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Authors' contributions Please specify the authors' role using this form.	Jiahe Guo: designed and performed most of the experiments. Jingjing Guo, Zhibo Wang and Zhiqing Yang: assisted in sample collection and analyzed the data. Guomin Zhang and Mingtian Deng: interpreted the data. Jiahe Guo drafted the manuscript with input from all the authors. Feng Wang: supervised the study and administrated the project. All authors read and approved the final manuscript.
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Abstract

The epithelial sodium channel (ENaC) plays a crucial role in reproductive physiology, yet its specific contributions to sheep endometrial function and embryo survival remain poorly understood. This study investigates the expression and functional significance of ENaC subunits in the endometrium of Hu sheep, focusing on their role in prostaglandin E2 (PGE2) synthesis. Quantitative real-time PCR and immunofluorescence staining revealed that SCNN1A, an ENaC subunit, is significantly upregulated in the endometrium of high-fertility Hu sheep during the luteal phase, while SCNN1B and SCNN1G expression remained unchanged. Notably, all three ENaC subunits (SCNN1A, SCNN1B, and SCNN1G) were localized in the uterine glandular epithelium. In vitro experiments using endometrial epithelial cells (EECs) demonstrated that trypsin activates ENaC, leading to increased intracellular calcium levels and enhanced PGE2 synthesis through the upregulation of CREB1 and PTGS2. This effect was abolished by ENaC inhibition, and neither mRNA nor protein levels of SCNN1A, SCNN1B, or SCNN1G were altered by trypsin treatment, indicating that ENaC activation, rather than expression changes, mediates PGE2 production. RNA knockdown experiments further highlighted the pivotal role of SCNN1A in trypsin-induced calcium influx and PGE2 synthesis, with SCNN1A knockdown significantly attenuating these processes. Overexpression of SCNN1A enhanced calcium influx and PGE2 production via the Ca^{2+}/P-CREB/PTGS2 signaling pathway. In vivo studies on Hu sheep with varying embryo survival rates revealed significantly higher uterine PGE2 concentrations and SCNN1A expression in high-survival rate groups, suggesting a correlation between SCNN1A expression, PGE2 synthesis, and embryo survival. These findings underscore the critical role of SCNN1A in

regulating trypsin-mediated PGE2 synthesis and its impact on embryo survival, providing new insights into the molecular mechanisms underlying fertility in Hu sheep

Keywords: Embryo survival; PGE2; Ca^{2+}/P-CREB/*PTGS2*; Fertility; Endometrial epithelial cells.

Introduction

The decline in livestock fertility can be attributed to various factors, among which endometrial function plays a crucial role in sustaining fertility (1, 2). Hu sheep, a high-reproductive breed native to China, are renowned for their high ovulation rates and multiple births, with an average lambing rate of up to 230% in multiparous individuals(3-5). However, despite these remarkable reproductive characteristics, a significant proportion of Hu sheep give birth to singletons. This phenomenon may be linked to reduced fecundity due to embryo loss, a condition influenced by alterations in uterine function(6).

In sheep, the function of the uterine endometrium is directly reflected in its secretion of various nutrients during early pregnancy to support embryo development(7-9). Insufficient secretion of these nutrients can lead to abnormal embryo development and subsequent embryo loss (10). Prostaglandin E2 (PGE2), a bioactive molecule secreted by uterine endometrial epithelial cells (EECs) during embryo implantation, plays a critical role in processes such as embryo adhesion (11), conceptus elongation(12), and corpus luteum maintenance(13). PGE2 is produced by prostaglandin-endoperoxide synthase 2 (*PTGS2*) from arachidonic acid, with significant expression in the endometrium starting on day 12 of pregnancy in sheep (14). Inhibition of *PTGS2* expression impairs PGE2 production and disrupts normal conceptus expansion during pregnancy (12). Several signaling pathways, including cytokine and Ca^{2+}/P-CREB/*PTGS2* signaling pathways, regulate *PTGS2* transcription and PGE2 synthesis in both model animals and humans(15, 16). However, the molecular regulatory mechanisms underlying PGE2 synthesis in the endometrium during early pregnancy in sheep remain unclear.

Understanding these mechanisms is essential for improving reproductive efficiency and reducing embryo loss in sheep.

Building on the crucial role of PGE2 in early pregnancy, recent studies have highlighted the importance of epithelial sodium channels (ENaC) in regulating its synthesis(17). ENaC, a protein complex composed of the Sodium channel alpha subunit (*SCNN1A*), beta subunit (SCNN1B), and gamma subunit (SCNN1G), is activated by serine proteases and facilitates PGE2 synthesis in mouse endometrial epithelial cells, playing a significant role in the decellularization of uterine stromal cells and influencing embryo adhesion rates(18-20). Reduced expression of ENaC has been observed in women who have experienced pregnancy loss compared to those with normal pregnancies(21). While the role of ENaC in PGE2 synthesis has been studied in mouse endometrial epithelial cells, its precise molecular regulatory mechanisms in sheep, particularly in relation to endometrial receptivity and embryo survival, remain unexplored.

Our laboratory previously identified a marked upregulation of *SCNN1A* expression in the endometrium of high-fecundity Hu sheep during the luteal phase compared to their low-fecundity counterparts (22). Notably, all these Hu sheep harbor the *FecB* gene, which is well-documented for its role in enhancing ovulation rates (23). This finding highlights a potential link between elevated *SCNN1A* expression and improved endometrial receptivity, suggesting that variations in reproductive performance among these sheep may be driven by differences in endometrial function rather than ovulatory capacity alone. The role of *SCNN1A* in regulating PGE2 synthesis in the endometrium of Hu sheep and its impact on embryo survival remains

unclear. To address this, we hypothesized that *SCNN1A* expression in endometrial epithelial cells modulates PGE2 synthesis. Using an in vitro model, we investigated the regulatory role of *SCNN1A* by altering its expression and evaluating its effects on PGE2 production, embryo survival rates, and associated signaling pathways. Our findings establish *SCNN1A* as a key regulator of PGE2 synthesis in sheep endometrial epithelial cells, providing insights into the molecular mechanisms underlying reproductive efficiency in sheep and offering broader implications for enhancing fertility in livestock through genetic and molecular interventions.

Material and Methods

2.1 Animals, experimental design and tissue Collection

This study utilized endometrial tissue from Hu sheep with varying fecundity, previously collected during the luteal phase by our laboratory, to investigate the expression and localization of *SCNN1A*(22). Specifically, we selected sixteen pluriparous ewes with $FecB^{BB}$ genotypes and eight pluriparous ewes with $FecB^{B+}$ genotypes from a large sheep breeding farm in Jiangsu, China. Based on their litter size records, the ewes were evenly divided into three groups: HBB (high prolificacy, average litter size 3), LBB (low prolificacy, average litter size 1), and LB+ (low prolificacy, average litter size 1). The sheep were slaughtered on the ninth day after estrus, and their uteri were subsequently collected.

To further explore the correlation between *SCNN1A* expression, intrauterine PGE2 synthesis, and embryo survival rates during early pregnancy in sheep, we randomly selected 20 healthy, disease-free ewes from a large Hu sheep farm in Jiangsu. The ewes,

aged 2 to 3 years and weighing 50.28 ± 1.37 kg, were all breeding-aged. Synchronized estrus treatment was conducted by inserting a vaginal sponge for 11 days. The estrus status of the ewes was monitored daily through interactions with a ram. On the day the ewes exhibited estrus, they were artificially inseminated with semen from a single ram, designated as day 0 (D0). On day 12 (D12), the ewes were humanely slaughtered, and both ovaries and uteri were meticulously collected, along with venous blood samples. The selection of D12 was based on the elevated progesterone levels during this period and the fact that the conceptus had developed into a filament, facilitating identification and easy flushing from the uterus before adhesion to the uterine lining(24, 25). Additionally, during this time, the endometrium secretes substantial amounts of secretions (26). The sample size was determined based on the breeding success rate of 80% in the sheep farm, and each experimental group in the animal studies consisted of at least 6-8 animals.

The ovulation rate was assessed by counting the number of corpora lutea in both ovaries of each ewe. The collected uteri were immediately transported to the laboratory, where the uterine lumen was gently flushed with 20 mL of sterile-filtered 1× PBS (Dulbecco's phosphate-buffered saline; pH 7.0), and the number of conceptuses in the uterine flushing fluid was quantified. The embryo survival rate was calculated by dividing the number of detected conceptuses by the total number of corpora lutea from both ovaries. Ewes were classified into two groups based on their ovulation and embryo survival rates: the high embryo survival rate group (HR, ewe IDs 1, 3, 5, 7, 11, 12, 14, 20), with ovulation rates ≥ 3 and embryo survival rates ≥ 66.7%, and the low embryo

survival rate group (LR, ewe IDs 4, 6, 8, 10, 13, 15, 17, 19), with ovulation rates ≥ 2 and embryo survival rates ≤ 50%. Four ewes (ewe IDs 2, 9, 16, and 18) were excluded from further assays due to failure to meet screening criteria (Table 1).

Following our laboratory's standard procedure for collecting uterine samples, one uterine horn from each ewe was excised and divided into two sections(22). One section was rapidly frozen in liquid nitrogen and stored at −80°C for future RNA and protein extractions, while the other was fixed in 4% paraformaldehyde for immunohistochemical analysis. After removing the conceptuses, the uterine luminal flush (ULF) was clarified by centrifugation at 3000×g for 15 minutes at 4°C. The resulting fluid was rapidly frozen in liquid nitrogen and stored at −80°C until analysis.

2.2 The in vitro experiments of endometrial epithelial cells

This in vitro study aimed to investigate the effect of ENAC on PGE2 synthesis in sheep EECs. The experiment consisted of two segments. The first segment examined the impact of ENaC subunit knockdown on PGE2 synthesis in uterine epithelial cells under the resting state and activated state of epithelial sodium channels (ENaC). The second segment evaluated the effect of *SCNN1A* knockdown and overexpression on PGE2 synthesis in uterine epithelial cells under the activated state of ENaC.

In this study, trypsin was used as an ENaC activator to treat uterine epithelial cells. Trypsin is known as an embryonic secretion factor and has been shown to enhance PGE2 synthesis in the endometrium by activating ENaC (27, 28). To ensure experimental rigor, a control group of uterine epithelial cells was treated with both trypsin and the serine protease inhibitor aprotinin to eliminate the potential influence of

other substances present in the trypsin solution. Additionally, to rule out the possibility of trypsin affecting other ion channels, we pre-treated uterine epithelial cells with amiloride hydrochloride, an ENaC inhibitor, before trypsin treatment. The sources of the reagents and drugs used in this study are as follows: 0.25% trypsin from bovine pancreas (Sigma Aldrich, T1426), amiloride (Sigma Aldrich, 129876-100MG), and serine protease inhibitor (Sigma Aldrich, A1153).

The knockdown of ENaC subunits was achieved using small interfering RNA (siRNA), as detailed in Table S1 (available in Supplementary Table 1). *SCNN1A* overexpression was facilitated by the use of specific plasmids (pcDNA3.1-SCNN1A and pcDNA3.1-NC), with the plasmid structure illustrated in Figure S2 (available in Supplementary Information). The siRNAs and plasmids were synthesized by Qinke (China) and GenePharma (Shanghai, China), respectively. Briefly, once the EECs reached 60% to 70% confluence in 6-well plates, Lipofectamine 3000 (Invitrogen, Shanghai) was used to transfect the siRNAs or plasmids into the sheep EECs. After 48 hours of transfection, the EECs were treated with trypsin for one hour. Finally, the treated EECs and culture media were separately collected for further analysis.

2.3 Isolation and culture of ovine endometrial epithelial cells

Uterine endometrial epithelial cells were isolated from the uteri of slaughtered sheep using modified procedures based on prior studies(27). Briefly, uterine tissues were washed with sterile PBS, cut into small pieces, and cultured in DMEM/F12 supplemented with 20% fetal bovine serum (FBS). Migrating cells were purified by

trypsin digestion. Uterine epithelial cells were identified by positive expression of keratin 18 (KRT-18) and negative expression of vimentin (VIM). The cells used in all subsequent experiments were F1 cells, which were passaged once from the same batch.

2.4 RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from uterine tissues and EECs using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The purity and concentration of the extracted RNA were assessed by measuring the absorbance at 260/280 nm using a UV spectrophotometer (GeneQuant 1300, GE Healthcare Life Sciences, UK). The RNA was then subjected to reverse transcription and fluorescence quantification using kits from TransGen Biotech (Beijing, China; AU341 and AQ602, respectively). Primer pairs were designed using Primer 5 software (Premier Biosoft, Palo Alto, CA, USA) and validated through the Basic Local Alignment Search Tool (BLAST; NCBI, USA). RNase/DNase-free water was used as a blank control in place of cDNA samples. mRNA expression levels were quantified using the 2-ΔΔCT method, with ACTB as the reference gene for normalization. The primer sequences are listed in Table S2 (available in Supplementary Information). Each experiment was repeated at least three times.

2.5 Western blotting assay

Western blot analysis was performed according to our previously described procedure, with minor modifications(28). Briefly, proteins were extracted from tissues

and cells using RIPA buffer and quantified with a BCA Protein Assay Kit (P0012S, Beyotime). A total of 20 μg of protein was loaded onto a 12% SDS-PAGE gel (Invitrogen, Shanghai, China) and transferred to PVDF membranes (Millipore, USA). After blocking with 5% non-fat milk, the membranes were incubated overnight at 4°C with primary antibodies (Table S3, Supplementary Material), followed by incubation with a secondary antibody for 1 hour. Protein signals were detected using an enhanced chemiluminescence kit and visualized with a detection system (Fujifilm, Tokyo, Japan), and the images were analyzed using ImageJ software. Each experiment was repeated at least twice.

2.6 Ca^{2+} imaging

Intracellular free calcium ions act as second messengers and regulate the transcription of *PTGS2* and the synthesis of PGE2 in endometrial epithelial cells (20). To evaluate the effect of various treatments on intracellular calcium levels, we performed Ca^{2+} imaging on sheep EECs during logarithmic growth. The cells were stained with Fluo-4 AM (S1061S, Beyotime), incubated at 37°C with 5% CO_2 for 20 minutes, and then analyzed using a laser scanning confocal microscope (LSM 900) with excitation at 506 nm and emission at 526 nm. Trypsin treatment was applied after the fluorescence intensity stabilized. Each experiment was repeated at least three times, with a minimum of 90 cells analyzed per trial.

2.7 ELISA assay

Venous blood samples were collected in EDTA tubes, and plasma was obtained by centrifuging the samples at 3000×g for 15 minutes at 4°C. The plasma was then rapidly frozen in liquid nitrogen and stored at −80°C until further analysis. Plasma levels of progesterone and estrogen were measured using ELISA kits (RD-RX74062-48T and RD-RX74879-48T, respectively). The performance parameters for the estrogen ELISA kit are as follows: intra-batch variation: CV < 10%, inter-batch variation: CV < 12%, sensitivity: 2.5 ng/L, and detection range: 2.5-400 ng/L. The progesterone ELISA kit has the following parameters: intra-batch variation: CV < 10%, inter-batch variation: CV < 12%, sensitivity: 3 ng/L, and detection range: 3-480 ng/mL. Levels of PGE2 and PGF2α in uterine flushing fluid were assessed using ELISA kits (RD-RX74882-48T and RD-RX74926-48T, respectively). The performance parameters for the sheep PGF2α ELISA kit include: intra-batch variation: CV < 10%, inter-batch variation: CV < 12%, sensitivity: 6 pg/mL, and detection range: 6-960 pg/mL. The sheep PGE2 ELISA kit has the following performance parameters: intra-batch variation: CV < 10%, inter-batch variation: CV < 12%, sensitivity: 10 pg/mL, and detection range: 10-1600 pg/mL. The concentration of PGE2 in the cell supernatant under various treatments was also measured using the same PGE2 ELISA kit. All ELISA kits were purchased from Beijing Ruida Henghui Technology Development Co., Ltd. Each experiment was repeated at least three times

2.8 Immunofluorescence assay

An immunofluorescence assay was performed on 5 μm tissue sections following

dehydration, paraffin embedding, and sectioning. The sections were deparaffinized, rehydrated, and subjected to antigen retrieval. Blocking was carried out with 5% bovine serum albumin (BSA), followed by overnight incubation at 4°C with *SCNN1A, SCNN1B and SCNN1G* primary antibodies (Table S3). Secondary antibodies and DAPI (1:1000) were then applied in the dark for two hours. PBS was used instead of primary antibodies as a negative control. After treatment with an anti-fade mounting medium, the sections were imaged using a laser scanning confocal microscope (LSM 900). The images were analyzed with ImageJ software.

2.9 Histomorphological analysis of uterine tissue

The uterine tissue structure was examined using the formalin-fixed paraffin-embedded (FFPE) technique, with 5 μm thick sections stained with hematoxylin and eosin (H&E) following standard protocols. All tissue slides were analyzed using a Nikon ECLIPSE Ti microscope (Tokyo, Japan), and uterine components were manually counted with ImageJ software. The density of uterine glands was assessed by counting the number of glands in five random fields per slide at 20× magnification (Figure S1A, Supplementary Material). Endometrial ductal gland invaginations were identified by distinct cavitation (Figure S1B, Supplementary Material). The mean thickness of the luminal epithelium and myometrium was measured from eight random fields (Figures S1C and S1D, Supplementary Material).

2.10 Statistical analysis

Data were analyzed using SPSS 28.0.1.1 and are presented as the means ±standard error of the mean (SEM) from at least three independent experiments. Comparisons between two groups were conducted using a *t*-test with statistical significance defined as $*p < 0.05$ and high significance as $**p < 0.01$. To compare between more than two groups, a one-way ANOVA followed by Tukey's post hoc test (statistical significance of $p < 0.05$) was used.

Results

3.1 Expression and localization of *ENAC* in the endometrium of Hu sheep

Quantitative real-time PCR analysis of endometrial tissues from Hu sheep with distinct fecundity ($FecB^{BB}$ genotype) during the luteal phase revealed a significant upregulation of SCNN1A, an epithelial sodium channel (ENaC) subunit, in high-fertility individuals ($P<0.01$). In contrast, the expression levels of SCNN1B and SCNN1G remained unchanged (Figure 1A). Immunofluorescence staining further demonstrated the co-localization of all three ENaC subunits (SCNN1A, SCNN1B, and SCNN1G) within the uterine glandular epithelium (Figure 1B). These findings suggest that the specific upregulation of SCNN1A in the endometrium is associated with enhanced reproductive performance in Hu sheep, potentially through its role in regulating uterine gland function.

3.2 Trypsin promotes PGE2 synthesis in endometrial epithelial cells through ENaC activation

To elucidate the role of ENaC in endometrial PGE2 synthesis, we isolated and

cultured KRT-18-positive/VIM-negative endometrial epithelial cells for in vitro experiments (Figure 2A). An ENaC activation model was established by treating EECs with 10 μg/ml trypsin to examine the regulatory effect of SCNN1A on PGE2 synthesis. Intracellular calcium imaging demonstrated that trypsin treatment significantly elevated calcium ion concentrations in EECs (Figure 3B), whereas this response was abolished in cells pretreated with the ENaC inhibitor amiloride (Figure 3C).

Quantitative analysis revealed that trypsin treatment markedly upregulated the expression of *PTGS2* and *CREB1* transcripts ($P < 0.05$) and enhanced PGE2 secretion ($P < 0.05$). Notably, these effects were significantly attenuated by pretreatment with either aprotinin (a trypsin inhibitor) or amiloride ($P < 0.05$) (Figure 3D and 3E). To further investigate the underlying mechanism, we assessed the expression profiles of ENaC subunits before and after trypsin treatment. Both qRT-PCR and Western blot analyses demonstrated that neither mRNA nor protein levels of SCNN1A, SCNN1B, or SCNN1G were significantly altered following trypsin treatment (Figure 2F and 2G).

These findings collectively indicate that trypsin-mediated enhancement of PGE2 synthesis occurs through functional activation of ENaC rather than modulation of its expression levels. The proposed mechanism involves ENaC-mediated sodium channel activation, which elevates intracellular calcium concentrations and subsequently stimulates PGE2 biosynthesis in ovine endometrial epithelial cells.

3.3 The impact of ENaC subunit knockdown on PGE2 synthesis in endometrial epithelial cells

To elucidate the functional contributions of individual ENaC subunits in regulating

PGE2 synthesis in endometrial epithelial cells, we employed RNA knockdown technology by designing specific small interfering RNAs (siRNAs) targeting *SCNN1A*, *SCNN1B*, and *SCNN1G*. The knockdown efficiency was rigorously validated, as detailed in the supplementary figure 3. Under basal conditions, siRNA-mediated silencing of individual ENaC subunits did not significantly alter intracellular calcium homeostasis, *CREB1* and *PTGS2* transcript levels, or PGE2 biosynthesis (Figure 3A-3C). Following trypsin stimulation, we observed that genetic knockdown of any ENaC subunit significantly attenuated the trypsin-induced calcium influx ($P < 0.05$). Notably, *SCNN1A* knockdown exerted the most substantial inhibitory effect, whereas *SCNN1B* and *SCNN1G* knockdown showed comparatively moderate impacts (Figure 3D). Mechanistically, trypsin treatment combined with *SCNN1A* silencing markedly suppressed both *CREB1* and *PTGS2* mRNA expression, resulting in a significant reduction in PGE2 synthesis ($P < 0.05$) (Figure 3E-3F). Interestingly, while *SCNN1B* knockdown under trypsin stimulation significantly decreased *CREB1* mRNA levels ($P < 0.05$), it failed to affect *PTGS2* expression or PGE2 production. Furthermore, *SCNN1G* knockdown demonstrated no significant regulatory effects on either *CREB1* or *PTGS2* mRNA levels, nor on PGE2 biosynthesis during trypsin treatment (Figure 3E-3F).

3.4 Under trypsin treatment, SCNN1A modulates PGE2 synthesis in endometrial epithelial cells through the Ca^{2+}/P-CREB/PTGS2 signaling pathway

Given the significant role of *SCNN1A* in trypsin-induced PGE_2 synthesis in EECs, we further investigated the underlying regulatory mechanisms of *SCNN1A*-mediated

308 PGE_2 production. To this end, we constructed an *SCNN1A* overexpression plasmid,
309 with transfection efficiency validated in the supplementary figure 3C-D.

310 Following trypsin treatment, *SCNN1A*-overexpressing cells exhibited a significantly
311 enhanced intracellular calcium influx compared to control cells (Figure 4A). Under
312 resting conditions, SCNN1A overexpression did not affect *CREB1* or *PTGS2* mRNA
313 expression levels or PGE_2 synthesis (Figure 4B, 4C). However, upon trypsin
314 stimulation, *SCNN1A* overexpression significantly increased *CREB1* and *PTGS2*
315 mRNA expression as well as PGE2 production.

316 Western blot analysis further revealed that SCNN1A overexpression markedly
317 upregulated CREB1 and PTGS2 protein levels following trypsin stimulation, whereas
318 SCNN1A knockdown significantly suppressed their expression (Figure 4D, 4E). These
319 findings demonstrate that under trypsin stimulation, SCNN1A regulates PGE2
320 synthesis in EECs via the Ca^{2+}/P-CREB/PTGS2 signaling pathway.

321 **3.5 The uterine PGE2 concentration was significantly higher in Hu sheep with**
322 **high versus low embryo survival rates**

323 To further investigate the correlation between *SCNN1A* protein expression in the
324 endometrium of Hu sheep during early pregnancy, embryo survival rate, and PGE2
325 concentration, we compared variations in reproductive hormones and uterine tissue
326 morphology among Hu sheep with different embryo survival rates on day 12 of
327 pregnancy. A quantitative analysis of the histomorphological characteristics of the
328 uterus in the low-survival rate (LR) and high-survival rate (HR) groups was performed
329 based on H&E staining (Figure S1, Supplementary Material). No significant differences

were observed in the uterine glands, endometrial ductal gland invaginations, luminal epithelium thickness, or myometrial thickness between the two embryo survival rate groups (Figure 5A-5D).

ELISA results of uterine contents revealed a significantly higher concentration of PGE2 in the HR group compared to the LR group ($P < 0.05$) (Figure 5E), whereas the difference in PGF2α concentration was not statistically significant (Figure 5F). In peripheral blood, estradiol and progesterone levels did not show significant differences between the groups (Figure 5G, 5H). These findings suggest that the concentration of PGE2 in uterine contents is a differential marker for Hu sheep with varying embryo survival rates.

3.6 *SCNN1A* is a differentially expressed gene in the endometrium of Hu sheep with varying embryo survival rates.

We measured the expression of several genes associated with PGE2 synthesis and endometrial receptivity in the endometrium of two groups of Hu sheep with differing embryo survival rates. Among these genes, *PTGS2*, *MMP-2*, and *SCNN1A*, which are directly involved in PGE2 synthesis and endometrial receptivity, showed significantly higher mRNA expression levels in the endometrium of the high-survival rate (HR) group (n = 8) compared to the low-survival rate (LR) group (n = 8) ($P < 0.01$) (Figure 6A). The protein expression of *SCNN1A* in the endometrium was also significantly higher in the HR group compared to the LR group ($P < 0.05$) (Figure 6B). These results indicate that *SCNN1A* is a differential gene in the endometrium of Hu sheep, correlating with varying embryo survival rates. A reduction in *SCNN1A* expression in the sheep

endometrium may lead to decreased PGE2 synthesis, which could contribute to embryo loss.

Discussion

Numerous studies have shown that prostaglandin E2 (PGE2) secreted by the endometrium is critical for embryo survival and subsequent development(11, 29). However, the molecular mechanisms regulating PGE2 secretion by endometrial epithelial cells in sheep during early pregnancy remain unclear. This study is based on the differential expression of *SCNN1A*, a gene previously identified in the endometrium of Hu sheep with varying fertility(22). Our in vitro experiments demonstrate that *SCNN1A* regulates PGE2 synthesis in endometrial epithelial cells through the Ca^{2+}/*P-CREB*/*PTGS2* signaling pathway. Furthermore, we show that *SCNN1A* is differentially expressed in the endometrium of sheep with differing embryo survival rates, with significant variations in PGE2 concentrations observed in their uterine contents. These findings highlight the critical role of *SCNN1A* in modulating PGE2 production in Hu sheep and provide new insights into the molecular mechanisms governing PGE2 release. Consequently, *SCNN1A* emerges as a promising molecular target for improving embryo survival rates and advancing sheep breeding practices.

SCNN1A is a key component of epithelial sodium ion channels, which play a crucial role in regulating the uterine fluid environment and the endocrine function of epithelial cells(30). Increased *SCNN1A* expression during pregnancy is essential for embryo implantation(31). In model organisms, *SCNN1A* has been shown to regulate the expression of *PTGS2* and the synthesis of PGE2. It can interact with inflammatory

374 factors to modulate PGE2 production under both acute and chronic inflammatory
375 conditions(32). Additionally, in mouse renal epithelial cells, *SCNN1A* expression is
376 positively correlated with *PTGS2* expression and PGE2 secretion(33). In this study, the
377 mRNA expression of SCNN1A was significantly upregulated in the endometrium of
378 high-fertility Hu sheep during the luteal phase. Additionally, its localization in the
379 uterine glands was confirmed. These findings suggest that SCNN1A may play a role in
380 regulating uterine gland function, thereby influencing fertility in Hu sheep.

381 Sodium ion channels exist in three distinct states: resting, activated, and
382 inactive(34). In the resting state, the epithelial sodium channel (ENaC) is relatively
383 inactive, with only a small fraction of ENaC on the epithelial membrane being in the
384 "open" state, allowing a limited influx of sodium ions into the cell(35). Our results first
385 demonstrated that the expression levels of ENaC subunits did not change significantly
386 under trypsin treatment. This finding indicates that the activation of downstream
387 PTGS2 and the synthesis of PGE2 induced by trypsin are mediated by functional
388 changes in ENaC rather than alterations in its expression levels. Furthermore, changes
389 in the expression of ENaC subunits did not affect PGE2 synthesis in EECs under resting
390 conditions, suggesting that the synthesis of PGE2 in EECs requires activation by trypsin.
391 Serine proteases secreted by embryos have been shown to interact with sodium
392 channels, activating them in epithelial cells(36, 37). When ENaC is activated, the
393 resulting increase in intracellular sodium concentration affects calcium ion transport
394 and membrane potential, subsequently promoting calcium ion influx (38). Intracellular
395 calcium ions often act as second messengers, triggering the activation of the

transcription factor *CREB1* in the nucleus, which in turn stimulates the expression of the downstream gene *PTGS2* and the synthesis of PGE2(39). *PTGS2*, the rate-limiting enzyme in PGE2 biosynthesis, is highly expressed in the uterine gland epithelium(40, 41). *PTGS2* knockout mice exhibit decreased PGE2 synthesis in the uterus, which correlates with reduced fecundity(42). In this study, trypsin treatment of sheep endometrial epithelial cells resulted in an increase in intracellular calcium ion concentration and PGE2 synthesis through the Ca^{2+}/P-CREB/*PTGS2* signaling pathway.

Trypsin treatment of mouse endometrial epithelial cells induces an increase in intracellular calcium ion concentration, thereby promoting the synthesis of prostaglandin E2 (PGE2)(20). Similarly, trypsin can also trigger calcium oscillations in human endometrial epithelial cells. However, in addition to the epithelial sodium channel (ENaC), other ion channels may contribute to these calcium dynamics(43). These findings suggest that the mechanism by which trypsin regulates PGE2 synthesis through ENaC exhibits a certain degree of conservation across species, including sheep, humans, and mice. Notably, PGE2 synthesis is activated only when an embryo enters the uterus or when external stimuli, such as trypsin, activate sodium ion channels in endometrial epithelial cells.

The activation of sodium ion channels in epithelial cells by trypsin is triggered by the direct cleavage of specific fragments of the ENaCα and γ subunits(36). In this process, changes in *SCNN1A* expression directly affect the activation of sodium ion channels by proteases (44). In this study, the knockdown of SCNN1A, SCNN1B, and SCNN1G subunits all resulted in a reduction of intracellular calcium levels upon trypsin

418 treatment. However, only the knockdown of SCNN1A significantly decreased PGE2
419 synthesis in EECs. This may be because SCNN1A knockdown led to the greatest
420 reduction in intracellular calcium concentrations, and the increase in intracellular
421 calcium directly correlates with PGE2 synthesis. As a result, SCNN1A knockdown had
422 a significant impact on PGE2 production in EECs, while the knockdown of SCNN1B
423 and SCNN1G showed a smaller effect on the calcium influx, and thus did not
424 significantly suppress PGE2 synthesis. This phenomenon may be attributed to the
425 significantly higher expression of SCNN1A compared to SCNN1B and SCNN1G,
426 which results in SCNN1A having the greatest impact on ENaC function during trypsin
427 cleavage.

428 The degree of sodium ion channel opening following activation significantly
429 influences the concentration of intracellular calcium ions (45). In our study, trypsin
430 increased intracellular calcium ion concentration by activating sodium ion channels.
431 Under these conditions, both knockdown and overexpression of *SCNN1A* significantly
432 regulated intracellular calcium ion concentration and PGE2 synthesis. These results
433 suggest that *SCNN1A* expression is closely associated with sodium ion channel function.
434 Specifically, in the activated state of epithelial cells, high *SCNN1A* expression enhances
435 intracellular calcium ion concentration and PGE2 synthesis, indicating that *SCNN1A*
436 plays a critical role in regulating PGE2 synthesis, dependent on sodium ion channel
437 activation.

438 Further in vivo experimental results revealed significant differences in PGE2
439 concentrations in the uterine contents of Hu sheep with varying embryo survival rates.

This finding mirrors the differences observed in cattle, where high-fertility cows exhibit significantly higher PGE2 concentrations compared to low-fertility cows(46). These results further support the role of PGE2 as a crucial factor in embryo survival and development in ruminants, regardless of whether they are single- or multiple-birthing species. Moreover, the expression levels of *SCNN1A* mRNA and protein were significantly higher in the uterus of Hu sheep with high embryo survival rates compared to those with low embryo survival rates. This observation indirectly supports our hypothesis that increased *SCNN1A* expression enhances trypsin-mediated PGE2 synthesis, as demonstrated in our in vitro experiments. A similar phenomenon has been reported in humans, where *SCNN1A* protein expression in the uterus of women with successful pregnancies is significantly higher than in those with failed pregnancies (47). Additionally, MMP-2 mRNA expression was found to differ between the endometrium of Hu sheep with varying embryo survival rates, suggesting that MMP-2 may be a key gene associated with early embryo survival and implantation in sheep. This finding is consistent with previous studies in mice, where increased MMP-2 expression has been observed during embryo implantation(48).

The limitations of this study should be acknowledged. The Hu sheep population used to examine the relationship between *SCNN1A*, PGE2 synthesis, and embryo survival rates was relatively small. Future studies involving a larger Hu sheep population and longer follow-up periods are needed to further investigate the dynamic changes in PGE2 levels and their correlation with embryo survival rates.

This study highlights the critical role of *SCNN1A* in regulating prostaglandin E2

(PGE2) synthesis in the uterine endometrial epithelial cells of Hu sheep. Our in vitro model demonstrates that *SCNN1A* modulates PGE2 production by influencing intracellular calcium levels during the activated state of epithelial cell sodium channels. These findings emphasize the importance of *SCNN1A* in embryo survival and provide valuable insights that could help enhance reproductive outcomes in Hu sheep.

Conclusion

This study underscores the critical role of *SCNN1A* in regulating prostaglandin E2 (PGE2) synthesis in the uterine endometrial epithelial cells of Hu sheep. Using an in vitro model, we demonstrate that *SCNN1A* modulates PGE2 production by influencing intracellular calcium levels during the activation of epithelial sodium channels. Our findings reveal that *SCNN1A* is differentially expressed in the endometrium of Hu sheep with varying embryo survival rates. These results highlight the significance of *SCNN1A* in embryo survival and offer valuable insights into improving reproductive outcomes in Hu sheep.

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Conflict of Interest Statement

The authors declare no competing financial interest.

Author contributions

Jiahe Guo: designed and performed most of the experiments and drafted the manuscript with input from all the authors.

Jingjing Guo, Zhibo Wang and Zhiqing Yang: assisted in sample collection and analyzed the data.

Guomin Zhang and Mingtian Deng: interpreted the data and provide methodological support.

Feng Wang: supervised the study and administrated the project.

Data Availability Statement

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval statement

All animal procedures were ethically approved by the Ethics Committee of Nanjing Agricultural University, China (SYXK2022-0031).

Reference

1. Diskin MG, Morris DG. Embryonic and early foetal losses in cattle and other ruminants. Reprod Domest Anim. 2008;43 Suppl 2:260-7. Epub 2008/07/25. doi: 10.1111/j.1439-0531.2008.01171.x. PubMed PMID: 18638133.

2. Spencer TE. Biological roles of uterine glands in pregnancy. Semin Reprod Med. 2014;32(5):346-57. Epub 2014/06/25. doi: 10.1055/s-0034-1376354. PubMed PMID: 24959816; PubMed Central PMCID: PMCPMC4198167.

3. H EE, Ma L, Xie X, Ma J, Ma X, Yue C, et al. Genetic polymorphism association analysis of SNPs on the species conservation genes of Tan sheep and Hu sheep. Trop Anim Health Prod. 2020;52(3):915-26. Epub 2020/02/07. doi: 10.1007/s11250-019-02063-1. PubMed PMID: 32026291; PubMed Central PMCID: PMCPMC7190689.

4. Wang W, Liu S, Li F, Pan X, Li C, Zhang X, et al. Polymorphisms of the Ovine BMPR-IB, BMP-15 and FSHR and Their Associations with Litter Size in Two Chinese Indigenous Sheep Breeds. Int J Mol Sci. 2015;16(5):11385-97. Epub 2015/05/21. doi: 10.3390/ijms160511385. PubMed PMID: 25993301; PubMed Central PMCID: PMCPMC4463706.

5. Yao X, Yang F, El-Samahy MA, Liu B, Zhao B, Gao X, et al. Identification and characterization of unique and common lncRNAs and mRNAs in the pituitary, ovary, and uterus of Hu sheep with different prolificacy. Genomics. 2022;114(6):110511. Epub 2022/10/26. doi: 10.1016/j.ygeno.2022.110511. PubMed PMID: 36283658.

6. Chu MX, Wang XL. [Estimation of phenotypic and genetic parameters for birth type and number of lambs born alive in hu sheep]. Yi Chuan Xue Bao. 2001;28(5):418-23. Epub 2001/07/10. PubMed PMID: 11441654.

7. Gray CA, Taylor KM, Ramsey WS, Hill JR, Bazer FW, Bartol FF, et al. Endometrial glands are required for preimplantation conceptus elongation and survival. Biol Reprod. 2001;64(6):1608-13. Epub 2001/05/23. doi: 10.1095/biolreprod64.6.1608. PubMed PMID: 11369585.

8. Gray CA, Burghardt RC, Johnson GA, Bazer FW, Spencer TE. Evidence that absence of endometrial gland secretions in uterine gland knockout ewes compromises conceptus survival and elongation. Reproduction. 2002;124(2):289-300. Epub 2002/07/27. PubMed PMID: 12141942.

9. Spencer TE, Gray CA. Sheep uterine gland knockout (UGKO) model. Methods Mol Med. 2006;121:85-94. Epub 2005/10/28. doi: 10.1385/1-59259-983-4:083. PubMed PMID: 16251736.

10. Kelleher AM, DeMayo FJ, Spencer TE. Uterine Glands: Developmental Biology and Functional Roles in Pregnancy. Endocr Rev. 2019;40(5):1424-45. Epub 2019/05/11. doi: 10.1210/er.2018-00281. PubMed PMID: 31074826; PubMed Central PMCID: PMCPMC6749889.

11. Niringiyumukiza JD, Cai H, Xiang W. Prostaglandin E2 involvement in mammalian female fertility: ovulation, fertilization, embryo development and early implantation. Reprod Biol Endocrinol. 2018;16(1):43. Epub 2018/05/03. doi: 10.1186/s12958-018-0359-5. PubMed PMID: 29716588; PubMed Central PMCID: PMCPMC5928575.

12. Dorniak P, Bazer FW, Spencer TE. Prostaglandins regulate conceptus elongation and mediate effects of interferon tau on the ovine uterine endometrium. Biol Reprod. 2011;84(6):1119-27. Epub 2011/01/29. doi: 10.1095/biolreprod.110.089979. PubMed PMID: 21270428.

13. Lee J, McCracken JA, Stanley JA, Nithy TK, Banu SK, Arosh JA. Intraluteal prostaglandin biosynthesis and signaling are selectively directed towards PGF2alpha during luteolysis but towards PGE2 during the establishment of pregnancy in sheep. Biol Reprod. 2012;87(4):97. Epub 2012/06/30. doi: 10.1095/biolreprod.112.100438. PubMed PMID: 22743300.

14. Charpigny G, Reinaud P, Tamby JP, Creminon C, Martal J, Maclouf J, et al. Expression of cyclooxygenase-1 and -2 in ovine endometrium during the estrous cycle and early pregnancy. Endocrinology. 1997;138(5):2163-71. Epub 1997/05/01. doi: 10.1210/endo.138.5.5148. PubMed PMID: 9112416.

15. Chen JJ, Wang Y, Meng X, Ruan YC, Zou F, Chan HC. MRP4 regulates ENaC-dependent CREB/COX-2/PGE(2) signaling during embryo implantation. Oncotarget. 2017;8(45):78520-9. Epub 2017/11/08. doi: 10.18632/oncotarget.19676. PubMed PMID: 29108246; PubMed Central PMCID: PMCPMC5667979.

16. Trudeau J, Hu H, Chibana K, Chu HW, Westcott JY, Wenzel SE. Selective downregulation of prostaglandin E2-related pathways by the Th2 cytokine IL-13. J Allergy Clin Immunol. 2006;117(6):1446-54. Epub 2006/06/06. doi: 10.1016/j.jaci.2006.01.049. PubMed PMID: 16751012.

17. Sun X, Ruan YC, Guo J, Chen H, Tsang LL, Zhang X, et al. Regulation of miR-101/miR-199a-3p by the epithelial sodium channel during embryo implantation: involvement of CREB phosphorylation. Reproduction. 2014;148(6):559-68. Epub 2014/09/05. doi: 10.1530/REP-14-0386. PubMed PMID: 25187622.

18. Zhou M, Fu J, Huang W, Shen L, Xiao L, Song Y, et al. Increased cystic fibrosis transmembrane conductance regulators expression and decreased epithelial sodium channel alpha subunits expression in early abortion: findings from a mouse model and clinical cases of abortion. PLoS One. 2014;9(6):e99521. Epub 2014/06/11. doi: 10.1371/journal.pone.0099521. PubMed PMID: 24914548; PubMed Central PMCID: PMCPMC4051784.

19. Di X, Duan Z, Ma Y, Song X, Hao Y, Li G, et al. Jiawei Shoutai Pill promotes decidualization by regulating the SGK1/ENaC pathway in recurrent spontaneous abortion. J Ethnopharmacol. 2024;318(Pt A):116939. Epub 2023/07/22. doi: 10.1016/j.jep.2023.116939. PubMed PMID: 37479068.

20. Ruan YC, Guo JH, Liu X, Zhang R, Tsang LL, Dong JD, et al. Activation of the epithelial Na+ channel triggers prostaglandin E(2) release and production required for embryo implantation. Nat Med. 2012;18(7):1112-7. Epub 2012/06/26. doi: 10.1038/nm.2771. PubMed PMID: 22729284.

21. Di X, Hao Y, Duan Z, Ma Y, Cao Y, Tan Z, et al. Activation of SGK1/ENaC Signaling Pathway Improves the Level of Decidualization in Unexplained Recurrent Spontaneous Abortion. Reprod Sci. 2023;30(11):3273-84. Epub 2023/06/07. doi: 10.1007/s43032-023-01273-1. PubMed PMID: 37280474; PubMed Central PMCID: PMCPMC10643273.

22. Li X, Yao X, Li K, Guo J, Deng K, Liu Z, et al. CREB1 Is Involved in miR-134-5p-Mediated Endometrial Stromal Cell Proliferation, Apoptosis, and Autophagy. Cells. 2023;12(21). Epub 2023/11/10. doi: 10.3390/cells12212554. PubMed PMID: 37947633; PubMed Central PMCID: PMCPMC10649013.

23. Mulsant P, Lecerf F, Fabre S, Schibler L, Monget P, Lanneluc I, et al. Mutation in bone morphogenetic protein receptor-IB is associated with increased ovulation rate in Booroola Merino ewes. Proc Natl Acad Sci U S A. 2001;98(9):5104-9. Epub 2001/04/26. doi: 10.1073/pnas.091577598. PubMed PMID: 11320249; PubMed Central PMCID: PMCPMC33171.

24. Thomas ES, Greg AJ, Fuller WB, Robert CB. Implantation mechanisms: insights from the sheep. Reproduction. 2004. doi: 10.1530/rep.1.00398.

25. Eggleston DL, Wilken C, Edward AVK, Slaughter RG, Tae HJ, William JM. Progesterone induces expression of endometrial messenger RNA encoding for cyclooxygenase (sheep). Prostaglandins. 1990. doi: 10.1016/0090-6980(90)90027-.

26. Spencer T, Johnson GA, Bazeru FW, Burghardt R. Fetal-maternal interactions during the establishment of pregnancy in ruminants. Bioscientifica proceedings. 2019. doi: 10.1530/biosciprocs.6.024.

27. Li X, Li K, Deng K, Liu Z, Huang X, Guo J, et al. LncRNA12097.1 contributes to endometrial cell growth by enhancing YES1 activating beta-catenin via sponging miR-145-5p. Int J Biol Macromol. 2024;256(Pt 2):128477. Epub 2023/12/01. doi: 10.1016/j.ijbiomac.2023.128477. PubMed PMID: 38035963.

28. Gao X, Yao X, Li X, Liang Y, Liu Z, Wang Z, et al. Roles of WNT6 in Sheep Endometrial Epithelial Cell Cycle Progression and Uterine Glands Organogenesis. Vet Sci. 2021;8(12). Epub 2021/12/24. doi: 10.3390/vetsci8120316. PubMed PMID: 34941843; PubMed Central PMCID: PMCPMC8708052.

29. Vilella F, Ramirez L, Berlanga O, Martinez S, Alama P, Meseguer M, et al. PGE2 and PGF2alpha concentrations in human endometrial fluid as biomarkers for embryonic implantation. J Clin Endocrinol Metab. 2013;98(10):4123-32. Epub 2013/08/28. doi: 10.1210/jc.2013-2205. PubMed PMID: 23979956.

30. Ruan YC, Chen H, Chan HC. Ion channels in the endometrium: regulation of endometrial receptivity and embryo implantation. Hum Reprod Update. 2014;20(4):517-29. Epub 2014/03/05. doi: 10.1093/humupd/dmu006. PubMed PMID: 24591147.

31. Zhou Y, Pei S, Qiu G, Zhang J, Guo H, Cui S, et al. Taurine is essential for mouse uterine luminal fluid resorption during implantation window via the SCNN1A and AQP8 signaling†. Biology of Reproduction. 2024. doi: 10.1093/biolre/ioae152.

32. Kantarci H, Elvira PD, Thottumkara AP, O'Connell EM, Iyer M, Donovan LJ, et al. Schwann cell-secreted PGE(2) promotes sensory neuron excitability during development. Cell. 2024;187(17):4690-712 e30. Epub 2024/08/15. doi: 10.1016/j.cell.2024.07.033. PubMed PMID: 39142281.

33. Hu C, Lakshmipathi J, Stuart D, Peti-Peterdi J, Gyarmati G, Hao CM, et al. Renomedullary Interstitial Cell Endothelin A Receptors Regulate BP and Renal Function. J Am Soc Nephrol. 2020;31(7):1555-68. Epub 2020/06/04. doi: 10.1681/ASN.2020020232. PubMed PMID: 32487560; PubMed Central PMCID: PMCPMC7351004.

34. Rook ML, Williamson A, Lueck JD, Musgaard M, Maclean DM. beta11-12 linker isomerization governs acid-sensing ion channel desensitization and recovery. Elife. 2020;9. Epub 2020/02/08. doi: 10.7554/eLife.51111. PubMed PMID: 32031522; PubMed Central PMCID: PMCPMC7041949.

35. Wisedchaisri G, Tonggu L, McCord E, Gamal El-Din TM, Wang L, Zheng N, et al. Resting-State Structure and Gating Mechanism of a Voltage-Gated Sodium Channel. Cell. 2019;178(4):993-1003 e12. Epub 2019/07/30. doi: 10.1016/j.cell.2019.06.031. PubMed PMID: 31353218; PubMed Central PMCID: PMCPMC6688928.

36. Anand D, Hummler E, Rickman OJ. ENaC activation by proteases. Acta Physiol (Oxf). 2022;235(1):e13811. Epub 2022/03/12. doi: 10.1111/apha.13811. PubMed PMID: 35276025; PubMed Central PMCID: PMCPMC9540061.

37. Garcia-Caballero A, Ishmael SS, Dang Y, Gillie D, Bond JS, Milgram SL, et al. Activation of the epithelial sodium channel by the metalloprotease meprin beta subunit. Channels (Austin). 2011;5(1):14-22. Epub 2010/10/19. doi: 10.4161/chan.5.1.13759. PubMed PMID: 20953144; PubMed Central PMCID: PMCPMC3052204.

38. Verkhratsky A, Trebak M, Perocchi F, Khananshvili D, Sekler I. Crosslink between calcium and sodium signalling. Exp Physiol. 2018;103(2):157-69. Epub 2017/12/07. doi: 10.1113/EP086534. PubMed PMID: 29210126; PubMed Central PMCID: PMCPMC6813793.

39. Zhou X, Li J, Yang W. Calcium/calmodulin-dependent protein kinase II regulates cyclooxygenase-2 expression and prostaglandin E2 production by activating cAMP-response element-binding protein in rat peritoneal macrophages. Immunology. 2014;143(2):287-99. Epub 2014/04/30. doi: 10.1111/imm.12309. PubMed PMID: 24773364; PubMed Central PMCID: PMCPMC4172144.

40. Simmons RM, Satterfield MC, Welsh TH, Jr., Bazer FW, Spencer TE. HSD11B1, HSD11B2, PTGS2, and NR3C1 expression in the peri-implantation ovine uterus: effects of pregnancy, progesterone, and interferon tau. Biol Reprod. 2010;82(1):35-43. Epub 2009/08/22. doi: 10.1095/biolreprod.109.079608. PubMed PMID: 19696010.

41. Siemieniuch MJ, Jursza E, Kowalewski MP, Majewska M, Skarzynski DJ. Prostaglandin endoperoxide synthase 2 (PTGS2) and prostaglandins F2alpha and E2 synthases (PGFS and PGES) expression and prostaglandin F2alpha and E2 secretion following oestrogen and/or progesterone stimulation of the feline endometrium. Reprod Domest Anim. 2013;48(1):72-8. Epub 2012/05/16. doi: 10.1111/j.1439-0531.2012.02031.x. PubMed PMID: 22583354.

42. Granger K, Fitch S, Shen M, Lloyd J, Bhurke A, Hancock J, et al. Murine uterine gland branching is necessary for gland function in implantation. Mol Hum Reprod. 2024;30(6). Epub 2024/05/25. doi: 10.1093/molehr/gaae020. PubMed PMID: 38788747; PubMed Central PMCID: PMCPMC11176042.

43. Hennes A, Devroe J, De Clercq K, Ciprietti M, Held K, Luyten K, et al. Protease secretions by the

invading blastocyst induce calcium oscillations in endometrial epithelial cells via the protease-activated receptor 2. Reprod Biol Endocrinol. 2023;21(1):37. Epub 2023/04/15. doi: 10.1186/s12958-023-01085-7. PubMed PMID: 37060079; PubMed Central PMCID: PMCPMC10105462.

44. Aufy M, Hussein AM, Stojanovic T, Studenik CR, Kotob MH. Proteolytic Activation of the Epithelial Sodium Channel (ENaC): Its Mechanisms and Implications. Int J Mol Sci. 2023;24(24). Epub 2023/12/23. doi: 10.3390/ijms242417563. PubMed PMID: 38139392; PubMed Central PMCID: PMCPMC10743461.

45. Clausen MJ, Poulsen H. Sodium/Potassium homeostasis in the cell. Met Ions Life Sci. 2013;12:41-67. Epub 2013/04/19. doi: 10.1007/978-94-007-5561-1_3. PubMed PMID: 23595670.

46. Moraes JGN, Behura SK, Geary TW, Spencer TE. Analysis of the uterine lumen in fertility-classified heifers: I. Glucose, prostaglandins, and lipidsdagger. Biol Reprod. 2020;102(2):456-74. Epub 2019/10/17. doi: 10.1093/biolre/ioz191. PubMed PMID: 31616913; PubMed Central PMCID: PMCPMC7331873.

47. Davoodi Nik B, Hashemi Karoii D, Favaedi R, Ramazanali F, Jahangiri M, Movaghar B, et al. Differential expression of ion channel coding genes in the endometrium of women experiencing recurrent implantation failures. Sci Rep. 2024;14(1):19822. Epub 2024/08/28. doi: 10.1038/s41598-024-70778-9. PubMed PMID: 39192025; PubMed Central PMCID: PMCPMC11349755.

48. Zhang S, Mesalam A, Joo MD, Lee KL, Hwang JY, Xu L, et al. Matrix metalloproteinases improves trophoblast invasion and pregnancy potential in mice. Theriogenology. 2020;151:144-50. Epub 2020/04/29. doi: 10.1016/j.theriogenology.2020.02.002. PubMed PMID: 32344273.

Table

Table 1: Classification Table of Sheep with Different Embryo survival rate

Sheep ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Conceptus Count	3	0	3	1	2	1	2	1	1	1	3	3	1	2	1	0	1	0	2	3
Corpus luteum of both ovaries	3	2	4	4	3	3	3	2	1	4	3	3	3	3	3	1	2	3	4	3
Embryo survival rate (%)[1]	100	0	75	25%	66.7	33.3%	66.7	50	100	25	100	100	33.3	66.7	33.3	0	50	0	50	100
GROUP[2]	HR	/	HR	LR	HR	LR	HR	LR	/	LR	HR	HR	LR	HR	LR	/	LR	/	LR	HR

1.The ratio of the number of conceptuses in the uterine flushing fluid to the total number of corpus luteum in both ovaries.

2HR：high embryo survival rate group LR：low embryo survival rate group

Figure Graphics

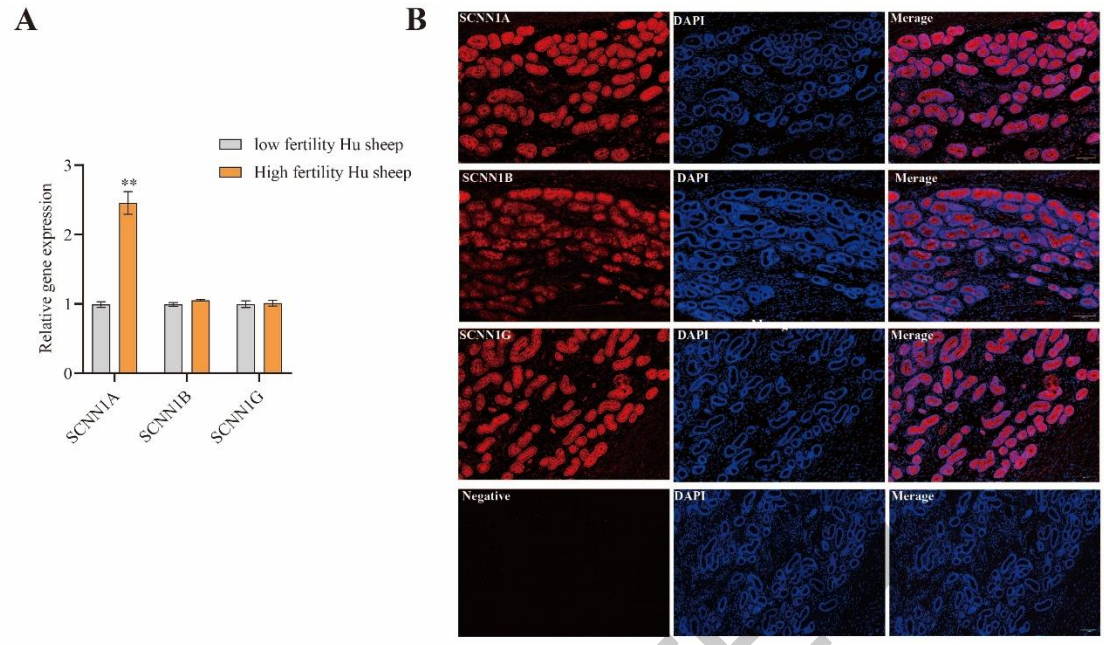


Figure 1. Expression and localization of ENAC in the endometrium of Hu sheep. A. Relative mRNA expression levels of ENaC subunits in the endometrium of Hu sheep with different fecundity. **B.** Immunofluorescence localization of SCNN1A, SCNN1B, and SCNN1G in ovine uterine tissues. Nuclei were counterstained with DAPI (blue). Scale bar = 100 μm. Data are presented as means ± SEM, ** $p < 0.01$.

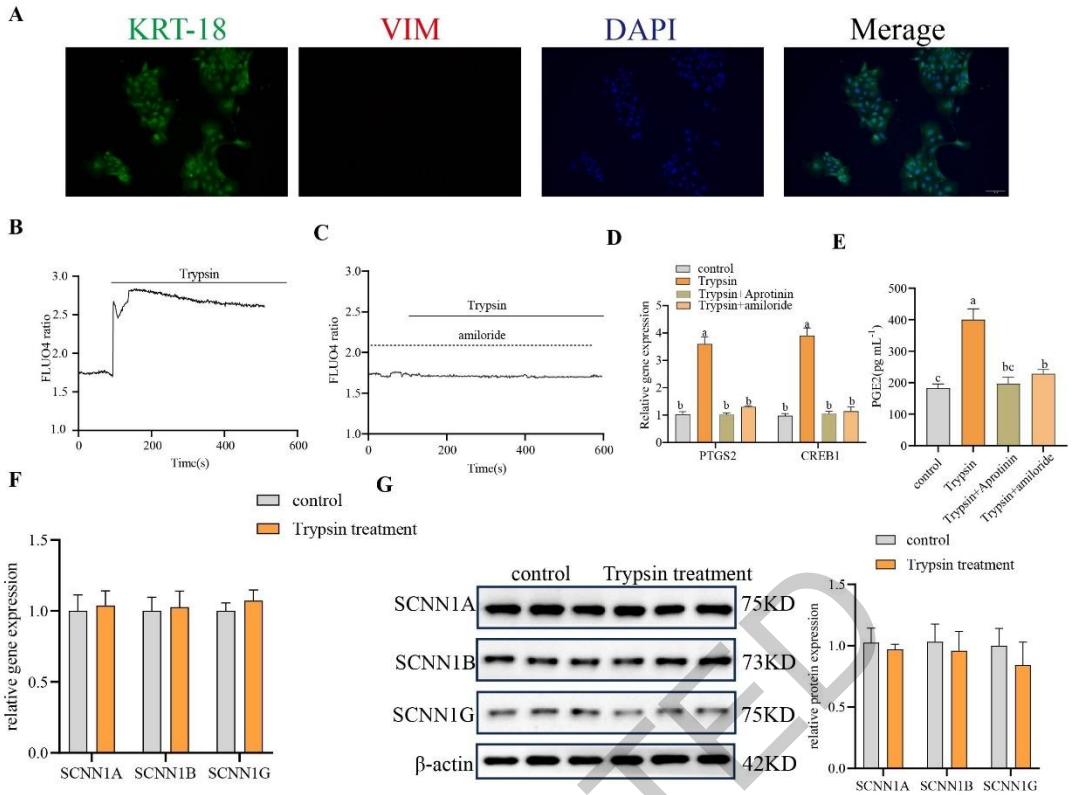


Figure 2. Trypsin promotes PGE2 synthesis in endometrial epithelial cells through ENaC activation. A. Identification of uterine endometrial epithelial cells. **B** and **C**. Calcium ion imaging in EECs treated with trypsin, with or without amiloride pretreatment. **D.** qRT-PCR analysis of PTGS2 and CREB1 mRNA levels in EECs treated with trypsin, with or without pretreatment with aprotinin or amiloride. **E.** Effect of different treatments on PGE2 release from EECs. **F.** qRT-PCR analysis of SCNN1A, SCNN1B, and SCNN1G mRNA levels in EECs treated with trypsin. **G.** Western blot analysis of SCNN1A, SCNN1B, and SCNN1G protein levels in EECs treated with trypsin. Each qRT-PCR experiment was performed with at least four replicates. Data are presented as mean ± SEM. Different superscript letters (a–c) denote statistically significant differences ($p < 0.05$).

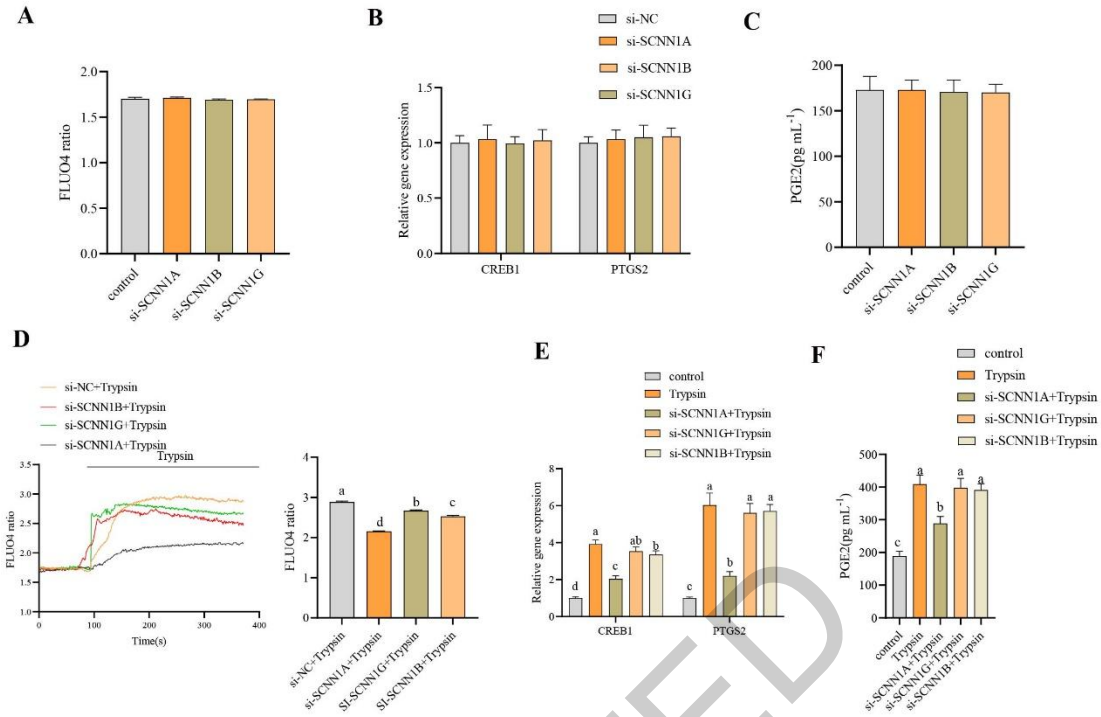


Figure 3. The impact of ENaC subunit knockdown on PGE2 synthesis in endometrial epithelial cells. A. Fluo-4 AM fluorescence intensity indicating intracellular calcium levels following ENaC subunit-specific knockdown. **B**. Expression levels of *CREB1* and *PTGS2* mRNA in EECs after knockdown of different ENaC subunits. **C.** PGE_2 concentration in the supernatant of EECs following knockdown of different ENaC subunits. **D.** Intracellular calcium concentration in EECs upon trypsin treatment after ENaC subunit knockdown. **E.** mRNA expression levels of CREB1 and PTGS2 in EECs treated with trypsin following ENaC subunit knockdown. **F.** PGE_2 concentration in the supernatant of EECs treated with trypsin after ENaC subunit knockdown. Each qRT-PCR experiment was performed with at least four replicates. Data are presented as mean ± SEM. Different superscript letters (a–c) denote statistically significant differences ($p < 0.05$).

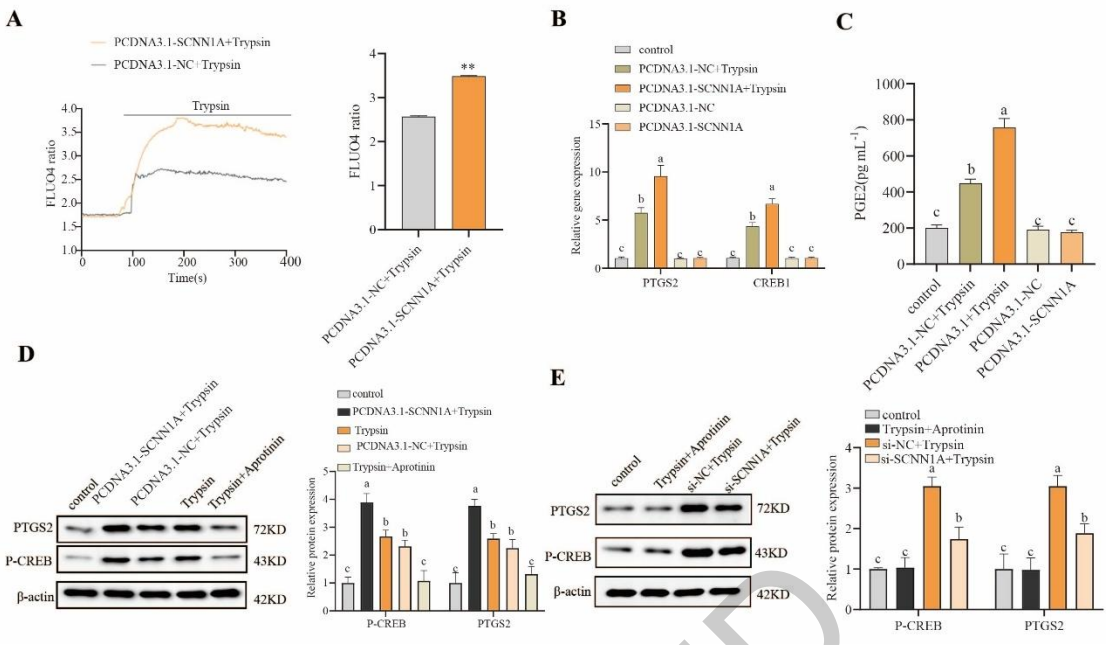
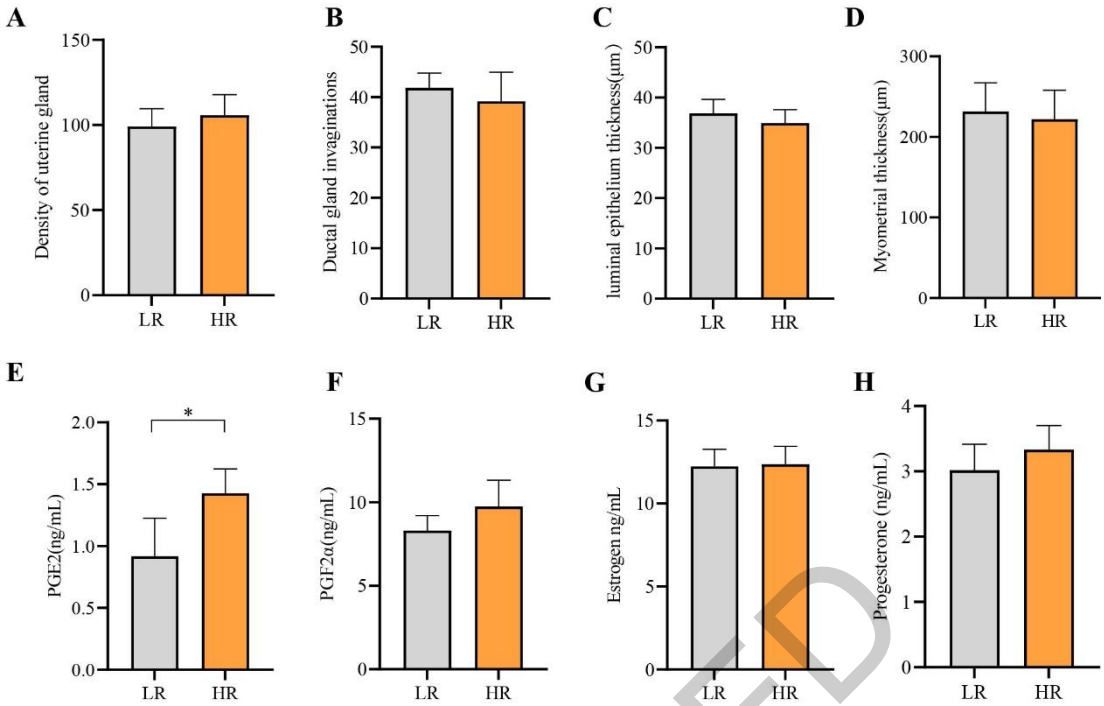


Figure 4. SCNN1A modulates PGE2 synthesis in EECs through the Ca^{2+}/P-CREB/PTGS2 signaling pathway under trypsin treatment. A. Intracellular calcium concentration changes in EECs transfected with an SCNN1A overexpression plasmid after trypsin treatment. **B**. qRT-PCR analysis of PTGS2 and CREB1 mRNA levels in SCNN1A-overexpressing EECs with or without trypsin treatment. **C**. PGE2 levels in the supernatant of SCNN1A-overexpressing EECs with or without trypsin treatment. **D.** Western blot analysis of PTGS2 and phosphorylated CREB1 (P-CREB1) protein levels in SCNN1A-overexpressing EECs with or without trypsin treatment. **E.** Western blot analysis of PTGS2 and P-CREB1 protein levels in EECs transfected with si-SCNN1A and treated with trypsin. Data are presented as means ± SEM (**$p < 0.01$). Different superscript letters (a–c) indicate statistically significant differences ($p < 0.05$). Each experiment included at least 4 replicates for qRT-PCR and 2 replicates for Western blot.



732

733 **Figure 5. Analysis of uterine histology and endocrinological analysis of Hu**

734 **sheep with low embryo survival rate group (LR) and high embryo survival rate**

735 **group (HR), on day 12 of pregnancy. A.** Analysis of uterine gland density. **B.** The

736 mean count of ductal gland invaginations. in five random fields of each slide. **C.**

737 Analysis of luminal epithelium thickness. **D.** Analysis of myometrial thickness. **E.**

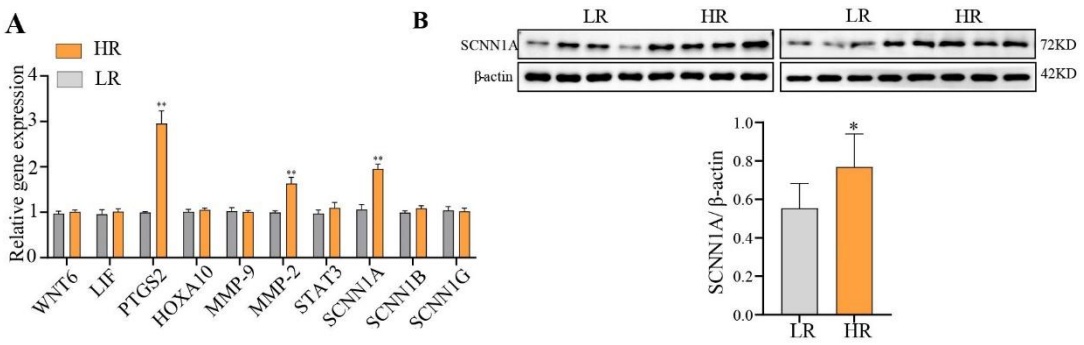
738 Analysis of PGE2 concentration in Uterine flush fluid. **F.** Analysis of PGF2 α

739 concentration in Uterine flush fluid**. G.** Analysis of estrogen concentration in venous

740 blood. **H.** Analysis of progesterone concentration in venous blood. Data are presented

741 as means ± SEM. *$p < 0.05$.

742



743

744 **Figure 6. The expression of *SCNN1A* in the uterine endometrium of Hu sheep**

745 **with different embryo survival rates. A.** qRT-PCR analysis of genes associated with

746 the secretion of PGE2 in the endometrium(n=8). **B.** Western blot analysis of *SCNN1A*

747 protein expression in the endometrium(n=8). Data are presented as means ± SEM. *p

748 < 0.05.

749