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Mechanism of collagen type IV regulation by focal adhesion kinase during retained fetal membranes in dairy cows

Chunhai Luo[1,3], Jinshui Chang[1,3], Weijia Yao[1], Weidong Qian[1], Yunlong Bai[1], Shixin Fu[1✉] & Cheng Xia[1,2✉]

Retained fetal membranes (RFM) is an important reproductive disease in dairy cows, caused by maternal and fetal placental tissue adhesion. The main collagen in maternal and fetal placenta tissues is collagen type IV (COL-IV) and its breakdown is the key to placental expulsion. Focal adhesion kinase (FAK) has been shown to regulate the hydrolysis of Col-IV by affecting the activity of MMP-2 and MMP-9 activity, but the regulation of the mechanisms involved in placenta expulsion in dairy cows after postpartum are still unclear. The aim of this study was to investigate the pathogenic mechanism of RFM by studying the relationship between the FAK signaling pathway and COL-IV regulation. Maternal placental tissues were collected from six healthy and six cows with RFM of similar age, parity, body condition and milk yield at 12 h postpartum. In vitro experiments were performed on bovine endometrial epithelial cells from three groups including a FAK inhibitor group, a FAK activator group and a control group without FAK inhibitor and activator. The abundance of molecules involved in the FAK signaling pathway and COL-IV was detected by immunohistochemistry, quantitative real-time polymerase chain reaction (qRT-PCR) and western blot. The immunohistochemical results showed that the key molecules of FAK signaling pathway FAK, Src, MMP-2 and MMP-9 and Col-IV were expressed in placental tissues. The expression level of FAK, Src, MMP-2, and MMP-9 were significantly down-regulated ($P < 0.05$) and the abundances of COL-IV were significantly up-regulated ($P < 0.05$) in maternal placental tissues of RFM cows compared with healthy cows. In the FAK inhibitor treatment group, the relative expression levels of FAK and other related proteins were significantly down-regulated ($P < 0.05$) and the relative expression levels of COL-IV were significantly up-regulated ($P < 0.05$) with the results of the FAK activation group the opposite. These results indicated that FAK in maternal endometrial epithelial cells could regulate the hydrolysis process of Col-IV through the expression of key factors of signaling pathways and promote collagen hydrolysis, which in turn facilitated the process of postpartum placenta expulsion in dairy cows.

Keywords Retained fetal membranes, Bovine endometrial epithelial cells, FAK, COL-IV, Dairy cow

In dairy cows, retained fetal membranes (RFM) are a common reproductive problem, where the fetal membranes cannot be naturally discharged from the body about 12 h after delivery[1]. Gohary et al. reported that each cow suffering from RFM will cause a loss of about 232 ± 58 dollars per year, for the dairy industry[2]. In addition, RFM can also induce other conditions including endometritis, mastitis and hoof leaf inflammation, which also affect the conception rate and milk yield of dairy cows[3]. In recent years, researchers have proposed many causative pathogenic factors for RFM, among which the hypothesis of adhesion between the placenta is generally accepted by scholars[4].

For the membranes of the fetus to be expelled, collagen is hydrolyzed between the fetal placenta and the maternal placenta. Sharpe et al. found that compared with healthy cows, the expression of collagen was higher in cows with RFM, suggesting that collagen regulated the separation process between uterus and placenta[5]. Collagen belongs to incomplete proteins and it does not contain tryptophan and cystine, but is rich in glycine,

[1]College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agriculture University, Daqing 16339, China. [2]Key Laboratory of Bovine Disease Control in Northeast China, Ministry of Agriculture and Rural affairs, Daqing 163319, China. [3]Chunhai Luo and Jinshui Chang contributed equally as the first authors. ✉email: fushixin@163.com; xcwlxyf2014@163.com

hydroxyproline, proline and lysine. The dense layer of the placental basement membrane is mainly composed of Collagen type IV (Col-IV)[6]. Col-IV is the first non-fiber-forming collagen, which provides structural support and regulates adhesion, migration and survival of cells[7]. Previous studies have reported that collagen plays an important role in RFM and its hydrolytic process is regulated by matrix metalloproteinases (MMPs).

Focal adhesion kinase (FAK) is an intracellular non receptor tyrosine kinase, located in the intercellular adhesion or intercellular matrix adhesion[8]. It is a multifunctional protein that integrates and transduces the signals sensed by integrin or growth factor receptor into cytoplasmic and nuclear reactions. Doherty et al. found that FAK played an extremely important role in the physiological processes of cell growth, development, apoptosis, adhesion, cytoskeleton reconstruction and tumor formation[9]. Marta Segarra et al. demonstrated that FAK in T lymphocytes affects the expression of MMPs belonging to zinc-dependent endopeptidase family, which is involved in the degradation of many proteins in extracellular matrix (ECM)[10]. In general, MMPs possess a propeptide sequence, a domain which catalyzes zinc metalloproteinases, a hinge or connecting peptide, and a Hopopexin domain[11], which are usually divided into collagenase, gelatinase, matrix protein, matrix protein, MT-MMPs and other MMPs according to their substrate and the organization of their structural domain. They are usually secreted in the form of inactive pro-MMP and are cut into active form by different proteases including other MMPs[12]. The degradation of ECM proteins (such as collagen and elastin) is caused by MPPs and may affect endothelial cell function, cell migration, proliferation, $Ca2+$ signaling, and contraction[12,13]. Given that FAK can regulate the activity of MMPs and participate in the decomposition process of collagen, it was hypothesized that FAK could participate in the process of placental exclusion in dairy cows during postpartum recovery.

Materials and methods
Sample collection
The procedures followed in this study were in accordance with the study protocol established by the Chinese Society of Laboratory Animals. Twelve Holstein cows were selected made up of six healthy cows and six with RFM, aged $5-6$ years, with an average weight of 570 ± 30 kg, a milk yield of 30 ± 3.5 kg who had previously calved three to four times. These cows were obtained from the experimental cattle farm of Jilin Agricultural University and parted into two groups, cows with normal fetal membranes discharged within 12 h after calving were the control group (N) and cows whose fetal membranes were not discharged within 12 h after calving were the experimental group (R). For the collection of Endometrial gangrene refer to our previous study[1,14,15]. In short, Endometrial gangrene was collected by sterile endometrial sampler at 12 h after delivery. Blood samples of 6 healthy cows and 6 RFM cows were collected at 6, 12 and 24 h after delivery.

Immunohistochemistry
Cow endometrium formed by dehydration and embedding in paraffin, preserved in 2.5% glutaraldehyde-polyoxymethylene solution, was prepared following routine procedures. Following routine procedures, paraffin sections were removed from the paraffin and immersed in distilled water. To ablate endogenous peroxidase, paraffin sections were rinsed in PBS-T, and then blocked with 3% peroxide-methanol at room temperature. Following this, the slides were rinsed with PBS for two min, blocked with 5% (w/v) BSA in PBS for half of hour, and then incubated overnight with rabbit polyclonal antisera. Anti-FAK, anti-Src (1:800, 1:3200, Cell Signaling Technology, MA, USA), anti-MMP-2, anti-Col-IV (1:500, 1;400, Abcam, Shanghai, China), and anti-MMP-9 (1:200, Cloud-Clone Corp, Wuhan, China) were diluted in PBS with 1% BSA. Closely followed by the slides were washed twice in PBS and then incubated with biotinylated anti-rabbit-IgG (1:100, Solarbio, Beijing, China) or anti-mouse-IgG (1:100, Solarbio, Beijing, China) for half of hours. The slides were then incubated with a preformed streptavidin/biotin-peroxidase (ABC) complex after being washed twice in PBS. Then washed in PBS, treated with DAB/ammonium nickel (III) sulfate-solution as above, and covered with a coverslip.

Culture of endometrial epithelial cells of bovine
We obtained a healthy uterus from a cow shortly after it had been slaughtered. We created primary cultures of endometrial epithelium cells using tissue explant adherents. In short, the caruncle tissue was cut into small sections and incubated in cell culture dishes with Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal calf serum (Cat: 11011$-$8611, Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China) and 1% penicillin/streptomycin (Cat: P1400, 10,000 IU/10,000 µg/mL; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at 37℃ in 5% CO2. Next, epithelioid cells were divided into two populations based on their enzyme sensitivity.

FAK inhibitor and activator treatment
Cells were seeded in a 6-well cell culture plate with RPMI-1640 containing 10% fetal calf serum without 1% penicillin/streptomycin and incubated at 37℃ in 5% CO2 for 12 h before treatment. For FAK inhibitor treatment, caruncular epithelial cells were divided into two groups, the FAK inhibitors group, where the cells were treated with 2.0 µM FAK inhabit 14 for 24 h and the control group whose cells were cultured in RPMI-1640 without FAK inhabit 14. For FAK activator treatment, FAK activator group had cells treated with 0.1 µM Angiotensin II for 24 h and control group had cells cultured in RPMI-1640 without Angiotensin II.

The qRT-PCR assay
The mRNA expression of COL-IV, Src, MMP-2, MMP-9 and FAK was detected by qRT-PCR. According to the manufacturer's instructions, total RNA was extracted from tissue samples and caruncular epithelial cells using TRIzol (Invitrogen Corporation, Carlsbad, CA, USA). In accordance with the manufacturer's instructions, RNA was reverse transcribed into cDNA using a reverse transcription kit (BioFlux, Hangzhou, China). The expression level of mRNA was detected using real-time polymerase chain reaction (PCR) analysis with SYBR

Green QuantiTect qRT-PCR Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) on the ABI PRISM 7000 quantitative real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). Gene-specific primers used for qRT-PCR are listed in Table 1. The relative expression levels were normalized to β-actin levels. The qRT-PCR was run at initial denaturation at 94 °C for two min, 30 cycles of amplification (denaturation at 94 °C for 30 s, annealing at 60 °C for 15 s, and extension at 72 °C for 30 s), and extension at 72 °C for 10 min. Primer sequences are described in the Supplementary Material The expression levels of genes were normalized to β-actin with the 2-ΔΔCt method.

Protein extraction and western blot analysis

The protein expression of FAK, Src, MMP-2, MMP-9 and COL-IV was detected by western blot. A commercial protein extraction kit (BestBio, Ltd., Shanghai, China) was used to extract cellular proteins from tissue samples and caruncular epithelial cells. Protein content was determined using the BCA Protein Assay Kit (Beyotime, Shanghai China). The protein (30 µg/lane) was separated in SDS-PAGE and electro-transferred onto polyvinylidene fluoride membranes. The membranes were blocked in bovine serum albumin/TBST buffer for 2 h. The blocked membranes were incubated overnight at 4℃ with primary antibodies against β-actin as the internal control (1:5,000, Abcam, Shanghai, China), FAK, Src (1:1,500, Cell Signaling Technology, Beverly, MA, USA), MMP-2, Col-IV (1:1,500, 1:1,000, Abcam, Shanghai, China), and anti-MMP-9 (1:1,000, Cloud-Clone Corp, Wuhan, China). The above antibodies both can react with proteins of cow origin. Membranes were washed three times and incubated with appropriate peroxidase-conjugated secondary antibody (1:5,000, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 45 min at room temperature. Immunoreactive bands were detected using an enhanced chemiluminescence solution (Pierce Biotechnology Inc, Chicago, IL, USA). Finally, the bands were visualized using a protein simple imager (Protein Simple, San Jose, CA, USA), and the intensities were quantified using Image J gel analysis software (representative images for each western blot result presented in Supplementary material).

Statistical analysis

Data are expressed as the mean ± standard error of mean (SEM) and analysed using SPSS (Statistical Package for the Social Sciences) 16.0 software (SPSS Incorporated, Chicago, IL, USA). Differences among groups were compared with one-way ANOVA. Compared to control group, a P value below 0.05 was considered significant, a P value below 0.01 was considered highly significant.

Results

Abundance of genes in the FAK signaling pathway in caruncle tissue of cows with RFM

The protein abundance of FAK, Src, MMP-2, and MMP-9 was lower and the expression of COL-IV was greater in cows with RFM than healthy cows (Fig. 1). The results of an in vivo study showed that the mRNA levels of FAK, Src, MMP-2, and MMP-9 ($P < 0.01$) were significantly down-regulated in RFM cows, compared to healthy cows, while COL-IV mRNA and protein levels were significantly up-regulated (Fig. 2).

Down-regulation of FAK expression could reduce the expression of related signaling pathway molecules and increase the abundance of COL-IV

The mRNA abundances of FAK, Src, MMP-2, and MMP-9 ($P < 0.01$) were significantly lower in the FAK inhibitor group than that in the control group, but the mRNA abundances of COL-IV ($P < 0.01$) were significantly up-regulated (Fig. 3). Treatment with FAK inhibitor sharply decreased the protein abundance of FAK ($P < 0.05$), Src ($P < 0.05$), MMP-2 ($P < 0.05$) and MMP-9 ($P < 0.01$). In contrast, FAK inhibitor treatment sharply increased the protein abundance of COL-IV ($P < 0.05$, Fig. 3).

Gene	Sequences (5 '-3')	Gene ID	Leng(bp)
FAK	forward: TCACACGCCGAGTTCAAGTA	506270	115
	reverse: TGGCTCACTGTTGCTTTCAA		
Src	forward: TCACATTCAGATGGCTGCAA	535742	116
	reverse: AGCCTGGATGGAGTCAGAGG		
MMP-2	forward: AGCCTGGATGGAGTCAGAGG	282872	125
	reverse: GTGCAGCTCTCGTGCTTGTT		
MMP-9	forward: CCATCGCGGAGATTAGGAAC	282871	117
	reverse: CAGGCCACTTGCTCTTGACA		
COL-IV	forward: CGTGCCACTACTACGCGAAC	282191	93
	reverse: CCTTGAGTGTGTCGGCAGAG		
β-actin	forward: GTCCACCTTCCAGCAGATGT	280979	90
	reverse: CAGTCCGCCTAGAAGCATTT		

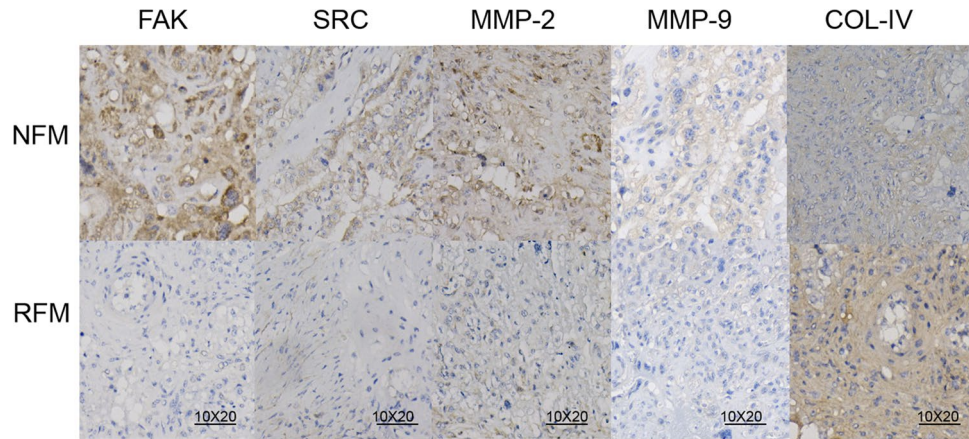**Table 1.** Sequence (5' to 3') of the primers that were used in the PCR.

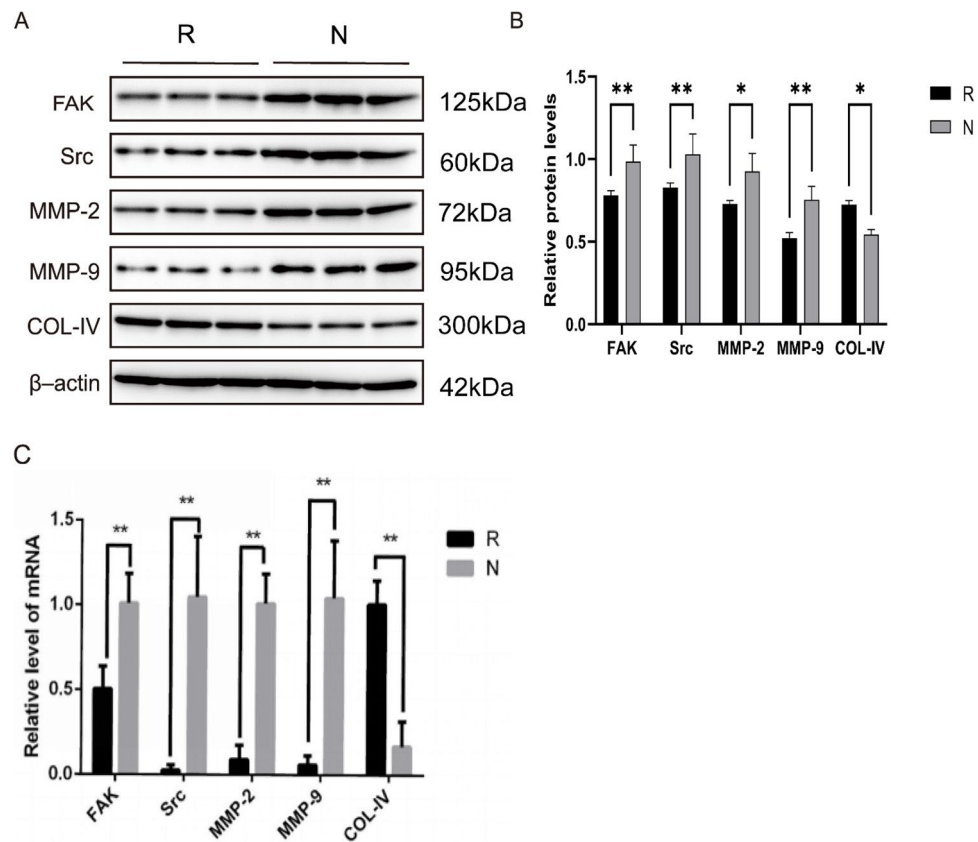**Figure 1.** Expression of FAK, Src, MMP-2, MMP-9 and COL-IV in maternal placenta. Note: NFM=6, RFM=6.

Figure 2. Results of relative mRNA and protein abundance of FAK, Src, MMP-2, MMP-9 and COL-IV in RFM and healthy dairy cows. Note: (**A**) Western blot analysis of FAK, Src, MMP-2, MMP-9 and COL-IV. (**B**) Protein expression of FAK, Src, MMP-2, MMP-9 and COL-IV. (**C**) mRNA expression of FAK, Src, MMP-2, MMP-9 and COL-IV. R: RFM cows. N: healthy dairy cows, N=3, RFM=3. Groups were compared using one-way ANOVA with a Duncan correction. This experiment was repeated 3 times, and the data presented are the mean ± SEM; * $P \leq 0.05$, ** $P \leq 0.01$. Results of mRNA and protein abundance of FAK, Src, MMP-2, MMP-9 and Col-IV in cow endometrial epithelial cells after 24 h of FAK inhibit 14 treatment (**A** and **B**: protein abundance level after FAK inhibit 14 treatment; **C**: mRNA expression level after FAK inhibit 14 treatment).

Up-regulation of FAK expression can increase the expression of related signaling pathway molecules and attenuate the abundance of COL-IV

The mRNA abundance of FAK, Src, MMP-2 and MMP-9 ($P < 0.01$) was significantly increased in the FAK activator group compared with the control group, while the mRNA abundances of COL-IV ($P < 0.01$) was
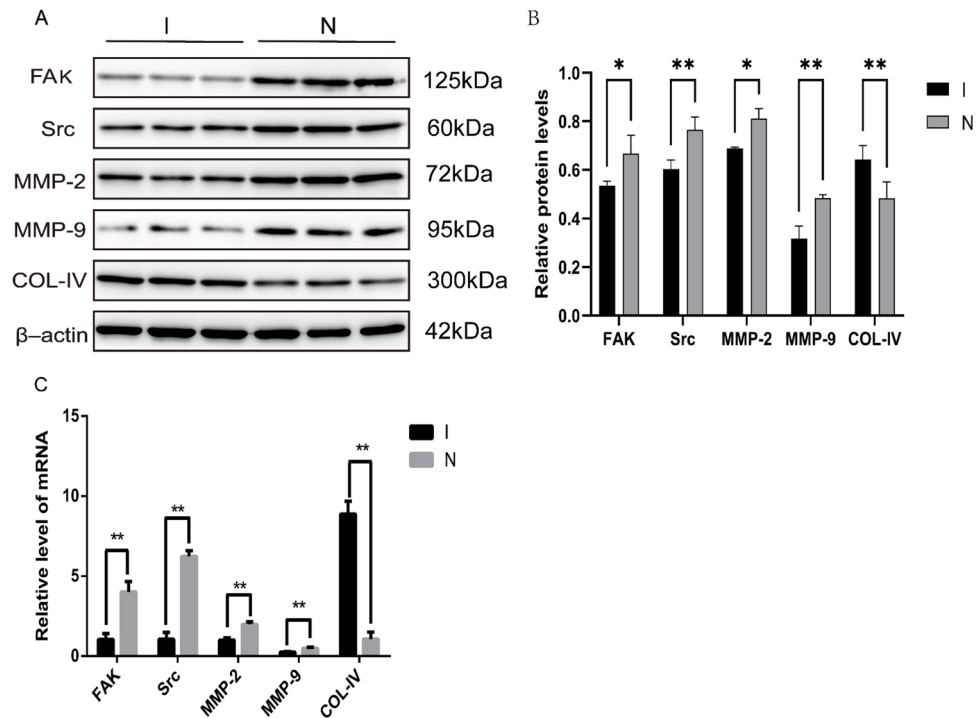
Figure 3. The effect of FAK inhibitor (FAK inhabit 14) on the relative expression of mRNA and protein in the bovine endometrial epithelium cells. Note: (**A** and **B**) protein abundance level after FAK inhabit 14 treatment; (**C**) mRNA expression level after FAK inhabit 14 treatment. I: inhibition group, N: control group, I=3, N=3. Groups were compared using one-way ANOVA with a Duncan correction. This experiment was repeated 3 times, and the data presented are the mean ± SEM; * $P \leq 0.05$, ** $P \leq 0.01$.

significantly down-regulated (Fig. 4). Compared with the control group, FAK activator treatment increased the protein abundance of FAK ($P < 0.05$), Src ($P < 0.05$), MMP-2 ($P < 0.01$) and MMP-9 ($P < 0.05$). In contrast, the protein abundance of COL-IV ($P < 0.05$) was markedly decreased in the FAK activator group.

Discussion

Compared to other animals, cows are more likely to suffer from RFM because of the unique cotyledon and caruncle structure. It is one of the main causes of RFM that collagenase and hyaluronidase fail to degrade the extracellular matrix of the placenta. As important collagenases, MMP-2 and MMP-9 are involved in the breakdown of extracellular matrix components such as collagen[16–19]. Alternatively, the activation of MMP-2 and MMP-9 is regulated by FAK through a series of cascades[20,21]. According to these findings, abnormal FAK concentrations are crucial to understanding RFM's mechanisms. This study suggested that the greater abundance of FAK in cows with RFM proved its involvement in the induction of RFM. The in vitro data revealed that the protein and mRNA expression of Col-IV was increased. Those responses were further accompanied by decreased expression of Src, MMP-2 and MMP-9, so data from the present study extended the study of processes related to RFM development in dairy cows by Zheng et al[1].

Placenta discharge is a complex process influenced in part by gene expression. This process involves loss of cell-cell junctions and cell-extracellular matrix (ECM) interactions, acquisition of migratory capacity, matrix metalloproteases (MMPs) secretion and degradation of ECM which leads to placenta discharge[22].

Cytoplasmic tyrosine kinase FAK plays a key role in the signal transduction of integrins and also in other cell surface receptor signaling[23]. During integrin-mediated cell adhesion, FAK is activated by disrupting its central kinase domain and amino-terminal FERM domain's autoinhibitory intramolecular interaction[24,25]. As FAK becomes activated, it forms a complex with kinases from the Src family to activate downstream signaling pathways. The FAK complex activates multiple downstream signaling pathways by phosphorylating other proteins that influence diverse cellular functions. A study of transgenic mice has suggested that FAK regulates cell invasion and migration genes through direct or indirect regulation[26]. It was demonstrated by Sanchez et al. that FAK directly interacted with and phosphorylated the actin regulatory protein N-WASP, which in turn promoted cell migration[27]. Previous studies demonstrated that activation of FAK gene increased MMP-2 expression in distinct types of cells[28]. However, there have been scarce data describing the regulatory network effects of FAK on the expression of collagen hydrolysis gene in dairy cows, so in this study, bovine endometrial epithelium cells were cultured in vitro. This study evaluated the effect of FAK on downstream genes Src, MMP-2, MMP-9 and Col-IV using the chemical inhibitor and activators FAK inhabit 14 and Angiotensin II. Inhibition or activation of FAK could lead to corresponding alteration in downstream genes, which in turn affected collagen hydrolysis.
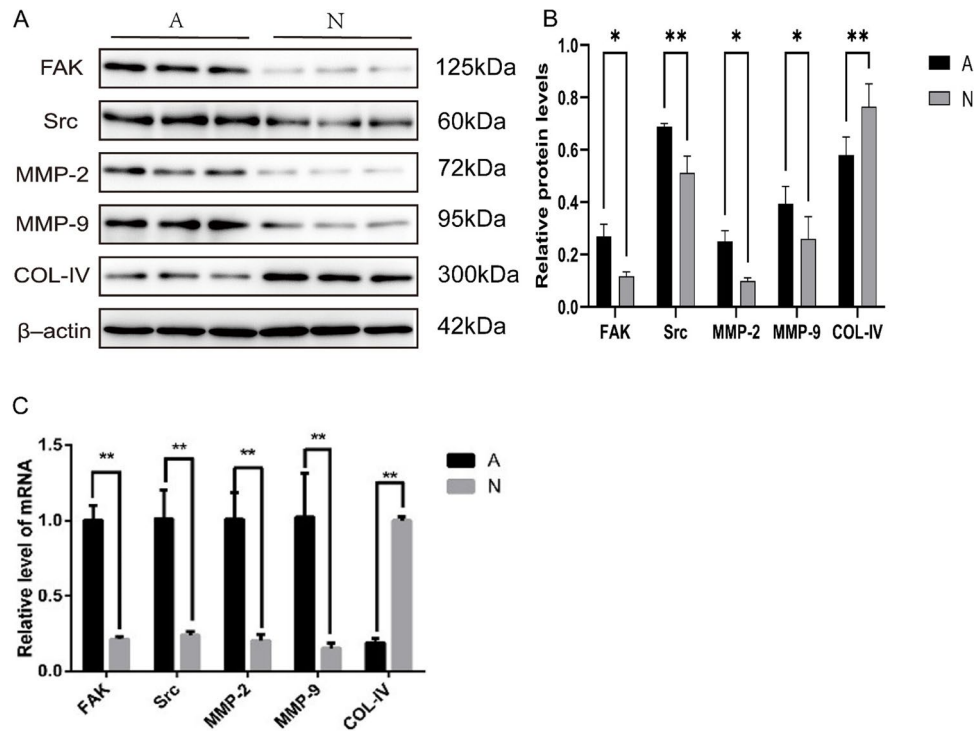
Figure 4. The effect of FAK activator (angiotensin II) on the relative expression of mRNA and protein in the endometrial epithelium cells of dairy cows. Note: (**A** and **B**) protein abundance level after angiotensin II treatment; (**C**) mRNA expression level after angiotensin II treatment. A: activation group, N: control group, A=3, N=3. Groups were compared using one-way ANOVA with a Duncan correction. This experiment was repeated 3 times, and the data presented are the mean $\pm$ SEM; * $P \leq 0.05$, ** $P \leq 0.01$.

According to previous studies, FAK is a key regulator of adhesion, and the exact mechanism of activation of adhesion signaling pathways has not been studied for many years. Studies including this one has shown that FAK played a crucial role in vertebrate cell adhesion and acted as an upstream regulator of collagenase, participating in the hydrolysis of extracellular matrix. Additionally, Chia-Ming Yeh showed that inhibition of the FAK/Src pathway inhibited the activation of MMP-2 and MMP-9[29]. In the present study, FAK activation increased the abundance of Src, MMP-2 and MMP-9 and decreased the abundance of COL-IV, which is consistent with the experimental results of Hiroaki Kawan in rat experiments[30]. The fact that FAK abundance altered the abundance of its downstream associated molecules and COV-IV led this study to hypothesize that FAK partially mediated the occurrence of RFM induced by abnormal collagen hydrolysis. Although the results of this experiment are significant, several limitations must be acknowledged. Without capturing what is occurring in the maternal tissues places an artificial constraint on what is happening at the fetal maternal interface during parturition. Additionally, the activity and spatial distribution of focal adhesion kinase (FAK) were not assessed, leaving a gap in determining the specific sites of FAK activity. Furthermore, the small sample size necessitates validation and expansion through larger-scale studies in future research.

Conclusions
This study provided the first evidence that the FAK regulated the abundance of Col-IV, influencing RFM in cows. The results demonstrated that FAK regulated the changes of Src, then regulated the expression of MMP-2 and MMP-9, which affected the hydrolysis process of Col-IV.

Data availability
Data is provided within the manuscript or supplementary information files.

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Institutional Review Board Statement

The Animal Ethics committee of Heilongjiang Bayi Agricultural University approved the experiment (20210022, Approval date: 3 May 2021). All methods were reported in accordance with ARRIVE guidelines.

Author contributions

Funding

Declarations

Competing interests
The authors declare that they have no competing interests.

Additional information
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Correspondence and requests for materials should be addressed to S.F. or C.X.

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