[*Original Article*]

Aquaporin-2 plays an important role in water transportation through the bladder wall in rats.

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Author contributions

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Abstract

Aim:

We investigated the role of the bladder urothelium in permeating water, focusing on aquaporins.

Methods:

Female Sprague-Dawley rats weighing 300 g were used to investigate the role of the bladder urothelium in saline permeation. Changes in intravesical fluid volume and sodium concentration were measured in the desmopressin acetate hydrate-loaded and control groups 3 h after administration. Bladders were resected to measure aquaporin-1, 2, and 3 gene expression using qRT-PCR. Additionally, the change of aquaporin-2 expression was measured using western blotting and immunohistochemistry in intravesical aquaporin-2 siRNA-treated and control groups.

Results:

Although the intravesical fluid volume and sodium concentration significantly decreased from 0 h to 3 h (1.00 ± 0.00 vs. 0.83 ± 0.08 mL, 157.80 ± 1.30 vs. 146.8 ± 1.92 mEq/mL, $p < 0.01$, respectively in the control group), administration of desmopressin did not affect the extent of volume change. Aquaporin-2 expression was significantly higher in the 3-h distended bladders than in the empty bladder. Aquaporin-2 siRNA treatment suppressed aquaporin-2 expression and the change of intravesical fluid volume from 0 h to 3 h (1.00 ± 0.00 and 0.99 ± 0.02 mL), which was related to the suppression of sodium concentration change in comparison with control siRNA treatment (149.6 ± 2.4 vs. 143.6 ± 3.67 mEq/mL, $p < 0.05$).

Conclusions:

Aquaporin-2 plays an important role in the transport of water under full-filled condition, accompanied by sodium concentration change. We demonstrated the bladder absorption mechanism, which may lead to development of a new method for regulating bladder storage function.

Key words: bladder, water transportion, aquaporin, desmopressin, intravesical fluid volume

Introduction

The bladder is generally perceived as a reservoir of urine that does not affect water metabolism. Therefore, the etiology of nocturnal enuresis or nocturia has been considered to be disordered storage function of the bladder, overproduction of nocturnal urine, or sleep-wake disorder. At bedtime, the urinary bladder gradually expands owing to urine production. Children with nocturnal enuresis do not wake up or elderly people with nocturia wake up when they feel the urge to urinate when the bladder reaches its functional capacity. However, in 2016, Watanabe et al. reported a new concept of bladder absorption to avoid nocturnal enuresis or nocturia. During the “steep-flat pattern,” the bladder reaches its functional capacity shortly after sleep onset and is maintained steadily at the functional capacity until wake time in the morning. In the process, a temporary reduction in bladder volume occurs, which is suggestive of water absorption from urine in the bladder [1]. Animal experiments demonstrated that intravesical fluid is absorbed in some conditions. Sugaya et al. reported that the infused saline was partially absorbed in the bladder, depending on the initial volume [2]. Other studies demonstrated that the urothelium actively transports ions alongside water and urea [3, 4]. In amphibians such as a frog, water is routinely absorbed in the urinary bladder, which occurs in response to dehydration and is enhanced by oxytocin and vasopressin [5]. There is an important phenomenon which suggests that the bladder absorption of water is essential in a certain condition related to sleep. Bears are thought to absorb water and solutions

in the bladder during hibernation [6, 7]. Spector et al. investigated the mechanism comparing urinary bladder architecture, channels and transporters of urothelial solute and/or water with other mammalians [8]. They could not demonstrate the differences of urothelium and tight junction including the expression of AQP-1 and -3, and sodium, potassium ATPase on urothelium. The mechanism of bladder absorption is still unknown.

Aquaporin (AQP) plays a vital role in regulating the water balance in the body, especially in the kidney. AQP-1, 2, and 3 are expressed on the rat bladder urothelium and are involved in the transport of water and salts [9]. AQP-2 in the collecting duct of the kidney, which is related to water reabsorption, is regulated by arginine vasopressin (AVP) through the AVP receptor [10]. In a human study, it was reported that freshly isolated urothelial consistently expressed transcripts for AQP-3, 4, 7, 9, and 11 [11], suggesting that the human bladder may have the ability to absorb water.

It is still too early to verify bladder reabsorption in humans because the control mechanism remains unclear. We started to investigate the detailed mechanisms underlying urine absorption using rat models. Although previous studies demonstrated the expression of water and solute channels and transporters which may regulate bladder absorptive function [9, 11-13], the functions have not been investigated. We aimed to confirm the hypothesis that AQPs function as transporters of water and AVP regulates water absorption in the rat bladder.

Materials and Methods

Animals

Female Sprague-Dawley rats weighing 300 g were obtained from Oriental Bio Service (Kyoto, Japan). They were housed in groups of 2 per cage and maintained under a 12-h light/12-h dark cycle (lights on automatically at 8:00 a.m.) with access to water and laboratory food *ad libitum*. Animal care was in compliance with the recommendations of The Guide for Care and Use of Laboratory Animals (National Research Council) and the study was approved by the animal facility committee at Nara Medical University.

Rats were anesthetized with urethane (1.0 g/kg body weight, intraperitoneal injection). Ureters were ligated bilaterally at the level of bifurcation of the abdominal aorta. The proximal urethra, into which the bladder catheter (PE50) was inserted, was ligated to prevent leakage of intravesical fluid.

Experiment 1

Twenty rats were divided into two groups—DDAVP-loaded (DDAVP+) and non DDAVP-loaded (DDAVP−). In the DDAVP+ group, 4 μg desmopressin acetate hydrate (1-desamino-8-D-arginine vasopressin: DDAVP) (16300AMY00153, Kyowa Kirin, Tokyo, Japan) was intravenously administered in each rat. In the DDAVP− group, 1.0 mL of saline was intravenously administered. The normal functional bladder capacity of female Sprague-Dawley rats is about 0.6 mL [14]. Sugaya et al.

infused saline (median volume, 1.24 mL) to induce isovolumetric bladder contractions [2]. Therefore, 1.0 mL of saline was used for filling the bladder to facilitate the transportation of the intravesical fluid through the urothelium for 3 h. The intravesical fluid was immediately shipped on ice to the Nagahama Life Oriental Yeast Co., ltd. (Shiga, Japan), where populations of sodium (Na) and chloride (Cl) were measured using ion-selective electrode method. Then, a total of 12 bladders were resected (n = 6 from the DDAVP- group, n = 6 from the DDAVP+ group) to measure gene expression of *aqp*-1, 2, and 3, which has been demonstrated in the rat bladder [9], using quantitative reverse-transcription-polymerase chain reaction (qRT-PCR). In 6 control rats, the same preparation was performed with the empty bladder, and the bladders were resected to measure gene expression levels of AQPs using qRT-PCR. We compared the expression levels between the empty and 3 h-distended bladders.

For qRT-PCR, after homogenization of the whole bladder of each rat, RNA was extracted using a QIAamp RNA Blood Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Conversion to cDNA was achieved using a High Capacity cDNA Reverse Transcription kit (Life Technologies). The gene-specific TaqMan primer and probe sets used in this study were Rn00562834_m1, Rn00563755_m1, Rn00581754_m1, and Rn00667869_m1 for rat AQP 1, 2, 3, and β-actin (Applied Biosystems, Foster City, CA, USA), respectively. qRT-PCR was performed using the cDNA, 0.2 μM of each primer, and 10 μL of AmpliTaq

Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA) under the following conditions: incubation at 95 °C for 10 min, and 40 cycles of denaturation at 95 °C for 15 sec and annealing and extension at 60 °C for 1 min. The expression level of target genes was normalized according to the expression level of ß-actin. Data of the treated group is shown by relative fold changes. The expression level of the control is defined as 1.

We evaluated the expression of AQP-2 by immunohistochemistry and western blotting in addition. For immunohistochemistry, resected bladders were filled with 300 μL 10% neutral buffered formalin and the entire specimens were placed in 10% neutral buffered formalin. Bladders in formalin were embedded in paraffin and then subjected to IHC staining for cell surface and immunological AQP-2 marker. Paraffin blocks were cut and placed on Superfrost Plus microslides (Thermo Fisher Scientific, Yokohama, Japan). Sections were deparaffinized and citric acid buffer (pH 6.0) antigen retrieval was carried out using an autoclave. IHC staining was performed using the Histofine ABC kit (Nichirei Biosciences, Tokyo, Japan) according to manufacturer instructions. Briefly, slides were incubated overnight at 4°C with a rabbit polyclonal antibody against Aquqporin-2 (dilution 1/500, LS-C312893, LSBio, Seattle, WA, USA). The slides were counterstained with Mayer's hematoxylin, dehydrated, and sealed with a cover slide. Finally, the tissue sections were examined and photographed under a light microscope. Western blotting was performed using the primary anti-AQP-2 rabbit polyclonal antibody (dilution 1/500, LS-C312893, LSBio, Seattle, WA, USA) following a previous report [15].

Experiment 2

Twelve rats were divided into two groups: intravesicular AQP-2 siRNA-treated and intravesicular control siRNA-treated. Eight rats were used to evaluate the inhibition by siRNA. Then, the bladders were resected and processed for AQP-2 expression using RT-PCR or western blotting. Western blotting was performed following the same methods used in Experiment 1. To knockdown AQP-2 expression on the urothelium, we utilized Stealth RNAi siRNA (AQP -2) (RSS352097, Thermo Fisher Scientific, Tokyo, Japan). AQP-2 siRNA was reconstituted in the provided buffer in accordance with the manufacturer's instructions to a concentration of 20 μM and then the siRNA was diluted in liposomal transfection reagent (DOTAP) (890890P , Avanti Polar Lipids, Alabaster, AL, USA) for use, following a previous report [16]. DOTAP was hydrated with nuclease-free water to a lipid concentration of 3 mM and diluted 10-fold with RNase-free PBS (containing Ca^{2+} and Mg^{2+}). Concentrated AQP-2 siRNA and control siRNA were diluted to a final concentration of 200 nM each. When this was done, the rats were anesthetized with isoflurane and 24 gauge angiocatheters (without the needle) were inserted into the bladder through the proximal urethra. Then, 0.5 mL of the siRNA-liposome complex was infused into the bladder through the urethral catheter with a 1 mL syringe and allowed to incubate for 1 h. The catheter was removed and the animals were allowed to recover from anesthesia before being returned to the animal facility. This procedure

was repeated 2 days after the initial treatment. On day 5, all 12 rats were anesthetized with urethane and the changes in intravesical fluid volume and the concentrations of sodium and chlorine were measured, following the same methods used in Experiment 1.

Statistical analysis

We used Mann-Whitney U test for all statistical analysis. PRISM software version 7.03 (GraphPad Software, Inc., USA) was used for all statistical analyses. $P < 0.05$ was considered statistically significant.

Results

Experiment 1

The mean volume of intravesical fluid and the mean concentrations of sodium and chlorine significantly decreased for 3 h in both the DDAVP− and DDAVP+ groups ($p < 0.01$) (Table 1). The expression change of AQP-2, but not AQP-1 and 3 was significantly larger in the distended bladder group than in the empty bladder group (Fig. 1 A1-3). Western blotting showed the intense band of AQP-2 in the distended bladder (Fig. 1B). Immunohistochemical labeling for AQP-2 was present in the urothelium. The labeling was stronger in the distended bladder than in the empty bladder, especially at the luminal side of urothelium (Fig. 1 C1-4).

Experiment 2

Western blot analysis of proteins extracted from the whole bladder showed that siRNA treatment knocked down AQP-2 expression, whereas control siRNA did not affect AQP-2 expression (Fig. 2A). qRT-PCR analysis showed that *aqp*-2 expression was significantly decreased in the siRNA+ group compared to that in the control group ($p = 0.0181$) (Fig. 2B). The mean intravesical fluid volume significantly decreased when the 1.0 mL-filled bladder condition was retained for 3 h in the control siRNA group ($p < 0.01$), but not in the AQP-2 siRNA group. The mean concentration of sodium significantly decreased when the 1.0 mL-filled bladder condition was retained for 3 h in both the control siRNA and the AQP-2 siRNA groups ($p < 0.001$ and $p < 0.01$). The mean sodium and chloride concentrations were significantly higher in the AQP-2 siRNA

group than in the control group ($p < 0.05$) (Table 2).

Discussion

Spector et al. reported that AQP-1 localizes to the endothelial cells of capillaries and arterioles in the rat bladder, whereas AQP-2 and -3 localize to the epithelial cell membranes of the urothelium. AQP-2 and -3 may play a role in the maintenance of water homeostasis due to water transport across the urothelium [9]. Some reports also suggested the potential role of AQPs in water homeostasis in the rat urinary bladder [17, 18]. In the present study, the expression of *aqp*-2 was higher in the full-filled bladder group than in the control group. The knockdown of *aqp* -2 with siRNA suppressed the change in intravesical fluid volume. The findings that expression of AQP-2 was higher and that the bladder of AQP-2 knock-down rats showed no change in intravesical fluid volume under full-filled (distended) conditions implied that AQP-2 may play a vital role in fluid absorption in the bladder urothelium. Dehydration resulted in increased AQP-2 and -3 expression in the rat bladder [9], which supports the importance of AQP-2 in the fluid absorption. In *in vitro* studies on normal human urothelium cells, AQP-3 expression was up-regulated by increased osmolality [19], which may be one of the reasons why the expression of AQP-3 did not increase in the present study. It has been reported in *in vitro* studies that AQP-2 is not expressed by normal human urothelium cells [11]. The expression and functions of AQP subtypes in the bladder may depend on surroundings and animal species.

In the collecting duct of the kidney, AVP regulates the expression

levels of AQP-2, which plays a key role in water absorption by regulating its transport [20]. In the present study, DDAVP administration had little effect on the decrease in intravesical saline volume. Although AQP-2 is involved in water absorption across the urothelium, our results suggested that AVP may not regulate water absorption in the rat urinary bladder. We expected that the administration of DDAVP would increase the absorption of water. However, it did not. We assume that the endogenous AVP could have already been fully secreted under the condition of the present study. Those rats were anesthetized with urethane at least 2 hours before the start of experiments. They did not take any water for totally about 6 hours until the end of experiments and should have been in a dehydrated state, which could have induced the secretion of AVP. It is possible that DDAVP did not work because AQP2 had already operated at its maximum with the endogenous AVP.

The concentrations of sodium and chlorine decreased as the volume of water decreased, which implies that both water and salts are transported across the urothelium. Additionally, the concentrations of sodium and chlorine decreased even when the water level did not decrease. Therefore, it was hypothesized that some channels in the urothelium function in the condition of full-filled (distended) bladder because AQPs transport water only. Urea, sodium, and chlorine move across rat urothelial cells [21, 22], but the underlying mechanism has not been elucidated. The epithelial sodium channel (ENaC) mediates sodium transport across the apical membrane of

the urinary bladder epithelium [12]. In the kidney, several subtypes of claudin, which is a family of proteins that are the most important components of the tight junctions, play important roles in paracellular ion absorption [23] and localize in the bladder urothelium [24, 25]. Some claudins and ENaCs, in addition to AQP-2, might be involved in the transport of water and ions. Further studies are needed to elucidate the detailed mechanisms.

This study has some limitations. First, the volume of saline (1.0 mL) infused into the bladder was high. Over-distension of the bladder might have damaged the urothelial layer. We histologically confirmed that the bladder urothelium had no obvious damages after 3 h of distention. Second, We could not distinguish the site where AQP-2 expression increased from the results of qRT-PCR and western blotting because we used whole bladders. However, immunohistochemistry suggested that the increase of AQ-2 expression may occurred mainly in the urothlium of distended bladder with saline. Third, we might perform an inconclusive experiment on the expression change of *aqp*-1. Although the difference of *aqp*-1 expression between the empty and the distended bladders was not significant, it may not indicate a lack of effect. If an about 50% difference does not reach statistical significance, it possibly occurs due to insufficient sample sizes. Lastly, we did not assess the expression of the vasopressin V2 receptor in the rat bladder. The administration of DDAVP did not affect the water absorption in the bladder, in contrast to the kidney, because

DDAVP is a selective agonist for the vasopressin V2 receptor [26].

Conclusions

The rat urinary bladder absorbs water and salts under the full-filled condition. AQP-2 plays an important role in the transport of water, accompanied by sodium concentration change. We demonstrated a part of the bladder absorption mechanism, which may lead to development of a new method for regulating bladder storage function.

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Figure legends

Fig. 1 The expression change of aquaporins by 3 h distention with saline

A: qRT-PCR for aquaporin-1 (A1), -2 (A2), -3 (A3). The fold mRNA expression of aquaporin-2 was significantly higher in the distended bladders (n = 12) than in the empty bladders (n = 6). Error bars represent standard deviation.

B: Western blotting for aquaporin-2.

C: Hematoxylin-eosin staining for the empty (C1) and the distended (C2) bladders. Immunohistochemistry for aquaporin-2 of the empty (C3) and the distended (C4) bladders. Photographs are at ×100 magnification.

Fig. 2 The knocked-down expression of aquaporin-2 by siRNA treatment

A: The expression of aquaporin-2 in normal bladder (Control), bladder treated with intravesical control siRNA (Control siRNA), and bladder treated with intravesical aquaporin-2 siRNA (Aquaporin-2 siRNA) were confirmed by western blotting.

B: qRT-PCR analysis (Control: n = 4, Control siRNA: n = 4, Aquaporin-2 siRNA: n = 4). Error bars represent standard deviation.

Table1. Comparison of intravesical fluid in the desmopressin acetate hydrate-loaded and control groups

	DDAVP- n = 10		DDAVP+ n = 10	
	0 h	3 h	0 h	3 h
Intravesical fluid volume (mL)	1.00 ± 0.00	0.83 ± 0.08**	1.00 ± 0.00	0.81 ± 0.09**
Sodium (mEq/mL)	157.80 ± 1.30	146.8 ± 1.92**	157.80 ± 1.30	149.0 ± 1.41**
Chlorine (mEq/mL)	153.80 ± 2.17	131.6 ± 2.97**	153.80 ± 2.17	135.6 ± 2.30**

DDAVP-: Control group, DDAVP+: Desmopressin acetate hydrate-loaded group

Statistical method: Mann-Whitney U test, Values are expressed as the mean ± standard deviation

** $p < 0.01$ vs. 0 h

Table2. Comparison of intravesical fluid in the intravesical aquqporin-2 siRNA-treated and control groups

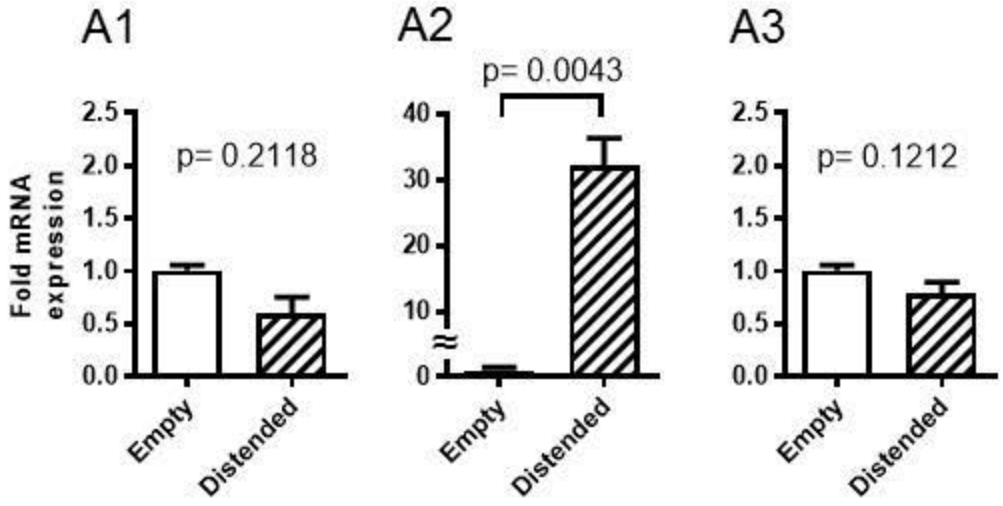
	Control siRNA n = 6		Aquaporin-2 siRNA n = 6	
	0 h	3 h	0 h	3 h
Intravesical fluid volume (mL)	1.00 ± 0.00	0.86 ± 0.07**	1.00 ± 0.00	0.99 ± 0.02##
Sodium (mEq/mL)	152.40 ± 1.12	143.6 ± 3.67***	152.40 ± 1.12	149.6 ± 2.4**#
Chlorine (mEq/mL)	153.00 ± 4.20	138.40 ± 4.18**	153.00 ± 4.20	145.50 ± 3.04**#

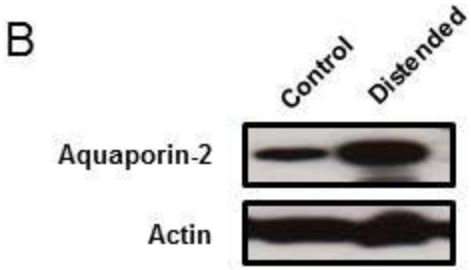
Statistical method: Mann-Whitney U test, Values are expressed as the mean ± standard diviation

** $p < 0.01$, *** $p < 0.001$ vs. 0 h

# $p < 0.05$, ## $p < 0.01$ vs. control siRNA

Fig. 1


A1
A2
A3
p= 0.0043
p= 0.2118
p= 0.1212
Fold mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
40
30
20
10
0
Empty
Distended


B
Control
Distended
Aquaporin-2
Actin

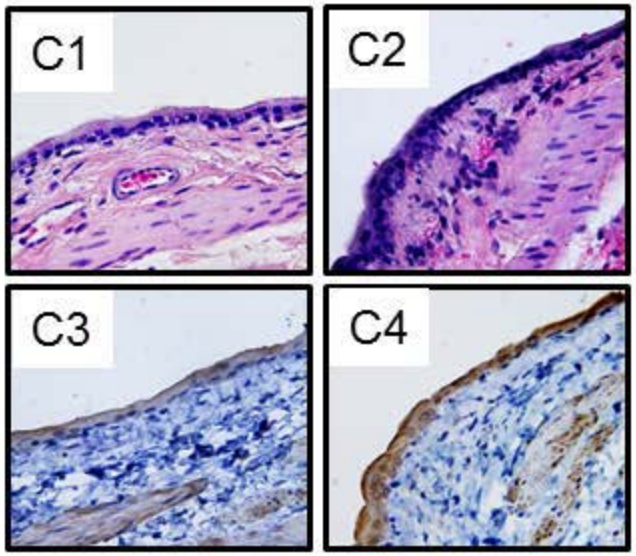
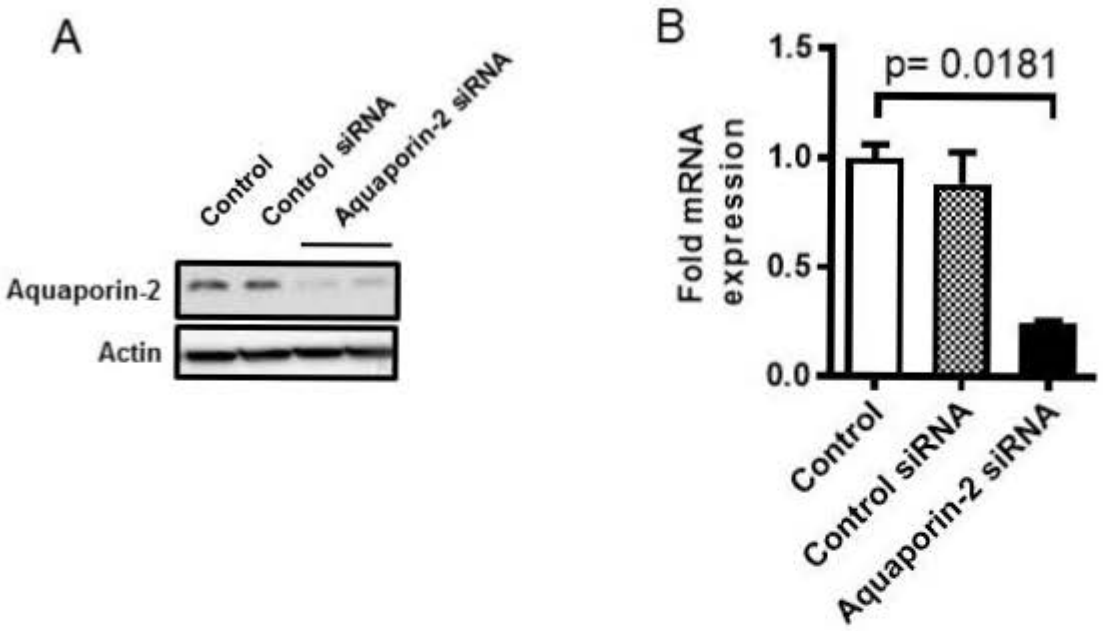

C1
C2
C3
C4

Fig. 2


A
Control
Control siRNA
Aquaporin-2 siRNA
Aquaporin-2
Actin
B
p= 0.0181
Fold mRNA expression
1.5
1.0
0.5
0.0
Control
Control siRNA
Aquaporin-2 siRNA