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Original Article

Mfsd2a Promotes the Proliferation, Migration, Differentiation and Adipogenesis of Chicken Intramuscular Preadipocytes

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■Keywords

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ABSTRACT

Intramuscular fat (IMF) content is a crucial parameter for estimating meat quality. Growing evidence indicates that gene regulation plays an important role in IMF deposition. This study aimed to determine the function of *Mfsd2a* in chicken intramuscular preadipocytes. In the present study, high *Mfsd2a* mRNA levels were observed in the liver and adipose tissues of broilers. Subsequently, we synthesized small interfering RNAs to silence the expression of *Mfsd2a* in chicken intramuscular preadipocytes. The following results suggested that *CDK2*, *PCNA*, *CCND1*, *CCND2* and *MKI67* were inhibited, with CCK-8 and EdU assays revealing that cell proliferation was inhibited. Scratch test showed that cell migration ratios were declined. We also found that *Mfsd2a* silencing decreased the mRNA levels of *PPARγ*, *RXRG* and their target genes. The similar results were found in some key genes that contribute to lipid synthesis, including *C/EBPα*, *C/EBPβ*, *FABP4*, *FASN*, *ACACA* and *ACSL1*. Finally, Oil red O staining showed that IMF accumulation was blocked after *Mfsd2a* silencing. In conclusion, our results implied that *Mfsd2a* promotes the proliferation and migration of chicken intramuscular preadipocytes, as well as the differentiation and adipogenesis through PPARγ signaling pathway, which may provide a potential target to improve chicken meat quality.

INTRODUCTION

Intramuscular fat (IMF) may serve as a marker reflecting health status and diseases progression of human body in clinical treatment (Ahmed*, et al.*, 2018). Increases in IMF proportion have been implicated in the various metabolic and endocrine dysfunction such as insulin resistance (Kitessa*, et al.*, 2016). In animal husbandry, however, IMF deposition is the final goal of a growing number of breeding strategies, because of its essential role in improving flavor and palatability of meat products (Li*, et al.*, 2020). Previous studies on IMF, or referred to as marbling fat, concentrated mainly on cattle (Troy*, et al.*, 2016) and pigs (Katsumata, 2011), but rarely on chickens, which are the second largest animal protein source in Chinese diets (Zhang*, et al.*, 2018).

The contents of IMF correlate with both hyperplasia and hypertrophy of intramuscular adipocytes (Park*, et al.*, 2018), because they provide sites for subsequent lipid deposition. Therefore, growing both number and size of adipocytes is an effective strategy to improve IMF deposition. Recent researches indicated that mesenchymal stem cells are committed to the myogenic and fibro-adipogenic progenitors (FAPs) during fetal muscle development (Du*, et al.*, 2013), with FAPs having dual potentials of adipogenic and fibrogenic differentiation (Uezumi*, et al.*, 2014). Intramuscular preadipocytes are formed in the adipogenic differentiation process of FAPs. Beyond doubt, IMF accumulation is influenced by the abilities of intramuscular preadipocytes to proliferate

and differentiate into mature adipocytes. These abilities are regulated by many factors, including the expression products of various genes (Chen*, et al.*, 2017; Qimuge*, et al.*, 2019; Xiong*, et al.*, 2018).

Major facilitator superfamily domain containing 2a (Mfsd2a), a sodium-dependent transporter, mediates docosahexaenoic acid and other polyunsaturated fatty acids esterified to lysophosphatidylcholine through the blood-brain barrier, thereby regulating lipogenesis in brain development (Chan*, et al.*, 2018). A partially inactivating mutation in *Mfsd2a* would lead to a non-lethal microcephaly syndrome (Alakbarzade*, et al.*, 2015). Besides, growing evidence has emerged to determine its regulatory role in various biological processes (Eser Ocak*, et al.*, 2020), for instance, adaptive thermogenesis (Angers*, et al.*, 2008), placenta development (Toufaily*, et al.*, 2013), tumor metastasis (Shi*, et al.*, 2018), immune response (Piccirillo*, et al.*, 2019), neurodegeneration (Sanchez-Campillo*, et al.*, 2020) and so on. Remarkably, while transporting lipids is a unique feature of Mfsd2a, most members of the major facilitator superfamily transport water-soluble substances (Quek*, et al.*, 2016). A lot of attention has recently been paid to Mfsd2a for its regulation in the maintenance of normal physiologic functioning of the blood-brain barrier (Wang*, et al.*, 2020), especially fatty acid uptake in the brain (Wong*, et al.*, 2020). Therefore, we wondered whether *Mfsd2a* has a significant effect on lipid metabolism in skeletal muscle tissues.

In this study, we synthesized small interfering RNAs to interfere the expression of *Mfsd2a*. Subsequently, we detected the changes concerning proliferation, migration, differentiation and adipogenesis of chicken intramuscular preadipocytes. In other words, we first determined the potential role of *Mfsd2a* in chicken intramuscular preadipocytes. These results may provide a novel insight to improve IMF deposition in broilers.

MATERIALS AND METHODS

Experimental animals

All animal experiments in this manuscript were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University (Approval No. DKY2020202025).

Six 90-day-old Daheng broilers (male and female, half of each) were used to collect thirteen tissue samples including heart, liver, spleen, lung, kidney, breast muscle, leg muscle, subcutaneous fat, abdominal fat, gizzard, glandular stomach, ovary and pituitary. Immediately, these samples were stored in liquid nitrogen for further analysis. Meanwhile, sufficient 14-day-old chicks were prepared for primary intramuscular preadipocytes isolation. All experimental birds, which were euthanized, came from Sichuan Daheng Poultry Breeding Co., Ltd (Chengdu, China).

Cell isolation, culture and differentiation

Chicken primary intramuscular preadipocytes were isolated from the breast muscle tissues of 14-day-old chicks. Briefly, the pectoral muscles were cut to pieces using ophthalmic scissors after the fascia and connective tissues were removed. The homogenate was digested for 1.5 h with the mixture of collagenase type I and type II (Biofroxx, Germany), next were passed through cell strainers (Biologix, China) with pore sizes of 70 μm and 45 μm. The cell precipitate was obtained after centrifugation at 1000 r/min for 10 min. Then these cells were maintained in DMEM/F12 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) and 1% penicillin/streptomycin (Invitrogen, USA) in an incubator at 37°C and a 5% CO_2 humidified atmosphere. After 2 h of differential adherence, the supernatant was removed and replaced with fresh complete medium to obtain relatively pure preadipocytes. Once the preadipocytes grew confluent, differentiation medium containing 10 μg/mL insulin, 250 μM oleic acid, 0.5 mM 3-isobutyl-1-methylxanthine and 1 μM dexamethasone was replaced to induce differentiation for two days. Subsequently, maintenance medium containing 10 μg/mL insulin and 250 μM oleic acid was replaced to maintain differentiation for two days. Finally, complete medium was replaced every two days until the cells were fully differentiated into mature adipocytes. The differentiation time set in this study was 10 days. All trials conducted for the cells contained at least three biological replicates, as shown in figure legends.

Immunofluorescence assay

Briefly, the preadipocytes and mature adipocytes were fixed with 4% paraformaldehyde for 30 min and then washed using PBS. Subsequently, they were permeabilized using 0.5% Triton X-100 for 20 min and blocked with goat serum for 30 min. The primary antibody PPARγ (ABclonal, China; 1: 200 dilution) was prepared to incubate with the cells at 4 °C overnight. After that, the cells were washed using PBST (0.05% Tween 20 + PBS) and incubated with fluorescence secondary antibody at 37 °C for 1 h. The nuclei were stained using 4', 6-diamidino-2-phenylindole (DAPI) for 5 min. The images were captured in randomly selected fields using IX53 biological microscope (Olympus, Japan).

Cell transfection

Three small interfering RNAs (siRNAs) were devised and synthesized by GenePharma (Shanghai, China) (Table 1), of which with the highest interference efficiency was selected for the present study according to the preliminary experiment results, named si-*Mfsd2a* (a). Then, si-*Mfsd2a* and negative control (NC) were diluted using Lipofectamine 3000 (Invitrogen, USA) and Opti-MEM medium (Gibco, USA) following the manufacturer's instructions in order to transfect into the cells.

Table 1 – The RNA oligonucleotides used for cell transfection.

siRNAs	Sense strands (5′→3′)	Antisense strands (5′→3′)
siRNA-195	GCUUCUUCCUCCAGAUCUATT	UAGAUCUGGAGGAAGAAGCTT
siRNA-1084	GAAGAAGACUGCUGUCUAUTT	AUAGACAGCAGUCUUCUUCTT
siRNA-1284	GCCACGAAGCCAUCUUCUUTT	AAGAAGAUGGCUUCGUGGCTT
NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

Quantitative real-time PCR

Total RNA from cells and tissues was isolated using Trizol reagent (TaKaRa, Japan), and its concentration and purity were examined by NanoDrop 2000C spectrophotometer (Thermo, USA). PrimeScript RT Reagent Kit (TaKaRa, Japan) was used to synthesize cDNA through reverse transcription of mRNA. Quantitative real-time PCR (qPCR) was performed in CFX Connect Real-Time System (Bio-Rad, USA) using TB Green Premix Ex Taq II (TaKaRa, Japan). Relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method with *GAPDH* as a reference gene. All the primers used in qPCR assay are presented in Table 2.

CCK-8 assay

Intramuscular preadipocytes were seeded in 96-well cell culture plates. Cell proliferation was monitored using Cell Counting Kit-8 (Meilun, China) by Microplate Reader (Thermo, USA) at 12 h, 24 h, 36 h and 48 h after transfection. The wavelength was 450 nm in absorbance determination.

EdU assay

After the cells in 96-well plates were transfected, EdU assay was performed using Cell-Light EdU Apollo567 In Vitro Kit (RiboBio, China) according to the manufacturer's protocol. Briefly, 50 μM 5-Ethynyl-2′-deoxyuridine (EdU) reagent was added to each well and incubated at 37 °C for 3 h. Subsequently, Hoechst 33342 reagent was used to stain cell nuclei. Randomly selected fields were captured using IX53 biological microscope (Olympus, Japan) and the number of stained cells was counted by Image-Pro Plus software (Media Cybernetics, USA).

Cell migration assay

Once the preadipocytes grew confluent, a uniform scratch was made using the tip of a medium-sized pipette. Then the cells were washed three times with PBS and cultured in serum-free medium. The required images were obtained in the same fields at 0 h, 24 h and 48 h after transfection. Relative migration ratios were calculated using Image J software.

Oil red O staining

Briefly, mature intramuscular adipocytes were washed once with PBS and fixed with 4% paraformaldehyde for 30 min. After washing twice with distilled water, the fixed samples were bathed with 60% isopropanol for 5 min and incubated with diluted Oil Red O (Solarbio, China) for 20 min. Then stained cells were washed with distilled water for several times. Images were captured by IX53 biological microscope (Olympus, Japan). Finally, the stained samples were incubated with 100% isopropanol for 15 min and the OD values were measured at 510 nm.

Western blotting

Total protein was isolated using Total Protein Extraction Kit (BestBio, China), with the concentration of protein samples determining by BCA Protein Quantitative Kit (BestBio, China). Then, briefly, 25 μg total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking with blocking buffer (Beyotime, China) on decolorization shaker for 1 h at room temperature, the membranes were incubated with anti-CDK2 (ZenBio, China; 1: 1000 dilution), anti-PCNA (ZenBio, China; 1: 1000 dilution), anti-PPARγ (ABclonal, China; 1: 1000 dilution) and anti-β-Tubulin (ZenBio, China; 1: 5000 dilution) overnight at 4°C. Subsequently, the membranes were washed three times with western wash buffer (Beyotime, China) and incubated with horseradish peroxidase conjugated IgG (ABclonal, China; 1: 2000 dilution) for 1 h at room temperature. Finally, specific bands were visualized using Ultra Hypersensitive ECL Chemiluminescence Kit (Beyotime, China). Image J software was utilized for the quantitative analysis of protein bands.

Statistical analysis

All data are presented as mean ± standard error (SEM). One-way ANOVA analysis or unpaired Student's *t*-test was performed by SPSS 26.0 software, and LSR method was used for multiple comparisons. The significant levels were set at * $p<0.05$, ** $p<0.01$ and $^{a\text{-}e}$ $p<0.05$.

Table 2 – The specific primers used for qPCR.

Accession No.	Gene symbol	Primer sequence (5′→3′)	Product length (bp)
XM_417826.6	*Mfsd2a*	F: CTCCACCCCATTTGCTGTCA R: GAAGCACGTCACAAGGGTCT	121
NM_001199857.1	*CDK2*	F: GCTCTTCCGTATCTTCCGCA R: ATGCGCTTGTTGGGATCGTA	192
NM_204170.2	*PCNA*	F: GGGCGTCAACCTAAACAGCA R: CTAGAGCCAACGTATCCGCA	101
NM_205381.1	*CCND1*	F: TACGAGCCTGCCAAGAACAG R: ATCTCGCACATCAGTGGGTG	137
XM_015292118.2	*CCND2*	F: CAACTGGAAGTGTGGGAGCA R: GGTTGCTTACTAGCACCGAC	165
XM_015289038.2	*MKI67*	F: GCAACAACAAGGAGGCTTCG R: TTCAGGTGCCATCCCGTAAC	204
NM_001001460.1	*PPARγ*	F: CATCAGGTTTGGGCGAATGC R: TAACTGGTCGATGTCGCTGG	76
NM_205294.1	*RXRG*	F: CATCCTTCTCCCATCGCTCC R: TTGGCGTCTGGGTTGAAGAG	211
NM_204542.2	*NR1H3*	F: ACCGAATGTGCAGGACCAGT R: AGAGCAGGGGAGGGAGTTTCT	212
NM_001005571.1	*CYP8B1*	F: TGGGTTACGCACTGGACTTC R: GCAGAAGGGGTCCATCACAA	128
NM_001030889.1	*FABP3*	F: GACGGTGAAGACCCATAGCA R: GCCGTGGTCTCATCGAACTC	78
NM_205282.1	*LPL*	F: CGTGCTCAGATGCCCTACAA R: GAAGAGACTTCAGGCAGCGT	158
NM_001127439.1	*PLIN1*	F: GACCTACACCAGCACCAAAGAG R: TCCATAGAGTTGCCGATGGT	291
NM_206991.1	*ADIPOQ*	F: GCAGAACCACTACGACAGCA R: TAGACCCCGTTGTTGTTGCC	258
NM_204194.1	*ILK*	F: GATATCTTCACGCAGTGCCG R: CGTTCACCGCATTGATGTCC	286
NM_001031352.3	*PDK1*	F: AAGTGGTGTATGTGCCGTCC R: CTCATAGGAACACCTCCGCC	184
NM_205471.1	*PCK1*	F: GGGTCGCTGGATGTCAGAAG R: ACATCCAACCGAGTGAAGGC	256
NM_001031459.1	*C/EBPγ*	F: GGAGCAAGCCAACTTCTACG R: GAGTGCTCGTTCTCGCAGAT	174
NM_205253.2	*C/EBPγ*	F: GACCACGAGAGAGCCATTGA R: AGGTGTAGTCGGGCTTCTTG	202
NM_204290.1	*FABP4*	F: CTGGGTGTGGGGTTTGCTAC R: TCAGTGTGCCACTGTCTAGG	208
NM_205155.3	*FASN*	F: ACACCCTAAGCCTCGTTC R: CCTCAAGATAGCCTGTAAGA	208
NM_205505.1	*ACACA*	F: TCCTGCCTGCTCATACTT R: GCGATACCTGTCCACTTC	227
NM_001012578.1	*ACSL1*	F: TGGAACGTGGCAAGAAGTGT R: CCCTTGGGGTTTCCTGTTGT	151
NM_204305.1	*GAPDH*	F: GGGGAAAGTCATCCCTGAGC R: AGCAGCCTTCACTACCCTCT	145

RESULTS

Relative *Mfsd2a* mRNA levels in chicken

To investigate the effects of *Mfsd2a* on fatty acid metabolism in intramuscular preadipocytes, we firstly determined *Mfsd2a* mRNA expression profiles in various tissues of broilers. The results showed that *Mfsd2a* was highly expressed in liver, abdominal fat and subcutaneous fat, indicating that *Mfsd2a* may regulate lipid deposition in broilers (Figure 1a). Through immunofluorescence assay, we found that PPARγ protein levels increased after intramuscular preadipocytes differentiated into mature adipocytes, suggesting that the primary cells we isolated from breast muscle were indeed preadipocytes (Figure 1b). Subsequently, we determined the changes of *Mfsd2a* mRNA levels in chicken intramuscular preadipocytes during differentiation process, which suggested that

the expression of *Mfsd2a* reached the peak level at the second day after differentiation (Figure 1c).

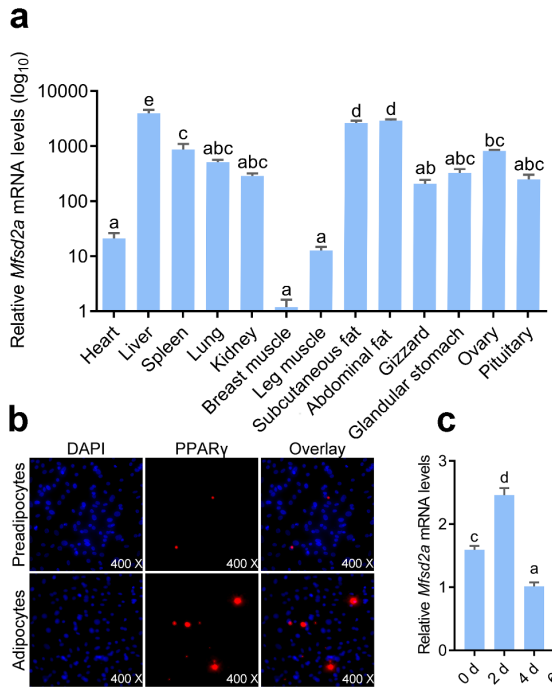

Figure 1 – Relative *Mfsd2a* mRNA levels in chicken. (a) *Mfsd2a* mRNA expression profiles in various tissues of Daheng broilers (n = 6). (b) Comparison of PPARγ protein levels before and after intramuscular preadipocytes differentiation (n = 3). (c) Changes of *Mfsd2a* mRNA levels at different time points during the differentiation process of chicken primary intramuscular preadipocytes into mature adipocytes (n = 3). All results are presented as mean ± SEM. Each column containing same letter means that the difference is not significant. $^{a\text{-}e}$ $p<0.05$.

Mfsd2a promotes the proliferation of chicken intramuscular preadipocytes

We transfected three siRNAs synthesized by GenePharma into chicken intramuscular preadipocytes. The most efficient siRNA was siRNA-195, named as si-*Mfsd2a*, and the interference efficiency hit 82.15% (Figure 2a). To investigate the potential role of *Mfsd2a* in regulating chicken intramuscular preadipocytes proliferation, we carried out qPCR assay and the results showed that *Mfsd2a* interference led to a decrease in the mRNA levels of proliferation-related genes (*CDK2*, *PCNA*, *CCND1*, *CCND2* and *MKI67*) (Figure 2b). Western blotting was used to investigate the protein levels of CDK2 and PCNA, which were inhibited after transfection (Figure 2c-d). CCK-8 assay presented that the proliferation of chicken intramuscular preadipocytes had significantly decreased after transfection (Figure 2e). In addition, we performed EdU assay to analyze the changes of cell number (Figure 2f-g). The results were consistent with that of CCK-8 assay. Finally, all these results demonstrate that *Mfsd2a* promotes the proliferation of chicken intramuscular preadipocytes.

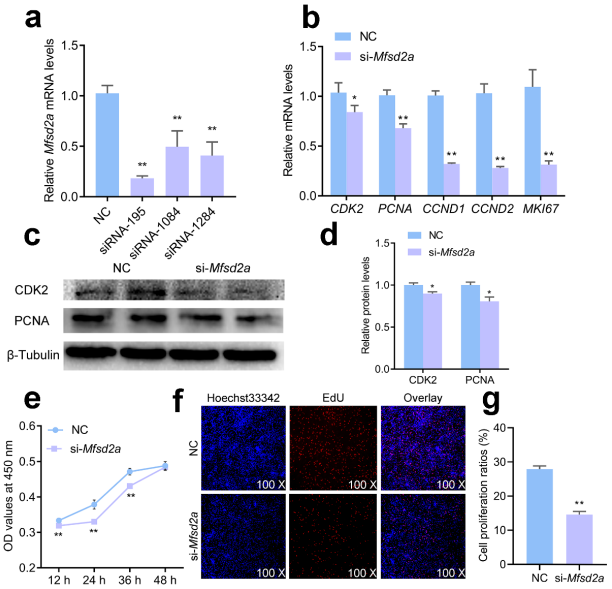

Figure 2 – *Mfsd2a* promotes the proliferation of chicken intramuscular preadipocytes. (a) *Mfsd2a* mRNA levels in the intramuscular preadipocytes after being transfected with three siRNAs and NC for 48 h (n = 3). The siRNA-195 with the highest interference efficiency was named si-*Mfsd2a*. (b) The relative mRNA levels of genes related to cell proliferation in chicken primary intramuscular preadipocytes after transfection (n = 3). (c) The protein levels of genes related to cell proliferation in chicken intramuscular preadipocytes after transfection (n = 3). (d) Quantitative results of protein bands relative to β-Tubulin (n = 3). (e) Cell growth curves determined by means of CCK-8 assay at 12 h, 24 h, 36 h and 48 h after transfection (n = 8). (f-g) Effects of *Mfsd2a* interference on the proliferation of chicken intramuscular preadipocytes assessed by EdU assay (n = 6). All results are presented as mean ± SEM. * $p<0.05$; ** $p<0.01$.

Mfsd2a promotes the migration of chicken intramuscular preadipocytes

Scratch test was performed to assess the effects of *Mfsd2a* on chicken intramuscular preadipocytes migration (Figure 3a). Migration ratios relative to 0 h were calculated by Image J software, indicating that *Mfsd2a* promotes the migration of chicken intramuscular preadipocytes (Figure 3b).

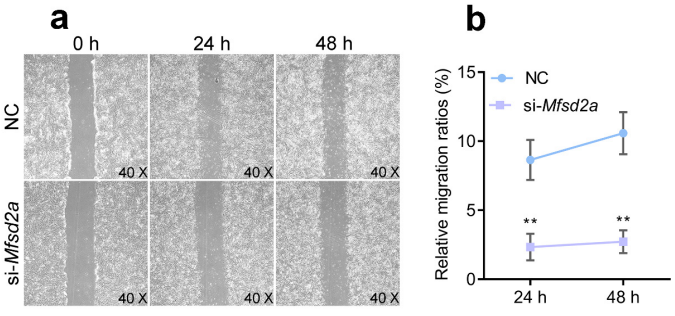

Figure 3 – *Mfsd2a* promotes the migration of chicken intramuscular preadipocytes. (a) Scratch test to observe cell migration at 0 h, 24 h and 48 h after transfection (n = 3). (b) Relative migration ratios of chicken intramuscular preadipocytes after *Mfsd2a* interference (n = 3). All results are presented as mean ± SEM. * $p<0.05$; ** $p<0.01$.

PPARγ signaling pathway is involved in the positive regulation of *Mfsd2a* on chicken intramuscular preadipocyte differentiation and adipogenesis

Finally, we explored the effects of *Mfsd2a* on chicken intramuscular preadipocytes differentiation. The cells transfected with siRNAs reduced the mRNA levels of *PPARγ*, *RXRG* and target genes in PPARγ signaling pathway compared with those in the control group (Figure 4a). The similar results lasted in the mRNA levels of other genes related to lipid metabolism (*C/EBPα*, *C/EBPβ*, *FABP4*, *FASN*, *ACACA* and *ACSL1*) (Figure 4b), as well as the protein levels of PPARγ (Figure 4c-d). In order to investigate the accumulation of lipid droplets, mature adipocytes were used to perform Oil red O staining assay (Figure 4e). The results showed that lipid deposition decreased significantly after interference of *Mfsd2a*, which were further verified by the results of triglyceride contents detection (Figure 4f). All these results demonstrate that PPARγ signaling pathway is involved in the positive regulation of *Mfsd2a* on chicken intramuscular preadipocyte differentiation and adipogenesis.

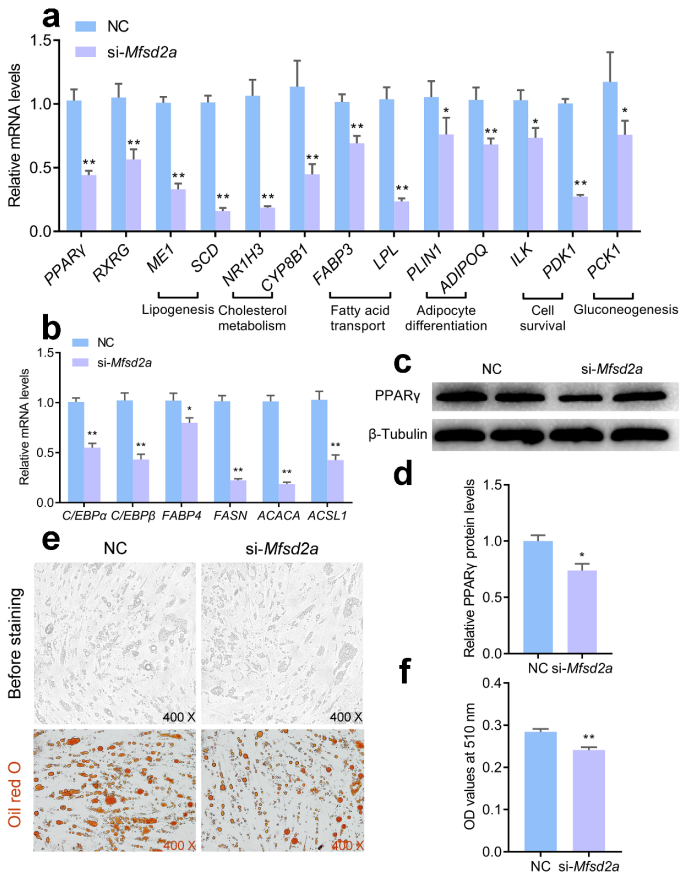

Figure 4 – PPARγ signaling pathway is involved in the positive regulation of *Mfsd2a* on chicken intramuscular preadipocyte differentiation and adipogenesis. (a) The relative mRNA levels of target genes in PPARγ signaling pathway (n = 3). (b) The relative mRNA levels of genes related to lipid metabolism in chicken primary intramuscular adipocytes after transfection (n = 3). (c-d) The PPARγ protein levels in chicken intramuscular adipocytes after transfection and quantitative results of protein bands relative to β-Tubulin (n = 3). (e) Chicken intramuscular adipocytes stained with Oil red O (n = 3). (f) Triglyceride contents measured by microplate reader (n = 3). All results are presented as mean ± SEM. * $p<0.05$; ** $p<0.01$.

DISCUSSION

Intramuscular fat (IMF) locates on epimysium, endomysium and perimysium, being one of the extremely important production traits in animal meat. Due to consumers' preference for high intramuscular fat meat (Realini, *et al.*, 2021), the underlying mechanism of IMF deposition is gradually explored. IMF contents directly depend on the proliferation and differentiation of intramuscular preadipocytes. In recent studies, *KLF13* (Chen, *et al.*, 2017), *FTO* (Chen, *et al.*, 2017), *FABP1* (Chen, *et al.*, 2017) and *GPR39* (Dong, *et al.*, 2016) were proved to promote the proliferation and differentiation of porcine intramuscular preadipocytes. While *FGF10* (Xu, *et al.*, 2018) was proved to have a negative regulatory effect on lipid accumulation in goat intramuscular adipocytes, *FGF21* (Xu, *et al.*, 2019), *LXR* (Xiong, *et al.*, 2018) and *GEM* (Xu, *et al.*, 2020) showed a positive effect. Only a few studies concentrated on chicken intramuscular preadipocytes, including *KLF9* for its inhibitory effect on the differentiation of preadipocytes (Sun, *et al.*, 2019). However, the specific mechanism of regulating intramuscular preadipocytes remains unclear. The related functional genes deserve further exploration and verification.

Mfsd2a is known intimately as a transporter for docosahexaenoic acid, an essential omega-3 fatty acid (Nguyen, *et al.*, 2014). But little is known about the effects of gene *Mfsd2a* on poultry IMF deposition. In the present study, the high mRNA levels of *Mfsd2a* in liver and adipose tissues implied that *Mfsd2a* plays an important role in poultry lipid metabolism. To further determine its function, we silenced the expression of *Mfsd2a* in chicken intramuscular preadipocytes using small interfering RNAs (siRNAs). Subsequently, we detected the mRNA and protein levels of downstream genes.

CDK2, PCNA, CCND1 and CCND2 are the key components of cell cycle signaling pathway (Marais, *et al.*, 2010). MKI67 regulates ribosomal synthesis of ribonucleic acid to affect cell proliferation (Giordano, *et al.*, 2020). Therefore, we selected these five genes to reflect the proliferation of cells. The silencing of *Mfsd2a* could influence the mRNA and protein levels of these genes, indicating that *Mfsd2a* may promote the proliferation of intramuscular preadipocytes. CCK-8 assay and EdU assay can quantify cell hyperplasia status and the determination results further confirmed above conjecture. Migration ability reflects stem cell potency of preadipocytes to a certain extent (Park, *et al.*, 2014). We found that the migration ratios of chicken intramuscular preadipocytes decreased after *Mfsd2a* expression was silenced.

PPARγ, a member of the nuclear receptor superfamily, functions as a leading role in adipocyte differentiation and metabolism (Qimuge, *et al.*, 2019). PPARγ is mainly expressed in adipose tissues and its protein levels gradually increased during the differentiation of preadipocytes into mature adipocytes (Tontonoz, *et al.*, 1994). Immunofluorescence assay presented similar results in this study (Figure 1b). RXRG, together with PPARγ, regulates the expression of target genes in PPARγ signaling pathway (Cheng, *et al.*, 2018). Our study found that the expression of PPARγ and RXRG decreased in chicken intramuscular adipocytes after transfection with siRNAs. At the same time, the mRNA levels of downstream target genes were also inhibited, and their functions covered lipogenesis (*ME1* and *SCD*), cholesterol metabolism (*NR1H3* and *CYP8B1*), fatty acid transport (*FABP3* and *LPL*), adipocyte differentiation (*PLIN1* and *ADIPOQ*), cell survival (*ILK* and *PDK1*) and gluconeogenesis (*PCK1*). In addition, other representative genes, including *C/EBPα*, *C/EBPβ*, *FABP4*, *FASN*, *ACACA* and *ACSL1*, have been shown to contribute to lipid anabolism (Zhang, *et al.*, 2020). The detection results for them showed that IMF synthesis was inhibited after the interference of *Mfsd2a*. As expected, the same result was observed in Oil red O staining assay, which was used to visualize lipid accumulation. Similarly, in recent studies, *NRF1* overexpression was found to inhibit adipogenesis of the immortalized chicken preadipocyte cell line (Cui, *et al.*, 2018). *LPIN1* was verified to inhibit abdominal fat deposition in broilers (Chao, *et al.*, 2020). These results showed that gene regulation plays an important role in lipid metabolism.

In conclusion, for the first time, *Mfsd2a* was found to promote the proliferation and migration of chicken intramuscular preadipocytes, as well as the differentiation and adipogenesis through PPARγ signaling pathway (Figure 5). These results conduce to further enrich our understanding to the underlying mechanism of intramuscular fat deposition.

CONFLICTS OF INTEREST

All authors have no declared conflict of interest.

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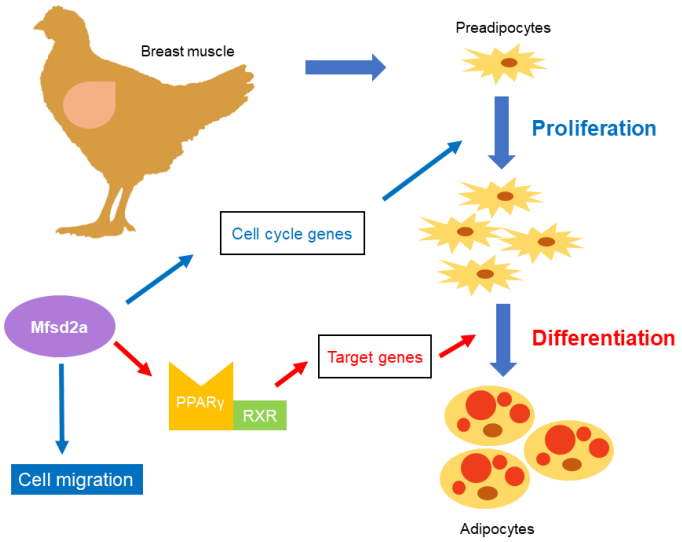

Figure 5 – A model depicting the role of *Mfsd2a* in regulating chicken intramuscular fat deposition. *Mfsd2a* promotes the proliferation and migration of chicken intramuscular preadipocytes, as well as the differentiation and adipogenesis through PPARγ signaling pathway.

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