miR-34a regulates adipogenesis in porcine intramuscular adipocytes by targeting ACSL4

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Abstract

Background: Intramuscular fat (IMF) content is an important factor in porcine meat quality. Previously, we showed that miR-34a was less abundant in liver tissue from pigs with higher backfat thickness, compared to pigs with lower backfat thickness. The purpose of this present study was to explore the role of miR-34a in adipogenesis.

Result: Bioinformatics analysis identified Acyl-CoA synthetase long chain family member 4 (ACSL4) as a putative target of miR-34a. Using a luciferase reporter assay, we verified that miR-34a binds the ACSL4 mRNA at the 3'UTR. To examine the role of the miR-34a-ACSL4 interaction in IMF deposition in the pig, mRNA and protein expression of the ACSL4 gene was measured in primary intramuscular preadipocytes transfected with miR-34a mimic and inhibitor. Our results showed that ACSL4 is expressed throughout the entire differentiation process in pig preadipocytes, similar to the lipogenesis-associated genes PPARγ and aP2. Transfection with miR-34a mimic reduced lipid droplet formation during adipogenesis, while miR-34a inhibitor increased lipid droplet accumulation. Transfection with miR-34a mimic also reduced the mRNA and protein expression of ACSL4 and lipogenesis genes, including PPARγ, aP2, and SREBP-1C, but increased the expression of steatolysis genes such as ATGL and Sirt1. In contrast, the miR-34a inhibitor had the opposite effect on gene expression. Further, knockdown of ACSL4 decreased lipid droplet accumulation.

Conclusions: Our results support the hypothesis that miR-34a regulates intramuscular fat deposition in porcine adipocytes by targeting ACSL4.

Keywords: IMF, miR-34a, ACSL4, Pig

Background

Intramuscular fat (IMF) content is a primary indicator of porcine meat quality [1]. An increase in IMF content can improve meat flavor [2]. However, substantial efforts have been made to improve production efficiency and select for lean growth, both of which impact IMF negatively. Selection for enhanced IMF has therefore become an important focus in pork production.

High-throughput methods, such as genome-wide association studies and transcriptome expression profiling, have been used to search for genes that potentially affect fat deposition in swine. Many genes associated with an extreme capacity for IMF deposition have been identified [3–9]. In addition to the multiple genes that influence adipogenesis, IMF is also likely to be under the control of post-transcriptional regulatory factors such as microRNAs. MicroRNAs are small non-coding RNA molecules that regulate gene expression by targeting mRNA transcripts for cleavage or translational inhibition [10, 11]. miRNAs play important roles in various biological processes, including cell differentiation, proliferation and apoptosis [12], organ development [13], lipid metabolism [14], and...
tumorigenesis [15]. Emerging evidence suggests that miRNAs are also involved in adipogenesis. For example, miR-196a induces preadipocyte differentiation by increasing adipocyte marker expression, lipid accumulation, and triglyceride content [16]. miR-27a-5p increases fat deposition in steers partly by targeting the calcium-sensing receptor (CASR) [17]. miR-30e regulates adipocyte differentiation by targeting the low-density lipoprotein receptor-related protein 6 [18]. miR-155, miR-130, and miR-210 inhibit adipocyte formation by targeting the key adipogenic transcriptional factors PPARγ, C/EBPα, and TCF712 in the Wnt/β-catenin signaling pathway [19, 20]. We previously identified potential miRNAs regulators of porcine fat deposition by using high-throughput sequencing to examine the transcriptomes in animals with extreme differences in backfat thickness. One of the miRNAs, miR-34a, is markedly less abundant in animals with higher backfat thickness (H group) compared with those with lower backfat thickness (L group) [9]. This result suggests that miR-34a may play important roles in porcine adipogenesis.

MicroRNA-34a has attracted interest recently because of its ability to modulate a myriad of oncogenic functions in different cancers [21–27]. Not only does it play a role in cancer metastasis [28, 29] and drug resistance [30], it is now being evaluated as a diagnostic as well as a prognostic biomarker [31–33]. In addition, a miR-34a inhibitor has been identified that may effectively protect against sevoflurane-induced hippocampal apoptosis by targeting Wnt1 and activating the Wnt/β-catenin pathway [34]. miR-34a is involved in the pathogenesis of non-alcoholic fatty liver disease [35] and is down-regulated in genetically improved farmed tilapia (Oreochromis niloticus) when they are fed a high-fat diet [36]. However, little is known about the role of miR-34a in porcine adipogenesis.

To explore the function of miR-34a in swine, we used bioinformatics analyses to predict its interactions, and conducted experiments to test our predictions using primary preadipocytes. The results provide insight into the ways in which non-coding RNAs affect IMF in pigs.

Results

Biological functions of miR-34a based on target analysis

To explore the possible biological functions of miR-34a, the TargetScan and miRDB algorithms were used to predict miR-34a targets. Seven hundred fifty-four and five hundred forty-seven targets were predicted with TargetScan and miRDB, respectively (Supplementary Tables S1 and S2). Two hundred ninety-eight genes overlapped with the targets (Supplementary Table S3), and were examined for potential biological roles using Gene Ontology (GO) term enrichment and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analyses. Several molecular function categories were enriched in the GO analysis (Fig. 1a). Fatty acid biosynthesis and the phosphatidylinositol signaling system were significantly enriched in the KEGG analysis (Fig. 1b).

The mature miR-34a sequence is highly conserved in various species, including human, mouse, and rat (Fig. 2a). miRNA target prediction identified ACSL4 as a potential target gene of miR-34a, with an estimated free energy of −29.2 kcal/mol for the interaction between them. ACSL4 encodes acyl-CoA synthetase long chain family member 4, which generates fatty acyl-CoA esters from long-chain fatty acids. The putative target site in the ACSL4 mRNA is shown in Fig. 2b.

Interaction between miR-34a and ACSL4

To verify that ACSL4 is a target of miR-34a, we tested their ability to interact in 293 T cells using a dual-luciferase reporter system. miR-34a mimic significantly decreased luciferase activity generated by the wild-type ACSL4 reporter vector, compared to the negative control (P < 0.01). In contrast, luciferase activity was not affected when a mutated version of the putative miR-34a interaction region was transfected into the cells (Fig. 3a). These results support the hypothesis that ACSL4 mRNA is targeted by miR-34a. We further detected the expression of ACSL4 in muscle tissue, which revealed a higher expression in the H group than that in the L group (Fig. 3b).

Expression of ACSL4 during porcine preadipocyte differentiation

To examine whether ACSL4 is a potential contributor to IMF deposition, expression of ACSL4 was measured by qRT-PCR during preadipocyte differentiation (0, 2, 4, 6, and 8 days after induction). Other marker genes that are widely used in studies of lipid metabolism [37] such as PPARγ, aP2, ATGL, and Sirt1 were also included. As shown in Fig. 4, expression of ACSL4 mRNA gradually increased after adipocytes were induced to differentiate. Expression peaked at 4 days, the time at which a majority of preadipocytes differentiated into mature adipocytes, and then declined steadily (Fig. 4). Interestingly, similar expression patterns were also observed for lipogenesis transcripts such as PPARγ and aP2 (Fig. 4). In contrast, expression of the steatolysis genes ATGL and Sirt1 increased steadily during preadipocyte differentiation (Fig. 4). The results are consistent with the hypothesis that ACSL4 is involved in lipogenesis.

miR-34a inhibits lipogenesis by targeting ACSL4

The results of the dual luciferase assay described earlier strongly suggested that miR-34a and ACSL4 mRNA interact. To test if miR-34a affects lipid metabolism, a mimic and an inhibitor of miR-34a were transfected into porcine preadipocytes. As shown in Fig. 5a, the miR-34a mimic was detected after transfection, with the highest
levels observed after 48 h. We then used qRT-PCR and western blotting to measure mRNA and protein expression of ACSL4 and other genes related to lipid metabolism 48 h after transfection of preadipocytes with miR-34a mimic and inhibitor (Fig. 5b, c, d). As expected, transfection with miR-34a mimic significantly suppressed mRNA and protein expression of ACSL4 and other lipogenesis genes, including PPARγ, aP2, and SREBP-1C, and increased expression of steatolysis genes, such as ATGL and Sirt1. In contrast, miR-34a inhibitor had the opposite effect on the mRNA and protein expression of lipogenesis and steatolysis-related genes, suggesting that miR-34a inhibits lipogenesis by targeting ACSL4. Consistent with this result, Oil Red O and triglyceride (TG) quantification assays revealed that the miR-34a mimic significantly decreased lipid droplet numbers, while the miR-34a inhibitor increased them (Fig. 5e, f).

Silencing of the ACSL4 gene decreases accumulation of lipid droplets
RNA interference was used to investigate the function of ACSL4 in adipogenesis. Three si-ACSL4 fragments (si1-ACSL4, si2-ACSL4, si3-ACSL4) were designed (Supplementary Table S4), and si3-ACSL4 was found to have the highest interference effect (Fig. 6a, b, c). Further, Oil Red O and TG quantification assays showed that
Fig. 2 Bioinformatics analysis of miR-34a. a Mature sequence of miR-34a is conserved among species including swine (ssc), human (hsa), mouse (mmu), and rat (rno). Data were obtained from miRBase (www.mirbase.org/). b Predicted interaction between ACSL4 3’UTR and miR-34a.

Fig. 3 a Dual luciferase reporter assay to detect targeting of ACSL4 by miR-34a in 293 T cells. b The relative expression of ACSL4 mRNA in muscle tissues obtained from animals in the H and L groups. Results are presented as means ± SE of three independent determinations. Labels (A vs. B) indicate significantly different values ($P < 0.01$).
knockdown of ACSL4 reduced lipid droplet accumulation (Fig. 6d, e), similar to the effect of miR-34a mimic (Fig. 5e, f). Taken together, these results demonstrated that miR-34a negatively regulates adipogenesis in porcine adipocytes by targeting ACSL4.

Discussion

Numerous miRNAs regulate adipogenesis by interacting with transcription factors or important signaling molecules that are involved in adipocyte differentiation [38]. Based on our previous studies, miR-34a is less abundant in liver tissue from pigs with higher backfat thickness, compared with pigs with lower backfat thickness [9]. Bioinformatics analysis suggested that potential miR-34a targets functioned in MAPK signaling, regulation of the actin cytoskeleton, galactose metabolism, and fatty acid biosynthesis (Fig. 1). The potential involvement of miR-34a in fatty acid biosynthesis, in combination with the high expression of miR-34a in pigs with low backfat thickness, led us to hypothesize that miR-34a functions in lipid metabolism.

In this study, we investigated the mechanism by which miR-34a affects lipid metabolism in pigs. Sequence analysis suggested that miR-34a targets the ACSL4 mRNA within the 3’UTR (Fig. 2). In a dual luciferase assay, overexpression of miR-34a inhibited luciferase activity generated by wild-type ACSL4, but did not affect activity of a construct containing a mutated version of the putative miR-34a interaction region (Fig. 3a). The porcine ACSL4 gene is a member of the long-chain acyl-CoA synthetase (ACSL) family of enzymes that catalyze the addition of a coenzyme-A (CoA) group to a fatty acid to form fatty acyl-CoAs [39, 40]. Five ACSL isoforms can each activate and channel various fatty acids to different metabolic fates [40]. Proposed functions of ACSL4 include intracellular lipid storage [41], cholesterol transport from the endoplasmic reticulum into the mitochondria [42], and regulation of arachidonic acid and its metabolites [43–46]. In addition, ACSL4 polymorphism is associated with IMF content and fatty acid composition in different pig breeds [47–49].

To verify that ACSL4 plays a role in the regulation of lipid metabolism, we measured the expression of ACSL4 during porcine preadipocyte differentiation. ACSL4 mRNA was expressed throughout the entire differentiation process, and abundant in middle term after adipocyte differentiation (Fig. 4). By studying adipocyte differentiation in vitro using various preadipose cell lines and primary preadipocytes, it has been possible to dissect the molecular and cellular events that occur during the transition from undifferentiated fibroblast-like preadipocytes into mature round fat cells [50]. As with primary human, mouse, and rat preadipocyte cell lines, primary pig preadipocytes also proliferate and differentiate, becoming adipocytes with lipid droplets in vitro (Supplementary Figure S1) [51]. We also analyzed the expression of PPARE and aP2, which are prominent adipocyte marker genes, and ATGL lipases and deacetylase Sirt1 [52]. Similar expression patterns were observed for ACSL4 and lipogenesis-associated genes, while the expression patterns for the steatolysis genes were different (Fig. 4). Also, the expression of ACSL4 mRNA in muscle

![Fig. 4 Expression of ACSL4 and other lipid metabolism-associated genes during preadipocyte differentiation in vitro (0, 2, 4, 6, and 8 days). The results are presented as means ± SE of three independent determinations. Labels (a, b, c) indicate significantly different values (P < 0.05)](image-url)
tissue is higher in the H group than that in the L group (Fig. 3b). This suggests that ACSL4 is involved in pig lipogenesis.

To further investigate whether interaction between miR-34a and ACSL4 mRNA plays a role in lipid deposition, miR-34a mimic, inhibitor, and NC were transfected into porcine preadipocytes. As expected, miR-34a mimic decreased the number of lipid droplets, resulting in lower lipid content levels compared to cells transfected with NC. In contrast, miR-34a inhibitor increased the lipid droplet number, resulting in higher lipid content levels (Fig. 5e, f). Our results are consistent
with one previous study [53], but are inconsistent with a recent report that overexpression of miR-34a increases lipid deposition in mouse liver and HepG2 cells [54]. The discrepancy may be due to differences between species or cell type. A particular miRNA may play different roles at different developmental stages within one cell type, or at the same developmental stage in different cell types [16]. We also investigated whether miR-34a regulates the expression of lipogenesis- and steatolysis-related genes. As shown in Fig. 5, miRNA-34a mimic decreased the expression of ACSL4 and lipogenesis genes (PPARγ, aP2 and SREBP-1C) and increased expression of steatolysis genes (ATGL and Sirt1). In contrast, the miR-34a inhibitor had the opposite effect on gene expression. Using RNA interference, we investigated the function of ACSL4 in adipogenesis. The results showed that knockdown of ACSL4 reduced lipid droplet accumulation (Fig. 6d, e), similar to the effect of miR-34a mimic (Fig. 5c, f). Taken together, the results demonstrate that miR-34a negatively regulates adipogenesis in porcine adipocytes by targeting ACSL4. A checklist for microRNA-target interactions (MTI) is presented in Table 1, following the recommended standards for an MTI report [55].

**Conclusions**
In this study, we investigated the mechanism by which miR-34a affects lipid metabolism in pigs. First, we demonstrated that miR-34a binds the ACSL4 mRNA at the 3’UTR using a luciferase reporter assay. We then showed that transfection of porcine preadipocytes with miR-34a mimic reduced lipid droplet formation, while transfection with miR-34a inhibitor increased the accumulation of lipid droplets. Further, knockdown of ACSL4 also decreased lipid droplet accumulation. Together, our data support the conclusion that miR-34a negatively regulates lipogenesis in porcine adipocytes by targeting ACSL4.

**Methods**

**miRNA target gene prediction and functional analyses**
miRNA targets were predicted using TargetScan 7.2 (http://www.targetscan.org/) [56] and miRDB (http://mirdb.org) [57]. The free energy of the miR-34a-A CSL4 interaction was calculated using RNAhybrid 2.2 (http://bibiserv.cebitec.uni-bielefeld.de/rnahybrid) [58]. GO term and KEGG enrichment analyses for overlapping target genes were performed using the R package ‘clusterProfiler’ [59], with p-values calculated using right-sided hypergeometric tests. Figures were prepared using the R package ‘ggplot2’ [60].

![Image](image-url)
Dual luciferase reporter assay
The mature miR-34a sequence was retrieved from miRBase (http://www.mirbase.org/). The ACSL4 3’UTR wild type (WT) and mutated (MUT) sequences were cloned into pmirGLO vectors using the SacI and SalI restriction sites. Primers for the luciferase reporter assay are listed in Supplementary Table S5. Two hundred ninety-three T cells in logarithmic growth phase were seeded into the wells of a 96-well plate. Upon reaching 80% confluence, the cells were co-transfected with ACSL4–3’UTR-Wild plasmid and miR-34a mimic using Lipofectamine 2000. ACSL4–3’UTR-Wild + miR-34a mimic NC, ACSL4–3’UTR-Mut + miR-34a mimic, and ACSL4–3’UTR-Mut + miR-34a mimic NC were also transfected for comparison. After 48 h, luciferase activity was determined with the dual-luciferase reporter assay system (Promega, USA). All luciferase assays were performed in triplicate and the experiment was performed three times.

Isolation, culture, differentiation, and transfection of porcine primary intramuscular preadipocytes
Six 7-day old Yorkshire were purchased from the experimental farm at the Chinese of Academy Agricultural Sciences. Animals were humanely euthanized by electrical stunning. The longissimus dorsi muscle (LD) was removed from piglets under sterile conditions. Visible connective tissue was removed, and the remaining tissue was finely minced. Following the protocol described in a previous study [53], preadipocytes were isolated using differential rate adherence by subjecting the tissues to digestion for 2 h with 0.1% type II collagenase. The digested sample was filtered aseptically through a 200 μm nylon mesh filter to isolate cells. The filtered and washed cells were seeded at a density of 2.5 × 10⁵ cells per 35-mm culture dish in DMEM/F12 medium with 10% fetal bovine serum (HyClone, Logan, UT, USA), supplemented with penicillin (100 U/mL) and streptomycin (100 U/mL). The cells were incubated at 37°C under a water-saturated atmosphere containing 95% air and 5% CO₂. After 2 h, the dishes were washed with a PBS solution 2–3 times to remove nonadherent cells and to obtain the precursor intramuscular-muscle fat cells. Subsequently, fresh complete culture solution was added and replaced every 2 days. After 2 days, the majority of cells had adhered to the culture dish walls (Supplementary Figure S1A). The number of adherent cells continued to increase with time, and spreading cells had triangular or fusiform shapes. After 8 days, cells had formed a single layer and were morphologically similar to primary cells (Supplementary Figure S1B). To examine IMF deposition and droplet morphology in cultured intramuscular adipocytes, cells were collected 2, 4, 6, and 8 days after induction of differentiation and then stained using Oil Red O. After 2 days of induction, a small number of lipid droplets were detected (Supplementary Figure S1C). The abundance of lipid droplets increased gradually from 4 to 6 days (Supplementary Figure S1D, E), and a large number of lipid droplets were apparent at 8 days (Supplementary Figure S1F). The results showed that the isolated cells were intramuscular preadipocytes.

When the cells reached 80% confluence, they were divided into two groups. The first group was induced to differentiate from preadipocytes to adipocytes. The differentiation medium (AIM; adipocyte-inducing medium) consisted of base medium supplemented with 0.5 mM isobutyl methylxanthine (IBMX, Sigma-Aldrich, St. Louis, MO, USA), 1.0 μM dexamethasone (DEX, Sigma, USA) and 1.0 μg/mL insulin (INS, Sigma). After addition of differentiation medium, cells were collected at 0, 2, 4, 6, and 8 days. The second group was used for overexpression and knockdown experiments. These cells were starved for 12 h in Opti-MEM (Gibco, USA), and then transfected with miR-34a mimic (artificially synthesized miR-34a mimic, 100 nM), mimic negative control (NC, 100 nM), inhibitor (anti-miR-34a, 100 nM), inhibitor NC (100 nM) and si1-ACSL4 (100 nM), si2-ACSL4 (100 nM).
μL working fluid. The standard group (SG) was added into 2.5 μL working fluid. The experiments were conducted in triplicate, and results are presented as means ± SE. Multiple comparisons were assessed with a one-way analysis of variance followed by Dunnett’s tests. P-values < 0.05 were considered to be statistically significant.

Western blotting
Cultured cells were washed two times with PBS, digested with 0.25% trypsin and then centrifuged at 1000 rpm for 15 min. The cells were homogenized in radioimmunoprecipitation assay (PIPA) lysis buffer (Beyotime, Shanghai, China) with phenylmethylsulfonyl fluoride (PMSF, Beyotime, Shanghai, China). Total protein was extracted from the supernatants after centrifugation at 12,000 rpm for 10 min. Protein concentration was determined using a BCA Protein Assay Reagent Kit (Beyotime, Shanghai, China). A protein sample of 50 μg was separated by SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membranes (Beyotime, Shanghai, China), and sealed overnight in 5% sealant. The membranes were washed three times for 10 min with 10 × TBST, blocked with Difco™ skim milk for 1 h at room temperature, and then incubated at 4°C overnight with the following rabbit primary antibodies: anti-ACSL4 polyclonal antibody (1:1000, Abclonal, Wuhan, China), anti-Sirt1 polyclonal antibody (1:1000, Abclonal, Wuhan, China), anti-ATGL polyclonal antibody (1:1000, Abclonal, Wuhan, China), anti-αP2 polyclonal antibody (1:1000, Abclonal, Wuhan, China), anti-SREBP-1c polyclonal antibody (1:1000, Abclonal, Wuhan, China), anti-PPARγ polyclonal antibody (1:1000, Abclonal, Wuhan, China), and anti-GAPDH polyclonal antibody (1:1000, Abclonal, Wuhan, China). After three washes with 10 × TBST, the second antibody (IgG 1:5000, ABclonal, Wuhan, China), conjugated with horseradish peroxidase, was added and the reaction was incubated at 37°C for 1 h. Binding was detected using an ECL chemiluminescence kit (Beyotime, Shanghai, China). A gel imaging instrument (Vilber Lourmat fusion FX 7 Spectra, France) was used to scan the immunoblots, and an image analysis application (FUSIONCAPT, France) was used to determine the relative density of each band. The results are presented as the ratios of the optical densities of targeted proteins to those of GAPDH.

Statistical analysis
Statistical analyses were performed using SAS 9.4. All experiments were conducted in triplicate, and results are presented as means ± SE. Multiple comparisons were assessed with a one-way analysis of variance followed by Dunnett’s tests. P-values < 0.05 were considered to be statistically significant.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12863-020-0836-7.

Additional file 1: Supplementary Figure S1. Identification of porcine primary intramuscular preadipocytes. (A–B) Morphology of primary intramuscular preadipocytes observed under an inverted microscope (× 400).
Supplementary Table S1. miR-34a target genes predicted using TargetScan. Supplementary Table S2. miR-34a target genes predicted using miRDB. Supplementary Table S3. miR-34a target genes predicted both by TargetScan and miRDB. Supplementary Table S4. The sequences of small interfering RNAs (siRNAs) specifically targeting ACSL4. Supplementary Table S5. Primers used for real-time quantitative PCR.

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| Authors’ contributions | HT, WW, and XL conceived this study and wrote the manuscript. XL, ND, and JT were responsible for animal care, sample preparation, and the performance of experiments. QZ and SZ performed the data processing. All authors reviewed and approved the final manuscript. |
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