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AQP5 Is Expressed In Type-B Intercalated Cells in the Collecting Duct System of the Rat, Mouse and Human Kidney

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Key Words

AQP5 • Kidney • Pendrin • Aquaporin • Intercalated cells • Stem cells

Abstract

We screened human kidney-derived multipotent CD133+/CD24+ ARPCs for the possible expression of all 13 aquaporin isoforms cloned in humans. Interestingly, we found that ARPCs expressed both AQP5 mRNA and mature protein. This novel finding prompted us to investigate the presence of AQP5 *in situ* in kidney. We report here the novel finding that AQP5 is expressed in human, rat and mouse kidney at the apical membrane of type-B intercalated cells. AQP5 is expressed in the renal cortex and completely absent from the medulla. Immunocytochemical analysis using segment- and cell type-specific markers unambiguously indicated that AQP5 is expressed throughout the collecting system at the apical membrane of type-B intercalated cells, where it co-localizes with pendrin. No basolateral AQPs were detected in type-B intercalated cells, suggesting that AQP5 is unlikely to be involved in the net trans-epithelial water reabsorption occurring in the distal tubule. An intriguing hypothesis is that AQP5 may serve an osmosensor for the composition of the fluid coming from the thick ascending limb. Future studies will unravel the physiological role of AQP5 in the kidney.

Introduction

AQUAPORINS (AQPs) are a family of water-selective transporting proteins sharing high level of homology with major intrinsic protein (MIP) of lens [1]. The aquaporins play different roles in body water homeostasis with respect to permeability and localization. Following the discovery of the first mammalian water channel, AQP1 [2, 3], twelve other isoforms have been identified on the base of sequence homology [4]. Of the known 13 aquaporin isoforms expressed in mammals (AQP0-12), it has been reported that at least 8 are expressed in the kidney at distinct sites along the nephron and collecting duct: AQP1-4, AQP6-8 and AQP11 [4-7].

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However, a single physiological role, in the process of urine concentration occurring along the kidney tubule, has been attributed only to AQP1-4.

AQP1 is abundant in the proximal tubule, descending thin limb and *vasa recta* and mediates isosmotic water reabsorption in the proximal nephron [8].

AQP2 is abundant in the collecting duct principal cells and is the chief target for the regulation of collecting duct water reabsorption by vasopressin during antidiuresis [9-12]. AQP3 and AQP4 are basolateral water channels located in the kidney collecting duct principal cells and act as exit pathways for water reabsorbed via AQP2 [13, 14].

AQP6 is present in the collecting duct intercalated type-A cells [15]. At subcellular level, AQP6 is exclusively present in an intracellular location, with no expression in the plasma membrane. The physiological role of AQP6 is still undefined, but its location and anion permeability have suggested a role in the acidification of vesicles. The physiological roles of AQP7-8 and 11 are still under investigation.

The primary physiological role of most renal AQPs in regulating water balance has been well clarified by generating KO animal models and by studying naturally-occurring mutations in humans [4]. However, the possibility that AQPs may exert new unexplored functions in kidney physiology cannot be ruled out. In this regard, it is interesting that AQP1 and AQP2 have been found in adult renal progenitor/stem cells (ARPCs) isolated from the S3 segment of rat kidney tubule, suggesting a role for AQPs in the regeneration processes taking place in the kidney tubules [16].

In the present study, we show, for the first time, that AQP5 is expressed in ARPCs from human kidney isolated as previously described [17].

The AQP5 gene was first cloned from salivary gland cDNA and then evidenced in eye, salivary and lacrimal glands, lung, trachea and cochlea, displaying an apical localization in each case [18-21].

Our original observation that AQP5 is expressed in ARPCs isolated from human kidney prompted us to explore the presence of AQP5 in mammalian kidney. Analysis of transcripts and protein expression, together with immunolocalization studies were performed *in situ* and revealed that AQP5 is actually expressed in the kidney of human, rat and mouse at the apical membrane of pendrin-positive intercalated cells. The fact that AQP5 expression and localization occur within the kidney, as established in this study, raises questions concerning its potential role in kidney function and dysfunction.

Materials and Methods

Antibodies

Anti AQP1 (Rabbit polyclonal purified IgG) and anti AQP5 (rabbit-anti human AQP5 or goat-anti human AQP5 purified IgG) antibodies were from Santa Cruz Biotechnology (www.scbt.com). Rabbit-anti-rat AQP5 antibodies were from Alomone Labs (www.alomone.com). Rabbit-rat-human AQP5 (Alomone) and Goat-anti-human AQP5 (Santa Cruz) were also pre-adsorbed to the corresponding immunizing peptides to verify the specificity of the obtained signals.

Rabbit affinity-purified polyclonal antibodies against rat AQP2 and human AQP3 were previously described [22, 23]. Anti-pendrin monoclonal antibodies were from MBL® (www.mblintl.com). Rabbit polyclonal anti V-ATPase, produced as previously described, was a kind gift from Prof. Dennis Brown (Massachusetts General Hospital, Boston, USA). The polyclonal R5 antibody against phospho-NKCC2, produced as previously described [24], was a kind gift from Prof. Biff Forbush (Yale University, New Haven, CT). Monoclonal anti Calbindin-D28k (clone CB-955) was from Sigma.

ARPC isolation and culture

CD133-positive ARPCs were isolated and characterized as previously described [17]. Briefly, Cortex renal fractions were dissected by the passage through a graded series of meshes steel sieves to remove the fibrous component. After several washes, the isolated fractions were cultured in EGM-MV medium (Lonza, Valais, Switzerland) supplemented with 20% FBS (Sigma-Aldrich). After 4-5 days, cultures were washed twice with Hanks' buffer to remove nonadherent cells and after about 1 week in culture, cell viability and number were checked. CD133-positive cells were then isolated by magnetic cell separation technology (MACS) by means of CD133Ab-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The CD133-positive cells retained inside the column were washed 3 times and then eluted once the magnetic field was removed. The eluted cells were resuspended and maintained in EGM-MV medium from Lonza (www.lonza.com) supplemented with 20% FBS from Sigma (www.sigmaaldrich.com) and incubated at 37°C with 5.0% CO2.

After cell expansion, ARPCs markers were checked by cytofluorimetric determination and by cell immunofluorescence microscopy. Cytofluorimetric analysis was performed using a Partec Flow-Max cytofluorimeter (Partec, Münster, Germany). Each determination was performed on 10^5 cells.

The following antibodies were used: PE-conjugated anti-CD133/2 (293C3), FITC-conjugated anti-CD34, and FITC-conjugated anti-CD45 (all from Miltenyi Biotec); FITC-conjugated anti-CD105 and FITC-conjugated anti-CD24 (all from Serotec, Oxford, UK); and FITC-conjugated anti-CD44 (from Instrumentation Laboratory, Milan, Italy). FITC-conjugated mouse IgG1 (Serotec) was used as an isotype control. In all cytofluorimetric determinations performed using Miltenyi antibodies, nonspecific sites were blocked with the FcR blocking reagent (Miltenyi Biotec). Immunofluorescence experiments were performed using the following primary antibodies: mouse anti-human CD133/1 mAb (clone AC133;

SPECIES	GENE	PRIMER SEQUENCE (5'-3')	PRODUCT LENGTH (bp)
human	*AQP1*	F:GTCCAGGACAACGTGAAGGT R:ACCCTGGAGTTGATGTCGTC	656
human	*AQP2*	F:CTGTGTTCGCAGAGTTCCTG R:GGGCAGGATTCATAGAGCAG	525
human	*AQP3*	F:GTCACTCTGGGCATCCTCAT R:GGCCAGCTTCACATTCTCTT	654
human	*AQP4*	F:AGATCAGCATCGCCAAGTCT R:GGTCAACGTCAATCACATGC	591
human	*AQP5*	F:CCACCCTCATCTTCGTCTTC R:TCACTCAGGCTCAGGGAGTT	643
human	*AQP6*	F:CGGGGCTGTATGTGTTCTTT R:TCTACGGTGCCTGTGAGGAT	661
human	*AQP7*	F:TCAACTTGGGTTTTGGCTTC R:ATAGGCACCCAGAAGTGGTG	590
human	*AQP8*	F:GCCTGTCGGTCATTGAGAAT R:CCATCTCCAATGAAGCACCT	598
human	*AQP9*	F:GTTCTTGGGCAGGTTCATCT R:AAAGGGCCCACTACAGGAAT	669
human	*AQP10*	F:GGTCAAGCTCCCCATTTACA R:CTGAGGCAGGAGTTTCCAAC	590
human	*AQP11*	F:TTCTAGCCACCTTCCAGCTC R:CCATGGAAGGAAAAAGCTGA	629
human	*AQP12*	F:ACTGTGTCCCTGCAGGAGTT R:TCTTCTGGCCGTAGAACAGG	520
human	*AQP0*	F:TGAGTTCTTTGCCACCCTCT R:CGGGGGAAGAGAAGAAAGTC	633
Mouse	*AQP5*	F:GGCCCTCTTAATAG GCAACC R:TTGCCTGGTGTTGTGTTGTT	177
Rat	*AQP5*	F:TTTCCAGCTAGCCCTCTGCATCTT R:CAGCTCGATGGTCTTCTTCC	369

Table 1. Primers used for RT-PCR analysis.

Miltenyi Biotec), mouse antihuman CD133/2 mAb (Miltenyi Biotec), rabbit anti-human Pax-2 pAb (Covance, Princeton, NJ), mouse anti-human CD105 mAb (Abcam, Cambridge, UK), mouse anti-human CD24 mAb (Dako, Glostrup, Denmark), mouse anti-human CD44 mAb (Chemicon, Temecula, CA, USA), mouse anti-human Bmi1 mAb (Upstate Biotechnology, Lake Placid, NY, USA), rabbit anti-human Oct-4 pAb (Abcam). The following secondary antibodies were used: Alexa Fluor 555 goat anti-mouse IgG, Alexa Fluor 488 goat anti-rabbit IgG, and Alexa Fluor 488 goat anti-mouse IgG1 (all from Molecular Probes).

AQP5/M-1 cells were obtained by stably transfecting the mouse collecting duct M-1 cell line [25] with the cDNA coding for the human AQP5 gene (www.origene.com) subcloned into the pcDNA3.1 vector obtained from Invitrogen (www.invitrogen.com). Cells were transfected by Lipofectamine®2000 (Invitrogen), according to the manufacturer's protocol and positive clones selected for 10-15 days in the presence of Geneticin (500 µg/ml, Invitrogen) in the culture medium. Cells were cultured in DMEM/F12, 1:1, supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 5 µM dexamethasone as previously described [26].

Animals

All the experiments involving animals were performed on male 3 month-old Wistar rats and 8 week-old c57BL/6 mice obtained from Harlan (www.harlan.com). The studies were performed in accordance with animal welfare laws and in conformity with the Italian Guidelines for the use of laboratory animals, which conforms to the European Community Directive published in 1986 (86/609/EEC).

Human samples

Human kidney samples were obtained from fresh human renal cortical tissue harvested from patients diagnosed with renal carcinoma undergoing a nephrectomy or undergoing a kidney biopsy with approval of the local ethics committee. All patients gave signed consent for the use of their tissue for research purposes at the time of radical nephrectomy. Portions of normal-appearing cortex were isolated surgically and examined histologically to exclude presence of carcinoma.

Animal samples

Kidneys and lungs were rapidly excised and either preserved in RNAlater (Ambion) for RNA extraction or stored at -80 °C for protein extraction and Western blotting experiments.

For immunofluorescence experiments, kidney samples were fixed in 4% paraformaldehyde in phosphate-buffered solution (PBS) overnight at 4 °C and infiltrated in 30% sucrose in PBS for 12 h. Tissues were then frozen in dry ice in Shandon Cryomatrix (www.thermoscientific.com), and 5 µm sections were cut with a cryostat. For western blotting analysis, the kidney cortex and inner medulla/papilla were dissected under a stereomicroscope before protein extraction.

RNA isolation, RT-PCR and cDNA sequencing

Total RNA was extracted from adult renal stem cells, human kidney, rat lung, rat kidney, mouse lung and mouse kidney by the TRIzol extraction method (TRIzol reagent, Life Technologies, Invitrogen). The RNA was then used to amplify fragments of the cDNA of the AQPs using SuperScriptTM One-Step RT-PCR with Platinum® Taq (Invitrogen). The primers (Table 1) were designed on the basis of the AQP nucleotide sequences available in the GenBank database (web site: www.ncbi.nlm.nih.gov/Entrez/nucleotide.html). All primer pairs were chosen to hybridize with cDNA sequences derived from different exons, thereby excluding those amplimers arising from genomic DNA contamination. A positive control was performed by using primers specific for human GAPDH cDNA and rat and mouse ß-actin cDNA (Table 1).

PCRs were performed with the following program:

(55°C, 30 min; 94°C, 5 min) x 1 cycle;

(95°C, 15 sec; 55°C, 1 min; 72°C, 1 min) x 40 cycles;

(72°C, 5 min) x 1 cycle.

The sequences of the cloned DNA fragments were assessed by sequencing.

Protein extraction and Western blotting analysis

ARPCs, AQP5/M-1 cells and kidney samples from humans, rats and mice were solubilized in RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% Deoxycholate, 5 mM EDTA) containing protease inhibitors (20 mmol/l pepstatin, 20 mmol/l leupeptin, and 1 mmol/l phenylmethylsulfonyl fluoride). Lysates were sonicated for 30 s and centrifuged at 13,000g for 15 min at 4 °C. The protein concentration of the supernatants was determined.

Protein extracts were separated by SDS-PAGE on 12% polyacrylamide gels under reducing conditions. Protein bands were electrophoretically transferred to ImmobilonP membranes (www.millipore.com) for Western blot analysis, blocked in TBS-Tween containing 3% BSA and incubated with primary antibodies. Immunoreactive bands were detected with secondary antibody conjugated to horseradish peroxidase (HRP). After each step, the membranes were washed with TBS-Tween. Membranes were developed with SuperSignal West Pico Chemiluminescent Substrate (www.piercenet.com) and chemiluminescence detected with Chemidoc™XRS detection system equipped with Image Lab™ Software for image acquisition (www.bio-rad.com).

Immunofluorescence on kidney cryosections

Sections were subjected to antigen retrieval procedure before immunostaining. Briefly, sections were treated with 1% SDS in PBS for 10 minutes at room temperature (RT). After three washes in PBS, sections were blocked in saturation buffer (1% bovine serum albumin in PBS) for 20 min at RT and incubated with the primary antibodies for 2 h at RT (Dilution: AQP1/AQP2/AQP3 1:500; AQP5/pendrin 1:250; calbindin-D28k 1:1000; V-ATPase 1:100) in saturation buffer. After three washes in PBS, sections were incubated with the appropriate Alexa Fluor®-conjugated secondary antibodies (www.invitrogen.com) for 1 h at RT. Fluorescent images were acquired on an inverted Leica LSM TSC SP2 AOBS confocal microscope (www.leica-microsystems.com) equipped with a 63× oil-immersion objective. Multiple color images were acquired by scanning in sequential mode to avoid cross-excitation of the different fluorophores.

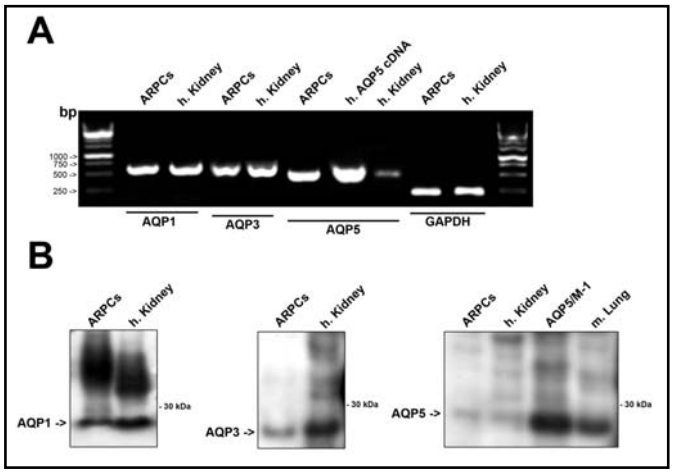

Fig. 1. Expression of AQPs in the adult renal progenitor/stem cells (ARPCs) by RT-PCR and Western blotting. A) Total RNA from cultured ARPCs and human kidney biopsy was probed for the presence of mRNA coding AQP0-12. Strong signals for AQP1, 3 and 5 were detected, and reported while the presence of mRNA for the other AQPs was not detected in ARPCs (not shown). Control RT-PCR was performed using primers amplifying human GADPH mRNA. The AQP5 amplimers obtained from ARPCs and human kidney were the same size as those obtained using human AQP5 cDNA as PCR template. B) Total protein extract from ARPCs and human kidney biopsy were separated by SDS-PAGE and immunoblotted for the presence of AQP1, AQP3 and AQP5. AQP5-transfected M-1 cells (AQP5/M-1) and mouse lung were used as additional controls for the size of the AQP5 band revealed in ARPCs and human kidney. Anti AQP5 antibodies used were Rabbit-anti-rat AQP5 (Alomone Labs.), see Methods.

Results

Isolation and Characterization of ARPCs

CD133-positive ARPCs were isolated by magnetic sorting, starting from healthy sections of kidney removed during resection for renal carcinoma. Confocal microscopy and FACS analysis showed that the recovered $CD133^+$ populations were homogeneously positive for CD133, CD24 and PAX-2 (supplemental Fig. 1 A, B, E), previously described as markers of adult renal progenitors. However, CD34, CD105, and CD45 membrane proteins were not detectable (data not shown). Moreover, these $CD133^+$ cells expressed the adult SC marker BMI-1, the blastocyst stem cell marker Oct-4, and the hyaluronic acid receptor CD44 (supplemental Fig. 1 C, D, F). See supplemental Fig. 1 at http://www.biologia.uniba.it/dfga/english/didattica/Fig.%201%20supplemental.tif.

Aquaporin expression in ARPCs

The presence of AQP transcripts was evaluated by RT-PCR. Human GAPDH amplification was the positive control in the PCR reactions. AQP1, AQP3, and AQP5 transcripts were detected in ARPC samples from all three patients (Fig. 1A). PCR products at the expected molecular size were obtained for AQP1 (656 bp), AQP3 (654 bp) and AQP5 (643 bp). As positive controls, AQP1, AQP3 and AQP5 mRNA were amplified by RT-PCR from total RNA extracted from human kidney biopsies. Interestingly, AQP5 transcripts were also amplified from human kidney RNA samples. The size of the AQP5 RT-PCR product was also compared to that obtained using human AQP5 cDNA as the template for a PCR reaction. This provides *prima facie* evidence that ARPCs express AQPs, at least at the transcript level. By contrast, transcripts for AQP0, 2, 4, and AQP6-12 were not detected in ARPCs (data not shown).

Having detected AQP expression at the mRNA level, the presence of the corresponding protein was examined in ARPCs by Western blotting.

AQP1, AQP3 and AQP5 protein bands were all immunodetected in cell lysates from ARPCs (Fig. 1B). AQP1 and AQP3 protein bands were also revealed, as

expected, in human kidney homogenates. Interestingly, a protein band corresponding to AQP5 was revealed in ARPCs and human kidney homogenate. As positive control we used M-1 renal cortical collecting duct cells [26], stably transfected with human AQP5 cDNA and mouse lung homogenate. The specificity of the anti AQP5 antibody (Alomone Labs; www.alomone.com) was tested by preadsorbing the antibody with the immunizing peptide (see Fig. 2B).

AQP5 is expressed in mouse, rat and human kidney

The original observation that AQP5 is expressed in human kidney and ARPCs isolated from human kidneys prompted us to investigate the endogenous expression of AQP5 in kidneys from other species. We isolated total RNA from mouse, rat and human kidney and from mouse and rat lung, and performed RT-PCR with specific primers for AQP5. Rodent beta-actin and human GAPDH amplification was the positive control for the efficiency of retrotranscription. Results, shown in Fig. 2A, indicated that RT-PCR products of the same size were obtained from both kidney and lung mRNAs of mouse and rat.

AQP5 protein expression was examined by Western blotting and reported in Fig. 2A. Forty μg of protein extracts from kidney papilla and cortex from mouse and rat, human kidney cortex were separated by SDS-PAGE and probed with anti AQP5 purified antibodies. In parallel, 10 μg of rat lung and AQP5/M-1 cells protein extracts were separated on the same gel as positive controls. A sharp band at the expected molecular size (≈28 kDa) was revealed in all samples. It has to be underlined that in the kidney fractions immunoreactive protein bands were obtained only when Western blotting detection was performed using the SuperSignal West Femto substrate (Thermo Fisher Scientific, Rockford, IL, USA). This indicates that the abundance of AQP5 in the native kidneys is very low.

In both mouse and rat AQP5 was detected exclusively in the kidney cortex and not in the kidney papilla. The specificity of the obtained signal was verified by preadsorbing the antibody on the immunizing peptide. In this condition the antibody failed to detect immunoreactive bands in the same samples (Fig. 2B; preadsorbed). Comparable results were obtained with a second commercial antibody against AQP5 (Rabbit-anti-human AQP5, Santa Cruz Biotechnology, see supplemental Fig. 2 for results). See supplemental Fig. 2 at http://www.biologia.uniba.it/dfga/english/didattica/Fig.2%20supplemental.tif.

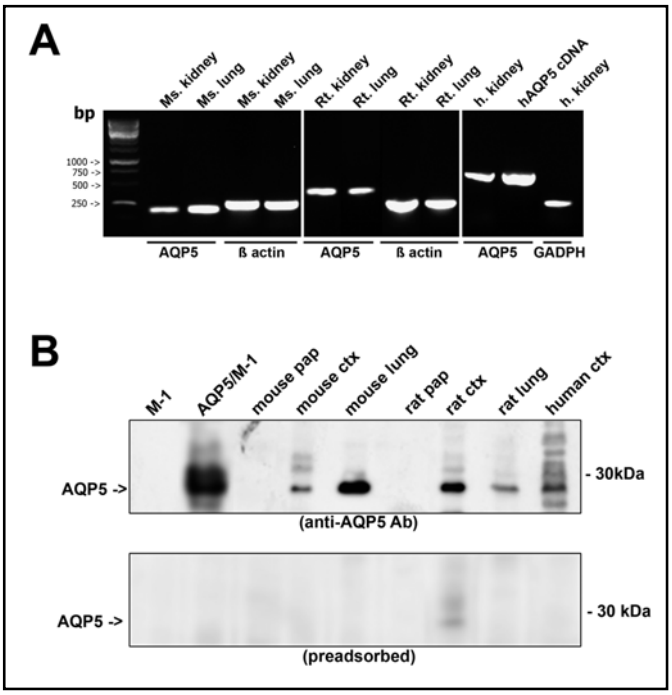

Fig. 2. Expression of AQP5 in mouse, rat and human kidney by RT-PCR and Western blotting. A) Total RNA from kidney from mice, rat and human was probed for the presence of mRNA coding AQP5. Amplimers obtained from kidney samples are the same size as those obtained from lung RNA samples. Control RT-PCR was performed using primers amplifying human GADPH and rodent beta-actin mRNAs. B) Total protein extract from kidney cortex and papilla from mouse and rat and human kidney biopsy were separated by SDS-PAGE and immunoblotted for the presence of AQP5. AQP5-transfected M-1 cells (AQP5/M-1), mouse lung and rat lung were used as additional controls for the size of the AQP5 band revealed in the kidney samples. AQP5 was only detected in the kidney cortex samples. Anti AQP5 antibodies used were Rabbit-anti-

For AQP5 subcellular localization, kidneys from rat and mouse were immunostained with anti-AQP5 antibodies and analyzed by confocal microscopy. Sections were immunostained with goat-anti-human AQP5 antibody (Santa Cruz Biotechnology). Figure 3 reports the superimposition of a single optical section of AQP5 staining, obtained with confocal laser scanning equipment, on the phase-contrast image obtained from rat and mouse kidneys. Pictures were taken in the renal cortex, outer medulla and inner medulla/papilla from rat and mouse samples. Figure 3 is representative of 10 randomly-chosen photographs taken in each kidney region from 5 different rats and mice. The first evidence arising from this analysis is that, in both species, AQP5 staining (Fig. 3, red signal) showed a discontinuous pattern associated with the apical plasma membrane of epithelial cells lining the kidney

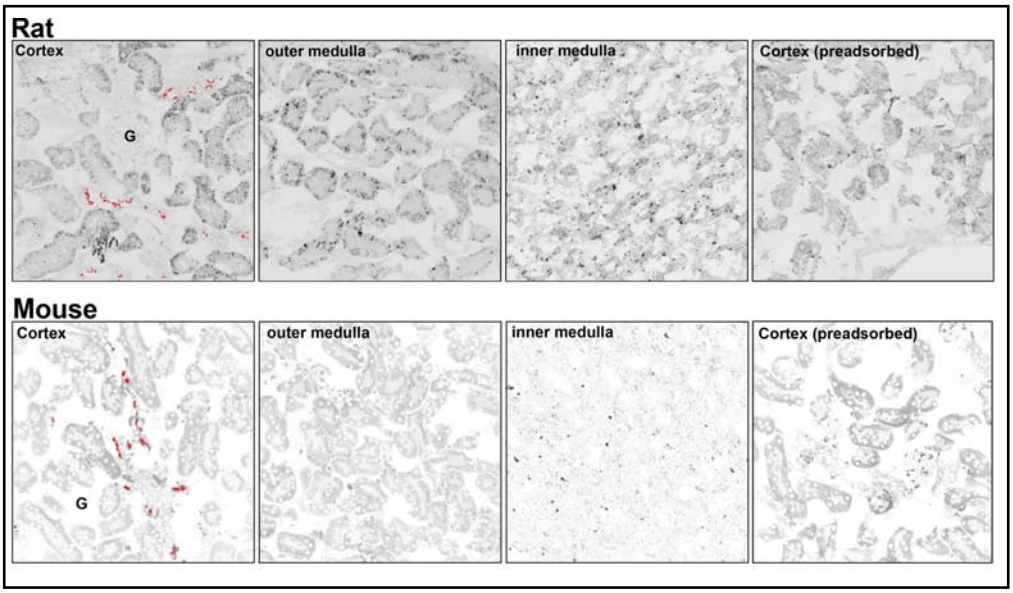

Fig. 3. Immunofluorescence detection of AQP5 in rat and mouse kidney. AQP5 staining (red signal) was observed only in the renal cortex of both rat and mouse kidney samples. No immunoreactivity was found in the kidney medulla. Labeling was completely absent in kidney slices incubated with preadsorbed antibody. Pictures are representative of the staining obtained in 3 independent experiments on 3 different animals for each species. G, glomeruli. Anti AQP5 antibodies used were Goat-anti-human AQP5 (Sant Cruz Biotechnology), see Methods.

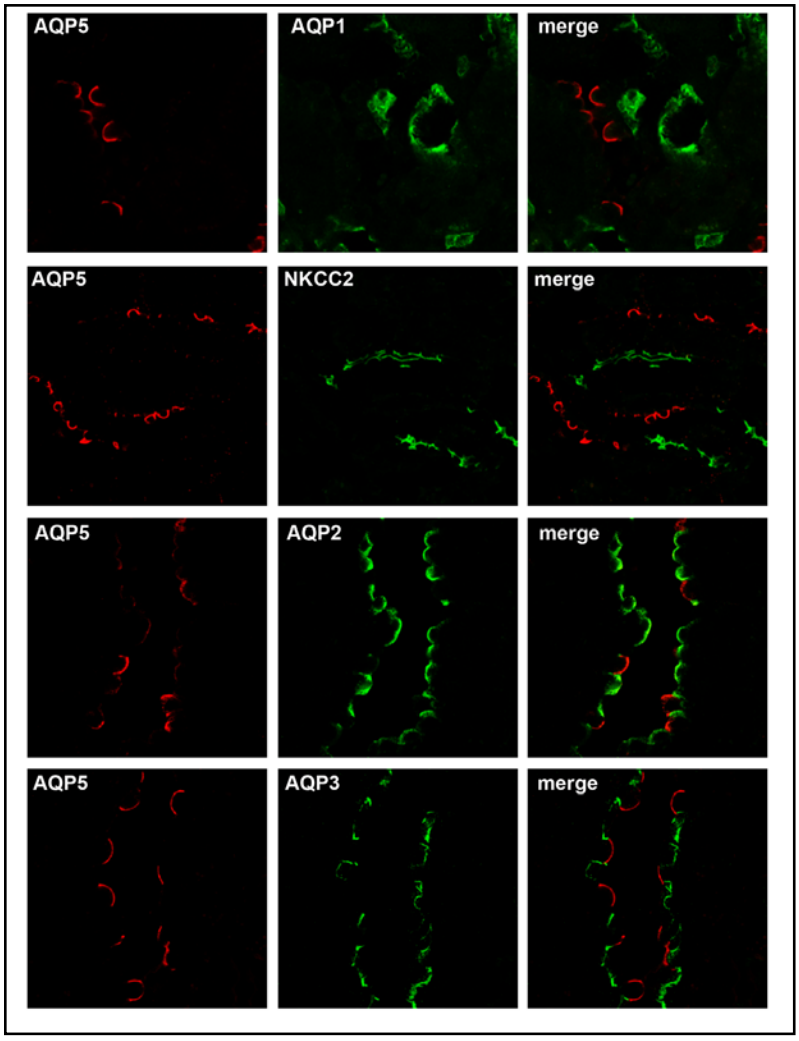

Fig. 4. Immunofluorescence detection of AQP5 in different nephron segments in rat kidney. No staining for AQP5 (red) is detectable either in AQP1-positive tubules (PT and TDL), or in NKCC2-positive tubules (TAL). AQP5 is present in the apical membrane of a subpopulation of epithelial cells in AQP2/AQP3-positive tubules (CNT and CD). AQP5-positive cells do not express either AQP2 or AQP3, indicating that they are intercalated cells. PT, proximal tubule; TDL, thin descending limb; TAL, thick ascending limb; CNT, connecting tubule; CD, collecting duct. AQP5 antibodies used were Goat-anti-human AQP5 (Sant Cruz Biotechnology), see Methods.

tubules. The second evidence is that the presence of AQP5-positive cells is exclusively confined to the cortical portion of the kidney, the staining being virtually absent from the outer and inner medulla. The immunoreactivity of the antibody was completely abolished after preadsorption with the immunizing peptide. Unfortunately,

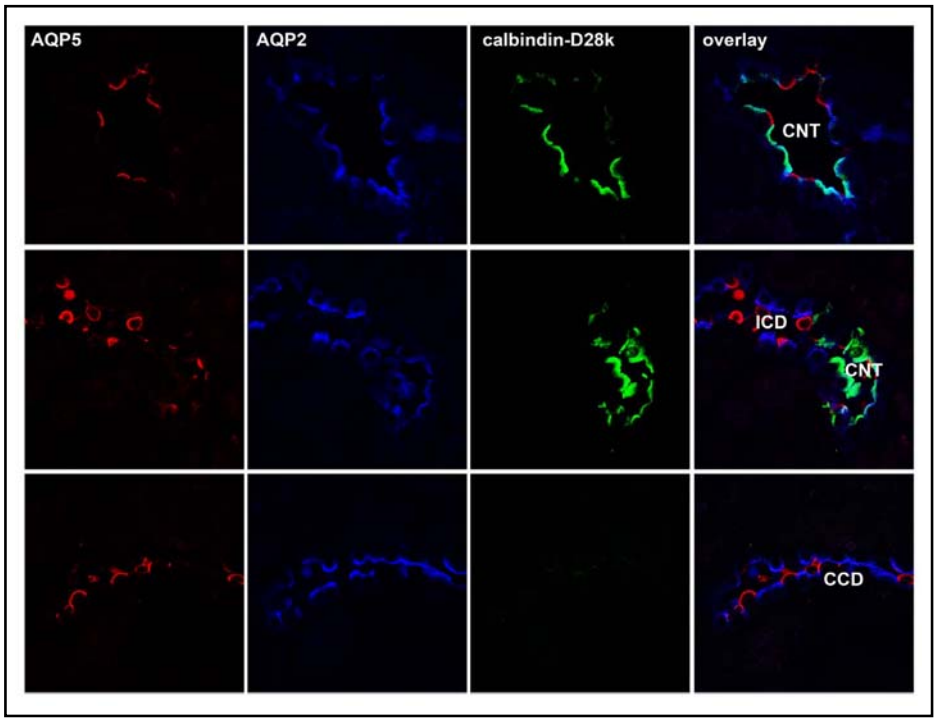

Fig. 5. Immunofluorescence detection of AQP5 in the rat collecting system. AQP5 (red) was co-localized with AQP2 (blue), expressed throughout the collecting system (CNT, ICD, CCD) and calbindin-D28k (green) selectively expressed in the CNT. AQP5-positive cells were found in CNT, ICD and CCD. CNT, connecting tubule; ICD, initial collecting duct; CCD, cortical collecting duct. AQP5 antibodies used were Goat-anti-human AQP5 (Sant Cruz Biotechnology), see Methods.

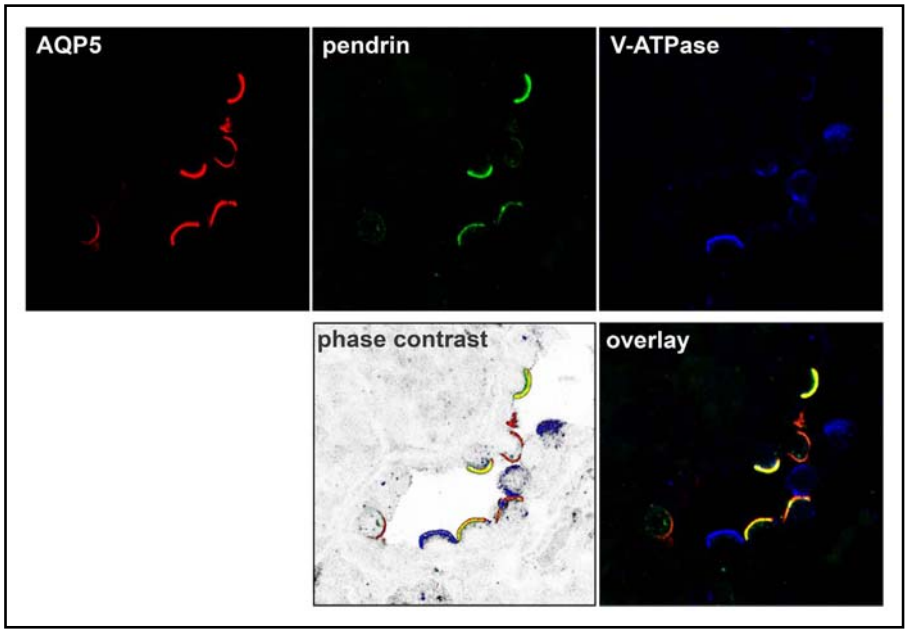

Fig. 6. Immunofluorescence detection of AQP5 in renal intercalated cells. AQP5 (red) was co-localized with pendrin (green) and V-ATPase (blue). High degree of co-localization (overlay, yellow) was found between AQP5 and pendrin, indicating that AQP5 is expressed in B-type intercalated cells. No co-localization of AQP5 was seen with V-ATPase at the apical membrane of A-type intercalated cells. Anti AQP5 antibodies used were Goat-anti-human AQP5 (Sant Cruz Biotechnology), see Methods.

the other two antibodies, used for the immunoblotting experiments described above, failed to recognize AQP5 by immunofluorescence.

We could not perform the analysis of AQP5 distribution in human medulla/papilla, since the biopsy samples had been taken from the cortex.

AQP5 is expressed in intercalated cells in the connecting tubule and collecting duct

We next examined the distribution of AQP5 staining in the whole kidney using immunofluorescence analysis combined with confocal scanning of the fluorescence. Semithin (5 µm) cryosections of whole rat kidneys were used. Tissues were immunostained with AQP5 antibodies and co-stained with antibodies recognizing markers of different tubule segments. In particular, AQP1 was used to stain the proximal tubule (PT) and thin descending limb (TDL), NKCC2 to stain the thick ascending limb (TAL), AQP2 and AQP3 for the connecting tubule/collecting duct (CNT/CD) system. Results obtained in rat are reported in Fig. 4, and are also representative of those obtained in mouse and human (not shown). AQP5-positive tubule segments were distinct from those stained by AQP1 (PT/TDL) and NKCC2 (TAL). Interestingly, AQP5 was

expressed in the AQP2-positive tubules (CNT/CD). Although both aquaporins were expressed at the apical plasma membrane of tubular cells, they never co-localized in the same cell, indicating that they were expressed in different cell types. When tubules were co-stained with AQP5 and AQP3, the two proteins were always expressed within the same tubule segments. In analogy with the results obtained with AQP2, AQP5-positive cells were negative for AQP3 and *vice versa*. Collectively, these observations indicate that, within the kidney, AQP5 is expressed in the CNT/CCD at the apical plasma membrane of intercalated cells (IC).

To discriminate between these two portions of the distal tubule, we stained rat kidney sections with antibodies against calbindin-D28k, selectively expressed in the CNT but not in the CD [27, 28]. Sections were co-stained with AQP5 and AQP2 antibodies. Results, reported in Fig. 5, indicate that AQP5-positive cells were expressed not only in the calbindin-D28k-positive CNT, but also in the initial collecting duct (ICD) and in the CCD. AQP2, which is expressed by principal cells, was present in the same tubules but the absence of co-localization between AQP5 and AQP2 within the same cell, indicated that AQP5 is expressed by intercalated cells (ICs)

To better identify the subtype of IC expressing AQP5, we performed a triple co-localization experiment staining AQP5 with specific markers of type A- and type B-ICs. To this end, we used antibodies against the Vacuolar-type H^+-ATPase (V-ATPase) [24], expressed in both IC types with opposite polarity, and the anion exchanger pendrin, expressed at the apical membrane of type B- ICs. A confocal reconstruction of the three stainings obtained in rat kidney is reported in Fig. 6. AQP5 is visualized in red, pendrin in green and V-ATPase in blue. Images are representative of 10 randomly-chosen photographs taken from 3 different rats. In all the tubules analyzed, AQP5 and pendrin completely co-localized (yellow signal in the overlay picture) at the apical plasma membrane of type B-ICs. AQP5 was not expressed in cells expressing V-ATPase but not pendrin at the apical membrane (type A-ICs). Identical results were obtained in mouse and human samples (not shown).

Discussion

In this work, we present the novel evidence that AQP5, a water channel having a prominent expression in salivary glands and lung [18-21], is also expressed both in human ARPCs and in intact kidneys of mouse, rat and human. The expression is however weak and restricted to the apical membrane of type-B intercalated cells (ICs) in the connecting tubule and cortical collecting duct.

In an attempt to discover new specific markers for the identification of ARPCs in the kidney, we screened human multipotent $CD133^+/CD24^+$ ARPCs isolated and characterized as recently described [17], for the possible expression of the 13 aquaporin isoforms expressed in humans [4]. Interestingly, we found that AQP1, AQP3 and AQP5 were expressed at both mRNA and protein levels in ARPCs. It is well known that AQP1 and AQP3 are expressed in the proximal tubule and collecting duct respectively [14], indicating that these ARPCs exhibit molecular features of epithelial cells from different portions of the kidney tubule. The expression of AQP1 and AQP2 in renal progenitor-like cells isolated from S3 segment of rat nephrons has already been reported [16].

Strikingly, we found that ARPCs also expressed low but measurable levels of AQP5 whose expression in human, mouse or rat kidney had never been reported before. The novel observation that AQP5 is expressed in kidney-derived cells prompted us to investigate the presence of AQP5 in the native kidney. A detailed analysis of the transcripts, conducted in parallel in mouse, rat and human, showed that AQP5 mRNA is indeed endogenously expressed in the kidney of all three species.

To the best of our knowledge, this is the first evidence of the expression of AQP5 transcript in human kidney.

In rat, a detailed analysis of AQP5 transcript had already been performed, by RNAase protection assay, in different tissues, showing negligible expression in the kidney [29].

In mouse, Krane and collaborators, performing a Northern-blot and Western-blot analysis of different tissues, found that the AQP5 mRNA was only expressed in the lung, salivary and lacrimal glands but not in the kidney [30].

Other authors have reported the presence of AQP5 in the kidney of a mammal, the Asian Musk Shrew (*Suncus murinus* L.) [31], showing both the AQP5 transcript and a single protein band around 25 kDa. However, they found complete co-localization of AQP5 and AQP2 in the collecting duct principal cells.

In the present contribution, we unambiguously provide evidence that AQP5 is expressed in intercalated cells.

The use of different sets of primers, antibodies and experimental protocols might explain the discrepancy between our data and those of other researchers. Another

possible explanation may reside in the low abundance of AQP5 in the kidney. In fact, according to our evidences, AQP5 is expressed only in a subpopulation of intercalated cells in the outermost region of the kidney. The percentage of B-IC in the kidney tubules ranges from 0.46 to 15.7% in rat and from 0.12 to 6.1% in mouse [32]. Among all the tested antibodies, only one (goat-anti-human AQP5, Santa Cruz Biotechnology) was able to detect AQP5 by immunofluorescence in the apical pole of B-IC. Preadsorption of the antibody with the immunizing peptide completely abolished the antibody immunoreactivity thus confirming the specificity of the staining (Fig. 3).

Immunolocalization experiments showed that AQP5 is exclusively detectable in B-IC in the renal cortex in rat and mouse (Fig. 3). We also detected AQP5 in human kidney biopsies isolated from the cortex (not shown). Co-localization with markers of different tracts of the kidney tubule confirmed that AQP5 was present in all species at the apical membrane of epithelial cells lining the CNT/CD. The absence of co-localization of AQP5 with AQP2 and AQP3, both expressed in the principal cells (PCs) of the CNT/CD [14] indicated a selective expression of AQP5 in the intercalated cells. The IC subtype in which AQP5 is expressed was identified using cell type-specific markers.

Different IC subtypes are expressed along the CNT/CD systems (see Brown *et al*. for review) [33] and can be identified by the presence of selective molecular signatures. A-type intercalated cells (A-IC) are identified by the presence of apical V-ATPase and basolateral anion exchanger AE1 [34]. B-type intercalated cells (B-IC) express V-ATPase at various locations, and the apical anion exchanger, pendrin [35]. A-IC and B-IC were proposed to be functional mirror images, with A-IC responsible for acid secretion into the tubule lumen and B-IC being involved in bicarbonate secretion into the lumen [36].

In this work, we found a systematic colocalization of AQP5 with pendrin, unambiguously indicating that AQP5 is specifically expressed at the apical plasma membrane of the CNT/CCD B-IC.

Strikingly, co-expression of AQP5 and pendrin is recurrent in other organs where the two proteins are expressed, such as in the cochlea [20, 37] and in the bronchial epithelial cells [38, 39].

The physiological role of the presence of AQP5 water channel in B-ICs is unknown. The apparent absence of basolateral aquaporins in B-ICs and the low abundance of AQP5 compared to that of AQP2 and AQP3 expressed by the PC in the same tubule, suggest that AQP5 might not participate to the net trans-epithelial water reabsorption occurring in the distal tubule. Interestingly, an unconventional role of AQPs is the osmosensation and such a role has been proposed for AQP5 in other tissues. Association between transient receptor potential vanilloid 4 (TRPV4) and AQP5, controls the regulatory volume decrease (RVD) in salivary gland cells exposed to hypotonicity [40]. We might speculate that AQP5 could act as an osmosensor regulating the cell volume of CNT/CCD intercalated cells, exposed to the luminal hypotonicity produced by the thick ascending limb and distal tubule.

Although we did not provide here experimental evidence supporting this possible function of AQP5 in the kidney, we suggest that it is worthy of further investigation.

In conclusion, we demonstrated for the first time that AQP5 is expressed in adult renal progenitor/stem cells isolated from humans. We also demonstrated that AQP5 is expressed in the kidney of rat, mouse and human at the apical plasma membrane of type-B intercalated cells and co-localizes with pendrin. Among the aquaporins expressed in the kidney, AQP5 therefore has a unique distribution and likely a distinct function.

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