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Knockdown of the BBS10 Gene Product Affects Apical Targeting of AQP2 in Renal Cells: A Possible Explanation for the Polyuria Associated with Bardet-Biedl Syndrome

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Abstract

Objective: Bardet-Biedl syndrome (BBS) is a rare genetic disorder whose clinical features include renal abnormalities, which ranges from renal malformations to renal failure. Polyuria and iso-hyposthenuria are common renal dysfunctions in BBS patients even in the presence of normal GFR. The mechanism underlying this defect is unknown and no genotype-phenotype correlation has yet been reported. Here we report four BBS patients showing different renal phenotypes: one had polyuria with hyposthenuria associated with mutation of BBS10, while three patients with normal urine concentrating ability had mutations in BBS1.

Methods: We measured aquaporin 2 (AQP2) urinary excretions in BBS patients and studied the possible role of BBS1 and BBS10 on AQP2 trafficking in a mouse cortical collecting duct cell line.

Results: We found that the BBS1-mutated patients showed a significant increase of water channel AQP2 urine excretion in antidiuresis. In contrast, the BBS10-mutated patient showed no difference in AQP2 excretion in antidiuresis and after an acute water load. In mouse kidney cortical collecting duct MCD4 cells, knockdown of BBS10, but not of BBS1, prevented the forskolin-dependent trafficking of AQP2 to the apical membrane, and induced the mis-trafficking to the basolateral membrane. Interestingly, BBS10 knockdown was associated with a dramatic reduction of tubulin acetylation without loss of cell polarity.

Conclusions: Therefore, the effect of BBS10 knockdown *in vitro* is consistent with the hyposthenuria observed in the patient with mutation of BBS10. This correlation between renal phenotype and genotype indicates that BBS10, but not BBS1, might control the trafficking of AQP2 and therefore plays a key role in the renal concentrating mechanism.

Keywords: Bardet-biedl syndrome; Polyuria; Hyposthenuria; Central cilium; Water channels; Aquaporin 2; Cell polarity; Kidney disease; Kidney epithelial cells; Protein trafficking; Cytoskeleton microtubules

Introduction

Bardet-Biedl syndrome (BBS, OMIM #209900) is an autosomal recessive disorder characterized by retinal dystrophy, obesity, hypogenitalism, polydactyly and renal dysfunction [1-5]. The prevalence ranges from 1/125,000 to 1/ 175,000 in Europe [6] and North America, and is higher in Kuwait (1/13,500) and Newfoundland (1/17,500). Eighteen genes have been associated with BBS, up to now [7-11]. The various BBS-associated genes encode proteins localized to the primary cilia, basal body, or centrosome, and are involved in the regulation of ciliary structure, biogenesis and function [11].

Proteins BBS1, BBS2, BBS4, BBS5, BBS7, BBS8 and BBS9 form the BBSome complex, which controls the intraflagellar trafficking, while BBS6, BBS10 and BBS12 form the chaperonin complex [11,12]. About 23% and 20% of BBS patients had pathogenic mutations in the BBS1 and BBS10 gene, respectively [6].

The spectrum of renal phenotype is highly variable in BBS; on ultrasound, kidney appearance ranges from retained fetal lobulations to loss of cortico-medullar differentiation, cysts and pelvic dilation. Either tubular defects, mainly a urinary concentrating defect leading to polyuria, and the decline of glomerular filtration rate have been described [4]. Approximately one-third of patients have polyuria/polydypsia due to a vasopressin-resistant urinary concentration defect [13-17]. Patients with mutation of BBS1 usually do not show renal involvement [18].

Vasopressin (AVP) promotes urine concentration by acting on the

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water channel aquaporin 2 (AQP2). In isolated collecting ducts (CD), AQP2 is primarily localized in intracellular vesicles. However, AVP perfusion causes its trafficking to the apical membrane, resulting in a dramatic increase in water permeability. As a result of the AVP washout, apical AQP2 is endocytosed with reversal of the water permeability [19]. Indeed, AQP2 has a critical role in several inherited and acquired water-balance disorders, including the nephrogenic diabetes insipidus (NDI), in which AQP2 expression and/or apical plasma membrane targeting are affected [19-22].

Here we report a BBS patient showing polyuria with hyposthenuria in the presence of normal estimated glomerular filtration rate (eGFR), who was homozygous for a missense mutation in BBS10. Three other BBS-affected patients with preserved renal concentrating ability harbored mutations in the BBS1 gene. We explored the role of BBS10 and BBS1 in the urine concentrating mechanism by measuring AQP2 urinary excretion in the four patients and by analyzing AQP2 trafficking to the apical membrane in a mouse cortical CD cell model after BBS1 and BBS10 knockdown.

Methods

Ethics statement

Ethical approval for this study was obtained from the Institution Review Board of the Azienda Ospedaliera Universitaria, Seconda Università degli Studi di Napoli.

The study did not deviate from current and accepted clinical practice. No any additional burden or risk has been posed to the patients. All procedures have been performed to generate potential benefits to the patients, and have been conducted in accordance with world guidelines for ethical research involving humans. This study complies with the Declaration of Helsinki. Written informed consent was obtained from all participants before genetic testing. For the 10-year old patient in this study written informed consent was obtained from his parents prior to conducting the study. The written informed consent for the study participants include information that their biological samples would be used for research purposes. The data used in this study were anonymized.

Reagents

siRNA specifically targeting mouse BBS1 (cat. # 4390771, I.D. S78733 GenBank accession number NM_001033128.3), BBS10 (Cat. # 4390771, I.D. S90017, GenBank accession number NM_027914) and Silencer® Select Negative Control #1 (Cat. # 4390843) were from Life Technologies™. EZ-Link Sulfo-NHS-Biotin and Streptavidin Agarose beads were from PIERCE (www.piercenet.com)

Antibodies

Rabbit polyclonal antibodies against human AQP2 were generated as previously described [44]. Antibody against acetylated tubulin (Cat.# T6793, clone 6-11B-1, ascite fluid) was from Sigma-Aldrich (www.sigmaaldrich.com); monoclonal antibody against Zo-1 (Cat.# 33-9100) was from Life Technologies. Monoclonal anti-Na/K-ATPase (Cat.# 05369) was from Millipore (www.millipore.com).

Osmolality measurements

Patients and control subjects were dehydrated and fasted overnight (water restriction). Second morning urine was collected and immediately stored at 80°C until use. Then, controls and patients drank 1L of water in 20 min (acute water load) and urine samples were then collected hourly thereafter for 2 h. Two hours after the water load, desamino-8-D arginine vasopressin (DDAVP) was administered intravenously in a time span of 20 min at the dose of 0.3 μg/kg body weight in 100 cc of 0.9% saline solution. Urine samples were collected every hour after DDAVP, for 2 hrs.

For all the collected urine samples, osmolality and creatinine were measured.

Urine AQP2 measurement

Urine protein precipitation: Urine samples were spun at 10,000 rpm for 10 min at 4°C. Cleared urine samples were mixed with methanol, chloroform and ddH_2O in a ratio of 1:4:1:3 and then centrifuged at 6,500 rpm for 20 min at 10°C. The upper phase was discarded; the lower phase was mixed with methanol and then centrifuged at 10,000 rpm for 60 min at 10°C. Sediment was suspended in 150 μl of lysis buffer (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 1 mg/ml leupeptin, 1 mM PMSF).

Western blot: Urine samples were diluted with the loading buffer and normalized to urinary creatinine. Samples were resolved by SDS polyacrylamide gradient gel electrophoresis (polyacrylamide gradient from 10 to 20%) and then transferred to Invitrolon PVDF membranes (Invitrogen). Filters were blocked in casein/10% Triton X-100 and then incubated overnight with ant-AQP2 antibody (dilution 1:1000). After washing, membranes were incubated with a phosphatase-conjugated anti-rabbit IgG secondary antibody for 1h and bands were visualized with the Tropix chemiluminescence kit and quantized by the Versadoc Imaging System.

Genetic study

Genomic DNA of patients was extracted from peripheral blood leukocytes by using the automated MagNA Pure LC system (Roche Diagnostics). All exons with flanking intronic sequences (50 bp at least) of BBS1 and BBS10 were amplified. The corresponding amplicons were analyzed by direct sequencing.

Cell cultures

Mouse cortical collecting duct MCD4 cells that stably express human AQP2 were generated and maintained as described elsewhere [45]. Briefly, mouse cortical collecting duct M-1 cells, originating from mice transgenic for the early region of simian virus 40, were purchased from the European Collection of Cell Culture (cat. #95092201) and transfected with the human AQP2 cDNA. One clone, positive for stable transfection (MCD4) was used for all the following studies. For immunofluorescence, RNAi and biotinylation experiments, cells were plated on six-well 0.4-μm pore size cell culture inserts and allowed to grow for 10 days before the experiment. In order to promote formation of central cilia, at day 8, medium was replaced by Opti-MEM reduced-serum medium (http://www.lifetechnologies.com) up to day 10.

RNAi

Cells were cultured in six-well-cell permeable inserts for 8 days, treated twice on 2 days with 5 μl of Lipofectamine 2000 reagent (http://www.lifetechnologies.com) alone or with 100 nM siRNA targeting BBS1, BBS10 or control siRNAs, in serum-free Opti-MEM. Cells were then subjected to total RNA extraction, immunofluorescence or biotinylation experiments.

RNA isolation, RT-PCR and cDNA sequencing

Total RNA was extracted from MCD4 cells by the TRIzol extraction method (TRIzol reagent, Life Technologies). The RNA was

then retrotranscribed using the SuperScript VILO cDNA Synthesis Kit (Life Technologies). cDNAs of BBS1 (#NM_024649.4; GI:29029553) and BBS10 (NM_024685.3; GI:100816406) were amplified by using specific primer pairs. For BBS1, we used the forward primer 5'-CAGGACGTTTGGAACCAAGCCAAA-3' and the reverse 5'-TCTTACCCTTGTGGGTGAAGCCAT-3'; for BBS10, the forward primer was 5'-CGGTGACGGCGGCTTTGCGG-3', the reverse 5'-ACTCTTCCACAAAAGTATGC-3'. Mouse β-actin cDNA served as housekeeping control gene. All primer pairs were chosen to hybridize with cDNA sequences derived from different exons. PCRs were performed as follows: 94°C, 3 min; N cycles at 94°C, 45 sec; 50°C, 30 sec; 72°C, 1,5 min; final extension at 72°C for 10 min. The appropriate number of cycles was optimized so that the amplimers were analyzed in the exponential phase. The PCR products were separated on a 3% agarose gel, visualized with ethidium bromide staining and then analyzed by direct sequencing.

Immunofluorescence

For immunofluorescence experiments, MCD4 cells were grown on porous filters as described above. siRNA-transfected cells were used 48 h after transfection. Cells were treated with 10-4 M forskolin in the culture medium for 20 min. Monolayers were then fixed with 4% PFA in PBS for 20 min at room temperature or cold methanol for 5 min. Cells were incubated for 2 h at room temperature with the primary antibodies. Bound antibodies were detected with Alexa-Fluor-conjugated donkey anti-rabbit IgG antibodies (Life Technologies). Filters were excised from the support, mounted on microscope slides and viewed with a Leica confocal microscope TSC-SP2.

Apical surface biotinylation and immunoblotting

Forty-eight h after transfection with or without siRNAs, cell monolayers were treated or not with 10-4M FK for 10 min at 37°C. Filters were then rapidly washed twice in ice-cold EBS buffer for biotinylation (10 mM triethanolamine pH 9.0, 150 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$) and the apical side was incubated with 650 µl of 2.5 mg/ml EZ-link Sulfo NHS-biotin (http://www.piercenet.com) in EBS buffer on ice for 30 min. Filters were washed twice in ice-cold PBS-CM and unbound biotin was quenched for 10 min in quenching buffer (50 mM NH4Cl in PBS-CM) on ice. Cells were scraped from filters in 500 µl of lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X- 100, 0.2% BSA, 1 mM PMSF, protease inhibitors cocktail), lysates were sonicated, clarified at 13,000 g for 10 min and biotinylated proteins were precipitated for 16 h with of Immunopure immobilized-streptavidin bead suspension (Pierce) at 4°C. Biotinylated proteins were extracted in NuPAGE LDS Sample buffer (Life Technologies), heated at 95°C and resolved on 4-12% NuPAGEBis-Tris gels. Protein bands were transferred to PVDF membranes, blocked in TBS-Tween containing 3% BSA and incubated with primary antibodies, with secondary antibody conjugated to horseradish peroxidase (HRP), revealed with an enhanced chemiluminescence detection system (SuperSignal West Pico Chemiluminescence Substrate, Pierce) and detected with Chemidoc™XRS detection system (www.bio-rad.com). Band densitometry was performed using ImageJ software. P values were calculated by Student's t-test for unpaired data.

Results

Patients: clinical features and genotype

Table 1 shows the clinical and genetic features of patients; their blood and urinary parameters are listed in Table 2. All patients had a clinical diagnosis of BBS based on Beals criteria. Based on molecular epidemiology study (personal observation), mutation of BBS1, BBS2 and BBS10 is a frequent feature of BBS in Italy (about 50% of cases). Therefore, in our routine clinical practice, we carry out the sequence analysis of these three genes. Patient 1 was a 39-year-old woman with normal eGFR and polyuria. Urine osmolality (u-Osm) 12 hrs after water restriction (WR) was 380-430 mOsm/kg. Renal ultrasound revealed 2 cysts on the right kidney and a dysmorphic left kidney. Molecular analysis of the BBS10 gene identified a new homozygous single nucleotide substitution, c.641T>A, leading to the missense variant p.V214E. No mutations were detected in the BBS1 gene. We did not detect the variant c.641T>A in 200 alleles from normal subjects; moreover, it was not reported in the NCBI SNPs database. Mendelian transmission was confirmed in the unaffected consanguineous parents, both heterozygous for the mutation, which was not present in the proband's healthy sister.

Patient 2 was a 32-year old man. He showed renal cysts and fetal lobulations on ultrasound; the eGFR and serum electrolyte levels

Patients	P1*	P2*	P3	P4*
Age	40	32	24	10
Sex	female	male	male	male
Retinal degeneration	yes	yes	yes	yes
Obesity	yes	no	no	yes
Childhood obesity	yes	yes	yes	yes
Polydactyly	yes	yes	yes	no
Learning disabilities	yes	no	yes	yes
Genotype	c.641T>A/ c.641T>A	c.1169T>G/ c.1169T>G	c.1169T>G/ c.1642delC	c.664G>C/ c.664G>C
Gene and mutation effect	BBS10, **p.V214E**	BBS1, **p.M390R**	BBS1, p.M390R and **p.L548Wfs*31**	BBS1, **p.G222R**

Table 1: Clinical and genetic features of BBS patients
*Consanguineous parents; in bold, new mutations
*The GFR has been estimated using CKD-EPI and Schwarts formulae in adults and children respectively.
Abbreviations: PCR: Urine Protein-creatinine Ratio; FeNa: Excretional Fraction of Sodium; FeCl: Exretional Fraction of Chloride; U-Osm: Urine Osmolality; WR: Water Restriction.

	P1	P2	P3	P4
eGFR* (ml/min/1.73m²)	102	108	122	185
PCR (mg/g)	260	110	160	102
Serum Na (mEq/L)	144	144	143	140
Serum K (mEq/L)	4.5	3.9	4	4
Serum Cl (mEq/L)	108	105	101	100
FeNa (%)	1.03	0.72	0.64	0.8
FeCl (%)	1.3	0.54	0.78	0.86
Urine Ca/Urine creatinine	0.02	0.03	0.11	0.08
Urine pH	5.5	6	7	6.5
U-Osm after 12h WR (mOsm/kg)	380	750	760	836
Plasma renin activity (ng/ml/h)	3.6	0.9	1.2	0.8

Table 2: Blood and urine analysis of BBS patients. Patient 1 showed hypostenuria, while the others did not. Consistent with polyuria, plasma renin activity was higher in pz 1 than the others.

were normal. U-Osm 12 hrs after WR was 750 mOsm/kg. Molecular analysis of BBS1 revealed the common missense variant p.M390R at the homozygous state. Parents and the proband's healthy sister were heterozygous carriers. No mutations were detected in BBS10.

Patient 3 was a 24-year old man. Renal sonography was unremarkable and u-Osm 12 hrs after WR was 760 mOsm/kg. Molecular analysis of BBS1 showed that the patient was compound heterozygote for the common missense variant p.M390R and for a new single nucleotide deletion c.1642delC that presumably shifted the reading frame thus leading to a truncated (p.L548Wfs*31), nonfunctional protein. BBS10 molecular analysis was negative. Mendelian segregation analysis revealed that the father was heterozygous for the frameshift mutation, the mother for the missense mutation, the healthy brother was wild type.

Patient 4 was a 10 year-old boy at the time of screening. Blood test showed an eGFR of 185 ml/min/1.73m^2 and no electrolytes imbalance. Renal ultrasound was unremarkable. U-Osm 12 hrs after WR was 836 mOsm/kg. Genetic testing showed that the proband was homozygous for a new missense variant c.664G>C (p.G222R) in BBS1. No mutation was found BBS10. The new BBS1 variant was not found in 200 alleles from normal subjects and it was not reported in the NCBI SNPs database. Mendelian transmission was confirmed in the unaffected consanguineous parents and in the proband's healthy sister: all were heterozygous for the mutation. Pathogenicity of the new missense variants was assessed using the online prediction tools: the polymorphism phenotyping v-2 (PolyPhen-2) and the sorting intolerant from tolerant (SIFT).

Dehydration, water load and DDAVP test

To address the pathophysiology of hyposthenuria of patient 1, u-Osm was measured 12 h after WR and 2 h after intravenous administration of desmopressin (DDAVP), a synthetic analog of AVP. Before DDAVP infusion, an acute water load (WL) has been given orally to suppress endogenous AVP. Patient 3 and four aged-matched healthy volunteers served as controls; the remaining two BBS patients underwent water restriction (WR) and WL analysis. As shown in Figure 1, baseline u-OSM was lower in patient 1 than in controls (380 mOsm/kg vs 970 mOsm/kg (mean controls), and in patients 2, 3 and 4 (mean 785 mOsm/kg); moreover, DDAVP infusion did not cause significant elevation of u-Osm (380 vs. 410 mOsm/kg) in patient 1, while in patient 3, as well as in the controls, u-Osm increased to over 750 mOsm/kg. Therefore, urine hyposthenuria of patient 1 depends on renal 'resistance' to DDAVP/ADH. Noteworthy patient 1, unlike the other three BBS patients we tested, was not able to dilute urine after the WL (-20.2% in patient 1 vs. -99.9% in the controls and in patients 2 and 3), suggesting the occurrence of a combined concentrating and diluting defect.

Urine AQP2

Urine AQP2 (u-AQP2) abundance was higher in WR than in WL in the controls and in the three patients carrying BBS1 mutations (Figure 2). This difference did not depend on dilution because we normalized urine samples to urine creatinine levels. In contrast, in patient 1, u-AQP2 did not differ between WR and WL, which is consistent with the presence of polyuria and hyposthenuria. These data suggest that BBS10 might be involved in urine concentrating mechanism, conceivably by regulating AQP2 trafficking to/from the apical membrane.

Role of BBS1 and BBS10 on central cilium formation and microtubule acetylation in MCD4 renal cells

As shown in Figure 3, MCD4 cells that grew on a permeable support developed a central cilium. In resting cells, confocal xy sections of the apical plasma membrane revealed very low AQP2 staining, which was scattered throughout the cytoplasm in the xz sections. Treatment of cells with 10^{-4} M forskolin (FK) resulted in an increased AQP2 staining to the apical microvilli of the epithelium (Figure 3).

In MCD4 cells, we silenced BBS1 and BBS10 by transfecting siRNAs specifically targeting both mRNAs (Figure 4A). Cells transfected with the anti-BBS siRNAs had a lower number of cilia than the control cells (Figure 4B); however, subapical acetylated tubulin was reduced only in BBS10-silenced cells. Silencing of BBS1 or BBS10 did not affect cell polarization because the formation of tight junctions and the basolateral distribution of Na+/K+-ATPase were unchanged (Figure 4B).

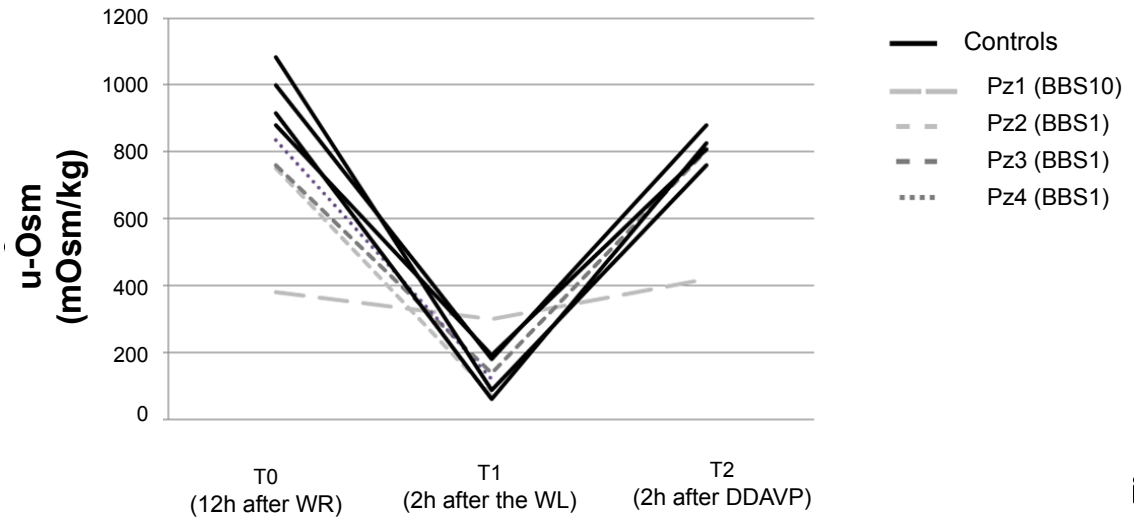

Figure 1: osmolality (U-Osm) measurements. Urine osmolality of BBS patients (Pz 1, 2, 3, 4) and age-matched volunteers (Controls) measured 12 hrs after water restriction (WR), 2 hrs after an acute water load (WL) and 2 h after intravenous DDAVP administration.

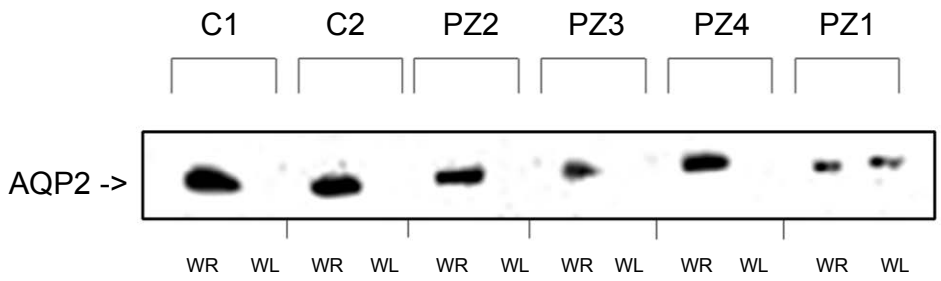

Figure 2: Urinary excretion of AQP2 in controls and BBS patients. Western blotting analysis of Urine AQP2/creatinine ratio after water restriction (WR) and water load (WL) in controls (C1, C2), BBS1 (PZ 2, 3, 4) and BBS10 (PZ1) patients. Each lane is representative of u-AQP2 abundance per 0.5 mg creatinine.

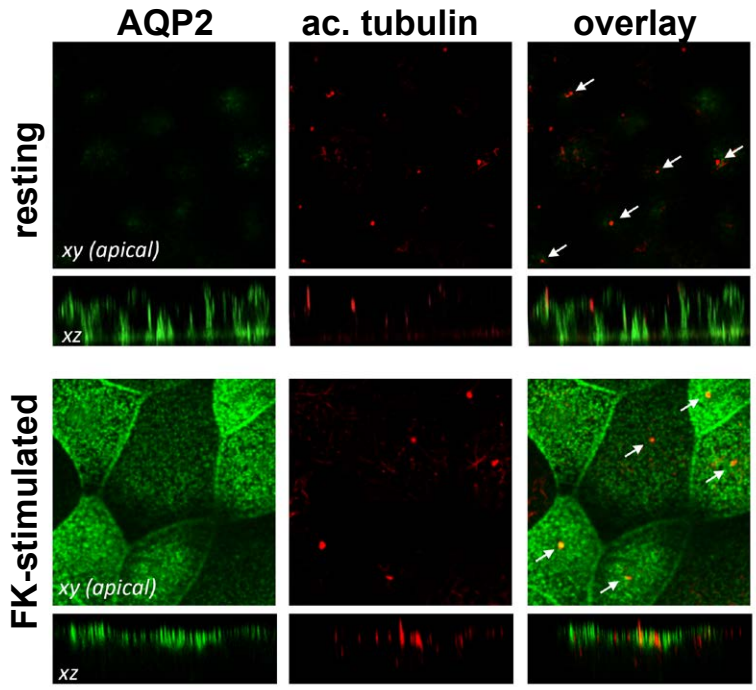

Figure 3: MCD4 cells express a central cilium and show polarized trafficking of AQP2. Confocal images of MCD4 cells under resting condition or stimulated with FK (see Methods for details). Sections were taken at the apical pole in the xy plan or in the xz plan. Acetylated tubulin (red) coincides with the central cilia, at the cell top. AQP2 (green) shuttled from a storage intracellular compartment to the apical membrane, upon FK stimulation. Overlay images show that AQP2 and acetylated tubulin may colocalize after FK stimulation.

BBS10, but not BBS1 knockdown, dramatically affected polarized trafficking of AQP2 in renal cells

To assess whether BBS1 or BBS10 inactivation perturbed the cAMP-dependent shuttling of AQP2, we conducted apical surface biotinylation experiments. In control and in BBS1-silenced cells, FK stimulation dramatically increased biotinylated AQP2 at the apical plasma membrane (Figure 5A). Interestingly, BBS10 knockdown completely prevented the FK-induced increase of biotinylated-AQP2 (Figure 5B).

To further investigate the effect of BBS10-silencing on AQP2 shuttling, we analyzed subcellular AQP2 level. In resting conditions, AQP2 was mainly localized in subapical vesicles in control and in BBS silenced cells (Figure 6A-C). Following the FK stimulation, AQP2 totally reallocated to the apical plasma membrane, in control cells (Figure 6A) and in BBS1 silenced cells (Figure 6B). Surprisingly, in BBS10-silenced cells, FK stimulation resulted in AQP2 mis-localization to the basolateral membrane (Figure 6C); moreover, specific staining indicated that AQP2 co-localized with the anti Na+/K+-ATPase (Figure 1, supplemental material).

Discussion

Ciliopathies, such as those caused by mutations of the BBS genes, are responsible for multiple organ dysfunctions with chronic kidney disease being one of the main clinical features. This applies also to BBS patients [23]. In addition to the high genetic heterogeneity, genetic complexity of the syndrome is also represented in the oligogenic transmission hypothesis, i.e. heterozygous mutations in certain BBS genes may have a potential epistatic effect on BBS patients with two recessive mutations at another BBS locus (triallelic inheritance) [12]. Nevertheless, biallelic mutations in the BBS1 and BBS10 genes are the most recurrent pathogenic molecular alterations [1,24]. Notably, BBS1

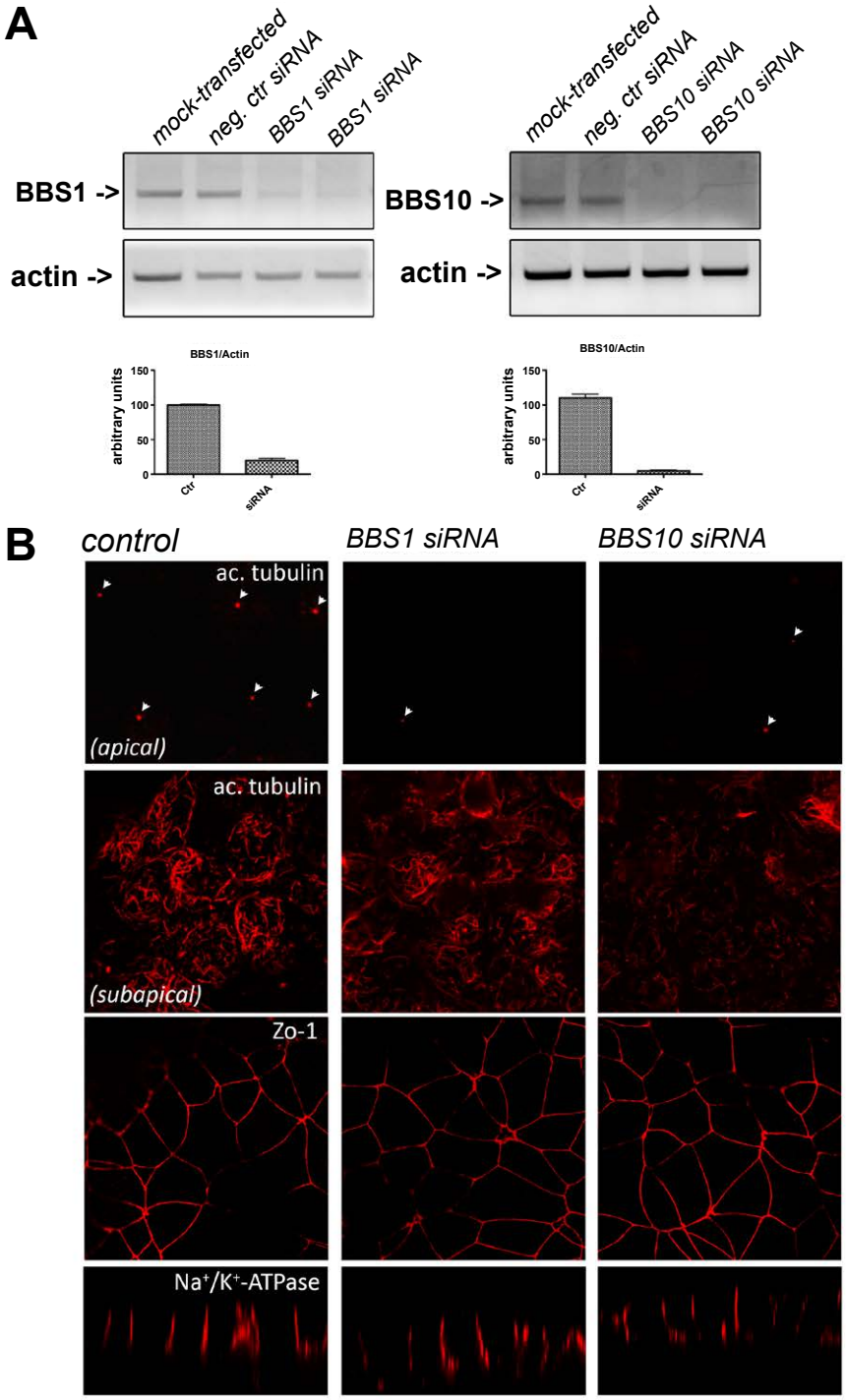

Figure 4: Effect of BBS1 and BBS10 knockdown on cilium integrity, microtubule organization and cell polarity in MCD4 cells. MCD4 cells were either mock-transfected or transfected with negative control siRNA or specific anti BBS1 or BBS10 siRNA. (A) Silencing efficiency was tested 48 h after transfection by semiquantitative RT-PCR and the effect of BBS1 or BBS10 silencing evaluated by densitometric analysis of the RT-PCR bands. (B) Compared to control siRNA-transfected cells, BBS1 and BBS10 silencing dramatically reduced the number of ciliated cells as indicated by the staining of acetylated tubulin at the top of the cells (white arrowheads, apical). In a subapical confocal section, the staining of acetylated tubulin was dramatically reduced in BBS10-silenced cells. The intensity and localization of the tight junction marker Zo-1 and the basolateral marker Na^+/K^+-ATPase were virtually unaffected by BBS silencing. Experiments were repeated at least three times with comparable results.

mutations usually associated with milder renal dysfunction than BBS10 [18].

Here we provide the first evidence that BBS10, but not BBS1, is a key element in the renal vasopressin-dependent urine concentrating process. Moreover, we report a correlation between the renal phenotype and the genotype in a BBS patient with mutation of BBS10.

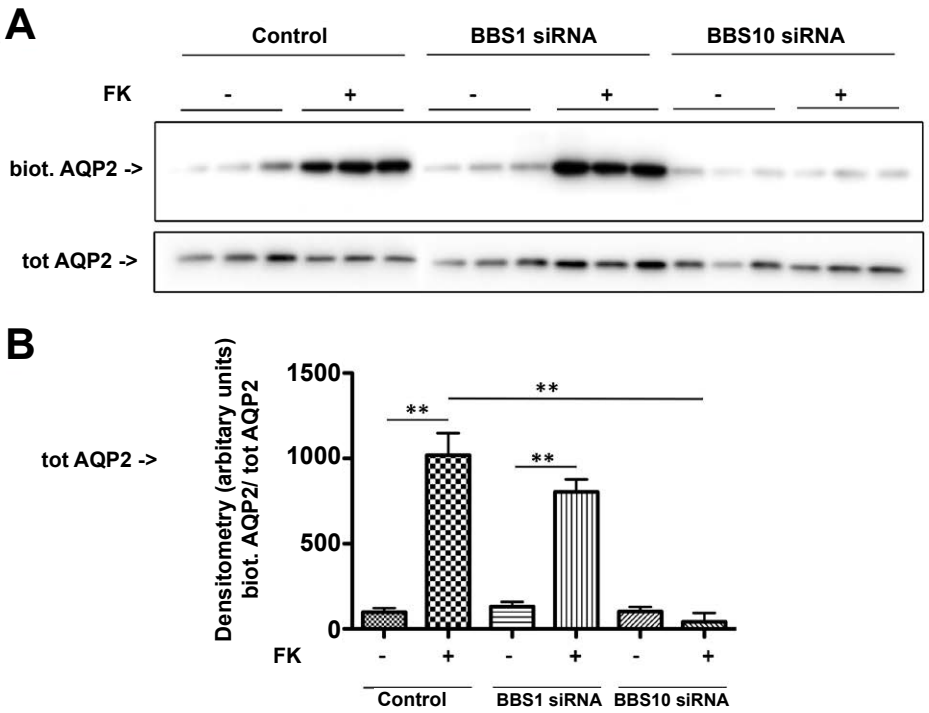

Figure 5: BBS10 silencing prevents FK-induced AQP2 accumulation at the apical plasma membrane in renal MCD4 cells. Both BBS1- and BBS10-silenced MCD4 cells were either left under resting condition or stimulated with FK. Biotinylated apical proteins were isolated and immunoblotted with anti-AQP2 antibodies. After normalization for total AQP2 amounts, apical AQP2 was quantified by densitometry. Experiments were repeated three times with similar results (** P<0.02).

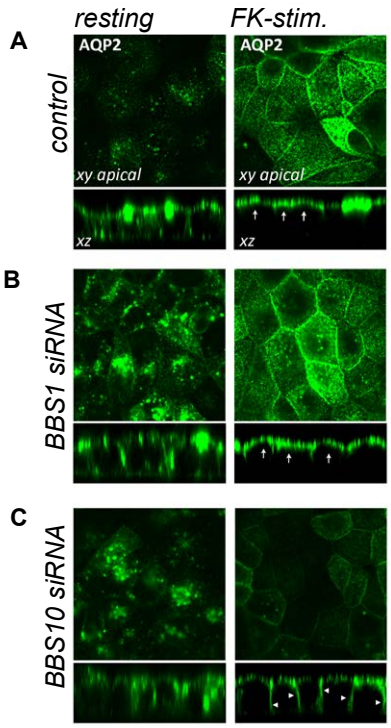

Figure 6: BBS10 silencing is associated with misrouting of AQP2 at the basolateral membrane after FK stimulation in renal cells. Both BBS1- and BBS10-silenced MCD4 cells were either left under resting condition or stimulated with FK. Cells were subjected to immunofluorescence with anti-AQP2 antibodies and analyzed by confocal laser-scanning microscopy. Compared to controls cells (Figure 6A), BBS1 silencing did not affect the FK-induced accumulation of AQP2 at the apical pole in MCD4 cells (Figure 6B, white arrows). Conversely, BBS10 silencing prevented the apical translocation of AQP2 and clearly increased AQP2 staining at the basolateral plasma membrane (Figure 6C, white arrowheads). All images are representative of three independent experiments giving similar results.

A recent analysis of 33 BBS patients revealed that impaired urinary concentrating ability is the most frequent manifestation (63%) in non-dialyzed and non-transplanted patients [25]. Interestingly, in that cohort of patients, mutations in the BBS6, BBS10 and BBS12 genes correlated with a higher incidence of abnormal urine concentrating mechanism, which actually depends on decreased kidney responsiveness to vasopressin [16,17,26-28].

Here, we describe the renal phenotype of four unrelated patients suffering from BBS. One of these patients had polyuria and hyposthenuria and was homozygous for the new missense variant p.V214E in BBS10. The remaining three patients, who showed mild renal phenotypes, had different mutations in BBS1.

We are aware that, by limiting our analysis to the sequencing of three, albeit major, BBS-causative genes, the triallelism could elude detection. Undoubtedly, given the wide genetic heterogeneity and complexity of this rare disease, a more realistic molecular diagnostic approach would be to sequence the entire exome. However, we can reasonably assume that the genetic data we obtained sufficiently support the experimental evidences here presented as well as the genotype-phenotype correlation in the aforementioned patients.

Consistent with the disease rarity and autosomal recessive transmission, three of our patients were homozygotes and had consanguineous parents (Table 1). Notwithstanding the small number of patients analyzed, we identified three new sequence variants, two in the BBS1 gene and one in the BBS10 gene. Both the new mutations in BBS1 lead to variant proteins, i.e. p.G222R and p.L548Wfs*31. The frameshift mutation p.L548Wfs*31 (c.1642delC) should produce an aberrant BBS1 protein, 15 amino acids shorter than the wild type. The novel mutation in BBS10 changes valine 214 into a glutamic acid residue (p.V214E). Both Polyphen-2 and SIFT predicted that all the new mutations we identified were "probably damaging". Although in silico analysis is an efficient analytical tool to assess the pathogenicity of new variants, functional validation should be mandatory. However, segregation analysis of the new alleles in the families supports their pathogenic role.

Two of our BBS patients carried the known BBS1 pathogenic variant, p.M390R. This is not surprising since this mutation is recurrent and accounts for approximately 27% of all cases of BBS [29]. It is usually associated with a very mild ocular phenotype and often correlates with very mild or no renal disease, in BBS patients [29]. Our BBS1 patients showed normal urine concentrating ability. In contrast, the patient 1, who was homozygote for the new BBS10 variant showed polyuria and renal resistance to DDAVP. Her inability to dilute urine even after an acute WL was conceivable with the presence of a complex defect in both renal diluting and concentrating ability.

In the nephron, the CD is a major site involved in the urine concentrating mechanism. AVP, upon binding to the V2 receptor (V2R), increases CD water permeability by promoting the trafficking of AQP2 from intracellular storage vesicles to the apical membrane, in the short term. AQP2 is a well-established urinary biomarker of CD responsiveness to AVP and is critical for the diagnosis of several disorders of renal water balance [31-35]. U-AQP2 reflects its abundance on the apical membrane [33-35]. Accordingly, u-AQP2 increases during thirsting and after DDAVP administration, and decreases after a WL [30]. To address the mechanism underlying the defective urine concentrating ability of the BBS10 patient, we measured u-AQP2 in all patients and in healthy volunteers, both in antidiuresis and after an acute WL. Interestingly, BBS1 patients and controls showed a greater increase in u-AQP2 levels after WR than during the acute WL, unlike the BBS10 patient. Therefore, we concluded that polyuria in the BBS10 patient may result from a defective vasopressin-dependent trafficking of AQP2 to the apical membrane.

The observation that BBS10 but not BBS1 mutation was associated with blunted responsiveness to AVP in the kidney prompted us to investigate whether BBS1 and/or BBS10 gene inactivation affected the AQP2 trafficking. Interestingly, silencing of BBS10, but not BBS1, dramatically reduced subapical acetylated tubulin. Tubulin post-translational modifications seem to provide navigational cues for molecular motors to deliver cargo to spatially defined subcellular domains [36]. Indeed, as kidney epithelial cells acquire apical-basal three-dimensional polarity, tubulin undergoes a switch from detyrosinated to acetylated [36]. In our experimental model, the three-dimensionally polarized MCD4 cells, secretory traffic is mostly directed toward the apical domain and the meshing of acetylated microtubule bundles serves as tracks for this traffic. Interestingly, the biotinylation experiments showed that BBS10 but not BBS1 knockdown prevents AQP2 accumulation at the apical plasma membrane after FK stimulation. Moreover, confocal immunofluorence images in the xz plan revealed that AQP2 was misdirected to the basolateral membrane after FK treatment, in BBS10-silenced cells. Therefore, the reduced acetylation of tubulin we observed in these cells may underlie the AQP2 misrouting. Taken together, our *in vitro* data correlate well with the excreted u-AQP2 amount in the BBS patients.

In polarized mouse kidney epithelial cells, MCDK, AQP2 is first targeted to the basolateral membrane [37]. Membrane AQP2 is stored in Rab5-positive clathrin-coated endosomes, which enter a microtubule-mediated transcytosis pathway toward the Rab11-positive subapical endosomes, whose exocytosis is under the control of vasopressin [37]. In this scenario, the reduced microtubule acetylation we observed in BBS10-silenced cells may affect the transcytosis pathway of AQP2 to the apical membrane thus explaining the accumulation of AQP2 at the basolateral membrane. It should be noted that BBS1 or BBS10 silencing did not affect the overall polarity of MCD4 monolayer, being preserved the Zo-1 and Na+/K+ ATPase staining.

The different effect of BBS1 and BBS10 knockdown on tubulin polymerization and AQP2 trafficking may reflect the different function of these two BBS proteins within the renal cell. BBS1 is an important component of the BBSome, a complex of proteins that promotes ciliary membrane biogenesis [38]. The chaperonin-like proteins, BBS10, BBS6 and BBS12, promote BBSome assembly [39-41]. Interestingly, clinical data show more severe renal phenotype in patients with mutations in BBS chaperonine-like proteins [23]. So far, only a BBSome protein, i.e. BBIP10, but none of the chaperonin-like BBS proteins, has a known role in regulating microtubule acetylation [42]. Our results strongly indicate a novel function of BBS10 in promoting tubulin acetylation.

It has recently proposed that BBS10 inactivation may affect the vasopressin/AQP2 transduction cascade in renal cells [25]. Our *in vitro* experiments evidence those BBS10 knockdown associates with loss of primary cilia, where the V2R localizes. Therefore, it is conceivable that cilia loss may prevent CD cells from sensing luminal vasopressin, thus inhibiting AQP2 exocytosis. This hypothesis may explain the high frequency of low-osmolar urine and polyuria in several ciliopathies, including autosomal dominant polycystic kidney disease (ADPKD), nephronophthisis (NPHP) and medullary cystic kidney disease [43]. However the loss of primary cilium in BBS1-deprived cells and the presence of a normal urine concentrating ability in BBS1 patients do not support this hypothesis. Here, in addition to the well-established role

of cilium loss in attenuating vasopressin response in BBS, we propose that loss of polarized trafficking of AQP2 as a novel explanation for the polyuria associated with mutations of the BBS10 gene. Whether this mechanism might explain the polyuria observed in other ciliopathies is worth further studies.

Taken together, our data highlight a possible unexplored physiological role of BBS10 in regulating the dynamics of microtubule post-translational modification, thus influencing the sorting machine that regulates the apical expression of AQP2 in renal cells.

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