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Specific Cre/Lox Recombination in the Mouse Proximal Tubule

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Abstract. The present work reports for the first time the construction of a transgenic mouse strain with specific expression of Cre recombinase in the kidney proximal tubule. A Cre/loxP strategy was developed using sglt2 promoter to drive Cre recombinase expression in transgenic mice. The mouse sglt2 5' region consisting of the first exon, the first intron, and part of the second exon was cloned upstream of a nucleotide sequence encoding the Cre recombinase. Transgenic mice were generated by pronuclear injection, and tissue specificity of Cre expression was analyzed using reverse transcription–PCR. The iL1-sglt2-Cre mouse line scored positive for kidney transcription of Cre but not for the other tissues analyzed. Within the kidney, Cre transcripts were demonstrated to be restricted to the proximal tubule only. iL1-sglt2-Cre mice were bred with ROSA26-LacZ reporter mice that contained a *loxP*-flanked stop sequence upstream of the LacZ gene. X-gal staining and immunohistochemistry using specific antibodies (anti-megalin, anti–Tamm-Horsfall, anti–NaCl co-transporter, and anti–aquaporin 2) revealed that sglt2 drives Cre functional expression specifically in proximal tubules. The iL1-sglt2-Cre mouse therefore represents a powerful tool for Cre-LoxP–mediated conditional expression in the renal proximal tubule.

The kidney is the organ that maintains body fluid homeostasis. Specific transport processes localized all along the highly differentiated segments of the nephron support this essential function. Because of this large functional heterogeneity, experiments performed at the whole-organ level cannot give precise information about the physiologic role of these transporters and associated proteins. By using the serial analysis of gene expression (SAGE) method, it was found that ~1000 transcripts could specifically participate in human renal function (1). Moreover, some of these transcripts are involved in genetic disorders such as hypertension, diabetes insipidus, Bartter and Fanconi syndromes, Dent's disease, and cystic fibrosis. Even if the genes involved in some of these pathologies have been identified, their physiologic roles together with their precise interactions need to be elucidated. Transgenic knockout animals obtained by gene targeting represent a potent tool to address this question because of the degree of specificity that can be reached by this technique. Thus, gene targeting is a powerful technique to analyze gene function. However, conventional knockouts are limited for several reasons. First, the knockout of a gene that possesses an important function can be embryonic or perinatal lethal in the homozygous state. For example, the homozygous knockout of the gene encoding the WT1 transcription factor, which is crucial for kidney development, leads to lethality very early in the embryonic stage (2). Second, several proteins play key roles in different organs, making it difficult to use generalized knockout approaches to establish a specific phenotype for a precise function in a given organ. This is the case for many different channels or transporters, which are expressed in both the intestines and the kidneys, where they mediate vectorial absorption or reabsorption of various solutes. To circumvent these limitations and to assess specifically gene function in the adult kidney, we decided to use a powerful approach enabling conditional and tissue-specific genetic invalidation. It consists of the generation of two transgenic lines, one expressing the target floxed gene and the second expressing Cre recombinase under the control of a tissue- and cell-specific promoter. The Cre gene of bacteriophage P1 encodes a site-specific recombinase. Generally, Cre recombinase mediates recombination between the loxP sites, leading to specific genome alterations such as deletion, insertion, translocation, or inversion of a DNA sequence depending on the location and orientation of the loxP sites (3,4). In particular, Cre recombinase efficiently excises DNA sequences located between two loxP sites in the same orientation, leaving one loxP site on the DNA (5,6). Therefore, choosing the appropriate promoters for the proximal, distal, and collecting tubules or for the Henle's loop makes it possible to target the expression of the Cre recombinase to a distinct cell type within the nephron (7).

The present work reports for the first time the engineering and the characterization of a transgenic mouse strain expressing Cre recombinase in the proximal tubule. For this purpose, we used the promoter sequences of the Sglt2 gene to drive the expression of the Cre recombinase. This promoter was chosen, because the glucose transporter Sglt2 is specifically expressed in the brush border membrane of the S1 segments of the

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proximal tubule. Furthermore, we demonstrate in this work that this Sglt2 promoter-Cre recombinase construct is fully functional when inserted in transgenic mice and allows for specific recombination in the proximal tubular cells. Therefore, this work allows for the first time specific targeting of the knockout of genes expressed in this particular segment. This is of high interest, because proximal tubule supports the major part of the transport of the filtered ions and solutes in the kidney.

Materials and Methods

Construction of the mSglt2 5pr-mut-Cre Transgene

A total of 2637 bp of the murine sglt2 5' region (nucleotides 55 to 2691 of GenBank accession no. AJ292928) were amplified from murine genomic DNA by PCR (Advantage Genomic PCR; Clontech Laboratories, Palo Alt, CA) using the following primers: sglt2 sense, 5'-ACTGGACATCACATGGTACC-3' and sglt2 antisense 5'-CACCAACTGTGCCTCTATTG-3'. The amplified sequence contained the sglt2-5' flanking region, exon 1 within the initiation codon ATG, intron 1, and the first part (23 bp) of exon 2. The PCR product was directly ligated into a pGEM vector, using the T/A cloning kit (Promega, pGEM-T Easy Vector Systems). For avoiding any potential interference with the initiation codon of Cre recombinase, site-directed mutagenesis was performed directly on double-stranded pGEM-sglt2–5pr plasmid DNA (Quickchange Site-Directed Mutagenesis kit; Stratagene, La Jolla, CA) with 5'- and 3'-homologous primers containing the desired mutation (5'-TGGAGAGAAT**TC**AGCAACACGT-3' and 5'-ACGTGTTGCT**GA**ATTCTCTCCA-3'). A 2.7-kb *Xho*I-*Hind*III fragment containing the Cre recombinase gene and the metallothionein-I polyA signal sequence was subcloned from pBS185 plasmid (Gibco BRL) into pGL2 Basic vector (Promega) to give pGL2-Cre vector. Three restrictions sites were inserted in this plasmid: *Sca*I-*Not*I sites upstream of the *Xho*I site and a *Sca*I site downstream of the *Hind*III site. The sglt2 5' flanking region with mutated ATG (pGEM-sglt2–5pr-mut) was inserted into the *Not*I site of pGL2-Cre vector. pGL2-sglt2–5pr-mut-Cre was digested by *Sca*I to release the mouse sglt2 5' region linked to the coding region of Cre recombinase. The linear transgene was isolated by gel electrophoresis, extracted (QIAGEN extraction kit), and purified by anion-exchange chromatography (Elutip-d; Schleicher & Schuell, Keene, NH). The purified DNA was suspended at a concentration of 2 μg/ml in water and sterilized by filtration through 0.22-μm filters.

Generation of Transgenic Mice

The linear purified transgene was injected into the pronuclei of fertilized oocytes of B6D2F1 animals, and the injected embryos were transferred into pseudopregnant mice according to standard techniques. Pups were analyzed for the presence of the transgene by PCR amplification of tail DNA using the following primers: Cre sense 5'-CCTGGAAAATGCTTCTGTCCG-3', Cre antisense 5'-CAGGGTGTTATAAGCAATCCC-3'. As a control of DNA quality, the myogenic DNA sequence was co-amplified using the following primers: sense 5'-TTACGTCCATCGTGGACAGC-3', antisense 5'-TGGGCTGGGTGTTAGTCTTA-3'. Founder mice were backcrossed with B6/D2 F1 animals to generate transgenic lines. For functional analysis of Cre expression, sglt2-Cre heterozygous mice were bred with ROSA26 reporter mice (8).

RNA Preparation and Reverse Transcription–PCR

Total RNA was prepared from various organs of transgenic mice or from microdissected tubules (9) using an RNeasy Mini Kit with an additional DNase I treatment (Qiagen, Studio City, CA). cDNA synthesis was carried out using Superscript First-Strand Synthesis System for reverse transcription–RT-PCR (RT-PCR; Invitrogen, San Diego, CA) according to the manufacturer's instructions. The following oligonucleotide primers were used to amplify Sglt2 (707 bp), Cre (625 bp), and GAPDH: sglt2 sense, 5'-AGGATCCATCTGTTGGCA-3'; sglt2 antisense, 5'-ACGGGGCACAAAGAGT-3' (10); Cre sense, 5'-CGACCAAGTGACAGCAATGCTGTTTCA-3'; Cre anti-sense, 5'-CACCAGCTTGCATGATCTCCGGTATT-3'; GAPDH sense, 5'-ACCACAGTCCATGCCATCAC-3'; GAPDH antisense, 5'-TCCACCACCCTGTTGCTGTA-3'.

X-gal Staining and Immunohistochemistry

Animals were anesthetized with Imalgene-xylasine ROMPUN and perfused transcardially with PBS. Kidneys were dissected, embedded in OCT compound (Tissue-Tek 4385), and immediately frozen in isopentane cooled in liquid nitrogen. Cryostat sections (12 μm) were prepared and fixed for 10 min in 0.5% glutaraldehyde at room temperature. The fixed sections were washed twice with PBS and subjected to X-gal staining overnight at 37°C (X-gal solution: 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 1 mg/ml X-gal in PBS [pH 7.4]). After staining, sections were rinsed in PBS and mounted. For immunohistochemical analysis, X-gal–stained slices were blocked with PBS-BSA 3% for 30 min and incubated overnight at 4°C with primary antibodies: anti-megalin (1:100) or anti-THP (1:100; both provided by Dr P. Ronco, Tenon Hospital, Paris, France) or anti–aquaporin 2 (AQP2; 1:250 #178612; Calbiochem, San Diego, CA). The NaCl co-transporter (NCC) antibody was donated by Dr. J. Loffing (Institute of Pharmacology and Toxicology, Lausanne, Switzerland). Sections were washed with 0.1 M PBS and incubated at room temperature with FITC-conjugated goat or horseradish peroxidase–conjugated secondary antibodies at a dilution of 1:100. Peroxidase deposit was visualized by incubating sections with 0.3 mg/ml diaminobenzidine–4HCl diluted in Tris-HCl buffer 100 mM (pH 7.6) containing 0.01% H_2O_2. Control sections were incubated with secondary antibodies alone.

Results

Sglt2 is Kidney Specific in Wild-Type Mice

To check for the specific renal expression of Sglt2, we extracted RNA from a variety of mouse tissues and carried out RT-PCR using oligonucleotide primers specific for mouse sglt2 cDNA (10). A 707-bp product representing the fragment expected from the published mouse sglt2 cDNA sequence (GenBank accession no. AY033886) was amplified only in the kidney (Figure 1A). No fragment was detected in other tested organs, including lung, intestine, colon, liver, heart, brain, cerebellum, spleen, and skeletal muscle. This result confirmed the kidney specificity of sglt2 gene expression in mice. To map precisely the distribution of sglt2 along the murine nephron, we used RT-PCR on microdissected nephron segments. Proximal convoluted tubule, distal convoluted tubules, and cortical collecting ducts were microdissected as described previously (9). The results obtained clearly showed that proximal tubules expressed sglt2 transcripts, whereas no expression has been detected in distal convoluted tubules and cortical collecting ducts (Figure 1B).

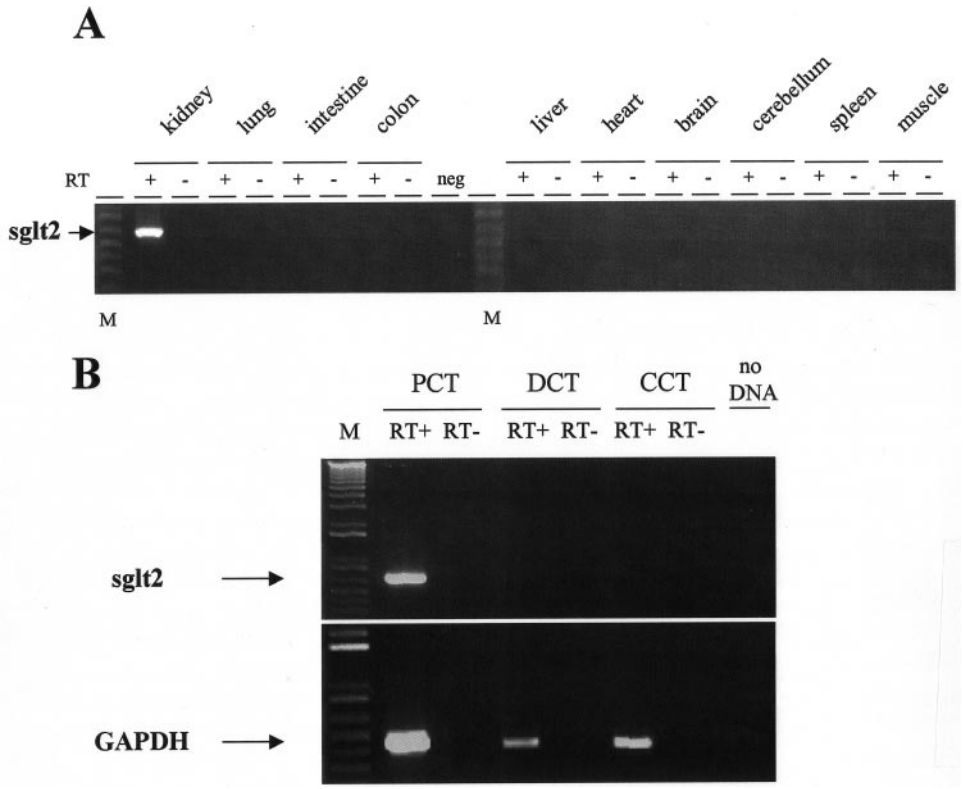

Figure 1. Tissue-specific expression of sglt2 gene in mouse organs and microdissected tubules revealed by reverse transcription–PCR (RT-PCR). RT-PCR was carried out using oligonucleotide primers specific for mouse sglt2 cDNA. (A) Gel representative of results obtained from three different analyses. (B) Total RNA was extracted from microdissected nephron segments, proximal convoluted tubule (PCT), distal convoluted tubule (DCT), and cortical collecting duct (CCD). RT-PCR was performed in the presence (+) or the absence (−) of reverse transcriptase to control for DNA contamination. M, markers.

Construction of Sglt2-Cre Transgene and Generation of Transgenic Mice

Using data available from the current mouse sequence databases (GenBank accession no. AJ292928), we amplified a sequence from genomic mouse DNA from −1952 bp upstream to 685 bp downstream of the transcription start site of the sglt2 gene. We decided to use this large stretch of sequence to maximize our chances to clone the sequences responsible for the tissue specificity of this promoter. For example, this region contained the two HNF1 binding sites that are necessary for the activation of the promoter (11). Moreover, the 3' end of the amplified product contained the first exon, the first intron, and part of the second exon (Figure 2). These regions have been reported frequently to contain tissue-specific and expression enhancers (12). For avoiding any potential interference with the initiation codon of Cre recombinase, the sglt2 initiation codon was eliminated by site-directed mutagenesis. The transgenic construct was engineered by inserting this sglt2 5' region upstream of Cre cDNA sequence. Transgenic mice were generated by conventional pronuclear microinjection using sglt2-Cre transgene. A total of 600 oocytes were injected and transferred into 31 pseudopregnant mice. Of these, 19 gave 64 pups that were analyzed for the integration of Cre by PCR analysis carried out on tail biopsies. Three founder transgenic mice were then selected for their ability to express germline transmission of the Cre recombinase transgene.

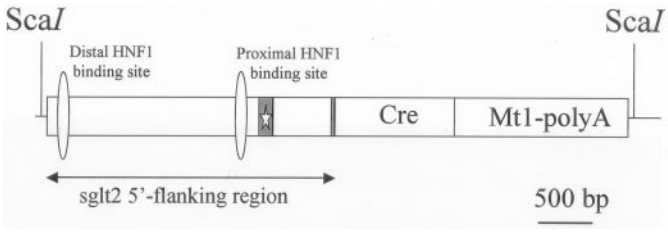

Figure 2. Structure of the sglt2-Cre transgene. Mouse sglt2 5' region containing 1.9 kb of the 5' upstream promoter region (open box), the first coding exon (filled box), the first intron (open box), and the first 23 bp of exon 2 was fused to the Cre coding sequence followed by the MT-I poly (A) tail. *ATG codon mutated in exon 1.

RT-PCR Analysis of Cre Recombinase in Transgenic Mice

Various organs from mice that genotyped positive for transgene integration (see Materials and Methods) were analyzed for Cre transcription using RT-PCR experiments. These experiments have been repeated at least three times on RNA samples taken independently from different animals. The number of PCR cycles was purposely kept high (35 cycles) to maximize the chances of detecting nonspecific expression in organs other than kidney. Of a total of three positive strains tested, two transgenic mouse lines showed a low-level transcription of the transgene. By contrast, the iL1-sglt2 strain showed a high level of Cre transcription in RNA prepared from kidney only. No expression could be detected in the other organs tested. A representative expression profile is shown in Figure 3A. For analyzing Cre expression in more detail along the nephron, RT-PCR experiments were carried out on microdissected segments of

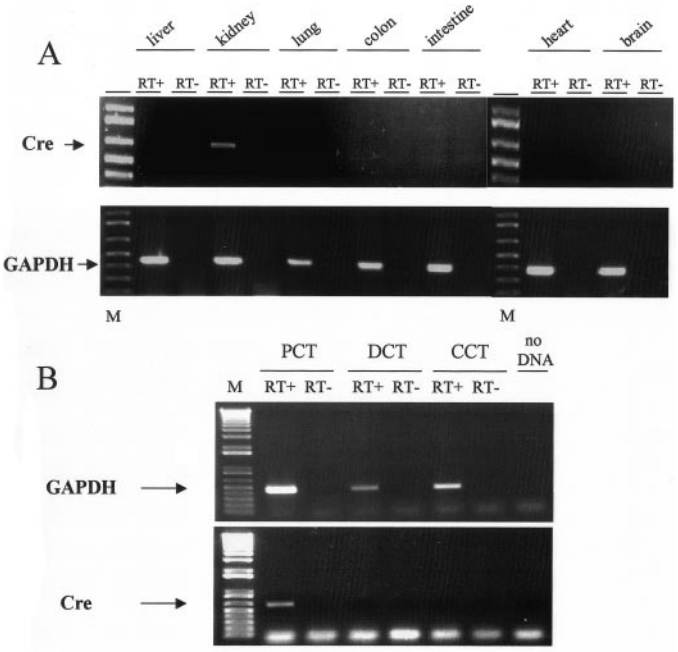

Figure 3. RT-PCR determination of Cre mRNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in mouse organs and microdissected tubules obtained from iL1-sglt2 Cre mice. RT-PCR analysis was performed with primers specific for Cre or GAPDH. RT-PCR was performed in the presence (RT+) or the absence (RT−) of reverse transcriptase to control for DNA contamination. (A) The gel is representative of results obtained from three different analyses. M, markers. (B) Cre expression along the nephron segments, PCT, DCT, and CCD.

the iL1-sglt2-Cre strain. Cre transcripts were detected in proximal tubules only. There was no detectable signal in distal tubules and cortical collecting ducts (Figure 3B).

Cre-Mediated Recombination in the Kidney

For determining whether the iL1-sglt2-Cre strain expressed a functional recombinase activity in kidney and which cell type was involved, mice were crossed with ROSA26 reporter mice. These mice carry a lacZ gene whose expression requires excision of loxP-flanked stop sequences (8). Analysis of X-gal–stained cryostat sections revealed a blue staining in the cortical part of kidneys originating from double-transgenic iL1-sglt2-Cre/ROSA26 mice (Figure 4A). Kidney sections from Cre-negative control mice did not reveal any labeling (Figure 4J). As shown in Figure 4, B through I, no detectable lacZ expression was present in other tissues (lung, intestine, colon, liver, heart, brain, spleen, and muscle) from double-transgenic iL1-sglt2-Cre/ROSA26 mice. This clearly demonstrates the kidney specificity of Cre expression.

To determine the identity of the blue-stained structures within the kidney, we performed co-localization experiments with known markers of the kidney cortex. Thus, immunohistochemistry experiments were performed on kidney sections stained by the X-gal reaction. For this purpose, antibodies directed against proteins specifically expressed in the different parts of the nephron were used. Anti-megalin antibody was used to identify proximal tubule (13), anti–Tamm-Horsfall antibody was used for the loop of Henle (13), antiserum against the thiazide-sensitive NCC was used to identify distal tubules (14), and anti-AQP2 antibody was used for collecting tubule (15). Results are shown in Figure 5. Co-localization of Cre activity and immunolabeling was clearly evidenced with anti-megalin antibody (Figure 5, A and B). Tubules labeled by anti–Tamm-Horsfall (Figure 5F), anti-NCC (Figure 5G), or anti-AQP2 antibodies (Figure 4, C through E) did not show any blue staining. Statistical analysis of the labeling obtained on cryostat sections from two different iL1-sglt2-Cre/ROSA26 mice revealed that on a total of 633 positive megalin tubules, $96.46 \pm 0.71\%$ ($n = 20$ microscope fields) exhibited a blue staining reflecting the expression of an active Cre recombinase. Together, these results unambiguously show that the iL1-sglt2 strain exhibits a fully functional Cre expression in the kidney and that the recombination events occur in the proximal tubule only.

Discussion

The elementary mechanisms involved in the epithelial ionic transport and in kidney homeostasis have been studied extensively from a functional point of view. Various mechanisms implicating K^+, Na^+, Ca^{2+}, Cl^-, and H^+ movements have been evidenced along the renal tubule (16–19). These data allowed for the establishment of models of ionic handling along the renal epithelium. Further studies are now required to reintegrate these functional data at the cellular and molecular levels and to construct a coherent picture of the physiologic functioning and regulation of the organ at whole. Such approaches will also facilitate the understanding of complex pathologic traits involving kidney function. In this context, the creation of transgenic models represents a particularly powerful approach, provided that sufficient care has been taken with respect to the specificity of the engineered genetic lesion. Transgene overexpression or classical knockout approaches are in this context limited for the analysis of gene function in a complex organ such as kidney, especially when essential ubiquitously expressed genes are concerned. To circumvent these limitations, we decided to use a conditional gene-targeting strategy, using site-specific recombinases, that has been developed to control gene inactivation at specific times and in specific tissues.

One essential part of the technique is the engineering of mouse strains that express Cre recombinase, in particular cells of the target organ. Concerning the kidney, very few mouse strains expressing Cre recombinase have been created. Two strains express Cre in the highly specialized podocytes by using Nphs1 and Nphs2 promoters (20,21). In another strain, the Tamm-Horsfall promoter (22) was used to drive Cre expression in the thick ascending limb. Shao *et al.* (23) used Ksp-cadherin promoter to control Cre expression in collecting ducts and thick ascending limbs of Henle's loops, and Nelson *et al.* (24) engineered a mouse strain that expresses a Cre-

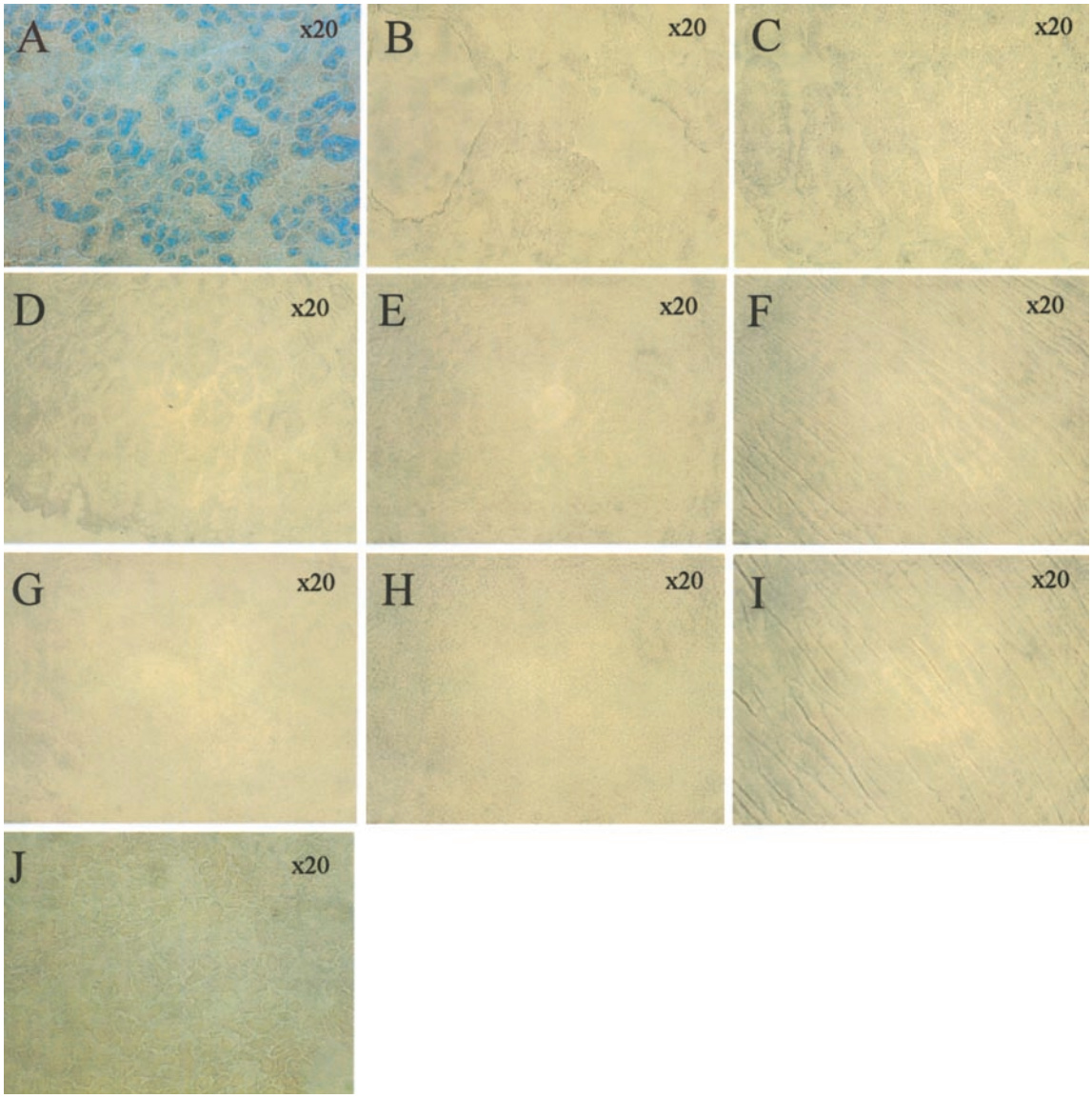

Figure 4. Cre-mediated recombination in organs isolated from double-transgenic iL1-sglt2-Cre/ROSA26 reporter mice. (A) X-gal staining on cortical kidney sections from iL1-sglt2-Cre/ROSA26 mice. No β-galactosidase activity was observed in lung (B), intestine (C), colon (D), liver (E), heart (F), brain (G), spleen (H), and muscle (I). (J) X-gal staining on cortical kidney sections from Cre-negative control mice. Photomicrographs are representative of those obtained from two offspring derived from iL1-sglt2-Cre mice.

tagged recombinase in collecting duct by using the AQP2 promoter. Finally, Yu *et al.* (25) used the HoxB7 promoter/enhancer to drive Cre recombinase specifically within the mesonephric duct. Concerning the proximal tubule that is in charge of the major part of the reabsorption of ions and solutes through highly differentiated cells, a γ-GT-Cre mouse strain (26) that achieved cortical tubular Cre expression has been produced. However, the precise cortical localization of Cre expression was not determined in detail in this strain.

To drive Cre expression in the proximal tubule, we chose the promoter that permits the renal expression of the low-affinity, high-capacity co-transporter Na^+/glucose sglt2 that is known to be mainly involved in glucose reabsorption along S1 segments (27,28). As stated in the Results section, we used a large stretch of sequence in our transgenic constructs, containing the nuclear factor HNF1α sites and the first intron, to maximize the chances of tissue specificity. This strategy allowed us to isolate a transgenic mouse strain that specifically expresses Cre in proximal tubule of the kidney. This indicates that the promoter sequence used in this work contains the necessary and sufficient elements to ensure the desired level of tissue specificity. To validate our strategy further, we crossed our proximal tubule–specific mouse strain with a reporter strain expressing the stop-floxed LacZ gene under the control of the ubiquitous promoter ROSA26 (8). β-Gal expression was restricted to tubular structures coexpressing megalin, a multiligand receptor localized only in the proximal tubule (13).

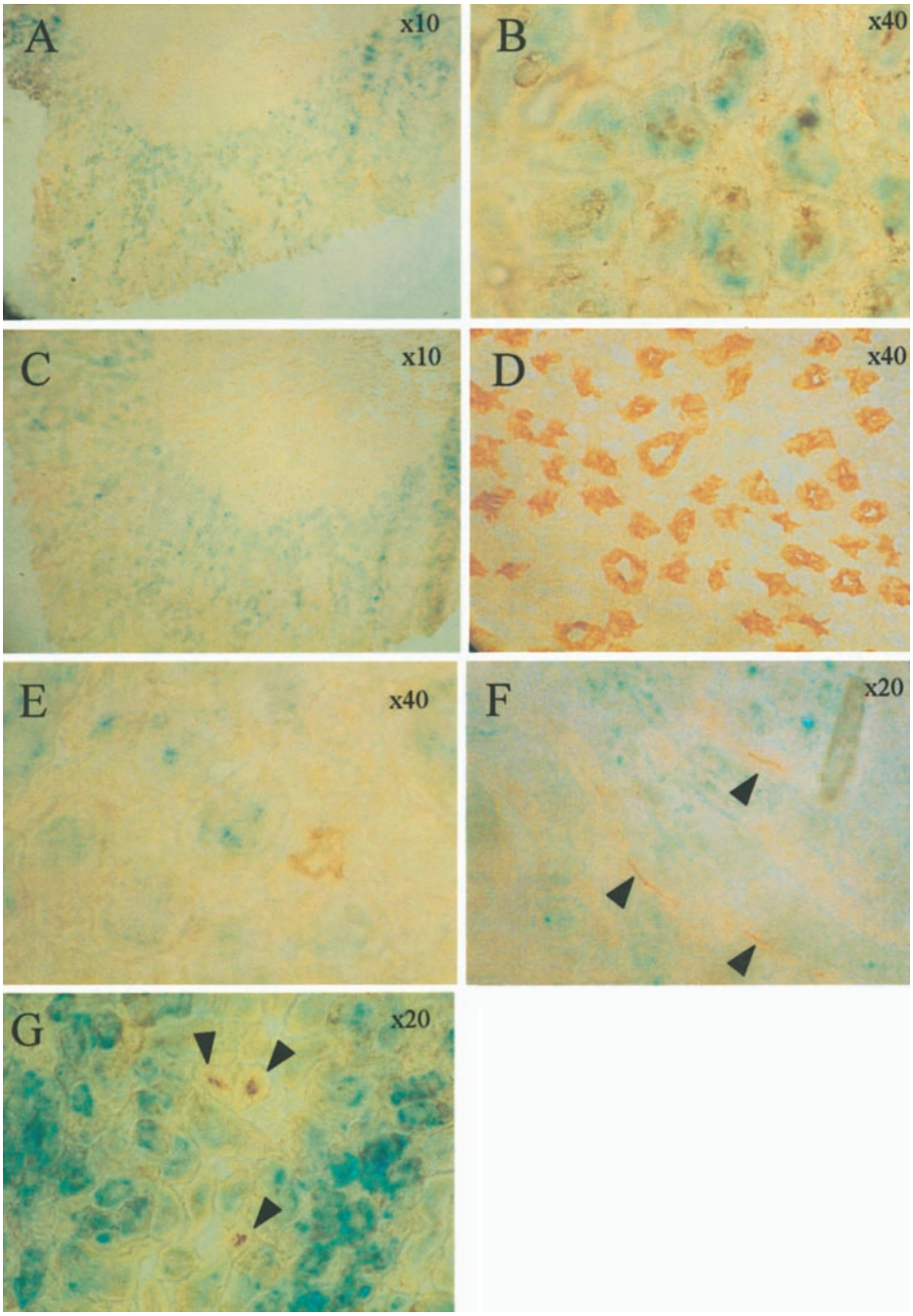

Figure 5. Localization of Cre-mediated recombination in kidney sections from double-transgenic iL1-sglt2-Cre/ROSA26 reporter mice. (A and B) X-gal staining on kidney sections followed by anti-megalin antibody labeling. (C through E) X-gal staining on kidney sections followed by anti–aquaporin 2 antibody labeling. (D and E) Enlargements of medullary and cortical zones, respectively. (F) X-gal staining on kidney section followed by anti–Tamm-Horsfall antibody labeling. (G) X-gal staining on kidney section followed by anti–NaCl co-transporter antibody labeling. Arrowheads indicate the specific labeling of loops of Henle (F) or distal tubules (G). Primary antibodies were revealed using horseradish peroxidase-conjugated second antibody followed by diaminobenzidine revelation. Photomicrographs are representative of those obtained from two offspring derived from iL1-sglt2-Cre mice.

In conclusion, we have established a transgenic mouse line that expresses Cre recombinase under the control of the sglt2 5' region specifically in renal proximal tubules. This transgenic strain, which is now available for all laboratories of the scientific community, will represent a valuable tool to direct proximal tubule–specific excision of a wide panel of genes of interest. This will enable the study of both normal and pathologic proximal tubule functions with an unprecedented level of resolution. For instance, this could be the case for the mechanism implicated in Dent or Fanconi syndromes that are characterized by a dysfunction of specific proteins of the endocytotic pathway (29).

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See related editorial, “Kidney-Specific Gene Targeting,” on pages 2237–2239.