miR-27a controls triacylglycerol synthesis in bovine mammary epithelial cells by targeting peroxisome proliferator-activated receptor gamma

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ABSTRACT

Growing evidence has revealed that microRNA are central elements in milk fat synthesis in mammary epithelial cells. A negative regulator of adipocyte fat synthesis, miR-27a has been reported to be involved in the regulation of milk fat synthesis in goat mammary epithelial cells; however, the regulatory role of miR-27a in bovine milk fat synthesis remains unclear. In the present study, primary bovine mammary epithelial cells (BMEC) were harvested from mid-lactation cows and cultured in Dulbecco's modified Eagle's medium/F-12 medium with 10% fetal bovine serum, 5 µg/mL of insulin, 1 µg/mL of hydrocortisone, 2 µg/mL of prolactin, 1 µg/mL of progesterone, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. We found that the overexpression of miR-27a significantly suppressed lipid droplet formation and decreased the cellular triacylglycerol (TAG) levels, whereas inhibition of miR-27a resulted in a greater lipid droplet formation and TAG accumulation in BMEC. Meanwhile, overexpression of miR-27a inhibited mRNA expression of peroxisome proliferator-activated receptor gamma (PPARG), CCAAT/enhancer-binding protein beta (C/EBPβ), perilipin 2 (PLIN2), and fatty acid binding protein 3 (FABP3), whereas miR-27a downregulation increased *PPARG*, *C/EBPβ*, *FABP3*, and CCAAT enhancer binding protein alpha (C/EBPα) mRNA expression. Furthermore, Western blot analysis revealed the protein level of PPARG in miR-27a mimic and inhibitor transfection groups to be consistent with the mRNA expression response. Moreover, luciferase reporter assays verified that PPARG was the direct target of miR-27a. In summary, these results indicate that miR-27a has the ability to control TAG synthesis in BMEC via targeting PPARG, suggesting that miR-27a could potentially be used to improve beneficial milk components in dairy cows.

Key words: miRNA-27a, PPARG, triacylglycerol synthesis, bovine mammary epithelial cell

INTRODUCTION

Milk is one of the most complete foods produced in the world, and its major nutritional value is attributed to its fats and proteins (Nickerson, 1960). Milk fat determines the quality of milk, and its production and is also a main target trait in dairy cow breeding (Li et al., 2015a). Recently, molecular mechanisms of milk fat synthesis and secretion have been widely studied; however, our understanding of the mechanisms underlying the regulation of milk fat synthesis still remains limited (Clegg et al., 2001; Bionaz and Looor, 2008b; Lin et al., 2013c; Liu et al., 2015).

MicroRNA (**miRNA**) are small regulatory molecules that are involved in the posttranscriptional regulation of gene expression (Bartel, 2004) and have an established role as central elements in many biological processes, such as development, differentiation, and metabolism (Shen et al., 2010; Ono et al., 2015; Martin et al., 2016). Additionally, recent studies have revealed important regulatory roles of miRNA in milk fat synthesis (Gu et al., 2007; Wang et al., 2012; Lin et al., 2013b). For instance, miR-103, miR-24, and miR-145 increase milk fat synthesis by enhancing fat droplet formation and triacylglycerol (**TAG**) accumulation in goat mammary epithelial cells (**GMEC**; Lin et al., 2013a; Wang et al., 2015, 2017). Similarly, miR-486 promotes TAG accumulation via a downregulation of *PTEN* gene expression in bovine mammary epithelial cells (**BMEC**; Li et al., 2015a), whereas in others, bta-miR-181a has been reported to have a negative effect on lipid synthesis in BMEC via targeting *ACSL1* (Lian et al., 2016).

Bovine miR-27a (bta-miR-27a) is located on the bovine chromosome 7 between 12,981,791 and 13,839,090 bp. Recent studies have indicated that miR-27a had different expression profiles during different stages of lactation in mammary glands of mice, goats, and cows (Alonso-Vale et al., 2009; Chen et al., 2010; Lin et al., 2013c; Shen et al., 2016), and have reported that miR-27a decreases milk fat synthesis by suppressing TAG

Received November 6, 2016.

Accepted January 20, 2017.

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accumulation in GMEC (Lin et al., 2013c). Furthermore, recent studies have indicated that *PPARG* is a prospective target gene of miR-27a (Lin et al., 2008; Ji et al., 2009; Kim et al., 2010) and found that overexpression of miR-27a reduces *PPARG* expression in GMEC (Lin et al., 2013c); *PPARG* is a member of the peroxisome proliferator-activated receptors (**PPAR**) family (consisting of PPAR α , β , and γ) and a key regulator of lipid accumulation (Lin et al., 2008). In the bovine mammary gland, *PPARG* mRNA expression changes during lactation, which was suggested to be central for the regulation of milk fat synthesis (Bionaz and Loor, 2008b; Kadegowda et al., 2009a). The *PPARG* knockout mice increased the utilization of fatty acids for synthesis of inflammatory lipids due to reduced TAG synthesis (Wan et al., 2007), and FABP3 affects *PPARG* expression toward stimulating milk fat synthesis in BMEC (Liang et al., 2014). Consequently, we hypothesized that miR-27a may be involved in the regulation of milk fat synthesis in BMEC. The specific objectives of our study sought to overexpress or inhibit bta-miR-27a in BMEC to establish functional relationships with *PPARG*.

MATERIALS AND METHODS

Ethics Statement

The animal care and use were approved by the Institutional Animal Care and Use Committee in the College of Animal Science and Technology, Northwest A&F University, Yangling, China.

Cell Culture and Transfection

Primary BMEC were harvested from mid-lactation cows following published protocols (Lu et al., 2012; Hou et al., 2016). Briefly, mid-lactation cows were slaughtered and several pieces of mammary parenchyma tissue were aseptically removed from the mid region of the mammary glands. Mammary epithelial cells were isolated from mammary tissues by collagenase digestion and cultured in Dulbecco's modified Eagle's medium/F12 medium (Gibco-Invitrogen, New York, NY) containing 10% fetal bovine serum, 100 U/mL of penicillin, 100 μ g/mL of streptomycin, 5 μ g/mL of insulin, 1 μ g/mL of hydrocortisone, and 1 μ g/mL of progesterone on cell culture dishes coated with 0.5% rat tail collagen (Sigma-Aldrich, St. Louis, MO). Cells were maintained at 37°C in an atmosphere containing 5% CO₂. When cells grew to 80% confluency, the primary mammary epithelial cells were trypsinized with 0.25% trypsin plus 0.02% EDTA and passaged. The pure mammary epithelial cells were isolated after 3 to 4 passages. Forty-

eight hours before BMEC treatment, the culture medium was replaced with lactogenic medium, which was prepared as the basal medium and supplemented with prolactin (2 μ g/mL). Cells were then transfected with a miR-27a mimic (50 nM), inhibitor (100 nM), and the respective controls (50 nM; Invitrogen, Carlsbad, CA) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Cells were collected for analysis 48 h after transfection.

Quantitative Real-Time PCR

Total RNA was extracted from cultured cells using the Trizol reagent (TaKaRa, Shiga, Japan) according to manufacturer's instructions. For miRNA expression analysis, cDNA was synthesized via the PrimeScript miRNA RT-PCR kit (Tiangen, Beijing, China), and *5S* and *U6* were used as endogenous control genes (Peltier and Latham, 2008; Bustin et al., 2009; Wang et al., 2012). The primers 5'-AGGGCTTAGCTGCT TGT-GAGCA-3' for miR-27a, 5'-ACTCTTAGCGGTGGAT-CATC-3' and 5'-AGTG ACGCTCAGAC AGGCA-TA-3' for *5S*, and 5'-CGCTTCGGCAGCACATATAC-3' and 5'-TCACGA ATTTGCGTGTGCAT-3' for *U6* were designed using Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA) and synthesized by ShengGong (Sangon Biotech, Shanghai, China). For mRNA expression analysis, first-strand cDNA was synthesized using a reverse transcription kit (TaKaRa), and *UXT* and *RPS9* were used as endogenous control genes (Bionaz and Loor, 2007; Kadegowda et al., 2009b; Bougarn et al., 2011). Real-time PCR primers for amplification of mRNA were designed by Primer Premier 5.0 and synthesized by ShengGong (Sangon Biotech; Table 1). Quantitative real-time (**RT**) PCR was performed using the SYBR Premix EX *Taq*II (TaKaRa) and miRcute miRNA qPCR detection kit (Tiangen) in a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA). The relative expression results were obtained using the $2^{-\Delta\Delta C_t}$ method, which were first normalized to the geometric mean of 2 endogenous control genes (Bionaz and Loor, 2008b; Huang et al., 2014).

Western Blot

For Western blot, cells were collected and lysed in radio immunoprecipitation assay buffer (Solarbio, Beijing, China) supplemented with phenylmethanesulfonyl fluoride (Pierce, Rockford, IL) after treatment for 48 h. Total protein concentrations were determined by bicinchoninic acid assays (Sangon Biotech). Proteins were separated on 12% polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). After overnight blocking with 5%

Table 1. Sequences of primer pairs and amplicon size for real-time PCR

Gene	Primer sequences (5'-3')	Amplicon size (bp)	Accession number
<i>C/EBPβ</i>	TTCTCTCCGACCTCTTCTC CCAGACTCACGTAGCCGTACT	74	NM_176788
<i>PPARG</i>	CGTGGACCTTTCTATGATGGATG GATACAGGCTCCACTTTGATTGC	90	NM_181024
<i>C/EBPα</i>	ATCTGCGAACACGAGACG CCAGGAACCTCGTCGTTGAA	69	NM_176784
<i>FATP1</i>	GAGAACGTCTCCACCACTGA ACAGCCACTCCATACACAGC	83	NM_001033625
<i>FASN</i>	ACTCAACGGGAACCTTACAG GTGGGGATGCGGGAATACA	201	NM_001012669
<i>FABP3</i>	GTCACCTCGGTGTCGGTTTGG TCCTGTCTATCTGCTGTGGTCT	173	NM_174313
<i>PLIN2</i>	GGCTGGTGACATCTACTCTG GGACGGGTTTAGGGTGTT	260	NM_173980
<i>ACSL1</i>	CCTGACTGTTGCTGGAGACT ACCACTTGCCAATGTCCC	230	NM_001076085
<i>ACCA</i>	CTCCAACCTCAACCACTACGG GGGGAATCACAGAAGCAGCC	171	NM_174224
<i>SREBP1</i>	CAATGTGTGAGAAGGCCAGT ACAAGGAGCAGGTACACAG	109	NM_001113302
<i>UXT</i>	TGTGGCCCTTGGATATGGTT GGTTGTCTGCTGAGCTCTGTG	101	NM_001037471
<i>RPS9</i>	CCTCGACCAAGAGCTGAAG CCTCCAGACCTCACGTTTGTTC	64	NM_001101152

dried nonfat milk in PBS containing 0.1% Tween 20, membranes were incubated with rabbit anti-PPARG polyclonal antibody (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and mouse anti-GAPDH monoclonal antibody (1:1000; Santa Cruz Biotechnology). Membranes were then washed 3 times with PBS-Tween and incubated for 1 h at room temperature with 3,500-fold diluted horseradish peroxidase-labeled goat or mouse anti-rabbit IgG (Santa Cruz Biotechnology). Immunoreactive bands were detected using the enhanced chemiluminescence detection kit (Amersham Biosciences, Little Chalfont, UK) and scanned on a chemiluminescent imaging system (MFCHEMI-BIS3.2, DNR Bio-Imaging Systems Ltd., Jerusalem, Israel).

Oil Red O Staining

The BMEC transfected with miR-27a mimic and inhibitor for 48 h were washed 3 times in PBS, fixed in 10% (vol/vol) paraformaldehyde for 40 min, and then washed again with PBS. The lipid droplets in the cells were stained with 5% oil red O (Sigma-Aldrich, St. Louis, MO) in isopropanol for 40 min and then washed with PBS and examined microscopically.

TAG Assay

The levels of cellular TAG in BMEC were assayed using the tissue triglyceride assay kit (Applygen Technologies, Beijing, China). Total protein concentrations

were determined by the BCA assay (Sangon Biotech). All of the experiments were performed according to the manufacturer's recommended protocol. The values obtained were normalized to the total cellular protein content and were expressed as micrograms per milligram of protein.

miRNA Target Validation Using Dual Luciferase Reporter Assay

TargetScan (www.targetscan.org) was used to predict biological targets of miRNA through scanning for seed matches between the bta-miR-27a seed region and the predicted gene. The mature sequence of bta-miR-27a was obtained from the miRBase Sequence Database (<http://www.mirbase.org/>). That sequence and the 3' untranslated region (UTR) mRNA of PPARG from *Bos taurus* were aligned using the UCSC Genome Bioinformatics program (<http://genome.ucsc.edu/>). The PPARG-3'UTR-wildtype (WT) or -mutation type (MUT) was cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI) using the XhoI and NotI restriction sites. The 293T cells were cultured in 24-well plates 1 d before transfection. Approximately 0.16 μ g of PPARG -3' UTR-WT or -MUT vectors were cotransfected with 5 pmol miR-27a mimics or negative control into 293T cells using Lipofectamine 2000 reagent (Invitrogen). After 48 h of transfection, luciferase activity was measured using the Dual-Luciferase Reporter Assay System

(Promega) according to the manufacturer's instructions. Firefly luciferase activity was normalized to the activity of Renilla luciferase.

Data Analysis

All the results are presented as means $\pm$ standard error. Statistical differences in 2 groups were assessed by Student's *t*-test via SPSS Statistics 17 software (SPSS Inc., Chicago, IL). Statistical significance was declared at $P < 0.05$ and $P < 0.01$. All experiments were performed in triplicate.

RESULTS

miR-27a Suppresses Milk Lipid Droplet Accumulation and Decreases TAG in BMEC

The miR-27a expression was significantly increased in the miR-27a mimic-transfected group compared with the negative control (Figure 1A). In contrast, the miR-27a inhibitor significantly suppressed miR-27a expression (Figure 2A). We measured the lipid droplet size and cellular TAG content in BMEC; miR-27a mimic significantly decreased the lipid droplet size (Figure 1B) and resulted in lower TAG level (Figure 1C) compared with the control group. However, the lipid droplet size and cellular TAG content were increased in the miR-27a inhibitor-transfected group compared with the control (Figure 2B and 2C).

miR-27a Regulates Expression of Lipid-Related Genes in BMEC

The expression of lipid metabolism-related genes was quantified via RT-PCR after transfection with the miR-27a mimic or miR-27a inhibitor. The results revealed that the miR-27a mimic significantly downregulated *C/EBP β* ($P < 0.05$), *FABP3* ($P < 0.01$), and *PLIN2* ($P < 0.05$) gene expression compared with the control (Figure 3A). In contrast, the miR-27a inhibitor transfection group significantly increased expression of *C/EBP β* ($P < 0.05$), *C/EBP α* ($P < 0.01$), and *FABP3* ($P < 0.01$; Figure 3B).

PPARG is a Target Gene of miR-27a

Compared with the control group, RT-PCR results showed that the miR-27a mimic significantly reduced *PPARG* mRNA expression (Figure 4A), but that miR-27a inhibitor transfection significantly increased *PPARG* mRNA expression (Figure 4B). In parallel, Western blot analysis revealed the protein level of *PPARG* in miR-27a mimic and inhibitor transfection

groups to be consistent with the mRNA expression response (Figures 4A and 4B). Furthermore, *PPARG* was predicted to be a potential target gene of miR-27a via TargetScan (Figure 5A).

miR-27a Specifically Inhibits PPARG Expression

The dual-luciferase reporter system verified the *PPARG* gene to be a direct target of miR-27a. The miR-27a mimics significantly decreased the normalized luciferase activity by 41% compared with the negative control ($P = 0.006$, Figure 5B), and the luciferase activity in the *PPARG*-3'UTR-MUT group returned to normal levels (Figure 5B).

DISCUSSION

Recently, numerous studies have confirmed the crucial regulatory roles of miRNA in regulating mammary gland development and protein synthesis in cows (Wright et al., 2010). Furthermore, increasing evidence has emerged that miRNA play a key role in regulating milk fat synthesis. Currently, although several miRNA have been found to be involved in the regulation of milk fat synthesis in BMEC (Li et al., 2015a; Lian et al., 2016), data about the role of miRNA in bovine milk fat synthesis remain limited.

The role of miR-27a in various cell types has been well established (Ji et al., 2009; Chen et al., 2010; Kim et al., 2010; Zhao et al., 2015; Huang et al., 2012). Ji et al. (2009) reported that overexpressed miR-27 restores the ability to accumulate cytoplasmic lipid droplets in hepatic stellate cells. Interestingly, miR-27a has been downregulated during adipogenesis of 3T3-L1 cells, and has thus been considered a negative regulator of adipocyte fat synthesis (Kim et al., 2010; Wang et al., 2011). Moreover, previous research in GMEC demonstrated that miR-27a affects fat metabolism-related gene expression and suppresses TAG accumulation (Lin et al., 2013c). The fact that miR-27a significantly altered lipid accumulation and TAG content in our study was consistent with a previous study on GMEC (Lin et al., 2013c). The observed decrease in cellular TAG was consistent with the decrease in the milk fat-related genes *PPARG*, *C/EBP β* , *PLIN2*, and *FABP3*. Both *PPARG* and *C/EBP β* genes are critical regulators of adipogenesis (Ntambi and Kim, 2000; Lane and Tang, 2005; Avril-Sassen et al., 2009) and have been revealed as positive regulators of milk fat synthesis in mammary epithelial cells (Liu et al., 2016). *FABP3* significantly promotes the lipid droplet accumulation via upregulating the expression of *PPARG* (Liang et al., 2014). The effects of miR-27a on the expression profile of *PLIN2* in this study were consistent with a previous study in

Figure 1. Changes in triacylglycerol (TAG) and lipid content in bovine mammary epithelial cells caused by miR-27a mimic. (A) MiR-27a expression after treatment; (C) cellular TAG; (B) lipid droplet after oil red O (Sigma-Aldrich, St. Louis, MO) treatment (one representative view); scale bar = 50 μm . Values are presented as the means $\pm$ SEM; $*P < 0.05$, $**P < 0.01$. NC = negative control. Color version available online.

Figure 2. Changes in triacylglycerol (TAG) and lipid content in bovine mammary epithelial cells caused by miR-27a inhibitor. (A) MiR-27a expression after treatment; (C) cellular TAG; (B) lipid droplet after oil red O (Sigma-Aldrich, St. Louis, MO) treatment (one representative view); scale bar = 50 μm . Values are presented as the means $\pm$ SEM; ** $P < 0.01$. NC = negative control. Color version available online.

Figure 3. The expression of lipid metabolism-related genes. (A) Bovine mammary epithelial cells were transfected with miR-27a mimic; (B) bovine mammary epithelial cells were transfected with miR-27a inhibitor. Values are presented as the means $\pm$ SEM; * P < 0.05, ** P < 0.01. miRNA = microRNA; NC = negative control.

GMEC (Lin et al., 2013c). Recent studies have demonstrated that *PLIN2* is a PPARG downstream target gene (Liang et al., 2014), playing a central role in the regulation of lipid synthesis and droplet formation in dairy cow mammary epithelial cells (Shimano, 2001; Ma and Corl, 2012). Consequently, our results revealed

that the ability of miR-27a to control lipid accumulation and TAG content by affecting fat metabolism-related gene expression in BMEC.

Previous studies have indicated PPARG to be a prospective target gene of miR-27a in 3T3-L1 cell lines (Kim et al., 2010), mouse adipose tissue (Lin et al.,

2008), and rat hepatic stellate cell lines (Ji et al., 2009). Furthermore, miR-27a overexpression reduced PPARG expression in GMEC; however, direct proof that PPARG is a target of miR-27a has not been published (Lin et al., 2013c). In this study, we confirmed that miR-27a targets PPARG in BMEC via RT-PCR, Western blot, and a luciferase reporter assay. In our study, PPARG was significantly upregulated following overexpression of miR-27a and significantly downregulated following inhibition of miR-27a, both at the mRNA and protein levels. Our results revealed that miR-27a directly af-

fects milk fat synthesis in BMEC by targeting PPARG. At present, popular molecular markers include RAPD, RFLP, SSR, AFLP, SRAP, TRAP, and SNP. Although these molecular markers contribute significantly to the field of molecular genetics, each marker type generally has one or more deficiencies in terms of polymorphism abundance, reproducibility, and ease of production (Jin et al., 2003; Blair et al., 2011). MicroRNA are a type of endogenous noncoding RNA prevalent in the genomes of many organisms, and have been considered as a new type of molecular marker with high reproducibility,

Figure 4. Peroxisome proliferator-activated receptor gamma (PPARG) mRNA and protein expression. (A) The mRNA of the *PPARG* gene in the miR-27a mimic or inhibitor treatment experiments; Western blot analysis of *PPARG* expression in the miR-27a mimic or inhibitor treatment experiments. Values are presented as the means $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$. NC = negative control.

2013). Thus, the above results indicated that miR-27a regulating target genes in fat synthesis might be used as a new pathway for better breeding in dairy cows.

Figure 5. (A) Sequence alignment of miR-27a and 3'-untranslated region (UTR) of peroxisome proliferator-activated receptor gamma (PPARG) using the TargetScan (www.targetscan.org) and miRDB algorithms (<http://mirdb.org/miRDB/>). (B) The 293T cells were co-transfected with miR-27a mimics and a luciferase reporter containing a fragment of the PPARG 3'-UTR harboring either the miR-27a binding site (PPARG-3'UTR-WT) or a mutant (PPARG-3'UTR-MUT). Values are presented as the means $\pm$ SEM; $^{**}P < 0.01$. SV40 = Simian virus 40; poly A = polyadenylic acid; bta = *Bos taurus*; Rluc/Fluc = Renilla luciferase/firefly luciferase.

However, these findings underscore the need for further studies, including animal experiments, before miR-27a can be used for this purpose.

CONCLUSIONS

In summary, we identified miR-27a as a key regulator of TAG accumulation in BMEC. We demonstrated *PPARG* to be a direct target gene that links miR-27a with a potential role in the control of fat synthesis in BMEC. The results indicate that miR-27a could potentially be used to improve beneficial milk components in dairy cows.

ACKNOWLEDGMENTS

Research supported by the National Natural Science Foundation of China (No. 31402044 and 31402077, Beijing), the Fundamental Research Funds for the Central Universities (no. Z109021619 and Z109021603, Beijing, China), and the Ministry of Science and Technology (MOST), Government of PR China (no. 2012BAD12B07, Beijing).

REFERENCES

- Alonso-Vale, M. I. C., S. B. Peres, C. Vernochet, S. R. Farmer, and F. B. Lima. 2009. Adipocyte differentiation is inhibited by melatonin through the regulation of c/EBP β transcriptional activity. *J. Pineal Res.* 47:221–227.
- Avril-Sassen, S., L. D. Goldstein, J. Stingl, C. Blenkiron, J. Quesne, I. Spiteri, K. Karagavrilidou, C. J. Watson, S. Tavaré, E. A. Miska, and C. Calds. 2009. Characterisation of microRNA expression in post-natal mouse mammary gland development. *BMC Genomics* 10:548.
- Bartel, D. P. 2004. MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* 116:281–297.
- Bionaz, M., and J. J. Looor. 2007. Identification of reference genes for quantitative real-time PCR in the bovine mammary gland during the lactation cycle. *Physiol. Genomics* 29:312–319.
- Bionaz, M., and J. J. Looor. 2008a. ACSL1, AGPAT6, FABP3, LPIN1, and SLC27A6 are the most abundant isoforms in bovine mammary tissue and their expression is affected by stage of lactation. *J. Nutr.* 138:1019–1024.
- Bionaz, M., and J. J. Looor. 2008b. Gene networks driving bovine milk fat synthesis during the lactation cycle. *BMC Genomics* 9:366.
- Blair, M. W., N. Hurtado, C. M. Chavarro, M. C. Munoz-Torres, M. C. Giraldo, F. Pedraza, J. Tomkins, and R. Wing. 2011. Gene-based SSR markers for common bean (*Phaseolus vulgaris* L.) derived from root and leaf tissue ESTs: An integration of the BMC series. *BMC Plant Biol.* 11:50.
- Bougarn, S., P. Cunha, F. B. Gilbert, F. Meurens, and P. Rainard. 2011. Technical note: Validation of candidate reference genes for normalization of quantitative PCR in bovine mammary epithelial cells responding to inflammatory stimuli. *J. Dairy Sci.* 94:2425–2430.
- Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele, and C. T. Wittwer. 2009. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55:611–622.
- Chen, X., C. Gao, H. Li, L. Huang, Q. Sun, Y. Dong, C. Tian, S. Gao, H. Dong, D. Guan, X. Hu, S. Zhao, L. Li, L. Zhu, Q. Yan, J. Zhang, K. Zen, and C. Y. Zhang. 2010. Identification and characterization of microRNAs in raw milk during different periods of lactation, commercial fluid, and powdered milk products. *Cell Res.* 20:1128–1137.
- Clegg, R. A., M. C. Barber, L. Pooley, I. Ernens, Y. Larondelle, and M. T. Traversa. 2001. Milk fat synthesis and secretion: Molecular and cellular aspects. *Livest. Prod. Sci.* 70:3–14.
- Fu, D., B. Ma, A. S. Mason, M. L. Xiao, L. J. Wei, and Z. H. An. 2013. MicroRNA-based molecular markers: A novel PCR-based genotyping technique in Brassica species. *Plant Breed.* 132:375–381.
- Gu, Z., S. Eleswarapu, and H. Jiang. 2007. Identification and characterization of microRNAs from the bovine adipose tissue and mammary gland. *FEBS Lett.* 581:981–988.
- Hou, X., L. Lin, W. Xing, Y. Yang, X. Duan, Q. Li, X. Gao, and Y. Lin. 2016. Spleen tyrosine kinase regulates mammary epithelial cell proliferation in mammary glands of dairy cows. *J. Dairy Sci.* 99:3858–3868.
- Huang, Y. Z., L. Z. Zhang, X. S. Lai, M. X. Li, Y. J. Sun, C. J. Li, X. Y. Lan, C. Z. Lei, C. L. Zhang, X. Zhao, and H. Chen. 2014. Transcription factor ZBED6 mediates IGF2 gene expression by regulating promoter activity and DNA methylation in myoblasts. *Sci. Rep.* 4:4570.
- Huang, Z., X. Chen, B. Yu, J. He, and D. Chen. 2012. MicroRNA-27a promotes myoblast proliferation by targeting myostatin. *Biochem. Biophys. Res. Commun.* 423:265–269.
- Jevsinek Skok, D., I. Godnic, M. Zorc, S. Horvat, P. Dovc, M. Kovac, and T. Kunec. 2013. Genome-wide in silico screening for microRNA genetic variability in livestock species. *Anim. Genet.* 44:669–677.
- Ji, J., J. Zhang, G. Huang, J. Qiana, X. Q. Wang, and S. Meia. 2009. Over-expressed microRNA-27a and 27b influence fat accumulation and cell proliferation during rat hepatic stellate cell activation. *FEBS Lett.* 583:759–766.
- Jin, Q., D. Waters, G. M. Cordeiro, R. J. Henry, and R. F. Reinke. 2003. A single nucleotide polymorphism (SNP) marker linked to the fragrance gene in rice (*Oryza sativa* L.). *Plant Sci.* 165:359–364.
- Kadegowda, A. K., M. Bionaz, L. S. Piperova, and J. J. Looor. 2009a. Peroxisome proliferator-activated receptor- γ activation and long-chain fatty acids alter lipogenic gene networks in bovine mammary epithelial cells to various extents. *J. Dairy Sci.* 92:4276–4289.
- Kadegowda, A. K., M. Bionaz, B. Thering, L. S. Piperova, R. A. Erdman, and J. J. Looor. 2009b. Identification of internal control genes for quantitative polymerase chain reaction in mammary tissue of lactating cows receiving lipid supplements. *J. Dairy Sci.* 92:2007–2019.
- Kim, S. Y., A. Y. Kim, H. W. Lee, Y. H. Son, G. Y. Leea, J. W. Leea, Y. S. Leea, and J. B. Kima. 2010. miR-27a is a negative regulator of adipocyte differentiation via suppressing PPAR γ expression. *Biochem. Biophys. Res. Commun.* 392:323–328.
- Lane, M. D., and Q. Q. Tang. 2005. From multipotent stem cell to adipocyte. *Birth Defects Res. A Clin. Mol. Teratol.* 73:476–477.
- Li, D., X. Xie, J. Wang, Y. Bian, Q. Li, X. Gao, and C. Wang. 2015a. MiR-486 regulates lactation and targets the PTEN gene in cow mammary glands. *PLoS One* 10:e0118284.
- Li, R., F. Beaudoin, A. A. Mmah, N. Bissonnette, C. Benchaar, X. Zhao, C. Lei, and E. M. Ibeagha-Awemu. 2015b. Deep sequencing shows microRNA involvement in bovine mammary gland adaptation to diets supplemented with linseed oil or safflower oil. *BMC Genomics* 16:884.
- Lian, S., J. R. Guo, X. M. Nan, L. Ma, J. J. Looor, and D. P. Bu. 2016. MicroRNA Bta-miR-181a regulates the biosynthesis of bovine milk fat by targeting ACSL1. *J. Dairy Sci.* 99:3916–3924.
- Liang, M. Y., X. M. Hou, B. Qu, N. Zhang, N. Li, Y. J. Cui, Q. Z. Li, and X. J. Gao. 2014. Functional analysis of FABP3 in the milk fat synthesis signaling pathway of dairy cow mammary epithelial cells. *In Vitro Cell. Dev. Biol. Anim.* 50:865–873.
- Lin, Q., G. G. Zhan, R. M. Alarcon, J. P. Ye, and Z. Yun. 2009. A role of miR-27 in the regulation of adipogenesis. *FEBS J.* 276:2348–2358.
- Lin, X., J. Luo, L. Zhang, W. Wang, and D. Gou. 2013a. MiR-103 controls milk fat accumulation in goat (*Capra hircus*) mammary gland during lactation. *PLoS One* 8:e79258.

- Lin, X., J. Luo, L. Zhang, and J. Zhu. 2013b. MicroRNAs synergistically regulate milk fat synthesis in mammary gland epithelial cells of dairy goats. *Gene Expr.* 16:1–13.
- Lin, X. Z., J. Luo, L. P. Zhang, W. Wang, H. B. Shi, and J. J. Zhu. 2013c. MiR-27a suppresses triglyceride accumulation and affects gene mRNA expression associated with fat metabolism in dairy goat mammary gland epithelial cells. *Gene* 521:15–23.
- Liu, L., L. Jiang, X. D. Ding, J. F. Liu, and Q. Zhang. 2015. The regulation of glucose on milk fat synthesis is mediated by the ubiquitin-proteasome system in bovine mammary epithelial cells. *Biochem. Biophys. Res. Commun.* 465:59–63.
- Liu, L., Y. Lin, L. Liu, L. N. Wang, Y. J. Bian, X. J. Gao, and Q. Z. Li. 2016. Regulation of peroxisome proliferator-activated receptor gamma on milk fat synthesis in dairy cow mammary epithelial cells. *In Vitro Cell Dev. Biol. Anim.* 52:1044–1059. <https://doi.org/10.1007/s11626-016-0059-4>.
- Lu, L. M., X. J. Gao, Q. Z. Li, R. Liu, and H. Li. 2012. Comparative phospho proteomics analysis of the effects of L-methionine on dairy cow mammary epithelial cells. *Can. J. Anim. Sci.* 92:433–442.
- Ma, L., and B. A. Corl. 2012. Transcriptional regulation of lipid synthesis in bovine mammary epithelial cells by sterol regulatory element binding protein 1. *J. Dairy Sci.* 95:3743–3755.
- Martin, E. C., A. Qureshi, V. Dasa, M. Freitas, J. Gimble, and T. Davis. 2016. MicroRNA regulation of stem cell differentiation and diseases of the bone and adipose tissue: Perspectives on miRNA biogenesis and cellular transcriptome. *Biochimie* 124:98–111.
- Nickerson, T. 1960. Chemical composition of milk. *J. Dairy Sci.* 43:598–606.
- Ntambi, J. M., and Y. C. Kim. 2000. Adipocyte differentiation and gene expression. *J. Nutr.* 130:3122S–3126S.
- Ono, K., T. Horie, T. Nishino, and O. Bab. 2015. MicroRNA-33a/b in lipid metabolism. *Circ. J.* 79:278–284.
- Peltier, H. J., and G. J. Latham. 2008. Normalization of microRNA expression levels in quantitative RT-PCR assays: Identification of suitable reference RNA targets in normal and cancerous human solid tissues. *RNA* 14:844–852.
- Shen, B., L. Zhang, C. Lian, C. Lu, Y. Zhang, Q. Pan, R. Yang, and Z. Zhao. 2016. Sequencing and screening of differentially expressed microRNAs related to milk fat metabolism in bovine primary mammary epithelial cells. *Int. J. Mol. Sci.* 17:200.
- Shen, E., X. Diao, C. Wei, Z. Wu, L. Zhang, and B. Hu. 2010. MicroRNAs target gene and signaling pathway by bioinformatics analysis in the cardiac hypertrophy. *Biochem. Biophys. Res. Commun.* 397:380–385.
- Shimano, H. 2001. Sterol regulatory element-binding proteins (SREBPs): Transcriptional regulators of lipid synthetic genes. *Prog. Lipid Res.* 40:439–452.
- Wan, Y., A. Saghatelian, L. W. Chong, and C. L. Zhang. 2007. Maternal PPAR gamma protects nursing neonates by suppressing the production of inflammatory milk. *Genes Dev.* 21:1895–1908.
- Wang, H., J. Luo, Z. Chen, W. T. Cao, H. F. Xu, D. M. Gou, and J. J. Zhu. 2015. MicroRNA-24 can control triacylglycerol synthesis in goat mammary epithelial cells by targeting the fatty acid synthase gene. *J. Dairy Sci.* 98:9001–9014.
- Wang, H., H. Shi, J. Luo, Y. Yi, D. Yao, X. Zhang, G. Ma, and J. J. Looor. 2017. MiR-145 regulates lipogenesis in goat mammary cells via targeting INSIG1 and epigenetic regulation of lipid-related genes. *J. Cell. Physiol.* 232:1030–1040. <https://doi.org/10.1002/jcp.25499>.
- Wang, M., S. Moisa, M. J. Khan, J. Wang, D. Bu, and J. J. Looor. 2012. MicroRNA expression patterns in the bovine mammary gland are affected by stage of lactation. *J. Dairy Sci.* 95:6529–6535.
- Wang, T., M. Li, J. Guan, and X. W. Li. 2011. MicroRNAs miR-27a and miR-143 regulate porcine adipocyte lipid metabolism. *Int. J. Mol. Sci.* 12:7950–7959.
- Wright, J. A., J. K. Richer, and G. J. Goodall. 2010. microRNAs and EMT in mammary cells and breast cancer. *J. Mammary Gland Biol. Neoplasia* 15:213–223.
- Zhao, Y., P. Wang, J. Meng, Y. Ji, and D. Xu. 2015. MicroRNA-27a-3p inhibits melanogenesis in mouse skin melanocytes by targeting Wnt3a. *Int. J. Mol. Sci.* 16:10921–10933.