Received: 2 April 2021 | Revised: 27 May 2021 | Accepted: 28 May 2021

DOI: 10.1096/fj.202100529RR

RESEARCH ARTICLE

THE FASEB JOURNAL

Rictor/mTORC2 is involved in endometrial receptivity by regulating epithelial remodeling

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Funding information
National Natural Science Foundation of China (NSFC), Grant/Award Number: 31801247

Abstract

Successful embryo implantation requires well-functioning endometrial luminal epithelial cells to establish uterine receptivity. Inadequate uterine receptivity is responsible for approximately two thirds of implantation failures in humans. However, the regulatory mechanism governing this functional process remains largely unexplored. A previous study revealed that the expression of Rictor, the main member of mTORC2, in mouse epithelial cells is increased on the fourth day of gestation (D4). Here, we provide the first report of the involvement of Rictor in the regulation of endometrial receptivity. Rictor was conditionally ablated in the mouse endometrium using a progesterone receptor cre (PR^{cre}) mouse model. Loss of Rictor altered polarity remodeling and the Na^+ channel protein of endometrial cells by mediating Rac-1/PAK1(pPAK1)/ERM(pERM) and Sgk1/pSgk1 signaling, respectively, ultimately resulting in impaired fertility. In the endometrium of women with infertility, the expression of Rictor was changed, along with the morphological transformation and Na^+ channel protein of epithelial cells. Our findings demonstrate that Rictor is crucial for the establishment of uterine receptivity in both mice and humans. The present study may help improve the molecular regulatory network of endometrial receptivity and provide new diagnostic and treatment strategies for infertility.

KEYWORDS

endometrial receptivity, epithelium remodeling, infertility, Na^+ channel function, Rictor

1 | INTRODUCTION

According to a WHO comparative report, at least one in four couples suffers from primary or secondary infertility in developing countries; in developed countries, the incidence of infertility is approximately 15% and has remained stable since the turn of the 20th century.[1] The probability of conception during one menstrual cycle is approximately 30%.

Abbreviations: BSA, bovine serum albumin; DiBAC4(3), bis-(1,3-dibutylbarbituric acid) trimethine oxonol; DNA, deoxyribonucleic acid; E2, estrogen; EECs, endometrial epithelial cells; ER, estrogen receptor; ESCs, endometrial stromal cells; GE, glandular epithelium; IF, immunofluorescence; IHC, immunohistochemistry; IIS, inter-implantation sites; IS, implantation sites; LE, luminal epithelium; mRNA, messenger RNA; mTORC2, the mammalian target of rapamycin complex 2; P4, progesterone; PCR, polymerase chain reaction; PR, progesterone receptor; RNA, ribonucleic acid; TEM, transmission electron microscopy; WB, western blot.

Yue Zhang and Xinman Du contributed equally to this work.

Only 50%–60% of all conceptions advance beyond 20 weeks of gestation. Nearly 75% of pregnancy losses in humans are believed to be due to implantation failure.[2,3] Although a variety of assisted reproductive technologies have been developed, failed implantation is a major limiting step in assisted reproduction as well. Embryo implantation is a multifactorial and complex event initiated by the adhesion between the embryo and epithelial cells of the uterine endometrium. The technology of in vitro embryo culture has been significantly improved. However, there is no effective way to control endometrial receptivity due to the unclear molecular mechanism. A better understanding of the molecular mechanisms contributing to failed pregnancies is important for treating infertility and early pregnancy loss.[3]

In mammals, implantation involves intricate molecular interactions that must be executed within an optimal period. During this period, called "implantation window," the implantation ability of embryo is synchronized with the establishment of uterine receptivity. Ethical concerns and technical issues limit the analysis of human reproduction; therefore, most of the studies exploring implantation mechanisms are carried out using animal models. In mice, the uterus becomes fully receptive following the actions of ovarian progesterone (P4) and estrogen (E2) on fourth day of gestation (D4) and beyond. As the first maternal contact for an implanting embryo, the uterine luminal epithelium (LE) undergoes extensive structural and functional remodeling to establish transient uterine receptivity. Before entering the receptive period, luminal epithelial cells cease proliferation and increase differentiation under the influence of the increasing levels of ovarian P4 and E2. Concomitantly, the epithelium undergoes a dynamic cell junction remodeling, resulting in a decrease in cell polarity, and eventually achieving a state where the blastocyst attaches to the receptive endometrium on D4.5 (20:00-24:00). Recent studies have demonstrated that Rac-1, via PAK1-ERM signaling, directs normal luminal epithelial integrity during embryo implantation. In addition, implantation may be greatly influenced by uterine fluid; excessive intrauterine fluid impairs embryo implantation in both mice and humans.[4] The LE also absorbs the fluid in the lumen. Disappearance of luminal fluid leads to the "closure" of the lumen, thereby enabling better contact between the embryos and the endometrium. Several ion channels have been identified in the endometrial epithelium.[5] Protease-induced epithelial sodium channel (ENaC) activation has been observed in the uterus during implantation in mice.[6]

The mammalian target of rapamycin (mTOR), an evolutionarily conserved serine/threonine kinase, plays a vital role in the regulation of cell growth and proliferation.[7] mTOR complexes (mTORCs) consist of numerous proteins that control mTOR signaling. mTORC1 is composed of mTOR, Raptor, GβL, and Deptor and controls many major processes, including protein and lipid synthesis and autophagy. Previous research by our group focused on the function of mTORC1 in reproduction and demonstrated that mTOR plays an important role in spermatogenesis by regulating p70s6k activation.[8] Compared with mTORC1, the functions of mTORC2 and its upstream and downstream regulatory mechanisms have rarely been explored. mTORC2 is composed of mTOR, Rictor (rapamycin-insensitive companion of mTOR), Lst8, Deptor, and Proter. As the main member of mTORC2, Rictor has been studied widely. It has been reported to inhibit the function of RhoGDI2 (endogenous Rac-1 inhibitor) via downstream PKCα, promoting the expression and function of Rac-1, and playing an important role in the occurrence and development of breast cancer.[9] Jing et al revealed that mTORC2/Rictor can inhibit the degradation of ENaC on the cell membrane by Nedd4-2 via $pSgk1^{s422}$, and control ion movement.[10] Further, mTORC2/Rictor regulates blood–testis barrier dynamics by affecting gap junction communications.[11] Shiota et al demonstrated that placental defects occurring in Rictor-null embryos and fibroblasts exhibit low proliferation rates, impaired AKT/PKB activity, and diminished metabolic activity.[12] Recent studies have shown that mTORC2 is distributed in the rat uterus (LE and decidua) during implantation, suggesting that mTORC2 may be involved in the regulation of endometrial function during embryo implantation.[13] A previous investigation by our group revealed the temporal and spatial expression of Rictor in wild-type mice uterus during the early pregnancy.[14] However, the regulatory mechanism of Rictor in the endometrium during the peri-implantation period is not yet clear.

This study aimed to clarify the regulation and molecular mechanism of Rictor in endometrial functioning during embryo implantation in mice and humans. The investigation will help improve the molecular regulatory network of endometrial receptivity and provide new diagnostic and treatment strategies for infertility.

2 | MATERIALS AND METHODS

2.1 | Mice and tissue collection

$Rictor^{f/f}$ mice (C57 BL/6J) were kindly provided by Prof. Liu Chaohong (Huazhong University of Science and Technology, Wuhan, China); the details of the model have been published earlier.[15] PR^{cre} mice (C57 BL/6J) were purchased from Jackson Laboratory (Stock 017915; USA). Male C57 BL/6J mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Mice carrying the $Rictor^{f/f}$ allele were bred with $PR^{cre/+}$ mice to generate $PR^{cre/+}$ $Rictor^{f/f}$ ($Rictor^{d/d}$) mice. All mice were housed in a specific pathogen-free animal room, under a controlled photoperiod (12-h light/12-h dark) at 22 ± 2°C and 55 ± 10% relative humidity, at the Laboratory Animal Center of Chongqing Medical University, Chongqing,

China (Certificate: SICXK (YU) 2007-0001). All mice had access to water and food ad libitum.

Female mice were mated with fertile males at a ratio of 2:1 to induce pregnancy, and the day of appearance of a vaginal plug was considered day 1 of pregnancy (D1). The mice were sacrificed at 08:00 or 22:00 hours on D4 and D5. Part of the endometrium and ovaries were collected and stored at −80°C for quantitative polymerase chain reaction (qPCR) and western blotting (WB). The remaining samples were fixed in 4% paraformaldehyde or glutaraldehyde for immunohistochemistry (IHC), immunofluorescence (IF), and transmission electron microscopy (TEM). The implantation sites (IS) on D5 tissues were identified by intravenous injection of trypan blue through the tail vein. Uteri without IS were flushed with saline to recover the unimplanted blastocysts.

All human samples were obtained from fertile and infertile women visiting the clinic at the Department of Gynecology at the First Affiliated Hospital of Chongqing Medical University between 2018 and 2020. The samples were obtained from healthy women (control group, n = 3) and infertile women (primary infertility or secondary infertility, n = 3), aged 20-35, who underwent hysteroscopy. Age, weight, and gestational weeks did not differ significantly among the participants. The inclusion criteria for selecting human participants for this study were as follows: (1) regular menstrual cycle; (2) no history of genetic diseases; (3) no sexually transmitted diseases; (4) non-immunodeficient; (5) no endocrine diseases; (6) no chromosomal abnormalities; (7) normal uterine anatomy (examined by ultrasound); and (8) normal ovarian anatomy and function (detected by ultrasound and serum hormone levels). The treatment of tissues has been described previously.

2.2 | Hormone assays

Blood samples from $Rictor^{d/d}$ and $Rictor^{f/f}$ mice were individually collected by vein puncture on D0, D4, D4.5, and D5. Serum concentrations of E2 and P4 were measured using enzyme-linked immunosorbent assay (ELISA) kits (Ruixinbio Quanzhou, China) following the manufacturer's instructions.

2.3 | TEM analyses

For TEM analyses, endometrial tissues were fixed in glutaraldehyde, post-fixed in 1% OsO_4, and transferred to propylene oxide. The samples were then infiltrated with propylene oxide resin. After polymerization, ultrathin (70 nm) sections were prepared, and stained with uranyl acetate and lead citrate. The samples were observed using a TEM Hitachi-7500 with an accelerating voltage of 100 kV.[16]

2.4 | RNA extraction and qPCR

Total RNA was extracted from endometrial tissues and cells using RNAiso Plus reagent (TaKaRa, China) following the manufacturer's protocol. cDNA was synthesized from an appropriate quantity of total RNA treated with DNase I in a 10-μL reaction system using an qPCR kit (TaKaRa, China). β-actin, a housekeeping gene, was used to normalize the data. The specific primers used for the relative genes are listed in Table S1. All primers were synthesized by Songon Biotech Co., Ltd. (Shanghai, China). Transcripts were quantified using SYBR Green, and PCR analyses were performed on a Bio-Rad CFX Manager 3.1 Detection System (USA). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels of genes in the endometrial tissues and cells. All reactions were performed in duplicate.

2.5 | WB analysis

Proteins were extracted from endometrial tissues and cells using lysis buffer. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China) following the manufacturer's protocol. Samples were boiled in 5× sodium dodecyl sulfate (SDS) sample loading buffer for 10 minutes and loaded onto a 10% SDS-PAGE gel. Samples were electrophoresed for 100 minutes at a constant voltage (80 V) and then transferred onto PVDF membranes. Membranes were blocked for 1 hour at room temperature in PBST containing 5% skim milk or bovine serum albumin (BSA) powder. Membranes were incubated with the following antibodies overnight at 4°C: anti-Rictor (1:1000 dilution; Cell Signaling Technology, MA, USA), anti-ENacα (1:1000 dilution; HUABIO, China), anti-Sgk1/pSgk1 (1:1000 dilution; Abcam, UK), anti-Rac-1 (1:1000 dilution; HUABIO, China), anti-ERM/pERM (1:1000 dilution; Cell Signaling Technology, MA, USA), anti-PAK1/pPAK1 (1:1000 dilution; Cell Signaling Technology, MA, USA), and anti-β-actin (1:2000 dilution; Sigma, USA). The membranes were subsequently washed three times (for 15 minutes each) with PBST. The washing process was repeated after incubation for 1 hour with goat anti-rabbit IgG or goat anti-mouse IgG. Protein expression was detected using the ECL Plus reagent according to the manufacturer's protocol, and analyzed using Quantity One 4.6 software.

2.6 | Immunohistochemistry and immunofluorescence

Tissue samples were embedded in paraffin according to standard histological procedures, and sectioned to obtain

continuous sections of 4 μm thickness. The sections were deparaffinized in xylene, rehydrated in descending concentrations of ethanol after antigen retrieval in sodium citrate buffer, and cooled to room temperature. The tissue sections were blocked in 10% normal goat serum for 30 minutes and incubated with anti-Rictor antibody (1:100 dilution; Sigma, USA), anti-Lif (1:500 dilution; Sigma, USA), anti-Ki67 (1:100 dilution; Abcam, UK), anti-E-cad (1:500 dilution; Santa Cruz Biotechnology, Inc., TX, USA), anti-CK8 (1:500 dilution; Abcam, UK), anti-ER (1:200 dilution; Santa Cruz Biotechnology, Inc., TX, USA), anti-pER (1:200 dilution; Abcam, UK), anti-PR (1:200 dilution; Santa Cruz Biotechnology, Inc., TX, USA), anti-Occludin (1:500 dilution; Abcam, UK), anti-Claudin-7 (1:500 dilution; Abcam, UK), and anti-Par3 (1:500 dilution; Abcam, UK) at 4°C overnight. The tissue sections were then incubated with a secondary antibody (goat anti-rabbit IgG or fluorescent secondary antibody) at 37°C for 30 minutes. Next, the sections were incubated with streptavidin-conjugated horseradish peroxidase at 37°C for 30 minutes. Staining was performed using DAB substrate for 5 minutes at room temperature and terminated by rinsing with water. The sections were subsequently stained with hematoxylin or DAPI. Images were captured using an Olympus microscope (BX40, Olympus, Tokyo, Japan).

2.7 | Isolation of endometrial epithelial cells (EECs) and culture of Ishikawa cells

The uteri obtained from Rictor$^{d/d}$ and Rictor$^{f/f}$ mice were sectioned longitudinally or cut into small fragments with sterile scissors, and subsequently washed thoroughly with D-Hanks' solution. The uterine tissues were placed in Hanks' basal salt solution (HBSS) containing an appropriate concentration of trypsin and dispase at 4°C for 1.5 hours and then incubated at room temperature and at 37°C for 30 minutes each. The digested uteri were gently agitated by pipetting to dislodge the sheets of luminal epithelial cells. After centrifugation, the supernatant containing sheets of epithelial cells was separated. Next, BSA (5%) was added to the supernatant, epithelial clumps were allowed to settle at the bottom, the supernatant was removed, and the epithelial clumps were washed with D-Hanks' solution. After the above process was repeated twice, the epithelial cells were resuspended in culture medium (Dulbecco's modified Eagle's medium-nutrient mixture F-12 Ham [DMEM-F12, Sigma] with 20% charcoal-stripped fetal bovine serum [BI, Israel]) and plated into a Petri dish covered with 10% gelatin. After 30 minutes, the unattached epithelial cell suspension was plated into 6-well cell culture plates.

Ishikawa cells, kindly provided by Prof. Su Renwei (South China Agricultural University, Guangzhou, China), was plated into 25-cm^2 culture bottles containing RPMI 1640 medium (Gibco, USA) supplemented with 10% charcoal-stripped fetal bovine serum (Gibco, USA).

2.8 | Cell transfection

Seed cells were 70%-90% confluent under normal growth conditions (37°C, 5% CO_2) before transfection. The shRNA specifically targeting mouse and human *Pdcd4* (HANBIO, China) were mixed with Poly Reagent in appropriate proportions. The mixture was added dropwise to the cells. The plates were then gently swirled to distribute the transfection complexes uniformly among all cells. The cells were incubated at 37°C and 5% CO_2 and were kept under observation. The complexes were removed after 12 hours, and fresh culture medium was added to the cells.

2.9 | Membrane potential measurement

Epithelial cells on a confocal dish were washed with HEPES buffer (HHBS). The voltage-sensitive dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC4(3)) (Sigma, USA) was dissolved in DMSO, and a working solution of 30 μM in HHBS was prepared at pH 7, with 0.04% Pluronic F-127 and 2-mM Trypan Red Plus. The samples were mixed well by vortexing. DiBAC4(3) dye (200 μL/well) was added to the cell plate after treatment with 20 μg/mL of trypsin, and the cells were incubated for 30-60 minutes. Fluorescence (495/520-nm excitation/emission) was monitored under a confocal microscope to determine the change in membrane potential.

2.10 | Statistics

GraphPad Prism software was used for Student's *t*-test analyses for two comparison tests and one-way ANOVA for multiple comparison tests. Each experiment was repeated at least three times in each group. Statistical significance was set at $P < .05$.

2.11 | Study approval

All mouse procedures were approved by the Ethical Committee of Chongqing Medical University. Written informed consent was obtained from all the patients before participation.

3 | RESULTS

3.1 | Mice lacking Rictor resulted in embryo implantation failure

The reproductive role of Rictor in the function of the adult uterus was investigated by breeding mice carrying the Rictor$^{f/f}$ allele with Pgr$^{cre/+}$ mice to generate Pgr$^{cre/+}$ Rictor$^{f/f}$ (Rictor$^{d/d}$) mice; female Pgr$^{cre/-}$ Rictor$^{f/f}$ (Rictor$^{f/f}$) mice were used as the control. Results of uterine IHC indicate that Rictor was knocked out in the whole uteri of Rictor$^{d/d}$ mice as compared with Rictor$^{f/f}$ mice (Figure 1A). WB also showed that Rictor protein was decreased in Rictor$^{d/d}$ mouse uteri (Figure 1B). Thus, the mouse model lacking Rictor was successfully established.

Embryo implantation was assessed by mating 8-week-old Rictor$^{d/d}$ females with wild-type C57BL/6J males. Mice were sacrificed on D5 (the presence of copulatory plug was recorded as D1) to determine the number of IS, identified by intravenous injection of trypan blue through the tail vein. As shown in Figure 1C,D, Rictor$^{f/f}$ mice showed normal embryo IS, whereas no embryo IS were observed in Rictor$^{d/d}$ mice. Subsequently, several embryos were flushed from the uteri of Rictor$^{d/d}$ mice using saline. This indicates that implantation failure was not due to the absence of embryos but because the embryos failed to attach and implant onto the endometrial epithelium on D4.5. As shown in Figure 1E, wet weight of Rictor$^{d/d}$ mice was significantly lower than that of Rictor$^{f/f}$ mice. These results suggest that the function of Rictor$^{d/d}$ mouse endometrium was impaired during the peri-implantation period.

3.2 | Rictor defines uterine receptivity

In humans and mice, the uterus differentiates into a receptive state when the blastocysts are ready for implantation. This state is called the "implantation window" and only lasts for a limited time. During this period, the uterine environment is beneficial for blastocyst attachment and other biological events associated with implantation. In the pre-implantation period, luminal epithelial cells cease proliferation and increase differentiation under the influence of increasing levels of ovarian P4 and E2. Thus, we next analyzed cell proliferation in the uterine epithelium using IHC. Rictor$^{d/d}$ mice uterine LE showed strong Ki67 staining, whereas stromal staining was markedly suppressed (Figure 2A). In addition, Lif, a marker of uterine receptivity, was decreased in Rictor$^{d/d}$ uteri (Figure 2A). IHC analyses of the Rictor$^{d/d}$ mice uteri on D4 and D4.5 showed an increase in estrogen receptor α (ERα) and p-ERα in the LE, and a reduction in glandular epithelium compared to Rictor$^{f/f}$ mice (Figure 2A). Concomitantly, as downstream targets of ER, mRNA levels of *Muc1* and *Ltf* (targeting epithelial cells) were elevated, whereas *Lgf* and *Hsd11b2* (targeting stromal cells) were decreased in Rictor$^{d/d}$ mice (Figure 2B). IHC analyses showed that the progesterone receptor (PR) signal was present in the nucleus of stromal cells, and was lower in the stromal cells of Rictor$^{d/d}$ uteri. The downstream targets of PR, viz., *Areg*, *Ihh*, and *Hand2*, were also downregulated in Rictor$^{d/d}$ mice (Figure 2B). Interestingly, we found that vigorous proliferation of epithelial cells was accompanied

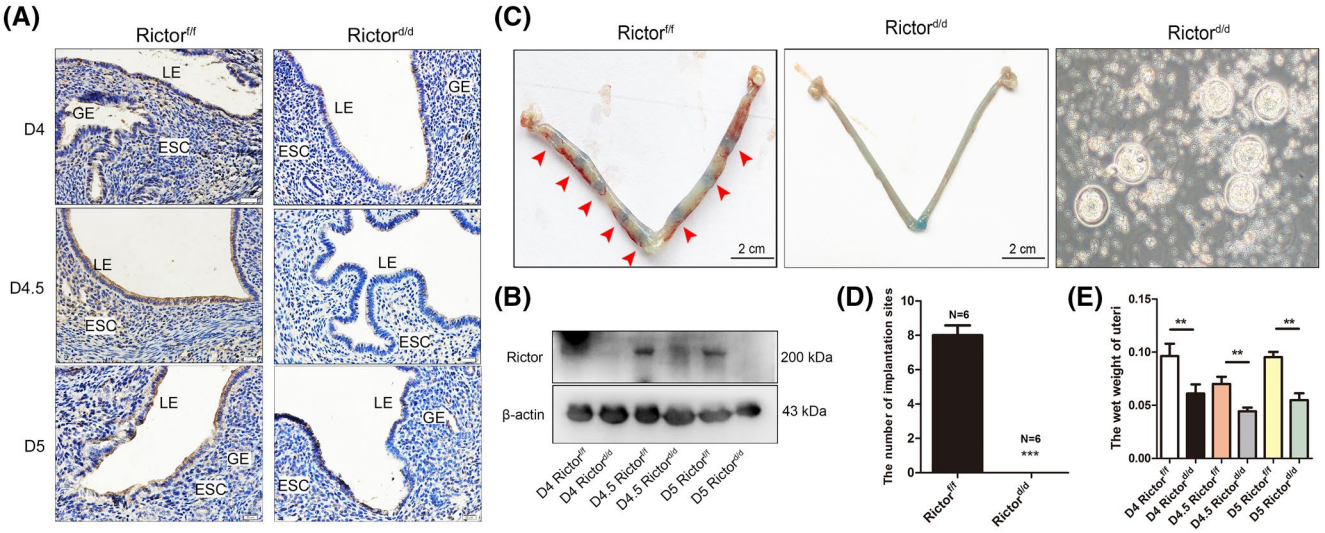

FIGURE 1 Phenotype of conditional deletion of Rictor in the mouse uterus. A, Immunohistochemical staining of Rictor in Rictor$^{f/f}$ and Rictor$^{d/d}$ mice uteri on D4, D4.5, and D5 (N = 3 per group). Scale bar: 20 μm. B, Western blotting analysis of Rictor in Rictor$^{f/f}$ and Rictor$^{d/d}$ mice uteri on D4, D4.5, and D5. β-actin was used as the control. C, Embryo implantation sites in Rictor$^{f/f}$ and Rictor$^{d/d}$ mice at D5, and embryos flushed from Rictor$^{d/d}$ mice uteri. Scale bar: 2 cm. D, Histogram of embryo implantation sites in Rictor$^{f/f}$ and Rictor$^{d/d}$ mice (N = 6 per group, analyzed by *t* tests). E, Histogram of uterine wet weight (N = 6 per group, analyzed by one-way ANOVA). ESCs, endometrial stromal cells; GE, glandular epithelium; LE, luminal epithelium. The values are shown as the mean ± SEM. $^*P < .05$, $^{**}P < .01$

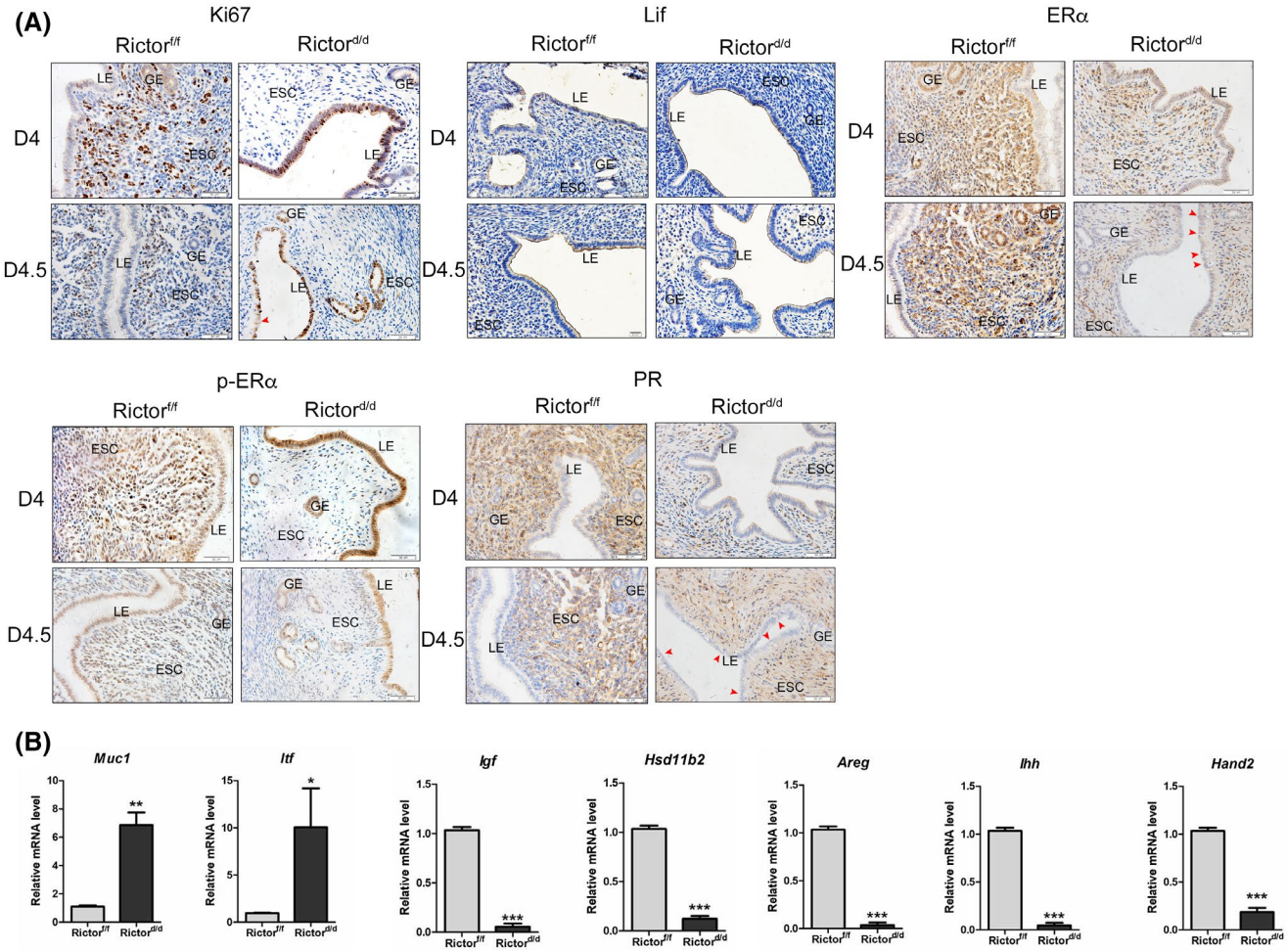

FIGURE 2 Impaired uterine receptivity in Rictor-deficient uterus. A, Immunohistochemical staining of Ki67, Lif, ERα, p-ERα, and PR in Rictor$^{f/f}$ and Rictor$^{d/d}$ mice uteri on D4 and D4.5 (N = 3 per group). Scale bar: 50 μm. B, Relative mRNA expression of *Muc1*, *Ltf*, *Lgf*, *Hsd11b2*, *Areg*, *Ihh*, and *Hand2* in Rictor$^{f/f}$ and Rictor$^{d/d}$ mice uteri on D4 (N = 6 per group, analyzed by *t* tests). ESCs, endometrial stromal cells; GE, glandular epithelium; LE, luminal epithelium. Data represent the mean ± SEM. $^{*}P < .05$, $^{**}P < .01$

with vacuolation/apoptosis of cells in Rictor$^{d/d}$ uteri. The results presented above indicate that loss of Rictor impairs uterine receptivity.

3.3 | Rictor regulates the morphological transformation of EECs by influencing tight junction-related proteins via Rac-1

Luminal epithelial cells undergo dynamic membrane reorganization and depolarization during the "implantation window." As mentioned above, Rictor defines uterine receptivity; therefore, we focused on the morphological transformation of EECs. Analysis of uterine epithelial micromorphology by TEM revealed that luminal epithelial cell microvilli of D4.5 Rictor$^{f/f}$ mice became shorter (black arrow), and some cell tight junctions opened; in Rictor$^{d/d}$ mice, luminal epithelial cell microvilli disappeared, tight junctions were still prominent, some of the perinuclear spaces widened (red arrow), and more apoptotic or necrotic cells (red *) were observed (Figure 3A). Analysis of uterine epithelial morphology following CK8 staining showed that loss of Rictor impacted the morphological transformation of uterine epithelial cells; unlike the epithelial cells of Rictor$^{f/f}$ mice, luminal epithelial cells in Rictor$^{d/d}$ mice were still columnar and had a multilayered structure. Some cells in Rictor$^{d/d}$ D4.5 mice were vacuolated (red arrow) (Figure 3B). Tight junctions between cells occurred frequently between epithelial cells. Occludin and Claudin-7 are the main proteins that constitute tight junctions. IF showed that the Occludin protein was markedly increased in the LE in D4 and D4.5 Rictor$^{d/d}$ mice. Interestingly, the positive signal was more obvious at the top of the epithelial cells towards the lumen. In addition, Claudin-7 was also increased in luminal epithelial cells, and the positive signal was more obvious between the cells. Par3 is a conserved group of membrane proteins that is segregated from the cell cortex. Cell polarity is defined by the domains of these proteins.[17] IF showed that Par3 was increased in D4 and D4.5 Rictor$^{d/d}$

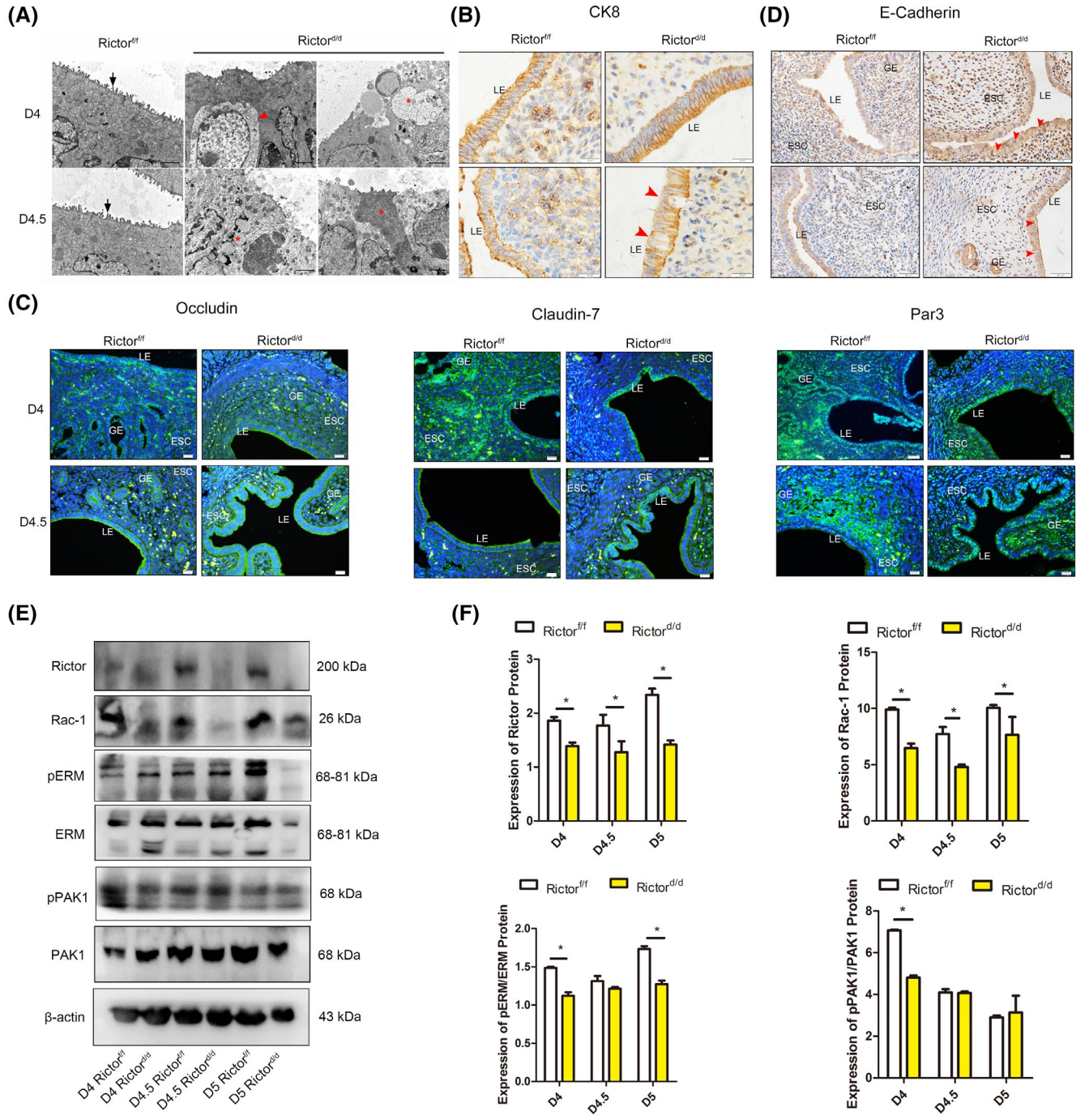

FIGURE 3 Dysregulated morphological transformation of endometrial epithelial cell in Rictor-deficient mice during the window of receptivity. A, Uterine epithelial micromorphology detected by TEM in D4.5 Rictor$^{f/f}$ and Rictor$^{d/d}$ mice endometrium. Black arrow: cell microvilli; Red arrow: perinuclear space; Red *: apoptotic cell. Scale bar: 2 μm. B, Immunohistochemical staining of CK8 in Rictor$^{f/f}$ and Rictor$^{d/d}$ mice uteri on D4 and D4.5 (N = 3 per group). Red arrow: vacuolating cell. Scale bar: 20 μm. C, Immunofluorescence staining of Occludin, Claudin-7, and Par3 in Rictor$^{f/f}$ and Rictor$^{d/d}$ mice uteri on D4 and D4.5 (N = 3 per group). Scale bar: 20 μm. D, Immunohistochemical staining of E-Cadherin in Rictor$^{f/f}$ and Rictor$^{d/d}$ mice uteri on D4 and D4.5 (N = 3 per group). Red arrow: vacuolating cell. Scale bar: 50 μm. E, Western blotting analysis of relative proteins in Rictor$^{f/f}$ and Rictor$^{d/d}$ mice uteri on D4, D4.5, and D5. β-actin was used as the control. F, Quantitative analysis diagram of Figure E. ESCs, endometrial stromal cells; GE: glandular epithelium; LE, luminal epithelium

mice. Notably, the positive signal was mainly located at the apical portion of the LE in Rictor$^{f/f}$ mice. However, in Rictor$^{d/d}$ mice, the location of Par3 was changed, and some of it was expressed between the cells (Figure 3C). IHC for E-cadherin showed a positive signal mainly at the top of luminal epithelial cells in D4 and D4.5 Rictor$^{f/f}$ mice; however, in D4 and D4.5 Rictor$^{d/d}$ mice, the expression of E-cadherin increased at both apices and the sides of the cells (Figure 3D).

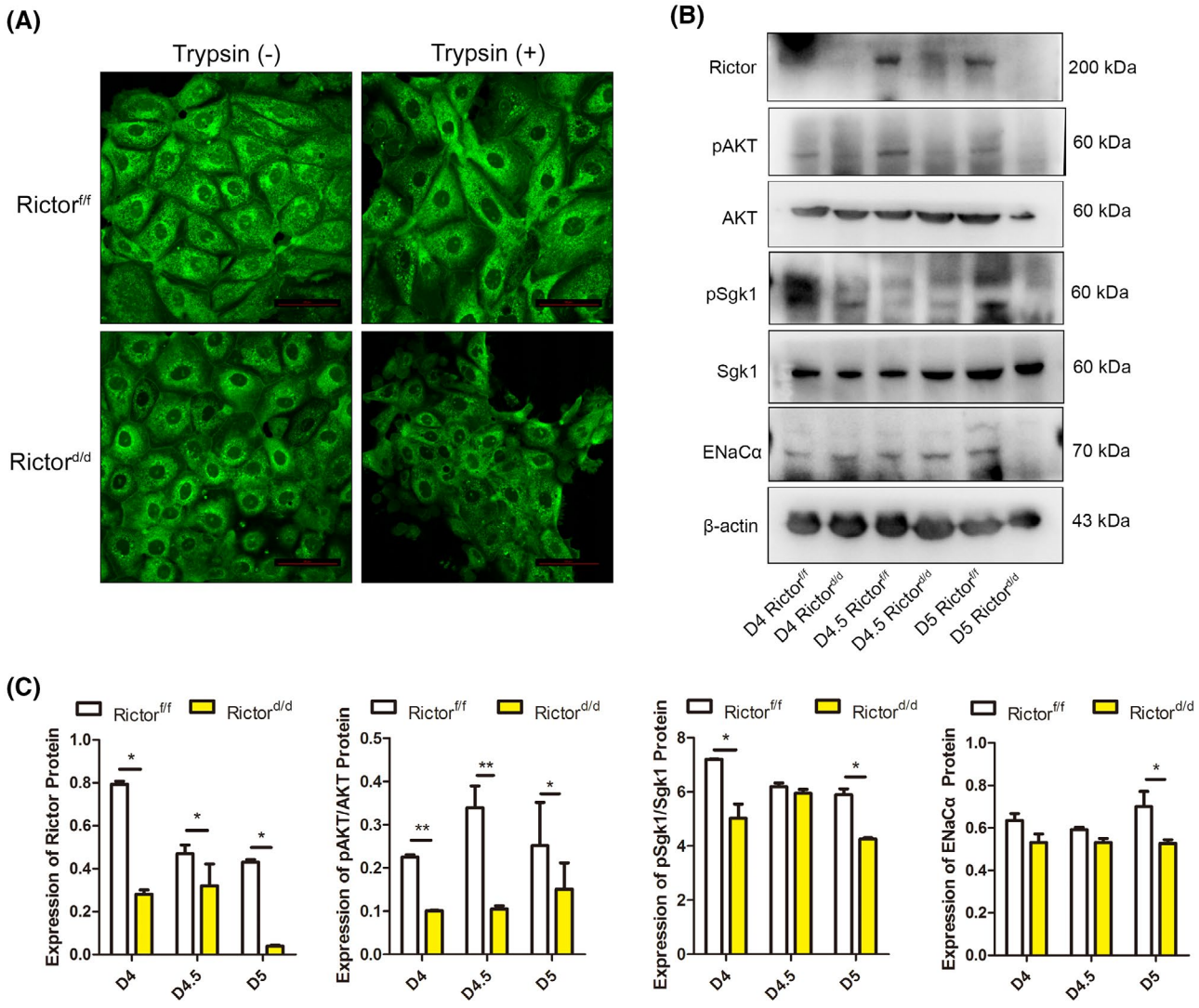

FIGURE 4 Na^+ channel function of epithelial cells was changed in $Rictor^{d/d}$ mice during the window of receptivity. A, Membrane potential measurement of epithelial cells performed under confocal microscope. Intensity of green fluorescence represents the strength of membrane potential. Scale bar: 50 μm. B, Western blotting analysis of relative proteins in $Rictor^{f/f}$ and $Rictor^{d/d}$ mice uteri on D4, D4.5, and D5. β-actin was used as the control. C, Quantitative analysis diagram of Figure B

All the results indicated that the morphological transformation of EECs was changed by deleting Rictor during the "implantation window."

Previous research has shown that uterine depletion of Rac-1 induces a decrease in epithelial polarity, defective tight junctions, and PAK1/ERM as the downstream signaling of Rac-1.[18] Therefore, we speculated that the Rac-1/PAK1(pPAK1)/ERM(pERM) signaling pathway plays an important role in the regulation of EEC transformation, which is regulated by Rictor. WB for $Rictor^{d/d}$ mice showed that Rictor was successfully deleted, and the expression of Rac-1 was reduced. The expression of pERM was reduced in D4 and D5 $Rictor^{d/d}$ mice, and the expression of pPAK1 was reduced in D4 $Rictor^{d/d}$ mice (Figure 3E).

3.4 | Rictor regulates Na^+ channel function in endometrial epithelium

Uterine fluid absorption acts as a rate-limiting process in reproduction and is secondary to ion movement.[19] Na^+ at the apical membrane of the epithelium is essential for electrolyte and fluid absorption. Previous research has indicated that ENaC mediates fluid absorption and immobilization of blastocysts.[20] WB showed that there was no significant difference in ENaCα protein between $Rictor^{d/d}$ and $Rictor^{f/f}$ mice. Measurement of membrane potential of the luminal epithelial cells of mice indicated that the membrane potential signal was enhanced in the cytoplasm after adding trypsin (20 μg/mL) to simulate blastocyst stimulation signal in EECs

of Rictor$^{f/f}$ mice. However, the membrane potential signal was not enhanced in Rictor$^{d/d}$ mice (Figure 4A). It has been reported that Sgk1 activates the expression of ENaC by suppressing NEDD4-2.[21] WB analysis showed that in Rictor$^{d/d}$ mice, pAKT and pSgk1 were downregulated (Figure 4B). These observations suggest that Rictor is involved in the regulation of Na^+ channel function in EECs via Sgk1/pSgk1.

3.5 | Rictor regulates human endometrial receptivity by regulating uterine epithelium remodeling

Clinical endometrium samples were collected for further exploration; the information of representative samples is provided in Table 1. IHC staining showed that Rictor was mainly located in the cytoplasm of epithelial and stromal cells. In samples with infertility caused by endometrial defects, Rictor staining was markedly reduced compared to that in normal samples (Figure 5A). IF analysis of Occludin and Claudin-7 showed that tight junctions between luminal epithelia were enhanced in infertile samples (Figure 5B). For the Ishikawa cells, results of WB showed that the expression of Rictor, Rac-1, pERM, and pPAK1 was decreased after transfection with shRNA-Rictor (Figure 5D). Measurement of membrane potential for Ishikawa cells showed that the fluorescence signal was enhanced in the cytoplasm after trypsin treatment. However, when the cells were transfected with shRNA-Rictor, the membrane potential was significantly reduced (Figure 5C). Further, WB revealed that pAKT, pSgk1, and ENaCα were also reduced after transfection with shRNA-Rictor (Figure 5E). Thus, it may be inferred that Rictor regulates human endometrial receptivity by regulating epithelial remodeling.

4 | DISCUSSION

Poor uterine receptivity is responsible for approximately two thirds of implantation failures, whereas the quality of the embryo is responsible for only one-third of such cases.[22] Aberrant implantation leads to unsuccessful pregnancy outcomes. Previous studies have revealed that a large number of transcription factors and cytokines are involved in regulating this complicated and delicate process. Little is known about the Rictor/mTORC2 pathway in this regard. mTORC2 regulates survival/metabolism and the cytoskeleton through the phosphorylation of many AGC kinases, including AKT, Sgk1, and PKC-α.[23] Previous studies have focused on the function of Rictor in tumors, because it can promote cell survival and inhibit apoptosis. Here, we demonstrate for the first time that Rictor is crucial for regulating uterine epithelial cell remodeling and embryo implantation in mice and humans. The uterine LE is the first maternal contact for a blastocyst. LE membrane remodeling and uterine fluid resorption are prerequisites for establishing transient uterine receptivity for embryo implantation.[24] On the morning of D4, mouse epithelial cells undergo dynamic junction remodeling, leading to a decrease in epithelial cell polarity as well as a cell-shape transition from columnar to cubic.[18,25] In this study, we found that the endometrial LE cells in Rictor-deleted mice were still columnar with polarity on D4, and there was a defect in junction remodeling. These phenotypes indicated that Rictor is involved in the regulation of epithelial cell remodeling, which ultimately affected uterine receptivity during the "implantation window." However, the regulatory mechanism remains unclear.

Recent studies revealed that some transcription factors and signaling molecules, such as Msx1, KLF5, Wnt5a, Stat3, and Rac-1, participate in the regulation of uterine epithelial transformation.[26,27] Ras-related C3 botulin toxin substrate 1 (Rac-1) is a multifunctional key factor involved in epithelial development, differentiation, apoptosis, and cell migration.[18,28-31] Tu et al found that uterine depletion of Rac-1 induces a premature decrease in epithelial apical-basal polarity and defective junction remodeling via PAK1-ERM signaling, leading to disrupted uterine receptivity and implantation failure.[18] Consistently, we found that the loss of Rictor in the endometrium inhibited the Rac-1 signaling pathway significantly. However, unlike the cellular experiment, there were no significant differences in the expression of PAK1-ERM signaling-related proteins between D4.5 Rictor$^{f/f}$ and Rictor$^{d/d}$ mice uteri. This may be attributed to the diverse cell types that exist in proteins extracted from the uterus, wherein luminal epithelial cells account for only a small proportion. The LE microvilli are the initial contact for blastocysts and are also considered to be morphological indicators for the establishment of uterine receptivity.[32,33] The LE microvilli in mice are long and thin under the influence of ovarian E2 on D1. In the subsequent days, the microvilli become shorter and more irregular with increasing P4 levels to make the LE surface smoother.[34] The shortening of microvilli is a prerequisite for the establishment of endometrial receptivity; however, excessive shortening or even disappearance of microvilli caused by the loss of Rictor may lead to the failure of endometrial receptivity. The regulation of cell microvilli depends on cytoskeletal function. The cytoskeleton,

TABLE 1 Information of representative clinical endometrial samples

ID	Age	Menstrual cycle	Note
Con	32	Proliferation	Normal endometrium
Infertility 1	28	Pre-secretion	Primary infertility
Infertility 2	29	Mid-secretion	Secondary infertility

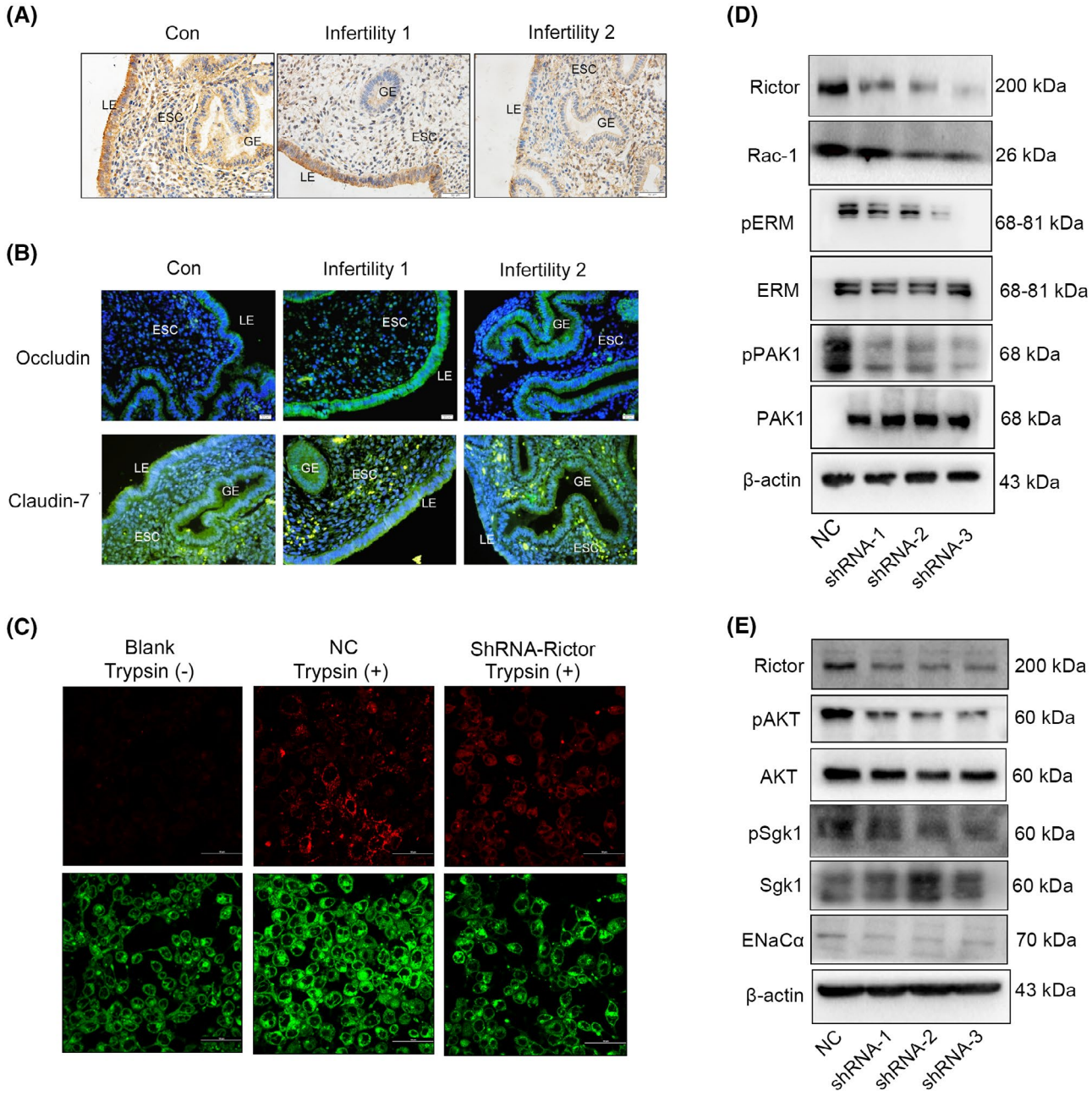

FIGURE 5 Rictor regulates human endometrial receptivity. A, Immunohistochemical staining of Rictor in Con and infertile endometrial tissues (N = 3 per group). Scale bar: 50 μm. B, Immunofluorescence staining of Occludin and Claudin-7 in Con and infertile endometrial tissues (N = 3 per group). Scale bar: 20 μm. C, E, Western blotting analysis of relative proteins in Ishikawa cells. β-actin was used as the control. D, Membrane potential measurement of Ishikawa cells performed under confocal microscope. Intensity of green fluorescence represents the strength of membrane potential, and the red fluorescence was carried by shRNA. Scale bar: 50 μm. ESCs, endometrial stromal cells; GE, glandular epithelium; LE, luminal epithelium

which includes microfilaments, microtubules, and intermediate filaments, is a complex and dynamic protein network that connects the nucleus and plasma membrane. PKC-α is an AGC kinase that is activated by mTORC2. Along with other effectors, such as paxillin and Rho GTPases, the activation of PKC-α by mTORC2 regulates cell shape in a cell type-specific manner by affecting the actin cytoskeleton.[35,36] As an important component of the cytoskeleton, microtubules play an important role in mediating the dialog between epithelial cells and blastocysts or stromal cells through autocrine and paracrine mechanisms. A recent study using a rat model revealed that microtubule depolymerization occurred in EECs during the establishment of receptivity.[37] Further, excessive depolymerization of microtubules led to the failure of uterine receptivity in a mouse model.[38] These data suggest that dynamic changes in epithelial microtubules are precisely

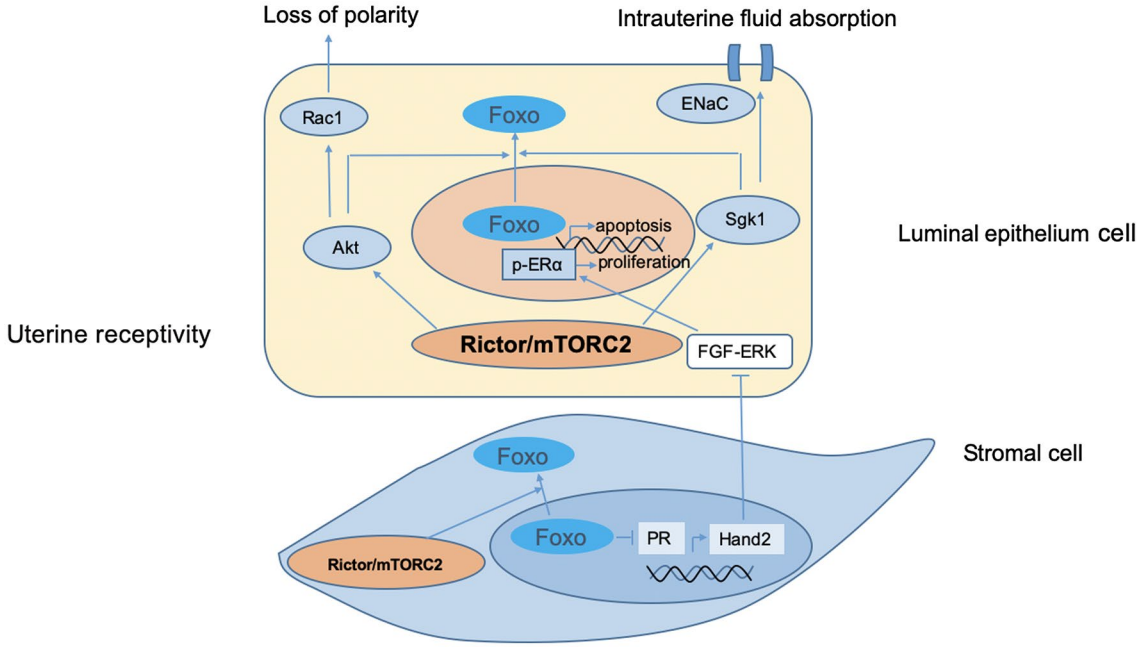

FIGURE 6 Summary chart illustrating how Rictor is essential for uterine receptivity

controlled by complex and fine regulatory networks during the establishment of receptivity. We speculate that Rictor may regulate epithelial remodeling by regulating the cytoskeleton, which is worthy of in-depth study in the future.

Ion channels are a group of transmembrane proteins that allow ions to flow across cell membranes.[19,39] Ion flow changes membrane potential and pH, making ion channels crucial for many physiological processes.[40] Several ion channels have been discovered in the endometrium. Cystic fibrosis transmembrane conductance regulator (CFTR) and ENaC are the main ion channels involved in regulating endometrial receptivity and embryo implantation. CFTR is essential for fluid secretion. It mediates Cl^- efflux and drives water to move into the lumen. In contrast, ENaC plays a key role in fluid absorption and is significantly upregulated in EECs during the receptive phase to accelerate the closure of the LE and embryo implantation.[41] In addition, ENaC also promotes decidualization and activates CREB and Cox2, which regulate downstream genes involved in implantation.[42,43] ENaC is encoded by sodium channel non-neuronal (SCNN1) and is composed of α, β, and γ subunits.[44] Recent studies have indicated that α-ENaC is expressed in the apical membrane of the human endometrial epithelium,[45] but the upstream regulatory mechanism is still unclear. In this study, we revealed that Rictor regulates ENaC in EECs.

Salker et al demonstrated that serum and glucocorticoid-induced kinase-1 (Sgk1) regulate the fluid environment in the uterus by promoting the expression of ENaC in EECs.[21] Previous studies have shown that Rictor promotes the occurrence and development of pancreatic cancer directly by activating the phosphorylation of Sgk1[46]; therefore, we focused on Sgk1 in our studies. Results showed that the regulation of ENaC by Rictor depends on Sgk1. In addition, apoptosis of epithelial cells was observed in $Rictor^{d/d}$ mice (Figures 3A and S1A). Previous reports indicate that mTORC2 regulates cell survival and apoptosis through the phosphorylation of Sgk1 and AKT. Rictor/mTORC2 directly activates AKT by phosphorylating its hydrophobic motif (Ser473), a site required for maximal activation.[47] AKT regulates cellular processes such as metabolism, survival, apoptosis, growth, and proliferation through the phosphorylation of several effectors. Defective AKT-Ser473 phosphorylation associated with mTORC2 depletion impairs the phosphorylation of some AKT targets, including forkhead box O1/3a (FOXO1/3a).[48] PI3K/AKT-dependent phosphorylation of FOXO proteins triggers their export from the nucleus and promotes the binding of these transcription factors to the 14-3-3 chaperone proteins in the cytosol. Sgk1 has also been implicated in FOXO phosphorylation and nuclear export. FOXO1 has been demonstrated to induce apoptosis through its localization to the nucleus and to enhance subsequent transcription of several genes involved in the apoptotic pathway.[49] We also confirmed that FOXO1 localized to the nucleus in the endometrial epithelium of $Rictor^{d/d}$ mice (Figure S1B), and activation of FOXO1 transcriptional activity following the inhibition of AKT and Sgk1 may be responsible for epithelial cell apoptosis. Thus, the absence of Rictor in endometrium directly leads to the failure of receptivity establishment via FOXO1. Previous studies have shown that FOXO1 was localized to the nucleus in EECs during the receptive phase,[50] and the regulatory mechanism of FOXO1 localization in epithelial cells is worthy of exploring deeply.

In our study, human endometrial tissue samples and EEC lines were used for preliminary exploration to reveal the regulation of Rictor in the human endometrium. Reduced expression of Rictor accompanied by abnormal tight junction-related proteins was observed in the endometrium of infertile patients. Consistently, Rictor also affected the function of ENaC in human EECs. These data suggest that Rictor plays an important role in the regulation of human endometrial function.

The PR^{Cre} model ablates genes in the pituitary gland, the preovulatory granulosa cells of the ovary, and the uterus starting at birth. In order to rule out changes in ovarian function and hormone levels in the established mouse model, the involvement of the pituitary–ovary axis in the sterile phenotype $Rictor^{d/d}$ mice was assayed by determining whether the ovary could ovulate, produce normal embryos, and maintain serum P4 levels during the preimplantation period. Previous results of embryo flushing indicated that the ovary was capable of ovulation. A mouse serum ELISA kit was used to detect the E2 and P4 levels. Results showed that there was no difference in the levels of serum E2 and P4 on D0 between the two groups of mice. So, the altered implantation is not caused by developmental abnormalities. Interestingly, the levels of E2 and P4 in $Rictor^{d/d}$ mice were significantly upregulated compared to $Rictor^{f/f}$ mice on D4 ($P < .05$) (Figure S2A,B). This suggests that the failure of receptive establishment in D4 $Rictor^{d/d}$ mice was not due to hormone deficiency; however, the mechanism of regulating hormone fluctuations needs to be explored further. The effects of E2 and P4 are mediated by nuclear estrogen receptor (ER) and PR.[51] The PR signaling pathway in the endometrium is activated on D4 in mice to antagonize the proliferation of epithelial cells induced by the ER pathway, thus, promoting the closure of the endometrial cavity and preparing for embryo implantation. It is interesting to note that uterine expression of P4-target epithelial (*Areg*, *Ihh*) and stromal genes (*Hand2*) was largely reduced in $Rictor^{d/d}$ mice. *Hand2*, a P4-induced transcription factor in the stroma, has been reported to participate in uterine receptivity by inhibiting epithelial proliferation via downregulation of fibroblast growth factor–extracellular signal regulated kinase (FGF-ERK) signaling.[52] The inhibition of PR and its target genes may be the cause of abnormal activation of the ER signaling pathway and the subsequent abnormal proliferation of LE in response to the loss of Rictor in the endometrium. In this study, the loss of Rictor leaded to dysregulation of hormone signaling and the obvious abnormality of epithelial function. Dysregulation of hormone signaling may be an important reason for the implantation failure. We will pay more attention to it in future research. FOXO1 and PR are involved in a mutual regulatory relationship in the endometrium during implantation, and the nuclear presence of FOXO1 coincides with the downregulation of PR in the same compartment.[50] These observations led us to hypothesize that downregulation of PR may be the result of FOXO1 nuclear localization following the inhibition of AKT and Sgk1 observed in $Rictor^{d/d}$ mice.

Here, we provide evidence that Rictor is a key factor in the establishment of uterine receptivity (Figure 6). Aberrantly low Rictor was closely associated with disorders of epithelial transformation and fluid absorption and caused adverse pregnancy outcomes. These findings complement the molecular regulatory network of endometrial receptivity. The data not only lay the foundation for the development of clinical strategies for boosting endometrial receptivity but also provide clues for the diagnosis and treatment of infertility.

ACKNOWLEDGMENTS

We are grateful to Prof. Liu Chaohong (Huazhong University of Science and Technology, Wuhan, China) for providing the $Rictor^{f/f}$ mice. We would like to thank Prof. Su Renwei (South China Agricultural University, Guangzhou, China) for providing the Ishikawa cells and his technical support. This work was supported by the National Natural Science Foundation (No. 31801247) and the Natural Science Foundation Project of CQ CSTC (No. cstc2019jcyj-msxmX0478).

CONFLICT OF INTEREST

The authors have declared that no conflict interest exists.

AUTHOR CONTRIBUTIONS

Y. Geng and X. Liu designed the experiments and interpreted the data. Y. Zhang, X. Du, and H. Tang performed the experiments. Q. Zhou, X. Chen, J. He, and Y. Ding provided technical support. Y. Zhang wrote the manuscript. X. Liu, Y. Wang, and Y. Geng acquired the funding.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Zhang Y, Du X, Chen X, et al. Rictor/mTORC2 is involved in endometrial receptivity by regulating epithelial remodeling. *The FASEB Journal*. 2021;35:e21731. https://doi.org/10.1096/fj.202100529RR