Mild Nephrogenic Diabetes Insipidus Caused by Foxa1 Deficiency

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Foxa1 is a member of the winged helix family of transcription factors and is expressed in the collecting ducts of the kidney. We investigated its potential contribution to renal physiology in Foxa1-deficient mice on a defined genetic background. Foxa1−/− mice are dehydrated and exhibit electrolyte imbalance as evidenced by elevated hematocrit and plasma urea levels, hypernatremia, and hyperkalemia. This phenotype is the consequence of decreased urine osmolality secondary to renal vasopressin resistance. Mutations of the human genes encoding the vasopressin 2 receptor and aquaporin 2 cause nephrogenic diabetes insipidus; however, expression of these genes is maintained or even increased in Foxa1−/− mice. Likewise, expression of the genes encoding the Na-K-2Cl cotransporter (NKCC2), the calcium channel CLCNKB, barttin (BSND), and the calcium-sensing receptor (CASR), each of which is important in sodium reabsorption in the loop of Henle, is maintained or even increased in Foxa1-deficient mice. Thus, we have shown that Foxa1−/− mice represent a new model of nephrogenic diabetes insipidus with unique molecular etiology, and we have identified the first transcription factor whose mutation leads to a defect in renal water homeostasis in vivo.

Maintenance of water homeostasis is central to mammalian survival and therefore under tight hormonal and metabolic control. Water homeostasis is achieved by balancing fluid intake with water excretion, governed by the antidiuretic action of arginine-vasopressin (AVP, also known as antidiuretic hormone, ADH) (for recent reviews, see Refs. 1 and 2). Increases in plasma osmolality are sensed by osmoreceptors in the circumventricular organ in the anterior hypothalamus. Stimulation of these receptors causes release of AVP from the posterior pituitary. AVP is synthesized and initially secreted as a neurophysin-AVP complex but is uncoupled before it reaches its target, the AVP V2R receptor, on the basolateral surface of tubular cells in the renal collecting ducts.

Binding of AVP to its receptor activates adenylate cyclase, leading to elevated intracellular cAMP levels. AVP binding triggers both short term and long term responses. Immediately following activation of the receptor, preexisting stores of the water channel protein aquaporin 2 translocate to the apical surface of the cell, allowing for reabsorption of water from the collecting duct (3). Water exits the duct cell via the basolaterally located aquaporins 3 and 4, enters the interstitial space, and ultimately returns to the circulation (4, 5). Over the longer term, elevated cAMP levels lead to activation of protein kinase A and the phosphorylation of the transcription factor CREB (cyclic AMP-response element-binding protein) (6). Thus activated, CREB increases transcription of the aquaporin 2 message and ultimately increases aquaporin 2 protein levels (7).

The central importance of the AVP-aquaporin 2 pathway is most clearly illustrated by the condition of diabetes insipidus (DI), which is characterized by polyuria and polydipsia secondary to a lack of AVP secretion (central DI) or resistance of the kidney to its action (nephrogenic DI, or NDI). Inherited nephrogenic DI can be caused by mutations in the genes encoding either the vasopressin 2 receptor or aquaporin 2, highlighting the central role of these two proteins in the pathway (7–11). The ability of the kidney to concentrate urine also depends on the interstitial osmotic gradient, as illustrated by the molecular defects in Bartter’s syndrome. In this condition, sodium reabsorption in the loop of Henle is impaired as a result of mutations in various ion channels (12–14).

The transcription factor Foxa1 was originally identified as the DNA binding protein hepatocyte nuclear factor 3α (HNF3α) and shown to function in hepatic gene regulation (15, 16). Analysis of the promoter of the rat Foxa1 gene identified binding sites for a kidney-enriched transcription factor and prompted the re-evaluation of the expression domain of Foxa1 (17). Foxa1 transcripts were localized to the collecting duct in embryonic and adult kidney. Data base analysis identified potential Foxa1 binding sites in the promoters of several genes prominently expressed in the kidney, including the vasopressin receptor and several subunits of the Na,K-ATPase (17). These findings prompted us to evaluate the renal physiology of mice deficient for Foxa1. Here we show that Foxa1−/− mice exhibit all of the hallmarks of nephrogenic diabetes insipidus, identifying the Foxa1 gene as the first transcription factor whose inactivation results in a defect in renal water homeostasis.

MATERIALS AND METHODS

Animals—The derivation of the Foxa1−/− mice has been described previously (18). The mutation has since been backcrossed for 12 generations to the C57BL/6 and 129SvEv inbred mouse strains to obtain inbred congenic lines. All mutant mice were analyzed on postnatal...
day 8 (P8) as F1 hybrids (C57BL/6 × 129SvEv) along with littermate controls. Using F1 hybrids has the advantage of hybrid vigor by complementation of recessive mutations from parental strains (19). The F1 hybrid is a defined genetic background, as all mice are genetically uniform with the exception of the Foxa1 locus. Mice were housed in 12-h light/dark cycles and fed standard rodent chow ad libitum. All experiments were conducted in accordance with the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Blood and Urine Chemistry—Trunk blood and urine were collected from P8 mice after sacrifice by decapitation. Blood and urine chemistries were determined at Anilytics Inc. (Gaithersburg, MD). Urine osmolality was determined using an Advanced Micro osmometer (Advanced Instrument Inc., Norwood, MA).

Histology and Immunofluorescence—For histology, kidneys were collected from fetuses (day 17.5 postcoitum), on postnatal day 1, and on postnatal day 8. After fixation in 4% paraformaldehyde, tissues were paraffin-embedded, sectioned, and stained with hematoxylin and eosin. For immunofluorescence, coronal sections of mouse kidneys were fixed in 4% paraformaldehyde prepared in 5% sucrose in PBS for 1 h. The tissue was quenched with 50 mM NH₄Cl in 5% sucrose, PBS for 10 min. After washing twice with 5% sucrose, PBS, the tissue was immersed in graded sucrose/PBS solutions to a final sucrose concentration of 2.3 M for cryoprotection. Blocks of tissue were embedded in OCT compound (Sakura Finetek, Torrance, CA), diluted 1:2 with 20% sucrose, frozen, and stored at −80 °C. Tissue sections (4 µm) were cut by using a Reichter-Jung cryostat (model 2800) and collected on Probe-on-Plus (Fisher) microscope slides.

Sections were hydrated with PBS, blocked with 10% goat serum in PBS for 1 h, and then incubated for 3 h with an anti-aquaporin 2 (anti-AQP2) antibody (stock, 0.2 mg/ml) raised in rabbit (Alomone Labs, Jerusalem, Israel) diluted 1:25 in PBS containing 0.1% bovine serum albumin and 0.2% Triton X-100. Slides were then washed three times for 5 min with PBS and incubated in the dark for 1 h at room temperature with a 1:100 dilution of Texas Red-conjugated affinity-purified goat anti-rabbit IgG (H+L) secondary antibody (Jackson ImmunoResearch Labs).

Immunofluorescence localization with one of the following primary antibodies to AQP3, AQP4, Na,K-ATPase α subunit, ROMK, Tamm Horsfall, and NKCC2 was conducted in a manner similar to the anti-AQP2 staining above with the noted exceptions below. Anti-AQP3 fraction 3 antibody (stock, 0.2 mg/ml) raised in rabbit (a generous gift from Mark Knepper) was diluted 1:100 in PBS, and anti-AQP4 antibody (stock, 0.2 mg/ml) raised in rabbit (Alomone Labs) was diluted 1:100. Anti-Na,K-ATPase α subunit antibody (stock, 1 mg/ml) raised in rabbit (Upstate Biotechnology, Lake Placid, NY) was diluted 1:200, and anti-ROMK antibody (stock, 0.24 mg/ml) raised in rabbit (Alomone Labs) was diluted 1:15. Anti-Tamm Horsfall glycoprotein antibody that recognizes TALH (stock, 0.05 mg/ml) raised in goat (ICN Cappel Pharmaceuticals, Aurora, OH) was diluted 1:1000, and slides were blocked with 10% bovine serum albumin in PBS for 1 h. Anti-NKCC2 antibody (stock,
0.25 mg/ml) raised in rabbit (a generous gift from Mark Knepper) was diluted 1:100. The slides were washed three times and incubated with Texas Red anti-rabbit secondary antibody at a 1:100 dilution as described above for anti-AQP2 staining to label anti-AQP3, anti-AQP4, and anti-NKCC2 antibodies. Texas Red anti-rabbit IgG (H+L) secondary antibody at a dilution of 1:200 was used for the anti-Na,K-ATPase subunit, and a 1:400 dilution was used for anti-ROMK visualization. Texas Red-conjugated affinity-purified donkey anti-goat IgG (H+L) secondary antibody (Jackson ImmunoResearch Labs) at a 1:100 dilution was used to incubate the slides in the dark for 1 h to visualize the anti-Tamm Horsfall glycoprotein labeling. Control experiments consisted of either omitting the primary antibodies or substituting the primary antibody with non-immune chicken or rabbit antibody, as appropriate. All controls were negative for specific primary antibody labeling. Fluorescence microscopy was performed using a Nikon Microphot-FX microscope with a CoolSNAP digital camera (Roper Scientific).

**RESULTS AND DISCUSSION**

**Foxa1-deficient Mice Are Dehydrated**—During the initial characterization of Foxa1<sup>−/−</sup> mice, we had noticed the dehydrated appearance of the mutant pups in addition to their hypoglycemic phenotype (18). To investigate this further, we analyzed blood chemistries of these mice on a defined genetic background to minimize the variability of the phenotype. Heterozygous mice were backcrossed to both 129SvEv and C57BL/6 inbred strains of mice for more than 10 generations to derive incipient congenic strains with greater than 99% homozygosity, and all mutants were analyzed in F1 matings between the two parental strains to ensure genetic homogeneity (see "Materials and Methods" for details). The hematocrit, plasma urea, sodium, and potassium in postnatal day 8 mutant mice were all elevated in comparison with littermate controls (Fig. 1, A–D). Thus, Foxa1<sup>−/−</sup> mice were significantly dehydrated and displayed elevated plasma osmolality. However, plasma creatinine levels were normal, excluding the possibility of global renal failure (Fig. 1E).

As water balance is maintained by controlling both fluid
intake and water excretion, we first considered the possibility that Foxa1−/− mice might not drink sufficiently. However, we frequently found milk in the stomach of these mice, making this unlikely (data not shown). Next we considered that the Foxa1 mutant mice might not be able to concentrate urine to a sufficient degree to prevent water loss. When we determined urine osmolality, we indeed found that Foxa1−/− mice have significantly more dilute urine than their control littersmates, despite the fact that they are dehydrated (Fig. 2A). We confirmed this finding using two other measures. Kidney weight was significantly increased in Foxa1−/− mice, despite the fact that these mice are smaller than their littermates, indicative of water retention (Fig. 2A). Polyuria was suggested by the presence of dilated collecting ducts in Foxa1−/− mice as determined by immunofluorescence staining with antibodies to aquaporin 2 (Fig. 2B, C and D). Thus, Foxa1 mice failed to concentrate urine in the face of severe dehydration.

**F Foxa1−/− Mice Have Nephrogenic Diabetes Insipidus—**As Foxa1 has been shown to be expressed in the developing central nervous system as well as the kidney (17, 22–24), we considered the possibility that Foxa1−/− mice might suffer from central diabetes insipidus. Therefore, we investigated whether expression of arginine-vasopressin in the hypothalamus is dependent on Foxa1. We isolated total RNA from the hypothalami of P8 control and mutant mice and determined the expression of the AVP message by RNase protection assay (data not shown) and quantitative reverse transcription-PCR. As shown in Fig. 3, AVP mRNA is still expressed in the hypothalamus of Foxa1−/− mice; in fact, its expression is increased 2.5-fold over controls (p < 0.05). This increase in the AVP message is likely not a direct effect of Foxa1 deficiency in the hypothalamus, i.e. the removal of Foxa1 as a transcriptional repressor of the AVP gene, but rather a response of the hypothalamus to the state of dehydration. Osmotic stimulation and water retention have been shown previously to increase AVP expression in the hypothalamus (25).

Next we investigated whether the diabetes insipidus of Foxa1−/− mice could be nephrogenic. To address this question, we injected both control and Foxa1−/− mice with AVP and determined urine osmolality 2 h later. As shown in Fig. 4, injection of AVP into P8 control mice led to a significant increase in urine osmolality compared with saline-injected controls, demonstrating for the first time that preweaning mice have the capacity to concentrate urine in response to AVP. In contrast, Foxa1−/− mice showed no response to exogenous AVP (Fig. 4). Thus, Foxa1−/− mice showed all of the hallmarks of nephrogenic diabetes insipidus. Given the facts that Foxa1 is expressed in many other tissues besides the kidney (22) and that Foxa1−/− mice display multiple metabolic defects (18), it is possible that the renal phenotype described here is an indirect consequence of the overall metabolic state of the animals.

Given the facts that nephrogenic DI has been shown to be caused by mutations in the genes encoding the vasopressin receptor V2R and aquaporins 1–3 as well as all of the subunits of the Na,K-ATPase (17), we investigated whether the expression of these genes might be altered in Foxa1−/− mice. We developed specific probes for the vasopressin V2R receptor and aquaporins 1–3 as well as all of the subunits of the Na,K-ATPase and measured their mRNA levels by RNase protection assay (Fig. 5 and data not shown). As is demonstrated in Fig. 5A, expression of the vasopressin 2 receptor and aquaporin 1 is unchanged in Foxa1−/− mice. Interestingly, mRNA levels for both aquaporin 2 (apical membrane) and aquaporin 3 (basolateral membrane) are increased —3-fold in Foxa1 mutant mice (Fig. 5, B and C). Thus, a deficiency in vasopressin receptor or aquaporin gene expression cannot be the cause for the defect in water retention in Foxa1 mutant mice. In fact, the increased renal expression of aquaporin 2 documents that both sensing of and response to dehydration by the AVP-aquaporin system are intact in Foxa1−/− mice. In addition, expression of all of the subunits of the Na,K-ATPase was maintained in Foxa1-deficient mice (data not shown).

Water reabsorption in the kidney is also dependent on the interstitial solute concentration as evidenced by the various forms of Bartter’s syndrome. Bartter’s syndrome is characterized by electrolyte imbalance and severe polyuria. So far, five genes expressed in the thick ascending limb of Henle’s loop in the kidney have been demonstrated to contribute to the interstitial solute concentration. These encode the Na-K-2Cl cotransporter (NKCC2), the potassium channel ROMK, the β
subunit of the chloride channels ClC-KA and ClC-KB, barttin (BSND), the calcium-sensing receptor (CASR), and the chloride channel CLCNKB (12–14, 26, 27). To investigate whether any of these genes might be dependent on Foxa1 and thus contribute to the Foxa1 mutant phenotype, we determined their transcript levels by quantitative real time PCR. As shown in Fig. 6, expression of ROMK, BSND, and the calcium-sensing receptor was not changed, whereas NKCC2 and CLCNKB were upregulated by 69% (p < 0.0005) and 154% (p < 0.02), respectively, in Foxa1-deficient kidneys. As discussed above for the changes in aquaporin 2 and 3 levels, we propose that these increases are not direct transcriptional responses to Foxa1 but rather an attempt by the kidney to compensate for the water loss. In addition, no significant differences in immunofluorescent localization of AQP3, AQP4, Na,K-ATPase subunit, Tamm Horsfall protein, and NKCC2 were seen between the

**Fig. 5.** *Foxa1*−/− mice have increased renal expression of aquaporins 2 and 3. Total kidney RNA was isolated from P8 control and mutant mice and subjected to RNase protection assay with probes specific to the vasopressin 2 receptor and aquaporins 1–3 as described under “Materials and Methods.” A, autoradiogram of RNase protection assay for vasopressin 2 receptor (VR) and aquaporin 1 (AQP1). M, marker lane, sizes in nucleotides; P, undigested antisense RNA probes; T, tRNA (negative control demonstrating complete digestion of unhybridized probe). Control and mutant samples contain equal levels of vasopressin 2 receptor and AQP1 mRNAs. B, a similar RNase protection assay was performed with a probe specific for mouse aquaporin 2 (AQP2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard. Radioactive bands were quantified using phosphorimaging analysis and are expressed as expression levels of AQP2 relative to glyceraldehyde-3-phosphate dehydrogenase. AQP2 levels are increased 3.5-fold in *Foxa1*−/− mice compared with control littermates. C, RNase protection assay for aquaporin 3 (AQP3), a basolateral water channel in the kidney. AQP3 mRNA levels are increased ~3-fold in *Foxa1*−/− mice compared with control littermates. Data in B and C are presented as mean ± S.E. (n = 3 for control and n = 3 for mutant). Statistical significance was determined by Student’s t test. *, p < 0.02; ***, p < 0.001. **CASR, calcium-sensing receptor.**

**Fig. 6.** Expression of ion channel and transporter genes in *Foxa1* mutant mice. Real time PCR was performed on kidney RNA isolated from postnatal day 8 control or *Foxa1*−/− mice as described under “Materials and Methods.” Hypoxanthine phosphoribosyltransferase (HPRT) was used as the internal control. All expression data were normalized to hypoxanthine phosphoribosyltransferase expression in the same sample and scaled to an expression level of 1 in the control. Data are presented as mean ± S.E. (n = 5 for control and n = 6 for mutant). Statistical significance was determined by Student’s t test. *, p < 0.02; ***, p < 0.001. **CASR, calcium-sensing receptor.**

**Fig. 7.** Fetal and newborn renal morphology of *Foxa1* mutant mice. Shown are histological images obtained from control (A, B, and C) and *Foxa1*−/− mice (D, E, and F) kidneys harvested on gestational day 17.5 (A and D), postnatal day 1 (B and E), and postnatal day 8 (C and F). dpc, days postcoitum.
wild type and the Foxa1-deficient kidneys (data not shown).

Fetal Kidney Development and Postnatal Morphology Are Normal in Foxa1−/− Mice—As Foxa1 has been shown to be expressed in the developing kidney, we considered the possibility that the physiological defects described above might be secondary to a delay or defect in renal development. To address this issue, we analyzed renal morphology in late fetal stages as well as postnatal days 1 and 8, the latest stage to which the Foxa1−/− mice survive. As is shown in Fig. 7, renal morphology is comparable between control and mutant mice, suggesting that the physiological defect is not caused by a defect in embryogenesis.

CONCLUSION

In summary, we have demonstrated that mice homozygous for a null mutation for the winged helix transcription factor Foxa1 develop nephrogenic diabetes insipidus shortly after birth, identifying Foxa1 as the first transcription factor that when mutated leads to defects in renal water reabsorption. Analysis of the expression levels for all genes encoding hormone receptors, channels, and ion transporters known to affect water balance in the kidney failed to identify a direct downstream target for Foxa1 in that could account for the phenotype. Thus, Foxa1−/− mice represent a new and unique model of diabetes insipidus that will form the basis of identifying new pathways important in renal physiology. We are currently developing a conditional allele of Foxa1 that will enable us to delete the gene only in the kidney, as the current model does not differentiate between a primary defect in the kidney or one caused by the other metabolic abnormalities in the mice. Future work will be directed at identifying the transcriptional targets of Foxa1 in the kidney and investigating whether the human FOXA1 gene is mutated in certain patients suffering from nephrogenic diabetes insipidus.

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