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MED4 gene positively affects preadipocyte differentiation in Chinese red steppe cattle

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Key words: Chinese red steppe cattle, *MED4*, *PPARγ*, Predipocyte differentiation, Lipid metabolism, Meat quality trait

Abstract: Background: The regulatory genes related to lipid metabolism affect the intramuscular fat (IMF) content and improve meat quality traits. Mediator Complex Subunit 4 (MED4), a vitamin D receptor protein, affects vitamin D, livestock growth, carcass traits, and triglyceride deposition. However, the physiological function of the *MED4* gene on bovine adipocyte differentiation remains unknown. **Methods:** This study explored the function of the *MED4* gene in preadipocyte differentiation in Chinese Red Steppe cattle. The overexpression plasmid and the interference sequences of the *MED4* gene were constructed to detect the effects of the *MED4* gene on adipogenesis and biomakers using quantitative polymerase chain reaction and western blotting. **Results:** The *MED4* gene had significantly high expression during preadipocyte differentiation ($p < 0.05$). Overexpression of the *MED4* gene increased the expression of the *PPARγ* gene, a preadipocyte differentiation biomarker, improved cellular lipid droplets and triglycerides accumulation, and positively accelerated adipocyte maturation ($p < 0.05$). Interference of the *MED4* gene can negatively regulate preadipocyte differentiation. **Conclusion:** This study showed that the *MED4* gene may affect the preadipocyte differentiation and adipogenesis in Chinese Red Steppe cattle by regulating the *PPARγ* gene.

Introduction

Chinese Red Steppe cattle, a special breed with roughage tolerance, strong stress resistance, and high meat quality in China, is widely raised in northern China (Fang *et al.*, 2021). As the economy and livestock industry continues to develop, higher meat quality is becoming more popular with consumers. However, the growth rate and meat quality of the Red Steppe cattle are not able to meet the rising demand, making it crucial to improve the growth rate and meat quality trait under keeping the original characteristics of Red Steppe cattle. The intramuscular fat (IMF) content significantly affects meat quality traits, such as taste, flavor, tenderness, juiciness, and nutrients (Hocquette *et al.*, 2010; Ahmed *et al.*, 2022). High IMF content stored in the muscle is also called marbling, which provides more fatty acids composition and significantly improves the meat trait, and is highly appealing to consumers (Wen *et al.*, 2020; Yao *et al.*, 2022). Genetics plays a vital role in IMF formation and accumulation under the same feeding condition; therefore, it is important to explore genes affecting adipocyte differentiation and lipid metabolism (Estany *et al.*, 2017; Miao *et al.*, 2021).

IMF comprises white adipose tissue, an endocrine organ that stores energy in the form of triglycerides (TG), regulates the endocrine balance, and affects insulin sensitivity (Wang *et al.*, 2013; Kawai *et al.*, 2021). Preadipocyte derived from mesenchymal stem cells near the stromal vascular fraction (SVF) differentiate into mature adipocytes; this process is regulated by various biomarkers, for example, peroxisome proliferator-activated receptorγ (PPARγ) (Mota de Sá *et al.*, 2017; Wu *et al.*, 2022). PPARγ, a member of the nuclear hormone receptor superfamily, is a transcription factor that plays a vital and indispensable role in initiating and inducing adipocyte differentiation, which fails if PPARγ is not expressed (Yang *et al.*, 2018).

A mediator complex, comprising mediator complex subunit 4 (MED4) and another subunit, regulates the transcriptional function and influences the proliferation and differentiation of the cells (Verger *et al.*, 2019). Some studies

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have shown that the *MED4* gene affects poultry growth and carcass traits, and regulates IMF deposition in livestock (Zhang *et al*., 2021). However, the function of the *MED4* gene and its regulatory mechanism in bovine preadipocytes are not fully elucidated. This study explored the consequences of the *MED4* gene on adipogenesis, biomarker gene, lipid droplets, and TG accumulation in Chinese Red Steppe cattle. The study may provide a candidate gene or reference for improving the meat quality in cattle.

Materials and Methods

Animals

Male healthy Chinese Red Steppe calves at 2 months of age were obtained from the Jilin Academy of Agricultural Sciences and slaughtered for preadipocyte isolation. The animal experiment strictly complied with ethical requirements and operating rules. All animal procedures, such as welfare and ethical issues, were approved by the Committee for the Ethics of Animal Experiments by AWEC 2019A05, 16 May 2019.

Preadipocyte isolation, proliferation, and maturation

Subcutaneous white adipose tissue of calve was isolated and washed with PBS (Sigma-Aldrich, St. Louis, MO, US) containing 6% penicillin/streptomycin (Sigma). The samples were sliced into 1 mm^2 piece with sterile scissors and were digested with collagenase Type II (Sigma) and 0.25% trypsin (Sigma) for 1 h. The solution containing blocks and cells was filtered with a 40 μm filter to remove the undigested tissue pieces and obtain the preadipocytes. The cells were placed in a 37°C incubator (Thermo Fisher Scientific, Waltham, MA, United States) with 5% CO_2 to culture in a complete culture medium consisting of 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA, United States), 1% penicillin/streptomycin (Sigma), and Dulbecco's modified Eagle medium-basic medium (Sigma), the fresh culture medium was replaced once 48 h. After culturing for 6 h, the preadipocyte proliferation and differentiation at the end of proliferation was induced into the mature adipocyte with the exogenous supply of inducer containing inducer I and II. Inducer I solution consisted of 10 mg/mL insulin (Sigma), 1.0 mM dexamethasone (Sigma), 0.5 mM IBMX (Sigma) and was added to the culture medium to treat the cells for 2 days. Inducer II solution consisted of 10 mg/mL insulin (Sigma) and was added to the culture medium to treat the cells for 2 days. The cells were cultured in a fresh culture medium until preadipocyte maturation.

Oil red O staining

The lipid droplets in the mature adipocytes can be stained by Oil Red O dye to red color, and it is a general way to verify adipocyte maturation. The adipocytes were washed with cold PBS three times, treated with 4% paraformaldehyde for 30 min in a 37°C incubator, and then washed again. Oil Red O solution containing Oil Red O dye and ddH_2O in a volume ratio of 3:2 was used to treat cells for 30 min in an incubator. The mature adipocytes were photographed and analyzed under a microscope after being washed with PBS. Finally, an isopropanol solution was used to absorb the lipid droplets, then the solution was mixed with the lipid to determine the lipid droplet content at an OD value of 490 nm.

Construction of overexpression plasmid and synthesis of interference sequences

The primers to amplify the coding-sequence region (CDs) region of the *MED4* gene were designed according to the bovine sequences (NM_001034486.1) available on the NCBI website. Overexpression plasmid pEX4 vector was used to construct the recombination vector containing MED4 CDs sequence at *XhoI* and *EcoRI* restriction endonuclease, and pEX4 empty plasmid was the negative control. Based on the sequence of *MED4* mRNA, three small interference RNA sequences and negative control were designed and synthesized by GenePharma Company, Shanghai (Table 1). The gel image mapping the CDs region of *MED4* gene and sequencing comparison results are showed in the Suppl. Fig. S1.

Transfection and verification of transformants

The plasmid or interference sequences were transfected into the preadipocytes when the cells fused 70% culture dishes. Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) reagent gets the room temperature before use. One hundred picomoles of interference sequences, 5 μL lip2 000, and 200 μL Opti-MEM (Invitrogen, Carlsbad, CA, USA) were mixed well for 10 min. The mixture was added into the cells with 1.8 mL fresh medium for 6 h and replaced with a fresh complete culture medium. Transfection efficiency was determined by quantitative polymerase chain reaction (qPCR) test after 48 h. FuGENE reagent (Roche Applied Science, Indianapolis, IN, USA) was used to mix with 400 μL Opti-MEM, 3 ng plasmid; it was placed at room temperature for 20 min and added into the cells for 48 h, the transfection efficiency was determined by qPCR test.

RNA extraction and verification through the quantitative polymerase chain reaction

TRIzol reagent (Thermo Fisher Scientific) was used to extract total cellular RNA following the procedure. RNA contamination and degradation were validated via 1%

TABLE 1

Related sequences

Groups	Sequences 5′→3′
MED4-CDS	Sense: 5′-ATGGCGGCGGCGTCGAGC-3′
	Anti-sense: 5′-TCTACCACAGCCAACATCACC-3′
Negative control (NC)	Sense: 5′-UUCUCCGAACGUGUCACGUTT-3′
	Anti-sense: 5′-ACGUGACACGUUCGGAGAATT-3′
MED4-Bos-197	Sense: 5′-GGUCCUGGAGUUGUUAAUUTT-3′
	Anti-sense: 5′-AAUUAACAACUCCAGGACCTT-3′
MED4-Bos-354	Sense: 5′-GCAGAACAGAUACUGGCAATT-3′
	Anti-sense: 5′-UUGCCAGUAUCUGUUCUGCTT-3′
MED4-Bos-459	Sense: 5′-GCACAUAGGAUUAGUGCAATT-3′
	Anti-sense: 5′-UUGCACUAAUCCUAUGUGCTT-3′

TABLE 2

Primer sequences for the quantitative polymerase chain reaction

Genes	Primer sequence 5′→3′	Length/bp	Temperature/°C
MED4	Sense: 5′-CCTTGCTCCACAGTATCCA-3′	125	60
	Anti-sense: 5′-CCTCTACATCATCCTCGTTTT-3′		
PPARγ	Sense: 5′-CGGAAGCCCTTTGGTGACTTTATG-3′	175	60
	Anti-sense: 5′-GCAGCAGGTTGTCTTGGATGTC-3′		
Beta-Actin	Sense: 5′-GTCCACCTTCCAGCAGAT-3′	96	60
	Anti-sense: 5′-GCTAACAGTCCGCCTAGAA-3′		

agarose gels, and concentration was detected via a Quawell Q5000 spectrophotometer (Quawell Technology, San Jose, CA, USA). The reverse transcription kit (Takara, Japan) reversed the total RNA into cDNA. The reaction volume includes 2 μL of 5 × PrimeScript RT Master Mix, 500 ng of total RNA, and RNase Free ddH_2O up to 10 μL. Reaction condition: 37°C for 15 min, 85°C for 5 s, and the PCR products were normalized with ddH_2O and stored in a 4°C fridge. Roche LightCycler® 480 (Roche Applied Science), a quantitative fluorescent PCR device, detected the relative expression of the mRNA. *Beta-actin* was an internal control. The reaction volume: 10 μL of 2 × Light Cycler 480 SYBR Green I Master, 8 μL of ddH_2O, 1 μL of cDNA, and 0.5 μL of sense and anti-sense primers. The reaction condition: 40 cycles of 95°C for 5 min, 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s, followed by 95°C for 5 s, 65°C for 1 min, and 40°C for 10 s. The detailed information on primers was showed in Table 2.

Triglyceride content determination

Cell TG content was determined using a Triglyceride kit (Prilax, Beijing, China) following the manufacturer's procedure. Clean mature adipocytes without medium were digested with 0.25% trypsin (Sigma-Aldrich), and the mixture was centrifuged at 1500 rpm for 20 min. The sediments were digested for 10 min with lysis solution, heated at 70°C for 10 min, then centrifuged at 2000 rpm for 5 min. The OD of the enzyme-labeled supernatant was detected using an instrument at 550 nm wavelength and TG content was calculated.

Western blotting

Total protein from the cells was extracted using a Protein extraction kit (Solarbio, Shanghai, China). Adipocytes without medium were lysed for 25 min on ice, and the mixture was transferred into a tube and centrifuged at 12000 rpm at 4°C for 30 min to collect the protein. The BCA assay kit was used to determine the protein concentration. All proteins of samples were normalized with those in the lysate solution. The proteins were heated at 99°C for 8 min and mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer (Beyotime, Jiangsu, China). The upper and lower gels were made with the PAGE Gel Fast Preparation kit (EpiZyme, Shanghai, China). Thirty micrograms of protein in each sample and 6 μL standard marker (Thermo Fisher Scientific) were loaded to the gel wells and separated by electrophoresis at 80 V for 20 min. Then 120 V for 40 min, the gel with samples was transferred into a 0.45 mm Immobilon poly-vinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) at 200 mA for 1 h. PVDF membrane was washed with TBST (Tris-HCl buffered saline with tween 20; Beyotime, Jiangsu, China), blocked with 5% skimmed milk for 2 h, then washed again, and incubated with antibody (dilution ratio at 1:1 500; Bioss Beijing, China) and placed overnight on a shaker at 4°C. The clean membrane was incubated with a secondary antibody (anti-rabbit or anti-mouse IgG-HRP, 1:5000) at room temperature for 2 h and washed again. ECL hypersensitive photoluminescence solution (Pripril, Beijing, China) was used for a chromogenic reaction with the proteins. Then, protein bands were determined using the ChemiScope 6000 Touch imaging system (Clinx Science Instruments, Shanghai, China), and quantified using the ImageJ software. β-actin was an internal reference protein.

Statistical analysis

SPSS 17.0 software was used for statistical analyses. Each experiment was repeated three times. All data was described as means ± SD. The results compared two groups by *t*-test, and multiple group comparisons by one-way ANOVA. The statistical significance levels were set at $p < 0.05$. The raw data and images are presented in the Suppl. Table S1 and Fig. S1.

Results

Preadipocyte culture and differentiation

The preadipocytes were isolated and cultured in a normal incubator, and they started proliferating until full fusion in the plates. Then, the inducer solution was added to promote the preadipocyte differentiation into mature adipocytes. The whole process of differentiation took around 10 days. The mature adipocytes were shown in Fig. 1.

Expression of the MED4 gene increased during preadipocyte differentiation

To investigate the changing pattern of the *MED4* gene in preadipocyte differentiation. RNA in different periods (days 0, 2, 4, 6, 8) was extracted to verify the level of *MED4* gene

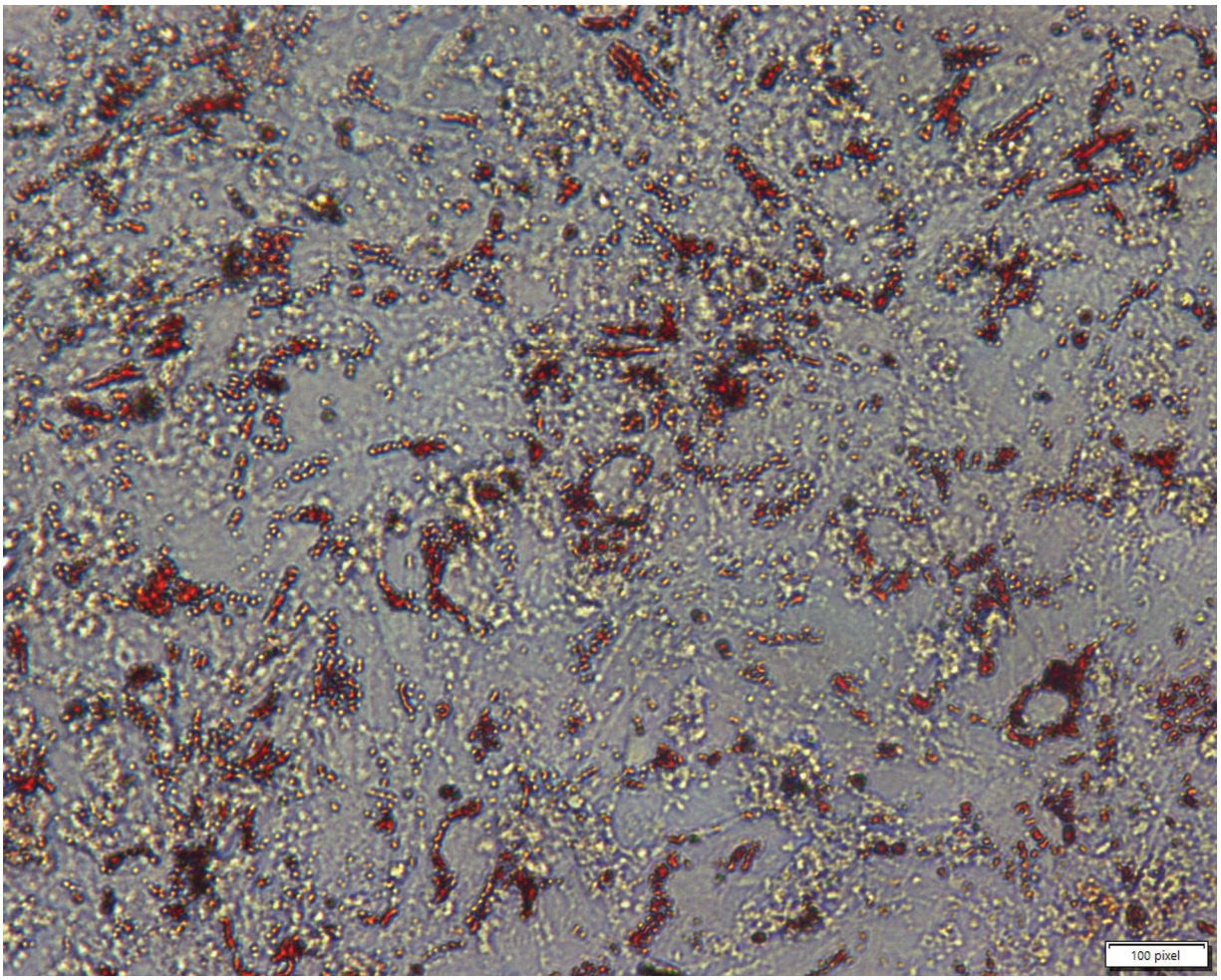

FIGURE 1. Morphology of mature adipocytes. Red dots represent lipid droplets in the adipocytes. Scales bar: 100 pixel.

expression by qPCR. The inducer I solution was added to the preadipocytes on day 0 and inducer solution II was added on day 2. On days 4 and 6, fresh medium was added into the preadipocytes. Some small lipid droplets we noticed in the preadipocytes on day 8 and the majority of preadipocytes became mature adipocytes on day 10. These results showed that the expression of the *MED4* gene was constant from day 0 to day 6 and significantly increased on day 8 (Fig. 2).

Verification of transfection efficiency

After 48 h of transfection, the efficiency was determined by qPCR test. The recombination plasmid exhibited significantly increased expression of the *MED4* gene compared to the empty plasmid ($p < 0.05$) (Fig. 3A). Subsequent assessment showed that three interference sequences had different effects, of which siR354 significantly decreased the *MED4* gene expression compared to the negative control ($p < 0.05$) (Fig. 3B). The results of western blotting had a similar trend as the qPCR results (Figs. 3C–3F).

MED4 overexpression promotes adipocyte differentiation

To assess the effect of the *MED4* gene on adipocyte differentiation, the expression of differentiation-related biomarker, was detected by qPCR test in different periods (days 0, 4, 8), and the TG content and lipid droplets were measured on day 10. With the increase in the *MED4* gene, the *PPARγ* gene expression significantly increased ($p < 0.05$). Meanwhile, the *MED4* gene also improved the accumulation of the TG content and lipid droplets ($p < 0.05$) (Fig. 4) and may improve adipocyte differentiation by affecting the *PPARγ* gene expression.

MED4 interference inhibits adipocyte differentiation

To investigate the effect of the *MED4* gene on adipocyte differentiation. The differentiation-related biomarker expression in different periods (days 0, 4, 8) was detected by qPCR, and the TG content and lipid droplets were measured on day 10. Inhibition of the *MED4* gene significantly reduced the *PPARγ* gene expression ($p < 0.05$) and decreased the OD value of TG content and lipid droplets ($p < 0.05$) (Fig. 5). Thus, the *MED4* gene may inhibit adipocyte differentiation by affecting the expression of the *PPARγ* gene.

Discussion

Chinese Red Steppe cattle, a unique breed resource in China, mainly provides meat products in the northeast of China, but its meat quality needs to be improved to meet the consumption demand (Li *et al.*, 2022). Visible adipose tissue in the muscle forms marbling, and is positively correlated with beef juiciness, tenderness, and palatability, and

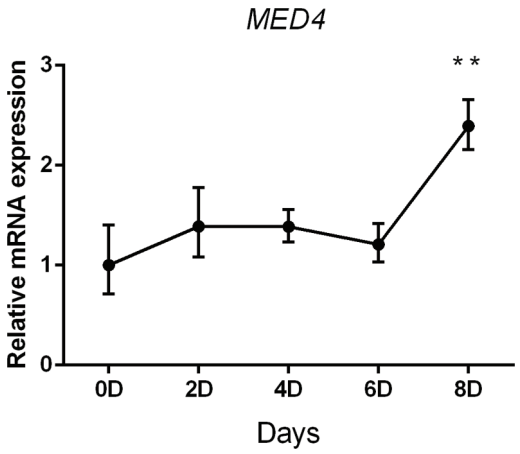

FIGURE 2. The change in the pattern of the *MED4* gene expression during preadipocyte differentiation $^{**}p < 0.05$.

significantly improves the meat quality (Shahrai *et al.*, 2021). Albrecht reported that compared to Holstein, the Japanese black cattle have a stranger ability to accumulate the IMF content (marbling), even under the same feeding condition (Albrecht *et al.*, 2011). Thus, the exploration mechanism of adipogenesis and lipid metabolism is crucial for increasing the IMF content and promoting meat quality (Nematbakhsh *et al.*, 2021). The process of adipocyte differentiation and IMF accumulation involves several genes, molecules, transcription factors, and so on (Tong and Sun, 2015; Xiao *et al.*, 2021), and this study aimed to find new candidate genes regulating the adipogenesis and provide a valuable reference for improving the meat quality by molecular breeding.

We investigated the physiological function of MED4 in the process of adipocyte differentiation. MED4, one of the subunits in a mediator complex protein, supports the cellular structure, affects the transcription and activity of RNA polymerase II, and also encodes vitamin D receptor protein to interact with nuclear receptors (Rachez *et al.*, 1999; Koschubs *et al.*, 2010; Balykova *et al.*, 2022). Only a few studies are reported on the *MED4* gene for adipogenesis; in this study, changing pattern of *MED4* in the differentiation process was detected. *MED4* gene expression first increased (day 2) and became constant (day 2–6), and then increased in the later stage of differentiation

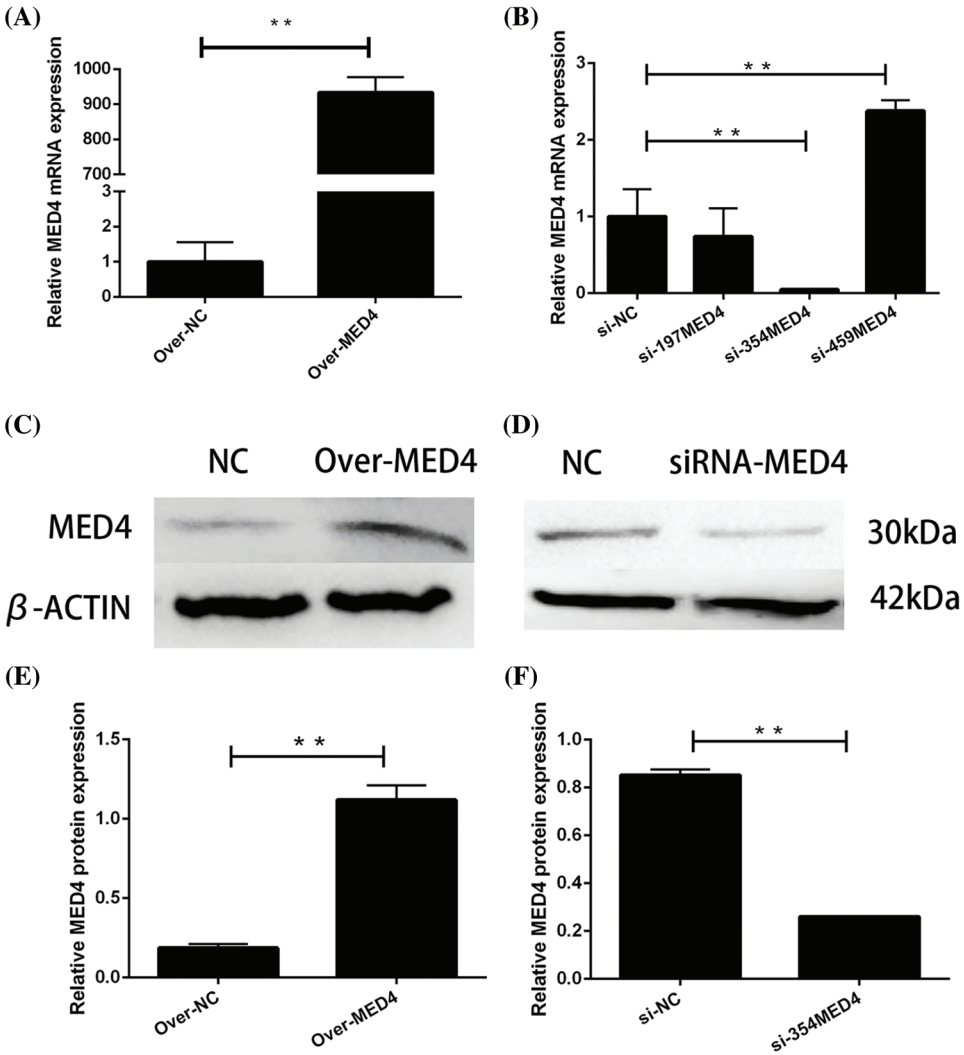

FIGURE 3. Verification of transfection efficiency. (A) Overexpression plasmid verification by quantitative polymerase chain reaction (qPCR); (B) verification of the interference sequence by qPCR; (C) overexpression plasmid verified by western blotting; (D) verification of the interference sequence by western blotting; (E and F) gray value analysis $^{**}p < 0.05$.

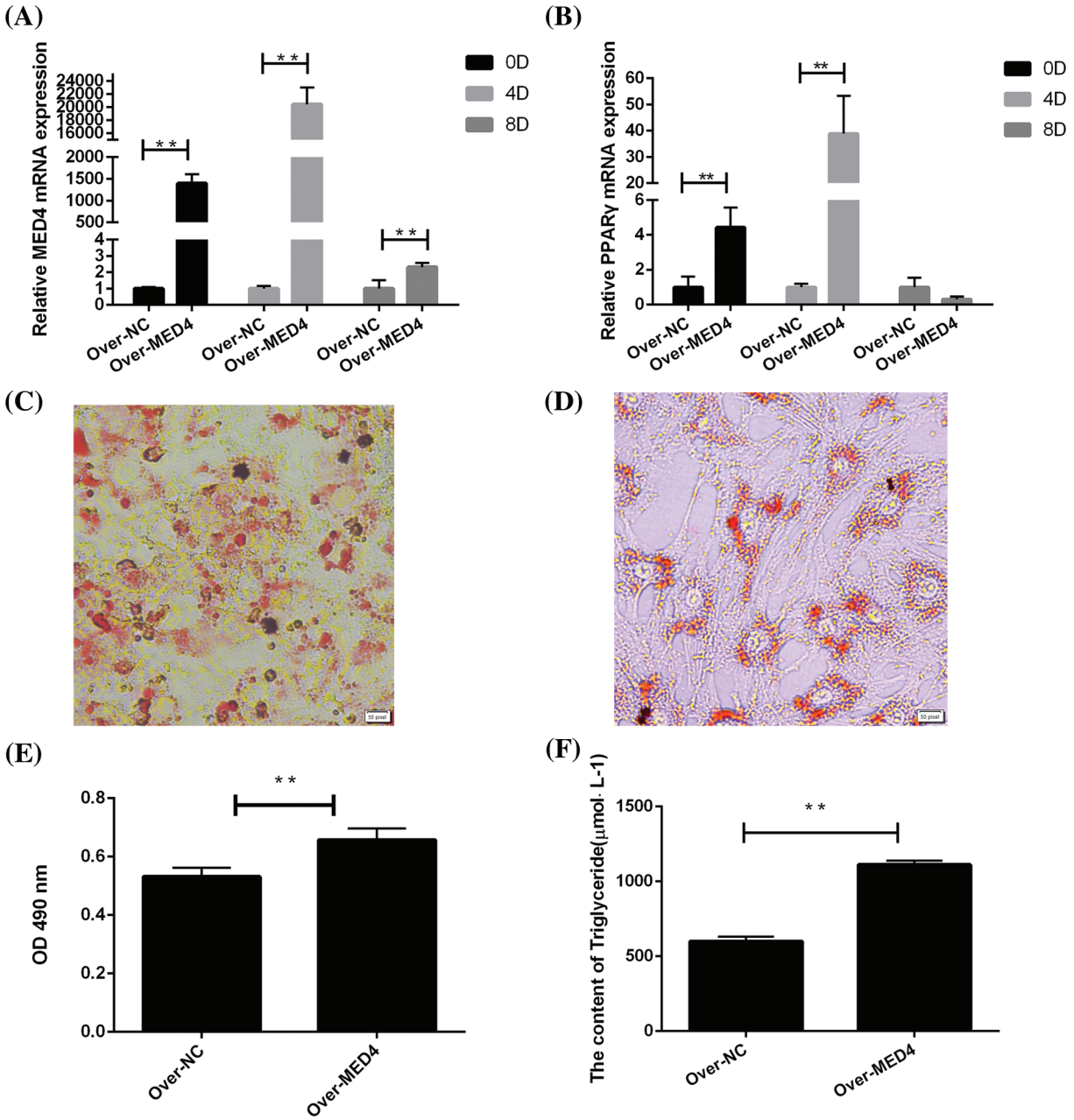

FIGURE 4. *MED4* overexpression affects the differentiation of adipocytes. (A) *MED4* gene expression for different periods; (B) *PPARγ* gene expression in the different periods; (C) oil red O staining in the *MED4* overexpression group; (D) oil red O staining in the control group; scale bar: 50 pixel; (E) lipid droplets in the adipocytes test; (F) the triglyceride content test; $^{**}p < 0.05$.

(day 8). Therefore, the *MED4* gene may be involved in adipogenesis and lipid formation. PPARγ, an important transcription factor for adipocyte differentiation, can activate several genes that play vital roles in adipogenesis (Wafer *et al.*, 2017), such as those coding for CCAAT/enhancer-binding protein alpha (C/EBPA), lipoprotein lipase (LPL), fatty acid-binding protein 4 (FABP4), Krüppel-like factor (KLF) family members to initiate the preadipocyte differentiation (Moseti *et al.*, 2016; Salmerón, 2018). The activated *PPARγ* gene is necessary for preadipocyte differentiation, even in cattle, sheep, pigs, poultry, and rodents (Soret *et al.*, 2016; Martínez Del Pino *et al.*, 2017; Stachecka *et al.*, 2019; Sun *et al.*, 2019; Guru *et al.*, 2021). Knockout or interference of the *PPARγ* gene leads to differentiation failure. Nonetheless, exogenous PPARγ recovers the ability of preadipocyte differentiation (Ren *et al.*, 2002), and is thus used as a biomarker for differentiation in general. The trend of *MED4* expression is similar to that of *PPARγ* and *C/EBPα* in preadipocytes. The change in *MED4* can influence the expression of the *PPARγ* gene, and may have a positive correlation (Wallberg *et al.*, 2003). With the increase in *MED4*, the *PPARγ* expression constantly increased; consequently, the accumulation of the TG content and lipid droplets significantly improved. However, *MED4* interference assay, Oil Red O staining, and TG detection showed a negative effect. In one study, the *MED1* gene was found to bind to the *PPARγ* gene and regulate adipogenesis (Ge *et al.*, 2002; Grøntved *et al.*, 2010). MED1 and MED4 can form a mediator complex and have a close relationship, suggesting that MED4 may affect the

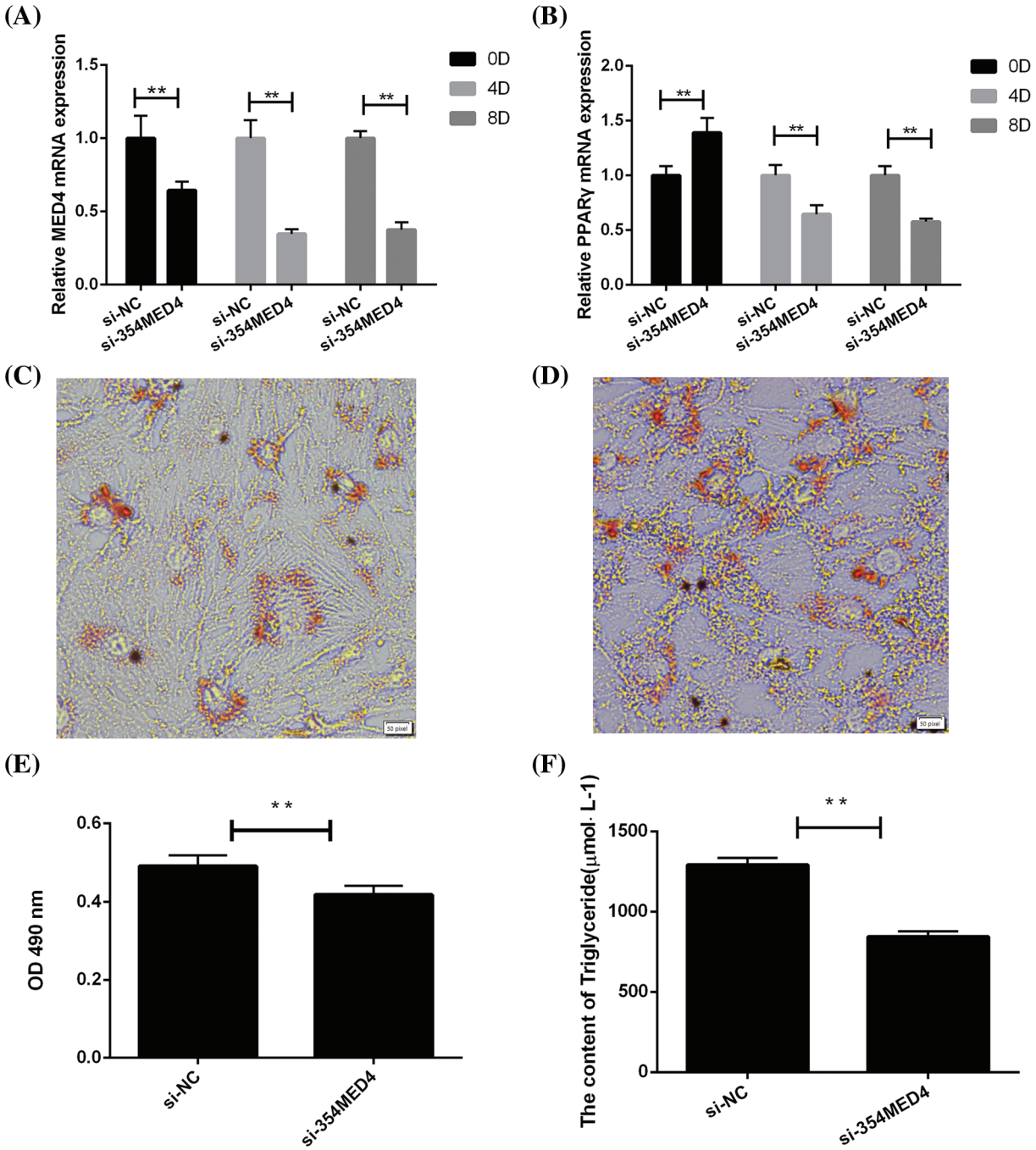

FIGURE 5. *MED4* inhibition affects adipocyte differentiation. (A) *MED4* gene expression in the different periods; (B) *PPARγ* gene expression in the different periods; (C) oil red O staining in the *MED4* inhibited group; (D) oil red O staining in the control group; scale bar: 50 pixel; (E) lipid droplets in the adipocytes test; (F) the triglyceride content estimation test; $^{**}p < 0.05$.

expression of PPARγ and lipid metabolism; one study described higher expression of the *MED4* gene in high TG content samples than in low TG samples in Jinghuang chicken (Li *et al.*, 2020). Another study a conducted genome-wide association study in Gushi chicken and reported the association of *MED4* with growth and carcass traits (Zhang *et al.*, 2021). *MED4* gene may regulate the adipocytes differentiation by affecting *PPARγ* expression. Unfortunately, in this study, we did not detect the *PPARγ* protein expression due to technical limitations; however, the results provide useful proof of *MED4*-mediated regulation of adipogenesis.

Conclusion

MED4 gene significantly increases in the late-stage duration of adipocyte differentiation and positively regulates lipid droplets and TG accumulation.

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Availability of Data and Materials: All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Ethics Approval: All procedures involving animals, such as welfare and ethical issues, were approved by the Committee for the Ethics of Animal Experiments (AWEC2017A01, 9 March 2017).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

Supplementary Materials: The supplementary materials are available at https://doi.org/10.32604/biocell.2023.030364.

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