

Downregulation of AQP2 in the anterior vaginal wall is associated with the pathogenesis of female stress urinary incontinence

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Abstract. The pathogenesis of stress urinary incontinence (SUI) is unclear. Aquaporins (AQPs) are a family of transmembrane proteins that transport water and small solutes, including glycerol, across cell membranes. AQPs have been demonstrated to serve a role in skin hydration, cellular proliferation, migration, immunity, wound healing and vascular remodeling in multiple organs. Furthermore, studies have confirmed that abnormal synthesis and degradation of collagens in extracellular matrix (ECM) remodeling contributes to SUI, by altering normal tissue architecture and mechanical properties. The authors previously demonstrated that AQP2 expressed in the human endometrium varies during the menstrual cycle. However, it is unknown whether AQP2 serves a role in the pathogenesis of SUI in the urethral supporting tissue. In the present study, AQP2 location and expression was examined in the anterior vaginal wall, and investigated the association between AQP2 and collagen I/III in female SUI. Western blotting, immunohistochemistry and immunofluorescence were used to measure AQP2 expression levels, and to reveal the location of AQP2 in the anterior vaginal wall, as well as fibroblasts in SUI and non-SUI. The association between AQP2 and collagen I/III was subsequently investigated by AQP2-small interfering RNA knockdown and overexpression in fibroblasts. AQP2 expression in the anterior vaginal wall was significantly increased in women without SUI compared with those with SUI ($P<0.05$). Downregulation of AQP2 significantly decreased the mRNA and protein expression of collagen I/III, while AQP2 overexpression significantly increased the mRNA and protein

expression of collagen I/III in fibroblasts ($P<0.05$). AQP2 was demonstrated to be expressed in the anterior vaginal wall and fibroblasts, and to regulate the expression level of collagen I/III in the anterior vaginal wall and fibroblasts, suggesting that AQP2 is associated with the pathogenesis of female SUI through collagen metabolism during ECM remodeling.

Introduction

Stress urinary incontinence (SUI) is defined as the involuntary loss of urine that occurs when intra-abdominal pressure exceeds urethral pressure during coughing, sneezing or physical exertion. SUI is a distressing problem with a profound impact on health-associated quality of life (1). The etiology of SUI is unclear. Several studies have proved that its onset is associated with the response of tissue to overstress, inflammation and hypoxia-associated injury (2-6).

Aquaporins (AQPs) are a family of transmembrane proteins that transport water and small solutes, including glycerol, across cell membranes. AQPs are mediators of trans-cellular water flow and serve an important role in maintaining intra/extracellular fluid homeostasis by facilitating water transport in response to altering osmotic gradients. AQPs have been demonstrated to serve a role in skin hydration, cellular proliferation, migration, immunity, wound healing and vascular remodeling in multiple organs, and during acute lung injury and cancer (7-12). Furthermore, a previous study confirmed that abnormal synthesis and degradation of collagens during extracellular matrix (ECM) remodeling contributes to SUI, by altering normal tissue architecture and mechanical properties (13), and that ECM repair and/or remodeling-associated proteins serve a role in the development of SUI.

The authors previously demonstrated that AQP2 expression in the human endometrium varies during the menstrual cycle (14). The present study used anterior vaginal wall tissue as this is an important part of the periurethral support structure (15,16). The vaginal wall consists of a dense ECM, rich in collagen, that is maintained and remodeled by fibroblastic cells. Fibroblasts are involved in normal and pathological soft tissue repair processes (17). Therefore, the present study was designed to determine AQP2 location and expression in the anterior vaginal wall, and investigate the association between AQP2 and the ECM in the pathogenesis of SUI.

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Materials and methods

Ethics statement. Ethical approval for the present study was granted by the Ethics Committee of the School of Medicine of Zhejiang University (Hangzhou, China). All participants provided written informed consent to participate in the study, and the ethics committee approved the consent procedure.

Subjects and sample collection. A total of 12 women aged 47.5 ± 9.2 years, diagnosed with SUI according to the recommendations of the International Continence Society (18), who underwent tension-free vaginal tape surgery at the Department of Gynecology (Women's Hospital, School of Medicine, Zhejiang University) between March 2013 and March 2014 were recruited to the present study. A total of 12 female patients aged 47.6 ± 9.9 in the Department of Gynecology (Women's Hospital, School of Medicine, Zhejiang University) without SUI or pelvic organ prolapse (POP) who were undergoing intravaginal cystectomy for vaginal wall cyst between March 2013 and March 2014 were recruited as controls. The study groups were as follows: i) SUI patients were divided into two groups, the premenopausal group (6 cases) and postmenopausal group (6 cases); and ii) the control group (12 cases), which were also divided into a premenopausal group (6 cases) and postmenopausal group (6 cases). There were no statistically significant differences in ages, body mass indices and parity among the groups. Criteria for exclusion from the control group were: Hormone replacement therapy within the previous 3 months; signs of urinary infection; estrogen-associated disease (endometriosis, myoma or functional ovarian tumor); clear clinical evidence of POP (grade 2 or higher); and urge incontinence. All participants were diagnosed via a combination of medical history, gynecological examination, urinary stress test, ultrasonography and urodynamic examination, including a POP-quantification test.

Biopsy samples of the anterior vaginal wall were taken 1–2 cm from the uterine cervix, and included mucosa, submucosa, connective tissue and smooth muscle.

Each sample (24 cases) was divided into two parts; one part of the tissue was processed for paraffin embedding for immunohistochemical analysis, and the tissue was fixed in 10% neutral buffered formalin at 25°C for 12–24 h followed by paraffin embedding. Another part of the tissue (from each of the 24 cases) was frozen at -80°C for western blotting, and 3 cases of SUI tissue (premenopausal, $n=2$; postmenopausal, $n=1$) was separated for cell culture; the tissues were washed with PBS, cut up into pieces and digested by collagenase for cell culture.

Immunohistochemical analysis of AQP2 expression and localization. The tissue was processed for paraffin embedding. Sections of 3 μ m were prepared and blocked in 3% hydrogen peroxide [cat. no. GK600711; Gene Tech (Shanghai) Co., Ltd., Shanghai, China] for 10 min at 25°C. The immunohistochemical analysis of AQP2 expression and localization was performed on the anterior vaginal wall using an anti-AQP2 antibody (1:400; cat. no. ab15081; Abcam, Cambridge, UK) incubated for 1 h at 20°C. Anti-immunoglobulin (Ig) G-horseradish peroxidase (HRP)

[cat. no. GK600711.A; Gene Tech (Shanghai) Co., Ltd.] and 3'-3'-diaminobenzidine kit immunohistochemical detection reagent [cat. no. GK600711.B; Gene tech (Shanghai) Co., Ltd.] were used for immunohistochemical staining according to the manufacturer's protocol. The results of the immunohistochemistry for AQP2 were examined with a light microscope (LEICA DM 2000).

Western blotting for AQP2. Total protein from the anterior vaginal walls of each group was extracted using Radioimmunoprecipitation Assay Lysis and Extraction Buffer (containing a protease inhibitor cocktail; Haoji Biological Co., Ltd., Hangzhou, China) and the protein concentration was determined using a Bicinchoninic Acid Protein assay kit (Haoji Biological Co., Ltd.). A total 60 μ g proteins/lane were separated by SDS-PAGE on a 10% gel and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked in 5% nonfat milk for 12 h at 4°C (cat. no. 000105; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Protein signals were detected by incubating membranes with 1:500 dilution of anti-AQP2 antibody (cat. no. SC-28629; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti- β -actin as an internal control (cat. no. SC-47778; Santa Cruz Biotechnology, Inc.) at 22°C for 2 h. The membranes were subsequently incubated with goat anti-rabbit IgG-HRP (1:500; cat. no. 32430; Thermo Fisher Scientific, Inc.) antibody and goat anti-mouse IgG-HRP (1:500; cat. no. 32430; Thermo Fisher Scientific, Inc.) antibody at 22°C for 1 h. Protein signals were visualized using Enhanced Chemiluminescence Plus™ Western Blotting Reagents (GE Healthcare Life Sciences, Little Chalfont, UK) and Kodak X-OMAT film (Kodak, Rochester, NY, USA). Band intensities were quantitated using Quantity one software, version 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell culture and identification. The tissue size was $\sim 0.5 \times 0.5 \times 1.0$ cm, with blood and mucus in the SUI and non-SUI groups. The tissues were washed with PBS and cut up into pieces. The tissues were subsequently centrifuged for 5 min at 250 \times g at 25°C, followed by successive digesting with collagenase for 120 min and DNase I for 20 min. The cells were washed with PBS and cultured in Dulbecco's modified Eagle's medium (DMEM)/high glucose (cat. no. SH30022.01; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) containing 20% bovine serum (cat. no. SH30406.02; HyClone; GE Healthcare Life Sciences). After 3–5 days of primary culture, fibroblasts were digested with pancreatin for 2 min and passaged in culture bottles. The third generation of the cells were identified to be fibroblasts by morphological and immunohistochemical analysis, according to previous methods (15). The fibroblasts in the 3rd–6th generation were taken for experiments for identifying AQP2 by immunofluorescence. The fibroblasts were blocked in 1% bovine serum albumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 30 min at 25°C and subsequently incubated with a 1:50 dilution of the primary anti-AQP2 antibodies at 4°C overnight. The cells were incubated with a 1:100 dilution of the secondary antibody goat anti-rabbit IgG-fluorescein isothiocyanate (cat. no. 65-6111; Thermo Fisher Scientific,

Inc.) for 30 min at 25°C. Fibroblasts incubated without the primary antibodies were used as the control. A portion of the cells were also used for identifying AQP2 expression by western blotting as described above.

Small interfering RNA (siRNA) knockdown and vector-mediated overexpression. Following seeding of 200,000 cells/well in 6-well plates, cell transfection was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Cells were harvested subsequent to two days of culture. For knockdown experiments, 100 pmol/well siRNA targeting the AQP2 gene (target sequence, AGCTGTCGTCAGTGGCAAATT) and the siRNA negative control (cat. no. siN05815122147-1-5; Guangzhou RiboBio Co., Ltd., Guangzhou, China) were added. For overexpression experiments, AQP2 overexpression vector pcDNA-AQP2 constructed from pcDNA3.1 was purchased from OriGene Technologies, Inc. (Rockville, MD, USA), and 2 µg/well vector was used for transfection, with pcDNA3.1 as the control. Negative control cells were untransfected.

ELISA. The fibroblasts in the 3rd-6th generation were cultured for 24 h with DMEM/high glucose medium containing 20% bovine serum. The culture medium was collected and collagen I/III expression levels were tested using Human Pro-Collagen I alpha I DuoSet ELISA kit (cat. no. DY6220-0; R&D Systems, Inc., Minneapolis, MN, USA) and Human Pro-Collagen III ELISA kit (cat. no. E-EL-H0182; Elabscience Biotechnology Co., Ltd., Wuhan, China).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNAs were extracted from fibroblasts using RNAiso Plus (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. Then cDNA was synthesized using PrimeScript RT Master Mix (Takara Biotechnology Co., Ltd.). qPCR was carried out using SYBR® Premix Ex Taq™ (Takara Biotechnology Co., Ltd.) in an Applied Biosystems 7900 Fast (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following primers: AQP2-F, 5'-GGCCCCGACGGACGCTTGT-3' and AQP2-R, 5'-TGCGCTGGGGGCCAACTT-3', as the sense primer and anti-sense primer, respectively. GAPDH was used as the internal control. The primers were GAPDH-F, 5'-CCATGA CACTTTGGTATCGTGGAA-3' and GAPDH-R, 5'-GGC CATCACGCCACAGTTTC-3'. The samples were run under the following conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 25 sec. A total of three replicates were performed. Data were analyzed using the $2^{-\Delta\Delta C_q}$ method (19).

Statistical analysis. Statistical analyses were performed using SPSS statistical software (version 20.0; IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference. The results are presented as the mean \pm standard deviation. Fisher's exact test significant difference test was used to analyze the immunohistochemical analysis, and independent samples t-tests were used to analyze the AQP2 protein and mRNA expression, and the collagen I/III protein and mRNA expression.

Results

Expression of AQP2 in the anterior vaginal wall. Expression of AQP2 was detected in the anterior vaginal wall of patients with and without SUI via immunohistochemical analysis (Fig. 1).

AQP2 expression in the anterior vaginal wall is decreased in patients with SUI. Expression of AQP2 was detected in the anterior vaginal wall in patients with and without SUI by western blotting, and the expression of AQP2 was significantly decreased in patients with SUI compared with patients without SUI (non-SUI AQP2/ β -actin ratio, 8.13 ± 0.85 ; SUI AQP2/ β -actin ratio, 2.02 ± 0.52 ; $P < 0.01$; Fig. 2).

Identification of AQP2 expression in fibroblasts by immunofluorescence. As immunohistochemical and western blot analysis demonstrated AQP2 to be expressed in the anterior vaginal wall, it was confirmed whether AQP2 is expressed in fibroblasts. Fibroblasts were separated from the anterior vaginal wall, cultured *in vitro* and identified by their spindle-shaped morphology (Fig. 3A). Fibroblasts are widely distributed in the majority of tissue types, particularly connective tissues. These cells are of mesenchymal origin and express vimentin (Fig. 3B), but not keratin (Fig. 3C) or α -smooth muscle actin (Fig. 3D) as described previously (15). In addition, immunofluorescence was performed to identify the location and expression of AQP2 in fibroblasts, as shown in Fig. 3E and F. AQP2 stained positively in the cell membrane and cytoplasm.

Inhibition of AQP2 downregulated the secretion of collagen I and III in SUI fibroblasts. In order to determine the role of AQP2 in ECM secretion in SUI fibroblasts, fibroblasts were transfected with siRNA targeting AQP2. Compared with the scrambled siRNA, treating fibroblasts with AQP2 siRNA for 36 h reduced the expression level of AQP2 mRNA by 67.4% (Fig. 4A). Inhibition of AQP2 with AQP2 siRNA significantly reduced the relative expression of collagen I mRNA (25.78 ± 0.73 vs. 41.12 ± 1.28 ; $P < 0.01$; Fig. 4B) and protein (93.66 ± 6.74 vs. 115.68 ± 10.32 ; $P < 0.05$; Fig. 4C) compared with the scrambled siRNA. Furthermore, inhibition of AQP2 with AQP2 siRNA significantly reduced the relative expression of collagen III mRNA (6.64 ± 0.20 vs. 12.20 ± 0.32 ; $P < 0.01$; Fig. 4D) and protein (62.01 ± 8.47 vs. 70.36 ± 3.33 ; $P < 0.05$; Fig. 4E) compared with the scrambled siRNA. Therefore, inhibition of AQP2 expression may reduce the secretion of collagen I/III in the anterior vaginal wall-associated fibroblasts in patients with SUI.

Overexpression of AQP2 enhanced the secretion of collagen I/III into the ECM by fibroblasts in SUI. In order to confirm that high AQP2 expression serves an important role in the secretion of ECM proteins by fibroblasts in SUI, AQP2 was overexpressed in fibroblasts. After 48 h of AQP2 overexpression vector transfection, the AQP2 mRNA level was significantly increased compared with the control (684.80 ± 36.13 vs. 14.75 ± 1.21 ; $P < 0.01$; Fig. 5A). AQP2 overexpression significantly increased the relative expression of collagen I mRNA (56.32 ± 1.82 vs. 41.12 ± 1.28 ; $P < 0.05$; Fig. 5B) and protein (163.55 ± 16.19 vs. 115.68 ± 10.32 ; $P < 0.01$; Fig. 5C)

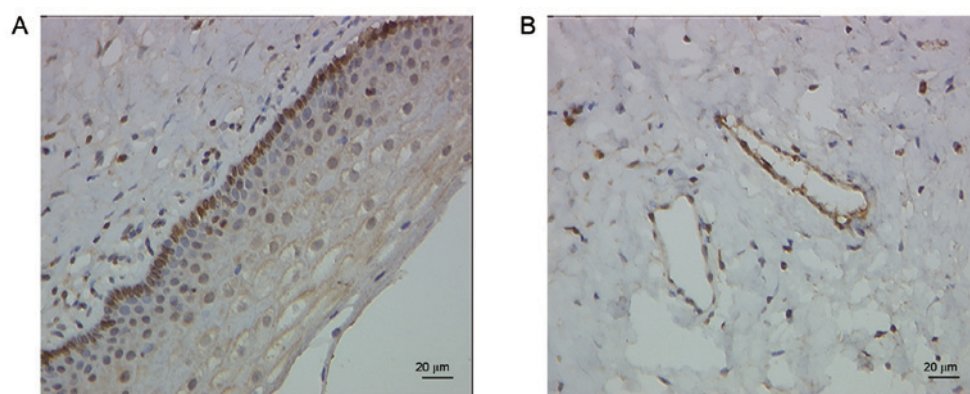


Figure 1. Identification of the expression of AQP in the anterior vaginal wall. (A and B) Immunohistochemistry demonstrated the strong expression of AQP2 in fibroblasts. AQP, aquaporin.

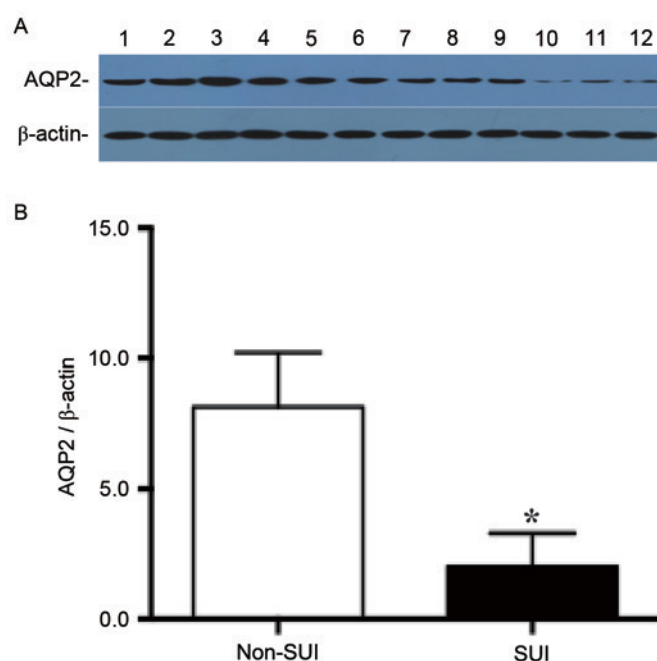


Figure 2. Western blotting of AQP2. (A) Protein levels of AQP2 expression in the anterior vaginal wall of patients with (n=6) and without (n=6) SUI. Lanes 1-6, non-SUI group; lanes 7-12, SUI group. (B) Quantification of AQP2 expression in the anterior vaginal wall of patients with and without SUI. AQP2 expression was decreased in the anterior vaginal wall of the SUI group compared with the non-SUI group. Values are presented as the mean ± standard deviation. *P<0.01 vs. non-SUI group. AQP, aquaporin; SUI, stress urinary incontinence.

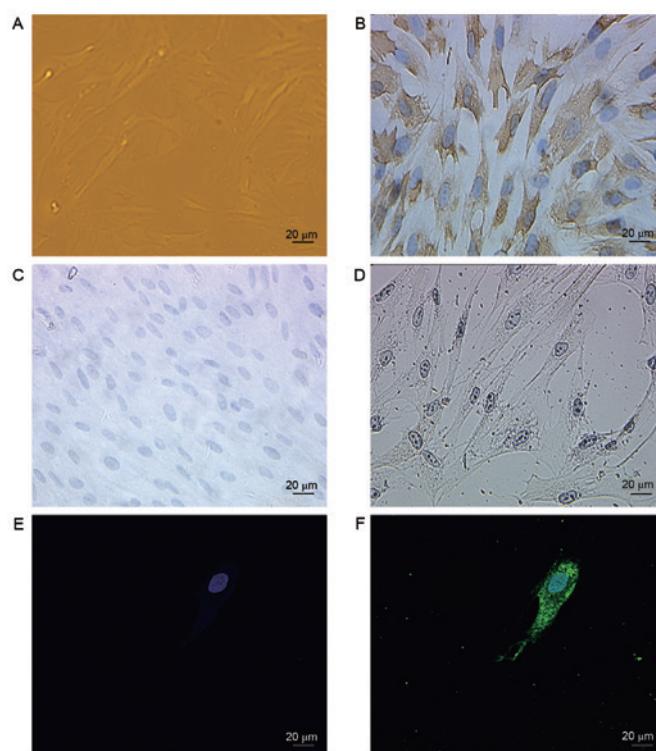


Figure 3. Identification of fibroblasts and expression of AQP2 in fibroblasts. (A) Fibroblasts exhibit a spindle-shaped morphology. (B) Positive staining of vimentin in cultured cells. (C) Negative staining of keratin in cultured cells. (D) Negative staining of actin in cultured cells. (E) The negative control fibroblasts incubated without primary antibodies. (F) Positive staining of AQP2 in the cell membrane and cytoplasm of fibroblasts. AQP, aquaporin.

compared with the control. In addition, AQP2 overexpression significantly increased the relative expression of collagen III mRNA (21.80 ± 0.57 vs. 12.20 ± 0.32 ; $P < 0.01$; Fig. 5D) and protein (101.46 ± 12.42 vs. 70.36 ± 3.33 ; $P < 0.01$; Fig. 5E) compared with the control.

Discussion

The results of the present study indicated that AQP2 is expressed in anterior vaginal wall tissue, including fibroblasts, and that the expression of AQP2 in the anterior vaginal wall was significantly decreased in patients with SUI compared with those without SUI. In addition, AQP2 expression was

demonstrated to be associated with collagen I/III metabolism in fibroblasts of the anterior vaginal wall. Inhibition of AQP2 downregulated collagen I/III secretion in fibroblasts, while overexpression of AQP2 upregulated it.

Previous studies have confirmed the abnormal synthesis and degradation of collagens in ECM remodeling contributes to SUI, by altering normal tissue architecture and mechanical properties (13); tissue maintenance and remodeling is performed by fibroblasts, therefore altered cellular functionality may influence tissue quality (20). However, the mechanism of regulation of fibroblastic cells in the ECM is unclear.

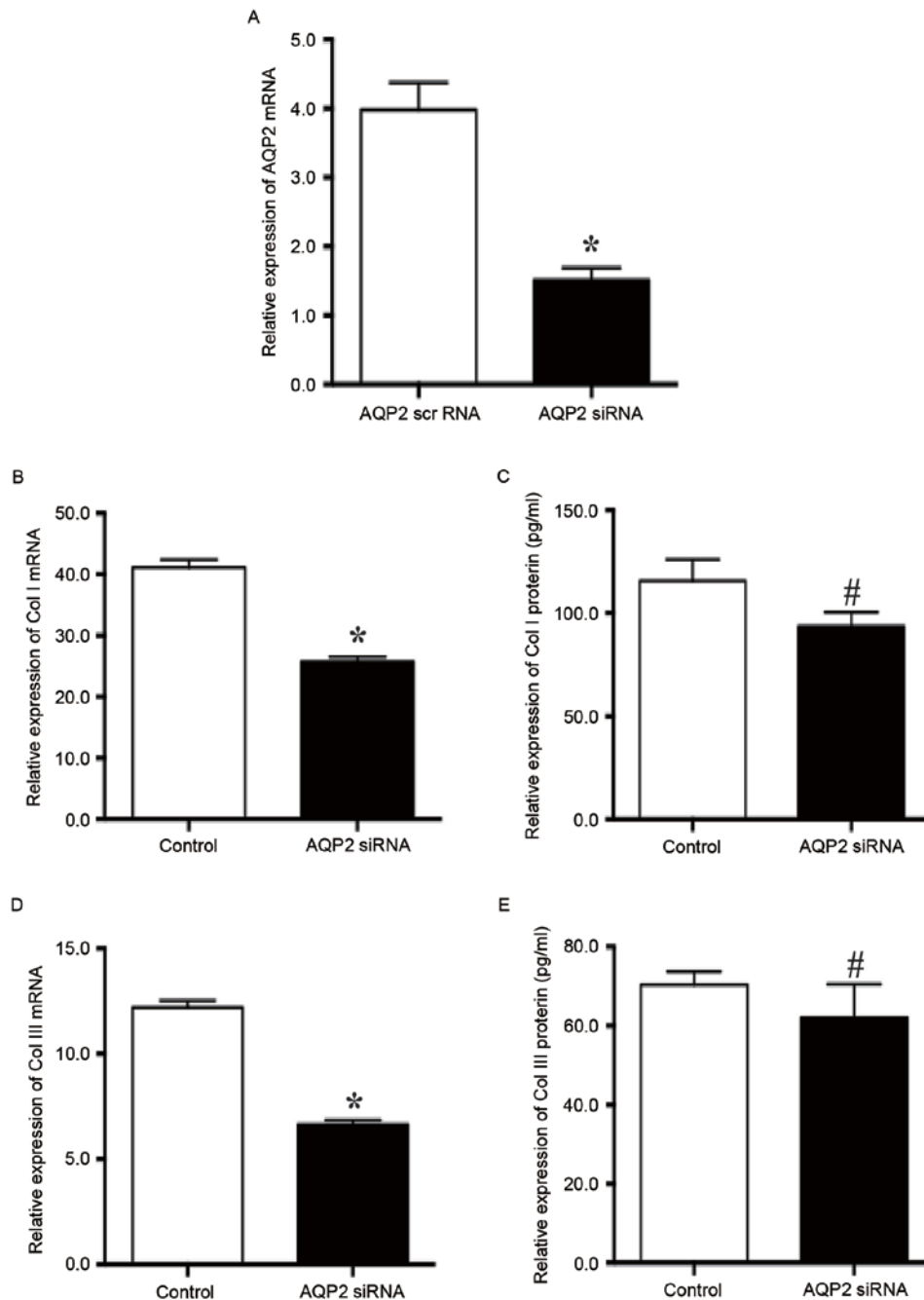


Figure 4. Knockdown of AQP2 downregulated the secretion of col in the extracellular matrix by fibroblasts in stress urinary incontinence. (A) AQP2 interference reduced the expression level of AQP2 mRNA by 67.4%. (B) Knockdown of AQP2 reduced the relative expression of col I mRNA. (C) Knockdown of AQP2 reduced the relative expression of col I protein. (D) Knockdown of AQP2 reduced the relative expression of col III mRNA. (E) Knockdown of AQP2 reduced the relative expression of col III protein. * $P < 0.01$, # $P < 0.05$ vs. the control. AQP, aquaporin; col, collagen; siRNA, small interfering RNA.

AQPs are widely expressed in human tissue, including the skin (7), lung (8), vascular (9), and overexpression of AQPs has been reported in several types of human cancer (10-12). The roles of AQPs in pelvic support tissues and the pathology of female SUI remain unknown; however, the present study used anterior vaginal wall tissue as this is an important part of the periurethral support structure and has been previously examined (15,16). In the present study, immunohistochemical and immunofluorescence analysis revealed that AQP2 is expressed in the vaginal wall, including the fibroblasts, and that AQP2 protein expression in the anterior vaginal wall is decreased in patients with SUI. This indicates that

abnormalities in the AQP2 expression may serve a role in the pathogenesis of SUI.

In healthy women, the bladder is kept in place by the connective-tissue layer of the anterior vaginal wall which is a dense ECM with relatively few cells. The ECM obtains its strength from fibrillar proteins (collagen I and III), and is produced and maintained by fibroblastic cells (17,20). Abnormal collagen metabolism in SUI is well documented (21,22). Collagen types I and III are important in maintaining tissue tensile strength and the mechanical stability of pelvic support tissues. In the present study, knockdown of AQP2 reduced the relative expression of collagen I/II mRNA and protein, while AQP2

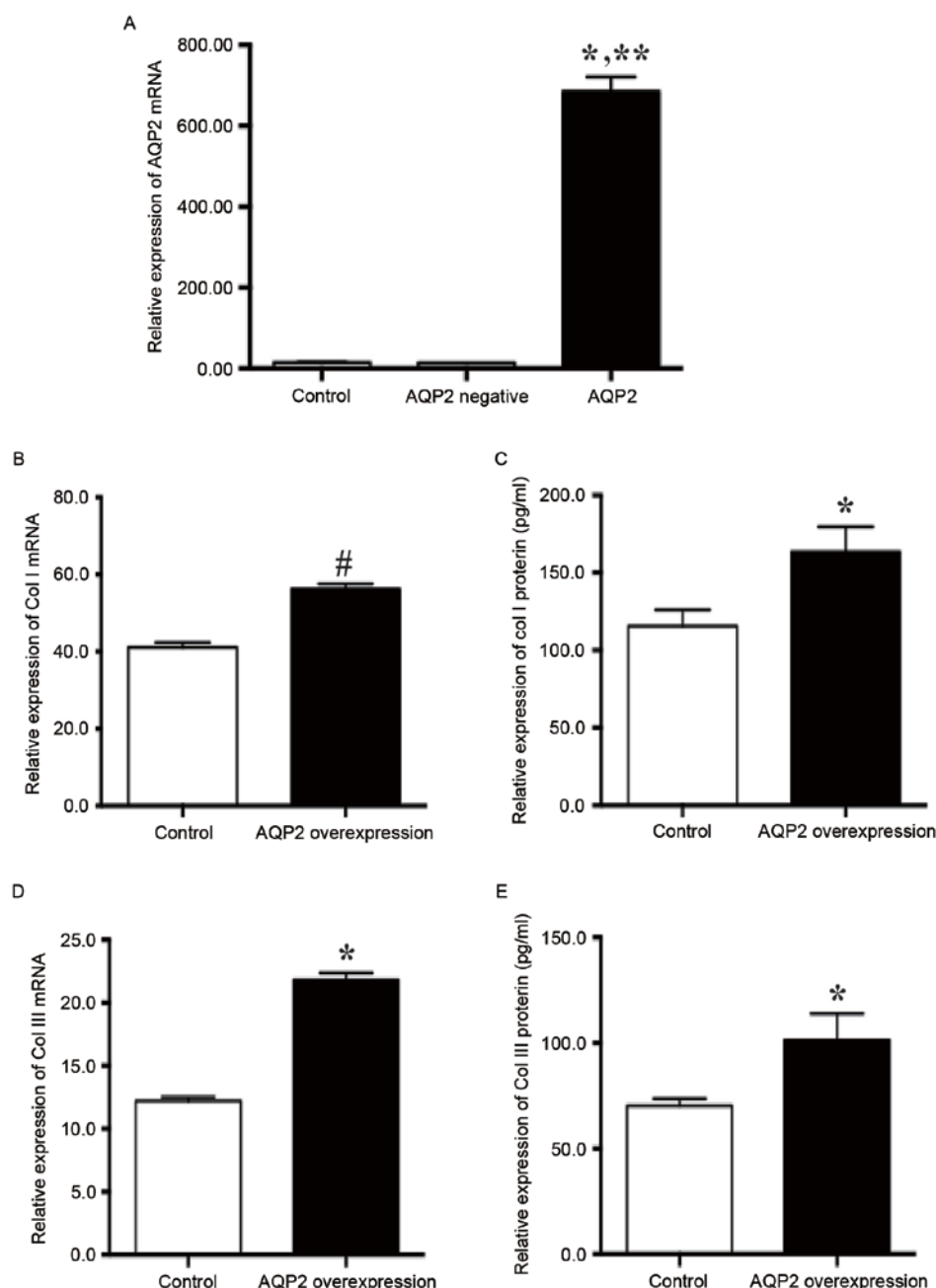


Figure 5. AQP2 overexpression enhanced the secretion of col I/III in the anterior vaginal wall of patients with stress urinary incontinence. (A) AQP2 overexpression increased AQP2 mRNA expression. (B) AQP2 overexpression enhanced the relative expression of col I mRNA. (C) AQP2 overexpression enhanced the relative expression of col I protein. (D) AQP2 overexpression enhanced the relative expression of col III mRNA. (E) AQP2 overexpression enhanced the relative expression of col III protein. * $P < 0.01$, # $P < 0.05$ vs. the control; ** $P < 0.01$ vs. the AQP2 negative group. AQP, aquaporin; col, collagen.

overexpression significantly increased the relative expression of collagen I/III mRNA and protein, suggesting that AQPs are crucial for collagen I/III synthesis in the pelvic floor. AQP2 promotes collagen synthesis in the ECM and inhibits collagen decomposition. The results suggested that AQP2 may be associated with the pathogenesis of female SUI through collagen metabolism regulated by fibroblasts.

In conclusion, female SUI is associated with reduced levels of AQPs compared with non-SUI controls and inhibition of AQP2 inhibits collagen I/III synthesis in vaginal wall fibroblasts. AQP2 regulates the expression level of collagen I/III in the anterior vaginal wall and fibroblasts. It is possible that abnormalities of AQP2 expression may be

involved in the pathogenesis of female SUI through ECM metabolism. Furthermore, larger scale investigations are required to fully elucidate the influence of AQP2 on ECM metabolism in SUI.

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