AQP2 is regulated by estradiol in human endometrium and is associated with spheroid attachment in vitro

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Abstract. 17β-estradiol (E2) and aquaporin 2 (AQP2) are associated with endometrial receptivity, and E2 directly regulates AQP2 expression in endometrial cancer cells. The present study aimed to investigate the role of AQP2 in embryo implantation. Normal endometrial samples were collected at the Women's Hospital (Hangzhou, China) from women seeking in vitro fertilization and embryo transfer; women with endometrial abnormalities were excluded from the study. Samples were categorized into early-mid proliferative, late proliferative, early secretory, mid-secretory and late secretory phase groups, according to the menstrual cycle. The mRNA and protein expression levels of AQP2 were assessed in normal human endometrium in response to E2 via reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. The effects of AQP2 on spheroid attachment were assessed using an in vitro co-culture assay with small interfering (si)RNA against AQP2. The highest expression levels of AQP2 were observed in the late proliferative and mid-secretory phases, with the lowest levels detected in the early proliferative and late secretory phases. In addition, treatment with 10^{-9} or 10^{-7} M E2 for 24 h upregulated AQP2 in the cultured endometrium. Knockdown of AQP2 by siRNA significantly decreased JAr spheroid attachment; however, this effect was significantly reversed when AQP2 siRNA-transfected cells were treated with 10^{-7} M E2. The results of the present study suggested that AQP2 expression levels in human endometrium may be mediated by estrogen, and low AQP2 expression levels may be a potential cause of impaired uterine receptivity.

Introduction

In vitro fertilization (IVF) is a first-line treatment for female infertility. It involves egg collection, fertilization of the eggs with sperm in the laboratory, followed by the transfer of one or more viable embryos to the uterus, with the hope of successful pregnancy. In 2012, an estimated 5 million children were born from IVF (1). During IVF, it is necessary to collect and fertilize numerous eggs in order to increase the number of high-quality embryos available for transfer, and multi-follicular development is induced by hormonal stimulation. In women without ovarian function, 17β-estradiol (E2) and progesterone are the two hormones that need to be provided to achieve a receptive endometrial environment (2). Clinical evidence has suggested that endometrial receptivity during controlled ovarian hyperstimulation is reduced, which may increase the circulating levels of E2 to supraphysiological levels (3,4). Supraphysiological serum levels of E2 may exert a negative effect on endometrial receptivity, but without affecting embryo quality (5-7). Using a step-down protocol to decrease the plasma levels of E2 prior to embryo transfer may improve the implantation rate (8). Nevertheless, the mechanisms underlying the effects of supraphysiological levels of E2 on endometrial receptivity remain unclear. Proteomic studies have revealed the differences between receptive and non-receptive endometria during the implantation window (9); however, the effect of E2 is still poorly understood (8).

The roles of aquaporins (AQPs) in the reproductive system have previously been examined (10-12). AQPs serve functional and distinctive collaborative roles in regulating the amount of water in the mammalian uterus and oviduct (13,14). It has previously been demonstrated that in the human endometrium, AQP2 is expressed in a menstruation-dependent manner, suggesting that AQP2 may serve physiological roles in uterine receptivity (15). In addition, AQP2 expression in human endometrium is positively correlated with serum E2 levels, suggesting that E2 may regulate AQP2 expression (15,16). A previous study strongly suggested that AQP2 may be regulated by E2 via an estrogen receptor-estrogen response element (ER-ERE) in endometrial carcinoma cells (17). However,
whether E2 regulates AQP2 expression in normal human endometrium has yet to be determined. In addition, the role of AQP2 in embryo implantation is largely unknown. Therefore, the present study aimed to investigate whether E2 regulates AQP2 expression in normal human endometrial tissues, and to determine the role of AQP2 in embryo implantation.

Patients and methods

Patients and sample collection. Normal endometrial samples were collected from women seeking IVF and embryo transfer (n=113; median age, 32 years; age range, 25-38 years) at the Women’s Hospital (Hangzhou, China). Women with endometrial abnormalities were excluded from this study. Endometrial samples were collected from women undergoing hysterectomies between August 2015 and February 2016 on days 6-15 (proliferative phase, n=38) or 16-28 (secretory phase, n=75) of the menstrual cycle. None of the women were treated with cycle-altering medication. Shortly after collection, the endometrial samples were cut with a scalpel into pieces (size, 1.0x0.5 cm), and either snap frozen in liquid nitrogen and stored at -70°C for the extraction of protein and mRNA, or fixed with 4% paraformaldehyde for 24 h at 4°C prior to pathological examination. According to Noyes pathological diagnostic criteria (18), endometrial samples were categorized as early-mid proliferative (days 5-10), late proliferative (days 11-14), early secretory (days 15-18), mid-secretory (days 19-24) and late secretory (days 25-28) phase groups following hematoxylin and eosin staining. Briefly, the sections were prepared at room temperature and were stained with hematoxylin (0.5%) and eosin (0.5%) for 25-28 min at room temperature, after which, they were examined using a light microscope (Olympus Corporation, Tokyo, Japan).

The present study was approved by the Ethics Committee of the School of Medicine of Zhejiang University (Hangzhou, China). Written informed consent was obtained from each patient prior to tissue collection and enrolment in the present study.

Tissue culture. Endometrial explant culture was performed as previously described (19). Briefly, endometrial explants in the proliferative phase from hysterectomy specimens were minced using a scalpel (2-3 mm), placed in a 12-well dish with the same volume as in the e2 groups) or various concentrations of serum (FBS; Hyclone; Ge Healthcare life Sciences, Logan, UT, USA) at 37°C in a humidified environment containing 5% CO2 at 95% air volume, after which, they were examined using a light microscope.

Immunofluorescence. Endometrial samples (1.0x0.5 cm) were fixed as aforementioned, sectioned at 4 μm, permeabilized with Triton-X, blocked with 1% bovine serum albumin (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) at 4°C for 5 min; 3 cycles of denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec and extension at 72°C for 30 sec, and final extension step at 72°C for 10 min. Data were obtained using the comparative quantification cycle (Cq) method (21,22). Relative expression = 2^(-ΔΔcT) where ΔΔcT = cT (treated group) - cT (control group) and ΔcT = Cq (sample) - Cq (internal control).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Following treatment, endometrial tissues were either snap frozen in liquid nitrogen and stored at -70°C for the extraction of RNA and protein, or processed to obtain 4-μm sections. For RNA extraction, endometrial tissues (50-100 mg) were homogenized in 1 ml RNAiso Plus (Sangon Biotech Co., Ltd., Shanghai, China). The supernatant was collected, mixed with 200 μl trichloromethane and agitated for 30 sec. After centrifugation at 240 x g for 15 min at 4°C, the supernatant was collected and mixed with an equal volume of isopropanol alcohol. The mixture was centrifuged at 240 x g for 10 min at 4°C, and the precipitate was dissolved in 75% ethanol. Total RNA was extracted after being centrifuged at 120 x g for 5 min at 4°C, and was dissolved in DEPC water. Total RNA was processed using DNase to remove DNA contamination. Subsequently, cDNA was synthesized using the Reverse Transcripase kit (Takara Bio, Inc., Otsu, Japan); the RNA was heated to 37°C for 60 min, followed by incubation at 95°C for 5 min. Eventually, the cDNA was stored at -20°C for further analysis. RT-qPCR was conducted on an Icycler™ Optical Module using the IQ™ SYBR® Green Supermix (both Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primers were used as follows: AQP2 forward, 5'-TGGGCGCATATGTGCTATGGAGA-3' and reverse, 5'-AAGGACACTCAGGTCGCCAGGA-3' (amplicon size, 142bp); and as an internal reference, GAPDH forward, 5'-CAGGGCCTGTATTAA CTCCTGG-3' and reverse, 5'-TGGGGTGAATCATATGGAACA-3' (amplicon size, 102bp). For qPCR, the following thermocycling program was adopted: Initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec and extension at 72°C for 30 sec, and final extension step at 72°C for 10 min. Data were analyzed using the comparative quantification cycle (Cq) method (21,22); Relative expression = 2^(-ΔΔcT), where ΔΔcT = cT (treated group)-cT (control group), and ΔcT = Cq (sample)-Cq (internal control).

Western blot analysis. Western blotting was performed as described previously, without modification (15). Briefly, the proteins were separated by 8% SDS-PAGE and were transferred onto nitrocellulose membranes. The membranes were blocked in skimmed milk at 4°C for 1 h and incubated with goat anti-AQP2 primary antibody (1:100; cat. no. sc-9882; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C overnight. The samples were subsequently incubated with Alexa Fluor® 488 rabbit anti-goat immunoglobulin G (IgG) secondary antibodies (1:200; cat. no. A27012; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 2 h at room temperature. No counterstain was used. The slides were analyzed under a Zeiss LSM 510Meta laser scanning confocal microscope (Carl Zeiss AG, Oberkochen, Germany) or an Olympus BX51TF fluorescence microscope (Olympus Corporation).
AQP2 silencing by small interfering RNA (siRNA) technology. siRNA sequences against AQP2 were generated using the Silencer siRNA Construction kit (Ambion; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. The siRNA sequences against AQP2 were as follows: Forward, 3'-GGUGGGUGUAAGGGATT-5' and reverse, 3'-UUCUCCUACACCACCTG-5'. Control scrambled siRNAs were purchased from Ambion (cat. no. AM4611; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. The siRNA sequences against AQP2 were as follows: Forward, 3'-GGUGGGUGUAAGGGATT-5' and reverse, 3'-UUCUCCUACACCACCTG-5'. Control scrambled siRNAs were purchased from Ambion (cat. no. AM4611; Thermo Fisher Scientific, Inc.). Ishikawa cells (cat. no. 99040201) were inoculated at 100 cells/cm² in minimum essential medium (MEM; both Sigma-Aldrich; Merck KGaA) supplemented with 2 mM glutamine, 1% non-essential amino acids and 5% FBS (Hyclone; GE Healthcare Life Sciences) at 37°C in an atmosphere containing 5% CO₂. After reaching 70-80% confluence, the cells were transfected with siRNA duplexes for 24 h using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. A final concentration of 5 nM AQP2 siRNA was determined to maximally suppress target RNA expression. The same concentration was used for scrambled siRNA transfection.

Evaluation of JAr spheroid attachment onto Ishikawa cells. Attachment of the choriocarcinoma cell line, Jar (HTB-144; American Type Culture Collection, Manassas, VA, USA) to endometrial cells (Ishikawa) was quantified according to an adhesion assay, as previously described (23-25). Briefly, a suspension of 6x10⁴ JAr cells in 6 ml RPMI-1640 medium supplemented with 10% FBS was incubated at 37°C on a gyratory shaker at 110 rpm, in order to generate multicellular spheroids 3 days following culture initiation. Ishikawa cells were inoculated at 100 cells/cm² in 12-well plates containing MEM supplemented with 2 mM glutamine, 1% non-essential amino acids and 5% FBS (Hyclone; GE Healthcare Life Sciences) at 37°C in an atmosphere containing 5% CO₂. At 40% confluence, Ishikawa cells were transfected with scrambled siRNA or siRNA (6 nM) duplexes targeting AQP2 for 24 h, as mentioned, and were then treated with or without E2 (10⁻⁷ M) for 24 h at 37°C in an atmosphere containing 5% CO₂. Subsequently, spheroids (60-200 µm) were transferred onto a confluent monolayer of Ishikawa cells. The cultures were maintained in culture medium (RPMI-1640 medium with 10% FBS) for 1 h at 37°C in a 5% CO₂ incubator. After 1 h, spheroid adhesion to the endometrial monolayer was quantified by centrifugation of the 12-well plates with the cell-spheroid surface facing down at 10 x g for 10 min at 4°C. The unattached spheroids were removed by centrifugation. Attached spheroids were visualized under an anatomical microscope (magnification, x100) and were counted under a phase contrast microscope (magnification, x400). Results were expressed as a percentage of the total number of spheroids (attachment rate (%) = number of attached spheroids/total spheroids) x 100.

Results

Menstrual cycle-dependent expression of AQP2 in human endometrium. AQP2 mRNA and protein were expressed in human endometrium, in a menstrual cycle-dependent manner (Fig. 1A). The lowest levels of AQP2 expression were observed in the early-mid proliferative and late secretory phases (Fig. 1A and B). The highest AQP2 expression levels were observed in the late proliferative and mid-secretory phases (Fig. 1A and B). E2 levels are altered throughout the menstrual cycle (with a peak at day 12-13) (26) and these results suggested that AQP2 expression in the human endometrium may be E2-dependent.

Treatment with 10⁻⁷M E2 increases AQP2 expression in cultured human endometrium. Immunofluorescence revealed that AQP2 was mainly located in the glandular and luminal epithelia (Fig. 2Aa-d), in accordance with our previous report (15). Stronger immunofluorescence signals for AQP2 were observed in the 10⁻⁷ M E2 group (Fig. 2Ac); however, treatment with 10⁻⁵ M E2 notably decreased the fluorescence of AQP2 (Fig. 2Ad) compared with 10⁻⁷ M E2 (Fig. 2Ac). Western blotting (Fig. 2B) and RT-qPCR (Fig. 2C) analyses revealed that endometrial tissues in the 10⁻⁹ and 10⁻⁷ M E2-treated groups exhibited significantly higher expression levels of AQP2 compared with the control group. AQP2 expression levels were significantly reduced in response to treatment with 10⁻⁴ M E2 compared within response to 10⁻⁷ M E2 (Fig. 2B and C). Therefore, 10⁻⁷ M E2 was selected for subsequent experimentation to assess the effects of E2 on the endometrium.

Effects of AQP2 RNAi on Ishikawa cells. To confirm the silencing efficiency of AQP2 siRNA by RT-qPCR and western blotting, Ishikawa cells were harvested 24 h post-transfection with scrambled or AQP2 siRNA duplexes. Compared with the scrambled siRNA group, Ishikawa cells transfected with AQP2 siRNA exhibited significant reductions in the mRNA and protein expression levels of AQP2 [-81.0±2.1% (P<0.05) and -72.0±1.9% (P<0.05), respectively] (Fig. 3).

AQP2 siRNA reduces spheroid attachment. JAr spheroids at a diameter of 60-200 µm were used to assess JAr cell attachment onto endometrial Ishikawa cells (Fig. 4A). As presented in Fig. 4B, knockdown of AQP2 by siRNA resulted in a decreased attachment rate in the in vitro attachment model. Attachment rates were: 80.6±2.2, 62.4±1.5, 82.1±2.2 and 85.4±2.4% in the scrambled siRNA (control), AQP2 siRNA, 10⁻⁷ M E2 + AQP2 siRNA, and scrambled siRNA + 10⁻⁷ M E2, respectively.
siRNA, and $10^{-7}$ M E2 groups, respectively. There were no differences between the $10^{-7}$ E2 and $10^{-7}$ E2 + siRNA groups, between the $10^{-7}$ E2 and control groups, and between the $10^{-7}$ E2 + siRNA groups and control group (all $P>0.05$); however, a significant difference was detected between the siRNA and $10^{-7}$ E2 groups, between the siRNA and $10^{-7}$ E2 + siRNA groups, and between the siRNA and control groups (all $P<0.05$). These results revealed that knockdown of AQP2 by siRNA significantly decreased JAr spheroid attachment compared with in the control group; however, this effect was significantly reversed when AQP2 siRNA-transfected cells were treated with $10^{-7}$ M E2 (Fig. 4C). These results indicated that the expression of AQP2 may be associated with spheroid attachment and E2 could improve spheroid attachment since significant differences were observed between the siRNA and control groups, and between the siRNA and $10^{-7}$ E2 + siRNA groups.

**Discussion**

E2 and AQP2 have been associated with endometrial receptivity (5-7,10-12), and E2 can directly regulate AQP2 expression in endometrial cancer cells (15). Therefore, the present study aimed to assess the role of AQP2 in embryo implantation. The results revealed that AQP2 expression in human endometrium maybe mediated by E2. These findings suggested that AQP2 may serve physiological roles...
in uterine receptivity and affect embryo attachment, which is the initial step of implantation. This hypothesis was tested in vitro by knocking down AQ2P2 via siRNA in the JAr-Ishikawa cell attachment assay (23-25). In the present study, the Ishikawa cell line was selected as a model for receptive endometrium as these cells possess well-reported receptivity and express various implantation-associated molecules (27), as well as estrogen and progesterone receptors (28). JAr is a trophoblastic choriocarcinoma cell line, which exhibits cytotrophoblastic characteristics (29) and is able to attach to Ishikawa cells in vitro (30). The present study demonstrated that knockdown of AQ2P2 by siRNA in Ishikawa cells significantly suppressed embryo attachment; this finding further supported the hypothesis that AQ2P2 serves a key role in uterine receptivity and embryo implantation. Our previous study reported an association between AQ2P2 and E2 in the human endometrium (15). Additionally, the present study revealed the regulatory effects of E2 on AQ2P2. Specifically, E2 had an effect on the expression of AQ2P2, but not on JAr attachment to Ishikawa cells compared with the control group. Therefore, these results suggested that E2 may have an indirect effect on endometrial receptivity. These findings in healthy women were in accordance with those of a recent report, which demonstrated that impaired endometrial receptivity in patients following controlled ovarian stimulation is associated with reduced AQ2P2 expression (31).

Estrogens serve crucial roles in endometrial cellular functions, including cell homeostasis, proliferation, differentiation and vascularization (32,33). ERs are expressed in endometrial glands and the stroma, and genomic experiments have
demonstrated that estrogens mainly exert their effects via putative ER-EREs in promoter regions (34). A putative ER-ERE was identified in the promoter region of the AQP2 gene (17). Recently, Chai et al (35) compared the effects of high serum E2 levels on endometrial steroid receptors in gonadotropin-stimulated and natural cycles. Analysis revealed that ER expression is significantly reduced in stimulated cycles (35). Therefore, it may be inferred that AQP2 expression and function may be attenuated in the presence of highly elevated serum E2 levels; however, further investigation is required.

Studies have reported significantly lower implantation and pregnancy rates in cycles with high serum E2 concentrations (3-7); however, high serum E2 levels in fresh IVF cycles may not affect implantation and pregnancy rates in subsequent frozen-thawed embryo transfer cycles (36). Therefore, reduced implantation may be due to an adverse endometrial environment resulting from high serum E2 levels and not by the direct action of E2, suggesting that high serum E2 levels may be an indirect cause of reduced implantation (25,31,37-39). The endometrium undergoes a series of precisely regulated alterations under highly elevated serum estrogen levels induced by the application of gonadotrophin in IVF cycles compared with natural cycles (40,41). These changes include impaired development of the endometrial glands (42), advanced stromal development (39), desynchrony between glands and the stroma (36), and early expression of pinopodes (43). The present study revealed that AQP2 expression was decreased under abnormally high E2 levels in human endometrium. This finding suggested a novel molecular mechanism may be involved in reducing endometrial receptivity by elevating serum E2 levels in assisted reproductive technologies, such as IVF. Physiological E2 levels are closer to 10⁻⁷ M; however, at 10⁻⁵ M, E2 may reduce AQP2 expression via ERs (44), which may indicate a different mechanism from that of 10⁻⁷ M E2 used in the present study. Conversely, a previous study proposed that very high serum E2 levels may not be detrimental to the clinical outcomes of IVF within a small sample (45). Further investigation into the underlying mechanism of impaired endometrial receptivity induced by AQP2 is required.

In conclusion, the present study demonstrated that AQP2 expression in human endometrium may be regulated by E2. The in vitro co-culture model using JAr and Ishikawa cells suggested a notable role of AQP2 in uterine receptivity and embryo implantation. Additionally, abnormal AQP2 expression may be a potential cause of impaired uterine receptivity. The results of the present study provided information regarding the causes of decreased uterine receptivity in the context of controlled ovarian hyperstimulation. These findings may provide an important marker (AQP2) for endometrial receptivity in women undergoing IVF. Further studies on the effects of AQP2 expression or polymorphisms on female fertility are required.

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Availability of data and materials

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

Authors’ contributions

YZ and RH designed the study. WH, XC and XH conducted the experiments. YH and RH participated in data analysis. RH and WH wrote the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the School of Medicine of Zhejiang University. Written informed consent was obtained from each subject prior to participation in this study.

Patient consent for publication

Written informed consent was obtained from each patient prior to tissue collection and enrolment in the present study.

Competing interests

The authors declare that they have no competing interests.

References


