MicroRNA-221 regulates proliferation of bovine mammary gland epithelial cells by targeting the *STAT5a* and *IRS1* genes

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ABSTRACT

MicroRNAs (miRNA) play an essential role in mammary gland development and lactation. Previous studies in cattle have shown that miR-221 is highly expressed in peak compared with early lactation. However, the functions of miR-221 in bovine mammary gland epithelial cells and the mechanisms by which this miRNA affects cell proliferation and milk synthesis remain unclear. We hypothesized that miR-221 targets and modulates the expression of specific genes in the Janus kinase-signal transducer and activator of transcription (JAK-STAT) and phosphatidylinositol 3-kinase-protein kinase B/mammalian target of rapamycin (PI3K-Akt/mTOR) signaling pathways, which have crucial roles in lactation in cattle. Following transfection of miR-221 into cultured bovine mammary gland epithelial cells, inhibition of cell proliferation and reduced viability of these cells were observed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry analysis. To elucidate the molecular mechanisms of the effects of miR-221 on cell proliferation, we selected potential candidate genes that can be targeted by miR-221 using bioinformatics prediction tools. The dual luciferase assay revealed that *STAT5a*, *STAT3*, and *IRS1* interact with miR-221 by its direct binding to the 3'-untranslated regions (UTR) of these genes. Subsequent analysis showed that transfection of a miR-221 mimic resulted in significantly decreased expression of *STAT5a* and *IRS1* at both the RNA and protein levels using quantitative real-time PCR and Western blot analyses. Furthermore, expression levels of the downstream genes *SOCS3*, *AKT3*, and *mTOR* that are regulated by *STAT5a* and *IRS1* in the JAK-STAT and PI3K-Akt/mTOR signaling pathways, were also altered after miR-221 transfection. This is the first study to reveal the mechanisms by which miR-221 in-

hibits mammary gland epithelial cell proliferation by targeting *STAT5a* and *IRS1*, key genes in the PI3K-Akt/mTOR and JAK-STAT signaling pathways.

Key words: miR-221, *STAT5a*, *IRS1*, bovine mammary gland epithelial cell, cell proliferation

INTRODUCTION

The mammary gland is a unique organ that can pass through several distinct stages of development, including embryonic, pubertal, pregnancy, lactation, and involution (Macias and Hinck, 2012). Consequently, the tissues of the mammary gland experience cell proliferation, differentiation, and apoptosis cycles during adult life that are under the regulation of distinct hormones, growth factors, and coding genes (Topper and Freeman, 1980; Oka et al., 1991; Liu et al., 1997; Wiseman and Werb, 2002; Shackleton et al., 2006). These stage-based processes require numerous signaling pathways that have distinct regulatory functions (Hennighausen and Robinson, 2001; Robinson, 2004; Howard and Ashworth, 2006). The primary signaling pathways Janus kinase-signal transducer and activator of transcription (JAK-STAT; Watson and Burdon, 1996; Mapes et al., 2017) and phosphatidylinositol 3-kinase-protein kinase B/mammalian target of rapamycin (PI3K-Akt/mTOR) are essential for mammary gland development and milk protein synthesis (Sobolewska et al., 2009). The *STAT5a* gene, a member of the JAK-STAT signaling pathway, plays a pivotal role in the proliferation, secretory differentiation, and survival of mammary epithelial cells (Cui et al., 2004; Yamaji et al., 2009; Furth et al., 2011; Liu et al., 2012b). The PI3K-Akt/mTOR signaling pathway regulates a broad range of cellular processes including survival, cell proliferation, growth, and metabolism (Ersahin et al., 2015). However, the mechanisms by which these pathways are regulated in cattle are poorly understood.

MicroRNAs (miRNA) are small non-coding RNA sequences of 22 to 25 nt that play roles in the regulation of gene expression by binding to the 3'-untranslated

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region (**UTR**) of target mRNA (Huang et al., 2011). Several studies have reported miRNA involvement in mammary gland development (Silveri et al., 2006; Tanaka et al., 2009; Wang et al., 2018), maintenance of mammary epithelial progenitor cells (Ibarra et al., 2007), outgrowth of epithelial ducts (Ucar et al., 2010), and in proliferation and differentiation of mammary epithelial cells (Li et al., 2010; Howe et al., 2012; Liu et al., 2012a). In addition, mammary gland tissue-specific and developmental stage-specific miRNA have been found in different species including human, mouse, and bovine (Liu et al., 2004; Avril-Sassen et al., 2009; Wang et al., 2012; Phua et al., 2015). For example, expression of miR-205 was found to be associated with an increase in the number of mammary gland stem cells in the mouse by acting on the *PTEN* gene, and it plays an important role in the regulation of mammary stem cell fate and tumorigenesis (Greene et al., 2010; Chao et al., 2014). Overexpression of miR-101a and miR-126-3p led to inhibition of mammary gland epithelial cell differentiation (Tanaka et al., 2009; Wei et al., 2011). In cattle, miR-15a was found to inhibit mammary gland epithelial cell vitality and lactation by targeting the *GHR* gene (Li et al., 2012).

Recent studies have reported that miR-221 regulates cell growth and cell cycle progression in various cell types including cancer cells (Galardi et al., 2007; Pineau et al., 2010; Rong et al., 2013; Chu et al., 2018). In cattle, expression of miR-221 was found to be higher in peak lactation than in early lactation (Wang et al., 2012). However, the specific mechanisms by which miR-221 affects bovine mammary gland development remain poorly understood. Thus, the objectives of this study were to investigate whether miR-221 affects the proliferation of mammary epithelial cells and to characterize the target genes of miR-221 in the JAK-STAT and PI3K-Akt/mTOR signaling pathways and to evaluate the regulatory effects of miR-221 on these genes.

MATERIALS AND METHODS

Effects of miR-221 Transfection on Cell Proliferation

Cell viability and cell cycle analyses were performed to study the effects of miR-221 on cell proliferation. The bovine mammary epithelial cell (**BMEC**) line was donated by H. B. Zhao's laboratory (Northwest A&F University, Shaanxi, China). The cell line was transfected with SV40 large T antigen, which confers immortality to BMEC. The cell line is responsive to manipulations both in extracellular matrix and in the presence of lactogenic hormones. When differentiated, the cells synthesize and secrete α - and β -casein. As such, BMEC cells represent an in vitro model for

bovine lactation (Huynh et al., 1991). To determine cell viability, BMEC were cultured in DF-12 growth medium (a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium) supplemented with 10% heat inactivated fetal bovine serum (**FBS**), and 1% penicillin streptomycin (all from Gibco, Grand Island, NY) at 37°C with 5% CO₂. Single cell pellets were seeded in a 96-well plate at a concentration of 1×10^4 cells per well. For transfections of BMEC, miR-221 mimic, miR-221 inhibitor, and their corresponding negative control oligonucleotides were synthesized by Shanghai GenePharma Co. Ltd. (Shanghai, China). The miR-221 mimic (40 nM) and miR-221 inhibitor (80 nM) were transfected into BMEC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After transfection for 48 h, the culture medium was aspirated, 50 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (**MTT**; 0.5 mg/mL, Sigma, St. Louis, MO) was added to each well, and the culture plates were further incubated for 4 h in the dark at 37°C. Then, 150 μ L of dimethyl sulfoxide (Amersco, Solon, OH) was added to each to dissolve the formed formazan crystals. A SynergyH1 multi-detector microplate reader (BioTek, Winooski, VT) was used to measure the absorbance of each well at a wavelength of 570 nm. The relative cell viability was calculated using optical density at 570 nm (OD₅₇₀) absorbance value ratios.

For cell cycle analysis, single cell pellets were fixed with 70% ethanol and incubated with PBS supplemented with 50 μ g/mL propidium iodide (Abcam, Shanghai, China) and 100 μ g/mL RNase A (Beyotime, Beijing, China) at 37°C for 30 min. Cell cycle was measured with BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Data were analyzed using ModFit LT 4.0 software (Becton Dickinson).

Interactions Between miR-221 and Target Genes

Based on the seed sequence of miR-221 and on their roles in the JAK-STAT and PI3K-Akt/mTOR signaling pathways, *mTOR*, *IRS1*, *PI3KR1*, *STAT3*, *IGF1*, *STAT5a*, *PRLR*, and *AKT3* were considered potential targets of miR-221. To identify the interactions between miR-221 and target genes, the dual luciferase reporter system was used. First, 3'-untranslated region (**UTR**) fragments of the targets were amplified by PCR and ligated into the psi-CHECK-2 vector (Promega, Madison, WI) using *XhoI* and *NotI* restriction enzymes. Primers used for PCR amplification were synthesized by Invitrogen. All the qPCR experiments were performed according to the MIQE checklist (<http://www.rdml.org/miqe.php>). The vectors Psicheck-2-*mTOR*, Psicheck-2-*IRS1*, Psicheck-2-*PI3KR1*, Psicheck-2-*STAT3*, Psicheck-

Table 1. Real-time (RT) quantitative (q)PCR primer information for microRNA (miR)-221 and target genes

Gene	Accession number	Sequence (5' to 3'; F = forward, R = reverse)	Product size (bp)
<i>IRS1</i>	XM_003585773	F: TTGGTCAAGGACTTCAAACAG R: TTGCTGAGGTCATTTAGGTCT	227
<i>STAT5a</i>	NM_001012673	F: ACGGTACCTTCTTGTTCGCG R: CAGGTTACGGTCAGAGAGTCAAAC	85
<i>STAT3</i>	NM_001012671	F: AGTGATGAACATGGAAGAGTC R: TGATACACCTCAGTCTCAAAG	156
<i>SOCS3</i>	XM_005221139	F: CCCCAGGAGAGCCTATTACAT R: GGGTGACTTTCTCGTAGGAGT	146
<i>mTOR</i>	XM_002694043	F: AGATTGACACTTGGTTACAGG R: ATGTTCTTCAGGATTTTGTG	202
<i>AKT3</i>	XM_024975966	F: TACCTTATCCCTCAACAAC R: ATTCTCTCTTCTTCTTGCCCTC	211
<i>GAPDH</i>	NM_001034034	F: AGATGGTGAAGGTCGGAGTG R: CGTTCTCTGCCTTGACTGTG	189
miR-221 RT primer		CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAAACCCAG	60
miR-221 forward primer		F: ACACTCCAGCTGGGAGCTACATTGTCTGCT	
Universal reverse primer		TGGTGTCTGGAGTCG	

2-*IGF1*, Psicheck-2-*STAT5a*, Psicheck-2-*PRLR*, and Psicheck-2-*AKT3* were Sanger sequenced to verify the presence of 3'-UTR fragments. Then, the dual luciferase reporter system was used to measure the interactions between miR-221 and its targets using HEK293T cells. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco) with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C with 5% CO₂. The dual luciferase transfection was performed in 24-well plates where each well was co-transfected with 300 ng of dual luciferase reporter vector and 20 pmol of miR-221 mimic or 40 pmol of miR-221 inhibitor with Lipofectamine 2000 for 36 h (Invitrogen). Transfections were performed in triplicate. To assess the interactions between miR-221 and the potential target genes, the firefly and Renilla luciferase activities were measured using the dual luciferase reporter kit (Promega).

Effects of miR-221 on the Expression of Its Target Genes

To study the effects of miR-221 on the activity of its target genes, miR-221 mimic or inhibitor were transfected into BMEC as described in the previous section. Cells were collected 48 h after transfection. The cells were harvested when the confluence reached 90%. All transfections were performed in triplicate. Total RNA was extracted using Trizol (Invitrogen) following the manufacturer's instructions. To test whether miR-221 was taken up by BMEC, cDNA was synthesized using a specific stem-loop RT primer for miR-221 through PrimerScript RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China). Expression of miR-221 was quantified with *U6* as an endogenous control using the SYBR Premix Ex Taq™ II kit (TaKaRa) according

to the manufacturer's protocol. The miR-221 primers are listed in Table 1, and the *U6* primer sequences were synthesized according to Guan et al. (2017). The real-time quantitative (q)PCR reactions consisted of 10 µL of SYBR Premix Ex Taq, 0.6 µL of miR-221 forward primer (10 µM), 0.6 µL of universal reverse primer (10 µM), 2 µL of miRNA RT product (100 ng/µL), and 6.8 µL of double-distilled H₂O to a final volume of 20 µL. The qPCR protocol was initiated with an incubation of 3 min at 95°C, followed by 40 cycles of 95°C for 12 s and 62°C for 40 s using a Bio-Rad CFX96 Touch Real-time PCR detection system (Bio-Rad, Hercules, CA).

For coding genes, the cDNA was synthesized using PrimerScript RT reagent kit with gDNA Eraser (TaKaRa) according to the manufacturer's protocol. Then, the expression levels of the target genes *STAT5a*, *IRS1*, *STAT3*, and the downstream genes *SOCS3*, *AKT3*, and *mTOR* in the JAK-STAT and PI3K-Akt/mTOR signaling pathways were quantified with SYBR Premix Ex Taq kit (TaKaRa) using *GAPDH* as a reference gene. All qPCR reactions were run in triplicate in a 20-µL volume. Primers for qPCR are listed in Table 1. The initial cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 12 s and 58°C for 30 s.

Western Blot Analysis of *IRS1* and *STAT5a*

Genes found to be regulated by miR-221 at the transcriptional level were further analyzed by Western blot. Total protein was extracted from the BMEC using RIPA (radioimmunoprecipitation assay) cell lysis solution (Applygen, Beijing, China) after transfection for 72 h. The samples were separated on a 10% SDS-PAGE gel (30 µg of protein per sample) and transferred onto nitrocellulose membranes (Bio-Rad, Shanghai, China).

After blocking with 5% skim milk powder solution for 1 h at 37°C, the membranes were incubated overnight at 4°C with a murine polyclonal rabbit antibody against IRS1 and STAT5a (Santa Cruz Biotechnology Inc., Dallas, TX), and then incubated with a horseradish peroxidase (HRP)-conjugated secondary goat-anti-rabbit IgG (Zhongshan-Bio, Beijing, China). The protein bands were visualized with Super ECL Plus (Apply-GEN), and GAPDH (Abcam) was used as an internal reference control.

Data Analysis

To determine the effect of miR-221 mimic or inhibitor on gene expression, the relative expression of potential target genes was estimated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). The miR-221 mimic group was compared with the mimic control group, and the miR-221 inhibitor group was compared with the inhibitor control group. To detect interactions between miR-221 and potential target genes, the Renilla/firefly activity was compared between mimic and mimic control group, and between inhibitor and inhibitor group in HEK293T cells. Data were expressed as means $\pm$ standard error of the mean. Statistical analysis was performed using unpaired *t*-test in GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA). Statistical significance was declared as $P < 0.05$, $P < 0.01$, and $P < 0.001$.

RESULTS

Functional Analysis of miR-221 Effects on BMEC Proliferation

To explore the effect on cell proliferation, different concentrations of miR-221 mimic and inhibitor were supplemented to BMEC in 6-well plates and incubated for 48 h. The qPCR analysis revealed that supplementation of 100 pmol/L miR-221 mimic significantly increased the level of miR-221 in BMEC compared with the mimic-negative control (Figure 1). In contrast, supplementation of 80 pmol/L inhibitor resulted in a significant decrease of miR-221 level in BMEC compared with other concentrations used (Figure 1).

Proliferation of BMEC was assayed by the MTT assay 48 h following transfection of miR-221 mimic or inhibitor. Cell viability was significantly reduced in BMEC transfected with miR-221 mimic compared with the control cells ($P < 0.001$), whereas supplementation of miR-221 inhibitor led to a significant increase ($P < 0.001$) in cell viability compared with controls (Figure 2A).

To further validate the effect of miR-221 on cell proliferation, cell cycle analysis was performed using the flow cytometry method. As shown in Figure 2B, most BMEC were in G₁ phase. Figure 2B shows that 18.15% of cells in the miR-221 mimic group were in the DNA post-synthesis phase (G₂) or synthesis phase (S) compared with 31.27% of cells in the mimic-control group, representing a cell reduction of 13.12 percentage units in the mimic group. In contrast, cells in the G₂ and S phases were increased by 10.57% in the miR-221 inhibitor group compared with the inhibitor control group (Figure 2B).

Interactions Between miR-221 and Its Targets

To better understand the mechanisms by which miR-221 affects cell viability and cell proliferation, we

Figure 1. Quantitative real-time PCR of microRNA (miR)-221 following transfection of miR-221 mimic and inhibitor into bovine mammary epithelial cells (BMEC). Transfection with 100 pmol/well of miR-221 mimic led to the highest expression of miR-221 in BMEC compared with the mimic control group. Transfection of 200 pmol of inhibitor/well for 48 h resulted in a significant decrease in miR-221 expression level in BMEC compared with the nonspecific scrambled control (NC). Data are from 3 independent experiments and presented as mean $\pm$ SEM ($n = 3$); * $P < 0.05$ and ** $P < 0.01$: significant difference between mimic or inhibitor and respective controls (NC). Mimic_80 and mimic_100 indicate mimic concentrations of 80 and 100 pmol/well, respectively. Inhibitor_100, inhibitor_150, and inhibitor_200 indicate inhibitor concentrations of 100, 150, and 200 pmol/well, respectively.

tested the activities of genes targeted by miR-221. Target genes of miR-221 were predicted using TargetScan (http://www.targetscan.org/vert_71/) and DNAMAN software (Lynnon Corp., San Ramon, CA) based on the mature sequence of miR-221 (<http://www.mirbase.org>). The corresponding psi-check2 vectors were constructed and transferred into HEK293T cells with miR-221 mimic/inhibitor. The Renilla/firefly luciferase assay showed that the activities of *mTOR*, *IRS1*, *STAT3*, *IGF1*, *STAT5a*, and *PRLR* genes were reduced after transfecting miR-221 mimic (Figure 3), whereas the activities of *IRS1*, *STAT3*, *STAT5a*, and *AKT3* were increased by transfecting miR-221 inhibitor (Figure 3). The activity of *PI3KR1* did not change significantly following miR-221 mimic or inhibitor transfections into HEK293T cells. Thus, the dual luciferase results indicate that miR-221 interacts with *IRS1*, *STAT5a*, and *STAT3* genes by binding to their 3'-UTR. Therefore, these genes were selected for further analysis to study their expression activities in BMEC following miR-221 binding.

MiR-221 Regulates the Expression of *STAT5a* and *IRS1*

To investigate the potential regulatory role of miR-221 on *IRS1*, *STAT5a*, and *STAT3* genes, miR-221 mimic and inhibitor were transfected into the cultured BMEC. Figure 4 shows the expression levels of *IRS1*, *STAT5a*, and *STAT3* following transfection of miR-221 mimic and its inhibitor. Overexpression of miR-221 in BMEC was associated with a decrease in *IRS1* ($P < 0.05$) and *STAT5a* ($P < 0.001$) expression levels (Figure 4A), whereas supplementation of miR-221 inhibitor led to increased expression of *IRS1* ($P < 0.01$) and *STAT5a* ($P < 0.05$; Figure 4A). Although *STAT3* showed similar trends of expression to those of *IRS1* and *STAT5a*, the difference compared with control cells was not statistically significant.

To test whether *IRS1* and *STAT5a* are regulated by miR-221 at the protein level, a Western blot was performed (Figure 4B). Transfection of miR-221 mimic into BMEC led to reduced expression of *IRS1* ($P <$

Figure 2. Cell viability and proliferation assays of bovine mammary epithelial cells (BMEC) transfected with microRNA (miR)-221. (A) Transfection of miR-221 mimic and miR-221 inhibitor resulted in a decrease and increase, respectively, in the activity of BMEC using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; (B) cell proliferation analysis using flow cytometry showed that miR-221 mimic-transfected BMEC in the G₂ and S phases were reduced by 13.12 percentage units compared with the control group, whereas cells transfected with miR-221 inhibitor were increased by 10.57 percentage units in the G₂ and S phases compared with the inhibitor control. The data were derived from 3 independent experiments on different days and are presented as mean $\pm$ SEM ($n = 3$). *** $P < 0.001$: significant difference between mimic or inhibitor and respective controls (NC).

0.01) and STAT5a ($P < 0.001$) proteins (Figure 4C), whereas expression levels of these proteins were increased in BMEC transfected with miR-221 inhibitor (Figure 4C).

Expression of Downstream Genes in the JAK-STAT and PI3K-Akt/mTOR Signaling Pathways

A previous study reported that overexpression of *SOCS3* in bovine mammary epithelial cells significantly inhibited cell growth and viability and that *SOCS3* could be targeted by *STAT5a*, a member of the JAK-STAT signaling pathway (Huang et al., 2013). The *AKT3* and *mTOR* genes are located in the PI3K-Akt/mTOR signaling pathway, which is activated by phosphorylation of *IRS1* (Law et al., 2016). Therefore, the expression of *SOCS3*, *AKT3*, and *mTOR* was assessed in BMEC by qPCR following miR-221 transfection. Figure 5 shows that overexpression of miR-221 significantly decreased the expression of *SOCS3*, *AKT3*, and *mTOR* genes, whereas supplementation of miR-221 inhibitor resulted in increased expression of *SOCS3* and *mTOR* compared with control cells. The expression of *AKT3* was also increased after miR-221 inhibitor transfection but not significantly compared with controls.

DISCUSSION

Previous studies have shown that miR-221 plays roles in disease, cell differentiation, and cell proliferation processes (Felli et al., 2005; Chen et al., 2018), mammary gland tumorigenesis (Lv et al., 2017), and milk production traits (Wang et al., 2018). Furthermore, miR-221 has been found to be highly expressed in early lactation compared with fresh and dry periods, suggesting a role in the control of endothelial cell growth and proliferation (Wang et al., 2012). Thus, the objective of this study was to elucidate the molecular mechanisms by which miR-221 affect mammary gland development. The results of the MTT and flow cytometry assays revealed that overexpression of miR-221 in cultured BMEC decreased cell viability and inhibited cell proliferation.

Given the roles of the JAK-STAT and PI3K-Akt/mTOR signaling pathways in mammary gland development and lactation, we sought to test whether genes in these pathways are potential targets of miR-221 binding. Indeed, using the dual luciferase assay in HEK293T cells, we found that miR-221 interacts with the 3'-UTR of *STAT5a*, *STAT3* (members of the JAK-STAT pathway), and *IRS1* (a member of the PI3K-Akt/mTOR

Figure 3. Dual luciferase assay to detect interactions between microRNA (miR)-221 and potential target genes. The Renilla/firefly activity was measured in HEK293T cells co-transfected with the vector constructs of the target genes and with miR-221 mimic and miR-221 inhibitor. Binding sites of the 3'-untranslated regions (UTR) of the candidate target genes and miR-221 are shown. Data are presented as mean $\pm$ SEM ($n = 3$); * $P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$: significant difference between mimic or inhibitor and respective controls (NC).

Figure 4. Expression of insulin receptor substrate 1 (IRS1) and signal transducer and activator of transcription 5a (STAT5a) at the mRNA and protein levels in bovine mammary epithelial cells transfected with microRNA (miR)-221 mimic or miR-221 inhibitor. (A) The relative expression of *IRS1* and *STAT5a* was significantly decreased after miR-221 mimic transfection and increased after miR-221 inhibitor transfection; (B) Western blot of IRS1 and STAT5a and their negative controls using GAPDH as a reference control; (C) relative expression of IRS1 and STAT5a proteins after miR-221 mimic or miR-221 inhibitor transfections into bovine mammary epithelial cells (BMEC). Data are presented as mean $\pm$ SEM ($n = 3$); * $P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$: significant difference between mimic or inhibitor and respective controls (NC).

pathway). Further analysis revealed that *STAT5a* and *IRS1* are regulated by miR-221 at both the transcriptional and translational levels. Overexpression of miR-221 led to decreased expression, and its inhibition led to increased expression of *STAT5a* and *IRS1*. Interestingly, it has been found that tyrosine-phosphorylated STAT5 interacts with the PIK3R1 regulatory subunit of PI3K, a member of PI3K-Akt/mTOR, to activate the JAK-STAT pathway, which in turn promotes cell survival and proliferation (Rädler et al., 2017). Thus, the regulation of *STAT5a* by miR-221 sheds new light on the combined effects of both signaling pathways, JAK-STAT and PI3K-Akt/mTOR, on mammary gland

development. Additionally, *STAT5a* can mediate a signal-transduction pathway to regulate milk protein synthesis, to regulate mammary gland epithelial cell differentiation in pregnancy, and to maintain survival of breast epithelial cells (Faraci-Orf et al., 2006; Buser et al., 2007).

Insulin has been reported to stimulate cell proliferation and the expression of milk protein genes in bovine mammary epithelial cells and in mouse mammary glands (Lanna and Bauman, 1999; Menzies et al., 2009, 2010). The IRS1 protein is a signaling adapter for insulin-like growth factor 1 receptor (*IGF1R*) and insulin receptor (*IR*), which play a central role in cell

signaling molecule networks, including the PI3K-Akt/mTOR signal pathway (Yu and Rohan, 2000; Baserga et al., 2003; Cao et al., 2007). Interestingly, Chen et al. (1997) reported that the major insulin receptor substrate IRS-1 activates STAT5 by phosphorylation. Furthermore, *IRS1* is involved in insulin-dependent activation of Akt signaling within the lactating mammary gland, although the loss of IRS1 protein has only a modest effect on normal milk synthesis (Hadsell et al., 2007). In this study, *IRS1* was identified as a target of miR-221, and overexpression of miR-221 led to a decreased expression of *IRS1* at both the transcriptional and translational levels. Thus, the finding that miR-221 regulates both *STAT5a* and *IRS1* indicates a major role for miR-221 in mammary gland development.

For a deeper understanding of the subsequent effects of *STAT5a* and *IRS1* regulation by miR-221, we analyzed the downstream genes *SOCS3*, *AKT3*, and *mTOR* located in the JAK-STAT and PI3K-Akt/mTOR pathways for gene expression changes. Overexpression of miR-221 led to a reduced expression of *SOCS3*, and inhibition of miR-221 resulted in increased expression of *SOCS3*, which is a suppressor of cytokine signaling and a downstream gene in the JAK-STAT signaling pathway. It has been reported that the functions of JAK2-STAT5 in breast development and lactation could be modulated by the *SOCS* family (Huang et al., 2013; Arun et al., 2015). Huang et al. (2013) found that reduced activity of *STAT5a* was associated with a decreased expression of *SOCS3* and concluded that *SOCS3* could be a target of *STAT5a*. Thus, our results on the expression analysis of these 2 genes support the

findings of Huang et al. (2013) and provide an additional layer of information regarding the role of miR-221 in regulating *STAT5a* expression. Thus, miR-221 could be involved in mammary epithelial cell proliferation by regulating the expression of *STAT5a*, which in turn suppresses *SOCS3* expression. Indeed, it has been reported that low expression of *SOCS3* is required for milk synthesis and proliferation of dairy cow mammary epithelial cells in vitro (Huang et al., 2013).

Given that the PI3K-Akt-mTOR signaling pathway is activated by *IRS1* (Yu and Rohan, 2000) and that *IRS1* is regulated by miR-221, the downstream genes *mTOR* and *AKT3* were evaluated for gene expression following miR-221 transfection. The expression of *AKT3* and *mTOR* was significantly reduced after transfecting miR-221 mimic into BMEC (Figure 5). Because miR-221 had no observed interaction with the 3'-UTR of *AKT3* using the dual luciferase assay (Figure 3), we conclude that the effect of miR-221 on *AKT3* expression is modulated through *IRS1*. Different knockout mice of *AKT1*, -2, and -3 have shown that *AKT* is necessary for *STAT5* activation and mammary gland cell proliferation (Chen et al., 2010). Thus, our results provide further validation that miR-221 might regulate bovine mammary gland epithelial cell proliferation by regulating some key genes in the JAK-STAT and PI3K-AKT/mTOR signaling pathways.

CONCLUSIONS

This study is the first to illustrate the functional and regulatory mechanism of miR-221 in cultured bovine

Figure 5. Relative expression of the downstream genes *SOCS3*, *AKT3*, and *mTOR* in the Janus kinase-signal transducer and activator of transcription (JAK-STAT) and PI3K-Akt/mammalian target of rapamycin (mTOR) signaling pathways. Expression levels of *SOCS3*, *AKT3*, and *mTOR* were significantly decreased after microRNA (miR)-221 mimic transfection into bovine mammary epithelial cells ($P < 0.05$), and the expression of *SOCS3* and *mTOR* was significantly increased after transfection of miR-221 inhibitor ($P < 0.05$). Data are presented as mean $\pm$ SEM ($n = 3$); * $P < 0.05$: significant difference between mimic or inhibitor and respective controls (NC).

mammary epithelial cells. The MTT assay and flow cytometry analysis revealed that miR-221 could inhibit mammary gland epithelial cell proliferation by targeting *STAT5a* and *IRS1*, key genes in the PI3K-Akt/mTOR and JAK-STAT signaling pathways, respectively. The finding that miR-221 is involved in the regulation of these pathways would be valuable to enhance our understanding of the mechanisms of mammary gland development and milk synthesis.

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