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Reduced migration of Ishikawa cells associated with downregulation of aquaporin-5

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Abstract. Aquaporin (AQP)-dependent cell migration has broad implications in angiogenesis, tumor metastasis, wound healing, glial scarring and other events requiring cell movement. There are 13 isoforms of AQP (0-12) that have been identified in mammals. It is unclear whether AQP5 plays a role in the development of endometrial cancer. We recently demonstrated that ovarian steroids may affect the expression of AQP5 in the female genital tract. In this study, we considered whether AQP5 may affect cell migration in Ishikawa cells, an adenocarcinoma cell line derived from the endometrium. The results showed that the downregulation of AQP5 results in reduced Ishikawa cell migration. The estrogen (E2) receptor in the promoter of AQP5 mediated the regulation of AQP5 expression in the normal endometrium and endometrial cancer. By contrast, the upregulation of AQP5 by E2 increased cell migration, invasion and adhesion through increased annexin-2, which is responsible for F-actin remodeling and rearrangement. E2 regulates Ishikawa cell migration by regulating the AQP5 expression.

Introduction

Aquaporins (AQPs) are membrane glycoproteins, embedded in cell membranes, that allow water to move in response to osmotic and hydrostatic differences (1). There are at least 13 AQPs (0-12) in mammals expressed in a number of epithelia, endothelia and other types of cells. At least 12 different tumor cell types have been reported to express AQPs *in vivo* in humans and rodents. Accumulating evidence suggests that ovarian steroids may affect the expression of several AQPs in the female genital tract (2-3). AQP-dependent cell migration has broad implications in angiogenesis, tumor metastasis, wound healing, glial scarring, embryonic development and other events requiring cell movement (4).

AQP5 is expressed in the mouse, rat, pig and human uterus (5-7). AQP5 has been shown to be involved in cell proliferation and carcinoma genesis in lung and colon tissues (8,9). Results of previous studies demonstrated that in ovarian cancer, AQP5 is important in a variety of pathological conditions, including ascites formation (10). In addition, AQP5 is expressed in normal human endometrium (11) and endometriosis ectopic endometrium (7). The expression of human endometrial AQP5 is menstrual cycle-dependent (7). High levels of AQP5 are found at the proliferative and mid-secretory phases and are positively correlated with serum levels of estradiol (E), suggesting that estrogen (E2) may regulate the expression of AQP5 in endometrial cells (7).

Migration is crucial in endometriosis and endometrial cancer development. However, it is unclear whether the downregulation of AQP5 is directly correlated with endometrial disease. In this study, we examined the role of AQP5 in cell migration in human Ishikawa cells, an endometrial adenocarcinoma cell line (12).

Endometrial carcinoma (EC) is the most common type of gynecological cancer in developed countries. EC is divided into two subtypes: type I, endometrioid-type EC, which accounts for 80-90% of EC, is of endometrial origin and is estrogen-dependent, and type II, non-endometrioid type EC, is mostly presented by papillary serous and clear-cell adenocarcinomas, accounts for 10-20% of EC and is usually estrogen-independent (13). Although it is a common malignancy, the molecular aspects of EC are poorly understood. For certain tumors, positive correlations have been established between histological tumor grade and the amount of AQP expression (14,15). AQP-dependent cell migration has broad implications in angiogenesis, tumor metastasis, wound healing, glial scarring and other events requiring rapid cell movement (16-18). It remains unclear whether AQP5 mediates these processes. Direct mechanistic evidence for AQP regulation of EC cell migration and invasion in the context of EC is limited. Therefore, we aimed to determine whether AQP5 is involved in EC development by migration.

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Materials and methods

Cells culture. Ishikawa cells (American Type Culture Collection, Manassas, VA, USA), an endometrial adenocarcinoma cell line, provided by the Laboratory of Gynecology and Obstetrics, Women's Hospital, School of Medicine, Zhejiang University, China, were cultured in phenol red RPMI-1640 medium (Thermo Scientific HyClone, South Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS; vol/vol), 100 U/ml penicillin and 100 U/ml streptomycin at 37˚C plus 5% CO_2.

RNA interference (RNAi) experiments. Cells were seeded at $1x10^{-6}$/well in 6-well plates. When the cells reached 80-90% confluence, cationic lipid complexes, prepared by incubating RNAi or negative RNAi with 5 nM Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) in 500 μl RPMI-1640 medium, were added into the wells according to the manufacturer's instructions. Cells were suspended and cultured in RPMI-1640. The study groups were experimental [group 1: specific small interfering RNA (siRNA)], control (group 2: non-specific siRNA) and blank (group 3: no siRNA). After 48 h, green fluorescence was quantified by a fluorescence-activated cell sorting (FACS) analysis to assess the transfection efficiency. The efficiency of transfection was confirmed by real-time PCR (RT-PCR) and western blotting. AQP5 siRNA duplexes were chemically synthesized by Thermo Scientific Dharmacon (Lafeyette, CO, USA). Four siRNAs targeting human AQP5 and four non-specific siRNAs each combined into one pool were designed and synthesized by Dharmacon: Duplex 1, 3'-GCUCCGGGCUUUCUUCUA CUU-5'; duplex 2, 3'-GAACCCAGCCCGCUCUUUUUU-5'; duplex 3, 3'-CGUAUGAGCCUGACGAGGAUU-5'; duplex 4, 3'-GCGCUCAACAACAACACAAUU-5'. Non-specific control duplexes: Duplex 1, 3'-AUGAACGUGAAUUGCU CAA-5'; duplex 2, 3'-UAAGGCUAUGAAGAGAUAC-5'; duplex 3, 3'-AUGUAUUGGCCUGUAUUAG-5'; duplex 4, 3'-UAGCGACUAAACACAUCAA-5'.

Quantitative RT-PCR. Two days after transfection, total RNA was extracted at the indicated times using an EZ spin column RNA extraction kit (Sangon, Shanghai, China). cDNA was reverse transcribed using an MMLV first-strand synthesis kit (BBInternational, Madison, WI, USA). PCR reactions were conducted in a 25 μl volume, containing 1l μl cDNA, 1 mM of each forward and reverse primer and 0.25xSyBr green mix. β-actin was used as the internal control to quantitate initial cell transcripts. Primer sequences included: β-actin; sense, 5'-CCTGTACGCCAACACAGTG-C-3'; antisense, 5'-ATAC TCCTGCTTGCTGATCC-3'; AQP5; sense, 5'-CTGTCCATT GGCCTGTCTGTC-3'; antisense, 5'-GGCTCATACGTGCC TTTGATG-3'. Quantitative real-time PCR analysis was conducted using an Applied Biosystems 7500 fast RT-PCR system (ABI; Carlsbad, CA, USA).

Western blot analysis. Cells were harvested and re-suspended in PBS 2 days after transfection. Total protein was extracted using a RIPA kit. GADPH (EarthOx, San Fransisco, CA, USA) was used as reference for the normalized expression level. The protein was electrophoresed on a polyacrylamide gel and transferred to a Hybond-C nitrocellulose membrane. Briefly, the separated samples were transferred to nitrocellulose membranes and exposed to mouse anti-AQP5 antibody (1:1000) for 2 h, followed by horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:10,000) for 1.5 h at room temperature. Proteins were visualized using enhanced chemiluminescence (ECL) detection reagents (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The antibodies were obtained from Abcam.

Transwell assay. Ishikawa cells ($1x10^5$/well) were loaded and cultured in 24-well plates. Cells were vaccinated in the transwell upper chamber in a small room at 37˚C with a 5% CO_2 incubator; the upper chamber contained 1% serum and the lower chamber contained 10% serum. Incubated cells were removed from the small room at 24, 48, 72 and 96 h. The lower cell medium was removed and crystal violet was stained before the cells were counted. Each count of five high-power field was averaged and the number of cell perforations formed in each group was calculated.

Statistical analysis. Data were normally distributed and were shown as the mean ± SEM. The independent-samples t-test was used to evaluate the statistical significance of the differences between two groups, and the one-way ANOVA test was used to evaluate the statistical significance of the differences between more than two groups. SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

AQP5 gene expression in Ishikawa cells. After 48 h, cultured cells were attached onto the thin film. RT-PCR amplification of the AQP5 gene in the Ishikawa cells was examined by 1% agar gel electrophoresis, which showed the expression of AQP5 mRNA in Ishikawa cells. The AQP5 molecular length was approximately 250 bp. We detected AQP5 protein levels in Ishikawa cells using western blotting. The results demonstrated a higher amount of AQP5 protein expression in Ishikawa cells.

Effect of RNAi on AQP5 gene expression levels in Ishikawa cells. RT-PCR demonstrated the level of interference effects in AQP5 mRNA. Compared with the control and blank groups, mRNA expression levels in the experimental group were significantly decreased (P=0.03) (Table I). In the Ishikawa cells, after AQP-5 RNAi, the protein expression level was significantly decreased (group 1 vs. groups 2 and 3, P=0.007), while AQP5 protein expression in the control and blank group was not significantly different (group 2 vs. 3, P=0.507) (Table II).

Determination of Ishikawa cell migration after RNAi. Following a comparison of the experimental and negative group, we determined that an optimal transfection efficiency of 80% could be achieved at 48 h; the best concentration was 100 nM. When cells were transfected with 100 ng siRNA in the transwell chamber after 24, 48, 72 and 96 h, the number of perforated cells increased with time. Following a comparison

Table I. AQP5 mRNA expression in Ishikawa cells after RNA interference.

Group	Sample (mean ± SD)	β-actin (mean ± SD)	P-value
Experimental (group 1)	21.638±0.078	21.778±0.028	0.03[a]
Control (group 2)	27.324±0.037	21.155±0.069	0.48[b]
Blank (group 3)	29.136±0.046	20.672±0.083	

[a]Comparison of group 1 vs. groups 2 and 3. [b]Comparison of group 2 vs. group 3. AQP, aquaporin; SD, standard deviation.

Table II. AQP5 protein levels in Ishikawa cells after RNA interference examined by western blotting.

Group	Expression amount (mean ± SD)	Inhibition rate (AQP5/GADPH) (%)	P-value
Experimental (group 1)	0.222±0.108	75.84	0.007[a]
Control (group 2)	0.844±0.166	8.16	0.507
Blank (group 3)	0.919±0.129		

[a]Comparison of group 1 vs. groups 2 and 3. [b]Comparison of group 2 vs. 3. AQP, aquaporin; SD, standard deviation.

Table III. Cell perforation ability after RNA interference.

	Number of cell perforations							
Group	24 h	48 h	72 h	96 h	P-value[a]	P-value[b]	P-value[c]	P-value[d]
1	35.7±4.8	54.6±1.1	70.4±2.3	76.7±4.4	0.37	0.04	0.03	0.01
2	34.33±4.3	96.6±3.1	140.5±2.3	160. 9±5.2	0.74	0.83	0.66	0.58
3	32.3±4.5	98.5±2.3	150.2±7.1	179.6±6.3				

Group 1 vs. groups 2 and 3 at [a]24 h, [b]48 h, [c]72 h and [d]96 h.

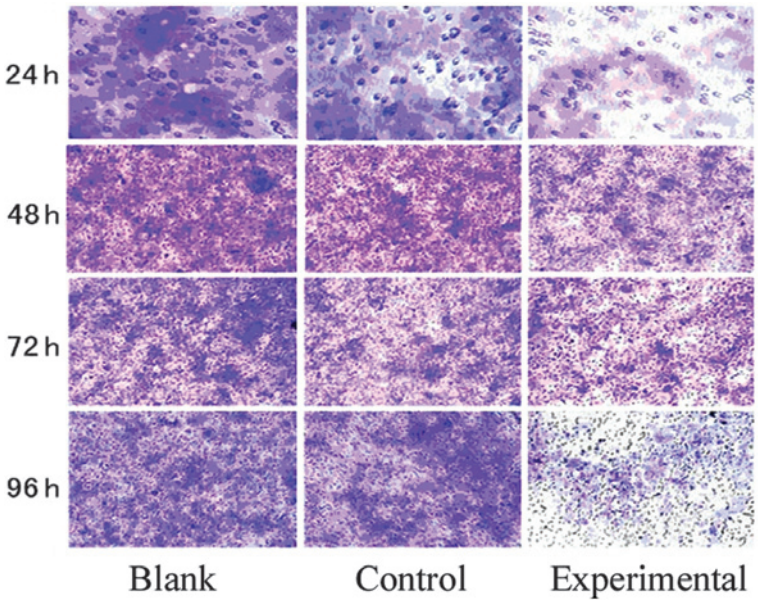

Figure 1. Increased migration of AQP5-expressing Ishikawa cells. Control and AQP5-knockdown tumor cells were applied to transwell filters, were stained in crystal violet in lower room cells on different days and counted in low/high-power fields. At 24 h; original magnification, x400; at 48-96 h; original magnification, x200.Calculated as the average number of cells. AQP, aquaporin.

of the experimental group with control and blank groups at 48, 72 and 96 h, the number of perforated cells in the experimental group decreased (Table III, Fig. 1).

Discussion

Accumulating evidence has demonstrated that AQPs are important for cell migration, invasion and spread in malignancy (19). Expression of AQP1 has been associated with colon cancer, mammary carcinoma, brain tumor, hemangioblastoma and multiple myeloma (20). Other studies have demonstrated an increased expression of AQP3 in skin carcinoma, increased AQP4 expression in glioblastoma and increased AQP5 expression in pancreatic and colon cancer (21-24).

Cell migration is affected by numerous factors; it is time-dependent (25), significantly enhanced by collagen IV in embryonic stem cells (26), and it has been observed that even a lesion can induce an increase in the migration of human neural progenitor cells (27). The findings from this study demonstrate that AQP5 knockdown reduced the migration of EC cells, suggesting that AQP5 is involved in the development of EC. The mechanisms underlying AQP-facilitated migration, invasion and proliferation remain unclear. Certain data suggest that changes in the cell volume and shape mediated by AQP may contribute to migration and invasion (28). The present study demonstrated that inhibition of the endogenous AQP5 expression attenuated migration of EC cells as examined by a transwell assay, providing evidence that supports our hypothesis (3).

In a previous study, we found that AQP5 was highly expressed in endometriosis patients in the eutopic and ectopic endometria, and expression levels changed with the E2 level. The present study provides evidence that AQP5 is expressed in endometroisis and endometrial cancer cells, both of which are E2-dependent diseases (29).

Several studies have revealed an estrogen response element (ERE) in the promoter of AQP2, which mediates the regulation of AQP2 expression by E in the normal endometrium and EC. By contrast, the upregulation of AQP2 by E2 increased cell migration, invasion and adhesion through increased annexin-2, which is responsible for F-actin remodeling and rearrangement (3). We consider that these results may provide insights into the potential mechanism in which AQP5 regulates migration by E2.

AQP-facilitated cell migration to different cell types suggests that AQP expression in tumor cells may increase local tumor invasiveness and the ability of tumor cells to metastasize by crossing plasma membrane barriers. To test this possibility, tumor cell migration, invasiveness and metastatic potential were evaluated by comparing Ishikawa cell lines with and without AQP5 expression. *In vitro* analysis of cell migration using transwell migration assays demonstrated a greatly increased migration of AQP5-expressing tumor cells, as predicted. From counting numerous cells on multiple filters, the ratio of AQP5-expressing cells compared with control cells was significantly increased after 48 h, indicating increased migration of AQP5-expressing cells. The involvement of AQP5 in tumor cell migration has potentially important clinical implications. It provides a functional explanation for AQP expression in tumor cells and for the correlations between tumor shift and AQP expression. Additionally, it provides a rational basis to evaluate AQP inhibitors, when available, for tumor therapy, both for reduction of tumor development and tumor spread (30).

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