Aquaporin Expression in the Fetal Porcine Urinary Tract

Changes During Gestation

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Short title: AQP expression in the urinary tract during gestation

Summary

Background

The expression of aquaporins (*AQPs*) in the fetal porcine urinary tract and its relation to gestational age has not been established.

Methods

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.

Results

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.

Conclusion

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, protein expression, pig

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; 2005; 2004; Walser et al. 1988). Furthermore, there is evidence that the urothelium might be involved in sensing and signalling 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; 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; 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; 2010; 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^{o}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™) 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 seconds of denaturation at 95°C followed by 1 minute 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 ($E = 10^{-1/slope}$) 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:

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

REF = Geometric mean RNA quantity of four reference genes

AQP = Aquaporin of interest RNA quantity

Immunohistochemistry

Tissue was immersed in 10 % formalin for 24 hours, washed in PBS buffer, dehydrated and embedded in paraffin wax. Sections of 3 µm were cut, deparaffinised 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^{o}C for

a total of 44 minutes with addition of further CC2 every four minutes. Primary antibody (*AQP1*, Alomone Labs, Jerusalem, Israel, cat.no: *AQP*-001) was diluted 1:1000 in DAKO diluent and sections were incubated with this for 32 minutes. 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 minutes at 50 Hz by a TissueLyser LT (Qiagen, Hilden, Germany) in RIPA buffer (50mM Tris-HCl, 150 mM NaCl, 1mM Na_2EDTA, 1% triton X-100, 0.5% sodium deoxycholate, pH 7.4). Homogenates were centrifuged for 10 min at 1000 G at 4^{o}C. Using a Pierce BCA protein assay kit (Roche) 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 Na_2HPO_4, 20 mM NaH_2PO_4, 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^{o}C (*AQP1*, Alomone Labs, Jerusalem, Israel, cat.no: AQP-001, 1:500). Horseraddish peroxidase-conjugated secondary antibody (p448 goat anti-rabbit immunoglobulin, DAKO, Glostrup, Denmark) was added and incubated for 1

hour at room temperature. Antigen-antibody reactions were visualized using an enhanced chemiluminescence system (Amersham ECL Plus, GE Healthcare) 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, 1:2000, 20 µg of protein mounted). Pre-absorption with the control peptide (*AQP1*, 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.

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 (Figure 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 confirms 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 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. (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, we also observed *AQP1* staining in the vessels. Furthermore, this study shows for the first time, that the same four *AQPs* are expressed in the upper urinary tract and the urethra as well. Rubenwolf et al. 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* (Rubenwolf et al. 2009). 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. we demonstrated expression of *AQP1*. This difference may very well be explained by the fact that Rubenwolf et al. 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 et al. have described increasing expression of *AQP1, 2* and *3* in the fetal porcine kidney during gestation (Xing and Nørregaard 2016). 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 et al in the 1970ies on the American black bears, showing urine reabsorption from the bladder during hibernation (Nelson 1973; Nelson et al. 1975; 1973; Spector et al. 2015). Spector et al. 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 (Spector et al. 2002). Kim et al., 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 (Kim et al. 2010; 2013a).

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; 2010; Spector et al. 2002). Further supporting trans-urothelial transport, Rubenwolf et al. created a model with cultured human urothelial cells and demonstrated water and urea flux through *AQPs*, sensitive to osmolality (Rubenwolf et al. 2012b).

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; 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. Whether the function of the urinary tract *AQPs* change from fetal to adult life is also an interesting question. 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 et al. 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.

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

Table 1:

Primer sequences and positive control tissues for PCR

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.

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

Table 2:

Material

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

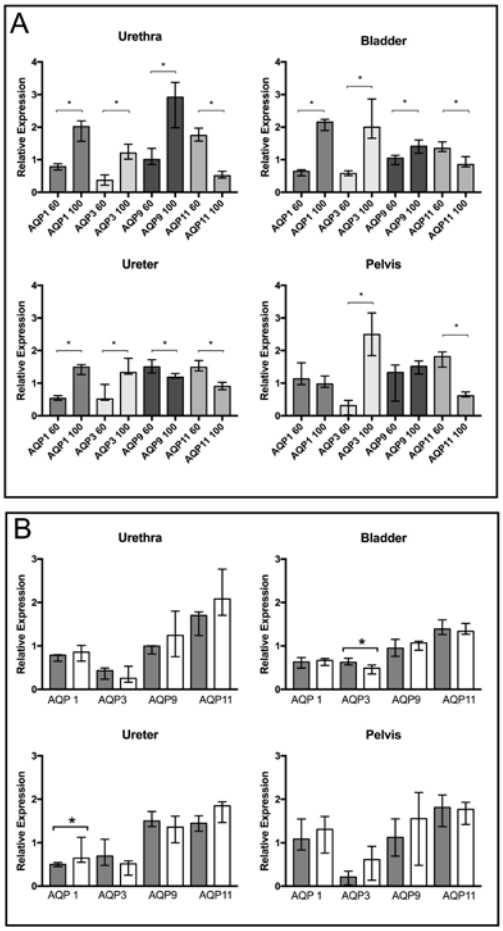

Figure 1:

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

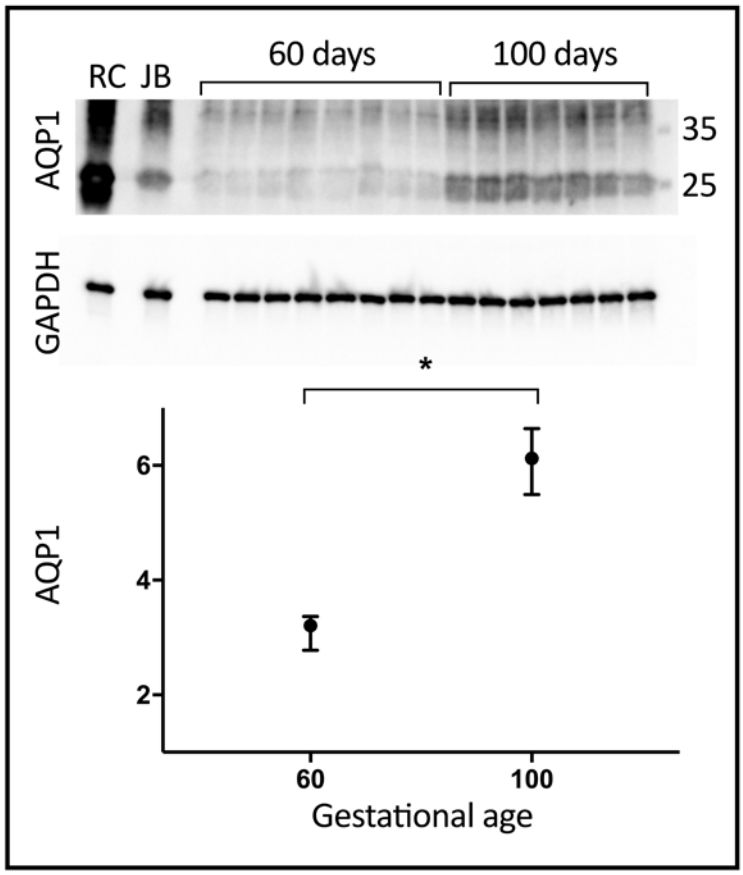

Figure 2:

Results from Western Blotting

Developmental expression of *AQP 1* 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.

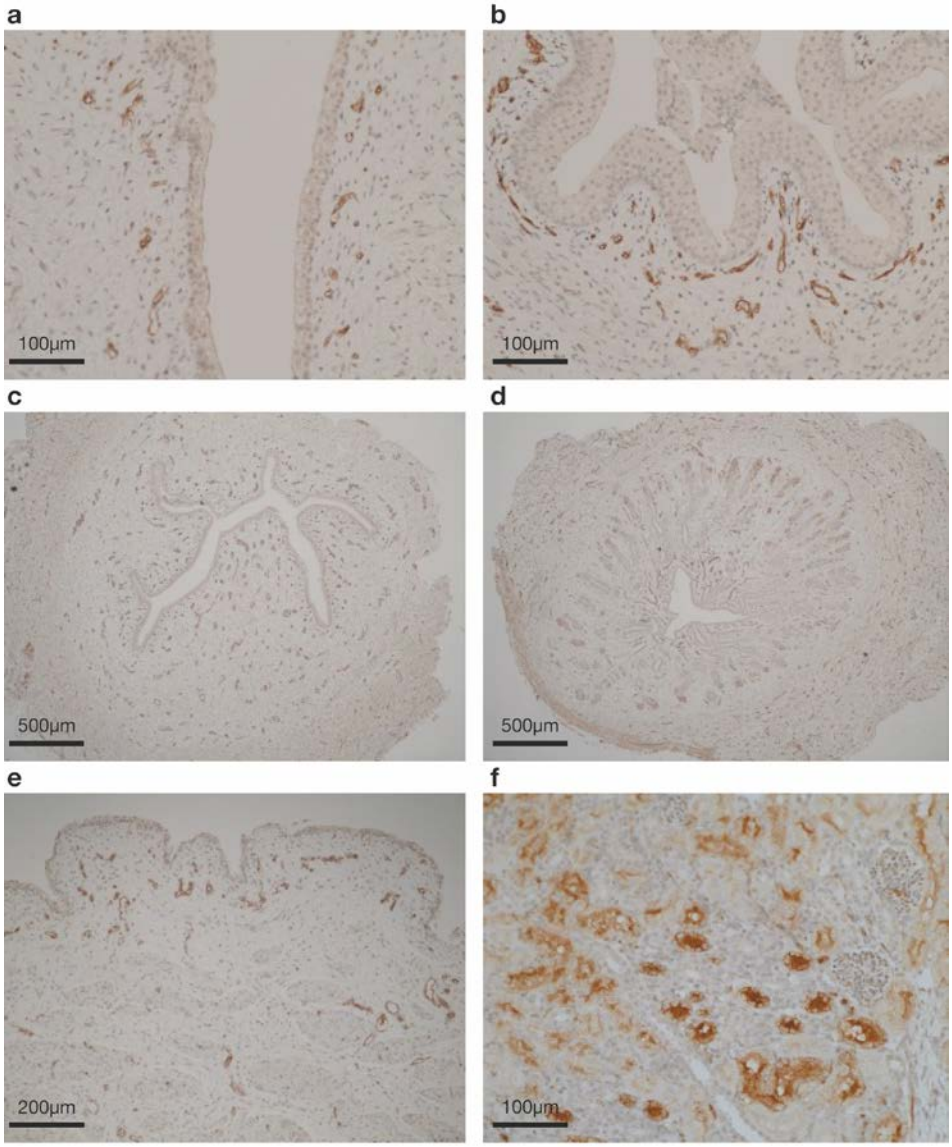

Figure 3:

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.

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