Adiponectin receptor 1 regulates endometrial receptivity via the adenosine monophosphate-activated protein kinase/E-cadherin pathway

BOLOR-ERDENE SARANKHUU[1], HYE JIN JEON[1], DA-UN JEONG[1], SEOK-RAE PARK[1,2], TAE-HYUN KIM[1,3], SUNG KI LEE[1,3], AE RA HAN[4,5], SEONG-LAN YU[1] and JAEKU KANG[1,6]

[1]Priority Research Center, Myunggok Medical Research Institute, College of Medicine, Konyang University, Daejeon 35365, Republic of Korea; [2]Department of Microbiology, College of Medicine, Konyang University, Daejeon 35365, Republic of Korea; [3]Department of Obstetrics and Gynecology, College of Medicine, Konyang University Hospital, Daejeon 35365, Republic of Korea; [4]I-Dream Clinic, Department of Obstetrics and Gynecology, Mizmedi Hospital, Seoul 07639, Republic of Korea; [5]Daegu CHA Fertility Center, CHA University, Daegu, North Gyeongsang 42469, Republic of Korea; [6]Department of Pharmacology, College of Medicine, Konyang University, Daejeon 35365, Republic of Korea

Received May 2, 2024; Accepted July 26, 2024

DOI: 10.3892/mmr.2024.13308

Abstract. Endometrial receptivity is essential for successful embryo implantation and pregnancy initiation and is regulated via various signaling pathways. Adiponectin, an important adipokine, may be a potential regulator of reproductive system functions. The aim of the present study was to elucidate the regulatory role of adiponectin receptor 1 (ADIPOR1) in endometrial receptivity. The endometrial receptivity between RL95-2 and AN3CA cell lines was confirmed using an *in vitro* JAr spheroid attachment model. 293T cells were transfected with control or short hairpin (sh)ADIPOR1 vectors and RL95-2 cells were transduced with lentiviral particles targeting *ADIPOR1*. Reverse transcription-quantitative PCR and immunoblot assays were also performed. *ADIPOR1* was consistently upregulated in the endometrium during the mid-secretory phase compared with that in the proliferative phase and in receptive RL95-2 cells compared with that in non-receptive AN3CA cells. Stable cell lines with diminished *ADIPOR1* expression caused by shRNA showed reduced E-cadherin expression and attenuated *in vitro* endometrial receptivity. ADIPOR1 regulated AMP-activated protein kinase (AMPK) activity in endometrial epithelial cells. Regulation of AMPK activity via dorsomorphin and 5-aminoimidazole-4-carboxamide ribonucleotide affected E-cadherin expression and *in vitro* endometrial receptivity. The ADIPOR1/AMPK/E-cadherin axis is vital to endometrial receptivity. These findings can help improve fertility treatments and outcomes.

Correspondence to: Professor Seong-Lan Yu, Priority Research Center, Myunggok Medical Research Institute, College of Medicine, Konyang University, 158 Gwanjeodong-ro, Seo, Daejeon 35365, Republic of Korea
E-mail: yusl73@konyang.ac.kr

Professor Jaeku Kang, Department of Pharmacology, College of Medicine, Konyang University, 158 Gwanjeodong-ro, Seo, Daejeon 35365, Republic of Korea
E-mail: jaeku@konyang.ac.kr

Key words: endometrial receptivity, adiponectin receptor 1, AMP-activated protein kinase, E-cadherin, dorsomorphin, 5-aminoimidazole-4-carboxamide ribonucleotide

Introduction

Human embryo implantation is a highly regulated process. It begins with the implantation of trophoblast cells from a competent blastocyst onto the maternal endometrium. This process is pivotal for a successful pregnancy (1). This adjustment occurs during the 'window of implantation (WOI)', a short period of uterine receptivity corresponding to the mid-secretory phase of the menstrual cycle. Successful embryo implantation is highly dependent on the endometrial cycle, ovarian steroid hormones and endometrial receptive adhesion molecules. Receptive adhesion molecules, such as integrins, cadherins and selectins, are differentially expressed in endometrial epithelial cells throughout the menstrual cycle and play important roles during implantation (1).

Adiponectin, an important adipokine secreted from the adipose tissues, exerts diverse physiological effects and influences carbohydrate metabolism, lipid metabolism, insulin sensitization, anti-inflammatory responses and cardiovascular health (2). In the human endometrium, adiponectin functions as a hormonal regulator of energy homeostasis and has anti-inflammatory effects associated with events such as implantation and endometriosis (3,4). Mice lacking adiponectin show impaired fertility potential and ovarian folliculogenesis (5). Adiponectin exerts its effects through the activation of two receptors; adiponectin receptor 1 (ADIPOR1), which is highly expressed in the skeletal muscle and acts via AMP-activated protein kinase (AMPK) and adiponectin receptor 2 (ADIPOR2), which is mostly expressed in the liver

and acts through the proliferator-activated receptor α (PPAR α) pathway to regulate glucose and lipid metabolism (6,7). These two adiponectin receptors are expressed in the epithelial and stromal cells of the endometrium, markedly increasing in the mid-secretory phase and aligning with the period of receptivity of the endometrium to the embryo. Additionally, adiponectin receptors are downregulated in the endometria of women with recurrent implantation failure compared with those in fertile women (8). AMPK comprises three subunits; the catalytic α (α1 and α2) subunit and the regulatory β (β1 and β2) and γ (γ1, γ2 and γ3) subunits, forming a heterotrimeric complex. The AMPK α subunit is activated via phosphorylation by liver kinase B1 (LKB1) or calmodulin-dependent protein kinase. Activated AMPK regulates the cellular metabolism (9,10). A previous study suggested that AMPK is a mediator of endometrial receptivity due to elevated estrogen expression and delayed embryo implantation in mice lacking AMPK (11). The relationship between AMPK and energy metabolism is critical in uterine receptivity (12). AMPK activity is closely associated with placental development (13,14). E-cadherin, a type of cadherin, is crucial for cell-cell contact via adherens junctions in epithelial cells. Its fundamental molecular function is implicated in the epithelial-mesenchymal transition. E-cadherin serves as a biomarker of endometrial receptivity and is closely linked to implantation, facilitating the initial communication between mothers and fetuses. It actively contributes to early adhesion during human embryo implantation (15). Adhesion molecules, pivotal proteins in embryo implantation, also play a crucial role in cancer progression. In endometrial cancer, the L1 cell adhesion molecule is particularly significant for predicting prognosis and recurrence due to its association with invasiveness and metastatic potential (16,17). However, advancements in studies on endometrial receptivity to improve pregnancy success are impeded by the lack of knowledge of the regulation of various signaling pathways.

The present study explored the effects of ADIPOR1 on the regulation of endometrial receptivity in human endometrial tissues and cell lines (RL95-2 and AN3CA) with different receptivity. The results showed that ADIPOR1 regulated AMPK activity and E-cadherin expression, which are closely related to endometrial receptivity. ADIPOR1 downregulation attenuated JAr spheroid attachment *in vitro*.

Materials and methods

Reagents. Dorsomorphin dihydrochloride, an AMPK inhibitor and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), an AMPK activator, were obtained from Tocris Bioscience and puromycin dihydrochloride was purchased from InvivoGen, Inc.

Collection of human endometrial tissues. Human endometrial tissues were obtained from fertile participants in the proliferative [9-11 menstrual cycle days (mcd); n=11; mean age, 36.7±2.8 years] and secretory (20-24 mcd; n=9; mean age, 37.8±2.6 years) phases between April and June 2019 at the Konyang University Hospital. Samples from patients with infertility were collected from the secretory phase (20-22 mcd; n=7; mean age, 38.9±3.5 years) between April 2018 and April 2020 at the MizMedi Hospital (Seoul, South Korea). Patients with infertility did not receive any medication, including hormone treatments, during the sampling period. Endometrial sampling was performed using a disposable uterine sampler (Rampipella; Ri.mos. (S.r.l.). The menstrual stages of the samples were determined using the Noyes criteria by an experienced gynecological pathologist (18). The present study was approved by the Bioethics Committee of Konyang University Hospital [Institutional Review Board (IRB) file no. KYUH 2018-11-007] and MizMedi Hospital (IRB file no. MMIRB 2018-3). Signed informed consent was obtained from each patient.

Cell culture. Human endometrial cell lines (RL95-2 and AN3CA) were acquired from the American Type Culture Collection. RL95-2 and AN3CA cells were cultured in DMEM/F-12 and MEM (HyClone; Cytiva) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (HyClone; Cytiva). Mycoplasma contamination tests were performed with MycoStrip (InvivoGen, Inc.).

Human choriocarcinoma JAR cells were obtained from the South Korean Cell Line Bank and cultured in RPMI-1640 medium (HyClone; Cytiva) supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. All cells were maintained at 37˚C in a humidified atmosphere containing 5% CO_2. RL95-2 cells were treated with dorsomorphin dihydrochloride for 24 h. AN3CA cells were treated with AICAR (AMPK activators; Tocris Bioscience) for 24 h.

Transfection and transduction. For the generation of lentivirus particles, 293T cells were transfected with 400 ng of either a control (pLKO1; SHC016) or a short hairpin (sh)RNA targeting adiponectin receptor 1 (shADIPOR1; sense: 5'-CGTCTATTG TCATTCAGAGAA-3', antisense: 5'-TTCTCTGAATGACAA TAGACG-3') expression vector (MilliporeSigma) alongside 400 ng pLP1, 100 ng pLP2 and 300 ng pLP/VSVG vectors (all from Thermo Fisher Scientific, Inc.) based on a 3rd generation system using Lipofectamine® 3,000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's recommended protocol. Lentiviral particles targeting *ADIPOR1* and control lentiviral particles were collected from media following transfection for 48 h. Subsequently, RL95-2 cells were transduced with 10 MOI control lentiviral particles or those carrying shRNA against the *ADIPOR1* mRNA. RL95-2 cells with ADIPOR1 knockdown were isolated and selected using 0.5 μg/ml puromycin dihydrochloride.

RNA isolation and reverse transcription-quantitative (RT-q) PCR. Total RNA extraction from both $1x10^6$ AN3CA or $2x10^6$ RL95-2 cells and endometrial tissues was performed using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 2 μg RNA for cells and 5 μg for tissues using Moloney Murine Leukemia Virus reverse transcriptase (Promega Corporation) according to the manufacturer's recommended protocol.

RT-qPCR was performed to analyze mRNA expression using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.) on a CFX Connect Real-time PCR Detection System (Bio-Rad Laboratories, Inc.) according to the

Table I. Primer sequences for reverse transcription-quantitative PCR.

Gene	Primer sequences	PCR product size, bp	Annealing temperature, ˚C
ADIPOR1	Forward 5'ACGTTGGAGGGTCATCCCATA 3' Reverse 5'AAACAGCACGAAACCAAGCAG 3'	175	56
ADIPOR2	Forward 5'CTGGATGGTACACGAAGAGGT 3' Reverse 5'TGGGCTTGTAAGAGAGGGGAC 3'	176	56
E-SEL	Forward 5'CCGAGCGAGGCTACATGAAT 3' Reverse 5'GCCACATTGGAGCCTTTTGG 3'	122	56
L-SEL	Forward 5'ATTTCCTGGCACATCATG 3' Reverse 5'ATTGTCTCGGCAGAATCT 3'	95	56
ITGB5	Forward 5'ACCTGGAACAACGGTGGAGA 3' Reverse 5'AAAAGATGCCGTGTCCCCAA 3'	217	60
E-cadherin	Forward 5'GGCCTGAAGTGACTCGTAACG 3' Reverse 5'TCAGACTAGCAGCTTCGGAACC 3'	201	60
AMPK	Forward 5'AGGAAGAATCCTGTGACAAGCAC 3' Reverse 5'CCGATCTCTGTGGAGTAGCAGT 3'	145	56
β-Actin	Forward 5'CAAGAGATGGCCACGGCTGCT 3' Reverse 5'TCCTTCTGCATCCTGTCGGCA 3'	275	56 or 60

ADIPOR1, adiponectin receptor 1; *ADIPOR2*, adiponectin receptor 2; *E-SEL*, E-selectin; *L-SEL*, L-selectin; *ITGB5*, integrin β5; AMPK, AMP-activated protein kinase.

manufacturer's recommended protocol. Specific gene primers and their annealing temperatures are listed in Table I. The following amplification conditions were used for select genes encoding *ADIPOR1*,*ADIPOR2*,*ITGB5*,*L-Selectin*,*E-Selectin*, *E-cadherin*, *AMPK* and *β-actin*: An initial denaturation step at 95˚C for 3 min, followed by 49 cycles of denaturation at 95˚C for 30 sec, annealing at 58˚C for 15 sec and extension at 72˚C for 15 sec. Gene expression levels were normalized to those of β-actin, an internal control. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (19) and fold change was evaluated compared with the control.

Immunoblot analysis. Cells were lysed using ice-cold radioimmunoprecipitation assay (RIPA) buffer on ice (JuBiotech), supplemented with protease and phosphatase inhibitors (Roche Diagnostics GmbH), and protein concentration was quantified using the bicinchoninic acid assay (Thermo Fisher Scientific, Inc.). Subsequently, 50 μg (for p-AMPK, AMPK, E-cadherin, integrin β5 and β-actin detection) or 70 μg (for ADIPOR1, ADIPOR2, L-selectin, E-selectin, E-cadherin and β-actin detection) proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis on 8 or 10% gels and transferred onto polyvinylidene difluoride membranes. The blots were blocked with 5% skimmed milk (Difco; BD Biosciences) for 2 h at room temperature, washed with 1X TBS containing Tween 20 buffer (Biosesang) and then incubated with primary antibodies against ADIPOR1 (1:500; cat. no. ab70362; Abcam), ADIPOR2 (1:500; cat. no. ab77612; Abcam), L-selectin (1:1,000; cat. no. PAA086Hu01; Cloud-Clone Corp.), E-selectin (1:1,000; cat. no. ab18981; Abcam), integrin β5 (1:1,000; cat. no. ab184312; Abcam), Phospho-AMPKα (Thr172) (1:1,000; cat. no. 2531; Cell Signaling Technology, Inc.), AMPKα (1:1,000; cat. no. 2532; Cell Signaling Technology, Inc.), E-cadherin (1:1,000; cat. no. 3195; Cell Signaling Technology, Inc.) and β-actin (1:3,000; cat. no. 4967; Cell Signaling Technology, Inc.) were incubated overnight at 4˚C. The following day, blots were probed with horseradish peroxidase-conjugated secondary antibodies (1:3,000; cat. no. AP132P; MilliporeSigma) for 2 h at room temperature and bands were visualized using an Enhanced Chemiluminescence Kit (Thermo Fisher Scientific, Inc.). Quantification of western blot images was performed using the ImageJ software 1.50b (National Institutes of Health).

In vitro assay for JAr spheroid implantation. JAr cell spheroids were prepared by seeding onto a V-bottom microplate (Greiner Bio One Ltd.) and incubating in DMEM supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (HyClone; Cytiva) for 24 h in a humidified atmosphere with 5% CO_2. Endometrial cells were cultured in 24-well plates until they reached 90% confluency. The harvested spheroids were co-cultured with either RL95-2 cell monolayers (for 1 h) or AN3CA cell monolayers (for 2 h), These cells were pre-treated with either dorsomorphin, AICAR, *ADIPOR1* shRNA vector, or respective controls. The attached spheroids were quantified by inverting the microplate and centrifuging at 10 x g for 10 min. The attached spheroids were then counted under a microscope (Olympus Corporation). The percentage of spheroid attachment was calculated by dividing the number of attached spheroids after centrifugation by the total number of spheroids. The implantation assay was conducted at least three times for validation.

Statistical analysis. Data are presented as mean ± standard error of the mean. The results were analysed using unpaired Student's t-test or one-way ANOVA with Tukey's post hoc test with GraphPad Prism 5 (Dotmatics). $P<0.05$ was considered to indicate a statistically significant difference.

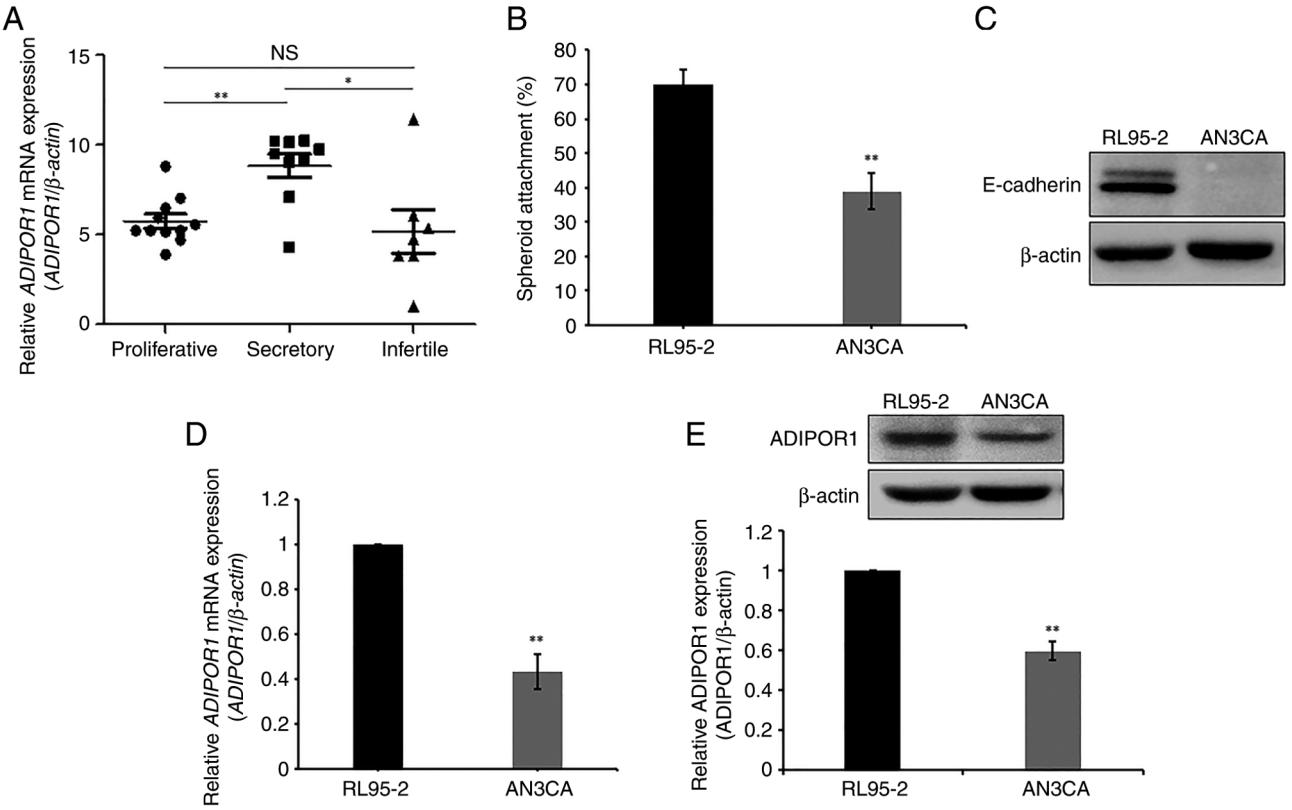

Figure 1. ADIPOR1 is associated with the endometrial receptivity. (A) *ADIPOR1* mRNA expression during the proliferative and secretory phases in endometrial tissues obtained from fertile patients, and during the secretory phase in endometrial tissues obtained from infertile patients. (B) Spheroid attachment to receptive RL95-2 cells and non-receptive AN3CA cells. (C) E-cadherin expression in RL95-2 and AN3CA cells. (D) *ADIPOR1* mRNA expression in RL95-2 and AN3CA cells. (E) Analysis of ADIPOR1 expression in RL95-2 and AN3CA cells. Data are presented as the mean ± standard error of the mean of values from three independent experiments (n=3). *P<0.05, **P<0.01. NS, not significant; ADIPOR1, adiponectin receptor 1.

Results

Examination of the dynamics of ADIPOR1 expression and its role in endometrial receptivity. To investigate the role of ADIPOR1 in the endometrial cycle, the present study first quantified its expression in the endometria during the proliferative and secretory phases of fertile women as well as the secretory phase of infertile women. The mRNA levels of *ADIPOR1* were markedly higher in the secretory phase compared with the proliferative phase in fertile women. *ADIPOR1* expression was markedly reduced during the secretory phase in infertile women compared with that in fertile women (Fig. 1A). Based on these results, to explore the function of ADIPOR1 in the endometrial receptivity process, the receptive RL95-2 and non-receptive AN3CA cell lines were selected. Endometrial receptivity between RL95-2 and AN3CA cell lines were confirmed using an *in vitro* JAr spheroid attachment model. The frequency of spheroid attachment was higher in receptive RL95-2 cells compared with non-receptive AN3CA cells, consistent with previous data (20) (Fig. 1B). For further validation, the expression patterns of proteins that were biomarkers of endometrial receptivity were examined. The expression levels of E-selectin, L-selectin and ITGB5 were significantly higher in the receptive RL95-2 cells compared with the non-receptive AN3CA cells (data not shown). In particular, the expression of E-cadherin was significantly higher in receptive RL95-2 cells compared with non-receptive AN3CA cells (Fig. 1C). Based on these results, the expression pattern of ADIPOR1 in cells with different receptivity were investigated. Lower mRNA levels of *ADIPOR1* were observed in non-receptive AN3CA cells compared with receptive RL95-2 cells (Fig. 1D). ADIPOR1 protein levels were reduced in AN3CA cells (Fig. 1E). By contrast, ADIPOR2 was more highly expressed in the endometria of infertile women compared with those of fertile women and in non-receptive epithelial cells compared with receptive epithelial cells in the present study (Fig. S1). These results suggested that differences in ADIPOR1 expression may be associated with endometrial receptivity.

Role of ADIPOR1 in regulating endometrial receptivity-related biomarkers. Due to the differences in ADIPOR1 expression in cells with different receptivity, the changes in endometrial receptivity caused by ADIPOR1 downregulation were investigated in relatively upregulated recipient RL95-2 cells. Stable cells with downregulated ADIPOR1 (shADIPOR1) were established using shRNA-mediated gene silencing. The expression levels of ADIPOR1 were examined to verify the establishment of stable cell lines. The mRNA levels of *ADIPOR1* and corresponding protein levels were significantly lower in sh*ADIPOR1* cells compared with the pLKO1 control cells (Fig. 2A and B). Next, an in vitro implantation assay using the JAr spheroid attachment method was performed to investigate the relationship between the changes in ADIPOR1

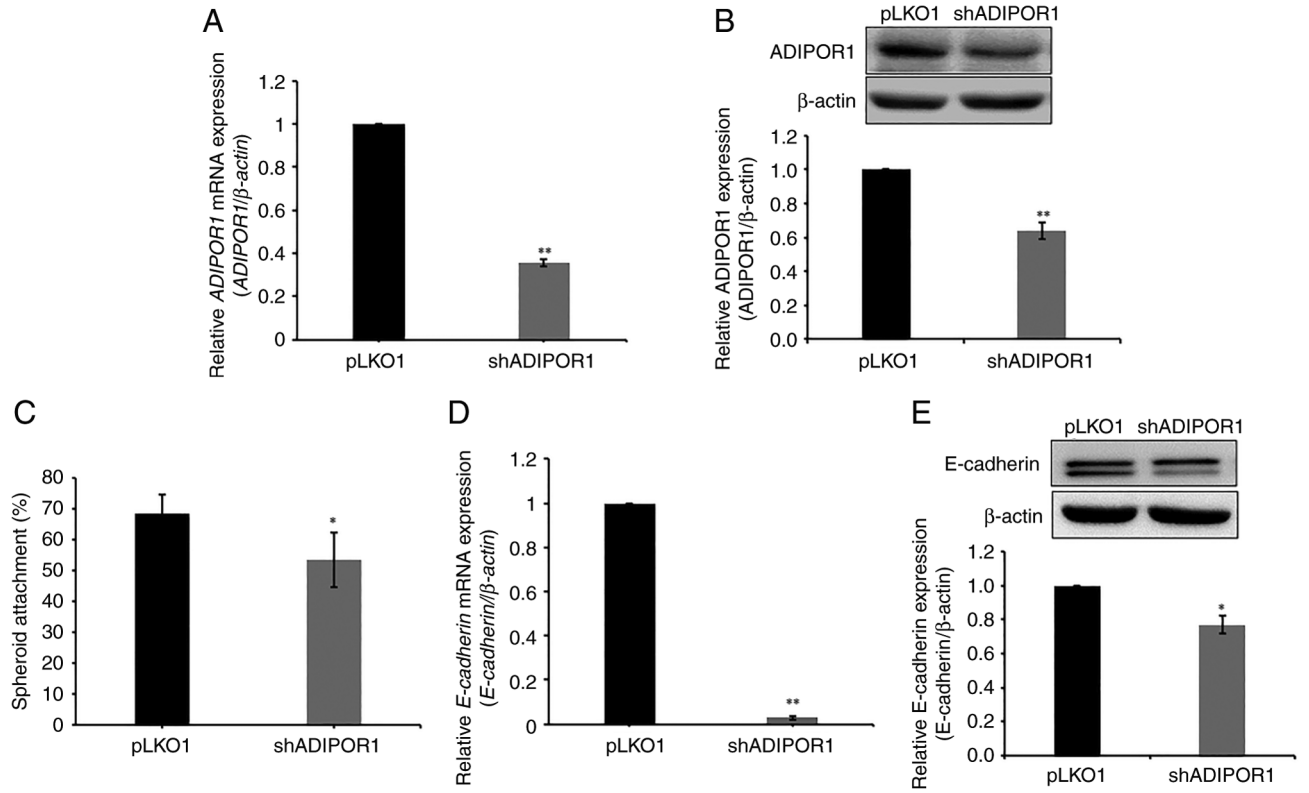

Figure 2. ADIPOR1 regulates E-cadherin mRNA and protein expression in endometrial epithelial cells. (A) Effect of *ADIPOR1* knockdown on corresponding mRNA expression in RL95-2 cells. (B) Effect of ADIPOR1 knockdown on corresponding protein expression in RL95-2 cells. (C) Rate of spheroid attachment to ADIPOR1-knockdown RL95-2 cells. (D) Effect of *ADIPOR1* knockdown on downregulation of -E-cadherin in RL95-2 cells. (E) Effect of ADIPOR1 knockdown on downregulation of E-cadherin in RL95-2 cells. Data are presented as the mean ± standard error of the mean of values from three independent experiments (n=3). *P<0.05, **P<0.01. ADIPOR1, adiponectin receptor 1; sh, short hairpin.

expression and embryo implantation. As shown in Fig. 2C, ADIPOR1 downregulation attenuated spheroid attachment in JAr cells compared with that in the control cells. The E-cadherin protein is important for successful embryo implantation (21). Therefore, the effect of *ADIPOR1* knockdown on E-cadherin expression was examined. *E-cadherin* mRNA expression was reduced in shADIPOR1 cells compared with that in the control cells (Fig. 2D). Consistent with the changes in mRNA expression, E-cadherin protein levels were reduced in shADIPOR1-treated cells (Fig. 2E). E-selectin, L-selectin and ITGB5 expression was reduced following ADIPOR1 knockdown (Fig. S2). These results suggest that ADIPOR1 positively regulates E-cadherin expression and may play an important role in the regulation of endometrial receptivity during embryo implantation.

ADIPOR1 regulates AMPK activity in endometrial epithelial cells. The present study examined the AMPK phosphorylation state in receptive and non-receptive cells showing differential ADIPOR1 expression to investigate the role of the association between ADIPOR1 and AMPK activity in regulating endometrial receptivity. AMPK phosphorylation was lower in non-receptive AN3CA cells compared with receptive RL95-2 cells (Fig. 3A). Knockdown of ADIPOR1 induced decreased AMPK phosphorylation in RL95-2 cells (Fig. 3B). These results indicated that ADIPOR1-mediated AMPK regulation may be associated with the receptivity of endometrial epithelial cells.

AMPK inhibition via dorsomorphin attenuates endometrial receptivity. The present study used dorsomorphin, which inhibits AMPK activity, to investigate whether the AMPK pathway regulates the receptivity of endometrial epithelial cells. Dorsomorphin did not affect cell viability in cytotoxicity tests. The inhibition of AMPK phosphorylation was also confirmed via western blotting in RL95-2 cells treated with various concentrations (Fig. S3A). AMPK phosphorylation was reduced upon dorsomorphin treatment in a dose-dependent manner (Fig. 4A). Next, JAr cell spheroid attachment-mediated endometrial receptivity in RL95-2 cells following dorsomorphin treatment was investigated. Dorsomorphin-induced reduction in spheroid attachment was proportional to the inhibition of AMPK phosphorylation (Fig. 4B). The expression patterns of biomarkers associated with endometrial receptivity was confirmed. Silencing AMPKa1 triggers the disruption of cell-cell adhesion via the Twist/E-cadherin pathway in breast cancer (22). Therefore, E-cadherin expression in receptive RL95-2 cells treated with dorsomorphin was examined. The mRNA expression of *E-cadherin* decreased in the cells treated with dorsomorphin (Fig. 4C). Dorsomorphin treatment also reduced E-cadherin protein expression in a dose-dependent manner (Fig. 4D). These results suggest that AMPK inhibition is closely associated with decreased endometrial receptivity.

AMPK activation via AICAR improves endometrial receptivity. The AMPK activator AICAR was used to further investigate the relationship between AMPK activity and

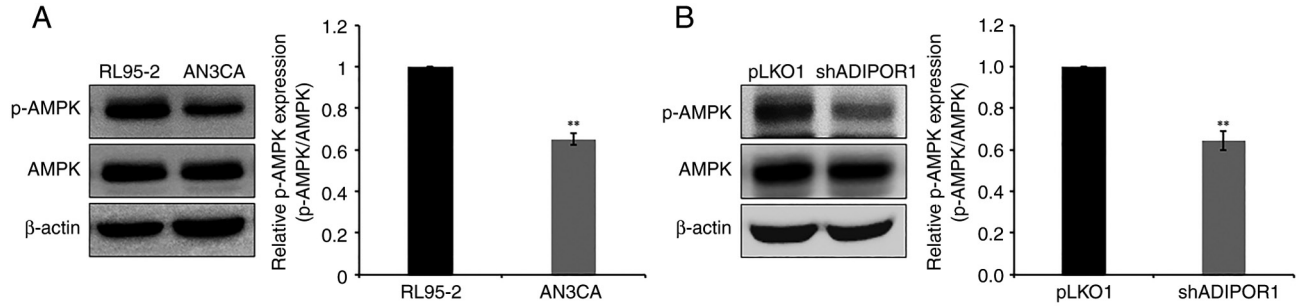

Figure 3. ADIPOR1 regulates AMPK activity in endometrial epithelial cells. (A) p-AMPK/AMPK protein expression in RL95-2 and AN3CA cells. (B) p-AMPK/AMPK protein expression ADIPOR1-knockdown RL95-2 cells. Data are presented as the mean ± standard error of the mean of values from three independent experiments (n=3). **P<0.01. ADIPOR1, adiponectin receptor 1; AMPK, AMP-activated protein kinase; p-, phosphorylated; sh, short hairpin.

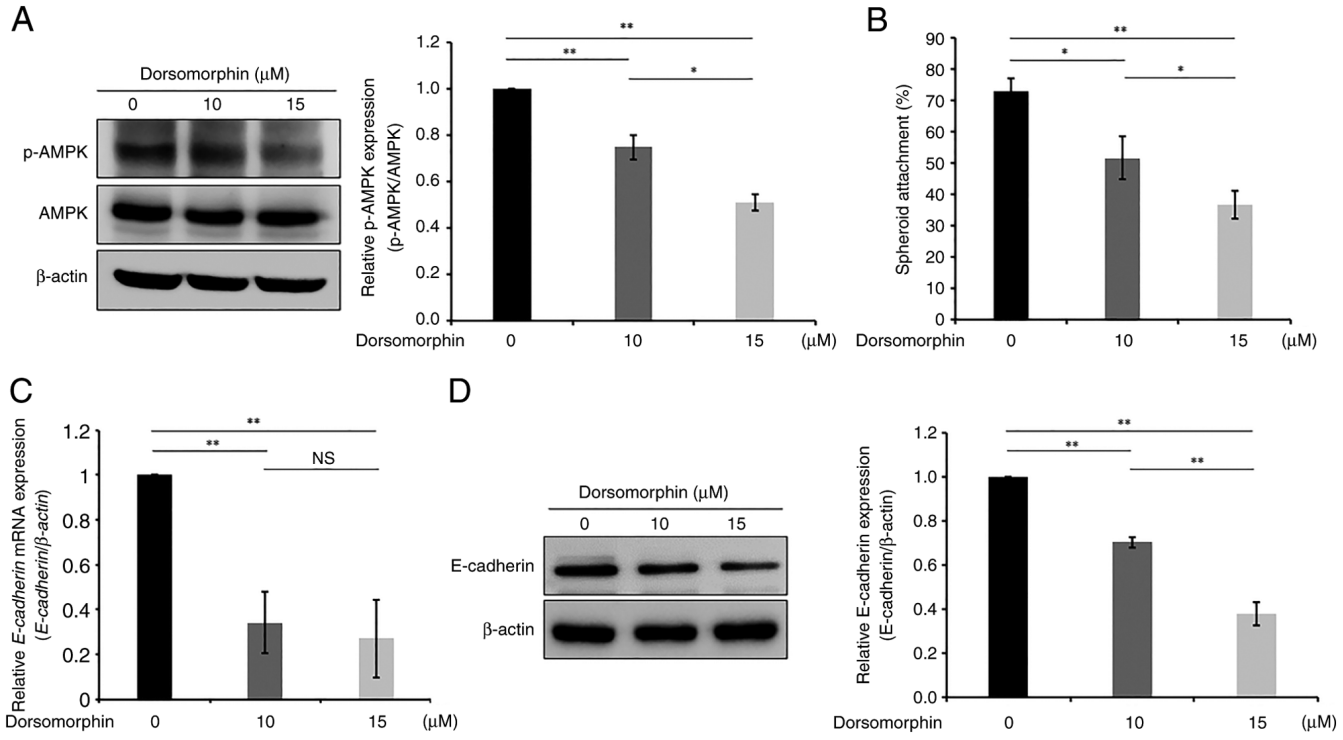

Figure 4. AMPK inhibition by dorsomorphin attenuates endometrial receptivity in receptive RL95-2 cells. (A) Analysis of AMPK inhibition by dorsomorphin via immunoblotting. (B) Effect of dorsomorphin on the spheroid-attachment rate in RL95-2 cells. (C) *E-cadherin* mRNA expression in RL95-2 cells treated with dorsomorphin. (D) E-cadherin expression in RL95-2 cells treated with dorsomorphin. Data are presented as the mean ± standard error of the mean of values from three independent experiments (n=3). *P<0.05, **P<0.01. NS, not significant; AMPK, AMP-activated protein kinase.

endometrial receptivity. The toxicity following AICAR treatment was assessed using the MTT assay for cell viability and the phosphorylation state of AMPK was verified via immunoblot analysis at different concentrations in non-receptive AN3CA cells (Fig. S3B). The phosphorylation level of AMPK increased proportionally with AICAR (250 and 500 μM) (Fig. 5A). The rate of JAr cell-spheroid attachment was significantly increased following AICAR treatment, consistent with enhanced AMPK phosphorylation (Fig. 5B). Next, *E-cadherin* expression in non-receptive AN3CA cells following AN3CA treatment was examined. AICAR treatment significantly increased the mRNA expression of E-cadherin in a dose-dependent manner (Fig. 5C). E-cadherin protein levels increased in a dose-dependent manner in the AICAR-treated cells (Fig. 5D). These results strongly suggested that AMPK activity affects endometrial receptivity by regulating E-cadherin expression.

Discussion

The endometrium undergoes morphological and functional changes throughout the phases of menstrual cycle. Specifically, the WOI represents a distinct period that leads to a dynamic transition from a non-receptive endometrium to a receptive endometrium for embryo implantation. Numerous researchers have explored the differences between the endometria of fertile and infertile women during WOI, aiming to identify key factors associated with endometrial receptivity and propose potential biomarkers for WOI (23,24). However, advancements in studies on endometrial receptivity to improve pregnancy

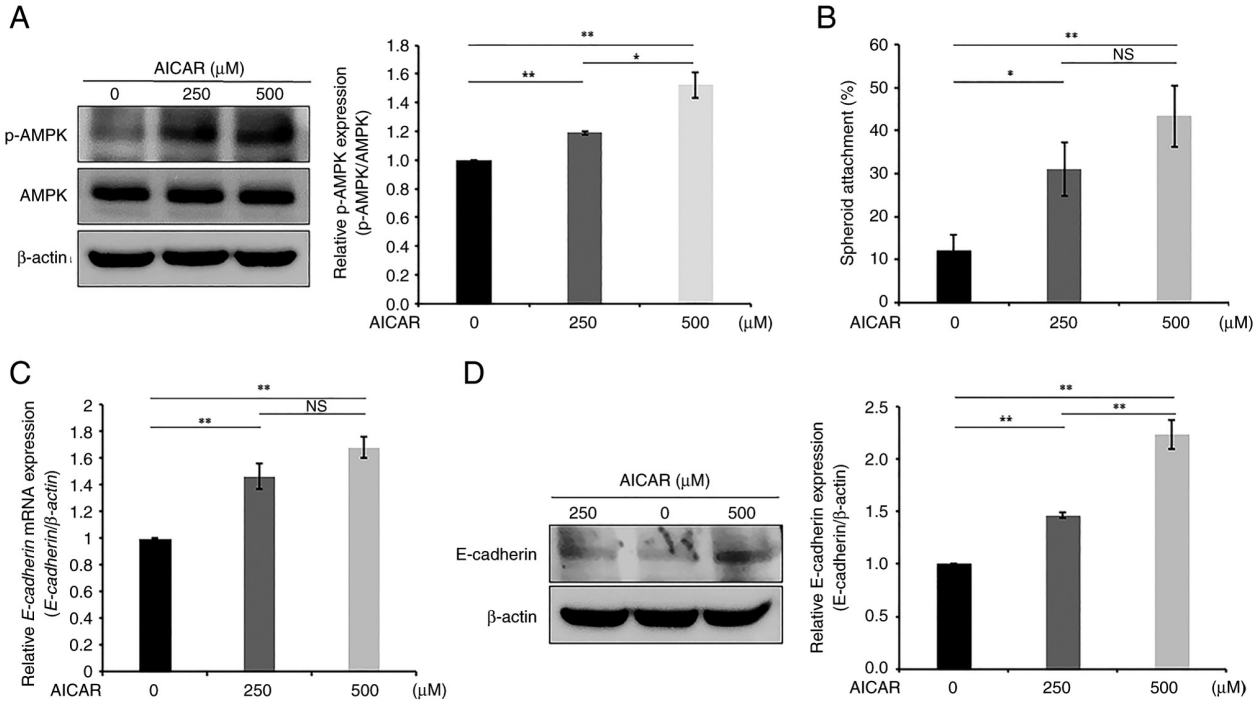

Figure 5. AMPK activation by AICAR induces endometrial receptivity in non-receptive AN3CA cells. (A) AMPK activation by AICAR assessed via immunoblot assay. (B) Effect of AICAR on the spheroid-attachment rate in AN3CA cells. (C) *E-cadherin* mRNA expression in AN3CA cells treated with AICAR. (D) E-cadherin expression in AN3CA cells treated with AICAR. Data are presented as the mean ± standard error of the mean of values from three independent experiments (n=3). *P<0.05, **P<0.01. NS, not significant; AMPK, AMP-activated protein kinase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; p-, phosphorylated.

success are impeded by the lack of knowledge of the regulation of various signaling pathways.

The present study investigated the correlation between endometrial receptivity and adiponectin signaling. Adiponectin is a potential regulator of reproductive system function. While plasma adiponectin was negatively associated with progressive gestational age in non-obese women, no significant difference was observed in plasma adiponectin levels between non-obese and overweight pregnant women (25). Another study found no significant difference in adiponectin expression between the endometria of fertile and infertile groups (26). However, both ADIPOR1 and ADIPOR2 are markedly upregulated in the endometrium during the mid-secretory phase, a critical stage of embryo implantation during the menstrual cycle (3). Furthermore, both receptors are expressed at lower levels in the endometria of the infertile group than in those of the fertile group (8).

The present study specifically focused on the potential association between the two adiponectin signaling pathway receptors and endometrial receptivity. It confirmed that ADIPOR1 was consistently upregulated in the endometrium during the mid-secretory phase compared with that in the proliferative phase, as previously reported (8). However, ADIPOR1 was expressed at lower levels in the endometria of infertile women. Additionally, ADIPOR1 expression was higher in receptive epithelial cells compared with non-receptive epithelial cells, as evident from the *in vitro* implantation assay.

Therefore, the correlation between ADIPOR1 expression and endometrial receptivity was examined. Stable cell lines with diminished *ADIPOR1* expression caused by shRNA showed attenuated JAr spheroid attachment in an *in vitro* implantation assay compared with that in the control cells. Moreover, sh*ADIPOR1* downregulated E-cadherin, a vital marker of endometrial receptivity. These findings indicate that ADIPOR1 may be important in regulating endometrial receptivity during embryo implantation.

In liver cells, adiponectin enhances AMPK activity through the ADIPOR1-mediated signaling pathway and PPARa activity through its other receptor ADIPOR2-mediated signaling pathway (7). Adiponectin receptors activate multiple signaling pathways and play various regulatory roles in cellular functions (27). ADIPOR1 stimulates the phosphorylation of AMPK, directly regulating glucose metabolism and insulin sensitivity in skeletal muscle cells (28,29). Kim *et al* (30) demonstrated a reduction in AMPK phosphorylation in the brains, livers, kidneys and spleens of mice with ADIPOR1 knockdown injected with an *ADIPOR1* shRNA mixture. Therefore, the present study investigated the correlation between ADIPOR1 and AMPK expression in the endometrial epithelial cells. The phosphorylation level of AMPK was lower in non-receptive AN3CA cells with downregulated ADIPOR1 compared with receptive RL95-2 cells and stable cells with ADIPOR1 knockdown showed a decrease in AMPK phosphorylation. These results demonstrated that ADIPOR1 mediated AMPK signaling in endometrial epithelial cells.

AMPK is intricately involved in steroid hormone signaling within the uterus and its activity, regulated by progesterone, is crucial in uterine receptivity for embryo implantation through the regulation of energy or glycogen in endometrial epithelial cells (11,31). In addition to its role in uterine receptivity, AMPK is vital in placental and embryonic development (32). Recent reports have indicated that the suppression of AMPKa1

induces breast cancer metastasis through the disruption of cell-cell adhesion mediated by E-cadherin. In contrast, the elevation of AMPKa1 expression induces E-cadherin expression in cervical cancer cells (22,33). AMPKa2 plays a role in regulating E-cadherin expression. When PHD finger protein 2 is phosphorylated by AMPKa2, it enhances demethylation activity, resulting in reduced methylation of E-cadherin at H3K9me2 (34). These observations indicate that AMPK activity may affect endometrial receptivity by regulating E-cadherin expression, which is vital in endometrial receptivity. Therefore, the present study examined the association between the AMPK/E-cadherin pathway and endometrial receptivity through the regulation of AMPK activity using two pharmacological agents; dorsomorphin and AICAR. Dorsomorphin reduced *in vitro* endometrial receptivity as well as E-cadherin expression by inhibiting AMPK phosphorylation in receptive RL95-2 cells. AICAR induced *in vitro* endometrial receptivity and E-cadherin expression through the activation of AMPK phosphorylation in non-receptive AN3CA cells. Accordingly, it was hypothesized that AMPK activity plays an important role in endometrial receptivity by regulating E-cadherin expression.

AICAR is a pharmacological agent that exhibits numerous beneficial effects on metabolism, hypoxia, exercise and cancer in the human body, with the potential for further research (35). Turner *et al* (36) reported that AICAR reduces the LPS-stimulated inflammatory response in bovine endometrial tissue. AICAR has been shown to prevent hypoxia-induced fetal growth restriction by improving the uterine artery blood flow during murine pregnancy (37). Therefore, it was hypothesized that AICAR treatment of non-receptive endometrium may improve endometrial receptivity. However, it is essential to validate this hypothesis through *in vivo* experiments because the present investigation focused solely on *in vitro* endometrial receptivity.

The present study revealed the intricate interplay among endometrial receptivity, the adiponectin signaling pathway and AMPK activity. ADIPOR1 is a key regulator of endometrial receptivity in successful embryo implantation. The ADIPOR1/AMPK/E-cadherin axis mediates key cellular processes in endometrial epithelial cells. Therefore, the findings of the present study provided insights into the molecular mechanisms underlying endometrial receptivity and can be used to design potential therapeutic approaches to improve pregnancy success. However, the present study has some limitations. The sample size for comparing ADIPOR1 expression between fertile and infertile women was small and tissue heterogeneity may have limited the applicability of the findings. Further validation using *in vivo* experiments is crucial to bridge the gap between these findings and their clinical applications.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Priority Research Centers Program through the National Research Foundation of Korea (NRF), the Ministry of Education (grant no. NR F-2021R1I1A3059211) and Konyang University Myunggok Research Fund (grant no. 2023-03).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author

Authors' contributions

BES, SLY and JK were responsible for conceptualization, validation, investigation, data curation and formal analysis. HJJ, DUJ and SRP were responsible for formal analysis. BES and SLY wrote, reviewed and edited the manuscript. THK, SKL and ARH were responsible for providing human endometrial samples and formal analysis. JK was responsible for project administration and funding acquisition. SLY and JK confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Bioethics Committee of Konyang University Hospital [Institutional Review Board (IRB) file no. KYUH 2018-11-007] and MizMedi Hospital (IRB file no. MMIRB 2018-3). Signed informed consent was obtained from each patient.

Patient consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

References

1. Achache H and Revel A: Endometrial receptivity markers, the journey to successful embryo implantation. Hum Reprod Update 12: 731-746, 2006.
2. Nguyen TMD: Adiponectin: Role in physiology and pathophysiology. Int J Prev Med 11: 136, 2020.
3. Takemura Y, Osuga Y, Yamauchi T, Kobayashi M, Harada M, Hirata T, Morimoto C, Hirota Y, Yoshino O, Koga K, *et al*: Expression of adiponectin receptors and its possible implication in the human endometrium. Endocrinology 147: 3203-3210, 2006.
4. Takemura Y, Osuga Y, Harada M, Hirata T, Koga K, Morimoto C, Hirota Y, Yoshino O, Yano T and Taketani Y: Serum adiponectin concentrations are decreased in women with endometriosis. Hum Reprod 20: 3510-3513, 2005.
5. Cheng L, Shi H, Jin Y, Li X, Pan J, Lai Y, Lin Y, Jin Y, Roy G, Zhao A and Li F: Adiponectin deficiency leads to female subfertility and ovarian dysfunctions in mice. Endocrinology 157: 4875-4887, 2016.
6. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, *et al*: Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature 423: 762-769, 2003.
7. Yamauchi T, Nio Y, Maki T, Kobayashi M, Takazawa T, Iwabu M, Okada-Iwabu M, Kawamoto S, Kubota N, Kubota T, *et al*: Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions. Nat Med 13: 332-339, 2007.

8. Dos Santos E, Serazin V, Morvan C, Torre A, Wainer R, de Mazancourt P and Dieudonné MN: Adiponectin and leptin systems in human endometrium during window of implantation. Fertil Steril 97: 771-778.e771, 2012.
9. Riek U, Scholz R, Konarev P, Rufer A, Suter M, Nazabal A, Ringler P, Chami M, Müller SA, Neumann D, *et al*: Structural properties of AMP-activated protein kinase: Dimerization, molecular shape, and changes upon ligand binding. J Biol Chem 283: 18331-18343, 2008.
10. Ross FA, MacKintosh C and Hardie DG: AMP-activated protein kinase: A cellular energy sensor that comes in 12 flavours. FEBS J 283: 2987-3001, 2016.
11. Griffiths RM, Pru CA, Behura SK, Cronrath AR, McCallum ML, Kelp NC, Winuthayanon W, Spencer TE and Pru JK: AMPK is required for uterine receptivity and normal responses to steroid hormones. Reproduction 159: 707-717, 2020.
12. Qi Y, Wang X, Hou S, Wu Z, Xu X and Pang C: Intracavitary physiotherapy combined with acupuncture mediated AMPK/mTOR signalling to improve endometrial receptivity in patients with thin endometrium. Eur J Obstet Gynecol Reprod Biol 277: 32-41, 2022.
13. Carey EA, Albers RE, Doliboa SR, Hughes M, Wyatt CN, Natale DR and Brown TL: AMPK knockdown in placental trophoblast cells results in altered morphology and function. Stem Cells Dev 23: 2921-2930, 2014.
14. Waker CA, Albers RE, Pye RL, Doliboa SR, Wyatt CN, Brown TL and Mayes DA: AMPK knockdown in placental labyrinthine progenitor cells results in restriction of critical energy resources and terminal differentiation failure. Stem Cells Dev 26: 808-817, 2017.
15. Jha RK, Titus S, Saxena D, Kumar PG and Laloraya M: Profiling of E-cadherin, beta-catenin and Ca(2+) in embryo-uterine interactions at implantation. FEBS Lett 580: 5653-5660, 2006.
16. Giannini A, D'Oria O, Corrado G, Bruno V, Sperduti I, Bogani G, Laganà AS, Chiantera V, Caserta D and Vizza E: The role of L1CAM as predictor of poor prognosis in stage I endometrial cancer: A systematic review and meta-analysis. Arch Gynecol Obstet 309: 789-799, 2024.
17. Vizza E, Bruno V, Cutillo G, Mancini E, Sperduti I, Patrizi L, Certelli C, Zampa A, Giannini A and Corrado G: Prognostic role of the removed vaginal cuff and its correlation with L1CAM in low-risk endometrial adenocarcinoma. Cancers (Basel) 14: 34, 2021.
18. Noyes RW, Hertig AT and Rock J: Reprint of: Dating the endometrial biopsy. Fertil Steril 112: e93-e115, 2019.
19. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
20. Yu SL, Kang Y, Jeong DU, Lee DC, Jeon HJ, Kim TH, Lee SK, Han AR, Kang J and Park SR: The miR-182-5p/NDRG1 axis controls endometrial receptivity through the NF-κB/ZEB1/E-cadherin pathway. Int J Mol Sci 23: 12303, 2022.
21. Rahnama F, Thompson B, Steiner M, Shafiei F, Lobie PE and Mitchell MD: Epigenetic regulation of E-cadherin controls endometrial receptivity. Endocrinology 150: 1466-1472, 2009.
22. Yi Y, Chen D, Ao J, Zhang W, Yi J, Ren X, Fei J, Li F, Niu M, Chen H, *et al*: Transcriptional suppression of AMPKα1 promotes breast cancer metastasis upon oncogene activation. Proc Natl Acad Sci USA 117: 8013-8021, 2020.
23. Messaoudi S, El Kasmi I, Bourdiec A, Crespo K, Bissonnette L, Saint CL, Bissonnette F and Kadoch IJ: 15 years of transcriptomic analysis on endometrial receptivity: What have we learnt? Fertil Res Pract 5: 9, 2019.
24. Rubin SC, Abdulkadir M, Lewis J, Harutyunyan A, Hirani R and Grimes CL: Review of endometrial receptivity array: A personalized approach to embryo transfer and its clinical applications. J Pers Med 13: 749, 2023.
25. Nien JK, Mazaki-Tovi S, Romero R, Erez O, Kusanovic JP, Gotsch F, Pineles BL, Gomez R, Edwin S, Mazor M, *et al*: Plasma adiponectin concentrations in non-pregnant, normal and overweight pregnant women. J Perinat Med 35: 522-531, 2007.
26. Pandey N, Kriplani A, Yadav RK, Lyngdoh BT and Mahapatra SC: Peritoneal fluid leptin levels are increased but adiponectin levels are not changed in infertile patients with pelvic endometriosis. Gynecol Endocrinol 26: 843-849, 2010.
27. Barbe A, Bongrani A, Mellouk N, Estienne A, Kurowska P, Grandhaye J, Elfassy Y, Levy R, Rak A, Froment P and Dupont J: Mechanisms of adiponectin action in fertility: An overview from gametogenesis to gestation in humans and animal models in normal and pathological conditions. Int J Mol Sci 20: 1526, 2019.
28. Kadowaki T and Yamauchi T: Adiponectin and adiponectin receptors. Endocr Rev 26: 439-451, 2005.
29. Zhou L, Deepa SS, Etzler JC, Ryu J, Mao X, Fang Q, Liu DD, Torres JM, Jia W, Lechleiter JD, *et al*: Adiponectin activates AMP-activated protein kinase in muscle cells via APPL1/LKB1-dependent and phospholipase C/Ca2+/Ca2+/calmodulin-dependent protein kinase kinase-dependent pathways. J Biol Chem 284: 22426-22435, 2009.
30. Kim MW, Abid NB, Jo MH, Jo MG, Yoon GH and Kim MO: Suppression of adiponectin receptor 1 promotes memory dysfunction and Alzheimer's disease-like pathologies. Sci Rep 7: 12435, 2017.
31. Nie L, Zhang LX, Wang YC, Long Y, Ma YD, Liao LC, Dai XH, Cui ZH, Liu H, Wang ZQ, *et al*: Consistency and synchronization of AMPK-glycogen in endometrial epithelial cells are critical to the embryo implantation. Reproduction 163: 293-307, 2022.
32. Kaufman MR and Brown TL: AMPK and placental progenitor cells. Exp Suppl 107: 73-79, 2016.
33. Konieczny P, Adamus T, Sułkowski M, Skrzypek K and Majka M: Impact of AMPK on cervical carcinoma progression and metastasis. Cell Death Dis 14: 43, 2023.
34. Dong Y, Hu H, Zhang X, Zhang Y, Sun X, Wang H, Kan W, Tan MJ, Shi H, Zang Y and Li J: Phosphorylation of PHF2 by AMPK releases the repressive H3K9me2 and inhibits cancer metastasis. Signal Transduct Target Ther 8: 95, 2023.
35. Višnjić D, Lalić H, Dembitz V, Tomić B and Smoljo T: AICAr, a widely used AMPK activator with important AMPK-independent effects: A systematic review. Cells 10: 1095, 2021.
36. Turner ML, Cronin JG, Noleto PG and Sheldon IM: Glucose availability and AMP-activated protein kinase link energy metabolism and innate immunity in the bovine endometrium. PLoS One 11: e0151416, 2016.
37. Lane SL, Houck JA, Doyle AS, Bales ES, Lorca RA, Julian CG and Moore LG: AMP-activated protein kinase activator AICAR attenuates hypoxia-induced murine fetal growth restriction in part by improving uterine artery blood flow. J Physiol 598: 4093-4105, 2020.