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MicroRNA-214 regulates immunity-related genes in bovine mammary epithelial cells by targeting NFATc3 and TRAF3

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ABSTRACT

In human, microRNA-214 (miR-214) plays crucial roles in mechanisms of immunity. However, the potential importance of miR-214 in immune mechanisms in dairy cows has not been investigated. In this study, we assessed potential immunity-related functions of miR-214 in human 293A cells and in bovine mammary epithelial cells (BMECs). We found that NFATc3 and TRAF3 could be targeted by miR-214 in both 293A cells and BMECs. We also found that miR-214 indirectly inhibited the expression of MAP3K14, TBK1 and inflammatory cytokines IL-6 and IL-1β. Taken together, our data revealed miR-214 regulated immunity-related genes by targeting NFATc3 and TRAF3, which provides insight into the molecular basis of immunity.

1. Introduction

A diversity of microRNAs (miRNAs) negatively regulate gene expression through directly binding with the target mRNA. miRNAs can inhibit transcription or translation of target mRNAs. Numerous recent studies have shown that mammary gland tissue expressed a diversity of miRNAs that played an important role in immune system [1–3]. For example, miR-296, miR-2430 and miR-671 regulated expression of the BoLA-DQA2 gene; miR-17-5p, miR-20b and miR-93 limited expression of the HMGB3 gene in mammary gland tissue [4,5]. In addition, single nucleotide polymorphisms (SNPs) within the HMGB1 and HSF1 transcribed region that affect the miRNA binding site were associated with gene expression [6]. Furthermore, a large set of miRNAs, including miR-214, were found to be differentially expressed in peripheral blood from mastitic cows [7].

In zebrafish, miR-214 was expressed during early segmentation, and inhibition of miR-214 resulted in a loss of slow-muscle cell types [8]. In human embryonic stem (ES) cells, miR-214-mediated enhancer of zeste 2 (Ezh2) protein accelerated skeletal muscle cell differentiation and led to ectopic expression of developmental regulatory genes [9]. Deregulation of miR-214 was a recurrent event in human ovarian cancer, where it was associated with cell survival and cisplatin resistance through targeting the PTEN/Akt pathway [10]. Moreover, miR-214 was also involved in the immune and inflammatory response. MiR-214 downregulated the expression of lactoferrin, thus promoting apoptosis in human mammary epithelial cells (HMECs) [11]; MiR-214 also regulated expression of the BAX gene and inhibited apoptosis in nasopharyngeal carcinoma cells [12]. When miR-214 combined with receptors of TLR2, TLR3, TLR4 and TLR9 in peripheral blood mononuclear cells (PBMCs), its expression was significantly increased [13]. This suggested that miR-214 participated in the TLRs signaling pathway, thus affected the immunity of pathogen infection. MiR-214 was associated with the immune response in HMECs [14].

In order to clarify the function of miR-214 in bovine immunity, target genes were predicted and characterized through a dual luciferase reporter assay in 293A cells. Additionally, the potential target genes of miR-214 and immunity-related genes were analyzed by qRT-PCR in BMECs.

2. Materials and methods

2.1. 3′UTR-psiCHECK-2 construction

Target genes of bovine miR-214 were identified by TargetScan in

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Table 1
Prediction of target genes of bovine miR-214 on immunity.

Gene	Possible role location of gene and miR-214
TOLLIP	Position 608-614 of TOLLIP 3' UTR 5' ...CCUUUGUUAUGGACGCCUGCUGC...
	bta-miR-214 3' UGACGGACAGACACGGACGACA
	Position 668-674 of TOLLIP 3' UTR 5' ...AAGACGUCCGAUGGCCCUGCUGU...
	bta-miR-214 3' UGACGGACAGACACGGACGACA
TRAF3	Position 1825-1831 of TRAF3 3' UTR 5' ...GCCUUCGCCCCAGUGCCUGCUGG...
	bta-miR-214 3' UGACGGACAGACACGGACGACA
IGDCC3	Position 337-343 of IGDCC3 3' UTR 5' ...CUCCAAAAAUAUGCCCCUGCUGU...
	bta-miR-214 3' UGACGGACAGACACGGACGACA
NFATc3	Position 1375-1381 of NFATC3 3' UTR 5' ...AGCAAGAAACUCCAGCCUGCUGU...
	bta-miR-214 3' UGACGGACAGACACGGACGACA
NFATc4	Position 393-400 of NFATC4 3' UTR 5' ...ACACCCACUUCCGGCCCUGCUGA...
	bta-miR-214 3' UGACGGACAGACACGGACGACA
IL17RD	Position 4110-4117 of IL17RD 3' UTR 5' ...GCUUCUGUAACCUCCCCUGCUGA...
	bta-miR-214 3' UGACGGACAGACAC--GGACGACA

Table 1 (http://www.targetscan.org) [15]. The 3'-untranslated regions (3′-UTRs) of bovine TOLLIP, IGDCC3, NFATc3, NFATc4, TRAF3 and IL17RD containing the predicted miR-214 binding sites were amplified by PCR using the oligonucletied primers listed in Table 2. PCR products were cloned into the restriction enzyme XhoI and NotI sites of the psiCHECK-2 plasmid (Promega, Madison, WI, USA). Here, TRAF3, IGDCC3 and NFATc3 were randomly selected to make XhoI and NotI double enzymes digestion verification. All constructs were verified by sequencing.

2.2. 293A cells culture and transfections

The 293A cells were obtained from American Type Culture Collection (ATCC) and cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Beijing, China) supplemented with 10% fetal bovine serum (FBS, Hyclone, Beijing, China). MiR-214 mimics (miR-214-mim) and negative control (NC-mim) were purchased from Ribobio Biotech company (Guangdong, China). Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere.

For transfection experiments, 293A cells were plated in DMEM medium without antibiotics when they were 50–60% confluent (24-well plates, at the density of 1×10^5/well). Transfection was carried out with X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Co-transfected utilized 50 nM miR-214-mim (or NC-mim) and 500 ng 3′UTR-psiCHECK-2 vectors. The cells were treated for further experiments 48 h after transfection.

Table 2
Primers used to amplify potential target genes' 3′ UTR of bovine miR-214.

Primer	Sequence (5′→3′)	Tm (°C)	Size (bp)
TOLLIP	F: CTCGAGCCTCCACGTAGGCTTAATGG	62	552
	R:GCGGCCGCCTCCGACACCAAGCAACGAC		
IGDCC3	F: CTCGAGCTGTCCTGACCGATCCTC	63	330
	R: GCGGCCGCTAGGTGCTGAAAGCCAAA		
NFATc3	F: CTCGAGTCAGAAGCCAAGCACAAA	63	391
	R: GCGGCCGCAACAGGAAACTAATCCCACT'		
NFATc4	F: CTCGAGGGCTGGAGTTTGGCTTTC	61	543
	R: GCGGCCGCCTGCACTGGCTGGCTTCT		
TRAF3	F: CTCGAGTGTGCCGCTCGTCCAAGA	56	481
	R: GCGGCCGCAGTGAAGCAGGAGGAAGTGGC		
IL17RD	F: CTCGAGTCATGGAGTTGGGTCATC	54	528
	R: GCGGCCGCAGGCAGCACATTCTATTT'		

2.3. Luciferase reporter assay

Luciferase activity assay were performed using the Dual-Luciferase® Reporter Assay System (Promega) according to the instructions. Transfected 293A cells were lysed in Passive Lysis Buffer. Cell lysate (20 μL) was distributed into a 96-well enzyme label plate and optical density (OD) reading was initiated by the injection of 50 μL of Luciferase Assay Reagent on a M200 Pro (Tecan, Mannedorf, Switzerland). Results were presented as normalized relative luciferase activity (Firefly/Renilla, OD/OD). Each experiment was repeated at least three times.

2.4. BMECs culture and identification

The primary BMECs (passage of 4) were purchased from Tongpai Biotech company (Shanghai, China) and stored at the laboratory of National Beef Cattle Improvement Center. BMECs were cultured in DMEM/F12 medium (Gibco, Beijing, China) supplemented with 100U/mL penicillin/streptomycin (Harbin Pharmaceutical Group, Harbin, China), 10 μg/mL bovine insulin (Sigma-Aldrich, St. Louis, MO), 10 ng/mL epidermal growth factor (Sigma-Aldrich), 5 μg/mL hydrocortisone (Sigma-Aldrich), 2 μg/mL prolactin (Sigma-Aldrich) and 10% FBS. To ensure the reproducibility, BMECs were only used between passages 8 and 12.

The expression of epithelial marker, cytokeratin 18, in BMECs was identified by immunofluorescence analysis [16–18]. Cells were seeded in 12-well plates and cultured until they reached 80–90% confluent. The cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS), fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 min, and treated with 1% Triton X-100 (Sigma-Aldrich) for 5 min. The cells were subsequently blocked at 37 °C for 1 h in 1% goat serum (Boster, Wuhan, China). The cells were incubated overnight with the primary antibody for cytokeratin 18 (1:500, Santa Cruz, Dallas, TX, USA). The cells were washed three times for 5 min each with DPBS and then incubated with secondary antibody, Cy3-conjugated monoclonal goat anti-mouse IgG (1:200, Beyotime, Shanghai, China), for 1 h in the dark. Lastly, the cells were washed three times and visualized with a fluorescent phase-contrast microscope (Olympus, IX71, Japan).