MicroRNA-24 can control triacylglycerol synthesis in goat mammary epithelial cells by targeting the fatty acid synthase gene

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ABSTRACT

In nonruminants it has been demonstrated that microRNA-24 (miR-24) is involved in preadipocyte differentiation, hepatic lipid, and plasma triacylglycerol synthesis. However, its role in ruminant mammary gland remains unclear. In this study we measured miR-24 expression in goat mammary gland tissue at 4 different stages of lactation and observed that it had highest expression at peak lactation when compared with the dry period. Overexpression or downregulation of miR-24 in goat mammary epithelial cells (GMEC) strongly affected fatty acid profiles; in particular, miR-24 enhanced unsaturated fatty acid concentration. Additional effects of miR-24 included changes in triacylglycerol content and the expression of fatty acid synthase, sterol regulatory element binding transcription protein 1, stearoyl-CoA desaturase, glycerol-3-phosphate acyltransferase mitochondrial, and acetyl-CoA carboxylase. Luciferase reporter assay confirmed that fatty acid synthase is a target of miR-24. Taken together, these results not only highlight the physiological importance of miR-24 in fatty acid metabolism in GMEC, but also laid the foundation for further research on regulatory mechanisms among miR-24 and other microRNA expressed in GMEC.

Key words: microRNA-24, milk fat metabolism, fatty acid synthase

INTRODUCTION

The mammary glands are specialized organs whose main function is to produce milk, the primary source of nutrition for neonates. Compared with cow milk, goat milk contains higher levels of fat and long-chain FA (Juarez and Ramos, 1986). Milk fat secreted from mammary epithelial cells is in the form of lipid droplets,

which are almost completely composed of triacylglycerol (TAG; Hansen et al., 1984; Bionaz and Looor, 2008). A network of genes with complementary functions coordinates the overall process of ruminant milk fat synthesis, which encompasses de novo synthesis (lipogenesis, FA uptake and transport, TAG synthesis, and lipid droplet formation; Bionaz and Looor, 2008).

MicroRNA (miRNA) are a subclass of noncoding RNA that direct mRNA degradation or disrupt its translation in a sequence-dependent manner (Bartel, 2004). They play an important role in various biological processes, for instance tissue development (Yang et al., 2015), cell differentiation (Gay et al., 2014; Vimalraj and Selvamurugan, 2014), cell proliferation and apoptosis (Chen et al., 2010; Adlakha and Saini, 2011), as well as cellular lipid metabolism (Fernández-Hernando et al., 2011; Shirasaki et al., 2013).

Recent studies have revealed the importance of certain miRNA as key regulators in lactating mammary gland of several species (Gu et al., 2007; Wang et al., 2012). For instance, it has been demonstrated that miR-103 can increase milk fat synthesis by enhancing TAG accumulation and lipid droplet formation in goat mammary epithelial cells (GMEC; Lin et al., 2013b). Likewise, miR-27a can decrease mRNA expression of genes associated with TAG synthesis by suppressing peroxisome proliferator-activated receptor gamma (PPARG) protein levels in GMEC (Lin et al., 2013a).

Besides the key role of miR-103 and miR-27a in GMEC, recent data utilizing mice revealed that miR-24 enhanced hepatic lipid accumulation by inhibiting expression of insulin induced gene 1 (*INSIG1*; Ng et al., 2014) and also by regulating 3T3-L1 preadipocyte differentiation (Kang et al., 2013). Comparative miRNA profiling of goat mammary gland tissue revealed that the expression of miR-24 differs between the peak-lactation and the dry period (Li et al., 2012a). Thus, these combined data led us to hypothesize that miR-24 may also play a role in the regulation of FA metabolism in GMEC. Specific objectives were to evaluate the functional relevance of miR-24 in milk fat synthesis via treatment of GMEC with a miR-24 mimic or inhibitor

Received February 4, 2015.

Accepted July 25, 2015.

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coupled with FA profiling, lipid droplet content, and lipogenic mRNA expression analyses.

MATERIALS AND METHODS

Tissue Samples

Thirty-nine healthy second-lactation Xinong Saanen goats (3 yr old) of similar weight were selected from the Northwest A&F University herd. Twenty-four goats in mid lactation (120 d after parturition) and 5 goats in early lactation (15 d after parturition), peak lactation (60 d after parturition), and the dry period (60 d before parturition) were euthanized and mammary gland tissue samples were collected. Total RNA was isolated from each tissue sample using Trizol reagent (Invitrogen, Waltham, MA) according to the manufacturer’s instructions. The RNA quality and quantity were determined using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). The peak lactation tissue samples also were used for cell culture as described below.

Cell Culture and Transfection

The GMEC were isolated and cultured according to published protocols (Wang et al., 2010; Shi et al., 2013b, 2014). Briefly, the mammary tissue was cultured for 4 to 7 d until cells were detached. The fibroblast-like cells and adipocytes were selected out after 5 passages and purification. The passage of 8 to 12 GMEC were cultured to confluence in a basal DMEM/F12 medium (Invitrogen) supplemented with insulin (5 µg/mL; Sigma-Aldrich, St. Louis, MO), penicillin and streptomycin (100 U/mL; Harbin Pharmaceutical Group, Harbin, China), epidermal growth factor 1 (10 ng/mL; Invitrogen), hydrocortisone (1 µg/mL; Sigma-Aldrich), and 10% fetal bovine serum (Hyclone, Logan, UT) at 37°C in a humidified atmosphere with 5% CO₂. To induce lactogenesis, GMEC were cultured in a lactogenic medium for 48 h before initial experiments, as reported previously (Peterson et al., 2004; Kadegowda

et al., 2009). The lactogenic medium was prepared as the basal medium supplemented with prolactin (2 µg/mL; Sigma-Aldrich).

The GMEC were plated before transfection at a density of 2.5 × 10⁵ cells in 6-well plates, and these were used for quantitative real-time PCR (RT-qPCR), oil red O, and FA synthase activity experiments. For Western blot, FA extraction, and cellular TAG content assays, cells were plated at a density of 1 × 10⁶ cells in 60-mm dishes. The luciferase reporter experiments were performed with GMEC at a density of 50,000 cells in 48-well plates. Cells were transfected with a miR-24 mimic (60 nM), inhibitor (60 nM), and the respective controls (60 nM; Invitrogen) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. Cells were harvested 48 h after transfection.

The miRNA mimic is a small chemically modified double- or single-stranded RNA molecule designed to mimic mature endogenous miRNA after transfected into cells, whereas a miRNA inhibitor is a single-stranded RNA molecule designed to specifically bind to and inhibit endogenous miRNA molecules. We designed a double-stranded RNA molecule as a miR-24 mimic and a single-stranded RNA molecule as a miR-24 inhibitor. The negative control for the mimic treatment was double-stranded and the inhibitor (miRNA inhibitor negative control) was single-stranded. The sequences of mimic, inhibitor, and controls are listed in Table 1.

RT-qPCR and Western Blot

Total RNA was extracted from cultured cells with Trizol reagent (Invitrogen) according to the manufacturer’s instructions. The mature miRNA expression level was determined with S-Poly(T) assay (Kang et al., 2012). Briefly, a 10-µL reaction included 0.2 µg of total RNA, 2.5 µL of 4 × reaction buffer, 1 µL of polyA/reverse transcriptase enzyme mix, and 1 µL of 0.5 µM reverse transcription (RT) primer. The reaction was performed at 37°C for 30 min, followed by 42°C for 30 min, and subsequently at 75°C for 5 min. The RT products were amplified and detected with

Table 1. The sequences of microRNA-24 (miR-24) mimic, inhibitor, and controls

Name	Sequence (5'-3')
MiR-24 mimic	Sense: 5'-UGGCUCAGUUCAGCAGGAACAG-3' Antisense: 5'-GUUCCUGCUGAACUGAGCCAUU-3'
Negative control (NC)	Sense: 5'-UUCUCCGAACGUGUCACGUTT-3' Antisense: 5'-ACGUGACACGUUCGGAGAATT-3'
MiR-24 inhibitor	Sense: 5'-CUGUCCUGCUGAACUGAGCCA-3'
MicroRNA inhibitor NC	Sense: 5'-CAGUACUUUUGUGUAGUACAA-3'

Table 2. Characteristics of primers in the microRNA (miRNA) quantitative real-time PCR

MiRNA	Sequence (5'-3')
MiR-24	Reverse (R): 5'-GTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTTGTTCCT-3' Forward (F): 5'-TTCGGTGGCTCAGTTCAGC-3'
MiR-27a	R: 5'-GTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTTGC GGAA-3' F: 5'-TTCGGTTCACAGTGGCTAAG-3'
MiR-103	R: 5'-GTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTTCATAGC-3' F: 5'-GGAGCAGCATTGTACAGGG-3'
5S rRNA	R: 5'-GTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAAAAGCCTACAGCACCCG-3' F: 5'-TCTCAGAAGCTAAACAGGGTCG-3'
Universal reverse primer	5'-CAGTGCAGGGTCCGAGGT-3'

an universal Taqman probe (IDT, Coralville, IA) in a 20- μ L PCR reaction that contained 0.3 μ L of RT products, 4 μ L of 5 $\times$ qPCR probe Mix, 0.5 U of GoTaq Hot Start Polymerase (Promega, Madison, WI), 0.2 mM universal Taqman probe, and 0.5 μ M forward primer and universal reverse primer, respectively. The PCR reaction was performed at 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. The 5S rRNA was used as an internal control. All the primers used for miRNA real-time quantitative PCR (**RT-qPCR**) are listed in Table 2.

For mRNA, 0.5 μ g of total RNA was used to synthesize cDNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time, Takara, Shiga, Japan). The RT-qPCR assays were performed according to the manufacturer's instructions (SYBR Premix Ex TaqII, Perfect Real Time, Takara). Specific primers were designed for several target genes: fatty acid synthase (*FASN*), acetyl-CoA carboxylase α (*ACACA*), and acyl-CoA synthase short-chain family member 2 (*ACSS2*) play a key role in de novo FA synthesis (Bionaz and Loor, 2008); stearoyl-CoA desaturase 1 (*SCD1*), glycerol-3-phosphate acyltransferase mitochondrial (*GPAM*), 1-acylglycerol-3-phosphate O-acyltransferase 6 (*AGPAT6*), and lipin 1 (*LPIN1*) are involved in desaturation and TAG synthesis (Coleman, 2004); perilipin 2 (*PLIN2*) and patatin-like-phospholipase domain containing 2 (*PNPLA2*) are important in lipid droplet formation; lipoprotein lipase (*LPL*) hydrolyzes circulating TAG and the FA released are transported into cells by thrombospondin receptor (*CD36*) and fatty acid-binding protein 4 (*FABP4*). In addition, *PPARG*, sterol regulatory element binding transcription factor 1 (*SREBF1*), and *INSIG1* are key transcription regulators controlling the expression of genes related to FA and TAG synthesis (Bionaz and Loor, 2008). All the primers used for mRNA RT-qPCR are listed in Table 3. The mRNA expression was normalized using the geometric mean (Bionaz and Loor, 2008) of the 3 internal controls (ubiquitously expressed transcript, mitochondrial ribosomal protein L39, and ribosomal protein S9;

Bionaz and Loor, 2007; Kadegowda et al., 2009; Bonnet et al., 2013). All reactions, including controls without template, were carried out on a Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA). After amplification, the products were confirmed by agarose gel electrophoresis and sequencing.

For Western blot, cells were collected and lysed in RIPA buffer (Solarbio, Beijing, China) supplemented with PMSF (Pierce, Rockford, IL). Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane (Millipore, Billerica, MA), and probed with the primary antibodies polyclonal rabbit anti-*FASN* (s-1498R, 1:500; Bioss Inc., Woburn, MA) and monoclonal mouse anti-*GAPDH* (CWBIO, Beijing, China; 1:1000). Polyclonal goat anti-rabbit horseradish peroxidase-conjugated IgG (Tiagen, Beijing, China; 1:1000) and polyclonal goat anti-mouse HRP-conjugated IgG (Tiagen; 1:1000) were used as secondary antibody. Signals were detected by the chemiluminescent ECL Western blot detection system (Pierce).

Oil Red O Staining

The GMEC transfected with miR-24 mimic and inhibitor were washed 3 times in PBS, fixed in 10% (vol/vol) paraformaldehyde for 1 h, and then washed again with PBS. The lipid droplets in the cells were stained with 5% oil red O in isopropanol for 20 min and then washed with PBS and examined microscopically.

Cellular TAG Content Assay

Cellular TAG assays were performed as previously described (Bartholomew et al., 2012; Kang et al., 2015). Briefly, GMEC were transfected with miR-24 mimic, inhibitor, and respective controls for 48 h and then cells were harvested in lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl, 1% Triton X-100; Amresco, Solon, OH). A TAG colorimetric assay kit was used according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI) on an XD 811G Biochemistry Ana-

Table 3. Characteristics of primers in the quantitative real-time PCR

Gene and accession number ¹	Primer ² (5'-3')	Efficiency ³	Source
<i>FASN</i> DQ915966.3	F: GGGCTCCACCACCGTGTTC R: GCTCTGCTGGGCCGTGCAGCTG	2.13	Shi et al. (2013a)
<i>ACACA</i> JN236219.1	F: CTCCAACCTCAACCACTACGG R: GGGGAATCACAGAAGCAGCC	2.09	Shi et al. (2013a)
<i>ACSS2</i> BC134532	F: GGCGAATGCCTCTACTGCTT R: GGCCAATCTTTTCTCTAATCTGCTT	2.08	Bionaz and Loor (2008)
<i>GPAM</i> AY515690	F: GCAGGTTTATCCAGTATGGCATT R: GGACTGATATCTTCCTGATCATCTTG	2.09	Bionaz and Loor (2008)
<i>AGPAT6</i> J1861797.1	F: AAGCAAGTTGCCCATCCTCA R: AAAGTGTGGCTCCAAATTTTCA	2.17	Shi et al. (2013a)
<i>LPIN1</i> NM_00120615.1	F: TCCCTGCTCGGAGTATGTTG R: TGGCCACCAGAAATAAGCATG	2.15	Lin et al. (2013c)
<i>PLIN2</i> HQ846826	F: TACGATGATACAGATGAATCCCAC R: CAGCATTCGGAAGCACAGAGT	2.16	Shi et al. (2013a)
<i>LPL</i> DQ997818	F: AGGACACTTGCCACTCATTC R: TTGGAGTCTGGTTCCCTCTTGTA	2.18	Shi et al. (2013a)
<i>FABP4</i> EF105407.1	F: ACTGGGATGGGAAATCAAC R: GTGGTAGTGACACCGTTCA	1.99	This article
<i>CD36</i> X91503	F: GTACAGATGCAGCCTCATTTCC R: TGGACCTGCAAATATCAGAGGA	2.18	Shi et al. (2013a)
<i>SCD</i> GU947654	F: CCATCGCCTGTGGAGTCAC R: GTCGGATAAATCTAGCGTAGCA	2.10	Shi et al. (2013a)
<i>INSIG1</i> JQ665439.1	F: AGCCTCACAAGTTCAAGCG R: ACAGTGCTGCTAATGTCAAGG	2.03	This article
<i>PNPLA2</i> GQ918145	F: GGAGCTTATCCAGGCCAATG R: GCGGGCAGATGTCACTCT	2.04	Lin et al. (2013a)
<i>SREBF1</i> HM443643.1	F: CTGCTGACCGACATAGAAGACAT R: GTAGGGCGGGTCAAACAGG	2.20	Shi et al. (2013a)
<i>PPARG</i> HQ589347.1	F: CCTTCACCACCGTTGACTTCT R: GATACAGGCTCCACTTTGATTGC	2.21	Shi et al. (2013a)
<i>UXT</i> BQ676558	F: TGTGGCCCTTGGATAGGTT R: GGTGTGCTGCTGAGCTCTGTG	2.10	Bionaz and Loor (2007)
<i>MRPL39</i> NM017446	F: AGGTTCTCTTTTGTGGCATCC R: TTGGTCAGAGCCCCAGAAGT	2.13	Bionaz and Loor (2007)
<i>RPS9</i> DT860044	F: CCTCGACCAAGGCTGAAG R: CCTCCAGACCTCACGTTTGTTC	2.07	Bionaz and Loor (2007)

¹*FASN* = fatty acid synthase; *ACACA* = acetyl-CoA carboxylase α ; *ACSS2* = acyl-CoA synthase short-chain family member 2; *GPAM* = glycerol-3-phosphate acyltransferase mitochondrial; *AGPAT6* = 1-acylglycerol-3-phosphate O-acyltransferase 6; *LPIN1* = lipin 1; *SCD1* = stearoyl-CoA desaturase 1; *PLIN2* = perilipin 2; *PNPLA2* = patatin-like-phospholipase domain containing 2; *LPL* = lipoprotein lipase; *FABP4* = fatty acid binding protein 4; *CD36* = thrombospondin receptor; *SREBF1* = sterol regulatory element binding transcription factor 1; *INSIG1* = insulin induced gene 1; *PPARG* = peroxisome proliferator-activated receptor gamma; *UXT* = ubiquitously expressed transcript; *MRPL39* = mitochondrial ribosomal protein L39; *RPS9* = ribosomal protein S9.

²F = forward primer; R = reverse primer.

³The PCR efficiency was determined by $[10^{(-1/\text{slope})}]$; the slope was obtained by the 7-point standard curve (with a minimum R^2 of 0.99).

lyzer (Shanghai Odin Science & Technology Company, Shanghai, China). Total protein was measured with the BCA protein assay kit according to the manufacturer's instructions (Thermo-Fisher Scientific, Waltham, MA) using an Agilent 2100 bioanalyzer (Agilent Technologies). The values obtained were normalized to the total cellular protein content and were expressed as micrograms per milligram of protein.

Cellular FASN Activity Assay

The enzyme activity of cellular FASN was analyzed as previously described (Chuang et al., 2011). Briefly, cells in 6-well plates were washed with cold PBS and

collected. Ultrasonic disruption was achieved in assay buffer with 0.6 mM PMSF (Pierce) on ice and then centrifuged at $18\,000 \times g$ for 30 min at 4°C. The assay buffer contained a 100 mM potassium phosphate buffer, 1 mM EDTA (Sigma-Aldrich), and 1 mM dithiothreitol (Sigma-Aldrich) at pH 7. The supernatant was used to evaluate the *FASN* activity at 340 nm (before and after malonyl-CoA). The assay solution without malonyl-CoA served as a background for the assay.

Luciferase Reporter Assay

To generate reporter constructs for luciferase assays, a 715-bp segment containing predicted miRNA target

site in the 3' untranslated regions (UTR) of *FASN* was cloned into the psiCHECK-2 vector (Promega) between the *XhoI* and *NotI* sites immediately downstream of the *Renilla* luciferase gene. The forward primer was 5'-cc-gctcgagACAATCTGTCCCAGGTGTGCGAT-3' and the reverse primer was 5'-taagaatgcggccgcCCAGAGC-GAACGAAGGATTT-3'; the lowercase nucleotides of primers were added with restriction sites *XhoI* or *NotI*. To generate reporter constructs with mutant 3'UTR of *FASN*, primers were designed for overlap PCR as follows: forward primer: 5'-GAAGAGTCGGggtgtCCGC-3'; reverse primer: 5'-TAGCGGacaccCCGACTCT-3' (the lower-case nucleotides of primers were mutant for the miR-24 target site). Five nucleotides (CTGAG) in the target site that were complementary to the miR-24

seed region were mutated to (GGTGT). All constructs were confirmed by sequencing.

The GMEC were seeded in 48-well plates at a density of 50,000 cells per well 1 d before transfection. A total of 0.33 μ g of each reporter construct were transiently transfected using the X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer's protocols. After a 6-h recovery period in medium, cells were transfected with miR-24 mimic (60 nM), inhibitor (60 nM), and respective controls (60 nM) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocols. At 48 h posttransfection, firefly (*hluc*⁺) and *Renilla* (*hRluc*) luciferase activities were measured with the Dual-Glo luciferase assay system according to the manufacturer's instructions (Promega) by a Varioskan flash (ThermoFisher Scientific); the *hluc*⁺ gene was used as reference gene to correct the variation of transfection efficiency and the relative luciferase activity was calculated as *hRluc*/*hluc*⁺.

FA Extraction and Analysis

Extraction and analyses of FA was according to published methods (Shi et al., 2013b; Zhu et al., 2014). Briefly, total FA were extracted and methylated from approximately 100 mg of cells using 2 mL of vitriol or methanol (2.5:1 vol/vol). Methylated samples were analyzed using GC-MS with an HP-5 column (Agilent Technologies). A 2- μ L sample at a 1:10 split ratio was injected and held for 2 min at 40°C, then the temperature was increased to 240°C for 15 min at a rate of 8°C/min. For analysis, the temperature of the injector and detector was 250 and 280°C, respectively, and the ionization energy was 70 eV with 35 to 500 amu mass number. Helium was the carrier gas (0.8 mL/min).

Statistical Analysis

Data of qPCR was analyzed relative to the control using the $2^{-\Delta\Delta Ct}$ method, where Ct is the cycle threshold. The Western blot results were quantified after normalization with GAPDH. The data for each FA was analyzed as a proportion of the total FA. The SPSS 19.0 software (IBM Corp., Armonk, NY) was used for statistical analysis. Data were analyzed by ANOVA using the general linear model procedure and mean separation was performed via Tukey; Pearson analysis was performed to identify the correlation coefficient and intensity scatter plot shows comparison of 2 miRNA profiles in mammary gland. Results are expressed as mean $\pm$ standard error of the means. Differences were considered statistically significant at $P < 0.05$.

Figure 1. MicroRNA-24 (MiR-24) expression in dairy goat mammary gland tissue. All experiments were performed in quintuplicate and repeated 3 times ($n = 15$). The expression of 5S rRNA was used as a normalization control. Values are presented as means $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$.

RESULTS

miR-24 Expression Level at Different Stages of Lactation

MicroRNA-24 was detected at all stages of lactation in whole mammary gland tissue. Relative to the dry period, the expression level of miR-24 in early, peak, and mid lactation was increased by 1.80 ($P < 0.05$),

2.43 ($P < 0.01$), and 1.65 fold ($P < 0.05$), respectively (Figure 1).

miR-24 Increases TAG Level and Milk Fat Droplet Accumulation in GMEC

The level of miR-24 in cells transfected with miR-24 mimic was over 40 times higher than the negative

Figure 2. Changes in triacylglycerol (TAG) and lipid content in goat mammary epithelial cells caused by microRNA-24 (miR-24) mimic (60 nM) and miR-24 inhibitor (60 nM). The TAG level and lipid droplet were measured at 48 h post-transfection. (A and D) MiR-24 expression after treatment; (B and E) cellular TAG; (C and F) lipid droplet after oil red O treatment (one representative view); scale bar = 100 μm. (A to C) MiR-24 mimic treatment; (D to F) miR-24 inhibitor treatment. The experiments of miR-24 expression were performed in sextuplicate and repeated 3 times (n = 18), and the other experiments were performed in duplicate and repeated 3 times (n = 6). Values are presented as means ± SEM; * $P < 0.05$, ** $P < 0.01$. Color version available online.

control (Figure 2A). For the inhibition experiments, use of the miR-24 inhibitor resulted in more than a 90% decrease in miR-24 (Figure 2D).

As depicted in Figure 2B and 2C, both TAG level and lipid droplets content increased in GMEC transfected with miR-24 mimic. The TAG content of cells transfected with miR-24 mimic was increased by 1.74

fold ($P < 0.05$) compared with the negative control. We detected an accumulation of lipid droplets using oil red O staining after ectopic overexpression of miR-24 compared with the negative control. In addition, TAG and lipid droplets content decreased in the miR-24 knockdown group when compared with the control (Figure 2E and 2F).

Figure 2 (Continued). Changes in triacylglycerol (TAG) and lipid content in goat mammary epithelial cells caused by microRNA-24 (miR-24) mimic (60 nM) and miR-24 inhibitor (60 nM). The TAG level and lipid droplet were measured at 48 h post-transfection. (A and D) MiR-24 expression after treatment; (B and E) cellular TAG; (C and F) lipid droplet after oil red O treatment (one representative view); scale bar = 100 µm. (A to C) MiR-24 mimic treatment; (D to F) miR-24 inhibitor treatment. The experiments of miR-24 expression were performed in sextuplicate and repeated 3 times ($n = 18$), and the other experiments were performed in duplicate and repeated 3 times ($n = 6$). Values are presented as means $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$. Color version available online.

miR-24 Increases the Percentage of Unsaturated FA in GMEC

Compared with the control, miR-24 mimic had a significant positive effect on unsaturated FA and resulted in increases of palmitoleate (C16:1), oleate (C18:1), and linoleate (C18:2) by 7.22, 9.52, and 0.76%, respectively, relative to the control (Table 4). In contrast, the miR-24 inhibitor had a marked negative effect on C16:1 ($P < 0.05$) and C18:2 ($P < 0.05$). The SFA palmitate (C16:0) and stearate (C18:0) decreased significantly in cells transfected with miR-24 mimic ($P < 0.05$); however, C16:0 increased in cells containing the miR-24 inhibitor ($P < 0.05$).

miR-24 Regulates Expression of Genes Related to Lipid Metabolism in GMEC

Real-time qPCR results revealed that, relative to the control, ectopic overexpression of miR-24 strongly upregulated the expression of *GPAM* (2.09 fold; $P < 0.01$), *AGPAT6* (2.06 fold; $P < 0.01$), *PLIN2* (1.54 fold; $P < 0.01$), *LPL* (1.28 fold; $P < 0.05$), *SCD1* (1.34 fold; $P < 0.05$), *CD36* (1.45 fold; $P < 0.01$), and *SREBF1* (1.65 fold; $P < 0.01$), but downregulated *FASN* (0.50 fold; $P < 0.01$), *ACACA* (0.71 fold; $P < 0.05$), *ACSS2* (0.70 fold; $P < 0.05$), *INSIG1* (0.60 fold; $P < 0.01$), and *PNPLA2* (0.37 fold; $P < 0.01$; Figure 3A). In contrast, cells transfected with miR-24 inhibitor displayed marked downregulation of *AGPAT6* (0.74 fold; $P < 0.05$), *SCD1* (0.56 fold; $P < 0.01$), *PLIN2* (0.47 fold; $P < 0.01$), *LPL* (0.52 fold; $P < 0.01$), *CD36* (0.78 fold; $P < 0.05$), and *SREBF1* (0.54 fold; $P < 0.01$), but upregulated *FASN* (1.89 fold; $P < 0.01$), *ACACA* (1.46 fold; $P < 0.01$), and *INSIG1* (1.68 fold; $P < 0.01$; Figure 3B). Furthermore, the protein level of *FASN* in GMEC containing the miR-24 mimic and inhibitor was consistent with the mRNA expression response (Figure 3C).

As described in Figure 4A and 4B, we found a strong correlation between miR-24 and miR-27a expression ($r = 0.64$; $P = 0.001$), but not between miR-24 and miR-103 expression ($r = -0.412$; $P = 0.045$). Overexpression of miR-24 downregulated miR-27a expression (3.13 fold; $P < 0.01$), but no significant relationship was found between miR-24 and miR-103 (1.24 fold; $P > 0.05$; Figure 4C).

miR-24 Suppresses FASN Activity in GMEC

The GMEC with the miR-24 mimic had lower FASN activity (0.61 fold; $P < 0.01$; Figure 5A), whereas the GMEC with the miR-24 inhibitor had greater FASN

Table 4. Effects of microRNA-24 (miRNA-24) mimic and inhibitor on FA composition in goat mammary epithelial cells

FA ¹	MiR-24 mimic	Negative control (NC)
C16:0 (%)	25.49 ± 1.24 ^b	31.59 ± 1.20 ^a
C16:1 (%)	9.32 ± 1.18 ^A	2.10 ± 0.24 ^B
C18:0 (%)	3.64 ± 0.43 ^B	15.04 ± 0.95 ^A
C18:1 (%)	59.55 ± 2.32 ^A	50.03 ± 2.78 ^B
C18:2 (%)	2.00 ± 0.14 ^a	1.24 ± 0.18 ^b
SFA (%)	26.13	46.63
UFA ² (%)	73.87	53.37
UFA/SFA	2.83	1.14
	MiR-24 inhibitor	MiRNA inhibitor NC
C16:0 (%)	30.21 ± 0.79 ^a	27.85 ± 0.53 ^b
C16:1 (%)	3.28 ± 0.15 ^b	4.43 ± 0.21 ^a
C18:0 (%)	13.50 ± 0.42	12.86 ± 0.31
C18:1 (%)	51.72 ± 0.69	53.01 ± 0.56
C18:2 (%)	1.29 ± 0.19 ^b	1.85 ± 0.11 ^a
SFA (%)	43.71	40.71
UFA ² (%)	56.29	59.29
UFA/SFA	1.29	1.46

^{a,b}Means in a row with different superscripts differ at $P < 0.05$.

^{A,B}Means in a row with different superscripts differ at $P < 0.01$.

¹Relative FA composition is calculated as percentages relative to the total of these FA identified. Difference was determined with Tukey's test. All experiments were performed in duplicate and repeated 3 times ($n = 6$).

²UFA = unsaturated fatty acids.

activity (1.85 fold; $P < 0.01$) compared with the control (Figure 5B).

miR-24 Specifically Inhibits FASN Expression

Luciferase reporter assay revealed that the miR-24 mimic decreased the relative luciferase activity (0.59 fold; $P < 0.01$) of the reporter containing the wild-type 3'UTR of *FASN*. In contrast, the miR-24 inhibitor increased luciferase activity by 1.62 fold ($P < 0.01$); however, the miR-24 mimic and inhibitor had no effect on the relative luciferase activity of the reporter containing the mutated 3'UTR of *FASN* (Figure 5D). These data demonstrated that miR-24 interacts directly with the predicted binding site of the *FASN* mRNA in GMEC.

DISCUSSION

Several candidate miRNA play important roles in the biology of the lactating goat mammary gland (Li et al., 2012a,b). For example, miR-27a and miR-103 expression levels were associated with lactating stages being higher in mid lactation than the dry period (Lin et al., 2013b,c). To investigate the role of miR-24, we first demonstrated that miR-24 is expressed in goat

mammary tissue, with expression level being highest at peak lactation. The enrichment of miR-24 at the peak lactation stage may be a reflection of its physiological role in the regulation of milk component synthesis or development of the adult mammary gland.

Previous research in GMEC demonstrated that miR-27a could suppress TAG accumulation and miR-103 could increase milk fat synthesis, TAG accumulation, and lipid droplet formation in GMEC (Lin et al., 2013a,b). The fact that the lipid accumulation and

Figure 3. Expression of lipid metabolism-related genes. (A and B) Goat mammary epithelial cells were transfected with microRNA-24 (miR-24) mimic or inhibitor at 48 h post-transfection, and *FASN*, *ACACA*, *ACSS2*, *GPAM*, *AGPAT6*, *LPIN1*, *SCD1*, *PLIN2*, *PNPLA2*, *LPL*, *FABP4*, *CD36*, *INSIG1*, *SREBF1*, and *PPARG* expression levels were quantified by quantitative real-time PCR ($n = 6$). (C) Western blot analysis of *FASN* expression in the miR-24 mimic or inhibitor treatment experiments (one representative replicate). All experiments were performed in duplicate and repeated 3 times ($n = 6$). Values are presented as means $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$.

Figure 4. Correlation between microRNA (miR) expression in mammary gland of lactating goats and expression of miR in goat mammary epithelial cells transfected with miR-24 mimic. (A and B) The expression of miR-24, miR-27a, and miR-103 were measured in each of 24 goats (midlactation) and repeated 3 times; (A) Correlation between miR-24 and miR-27a; (B) correlation between miR-24 and miR-103. (C) Expression level of miR-24, miR-27a, and miR-103 was determined in GMEC at 48 h post-transfection (with experiments were performed in sextuplicate and repeated 3 times ($n = 18$)). The expression of miR was normalized to internal control (5S rRNA). Values are presented as means $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$.

TAG content were significantly altered by miR-24 in our study was consistent with the reported effect of miR-103 in GMEC (Lin et al., 2013b). However, the average miR-24 expression change in mammary tissue between the lactation and dry period was ~ 1.96 -fold upregulation, which is modest compared with the ~ 40 -fold upregulation detected in GMEC with the overexpression of miR-24. More importantly, overexpression of miR-24 only induced a 1.74-fold change in TAG. Such discrepancy in response could be partly explained by the lack of exogenous availability of 16:0 and 18:0 for TAG synthesis, as reported in previous in vitro studies (Waters and Ntambi, 1996; Zhang et al., 2006). Although data from in vitro studies cannot be directly extrapolated to the in vivo response, the link between miR-24 and TAG synthesis appears to be biologically relevant.

The inhibition of *FASN* expression and greater lipid accumulation detected in the present study with miR-24 are similar to data from HepG2 cells and primary hepatocytes in which miR-107 induced lipid accumulation by posttranscriptional negative regulation by *FASN* (Bhatia et al., 2014). In contrast, these data are inconsistent with the reported inhibition of *FASN* in study utilizing small hairpin RNA (Zhu et al., 2015). The contrasting results might be due to multiple regulatory roles of miR-24, which is supported in part by the fact that it also altered expression of other genes in the lipid synthesis pathway (e.g., *INSIG1*, *PNPLA2*, and *CD36*). The changes detected for other genes are highly suggestive that miR-24 can induce lipid accumulation in GMEC partly via recycling of FA derived from breakdown of phospholipids or from extracellular uptake. In addition, because a previous study reported that *FASN* is regulated by *SREBF1* (Li et al., 2015), the finding that miR-24 decreased the expression of *FASN* but increased *SREBF1* suggests a feedback regulatory pathway may exist among miR-24, *SREBF1*, and *FASN* (Figure 6). As a terminal enzyme complex for de novo FA synthesis, *FASN* undoubtedly is subject to regulation by various mechanism including changes in gene expression.

Both *FABP4* and *INSIG1* are targets of miR-24 in humans and mice (Kang et al., 2013; Ng et al., 2014). We confirmed that miR-24 targets *INSIG1* in GMEC using RT-qPCR and a Luciferase reporter assay (data not shown), which is in agreement with miR-24 targeting *INSIG1* in mouse HepG2 cells (Ng et al., 2014). The fact that miR-24 had no significant effect on the *FABP4* mRNA expression agrees with previous data in 3T3-L1 adipocytes (Kang et al., 2013). The current study also revealed that miR-24 could directly target the 3'UTR of *FASN* directly.

Figure 5. MicroRNA-24 (miR-24) targets *FASN*. Regulation of *FASN* activity by miR-24 mimic (A) and inhibitor (B). (C) MiR-24 site in *FASN* 3' untranslated regions (UTR) and the construction of the luciferase (Luc) expression vector fused to the *FASN* 3'UTR. Bta = bovine; WT = Luc reporter vector with the WT *FASN* 3'UTR (7,482 to 8,197 bp); MU = Luc reporter vector with the mutation at miR-24 site in *FASN* 3'UTR. (D) Induction or suppression of Luc activity with the WT or MU of the *FASN* 3'UTR by miR-24 mimic or inhibitor. Goat mammary epithelial cells were transfected with miR-24 mimic, inhibitor, the respective controls, and WT and MU. The Luc activity was measured at 48 h post-transfection. All experiments were performed in duplicate and repeated 3 times (n = 6). Values are presented as means $\pm$ SEM; * P < 0.05, ** P < 0.01. NC = negative control; Ctrl = control.

Figure 6. Gene networks related to lipid metabolism driven by microRNA in goat mammary epithelial cells. Dashed lines indicate the suppression and solid lines indicate activation.

As key transcription factors involved in lipid synthesis in bovine mammary gland, *SREBF1* and *PPARG* can regulate the expression of the same genes, including *FASN* (Shi et al., 2013b; Li et al., 2015). To maintain intracellular homeostasis, the lower synthesis of de novo FA when miR-24 was overexpressed could have induced the expression of some lipid metabolism mRNA, an idea supported by the upregulation of *SREBF1* (but not *PPARG*) when miR-24 was overexpressed. This specific response suggested that *SREBF1*, but not *PPARG*, is the major effector of miR-24 in GMEC, and this is supported by a previous study reporting that upstream transcription factors, such as *PPARG* and *SREBF1*, have different effects on downstream genes (Duplus et al., 2000).

From previous studies and our research, miR-27a, miR-103, and miR-24 are all expressed in GMEC and may interact with each other to regulate FA synthesis and metabolism (Lin et al., 2013a,b). The current study revealed a strong positive relationship between the expression level of miR-24 and miR-27a in goat mammary gland tissue, which is consistent with a previous study reporting that in human the miR-23a-miR-27a-miR-24-2 cluster is transcribed by PolIII (Lee et al., 2004). The correlation in the expression of miR-24 and miR-27a was confirmed in GMEC by the fact that miR-24 upregulated miR-27a expression; however, little correlation was observed between miR-24 and miR-

103, which agrees with the fact that miR-24 did not alter the expression of miR-103 in GMEC. Based on the significant effects of miR-24, miR-27a, and miR-103 on the expression of the same gene (i.e., *DGAT1*, *SCD*, and *PLIN2*; Figure 3; Lin et al., 2013a,b), we speculated that the changes in FA synthesis in GMEC may have been related to the existence of co-regulation among various miRNA and target genes, underscoring the importance of molecular synergism.

The fact that ectopic expression of miR-24 led to marked upregulation of *SCD* expression and increased the unsaturated FA concentration is consistent with the function of *SCD* protein, which is to catalyze the conversion of C16:0 and C18:0 to C16:1 and C18:1. (Miyazakia and Ntambi, 2003). Besides the control of unsaturated FA synthesis at the level of *SCD*, other factors [such as *AGPAT6* or diacylglycerol acyltransferase 1 (**DGAT1**) mRNA and protein expression] may exist that in coordination with the desaturation process affects the cellular FA composition. Both *DGAT1* and *AGPAT6* are key components of the overall pathway leading to synthesis of milk lipid droplets and exert an effect on milk fat yield and composition (Schennink et al., 2007, 2008; Littlejohn et al., 2014). In the present study, miR-24 had a significant role in regulating *AGPAT6* mRNA expression and, although we did not measure the expression of *DGAT1*, the mechanism leading to an overall increase in unsaturated FA synthesis by miR-24

overexpression likely involves changes in expression of *SCD* and other genes, or possibly increases the activity of encoded enzymes. Our data highlight the role of miR-24 in regulating the synthesis of unsaturated FA in GMEC and suggest that it might be a crucial target for regulating the composition of unsaturated FA in goat milk by nutritional means. Further studies are necessary to uncover the detailed mechanisms.

CONCLUSIONS

We identified miR-24 as a key player in TAG accumulation and unsaturated FA concentration in GMEC. The *FASN* gene is a novel direct target that links miR-24 with a potential role in control of lipid metabolism in ruminant mammary cells. In light of the results presented herein, miR-24 may potentially play a role in milk production and synthesis of beneficial milk components in dairy ruminants.

ACKNOWLEDGMENTS

This research was jointly supported by the National Natural Science Foundation of China (No. 31372281; Beijing, China), the Special Fund for Agro-scientific Research in the Public Interest (No. 201103038; Beijing, China), and the Transgenic New Species Breeding Program of China (No. 2014ZX08009-051B; Beijing, China).

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