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RESEARCH ARTICLE

Molecular Mechanism of miR-184 Targets ELK1 to Regulate LPS-Induced Inflammation in Yak Endometrial Epithelial Cells

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ABSTRACT

Yak endometritis is a common postpartum disease that not only harms the reproductive health of yaks but also causes significant economic losses to yak farming. MicroRNAs (miRNAs) have been found to play vital roles in numerous inflammatory diseases. This study aims to investigate the role of miR-184 in modulating LPS-induced inflammation in the yak endometrial epithelial cells and to elucidate the underlying molecular mechanisms involved. Uterine tissues were obtained from three healthy yaks and three yaks diagnosed with endometritis, almost 21 days postpartum. The results demonstrated that inflammatory cell infiltration and structural damage of the endometrium in yak endometritis tissues, with upregulated levels of IL-6, IL-1β, TNF-α expression, and downregulation of miR-184. Similarly, in LPS-induced yak endometrial epithelial cells, miR-184 expression was significantly downregulated, while overexpression of miR-184 significantly declined the levels of IL-6, IL-1β, TNF-α expression and inhibited the activation of the MAPK signaling pathway. Moreover, silencing ELK1 yielded similar results while the targeting relationship between miR-184 and ELK1 was confirmed by dual luciferase reporter assay. Additionally, the overexpression of miR-184 was found to inhibit ELK1 expression. This work reveals the Latent regulatory mechanism of miR-184 in the inflammatory immune response of the yak endometrial epithelial cells and proposes that miR-184 might operate as a useful target for treating inflammation in the yak endometrial epithelial cells.

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INTRODUCTION

Endometritis is one of the most common diseases in dairy cows which is observed during the early postpartum period (Perez-Baez *et al.*, 2021). Clinical endometritis is characterized by the presence of purulent or mucopurulent discharge in the vaginal area at 21 days or later after the calving. Subclinical endometritis is diagnosed by the presence of neutrophils in endometrial biopsies or histological examinations without any clinical symptoms (Mandhwani *et al.*, 2017; Wang *et al.*, 2023). Bacterial contamination of the bovine uterus postpartum is a primary cause of postpartum endometritis in dairy cows (LeBlanc, 2023; Li *et al.*, 2023). The yak is a herbivorous animal that lives at altitudes ranging from 3,000 to 5,000 meters (Joshi *et al.*, 2020; Jing *et al.*, 2022) and postpartum endometritis is commonly observed in yaks which severely impacts their reproductive performance and poses a threat to the steady health and sustainable development of yak farming. Currently, antibiotics are the most commonly used treatment protocol for it, however, issues such as drug residue and bacterial resistance remain unresolved. Therefore, there is an urgent need to explore new therapeutic targets.

Bacterial lipopolysaccharide (LPS) can be recognized by mammalian cells as a signal of bacterial invasion, thereby initiating an innate immune response (Matsuura, 2013), Thus, it is regarded as a key factor in the onset of inflammation. LPS activates TLR4, which stimulates the NF-κB and MAPK pathways, bringing about the release of pro-inflammatory cytokines (Hu *et al.*, 2013). Therefore, LPS is commonly used to stimulate cells and establish inflammation models, including models of bovine endometritis (Huang *et al.*, 2022; Meng *et al.*, 2022).

MicroRNAs (miRNAs) are minute, non-coding endogenous RNAs composed of 19-25 nucleotides. miRNAs chiefly impact gene expression levels by targeting the mRNAs (Friedman *et al.*, 2009), any change in miRNA expression affects the degree of target regulation, thereby impacting cellular homeostasis (Carthew and Sontheimer, 2009; Macfarlane and Murphy, 2010). miRNAs are not only crucial factors in regulating several processes such as cell development, differentiation, and signal transduction (Ambros, 2004; Hwang and Mendell, 2006; Lee *et al.*, 2016), but also participate in modulating innate immune responses and inflammation (O'Neill *et al.*, 2011). They play a crucial role in pathogen clearance while ensuring the rapid restoration of homeostasis in the body (Nejad *et al.*, 2018).

miR-184 is a highly conserved miRNA that has evolved across the species, from fruit flies to humans. One study established an H9c2 cell model treated with H_2O_2 and found that inhibiting miR-184 regulates FBXO28, thereby suppressing oxidative stress and inflammation in cardiomyocytes (Zou *et al.*, 2020). A previous study found that during ocular *Chlamydia trachomatis* infection, the expression of miR-155 and miR-184 was inversely allied with the severity of conjunctival inflammation (Derrick *et al.*, 2016). These findings indicate that miR-184 participates in various inflammatory responses and plays an important role in regulating pro-inflammatory responses.

ELK1 consists of four domains, with domain C serving as a phosphorylation site for mitogen-activated protein kinase (MAPK), thereby playing a portion in activating transcription (Eggeling, 2018). Domain D serves as the binding site for MAPK (Whitmarsh *et al.*, 1995). Many investigations have demonstrated that ELK1 plays a vital part in regulating inflammatory responses. Liang *et al.* (2024) reported that knocking out ANXA3 blocks ERK/ELK1 signaling, thereby suppressing inflammation and apoptosis in sepsis-induced acute lung injury while Yu *et al.* (2021) found that upregulation of miR-150 resulted in reduced expression levels of its downstream target ELK1, which in turn alleviated inflammation in diabetic retinopathy. The above results indicate that miR-184 and ELK1 are closely associated with inflammation. However, the functions of miR-184 and its regulatory relationship with ELK1 in LPS-induced endometritis in yaks have not been studied. Therefore, this study reveals the regulatory functioning of miR-184 and ELK1 in LPS-induced inflammation in yak endometrial epithelial cells.

MATERIALS AND METHODS

Sample Collection and Source: Six female yaks, three healthy and three diagnosed with endometritis (all approximately 6 years old and in their second parity), were sourced from the Linhe slaughterhouse in Bayi District, Linzhi City, Xizang, around 21 days postpartum. Thereafter, cell cultures were performed with uterine tissues from healthy yaks for additional cellular experiments. Healthy yaks were characterized by the absence of evident systemic disease, the presence of clear uterine mucus secretions approximately 21 days postpartum, and a polymorphonuclear neutrophil (PMN) ratio of less than 18%. Diseased yaks were characterized by the absence of apparent systemic disease following parturition but with purulent uterine secretions and a PMN ratio exceeding 18% at about 21 days (LeBlanc *et al.*, 2002; Kasimanickam *et al.*, 2004).

Histopathological investigations: Uterine tissues from healthy yaks and those diagnosed with endometritis were fixed in 4% paraformaldehyde for 48 hours. The tissues from the uterine horns were subsequently embedded in paraffin, sectioned into 4-micron-thick slices, and stained with a hematoxylin-eosin staining kit for histological analysis. The pathological analysis of the uterine tissues was performed under an optical microscope.

Cell Culture and Inflammation Model Construction: Methods of yak endometrial epithelial cell culture was followed according to Wang *et al*. (2024). To induce inflammation, the cells were exposed to varying concentrations of LPS (0, 0.5, 1, 3, 5, and 10μg/mL) for 6, 12, and 24 hours. Cell viability was rated using the CCK-8 assay (Elabscience, China) to determine the optimal concentration and duration of LPS exposure that elicits inflammatory effects.

Fluorescence *In Situ* Hybridization: When the cells reached 80% confluence, they were fixed with an in-situ hybridization fixative (Zoonbio, China), and then digested with proteinase K at 40°C. Hybridization was performed using a probe for miR-184 (5'-ACCCTTATCAGTTCTCCGTCCA-3'), and cells were blocked with rabbit serum (Zoonbio, China) at ambient temperature. Subsequently, cells were incubated with anti-digoxigenin-HRP antibody (Jackson, USA) at 37°C, followed by incubation with TSA at ambient temperature and protected from light for 10 minutes. Finally, images were obtained using the fluorescence microscope.

ELISA: The levels of cytokine expression IL-6 (ml064296), IL-1β (ml064295), and TNF-α (ml077389) were measured using the relevant ELISA kits (mlbio, China) as per the protocol described by Yin *et al.* (2019).

miRNA Mimics, Inhibitors, and ELK1 Constructs: The miR-184 mimics, mimics NC, miR-184 inhibitor, inhibitor NC, and ELK1 overexpression (h-ELK1) and silencing sequences (si-ELK1), as well as h-NC and si-NC, were synthesized by Beijing Tsingke Biotech Co., Ltd. China.

Cell Transfection: The aforementioned plasmids were transfected into yak endometrial epithelial cells, following the manufacturer's instructions. After an incubation period of 72 hours, the transfected cells were processed based on the protocol provided by the apoptosis kit (Pricella, China), and the rate of apoptosis was assessed via flow cytometry (Beckman, USA).

Prediction of Target Genes and Dual-Luciferase Reporter Assay: The target genes of bta-miR-184 were identified through predictions by using the miRNA target prediction platforms, miRWalk and RNAhybrid, revealing binding sites between bta-miR-184 and ELK1. The pYr-MirTarget- vector plasmid was used, and double digestion was performed with XhoI and NotI. Two plasmids, pYr-MirTarget- Bos ELK1 -wt and pYr-MirTarget- Bos ELK1

-mut, were designed and synthesized by a biotechnology company (Fig. 8B). The luciferase reporter plasmids, together with miR-184 mimics and mimic NC, were co-transfected into HEK293T cells. Dual-luciferase activity was measured by a dual-luciferase reporter kit (Vazyme, USA).

RT-qPCR and Western Blot: RT-qPCR was performed using an ABI7500 system (Thermo Fisher, USA), with GAPDH and U6 serving as internal reference genes. Protein samples were separated by 10% SDS-PAGE. The membrane was blocked with 5% non-fat milk, specific primary antibody incubated overnight at 4°C. After washing, the membrane was incubated with secondary antibodies for one hour to allow for antigen-antibody binding. Imaging was performed using an automated chemiluminescence imaging system. Image analysis was carried out using ImageJ software with β-actin as the internal control (Zhang *et al.*, 2024).

Statistical Analysis: The consequences are presented as mean ± standard error. Group judgments were made using Student's t-test for two groups and one-way ANOVA for multiple groups. Statistical significance was defined as *P< 0.05, with **P<0.01 considered highly significant, indicating.

RESULTS

Expression Level of miR-184 is Downregulated in Endometrial Inflammation Tissue: Compared to healthy tissues, we observed significant inflammatory cell infiltration, severe congestion, and damage to the endometrium in the uterine tissues affected by endometrial inflammation (Fig. 1A, B). Subsequent RT-qPCR analysis revealed that the expression level of miR-184 (Fig. 1C) was meaningfully decreased in tissues exhibiting endometrial inflammation compared to healthy tissues (P<0.05). Additionally, the levels of IL-6, IL-1β, and TNF-α (Fig. 1D, E, F) were also significantly increased in the endometrial inflammatory tissues (P<0.05). These discoveries submit that the dysregulation of miR-184 expression is associated with the development of endometrial inflammation.

Downregulation of miR-184 Expression in LPS-Induced Endometrial Epithelial Cell Inflammation: To explore the expression of miR-184 in LPS-induced endometrial inflammation, we first isolated and cultured endometrial epithelial cells from healthy yaks. Cell viability was assessed using the CCK-8 assay across various LPS concentrations (0, 0.5, 1, 3, 5, and 10μg/mL) and exposure times (6, 12, and 24 h) (Fig. 2A). To evaluate the expression levels of IL-6, IL-1β, TNF-α, RT-qPCR was conducted following treatments with 1 μg/mL LPS for 12 h and 0.5μg/mL LPS for 24 h (Fig. 2B). The results revealed no considerable variations in the concentrations of IL-6 and IL-1β, and TNF-α between the control group and the group given 0.5μg/mL LPS for 24 hours. However, the levels of these cytokines were considerably downregulated in the 1μg/mL LPS for the 12 h group (P<0.05). Therefore, 1μg/mL LPS for 12 hours is the best concentration and time for modeling.

We did RT-qPCR and Western blot tests to check the levels of proteins like P-JNK, P-P38, P-ERK, and cytokines like IL-6, IL-1β, and TNF-α. The test results showed a significant increase in P-JNK, P-P38, and P-ERK levels (P<0.05) (Fig. 2D) confirming the activation of the MAPK pathway. The levels of IL-6, IL-1β, and TNF-α (Fig. 2C) also significantly upregulated (P<0.05). The RT-qPCR and in situ hybridization fluorescence results showed that miR-184 expression in the LPS group (Fig. 3A, B) was lower than that of the control group (P<0.05).

Effects of miR-184 on MAPK Pathway and Cytokine Expression Levels: We wanted to see how miR-184 affects the MAPK pathway and inflammation. So, we added miR-184 mimics and inhibitors to yak endometrial epithelial cells. Flow cytometry indicated that the apoptosis rate (Fig. 4) did not exhibit marked variations compared to the control group (P>0.05), which indicates that transfection with miR-184 mimics and inhibitors did not affect cell viability. RT-qPCR was employed to assess transfection efficiency. The results demonstrated that transfection with miR-184 mimics led to a significant increase in miR-184 expression (Fig. 5A) (P<0.01); conversely, after transfection with miR-184 inhibitors, miR-184 expression levels (Fig. 5B) were significantly decreased (P<0.05), confirming successful transfection. On the other hand, 72 hours after transfection with miR-184 mimics, the levels of IL-1β, TNF-α, and IL-6 reduced, while these cytokine levels (Fig. 5C, Fig. 6A) increased after transfection with the inhibitors. However, no distinct variations were noted (P>0.05). After the completion of the above transfections, cells were elicited by 1μg/mL LPS for 12 hours. IL-1β, TNF-α, and IL-6 levels (Fig. 5D, Fig. 6B) were significantly diminished in the miR-184 mimics-transfected cells, whereas their levels were significantly increased in the miR-184 inhibitor-transfected cells, which exacerbating the inflammation.

To study the role of miR-184 in MAPK pathway activation, proteins were extracted from the transfected experimental groups and investigated by Western blot. The observations indicated that the levels of expression of P-JNK, P-P38, and P-ERK in the miR-184 mimics-transfected cells, non-LPS-treated group (Fig. 7A) tended to decrease, while in the miR-184 mimics-transfected, LPS-treated group, the levels of expression of P-JNK, P-P38, and P-ERK (Fig. 7B) were meaningfully diminished (P<0.05), reflecting a marked reduction in their activation. These outcomes demonstrate that miR-184 mimics not only inhibit LPS-induced inflammatory cytokine production but also suppress the initiation of the MAPK signaling cascade.

ELK1 as a Functional Target of miR-184: Using online prediction databases miRWalk and RNAhybrid, we identified a binding site between ELK1 and miR-184, suggesting that ELK1 may be a potential target gene of miR-184 (Fig. 8A). To verify this interaction, we conducted a dual-luciferase reporter assay. The conclusions indicated that compared to the miR-184 mimics negative control group, the luciferase activity in the ELK1-WT+miR-184 mimics group was meaningfully lowered (Fig. 8C) (P<0.05), while the luciferase activity in the ELK1-MUT + miR-184 mimics group remained unchanged, indicating that ELK1 is a downstream target of

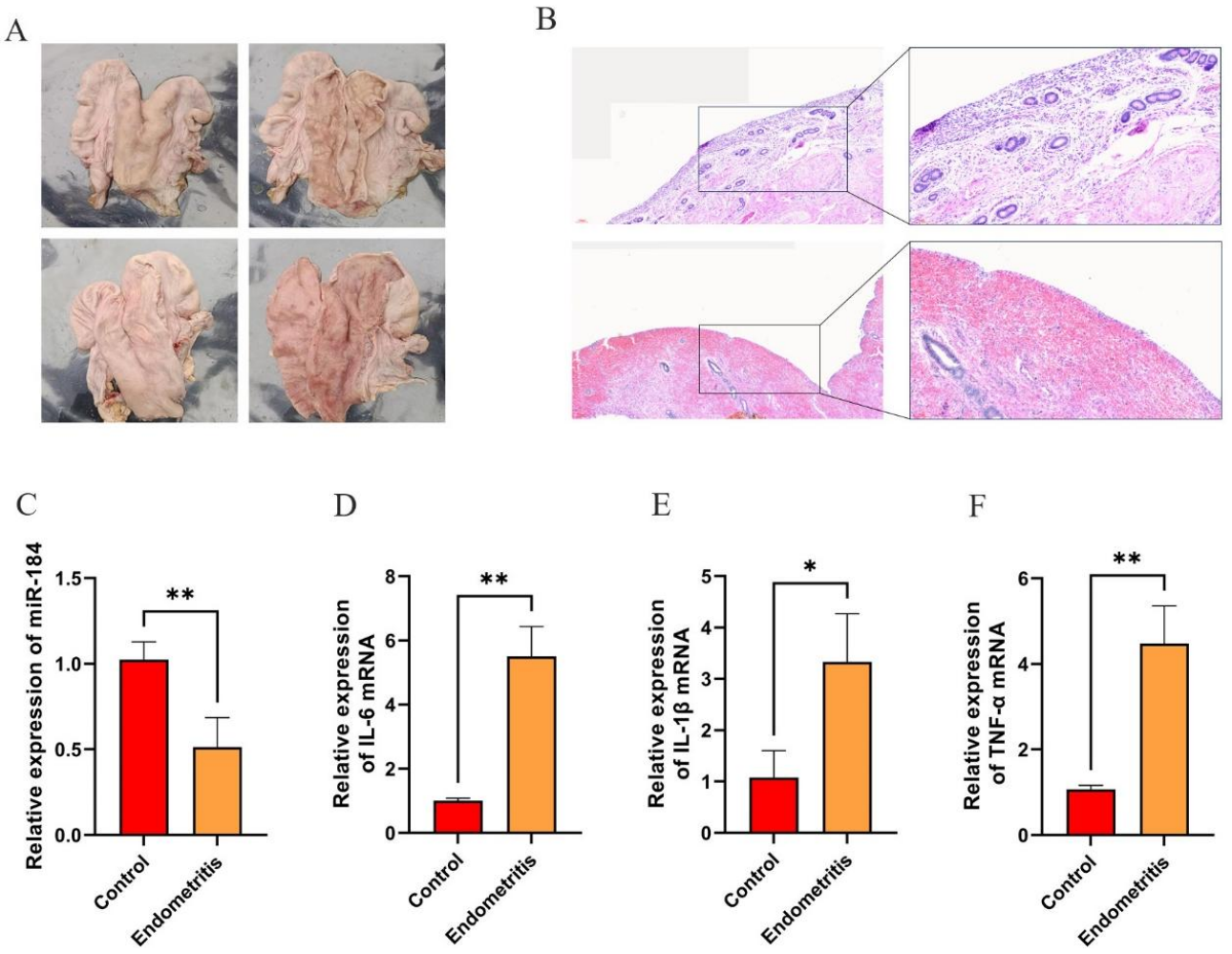

Fig. 1: Downregulation of miR-184 Expression in Endometrial Inflammation Tissue.

miR-184. To verify the regulatory relationship between ELK1 and miR-184, we transfected miR-184 mimics and inhibitors into yak endometrial epithelial cells. RT-qPCR and Western blot analyses exhibited that miR-184 overexpression generated a pronounced diminution in ELK1 expression ($P<0.05$), whereas inhibition of miR-184 resulted in increased ELK1 expression (Fig. 8D, E). This phenomenon was observed at both the mRNA and protein levels, suggesting that miR-184 likely exerts its effect at the transcriptional level.

Effect of ELK1 on MAPK Pathway and Cytokine Expression Levels: To examine the effect of ELK1 on the MAPK pathway and the expression of cytokines, we transfected yak endometrial epithelial cells with plasmids overexpressing ELK1 (h-ELK1) and silencing ELK1 (si-ELK1). After 72 hours of transfection, we assessed cell apoptosis rates by flow cytometry. No substantial difference in apoptosis rates was detected between the transfected cells and the control group (Fig. 9), which indicates that ELK1 overexpression and knockdown did not affect cell viability allowing us to proceed with further experiments.

Western blot analysis confirmed the transfection results, showing a significant decrease in ELK1 expression following si-ELK1 transfection (Fig. 10A) ($P<0.05$), and a significant increase in ELK1 expression following h-ELK1 overexpression (Fig. 10B) ($P<0.05$), indicating successful transfection. Furthermore, 72 hours after si-ELK1 transfection, the levels of IL-1β, TNF-α, and IL-6 (Fig. 10C, Fig. 11A), and P-JNK, P-P38, and P-ERK (Fig. 12A) showed a decreasing trend, whereas the levels of these cytokines increased following h-ELK1 transfection, though no noteworthy differences were detected ($P>0.05$). After the transfections, cells were stimulated with 1μg/mL LPS for 12 hours. The expression levels of IL-1β, TNF-α, IL-6 (Fig. 10D, Fig. 11B), and P-JNK, P-P38, and P-ERK (Fig. 12B) were meaningfully decreased in the si-ELK1 group ($P<0.05$).

DISCUSSION

Endometritis poses a substantial concern within the global cattle breeding industry. To minimize economic losses, antibiotics are often administered for the treatment purpose. However, the overuse of antibiotics and the subsequent emergence of resistant strains present new challenges in managing bovine endometritis. Studies have shown that miRNAs play a key character in controlling inflammation in many diseases. Our research suggests that miR-184 alleviates inflammation by influencing ELK1 it prevents the activation of the MAPK pathway and reduces the decrease of inflammatory cytokines. Nearly 90% of cows were infected with uterine inflammation after delivery, and one of the main bacteria causing endometritis is *Escherichia coli*. (Dohmen *et al.*, 2000). LPS can bind to

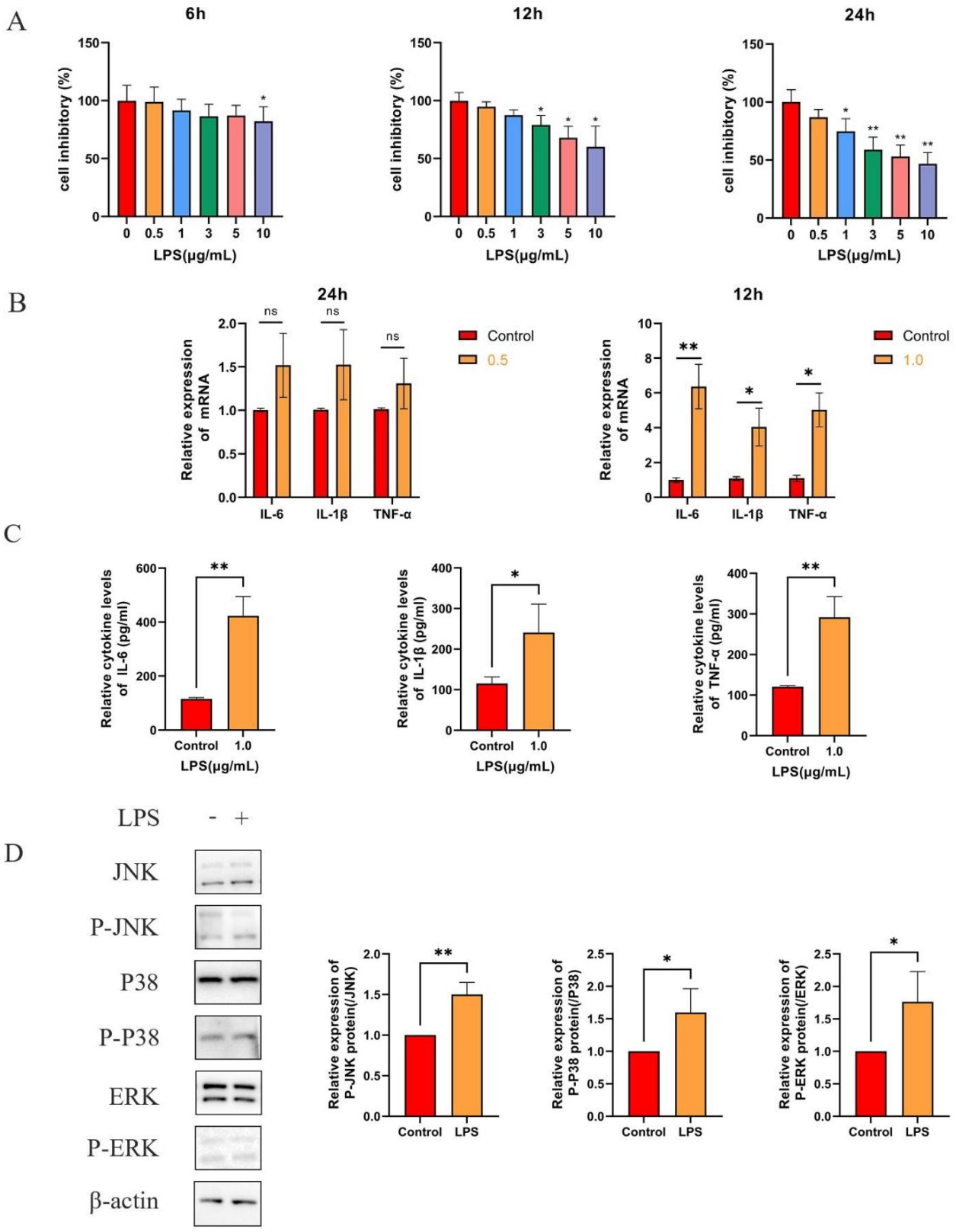

Fig. 2: Construction of the LPS Model and Upregulation of Pro-inflammatory Cytokines and MAPK Pathway-Related Factors.

TLR4 receptors, causing endometrial cells to release certain chemicals such as IL-6, IL-1 β, and TNF - α. LPS has been used to design different inflammatory models, such as acute kidney injury, mastitis, and endometritis (Yin *et al.*, 2019; Li *et al.*, 2022; Gong *et al.*, 2024). In this study, we induced yak endometrial epithelial cells with different concentrations of LPS and examined cell viability using the CCK-8 assay. The results showed that as the amount of LPS and the duration of cell exposure to LPS increased, the number of surviving cells significantly decreased ($P<0.05$). Then, we used RT qPCR and ELISA to measure the expression levels of IL-6, IL-1 β, and TNF - α. We found that exposure to 1μg/mL LPS for 12 hours significantly increased the levels of inflammatory cytokines ($P<0.05$) and ensured the normality of cell viability.

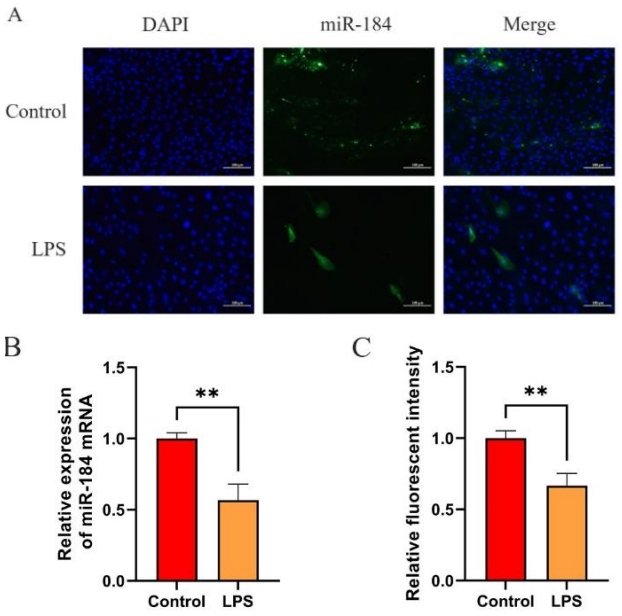

Fig. 3: Downregulation of miR-184 Expression in Endometrial Inflammation Tissue.

miRNAs play crucial roles in many cellular activities (Sun *et al.*, 2023; Wu *et al*., 2024). In LUAD, reducing the levels of miR-184, which targets C1QTNF6, slows the growth of these cells (Rao and Lu, 2022). Recent studies have shown that reducing miR-184 levels can reduce CaOx-induced inflammation, apoptosis, and oxidative stress in renal cells (Han *et al.*, 2023). However, we do not know what role miR-184 plays in yak endometritis. To explore the function of miR-184 in greater detail in yak endometritis, we obtained uterine tissues of healthy and endometritized yaks from slaughterhouses. H&E staining and RT-qPCR results confirmed the presence of inflammation in the tissues. Compared to healthy uterine tissues, miR-184 levels were significantly decreased in endometritis tissues. Furthermore, in an *in vitro* inflammation model of yak endometrial epithelial cells induced by LPS, we observed a significant downregulation of miR-184 levels. Transfection with miR-184 mimics led to a marked reduction in the levels of LPS-induced IL-6, IL-1β, and TNF-α. Furthermore, the expression levels of related proteins in the MAPK pathway were also notably downregulated, suggesting that overexpression of miR-184 can effectively inhibit the stimulation of the MAPK signaling pathway.

To investigate the molecular mechanism of miR-184 in LPS-triggered inflammation in yak endometrial epithelial tissue cells, we used online bioinformatics prediction tools miRWalk and RNAhybrid. Predicted outcomes showed that ELK1 might be a target of miR-184. We used a dual luciferase reporter gene assayto confirm that miR-184 targets ELK1. ELK1 is a transcription factor that is crucial in regulating cell growth, differentiation, and survival. It is vital for controlling cancer and inflammation (Kasza, 2013). ELK1 is highly expressed in various cancers, including gastric cancer, liver cancer, cervical cancer, endometrial cancer, and colorectal cancer (Zhang *et al.*, 2017; Huang *et al.*, 2019; Ma *et al.*, 2021). ELK1 is also a direct target of the MAPK pathway. When ELK1 is

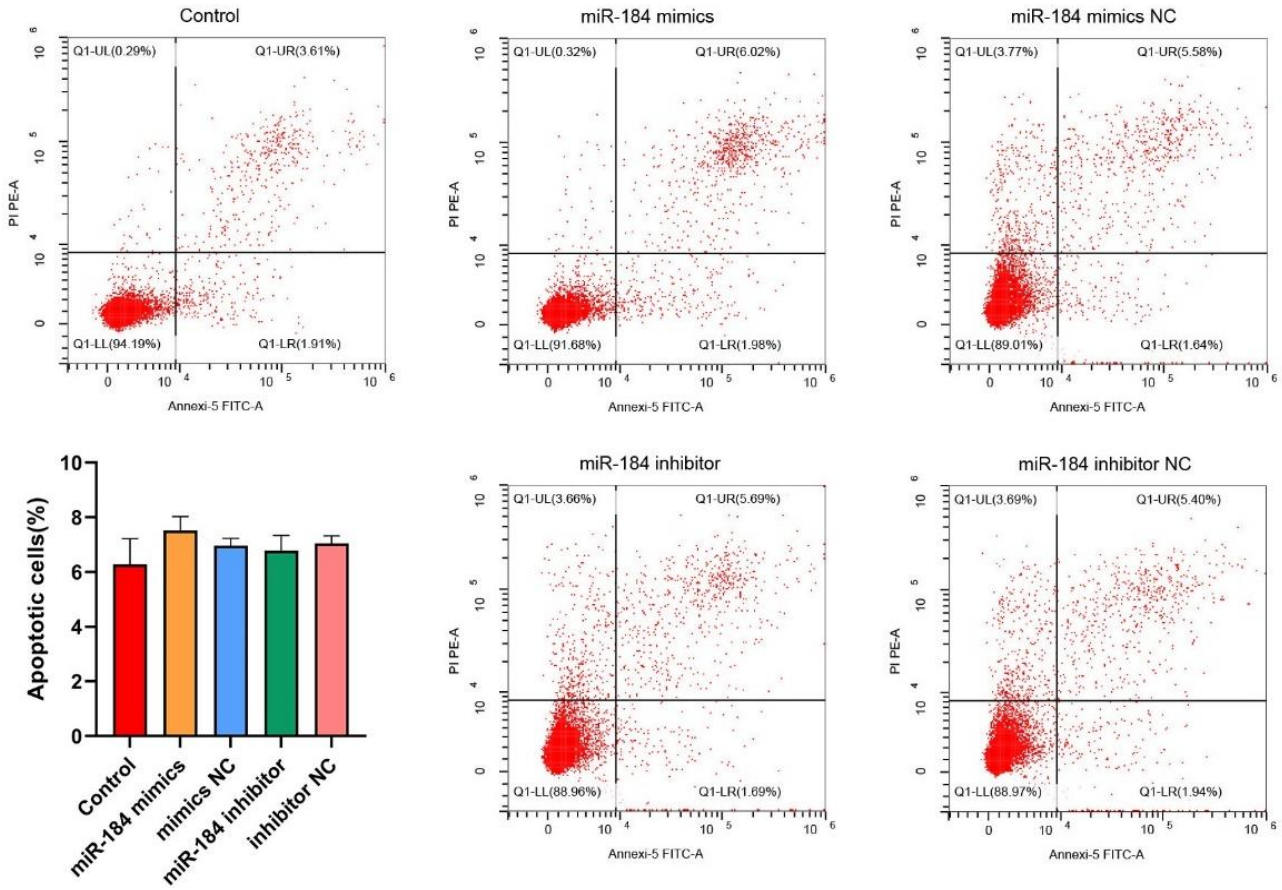

Fig. 4: Effects of apoptosis in yak endometrial epithelial cells transfected with miR-184.

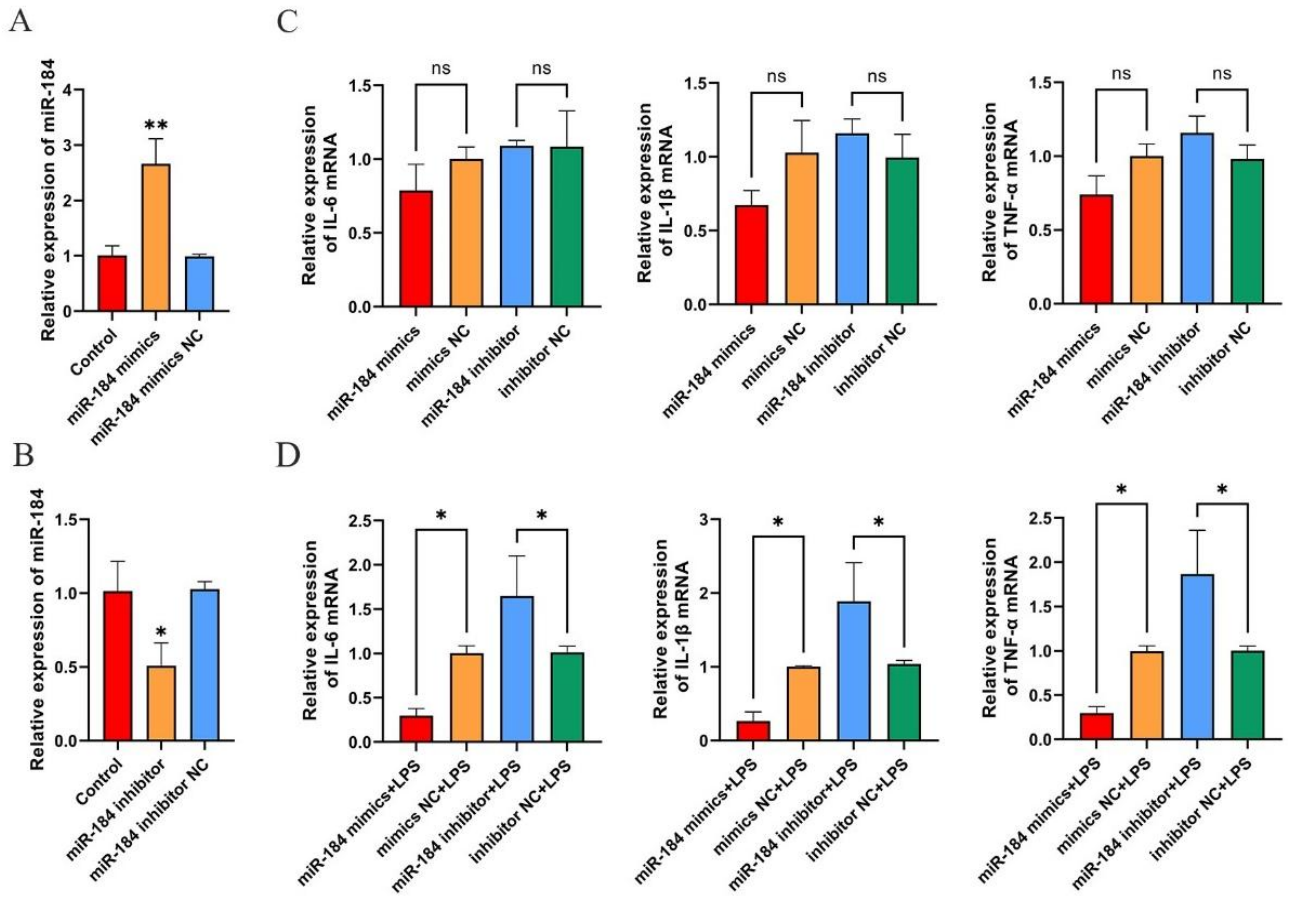

Fig. 5: Effect of miR-184 on Pro-inflammatory Cytokines.

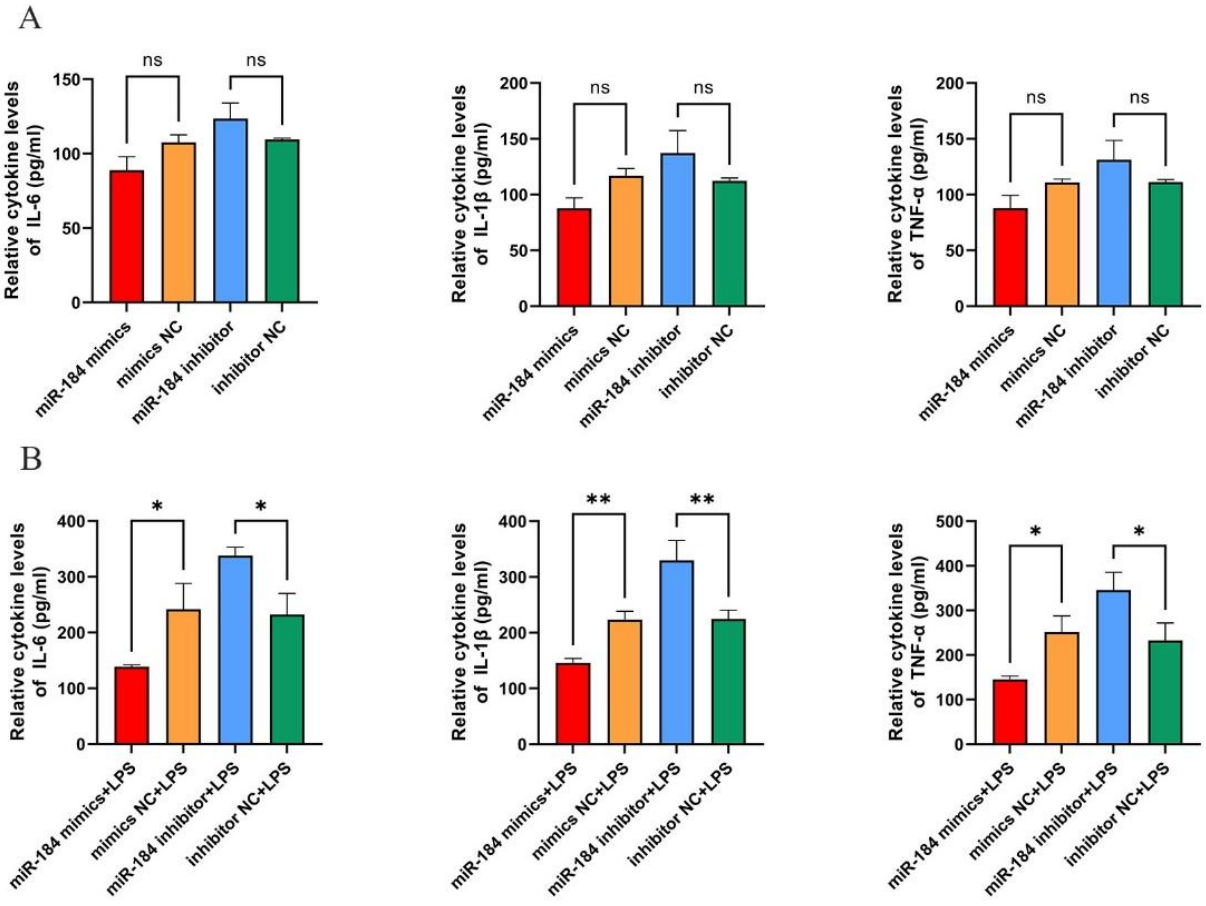

Fig. 6: Effect of miR-184 on Pro-inflammatory Cytokines.

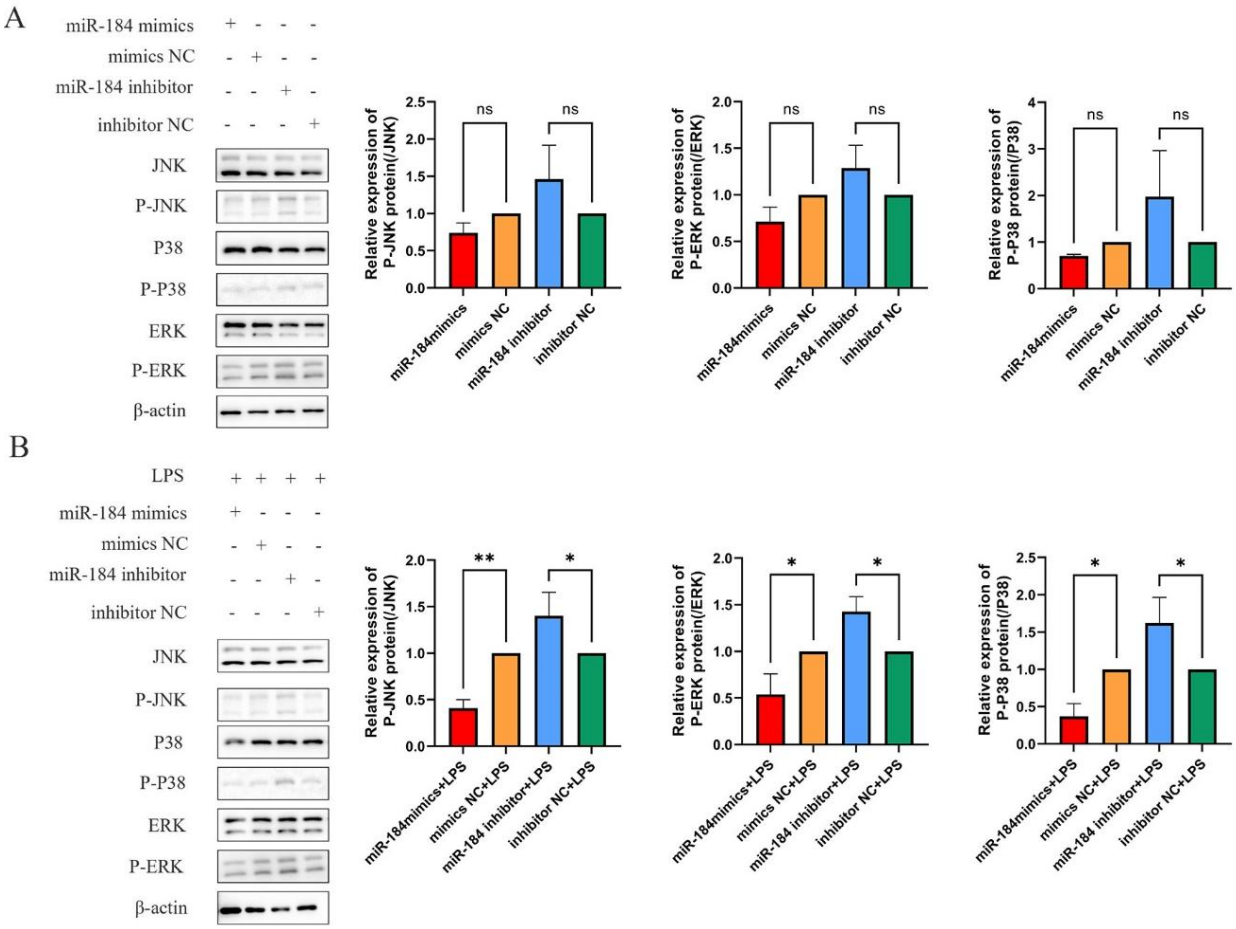

Fig. 7: Effect of miR-184 on MAPK Pathway Activation.

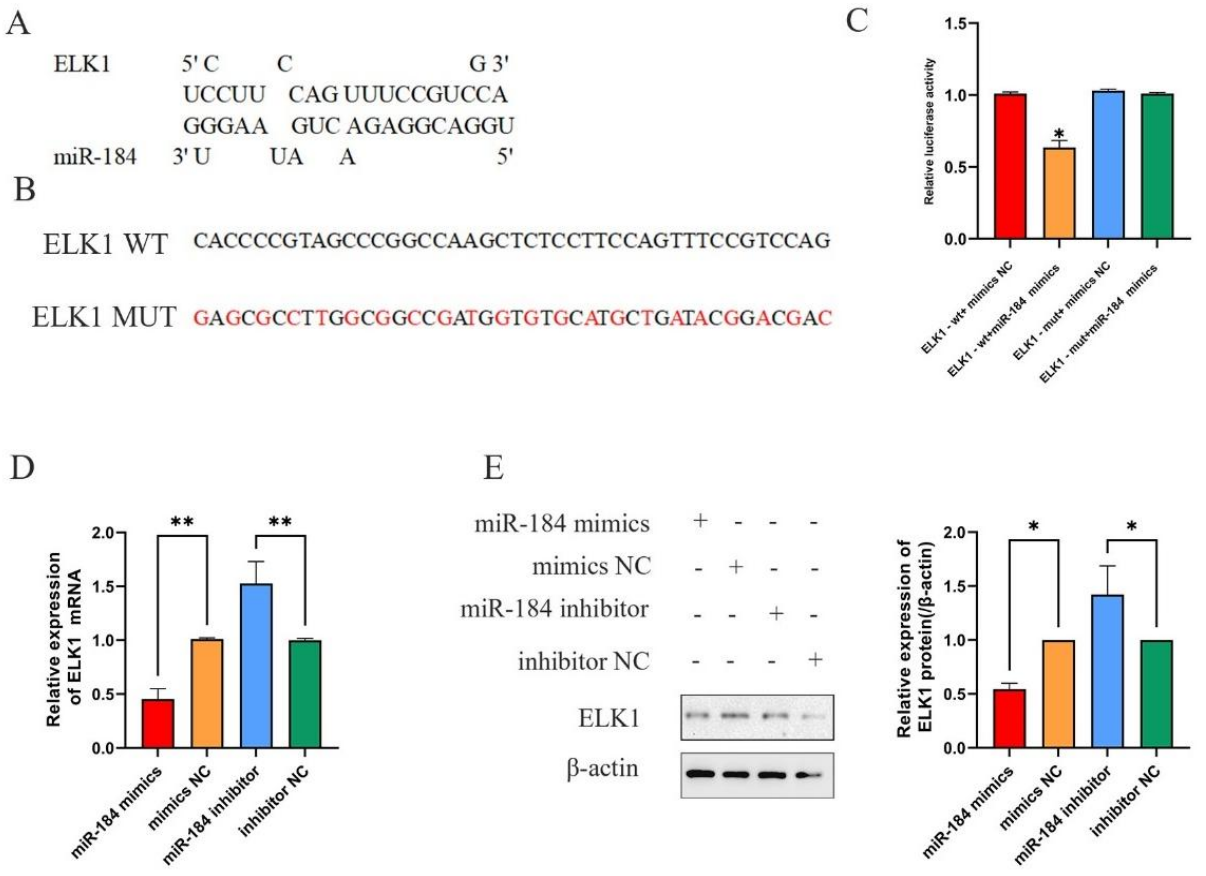

Fig. 8: ELK1 is a Functional Target of miR-184.

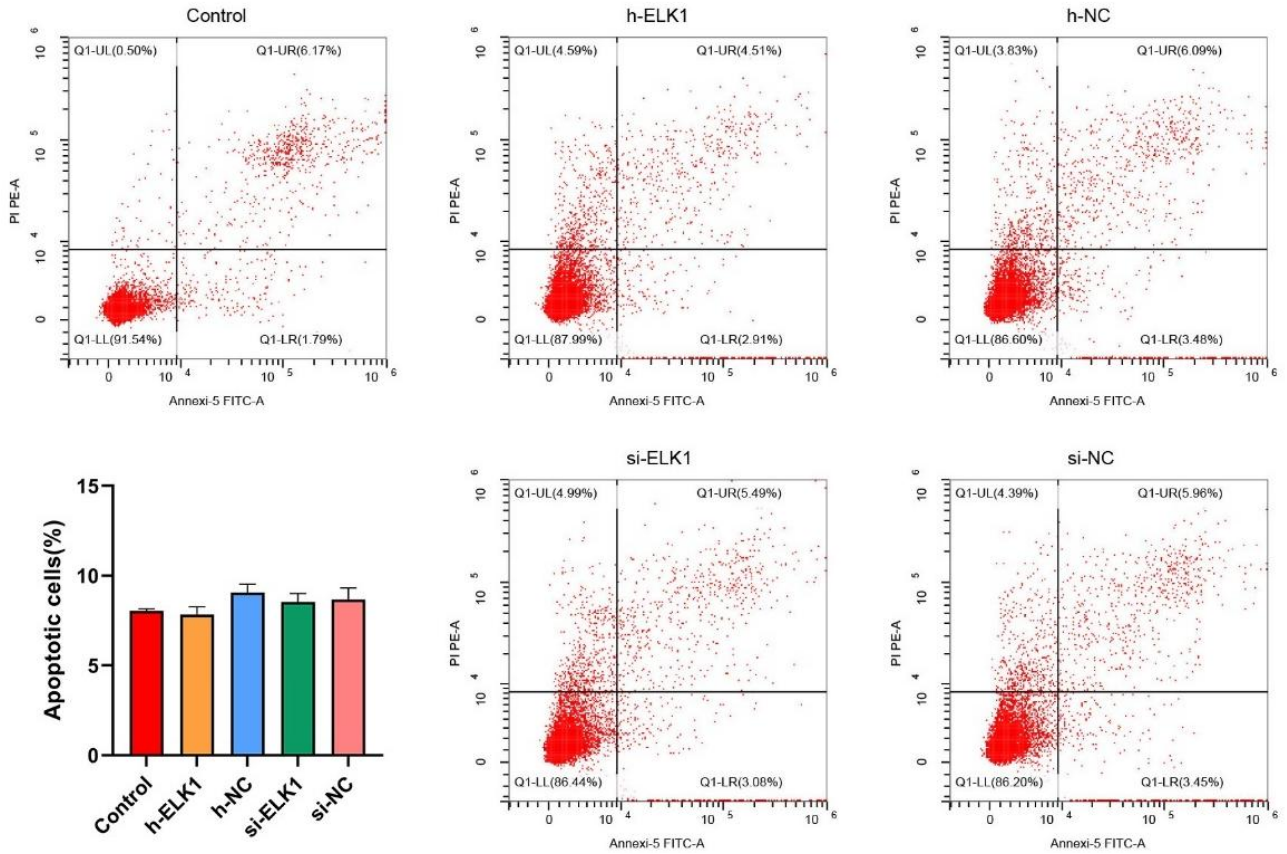

Fig. 9: Effect of apoptosis in yak endometrial epithelial cells transfected with ELK1.

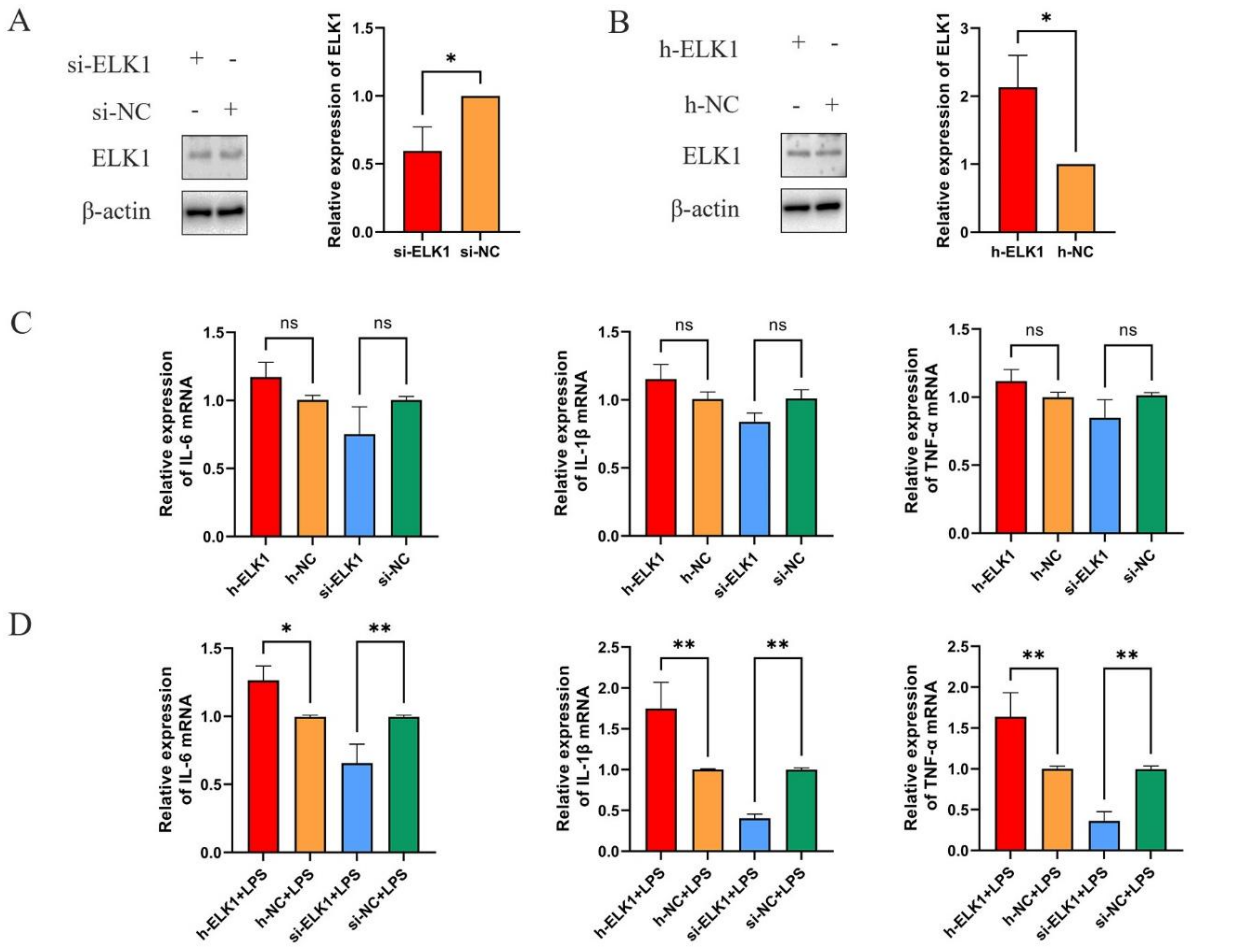

Fig. 10: Effects of ELK1 on pro-inflammatory cytokines.

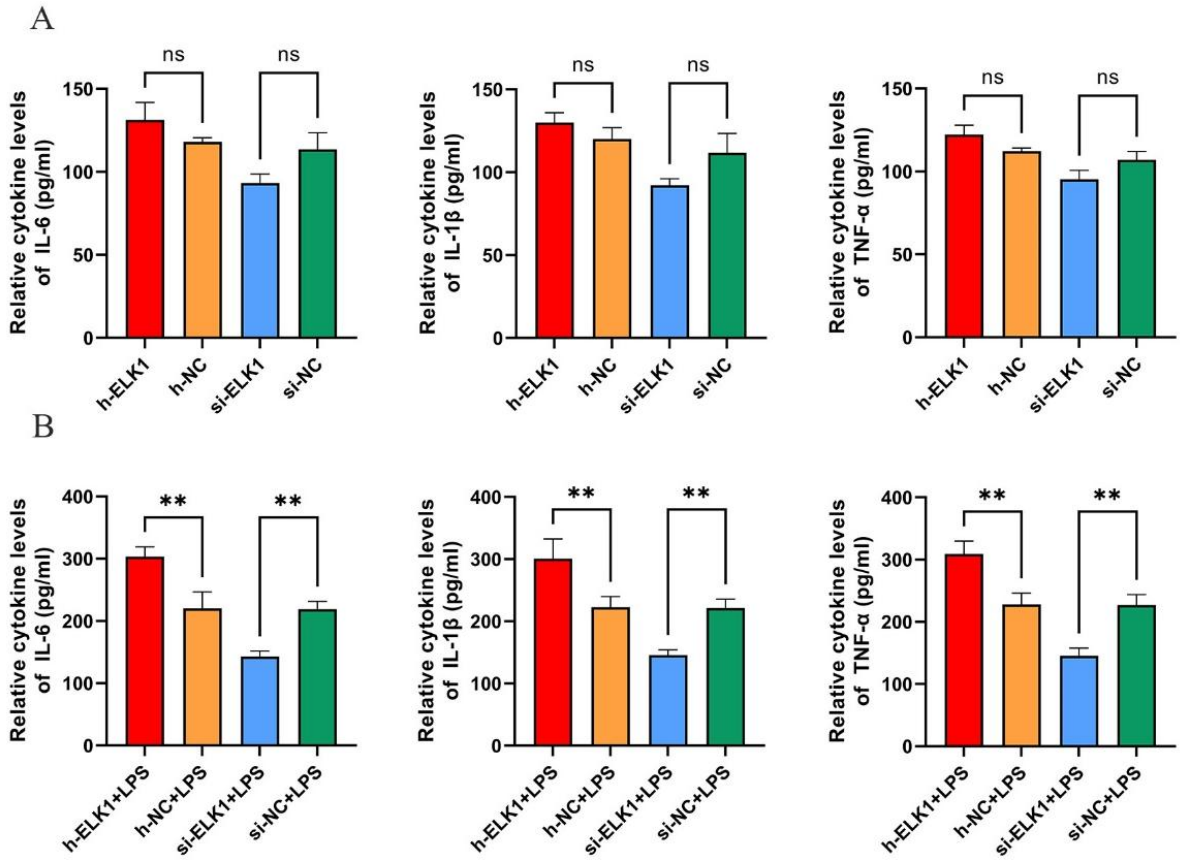

Fig. 11: Effects of ELK1 on pro-inflammatory cytokines.

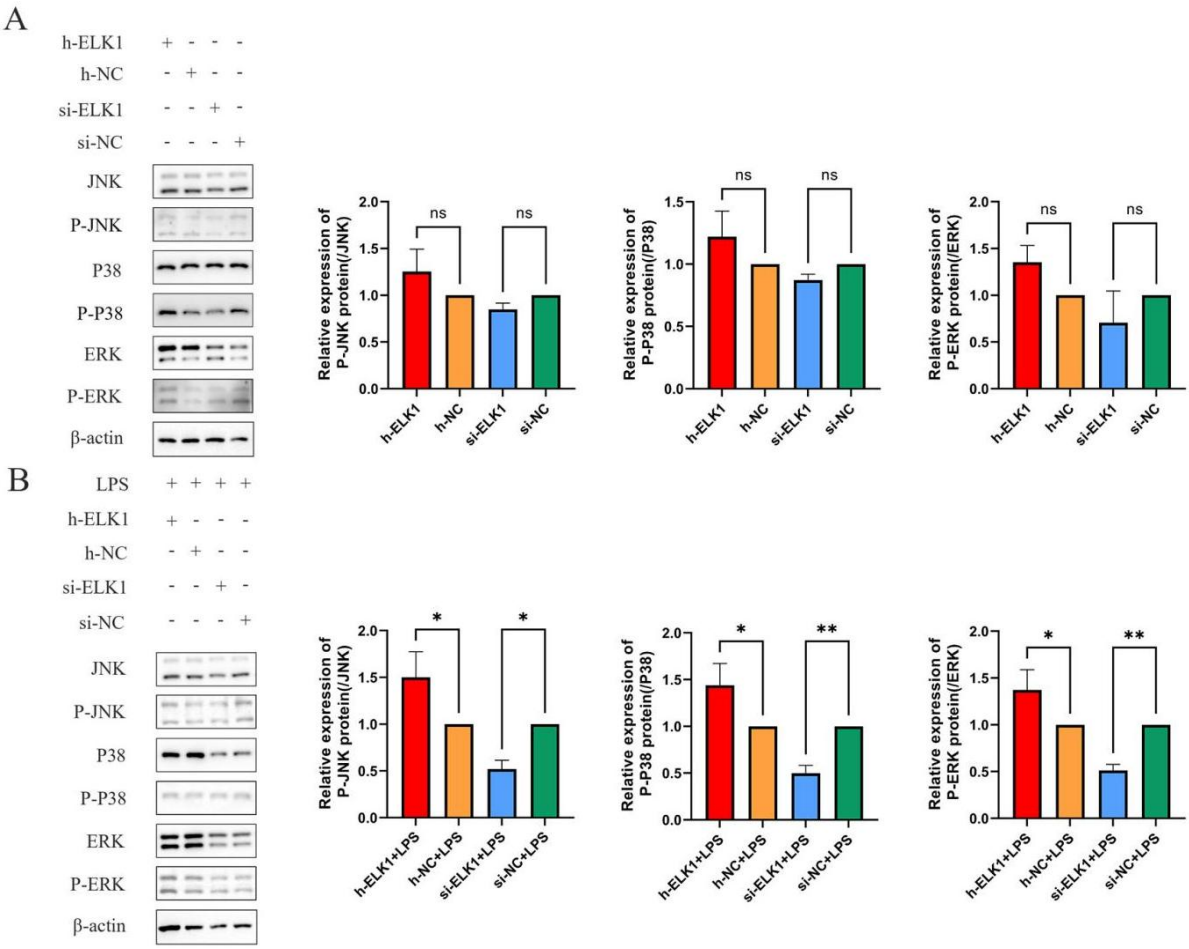

Fig. 12: Effects of ELK1 on MAPK pathway proteins.

reduced in acute lung injury, it lessens Th17 cell infiltration and lung damage in acute respiratory distress syndrome. It also helps to reduce damage caused by LPS in a model of pulmonary microvascular (Wei *et al.*, 2024). Flow cytometry results confirmed that transfection with either overexpressed or silenced ELK1 did not affect apoptosis rates. Therefore, we can take the next step in the experiment. When ELK1 was silenced in yak endometrial epithelial cells using si-ELK1, the levels of inflammatory factors decreased, and the phosphorylation of MAPK pathway-related proteins also decreased. Western blot results showed that overexpression of miR-184 significantly reduced ELK1 expression, signifying a negative regulatory relationship between miR-184 and ELK1. Therefore, the silencing of ELK1 mimicked the effects of miR-184 overexpression, suggesting that miR-184 exerts its anti-inflammatory effects on LPS-induced inflammation in yak endometrial epithelial cells by targeting ELK1.

Conclusions: In this study, we focused on elucidating the molecular mechanism by which miR-184 targets ELK1 in an in vitro model of LPS-induced inflammation. The outcomes indicate that in LPS-induced yak endometrial epithelial cell inflammation, overexpression of miR-184 can downregulate the expression of inflammation cytokines and inhibit the activation of the MAPK cascade. Additionally, high expression of miR-184 also suppresses the level of ELK1 expression. Furthermore, silencing ELK1 led to similar reductions in inflammation cytokines, confirming the results observed with miR-184 overexpression. In summary, miR-184 negatively regulates ELK1 and inhibits the initiation of the MAPK signaling cascade, Overexpression of miR-184 in LPS-induced yak endometrial epithelial cells effectively downregulates pro-inflammatory cytokines, thereby mitigating the inflammatory response by inhibiting the MAPK signaling pathway. This mechanism reduces the levels of key inflammatory mediators, providing evidence that miR-184 may offer a promising therapeutic approach for managing endometritis and related inflammatory conditions. Thus, miR-184 represents a novel potential target for future treatments to modulate inflammatory processes in yak and potentially other mammalian species.

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Ethics approval: All experiments were conducted under the guidance of the Animal Welfare and Research Ethics Committee of Xizang Agriculture and Animal Husbandry College in China.

Conflict of Interest: The authors declare that they have no competing interests.

Authors contribution: HD and QW provided the research idea. RL and SW performed the experiments. SW, XR, and MS wrote the manuscript. FSA handled the revision.

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