IL-1β mediates miR-34a promotes bovine endometritis via LGR4-NF-κB axis

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Research

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Abstract

Background

Persistent endometritis lead by bacterial infections has lethal effects on the reproductive performance of dairy cattle, which not only compromise animal welfare but also delay or prevent pregnancy. MicroRNA (miRNA) miR-34 family plays a pivotal role in the inflammatory process; however, the precise mechanism of miR-34a in endometritis is still not thoroughly revealed.

Methods

In this study, we established bovine endometrial epithelial cell (BENDs) inflammation model and mouse model stimulation with Lipopolysaccharide (LPS) in vitro and in vivo. CCK-8 was used to assess cell viability. H&E was used to characterize morphology. Immunohistochemistry, immunofluorescence, qRT-PCR and western blot assays were performed to measure the mRNA or protein expression of related genes. Online database, plasmid construction and dual-luciferase Reporter gene assays were applied to predict and validate the interaction between miR-34a and its target gene LGR4, and mice were injected vaginally with local antagomir to validate the role of miR-34a in murine uterine inflammation.

Results

Here, we report that miR-34a suppresses LGR4 gene expression by targeting its 3'untranslated regions (3'UTR). The miR-34a was up-regulated in cow uterine tissues and bovine endometrial epithelial cell (BENDs) stimulation with LPS. It further induces the release of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α by activating the phosphorylation level of NF-κB p65. Furthermore, we also revealed that IL-1β was responsible for the upregulation of miR-34a transcription and downregulation of LGR4 in an IL-1β-dependent manner.

Conclusions

Taken together, our study confirmed that miR-34a is regulated by IL-1β and suppress the level of LGR4 3'UTR which in turn exacerbates the inflammatory response. Thus knockdown miR-34a might be a new indirection for treatment endometritis.

Background

Persistent endometritis is one of the most common and important problem observed in dairy cows. Figures in the United Kingdom suggest that around 10-15% of cows develop endometritis[1]. It has a deleterious effects on the cow's subsequent reproductive performance, such as calving numbers and milk yield[2]. Studies confirmed that excessive intrauterine infection mediated by pathogenic bacteria such as
lipopolysaccharide (LPS), staphylococcus aureus and streptococcus often results in endometritis[3], which causes damage to the lining of the uterus[4], delays uterine regeneration[5], perturbs embryo implantations failure and survival[6]. Normal and active uterine defense mechanisms was one of the most critical factors for the elimination of bacterial infection and postpartum recovery[2, 7]. It has reported that antibiotic (local or systemic), disinfectant, sulfonamide, and hormonal treatments have not significantly improved the recovery rate of endometritis or pregnancy of cattle. Therefore, it is necessary to understand the precise mechanism of bovine endometritis.

MicroRNAs (miRNA) are a type of small non-coding endogenous RNAs with a length of 19-25 bp [8]. that down-regulate gene expression at pre-transcriptional, intra-transcriptional and post-transcriptional via complementary pairing with the 5' or 3' UTR of its target genes[8, 9]. It is highly species conserved and spatiotemporally expression specific[10]. Many studies have confirmed the microRNAs are involved in various physiological and pathological processes [11, 12], and such as cell development[13], cell apoptosis and cell proliferation[14]. The miR-34/449 family is conserved in mammalian organisms and generally consists of six homologous genes: miR-34a, miR-34b, miR-34c, miR-449a, miR-449b and miR-449[15], among which miR-34a being the first to be identified[16]. Increasing evidence shows that miR-34a is an essential regulator of inflammatory responses [17, 18]. miR-34a and miR-34c also promote the production of inflammatory chemokines and cytokines, which can enhance inflammation and delay healing [19]. Simultaneously, NF-κB-driven miR-34a impairs Treg/Th17 balance for autoimmune diseases[20]. However, the expression of miR-34a in the uterine tissue of dairy cow reported, while its specific function during endometritis is still unknown. Based on this information, we proposed the scientific hypothesis that miR-34a may also be involved in the regulation of endometrial inflammation.

Leucine-Rich Repeat Containing G Protein-Coupled Receptor 4 (LGR4), known as GPR48, is a member of the G protein-coupled receptor family [21]. In recent years, LGR4 has become a hot topic due to its potential as a pharmacological target. LGR4 expressed in humans and mice placenta, uterus, ovaries and fallopian tube epithelium [22, 23], was found to signal through both G protein-coupled as well as wnt-signaling pathways[24] and participate in the regulation of cell proliferation[25], cell apoptosis and cell invasion[26]. Especially, LGR4 plays a role in inflammatory bowel disease[27] and embryonic development[28]. Besides that many studies confirmed that LGR4-deficient mice exhibit excessive activation of innate immunity, which was considered to be a negative regulator of TLR2 and TLR4-related immune responses[29]. However, the specific mechanism of action of LGR4 in regulating LPS-induced inflammatory responses has not been reported and needs further investigation. This study aimed to explore the expression pattern of LGR4 in the infected cow uterus and further elucidate its precise molecular mechanisms that regulate endometrial inflammation.

Based on the aforementioned findings, we hypothesized that miR-34a may interact with LGR4 to regulate LPS-induced inflammatory responses. Thus, in the present study, we investigated the expression patterns and regulatory mechanisms of miR-34a and its downstream effects in BENDs.

**Results**
2.1 The expression of LGR4 in cow uterus tissues.

Endometritis leads to endometrium injury and early loss of embryos [30]. Fig. 1a showed apparent inflammatory damage in infected cow uterine tissues, with a large number of inflammatory cell infiltration, visible swelling. qRT-PCR results showed more secretion of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, while decrease of anti-inflammatory mediators IL-10 in endometritis tissue (Fig. 1b). To confirm the expression of LGR4 in cow uterus, immunohistochemistry displayed that LGR4 is mainly placed in plasma membrane, extracellular, cytoskeleton, and nucleus (Fig. 1c). Meantime, our western blot protein expression and qRT-PCR mRNA levels of LGR4 were reduced in infected uterus relative to control uterus tissues (Figs. d-f). Thus, our results reflected that LGR4 was down-regulated in the presence of uterus inflammation, suggesting that lack of LGR4 may lead to enhancing inflammation.

2.2 LGR4 suppresses the secretion of inflammatory cytokines in BENDs.

Given the above results, to further investigate whether the reduction of LGR4 will induce the secretion of large number of inflammatory cytokines. Different doses of LPS (0, 0.5, 1.0, 1.5, 2.0 μg/ml) treated on the BENDs at different time points (0, 3, 6, 12, 24 h). The data indicated that LGR4 decreased both in a dose- and time-dependent manner (Figs. 2a-b). In addition, CCK-8 was used to analyzed the effect of LPS on BENDs viability. The cell viability of BENDs was found to be largely unaffected with LPS (1.0 μg/ml) stimulation, as detected by CCK-8 assay in Fig. 2c. The result appeared that the level of LGR4 was dramatically lowered 3-fold change was verified by qRT-PCR in Fig. 2f. It also revealed that knockdown LGR4 up-regulated the production of IL-1β, IL-6 and TNF-α (Fig. 2g).

2.3 LGR4 inhibits the inflammatory response by blocking NF-κB phosphorylation.

Given that LGR4/Gpr48 was a negative regulator in TLR2/4-associated immune responses [29]. Therefore, we speculate that LGR4 negative feedback regulates the activation of NF-κB p65 phosphorylation, enhance the transcription of pro-inflammatory cytokines to cause inflammation. To verify the above hypothesis, si-LGR4 or si-NC was transfected into cells to knock down LGR4 expression, and the transfection efficiency was verified by western blot (Figs. 3a-b). Fig. 3a and Fig.3c showed that the phosphorylation level of NF-κB p65 after transfection of si-LGR4 was apparent inhibited. Meantime, immunofluorescence also confirmed this result (Figs. 3d-e). Taken together, these data confirmed that knockdown of LGR4 significantly suppresses the activity of phosphorylated NF-κB p65.

2.4 MiR-34a inversely correlates with the expression of LGR4.

Recent studies have shown that miRNA is involved in the embryonic development and various biological processes of ruminants [31]. Based on the finding that LGR4 has anti-inflammatory properties, four online websites (miRcode, miRDB, Targetscan and ENCORI) were used to predict the putative microRNA that may target LGR4. By taking the intersection of the prediction results of all websites, as shown in Fig. 4a, it was found that 5 microRNAs (miR-34a, miR-34a, miR-34c, miR-302a and miR-218) have the potential to target LGR4.
Based on this result, qRT-PCR was performed to verify the level of putative microRNA under the stimulation of LPS (1.0 μg/ml) (Fig. 4b) and in infected cow uterus tissues (Fig. 4c). The findings indicated that miR-34a, miR-34b, miR-34c, miR-218 and miR-302a were up-regulated, particularly, miR-34a was increased about 5-fold change.

To further determine the regulatory relationship between miR-34a and LGR4, miR-34a mimic or inhibitor were transfected into BENDs and then stimulated the cells with LPS (1.0 μg/ml) for 6 h. The transfection efficiency was verified by qRT-PCR (Fig. 4d). Figs. 4e-f appeared that the expression of LGR4 mRNA and protein in the mimic group was markedly lowered, but the inhibitor group achieved the opposite result. The data further displayed that miR-34a may directly negatively regulates LGR4 mRNA and then inhibits its translational level, which also confirmed the interaction between miR-34a and LGR4.

2.5 MiR-34a directly targets the 3'UTR of the LGR4.

LGR4 is a member of the leucine-rich repeat domain-containing G protein-coupled receptors, and it has been predicted that its interaction with miR-34a is highly conserved among species as shown in Fig. 5a. Based on website prediction results, miR-34a may target the 3'UTR of LGR4, and the interaction map is shown in Fig. 5b. Furthermore, RNAhybrid 2.2 was also shown that miR-34a has the potential to bind LGR4 3'UTR according to the calculation of minimum free energy (Fig. 5c). To further explore the interaction mechanism between miR-34a and LGR4, LGR4 3'UTR (WT-3'UTR or MuT-3'UTR) were amplified and cloned into psiCHECK-2 vector to synthesis the psiCHECK-2-LGR4 3'UTR plasmid (Fig. 5d), then co-transfected with miR-34a mimics or mimics NC or negative control into the 293T cells, the results of the dual-luciferase reporter gene assay described that the fluorescent activity of the WT-3'UTR was drastically decreased, while MuT-3'UTR group had no notable difference (Fig. 5e), reflecting that miR-34a directly targets the 3'UTR of LGR4 mRNA.

2.6 MiR-34a regulates inflammation through NF-κB in BENDs.

In this study, we determined that miR-34a was up-regulated in both the infected cow uterine tissue and the endometrial epithelial cells stimulated by LPS (1.0 μg/ml), contrary to the expression of LGR4. Based on the anti-inflammatory potential of LGR4. To further explain the function of miR-34a in the inflammatory response, miR-34a gain-of-function or loss-of-function experiments were performed. The data displayed that overexpression of miR-34a not only suppressed the expression of LGR4 (Figs. 6a-b), but also promoted the phosphorylation of NF-κB p65(Figs. 6c and 6d), conversely, the miR-34a inhibitor group had the opposite result, in line with the results of immunofluorescence assay (Figs. 7a-b). At the meantime, the production of IL-1β, IL-6 and TNF-α were strongly higher in miR-34a mimic group (Fig. 7c), but remarkably lower in miR-34a inhibitor group (Fig. 7d). Taken together, it further implied that miR-34a has the potential to promote the progression of inflammation.

2.7 Inhibition of miR-34a suppresses endometritis in mice.
miR-34a is highly conserved among species. To further investigate the role of miR-34a on endometritis in vivo, we established a mouse endometritis model following the previous laboratory method[32]. The groups as follows: blank group, LPS group, LPS+ miR-34 antagonist NC , and LPS + miR-34a antagonist. As shown in Fig. 8a, Kunming mice were infused with LPS ( 50 μl, 1 mg/ml ) in each uterine horn, and the day of injection was recorded as day 0 (D0). 24 h later, miR-34a antagonist or NC (intrauterine injection of 0.5 μmol/ kg) [33] was treated into the uterus on D1, D4, D7 and uterine tissues were collected on D10. qRT-PCR (Fig. 8b) verified the expression of miR-34a, showing a significant reduction after miR-34a antagonist treatment. Furthermore, LGR4 was remarkably inhibited after LPS stimulation, while the expression was upregulated after miR-34a antagonist injection (Fig. 8c-d). Consistently, miR-34a antagonist suppressed the entry of NF-κB into the nucleus (Fig. 8e-f) and lowered the production of IL-1β, IL-6 and TNF-α (Fig. 8g). Overall, our data reveal that knockdown of miR-34a could suppress the inflammatory cytokines and thus alleviate endometritis.

2.8 IL-1β suppresses LGR4 expression by enhancing miR-34a.

A et al.[34] has shown that IL-6 and TNF-α-activated p65 to bind to the miR-34a promoter and promote its transcription to enhance its activity. Therefore, we speculate that LPS induced the high expression of miR-34a in BENDs, which is most likely caused by the regulation of the pro-inflammatory cytokines IL-1β induced by activated NF-κB p65. To evaluate the effect of IL-1β on the expression of miR-34a and LGR4, different concentrations of recombinant IL-1β (0, 1, 5, 10 ng/ml) were used to treat BENDs. As shown in the Fig. 9a, miR-34a was enhanced under the incubation of IL-1β for 6 h in a dose manner (0, 1, 10 ng/ml). Moreover, BENDs was transfected with miR-34a inhibitor in the presence or absence of 5 ng/mL IL-1β treatment to further investigate the effect of IL-1β on miR-34a and LGR4. qRT-PCR (Fig. 9b) showed that down-regulation of miR-34a by inhibitor was partially reversed by IL-1β stimulation. As we expected, the mRNA and protein expression of LGR4 were notably aggravated by miR-34a inhibitor, which was rescued in the incubation of IL-1β, evidenced by Figs. 9c-e. Immunofluorescence results showed that IL-1β induced NF-κB p65 into the nucleus, while silencing miR-34a apparently rescued this change (Figs. 9f-g). Consequently, these above data together implied that IL-1β is an inducer of miR-34a enhancement which can also mediate LGR4 expression.

In summary, our results demonstrated that IL-1β mediates the transcription of miR-34a, and further positively regulates the secretion of inflammatory mediators via the LGR4-NF-κB pathway, causing an excessive inflammatory response, which in turn damages the endometrium (as shown Fig. 10).

Discussion

Embryo loss due to endometritis is a key element in low calving rates on farms[35]. Bacterial infections are the most common cause of postpartum endometritis in dairy cows[36], which leads to failure in embryo implantation and growth of embryos [37]. So far, antibiotics are universally accepted when it comes to clinical approaches to endometritis. However, antibiotic treatment brings serious drug residues and drug resistance. Therefore, it is important to understand the specific pathogenesis of the disease and
to discover new methods of treatment. MicroRNA expressions are prominently expressed on LPS induced endometrial inflammation\cite{38, 39}. However, the exact role in microRNA upstream and downstream regulation during endometrial inflammation, remains to be further investigated. Here, our study results demonstrate that miR-34a is a conservative repressor of LGR4, which can induce the release of pro-inflammatory factors through the LGR4-NF-κB axis and participate in the pathogenesis of endometritis. Additionally, we confirmed that the release of inflammatory environment IL-1β can increase the expression level of miR-34a, down-regulate LGR4, and form a positive regulatory chain that causes further deterioration of the inflammatory response.

Expectedly, we found that LGR4 is a negative regulator for inhibiting inflammation via the NF-κB signaling pathway. Whereas that LGR4 expression decreased in the uterine tissues of naturally infected cows. Moreover, to reveal the potential role of LGR4 in cells treated with LPS, si-LGR4 was transfected into cells in BENDs. As we expected, LGR4 was also lowered after LPS stimulated BENDs, which implied that knocking down LGR4 activated downstream NF-κB p65 phosphorylation and transcription into the nucleus, and induced the secretion of pro-inflammatory mediators, thus, further displayed LGR4 has the potential to inhibit inflammation, which was in accord with the result of \cite{40} Wu's findings that LGR4 modulate the inflammatory response of keratinocytes.

Moreover, we also predicted that microRNAs may target bovine and murine LGR4 3'UTR and take the intersection from four recognized databases: miRcode, miRDB, TargetScan and ECORI. The prediction results appeared that miR-34a, miR-34b, miR-34c, miR-302a and miR-218 were most likely to be target molecule for binding LGR4 3'UTR. Next, we determined the differential expression of the five microRNAs by qRT-PCR, which eventually screened for miR-34a. Interestingly, out of the five microRNAs, miR-34a was found the most notably upregulated expression. Subsequently, the relationship between mi-34a and LGR4 was identified by the dual-luciferase reporter assay, which showed that miR-34a drastically lowered the activity of LGR4 3'UTR. Similarly, it has been recently reported that LGR4 is a common target of miR-34a and miR-34c in mice\cite{30}. In conjunction with the above study results, that LGR4 inhibits the production of pro-inflammatory mediators, subsequently, we transfected miR-34a mimic or inhibitor to BENDs to carry out function gain and function loss experiments in order to further investigate whether there was a suppression effect of miR-34a on LGR4. Our data revealed that overexpression miR-34a indeed inhibited the levels of LGR4 mRNA, meanwhile, lowered the transcription of NF-κB p65 into the nucleus. Thus, we concluded that upregulation or down expression in LPS treated could strengthen or relieve inflammatory response by impairing with LGR4 via altering the phosphorylation level of NF-κB p65 and modulating the release of inflammatory cytokines.

Collectively, these data suggest that differentially expressed miR-34 family has been shown to play a pivotal role in autoimmune and other inflammatory responses\cite{41, 42}. Most interestingly, NF-κB was reported to drive miR-34a transcription, suggesting that chemokines or inflammatory cytokines produced by NF-κB induction may target the promoter that binds miR-34a, thereby mediating its transcription to affect the expression of downstream targets\cite{43}. 


Consistent with our findings, it has been reported that miR-34a aggravates wound inflammation[40]. Nevertheless, it is worth noting that studies have shown that enhance miR-34a expression may hold a promise in anti-inflammatory drugs development[44], which confirms the spatiotemporal expression specificity of microRNAs and suggests that miR-34a may be regulated by upstream molecules, such as long non-coding RNA, transcription factors, and pro-inflammatory mediators. Furthermore, it is also possible that it is related to the regulation of miRNA's multi-target network, which indicating that it is necessary to search for upstream molecules of miRNA and investigate its specific regulatory mechanisms.

Finally, we aimed to elucidate the upstream factors regulating miR-34a upregulation. p53 has been shown to regulate miRNAs including miR-34a/b/c at the transcriptional level as well as the processing and maturation of certain miRNAs[45-47], but its role has been shown to be primarily in tumor suppression. There are few reports on the upstream regulators of miR-34a in the inflammatory response. Fortunately, IL-6 and TNF-α have recently been shown to regulate miR-34a transcription and participate in rheumatoid arthritis[20].

Sustained activation of NF-κB can increase the expression levels of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α. Among them, IL-6 and TNF-α are multifunctional pro-inflammatory mediators that play an important role in the development of chronic inflammatory responses. Importantly, IL-1β is a crucial mediator of the inflammatory response which is essential for the host response and resistance to pathogens[48]. Therefore, we chose IL-1β as a candidate to investigate its effect on miR-34a transcriptional activity. The study data showed that IL-1β significantly promoted miR-34a transcription and suppressed LGR4 expression. Next, more research should pay attention to the specific mechanism of IL-1β involved in regulating miR-34a transcription. Finally, our investigation shows that IL-1β induces transcription of miR-34a, which can directly target the 3'UTR of LGR4, further promoting NF-κB phosphorylation and inflammatory response.

In conclusion, our investigation identified IL-1β acts as an agonist of miR-34 transcription, mediates an increase in miR-34a expression, further suppresses LGR4 expression levels, promotes the activity of phosphorylated p65, triggers the secretion of numerous inflammatory cytokines to induce an excessive inflammatory response.

Therefore, knockdown miR-34a may be a new direction to alleviate excessive inflammation.

**Conclusion**

In conclusion, our study provided the first evidence that a novel mechanism of miR-34a augmenting inflammatory response through triggering IL-1β/LGR4/NF-κB feedforward loop via LGR4 in BENDs. Thus, inhibition of miR-34a may be a novel therapeutic method for protection against endometritis.

**Materials And Methods**
5.1 Chemical reagents and antibodies

Bovine monoclonal antibodies against LGR4 was purchased from LifeSpan BioSciences, Inc (Seattle, WA, USA). The antibody NF-κB p65, phospho-NF-κB p65 (p-p65), β-actin and the horseradish peroxidase (HRP) goat anti-rabbit and goat anti-mouse antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Sigma Inc (St. Louis, MO, USA) provided fetal bovine serum (FBS); LPS (E. coli 055: B5) was obtained from Sigma (St. Louis, MO). Recombinant bovine IL-1β protein from Abcam (Ab88013, Cambridge, MA) was used in the study. All other chemicals were reagent grade.

5.2 Animals and tissues collection

The bovine uterus tissues in this study collected from dairy cows in the slaughterhouse (Wuhan, China). Kunming mice (6~8 weeks old, 19-22 g weight) were purchased from the Experimental Animal Center of Huazhong Agricultural University (Wuhan, China). The mice were kept at room temperature for 12 hours in a dark-light cycles with free access to food and water. All procedures followed the guidelines provided by the Laboratory Animal Research Center of Hubei province, and authorized by the Huazhong Agricultural University Animal Care and Use Committee (HZAUMO-2015-12).

5.3 Cell culture

Bovine endometrial epithelial cell line (BENDs) and Human embryonic kidney cell line (HEK293T cells) purchased from the American Type Culture Collection (ATCC TIB-71™). Both cell lines cultured in Dulbecco's modified Eagle's medium (DMEM, High Glucose) supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/ml streptomycin Base at 37°C under 5% CO2.

5.4 MicroRNA mimic/inhibitor or siRNA transfection

Cells (1.5 × 10^5/ml) were propagated into 96-well plates until the density reached about 60%-70%, 200nM miR-34a mimic or inhibitor or their respective negative control duplexes (Gene Pharma, Shanghai, China), and their sequences were shown in S-Table 2, were transfected into BENDs with the Lipofectamin™ 2000 (Invitrogen, Carlsbad, California, USA). After 24 h in an incubator, the transfection efficiencies were validated by either qRT-PCR or western blot analysis. Each experiment was independently repeated for three times.

5.5 Histological analysis of H&E staining

The uterine tissue of cows was washed with physiological saline and immediately fixed in 4% paraformaldehyde for 24 h. It was embedded in paraffin and cut into 4-micron thickness with a microtome, dehydrated with graded alcohol (100%, 95% and 90%), then stained with hematoxylin and eosin (H&E). Subsequently, observed and photographed under an optical microscope (Olympus Shinjuku-ku, Tokyo, Japan).

5.6 Immunohistochemistry (IHC) assays
The detailed procedures of tissue fixation, paraffin embedding, and sectioning are the same as H&E staining in 2.5. Sections were deparaffinized with xylene to water and incubated with 3% H$_2$O$_2$ for 10 min at room temperature. Next, Sections were blocked with normal goat serum at 37°C for 30 minutes, and incubated with the primary antibody overnight at 37°C or the secondary antibody for 1 h successively. After DAB color development, it was counterstained with hematoxylin and observed under a microscope.

5.7 Cell counting kit (CCK-8) assay

In order to detect the cell viability treated with LPS (1.0 ug/ml) or recombinant IL-1β (5 ug/ml), a Cell Counting Kit-8 (CCK-8) assay kit (Beyotime, Shanghai, China) was used. Briefly, the cells (4.5×10$^4$ cells) were seeded in a 96-well plate and stimulated with LPS or IL-1β for 6 h and then added 10 ul CCK-8 reagent, incubated for 3 h at 450 nm to measure the OD value with a microplate reader (Thermo Scientific Multiskan MK3, USA).

5.8 Immunofluorescence (IF) assay

The slide fixed with 4% paraformaldehyde for 20 min and then incubated with 0.5% Triton X-100 for 20 min. 10% normal goat serum was added to permeabilize the cells and block interactions with nonspecific proteins for 30 min, after which p-p65 (1:1000) and LGR4 (1:500) antibodies were incubated 12 h. The fluorescent antibody and DAPI were incubated in the dark box for 1 h and 5 min respectively. The slides were viewed under a fluorescence microscope.

5.9 Bioinformatics analysis

The possible microRNA for LGR4 were predicted with four algorithms from miRcode http://www.mircode.org/, miRDB http://mirdb.org/, Targetscan http://www.targetscan.org/vert_72/ and ENCORI http://starbase.sysu.edu.cn/. Then take the intersection of the prediction results of these four websites by https://bioinfogp.cnb.csic.es/tools/venny/index.html.

5.10 Western blot assay

Cells and tissues were lysed by RIPA (Biosharp, China) on ice for 30 min then extracted the total protein. The protein concentration was measured by BCA kit (Thermo Scientific, MA, USA) according to the manufacturer's protocols. Then protein loading buffer was added in a 4:1 ratio to denature at 95°C for 10 min. Equal amounts of cell lysate proteins were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, USA). 5% skimmed milk used to block the membranes for 2 h, incubated with primary antibodies (1:1000 dilutions) at 4°C overnight. Bands incubated with secondary antibody (1:4000 dilutions) for 2 h, proteins analyzed using Image J gel analysis software.

5.11 RNA extraction and qRT-PCR analysis

Total RNA was extracted from the uterus tissues and cells using Trizol (Invitrogen, USA). The PrimeScript RT reagent Kit and miRNA Reverse Transcription System TaqMan MicroRNA assay purchased from
Applied Biosystems (Foster City, USA) Reverse transcription and quantification of total RNA and miRNA were measured as previously described[30]. Data normalized to levels of U6 or GAPDH. All primers showed as S-Table 3.

5.12 Plasmid constructs and luciferase reporter assay

Amplification of the LGR4 3'UTR was synthesized and insert its wild-type or mutant-type LGR4 3'UTR plasmids into a psiCHECK™-2 reporter vector (Promega, Madison, WI, United States) using Xhol and Notl to construct psi-WT-LGR4-3’UTR (WT) and psi-MUT-LGR4-3’UTR (MuT), which were co-transfected with miR-34a mimics or negative control into 293T cells using Lipofectamine 2000™. The fluorescence activity assessed by the dual-luciferase reporter assay system (Promega, Madison, WI, USA). The rate of firefly luciferase activity to Renilla luciferase activity served as the relative luciferase activity.

5.13 Statistical analysis

Results expressed as mean ± SEM. The statistical significance of the differences between various treatments was determined by either the two-tailed Student’s t-test or one-way ANOVA with Bonferroni post-test, with a probability value of <0.05 was considered statistically significant. Data analyses performed using GraphPad Prism software version 8.0. All experiments repeated three times.

Declarations

Additional file 1: S-Table 1. Abbreviations used in this articalcs. S-Table 2. Primers used for qRT-PCR. S-Table 3. Primers for miR-34a and LGR4 siRNA.

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- Author Contributions

XM conceived and designed the experiments. XM, SG, and BY conducted the experiments. XM, ZW and JL analyzed and counted the data. QZ, AZ and TD wrote and revised the manuscript. All authors agreed to be responsible for the content of the work.

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- Availability of data and materials
The data used in the current study are available from the corresponding author on reasonable request.

- **Ethic approval and consent to participate**

The animal procedures followed the guidelines provided by the Laboratory Animal Research Center of Hubei province, and authorized by the Huazhong Agricultural University Animal Care and Use Committee (HZAUMO-2015-12).

- **Consent for publication**

Not applicable.

- **Competing interests**

The authors declare that the study was conducted in the absence of any commercial or economic relationship that could be interpreted as a possible conflict of interest.

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**Figures**
Figure 1

The expression of LGR4 in cow uterus tissues. (a) H&E pathology of cow uterine tissues (HE, ×200), scalebar=100 μm. (b) The mRNA levels of inflammatory cytokines IL-1β, IL-6, TNF-α and IL-10 detected by qRT-PCR for assessing the extent of inflammation in uterus tissue. (c) The expression and location of LGR4 in uterine tissue by immunohistochemistry (HE, ×100), scalebar=100 μm. (d, e) The expression of LGR4 protein was determined by western blot and its level was quantified by IPP 6.0. β-actin was used as an internal control. (f) The LGR4 mRNA was analyzed by qRT-PCR. GAPDH was used as a control. Control is the control group, Inf is the naturally infected cow uterine tissue. Data are expressed as means ± SEM of three independent experiments (n=3). *P < 0.05; **P < 0.01 compared with the control group (Student’s t-test).
LGR4 suppresses the secretion of inflammatory cytokines in BENDs. (a) BENDs were treated with LPS (0, 0.5, 1.0, 1.5, 2.0 ug/ml) for 6 h. The LGR4 mRNA levels were analyzed. (b) Cells were stimulated with LPS (1.0 ug/ml) at 0, 3, 6, 12 and 24 h. The expression level of LGR4 was measured by qRT-PCR. (c) The effects of LPS (1.0 ug/ml) on the viability of BENDs. Cell viability was determined with a CCK-8 assay kit. (d, e) Western blot analysis of LGR4 protein in BENDs after treatment with si-LGR4 or negative control (si-NC). β-actin was used as an internal control. (f) qRT-PCR analysis of LGR4 mRNA expression in BENDs after treatment with si-LGR4 or negative control (si-NC). GAPDH was used as a control. (g) Cells were transfected with si-LGR4 or si-NC for 24 h and then stimulated with LPS (1.0 ug/ml) for 6 h. The production of IL-1β, IL-6 and TNF-α were measured with qRT-PCR. GAPDH was used as a control. The data are presented as the means ± SEM of three independent experiments (n=3). *P < 0.05; **P < 0.01 versus the si-NC group; #P< 0.05; ##P < 0.01 versus the si-NC and LPS group (cells transfected with si-NC after stimulation with LPS) (Student's t-test).
Figure 3

LGR4 inhibits inflammatory response by blocking NF-κB phosphorylation. Cells were transfected with 200 nM si-LGR4 or si-NC for 24 h and then treated with LPS (1.0 ug/ml) for 6 h. (a) The expression of LGR4 and phosphorylated NF-κB p65 were determined by western blot in BENDs. (b, c) The levels of LGR4 and phosphorylated NF-κB p65 were determined by IPP in BENDs. (d, e) Translocation of the p65 subunit from the cytoplasm into the nucleus was evaluated by immunofluorescence. Blue spots represent cell nuclei, and green spots represent p-p65 staining. Data are presented as the means ± SEM of three independent experiments (n=3). *P < 0.05 versus the si-NC group; #P < 0.05 versus the si-NC and LPS group (cells transfected with si-NC after stimulation with LPS) (Student's t-test).
Figure 4

MiR-34a inversely correlates with the expression of LGR4. (a) The Venn diagram shows the predicted intersection of microRNAs that target cow and murine LGR4 3′UTR by miRcode, miRDB, TargetScan and ENCORI. (b, c) The levels of putative microRNAs: miR-34a, miR-34a, miR-34c, miR-218 and miR-302a were detected in BENDs treated with LPS (1.0 ug/ml) and cow uterus tissues by qRT-PCR, respectively. U6 snRNA was used as an endogenous control. (d) Cells were transfected with bovine miR-34a mimics, miR-34a inhibitor, or negative control (mimic NC or inhibitor NC) for 48 or 72 h then, miR-34a transcription levels were detected by qRT-PCR. (e, f) LGR4 mRNA and protein levels were determined by qRT-PCR and western blot, respectively. β-actin was used as an internal control. Data are presented as the means ± SEM of three independent experiments (n=3). *P < 0.05; **P < 0.01 versus the control group (4b-4d) ; #P < 0.05 versus the mimic NC group , *P < 0.05 versus the inhibitor NC group (4e and 4g, cell transfected with miR-34a mimic) (Student’s t-test).
Fig. 5 MiR-34a directly targets the 3’UTR of the LGR4. (a) Targetscan predicts the interaction of miR-34 with LGR4 among species. (b) Schematic of the design of the luciferase reporters with the wild type WT-LGR4 3’UTR (WT) or the site-directed mutant type MuT-LGR4 3’UTR (MuT). The nucleotides in red represent the ‘seed sequence’ of miR-34a; the mutation nucleotides are marked in blue. (c) Alignment of the 3’UTR of the LGR4 with miR-34a by RNAhybrid 2.2. (d) psicHECK-2 vector map (the insertion site of LGR4 3’UTR is marked in light blue). (e) 293T cells were co-transfected with WT-LGR4 3’UTR or MuT-LGR4 3’UTR luciferase reporter vectors, together with miR-34a mimics or mimics NC (final concentration: 20 nM) as indicated. After 24 h, firefly luciferase activity was measured and normalized to Renilla luciferase activity. WT, wild type; Mut, mutant type. Data are presented as the means ± SEM of three independent experiments (n=3). *P < 0.05 versus the control or mimic NC group (Student’s t-test).
Overexpression of miR-34a promotes NF-κB p65 into the nucleus in BENDs. (a-b) Cells were transfected with miR-34a mimics or miR-34a mimic NC for 24 h, followed by exposure to LPS for another 6 h. The relative protein expression of LGR4 and NF-κB p65 were measured by western blot. β-actin was used as an internal control. (c) Immunofluorescence staining was performed to identify the expression of p-p65 (×400), scale bar=50μm. Blue spots represent cell nuclei, and red spots indicate p-p65 staining. (d) The intensity of p-p65. Values are given as means ± SEM of three experiments (n=3). *P < 0.05; **P < 0.01 versus the control (6b and 6d) or mimic and LPS (6d) group; #P< 0.05; ##P < 0.01 versus the LPS group.
**Figure 7**

Inhibition of miR-34a suppresses inflammation in BENDs. (a) Cells were transfected with miR-34a inhibitor or negative control (inhibitor NC) for 24 h and then stimulated with LPS (1.0 ug/ml) for 6 h. The relative protein expression of LGR4 and NF-κB p65 were measured by western blot. β-actin was used as an internal control. (b) Gray values of LGR4 and NF-κB p65 protein were quantified by Image J software. (c, d) The secretion of IL-1β, IL-6 and TNF-α were detected by qRT-PCR. Values are given as means ± SEM of three experiments (n=3). *P < 0.05; **P < 0.01 versus the inhibitor NC (cells transfected with miR-34a inhibitor NC) or mimic NC (cells transfected with miR-34a mimic NC) group; #P < 0.05 versus the inhibitor group (7b and 7d); ▲p < 0.05 compared with the inhibitor group (7b).
Inhibition of miR-34a alleviates endometritis in mice. (a) Schematic illustration of intrauterine injection of miR-34a antagonir or negative control. (b) The mRNA level of miR-34a in mouse uterus tissues by qRT-PCR. (c, d) Western blot was used to determine the LGR4 protein level. Gray values of the indicated proteins were measured by Image J software. (e-f) Translocation of the p65 subunit from the cytoplasm into the nucleus was evaluated immunofluorescence (×400). Blue spots represent cell nuclei, and red spots represent p-p65 staining; scale bar=50 μm. (g) Effect of in vivo injection of miR-34a antagonir on the mRNA levels of L-1β, IL-6 and TNF-α in the uterus. Data are expressed as mean ± SEM of three independent experiments (n=3). *p < 0.05; **p < 0.01 versus blank group; #p < 0.05 compared with the LPS and antagonir NC group. Comparisons among multiple groups were analyzed by one-way analysis of variance with Bonferroni post-test.
IL-1β suppresses LGR4 expression by enhancing miR-34a. (a) BENDs were treated with recombinant IL-1β (0, 1, 10 ng/ml) for 12 h, then miR-34a transcription levels were analyzed by qRT-PCR. (b) Cells were transfected with miR-34a inhibitor or the negative control and then stimulated with recombinant IL-1β (10 ng/ml). The relative expression of miR-34a was normalized to U6 snRNA. (c) qRT-PCR assay was used to determine the LGR4 mRNA. (d) The expression of LGR4, p-p65 was determined using a western blot. (e) Gray values of the indicated proteins were measured by Image J software. (f) Translocation of the p65 subunit from the cytoplasm into the nucleus was evaluated immunofluorescence (×400). Blue spots represent cell nuclei, and red spots represent p-p65 staining; scale bar=50 μm. (g) The fluorescence intensity of p-p65. The integrated optical density (IOD) of DAPI was used as an internal control. The IOD and area of cells were measured by IPP 6.0 software, and the fluorescence intensity of p-p65 was expressed as IOD/area. Data are expressed as mean ± SEM of three independent experiments (n=3). *p < 0.05 versus the inhibitor negative control group (untreated with IL-1β); #p < 0.05 compared with the inhibitor group (untreated with IL-1β); ·p < 0.05 compared with the inhibitor group. Comparisons among multiple groups were analyzed by one-way analysis of variance with Bonferroni post-test.
Figure 10

Schematic diagram depicting the signaling pathways for miR-34a in the regulation of LPS-triggered inflammatory responses in bovine endometrial cell.

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