

Glomerular-Specific Gene Excision *In Vivo*

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Abstract. Podocytes (glomerular visceral epithelial cells) are highly specialized cells that are found in the renal glomerulus and make up a major portion of the filtration barrier between the blood and urinary spaces. Recently, the identification of a number of genes responsible for both autosomal dominant and recessive forms of human nephrotic syndrome has provided insight into a number of molecules responsible for unique features of the podocyte such as the slit diaphragms. Despite these major advances in our understanding of podocyte biology, the function of many genes expressed in the podocyte remains unknown. Targeted gene disruption using homologous recombination in murine embryonic stem cells (ES cells) is a

powerful tool to determine the biologic function of genes *in vivo*. However, resulting embryonic lethal or pleiotropic phenotypes often preclude the analysis of genes in specific renal cell types. To overcome this problem, a glomerular-specific Cre-recombinase transgenic murine line under the control of the Nphs1 (nephrin) promoter (Neph-Cre) was generated. This article reports successful Cre-mediated excision of a 'floxed' transgene specifically in podocytes *in vivo*. This murine founder line represents a powerful new tool for the manipulation of the expression of genes in podocytes and will provide valuable insight into podocyte biology in the whole animal.

Podocytes are highly specialized cells that are fundamental for glomerular filtration (1). Although water and small molecules pass freely through this filter, critical proteins, such as albumin and blood clotting factors, must not. In addition, podocytes are believed to play a pivotal role in the progression of chronic renal dysfunction due to a variety of causes such as diabetes, aging, and inflammation (2–4).

Despite the importance of the podocyte for development and function of the kidney, little is known about its biology due to the lack of appropriate model systems. Although it has been possible to isolate podocyte cell lines, these cells tend to dedifferentiate in culture. Furthermore, a model system that enables manipulation of gene expression specifically in podocytes *in vivo* would be valuable.

Recently, we and others have identified and characterized the first glomerular and podocyte-specific promoter from the human and murine nephrin (NPHS1, Nphs1) genes (5,6). NPHS1 encodes a member of the Ig superfamily and is the gene responsible for congenital Finnish nephropathy (7). 1.25 kb of the proximal region of the human NPHS1 promoter and 5.4 kb of the proximal region of the murine promoter are capable of directing transgene expression to the podocyte (5,6). In this article, we also demonstrate that 4.125 kb of the 5'

flanking region of murine Nphs1 is capable of directing podocyte-specific expression.

In an attempt to generate murine founder lines that express Cre recombinase specifically in podocytes, 22 independent founder lines with integration of the nephrin-Cre transgene (12 human and 10 murine promoter lines) were produced. To determine if any of these founder lines were capable of podocyte-specific Cre-mediated excision *in vivo*, they were bred with the Z/EG double reporter mouse line (8). Upon Cre-mediated excision of a β -geo cassette, these mice express enhanced green fluorescent protein. Green fluorescence podocytes were observed in the offspring of two of these founder lines (mNeph-Cre PC1 and PC2), which were doubly heterozygous for both transgenes and demonstrate successful podocyte-specific Cre-mediated excision *in vivo*.

Materials and Methods

Generation of Murine-Nephrin Transgenic Lines

A 4.125-kb fragment of the murine promoter (4145 to 8270 bp of accession number AF296764) was amplified from murine genomic DNA by PCR using the following primers: 5'NephPacI 5'CCTTAATTAAGGGGCGCGGGT3' and 3'NephXhoI 5'CCGCTCGAGATTCCTGAGCCTCTCT3'. The predicted initiation codon begins at bp 8296. A PacI and a XhoI site were added to the 5' and 3' ends during the PCR. This 4.125-kb fragment was isolated on a 1% agarose gel, gel-purified by using the Bio101 GeneClean kit (Qbiogene, Carlsbad, CA), and ligated to the PacI and XhoI sites of the pKO (knockout) vector (kind gift of J. Rossant, The Samuel Lunenfeld Research Institute, Toronto, Canada). The SalI fragment from the pSDK-lacZ cassette, which contains a Kozak consensus sequence and a cytoplasmic β -galactosidase gene (9) (Figure 1A) was ligated to the XhoI site of the pNeph-KO vector. The sequence for the SDK oligonucleotide is 5'GGAGGCCACCATGGCTCGC-GATATCGGTTCGAC3'. The final construct was digested with PacI and

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PmeI and the 9.8-kb fragment was injected into 1-cell mouse embryos as described elsewhere (10). Kidneys from postnatal day 0 mice were fixed in 4% paraformaldehyde and 1.5% glutaraldehyde and stained for β -galactosidase activity as previously described (11).

To make the pNeph-Cre transgene, a fragment containing the Cre recombinase cassette and beta-actin polyA signal was excised from the NLS-Cre plasmid (kind gift of Brian Sauer, Oklahoma Medical Research Foundation, Oklahoma City, OK) using *KpnI* and *SalI*. The *KpnI* site was blunted, and this fragment was ligated to the *XhoI* and *PmlI* sites of pNeph-pKO to generate the mNeph-Cre transgene (Figure 1B). Orientation of the construct was confirmed through sequence analysis. This construct was digested with *PacI* and *PmeI*, and an 8.4-kb fragment was gel purified (Bio101 GeneClean) and injected into 1-cell murine embryos as previously described (10).

The human Neph-Cre transgene was constructed by using the Neph-lacZ plasmid described elsewhere (5). The β -galactosidase cassette was excised by using *EcoRI*, and an *EcoRI* fragment containing the NLS-Cre cassette was digested from the NLS-Cre plasmid described above and ligated to the 1.125-kb human nephrin promoter. This transgene was linearized with *AflIII* and injected into 1-cell murine embryos as described above.

Genotypic Analysis of Transgenic Mice

Genomic DNA was isolated from tails of transgenic mice and used for genotypic analysis as described elsewhere (9). To detect the presence of the lacZ transgene in murine nephrin SDKlacZ mice, genomic DNA was digested with *EcoRI* and Southern blot analysis was performed using a probe for the β -galactosidase gene. The Cre transgene was detected by PCR using the following primers: Cre5' 5'ATGTCCAATTTACTGACCG3' and Cre3' 5'CGCCGCATAAC-CAGTGAAAC3', which amplified a band of approximately 300 bp (Figure 2A). Conditions for the Cre PCR were as follows: a hot start at 95°C for 1 min followed by 35 cycles, which included 30 s at 92°C, 30 s at 50°C, and 40 s at 72°C. Each reaction mix contained 50 μ l total volume with 1 μ l of purified genomic DNA, 10 mM each dNTP, 1 μ l of each primer (10 μ M concentration each), 5 μ l of 10X buffer #1 (Boehringer Mannheim Expand Long System; Boehringer Mannheim, Indianapolis, Indiana), and 1 μ l of Expand Long. To detect the GFP transgene, DNA was digested with *EcoRI* and Southern blot analysis was performed as described (9) using a 720-bp GFP probe (Figure 2B). To determine if the transgene had inserted in a single or multiple integration sites, the DNA was also digested with *XbaI* and Southern blot analysis was performed using a Cre recombinase probe.

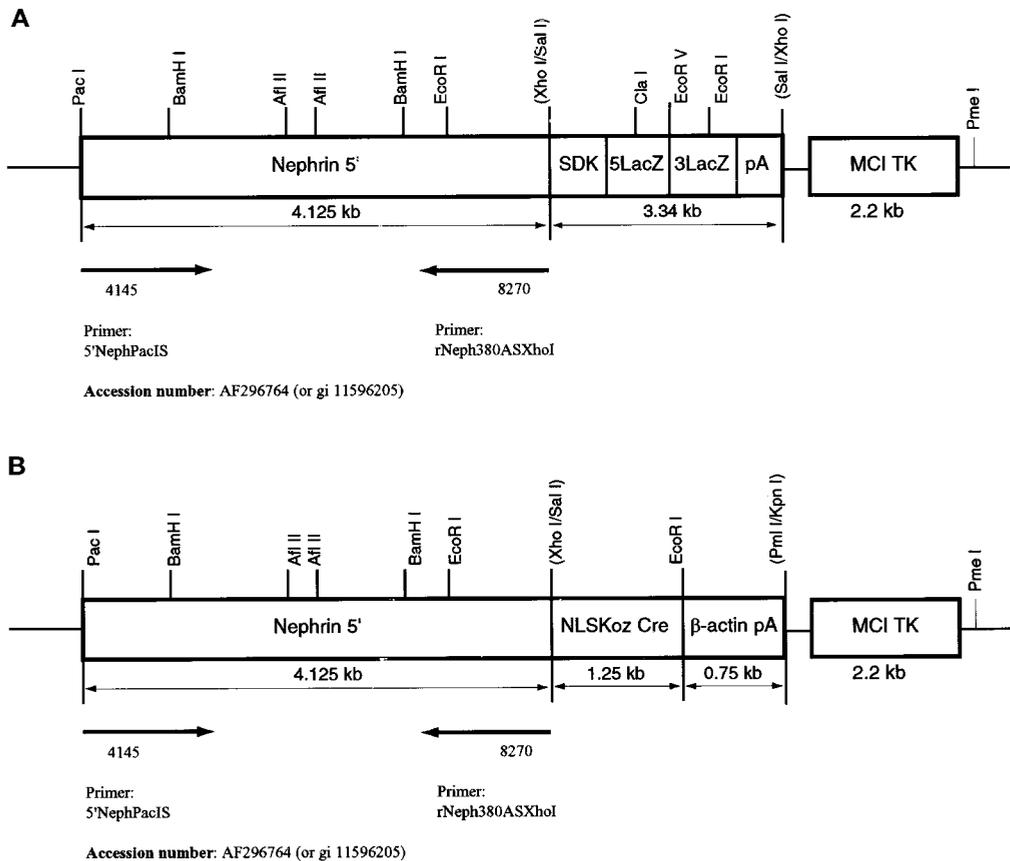


Figure 1. Transgenic constructs. 4.125-kb of the 5' flanking and proximal region of the murine NPHS1 gene excluding the predicted initiation codon was amplified, and *PacI* and *XhoI* sites were added to the 5' and 3' ends, respectively. This fragment was inserted into the pKO vector (not shown). (A) The SDK-lacZ reporter gene, which contains a Kozak consensus sequence and an initiation ATG, was inserted downstream of the nephrin promoter as a *SalI* fragment. (B) The NLS-Cre cassette was inserted as a *XhoI/KpnI* fragment into the NPHS1/pKO construct. SDK, Shine-Dalgarno sequence and Kozak consensus sequence; 5'LacZ, encodes the amino-terminal 146 amino acids of the β -galactosidase gene; 3'LacZ, encodes the carboxy-terminal region of the β -galactosidase gene; MCI TK, herpes simplex thymidine kinase gene; PA, polyadenylation signal.

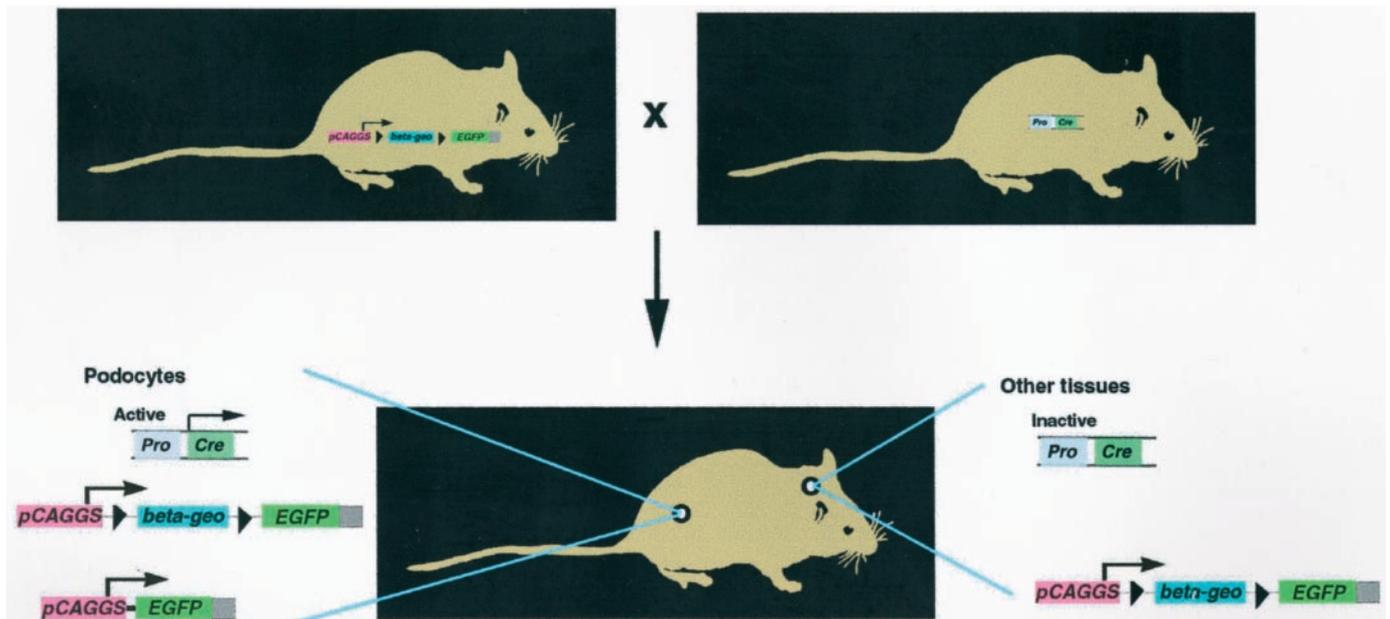


Figure 2. Scheme for detection of Cre-mediated excision *in vivo*. One of the nephrin-Cre founder lines, PC1, was crossed to the Z/EG reporter murine strain. *In vivo* excision of the floxed β -geo cassette allows transcription of the enhanced green fluorescent protein gene and should produce green fluorescent podocytes specifically. In other tissues, lack of Cre recombinase activity leaves the Z/EG transgene intact and the absence of fluorescence. Pro-Cre, Neph-Cre transgene; black arrowheads, loxP sites.

Cross of Nephrin-Cre Founder Lines with the Z/EG Reporter Mouse Line

All of the human neph-Cre and four of the murine neph-Cre transgenic founder lines (PC1-PC4) were selected to begin breeding with the Z/EG reporter murine line (kind gift of Dr. A. Nagy, The Samuel Lunenfeld Research Institute) (8). The Z/EG reporter transgene consists of the strong pCAGGS promoter directing expression of a loxP-flanked β -geo (lacZ/neomycin-resistance and STOP codon) fusion gene and three SV40 polyadenylation sequences. Site-specific recombination with Cre-recombinase between the loxP sites leads to expression of enhanced green fluorescent protein (EGFP) (Figure 3). Previously, we had demonstrated that the pCAGGS promoter expresses in podocytes (data not shown).

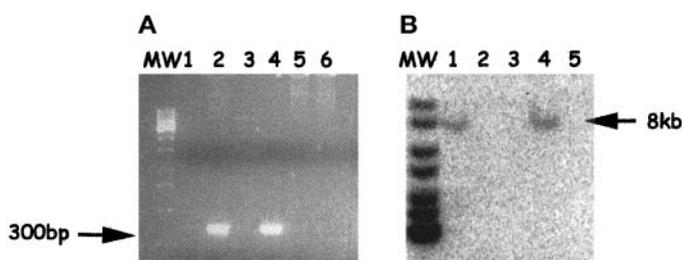


Figure 3. Genotypic analysis of the Neph-Cre PC1 X Z/EG offspring. (A) PCR analysis of Cre expression. Offspring 2 and 4 from the Neph-Cre founder line PC1 were positive for the Cre transgene. The PCR primers amplified a Cre fragment, which measures approximately 300 bp. (B) Southern blot analysis of green fluorescent protein (GFP) presence. Genomic DNA was digested with *Eco*RI and hybridized with a 720-bp GFP probe. Offspring 1 and 4 were positive for the GFP transgene. The *Eco*RI fragment measures approximately 8 kb.

Single litters (six pups each) were sacrificed at day 2 after birth (P2) from the four founder lines. All kidneys and tissues were visualized using FITC optics with a 488-nm filter to detect GFP fluorescence. In addition, 30- μ m cryosections of kidneys, pancreas, and brain were prepared as described (12) and stained with a polyclonal rabbit anti-GFP antibody (kind gift of J. Kahana, Dana-Farber Cancer Institute, Boston, MA) at a dilution of 1:1000 (0.4 mg/ml stock solution). The secondary antibody and detection system used was the Vectastain ABC rabbit IgG elite kit (PK 6101) (Vector Labs, Burlingame, New York). All animal experimentation was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Results

The 4.125-kb Murine Nephrin Promoter Directs Expression Specifically to Podocytes

Four Neph-SDK-lacZ founder lines were identified by Southern blot analysis. All of these founder lines expressed the β -galactosidase transgene in glomeruli (Figure 4A) and specifically in podocytes (data not shown). These results are similar to results by Moeller *et al.* (6), although we used a smaller genomic promoter fragment.

The 4.125-kb Nephrin-Cre Transgene Excises DNA Specifically in Podocytes

Offspring from the murine nephrin-Cre lines PC1–4 and Z/EG reporter strain were sacrificed, and their kidneys were observed under FITC optics with the 488-nm filter. One out of 6 F1 offspring from 3 of the 4 founder lines were doubly heterozygous for both transgenes (Figure 3). No bitransgenic offspring were identified from the fourth line. In founder lines,

PC1 and PC2, the bitransgenic kidneys demonstrated green fluorescent protein expression specifically in glomeruli (Figure 4B). Both of these lines had single chromosomal integration sites (data not shown).

Furthermore, cryosections demonstrated fluorescence consistent with podocyte-specific expression from the capillary loop stage of glomerulogenesis onwards (Figure 4C). To determine the number of glomeruli and podocytes that are fluorescing, we performed immunohistochemical analysis using an antibody that detects EGFP. These results demonstrated that all of the glomeruli undergo site-specific recombination. Similarly, within each glomerulus, the diffuse staining pattern demonstrated that the Cre recombinase was active in every podocyte (Figure 4D). Wholemout and cryosection analysis of other tissues demonstrated Cre activity in the rhombic lip but not in the pancreas of these bitransgenic mice, two tissues, which also express nephrin mRNA.

None (0 out of 12) of the human Neph-Cre founder lines

demonstrated Cre-mediated excision *in vivo*. To date, only 4 out of 10 murine founder lines have been crossed with the Z/EG reporter strain. Analysis of the remaining 6 lines is ongoing.

Discussion

Podocytes make up a major portion of the filtration barrier between the blood and urinary spaces (1). Elegant studies by Reeves *et al.* (13,14) and Abrahamson *et al.* (15–17) have provided fundamental knowledge about the unique structure and function of the podocyte and the glomerular filtration barrier. In addition, careful morphologic studies performed by Kriz *et al.* (18,19) suggest that podocytes play a pivotal role in the progression of glomerulosclerosis in a variety of renal diseases. More recently, the identification of a number of genes that cause inherited glomerular disease in humans and studies of knockout mice have provided a molecular foothold into podocyte biology (7,20–23).

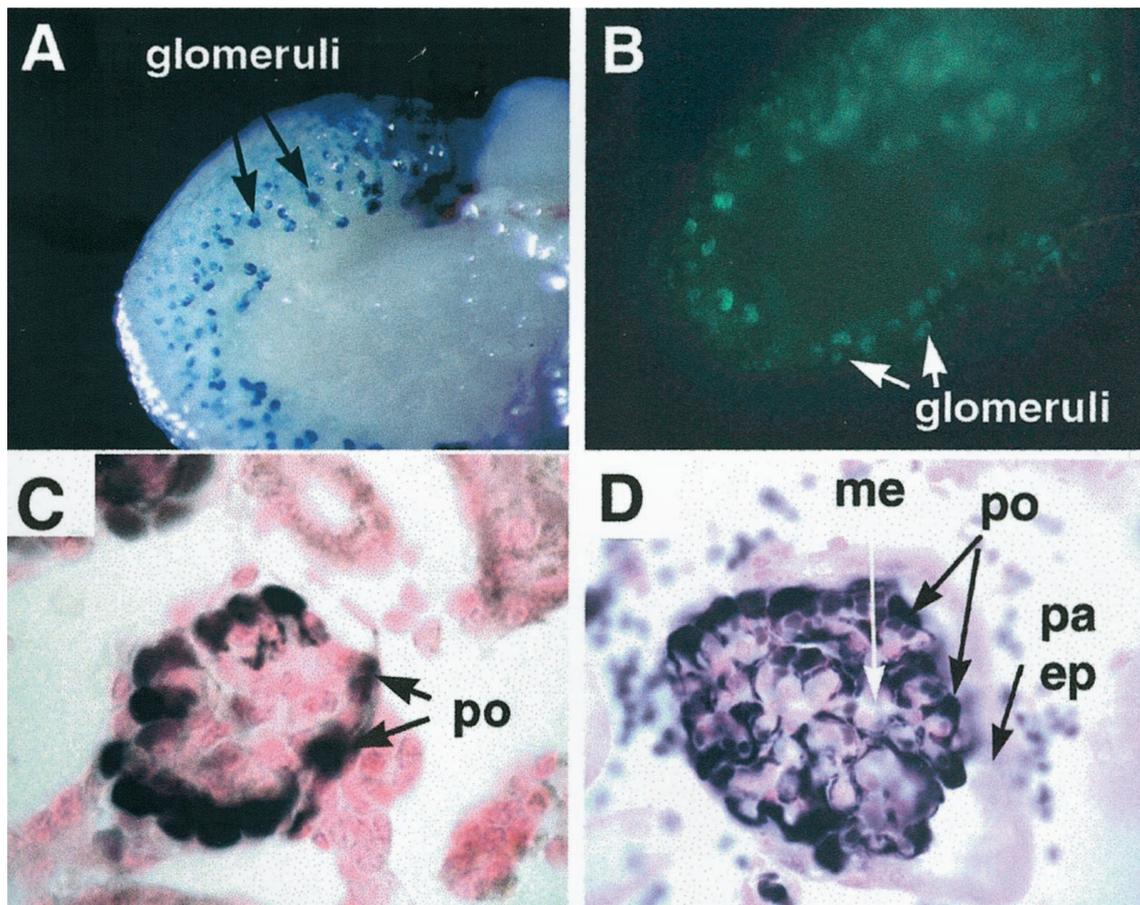


Figure 4. Transgenic expression under control of the 4.125-kb murine NPHS1 promoter. (A) The 4.125-kb murine NPHS1 promoter directs expression of the lacZ gene specifically to glomeruli in transgenic mouse kidneys. All founder lines (4 of 4) expressed the transgene in glomeruli. No ectopic expression was observed. (B) A wholemount photomicrograph of a mouse kidney at postnatal day 2 demonstrates GFP expression specifically in glomeruli. This kidney was taken from a mouse doubly heterozygous for the nephrin-Cre and pCaggs-lacZ-GFP transgenes. All fluorescence was detected using FITC optics and a 488-nm filter. GFP is expressed in all glomeruli and demonstrates that the nephrin-Cre recombinase is capable of excising DNA *in vivo*. (C) 30- μ m cryosections were immunostained with an antibody directed against EGFP and demonstrate that the nephrin-Cre recombinase is capable of excising DNA *in vivo* in podocytes in a late capillary-loop stage glomerulus. (D) Every podocyte stains in a mature glomerulus. In contrast, parietal epithelial cells, mesangial cells, and tubular epithelial cells do not express the Cre-recombinase or EGFP. po, podocyte; pa, parietal epithelial cell; me, mesangial cell, tu, tubular epithelial cell.

Despite these advances, the role of many genes, which are thought to be important for podocyte function, are still unknown. The ability to disrupt gene function in specific cell types using the Cre-loxP system has been invaluable in studying the role of a number of genes in the heart, germline, and central nervous system. Cre recombinase is a bacteriophage enzyme that causes site-specific recombination between loxP sites (24). We and others have identified the promoter region from the murine NPHS1 gene that is capable of driving the expression of transgenes specifically in podocytes from the capillary loop stage of development onwards. We used 4.125 kb of the murine NPHS1 promoter to drive expression of a nuclear-localized Cre recombinase specifically to podocytes in the kidney. In this article, we demonstrate that two of these founder lines are capable of excising DNA surrounded by loxP sites in podocytes *in vivo*. Although we have not bred this line to homozygosity, it excises DNA in every podocyte within the transgenic kidney. One of the four lines tested to date did not demonstrate excision *in vivo*, and a second line has not provided any bitransgenic offspring to study. It follows that transcriptional activity of the 4.125-kb promoter to drive adequate levels of Cre recombinase for *in vivo* excision is influenced by the chromosomal integration site. Furthermore, none of the human nephrin founders were capable of excising DNA *in vivo*, and this is likely the result of too low a level of transcriptional activity. The excising founder lines will be a useful resource to disrupt the expression of a variety of genes in the podocyte to unravel the biology of this fascinating cell type.

We also performed an analysis to determine activity of Cre recombinase in other tissues of the mouse and found fluorescence in the rhombic lip of the developing brain but no expression in the pancreas. In-depth molecular marker studies of the neural cells that express Cre-recombinase in these animals are in progress. Investigators who wish to use these murine lines to manipulate gene expression in the podocyte will also need to determine if their gene of interest is expressed in this small subset of specialized neurons.

Finally, the production of inducible versions of podocyte-specific Cre-recombinase murine lines will enable the temporal as well as spatial manipulation of genes and will be particularly useful in understanding the role of genes in acquired diseases of the podocyte.

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References

- Mundel P, Kriz W: Structure and function of podocytes: An update. *Anat Embryol (Berl)* 192: 385–397, 1995
- Abrass CK: The nature of chronic progressive nephropathy in aging rats. *Adv Ren Replace Ther* 7: 4–10, 2000
- Kriz W, Lemley K: The role of the podocyte in glomerulosclerosis. *Curr Opin Nephrol Hypertens* 8: 489–497, 1999
- Pagtalunan ME, Miller PL, Jumping-Eagle S, Nelson RG, Myers BD, Rennke HG, Coplon NS, Sun L, Meyer TW: Podocyte loss and progressive glomerular injury in type II diabetes. *J Clin Invest* 99: 342–348, 1997
- Wong MA, Cui S, Quaggin SE: Identification and characterization of a glomerular-specific promoter from the human nephrin gene. *Am J Physiol Renal Physiol* 279: F1027–F1032, 2000
- Moeller MJ, Kovari IA, Holzman LB: Evaluation of a new tool for exploring podocyte biology: Mouse Nphs1 5' flanking region drives LacZ expression in podocytes. *J Am Soc Nephrol* 11: 2306–2314, 2000
- Kestila M, Lenkkeri U, Mannikko M, Lamerdin J, McCready P, Putaala H, Ruotsalainen V, Morita T, Nissinen M, Herva R, Kashtan CE, Peltonen L, Holmberg C, Olsen A, Tryggvason K: Positionally cloned gene for a novel glomerular protein—nephrin—is mutated in congenital nephrotic syndrome. *Mol Cell* 1: 575–582, 1998
- Novak A, Guo C, Yang W, Nagy A, Lobe CG: Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis* 28: 147–155, 2000
- Puri MC, Rossant J, Alitalo K, Bernstein A, Partanen J: The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. *Embo J* 14: 5884–5891, 1995
- Joyner AL: *Gene Targeting: A Practical Approach*. New York, Oxford University Press, 2000
- Partanen J, Puri MC, Schwartz L, Fischer KD, Bernstein A, Rossant J: Cell autonomous functions of the receptor tyrosine kinase TIE in a late phase of angiogenic capillary growth and endothelial cell survival during murine development. *Development* 122: 3013–3021, 1996
- Quaggin SE, Schwartz L, Post M, Rossant J: The basic-helix-loop-helix protein Pod-1 is critically important for kidney and lung development. *Development* 126: 5771–5783, 1999
- Reeves W, Caulfield JP, Farquhar MG: Differentiation of epithelial foot processes and filtration slits: Sequential appearance of occluding junctions, epithelial polyanion, and slit membranes in developing glomeruli. *Lab Invest* 39: 90–100, 1978
- Reeves WH, Kanwar YS, Farquhar MG: Assembly of the glomerular filtration surface. Differentiation of anionic sites in glomerular capillaries of newborn rat kidney. *J Cell Biol* 85: 735–753, 1980
- Abrahamson DR: Glomerulogenesis in the developing kidney. *Semin Nephrol* 11: 375–389, 1991
- Robert B, Zhao X, Abrahamson DR: Coexpression of neuropilin-1, flk1, and VEGF(164) in developing and mature mouse kidney glomeruli. *Am J Physiol Renal Physiol* 279: F275–F282, 2000
- Robert B, St. John PL, Hyink DP, Abrahamson DR: Evidence that embryonic kidney cells expressing flk-1 are intrinsic, vasculogenic angioblasts. *Am J Physiol* 271: F744–F753, 1996
- Kriz W, Elger M, Nagata M, Kretzler M, Uiker S, Koepfenhagemann I, Tenschert S, Lemley KV: The role of podocytes in the development of glomerular sclerosis. *Kidney Int* 45: S64–S72, 1994

19. Kriz W, Kretzler M, Nagata M, Provoost AP, Shirato I, Uiker S, Sakai T, Lemley KV: A frequent pathway to glomerulosclerosis: deterioration of tuft architecture-podocyte damage-segmental sclerosis. *Kidney Blood Press Res* 19: 245–253, 1996
20. Kaplan JM, Kim SH, North KN, Rennke H, Correia LA, Tong HQ, Mathis BJ, Rodriguez-Perez JC, Allen PG, Beggs AH, Pollak MR: Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat Genet* 24: 251–256, 2000
21. Boute N, Gribouval O, Roselli S, Benessy F, Lee H, Fuchshuber A, Dahan K, Gubler MC, Niaudet P, Antignac C: NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat Genet* 24: 349–354, 2000
22. Shih NY, Li J, Karpitskii V, Nguyen A, Dustin ML, Kanagawa O, Miner JH, Shaw AS: Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science* 286: 312–315, 1999
23. Noakes PG, Miner JH, Gautam M, Cunningham JM, Sanes JR, Merlie JP: The renal glomerulus of mice lacking s-laminin/laminin beta 2: Nephrosis despite molecular compensation by laminin beta 1. *Nat Genet* 10: 400–406, 1995
24. Rossant J, Nagy A: Genome engineering: The new mouse genetics. *Nat Med* 1: 592–594, 1995