Bovine miR-146a regulates inflammatory cytokines of bovine mammary epithelial cells via targeting the *TRAF6* gene

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ABSTRACT

It has been reported previously that bovine miR-146a (bta-miR-146a) is significantly differentially expressed in mammary glands infected with mastitis, compared with healthy udders. This suggests that bta-miR-146a plays an important role in the regulation of mammary inflammation. However, the specifics of this function have yet to be elucidated. Bovine mammary epithelial cells (bMEC) represent the first line of defense against pathogens and have important roles in initiating and regulating inflammatory responses and innate immunity during infection. In this study, a double luciferase reporter assay was used to confirm that bta-miR-146a directly targets the 3' UTR of the tumor-necrosis factor receptor-associated factor 6 (*TRAF6*) gene. To elucidate the role of bta-miR-146a in innate immune responses, either a mimic or inhibitor of bta-miR-146a was transfected into bMEC stimulated with lipopolysaccharide, which activates the innate immune response through the toll-like receptor (TLR) 4/nuclear factor (NF)- κ B signaling pathway. Forty-eight hours post-transfection, quantitative real-time PCR and Western blots were used to detect the expressions of the related genes and proteins, respectively. An ELISA was used to measure the quantity of inflammatory factors in culture supernatants. The results showed that bta-miR-146a significantly inhibits both mRNA and protein expression levels of bovine TRAF6, and ultimately suppresses downstream expression of NF- κ B mRNA and protein. As a result, production of NF- κ B-dependent inflammatory mediators such as tumor necrosis factor α , IL-6, and IL-8 are suppressed following lipopolysaccharide stimulation of bMEC. Thus, we concluded that bta-miR-146a acts as a negative feedback regulator of

bovine inflammation and innate immunity through downregulation of the TLR4/TRAF6/NF- κ B pathway. This study presents a potential regulatory mechanism of bta-miR-146a on immune responses in bovine mammary infection and may provide a potential therapeutic target for mastitis.

Key words: miR-146a, bovine mammary epithelial cell, inflammation, innate immunity

INTRODUCTION

Bovine mastitis, an inflammation of the mammary gland caused by bacterial infection, is a highly prevalent and costly disease of dairy cattle (Seegers et al., 2003). Infection of the bovine mammary gland by pathogens modulates inflammatory cytokines in bovine mammary epithelial cells (bMEC), which recruit white blood cells to the sites of infection (Sordillo et al., 1997; Rainard and Riollot, 2006). During infection of bovine mammary glands, bMEC serve as the first line of defense against invading pathogens, and play a major role in the initiation of immune responses (Griesbeck-Zilch et al., 2008). These cells are capable of detecting pathogen-associated molecular patterns (PAMP) of invading pathogens through toll-like receptors (TLR), activating the nuclear factor (NF)- κ B pathway and the associated innate immune responses (Huang et al., 2012; Wellnitz and Bruckmaier, 2012; Gilbert et al., 2013; Gunther et al., 2016). Lipopolysaccharide, the major component of the outer membrane of gram-negative bacteria, is a common trigger of inflammation. It has been widely used to model bacterial interactions with mammalian cells, including bMEC (Wellnitz and Kerr, 2004; Strandberg et al., 2005; Gilbert et al., 2013; Wang et al., 2016a). Previous studies have indicated that stimulation of bMEC with LPS elicited a marked increase in the gene expression of inflammatory cytokines and chemokines, such as tumor necrosis factor (TNF) α , IL-6, and IL-8 (Strandberg et al., 2005; Gilbert et al., 2013; Wang et al., 2016a).

MicroRNA (miRNA) are endogenous, noncoding, small RNA molecules that regulate innate and adaptive

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immunity by directly binding to the 3' untranslated region (**UTR**) of the target mRNA (O'Connell et al., 2010; Lawless et al., 2014). Previously, we reported that the expression of bovine miR-146a (**bta-miR-146a**) was significantly increased in cases of bovine mastitis caused by *Staphylococcus aureus*, *Escherichia coli*, or clinical mixed bacteria (Wang et al., 2016b). However, Naeem et al. (2012) reported the expression of bta-miR-146a was not altered in response to *Streptococcus uberis* infection of mammary glands in vivo. These referenced studies suggest that the expression of bta-miR-146a varies in response to mammary tissue infection with the specific invading pathogen. Interestingly, it has been reported that induction of human miR-146a (**has-miR-146a**) is NF- κ B-dependent and regulates innate immune responses by targeting the tumor-necrosis factor receptor-associated factor 6 (*TRAF6*) and IL-1 receptor-associated kinase (*IRAK1*) genes (Taganov et al., 2006). Notably, the sequences of miR-146a, *TRAF6*, and *IRAK1* between bovine and human exhibit a high degree of nucleotide identity. However, whether or not bta-miR-146a has the same function as has-miR-146a requires further study. Moreover, the role and the detailed mechanisms of bta-miR-146a functions in response to infection of bMEC remain unclear. In this study, we sought to identify the target genes of bta-miR-146a and elucidate its potential mechanism on inflammatory responses in LPS stimulated-bMEC.

MATERIALS AND METHODS

Cell Culture

The HEK293 cells were cultured in high-glucose Dubecco's modified Eagle medium (HyClone Laboratories, South Logan, UT) supplemented with 10% fetal bovine serum (Clark Bioscience, Richmond, VA). The bMEC were cultured in Dubecco's modified Eagle medium/F12 containing 10% exosome-depleted fetal bovine serum (System Biosciences, Mountain View, CA). All cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C. For cytokine analysis, bMEC were incubated with 50 ng/ μ L of LPS for 3 h (Strandberg et al., 2005), which is capable of inducing an inflammatory response in bMEC, and to an extent, the bMEC model can reflect the in vivo response of mastitis.

miRNA Mimics and Inhibitors

Mimics of miR-146a/b (dsRNA oligonucleotides), mismatched negative control mimics (**NC_mimic**), and miR-146a/b inhibitor (single-stranded oligonucleotides), mismatched negative control inhibitor (**NC_inhibitor**), each labeled with Cy3, were synthesized by

GenePharma Co., Ltd. (Shanghai, China). Mimics and inhibitors were used to assess the effects of overexpression and inhibition of miR-146a/b on its activity in HEK293 cells or bMEC, respectively.

Target Prediction Analysis

Bioinformatics-based target prediction analysis was performed using TargetScan 7.0 (http://www.targetscan.org/vert_71/) and miRBase (<http://microrna.sanger.ac.uk/>).

3' UTR Luciferase Reporter Assays

Total RNA was extracted from bMEC using the Trizol reagent (Invitrogen, Carlsbad, CA) and was used to synthesize first strand cDNA using a PrimeScript II 1st Strand cDNA Synthesis Kit (Takara Biotechnology Dalian Co., Ltd., Dalian, China). Double-strand cDNA fragments of 3' UTR of the potential target gene containing the putative miRNA binding site were amplified by RT-PCR. Site-specific mutated forms of the 3' UTR were created using overlap extension PCR (primers presented in Supplemental Table S1; <https://doi.org/10.3168/jds.2017-12630>). The double-strand cDNA fragment of the 3' UTR and associated mutants were digested and cloned into the XhoI-NotI sites of the psiCHECK-2 vector, containing both renilla and firefly luciferase reporter genes (Promega Corp.), respectively. The recombinant plasmid was extracted with an Endo-Free Plasmid Mini Kit (Omega Bio-tek, Norcross, GA) and constructs were verified by DNA sequencing.

The human embryonic kidney (**HEK**) 293 cells were plated at a density of 1.0×10^5 cells/well in 24-well plates. Twenty-four hours later, HEK293 cells were co-transfected with plasmids (0.5 μ g) or mimic (or NC_mimic; 0.5 μ g, the final work concentration 75 nM) using 2 μ L of X-tremeGENE HP DNA Transfection Reagent (Roche, Penzberg, Germany) following the manufacturer's protocol (<https://pim-eservices.roche.com/LifeScience/Document/e36981cf-e722-e411-ed82-00215a9b0ba8>). At 48 h posttransfection, renilla luciferase activity, normalized to firefly luciferase activity, was detected using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions (<https://cn.promega.com/resources/protocols/technical-manuals/0/dual-luciferase-reporter-assay-system-protocol/>).

Transfection of bMEC

Six-well plates were seeded with bMEC at 50 to 60% confluence. The cells were then transfected with mimic, NC_mimic, inhibitor, or NC_inhibitor (each

labeled with Cy3) using the X-tremeGENE HP DNA Transfection Reagent (Roche, Germany) according to the manufacturer's instructions (<https://pim-eservices.roche.com/LifeScience/Document/e36981cf-e722-e411-ed82-00215a9b0ba8>). Briefly, X-tremeGENE HP DNA Transfection Reagent (4 μ L) was diluted in 100 μ L of Opti-MEM (Invitrogen) with mimic, NC_mimic, inhibitor, or NC_inhibitor to yield final concentrations of 75, 75, 150, and 150 nM, respectively, with gentle agitation. The mixture was incubated at room temperature for 20 min, then added to the bMEC. Transfection efficiency was assessed by fluorescence microscopy (Supplemental Figure S1; <https://doi.org/10.3168/jds.2017-12630>).

RNA Isolation and Quantitative Real-Time PCR

All quantitative real-time PCR (qPCR) experiments were designed and conducted in compliance with the MIQE guidelines (Bustin et al., 2009), the details of which, including PCR validation, are provided in Supplemental File S1 and Supplemental Figure S2 (<https://doi.org/10.3168/jds.2017-12630>). Briefly, total RNA and miRNA were extracted from the transfected bMEC using the Trizol reagent (Invitrogen) and the miRcute miRNA isolation kit (Tiangen Biotech Co., Ltd. Beijing, China), respectively. The concentrations and quality of miRNA and mRNA were measured using a BioSpec-nano Micro-volume UV-Visible Spectrophotometer (Shimadzu Scientific Instruments, Shimadzu, Japan). Quantitative PCR of miRNA and mRNA were performed using the miRcute miRNA qPCR Detection Kit with SYBR Green chemistry (Tiangen Biotech Co., Ltd.) and the SYBR Premix Ex Taq II kit (TaKaRa, Biotechnology Co. Ltd., Dalian, China), respectively, according to the manufacturer's instructions (<http://www.takarabiomed.com.cn/Download/RR820A.pdf>). Both a no-template control and no-reverse transcriptase control were included in all plates to confirm that amplification was not due to contamination or primer dimers. All reactions were performed using the ABI 7500 RT-PCR system (Applied Biosystems, Waltham, MA). The primers used in the study are listed in Supplemental Table S2 (<https://doi.org/10.3168/jds.2017-12630>). The qPCR amplification efficiencies of the primer sets ranged from 100.16 to 104.80% with an $R^2 > 0.99$ (Supplemental Figure S2; <https://doi.org/10.3168/jds.2017-12630>).

The relative expression quantification (fold changes) were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). *Cel-miR-39* was used as a reference gene to normalize the expression of the detected miRNA. The geometric mean of the 3 reference genes (β -actin, *18s rRNA*, and *UXT*), which have been validated as

reference genes of qPCR in the LPS-stimulated bMEC (Bougarn et al., 2011), was used to normalize the expression of mRNA, according to the method previously described by Vandesompele et al. (2002).

Western Blot Analysis

Western blotting was performed to detect expression of proteins related to miR-146a in bMEC. Control or transfected cells were lysed in RIPA buffer (Sangon Biotech Co. Ltd., Shanghai, China) containing 1% phenylmethane sulfonyl fluoride. Total protein concentrations were quantified using the Bicinchoninic Acid Protein Assay kit (Sangon Biotech Co. Ltd.).

Equal quantities of total protein (25 μ g) from each sample were fractionated by 12% SDS-PAGE and transferred to nitrocellulose protran membranes (GE Healthcare/Amersham, Pittsburgh, PA). Membranes were blocked with 5% nonfat milk, and then probed with an appropriate dilution of primary antibody at 4°C overnight. All of the primary and the secondary antibodies, including TRAF6 (1:500, catalog no. sc-7221), NF- κ B (1:500, catalog no. sc-8414), β -actin (1:500, catalog no. sc-47778), goat anti-mouse IgG-HRP (1:5,000, catalog no. sc-2005), and goat anti-rabbit IgG-HRP (1:5,000, catalog no. sc-2030), were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX). Bands were visualized using ECL Substrates (Millipore, Billerica, MA). β -Actin was used as the loading control for normalization. Western blot images were analyzed using ImageJ software (<http://imagej.nih.gov/ij/>).

ELISA for Cytokines

Cell culture supernatants from mimic- or inhibitor-transfected and LPS-stimulated bMEC were collected and stored at -80°C . Secretion of the cytokines IL-6, TNF α , and IL-8 in bMEC culture supernatants were measured by ELISA using the relative ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions (<http://en.mlbio.cn/>).

Statistical Analysis

The expression levels of mRNA and protein were assayed in triplicate. All data are expressed as mean $\pm$ standard deviation. Statistical analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, IL). Statistical significance in the data between treatment groups (mimic or inhibitor transfected) and control groups (mimic negative control or inhibitor negative control transfected) was determined by the Student's *t*-test.

P-values of <0.05 were considered to be statistically significant.

RESULTS

Bta-miR-146a Directly Targets the TRAF6 Gene

Previously, we reported that the expression of bta-miR-146a and bta-miR-146b in mammary gland tissue infected with *E. coli* and *S. aureus*, or mixed clinical bacteria were significantly upregulated in comparison to healthy cows (Wang et al., 2016b). This suggests that bta-miR-146a and bta-miR-146b participate in the inflammatory process in bovine mammary glands. Thus, we conducted a more in-depth exploration of the target genes of bta-miR-146a and bta-miR-146b influencing bovine immune responses in this study.

Using the online miRNA databases (TargetScan 7.0 and miRBase), we predicted that the 3' UTR of the *TRAF6* gene contains both bta-miR-146a and bta-miR-146b target sequences (Figure 1A). Interestingly, this was also reported to be a target of miR-146a in humans (Taganov et al., 2006).

To further investigate if the predicted binding sites of bta-miR-146a/b in the 3' UTR of *TRAF6* are responsible for the regulation, we cloned the 3' UTR of *TRAF6* (named TRAF6-wt) to the psiCHECK-2 vector (CHECK2_3'UTR-TRAF6-wt), and co-transfected CHECK2_3'UTR-TRAF6-wt and bta-miR-146a mimics (or negative control) into HEK293 cells. It was observed that in HEK293 cells co-transfected with CHECK2_3'UTR-TRAF6-wt and mimic exhibited a significant decrease in the relative luciferase activity compared with control groups (co-transfected with CHECK2_3'UTR-TRAF6-wt and NC_mimic; $P < 0.001$; Figure 1B). Moreover, when the *TRAF6* 3' UTR was mutated (TRAF6-mut), it was found that bta-miR-146a-mediated repression of luciferase activity was abolished (Figure 1B). These results indicated that bta-miR-146a directly targets 3' UTR of *TRAF6* gene, leading to an inhibition of luciferase activity.

Bta-miR-146b Does Not Directly Target the TRAF6 Gene

As described above, the 3' UTR reporter assay was performed, this time using bta-miR-146b. The results showed that the relative luciferase activities of HEK293 cells transfected with bta-miR-146b and CHECK2_3'UTR-TRAF6-wt were not significantly different compared with control groups ($P > 0.05$). Therefore, it is not believed that bta-miR-146b directly targets the *TRAF6* gene (Figure 1C).

Bta-miR-146a/b Does Not Directly Target the IRAK1 Gene

The 3' UTR of bovine *IRAK1* containing the 3 predicted binding sites of bta-miR-146a and bta-miR-146b (Figure 2A) were obtained. It has been previously reported that IRAK1_site#1 and IRAK1_site#2 had also been identified as human miR-146a targets (Taganov et al., 2006), and IRAK1_site#3 is the target of mouse miR-146a (Jiang et al., 2014). Primers for IRAK1-wt (Supplemental Table S1; <https://doi.org/10.3168/jds.2017-12630>) were used to amplify the 3' UTR of bovine *IRAK1* containing the 3 target sites mentioned above for 3' UTR luciferase reporter assays. Although human and bovine miR-146a/b, and their putative target sequences of *IRAK1*, are highly homologous, the current luciferase reporter assays (Figure 2B) indicated that bovine *IRAK1* is not a target gene of either bta-miR-146a or bta-miR-146b.

Bta-miR-146a Suppresses TRAF6 and NF- κ B Expression in bMEC

To explore the possible roles and associated molecular mechanism of bta-miR-146a in bMEC, we set out to determine whether bta-miR-146a regulates the post-transcriptional expression levels of *TRAF6* and downstream *NF- κ B* in bMEC. The qPCR analysis indicated that the bta-miR-146a mimic significantly increased the levels of mature bta-miR-146a (Figure 3A), and the bta-miR-146a inhibitor significantly decreased the level of bta-miR-146a in bMEC (Figure 3B). We then detected mRNA and protein expression of TRAF6 and NF- κ B in bMEC at 48 h posttransfection with either bta-miR-146a mimics, inhibitor, or scrambled controls. The results showed that overexpression of bta-miR-146a in bMEC significantly reduced the levels of TRAF6 and NF- κ B at both mRNA and protein level in comparison with negative controls (NC_mimic) group ($P < 0.01$; Figure 3C and 3D). Conversely, downregulation of bta-miR-146a resulted in a significant increase in both mRNA and protein expression of TRAF6 and NF- κ B ($P < 0.01$; Figure 3E and 3F). These results indicate that bta-miR-146a inhibits *TRAF6* and *NF- κ B* expression at the posttranscriptional level.

Bta-miR-146a Negatively Modulates Expression and Secretion of TNF α , IL-6, and IL-8 in LPS-Stimulated bMEC

TRAF6 and NF- κ B are involved in innate immune responses to infection. To investigate whether bta-miR-146a regulates the production of the pro-inflammatory

cytokines TNF α , IL-6, and the chemokine IL-8, which are key mediators in the animal inflammatory response, we transfected bMEC with bta-miR-146a mimic or inhibitor. Twenty-four hours posttransfection, bMEC were stimulated with 50 ng/ μ L of LPS. After 3 h, mRNA expression and secretion of TNF α , IL-6, and IL-8 were determined by qPCR and ELISA, respec-

tively. As shown in Figure 4, the bta-miR-146a mimic significantly decreased the mRNA expression and production of TNF α , IL-6, and IL-8 compared with the control group (Figure 4A, 4C, and 4E). In contrast, the bta-miR-146a inhibitor significantly increased expression of the cytokines (Figure 4B, 4D, and 4F). These data indicate that bta-miR-146a suppresses mRNA

Figure 1. Bovine miR-146a (bta-miR-146a) directly targets the tumor-necrosis factor receptor-associated factor 6 (*TRAF6*) gene by binding to its 3' untranslated region (UTR). (A) The predicted bta-miR-146a/b binding sites within 3' UTR of *TRAF6* and the experimentally introduced mutations are shown. (B) Verification of the bta-miR-146a target sites in the 3' UTR of *TRAF6* by dual-luciferase reporter assay. (C) Bta-miR-146b does not directly target the *TRAF6* gene. This was confirmed through the 3' UTR dual-luciferase reporter assay. Renilla luciferase activity was normalized to the activity of firefly luciferase in the same recombination vector. Results are mean $\pm$ SD. ** P < 0.001; NS: P > 0.05, as determined by paired 2-tailed Student's t -tests. CDS = coding sequence; NC_mimic = negative control, compared with bta-miR-146a/b mimics; TRAF6-wt = wild type of target sites in *TRAF6*; TRAF6-mut = site-directed mutagenesis of *TRAF6*; CHECK2_3'UTR = dual luciferase report recombinant vector of TRAF6-wt-3' UTR or TRAF6-mut-3' UTR and psi-CHECK-2; Rluc/Fluc = Renilla luciferase/firefly luciferase.

onstrated that expression of miR-146a is significantly increased in infected human or mouse tissues (Gao et al., 2015; Dai et al., 2016), LPS-induced cells (Taganov et al., 2006), human monocytes and macrophages (Zeng et al., 2013), RAW264.7 cells (Li et al., 2013), dendritic cells (Park et al., 2015), and keratinocytes (Meisgen et al., 2014) during inflammatory responses. In agreement with these findings, our previous work found that expression of bta-miR-146a was increased significantly in mammary glands challenged with *S. aureus* or *E. coli* compared with the glands of healthy cows (Wang et al., 2016b). However, Naeem et al. (2012) reported that the expression of bta-miR-146a did not change in high-dose *S. uberis*-infected mammary tissue, as compared with the expression in the mammary glands of healthy cows. The results of the above studies indicated that

DISCUSSION

In humans and mice, miR-146a has been shown to be a key gene involved in host immunity. Studies have dem-

Figure 3. Bovine miR-146a (bta-miR-146a) suppresses the expression of the tumor-necrosis factor receptor-associated factor 6 (*TRAF6*) and nuclear factor (NF)-κB in bovine mammary epithelial cells (bMEC). The bMEC were transfected with bta-miR-146a mimic or negative control mimic (NC_mimic), or either inhibitor or negative control inhibitor (NC_inhibitor). At 48 h posttransfection, quantitative real-time PCR (qPCR) and Western blot were used to measure the expression levels of genes and protein, respectively. (A) The transfection efficiency of bta-miR-146a mimic as measured by qPCR. (B) The transfection efficiency of bta-miR-146a inhibitor as measured by qPCR. (C) qPCR analysis of *TRAF6* and *NF-κB* in bMEC transfected with bta-miR-146a mimic. (D) Western blot analysis of *TRAF6* and *NF-κB* in bMEC transfected with bta-miR-146a mimic. (E) qPCR analysis of *TRAF6* and *NF-κB* in bMEC transfected with bta-miR-146a inhibitor. (F) Western blot analysis of *TRAF6* and *NF-κB* in bMEC transfected with bta-miR-146a inhibitor. Western blot images were analyzed using the ImageJ software (<http://imagej.nih.gov/ij/>), and densitometry quantification of Western blots were normalized to β-actin. Data are presented as means ± SD. Statistically significant differences are indicated: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All experiments were repeated 3 times.

Figure 4. Bovine miR-146a (bta-miR-146a) suppresses the production of tumor necrosis factor (TNF) α , IL-6, and IL-8 in bovine mammary epithelial cells (bMEC). The bMEC were transfected with bta-miR-146a mimic or scrambled control mimic (NC_mimic); bta-miR-146a inhibitor or scrambled control inhibitor (NC_inhibitor). Twenty-four hours posttransfection, bMEC were challenged with LPS for 3 h. Cells were then collected to measure mRNA expression and production of TNF α (A, B), IL-6 (C, D), and IL-8 (E, F). Levels of mRNA expression and production of inflammatory cytokines were determined by quantitative real-time PCR (qPCR) and ELISA, respectively. Data are shown as mean $\pm$ SEM of 3 independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001.

the expression of bta-miR-146a in the bovine mammary gland might be affected by multiple factors, such as bacterial type, bacterial dose, and infection time. Taken together, this suggests that the molecular mechanism of bta-miR-146a in bovine mastitis is highly complex and tightly regulated.

Regulation of gene expression by miRNA occurs through specific binding to the target gene that contains the complementary nucleotide sequence. It has been demonstrated that miR-146a inhibits the expression of *IRAK1* and *TRAF6* in humans and mice by directly targeting their 3' UTR (Taganov et al., 2006; Dai et al., 2016). TRAF6 and IRAK1 are key adaptor molecules in the TLR/IL-1 receptor signaling pathway, resulting in the mediation of NF- κ B signaling pathway activation in mammals. Notably, the sequences of miR-146a, *TRAF6*, and *IRAK1* genes between bovine and human are highly homologous, but not identical (Figure 1A and 2A). Although miR-146a has been recognized to function in regulation expression of the *TRAF6* and *IRAK1* genes in human and mouse (Taganov et al., 2006; Dai et al., 2016), the mechanistic role of bta-miR-146a underlying bovine mammary inflammation remains unclear. In this study, we predicted multiple putative bta-miR-146a binding sites within *IRAK1* and *TRAF6*, and performed 3' UTR luciferase reporter assays. Our results indicated that bta-miR-146a directly targets the *TRAF6* gene, a well-known mediator for NF- κ B activation. However, we did not find any evidence indicating that bta-miR-146a targets the *IRAK1* gene. Furthermore, our data indicated that bta-miR-146a mediated suppression of TRAF6 expression inhibits NF- κ B activity. This finding is in agreement with previous studies in humans and mice (Taganov et al., 2006; Sha et al., 2013; Gao et al., 2015; Park et al., 2015). In the case of bta-miR-146b, we were only able to demonstrate that bta-miR-146b does not directly target the *TRAF6* and *IRAK1* genes. Further screening is required to elucidate target genes against which bta-miR-146b acts.

The TRAF6 functions as an adaptor molecule in the NF- κ B pathway, and plays a crucial role in the initiation of the inflammatory response. This induction occurs through the phosphorylation of I κ B, which results in activation of NF- κ B, an important transcription factor that regulates expression and secretion of inflammatory cytokines (Zhang and Ghosh, 2001; Griesbeck-Zilch et al., 2008; Fu et al., 2013). Interestingly, activated NF- κ B increases miR-146a expression by binding its promoter (Taganov et al., 2006). Once induced by NF- κ B, increased expression of bta-miR-146a prevents the production of TRAF6 and NF- κ B. Thus, we propose that expression of bta-miR-146a and

Figure 5. Schematic summary of the roles of bovine miR-146a (bta-miR-146a) in bovine mammary epithelial cells (bMEC). In response to LPS or *Escherichia coli* in bMEC, the toll-like receptor (TLR) 4 signaling pathway is activated, as is the tumor-necrosis factor receptor-associated factor 6 (TRAF6), which leads to the activation of the transcription factor nuclear factor (NF)- κ B. Activated NF- κ B subsequently results in transcription of tumor necrosis factor (TNF) α , IL-6, and IL-8, as well as bta-miR-146a. The substantial increase in bta-miR-146a expression downregulates the expression of *TRAF6* in a negative-feedback cycle, leading to a suppression of TNF α , IL-6, and IL-8.

its targeted degradation of *TRAF6* mRNA functions as a negative feedback loop to control the expression of NF- κ B, which is necessary for expression of inflammatory cytokines IL-6, TNF α , and IL-8 (Figure 5).

Once the pathogen enters the udder, bMEC respond to bacterial invasion and play an important role in the initiation of the antibacterial response (Rainard and Riollot, 2003). It has been demonstrated that heat-inactivated *E. coli* or LPS activates NF- κ B via the TLR4/NF- κ B signaling pathway. In response to activation of the TLR signaling pathway, NF- κ B binding to the promoters of inflammatory cytokine genes results in increased expression of the cytokines TNF α , IL-6, IL-1, and IL-8 in bMEC at both mRNA and protein levels within 1 to 3 h of activation (Fu et al., 2013; Liu et al., 2014; Wang et al., 2016a). The TNF α and IL-6 are major pro-inflammatory cytokines that mediate the inflammatory response, especially IL-6, which plays an important role in the acute-phase response of inflammation (Le and Vilcek, 1989; Fu et al., 2013). Interleukin-8 is an important chemokine that recruits neutrophils and T-lymphocytes to the infection site, and is also involved in the activation of neutrophils

(Harada et al., 1994; Strandberg et al., 2005). To clarify whether bta-miR-146a regulates the expression of bovine inflammatory cytokines, we used LPS-stimulated bMEC as a model for the process of inflammation. Our results demonstrated that bta-miR-146a can inhibit mRNA expression and secretion of TNF α , IL-6, and IL-8. The data presented here are indeed consistent with several previous reports in humans and mice (Li et al., 2013; Meisgen et al., 2014; Park et al., 2015).

In conclusion, we observed that bta-miR-146a down-regulates *TRAF6* and *NF- κ B* expression posttranscriptionally by directly targeting the 3' UTR of *TRAF6*, consequently suppressing the production of the inflammatory mediators TNF α , IL-6, and IL-8. This finding suggests that bta-miR-146a acts as a negative feedback regulator of inflammation through the downregulation of the TLR4/TRAF6/NF- κ B pathway. This study presents a regulatory mechanism of bta-miR-146a on immune responses of bovine mammary infection, and may also provide a potential therapeutic target for mastitis.

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