Potential roles of aquaporin 9 in the pathogenesis of endometriosis

Running Title: Aquaporin 9 and endometriosis

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ABSTRACT

Aquaporins (AQPs) are involved in cell migration, proliferation, and carcinogenesis in tumor development and physiologic inflammatory processes, but their associations with endometriosis has not been fully evaluated. In this study, tissue samples were obtained from women undergoing laparoscopic surgery for endometriosis and other benign conditions. Analysis of expressions of AQP subtypes in eutopic and ectopic endometrium of patients with endometriosis (Eu-EMS and Ect-EMS, respectively) and eutopic endometrium of control patients without endometriosis (Eu-CTL) were performed using the NanoString nCounter System and western blotting. Human endometrial stromal cells (HESCs) were cultured and transfected with the siRNA of the AQP of interest. Among the AQP1–9 subtypes, endometrial expression of AQP2 and AQP8 was significantly increased, whereas AQP9 expression was significantly decreased in the Eu-EMS group compared to the Eu-CTL group. Comparison of expression of AQP2, AQP8, and AQP9 among Eu-EMS, Ect-EMS, and Eu-CTL groups revealed significant differences for only AQP9. Expression of AQP9 in the Eu-EMS group was decreased compared with that in Eu-CTL. After transfection of AQP9 siRNA in HESCs, expression of MMP2 and MMP9 were significantly elevated. Increased expression of phosphorylated ERK 1/2 (p-ERK 1/2) and phosphorylated p38 MAPK (p-p38 MAPK) proteins after transfection was also confirmed using western blot analysis. Increased migration and invasion potentials of HESCs after transfection were determined by migration and wound healing assays. These findings suggest that AQP9 may be involved in the pathogenesis of endometriosis and warrant further investigation as a potential therapeutic target for treating endometriosis.

Keywords: aquaporins, AQP9, endometriosis, extracellular signal-regulated kinase 1/2, matrix metalloproteinase, migration, pathogenesis, p38 mitogen-activated protein kinases, siRNA, transfection

Introduction

Endometriosis is characterized by the presence of endometrium-like epithelium and stroma outside the endometrium and myometrium (Zegers-Hochschild *et al.* , 2017). It is one of the most common gynecologic disorders affecting approximately 10% of all reproductive-aged women and 20-50% of women with chronic pelvic pain and/or infertility (Giudice and Kao, 2004). Despite the relatively high prevalence of the disease among young reproductive-aged women, the exact pathogenesis of the disease is not yet clearly understood and optimal treatment of the disease is still elusive. Many different pathogenic theories have been proposed for this estrogen-dependent chronic inflammatory condition, including retrograde menstruation, coelomic metaplasia, Müllerian remnants, and endometrial stem cell implantation (Du and Taylor, 2007, Giudice and Kao, 2004, Taylor, 2004, Vercellini *et al.* , 2014). However, endometriosis is often characterized by its heterogeneity, with a well-documented discrepancy between the severity of the disease and symptoms. None of these theories adequately explain the etiology of all the different phenotypes of endometriosis. It appears that the interaction of the amount of menstrual flows with genetic and environmental factors, as well as additional unknown underlying mechanisms, contribute to both the likelihood of developing and the phenotypic manifestation of the disease (Vercellini *et al.*, 2014).

Aquaporins (AQPs) are a family of small (25–34 kDa), hydrophobic and integral membrane channel proteins that facilitate rapid passive movement of water (Agre and Kozono, 2003). Since the first discovery of AQP1 by Agre and his colleagues (Preston *et al.* , 1992), 13 isoforms of AQPs (AQP0–AQP12) have been identified in mammals (Agre *et al.* , 2002) and at least nine AQP isoforms (AQP1–AQP9) have been shown to be expressed in various compartments of the female mammalian reproductive organs (Huang *et al.* , 2006). Although the major function of these AQPs in female reproduction is regulation of the passage of water and neutral solutes (Zhang *et al.* , 2012), recent evidence indicates that AQPs are involved in other processes like cell migration, proliferation, and carcinogenesis in tumor development and physiologic inflammatory process (Meli *et al.* , 2018). In addition, AQPs mediate signals via transporting signaling molecules or coupling with other proteins

(Kitchen *et al.* , 2015). It has been suggested that AQPs 1, 3, and 5 are involved in lung cancer differentiation, whereas reduced expression of AQPs 1, 3, 7, and 8 has been shown to play a role in the pathophysiology of inflammatory bowel disease (Ricanek *et al.* , 2015, Wang *et al.* , 2015). Previous studies also documented that overexpression of AQP5 may facilitate Ishikawa (IK) cell migration (an endometrial carcinoma cell line) and knockdown of AQP5 expression reduces cell migration of these cells (Jiang *et al.* , 2012). It was suggested that may play a role in estrogen-induced ectopic implantation of endometrial stromal cells in endometriosis (Jiang *et al.* , 2015).

Considering that chronic inflammatory processes and increased potentials of cell invasion, migration, and proliferation are pivotal processes involved in the pathogenesis of endometriosis, we hypothesized that AQPs are differentially expressed in the endometrium of the patients with endometriosis than those without the disease. In this present study, we evaluated expressions of AQP1–9 in patients with and without endometriosis and investigated whether modulation of specific AQPs is associated with the pathogenesis of endometriosis

Materials and methods

Study population and sample collection

After giving written informed consent, fifty-one women (aged 19 to 44 years) participated in this study, and the study was approved by the Institutional Review Board of Gangnam Severance Hospital. Between June 2015 and July 2017, participants were recruited from patients who underwent laparoscopic surgery for various indications such as endometriosis, pelvic pain, ovarian cysts, and diagnostic evaluation of benign gynecologic disease. Patients with postmenopausal status, use of hormonal agents or gonadotropin-releasing hormone (GnRH) agonist within 3 months of surgery, adenomyosis, endometrial diseases including polyps and hyperplasia, infectious diseases, acute or chronic inflammatory diseases, malignancies, autoimmune diseases, or cardiovascular disease were excluded from the study.

At the time of surgery, possible endometriotic lesions were removed and sent for pathologic examination. Patients were only assigned to the endometriosis group after pathologic confirmation of the condition. The extent of the disease was determined using the American Society of Reproductive Medicine (ASRM) revised classification (Canis *et al.* , 1997). Thirty-two patients were histologically confirmed to have moderate-to-severe (stages III and IV) peritoneal and/or ovarian endometriosis. Of the 19 patients who participated as controls, 14 had ovarian dermoid cysts, three had ovarian serous cystadenoma, and two had ovarian mucinous cystadenoma. Endometrial biopsies were obtained from the patients using a Pipelle aspiration catheter (Cooper Surgical, Trumbull, CT, USA).

Culture of primary endometrial stromal cells

We utilized a previously published method to culture endometrial stromal cells (Cho *et al.* , 2016). Endometrium was finely minced, and the cells were dispersed by incubation at 37 °C for 60 min with agitation and pipetting in Hanks balanced salt solution (HBSS) containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 2 mmol/mL), penicillin(100

U/mL)/streptomycin (100 μg/mL), and collagenase (1 mg/mL, 15 U/mg). The cells were pelleted, washed, suspended in Ham's F12:Dulbecco's Modified Eagle Medium (DMEM) in a 1:1 ratio containing10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, passed through a 40-μm cell strainer (Falcon, Corning, NY, USA), and plated onto 75-cm^2 Falcon tissue culture flasks (BD Biosciences, Bedford, MA, USA). Cultured primary human endometrial stromal cells (HESCs) at 3–5 passages were used for analysis.

RNA extraction and quantification

Total RNA was then extracted using an eCube RNA Mini Kit for cultured cells (Philekorea Technology, Seoul, Korea). RNA yield and purity were assessed using a DS 11 Spectrophotometer (Denovix Inc., Wilmington, DE, USA). 100 ng of total RNA was added to the sample preparation reaction in a 5-μL volume as recommended by the manufacturer. RNA quality was checked using a fragment analyzer (Advanced Analytical Technologies, Ankeny, IA, USA).

mRNA expression profiling

The expression levels of AQP1–9 were tested in eutopic endometrium and ectopic endometrium of the study patients using a NanoString nCounter Analysis System (NanoString Technologies, Seattle, WA, USA) as previously described (Jung *et al.* , 2016). In brief, the digital multiplexed NanoString nCounter human mRNA expression assay (NanoString Technologies) was performed with 100 ng total RNA isolated from cultured cells. Hybridizations were carried out by combining 5 μL of each RNA sample with 8 μL of nCounter Reporter probes in hybridization buffer and 2 μL of nCounter Capture probes (for a total reaction volume of 15 μL) overnight at 65 °C for 16–20 h. Excess probes were removed using a two-step magnetic bead-based purification on the nCounter Prep Station (NanoString Technologies). Abundances of specific target molecules were quantified on the nCounter Digital Analyzer by counting the individual fluorescent barcodes and assessing the target molecules. For each assay, a high-density scan encompassing 280 fields of view was performed. The data were

collected using the nCounter Digital Analyzer after taking images of the immobilized fluorescent reporters in the sample cartridge with a charge-couple device (CCD) camera.

Cell transfection

After being seeded into 6-well plates, cells were cultured to 70–80% confluence and were transfected with AQP9 siRNA or control siRNA-A (Santa Cruz Biotechnology, Dallas, TX, USA) with the use of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) at a final concentration of 50 nM. The transfected cells were harvested 48 hours after transfection.

RNA isolation from cultured HESCs and quantitative real-time polymerase chain reaction (qRT-PCR)

To quantify mRNA levels, total RNAs were isolated from cultured HESCs to use the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA sample concentrations were calculated with a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Using 1 μg of total RNA, cDNA was synthesized with oligo-dT in a Superscript III kit (Invitrogen) with the use of C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The resultant cDNA mixtures were stored at -20 °C. Then, using 2 μl of synthesized cDNA template, qRT-PCR amplification was performed with the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed with the Power SYBR Green PCR master mix (Applied Biosystems by Thermo Fisher Scientific, Woolston Warrington, UK). The reaction mixture included a cDNA template, forward and reverse primers, ribonuclease-free water, and the SYBR Green PCR master mix in a final reaction volume of 20 μL. The thermal cycling conditions were performed by procedures at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. The threshold cycle (Ct) and melting curves were acquired using the 7300 software program from the Applied Biosystems. Each reaction was performed in triplicate. The mRNA level of each sample was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels.

Primers for matrix metalloproteinase 2 (MMP2), MMP9, and GAPDH were as follows: MMP2 forward, 5'-ACCGCGACAAGAAGTATGGC-3' and reverse, 5'-CCACTTGCGGTCATCATCGT-3'; MMP9 forward, 5'-CGATGACGAGTTGTGGTCCC-3' and reverse, 5'-TCGTAGTTGGCCGTGGTACT-3'; GAPDH forward, 5'-ACCACAGTCCATGCCATCAC-3', and reverse, 5'-TCCACCACCCTGTTGCTGTA-3'.

Protein extraction and western blot analysis

For protein extraction we used RIPA buffer (Thermo Scientific, Rockford, IL, USA) with freshly added protease and phosphatase inhibitor cocktail (Thermo Scientific). Total cell lysate protein concentrations were measured with a bicinchoninic acid (BCA) assay kit (Thermo Scientific). A total of 20 μg of protein was mixed with 5× sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) loading buffer (Biosesang, Seongnam, Gyeonggi, Korea) and heated at 95 °C for 5 min. Samples were loaded on 12% SDS-PAGE and electrotransferred to a polyvinylidene fluoride membrane (PVDF) (Millipore, Billerica, MA, USA) with the use of a Transblot apparatus (Bio-Rad). Membranes (Millipore) were blocked with 5% non-fat skim milk in TBST [Tris-buffered saline solution (10 mmol/L Tris-HCl [pH 7.4] and 0.5 mol/L NaCl) with Tween-20 (0.1% v/v)]. The blots were probed with primary antibodies: MMP2 (1:250; Santa Cruz Biotechnology), MMP9 (1:300; Santa Cruz Biotechnology), AQP9 (1:1,000; Santa Cruz Biotechnology), extracellular signal-regulated kinase 1/2 (ERK 1/2) (1:1,500 with 5% skim milk; Cell signaling technology, Danvers, MA, USA), phosphorylated ERK 1/2 (p-ERK 1/2) (1:1,000 with 5% skim milk; Cell signaling technology), p38 mitogen-activated protein kinases (p38 MAPK) (1:1,500 with 5% skim milk; Cell signaling technology), phosphorylated-p38 MAPK (p-p38 MAPK; 1:1,000 with 5% skim milk; Cell signaling technology), and GAPDH (1:1,000, Abcam, Cambridge, UK) followed by horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibody (1:2000; Thermo Scientific). Proteins were detected using enhanced chemiluminescence (Santa Cruz Biotechnology). For representative samples drawn from 8 different patients, each protein was measured in triplicate. The data shown are representative.

Migration assay

The migration assay for transfected cultured cells was performed using 8-mm pore size polycarbonate membranes (Millipore) and 24-well plates. Freshly trypsinized cells were washed and suspended in serum-free medium. Cells (200 mL, $5x10^4$ cells/well) were placed in the top chamber of each insert; medium (600 mL) containing 10% FBS was added into the lower chambers. After incubating period of 24 hours at 37 °C in a 5% CO_2/95% air humidified incubator, cells were fixed, and stained with hematoxylin. Cells in the inner chamber were removed with a cotton swab, and cells attached to the bottom side of the membrane were counted and imaged under an inverted microscope (Olympus Corp., Shinjuku, Tokyo, Japan) at 200× magnification over 10 random fields in each well.

Wound healing assay

HESCs, either control or AQP9 siRNA transfected for 48 h, were seeded using DMEM/F12(1:1) with 10% FBS and antibiotics in 24-well culture plates and maintained in a humidified atmosphere containing 5% CO_2/95% air at 37 ºC for 24 h. A linear wound (scratch) was generated using a sterile 100-µL pipette tip, and debris was washed away twice with phosphate-buffered saline (PBS). Culture media was added to cells and incubated for 18–24 h at 37ºC with 5% CO_2. Images from each well of the scratched area were taken with an EVOS inverted microscope (Advanced Microscopy Group, Mill Creek, WA, USA) to estimate the migration ability of the APQ9 knockdown. Three representative images per well were captured along the wound at time 0 and after 24 h and analyzed with ImageJ (http://rsbweb.nih.gov/ij/, 13 March 2018, date last accessed). The percentage of wound closure was calculated by measuring on each image a rectangular area of 400 pixels width without cells at 16 h and just after generating the wound.

Immunofluorescence staining

Immunofluorescence staining of AQP9 was performed on endometrial biopsies from 5 participants of each group. Selected paraffin sections on glass slides were hydrated using a series of xylene and ethanol (100%, 95%, 80%, 70% v/v in distilled water [D.W.]) and washed in D.W. Tissue sections were incubated with antigen retrieval solution (pepsin

reagent; Sigma-Aldrich, MO, USA) in 37°C for 10 minutes for antigen retrieval. After being washed in PBS three times, tissue sections were then incubated with blocking solution (5% BSA/PBS + 0.1% triton-X) in room temperature for 30 min. For immunostaining, slides were treated overnight at 4 °C with mouse anti-AQP9 monoclonal antibody as primary antibody (sc-74409; Santa Cruz Biotechnology) and visualized using goat anti-mouse secondary antibody conjugated with Alexa Fluor® 594 (Jackson Laboratories, PA, USA). The immunostained slides were mounted and counterstained using DAPI (H-1500; Vector Laboratories, CA, USA). The fluorescence signals were imaged by confocal microscopy (LSM700, ZEISS, Germany) and processed using Zen software (ZEISS).

Statistical analysis

All of the data were assessed by Kolmogorov-Smirnov test or Shapiro-Wilk test to evaluate whether they were normally distributed and were compared using Student's *t*-test or Mann-Whitney *U* test, where appropriate. One-way analysis of variance (ANOVA) was performed to evaluate differences between the groups, followed by Tukey's post hoc test. The SPSS 16.0 program (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. nSolver software (freely available from NanoString Technologies), was used for mRNA data analysis, and the mRNA profiling data were normalized using housekeeping genes (GAPDH and beta-actin). A value of $P < 0.05$ was considered statistically significant.

Results

Clinical characteristics

The clinical characteristics of the participants are shown in Table I. There was no significant difference in age, parity, and body mass index between the endometriosis group and controls. However, the endometriosis group had significantly higher serum CA-125 levels than controls (103.03 ± 22.95 versus 14.08 ± 1.69, $P < 0.001$). All endometriosis patients had moderate-to-severe disease, with mean revised American Fertility Society (rAFS) scores of 57.37 ± 3.61.

Expression of AQP subtypes between endometriosis group and controls

Expression of AQPs in endometrial stromal cells between the endometriosis group and controls are shown in Table II. AQP2 and AQP8 showed moderately but statistically significantly higher expression in the endometriosis group than in controls (1.64 ± 0.25 versus 1.05 ± 0.28, $P = 0.020$; 1.64 ± 0.25 versus 1.09 ± 0.05, $P = 0.029$, respectively), whereas markedly lower expression of AQP9 was noted in the endometriosis group compared to controls (30.27 ± 5.35 versus 141.84 ± 31.97, $P = 0.005$). Expression of these three AQP subtypes were further analyzed between the endometrium of patients without endometriosis and the eutopic and ectopic endometrium of the patients with endometriosis. As shown in Figure 1, only AQP9 showed a significant difference between ectopic and eutopic tissue from patients with endometriosis. Endometrial expressions of APQ2, AQP8 and AQP9 were evaluated according to the menstrual cycle, no significant differences were noted in both endometriosis group and controls (Figure 2). Endometrial expression of AQP9 in the endometrium of the patients with and without endometriosis was further validated using western blotting. The results coincided well with mRNA expressions, indicating significantly lower APQ9 expression in the endometrium of the patients with endometriosis (Figure 3).

When clinical characteristics and expression of endometrial AQP2, AQP8, and AQP9 were evaluated, expression of AQP2 and AQP8 showed significant positive correlations with serum CA-125 levels (correlation coefficient = 0.529, $P = 0.002$; correlation coefficient = 0.503, $P = 0.003$,

respectively), whereas AQP9 showed negative correlation with marginal significance (correlation coefficient = -0.337, P = 0.059). Also, when correlations between AQP subtypes were evaluated, expression of AQP2 and AQP8 displayed strong correlations (correlation coefficient = 0.996, P<0.001), whereas no significant correlations were noted between AQP9 and other AQP subtypes.

Immunofluorescence staining

We evaluated AQP9 expression in eutopic and ectopic endometrial tissues of patients with or without endometriosis by immunofluorescence staining. Representative images are shown in Figure 4. Expression of AQP 9 was decreased in endometrial stroma as well as epithelial gland in eutopic endometrium of patients with endometriosis compared to those of controls. Expression of AQP9 was also decreased in ectopic endometrium of patients with endometriosis compared to those of ovarian tissues of controls.

Cellular characteristics of HESCs after AQP9 siRNA transfection

After AQP9 siRNA transfection using samples obtained from 8 different patients, expression of MMP2 and MMP9 in HESCs were evaluated using qRT-PCR (Figure 5A). The mRNA expression of MMP2 and MMP9 significantly increased after AQP9 siRNA transfection in comparison to vehicle treatment (1.46-fold change, P = 0.037; 2.43-fold change, P = 0.037, respectively). Expression of MMP2 and MMP9 was further validated using western blot analysis (Figure 5B, 5C). After 48 h of AQP9 siRNA transfection, MMP2 expression showed a statistically significant ~1.4-fold increase (P = 0.022) in HESCs compared with the vehicle-treated group, whereas MMP2 expression displayed a non-significant 1.3-fold increase (P = 0.093).

Migration and wound healing assay of HESCs after AQP9 siRNA transfection

Migration potential of HESCs was evaluated at 48 h after AQP9 siRNA transfection. The number of migrating cells was significantly higher in the AQP9 siRNA transfection-treated group than that in the vehicle-treated group (39.7 ± 1.74 versus 83.2 ± 3.60, respectively; P < 0.001; Figure 6A).

The wound healing assay revealed similar trends, showing wound closure (expressed as a percentage of the area of the initial wound) significantly increased after AQP9 siRNA transfection compared with vehicle treatment (8.2 ± 1.07% versus 28.9 ±8.92, respectively; $P = 0.026$; Figure 6B).

Expressions of ERK 1/2 and p38 MAPK proteins and their phosphorylated forms after AQP9 siRNA transfection of HESCs

Expressions of ERK 1/2 and p38 MAPK proteins and their phosphorylated forms after AQP9 siRNA transfections in HESCs were evaluated using western blot analysis. There were no significant differences in ERK 1/2 and p38 MAPK protein expression between the vehicle treatment group and the AQP9 siRNA treatment group. However, significantly higher expression of phosphorylated forms of both ERK 1/2 and p38 MAPK proteins were noted after AQP9 siRNA transfection (Figure 7). When the relative ratio of p-ERK 1/2 to ERK 1/2 and p-p38 MAPK to p38 MAPK was compared between the vehicle treatment group and the AQP siRNA transfection group, significantly higher ratios of p-ERK 1/2/ERK 1/2 and p-p38 MAPK/p38 MAPK were noted in the APQ9 siRNA transfection group (3.84-fold increase, $P = 0.001$; 2.51-fold increase, $P < 0.001$, respectively).

Discussion

In the present study, we demonstrated that AQP2, AQP8, and AQP9 were aberrantly expressed in endometriosis. It was also demonstrated *in vitro* that down-regulation of AQP9 in HESCs by siRNA transfection significantly increased migration and invasion potentials through modulation of MMP2 and MMP9 and involvement of the ERK/p38 MAPK signaling pathway. To our knowledge, this is the first study to evaluate the role of AQP9 in the pathogenesis of endometriosis. There have been several studies on the changes of AQP9 mRNA and protein expression in cancers (Yang *et al.* , 2011, Zhang *et al.* , 2016) and inflammatory diseases (Mesko *et al.* , 2010, Nagahara *et al.* , 2010). However, the role of AQP9 in the development of endometriosis has not been studied. AQP9 is down-regulated in hepatocellular carcinoma and it has been suggested that its overexpression suppresses cell invasion by inhibiting epithelial–mesenchymal transition (EMT) (Zhang *et al.*, 2016). Studies on cancer indicate that reversible EMT programs are manifested during tumor metastasis (Thiery, 2002). Similar transitions have been reported in cases of endometriosis (Yang and Yang, 2017). Recently, it was observed that AQP3, which is regulated by the combination and estrogen and progesterone, induced EMT of endometrial epithelial cells (Cui *et al.* , 2018).

In addition to water transport, AQP can facilitate cell migration, invasion, and proliferation in tumor development (Nico and Ribatti, 2010, Papadopoulos *et al.* , 2008, Verkman *et al.* , 2008). In the present study, endometrial mRNA expressions of AQP2 and AQP8 were significantly higher and that of AQP9 was significantly lower in the endometriosis group than the control group. These findings suggest that aberrant expressions of AQP are associated with the pathogenesis of endometriosis. Previously, semi-quantitative analysis by immunohistochemistry showed that AQP2, AQP5, and AQP8 are expressed with greater frequency in eutopic endometrial cells than in ectopic endometrial cells, suggesting that eutopic endometrial cells have stronger migration activity than ectopic endometrial cells in women with endometriosis (Jiang *et al.* , 2010). In the present study, however, mRNA expressions of AQP2 and AQP8 were not different between ectopic and eutopic endometrium in patients with endometriosis. On the contrary, endometrial mRNA expression of AQP9 was

significantly different among eutopic endometrium, ectopic endometrium, and control groups. In particular, mRNA expression of AQP9 was significantly lower in the eutopic endometrium than ectopic endometrium in women with endometriosis. Decreased expression of AQP9 protein in eutopic endometrium of the endometriosis group was confirmed using western blotting and immunofluorescence staining. We, therefore, hypothesize that decreased expression of AQP9 in eutopic endometrium of women with endometriosis may play a role in the development of endometriosis.

To determine the pathophysiologic mechanisms of how AQP9 contributes to the development of endometriosis, several experiments were performed after knockdown of AQP9 by siRNA transfection in HESCs. After the knockdown of AQP9 in HESCs, mRNA and protein expressions of MMP2 and MMP9 were significantly elevated compared to the vehicle control treatment group. In addition, after knockdown of AQP9 in HESCs, increased migratory and invasive capability was confirmed by migration and wound healing assays. MMPs hydrolyze the basement membrane and other components of extracellular matrix (ECM) and interact with cell surface receptors to regulate their biological activities including migration, angiogenesis, and apoptosis (Nagase *et al.* , 2006). Inhibition of MMP was shown to suppress formation of ectopic endometriotic lesions in a nude mouse model (Bruner *et al.* , 1997) and increased activity of MMP2 and MMP9 have been reported in the endometrial tissues of women with endometriosis (Chung *et al.* , 2002, Collette *et al.* , 2006). These study results suggest that dysregulation of the MMP system can enhance the cellular invasiveness of regurgitated endometrial cells and lead to the acquisition of vascular supply and establishment of endometriotic lesions (Osteen *et al.* , 2003). Here we suggest that decreased expression of AQP9 in eutopic endometrium may facilitate the dysregulation of the MMP system, enhance cellular migratory and invasive capability of regurgitated endometrial cells, and lead to the establishment of endometriotic lesions.

The ERK pathway is the most comprehensively studied of the mammalian MAPK pathways and regulates other cellular responses as well as cell proliferation (Dhillon *et al.* , 2007). The ERK 1/2

pathway plays an important role in cell motility including the regulation of the transcriptional levels of MMPs (McCawley *et al.* , 1999). The increased ERK activation in endometriotic tissue may have a role in the pathogenesis of endometriosis. It has been suggested that up-regulation of ERK phosphorylation might play a role in the pathogenesis of endometriosis by increasing proliferation and reducing apoptosis of endometrial cells (Murk *et al.* , 2008, Velarde *et al.* , 2009). In endometriosis, several factors may contribute to ERK activation, including the endometriotic microenvironment (Yoshino *et al.* , 2004), oxidative stress (Andrade *et al.* , 2013, Yoshino *et al.*, 2004), endocrine disruptors (Kim *et al.* , 2015), and estrogens (Cheng *et al.* , 2012). Treatment of endometrial stromal cells with E_2 conjugated to bovine serum albumin (E_2-BSA) also increases phosphorylated ERK expression in a dose-dependent manner (Cheng *et al.*, 2012). Because E_2-BSA cannot penetrate cells, these results indicate the effects of E_2-BSA are mediated at the cell membrane. Environmental stress stimuli, including heat, osmotic shock, and inflammatory cytokines influence the p38 MAPK pathway (Zarubin and Han, 2005). At present, there is little data to confirm an over-activation of p38 in endometriotic cells. The endometriotic microenvironment, however, contains high concentrations of numerous molecules that activate this pathway, suggesting that constitutive activation in ectopic endometrial cells is possible (McKinnon *et al.* , 2016). There are no data on a role of AQP9 in these signaling pathways in women with endometriosis. Following AQP9 siRNA transfection in the present study, protein expression of both p-ERK 1/2 and p-p38 MAPK p and their ratios (p-ERK 1/2/ERK 1/2 and p-p38 MAPK/p38 MAPK) increased. These findings indicate that decreased expression of AQP9 in eutopic endometrium may increase levels of MMPs through the ERK 1/2 and p38 MAPK signaling pathway, which in turn enhances the invasive and migratory capability of eutopic endometrial cells and results in the development of endometriosis.

Eutopic endometrium of women with endometriosis shows fundamental differences compared with that of healthy control (Brosens *et al.* , 2012). These differences may contribute to the survival of regurgitated endometrial cells in the peritoneal cavity and the development of endometriosis. Regurgitated endometrial cells undergo attachment, invasion, and angiogenesis to establish endometriotic lesions. Here we demonstrated that decreased expression of AQP9 from eutopic

endometrium likely play an important role in the migration and invasion of regurgitated eutopic endometrial cells. Of note, expression of AQP9 was decreased in eutopic endometrium versus ectopic endometriosis lesions of endometriosis patients indicating the molecular differences between eutopic endometrium and ectopic endometriotic lesions in endometriosis. Further study is warranted to elucidate the exact role of AQP9 in different ectopic lesions including peritoneal lesions and ovarian endometriomas.

There are several limitations in our study. Firstly, we performed *in vitro* single cell culture experiments using endometrial stromal cells without epithelial cells. Our results obtained from stromal cell only culture could be different from those from *in vivo* environment since there was no cell-to-cell interaction between these two types of cells. Since, in addition, cell physiology may have changed during *in vitro* culture, our results may not reflect *in vivo* phenomenon. However, we validated lower expression of AQP9 in eutopic endometrium of patients with endometriosis using western blot. Immunofluorescence staining also revealed lower expression of AQP9 in epithelial gland as well as stroma in eutopic endometrium of patients with endometriosis. These findings indicate that results of *in vivo* experiments were in line with those of *in vitro* experiments. Secondly, although it was observed that decreased expression of AQP9 facilitated the dysregulation of MMP in the *in vitro* study, a lack of *in vivo* data could weaken our data to draw conclusion. Further research with *in vivo* experiment using AQP9 knock-out mice is warranted to enrich our current findings.

In conclusion, we demonstrated that several AQP subtypes were aberrantly expressed in endometriosis. It was also demonstrated that down-regulation of AQP9 in HESCs significantly increased migration and invasion potentials through the modulation of MMP2 and MMP9 and involvement of the ERK/p38 MAPK signaling pathway in *in vitro study* through siRNA transfection. These findings suggest that AQP9 may play a role in the pathogenesis of endometriosis. Further researches will be warranted to confirm how AQP9 is involved in the pathogenesis of endometriosis and whether it will be a potential therapeutic target for treating endometriosis.

403 **Authors' roles**

404 Young Sik Choi, SiHyun Cho, Byung Seok Lee and Hugh S. Taylor were responsible for conception
405 and design of the work and analysis and interpretation of data. Young Sik Choi, and SiHyun Cho
406 drafted the paper. All authors took part in acquisition and analysis of data for the work, revising the
407 manuscript for important intellectual content and final approval of the version to be published.

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412 **Conflicts of Interest:**

413 None

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Figure legends

Figure 1. Multiple comparisons of endometrial mRNA expressions of aquaporin (AQP) 2, 8 and 9, relative to GAPDH, between eutopic endometrium of the patients with and without endometriosis and ectopic endometrium of endometriosis patients. Results are presented as mean + SEM values. CTL_EM, endometrium without endometriosis; EMS_EM, eutopic endometrium from the patients with endometriosis; EMS_Ect, ectopic endometrium from the patients with endometriosis. *one way ANOVA test, **Tukey's post-hoc test. N=19, 31 and 26 for CTL_EM, EMS_EM and EMS_Ect, respectively.

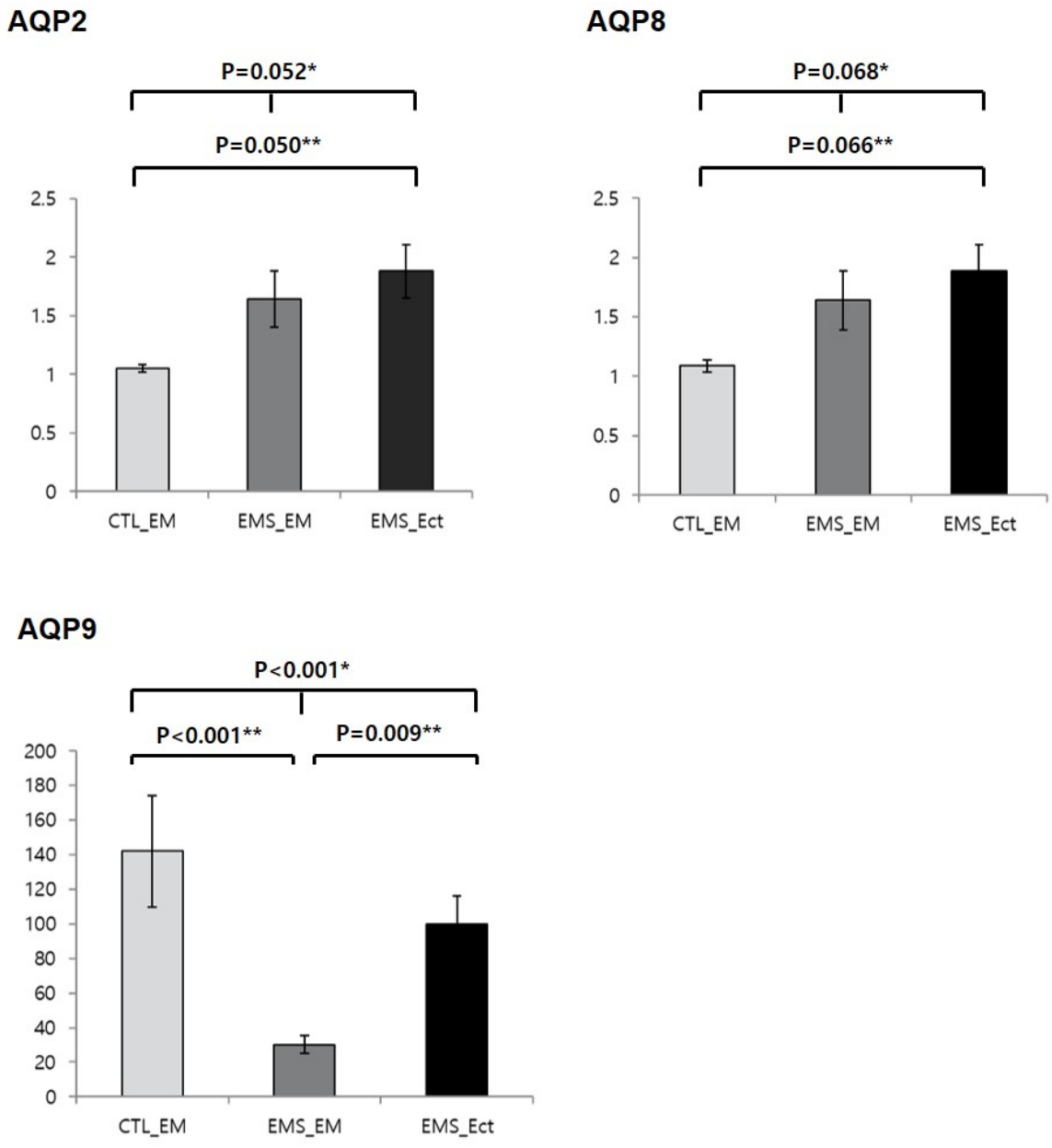


Figure 2. Endometrial expressions of aquaporin (AQP) 2, 8 and 9 according to the menstrual cycle in patients with endometriosis (A) and those without the disease (B).

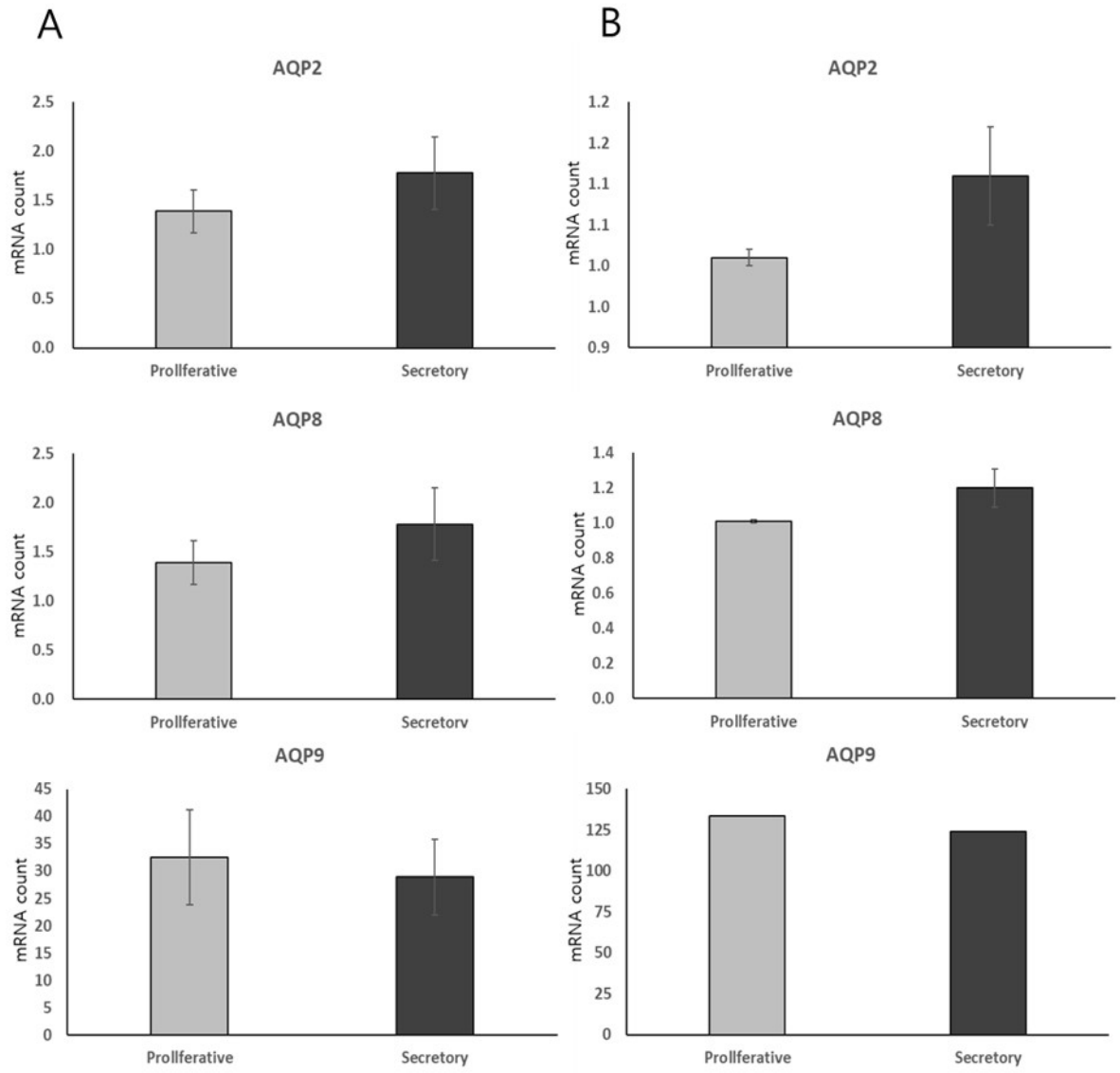


Figure 3. Endometrial expressions of aquaporin (AQP) 9 between endometriosis group and controls using Western blotting. *P< .01. Results are presented as mean + SEM values. CTL-EM, endometrium without endometriosis; EMS-EM, eutopic endometrium from the patients with endometriosis; EMS-OV, ectopic endometrium from patients with endometriosis. N=8.

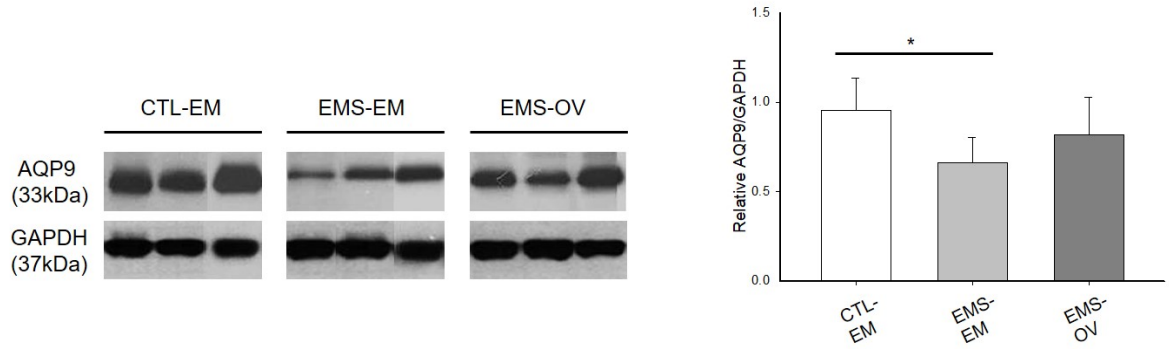


Figure 4. Immunofluorescence staining of aquaporin (AQP) 9 in eutopic endometrium and ovarian tissue of patients with endometriosis and controls. CTL, control; EMS, endometriosis (original magnification X100; inset, X200).

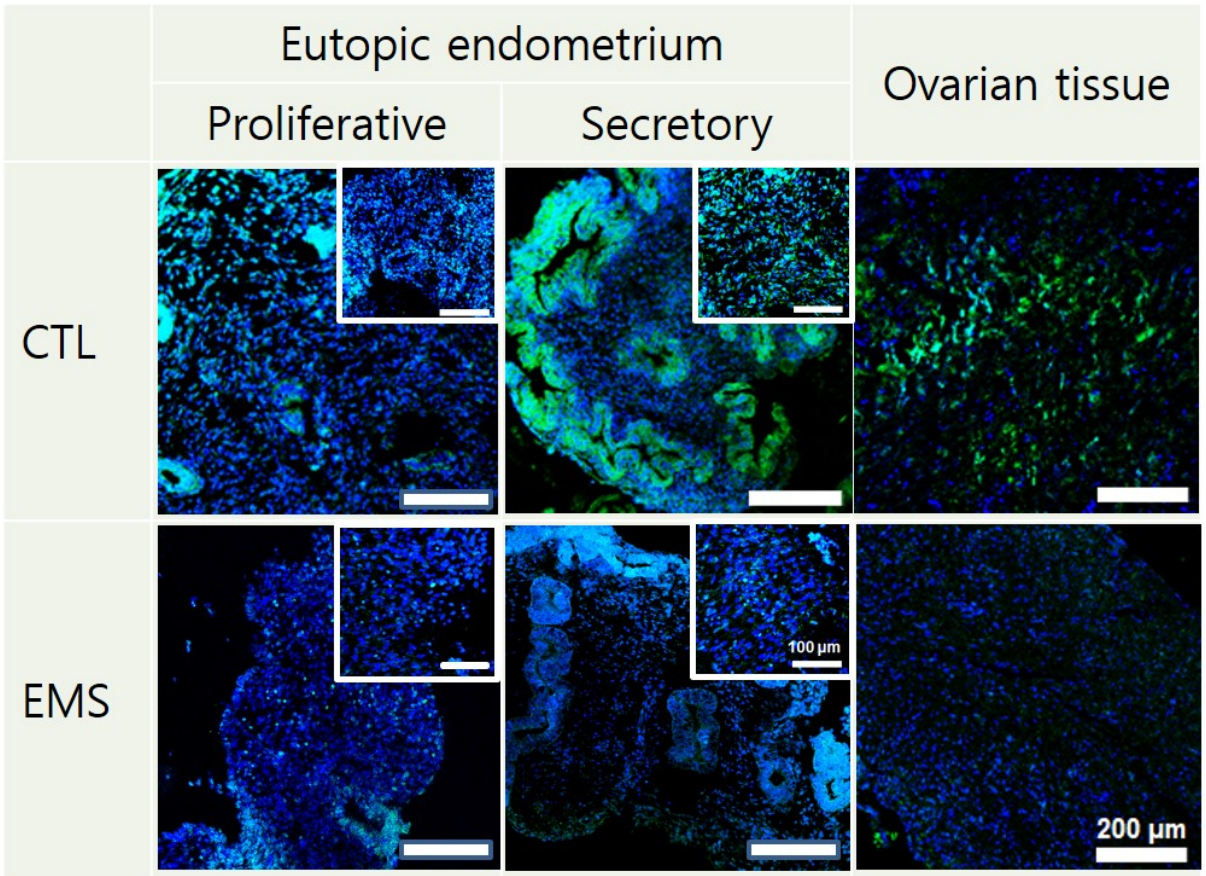


Figure 5. Expressions of matrix metalloproteinase (MMP) 2 and MMP9 of human endometrial stromal cells (HESCs) after siRNA control or aquaporin (AQP9) siRNA transfection. A, MMP-2 and MMP-9 mRNA expression. B and C, Western blot analysis of AQP9, MMP2 and MMP9. *P< .05. Results are presented as mean + SEM values. N=8.

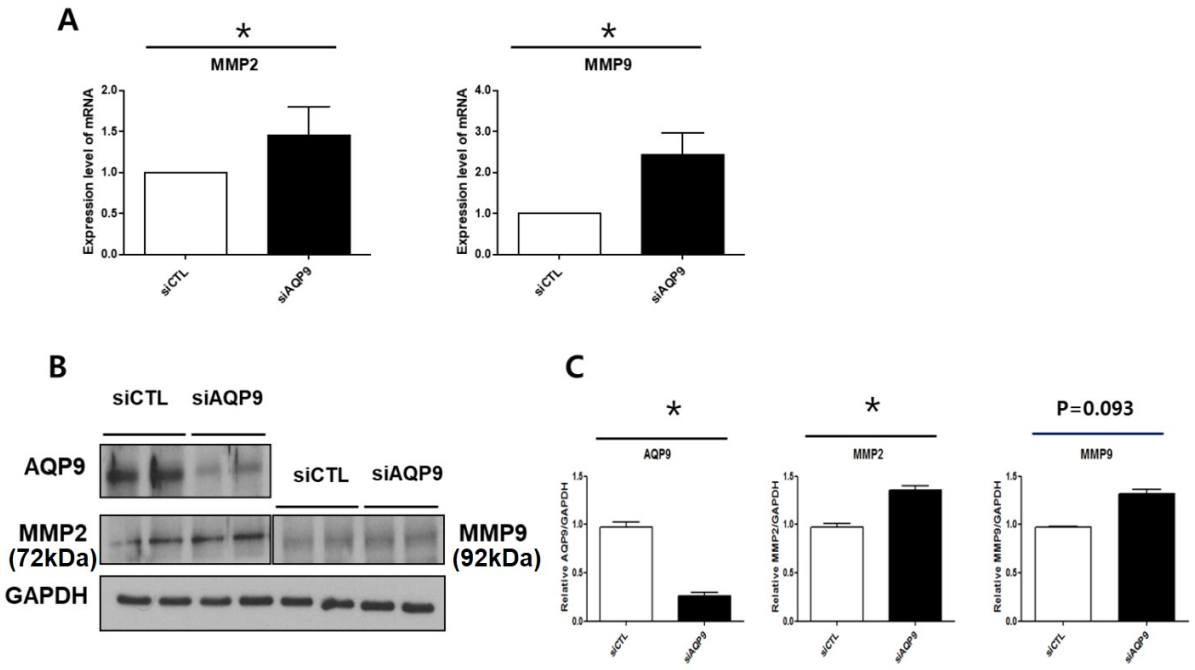


Figure 6. Effects of siRNA control (siCTL) or aquaporin 9 siRNA transfection (siAQP9) on cell migration in human endometrial stromal cells. Migration assay was performed with the use of Millicell cell culture insert system and representative fields of migration cells on the membrane were obtained with magnification xX200 (A). Wound healing assay was evaluated by cell migration determined by the rate of cells moving towards the scratched area. The percentage migration was calculated by ImageJ (B). *P < .05, **P < .01. Results are presented as mean + SEM values. N=8.

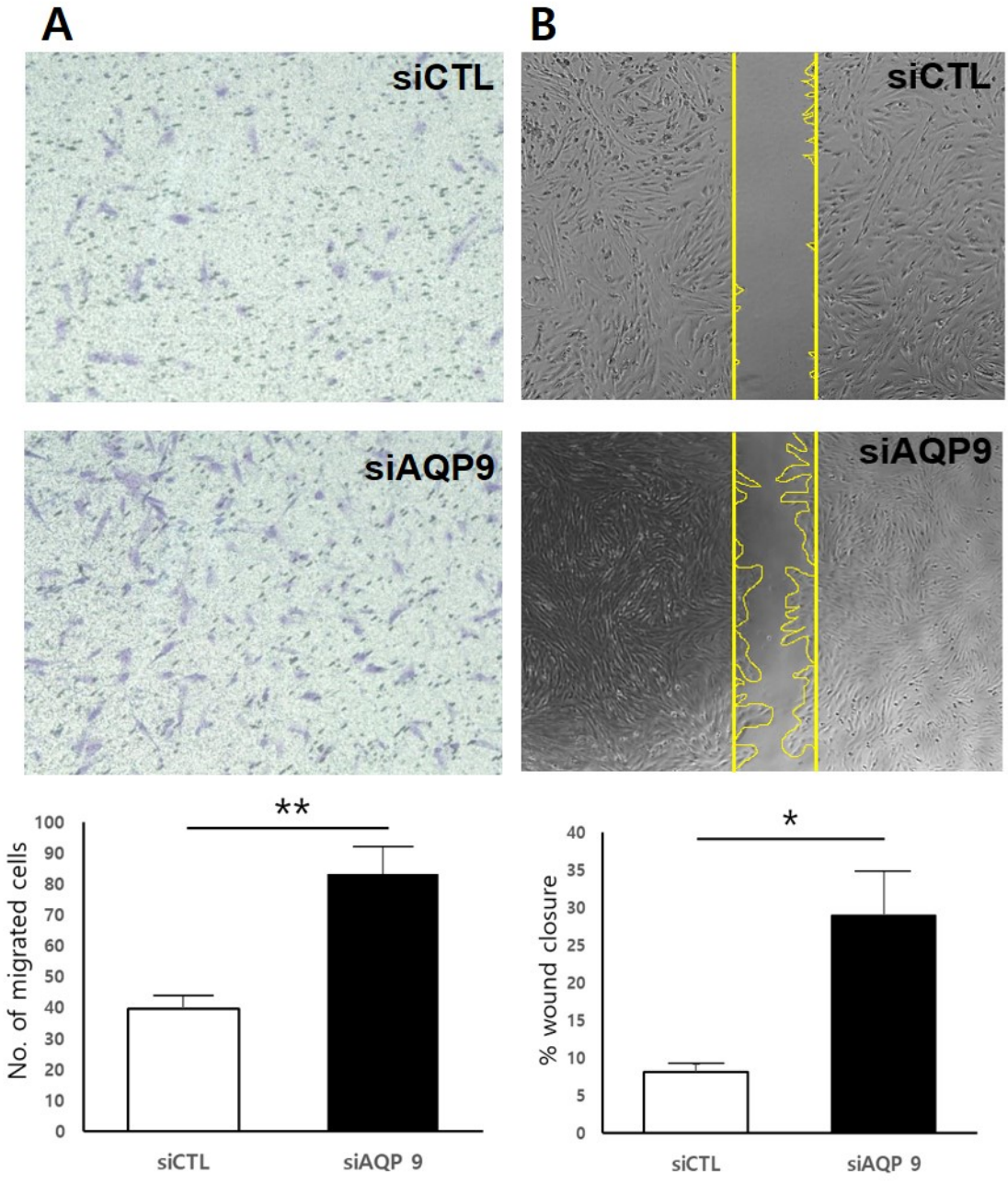
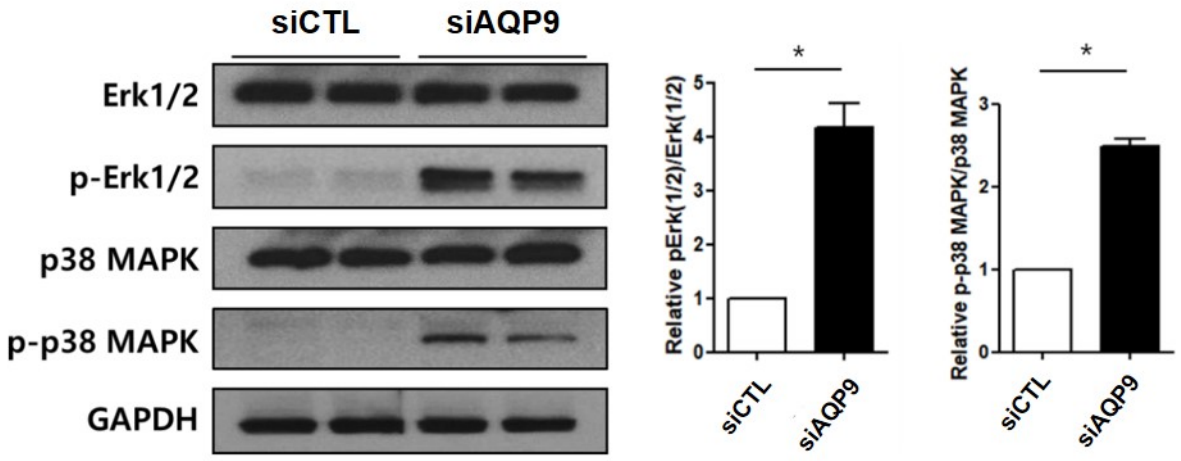


Figure 7. Western blot analysis of extracellular signal-regulated kinase (ERK) 1/2 and p38 mitogen-activated protein kinases (MAPK) proteins and their phosphorylated forms after siRNA control (CTL) or aquaporin (AQP) 9 siRNA transfection of human endometrial stromal cells (HESCs). *P< .01. Results are presented as mean + SEM values. P, phosphorylated. N=8.



572 **Table I.** Clinical characteristics of study participants with and without endometriosis.

	Endometriosis (n=32)	**Control (n=19)**	***P* value**
Age (years)	36.07 ± 1.07	34.42 ± 1.66	0.424
At least one pregnancy (n)	15 (47%)	8 (42%)	0.485
At least one child (n)	10 (32%)	8 (42%)	0.313
BMI (kg/m^2)	21.04 ± 0.27	21.16 ± 0.51	0.839
CA-125 (U/mL)	103.03 ± 22.95	14.08 ± 1.69	<0.001
Endometriosis stage			
III	14 (44%)	N/A	
IV	18 (56%)		
rAFS scores	57.37 ± 3.61	N/A	

573 Data are expressed as mean ± SEM or n (%)

574 rAFS, revised American Fertility Society.

575

576 **Table II.** Eutopic endometrial mRNA expressions AQP subtypes between endometriosis group and
577 controls. Expression was measured using a NanoString nCounter Analysis system and is expressed in
578 arbitrary fluorescence units.

579

	Endometriosis (n=32)	**Control (n=19)**	***P* value**
AQP1	14.75 ± 4.84	17.63 ± 8.70	0.306
AQP2	1.64 ± 0.25	1.05 ± 0.28	0.020
AQP3	281.36 ± 41.51	1163.54 ± 621.10	0.546
AQP4	1.87 ± 0.28	1.71 ± 0.49	0.167
AQP5	7.43 ± 1.79	43.61 ± 25.03	0.157
AQP6	1.76 ± 0.26	9.89 ± 6.11	0.784
AQP7	1.97 ± 0.28	1.60 ± 0.35	0.201
AQP8	1.64 ± 0.25	1.09 ± 0.05	0.029
AQP9	30.27 ± 5.35	141.84 ± 31.97	0.005

580 Data are expressed as mean ± SEM.

581 AQP, aquaporin