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Role of Collecting Duct Urea Transporters in the Kidney – Insights from Mouse Models

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Abstract. Urea movement across plasma membranes is modulated by specialized urea transporter proteins. These proteins are proposed to play key roles in the urinary concentrating mechanism and fluid homeostasis. To date, two urea-transporter genes have been cloned; UT-A (*Slc14a2*), encoding at least five proteins and UT-B (*Slc14a1*) encoding a single protein isoform. Recently we engineered mice that lack the inner medullary collecting duct (IMCD) urea transporters, UT-A1 and UT-A3 ($UT\text{-}A1/3^{-/-}$ mice). This article includes 1) a historical review of the role of renal urea transporters in renal function; 2) a review of our studies utilizing the $UT\text{-}A1/3^{-/-}$ mice; 3) description of an additional line of transgenic mice in which beta-galactosidase expression is driven by the alpha-promoter of the UT-A gene, which is allowing better physiological definition of control mechanisms for UT-A expression; and 4) a discussion of the implications of the studies in transgenic mice for the teaching of kidney physiology.

Key words: UT-A — Urinary concentrating mechanism — Countercurrent multiplication

Introduction

Historical Background

In the 1930's, clearance studies by James Shannon demonstrated that urea was reabsorbed by at least two distinct mechanisms within the kidney (Shannon, 1936; Shannon, 1938). One process, postulated to occur in the proximal tubule, and accounting for the reabsorption of approximately 40% of the filtered load of urea, was constitutive and could be strongly inhibited by the induction of an osmotic diuresis. The second reabsorption process, which occurs in the distal nephron, is dependent on the state of antidiuresis, showing greater urea reabsorption with higher levels of antidiuretic hormone (vasopressin) stimulation. Subsequent work by Morgan and Berliner in the 1960's identified the distal urea transport process identified by Shannon's studies (Morgan & Berliner, 1968; Morgan, Sakai & Berliner, 1968). In their newly developed isolated papilla preparation, they demonstrated that transepithelial urea transport across the inner medulla collecting duct (IMCD) epithelium was relatively fast and was accelerated by the addition of vasopressin. At the same time as these studies, studies by Burg and Grantham using isolated perfused tubules (Grantham & Burg, 1966) demonstrated that an earlier part of the collecting duct, the cortical collecting duct, has a very low permeability to urea that is not increased by vasopressin. Thus, rapid vasopressin-regulated urea transport was limited to the most distal part of the collecting duct system.

Morphometric studies (Knepper et al., 1977) demonstrated that the IMCD is composed of two morphologically distinct segments, prompting separate measurements of urea permeability in the initial and terminal parts of the IMCD. These measurements determined that the terminal part, but not the initial part, of the IMCD possesses extraordinarily high urea permeability, much greater than could be accounted for by simple lipid-phase permeation (Sands & Knepper, 1987). Furthermore, vasopressin increased urea permeability only in the terminal IMCD (Sands, Nonoguchi & Knepper, 1987). Thus, based on the prior demonstration of phloretin-sensitive urea transport in red blood cells (Macey & Farmer, 1970), it was proposed that an increase in urea permeability due to vasopressin required a specific transporter

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protein in the IMCD to act as a target. Chou and colleagues subsequently performed the critical studies that demonstrated that urea transport in the IMCD has properties consistent with a transporter-mediated (facilitated) mechanism, *viz*. inhibition by phloretin and urea analogues, and saturability of transport (Chou & Knepper, 1989; Chou et al., 1990a). In fact, the transport properties demonstrated by the IMCD were remarkably similar to those observed by Macey and Farmer for the red blood cell urea transporter more than a decade earlier. It was concluded that the terminal portion of the IMCD possesses a specialized urea transporter similar to the urea transporter in red blood cells.

Further studies in isolated perfused tubules (before the cloning of the aquaporins and urea transporters) addressed whether vasopressin-regulated urea transport and vasopressin-regulated water transport were mediated by the same transporter protein in the IMCD. Both processes were shown to be dependent on a rise in intracellular cyclic AMP (Star et al., 1988). However, careful reflection-coefficient measurements showed that the urea and water pathways were distinct, indicating separate water and urea channels (Chou et al., 1990b; Knepper, Sands & Chou, 1989). Nevertheless, kinetic measurements by Wall et al. demonstrated that the time courses of increased urea and water permeability in the IMCD in response to vasopressin were virtually indistinguishable (Wall et al., 1992). These results suggested that either the signaling processes were rate-limiting for the response to vasopressin, or that the as yet unidentified water channels and urea transporters were co-regulated, perhaps by shuttling to the plasma membrane on the same vesicles. However, additional studies of the activation and inactivation of water and urea transport in perfused IMCDs showed that if the osmotic gradient was reversed, the time courses of regulation of the two transport processes clearly differed, demonstrating separate transport mechanisms for urea and water (Nielsen & Knepper, 1993).

Role of Urea in Systemic Water Balance

In mammals, greater than 90% of waste nitrogen is normally excreted by the kidney as urea, the balance being attributable to ammonium and uric acid. The majority of this urea is generated in the liver, as a product of protein metabolism, from the urea-ornithine cycle. In humans and animals, under most circumstances, dietary protein intake greatly exceeds that necessary for the support of anabolic processes, thus excess quantities of urea are generated. For example, in humans, daily urea excretion is usually on the order of 0.5 to 1 mole per day. This excreted urea constitutes a large osmotic load to the kidney. Most solutes excreted in such large amounts, for example mannitol (Atherton, Hai & Thomas, 1968), would obligate large amounts of water excretion by causing an osmotic diuresis. However, as emphasized as early as the 1930s by Gamble et al. (Gamble, Putnam & McKhann, 1929; Gamble et al., 1934), it is evident that the kidney possesses specialized mechanisms that allow large amounts of urea to be excreted without obligating excessive water excretion.

In the 1950's, Ullrich and Jarausch (Ullrich, Drenckhan & Jarausch, 1955; Ullrich & Jarausch, 1956) showed that the concentration of urea and NaCl rose progressively from the corticomedullary junction to the tip of the papilla, and in particular that urea is accumulated in large amounts in the inner medulla of the kidney. Based on these observations, Berliner et al. (Berliner et al., 1958) proposed a mechanism for the excretion of large amounts of urea without obligating water excretion. They hypothesized that urea would not cause an osmotic diuresis if it was accumulated in the inner medullary interstitium to concentrations comparable to the urea concentration in the urine. Thus, interstitial urea was proposed to osmotically balance luminal urea, preventing a large secretory flux of water that would otherwise occur if urea did not accumulate in the renal inner medulla.

The process for urea accumulation in the medulla has been thoroughly studied and it is generally accepted that the accumulation is dependent on urea transport across the epithelium of the IMCD (Knepper & Star, 1990). As detailed above, several studies have documented the high urea permeability of the IMCD, which allows rapid urea equilibration across the IMCD epithelium. Two general mechanisms exist that account for the retention of the urea delivered from the IMCD to the inner medulla interstitium. First, countercurrent exchange processes by the vasa recta allow blood perfusion of the inner medulla without washout of the accumulated urea (Berliner & Bennett, 1967; Jamison, Bennett & Berliner, 1967). Second, urea from the inner medullary interstitium is transported back into the lumen of Henle's loop to be recycled back to the inner medullary collecting duct lumen where it can be reabsorbed (Knepper & Roch-Ramel, 1987; Trinh-Trang-Tan & Bankir, 1998).

In addition to the role of urea in nitrogen excretion and the requirements of the kidney to excrete it without obligating water, other roles have been proposed for urea transporters in the regulation of water balance. Most notable is a hypothesis proposed in the 1970s by Stephenson (Stephenson, 1972) and by Kokko and Rector (Kokko & Rector, 1972), suggesting a direct role for urea in the concentration of NaCl in the inner medulla. This hypothesis will be discussed later in this review.

Urea Transporters

The rich body of descriptive physiological observations described above facilitated the cloning of urea transporters from the kidney and from red blood cells. At present, several cDNAs (You et al., 1993; Olives et al., 1994; Smith et al., 1995; Shayakul, Steel & Hediger, 1996; Fenton et al., 2000; 2002c; Smith et al., 2004;) encoding urea transporters have been isolated and characterized and these transporters are members of two distinct, but closely related, urea transporter genes, UT-A (*Slc14a2*) and UT-B (*Slc14a1*) (Lucien et al., 1998; Nakayama et al., 2001; Fenton et al., 2002b;). In the mouse kidney, three cDNAs representing distinct UT-A urea transporter isoforms have been cloned and characterized. Two isoforms, UT-A1 and UT-A3, are expressed exclusively in IMCD cells. Immunochemical methods have localized UT-A1 to the cytoplasm and apical region of the IMCD (Nielsen et al., 1996; Fenton et al., 2002c;), whereas UT-A3 is localized both intracellularly and in the basolateral membrane (Terris, Knepper & Wade, 2001; Stewart et al., 2004) (*see* Figure 1). In contrast, UT-A2 is expressed in both the inner medulla and the inner stripe of the outer medulla, where it is localized to both the long and short limbs of Henle's loop, respectively (Fenton et al., 2002c; Shayakul et al., 1997; Wade et al., 2000) (*see* Fig. 1). In contrast to the multiple UT-A isoforms, the mouse UT-B gene encodes only a single protein. UT-B is expressed exclusively throughout the kidney medulla in the basolateral and apical (luminal) regions of the descending vasa recta (DVR) endothelial cells (Promeneur et al., 1996; Tsukaguchi et al., 1997; Xu et al., 1997).

Recently we generated mice in which the collecting duct-specific urea transporters, UT-A1 and UT-A3, were knocked out in tandem (Fenton et al., 2004). The initial aspect of this short review is to summarize our studies with these mice and discuss the conclusions of these studies with respect to existing concepts in renal physiology. The second aspect of the review summarizes results from an additional line of transgenic mice that has allowed us to examine the transcriptional regulation of the UT-A urea transporter in vivo (Fenton, Shodeinde & Knepper, 2006).

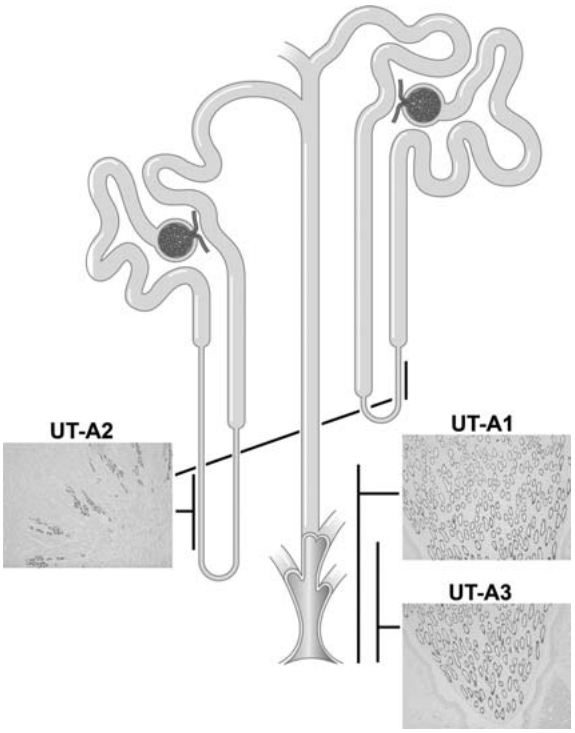

Fig. 1. Localization of UT-A urea transporters in the mouse renal tubule. A schematic representation of the mouse nephron is shown. UT-A1 is localized to the terminal portion of the IMCD and is both intracellular and in the apical domain. UT-A2 is localized to the thin descending limbs of Henle's loop in both the outer medulla and inner medulla. UT-A3 is localized to the terminal portion of the IMCD and is both intracellular and in the basolateral domains.

UT-A1 and UT-A3 Knockout Mice

In 2004, we developed a mouse model that allowed us to specifically assess the role of inner medullary urea transport in kidney function (Fenton et al., 2004). These UT-A1/UT-A3 knockout mice were produced by standard gene-targeting techniques. Briefly, the animals (termed *UT-A1/3*$^{-/-}$ mice) were generated by deletion of 3 kb of the UT-A gene containing a single 148-bp exon, exon 10. Exon 10 codes for amino acids 291-339 of UT-A1 and is situated in a large hydrophobic region, hypothesized to be membrane-spanning (Sands, 2003). Thus, it was predicted that deletion of this segment would completely disrupt the urea transport properties of the UT-A1 and UT-A3 proteins (due to the complex nature of the differential splicing within the UT-A gene, deletion of exon 10 putatively resulted in the deletion of a testis-specific isoform UT-A5 (Fenton et al., 2002b); *see below*). Successful deletion of the transporters from the IMCD was confirmed by immunoblotting and immunocytochemistry with several isoform-selective polyclonal antibodies, demonstrating that UT-A1 and UT-A3 proteins were absent from the IMCD of *UT-A1/3*$^{-/-}$ mice. Furthermore, a functional assessment of *UT-A1/3*$^{-/-}$ mice was performed using isolated perfused tubule studies and showed a complete absence of phloretin-sensitive and vasopressin-regulated urea transport in IMCD segments (*see* Fig. 2).

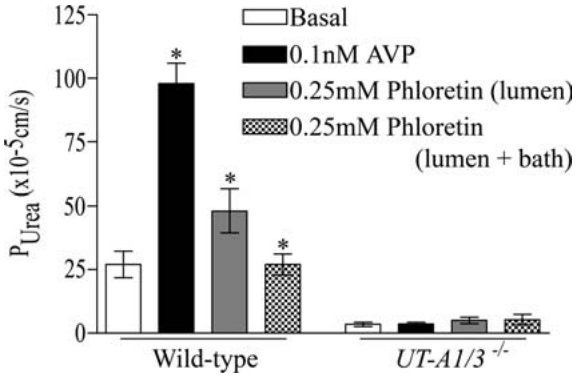

Fig. 2. Summary of the urea permeability (P_{urea}) in the IMCD of *UT-A1/3*$^{-/-}$ mice. Five tubules from age-matched wild-type and *UT-A1/3*$^{-/-}$ littermates were perfused for each group. Initially, the effect of 0.1 nM AVP on the basal P_{urea} was determined. Subsequently, the effect of 0.25 mM phloretin on P_{urea} in the presence of AVP was examined. Values are mean ± SEM and * represents a significant change in P_{urea} from the preceding perfusion group, as determined by two-way ANOVA. The conclusion from this data is that the urea transporters UT-A1 and/or UT-A3 are responsible for the vasopressin-stimulated and phloretin-sensitive urea permeability observed in the IMCD. Figure is adapted from Fenton et al., 2004.

General Observations of UT-A1 and UT-A3 Knockout Mice

UT-A1/3$^{-/-}$ mice have no instantly noticeable phenotype. Compared to normal wild-type animals they have no differences in appearance, body weight or behavior. The mice show complete sensory function and physical attributes. A comprehensive pathologic and histological survey of thirty three different tissues from *UT-A1/3*$^{-/-}$ mice determined that, apart from the kidney and testis, no abnormalities were present (Fenton et al., 2005). In the knockout animals, the kidneys are significantly smaller and the testis significantly larger compared to wild-type controls. The kidneys of *UT-A1/3*$^{-/-}$ mice also have greater blood congestion than the kidneys of wild-type animals, especially in the renal medulla. However, despite the increased blood congestion, total renal blood flow in the *UT-A1/3*$^{-/-}$ mice was not significantly different from that observed in age-matched wild-type control mice. However, excretion of nitric oxide was markedly increased in the *UT-A1/3*$^{-/-}$ mice relative to WT controls.

Role of IMCD Urea Transporters in the Urinary Concentrating Mechanism

The selective deletion of UT-A1 and UT-A3 provides an ideal model to address the role of inner medullary urea transporters in the urinary concentrating process. Contemporary thinking regarding the contribution of urea transporters to the urinary concentrating mechanism is based largely on a fundamental model of urea handling proposed in the 1950's (Berliner et al., 1958), *see* Introduction. Berliner *et al.* hypothesized that luminal urea in the IMCD is osmotically ineffective because of a high IMCD urea permeability that, abetted by countercurrent exchange processes, allows urea to accumulate to high concentrations in the inner medullary interstitium, thus preventing an osmotic diuresis. Therefore, the deletion of specialized urea transporters from the IMCD should result in an impaired capacity to conserve water, owing to the osmotic effect of urea in the lumen.

To test the 'Berliner hypothesis', the urinary concentrating function of *UT-A1/3*$^{-/-}$ mice on three different levels of protein intake was examined (Fenton et al., 2004; Fenton et al., 2005). *UT-A1/3*$^{-/-}$ mice fed either a normal-protein (20% protein by weight) or high-protein (40%) diet had a significantly greater fluid intake and urine flow, resulting in a decreased urine osmolality, than wild-type animals (*see* Fig. 3). However, *UT-A1/3*$^{-/-}$ mice on a low-protein diet (4% protein) did not show a substantial degree of polyuria. In this latter condition, hepatic urea production is low and urea delivery to the IMCD is predicted to be low, thus rendering the absence or presence of collecting duct urea transport immaterial with regard to water balance. In addition to the studies performed under basal conditions, when "challenged" by an 18-h water restriction, *UT-A1/3*$^{-/-}$ mice on a 20 or 40% protein intake are unable to reduce their urine flow to levels below those observed under basal conditions, resulting in volume depletion and loss of body weight. In contrast, *UT-A1/3*$^{-/-}$ mice on a 4% protein diet were able to maintain fluid balance without a marked loss of body weight.

We conclude from these findings that the concentrating defect in *UT-A1/3*$^{-/-}$ mice is caused by a urea-dependent osmotic diuresis; greater urea delivery to the IMCD results in greater levels of water excretion. Overall, the results are consistent with a role for IMCD urea transporters in the maintenance of water balance through their ability to prevent a urea-induced osmotic diuresis and are in agreement with the Berliner model.

Role of IMCD Urea Transporters in the Accumulation of NaCl in the Inner Medulla

In 1959, Kuhn and Ramel proposed the classical countercurrent multiplier model (Kuhn & Ramel, 1959), the basis for the urinary concentrating mechanism. The concentration gradient that drives the countercurrent multiplier system in the outer medulla, and thus concentrates the urine (and interstitial NaCl), relies on the active reabsorption of sodium chloride in the water-impermeable thick ascending limb of the loop of Henle. However, in the inner

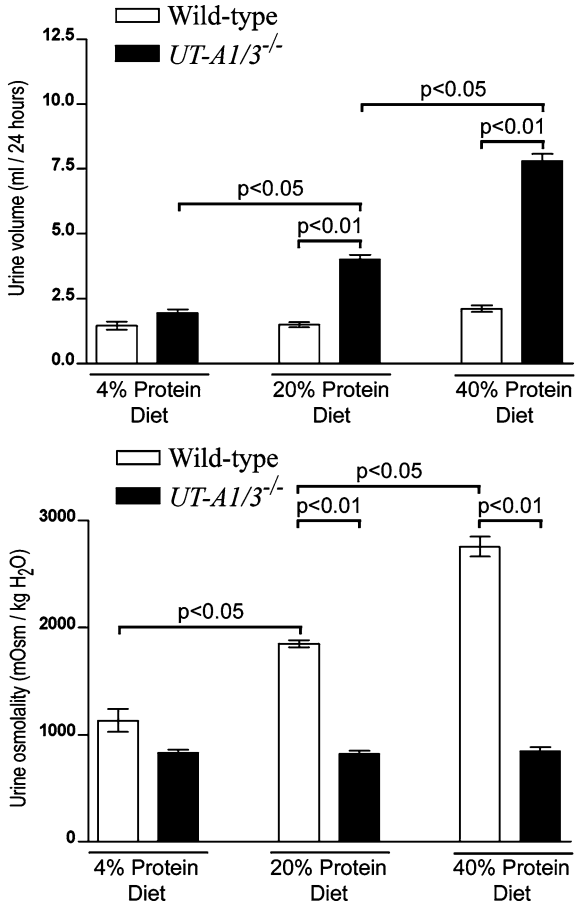

Fig. 3. Water conservation and urinary concentrating ability of *UT-A1/3*$^{-/-}$ mice. For all graphs, values are mean ± SEM and a significant difference (two-way ANOVA) between wild-type mice (*white bars*) and *UT-A1/3*$^{-/-}$ mice (*black bars*) is indicated. Mice received 4, 20, or 40% protein intake for 7 days before and throughout the duration of the study. Graphs show the urine output under basal conditions (free access to drinking water) and the urine osmolality under basal conditions. The conclusion from this data is that the role of IMCD urea transporters in water conservation is to prevent a urea-induced osmotic diuresis. Figure is adapted from Fenton et al., 2005.

medulla, the mechanism that concentrates NaCl in the interstitium, and thus water absorption from the collecting ducts, remains controversial, as the thin ascending limb of Henle's loop seems incapable of active NaCl transport (Imai & Kokko, 1974; Kondo et al., 1993). Various mechanisms have been offered to explain NaCl accumulation in the inner medulla (Knepper, Chou & Layton, 1993; Knepper et al., 2003; Schmidt-Nielsen, 1995; Thomas, 2000). The most widely recognized and influential model has been the "passive" mechanism of NaCl transport proposed independently by Stephenson and by Kokko and Rector in 1972 (Kokko & Rector, 1972; Stephenson, 1972). In this mechanism, the rapid reabsorption of urea from the IMCD generates and maintains a high urea concentration in the inner medullary interstitium, resulting in a transepithelial gradient favoring the passive absorption of NaCl from the thin ascending limb of Henle's loop. Furthermore, if the urea permeability of the ascending limbs is extremely low (virtually zero), then any NaCl that has been reabsorbed from the ascending thin limb will not be replaced by urea and the ascending limb fluid will be dilute relative to the fluid in other nephron segments. This dilutional process is proposed to constitute a "single effect" analogous to that in the outer medulla that can be multiplied by the counterflow between the ascending and descending limbs of Henle's loops.

The passive model of NaCl accumulation in the inner medullary interstitium relies on rapid urea transport from the IMCD, facilitated by the urea transporters UT-A1 and UT-A3. Thus, if the passive model is correct (in the form proposed by Stephenson and by Kokko and Rector), we would predict that in *UT-A1/3*$^{-/-}$ mice the lack of specific urea transporters from the IMCD would impair the concentration of NaCl in the IM. However, two independent experiments in the *UT-A1/3*$^{-/-}$ mouse line failed to corroborate the view that inner medullary Na^+ accumulation depends on facilitated urea transport in the IMCD. In the first experiment, the mean urea, Na^+, Cl^-, and K^+ concentrations were measured in whole inner medulla tissue isolated from water-restricted *UT-A1/3*$^{-/-}$ mice and wild-type littermates (Fenton et al., 2004). In *UT-A1/3*$^{-/-}$ mice there was a significantly lower inner medullary urea concentration, however, there was no reduction in the mean Na^+, Cl^- or K^+ concentrations.

In a separate experiment, the osmolality, urea and Na^+ concentrations were measured in the cortex, outer medulla, and two levels of the inner medulla from *UT-A1/3*$^{-/-}$ and wild-type mice (Fenton et al., 2005). These measurements were performed after the mice had been fed either a low (4%) or high (40%) protein diet, in order to assess the separate effects of changes in dietary protein intake and/or deletion of the collecting duct urea transporters on corticomedullary solute gradients (*see* Fig. 4). From this experiment, several striking observations were apparent. Firstly, in wild-type mice, changing the dietary protein intake from 4 to 40% resulted in an increase in tissue osmolality that was caused solely, of the solutes measured, by a greater urea accumulation in the inner medulla. However, sodium concentrations at all levels of the corticomedullary axis were unaffected by changes in the dietary protein intake. Secondly, in contrast to wild-type mice, in *UT-A1/3*$^{-/-}$ mice there was a substantially attenuated corticomedullary osmolality gradient and no urea gradient on either diet. However, the corticomedullary sodium gradients were virtually equivalent in wild-type and knockout mice on either level of dietary protein intake. Thus, neither marked medullary urea depletion caused by

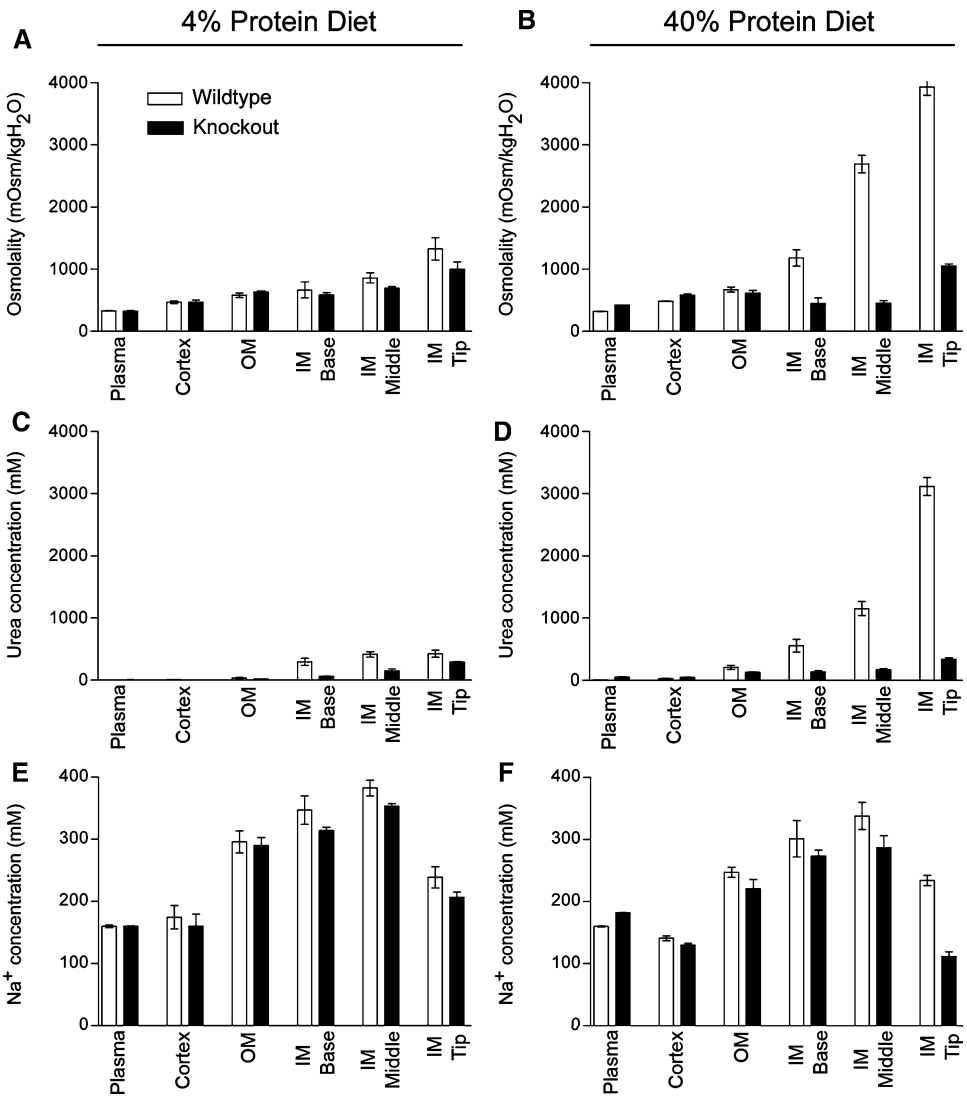

Fig. 4. Comparison of kidney solute composition of wild-type and *UT-A1/3*$^{-/-}$ mice. For all graphs, values are mean ± SEM. Wild-type mice (*white bars*) and *UT-A1/3*$^{-/-}$ mice (*black bars*) received either a 4 or 40% protein intake for 7 days before and throughout the duration of the study. Graphs show osmolality on either a 4% protein intake (*A*) or 40% protein intake (*B*); urea concentration on either a 4% protein intake (*C*) or 40% protein intake (*D*); Na concentration on either a 4% protein intake (*E*) or 40% protein intake (*F*). The conclusion from this data is that the absence of IMCD urea transport does not prevent the concentration of NaCl in the inner medulla. Figure is adapted from Fenton et al., 2005.

dietary protein restriction nor marked medullary urea depletion caused by deletion of collecting duct urea transporters affected the ability of the kidney to form a corticomedullary sodium gradient. We conclude from these two independent studies in *UT-A1/3*$^{-/-}$ mice that NaCl accumulation in the inner medulla is not reliant on either IMCD urea transport or the accumulation of urea in the IMCD interstitium. Thus, the passive concentrating model in the form originally proposed by Stephenson and by Kokko and Rector, where NaCl reabsorption from Henle's loop depends on a high IMCD urea permeability, is not the mechanism by which NaCl is concentrated in the inner medulla.

Unfortunately, the studies in *UT-A1/3*$^{-/-}$ mice do not provide an explanation or mechanism for NaCl accumulation in the inner medulla. However, the measurements of tissue osmolality, urea concentration, and sodium concentration do suggest that there is an "osmotic gap" between the measured osmolality and the additive concentrations of urea and NaCl, indicating that there are substantial amounts of unmeasured solutes. Numerous studies have reported that trimethylamines (e.g., glycerophosphorylcholine and betaine), polyhydric alcohols (e.g., inositol and sorbitol), amino acids, lactate, ammonium, and potassium are all accumulated in the inner medulla and it is likely that these unmeasured solutes

contribute to the osmotic gap. It is plausible that these unmeasured solutes play a role in both NaCl accumulation in the inner medulla and the urinary concentrating mechanism, and these possible mechanisms have been discussed in detail elsewhere (Knepper et al., 2003; Thomas, 2000).

Role of IMCD Urea Transporters in the Regulation of Glomerular Filtration Rate (GFR)

The consumption of diets rich in protein results in an increase in whole kidney GFR (Dicker, 1949; Mackay, Mackay & Addis, 1928). The mechanism behind this response is not fully understood, although microperfusion studies by Seney and Wright in the 1980's determined that this protein-induced increase in GFR results from changes in the tubuloglomerular feedback (TGF) system (Seney, Persson & Wright, 1987). Their studies, performed in rat, determined that after feeding for approximately 10 days with either a low or a high protein diet, there was an approximately 30% increase in the GFR of the high-protein diet-fed animals. Furthermore, simultaneous measurements of single nephron GFR (SNGFR) in the distal tubule were 20% higher in the rats fed the high-protein diet, due to a 50% smaller suppression of SNGFR by TG feedback, whereas proximally measured SNGFR was not different between the groups. They concluded that the sensing mechanism of the TG feedback system was rendered less responsive by a high protein intake, and that this change allowed GFR to increase. In further studies, they also determined that this diminished TGF response was caused, at least in part, by a reduced early distal NaCl concentration, without a change in early distal tubule osmolality (Seney & Wright, 1985). The exact cause of the reduced early distal NaCl concentration remained unknown.

One mechanism for the reduction in the early distal NaCl concentration, and thus the reduced GFR, was proposed by Bankir et al. (Bankir et al., 1993; Bankir, Bouby & Trinh-Trang-Tan, 1991). They suggested that the increased concentrations of urea consequent to a high protein intake allowed the concentration of NaCl to be lower in the early distal tubule, without rendering an osmotic effect. This increased urea concentration in the late thick ascending limb and early distal tubule would be dependent both on the urea concentration of the glomerular filtrate and the extent of urea recycling, a result of passive urea secretion into the loop of Henle from urea reabsorption in the IMCD (Klumper, Ullrich & Hilger, 1958; Lassiter, Gottschalk & Mylle, 1961; Lassiter, Mylle & Gottschalk, 1966). However, in *UT-A1/3*$^{-/-}$ mice, the lower inner medullary interstitial urea concentration is likely to result in the elimination of urea recycling and thus, if the mechanism proposed by Bankir *et al.* is correct, it would be predicted that the increase in GFR in response to high protein feeding would be markedly attenuated in these knockout mice.

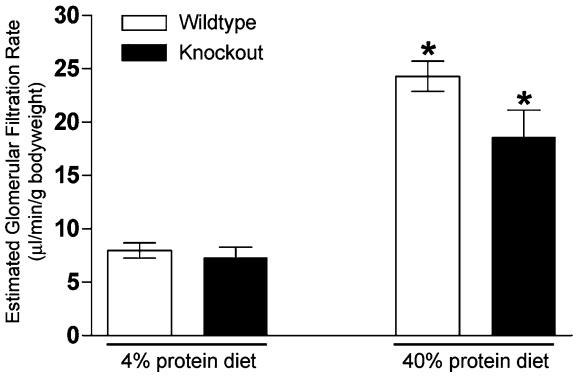

Fig. 5. Estimated GFR in conscious male mice. FITC–inulin clearance, as an estimate of GFR, for wild-type (*white bars*) and *UT-A1/3*$^{-/-}$ mice (*black bars*). Representative values are mean ± SEM and significant differences (two-way ANOVA) are indicated. Administration of a high-protein diet (40%) for 7 days dramatically increased GFR in both groups of animals ($n = 5$). However, no significant difference was observed in GFR between wild-type and *UT-A1/3*$^{-/-}$ mice. The conclusion from this data is that urea reabsorption from the IMCD and the process of urea recycling are not important determinants of protein-induced increases in GFR. Figure is adapted from Fenton et al., 2005.

To examine this hypothesis, we conducted a series of FITC–inulin clearance studies in conscious *UT-A1/3*$^{-/-}$ and wild-type mice on two levels of protein intake, low protein (4%) and high protein (40%). These experiments showed that increasing the protein content of the diet more than doubled the FITC–inulin clearance in both *UT-A1/3*$^{-/-}$ mice and wild-type controls, similarly to that observed in other studies. However, no significant differences were observed in inulin clearance between *UT-A1/3*$^{-/-}$ and wild-type mice under either dietary condition (*see* Fig. 5). Therefore, the conclusion from these studies using the *UT-A1/3*$^{-/-}$ mouse model is that urea reabsorption from the IMCD, and more specifically the process of urea recycling, is not an important determinant of protein-induced increases in GFR.

Role of IMCD Urea Transporters in the Regulation of Water Permeability

In isolated perfused IMCD tubules from *UT-A1/3*$^{-/-}$ mice, despite a complete absence of facilitated urea transport, there is no reduction in the basal, or the AVP stimulated, osmotic water permeability (Fenton et al., 2004). Thus, not only is the vasopressin signaling cascade intact in these animals, but their potential ability to transport water is not affected. Indeed, after water restriction, the expression of aquaporin 2 and aquaporin 3 in the kidney medulla of knockout mice is greater than in wild-type controls. Since previous studies have established that

increased levels of circulating vasopressin result in higher expression levels of the AQP2 and AQP3 in the kidney (DiGiovanni et al., 1994; Ecelbarger et al., 1995), the effect of water restriction in *UT-A1/3*$^{-/-}$ mice reflects a response by the kidney to the greater degree of water depletion. Therefore, the urinary concentrating defect in these animals arises solely from the urea-induced osmotic diuresis.

It therefore seems feasible that by regulating the expression or function of urea transporters in the IMCD, the kidney could indirectly regulate water and NaCl excretion by modulating the extent of urea-induced osmotic diuresis. Although this concept may seem both controversial and secondary to the regulation of, for example, the aquaporin water channels, it may also provide a mechanism that allows the kidney to maximize either its concentrating or diluting capacity. For example, in extracellular fluid volume expanded states, urea transporter expression is down-regulated and thus may be a homeostatic response to increase water and salt excretion (Wang et al., 2002). In addition, in other volume-expanded states, such as the Dahl rat model, the higher expression of urea transporters may be additive to the sodium retention observed (Fenton et al., 2003). Furthermore, some of the effects of glucocorticoids on water balance may result from the glucocorticoid-induced down-regulation of urea transporter expression in the IMCD (Naruse et al., 1997; Peng, Sands & Bagnasco, 2002).

Possibility of Active Urea Secretion along the Renal Tubule

Classically, the excretion of urea by the kidney is dependent on two elements; primarily on the filtered load of urea and secondarily on the amount of urea reabsorption that occurs along the nephron. During the process of urea recycling, the urea that is reabsorbed in the IMCD (via UT-A1 and UT-A3) is passively secreted into the loop of Henle (via UT-A2) (Klumper et al., 1958; Lassiter et al., 1961; 1966). In addition, several independent studies have proposed that active urea secretion may also occur somewhere along the nephron. Early evidence for active urea secretion in rodents was provided by Bodil Schmidt-Nielsen (Schmidt-Nielsen, 1955). Furthermore, a low rate of active urea secretion was detected in the rabbit proximal tubule by Kawamura and Kokko using microperfusion (Kawamura & Kokko, 1976), although net urea secretion was not detectable in another study of the rabbit proximal straight tubule (Knepper, 1983). More recently, isolated perfused tubule studies by Kato and Sands showed that urea can be actively secreted in the terminal IMCD (Kato & Sands, 1998; Kato & Sands, 1999).

To examine the possibility of urea secretion in the mouse nephron, careful measurements of fractional urea excretion (FE_{urea}) were performed in *UT-A1/3*$^{-/-}$ mice on either a low (4%) or a high (40%) protein intake. The *UT-A1/3*$^{-/-}$ mice provide an ideal model for examining urea secretion, as the main urea reabsorptive mechanism beyond the proximal tubule has been deleted. In *UT-A1/3*$^{-/-}$ mice, the FE_{urea} was approximately 77% on a low-protein diet, and 102% on a high-protein diet. Considering that at least 30 to 40% of the filtered load of urea is normally reabsorbed in the proximal tubule (Clapp, 1965, 1966; Lassiter et al., 1961), these findings suggest the presence of active urea secretion in the mouse renal tubule. For full confidence in the conclusion that active urea secretion occurs, micropuncture studies are needed in *UT-A1/3*$^{-/-}$ mice. The role of this active secretion and where this postulated active urea secretion occurs in the kidney nephron has been discussed in detail elsewhere (Yang & Bankir, 2005).

UT-A2 and UT-B Knockout Mice

In addition to the *UT-A1/3*$^{-/-}$ mice detailed above, two other urea transporter knockout mice have been developed and exploited in physiological studies (Bankir, Chen & Yang, 2004; Uchida et al., 2005; Yang & Verkman, 2002). These mouse models have selective deletion of either UT-A2 or UT-B. Space limitations prevent a detailed discussion of these mice in this article. Briefly, the UT-A2 knockout mice were generated by deleting both the internal promoter (UT-Aβ) and the first non-coding exon of UT-A2 (*see* Fenton et al., 2002b and article in this issue for organization of the mouse *UT-A* gene). UT-A2 is the major urea transporter of the thin descending limb of the loop of Henle and has been implicated in urea recycling in the medulla (Trinh-Trang-Tan & Bankir, 1998; Yang & Bankir, 2005). Surprisingly, unlike the *UT-A1/3*$^{-/-}$ mice, on a normal level of protein intake (20% protein), the UT-A2 knockout mice do not manifest a reduced urinary concentrating ability relative to wild-type mice or an impairment of urea accumulation in the inner medulla (Uchida et al., 2005). However, on a low-protein diet (4% protein), the animals have a reduced maximal urinary concentrating capacity compared to wild-type controls and a significant reduction in urea accumulation in the inner medulla. Thus, it seems from these initial studies that UT-A2 is important for maintaining a high concentration of urea in the inner medulla when urea supply to the kidney is limited. The other urea transporter knockout mouse model was generated by deletion of the UT-B gene. UT-B is expressed in both red blood cells and throughout the kidney medulla in the descending vasa recta endothelial cells (Promeneur et al., 1996; Tsukaguchi et al., 1997; Xu et al., 1997). UT-B knockout mice have a normal GFR, reduced urea clearance and a reduced urinary concentrat-

ing ability. Furthermore, they also have a reduced capacity to concentrate urea compared to other solutes. It is as yet unclear whether the defects observed in the UT-B knockout mice are due to the loss of urea transport in the vasa recta, in red blood cells, or both. The physiology of the UT-B knockout mice has been discussed in detail elsewhere (Yang & Bankir, 2005).

Role of Urea Transporters in Reproductive System

In 2000, we cloned a novel member of the UT-A urea transporter family, termed UT-A5, which is expressed exclusively within the testis and is localized to the periphery of the seminiferous tubules (Fenton et al., 2000). Furthermore, other studies have shown that multiple different UT-A and UT-B mRNA transcripts and proteins are also expressed in the testis (Tsukaguchi et al., 1997; Timmer et al., 2001; Fenton et al., 2002a). These isoforms (the molecular identity of which remains unknown as the cDNAs have yet to be isolated) are localized to various cell types within the seminiferous tubules at different stages of spermatogenesis. Several groups, including our own, hypothesized that because of both the high expression of these transporters and their cell-specific localization, the proteins were likely to play a role in testicular function, a seemingly plausible possibility since the first step in generation of polyamines, such as spermidine, involves generation of urea and ornithine by arginase. However, neither the *UT-A1/3*$^{-/-}$ mice, the UT-B knockout mice nor the recently developed UT-A2 knockout mice have been reported to have either impaired breeding or reproductive capabilities. Thus, it seems unlikely that either UT-A or UT-B proteins play any major role in the testis, although further studies would be required to confirm this.

UT-A Transgenic Mice

The generation of UT-A and UT-B knockout models has greatly enhanced our understanding of urea transporter function in the kidney. The mouse models discussed throughout this article have been developed using "classical" gene-targeting techniques to disrupt a particular region of either the *UT-A* or *UT-B* gene in the germ line of an animal, and these models were subsequently used to assess the renal phenotype of the ablated gene. However, gene deletion throughout the body often results in difficulties assessing the role of a gene in renal function, or a particular nephron segment or cell type. In its most extreme scenario, deletion of a gene results in mortality of the mice, rendering the model unsuitable for characterization (Hummler et al., 1997; Berger et al., 1998; Yun et al., 2000). In these cases, there is a need to delete the gene in a specific nephron or cell type in order to better understand its function. The first requisite to generating these cell- or tissue-specific knockouts is to characterize the promoter of a particular gene to determine if this region contains all the necessary elements that confer expression in a cell-specific manner.

As previously discussed, the urea transporters, UT-A1 and UT-A3, are two alternative splice products of a single gene. The *UT-A* gene contains two promoter regions; one promoter, situated at the 5′-end of the gene (UT-Aα promoter), drives the transcription of *UT-A1*, *UT-A3* and *UT-A5*; whereas an internal promoter (UT-Aβ) drives the transcription of *UT-A2* (*see* Fenton et al., 2002b and the article by Smith and Fenton in this issue). Recently, we generated a line of transgenic mice (termed UT-Aα-βGal mice) that expressed the reporter gene β-galactosidase (β-Gal) under the control of the mouse UT-Aα promoter (Fenton et al., 2006). This mouse line was analyzed to determine if the UT-Aα promoter region contained all the elements necessary to confer IMCD-specific gene targeting.

The UT-Aα Promoter Confers IMCD-specific Expression in the Kidney

Within the kidney of UT-Aα-βGal mice, β-Gal expression was specific to the terminal portion of the papillary tip and co-localization studies with AQP2 determined that expression was localized to the principal cells of the IMCD (*see* Fig. 6). Furthermore, we only detected β-Gal activity in the terminal portion of the IMCD, and in contrast to AQP2, there was no staining of the initial IMCD, the OMCD and the CCD. Importantly, no expression was observed in the cells of the thin descending limb of Henle's loop. The pattern of β-Gal expression was consistent with the localization of UT-A1 and UT-A3 in the mouse kidney (Fenton et al., 2002c; Stewart et al., 2004). Therefore, we concluded from this transgenic mouse line that the UT-Aα promoter is capable of IMCD principal cell-specific targeting, which should facilitate future studies aimed at gene deletion within the IMCD.

Regulation of the UT-Aα Promoter Activity *in vivo*

The promoter fragment used to generate the UT-Aα-βGal mice contains numerous transcription factor binding sites, including a tonicity-enhancer element, and previous studies in cell culture have shown that the promoter can be up-regulated in response to either hypertonicity or increased cAMP levels (Fenton et al., 2002b). However, despite evidence that prolonged antidiuresis can result in an increase in UT-A3 mRNA expression in the inner medulla (Bagnasco et al., 2000; Fenton et al., 2002b), it is uncertain whether this results from increased gene transcription or a difference in mRNA

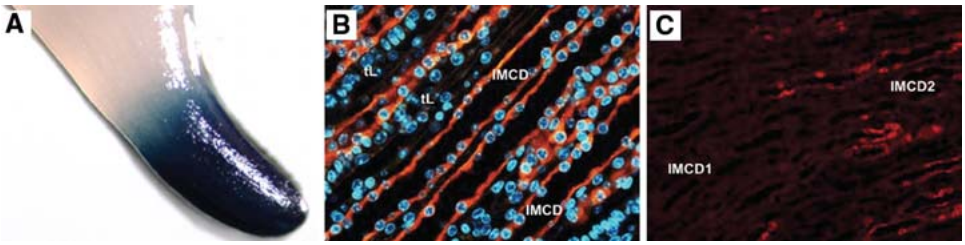

Fig. 6. Localization of β-Gal enzyme activity in the kidney of UT-Aα-βGal mouse**.** (*A*); X-Gal staining of the kidney inner medulla demonstrates that β-Gal activity was localized to the papillary tip and was strongest in the terminal portion of this region. (*B*) Localization of transgene in kidney inner medulla by immunohistochemistry. Low power magnification shows expression of β-Gal transgene (*red*) is confined to the cells of the IMCD and is not apparent in the thin descending limbs of Henle's loop (tL). Nuclei are stained blue. (*C*) Staining of β-Gal is localized to the IMCD2 and IMCD 3 regions, with little staining of the base of the inner medulla, IMCD1. The conclusion from this data is that the UT-Aα promoter is capable of targeting transgene expression to the principal cells of the IMCD. Figure is adapted from Fenton et al., 2006.

stability. Therefore, we used the UT-Aα-βGal mouse line to investigate whether the UT-Aα promoter could be regulated by prolonged antidiuresis *in vivo*. Inner medulla β-Gal activity was increased approximately two-fold in response to 3 days water restriction, thus we concluded that *UT-A* gene transcription is regulated in vivo by osmolality and/or vasopressin.

Glucocorticoids have been shown to increase the fractional excretion of urea and decrease both urea permeability and UT-A1 protein abundance in the IMCD (Naruse et al., 1997). Other studies have shown that dexamethasone administration significantly decreases the activity of the rat UT-Aα promoter in cell culture (Peng et al., 2002). Thus, we used the UT-Aα-βGal mouse line to investigate whether the same effects of glucocortcoids could be observed in vivo. Our studies demonstrated that the administration of dexamethasone to UT-Aα-βGal mice for 6 days significantly reduced β-Gal activity in the inner medulla. We conclude from these studies that the UT-Aα promoter is down-regulated by glucocorticoids in vivo, potentially providing a mechanism for the reduced UT-A1 and UT-A3 mRNA expression seen after glucocorticoid administration.

Non-Renal Targeting by the UT-Aα Promoter

A novel finding from the characterization of the UT-Aα-βGal mouse line was that the UT-Aα promoter also targeted expression of β-Gal to the columnar epithelial principal cells within the vas deferens, a site not previously known to express urea transporters (Fenton et al., 2006). The possible role of a urea transporter in this region of the reproductive tract is unknown. However, in view of the findings from the UT-A and UT-B knockout mice, it seems unlikely that it plays a major role in reproductive function. In contrast, no β-Gal expression was observed in the testis. The lack of expression in the testis, where UT-A5 is expressed, was unexpected, as the UT-Aα promoter was thought to drive the expression of this isoform. Two possible explanations for the lack of β-gal activity in the testis are that the *UT-Aα* transgene did not contain all the necessary "testis-specific" enhancer elements, or that a completely different internal promoter drives its transcription.

Perspectives

In recent years, our understanding of the role of urea transporters in renal function has been greatly enhanced by the development of knockout mouse models. The studies that we have reviewed here using $UT\text{-}A1/3^{-/-}$ mice attempt to specifically address observations made in the 'pre-molecular' era of renal physiology and have greatly influenced the way we teach kidney physiology. The major findings from the $UT\text{-}A1/3^{-/-}$ mouse model can be summarized as follows; 1) the urea transporters UT-A1 and/or UT-A3 are responsible for the vasopressin-stimulated and phloretin-sensitive urea permeability observed in previous isolated perfused tubule studies; 2) urea accumulation in the inner medullary interstitium depends on rapid transport of urea from the IMCD lumen via UT-A1 and/or UT-A3; 3) the role of IMCD urea transporters in water conservation is to prevent a urea-induced osmotic diuresis; 4) the absence of IMCD urea transport does not prevent the concentration of NaCl in the inner medulla, contrary to what would be predicted from the passive model in the form proposed by Kokko and Rector and Stephenson; 5) urea reabsorption from the IMCD and the process of urea recycling are not important determinants of protein-induced increases in GFR; 6) active urea secretion appears to occur somewhere along the mouse renal tubule.

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