

## SGLT2 Mediates Glucose Reabsorption in the Early Proximal Tubule

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### ABSTRACT

Mutations in the gene encoding for the Na<sup>+</sup>-glucose co-transporter SGLT2 (*SLC5A2*) associate with familial renal glucosuria, but the role of SGLT2 in the kidney is incompletely understood. Here, we determined the localization of SGLT2 in the mouse kidney and generated and characterized SGLT2-deficient mice. In wild-type (WT) mice, immunohistochemistry localized SGLT2 to the brush border membrane of the early proximal tubule. *Sglt2*<sup>-/-</sup> mice had glucosuria, polyuria, and increased food and fluid intake without differences in plasma glucose concentrations, GFR, or urinary excretion of other proximal tubular substrates (including amino acids) compared with WT mice. SGLT2 deficiency did not associate with volume depletion, suggested by similar body weight, BP, and hematocrit; however, plasma renin concentrations were modestly higher and plasma aldosterone levels were lower in *Sglt2*<sup>-/-</sup> mice. Whole-kidney clearance studies showed that fractional glucose reabsorption was significantly lower in *Sglt2*<sup>-/-</sup> mice compared with WT mice and varied in *Sglt2*<sup>-/-</sup> mice between 10 and 60%, inversely with the amount of filtered glucose. Free-flow micropuncture revealed that for early proximal collections, 78 ± 6% of the filtered glucose was reabsorbed in WT mice compared with no reabsorption in *Sglt2*<sup>-/-</sup> mice. For late proximal collections, fractional glucose reabsorption was 93 ± 1% in WT and 21 ± 6% in *Sglt2*<sup>-/-</sup> mice, respectively. These results demonstrate that SGLT2 mediates glucose reabsorption in the early proximal tubule and most of the glucose reabsorption by the kidney, overall. This mouse model mimics and explains the glucosuric phenotype of individuals carrying *SLC5A2* mutations.

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Glucose is the main source of energy in eukaryotic organisms. The homeostasis of glucose is maintained by intestinal glucose absorption and the coordinated regulation of hepatic and renal glucose production, as well as tissue consumption of glucose. As a consequence of renal glomerular filtration, approximately 180 g/d glucose enter the tubular system of the kidneys in a healthy individual with normoglycemia, which is equivalent to approximately one third of the total energy consumed by the human body. Glucose in urine, however, is absent or at very low concentrations in healthy adults (range 0.03 to 0.30 g/d) as a result of near

complete reabsorption along the nephron segments, primarily in the proximal tubule. The genes encoding transporter proteins participating in renal

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glucose reabsorption have been cloned and identified<sup>1-5</sup>; however, direct experimental *in vivo* evidence on their tubular sites of action and quantitative contributions is still sparse.

The concept has been developed that the low-affinity/high-capacity Na<sup>+</sup>-glucose co-transporter SGLT2 (*Slc5a2*) mediates the bulk uptake of glucose across the apical membrane of the early proximal tubule, whereas the high-affinity/low-capacity SGLT1 (*Slc5a1*) reduces luminal glucose concentrations to very low concentrations in further distal parts of the proximal tubule.<sup>1,5-7</sup> This concept is based on mRNA and transport studies in isolated nephron segments and membrane vesicles from various tissue zones of rat and rabbit kidneys.<sup>6-14</sup> Real-time PCR suggested SGLT2 mRNA expression in many human tissues,<sup>15</sup> but, because of the lack of good antibodies (Ab), the implication for extrarenal protein expression of SGLT2 is unclear. Another low-affinity Na<sup>+</sup>-D-glucose co-transporter named NaGLT1 was cloned from rat,<sup>2</sup> which has no amino acid sequence similarity to transporters of the SGLT family and does not transport D-galactose, as has been described for SGLT2.<sup>6,7</sup> NaGLT1 is highly expressed in rat kidney, where it was located to the brush border membranes of proximal tubules.<sup>2</sup> Its quantitative contribution to glucose reabsorption, however, is not known.

After the apical uptake, the glucose transporters GLUT2 and GLUT1 facilitate the basolateral exit of glucose.<sup>16</sup> In accordance with a primary role in the late proximal tubule, the expression of SGLT1 protein in the brush border membrane increases along the proximal tubule of the rat (S1 < S2 < S3).<sup>17</sup> The exact localization of SGLT2 protein along the proximal tubule, however, has been hampered by the lack of suitable Ab.

Mutations in SGLT1 are associated with intestinal glucose malabsorption, but these individuals with these mutations have little or no glucosuria.<sup>18</sup> In comparison and providing strong evidence for a prominent role in renal glucose reabsorption, individuals with gene mutations in SGLT2 have persistent renal glucosuria.<sup>19-22</sup>

To determine in an experimental and direct way the role of SGLT2 in proximal tubular glucose reabsorption, we generated and studied gene-targeted mice lacking *Sglt2* and applied a new Ab for SGLT2. The results demonstrate for the first time that the SGLT2 protein is localized to the brush border of the early proximal tubule, where it is responsible for all glucose reabsorption and is the major pathway of glucose reabsorption in the kidney, overall.

## RESULTS

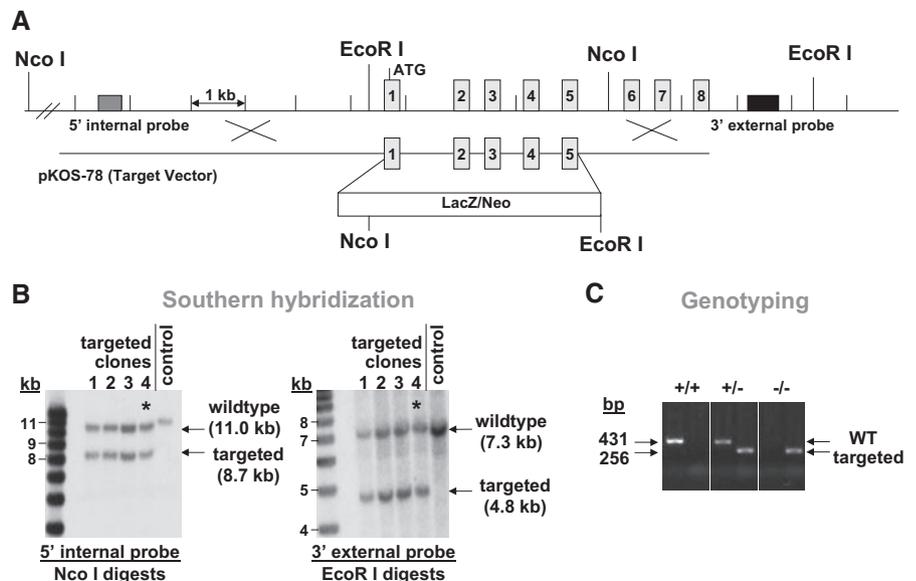
### Generation of *Sglt2*<sup>-/-</sup> Mice and Renal mRNA Expression of Glucose Transporters

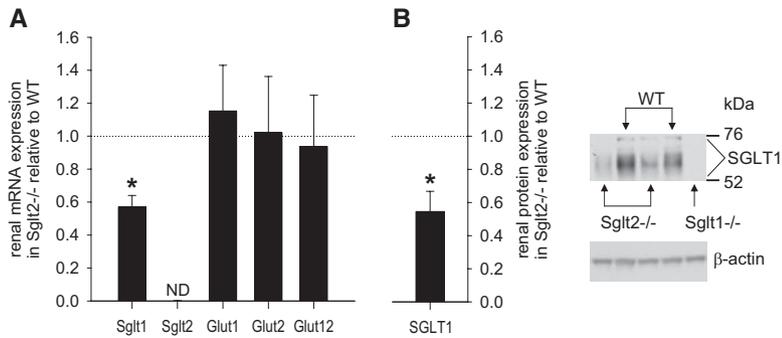
The strategy to generate gene-targeted mice lacking *Sglt2* is outlined in Figure 1. Real-time reverse transcriptase-PCR confirmed the knockout of *Sglt2* in the kidney of *Sglt2*<sup>-/-</sup> mice (Figure 2A). Whereas the renal mRNA expression of *Glut1*, *Glut2*, and *Glut12* were not significantly different between genotypes, the expression of *Sglt1* mRNA was reduced by approximately 40% in *Sglt2*<sup>-/-</sup> versus wild-type (WT) mice (Figure 2A). This was confirmed for SGLT1 expression at the protein level (Figure 2B).

### Localization of Renal SGLT2 Protein Expression in the Kidney

Western blot analysis of whole kidneys in WT and *Sglt2*<sup>-/-</sup> mice with an affinity-purified polyclonal Ab raised against rat SGLT2 showed a specific band with a molecular weight in the predicted range of SGLT2 (approximately 73 kD; Supplemental Figure S1). This band was absent from the *SGLT2*<sup>-/-</sup> mouse kidneys. Immunohistochemical analysis with the same SGLT2 Ab revealed specific staining of the apical brush border membrane of early proximal convoluted tubules, whereas no signal was detected in late proximal convoluted tubules (iden-

**Figure 1.** Generation of transgenic *Sglt2*<sup>-/-</sup> mice. (A) Targeting strategy used to disrupt the *Slc5a2* locus. Homologous recombination (represented by X) between the targeting vector and the *Slc5a2* gene results in the replacement of exons 1 through 5 with the selection cassette. (B) Southern hybridization indicating proper gene targeting in four ES cell clones. Clone 4 is transmitted through the germline; control represents untransfected ES cell DNA. (C) PCR amplification of tail-snip DNA from *Sglt2*<sup>-/-</sup>, WT (+/+), and heterozygous (+/-) mice. Separate PCRs are performed for the WT allele (left lane) and the targeted allele (right lane) for each sample.





**Figure 2.** Renal expression of Glut1, Glut2, and Glut12 is unaltered but the expression of Sglt1 is reduced in *Sglt2*<sup>-/-</sup> mice. (A) Renal mRNA expression of Glut1, Glut2, and Glut12 are unchanged whereas Sglt1 is reduced in *Sglt2*<sup>-/-</sup> relative to WT mice. Real-time PCR confirms the knockout of *Sglt2* in kidney of *Sglt2*<sup>-/-</sup> mice. ND, not detectable.  $n = 5$  per genotype. \* $P < 0.01$  versus WT mice. (B) Western blotting shows reduced renal SGLT1 protein expression (related to  $\beta$ -actin) in *Sglt2*<sup>-/-</sup> versus WT mice ( $n = 4$  per genotype). \* $P < 0.05$  versus WT mice. Gene knockout of *Sglt1* in mice (*Sglt1*<sup>-/-</sup>) confirms the specificity of the signal.

tified by reduced height of brush border), straight proximal tubules, or further downstream segments of the nephron and collecting duct system (Figure 3). In a similar manner, the Ab did not bind to any of the nephron segments in the knockout mice.

### Basal Parameters, Kidney Function, and Glucose Homeostasis in Awake Mice

Litter size and gender ratio were not different between *Sglt2*<sup>-/-</sup> and WT breeder pairs ( $6.5 \pm 0.7$  versus  $5.8 \pm 0.6$  pups per litter;  $1.2 \pm 0.2$  versus  $1.2 \pm 0.2$  male-female ratio;  $n = 12$  to 14 litters; NS). Weaned offspring showed a similar increase in body weight (Supplemental Figure S2). Spontaneous urine collection in awake adult mice revealed that absolute urinary glucose concentrations (Figure 4) as well as urinary glucose related to creatinine ( $1847 \pm 119$  versus  $6 \pm 1 \mu\text{mol/mg}$ ;  $P < 0.001$ ) were significantly greater in *Sglt2*<sup>-/-</sup> mice compared with WT mice, whereas simultaneously determined blood glucose levels were not different between genotypes ( $132 \pm 2$  versus  $134 \pm 4 \text{ mg/dl}$ ; NS; Figure 4).

GFR determined by FITC-inulin kinetics in awake mice revealed similar values in WT and *Sglt2*<sup>-/-</sup> mice (Figure 4). Glucosuria was associated with greater food and fluid intake in *Sglt2*<sup>-/-</sup> compared with WT mice (Figure 4), whereas systolic BP (Figure 4) and heart rate as well as body weight were not different between genotypes (Table 1). Greater fluid intake was associated with modestly greater plasma renin concentration and lower urine osmolality in *Sglt2*<sup>-/-</sup> mice, whereas plasma osmolality and urinary vasopressin (related to creatinine) were not different compared with WT mice (Table 1). Hematocrit and plasma concentrations of  $\text{Na}^+$  and  $\text{K}^+$  were not different between *Sglt2*<sup>-/-</sup> and WT mice, whereas plasma aldosterone levels were modestly lower in *Sglt2*<sup>-/-</sup> compared with WT mice (Table 1). Spontaneous urine collection (taken during the day; *i.e.*, not during time of eating, which mainly occurs at

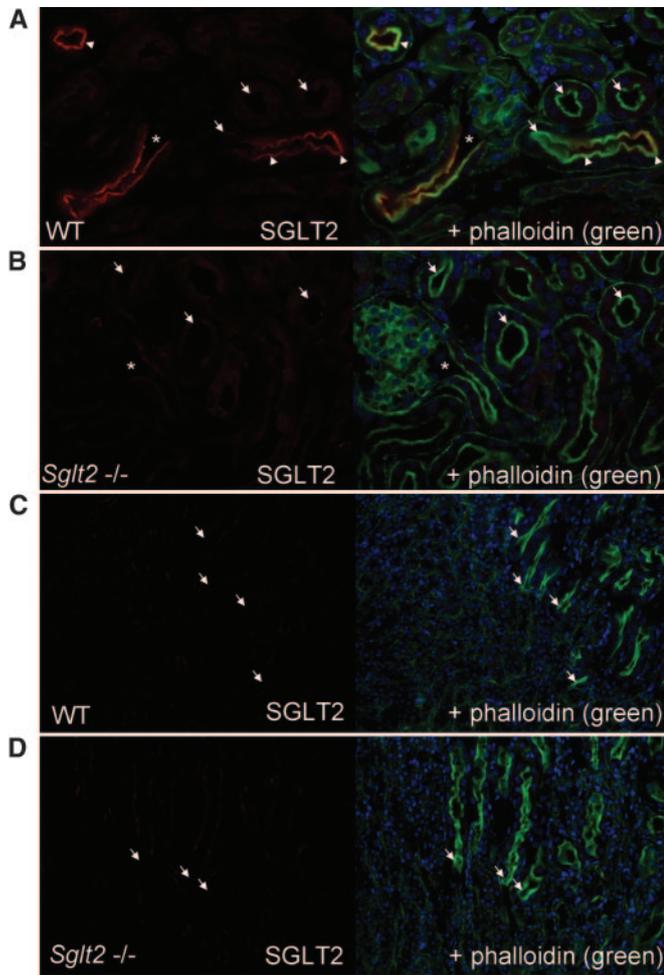
night) revealed that urinary concentrations of  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Ca}^{2+}$  were not different between *Sglt2*<sup>-/-</sup> and WT mice when related to creatinine (mmol/mmol creatinine:  $30 \pm 2$  versus  $30 \pm 3$ ,  $40 \pm 2$  versus  $37 \pm 3$ , and  $1.3 \pm 0.1$  versus  $1.1 \pm 0.2$ ;  $n = 9$  to 11; NS). Moreover, urinary excretion of amino acids was not significantly different between the genotypes (Supplemental Figure S3).

### Glucose Handling by the Whole Kidney and along the Proximal Convoluted Tubule in Anesthetized Mice

Renal clearance experiments under anesthesia confirmed a similar mean arterial BP and heart rate in *Sglt2*<sup>-/-</sup> versus WT mice ( $101 \pm 3$  versus  $101 \pm 8 \text{ mmHg}$ ;  $527 \pm 12$  versus  $520 \pm 14/\text{min}$ ;  $n = 6$  to 8 mice; NS).

Moreover, plasma glucose concentrations, GFR, and the amount of filtered glucose were similar in *SGLT2*<sup>-/-</sup> and WT mice ( $155 \pm 16$  versus  $139 \pm 10 \text{ mg/dl}$ ;  $187 \pm 19$  versus  $218 \pm 20 \mu\text{l/min}$ ;  $1683 \pm 321$  versus  $1713 \pm 274 \text{ nmol/min}$ ; NS); however, absolute and fractional renal reabsorption of glucose were significantly lower in *Sglt2*<sup>-/-</sup> compared with WT mice (Figure 5). Mean values for fractional glucose reabsorption were  $36 \pm 8\%$  in *Sglt2*<sup>-/-</sup> mice and  $99.7 \pm 0.1\%$  in WT mice ( $P < 0.001$ ). The amount of filtered glucose was a primary determinant of glucose excretion as well as fractional glucose reabsorption in *Sglt2*<sup>-/-</sup> mice, the latter varying inversely with the amount of filtered glucose (between 60 and 10%). Glucosuria in *Sglt2*<sup>-/-</sup> mice was associated with doubling of absolute and fractional excretion of fluid in *Sglt2*<sup>-/-</sup> mice compared with WT mice, whereas the absolute and fractional excretion of  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Cl}^-$  were not different (Table 2).

Free-flow collections of tubular fluid were performed along accessible proximal convoluted tubules at the kidney surface to establish a profile for fractional reabsorption of glucose (FR-glucose) versus fluid (FR-fluid; Figure 6A). Mean values for single-nephron GFR and the amount of glucose filtered per nephron were not different in *Sglt2*<sup>-/-</sup> versus WT mice ( $8.5 \pm 0.6$  versus  $8.7 \pm 0.6 \text{ nl/min}$ ;  $65 \pm 4$  versus  $66 \pm 6 \text{ pmol/min}$ ;  $n = 23$  nephrons in five *Sglt2*<sup>-/-</sup> mice and 18 nephrons in four WT mice; NS for both comparisons). For early proximal collections (confirmed by FR-fluid  $< 40\%$ : mean  $\pm$  SEM for FR-fluid  $21 \pm 3$  and  $26 \pm 4\%$  in WT and *Sglt2*<sup>-/-</sup> mice, respectively;  $n = 9$  to 10 nephrons; NS),  $78 \pm 6\%$  of the filtered glucose was reabsorbed in WT mice, whereas mean FR-glucose was not different from 0 ( $0.2 \pm 6.8\%$ ) in *Sglt2*<sup>-/-</sup> mice ( $P < 0.001$  versus WT mice; Figure 6B). For late proximal collections (confirmed by FR-fluid  $\geq 40\%$ : mean  $\pm$  SEM for FR-fluid  $59 \pm 3$  and  $52 \pm 3\%$  in WT and *Sglt2*<sup>-/-</sup> mice, respectively;  $n = 9$  to 13 nephrons; NS), FR-glucose was  $93 \pm 1\%$  in WT mice and  $21 \pm 6\%$  in *Sglt2*<sup>-/-</sup> mice ( $P < 0.001$  versus WT



**Figure 3.** Expression of SGLT2 protein in the apical brush border membrane of the early proximal convoluted tubule. (A through D) Renal cortical sections (A and B) and outer medullary sections (C and D) are shown for WT and *SglT2*<sup>-/-</sup> mice. Left pictures show staining with SGLT2 Ab (red fluorescence); right pictures show additional co-staining with phalloidin (green fluorescence) to label filamentous actin of the proximal tubular brush border. Nuclei are stained with the marker DAPI (blue fluorescence). (A and B) The SGLT2 Ab provides specific and selective staining of the apical brush border membrane of the early proximal convoluted tubules. The staining for SGLT2 begins where the proximal tubule initiates from Bowman space (\*) and ends along the proximal convoluted tubule (arrowhead). Further downstream sections of the proximal convoluted tubule (identified by reduced height of brush border) do not stain for SGLT2 (arrows). (C and D) No specific SGLT2 signal is detected in the proximal straight tubules of the outer medulla. Arrows mark the distal ends of proximal straight tubules.

mice). Subtracting mean fractional glucose reabsorption between groups of late and early superficial proximal collections indicated that WT and *SglT2*<sup>-/-</sup> mice reabsorbed approximately 15 and 21%, respectively, of the filtered glucose in between. In contrast to glucose, fractional reabsorption of chloride was not significantly different in early or late proximal

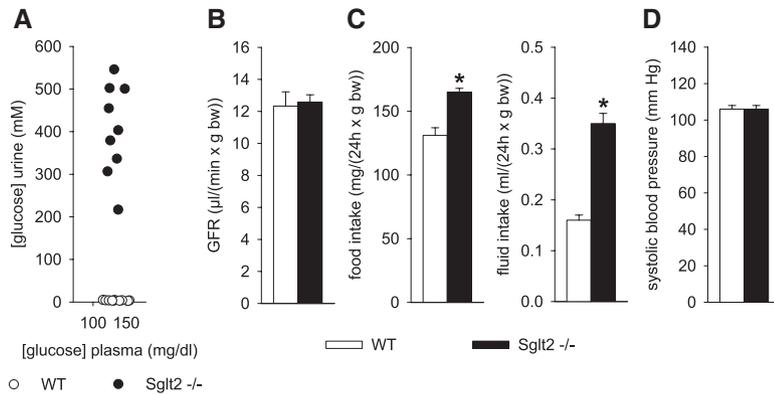
collections between WT and *SglT2*<sup>-/-</sup> mice (early  $22 \pm 5$  versus  $27 \pm 5\%$ ; late  $49 \pm 4$  versus  $49 \pm 3\%$ ; NS; Figure 6C).

## DISCUSSION

These studies demonstrate for the first time that SGLT2 protein is expressed in the brush border membrane of the early proximal convoluted tubule and that net glucose reabsorption in the early proximal tubule is absent in transgenic *SglT2* null mice. These data provide direct and unequivocal evidence that SGLT2 mediates all glucose reabsorption in the early proximal tubule. This is consistent with a previously proposed role of SGLT2 as the low-affinity/high-capacity glucose reabsorption pathway in the early proximal tubule.<sup>6</sup>

These data further show that on the whole-kidney level, SGLT2 is a major determinant of glucose reabsorption. These findings are consistent with the phenotype of individuals with familial renal glucosuria, who carry specific mutations in the *SLC5A2* gene.<sup>19–26</sup> A total of 44 different mutations have been described for the *SLC5A2* gene, including missense and nonsense mutations, small deletions (in-frame and frame shift), and splicing mutations, scattered throughout the gene.<sup>25</sup> Individuals with these mutations have normal blood glucose levels but persistent glucosuria. Heterozygous carriers often exhibit mild glucosuria ( $<10$  g/1.73 m<sup>2</sup> per d), whereas massive glucosuria is confined to the homozygous or compound heterozygous states ( $>10$  g/1.73 m<sup>2</sup> per d); glucosuria can range from  $<1$  to  $>150$  g/1.73 m<sup>2</sup> per d.<sup>18,19,27,28</sup> Similarly, *SglT2*<sup>-/-</sup> mice excreted much of the filtered glucose with urinary glucose to creatinine concentrations of approximately 1850  $\mu$ mol/mg; in comparison, urinary glucose concentrations of heterozygous *SglT2*<sup>+/-</sup> mice was 6  $\mu$ mol/mg ( $n = 3$ ) and thus not different from WT mice (approximately 6  $\mu$ mol/mg), indicating that one intact *SglT2* allele is sufficient to achieve normal renal glucose reabsorption.

These data show that *SglT2*<sup>-/-</sup> mice reabsorb glucose in nephron segments downstream of the early proximal tubule, including distal aspects of the proximal convoluted tubule. The whole-kidney clearance studies show that increasing glomerular filtration of glucose does not enhance absolute renal glucose reabsorption in *SglT2*<sup>-/-</sup> mice, indicating that the capacity of the remaining glucose transport system in these mice is saturated. This reabsorption may be mediated by SGLT1, the high-affinity/low-capacity co-transporter, and/or by NaGLT1, the low-affinity co-transporter. Further studies are needed to demonstrate conclusively the quantitative role of SGLT1 and NaGLT1 in renal glucose transport. Notably, renal SGLT1 mRNA and protein expression both are reduced (by approximately 40%) in mice lacking *SglT2*. The findings indicate that an upregulation of SGLT1 expression does not occur in the kidney when the amount of glucose delivered out of the early proximal tubule is increased. This does not exclude, however, that more glucose is transported *via* SGLT1 in *SglT2*<sup>-/-</sup> mice; in fact, the micropuncture data indicate that WT and *SglT2*<sup>-/-</sup> mice reabsorbed approximately 15 and 21%, respectively, of



**Figure 4.** Urinary glucose concentration is enhanced in *Sglt2*<sup>-/-</sup> mice despite normal blood glucose levels and GFR: Studies in awake mice. (A) Glucose measured in spontaneous urine collections and subsequent blood collections from tail vein. (B) GFR determined by plasma FITC-inulin kinetics. (C) Food and fluid intake assessed in regular cages. (D) BP measurements using a tail-cuff system in trained awake mice ( $n = 9$  to 10 per group). \* $P < 0.05$  versus WT mice.

the filtered glucose between early and distal sites of proximal tubular collections. Thus, downregulation of SGLT1 mRNA and protein may be a mechanism of these segments to limit the increase in glucose reuptake under conditions of increased luminal glucose delivery. Preliminary studies in gene-targeted mice heterozygous for *Sglt1* (which have approximately 50% of the *Sglt1* mRNA expression of WT mice) show no evidence of urinary glucose loss (urinary glucose/creatinine  $5 \pm 2$  versus  $5 \pm 3$   $\mu\text{mol}/\text{mg}$  in WT mice;  $n = 3$  per genotype; H.K., V.V., unpublished data, 2010), indicating that the downregulation of SGLT1 expression in *Sglt2*<sup>-/-</sup> mice does not explain their major urinary glucose loss.

Mice lacking SGLT2 have normal BP, GFR, plasma levels of  $\text{Na}^+$  and  $\text{K}^+$ , and no significant increase in urinary excretion of amino acids, arguing against a general proximal tubular dysfunction in these mice. Moreover, modest polyuria is compensated by increased fluid intake, and polyphagia may serve to compensate for urinary glucose and calo-

**Table 1.** Systemic parameters in age-matched male *Sglt2*<sup>-/-</sup> and WT mice

Parameter	WT	<i>Sglt2</i> <sup>-/-</sup>
Body weight (g)	30 $\pm$ 1	30 $\pm$ 1
Kidney weight (mg/g body wt)	11.9 $\pm$ 0.3	11.9 $\pm$ 0.2
Heart rate (/min)	576 $\pm$ 19	579 $\pm$ 19
Hematocrit (%)	42.4 $\pm$ 0.5	42.8 $\pm$ 0.5
Urine osmolality (mmol/kg)	1849 $\pm$ 96	1488 $\pm$ 73 <sup>a</sup>
Urine vasopressin (pmol/mmol creatinine)	36 $\pm$ 3	39 $\pm$ 4
Plasma aldosterone (pg/ml)	136 $\pm$ 14	80 $\pm$ 12 <sup>b</sup>
PRC (ng angiotensin I/ml per h)	445 $\pm$ 39	613 $\pm$ 61 <sup>a</sup>
Plasma $\text{Na}^+$ (mM)	155 $\pm$ 1	156 $\pm$ 2
Plasma $\text{K}^+$ (mM)	4.0 $\pm$ 0.1	4.0 $\pm$ 0.1
Plasma osmolality (mmol/kg)	328 $\pm$ 2	332 $\pm$ 2

Data are means  $\pm$  SEM;  $n = 6$  to 10 per group. PRC, plasma renin concentration.

<sup>a</sup> $P < 0.05$ .

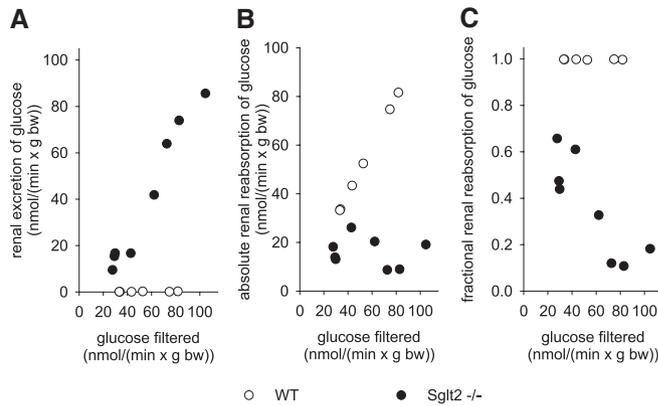
<sup>b</sup> $P < 0.01$ .

rie loss, thereby normalizing body weight increase. Similarly, individuals with homozygous mutations in *SLC5A2* (“type 0” according to the historical classification of renal glucosurias) can present with polyuria, polydipsia, and episodes of polyphagia<sup>19,28,29</sup>; moreover, these individuals with mutations in *SLC5A2* have normal GFR, plasma levels of  $\text{Na}^+$  and  $\text{K}^+$ , and BP and exhibit no manifestations of generalized proximal tubular dysfunction and, in general, do not seem to develop significant clinical problems over time.<sup>20,22,27,29</sup> Although some case reports of selective and mild aminoaciduria have been reported in patients with familial renal glucosuria,<sup>20,30,31</sup> this is not a general finding.<sup>22,24</sup>

Delayed growth and pubertal development has been described in one male individual with a homozygous mutation in *SLC5A2* and severe renal glucosuria (“type 0 glucosuria”), but the causal role of the mutation remained unclear.<sup>29</sup> This individual as well as five of seven male children with renal glucosuria from another study<sup>32</sup> also had modest hypercalciuria with urinary calcium-creatinine ratios less than double the upper normal value. Our studies did not detect delayed growth or significantly increased urinary calcium-creatinine ratios in mice lacking SGLT2.

An increase in plasma aldosterone and renin concentrations has been described in three individuals with mutations in *SLC5A2* and severe glucosuria (30.0 to 86.5 g/1.73 m<sup>2</sup> per d).<sup>25,26</sup> Our studies revealed modestly increased plasma renin concentrations associated with lower plasma aldosterone concentrations in *Sglt2*<sup>-/-</sup> mice compared with WT mice. This dissociation may reflect compensatory adaptations to changes in  $\text{NaCl}$ , fluid, and  $\text{K}^+$  balance. The increase in renin concentration is expected to increase angiotensin II activity and may serve to stabilize  $\text{NaCl}$  and volume homeostasis by increasing fluid intake and opposing the inhibitory influence of *Sglt2* deficiency on renal  $\text{NaCl}$  and water reabsorption. In accordance, *Sglt2*<sup>-/-</sup> mice have normal BP, body weight, hematocrit, and plasma osmolality, arguing against significant volume depletion. Moreover, reabsorption of chloride seemed unaltered along the nephron. We speculate that in *Sglt2*<sup>-/-</sup> mice, enhanced tubular flow in collecting ducts (as a result of osmotic diuresis) stimulates flow-induced urinary  $\text{K}^+$  secretion and, thus, facilitates renal  $\text{K}^+$  excretion; given that there is no increase in  $\text{K}^+$  excretion, the mice may suppress plasma aldosterone levels to maintain normal urinary  $\text{K}^+$  excretion and, thereby, plasma  $\text{K}^+$  concentrations. Further studies are necessary to test this hypothesis.

In summary, we have localized the SGLT2 protein to the brush border of the early proximal tubule. Studies in WT and gene-targeted mice lacking *Sglt2* provide direct *in vivo* evidence that SGLT2 mediates all glucose reabsorption in the early proximal tubule and is the major pathway of glucose reabsorption in the



**Figure 5.** Renal excretion of glucose in *Sglt2*<sup>-/-</sup> is a function of the amounts of glucose filtered: Clearance studies under anesthesia ( $n = 9$  to  $10$  per group).

kidney, overall. This new mouse model mimics and explains the glucosuric phenotype of individuals carrying mutations in *SLC5A2*.

## CONCISE METHODS

All animal experimentation was conducted in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD) and was approved by the local Institutional Animal Care and Use Committee.

### Generation of Mice Lacking *Sglt2* (*Slc5a2*)

*Slc5a2* mutant mice were generated at Lexicon Pharmaceuticals (The Woodlands, TX) as outlined in Figure 1A. The *Slc5a2* targeting vector was derived using the Lambda KOS system.<sup>33</sup> The Lambda KOS phage library, arrayed into 96 superpools, was screened by PCR using exon 1–specific primers Slc5-1 (5′-GTCCTGATTGATAATCCTGC-3′) and Slc5-2 (5′-TGCACAATCCAGAAGGTACG-3′). The PCR-positive phage superpools were plated and screened by filter hybridization using the 251-bp amplicon derived from primers Slc5-1 and Slc5-2 as a probe. Three pKOS genomic clones, pKOS-61, pKOS-78, and pKOS-91, were

**Table 2.** Renal clearance experiments in age-matched male *Sglt2*<sup>-/-</sup> and WT mice

Parameter	WT	<i>Sglt2</i> <sup>-/-</sup>
AE-glucose (nmol/min per g body wt)	0.14 ± 0.04	40.4 ± 10.7 <sup>a</sup>
AE-Na <sup>+</sup> (nmol/min per g body wt)	3.8 ± 0.6	3.6 ± 0.6
AE-K <sup>+</sup> (nmol/min per g body wt)	6.1 ± 0.6	6.0 ± 0.6
AE-Cl <sup>-</sup> (nmol/min per g body wt)	3.2 ± 0.4	3.2 ± 0.7
AE-H <sub>2</sub> O (nl/min per g body wt)	45 ± 10	77 ± 10 <sup>b</sup>
FE-glucose (%)	0.26 ± 0.04	63.6 ± 7.6 <sup>a</sup>
FE-Na <sup>+</sup> (%)	0.36 ± 0.03	0.36 ± 0.05
FE-K <sup>+</sup> (%)	17.7 ± 1.5	18.5 ± 2.3
FE-Cl <sup>-</sup> (%)	0.42 ± 0.05	0.44 ± 0.11
FE-H <sub>2</sub> O (%)	0.62 ± 0.09	1.20 ± 0.20 <sup>a</sup>

Data are means ± SEM;  $n = 6$  to  $8$  per group. AE, absolute renal excretion; FE, fractional renal excretion.

<sup>a</sup> $P < 0.01$ .

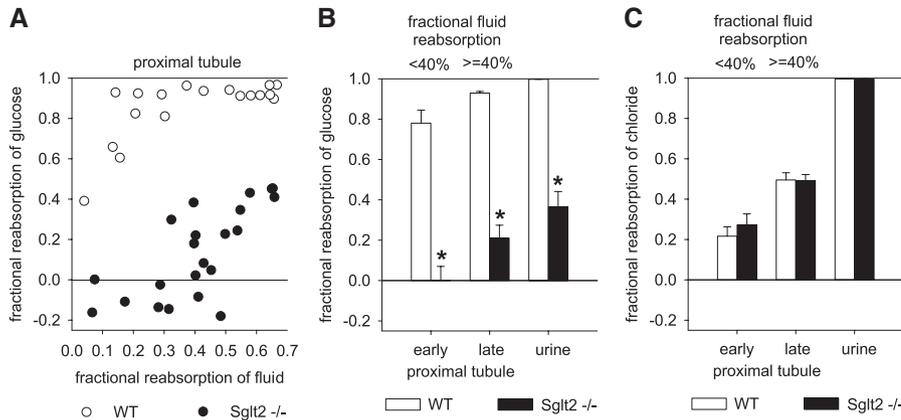
<sup>b</sup> $P < 0.05$ .

isolated from the library screen and confirmed by sequence and restriction analysis. Gene-specific arms (5′-ACTAGCCCCCTTGAGGGACAGATGCTGGAGAGA-3′) and (5′-GCTCTCTGTCACCACCCTCAAGCTCCTTGCC-3′) were appended by PCR to a yeast selection cassette containing the URA3 marker. The yeast selection cassette and pKOS-78 were co-transformed into yeast, and clones that had undergone homologous recombination to replace a 2419-bp region containing exons 1 through 5 with the yeast selection cassette were isolated. The yeast cassette was subsequently replaced with a LacZ/Neo selection cassette to complete the *Slc5a2* targeting vector. The NotI linearized targeting vector was electroporated into 129/SvEvBrd (*Lex-2*) embryonic stem (ES) cells. G418/FIAU-resistant ES cell clones were

isolated, and correctly targeted clones were identified and confirmed by Southern analysis using a 302-bp 5′ internal probe (15/16), generated by PCR using primers Slc5-15 (5′-TGTCTTTAGAGGCTGTATCC-3′) and Slc5-16 (5′-ACAGGCTGCATTTAGGAAGG-3′), and a 295-bp 3′ external probe (13/14), amplified by PCR using primers Slc5-13 (5′-CCATCTTTAACAGCAGTAGC-3′) and Slc5-14 (5′-AATGTAATCGAAGAGCTGCC-3′). Southern analysis using probe 15/16 detected a 11.0-kb WT band and an 8.7-kb mutant band in NcoI digested genomic DNA, whereas probe 13/14 detected a 7.3-kb WT and a 4.8-kb mutant band in EcoR I digested genomic DNA (Figure 1B). Four targeted ES cell clones were identified and microinjected into C57BL/6 blastocysts to generate chimeric animals that were bred to C57BL/6 females. Germline transmission was observed with one clone, and heterozygous offspring were backcrossed for 10 generations to the C57BL/6 background. Heterozygous mice were then interbred to produce homozygous *Sglt2*<sup>-/-</sup> mice and WT mice, which were used to establish breeding colonies for *Sglt2*<sup>-/-</sup> and WT mice. Genotypes were confirmed by PCR amplification of tail-snip DNA (Figure 1C). Male mice of matched age (3 to 5 months) were used in the experiments described. Mice were housed in a 12:12-hour light-dark cycle in standard rodent cages with free access to standard rodent chow (1% NaCl; Harlan Teklad, Madison, WI) and tap water.

### SGLT2 and SGLT1 Protein Expression in the Kidney

Polyclonal immune sera were raised in rabbits against amino acids 592 through 609 of rat SGLT2 (AMGIEEVQSPAPGLLRQC) and, as described previously,<sup>17</sup> against amino acids 585 through 600 of rat SGLT1 (PKDTIEIDAEAPQKEK). The peptide sequences have 83 and 81% identity with the corresponding peptides in SGLT2 and SGLT1 of the mouse, respectively, but no significant homology to other SGLT subtypes. The Ab were affinity-purified from the immune sera *via* the antigenic peptides as described previously.<sup>17,34</sup> Whole kidneys were prepared for Western blot analysis as described previously.<sup>35,36</sup> Briefly, kidneys were homogenized in a buffer containing 250 mM sucrose, 10 mM triethanolamine (Sigma-Aldrich, St. Louis, MO) and Complete Protease Inhibitor cocktail (Roche, Indianapolis, IN) using a tissue homogenizer (Tissumizer; Tekmar, Cincinnati, OH). Ho-



**Figure 6.** Reabsorption of glucose is absent in the early proximal tubule of *Sglt2*<sup>-/-</sup> mice: Micropuncture studies under anesthesia. (A) Free-flow collections of tubular fluid are performed along accessible proximal tubules at the kidney surface to establish a profile for FR-glucose versus FR-fluid. (B and C) Mean FR-glucose (B) and fractional reabsorption of chloride (C) for early (FR-fluid <40%) and late (FR-fluid ≥40%) proximal tubular collections and up to the urine (n = 18 to 23 nephrons in four to five mice). \*P < 0.001 versus WT mice.

mogenates were centrifuged at 16 000 × g for 30 minutes to obtain a membrane pellet. The pellet was resuspended in homogenizing buffer, and protein concentration was determined using a DC Protein Assay (Bio-Rad, Hercules, CA). Samples used for SGLT1-Ab were diluted in 2× SDS gel loading buffer (100 mM Tris, 4% SDS, 20% glycerol, 0.2% bromphenol blue) and heated for 15 minutes at 70°C. Samples used for SGLT2-Ab were diluted in 4× LDS reducing sample buffer (Invitrogen, Carlsbad, CA) and heated for 15 minutes at 65°C. Samples were loaded on precast 4 to 12% Bis-Tris SDS-PAGE gels (Invitrogen) using MOPS buffer. The membranes were blocked with 5% nonfat dry milk (Bio-Rad) in PBS (pH 7.4) containing 0.1% Tween 20 (PBS-T) for 1 hour. Immunoblotting was performed at 4°C overnight with the primary SGLT2-Ab (see previous section) diluted 1:1000 and SGLT1-Ab diluted 1:2000 in PBS-T containing 1% BSA. Chemiluminescent detection was performed using a 1:5000 dilution of ECL donkey anti-rabbit IgG linked to horseradish-peroxidase and ECL detection reagent (GE Healthcare, Buckinghamshire, UK). To verify equal protein loading between genotypes, the membrane was stripped (0.2 M NaOH for 5 minutes) and re probed with monoclonal anti-β-actin Ab (Sigma-Aldrich).

**SGLT2 Protein Localization in the Kidney**

Perfusion was performed in anesthetized mice *via* the left ventricle of the heart at a pressure of 120 to 140 mmHg using 1× PBS (pH 7.4) for 1 to 2 minutes followed by approximately 50 ml of 4% paraformaldehyde in PBS. After removal the kidneys were kept in 4% paraformaldehyde overnight, rinsed thoroughly with PBS, and dehydrated by a sucrose gradient (10, 20, and 30% sucrose in PBS, each overnight). Kidneys were embedded in Cryo-embedding compound (MICROM, Walldorf, Germany) and cut into 10-μm frozen sections at -25°C using an HM560 Cryostat (MICROM). Sections were mounted on microscope slides and air-dried for 3 to 4 hours before storage at -80°C. Sections were rehydrated in PBS (three times for 5 minutes), blocked at room temperature for 30 minutes in blocking buffer (0.1%

Triton X-100 and 5% normal donkey serum in PBS), and given three quick rinses with PBS. The primary Ab (SGLT2-Ab, see previous section) was applied at a dilution of 1:250 in blocking buffer and incubated overnight at 4°C. Slides were washed with PBS. The secondary Ab (CY3-conjugated donkey anti-rabbit IgG; Jackson ImmunoResearch Laboratories, West Grove, PA) was applied at a dilution of 1:250 in blocking buffer and incubated for 1 hour at room temperature in the dark. In addition to the secondary Ab, 1 U of Alexa Fluor 488-conjugated phalloidin (Invitrogen Molecular Probes, Eugene, OR) was applied to label filamentous actin of the proximal tubular brush border. The slides were washed with PBS before being mounted with ProLong Gold antifade reagent with DAPI (Invitrogen Molecular Probes). Slides were analyzed and pictures were taken on an Olympus IX81 Microscope.

**Reverse Transcription and Real-Time PCR**

Whole-kidney RNA was prepared with the RNeasy Mini Kit. DNase digestion was performed with Turbo DNase (Ambion, Austin, TX), and cDNA was prepared with the Superscript II First Strand Synthesis System. For quantification, specific primers were used with Power SYBR Green PCR Master Mix (10 minutes at 95°C with 50 cycles of 15 seconds at 95°C and 1 minute at 60°C) in a Chromo4 Real-time PCR Detector (MJ Research and Bio-Rad). Given the homology of the *Sglt* isoforms, we used Taqman PCR Universal Mastermix with the following primers to improve specificity and sensitivity of these reactions: *Sglt1* Mm00451203\_m1 and *Sglt2* Mm00453831\_m1 (Applied Biosystems). The primers used for *Glut1*, *Glut2*, and *Glut12* are listed in Table 3. Amplification efficiencies were normalized against the housekeeping gene RPL19, and relative fold increases were calculated using the Pfaffl technique of relative quantification, which accounts for real-time efficiencies.<sup>37</sup> Each experiment was performed in triplicate.

**Food and Fluid Intake**

Daily food and fluid intakes were determined over 3 days while the mice were maintained in their regular cages.

**Blood and Urine Analysis**

Urine was obtained by picking up the mice to elicit reflex urination, then holding them over a clean Petri dish for sample collection. For obtaining paired measurements, blood was collected by tail snip immediately after urine collection. Plasma and urine glucose was

**Table 3.** Real-time PCR primers used

Target	Forward	Reverse
<i>Glut1</i>	AACATGGAACACCGCTACG	GTGGTGAGTGTGGTGGATGG
<i>Glut2</i>	ATCGCCCTCTGCTCCAGTAC	GAACACGTAAGGCCCAAGGA
<i>Glut12</i>	CACAGGAGGCGTGCTCATAG	AGGATTATGGCGAGCCTTCTT
<i>Rpl19</i>	TGCTCAGGCTACAGAAGAGGCTTG	GGAGTTGGCATTGGCGATTTTC

determined by the hexokinase/glucose-6-phosphate dehydrogenase method (Infinity, Thermo Electron, Louisville, CO). Urinary calcium and creatinine were determined photometrically (Arsenazo III Method and enzymatically, respectively; Thermo Electron, Melbourne, Australia). Urinary amino acid concentrations were determined by HPLC. In a separate set of mice, blood was collected by puncturing the retrobulbar plexus under brief isoflurane anesthesia to determine hematocrit and plasma  $\text{Na}^+$  and  $\text{K}^+$  (measured by flame photometry; Cole-Parmer Instrument Co., Vernon Hills, IL). Plasma aldosterone was determined by RIA (Diagnostic Systems Laboratories, Webster, TX). Plasma renin concentration was determined by RIA (GammaCoat; DiaSorin, Stillwater, MN) as the generation of angiotensin I after addition of excess renin substrate from 24-hour nephrectomized rats.<sup>38</sup> As an indirect measure of circulating vasopressin, concentration of vasopressin in urine was measured using a commercial assay (IBL, Hamburg, Germany) and related to creatinine.<sup>39</sup> Urine and plasma osmolalities were measured by vapor pressure (Vapro; Wescor, Salt Lake City, UT).

### BP, Heart Rate, and GFR Measurement in Awake Mice

Systolic BP and heart rate were determined in awake animals over a period of 5 days using the tail-cuff system (BP-2000; Visitech-Systems, Apex, NC) after appropriate training (for 5 days), as described previously.<sup>38,40,41</sup> GFR measurements were performed in conscious mice using the plasma kinetics of FITC-inulin after a single-dose intravenous injection as described previously.<sup>42</sup>

### Whole-Kidney and Proximal Tubular Reabsorption of Glucose in Anesthetized Mice

Mice were anesthetized with thiobutabarbital (100 mg/kg intraperitoneally) and ketamine (100 mg/kg intramuscularly) and prepared for renal micropuncture.<sup>43–45</sup> A catheter was placed in the femoral artery for continuous BP recording. For assessment of two-kidney and single-nephron GFR, [<sup>3</sup>H]inulin was added to the infusion to deliver 20  $\mu\text{Ci/h}$ . Urine was quantitatively collected using a bladder catheter. For determination of glucose reabsorption along the proximal tubule, quantitative fluid collections were made from early and late surface loops of the proximal convoluted tubule. Tubular fluid volume was determined from transfer to a constant bore capillary. The concentrations of glucose in plasma, urine, and tubular fluid were determined enzymatically (Infinity; Thermo Electron, Melbourne, Australia) in a regular photometer and a flow-through microfluorometer (NanoFlo; WPI, Sarasota, FL), respectively.<sup>44</sup> The concentrations of chloride in plasma, urine, and tubular fluid were determined photometrically (Infinity, Thermo Electron) and by a microadaptation of the electrometric titration method,<sup>45</sup> respectively.

### Statistical Analysis

Data are presented as means  $\pm$  SEM. Statistical differences between *Sglt2*<sup>-/-</sup> and WT mice were analyzed by the unpaired two-tailed *t* test. *P* < 0.05 was considered to be statistically significant.

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