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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 Loor, 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

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(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 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 μg/mL of streptomycin, 5 μg/mL of insulin, 1 μg/mL of hydrocortisone, and 1 μg/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% CO_2. 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 μg/mL). Cells were then transfected with a miR-27a mimic (50 n*M*), inhibitor (100 n*M*), and the respective controls (50 n*M*; 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 TGTGAGCA-3′ for miR-27a, 5′-ACTCTTAGCGGTGGATCACTC-3′ and 5′-AGTG ACGCTCAGAC AGGCATA-3′ for *5S*, and 5′-CGCTTCGGCAGCACATATAC-3′ and 5′-TCACGA ATTTGCGTGTCAT-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 $2^{-\Delta\Delta Ct}$ 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