A Regulatory Circuit Orchestrated by Novel-miR-3880 Modulates Mammary Gland Development

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Milk casein and triglyceride content are important production traits in goats. Studies on mechanisms in milk casein secretion and mammary gland development is essential for milk goat breeding. miRNAs play an important role in goat lactation. While novel-miR-3880 is highly expressed at goat peak lactation stage, its molecular mechanism has not been studied. The purpose of the present study was to explore the relationship between novel-miR-3880 and lactation, as well as to construct a network among novel-miR-3880, ciRNA13761, and E74 like ETS transcription factor 2 (ELF2), thus further exploring their potential roles in milk components and mammary gland development. ELF2 was previously proven to be important in cell survival and proliferation, and 3′-UTR of ELF2 was predicted to have binding sites of novel-miR-3880. Our study found that the overexpression of novel-miR-3880 exerted anti-apoptotic and proliferative roles in GMEC, induced a boost in triglyceride synthesis, and caused a decrease in αs1-, αs2-, and β-casein, but an increase in κ-casein secretion. Furthermore, treatment in mice indicated that novel-miR-3880 could promote mammary gland development and extend the lactation period, while novel-miR-3880 expression was found to be suppressed by ciRNA13761 as a miRNA sponge. The present study explores a mechanism of triglyceride synthesis and casein secretion, and reveals a crosstalk between ciRNA13761/novel-miR-3880/ELF2 axis and PI3K/AKT/mTOR/S6K1 pathway, to gain a better understanding of lactation traits in dairy goats.

Keywords: ciRNA13761, novel-miR-3880, ELF2, PI3K/AKT/mTOR/S6K1 pathway, mammary gland development

INTRODUCTION

Goat’s milk is regarded as a hypoallergenic milk type with therapeutic functions and is accepted as a major functional food (Roncada et al., 2002) when casein, including κ-, β-, αs1-, and αs2-casein, is the main protein in goat’s milk (Singh and Singh, 1980; Chatchatee et al., 2001). MicroRNAs (miRNAs) are a class of endogenous non-coding RNAs of approximately 23 nt in length. They are reported to regulate gene expression by repressing translation or facilitating mRNA degradation (Ambros, 2004) and play an important role in goat lactation (Ji et al., 2017). Novel-miR-3880 was highly expressed during goat peak lactation period based on our previous sequencing data (Hou et al., 2017). The novel-miR-3880 expression data was selected from Supplementary File S1 (Hou et al., 2017). Our study explored effects of novel-miR-3880-related molecules on mammary

Frontiers in Cell and Developmental Biology | www.frontiersin.org 1 June 2020 | Volume 8 | Article 383
gland development in vivo and in vitro, thus providing a theoretical molecular basis for breeding and breast care.

Our study found that the overexpression of novel-miR-3880 induced anti-apoptotic and proliferative effects, promoted triglyceride synthesis, and caused a decrease in α s1-, α s2-, and β-casein but an increase in κ-casein secretion. Further, the regulation pattern was studied. Firstly, negative control (NC) and novel-miR-3880 were transfected into goat mammary epithelial cell (MEC) and RT-qPCR was applied to detect the expression of miRNAs, thus ensuring the efficiency of RNA sequencing. Differentially expressed genes (DEGs) of novel-miR-3880 were screened, and 67 downstream genes were found. E74 like ETS transcription factor 2 (ELF2), which is proven to be important in cell survival and proliferation (Qiu et al., 2008), might be essential for mammary gland development and lactation, and was found in the downregulated DEG list of sequencing results.

CircRNAs are another class of endogenous non-coding RNAs from non-classical alternative splicing that can expropriate miRNAs as a sponge to block miRNAs from binding to target genes (Hansen et al., 2013; Vicens and Westhof, 2014; Meng et al., 2017). To explore how novel-miR-3880 expression was regulated, circRNAs that have binding sites of novel-miR-3880 were screened according to circRNA sequencing data our laboratory acquired before. CircRNA13761 (ciRNA13761), whose structure is shown in Supplementary File S2, was selected and verified as a novel-miR-3880 sponge, while its source gene DOCK1 is reported to be involved in mammary gland involution (Bagci et al., 2014). Then, the network among ciRNA13761, novel-miR-3880, and ELF2 was constructed, and the role of DOCK1 in the network was detected.

As is known, mTOR is a central modulator in protein/lipid synthesis and cell growth processes and plays important roles in milk production (Osorio et al., 2016; Saxton and Sabatini, 2017). It serves as a crucial downstream signal of PI3K/AKT pathway to form a functional compound (Yang et al., 2014), which participates in lactation initiation (Chen et al., 2012). S6K1, a downstream effector of mTOR, is also critical for promoting protein and lipid synthesis (Yang et al., 2014); its activation relies on phosphorylation mediated by mTOR (Magnuson et al., 2012). Our study explored whether and how novel-miR-3880 regulates PI3K/AKT/mTOR/S6K1 pathway and participates in MEC biological processes and mammary gland development. In addition, MEC anti-apoptosis signaling was evaluated by the protein expression ratio of Bcl-2 and Bax, which is regarded as a cell survival signal (Basu and Haldar, 1998).

In this study, roles of ciRNA13761, novel-miR-3880, and ELF2 on mammary gland development and lactation traits were studied to provide a basis for molecular breeding of dairy goats. More precisely, the interaction among ciRNA13761, novel-miR-3880, and ELF2, and their effects on MEC triglyceride synthesis, lipid formation, casein secretion, viability, proliferation, and apoptosis were explored, as well as their participation in PI3K/AKT/mTOR/S6K1 pathway and Bcl-2/Bax pathway. Novel-miR-3880 and siELF2 were injected into C57BL/6 mice through the tail vein to examine the participation of PI3K/AKT/mTOR/S6K1 pathway in vivo and to observe the development of mammary glands affected by novel-miR-3880 and siELF2 with ultramicroscopic technique, to judge the availability of the molecular experiments and provide a theoretical basis for practice in dairy goat breeding and breast care.

**MATERIALS AND METHODS**

**Animals and Ethics**

Three-year old female Guanzhong dairy goats 90-day postpartum (peak lactation period) in a research-animal-keeping farm near Northwest A&F University of Shaanxi province in China were selected and anesthetized. Then, one cubic centimeter of mammary gland tissue was removed to PBS with penicillin/streptomycin (100 U/mL, Harbin Pharmaceutical Group, China) from the middle part of the mammary gland with a scalpel. The mammary gland tissue was used to isolate MECs. The wound was sewn and sterilized immediately and animals recovered after the surgical line was removed a week later. C57BL/6 mice used in this study were of a similar age, weight, parity, and litter size, and delivered newborns on the same day. The mice were raised in an SPF environment with natural drink and food in separate nests. Each group had six nests of mice. Injuctive novel-miR-3880 agomir and siELF2 with 2′OMe and 5′Chol in vivo modification were bought from Ribobio (Guangzhou, China). All surgical procedures conformed to institutional and national guidelines and were approved by the Animal Care and Use Committee of the Northwest A&F University (China).

**Cell Culture and Cell Treatment**

A previous method (Wang et al., 2010) was used to isolate goat MECs. Mammary epithelial cells were cultured in a basic DMEM/F12 medium (Hyclone, United States) with 10% bovine serum albumin (Gibco, United States) and penicillin/streptomycin, and incubated in 5% CO2 at 37°C in a humid atmosphere. Novel-miR-3880, siELF2, siDOCK1, and si-ciRNA13761 were synthesized at GenePharma Corporation (Shanghai, China), and the sequences were GGUCGGCCGGCCGGCCG, CCUAUGCUGCUUAGAGAGAU, GCUUCGUUCAUCCAUUUA, and CCUGCACAAGGAAUGUGAU. mammary epithelial cells transfection was performed with Lipopectamine 2000 reagent (Invitrogen, United States). PI3K inhibitor (TGX-221, Selleck, Shanghai, China) in 20 μM, AKT (GDC-0068, Selleck, Shanghai, China) inhibitor in 50 μM, mTOR (Everolimus RAD001, Selleck, Shanghai, China) inhibitor in 0.5 μM, and S6K1 inhibitor (WAY-600, Selleck, Shanghai, China) in 50 μM applied to treat MEC were dissolved in DMSO, and equal DMSO was applied in the control group. The concentration was selected as suggested.

**Isolation and Analysis of RNA**

Total RNA was isolated from MECs by Trizol Reagent (Invitrogen, United States) according to the manufacturer's protocol. miRcute Plus miRNA First-Strand cDNA Kit (Tiangen, Beijing, China) was used to perform reverse transcription...
of novel-miR-3880. The PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Japan) was used to acquire cDNA. Oligo dT primer was applied for mRNA reverse transcription; for cDNA of ciRNA, total RNA was dealt with RNase, and then random primer was used for reverse transcription. RT-qPCR was conducted using SYBR Green qPCR Master Mix (Takara, Japan) to analyze the mRNA, miRNA, or ciRNA expression levels. Primers used in RT-qPCR are shown below. ELF2: TGTGGCGGTTCAGAATTTGCGT (forward), CAGTAGAGGCTGGCATCACA (reverse); Dock1: CGGGGCTCAGAACTCATCGC (forward), CACCACATCGGCTTCATGTC (reverse); ciRNA13761: GAAGTGGTCGGAGGACGTGTTGTGCAGGTCACAGAGCTT (forward), CACCACATCGGCTTCATGTC (reverse); novel-miR-3880: TATATAGCCGCCGCTCAGAACTCATCGG (forward), CGGGAAGTTGACCTAGTTTGGTGT (reverse). The complete CDS region of ELF2 was amplified with primers ATGACATCAGCAGTGGTTGAC (forward) and CGAACAAGATACCACACTC (reverse) for cDNA of ELF2-1 and ELF2-2 wild type (Wt) vectors. Primers for ELF2-1 3′UTR were CTAGGTGTGTAGTGGCCGGTC (forward) and CGACAAGATACCACACTC (reverse); for ELF2-2 3′UTR were TCCCAACTGCTGCGTGAA (forward) and GAGTTACAGGACGTGTTGT (reverse). The vectors sequences documents are shown in Supplementary Files S3, S4. ELF2-1 Mutant (Mu) and ciRNA13761 Wt/Mu psiCHECK-2 vectors were provided by Tsingke Biological Technology Company (Beijing, China).

RNA-Sequencing
Goat MEC RNA quality was analyzed with an Agilent Bioanalyzer 2100, and mRNA was captured using the Poly (A) mRNA Magnetic Isolation Module (NEBNext, United States). The quality of the library was detected again using an Agilent Illumina (NEBNext, United States). After the library was purified by Agencourt AMPure XP beads (Beckman, United States), the quality of the library was detected again using an Agilent Bioanalyzer 2100 and Qubit. In addition, before Illumina HiSeq, cBOT automatic clustering was conducted using the TrueSeq PE Cluster Kit v4.

Vector Construction
PsiCHECK-2 empty vector was purchased from Promega (Madison, United States). ELF2-1 3′UTR and ELF2-2 3′UTR containing the seed site were amplified and inserted into psiCHECK-2 vector as ELF2-1 and ELF2-2 wild type (Wt) vectors. Primers for ELF2-1 3′UTR were CTAGGTGTGTAGTGGCCGGTC (forward) and CGACAAGATACCACACTC (reverse); for ELF2-2 3′UTR were TCCCAACTGCTGCGTGAA (forward) and GAGTTACAGGACGTGTTGT (reverse). The vectors sequencing documents are shown in Supplementary Files S3, S4. ELF2-1 Mutant (Mu) and ciRNA13761 Wt/Mu psiCHECK-2 vectors were provided by Tsingke Biological Technology Company (Beijing, China).

Dual-Luciferase Reporter Assays
Luciferase activities were measured in accordance with the protocol of Dual-Luciferase Reporter Assay System (Promega, Madison, WI, United States). The ratio of hRluc and hluc+ activities was used to calculate the relative luciferase activity.

Western Blot Analysis
Equal amounts of each protein sample was loaded to detect protein expression. The following primary antibodies were used in this experiment: β-actin (Beyotime, AA128, Shanghai, China), ELF2 (Proteintech, 12499-1-AP, United States), Bax (BBI, D220073, Shanghai, China), Bcl-2 (BBI, D260117, Shanghai, China), PI3K p110 beta (Bioss, bs-6423R, Beijing, China), p-PI3K p110 beta (Bioss, bs-6417R, Beijing, China), AKT (Cell Signaling Technology, 4685, United States), p-AKT (Cell Signaling Technology, 4060, United States), mTOR (Boster Biological Technology, BM4182, United States), and p-mTOR (Boster Biological Technology, BM4840, United States). Horse Radish Peroxidase-conjugated goat anti-rabbit IgG secondary antibodies were purchased from Beyotime (Shanghai, China).

Immunohistochemistry
Slides cut from paraffin-embedded tissues underwent drying, rehydration, antigen retrieval, and permeation before the samples were blocked in goat serum for 20 min and incubated in the primary antibody then in the second antibody. Color development was performed with a DAB Substrate kit (Solarbio, DA1010, Beijing, China) and counterstained with Hematoxylin (Solarbio, H8070, Beijing, China).

Flow Cytometry
MEC apoptotic rate was evaluated using the Annexin V-FITC/PI apoptosis kit (7Sea Biotech, Shanghai, China) and MEC cell cycle was measured by Cell cycle staining Kit (MultiSciences, Hangzhou, China) according to the manufacturer’s instructions.

Triglyceride Content Detection and Oil Red O Staining
Triglyceride content was measured by Tissue/Cell triglyceride assay kit (Applygen, Beijing, China) and normalized to protein concentration, which was tested by BCA protein assay kit. Oil red O used in staining was purchased from Solarbio (Beijing, China). Relative density of lipid drops was analyzed by Image-Pro Plus 6.0 Software.

ELISA
MEC cultivator was acquired to detect the contents of α s1-, α s2-, β-, and κ-casein respectively using casein ELISA Kits specialized for goats (mlbio, Shanghai).

Cell Counting Kit-8 (CCK-8) Assay
Ten microliter of CCK-8 solution (7Sea Biotech, Shanghai, China) was added into MECs seeded in 96-well plates and incubated at 37°C for 4 h to evaluate MEC viability. Then, the optical density value was measured at 450 nm with a Microplate Reader (Bio Tek, United States).
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**RESULTS**

Novel-miR-3880 Directly Suppresses the Expression of ELF2

After novel-miR-3880 was transfected into MECs with NC as control, RNA sequencing was conducted and DEGs are shown in Supplementary File S6. There were 57 downregulated DEGs and 10 upregulated DEGs in novel-miR-3880 group (Figure 1A). ELF2 selected from DEGs of RNA-sequencing was verified via RT-qPCR and western blotting to evaluate the sequencing accuracy (Figure 1B). A schematic diagram of dual luciferase reporter vectors was described in Figure 1C. It is shown in Figure 1D that the relative luciferase activity was lowered when novel-miR-3880 was co-transfected with psiCHECK2-ELF2-1 3′-UTR Wt vector. However, the relative luciferase activity stayed the same level when either NC or novel-miR-3880 were co-transfected with ELF2-2 3′-UTR Wt vector or psiCHECK2-ELF2-1 3′-UTR Mu vector (Figure 1D). In addition, the expression of ELF2 in different goat tissues was detected (Figure 1E) and pcDNA3.1-ELF2 overexpression vector efficiency is provided in Figures 1F,G.

**Statistical Analysis**

Sequencing data quality was evaluated using FastQC v0.10.1 and data filtering was performed with Cutadapt 1.9.1. Short read alignment was conducted using Hisat v2.0.14. SNV and InDel information was acquired from Samtools v0.1.18 analysis. The weight of female offspring in every nest was averaged and the data in six nests were set as one group. The acquired data of three groups were applied to analyze. Experiments in this study were conducted in three independent repeats. SPSS 22.0 was used to conduct one-way ANOVA and Student t-test. *p < 0.05, which was considered significant, and **p < 0.01, which was considered highly significant.

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

EdU fluorescence labeling was achieved by EdU Cell Proliferation Kit with Alexa Fluor 488 (Beyotime, Shanghai, China), and cell nucleuses were dyed with DAPI (Beyotime, Shanghai, China).

**CiRNA13761 Sponges Novel-miR-3880 and Induces a Regulation in ciRNA13761/Novel-miR-3880/ELF2 Network**

Relative luciferase activity shown in Figure 2A illustrates that the relative luciferase activity of Wt psiCHECK2-ciRNA13761 vector was reduced, but that of the Mu vector was not changed, which indicates novel-miR-3880 was absorbed by ciRNA13761. To explore the regulatory pattern of ciRNA13761, the network among ciRNA13761, novel-miR-3880, and ELF2 was established, and the role of ciRNA13761 source gene-DOCK1 in the network was determined. The efficiency of si- and pcDNA3.1-ciRNA13761 is clarified in Figures 2B,C; it could be seen in Figures 2C,D that novel-miR-3880 decreased the expression of ciRNA13761. Combined with the effect of ciRNA13761 on novel-miR-3880 shown in Figure 2E, it showed a mutual inhibition between novel-miR-3880 and ciRNA13761. Furthermore, ciRNA13761 induced an increase...
in ELF2 expression, while si-ciRNA13761 decreased ELF2 expression (Figures 2F,G). To determine the function of ELF2 in the network, the efficiency of siELF2 was verified (Figures 2H, I, J). It is evident that ELF2 inhibited novel-miR-3880 (Figure 2J), but promoted ciRNA13761 and DOCK1 expression (Figures 2K, L). Otherwise, novel-miR-3880 improved but ciRNA13761 restrained the expression of DOCK1 (Figures 2M, N). Interestingly, as DOCK1 was knocked down (Figure 2O), novel-miR-3880 and ciRNA13761 expression were enhanced while ELF2 expression was not affected (Figures 2P, R).

**Novel-miR-3880 Improves MEC Viability and Suppresses Apoptosis via ELF2**

As CCK-8 results revealed, si-ciRNA13761, novel-miR-3880, and siELF2 improved MEC viability (Supplementary File S7a) while novel-miR-3880 recovered MEC viability reduction caused by ciRNA13761 and ELF2 overexpression.
**CiRNA13761/Novel-miR-3880/ELF2 Axis Activates PI3K/AKT/mTOR/S6K1 Pathway**

C57BL/6 mice during the lactation period were injected with novel-miR-3880 or siELF2 and a schematic diagram about the operational details is shown in Figure 3A. Postpartum mice were injected with 10 nmol novel-miR-3880 or siELF2 per individual with six mice in a group,
while an interval of three days and four days was applied alternately. Mammary gland tissue and blood samples were collected at day 22. The phosphorylation levels of PI3K, AKT, mTOR, and S6K1 in mammary gland tissue were shown in Figures 3B,C, which showed a higher phosphorylation of PI3K/AKT/mTOR/S6K1 in novel-miR-3880 and siELF2 groups. In MEC, novel-miR-3880 and siELF2 enhanced the phosphorylation of PI3K, AKT, mTOR, and S6K1, and novel-miR-3880 balanced the reduction of phosphorylation caused by ELF2 overexpression (Figures 3D,E). Then PI3K, AKT, mTOR, and S6K1 inhibitors dissolved in DMSO were applied to demonstrate the role of novel-miR-3880 and siELF2 in PI3K/AKT/mTOR/S6K1 pathway. In Figure 3F, PI3K, AKT, mTOR, and S6K1 inhibitors show a negative effect to the phosphorylation of each other, and the level of Bcl2/Bax was decreased when PI3K, AKT, mTOR, and S6K1 were inhibited. The results indicated that novel-miR-3880 and siELF2 could alleviate the reduction of phosphorylated-PI3K/AKT/mTOR/S6K1 and Bcl2/Bax levels (Figures 3G–J). However, Figures 3K,L show that ciRNA13761 played a negative role in PI3K/AKT/mTOR/S6K1 pathway activation, but PI3K/AKT/mTOR/S6K1 pathway was reactivated when novel-miR-3880 was co-transfected or ciRNA13761/DOCK1 was knocked down.

**Novel-miR-3880 Enhances MEC Proliferation Through PI3K/AKT/mTOR/S6K1 and Bcl-2/Bax Pathway via Targeting ELF2**

The results show that MEC proliferation would be suppressed when PI3K, AKT, mTOR, or S6K1 were inhibited (Supplementary Files S8a,b), but the participation of novel-miR-3880 and siELF2 played a positive part in freeing MEC to proliferate (Supplementary Files S8a,c–g). In addition, results shown in Supplementary Files S8h–k provide evidence for the restraint of ciRNA13761 and ELF2 overexpression on MEC proliferation and the restraint was alleviated by novel-miR-3880, and further affirmed the modulation of ciRNA13761, novel-miR-3880, and ELF2 on MEC proliferation.

**ELF2 Contributes to the Modulation of Novel-miR-3880 to MEC Lipid Formation, Triglyceride Synthesis and Casein Secretion**

The Oil Red O staining and triglyceride content test revealed that novel-miR-3880, si-ciRNA13761, and siELF2 enhanced while ciRNA13761 and ELF2 overexpression reduced MEC lipid formation (Supplementary Files S9a–c) and triglyceride synthesis (Supplementary Files S9d–f), with the reduction weakened by novel-miR-3880 (Supplementary Files S9b,c,e,f). The participation of PI3K/AKT/mTOR/S6K1 pathway is illustrated in Supplementary File S9g; it is shown that MEC triglyceride content declined significantly when PI3K, AKT, mTOR, or S6K1 was inhibited. It is worthwhile to mention that novel-miR-3880 and siELF2 improved the triglyceride content in mouse mammary glands but did not change blood triglyceride content (Supplementary Files S9h,i).

As is shown in Supplementary File S10a, novel-miR-3880, si-ciRNA13761, and siELF2 increased MEC κ-casein secretion, but decreased α s1-, α s2-, and β-casein secretion. The ciRNA13761 and ELF2 overexpression and novel-miR-3880 co-transfection experiment demonstrated an interaction among ciRNA13761, novel-miR-3880, and ELF2 in MEC κ-, α s1-, α s2-, and β-casein secretion regulation (Supplementary Files S10b,c).

**Novel-miR-3880 Promotes Mammary Gland Development, Extends Lactation Period and Benefits Offspring Growth**

The ultrastructure of collected mammary gland tissues was observed. It is significant that lipid droplets became larger but fewer, as the acini cavities turned smaller in the control group (Figure 4A). However, animals injected with novel-miR-3880 or siELF2 were still lactating with plump acini filled with secreta and had more abundant mammary ducts (Figures 4B,C). More images can be found in Supplementary File S11. The weight of all female offspring in six nests of each group was measured, and it was found that newborns fed by mothers in novel-miR-3880 or siELF2 groups gained more weight (Figure 4D).
DISCUSSION

This research aimed to study the effects of novel-miR-3880 and relative molecules on mammary gland development and lactation traits, thus gaining a better understanding of lactation in dairy goats. A general view of our work is presented in Supplementary File S12. Novel-miR-3880 was selected from our previous study (Hou et al., 2017) for its higher expression in peak lactation stage. The present study reveals that novel-miR-3880 decreased content of most casein (α s1-, α s2-, and β-casein), which makes it closer to human milk protein composition (Kunz and Lonnerdal, 1992; Hao-Feng, 2012), and increased κ-casein content to make milk products easier to process (Brigid et al., 2003).

Importantly, it was confirmed that novel-miR-3880 and siELF2 work well in facilitating mammary gland development, improving triglyceride content in mammary glands but not in blood, extending lactation days, and promoting newborn growth through PI3K/AKT/mTOR/S6K1 pathway. Therefore, novel-miR-3880 application is promising in the goat breeding and milk industry.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of Northwest A&F University.

AUTHOR CONTRIBUTIONS

BC and XA guided the experiment and applied the funds. YZ designed and performed most of the experiment and wrote the manuscript. FC, WL, JL, and XD conducted some of experiments. GN and SJ revised the manuscript. All of the authors have read and approved the submitted version of the manuscript.

FUNDING

This study was supported by Shaanxi Key Research and Development Plan Project (2020ZDLNY02-01 and 2020ZDLNY02-02), National Natural Science Foundation of China (31601925), Natural Science Foundation of Shaanxi Province (2018JM3006 and 2015JM3087), China Postdoctoral Science Foundation (2016T90954 and 2014M552498) and Shaanxi Science and Technology Innovation Project Plan (2015KTCQ03-08, 2016KTDZNY02-04, 2017ZDM-NY-081, and 2018ZDCXL-NY-01-04).

ACKNOWLEDGMENTS

Thanks for Northwest A&F University’s Life Science Core Services Facility.
SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00383/full#supplementary-material

FILE S1 | Differentially expressed miRNAs between Colostrum period and peak period acquired from previous sequencing results in our laboratory (Hou et al., 2017).

FILE S2 | Source and structure of ciRNA13761.

FILE S3 | Result of psiCHECK2-ELF2-1 vector sequencing.

FILE S4 | Result of psiCHECK2-ELF2-2 vector sequencing.

FILE S5 | Result of pcDNA3.1-ELF2 vector sequencing.

FILE S6 | Novel-miR-3880 regulated differentially expressed genes in MEC acquired from RNA-sequecing.

FILE S7 | MEC viability and apoptosis regulated by ELF2, novel-miR-3880, and ciRNA13761. (a–c) MEC viability was regulated by novel-miR-3880, ciRNA13761 and ELF2. (d–f) The modulation of novel-miR-3880, ciRNA13761 and ELF2 to MEC apoptosis. (g) The effect of novel-miR-3880 on MEC cycle.

FILE S8 | Roles of P38/akt/mTOR/s6K1 pathway and ciRNA13761/novel-miR-3880/ELF2 axis in MEC proliferation. (a–g) In accordance with the EdU results (a), P38K, Akt, mTOR, and S6K1 inhibitors restrained MEC proliferation (b), while novel-miR-3880 and siELF2 aroused MEC proliferation (c); and novel-miR-3880 and siELF2 could suppress the inhibition come from P38K (d), Akt (e), mTOR (f) and S6K1 inhibitors (g). (h,i) Regulation of ciRNA13761 to MEC proliferation and the function of novel-miR-3880 on balancing reduced MEC proliferation caused by ciRNA13761 overexpression. (j,k) Regulation of ELF2 to MEC proliferation and the balance of novel-miR-3880 on reduced MEC proliferation caused by ELF2 overexpression.

FILE S9 | Effects of ciRNA13761, novel-miR-3880, and ELF2 on MEC lipid droplets formation and triglyceride synthesis. (a–c) Oil red O staining illustrating the amount of MEC lipid droplets. (d) MEC triglyceride content was improved by si-ciRNA13761, novel-miR-3880, siELF2. (e,f) ELF2 and ciRNA13761 overexpression restrained triglyceride synthesis while novel-miR-3880 eliminated some restraint. (g) P38K, Akt, mTOR and S6K1 inhibitors suppressed triglyceride synthesis in MEC. (h) Novel-miR-3880 and siELF2 improved triglyceride content in mouse mammary gland. (i) Novel-miR-3880 and siELF2 did not change triglyceride content in blood of mouse.

FILE S10 | Effects of ciRNA13761, novel-miR-3880, and ELF2 on -a s1-, -a s2- and -a casein secretion in MEC. (a) Effects of si-ciRNA13761, novel-miR-3880 and siELF2 on casein secretion. (b) Regulation of overexpressed ciRNA13761 to casein secretion. (c) Regulation of overexpressed ELF2 to casein secretion.

FILE S11 | General view of mutual regulation among ciRNA13761, DOCK1, novel-miR-3880, and ELF2.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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