Endometrial epithelial cell apoptosis is inhibited via a ciR8073-miR181a-neurotensin pathway during embryo implantation

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ABSTRACT

Development of receptive endometrium (RE) from pre-receptive endometrium (PE) is essential for embryo implantation, but the molecular mechanisms are not fully understood. In this study, lncRNA/circRNA-miRNA-mRNA networks were constructed to explore the functions of potential competing endogenous RNAs (ceRNA) during the development of RE in dairy goats. We observed that circRNA8073 (ciR8073) decreased levels of miR-181a by acting as a miRNA sponge. This effect indirectly increased expression of neurotensin in endometrial epithelial cells (EECs). Neurotensin in turn inhibited EEC apoptosis by increasing expression of BCL-2/BAX via the MAPK pathway, and also increased expression leukemia-inhibitory factor, cyclo-oxygenase 2, vascular endothelial growth factor A and homeobox A10. We have thus identified a ciR8073-miR181a-neurotensin pathway in the endometrium of dairy goats. Via this pathway, ciR8073 functions as a ceRNA that sequesters miR-181a, thereby protecting neurotensin transcripts from miR-181a-mediated suppression in EECs.

INTRODUCTION

The establishment of endometrial receptivity is known as the “window of implantation (WOI)”, which is a spatially and temporally restricted stage [1]. During this period, the endometrium undergoes pronounced structural and functional changes that prepare it to be receptive to adhesion by the qualified embryo [2, 3]. An abnormal receptive endometrium (RE) is one of the major reasons for the failure of embryo transplantation during assisted reproduction with good-quality embryos [4]. Notably, many studies have reported that attaining endometrial receptivity is a complex process involving numerous molecular mediators [1].

It has been speculated that non-coding (nc) RNAs serve as key regulators of gene expression under physiological and pathological conditions [5, 6]. These include miRNAs, lncRNAs and circRNAs (a class of RNAs derived mostly from non-canonical splicing in which the exon ends are joined to form a loop

[7, 8]. ncRNAs are reportedly crucial for a variety of physiological processes, including the establishment of endometrial receptivity [9–11]. A comprehensive forecasting and analysis of the ncRNAs underlying the progression of RE is essential for the development of effective strategies to develop the endometrium receptivity and improve the implantation rate.

LncRNAs and circRNAs contain miRNA response elements (MREs) and compete with mRNAs for MREs. This enables them to act as molecular sponges for miRNAs, and ultimately de-repress miRNA target genes [12] to influence the post-transcriptional regulation. An appealing aspect of the ceRNAs hypothesis provides a pathway for predicting a non-coding function of any uncharacterized RNA transcript by the identification of putative miRNA binding sites. This hypothesis is now supported by experimental evidence for an accumulating number of lncRNAs [13–15] and circRNAs [16, 17]. For example, lncRNA-MIAT effectively decreases expression of miR-150-5p and target gene

VEGF [13]; lncRNA-CCAT1 increases Bmi1 expression by competitively sponging miRNA-218-5p [14]; and lncRNA-MD1 acts as a miR-133 and miR-135 sponge [15]. Although the function of most circRNAs remains elusive, at least three circRNAs have been experimentally shown to act as ceRNAs: ciRS-7 (also known as CDR1-AS) inhibits miR-7 activity in the central nervous system [17], an Sry-derived circRNA acts as a sponge for miR-138 [18], and ciR-ITCH controls the level of itchy E3 ubiquitin protein ligase by sponging miR-7, miR-17 and miR-214 in oesophageal squamous cell carcinoma [19].

The results outlined above suggest a better understanding the ceRNA could provide new insight into the mechanisms of RE. Our findings will help us to better understand the molecular regulation of RE, and may provide essential information in support of further research on the development of endometrial receptivity.

RESULTS

Differentially expressed miRNA (DEmiR), mRNA (DEmR), lncRNA (DElncR), and circRNA (DEciR) between the RE and PE in dairy goat

In our previous study, a total of 145 DEmiRs meeting the designated criteria of “ P -values < 0.05 and fold change > 2” (Supplementary File 1), including 111 up-regulated and 34 down-regulated DEmiRs in RE compared with PE [10]. Moreover, miR-181a had the high expression level and the level decreased 0.38-fold in RE compare with PE.

And 810 mRNAs were found to differ significantly between the PE and RE, the full list of DEmR was provided in Supplementary File 2 meeting the designated

criteria of “ P -values < 0.05 and fold change > 2”. *NTS* mRNA levels decreased 6.08-fold in RE compared with PE in dairy goats, but this result was inconsistent with the previous what reported that *NTS* remarkably increased in RE [20]. What’s more, a total of 668 DElncRs were found and the full list was provided in Supplementary File 3, and TCONS_00376915 (lncR915) was significantly higher in RE compared with PE. There were 334 DEciRs (Supplementary File 4), 77 DEciRs increased and 257 DEciRs down-regulated in the RE compared with PE. It deserved to note that ciR8073 was expressed specifically in the RE with a higher expression level.

miRNAs-binding sites prediction and ceRNA network construction

To examine the molecular mechanism of ncRNAs involved in the development of RE, all possible interactions of each DEmiR with all DEmRs (Supplementary File 5), DElncRs (Supplementary File 6) and DEciRs (Supplementary File 7) were analyzed using Targetscan and miRanda. As a result, *NTS*, lncRNA915 (lncR915) and ciR8073 were predicted as a target of miR-181a.

Based on the theory of ceRNA, the DEmiR–DEmR, DEmiR–DEciR and DEmiR–DElncR pairs were used to construct the ceRNA networks. As shown in Supplementary File 8, 144 DEmiRs shared 239,385 MREs with 305 DEmRs, 105 DEciRs and 887 DElncRs in the ceRNA network. The ceRNA networks were visualized by importing the above interactions into the Cytoscape software to assemble the regulation network. For example, miR-181a shared MREs with 24 DEmRs, 105 DEciRs and 16 DElncRs in the ceRNA network (Figure 1A, Supplementary File 9). It deserved to note that ciR8073

Figure 1: The ceRNA network centered on miR-181a and ciR8073 in endometrium of dairy goats. Note: (A) The differentially expressed targets of miR-181a; (B) the ceRNA network centered on ciR8073. The red hexagon represented ciR8073, and yellow quadrangle represented *NTS*. The green triangle represented miRNAs; red circle represented circRNAs; blue circle represented lncRNAs; pink circle represented circRNAs.

shared MREs with 9 DEmiRs, including miR-181a (Figure 1B, Supplementary File 10). Indeed, as previously described above, the regulatory role of ncRNAs in RE was very complicated such that an in-depth study should be implemented in the future.

To explore the function of the ceRNA network, the interrelated genes within the network were imported to the GO terms and KEGG pathways. Based on GO enrichment analysis, 755 GO functions were found to be the significant component relating the genes in the network, involved in processes such as regulation of cell growth (GO:0001558), cell differentiation (GO:0030154), multicellular organismal development (GO:0007275), cell-cell signaling (GO:0007267), apoptosis (GO:0006915), focal adhesion (GO:0005925), metalloproteinase activity (GO:0008237) (Supplementary File 11, Supplementary Figure 1A). Functional pathways analysis demonstrated that the ceRNA network potentially modulated multiple signaling pathways, such as Focal adhesion (ko04510), mTOR signaling pathway (ko04150), Insulin signaling pathway (ko04910), Neurotrophin signaling pathway (ko04722), Apoptosis (ko04210) (Supplementary File 12, Supplementary Figure 1B).

miR-181a and NTS were differentially expressed in the PE and RE

Stem-loop qRT-PCR was used to validate the expression levels of miR-181a in various tissues of dairy goats, and the results showed it was widely expressed in dairy goats (Figure 2A). The highest expression level was

observed in the ovary, followed (in order) by the spleen, oviduct, liver, longissimus dorsi, kidney, lungs, heart and hypothalamus. In the endometrium of dairy goats, the expression level of miR-181a at D15 was lower than that at D5 (Figure 2B). The miRNA in situ hybridization (MISH) assay further showed miR-181a was significantly decreased in RE compared with PE (Table 1), especially in the EECs (Figure 2C-2E).

NTS widely expressed in a series of tissues, and the lowest expression levels were observed in the ovary, increased (in order) by the oviduct, lung, liver, spleen, kidney, longissimus dorsi, hypothalamus, and heart (Figure 3A). What's more, the expression level of *NTS* increased 195-fold in the endometrium in RE compared to PE (Figure 3B), which was in accordance with the previous sequencing data [20]. Immunohistochemistry (IHC) results further showed *NTS* protein increased in RE (Table 2), especially in the EECs (Figure 3C-3F).

The levels of miR-181a and NTS mRNA were response to estrogen (E2) and progesterone (P4) in EECs *in vitro*

To investigate the response of miR-181a and *NTS* levels to sex hormones in EECs *in vitro*, E2 and P4 were diluted in cell medium to different concentrations. The results showed that miR-181a levels were significantly and concentration-dependently decreased in the presence of E2 alone, and enhanced in the presence of P4 alone in EECs (Figure 4A). And the highest levels appeared in 20 ng/ml P4 alone, and lowest level appeared in 100 pg/ml

Figure 2: Expression levels of miR-181a in dairy goats. Note: (A) The miR-26a was expressed in various tissues of dairy goats; (B) the miR-181a levels in endometrium in PE and PE; The miR-181a levels were measured by Stem-loop RT-qPCR and normalized to U6, the values were showed as “means $\pm$ SD” (n = 3), ** indicates that p-value < 0.01 and * indicates that p-value < 0.05. (C) the NC for in situ hybridization; the miR-181a in the endometrium in PE (D) and RE (E) was detected by in situ hybridization; UC: Uterine cavity; LE: luminal epithelium; GE: glandular epithelium; SC: stroma cell; original magnification $\times 200$; the ruler was 100 μ m.

Table 1: The MISH staining results of miR-181a in uterus of dairy goats

Index	Area		Density		IOD	
	Mean	Sum	Mean	Sum	Mean	Sum
PE (D5)	758.416	1623011	0.156	333.794	210.80	451112.63
RE (D15)	160.026*	189631**	0.204	242.259	33.922**	40197.79*

Note: MISH was miRNA in situ hybridization, IOD was integrated optical density; * $p < 0.05$, ** $p < 0.01$.

E2 alone, suggesting that E2 decreased but P4 increased the miR-181a levels in EECs *in vitro*. What's more, the *NTS* mRNA levels were detected, and the results showed that E2 and P4 also regulated the *NTS* mRNA levels but without obvious regularity in EECs *in vitro*, and the highest levels appeared in a combination of 100 pg/ml E2 and 20 ng/ml P4 (Figure 4B).

miR-181a directly decreased *NTS* expression in EECs *in vitro*

Transfection with miR-181a mimics successfully led to a greater than 168-fold increase, at the same time, miR-181a inhibitors led to 0.39-fold decrease in EECs (Supplementary Figure 2A). To determine whether miR-181a directly targets goat *NTS* through the predicted

binding sites in the *NTS* 3S untranslated region (UTR), the psiCHECK™-2 reporter and mutated plasmids were constructed (Figure 5A). And the luciferase activity of the miR-181a group was significantly lower than that of the NC group ($p < 0.01$), but these reduction did not occur with the mutated plasmid (Figure 5B).

The *NTS* mRNA levels decreased in EECs after transfection with miR-181a mimics ($p < 0.05$), meanwhile, miR-181a inhibitors increased the mRNA levels ($p < 0.01$, Figure 5C) in EECs *in vitro*. *NTS* protein levels were evaluated by performing western blot (WB) analysis, and the quantification of the amount of protein expression was depicted in Figure 5D, what showed the *NTS* protein significantly decreased in miR-181a-transfected cells compared with NC ($p < 0.05$, Figure 5D). Additionally, miR-181a inhibitors significantly

Figure 3: The expression levels of *NTS* in dairy goats. Note: (A) The *NTS* was expressed in various tissues of dairy goats; (B) the *NTS* levels in endometrium in PE and PE; The *NTS* levels were measured by RT-qPCR and normalized to GAPDH, the values were showed as “means $\pm$ SD” ($n = 3$), ** indicates that p -value < 0.01 and * indicates that p -value < 0.05 . (C, D) the NC for Immunohistochemical staining; *NTS* levels were detected by Immunohistochemical staining in the endometrium in PE (E) and PE (F); LE, luminal epithelium; GE, glandular epithelium; SC, stroma cell. original magnification $\times 100$; the ruler was 50 μ m.

Table 2: The ISH staining results of NTS in uterus of dairy goats

Index	Area		Density		IOD	
	Mean	Sum	Mean	Sum	Mean	Sum
PE (D5)	76.71	181276	0.296	699.196	21.410	50592.813
RE (D15)	72.699	151650	0.432*	900.430	32.879*	68585.008*

Note: ISH was in situ hybridization, IOD was integrated optical density; *p < 0.05.

Figure 4: The effect of E2 and P4 on the expression levels of miR-181a and NTS in EECs. Note: (A) the levels of miR-181a; (B) the levels of NTS mRNA. The values were showed as “means ± SD” (n = 3).

Figure 5: miR-181a down-regulated the expression level of NTS via the 3' UTR. Note: (A) Schematic diagram illustrating the design of luciferase reporters with the WT- NTS 3' UTR (Wt-NTS) or the site-directed mutant NTS 3' UTR (Mu-NTS). The nucleotides in red represented “seed sequence” of miR-181a, the mutation nucleotides were in gray color. (B) The NTS-3' UTR luciferase reporter vectors were co-transfected with miR-181a mimic (or negative control) into 293T cells, luciferase assay was performed 24 h after transfection. (C) miR-181a down-regulated the NTS mRNA levels in EEC; NTS mRNA levels were measured by RT-qPCR and normalized to GAPDH. (D) NTS protein levels in EECs were measured by WB, the densitometry was normalized to the β-actin density from the same lane. Each experiment was repeated three times in triplicate, the results are represented as mean ± SD (n = 3); *p < 0.05, ** p < 0.01.

increased the *NTS* protein in EECs ($p < 0.05$, Figure 5D). Thus, these results suggested that goat *NTS* was a target of miR-181a in EECs, what were consistent with our speculation.

miR-181a did not regulate lncR915 in EECs *in vitro*

Transfection with pcDNA3.1(+)-lncR915 successfully led to a 89-fold increase of lncR915 in EECs *in vitro* ($p < 0.01$, Supplementary Figure 2B). The psiCHECK™-2 reporter and mutated plasmids were constructed (Figure 6A), and the luciferase activity of the miR-181a mimics and inhibitors groups did not significantly change compared to the NC group ($p > 0.05$, Figure 6B). And there was no significant change on the expression levels of lncR915 (Figure 6C) in EECs what were treated with miR-181a mimics and inhibitors. In addition, lncR915 did not change the miR-181a (Figure 6D) and *NTS* mRNA levels (Figure 6E) in EECs. Thus, these results primarily suggested that miR-181a did not target-regulate lncR915 in the EECs of dairy goats.

ciR8073 served as miR-181a sponge in EECs *in vitro*

Transfection with pcDNA3.1(+)-ciR8073 successfully led to a 27.57-fold increase of ciR8073 compared with NC in EECs *in vitro* ($p < 0.01$, Supplementary Figure 2C). To determine whether miR-

181a directly targets ciR8073 through the predicted binding sites, the psiCHECK™-2 reporter and mutated plasmids were constructed (Figure 7A), and the luciferase activity of the miR-181a mimics group was significantly lower than that of the NC group ($p < 0.01$), and these reductions did not occur with the mutated plasmids ($p > 0.05$, Figure 7B).

Further study indicated that the ciR8073 level decreased significantly when the cells were treated with miR-181a mimics (Figure 7C), and the level also was also decreased by ciR8073 in EECs (Figure 7D). On the contrary, the *NTS* mRNA significantly increased when the ciR8073 was over-expressed and decreased when ciR8073 was mutated in EECs *in vitro* (Figure 7E). What's more, the expression levels of *NTS* were increased by ciR8073, and this increase was counteracted by miR-181a at both the mRNA and protein levels (Figure 7E-7G). Thus, these results suggested that miR-181a targeted ciR8073 what were consistent with our speculation, and verified the accuracy of the network interaction of ciR8073-miR-181a-*NTS*.

miR-181a promoted cell apoptosis of EECs *in vitro*

Methyl thiazolyl tetrazolium (MTT) assays showed that miR-181a mimics inhibited the proliferation of EECs (Figure 8A, $p < 0.05$), meanwhile, the miR-181a inhibitor promoted the proliferation of EECs ($p < 0.05$). Flow cytometry (FCM) was used to analyze the changes in the

Figure 6: lncR915 was not a target of miR-181a in EECs. Note: (A) Schematic diagram illustrating the design of luciferase reporters with the Wt-lncR915 or the site-directed mutant (Mu-lncR915). The nucleotides in red represented “seed sequence” of miR-181a, the mutation nucleotides were in gray color. (B) The lncR915 luciferase reporter vectors were co-transfected with miR-181a mimic (or negative control) into 293T cells, luciferase assay was performed 24 h after transfection. (C) miR-181a did not regulate the lncR915 levels in EECs; lncR915 levels were measured by RT-qPCR and normalized to GAPDH. (D) lncR915 did not regulate the miR-181a levels in EECs. (E) lncR915 did not regulate the *NTS* mRNA levels in EECs. Each experiment was repeated three times in triplicate, the results are represented as mean $\pm$ SD ($n = 3$).

cell cycle, and the results showed that the numbers of cells in S-phase was higher in the miR-181a mimics treated EECs than in NC (Figure 8B, Supplementary Figure 3). Furthermore, an Annexin V-FITC/PI assay combined with FCM was used to detect the effects of miR-181a on cell apoptosis, and the results showed that miR-181a obviously induced the apoptosis of EECs ($p < 0.01$, Figure 8C), meanwhile, the miR-181a inhibitor inhibited the apoptosis ($p < 0.05$, Supplementary Figure 4).

The BCL-2 expression levels were decreased significantly by miR-181a mimics and increased significantly by inhibitors ($p < 0.05$, Figure 8D). On the contrary, miR-181a mimics increased and inhibitors decreased BAX protein levels ($p < 0.05$, Figure 8D). What's more, miR-181a mimics facilitated the phosphorylation JNK (p-JNK) and increased the levels of P27, miR-181a inhibitors decreased the levels of P27 and PI3K ($p < 0.05$, Figure 8D).

In addition, the changes in LIF, COX2, VEGFA, HOXA10 and osteopontin (OPN) protein levels were

investigated in endometrial cells after they were treated with miR-181a mimics or inhibitors. The LIF and COX2 protein levels increased in miR-181a mimics-treated EECs ($p < 0.05$), and decreased in the inhibitors group ($p < 0.05$, Figure 8D). However, the opposite results were detected on the OPN protein levels, which suggested miR-181a inhibited the OPN in EECs *in vitro* ($p < 0.05$, Figure 8D).

NTS inhibited cell apoptosis of EECs *in vitro*

To further investigate the function of NTS in EECs, an overexpression vector [pcDNA3.1(+)-NTS] was constructed and siRNA-NTS was synthesized, and NTS was overexpressed or inhibited successfully in EECs *in vitro* (Supplementary Figure 5). In addition, the levels of miR-181a decreased by NTS, and increased by siRNA-NTS (Supplementary Figure 6), suggested a negative relationship between NTS and miR-181a in EECs.

MTT assay showed that over-expressed NTS markedly promoted ($p < 0.05$, Figure 9A) and siRNA-

Figure 7: ciR8073 could decrease the levels of miR-181a served as a miRNA sponge in EECs. Note: (A) Schematic diagram illustrating the design of luciferase reporters with the Wt-ciR8073 or the site-directed mutant (Mu-ciR8073). The nucleotides in red represented "seed sequence" of miR-181a, the mutation nucleotides were in gray color. (B) The ciR8073 or its mutation luciferase reporter vectors were co-transfected with miR-181a mimic (or negative control) into 293T cells, luciferase assay was performed 24 h after transfection. (C) miR-181a decreased the ciR8073 levels in EECs, the levels were measured by RT-qPCR and normalized to GAPDH. (D) The miR-181a levels in EECs what were overexpressed or mutational ciR8073 (Mu-ciR8073). (E) The NTS mRNA levels in EECs what were overexpressed or mutational ciR8073 (Mu-ciR8073). (F) The NTS mRNA levels in EECs what were overexpressed ciR8073 and miR-181a. (G) NTS protein levels in EECs what were overexpressed ciR8073 and miR-181a, the densitometry was normalized to the β-actin density from the same lane. Each experiment was repeated three times in triplicate, * $p < 0.05$, ** $p < 0.01$.

Figure 8: miR-181a promoted EECs apoptosis *in vitro*. Note: (A) The effect of miR-181a on the proliferation of EECs what were measured by MTT. Values were showed as “means ± SD” (n = 7). The cell cycle (B) and apoptosis (C) analysis of EECs what were detected with FCM. (D) The levels of BCL-2, BAX, MAPK pathway proteins, and endometrial receptivity markers (LIF, COX2, VEGFA, HOXA10 and OPN) were measured by WB after the EECs were treated with miR-181a. Densitometry was normalized to the β-actin density from the same lane, ** indicates that p-value < 0.01 and * p < 0.05.

NTS significantly inhibited EECs proliferation ($p < 0.05$, Figure 9D). No notably change was observed in the analysis of cell cycle ($p > 0.05$, Figure 9B, 9E), but over-expressed inhibited ($p < 0.05$, Figure 9C) and siRNA-NTS dramatically induced EECs apoptosis ($p < 0.05$, Figure 9F; Supplementary Figure 6, Supplementary Figure 7). Thus, some apoptosis-related genes were detected when NTS was overexpressed in EECs, the results showed that NTS increased the protein levels of BCL-2, Caspase-8, FAS, and pleiotrophin (PTN), and decreased BAX and Sp1 transcription factor (SP1) ($p < 0.05$, Figure 10). In addition, NTS also increased p-ERK1/2, JNK, p-JNK, and p-P38, but decreased the total level of JNK. At the same time, NTS decreased the protein levels of P27 and PI3K ($p < 0.05$, Figure 10). What's more, the protein levels of BCL-2 and BAX were also detected in EECs when the NTS was silenced by siRNA-NTS, and the results showed that BCL-2 decreased and BAX increased (Supplementary Figure 9), what were in accordance with the function of miR-181a but contrary to overexpressed NTS in EECs *in vitro*. All these results suggested that miR-181a induced EECs apoptosis by targeted NTS.

As to the biochemical endometrial receptivity biomarkers, WB analysis showed NTS increased LIF, COX2, VEGFA and HOXA10 ($p < 0.05$, Figure 10). In addition, the endometrial receptivity biomarkers were detected in EECs when the NTS was knockout by siRNA-NTS, and the results showed siRNA-NTS increased the VEGFA levels in EECs *in vitro* (Supplementary Figure 10) what needed further studies.

DISCUSSION

ceRNA network in the endometrium of dairy goats

Accumulated studies revealed that ceRNAs could serve as post-transcriptional regulators of protein-coding gene expression by decoying miRNAs from other target transcripts, such as lncRNAs, mRNAs, pseudogenes and circRNAs

[21, 22]. In this study, based on the high-throughput RNA-sequencing data, we constructed putative ceRNA networks by integrating lncRNAs, circRNAs, mRNAs and miRNAs in the endometrium of dairy goats. This was the first study to comprehensively identify ncRNAs in the development of RE from PE by regulatory network analysis.

The mechanism of the ceRNA network was that all types of RNA transcripts could communicate with each other by competing for binding to shared MREs [23]. Some lncRNAs could be considered ceRNAs because they could serve as miRNA “sponges” that inhibit interaction with their miRNA targets in post-transcriptional regulation. Sun established that 51 miRNAs sharing 13,623 MREs with 2260 miRNAs and 82 lncRNAs were involved in the ceRNA networks of cell lines SHEE and SHEEC [24]. In this study, we constructed the DELncR/DEciR-DEmiR-DEmR network in the endometrium of dairy goats, and the results showed that 144 DEmiRs shared 239,385 MREs with 305 DEmRs, 105 DEciRs, and 887 DELncRs in the ceRNA networks. It deserved to note that ciR8073 shared MREs with 9 differentially expressed miRNA, including miR-181a. What's more, miR-181a shared MREs with 24 mRNAs, 105 circRNAs and 16 lncRNAs in the ceRNA networks, and NTS mRNA, ciR8073 and lncR915 shared the same MRE of miR-181a.

miR-181a and NTS were regulated by E2/P4 in EECs *in vitro*

Because E2 and P4 played leading roles in the uterus of animals, and the concentrations underwent dynamic changes under different physio-pathological conditions [25], we detected the miR-181a and NTS mRNA levels in EECs after the cells were treated with different concentrations of E2 and/or P4, and the data suggested that the levels of miR-181a and NTS mRNA were regulated by E2 and P4 *in vitro*. Further analysis found that miR-181a was widely expressed in different tissues of dairy goats, and the levels decreased in RE compared with PE. Under

Figure 9: NTS inhibited EECs apoptosis *in vitro*. Note: (A, D) The effect of NTS on the proliferation of EECs what were measured by MTT. Values were showed as “means $\pm$ SD” ($n = 7$). The cell cycle (B, E) and apoptosis (C, F) analysis of EECs what were detected with FCM. Values were showed as “means $\pm$ SD” ($n = 3$). ** indicates that p -value < 0.01 and * indicates that p -value < 0.05 .

Figure 10: NTS increased the expression levels of BCL-2/BAX, and increased LIF, COX2, VEGFA and HOXA10 in EECs *in vitro*. The levels of apoptosis-related proteins (BCL-2, BAX, Caspase-8, Caspase-3, FAS, SP1 and PTN), MAPK pathway proteins, and endometrial receptivity markers (LIF, COX2, VEGFA, HOXA10 and OPN) were measured by WB when the NTS was over-expressed in EECs. Densitometry was normalized to the β -actin density from the same lane, ** indicates that p -value < 0.01 and * $p < 0.05$.

the coordination of E2 and P4, EECs underwent structural and functional changes that established endometrium receptivity [26], which was absolute necessary for successful embryo implantation in all mammals. *NTS* was also widely expressed in different tissues of dairy goats, and its protein increased in the endometrium in RE compared to PE. Thus, we hypothesized that *NTS* expression was also regulated by E2 and P4 in the endometrium of dairy goats, and this conjecture was verified in the present study in which we observed a direct regulation of *NTS* by E2 and P4 in the EECs *in vitro*.

miR-181a decreased NTS levels in EECs

In pigs, miR-181a was found highly expressed on day 15 of gestation, followed by decreased expression on gestational days 26 and 50 in the endometrium during pregnancy [27]. And miR-181a could promote hESCs (human endometrial stromal cells) decidualization-related gene expression and morphological transformation; conversely, inhibition of miR-181a expression compromised hESC decidualization *in vitro* [28]. *NTS* was a 13 amino acid peptide, which was biologically active in the central nervous system and in the periphery [29, 30]. In addition, the higher levels of *NTS* expressions in the endometrial epithelial and glandular epithelial cells were detected in the bovine endometrium, and more abundant in the endometrium from summer than that from autumn [31]. In this study, miR-181a directly inhibited *NTS* expression levels at the mRNA and protein levels via its 3' UTR in EECs of dairy goats.

ciR8073 displayed a sponging effect for miR-181a in EECs

At present, some lncRNAs and circRNAs had been confirmed as ceRNAs to involve in a variety of physiological functions and pathological processes. For example, lncR-MD1 could control muscle differentiation by liberating the differentiation factors MEF2C and MAML1 from repression by decoying miR-133 and miR-135 [15], and lncR-CHRF regulated cardiac hypertrophy by targeting miR-489 [32]. However, miR-181a did not target lncR915 in EECs of dairy goats, what was inconsistent with our speculation.

Hansen identified a highly expressed ciRS-7, contained more than 70 selectively conserved miRNA target sites, markedly suppressed miR-7 activity, resulting in increased levels of miR-7 targets [16]. What's more, a testis-specific circRNA, sex-determining region Y (Sry), served as a miR-138 sponge suggesting miRNA sponge effects achieved by circRNA formation were a general phenomenon [18]. In EECs of dairy goats, ciR8073 displayed a sponging effect for miR-181a, and the *NTS* levels significantly increased when the ciR8073 was over-expressed and decreased when ciR8073 was mutated in EECs, suggesting a high degree of endogenous interaction.

This study served as the first, to our knowledge, functional analysis of a naturally expressed circRNA in dairy goats.

miR-181a promoted EECs apoptosis *in vitro*

In many mammals, endometrial cells were remodeled by apoptosis and proliferation throughout the estrous cycle [33], and studies had been performed in human [34], mice [35], pig [36] and so on. Furthermore, a coordinated regulation of the embryonic induction of EECs apoptosis was crucial for the embryo to breach the epithelial barrier *in vitro* [37]. Recently, it had been reported that inhibition of miR-181a might suppress proliferation and promote apoptosis of HeLa and CaSKi cells by modulating the PTEN/Akt/FOXO1 signaling pathway [12]. The balance between BCL-2 and BAX played a key role in determining apoptosis or cell survival [38], and a high BCL-2/BAX ratio made cells resistant to apoptotic stimuli, while a low ratio may predispose them to apoptosis [39]. In the present study, we found lower levels of BCL-2 in the miR-181a-treated EECs, meanwhile, BAX levels increased. Thus, we hypothesized that miR-181a plays a role in the regulation of endometrium cell apoptosis via BCL-2/BAX in EECs of dairy goats.

In this study, miR-181a promoted EECs apoptosis and a further study showed that miR-181a decreased the BCL-2 and increased the BAX, what was agree with previous study which reported that miR-181a could direct target BCL-2 in endothelial cells [40]. Furthermore, miR-181a increased p-JNK and P27, and these results suggested that miR-181a might play an important role as an apoptosis-regulating factor by MAPK pathway in EECs of dairy goat.

miR-181a might participate in the formation of endometrial receptivity

Recently, several morphological and biochemical biomarkers of endometrial receptivity were proposed, including LIF, COX2, VEGFA, HOXA10, and OPN. LIF was seen in the cytoplasm of the EECs [41], and it was widely acknowledged as biochemical markers of receptive endometrium [42]. COX2 was a rate-limiting enzyme for the synthesis of prostaglandin, which was a critical factor for maintaining the uterine environment during early pregnancy [43], and we found that the COX2 protein levels were decreased by the miR-181a in EECs. In addition, VEGFA was expressed in the mouse and rabbit endometrium and likely participated in the increased angiogenesis and vascular permeability necessary for implantation [44, 45]. Evidence suggested that the expression of VEGFA was highly regulated in a temporal and spatial manner at the early stage of implantation [46]. HOXA10, a homeobox-containing transcription factor, expressed cyclically during the menstrual cycle in the endometrium under the influence of steroid hormone, and

had the highest expression level during WOI [47, 48]. OPN played an important role in endometrial receptivity due to its consistent up-regulation during the WOI [49]. In this study, miR-181a significantly increased LIF and COX2 protein levels but decreased the levels of OPN in EECs *in vitro*. These results suggested that miR-181a might participate in the formation of endometrial receptivity.

NTS promoted EECs proliferation and inhibited apoptosis *in vitro*

Previously, much attention was devoted to the function of NTS in cancer [50], or as an important regulator of the fat absorption and obesity [51]. NTS was detected in bovine endometrium, and the expression levels showed difference between the breeding and non-breeding seasons [31]. However, there was no relevant literature on the function of NTS in the formation of endometrial receptivity in dairy goats, and this study was the first one to study the function of NTS in EECs of dairy goats.

At present study, NTS could promote EECs proliferation and inhibit cell apoptosis *in vitro*. Caspase-8 was crucial for apoptosis progression, when it was degraded or decreased, resulting in immortality of injured cells and induction of various cancers [52]. Furthermore, several studies had suggested that endometrial cell death was increased by the active Caspase-3 during the estrous

cycle [53]. In this study, we found that the NTS did not affect the Caspase-8 protein levels but increased the Caspase-3 protein levels in EECs. In addition, NTS did not change the protein levels of FAS, what was a critical factor for the progression of extrinsic apoptosis in human endometrium [34, 54].

Iwahori reported that SP1 might play a role in DNA repair at damage sites and inhibited cell apoptosis [55], however, silencing SP1 suppressed telomerase activity and promoted apoptosis of SW480 cells line in colorectal carcinoma [56]. In this study, NTS dramatically decreased the SP1 protein levels in EECs, which agreed with the FCM results. PTN was a secreted cytokines that played roles in diverse biology process, such as cell adhesion, migration, survival, growth, and differentiation [57]. In this study, the protein levels of PTN increased with the increase of NTS in EECs. Considering the fact that PTN mainly expressed in the caruncular areas of bovine endometrium [58], it might participate in the proliferation of endometrial cells in dairy goats.

Further investigation revealed that NTS decreased the total levels of MAPK and JNK, but increased phosphorylation levels of MAPK, JNK and P38, meanwhile, it decreased the protein levels of P27 and PI3K in EECs *in vitro*. Several studies had shown that the ERK1/2 activation usually protected neurons from apoptosis, whereas prolonged activation of P38 and/or JNK often caused death [59]. Moreover, Chen reported that P27 expression level

Figure 11: Proposed network of ciR8073-miR181a-NTS in the endometrium of dairy goats. ciR8073 regulates EECs by functioning as a ceRNA, which sequesters miR-181a, thereby relieving its repressive effect on NTS.

increased through increased SP1 binding to its promoter region, which in turn was mediated through increased SP1 protein levels and decreased inhibitory regulation by the MAPK pathway [60]. In this study, we postulated that NTS decreased SP1, and then decreased P27, activated MAPK pathway, finally inhibited cell apoptosis in EECs *in vitro*. In addition, many studies showed that PI3K also involved in the process of cell apoptosis [61], thus the PI3K protein level was also detected in this study, and the result showed that NTS decrease it in EECs. All these results suggested that NTS played very important and complex functions in the EECs apoptosis.

In EECs, NTS could inhibit the cells apoptosis by increasing BCL-2 and decreasing BAX. However, it was noteworthy that it also increased Caspase-3, FAS and PTN, decreased SP1 in EECs *in vitro*. The reasons for this difference might be that NTS prevented EEC apoptosis, what was contrary to the normal processes. Thus, we hypothesized that miR-181a plays a role in the regulation of endometrium cell apoptosis via BCL-2/BAX in EECs of dairy goats.

NTS might participate in the formation of endometrial receptivity

In this study, NTS could increase some biochemical markers of receptive endometrium, such as LIF, COX2, and HOXA10 in EECs *in vitro*. However, it should be noted that there was no significant changes on the protein levels of LIF, COX2, and HOXA10 when the NTS was knockdown partly by specific siRNA in EECs. All these results suggested that NTS was helpful to the formation of endometrial receptivity by increasing the protein levels of LIF, COX2, and HOXA10, but this function was not absolutely necessary in EECs *in vitro*. And more in-depth research was needed to study the functions of NTS in EECs of dairy goats.

Here we provided direct evidences that ciR8073 could function as a ceRNA, which sequestered miR-181a, thereby protecting NTS transcripts from miR-181a-mediated suppression (Figure 11). As it was well established that NTS could promote EECs proliferation and increased the expression levels of LIF, COX2, VEGFA and HOXA10, all these data suggested that ceRNA regulation was of crucial importance in formation of endometrial receptivity in dairy goats. This information will facilitate the further understanding of the ceRNA roles in the regulation mechanism of the RE, and help us to better understand the molecular regulation of endometrial receptivity.

MATERIALS AND METHODS

Ethics statement

All animals in this study were maintained according to the No. 5 proclamation of the Ministry of Agriculture,

P. R. China. And animal protocols were approved by the Review Committee for the Use of Animal Subjects of Northwest A&F University.

Study design and tissue collection

A total of 20 healthy 24-month-old multiparous dairy goats (Xinong Saanen) were induced to estrous synchronization for this study. The first day of mating was considered to be Day 0 of pregnancy, D5 (PE) and D15 (RE) were important time points for the embryo implantation in goats [62]. The goats were euthanized when the goats lost consciousness caused by intravenous injection of barbiturate (30mg/kg) at D5 and D15. Endometrium samples were obtained from the anterior wall of the uterine cavity. All tissue samples were washed briefly with PBS (Phosphate Buffered Saline) and then immediately frozen in liquid nitrogen.

miRNA and transcript differential expression analysis

miRNA library construction, sequencing, and identification of potential novel miRNAs were showed as before [63]. Expression levels of all of the transcripts, including putative mRNAs, lncRNAs and circRNAs, were quantified as fragments per kilobase of exon per million fragments mapped (FPKM) using the Cuffdiff program from the Cufflinks package [9]. Differential gene expression was determined using Cuffdiff with a p-value of <0.05.

Construction of ceRNA network

The putative miRNA-mRNA, miRNA-lncRNA, miRNA-circRNA interactions were evaluated using the algorithms of Targetscan version 6.2 (<http://www.targetscan.org/>) and miRanda version 3.3a (<http://www.microrna.org/microrna/home.do>). The miRNA binding-site prediction in lncRNAs was based on their full-length sequence in consideration of their non-coding properties. High-confidence miRNA-mRNA/lncRNA/circRNA pairs had a Targetscan context+ score percentile >50 and miRanda max energy < -20. The ceRNA relationships were integrated using an in-house Perl script. The information including all of the above interactions was imported into Cytoscape software version 2.8.0 (<http://www.cytoscape.org>) to construct a regulatory network.

Vector construction

Bioinformatic analysis of miRNA binding sequences in NTS, ciR8073, and ncR915 were performed to identify the target genes of miR-181a using Miranda and Targetscan. To construct reporters for luciferase assays, 3' UTR of NTS, ciR8073 and lncR915 respectively contained the miR-181a target site were cloned and

inserted downstream of the Renilla luciferase gene in the psiCHECK™-2 vector (Promega, Madison, USA), and the mutated plasmids were constructed.

To construct reporters for overexpression assays, CDS of NTS, full length of ciR8073 and lncR915 were cloned and inserted in the psiDNA3.1(+) vector (Promega, Madison, USA), and the target site of miR-181a were mutated and constructed mutant psiDNA3.1(+)-ciR8073(Mu) and psiDNA3.1(+)-lncR915(Mu).

Primary cell culture and purification

Primary EECs were isolated and purified by digestion of trypsin, centrifugation and difference tempo adherence, and observed by light microscope, and cell purity and identification was evaluated immunocytochemically as previously described [64]. The cells were cultured to the third generation with the same cell culture system to further purify the cells.

Cell transfection

Mature miR-181a mimics, inhibitors and siRNA-NTS were synthesized by GenePharma (Shanghai, China). Cells were plated at a density of 7.5×10^5 in 6-well plates, then the cells were transfected at fifty percent confluency with miR-181a mimics, miR-181a inhibitors, NC, or NC inhibitors at final concentrations of 100 nM using the X-tremeGENE siRNA Transfection Reagent (Roche, Switzerland) according to the manufacturer's concentrations of 100 nM using the X-tremeGENE Lip2000 liposome (Invitrogen, USA).

Cell proliferation and apoptosis assays

The MTT (Sigma, St. Louis, MO, USA) colorimetric assay was used to screen for cell proliferation as previously described [65]. To further investigate cell proliferation, cell cycle staining Kit was used (Liankebio, Hangzhou, China) according to the manufacturer's instructions. Cell apoptosis analysis was carried out using the Annexin V-FITC/PI apoptosis kit, Annexin-V-positive and PI-negative cells were defined as early apoptotic cells, and the late apoptotic cells were Annexin-V and PI positive cells. Analyses were performed using a flow cytometer (BD Biosciences, San Diego, CA).

Luciferase assay

The wild-type (psiCHECK2-Wt) or mutated (psiCHECK2-Mu) plasmids were co-transfected with the miR-181a mimic into 293T cells, respectively. At 24 h post-transfection, firefly (hluc+) and Renilla (hRluc) luciferase activities were measured with the Dual-Glo luciferase assay system (Promega, USA). Experiments were performed three times.

RNA extraction and RT-qPCR

Total RNA was extracted using Trizol reagent (TaKaRa, Dalian, China), RT-qPCR was performed using SYBR Green PCR Master Mix (TaKaRa, Dalian, China) in a 20 µl reaction. GAPDH was used as the reference for mRNA and U6 for miR-181a, and all primers for the RT-qPCR are shown in Supplementary Table 1. The relative expression levels were calculated using the equation $N = 2^{-\Delta\Delta C_t}$.

Another total RNA were incubated for 15 min at 37°C with or without (mock) 3 U/µg of RNase R (Epicenter Biotechnologies, Chicago, USA). To quantify the amount of circRNA, cDNA was synthesized with the Prime Script RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) using random hexamers. In particular, the divergent primers annealing at the distal ends of circRNA were used to determine the abundance of circRNA, and the outward-facing primer sets listed in Supplementary Table 1.

Protein extraction and WB analysis

Protein extraction was performed as described previously [66]. Thirty micrograms of protein from each treatment was used for 12% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA) at 100 V for 1.5 h in an ice bath. Non-specific binding sites were blocked with 5% fat-free powdered milk (blocking solution) as well as Tris-buffered saline plus Tween 20 [0.2% (vol/vol)] (TBST) at room temperature (RT) for 2 h. After three washes for 10 min each with TBST, the membranes were incubated overnight at 4°C with primary antibodies (as shown in Supplementary Table 2). After this incubation, the membranes were washed three times and then incubated at RT for 2 h with the horseradish peroxidases (HRP)-conjugated second antibody. After three washes for 5 min each with TBST, proteins were detected using enhanced chemiluminescence (Advansta, California, USA). Quantification was performed using the Quantity One program (Bio-Rad, California, USA). The experiments were performed three times.

Immunohistochemistry (IHC)

For IHC, the paraffin-embedded tissue sections were de-paraffinized in xylene for 15 min, and rehydrated in descending concentrations of ethanol (anhydrous ethanol for 5 min, 85% for 5 min, 75% for 5 min and then rinsed with distilled water). Antigen retrieval in sodium citrate buffer (pH= 9.0) was performed for 10 min in a microwave oven, then the tissue sections were placed in PBS (PH=7.4) and washed three times (each time 5 min) on a decoloring shaking bed. Endogenous peroxidases of IHC were inhibited by incubation with 3% hydrogen peroxide for 25 min at RT. The samples were blocked

in 5% bull serum albumin (BSA) for 30 min, incubated with a primary antibody (as be shown in Supplementary Table 2) at 4°C overnight, and then washed three times (each time 5 min) on a decoloring shaking bed. Then, the samples were incubated with HRP-labeled secondary antibody for 30 min at 37°C. After washing the cells three times in PBS, the color reaction was developed with the substrate diaminobenzidine according to the manufacturer's instructions, and the slides were washed under running water for 5 min before being counterstained with hematoxylin (Carnaxide, Oeiras, Portugal). After re-dyeing the nucleus with hematoxylin and ammonia, the slides were dehydrated with concentrations of ethanol and sealed with xylene for 5 min. The relative density of the positive cells (density/area) in each slide was analyzed using Image-Pro Plus 6.0 Software (Media Cybernetics, USA).

miRNA in situ hybridization (MISH)

An oligonucleotide probe complementary to the miR-181a sequence was purchased from RiboBio Co., Ltd. (Guangzhou, China). The sequence of the probe was 5' ACTCACCGACAGCGTTGAAGA-3' TCACCGACAGCGTTGAAGA, China). The sequencedigoxigenin (DIG), and some of these bases were modified with a DIG-labeled LNA (Locked Nucleic Acid). In situ hybridization for miR-181a (MISH) was performed on fixed paraffin-embedded sections as previously described [67]. The relative density of the positive cells (density/area) in each slide was analyzed using Image-Pro Plus 6.0 Software (Media Cybernetics, USA).

Statistical analysis

All the data were processed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to compare the differences, and the method of the least significant difference (LSD) was used for further analysis. Differences were considered significant when the p-value was < 0.05 and very significant when p-value was < 0.01.

Abbreviations

RE, receptive endometrium; PE, pre-receptive endometrium; ceRNA, competing endogenous RNAs; ciR8073, circRNA8073; NTS, Neurotensin; EECs, endometrial epithelium cells; LIF, leukaemia-inhibitory factor; COX2, cyclo-oxygen-ase 2; VEGFA, vascular endothelial growth factor A; HOXA10, homeobox A10; OPN, osteopontin; ncRNAs, non-coding RNA; miRNA, microRNA; lncRNA, long non-coding RNA; circRNA, circular RNA; WOI, window of implantation; MREs, miRNA response elements; DE miR, differentially expressed miRNA; DE mR, differentially expressed mRNA; DE lncR, differentially expressed lncRNA; DE ciR,

differentially expressed circRNA; FPKM, fragments per kilobase of exon per million fragments mapped; lncR915, lncRNA915; D15, gestational day 15; D5, gestational day 5; hESCs, human endometrial stromal cells; PRL, prolactin; IHC, immunohistochemical; E2, estrogen; P4, progesterone; UTR, untranslated regions; WB, western blot; p-JNK, phosphorylation JNK; p-MAPK, phosphorylation MAPK; PTN, pleiotrophin; SP1: Sp1 transcription factor; PBS, Phosphate Buffered Saline; MTT, methyl thiazolyl tetrazolium; TBST, Tris-buffered saline plus Tween 20; RT, room temperature; LSD, least significant difference; MISH, miRNA in situ hybridization; FCM, flow cytometry.

Author contributions

LZ and RXL gathered samples, conceived the report, participated in its design, performed data analysis, interpreted results and drafted the manuscript. BYC, YUS and ZQZ participated in intellectual discussion. YXL, XNM, YZ, XYD and JZL contributed to gathering samples. BYC and YUS designed experiments, analyzed data and wrote the manuscript.

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CONFLICTS OF INTEREST

The authors declare no competing financial interest.

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