Aquaporin Expression in the Fetal Porcine Urinary Tract Changes During Gestation

L. K. JAKOBSEN^{1,2}, K. F. TRELBOG¹, P. S. KINGO^{1,2}, S. HØYER³,
K.-E. ANDERSSON^{1,4}, J. C. DJURHUUS¹, R. NØRREGAARD¹, L. H. OLSEN^{1,2}

¹Department of Clinical Medicine, Aarhus University, Aarhus, Denmark, ²Department of Urology, Aarhus University Hospital, Aarhus, Denmark, ³Department of Pathology, Aarhus University Hospital, Aarhus, Denmark, ⁴Department of Gynecology and Obstetrics, Aarhus University Hospital, Aarhus, Denmark

Received November 2, 2016

Accepted September 12, 2017

On-line January 5, 2018

Summary

The expression of aquaporins (*AQPs*) in the fetal porcine urinary tract and its relation to gestational age has not been established. Tissue samples from the renal pelvis, ureter, bladder and urethra were obtained from porcine fetuses. Samples were examined by RT-PCR (*AQPs 1-11*), QPCR (*AQPs* positive on RT-PCR), and immunohistochemistry. Bladder samples were additionally examined by Western blotting. RNA was extracted from 76 tissue samples obtained from 19 fetuses. Gestational age was 60 (n=11) or 100 days (n=8). PCR showed that *AQP1*, *3*, *9* and *11* mRNA was expressed in all locations. The expression of *AQP3* increased significantly at all four locations with gestational age, whereas *AQP11* significantly decreased. *AQP1* expression increased in the ureter, bladder and urethra. *AQP9* mRNA expression increased in the urethra and bladder, but decreased in the ureter. *AQP5* was expressed only in the urethra. Immunohistochemistry showed *AQP1* staining in sub-urothelial vessels at all locations. Western blotting analysis confirmed increased *AQP1* protein levels in bladder samples during gestation. Expression levels of *AQP1*, *3*, *5*, *9* and *11* in the urinary tract change during gestation, and further studies are needed to provide insights into normal and pathophysiological water handling mechanisms in the fetus.

Key words

Aquaporins • Urinary tract • Fetal development • Pig • Protein expression

Corresponding author

L. K. Jakobsen, Department of Clinical Medicine, Department of Urology, Aarhus University, Palle Juul-Jensens Boulevard 99, 8200 Aarhus N, Denmark. E-mail: lottekj@clin.au.dk

Introduction

The urothelium covers the inner surface of the urinary tract and was for a long time considered an impermeable barrier, due to a very high trans-urothelial resistance. This is achieved by tight junctions between cells, as well as glycans, membrane lipids and uroplakins lining the umbrella cells (Hicks *et al.* 1974, Lewis 2000).

The functional role of the urothelium has been extensively investigated (Birder and Andersson 2013), and with respect to its barrier function studies have shown changes in urine composition along the urinary tract and during storage in the bladder, but also passage of substances between blood and urine (Cahill *et al.* 2003, Englund 1956, Levinsky and Berliner 1959, Shafik *et al.* 2006, Shafik *et al.* 2005, Shafik *et al.* 2004, Walser *et al.* 1988). Furthermore, there is evidence that the urothelium might be involved in sensing and signaling related to osmolality of the urine and bladder fullness (Andersson and McCloskey 2014, Birder and Andersson 2013, Rubenwolf *et al.* 2012b). The presence of membrane transporters in the urothelium, such as aquaporins (*AQPs*), has also been shown, suggesting a role in water reabsorption (Kim *et al.* 2010, Rubenwolf *et al.* 2012b, Rubenwolf *et al.* 2009, Spector *et al.* 2002). Such a role

for the *AQPs* has been demonstrated in the adult mammalian kidney (Nielsen *et al.* 2000), and in the skin and urinary bladder of amphibians (Suzuki and Tanaka 2009). Recent studies show changes in *AQP* expression in the urothelium and lamina propria of the adult mammalian bladder following urothelial carcinoma (Rubenwolf *et al.* 2015), bladder outlet obstruction or dehydration (Kim *et al.* 2010, Kim *et al.* 2012, Spector *et al.* 2002), emphasizing the need for better understanding the role of *AQPs* in the urinary tract.

Pigs are often utilized as experimental animals for studies on urinary tract function as they share many physiological and anatomical features with humans (Crowe and Burnstock 1989). However, little is known about the developmental physiology of the porcine urinary tract function. Olsen *et al.* (2001) studied bladder function urodynamically in porcine fetuses at 62 and 80 days of gestation (corresponding to mid second and early third trimester in human pregnancy), and demonstrated developmental changes in voiding function as the striated sphincter evolves. In adult rats a partial bladder outlet obstruction causes expression of *AQP1*, 2 and 3 to increase (Kim *et al.* 2012, Kim *et al.* 2010, Kim *et al.* 2013b). We speculate whether the sphincter development during fetal life has the same effect. Studies concerning the development of *AQPs* in the porcine kidney during gestation has been performed, showing an increased expression of *AQP1*, 2, and 3 (Xing and Nørregaard 2016), but studies on the urinary tract during development seem to be lacking and suitable animal models would be desirable.

The purpose of the present study was to gain basic knowledge about the expression of *AQPs* in the fetal porcine urinary tract, and how this expression relates to gestational age. We investigated the expression of *AQPs* in four different locations: the renal pelvis, the ureter, the bladder and the urethra, assuming that *AQPs* are expressed in the porcine urinary tract in a similar fashion as in humans.

Material and Methods

All procedures conformed to the Danish National Guidelines for care and handling of animals and to the published guidelines from the National Institute of Health. Experiments were carried out after ethical approval by the Danish Animal Experiments Inspectorate, Approval no. 2014-15-0201-00356. Pregnant sows, crossbred Danish Landrace/Yorkshire, inseminated with semen from Duroc boar, were obtained from

a commercial source and sacrificed at different occasions by intravenous injection of pentobarbital at Aarhus University Foulum at 60 and 100 days of gestation (corresponding to mid second and late third trimester in humans). Average gestation length in these pigs is 114 days. The uterus was excised and opened, and the fetuses were weighed and necropsied.

Tissue handling

Access to the fetal urinary tract was obtained through midline sagittal and transverse laparotomy. The urachus was cut and held, and the bladder was excised, along with proximal urethra and distal ureters. Samples were taken from the proximal urethra and distal ureters, and either snap frozen in liquid nitrogen, or placed in 10 % formalin. Both kidneys were excised. A sample from the renal pelvis of one kidney was snap frozen. The contralateral kidney, including pelvis, was placed in 10 % formalin. All snap frozen samples were stored at -80 °C until processing.

The bladders were handled in different ways:

- Whole wall tissue sample from the bladder dome was taken and snap frozen for RNA-extraction, whole bladders were snap frozen for protein extraction.
- Bladders for immunohistochemistry were either pin-mounted on a cork plate and placed in 10 % formalin, or filled with 1-2 ml of formalin, *via* a 17G catheter inserted through the urachus, after applying metal clips to close ureters and urethra. Filled bladders were subsequently submerged in 10 % formalin.
- Some of the bladders were selected for use in another project.

RNA extraction and PCR analysis

Total RNA was isolated from the frozen samples using TRIzol® (ThermoFischer Scientific™, Waltham, USA) and chloroform/isopropanol extraction. cDNA was synthesized from 0.5 µg RNA with the RevertAid First Strand cDNA Synthesis kit® (Thermo Scientific™, MBI Fermentas, Burlington, Canada). Primers were designed for *AQPs 1-11* and four reference genes. Reference genes were chosen based on the literature (Nygard *et al.* 2007, Xu *et al.* 2015), previous experience and results from validation of stable expression across gestation. RT-PCR was performed for all *AQPs* along with positive control tissue samples and distilled water serving as a negative control. Primer sequences and positive control tissues are shown in Table 1. PCR products were analyzed by electrophoresis in a 1 % agarose gel at 100 V for 60 min.

Supplementary quantitative PCR was performed for relevant *AQPs* and reference genes on all samples that were positive in RT-PCR, using the Maxima® SYBR® Green QPCR Master Mix (ThermoFischer Scientific™) according to manufacturer's instruction. Duplicate samples were amplified in 96-well plates, running 40 cycles with 30 s of denaturation at 95 °C followed by 1 min of annealing and polymerization at 60 °C. Fluorescence emission was detected during the annealing/extension step in each cycle. After PCR a melting curve analysis of the product was performed, which for all primer sets resulted in single product-specific melting curves with no primer-dimers. Threshold cycle (C_t values) from serial dilutions of cDNA was used

to construct a standard curve, and the individual real-time PCR amplification efficiency ($E = 10^{-1/\text{slope}}$) was calculated from this curve. The relative expression ratio of a target gene was based on its amplification efficiency (E) and the crossing point difference (ΔC_t) for an unknown sample vs. a control.

The geometric mean of the reference genes was used to normalize the raw value of the genes of interest:

$$2^{(REF - AQP)}$$

where *REF* means geometric mean RNA quantity of four reference genes and *AQP* means aquaporin of interest RNA quantity.

Table 1. Primer sequences and positive control tissues for PCR.

	Accession number	Forward sequence	Reverse sequence	Positive control tissue
Aquaporins				
<i>AQP1</i>	NM214454.1	TTG GGC TGA GCA TTG CCA CGC	CAG CGA GTT CAG GCC AAG GGA GTT	Kidney
<i>AQP2</i>	EU636238.1	CTG TGG AGC TTT TCC TGA CC	TAG TGG ATC CCG AGA AGG TG	Kidney
<i>AQP3</i>	EU024115.1	CTC ATG GTG GTT TCC TCA CC	CAA GGA TAC CCA GGG TGA CA	Kidney
<i>AQP5</i>	NM001110424.1	TAG TGG GCA ACC AGA TCT CC	CGT GTT GTT GTT GAG CGA GT	Lung
<i>AQP6</i>	NM001128467.1	TGG ATG ACT GTC AGC AAA GC	ATT TGC AGC ACA GAG GGA AG	Kidney
<i>AQP9</i>	EU194551.1	GCC TAC AGC CCA TTG TCA TT	AAA GGG CCC ACT ACA GGA AT	Liver
<i>AQP11</i>	NM001112682.1	CGC TTT CGT CTT GGA GTT TC	CCA GCA TCA TTT GCA TCA TC	Kidney
Reference genes				
<i>B2M</i>	DQ845172.1	AGG CTG TCT TTC AGC AAG GA	TCT TGG GCT TAT CGA GAG TCA	
<i>GAPDH</i>	AF017079.1	GGG CAT GAA CCA TGA GAA GT	TGT GGT CAT GAG TCC TTC CA	
β -actin	DQ845171.1	CAT CAC CAT TGG CAA TGA GCG	CTA GAA GCA TTT GCG GTG GAC	
<i>TBP</i>	DQ845178.1	GCC AGA GTT GTT TCC TGG TT	TCG TCT TCC TGA AAC CCT TT	

Primers were designed using Primer3 software specifically for porcine sequences found in the NCBI database. Porcine tissue with previously demonstrated expression of *AQPs* of interest were chosen as positive controls. All the listed primer sequences confirmed this expression.

Immunohistochemistry

Tissue was immersed in 10 % formalin for 24 h, washed in PBS buffer, dehydrated and embedded in paraffin wax. Sections of 3 μ m were cut, deparaffinized and rehydrated. H&E-staining was performed for orientation. Sections for immunohistochemistry were stained using the Ventana Benchmark Xt® (Ventana Medical Systems, Tucson, USA). Demasking was done using the CC2 protocol for heat induced epitope retrieval. Cell conditioning solution (CC2, Ventana) is a low pH

buffer solution which was added to the tissue sections. Sections were then heated to 94 °C for a total of 44 min with addition of further CC2 every four minutes. Primary antibody (*AQP1*, Alomone Labs, Jerusalem, Israel, cat. no: AQP-001) was diluted 1:1,000 in DAKO diluent and sections were incubated with this for 32 min. Reaction was visualized with the ultraview DAB v3 kit (Ventana). Nuclei were counterstained by hematoxylin and enhanced by bluing agent.

Western blotting

Bladder tissue was homogenized for 4 min at 50 Hz by a TissueLyser LT (Qiagen, Hilden, Germany) in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM Na₂EDTA, 1 % Triton X-100, 0.5 % sodium deoxycholate, pH 7.4). Homogenates were centrifuged for 10 min at 1000 G at 4 °C. Using a Pierce BCA protein assay kit (Roche, Basel, Switzerland) the total protein concentrations in the supernatant were measured at 562 nm. Gel samples were prepared using Laemmli sample buffer and loaded on a 12 % Criterion TGX Precast Gel (Bio-Rad Laboratories, Copenhagen, Denmark). From bladder samples 100 µg of protein was added for *AQP1* detection. From the renal cortex control a 20 µg sample of protein was added. After electrophoresis proteins were transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare, Hatfield, UK). Blocking was done in 5 % non-fat dry milk dissolved in PBS-Tween 20 (80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, 0.1 ml Tween 20; pH 7.4). Membranes were washed in PBS-T and incubated with primary antibody overnight at 4 °C (*AQP1*, Alomone Labs, Jerusalem, Israel, cat. no: AQP-001, 1:500). Horseradish peroxidase-conjugated secondary antibody (p448 goat anti-rabbit immunoglobulin, DAKO, Glostrup, Denmark) was added and incubated for 1 h at room temperature. Antigen-antibody reactions were

visualized using an enhanced chemiluminescence system (Amersham ECL Plus, GE Healthcare, Chicago, USA) and imaged using the Bio-Rad ChemiDoc-MP imaging system, Image Lab software v 5.2.1 (Bio-Rad Laboratories, Copenhagen, Denmark). All Western blots were normalized to *GAPDH* as a reference protein (*GAPDH* primary antibody, Cell signals #2118, 1:2,000, 20 µg of protein mounted). Pre-absorption with the control peptide (*AQPI*, Alomone Labs, Jerusalem, Israel, cat. no: AQP-001) was performed as a negative control.

Statistics

GraphPad Prism 7.0a (GraphPad Software Inc., La Jolla, USA) was used for statistical analyses. Group means were compared by Mann-Whitney test. The null hypothesis was rejected at p<0.05. Outcomes are reported as median and interquartile range (IQR).

Results

Forty-one fetuses at 100 days of gestation were obtained from two sows. Two fetuses from each sow were immediately discarded, due to small size and/or malformed appearance. Two sows at 60 days of gestation carried 26 and 23 fetuses, respectively. Specifications on sex, weight and tissue utilization are listed in Table 2.

Table 2. Material. Details on fetuses regarding sex, weight, gestational age and tissue utilization.

	60 days of gestation	100 days of gestation
<i>Total number of fetuses</i>	49	41
<i>Sex</i>	23 male 24 female 2 not recorded	16 male 21 female 4 discarded
<i>Weight, median (IQR)</i>	120 (96-131) g	808 (682-897) g
<i>Number of samples for PCR</i>		
<i>Urethra</i>	11	8
<i>Bladder</i>	11	8
<i>Ureter</i>	11	8
<i>Renal pelvis</i>	11	8
<i>Total</i>	44	32
<i>Number of samples for immunohistochemistry</i>		
<i>Urethra</i>	3	3
<i>Bladder</i>	3	6
<i>Ureter</i>	3	3
<i>Renal pelvis</i>	3	3
<i>Total</i>	12	15
<i>Number of samples for Western blotting</i>		
<i>Bladder</i>	8	7

Fig. 1. Expression of *AQPs* mRNA, results from QPCR. **A.** Bar graphs showing developmental expression of *AQPs* 1, 3, 9 and 11 mRNA in the 4 locations of the urinary tract. Gestational age is marked on the X-axis along with each of the four *AQPs*. Y-axis is relative *AQP* expression (median and IQR), normalized to the expression of 4 reference genes. It is the presence of *AQPs* and the change in expression level that is interesting rather than the absolute numbers. * $p < 0.05$. **B.** Relative expression levels comparing samples from male (gray) and female (white) fetuses at 60 days of gestation. There is no significant difference between sexes, except for *AQP3* in the bladder and *AQP1* in the ureter. * $p < 0.05$.

PCR: Expression of AQP mRNA during development

For PCR we selected fetuses with a complete set of snap frozen samples (urethra, bladder, ureter and renal pelvis). Eight fetuses at 100 days of gestation and eleven fetuses at 60 days of gestation met these criteria. Thus a total of 76 samples from 19 fetuses were included for RNA extraction. RNA was obtained from 71 of these.

AQP1, 3, 9 and 11 mRNA was expressed in all

locations. *AQP5* mRNA was expressed at low levels in urethra samples from both male and female fetuses, but not in any samples from other locations. We were not able to identify *AQP2*, 4, 6, 7, 8 and 10 mRNA in any samples. Concerning *AQPs* 2 and 6 we had adequate positive controls, suggesting that these *AQPs* were not expressed. With respect to *AQPs* 4, 7, 8 and 10 the primers did not work optimally. Results from QPCR are

shown in Figure 1. The expression of *AQP3* increased significantly at all four locations during gestation, whereas *AQP11* mRNA expression significantly decreased at all four locations. In the urethra and bladder we also found a significant increase in the expression of *AQP1* and *9* with gestational age. Expression of *AQP1* also increased significantly in the ureter whereas *AQP9* expression decreased. In the pelvis we did not find significant changes in expression of *AQP1* and *9* between gestation day 60 and 100. *AQP5* was expressed in the urethra at a low level that did not show significant change through gestation (data not shown). In the 60-day group we observed no significant difference in expression level between sexes, except for *AQP3* in the bladder where the male samples showed a slightly higher expression level and *AQP1* in the ureter, where the female samples showed a slightly higher expression level (Fig. 1B). In the 100-day group there were too few male samples to make a valid comparison.

Western blotting: *AQP1* in the fetal porcine bladder

For Western blotting we used 8 bladders from fetuses at 60 days of gestation and 7 bladders from fetuses at 100 days of gestation. Renal and bladder tissue from a juvenile pig was used as controls. Results are visualized in Figure 2 and confirm our QPCR data showing significantly higher *AQP1* protein levels at day 100 compared to day 60 of gestation. Pre-absorption with the control peptide was performed as a negative control in porcine kidneys and showed that pre-absorption of the *AQP1* antibody eliminates the binding of the antibody to the protein in the tissue.

Immunohistochemistry: *AQP1* localization in the fetal porcine urinary tract

In order to define how the *AQPs* were localized we performed immunohistochemistry. Figure 3 shows images from immunohistochemistry. Labelling with *AQP1*-antibody showed staining in the endothelial cells lining the vessels throughout the bladder wall, as well as vessels in the sub-urothelial layers of the urethra, the ureter and the renal pelvis. *AQP3*, *5*, *9* and *11* protein expression and localization could not be assessed due to the lack of suitable antibodies.

Discussion

The expression and function of *AQPs* has been examined widely in a number of tissues. However, the urinary tract has not been fully investigated in relation to

Fig. 2. Results from Western blotting. Developmental expression of *AQP1* in the bladder, confirming PCR results on the protein level. From the top: Immunoblot showing *AQP1* as two bands at 25 and 37 kDa (markers at 25 and 35 kDa). Protein samples from renal cortex (RC), juvenile bladder (JB), eight fetal bladders at 60 days of gestation and 7 fetal bladders at 100 days of gestation. Below is the immunoblot of *GAPDH* as the reference protein in identical samples. The graph at the bottom shows the calculated relative expression of *AQP1/GAPDH* (median and IQR) in the fetal bladders, with a clear increase in expression of *AQP1* from 60 to 100 days of gestation. * $p < 0.05$.

water channel expression and especially not during fetal development. The present study demonstrates in porcine fetuses the expression of *AQP1*, *3*, *9* and *11* mRNA at four locations along the urinary tract, at two time-points during gestation. In addition, *AQP5* mRNA transcript was detected in the urethra. Using Western blot analysis we confirmed increased *AQP1* protein level in the bladder with gestational age. Immunohistochemistry showed localization of *AQP1* in the endothelial cells lining the vessels throughout the wall of the entire urinary tract.

Previous studies on *AQPs* in the urinary tract have focused on the expression in the adult mammalian bladder. Vahabi *et al.* (2015) detected mRNA transcripts of *AQP1*, *3*, *9* and *11* in the juvenile porcine bladder, and confirmed the expressed proteins with immunohistochemistry. This is consistent with our findings showing that these same *AQPs* are expressed in the fetal porcine bladder already halfway through gestation. Similar to Vahabi *et al.* (2015) we also observed *AQP1* staining in the vessels. Furthermore, this study shows for the first time, that the same four *AQPs*

Fig. 3. Images from immunohistochemistry. *AQP1* labelling in endothelial cells lining vessels in the wall of the urinary tract in the same pattern at all four sample locations. Pelvis (**a**), ureter (**b**), female urethra (**c**), male urethra (note the prostatic tissue) (**d**), bladder (**e**). The kidney (**f**) serves as positive control, and we see a clear reaction in the brush border of proximal tubules, and a vague reaction in glomeruli, as expected.

are expressed in the upper urinary tract and the urethra as well. Rubenwolf *et al.* (2009) identified transcripts for *AQP3*, 4, 7, 9 and 11 in the urothelium of the adult human bladder and ureter, and confirmed findings on the protein level with immunohistochemistry for all but *AQP11*. Our findings are consistent with these results regarding *AQP3*, 9 and 11, but we were not able to identify *AQP4* and 7 in the porcine fetal bladder and

ureter. In contrast to Rubenwolf *et al.* (2009) we demonstrated expression of *AQP1*. This difference may very well be explained by the fact that Rubenwolf *et al.* (2009) only studied the urothelial layer – we found *AQP1* to be expressed in the vessels of the urinary tract wall, below the urothelium.

Studies on *AQP* expression during development are scarce. Xing and Nørregaard (2016) have described

increasing expression of *AQP1*, 2 and 3 in the fetal porcine kidney during gestation. In our study we also observed an increased expression with regards to most of the identified *AQPs*. In contrast, we found a significant decrease in the expression of *AQP11* mRNA at all locations of the urinary tract. We can only speculate on the reasons for this difference, since the functional importance of the *AQPs* in the urinary tract remains to be established. Involvement in the trans-urothelial transport of water seems to be a reasonable assumption. This assumption was generated through the work done by Nelson and coworkers on the American black bears, showing urine reabsorption from the bladder during hibernation (Nelson 1973, Nelson *et al.* 1975, Nelson *et al.* 1973, Spector *et al.* 2015). Spector *et al.* (2002) studied *AQPs* in adult rats and showed expression of *AQP2* and 3 in the urothelium and *AQP1* in the sub-urothelial vessels. Particularly *AQP3*, but also *AQP2*, were upregulated in the rats in response to dehydration. Kim *et al.* (2010, 2013a), investigating expression of *AQP1*, 2 and 3 in rat urinary bladder after partial bladder outlet obstruction (BOO), found that immunoreactivity of *AQP1* in both the control and the BOO groups was localized in the capillaries, arterioles, and venules in the lamina propria of the urinary bladder. The expression of *AQP1*, 2 and 3 was significantly increased in the BOO group.

It has been speculated whether the *AQPs* in the bladder are merely regulating local cell volume and tonicity, but these studies indicate that the *AQPs* might also mediate transport of water between urine and the bloodstream (Kim *et al.* 2013a, Kim *et al.* 2010, Spector *et al.* 2002). Further supporting trans-urothelial transport, Rubenwolf *et al.* (2012b) created a model with cultured human urothelial cells and demonstrated water and urea flux through *AQPs*, sensitive to osmolality.

Along with several other studies on changes in urine composition during passage and storage in the urinary tract (Cahill *et al.* 2003, Englund 1956, Levinsky and Berliner 1959, Shafik *et al.* 2005, Shafik *et al.* 2004), this justifies hypothesizing that adjusting water and salt homeostasis does not stop as the urine leaves the collecting tubules in the kidney, but continues as the urine is transported along the urinary tract and is exposed to the urothelium. It is also an interesting question whether the function of the urinary tract *AQPs* change from fetal to adult life.

Fetal urine production is greater than in the adult (Ervin *et al.* 1993). Furthermore, the urine pathway

being continuously “recycled” from the urinary tract into the amniotic cavity and then back into the fetus is certainly different from postnatal life, meaning that it is fair to assume that there may also be differences in the properties of the urinary tract surface from fetal to postnatal life.

Many questions remain unanswered concerning the role of *AQPs* in the urinary tract. Are they actors in regulating the universal water and salt homeostasis, or are they merely regulating local cell hydration and tonicity? Do they respond to changes in urine composition, stretch of the bladder wall or other factors? Do they have other functions not immediately related to water balance? For example, Rubenwolf and coworkers demonstrated downregulation of *AQP3* in urothelial carcinoma and a correlation between *AQP3* expression and prognosis (Otto *et al.* 2012, Rubenwolf *et al.* 2015, 2012a).

There is a need for better understanding not only the developmental aspects with respect to distribution and function of *AQPs* in the urothelium and sub-urothelial layers, but also their roles postnatally, in normal animals and in urinary tract diseases. Signaling pathways in lower urinary tract dysfunction might also include *AQPs*. From a clinical perspective, the fact that *AQPs* can be measured in the urine (Umenishi *et al.* 2002) and changes in response to bladder conditions, such as infra-vesical obstruction, render them a possible diagnostic or pharmacological target.

Conclusion

The present study shows that several *AQPs*, e.g. *AQP1*, 3, 5, 9 and 11 are expressed in the porcine urinary tract already during fetal life. Expression levels change during gestation: *AQP11* is downregulated, whereas other *AQPs* are upregulated in most locations. *AQP1* was demonstrated in endothelial cells of vessels in the bladder wall. The functional and developmental consequences of these findings have not been explored, but deserve further study.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

The expert technical assistance from Gitte Kall, Gitte Skou and Kristina Mortensen is highly appreciated. Thanks to The Lundbeck Foundation for granting financial support for the study.

References

- ANDERSSON K-E, MCCLOSKEY KD: Lamina propria: the functional center of the bladder? *Neurourol Urodyn* **33**: 9-16, 2014.
- BIRDER L, ANDERSSON K-E: Urothelial signaling. *Physiol Rev* **93**: 653-680, 2013.
- CAHILL DJ, FRY CH, FOXALL PJ: Variation in urine composition in the human urinary tract: evidence of urothelial function in situ? *J Urol* **169**: 871-874, 2003.
- CROWE R, BURNSTOCK G: A histochemical and immunohistochemical study of the autonomic innervation of the lower urinary tract of the female pig. Is the pig a good model for the human bladder and urethra? *J Urol* **141**: 414-422, 1989.
- ENGLUND SE: Observations on the migration of some labelled substances between the urinary bladder and the blood in the rabbit. *Acta Radiol Suppl*: 1-80, 1956.
- ERVIN MG, KULLAMA LK, ROSS MG, LEAKE RD, FISHER DA: Vasopressin receptors and effects during fetal development. *Regul Pept* **45**: 203-208, 1993.
- HICKS RM, KETTERER B, WARREN RC: The ultrastructure and chemistry of the luminal plasma membrane of the mammalian urinary bladder: a structure with low permeability to water and ions. *Philos Trans R Soc Lond B Biol Sci* **268**: 23-38, 1974.
- KIM S-O, SONG SH, AHN K, KWON D, PARK K, RYU SB: Changes in aquaporin 1 expression in rat urinary bladder after partial bladder outlet obstruction: preliminary report. *Korean J Urol* **51**: 281-286, 2010.
- KIM S-O, SONG SH, HWANG EC, OH KJ, AHN K, JUNG SI, KANG TW, KWON D, PARK K, RYU SB: Changes in aquaporin (AQP)2 and AQP3 expression in ovariectomized rat urinary bladder: potential implication of water permeability in urinary bladder. *World J Urol* **30**: 207-212, 2012.
- KIM S-O, CHOI D, SONG SH, AHN KY, KWON D, PARK K, RYU SB: Effect of detrusor overactivity on the expression of aquaporins and nitric oxide synthase in rat urinary bladder following bladder outlet obstruction. *Can Urol Assoc J* **7**: e268-e274, 2013a.
- KIM S-O, SONG SH, PARK K, KWON D: Overexpression of aquaporin-1 and caveolin-1 in the rat urinary bladder urothelium following bladder outlet obstruction. *Int Neurourol J* **17**: 174-179, 2013b.
- LEVINSKY NG, BERLINER RW: Changes in composition of the urine in ureter and bladder at low urine flow. *Am J Physiol* **196**: 549-553, 1959.
- LEWIS SA: Everything you wanted to know about the bladder epithelium but were afraid to ask. *Am J Physiol* **278**: F867-F874, 2000.
- NELSON RA: Winter sleep in the black bear. A physiologic and metabolic marvel. *Mayo Clin Proc* **48**: 733-737, 1973.
- NELSON RA, WAHNER HW, JONES JD, ELLEFSON RD, ZOLLMAN PE: Metabolism of bears before, during, and after winter sleep. *Am J Physiol* **224**: 491-496, 1973.
- NELSON RA, JONES JD, WAHNER HW, MCGILL DB, CODE CF: Nitrogen metabolism in bears: urea metabolism in summer starvation and in winter sleep and role of urinary bladder in water and nitrogen conservation. *Mayo Clin Proc* **50**: 1-9, 1975.
- NIELSEN S, KWON T-H, FRØKIAER J, KNEPPER MA: Key roles of renal aquaporins in water balance and water-balance disorders. *News Physiol Sci* **15**: 136-143, 2000.
- NYGARD A-B, JØRGENSEN CB, CIRERA S, FREDHOLM M: Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. *BMC Mol Biol* **8**: 67, 2007.
- OLSEN LH, DALMOSE AL, SWINDLE MM, JØRGENSEN TM, DJURHUUS JC: Male fetal pig lower urinary tract function in mid second and early third trimester of gestation. *J Urol* **165**: 2331-2334, 2001.
- OTTO W, RUBENWOLF PC, BURGER M, FRITSCHKE H-M, RÖBLER W, MAY M, HARTMANN A, HOFSTÄDTER F, WIELAND WF, DENZINGER S: Loss of aquaporin 3 protein expression constitutes an independent prognostic factor for progression-free survival: an immunohistochemical study on stage pT1 urothelial bladder cancer. *BMC Cancer* **12**: 459, 2012.
- RUBENWOLF PC, GEORGOPOULOS NT, CLEMENTS LA, FEATHER S, HOLLAND P, THOMAS DFM, SOUTHGATE J: Expression and localisation of aquaporin water channels in human urothelium in situ and in vitro. *Eur Urol* **56**: 1013-1023, 2009.

- RUBENWOLF PC, DENZINGER S, OTTO W: Aquaporin 3 protein expression in transitional cell carcinoma: a potential marker with regard to tumour progression and prognosis? *Eur Urol* **61**: 627-628, 2012a.
- RUBENWOLF PC, GEORGOPOULOS NT, KIRKWOOD LA, BAKER SC, SOUTHGATE J: Aquaporin expression contributes to human transurothelial permeability in vitro and is modulated by NaCl. *PLoS One* **7**: e45339, 2012b.
- RUBENWOLF P, THOMAS C, DENZINGER S, HARTMANN A, BURGER M, GEORGOPOULOS NT, OTTO W: Loss of AQP3 protein expression is associated with worse progression-free and cancer-specific survival in patients with muscle-invasive bladder cancer. *World J Urol* **33**: 1959-1964, 2015.
- SHAFIK A, SIBAI EL O, SHAFIK AA, AHMED I: Do vesical and voided urine have identical compositions? *Scand J Urol Nephrol* **38**: 243-246, 2004.
- SHAFIK A, SHAFIK I, SIBAI EL O, SHAFIK AA: Changes in the urine composition during its passage through the ureter. A concept of urothelial function. *Urol Res* **33**: 426-428, 2005.
- SHAFIK A, AHMED I, SIBAI EL O, SHAFIK AA: Does the composition of voided urine reflect that of the renal pelvis? *Urol Res* **34**: 261-264, 2006.
- SPECTOR DA, WADE JB, DILLOW R, STEPLOCK DA, WEINMAN EJ: Expression, localization, and regulation of aquaporin-1 to -3 in rat urothelia. *Am J Physiol Renal Physiol* **282**: F1034-F1042, 2002.
- SPECTOR DA, DENG J, COLEMAN R, WADE JB: The urothelium of a hibernator: the American black bear. *Physiol Rep* **3**: e12429, 2015.
- SUZUKI M, TANAKA S: Molecular and cellular regulation of water homeostasis in anuran amphibians by aquaporins. *Comp Biochem Physiol A Mol Integr Physiol* **153**: 231-241, 2009.
- UMENISHI F, SUMMER SN, CADNAPAPHORNCHAI M, SCHRIER RW: Comparison of three methods to quantify urinary aquaporin-2 protein. *Kidney Int* **62**: 2288-2293, 2002.
- VAHABI B, MANSO M, DRAKE MJ: Expression of aquaporin channels in pig urinary bladder. *Eur Urol Suppl* **14**: e796, 2015.
- WALSER BL, YAGIL Y, JAMISON RL: Urea flux in the ureter. *Am J Physiol* **255**: 244-249, 1988.
- XING L, NØRREGAARD R: Influence of sex on aquaporin1-4 and vasopressin V2 receptor expression in the pig kidney during development. *Pediatr Res* **80**: 452-459, 2016.
- XU H, BIONAZ M, SLOBODA DM, EHRLICH L, LI S, NEWNHAM JP, DUDENHAUSEN JW, HENRICH W, PLAGEMANN A, CHALLIS JR, BRAUN T: The dilution effect and the importance of selecting the right internal control genes for RT-qPCR: a paradigmatic approach in fetal sheep. *BMC Res Notes* **8**: 58, 2015.
-