

Modulatory effect of aquaporin 5 on estrogen-induced epithelial-mesenchymal transition in prostate epithelial cells

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Abstract

Background: Estrogen is involved in the pathophysiological process of benign prostatic hyperplasia (BPH), in which epithelial-mesenchymal transition (EMT) plays an important role. Upregulation of aquaporin (AQP) 5, which is directly activated by estrogen, has been reported to promote EMT in multiple cells. This study aimed to examine the effects of AQP5 on estrogen-induced EMT in the prostate.

Methods: Normal prostate (NP) tissue samples without any histopathological changes and BPH tissue samples with pathologically confirmed hyperplasia were obtained. An EMT cell model was subsequently established by adding estradiol (E2) to RWPE-1 cells, after which AQP5 knockdown was performed. Tissue morphological and immunohistochemical features were examined using hematoxylin-eosin and immunohistochemical staining. Western blot analysis was performed to determine the expression of AQPs, estrogen receptors, and EMT-related proteins. Cell proliferation was assessed and supernatants were collected for enzyme-linked immunosorbent assay to determine transforming growth factor- β 1 (TGF- β 1) concentrations. Immunofluorescence staining was performed to assess protein expressions in RWPE-1 cells.

Results: BPH tissues exhibited greater EMT (TGF- β 1: 1.362 ± 0.196 vs. 0.107 ± 0.067 , $P = 0.003$; vimentin: 1.581 ± 0.508 vs. 0.221 ± 0.047 , $P < 0.001$; E-cadherin: 0.197 ± 0.188 vs. 1.344 ± 0.088 , $P < 0.001$), higher AQP5 (1.268 ± 0.136 vs. 0.227 ± 0.055 , $P < 0.001$) and estrogen receptor (ER) α (1.250 ± 0.117 vs. 0.329 ± 0.134 , $P < 0.001$) expression but lower ER β (0.271 ± 0.184 vs. 1.564 ± 0.130 , $P < 0.001$) expression than NP tissues. E2-stimulated cells had higher AQP5 expression (1.298 ± 0.058 vs. 1.085 ± 0.104 , $P = 0.049$), increased cell proliferation (1.510 ± 0.089 vs. 1.000 ± 0.038 , $P < 0.001$), and EMT (TGF- β 1 concentration: 0.352 ± 0.021 ng/mL vs. 0.125 ± 0.014 ng/mL, $P < 0.001$; vimentin: 1.641 ± 0.120 vs. 0.188 ± 0.020 , $P = 0.002$; E-cadherin: 0.075 ± 0.030 vs. 0.843 ± 0.046 , $P < 0.001$) than controls. E2-stimulated cells with AQP5 knockdown exhibited decreased EMT (TGF- β 1 concentration: 0.223 ± 0.041 ng/mL vs. 0.352 ± 0.021 ng/mL, $P = 0.016$; vimentin: 0.675 ± 0.056 vs. 1.641 ± 0.120 , $P = 0.001$; E-cadherin: 0.159 ± 0.037 vs. 0.075 ± 0.030 , $P = 0.040$) than E2-stimulated cells with non-related small interfering RNA (siRNA).

Conclusion: Our findings suggest that estrogen induces BPH possibly by promoting AQP5 expression. Hence, AQP5 might be a novel target for modulating EMT in prostate epithelial cells.

Keywords: Aquaporin; Benign prostatic hyperplasia; Epithelial-mesenchymal transition; Estrogen; Prostate epithelial cells

Introduction

Benign prostatic hyperplasia (BPH) is a common benign proliferative disorder among older males. Although BPH is considered an androgen-dependent disease, one study conducted in 1990 discovered that estrogen administration could result in the development of canine BPH.^[1] Subsequently, a model of BPH had been successfully established with estrogen administration combined with androgen.^[2] Current evidence has indicated that changes in the estrogen to androgen ratio are closely related to the development of BPH.^[3] Estrogen and its receptors have

been considered to be involved in prostate tissue inflammation, stromal cell proliferation, and epithelial-mesenchymal transition (EMT).^[4,5] Although accumulating evidence has indicated that estrogen could trigger EMT,^[6,7] the mechanism involved in the pathophysiological process of BPH remains unclear.

EMT is an important conserved biological process in embryonic development, chronic inflammation, tissue remodeling, cancer metastasis, and various fibrotic diseases. Through this process, polarized epithelial cells can transform their phenotype into a migratory mesenchymal phenotype with various features, including reduced cell

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adhesion molecules (such as epithelial cadherin [E-cadherin], a cell-cell adhesion molecule) and transformation of the cytokeratin cytoskeleton into a vimentin (a myofibroblast cell marker)-based cytoskeleton.^[8] EMT has been noted in the development of benign and malignant prostate growths.^[6,7] According to previous studies, signaling from the surrounding reactive prostate stroma, such as transforming growth factor- β (TGF- β) and fibroblast growth factor, promotes further structural changes among epithelial cells leading to EMT.^[6]

Aquaporin (AQP) 5, an important protein that promotes EMT during embryonic development, has been reported to play an important role in estrogen-related diseases, including endometriosis, polycystic ovarian syndrome, and breast/ovarian/endometrium carcinoma.^[9-12] Endometrial AQP5 expression is dependent on the menstrual cycle. Accordingly, high AQP5 levels have been noted during the proliferative and mid-secretory phases, suggesting that estrogen might regulate AQP5 expression in endometrial glandular cells.^[9] Estrogen has also been found to induce a significant increase in AQP5 levels.^[13] This is associated with the activation of the *AQP5* gene soon after estrogen administration considering that the promoter region of *AQP5* contains a functional estrogen response element directly activated by estrogen.^[14] Unfortunately, no study has yet explored the role of AQP5 in the pathophysiological process of BPH.

Considering the important role of estrogen in the occurrence of BPH, the activation of *AQP5* after estrogen administration, and the promotional effect of AQP5 on EMT, we hypothesized that additional estrogen could increase AQP5 expression, thereby triggering EMT and leading to the occurrence of BPH. The present study therefore aimed to evaluate the potential effects of estrogen and AQP5 on BPH, focusing particular on the process of EMT.

Methods

Ethical approval

Human prostate tissues were obtained from the specimen repository of the Institute of Urology, West China Hospital of Sichuan University. Approval for the use of human samples was obtained from the Ethics Committee of West China Hospital of Sichuan University.

Patients

Three normal prostate (NP) tissue samples without any histopathological changes and five BPH tissue samples with pathologically confirmed hyperplasia that passed the specimen quality test were used. NP tissue samples were obtained from patients with bladder urothelial carcinoma (BUC), while BPH tissue samples were obtained from patients with BPH.

Tissue hyperplasia and sites/levels of protein expressions in prostate tissues

BPH and NP tissues were identified using hematoxylin-eosin (HE) staining, while the sites and levels of protein

expressions were determined using immunohistochemical (IHC) staining. Procedures were performed as previously described.^[15] Prostate tissue sections were stained with primary antibodies and corresponding secondary antibodies. The brown color under the microscope (Olympus Co., Ltd., Shinjuku, Tokyo, Japan) was considered to indicate relevant protein expression (see Supplementary File 1 for details of reagents, <http://links.lww.com/CM9/A348>).

RWPE-1 cell culture, RNA interference, and cell grouping

RWPE-1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured according to their recommended methods.^[15]

Three different AQP5 small interfering RNAs (siRNAs) were purchased from GenePharma (GenePharma Co. Ltd., Shanghai, China). Healthy RWPE-1 cells were added to 6-well plates at a density of 3×10^5 cells/well and cultured for 12 h until approximately 60% confluency. Thereafter, Lipofectamine 3000 and siRNAs were used for transfection (48 h) to obtain the following two types of cells: siAQP5 cells (*AQP5*-knockdown cells) and NR cells (control cells created using non-related siRNAs provided by the manufacturer). The cells were collected for quantitative real-time polymerase chain reaction (qRT-PCR) to select the most effective siRNA. Cell RNA was extracted using the TaKaRa MiniBEST universal RNA extraction kit (9767, TaKaRa Bio Inc., Kusatsu, Shiga, Japan), while cDNA was synthesized using the Primescript RT reagent kit with gDNA Eraser (Perfect Real Time) (RR047Q, TaKaRa Bio Inc.). After reverse transcription and dilution, primers were applied to the PCR reactions. The sequence of the selected siRNA was 5'-GCGCTCAA-CAACAACACAA-3'.

RWPE-1 cells with or without RNA interference (RNAi) were added to 6-well plates (3×10^5 cells/well), 24-well plates (2.5×10^4 cells/well), or 96-well plates (4×10^3 cells/well) and cultured with or without 10 nmol/L estradiol (E2) stimulation. Thereafter, cells were divided into the following six groups: NC (negative control: adding solvent without E2 to cells without RNAi), E2 (adding E2 to cells without RNAi), NR (adding solvent without E2 to NR cells), siAQP5 (adding solvent without E2 to siAQP5 cells), E2 + NR (adding E2 to NR cells), and E2 + siAQP5 (adding E2 to siAQP5 cells).

Cell proliferation assay

Cell proliferation was assessed using Cell Counting Kit-8 (CCK8) (CK04, Dojindo Co., Ltd., Kumamoto, Japan). Accordingly, cells were added to 96-well plates (4×10^3 cells/well) and cultured as described above. After adding 10 nmol/L of E2 for 24, 48, and 72 h each, the medium was changed to a reagent containing 10% CCK8, and the plates were incubated for 4 h. Optical density (OD) values for calculating cell proliferation were measured at 450 nm using a microplate reader (EnVision, PerkinElmer Co., Ltd., Shanghai, China).

Transforming growth factor- β 1 (TGF- β 1) concentration assay

After 10 nmol/L E2 stimulation for 24, 48, and 72 h each, supernatants of cells plated onto 24-well plates (2.5×10^4 cells/well) were collected for enzyme-linked immunosorbent assay (ELISA) to determine TGF- β 1 concentrations. Procedures were strictly performed according to the instructions of the TGF- β 1 ELISA kit (ab108972, Abcam Co., Ltd., Cambridge, MA, USA).

Western blot assay

Prostate tissues and RWPE-1 cells (plated on 6-well plates at a density of 3×10^5 cells/well) with or without E2 stimulation were collected for Western blot assay.^[15] Radioimmunoprecipitation assay lysis solution was used to prepare cell/tissue lysates, and protein concentrations were measured using the bicinchoninic acid (BCA) Protein Assay Kit (P0009, Beyotime Biotechnology Co., Ltd., Haimen, Jiangsu, China). After membrane transfer and blocking, membranes were incubated with primary antibodies followed by the corresponding secondary antibodies and assessed using chemiluminescence.

Immunofluorescence staining

Immunofluorescence (IF) staining was performed to assess AQPs, E-cadherin, and vimentin expressions in RWPE-1 cells. RWPE-1 cells were added to glass slides in 6-well plates at a density of 3×10^5 cells/well with or without E2 stimulation. After fixation, blockage, and permeabilization (when needed), prostate cell slides were stained with primary antibodies. The slides were then stained with fluorescent secondary antibodies and 4', 6-diamidino-2-phenylindole. Subsequently, sections were mounted with antifade mounting medium.

Statistical analysis

Data are presented as mean \pm standard deviation. All data were analyzed using Student's *t*-test. Average optical density (AOD) of positive staining was quantified using ImageJ version 1.52a (Wayne Rasband, National Institutes of Health, USA). All analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 7.0 (GraphPad Software Inc., La Jolla, CA, USA), with a *P* value of < 0.05 indicating statistical significance.

Results

BPH tissues exhibited greater EMT than NP tissues with more AQP5 and ER α expressions and lower ER β expression

Patients with BUC were significantly younger than patients with BPH (34.7 ± 4.7 years *vs.* 59.2 ± 2.8 years, $P = 0.005$). Both patients had comparable body mass index and no comorbidities (*eg.* diabetes mellitus, other cancers, urethrostenosis, urinary infection, bacterial prostatitis, and autoimmune disease) (Supplementary File 1, <http://links.lww.com/CM9/A348>). On hematoxylin-eosin staining (Figure 1A and Supplementary Figure 1,

<http://links.lww.com/CM9/A348>), the prostate glandular cavities of NP tissues were clear and had a neat shape. Epithelia were composed of monolayer columnar/cuboid epithelial cells. The nuclei located near the basal part were round and deeply stained. On the other hand, BPH tissues showed hyperplasia of both glandular epithelia and stroma. Epithelia were papillary and protruded into the cavity, with irregular inflation. Epithelial cells with lightly stained nuclei were tall columnar, clustered, and closely arranged.

On IHC staining (Figure 1A and Supplementary Figure 1, <http://links.lww.com/CM9/A348>), AQPs were expressed in the prostate epithelia and stroma, except for AQP8, which was expressed only in epithelia. Epithelial cells expressed AQP1, AQP4, and AQP8 in the plasma membrane, while AQP5 and AQP6 were mainly expressed in the nuclei of epithelial cells. Although heterogeneous AQP5 and AQP6 expressions were observed even in the nuclei of adjacent epithelial cells in the same acinus cavity, BPH tissues showed higher AQP5 expression in both the epithelia and stroma than NP tissues (0.420 ± 0.032 *vs.* 0.322 ± 0.001 , $P = 0.002$). Moreover, BPH tissues expressed lower AQP1 (0.429 ± 0.060 *vs.* 0.631 ± 0.097 , $P = 0.047$) and AQP4 (0.345 ± 0.087 *vs.* 0.650 ± 0.124 , $P = 0.047$) than NP tissues, while no significant difference in AQP6 (0.338 ± 0.054 *vs.* 0.333 ± 0.027 , $P = 0.858$) and AQP8 (0.203 ± 0.026 *vs.* 0.194 ± 0.008 , $P = 0.537$) expressions were observed between BPH and NP tissues. E-cadherin and vimentin were expressed in prostate epithelia and stroma, especially in the plasma membrane and cytoplasm of epithelial cells. Accordingly, BPH tissues exhibited lower E-cadherin (0.543 ± 0.054 *vs.* 0.683 ± 0.059 , $P = 0.028$) but higher vimentin (0.561 ± 0.036 *vs.* 0.383 ± 0.029 , $P < 0.001$) expression in the epithelia than NP tissues.

On Western blot analysis (Figure 1B and Supplementary Figure 2, <http://links.lww.com/CM9/A348>), BPH tissues exhibited higher AQP5 (1.268 ± 0.136 *vs.* 0.227 ± 0.055 , $P < 0.001$), TGF- β 1 (1.362 ± 0.196 *vs.* 0.107 ± 0.067 , $P = 0.003$), vimentin (1.581 ± 0.508 *vs.* 0.221 ± 0.047 , $P < 0.001$), and ER α (1.250 ± 0.117 *vs.* 0.329 ± 0.134 , $P < 0.001$) expression but lower AQP1 (0.321 ± 0.110 *vs.* 1.188 ± 0.084 , $P < 0.001$), AQP4 (0.226 ± 0.276 *vs.* 1.208 ± 0.028 , $P = 0.001$), AQP6 (0.425 ± 0.051 *vs.* 0.833 ± 0.025 , $P < 0.001$), AQP8 (0.347 ± 0.280 *vs.* 1.035 ± 0.032 , $P = 0.005$), E-cadherin (0.197 ± 0.188 *vs.* 1.344 ± 0.088 , $P < 0.001$), and ER β (0.271 ± 0.184 *vs.* 1.564 ± 0.130 , $P < 0.001$) expression than NP tissues.

E2 stimulation promoted AQP5 expression, cell proliferation, and EMT of RWPE-1 cells

The CCK8 test [Figure 2A] showed that the E2 group had a significantly greater relative OD value reflecting cell proliferation ratio after a 48-h (1.166 ± 0.686 *vs.* 1.000 ± 0.030 , $P < 0.001$) and 72-h (1.296 ± 0.669 *vs.* 1.000 ± 0.055 , $P < 0.001$) stimulation than the NC group. Moreover, in the E2 group, a 72-h stimulation promoted a significantly greater relative OD value than a 48-h (1.296 ± 0.669 *vs.* 1.166 ± 0.686 , $P = 0.003$) and 24-h (1.296 ± 0.669 *vs.* 1.074 ± 0.644 , $P = 0.002$) stimulation.

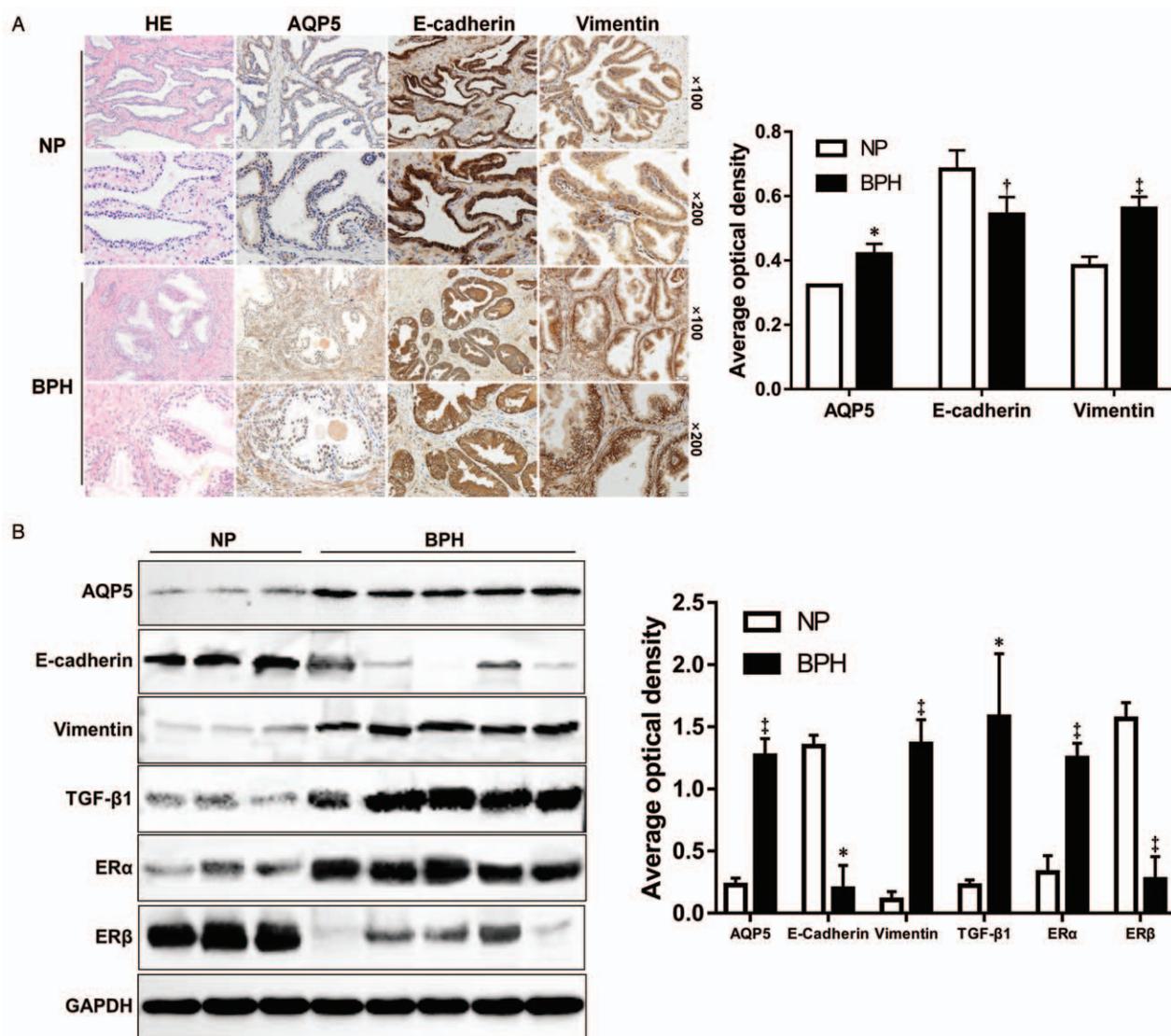


Figure 1: HE/IHC staining and Western blot analysis of NP and BPH tissues. (A) HE/IHC staining. AQP5, E-cadherin, and vimentin were expressed in the prostate epithelia (mainly) and stroma. AQP5 was especially expressed in the nuclei of epithelial cells, while heterogeneous AQP5 expression was observed even in the nuclei of adjacent epithelial cells within the same acinus cavity. E-cadherin and vimentin were mainly expressed in the plasma membrane and cytoplasm of epithelial cells. BPH tissues exhibited higher AQP5 expression but lower E-cadherin expression compared to NP tissues. Original magnification, $\times 100$ or $\times 200$ as indicated. (B) Western blot analysis. BPH tissues exhibited higher AQP5, TGF- $\beta 1$, vimentin, and ER α expression but lower E-cadherin and ER β expression compared to NP tissues. * $P < 0.01$, † $P < 0.05$, ‡ $P < 0.001$ vs. NP tissues. AQP5: Aquaporin 5; BPH: Benign prostatic hyperplasia; E-cadherin: Epithelial cadherin; ER: estrogen receptor; HE: Hematoxylin-eosin; IHC: Immunohistochemistry; NP: Normal prostate; TGF- $\beta 1$: Transforming growth factor- $\beta 1$.

ELISA findings [Figure 2B] showed that the TGF- $\beta 1$ concentration in the supernatants increased along with E2 stimulation duration. Meanwhile, we found that the E2 group (72-h stimulation) had a significantly greater TGF- $\beta 1$ concentration than the NC group (0.296 ± 0.074 ng/mL vs. 0.124 ± 0.024 ng/mL, $P = 0.045$).

Western blot analysis (Figure 2C and Supplementary Figure 4, <http://links.lww.com/CM9/A348>) indicated that the E2 group (24-h E2 stimulation) exhibited higher AQP5 (1.413 ± 0.095 vs. 1.190 ± 0.148 , $P = 0.023$) and AQP1 (0.929 ± 0.063 vs. 0.759 ± 0.022 , $P = 0.032$) expression than the NC group. With 72-h E2 stimulation, the E2 group exhibited higher AQP1 (1.158 ± 0.054 vs. 0.990 ± 0.070 , $P = 0.033$), AQP4 (1.132 ± 0.062 vs. 0.968 ± 0.034 , $P = 0.026$), and vimentin (1.281 ± 0.061 vs. 0.465 ± 0.008 , $P = 0.002$) expression but lower

E-cadherin expression (0.405 ± 0.234 vs. 1.155 ± 0.071 , $P = 0.023$) than the NC group. However, no significant difference in AQP6 (1.090 ± 0.113 vs. 1.061 ± 0.084 , $P = 0.743$) and AQP8 (1.142 ± 0.142 vs. 1.066 ± 0.055 , $P = 0.458$) expressions were found between both groups. Similar to Western blot analysis findings, IF staining (Figure 3 and Supplementary Figure 3, <http://links.lww.com/CM9/A348>) revealed that AQP1, AQP4, and AQP8 were expressed in the plasma membrane of RWPE-1 cells, AQP5 and AQP6 were expressed in the nuclei of RWPE-1 cells, and E-cadherin and vimentin were both expressed in the plasma membrane of RWPE-1 cells. Under the same exposure time, the E2 group (72-h stimulation) exhibited lower E-cadherin fluorescence intensity (0.058 ± 0.003 vs. 0.087 ± 0.010 , $P = 0.027$) and higher AQP1 (0.111 ± 0.003 vs. 0.051 ± 0.003 , $P < 0.001$), AQP4 (0.069 ± 0.008 vs. 0.050 vs. 0.004 , $P = 0.035$), AQP5 (0.120 ± 0.013 vs.

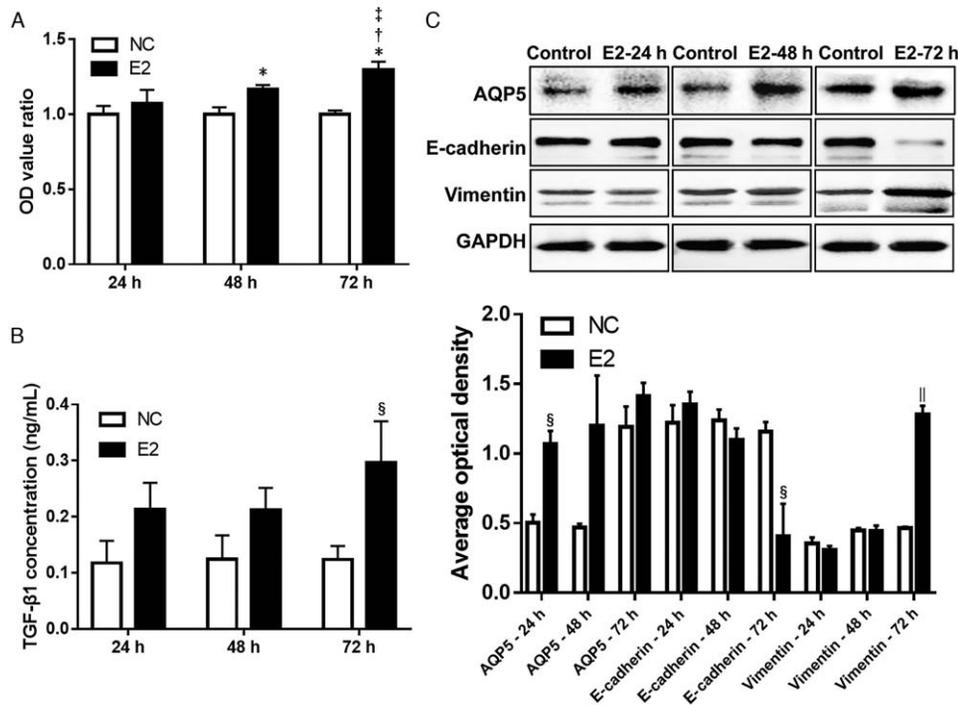


Figure 2: CCK8 test, ELISA, and Western blot analysis of RWPE-1 cells after E2 stimulation. (A) CCK8 test. The E2 group had a significantly greater OD value ratio (OD value of the NC group was set to 1) after 48 and 72 h of stimulation than the NC group. In the E2 group, OD value ratio was significantly greater with a 72-h stimulation than with 48- and 24-h stimulations. (B) ELISA. TGF-β1 concentration in the supernatants increased along with E2 stimulation, while the E2 group had a significantly greater TGF-β1 concentration after 72 h of stimulation than the NC group. (C) Western blot analysis. In the E2 group, AQP5 and vimentin expressions increase along with E2 stimulation duration, whereas E-cadherin expression gradually decreased. The E2 group exhibited significantly lower E-cadherin expression and greater vimentin expression than the NC group after 72 h of stimulation. [†]*P* < 0.01 vs. E2-24 h group; [§]*P* < 0.01 vs. E2-48 h group. AQP5: Aquaporin 5; CCK8: Cell counting kit-8; E2: Estradiol; E-cadherin: Epithelial cadherin; ELISA: Enzyme-linked immunosorbent assay; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; NC: Negative control; OD: Optical density; TGF-β1: Transforming growth factor-β1.

0.078 ± 0.001, *P* = 0.028), AQP6 (0.087 ± 0.002 vs. 0.071 ± 0.001, *P* = 0.001), AQP8 (0.037 ± 0.004 vs. 0.056 ± 0.006, *P* = 0.017), and vimentin (0.128 ± 0.006 vs. 0.064 ± 0.003, *P* < 0.001) intensities than the NC group.

AQP5 knockdown inhibited EMT of RWPE-1 cells after E2 stimulation

After validating three different types of AQP5 siRNAs, one of the selected siRNA types (siRNA2) achieved acceptable silencing efficiency according to qRT-PCR [Figure 4A], IF [Figure 3], and Western blot analysis [Figure 4B]. ELISA findings showed that the siAQP5 group had a lower OD value ratio (0.738 ± 0.035 vs. 1.000 ± 0.038, *P* < 0.001) than the NR group but comparable TGF-β1 concentrations (0.111 ± 0.007 ng/mL vs. 0.125 ± 0.014 ng/mL, *P* = 0.206). Western blot analysis revealed that the siAQP5 group had numerically lower E-cadherin (0.711 ± 0.079 vs. 0.843 ± 0.046, *P* = 0.083) and vimentin (0.128 ± 0.033 vs. 0.188 ± 0.020, *P* = 0.070) expressions than the NR group. The CCK8 test [Figure 4C] showed that the E2 + NR group (72-h E2 stimulation) exhibited a significantly greater OD value than the E2 + siAQP5 (1.510 ± 0.089 vs. 1.236 ± 0.096, *P* = 0.002) or NR (1.510 ± 0.089 vs. 1.000 ± 0.038, *P* < 0.001) groups. Meanwhile, the E2 + siAQP5 group exhibited a significantly greater OD value than the NR group (1.236 ± 0.096 vs. 1.000 ± 0.038, *P* = 0.003). AQP5 knockdown slowed the rapid proliferation of RWPE-1 cells under E2 stimulation. ELISA findings [Figure 4D] showed that the E2 + NR group

had a significantly greater supernatant TGF-β1 concentration than the E2 + siAQP5 (0.352 ± 0.021 ng/mL vs. 0.223 ± 0.041 ng/mL, *P* = 0.016) or NR (0.352 ± 0.021 ng/mL vs. 0.125 ± 0.014 ng/mL, *P* < 0.001) groups. Meanwhile, the E2 + siAQP5 group exhibited significantly greater supernatant TGF-β1 concentration than the NR group (0.223 ± 0.041 ng/mL vs. 0.125 ± 0.014 ng/mL, *P* = 0.042).

IF [Figure 3] and Western blot analysis [Figure 4B] produced consistent results. Fluorescence intensity and protein expression of AQP5 were lower in the siAQP5 group (0.060 ± 0.001 vs. 0.078 ± 0.003, *P* = 0.006 and 0.084 ± 0.006 vs. 1.085 ± 0.104, *P* = 0.003, respectively) or E2 + siAQP5 group (0.065 ± 0.005 vs. 0.128 ± 0.009, *P* = 0.001 and 0.150 ± 0.020 vs. 1.298 ± 0.058, *P* < 0.001, respectively) than in the NR group or E2 + NR group, respectively. Fluorescence intensity and protein expression of E-cadherin was lower in the E2 + NR (0.067 ± 0.003 vs. 0.115 ± 0.005, *P* < 0.001 and 0.075 ± 0.030 vs. 0.843 ± 0.046, *P* < 0.001, respectively) and E2 + siAQP5 (0.077 ± 0.006 vs. 0.115 ± 0.005, *P* < 0.001 and 0.159 ± 0.037 vs. 0.843 ± 0.046, *P* < 0.001, respectively) groups than in the NR group, while the E2 + siAQP5 group exhibited higher fluorescence intensity and protein expression of E-cadherin than E2 + NR group (0.077 ± 0.006 vs. 0.067 ± 0.003, *P* = 0.064 and 0.159 ± 0.037 vs. 0.075 ± 0.030, *P* = 0.040, respectively). Fluorescence intensity and protein expression of vimentin was greater in the E2 + NR group (0.122 ± 0.016

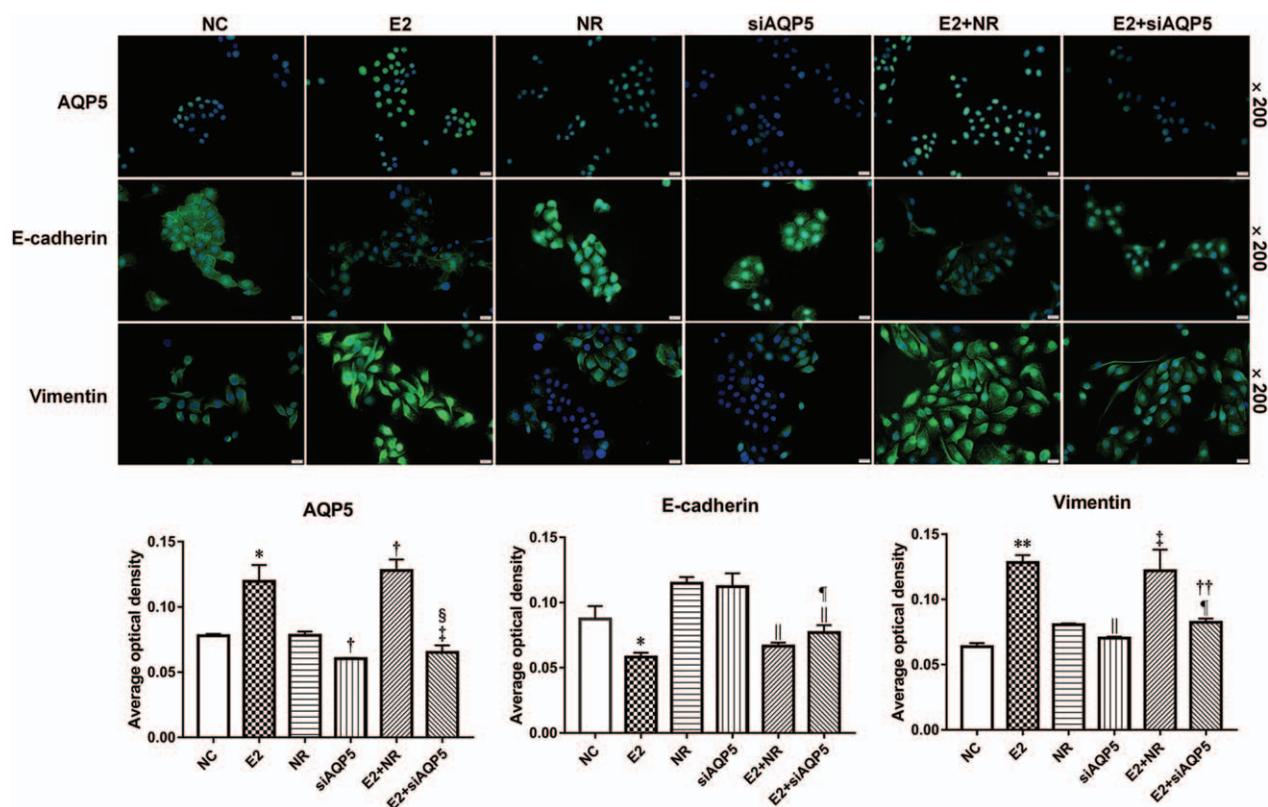


Figure 3: IF staining and quantitative analysis of RWPE-1 cells. E2, E2 + siAQP5 and E2 + NR group cells were all cultured with 10 nmol/L E2 stimulation for 72 h; NC, NR and siAQP5 group cells were all cultured without E2 stimulation for 72 h. AQP5 was expressed in the nuclei of RWPE-1 cells, whereas E-cadherin and vimentin were both expressed in the plasma membrane of RWPE-1 cells. The E2 group exhibited lower E-cadherin fluorescence intensity and higher AQP5 and vimentin intensities than the NC group. The siAQP5 group had a lower AQP5 fluorescence intensity than the NR group. Similarly, the E2 + siAQP5 group had a lower AQP5 fluorescence intensity than the E2 + NR group. The E2+NR and E2+siAQP5 groups had lower E-cadherin fluorescence intensities than the NR group. Although not statistically significant, the E2 + siAQP5 group had mildly higher E-cadherin fluorescence intensity than the E2 + NR group. Vimentin fluorescence intensity was higher in the E2 + NR group and lower in the siAQP5 group than in the NR group, while the E2 + siAQP5 group had lower vimentin fluorescence intensity than the E2 + NR group. * $P < 0.05$, ** $P < 0.001$ vs. NC group; † $P < 0.01$, ‡ $P < 0.05$, †† $P < 0.001$ vs. NR group; ††† $P < 0.001$, †††† $P < 0.05$ vs. E2+NR group; †† $P < 0.05$ vs. siAQP5 group. AQP5: Aquaporin 5; E2: Estradiol; E-cadherin: Epithelial cadherin; EMT: Epithelial-mesenchymal transition; IF: Immunofluorescent; NC: Negative control; NR: RNA interfere with non-related siRNA; siAQP5: AQP5 knockdown.

vs. 0.081 ± 0.001 , $P = 0.047$ and 1.641 ± 0.120 vs. 0.188 ± 0.020 , $P = 0.002$, respectively) and lower in the siAQP5 group (0.070 ± 0.001 vs. 0.081 ± 0.001 , $P < 0.001$ and 0.128 ± 0.033 vs. 0.188 ± 0.020 , $P = 0.070$, respectively) than in the NR group, while the E2 + siAQP5 group exhibited lower fluorescence intensity and protein expression of vimentin than the E2 + NR group (0.082 ± 0.003 vs. 0.122 ± 0.016 , $P = 0.048$ and 0.675 ± 0.056 vs. 1.641 ± 0.120 , $P = 0.001$, respectively).

Discussion

To date, 13 types of AQPs (AQP0–12), a family of small integral membrane proteins, have been found in mammals.^[10] Some studies have revealed that AQPs could be involved in the pathophysiological processes of tissue inflammation, oxidative stress, cell-to-cell adhesion, cell proliferation/migration/differentiation, EMT, and cellular water homeostasis.^[16,17] With regard to the human AQP family, the major physiological functions of AQP5 in the reproductive system include water and small uncharged solute molecule transport and early embryo development/implantation in females.^[10] Previous studies have documented the expression of AQP1, 3, 5, 7, and 9 in the human prostate, as well as heterogeneous AQP5 expression in the epithelia of both benign and malignant prostate

tissues on IHC staining.^[18] The presents study found that the human prostate expressed AQP1, 4, 5, 6, and 8. Accordingly, AQP5 and AQP6 were expressed in the prostate epithelia and stroma, especially in the nuclei of epithelial cells. Moreover, heterogeneous expressions of AQP5 and AQP6 had been observed in the nuclei of adjacent epithelial cells within the same acinus cavity. Generally, BPH tissues exhibited higher AQP5 expression, along with lower ERβ and higher ERα expression, compared to NP tissues.

Thus far, available evidence has indicated that ERα is expressed mainly in the stroma of NP and BPH tissues and mediates the proliferative effects of estrogens on prostate cells, whereas ERβ is expressed mainly by the prostate epithelium and mediates the apoptotic effects on prostate cells.^[4] However, no consensus exists regarding the difference in ERα and ERβ expression between BPH and NP tissues (eg., higher expressions of both ERα and ERβ,^[19] higher ERα expression and lower ERβ expression,^[20] or lower ERα expression and undifferentiated ERβ expression^[21] in BPH vs. NP tissues). The present study revealed that BPH tissues exhibited higher ERα and lower ERβ expression along with greater EMT (higher TGF-β1 and vimentin expressions and lower E-cadherin

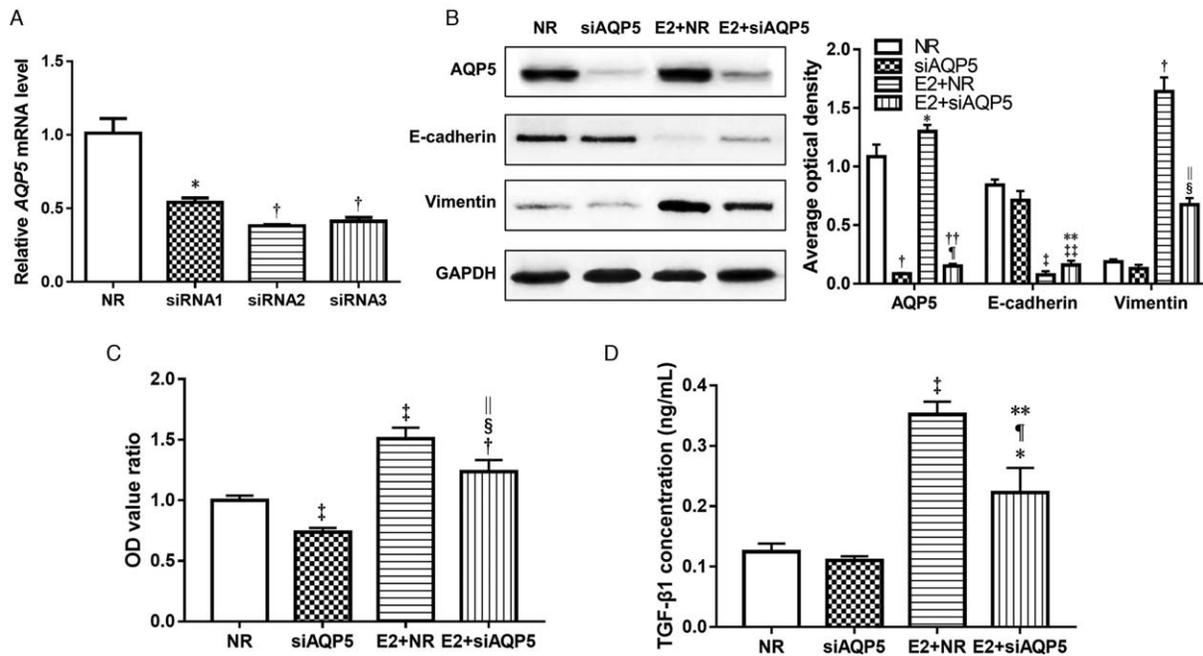


Figure 4: Knockdown of *AQP5* inhibits EMT in RWPE-1 cells after E2 stimulation. (A) qRT-PCR analysis. siRNA2 exhibiting the greatest *AQP5* gene knockdown was selected. (B) Western blot analysis. The siAQP5 group had lower *AQP5* expression than the NR group but comparable E-cadherin and vimentin expressions. The E2 + siAQP5 group had lower *AQP5* and vimentin expression but greater E-cadherin expression than the E2 + NR group. (C) CCK8 test. The E2+NR group had a significantly greater OD value ratio than the E2 + siAQP5 and NR groups. The E2 + siAQP5 group had a significantly greater OD value ratio than the NR group. The siAQP5 group had the lowest OD value ratio. (D) ELISA. The E2+NR group had a significantly greater supernatant TGF-β1 concentration than the E2 + siAQP5 and NR groups. The E2 + siAQP5 group had a significantly greater supernatant TGF-β1 concentration than the NR and siAQP5 groups. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ vs. NR group; § $P < 0.001$, ¶ $P < 0.05$, ** $P < 0.01$ vs. siAQP5 group; †† $P < 0.01$, ††† $P < 0.001$ vs. E2+NR group. AQP5: Aquaporin 5; CCK8: Cell counting kit-8; E2: Estradiol; E-cadherin: Epithelial cadherin; ELISA: Enzyme-linked immunosorbent assay; EMT: Epithelial-mesenchymal transition; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; NR: RNA interfere with non-related siRNA; OD: Optical density; qRT-PCR: Quantitative real-time polymerase chain reaction; siAQP5: *AQP5* knockdown; siRNAs: small interfering RNAs; TGF-β1: Transforming growth factor-β 1.

expression) compared to NP tissues. Shi *et al.*^[22] who identified E2 as a promoter of EMT in benign prostatic epithelial cell lines (RWPE-1 and BPH-1), found that E2 treatment downregulated E-cadherin expression and upregulated vimentin and snail expression. Moreover, pretreatment with an ER receptor antagonist abolished these effects.^[22] Considering the findings presented herein and in previous studies, we can hypothesize that increased EMT in BPH tissues promotes a higher proportion of mesenchymal phenotype cells that express mainly ERα and not ERβ. EMT plays an important role in the development of BPH through “embryonic awakening,” which involves cell phenotypical interconversion.^[6] Such “embryonic awakening” might be attributed to the increased expression of *AQP5* possibly occurring during the physiological process of embryo implantation.^[10]

Increased *AQP5* expression has been reported in a majority of pathological tissues (whether benign or malignant), with considerable evidence suggesting that *AQP5* upregulation could increase cell migration and proliferation in non-cancerous and malignant cells.^[9,11,12,18,23-26] For instance, Kumari *et al.*^[24] showed that corneal epithelial cell migration and proliferation increased significantly during corneal re-epithelialization and wound healing in the presence of *AQP5*. Jiang *et al.*^[9] noted that inducing *AQP5* expression by activating the estrogen response element in the promoter region of the *AQP5* gene activated the PI3K/AKT pathway and promoted endometrial cell invasion and

proliferation. Moreover, Chen *et al.*^[23] observed that *AQP5* overexpression promoted a mesenchymal-like phenotype and EMT in colorectal cancer cells. In contrast, the same study showed that *AQP5* silencing inhibited EMT and that the association between *AQP5* and EMT involved the TGF-β/Smad pathway. *AQP5* has been reported to play an important role in prostate cancer. After assessing 60 prostate cancer tissues, Li *et al.*^[25] found that *AQP5* expression was positively related to the tumor-node-metastasis (TNM) stage, lymph node metastasis, number of circulating tumor cells, and oncogene amplification detected on fluorescence *in situ* hybridization. Additionally, they found that *AQP5*-siRNA could significantly attenuate prostate cancer cell proliferation and migration. Pust *et al.*^[26] who analyzed 12,427 prostate cancer tissues via IHC, found that both *AQP5* negativity and strong positivity were linked to unfavorable disease outcomes. *AQP5* might influence cancer cells by regulating hydraulic motility and Ras/mitogen-activated protein kinase (MAPK) pathway activation. To the best of our knowledge, this has been the first study to explore the role of *AQP5* in the process of EMT among E2-stimulated NP epithelial cells. Our findings showed that *AQP5* knockdown inhibited EMT in RWPE-1 cells after E2 stimulation, indicating the promoting effects of *AQP5* in the pathophysiological process of BPH.

Some limitations of the current study include the limited number of tissues and insufficient *in vivo* testing.

Moreover, the exact mechanism of AQP5 still remains unknown. As such, further trials, especially those involving *in vivo* testing, are needed to address such crucial concerns.

Taken together, our study findings provide novel insights into how estrogen could induce BPH. Accordingly, our results indicate that estrogen can induce EMT in prostate epithelial cells, with AQP5 playing a significant role in this process. Estrogen can promote AQP5 overexpression, thereby increasing EMT in prostate epithelial cells, which might subsequently be involved in the pathogenesis of BPH. Hence, AQP5 might be a novel target for modulating EMT in prostate epithelial cells. Considering the broad potential clinical applications of drugs targeting aquaporins,^[27] AQP5 inhibitors as a treatment for BPH may be expected to transition from benchside to bedside in the foreseeable future.

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Conflicts of interest

None.

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