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Regulation of bta-miRNA29d-3p on Lipid Accumulation via *GPAM* in Bovine Mammary Epithelial Cells

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Abstract: MicroRNAs (miRNAs) are small RNA molecules consisting of approximately 22 nucleotides that are engaged in the regulation of various bio-processes. There is growing evidence that miR-29 is a key regulator of hepatic lipid metabolism. Mimics and inhibitors of bta-miRNA29d-3p were transiently transfected in bovine mammary epithelial cells (BMECs) to reveal the regulation of bta-miRNA29d-3p on lipid accumulation in BMECs. Results showed that overexpression of bta-miRNA29d-3p significantly inhibited the expression of genes related to triglyceride (TAG) synthesis, namely *DGAT1* and mitochondrial glycerol-3-phosphate acyltransferase (*GPAM*, $p < 0.01$) and down-regulated TAG levels in cells ($p < 0.05$). The expression of fatty acid synthesis and desaturation-related genes *FASN*, *SCD1*, and *ACACA*, and transcription factor *SREBF1* also decreased. Interference of bta-miRNA29d-3p significantly increased the expression of *GPAM*, *DGAT1*, *FASN*, *SCD1*, *ACACA*, and *SREBF1* ($p < 0.01$), and significantly upregulated the concentration of TAG in cells. Furthermore, a luciferase reporter assay confirmed that *GPAM* is a direct target of bta-miRNA29d-3p. In summary, bta-miRNA29d-3p modulates fatty acid metabolism and TAG synthesis by regulating genes related to lipid metabolism in BMECs and targeting *GPAM*. Thus, bta-miRNA29d-3p plays an important role in controlling mammary lipid synthesis in cows.

Keywords: miR-29; lipid metabolism; *GPAM*; milk fat; cow mammary cells

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1. Introduction

Milk has gradually become an indispensable part of the daily diet of humans. Milk is high in fat, and it contains unsaturated fatty acids that are effective in preventing high blood cholesterol and cardiovascular disease in humans [1]. The composition and content of fatty acids are also strong correlates of the nutrition of milk [2]. Increasing lipid content and ameliorating lipid composition could help improve milk quality. One of the essential organs of lactating animals is the breast. Its development and lactation are regulated by cytokines, hormones, and some natural biological functional substances [3]. MicroRNA (miRNA) research in terms of breast development and secretion control in mammals (cows, mice, etc.) has also progressed to a certain extent.

MiRNAs are small, siRNA-like molecules that bind to targeted mRNAs, inhibiting their translation and accelerating their degradation at the post-transcriptional level [4]. MiRNA regulates the biometabolism of most lactating animal tissues, such as the liver, fat, and skeletal muscle in humans, dairy cows, and dairy goats, as well as in mice [5]. The contributions of miRNAs to milk gland evolution are mainly achieved by regulating the proliferation and differentiation of mammary epithelial cells, the differentiation of

mammary stem cells, and the development of glandular vesicles and milk ducts [6]. The miR-29 family mainly includes miR-29a, miR-29b, miR-29c, miR-29d-3p, and miR-29e. Reports on the structure, function, and regulation of the miR-29 family are mainly focused on research related to humans and mice at present.

In ruminants, miR-29 was shown to target *PTX3* in goat granulosa cells through activation of PI3K/AKT/mTOR and Erk1/2 signaling pathways to stimulate granulosa cell multiplication and suppress steroidogenesis and apoptosis [7]. However, studies in non-ruminants have shown that miR-29 not only participates in fatty acid oxidation but also leads to the synthesis of TAG and, thus, performs an instrumental role in controlling fatty acid metabolism. He et al. used microarray technology to study rat skeletal muscle and discovered that miR-29 expression was significantly higher in a type II diabetic group than in healthy rats [8]. In addition, reduced fatty acid content and significantly decreased plasma cholesterol and triglyceride (TAG) levels were observed in the livers of miR-29 knockdown mice [9]. These findings offer the possibility that bta-miRNA29d-3p regulates fatty acid composition and TAG content in dairy cows.

MiRNAs have become significant regulators of glycolipid metabolism among many tissues [5]. However, to date, studies on bta-miRNA29d-3p in the dairy mammary gland have not been reported. This study explored the effect of bta-miRNA29d-3p on the expression of genes related to lipid production and TAG content in mammary epithelial cells of dairy cows and revealed its role in regulating milk lipids.

2. Materials and Methods

2.1. Cell Culture and Transfection

The BMECs used in this experiment were provided by Mr. Chen Zhi from the College of Animal Science and Technology, Yangzhou University. The mammary gland tissue samples used for BMEC isolation were collected from three healthy Holstein cows (4 years old, second parity, non-pregnant) at peak lactation. The psiCHECK-2 vector (psiCHECK-2 plasmid) was presented by Professor Jun Luo from Northwest A&S University.

On the basis of the available literature [10], BMECs were cultured by 60 mm cell dishes in a 5% CO_2 incubator at 37 °C. The medium contained 10% fetal bovine serum, 5 μg/mL insulin, 100 U/mL penicillin and streptomycin, 10 ng/mL epidermal growth factor, 1 μg/mL hydrocortisone, and 90% DMEM/F12. The medium was replaced once in 24 h.

When the cell confluence reached 80%, the cells were transfected with Lipofectamine® RNAiMAX Transfection Reagent, and the transfection complex was prepared by referring to the product's instructions. The total volume of transfection complexes was 100 μL per well. miR-29d mimic (miR-29d-3p) and its negative control (Con miR) and miR-29d inhibitor (anti-miR-29d-3p) and its negative control (Con Inh) were transiently transfected into mammary epithelial cells at a final concentration of 50 nM, and the remaining volume was replenished with DMEM/F-12. The transfection complexes were mixed well and incubated at room temperature for 20 min. The cultured BMECs were digested into separate suspensions and added to a 24-well plate. Finally, the incubated transfection complexes were added drop-wise and incubated at 37 °C in a 5% CO_2 incubator. After 48 h, intracellular miR-29d-3p mRNA levels were examined using real-time fluorescence quantification (RT-qPCR). Each treatment was performed in three biological replicates.

2.2. RNA Extraction and Real-Time Quantitative PCR

Cells were lysed to carry out RNA extraction after 48 h of transfection, and the RNA extraction method was carried out using the cultured cell/bacterial total RNA extraction kit from Tiangen Biochemical Technology (Beijing, China) Co., Ltd. (DP430). The extracted RNA was reverse transcribed with the PrimeScriptTM RT kit (Perfect Real time, Takara Bio Inc., Kusatsu, Japan), where the bta-miRNA29d-3p reverse transcription primers were designed using the stem–loop method. The bta-miRNA29d-3p reverse-transcribed cDNA was used as a template, and the 18s gene was used as an internal reference gene for RT-

qPCR amplification of the gene by using SYBR Green (SYBR Premix Ex Taq II, Perfect Real Time, Takara Bio Inc., Kusatsu, Japan). Then, a 20 µL system was designed as follows: SYBR GreenMix, 10 µL; PCR Forward Primer, 0.8 µL; PCR Reverse Primer, 0.8 µL; DNA template, 2 µL; and sterilized water, 6.0 µL. The PCR reaction program was 40 cycles of 95 °C pre-denaturation for 30 s, 95 °C for 5 s, and 60 °C for 30 s.

The genes detected included fatty acid synthase (*FASN*), glycerol-3-phosphate acyltransferase (*GPAM*), peroxisome proliferator-activated receptor γ (*PPARG1*), diacylglycerol acyltransferase 1 (*DGAT1*), sterol regulatory element-binding factor 1 (*SREBF1*), liver X receptor alpha (*LXRA*), and acetyl-coenzyme A carboxylase alpha (*ACACA*). The internal controls were ubiquitously expressed transcript (*UXT*) and mitochondrial ribosomal protein L39 (*MRPL39*). The sequences of primers with qPCR are shown in Table 1.

Table 1. Characteristics of primers used of the RT-qPCR reaction.

Gene1	Accession No.	Primer Sequences (5′-3′)	Source
UXT	XM_005700842.1	F: TGTGGCCCTTGGATATGGTT R: GGTTGTCGCTGAGCTCTGTG	[11]
MRPL39	XM_005674737.1	F: AGGTTCTCTTTTGTTGGCATCC R: TTGGTCAGAGCCCCAGAAGT	[11]
GPAM	AY515690	F: GCAGGTTTATCCAGTATGGCATT R: GGACTGATATCTTCCTGATCATCTTG	[12]
PPARG1	NM_001290893.1	F: CGTTTCCTTAAACAAGTG R: TCCCTCAAAATAATAGTGC	[13]
DGAT1	NM_174693	F: CCACTGGGACCTGAGGTGTC R: GCATCACCACACACCAATTCA	[14]
LXRA	NM_001014861.1	F: CATGCCTACGTCTCCATCCA R: TCACCAGTTTCATCAGCATCCT	[15]
SREBF1	TC263657	F: CCAGCTGACAGCTCCATTGA R: TGCGCGCCACAAGGA	[12]
FASN	CR552737	F: ACCTCGTGAAGGCTGTGACTCA R: TGAGTCGAGGCCAAGGTCTGAA	[14]
ACACA	AJ132890	F: CATCTTGTCCGAAACGTCGAT R: CCCTTCGAACATACACCTCCA	[14]
SCD1	GU947654	F: CCATCGCCTGTGGAGTCAC R: GTCGGATAAATCTAGCGTAGCA	[16]

2.3. Detection of Triglycerides in BMECs

The transfection operation was the same as above. Analyses were performed in triplicate. After the cells were transfected for 48 h and replaced with new medium, the cells were washed twice with PBS, and 1 mL of lysis buffer was added to each well. An appropriate amount of lysate was reserved for BCA protein quantitative determination. TAG content was detected and calculated in accordance with the instructions of the TAG test kit from Beijing Applygen. A TAG standard curve was constructed to specify the triglyceride content of the samples, after which the TAG content was corrected for total cellular protein concentration per mg.

2.4. Construction of psiCHECK-2-GPAM 3′UTR Recombinant Plasmid

To generate a reporter gene for luciferase assays, we amplified bovine *GPAM* 3′-UTR fragments by PCR and cloned them into the XhoI/NotI digest site of the dual-luciferase reporter gene vector psiCHECK-2 to construct a dual-luciferase reporter gene vector containing the wild-type bovine *GPAM* 3′UTR sequence. Meanwhile, a dual-luciferase expression vector containing a mutated *GPAM* 3′-UTR sequence was constructed by overlap PCR. The primer sequences are given in Table 2. All constructs were confirmed by sequencing.

Table 2. Primers used for site-directed mutation of bovine *GPAM* 3′UTR constructs.

Primer Name	Primer Sequences (5′-3′)
GPAM-F	TTGCCCTTTCAGTTGGTTT
GPAM-R	TTGCCCTTTCAGTTGGTTT
MiR-29d-3p-binding site	TGGTGCTTTG
Mutation site	CACGTACCCT
Overlap-F	GTAATTAACCACGTACCCTAA
Overlap-R	GGTTTTAGGGTACGTGGTTAATTAC

2.5. Dual Luciferase Assays

After mammary epithelial cells were transfected for 48 h, they were lysed and a dual-luciferase assay was performed by referring to the Promega dual reporter gene assay kit. The cells were washed twice using PBS. In each well of the 24-well opaque assay plate, 65 μL of cell lysate was added. The firefly luciferase activity value (F value) was first obtained, and then the firefly luciferase activity was terminated, the Renilla luciferase activity was activated, and the Renilla luciferase activity (R value) was read. Relative luciferase activity = R value/F value, which is the relative transcriptional activity.

2.6. Statistical Analysis

All data were analyzed in SAS 9.4 (Cary, NC, USA) using reusable two-factor analysis of variance and expressed as mean ± standard error of the mean (SEM). Each experiment was repeated at least three times. Relative quantitative analysis was performed using the $2^{-\Delta\Delta Ct}$ method, and the data were analyzed for significance. $p < 0.05$ indicated a significant difference (*, $p < 0.05$; **, $p < 0.01$).

3. Results

3.1. Detection of Overexpression and Interference Efficiency of bta-miRNA29d-3p in BMECs

According to Figure 1, the expression of bta-miRNA29d-3p increased by approximately 300-fold compared with that of Con miR ($p < 0.01$, Figure 1A), and the expression of anti-miR-29d-3p significantly decreased by approximately 80% compared with that of Con Inh ($p < 0.01$, Figure 1B). This result indicated that miR-29d-3p and anti-miR-29d-3p were transfected with good efficiency.

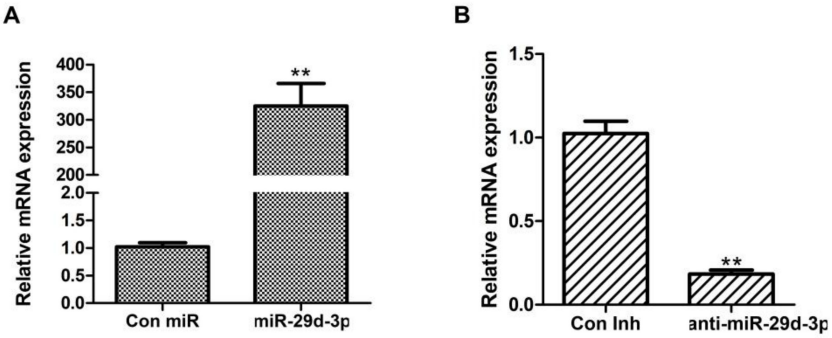

Figure 1. Detection of overexpression and interference efficiency of bta-miRNA29d-3p. Results are the mean ± SEM of three separate experiments. ** $p < 0.01$ versus control.

3.2. Overexpression of bta-miRNA29d-3p Affects the Expression of Genes Related to Lipid Metabolism in BMECs

On the basis of Figure 2, after bta-miRNA29d-3p was overexpressed and the expression of SREBF1 was clearly decreased, whereas that of LXRA was markedly upregulated ($p < 0.01$, Figure 2A). The synthesis and desaturation of fatty acid-related gene (ACACA, FASN, and SCD1) expression were remarkably reduced ($p < 0.01$, Figure 2B), and that of TAG synthesis-related genes DGAT1 and GPAM was significantly down-regulated ($p < 0.01$, Figure 2C).

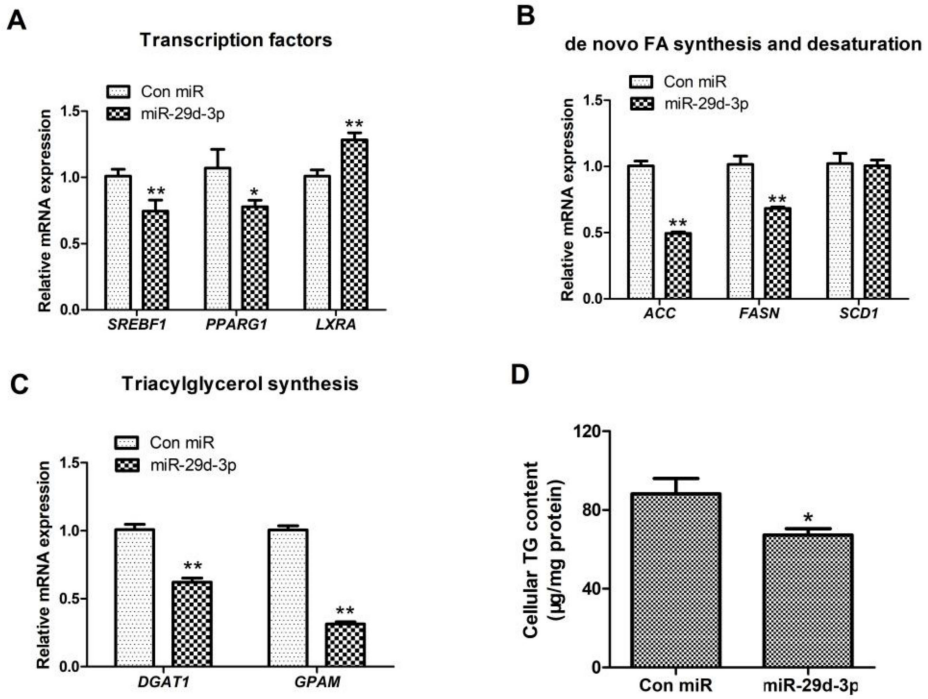

Figure 2. Effect of bta-miRNA29d-3p overexpression on genes related to lipid metabolism in BMECs. Panel (**A**): Effect of bta-miRNA29d-3p overexpression on the expression of transcription factors SREBF1, PPARG1, and LXRA. Panel (**B**): Effect of bta-miRNA29d-3p overexpression on the expression of fatty acid de novo synthesis and desaturation-related genes (ACACA, FASN, and SCD1). Panel (**C**): Effect of bta-miRNA29d-3p overexpression on TAG synthesis and expression of lipid droplet formation-related genes (DGAT1 and GPAM). Panel (**D**): Effect of bta-miRNA29d-3p overexpression on intracellular TAG content. Results are the mean ± SEM of three separate experiments. ** $p < 0.01$ versus control, * $p < 0.05$ versus control (Con miR).

3.3. Interference of bta-miRNA29d-3p Promotes the Expression of Genes Related to Lipid Metabolism in BMECs

As shown in Figure 3, interference with bta-miRNA29d-3p increased GPAM expression, and the expression of FASN, SCD1, ACACA, DGAT1, and PPARG1 was also significantly elevated ($p < 0.01$, Figure 3).

3.4. Effect of bta-miRNA29d-3p on TAG Content in BMECs

As illustrated in Figures 2 and 3, when bta-miRNA29d-3p was overexpressed, the TAG content in BMECs was significantly down-regulated ($p < 0.05$, Figure 2D). However, when bta-miRNA29d-3p was disrupted, the TAG content increased significantly ($p < 0.01$, Figure 3D).

3.5. Prediction of bta-miRNA29d-3p Target Genes

As shown in Figure 4, the target gene associated with fatty acid metabolism was GPAM, and bta-miRNA29d-3p was more conserved in human and chimpanzee sequences (Figure 4A). When the GPAM 3′-UTR was wild-type, bta-miRNA29d-3p overexpression significantly decreased the activity of the dual-luciferase reporter gene ($p < 0.01$, Figure 4B), whereas interference with the bta-miRNA29d-3p gene significantly increased the activity of the dual-luciferase reporter ($p < 0.01$, Figure 4C). When the GPAM 3′-UTR was mutant, bta-miRNA29d-3p showed an absence of effect towards the activity of dual-luciferase reporter gene expression. Therefore, bta-miRNA29d-3p acts directly on the cow GPAM 3′-UTR target site.

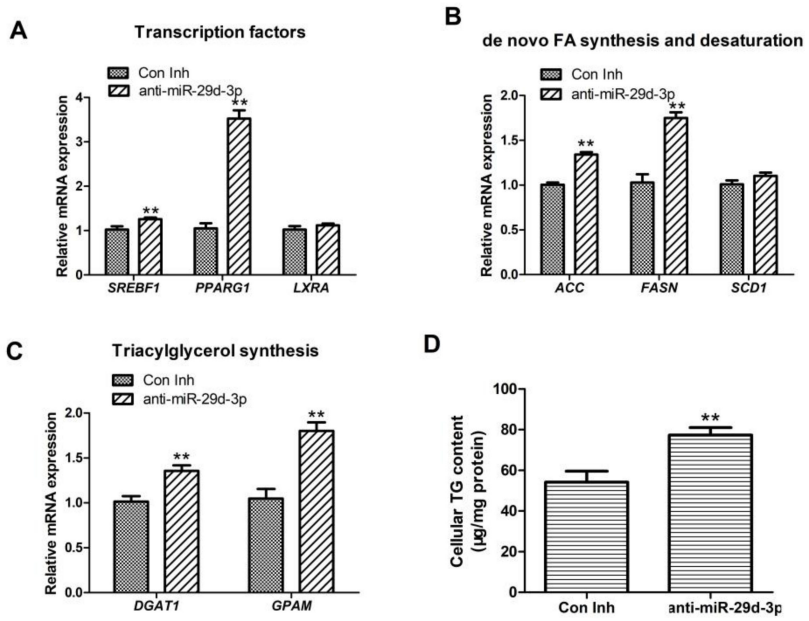

Figure 3. Effect of interference with bta-miRNA29d-3p with respect to lipid metabolism-related genes in BMECs. Panel (**A**): Effect of interfering with bta-miRNA29d-3p on the expression of transcription factors SREBF1, PPARG1, and LXRA. Panel (**B**): Effect of interfering with bta-miRNA29d-3p with reference to the expression of fatty acid ab initio synthesis and desaturation-related genes (ACACA, FASN, and SCD1). Panel (**C**): Effect of interfering with bta-miRNA29d-3p on the analysis of TAG synthesis and lipid droplet formation-related genes (DGAT1 and GPAM) expression. Panel (**D**): Effect of interfering with bta-miRNA29d-3p on intracellular TAG content. Results are the mean ± SEM of three separate experiments. ** $p < 0.01$ versus control (Con Inh).

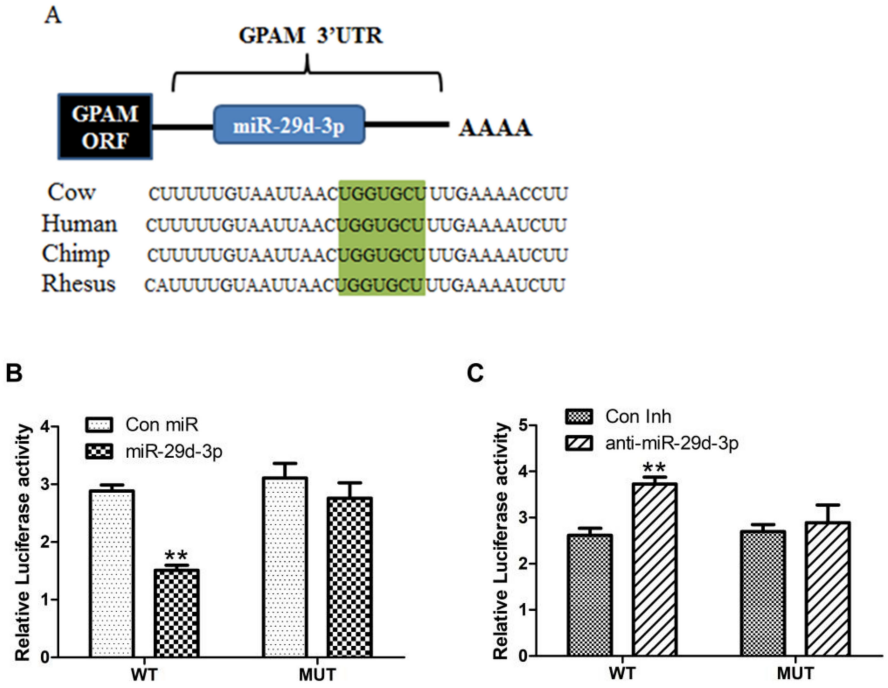

Figure 4. Bta-miRNA29d-3p target gene prediction and dual luciferase assay. Panel (**A**): bta-miRNA29d-3p target gene prediction and conservativeness analysis. Panel (**B**): Effect of bta-miRNA29d-3p overexpression on dual luciferase activity. Panel (**C**): Effect of interfering with bta-miRNA29d-3p on dual-luciferase activity. Results are the mean ± SEM of three separate experiments. ** $p < 0.01$ versus control (Con miR, Con Inh).

4. Discussion

The miR-29 family plays a core role in the etiology and pathogenesis of osteoarthritis (OA), osteoporosis, cardiac and renal diseases, and immune disorders [17]. Furthermore, miR-29 participates in the modulation of multiple cellular functions and in lipid metabolism [7,18,19]. In this study, combined with previous research of miR-29's effects on lactation in ruminants, the hypothesis was that bta-miRNA29d-3p has an effect on lipid metabolism in BMECs. In vitro experiments confirmed that pharmacological inhibition of miR-29 significantly down-regulated hepatic cholesterol and adipose synthesis de novo [9]. Moreover, miR-29 was found to be an important regulator of lipid oxidation by overexpression or deletion in human or primary human skeletal muscle cells [5]. Consequently, we investigated the regulatory mechanism of bta-miRNA29d-3p on milk lipids to provide a basis for further improvement of lactation performance of cows and their progeny.

Transcription factors (TF) are an important class of proteins involved in the regulation of gene transcription. They are key regulators of various signaling pathways. In this study, overexpression of bta-miRNA29d-3p dramatically down-regulated the expression of *SREBF1*, a member of a family of TFs that binds to the ER and acts centrally involved in fatty acid, phospholipid, and cholesterol synthesis [20]. Overexpression of nSREBP-1a or -1c was suggested to significantly upregulate the expression of its target genes *ACC*, *FASN*, and stearoyl coenzyme A desaturase 1 (*SCD1*) and remarkably enhanced the pri-miR-29 and mature miR-29 expression in glioblastoma (GBM) cells [21]. Overall, miR-29 suppressed SREBP-1 and the lipid synthesis pathway in GBM cells as a negatively regulated factor. The results of this research are in agreement with the findings reported in the literature above. Accordingly, bta-miRNA29d-3p may control lipid metabolism-related gene expression through *SREBF1*, which consequently impacts fatty acid synthesis.

Interestingly, we found extremely significant upregulation of *PPARG1* expression by interfering with bta-miRNA29d-3p. However, it has been demonstrated that inhibition of miR-29s can cause hypermethylation of overall DNA and augments the levels of methylation in the promoters of lactation-associated genes, including PPARG [22]. The peroxisome proliferator-activated receptor (PPAR) family is an important member of the nuclear hormone receptor superfamily, and their physiological functions are mainly associated with fatty acid metabolism, glucose metabolism, and cell proliferation and differentiation [16]. There is evidence that *PPARA* controls not only fatty acid oxidation-related genes, but also other genes concerned with its synthesis, including *ACACA*, *FASN*, and *SCD1* [16]. While *PPAR*γ is expressed at a high level in adipose tissue, it is a key coordinator of the transcriptional cascade response for adipocyte differentiation [23]. There are two homotypes of *PPARG*, *PPARG1* and *PPARG2*, which are splice variants of the *PPARG* gene via alternative promoters [24]. The results of the in vitro culture of mammary epithelial cell lines showed that PPARG-specific agonist ROSI treatment of mammary epithelial cells was able to upregulate fatty acid metabolism-related genes, such as *ACACA*, *FASN*, *DGAT1*, and *SREBF1*, and clarified that *PPARG* directly regulates the expression of some genes in mammary tissue and affects the fatty acid metabolic network [25–27]. To summarize the above reports, we presume that bta-miRNA29d-3p may have a vital effect in the control of lipid metabolism in BMECs by affecting the expression of *PPARG*.

Changes in *DGAT1* expression were also found in the current study. *DGAT1* is found to be expressed at a high level in adipose tissue, the liver, and the small intestine, all of which are more active in TAG synthesis [28]. For dairy cows, the non-conservative substitution of alanine for lysine in the *DGAT1* gene could have a significant effect on milk composition and yield. Evidence showed that mice with *DGAT1* gene deletion have reduced absorption of triacylglycerols [29]. Through interference with bta-miRNA29d-3p expression, *DGAT1* mRNA expression was significantly increased, along with an increase in intracellular TAG content. A combination of the above series of studies on lipid metabolism by bta-miRNA29d-3p showed that the synergistic effect of bta-miRNA29d-3p and *DGAT1* affects lipid synthesis in BMECs.

Screening and identifying miRNAs and their target genes are the key steps in studying the functional mechanism of miRNAs. The bioinformatics approach could provide theoretical guidance for the identification of miRNA target genes, and it has an essential role in studying the functional mechanisms of miRNAs. In this study, *GPAM* was identified as a target gene related to lipid metabolism by online study of the bta-miRNA29d-3p gene. *GPAM* is the limiting enzyme in the first step of TAG synthesis, and intracellular TAG levels are regulated by *GPAM* enzyme content. Knockdown of *GPAM* significantly reduced the expression of genes associated with TAG synthesis and lipid metabolism in bovine embryonic fibroblasts [30]. Furthermore, *GPAM* plays a dynamic role in TAG synthesis. For example, a 10-fold increase in *GPAM* activity in 3T3-L1 adipocyte differentiation and a 5-fold increase in *GPAM* activity during TAG synthesis were observed in neonatal liver hepatocytes [31]. The present findings on TAG synthesis have mainly focused on both *PPAR* and *SREBP* regarding the transcriptional regulation of the gene, but there are also reports confirming that *GPAM* may have a greater regulatory role on TAG synthesis [31,32]. To summarize, GPAM, as a target gene of bta-miRNA29d-3p, performs a key role in TAG regulation in cells, and its mutation can be used as an effective marker for selective breeding of dairy cows.

Taken together, the bta-miRNA29d-3p gene has a significant function in the control of lipid accumulation in dairy cows, and its regulatory mechanism is linked to the expression of lipid synthesis-associated genes in BMECs. However, the specific regulatory mechanism of the bta-miRNA29d-3p gene on lipid synthesis in mammary epithelial cells of dairy cows needs to be studied in depth. Additional studies on relevant non-coding RNAs in the mammary lipid metabolism pathway in dairy cows could help to better utilize functional miRNAs to regulate the fatty acid composition of milk in the future, which has important application value for dairy development.

5. Conclusions

In this study, overexpression of bta-miRNA29d-3p down-regulated the expression of *DGAT1* and *GPAM*, which are related to TAG synthesis ($p < 0.01$), and that of *FASN*, *ELOVL4*, *ACACA*, and *SREBF1*, which are related to FA synthesis and prolongation, significantly down-regulated intracellular TAG levels ($p < 0.05$). Interfering with the expression of the bta-miRNA29d-3p gene showed the opposite result. The downstream target gene of bta-miRNA29d-3p was predicted to be *GPAM*. The results provide a theoretical basis for the regulatory mechanism of the bta-miRNA29d-3p gene in the lipid synthesis of BMECs.

Author Contributions: Conceptualization, D.Y.; funding acquisition, D.Y.; supervision, Y.M.; formal analysis, M.Z. and L.C.; validation, S.Z.; writing—original draft, X.Z.; writing—review and editing, J.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data are available online at https://doi.org/10.6084/m9.figshare.21393204.v1, accessed on 25 October 2022.

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