \(\mu\)m were prepared from the Tissue-Tek embedded samples and were collected onto coated glass slides (Thermo Scientific SuperFrost Plus Adhesion slides, Fisher scientific, Brussels, Belgium) and stored at \(-20\) °C. The muscle cryosections were air-dried and then blocked for 120 min in 5% defat milk in PBS solution at room temperature. The slides were washed with permeabilization solution (BSA and 0.2% triton in PBS solution) and incubated overnight at \(-4\) °C with primary antibodies for MSTN (DF13273, Affinity, Hong Kong, China), MyoD1 (MA5-12902, Thermo Fisher), and MyoG (A17427, ABclonal, Wuhan, China) at a dilution of 1:500. After the slides were rinsed 5 min in PBS, they were washed briefly with permeabilization solution and incubated with the secondary antibodies dissolved in 0.5% BSA in PBS for 1 h at room temperature. Subsequently, after rinsing the slides for 5 min in PBS, the DAB Horseradish Peroxidase Color Development Kit (HZ-0010, Luzhen Biology, China) was applied. The staining intensity of 10 random units at a 200× amplification (with each muscle fascicle as the measure unit) was quantified by the well-established Image-J-software-based method indicated in the previous studies [27–29].

2.9. Statistics

There are at least three replicates of the parallel experiment, and the mean value of Mean ± S.D is indicated for the data. All data were analyzed by SPSS16.0 using two-way ANOVA with repeated measures, followed by Tukey’s test. A difference was deemed statistically significant if the \(p\)-value was less than 0.05.
3. Results

3.1. The Characteristics of Horse Muscle SCs

We were able to successfully separate and purify the Mongolian domestic horse muscle satellite cells (HMSC) in vitro using the methods of enzyme digestion and differential adhesion. The horse fetal fibroblast (HFF) is a polygonal cell type, as can be seen in (Figure 1A), whereas the HMSC have finer edges and are primarily spindle-shaped. Additionally, HMSC demonstrated a different rate of in vitro proliferation, demonstrating that its proliferation is slower than that of HFF ($p < 0.05$). (Figure 1B). Additionally, the results of qPCR (Figure 1C) and cell immunofluorescence test (Figure 1D–F) jointly demonstrated that HMSC, but not HFF, exhibits significant expression of genes specific to mammalian SCs, such as Desmin, Pax7, and MyoD1.

3.2. MSTN Knockdown Accelerates HMSC Cell Growth

The interference plasmids miR267 and miR364 were created in this investigation by designing two miRNAs (miR267, miR364) that target horse MSTN mRNA and cloning them into the Td Tomato-C1 plasmid (Figure 2A). The CtrlmiR plasmid containing a control miRNA was employed in this investigation as a control. Based on the red fluorescence of Td Tomato, the transfection efficiencies of three different plasmid types reached 80% after...
24 h (Figure 2B), and the expression of MSTN mRNA was dramatically repressed \((p < 0.05)\) (Figure 2C). Furthermore, we discovered that MSTN knockdown might greatly improve the cell proliferation rate of HMSC during subsequent culture, particularly during the first 48 to 72 h (Figure 2D).

3.3. MSTN Knockdown Alters the Transcriptome

Two samples of each cell line were sequenced, and RNA-seq libraries were created for the control (transfected with CtrlmiR) and experimental (transfected with miR267 and 364) groups to profile gene expression after MSTN knockdown. After eliminating adapters and removing low-quality reads, the sequencing provided a high-quality dataset (Figure 3A–C) and about 78,134,605 clean reads.

3.4. DEG Analysis Reveals the MSTN Regulatory Networks in HSMCs

To examine variations in gene expression, read count data from the transcriptome were used. There were 598 DEGs in all that showed a difference expression in the experimental and control groups. When the threshold values were \(q < 0.05\) and \(|\log_2\text{Fold change}| \geq 1\), 427 of them were up-regulated and 171 were down-regulated in the experimental groups compared to the control group (Figure 3D,E). Three categories were created from the results of the GO enrichment study of DEGs: biological process (BP), cellular component (CC), and molecular function (MF). The cellular activities, developmental processes, and biological regulation in terms of BP; the cell part, organelles, and membranes in terms of CC; and the catalytic activity, signal transduction activity, and binding in terms of MF were shown to be enriched in the down- and up-regulated genes (Figure 3F). According to KEGG analysis, DEGs are involved in 37 pathways, including the mitogen-activated protein kinase (MAPK) signaling pathway, the TGF-\(\beta\) signaling pathway, the signaling pathway that controls stem cell pluripotency, the signaling pathway that controls the actin skeleton, the PI3A-Akt signaling pathways, the cancer pathways, and the signaling pathway for cell adhesion molecules (Figure 3G). In total, 14 DEGs associated with myogenesis and the satellite cell cycle were chosen for qPCR experiments to confirm the RNA-seq results. Overall, the qPCR and RNA-seq results were well correlated, proving the accuracy and reliability of the RNA-seq data (Figure 3H).
Figure 3. Analysis of differentially expressed genes (DEGs) after MSTN knockdown in HMSC. (A) Clean base number of all sample readings. (B) Filtering distribution map indicates the high quality of readings. (C) The location of readings indicates the normal distribution ratio of read sequences. (D) Clustering of DEGs among samples. (E) Volcano plot for the DEGs between control group and MSTN knockdown group. (F) Histogram of GO enrichment of DEGs. (G) KEGG pathway analysis on DEGs. (H) qPCR validation of DEGs. Red line represents the gene expression level in control group, and all bars represent the expression levels of tested genes in experimental groups treated with different miRNA targeting MSTN. All tested genes in experimental groups showed significant changes at $p < 0.05$ compared to the control group.

3.5. The MSTN Regulatory Network in the Horse Muscle Was Verified by Immunohistochemistry Data

We used immunohistochemistry to examine the expression of MSTN, MyoG, and MyoD1 in adult and newborn equine muscle tissues because the in vivo expression of MSTN in muscle tissue declines with age. This allowed us to determine whether the expression of the positively regulated DEG MyoG, and the negatively regulated DEG MyoD1 also changed in a manner that was consistent with age. The immunohistochemistry results confirmed the findings of RNA-seq and qPCR, demonstrating that MSTN negatively regulates MyoD1 expression while positively regulating MyoG at the protein level (Figure 4).
Autonomous Region of China and Mongolia consider the Mongolian horse to be one of the most significant breeds of traditional long-distance races [30,31], is renowned for its exceptional endurance and robust genetic variety [30,32]. As a result, the Inner Mongolia Autonomous Region of China and Mongolia consider the Mongolian horse to be one of the most significant breeds of traditional long-distance races [32].

The procedure of harvesting SCs from equine muscles was first described in 1992 [33] and has been used to examine the impact of exercise on muscle tissue [34]. However, there haven’t been many investigations into the processes that underlie MSTN’s function in the horse model. When tissue is damaged or injured, SCs change to a proliferative state, which enables the production of a large cell pool suited for myogenic differentiation [35].

Our research shows that, similar to SCs from other species, SCs from horses exclusively express Pax7, MyoD1, and Desmin [36–38]. In the population of adult SCs, Pax7 is expressed in both quiescent and active conditions [39]. Equine SCs grown in culture have “wedge” morphologies and have condensed interphase chromatin, which is in line with the idea that the majority of SCs in resting muscles are quiescent and transcriptionally inactive [40,41].

The MSTN CDS region’s full-length sequence, which was cloned from a muscle sample from a Mongolian horse in the current work, is 1134 bp, and it is entirely consistent with the Thoroughbred MSTN CDS sequence supplied by NCBI (Accession number: NM_001081817.1). We discovered that MSTN knockdown could boost the SCs’ proliferation rate through the very effective RNAi studies. The MSTN knockdown investigations on the SCs of other animals likewise reported the same behavior. A previous mouse study discovered that MSTN functions through a complex regulatory network that includes PAX, Myosin family proteins, WNT, MAPKmTOR family members, and numerous genes related to CDKN1C (p57, Kip2) that control the cell cycle [42]. Similar to this, our RNA-seq analysis following MSTN knockdown revealed that MSTN is involved in the regulation of multiple pathways crucial for the activities of muscle cells in horse muscle SCs.

Figure 4. Immunohistochemistry staining of MSTN, MyoG, and MyoD1 expression in newborn and adult equine muscles. The positive signals are quantified in left bar charts. * Indicates the significance at the level of $p < 0.05$.

4. Discussion

An ancient horse breed known as the Mongolian horse, which has long been a staple of the nomadic pastoral herders’ culture in North Asia [30,31], is renowned for its exceptional endurance and robust genetic variety [30,32]. As a result, the Inner Mongolia Autonomous Region of China and Mongolia consider the Mongolian horse to be one of the most significant breeds of traditional long-distance races [32].

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According to our findings, we discovered that a number of DEGs are connected to the PI3K/AKT/mTOR signaling pathways, which are crucial for mediating a variety of cellular functions, including nutrition uptake, anabolic responses, cell development, and survival. Phosphatidylinositol 3-kinase (PI3Ks), AKT, and the mammalian target of rapamycin (mTOR, also known as mechanistic TOR), which make up the heart of this pathway, are frequently over-activated in most malignancies and have thus come to be the subject of research in this area [43]. Additionally, it has been noted that the MSTN-Smad pathway affects protein kinase AKT’s activity, preventing the mTOR pathway and protein synthesis [44]. DEGs belonging to WNT signaling also emphasize the critical functions of WNT members in muscles. The previous study found that WNT signaling might be activated after muscle damage and that myogenic cells’ TCF reporter activity increased two days after muscle damage [45]. Unlike Wnt1, Wnt3a, and Wnt5a, which promote SCs proliferation, Wnt4 and Wnt6 inhibit it [19]. Although Wnt4 has a beneficial role in controlling the proliferation of SCs, MSTN can operate as Wnt4’s upstream antagonist to prevent Wnt4-mediated SCs growth. Additionally, it has been demonstrated that MSTN increases the expression of the WNT signaling pathway inhibitors sFRP1 and sFRP2 [46]. We discovered that Wnt5a greatly increases following MSTN interference, suggesting that WNT is also involved in the SCs regulation controlled by MSTN. We also focused on aspects of cell cycle regulation in this work. SCs are in a quiescent state in the adult resting muscle [47], which is characterized by a low rate of metabolism, a lack of cell cycling (G0 phase), and low RNA concentration [48]. In cell-culture studies, mechanistically, MSTN interacts with the cell cycle machinery to induce the cell cycle exit during the gap phases (G1 and G2) [49]. In bovine skeletal muscle SCs, MSTN knockdown resulted in an increase in CDK2 expression, a decrease in P21 expression, and a stimulation of proliferation [50]. The increased production of the cyclin-dependent kinase inhibitor, P21, which further suppresses the gene essential for the G1/S transition, Cdk2, is thought to be the cause of the MSTN-triggered halt of the cell cycle and the inhibition of proliferation [51–53]. Compared with activated/cycling SCs, many negative regulators of the cell cycle and myogenic inhibitors, including cyclin-dependent kinase inhibitors 1B (Cdkn1b; also known as p27 or p27kip1 [54]) and 1C (Cdkn1c, also known as p57 or p57kip2); Rb [55] (also known as Rb1); and Rgs2, Rgs5, Pmp22, and FGF suppressor gene Spry1 [56], were highly up-regulated in quiescent SCs [57,58]. Our RNA-seq results mainly agree with those from studies on different species and models, suggesting that one of the main roles of MSTN is cell-cycle regulation.

Pax7 is robustly and consistently expressed in the SCs of adult muscle, whereas Pax3 is often expressed at very low levels, with the exception of muscles such as the diaphragm [59]. In our study, Pax7 and Pax3 expression did not alter significantly; however, Pax5 expression significantly decreased. This distinction leads us to believe that different species have different regulatory roles for PAX family proteins in the SCs. However, more research is required to make this clear. Myogenic cells can selectively express the genes Myf5 and MyoD1, whereas resting SCs cannot. When SCs enter the cell cycle again and differentiate into highly proliferating myoblasts, Myf5 and MyoD1 are elevated. Some cells exit the cell cycle during the proliferation phase, start to express MyoG, and then differentiate into new muscle fibers, while other cells lose their myogenic capabilities by lowering the level of MyoD1 expression and return to a resting state to refill the SCs pool [60]. In our research, after MSTN knockdown in horse muscle SCs, MyoD1 increased, whereas MyoG decreased. Together with the age-related MSTN downregulation-induced MyoD1 increase and MyoG decrease in horse muscle, the critical role of MSTN in the entire life cycle of muscle cells is highlighted here.

In conclusion, the MSTN regulatory network described here advances our understanding of the function and activity of equine muscle SCs and encourages the use of this information in tissue engineering, regenerative medicine, and related fields of study.
Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes13101836/s1, Table S1: Q-PCR primers for DEG validations.

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