Podocyte Cell–Specific Expression of Doxycycline Inducible Cre Recombinase in Mice

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Conventional silencing of many podocyte-specific genes in mice is associated with embryonic or perinatal lethality. Therefore, it would be of great importance to generate mouse models that allow the modification of genes that are expressed in podocytes at later stages of age. Herein is described a transgenic mouse with doxycycline-inducible podocyte-specific expression of Cre recombinase. For the generation of this binary system, a single transgenic construct that contained two separate genes was used: One encoding the optimized M2 version of the doxycycline-dependent transcription transactivator reverse tetracycline-controlled transcriptional activator (rtTA) under control of the human podocin (NPHS2) promoter and the other encoding the recombinase Cre under control of the rtTA/doxycycline-responsive minimal cytomegalovirus (CMV) Tet operator sequence 7 promotor. Microinjection of the JRC-CRE construct in fertilized oocytes from FVB/N mice resulted in 16 transgenic founders. Double-transgenic offspring from breeding of a selected founder with the Z/AP reporter mouse showed alkaline phosphatase staining only upon doxycycline administration and exclusively in podocytes. These data indicate that this new inducible Cre recombinase mouse line is an excellent tool in conditional, kidney glomerular podocyte-specific gene deletion in adult mice.

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The kidney glomerulus produces the primary urine. The essential structural element that is responsible for this function, the glomerular filtration barrier, consists of a fenestrated capillary endothelium, a layered glomerular basement membrane (GBM), and glomerular visceral epithelial cells called podocytes. Podocytes cover the GBM from the outside and present with highly ordered cellular extensions, foot processes, linked to each other by the slit diaphragm (1). This structure seems to be crucial in regulating the passage of circulating plasma proteins into primary urine. Glomerular diseases with proteinuria manifest with characteristic morphologic changes of the podocytes, including loss of the slit diaphragm and foot process effacement. The degree of these changes is associated with the severity of proteinuria (2). Children with congenital nephrotic syndrome of the Finnish type (CNF) have severely impaired podocyte function and, before current transplantation therapy, used to die within a few months after birth as a result of massive proteinuria. After a meticulous search for the responsible genetic defect in CNF, a nonfunctional NPHS1 gene was found (3). NPHS1 encodes the slit diaphragm–specific protein nephrin. Consistent with the human CNF disease phenotype, nephrin knockout (KO) mice die within a few days after birth, and the podocytes show closely similar morphologic abnormalities as the glomeruli of patients with CNF (4,5). Furthermore, other distinct single-gene mutations of genes that are expressed preferentially in the podocytes are associated with severe glomerular phenotypes in humans and mice. These include Wilms tumor gene (WT-1) (6), podocin (7,8), α-actinin-4 (9,10), and CD2AP (11). Conventional gene KO are not always useful tools to study podocyte-specific genes because deficiency of these genes results in perinatal death, thus preventing their detailed analysis within the fully functional kidney of the adult mouse.

To circumvent this, a conditional KO strategy was designed on the basis of the use of transgenic mice with doxycycline-inducible podocyte-specific Cre recombinase expression. The binary tetracycline-controlled transcriptional activation system is a powerful tool in achieving temporal control of transgene expression in mammals (12). For this purpose, two different tetracycline-controlled activators are widely used in transgenic mice. The original transcriptional activator (tTA) activates transcription of the target gene in the absence of tetracycline, after binding to the Tet operator sequence located in the 5′ region of the target gene (Tet-off system) (13). The reverse tetracycline-controlled transcriptional activator (rtTA) binds to Tet operator sequence 7 and activates transcription of the target gene in the presence of tetracycline (Tet-on system) (12). Recently, improved rtTA versions that require far lower tetracycline concentration for the induction together with minimal background expression of Cre recombinase in the absence of tetracycline were developed (14).

Here we present a podocyte-specific doxycycline-inducible Cre transgenic mouse generated by a single oocyte injection step based on a modified approach described by Utomo *et al.* (15). Within one transgene construct, all required DNA elements were combined: The Cre recombinase gene under control of the rtTA-M2 (14) transcription factor together with the

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rtTA-M2 encoding gene under control of a 2.5-kb fragment of the mouse NPHS2 (podocin) promoter. The use of this single-transgene construct obviates the need for multiple rounds of transgenesis and elaborate crossings. This mouse will enable studies of the phenotypic consequences of loss of defined proteins in the podocytes in the adult mouse by direct crossing with mice with appropriate floxed alleles.

Materials and Methods

Cloning of Doxycycline-Inducible Cre Recombinase Construct

The p2.5PodocinpnlacF plasmid, which contains 2.5 kb of genomic sequence of human NPHS2 gene located 5′ to the translation initiation codon, was provided by Dr. Lawrence Holzman (16,17). The "core" construct was provided by W.H. Lee (15). An EcoRI-HindIII fragment from the original rtTA of the core construct was replaced with the optimized version, rtTA-M2 (14). In our hands, several attempts to clone promoters into the designated cloning site failed as a result of instability of the construct. Therefore, the two functional EcoRI-ScaI fragments from the core construct were reconstituted in a pBR322-based, low-copy backbone after modification of the polylinker, generating NotI restriction sites instead of *ScaI* on both sides of the transgene. Finally, unique SalI and *SmiI* sites were introduced at the position of the EcoRI site. The resulting construct was stable and designated RRC-M2 (Figure 1).

The unique XbaI site in p2.5PodocinpnlacF was replaced by a SalI using a linker (sense 5′-ctagcagatctaagcagtcgaca-3′ and antisense 5′-ctagtgtcgactgcttagatctg-3′). The resulting SalI-NcoI fragment of the podocin promoter was cloned into the SalI-SmiI–digested RRC-M2 construct as a SalI-blunt fragment. The NcoI end was blunted using T4 polymerase (New England Biolabs, Ipswich, MA). Identity of the clones was verified by restriction mapping and sequencing. The final construct is referred to as JRC-CRE (Figure 1).

Generation and Identification of JRC-CRE Transgenic Mice

The podocin-rtTA construct was released from the plasmid vector backbone by digestion with NotI and purified by gel electrophoresis, and DNA was extracted using a QIAEX II Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). The purified construct DNA was introduced into the pronuclei of fertilized oocytes from the FVB/N mouse by microinjection using standard techniques. All experiments had approval of the local committee for laboratory animal welfare of the University of Helsinki.

Transgenic mice were identified by PCR on tail genomic DNA (50 to 100 ng), using CreForward (5′-gaccaggttcgttcactca-3′) and CreReverse (5′-tagcgccgtaaatcaat-3′) primers and 30 cycles of 95°C (30 s), 54°C (30 s), and 72°C (30 s) and a final extension of 72°C for 5 min. The reaction was performed with HotStar Taq DNase polymerase (Qiagen) in a total reaction volume of 20 μl, including 400 nM of both primers. Samples were analyzed by standard agarose gel electrophoresis (1.5% gel).

Phenotype characterization and behavioral screening was made using modification of the Irwin procedure (18). Observed parameters are listed in Table 1.

Doxycycline Administration in JRC-CRE Mice

F1 littermates were used to analyze Cre recombinase protein expression in mouse tissues. Doxycycline was administered in drinking water (0.2 mg/ml in 5% sucrose), and administration was started at the age of 8 to 10 wk. Water was changed twice a week, and the bottles were covered with aluminum foil to prevent degradation by light.

Reverse Transcription–PCR of Cre-Specific mRNA of JRC-CRE Mouse Kidneys

After cervical dislocation, mouse tissues were dissected immediately, snap-frozen in liquid nitrogen, and stored at −78°C until used. Total RNA was extracted from homogenized lysates of the cortex of frozen adult kidneys using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.

cDNA was prepared from 1 μg of RNA with the M-MLV reverse transcriptase (Promega, Madison, WI) using random primers (Roche Diagnostics GmbH, Mannheim, Germany; RT+). To confirm the RNA origin of the PCR signals, we also analyzed each sample without the reverse transcriptase reaction (RT−). Cre-specific cDNA was amplified by using the Cre thermocycling protocol (25 cycles; see above), and NPHS1 primers

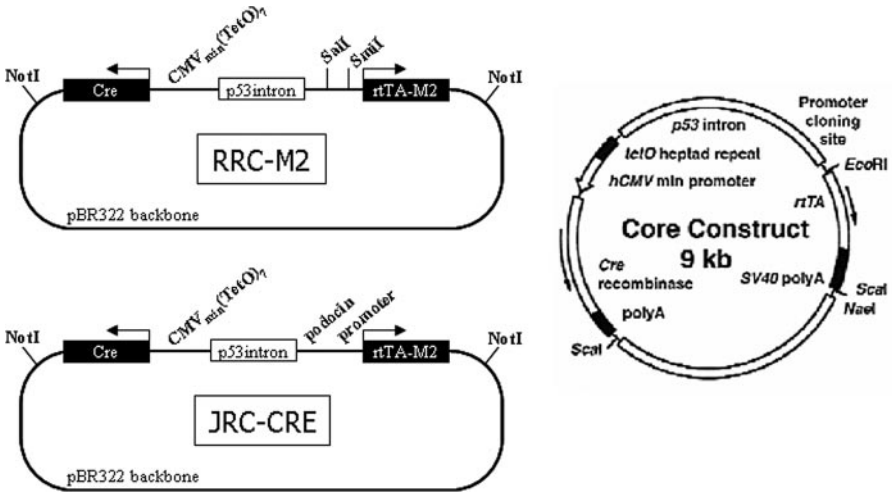

Figure 1. In a binary reverse doxycycline-regulated vector, an NPHS2 (podocin) promoter drives the expression of the recombinant inducible transcription factor reverse tetracycline-controlled transcriptional activator (rtTA; M2). In the presence of doxycycline, rtTA binds to the Tet operator sequence element linked to a minimal cytomegalovirus (CMV) promoter and drives the expression of Cre recombinase.

Table 1. Phenotype characterization of JRC-CRE mice[a]

Observed Parameters	Scaling	WT ($n = 6$)	JRC-CRE ($n = 6$)
Hair	Normal (0) or unfit (1)	0	0
Piloerection	None (0) or marked (1)	0	0
Body tone	Completely flaccid (0) to extreme resistance (2)	1	1
Spontaneous activity	None (0), normal (1), or repeated vigorous movement (2)	1	1
Provoked biting response	Absent (0) or present (1)	1	1
Tail elevated	Flattened (0), normal (1), or elevated (2)	1	1
Respiration	Gasping, irregular (0), normal (1), or hyperventilation (2)	1	1
Body weight	Weight increase after 2 wk of doxycycline administration	2.03 g	2.06 g

[a]WT, wild type.

were used as housekeeping gene controls: NPHS1Forward 5′-cctggagctaccctgcata-3 and NPHS1Reverse 5′-ggacttggtaaggcagcaaa-3′.

Generation of Double-Transgenic JRC-CRE × Z/AP Reporter Line

Generation of double-transgenic JRC-CRE × Z/AP reporter line JRC-CRE transgenic founder was bred with Z/AP (lacZ/human placental alkaline phosphatase) reporter mice (19) to obtain double-transgenic JRC-CRE × Z/AP reporter mice. The Z/AP reporter mouse line carries a β-geo cassette flanked by two loxP sites and an alkaline phosphatase (AP) gene (19). AP cannot be expressed in this transgene unless the β-geo cassette is excised. Thus, these mice express AP after Cre-mediated recombination. Double-transgenic mice were identified by PCR on tail genomic DNA for Z/AP using Z/APForward (5′-ctgctaaccatgttcatgcc-3′) and Z/APReverse (5′-ggcctcttcgctattacg-3′) primers and Cre primers (see above), using the same amplification protocol. Offspring that carried both transgenes (JRC-Cre × Z/AP) were administered 0.2 mg/ml doxycycline in the drinking water for 14 consecutive days.

AP Substrate Staining in JRC-CRE × Z/AP Mouse Kidney

For testing the presence of AP, the JRC-CRE × Z/AP and wild-type control mice either received ($n = 3$) or did not receive ($n = 3$) doxycycline, and tissues were collected as described above. The list of the tissues that were tested is provided in Table 2.

Before staining, slides were refixed in cold PBS that contained 0.2% glutaraldehyde for 10 min. After fixation, slides were washed in PBS for 5 min and endogenous AP was inactivated by incubating slides in PBS at 75°C for 30 min. Slides then were rinsed with PBS, washed in AP buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, and 10 mM $MgCl_2$) for 5 min, followed by incubation with BM purple (Roche) for 1 h at 4°C to detect AP activity. Gurr Aquamount (BDH, Poole, UK) was used for mounting.

Immunofluorescence Staining in Mouse Tissues

Immunofluorescence staining was performed on frozen tissue sections (6 μm). The sections were fixed in acetone (−20°C) for 10 min on ice, washed with PBS, and blocked with CAS-block solution (Zymed Laboratories Inc., San Francisco, CA) for 10 min. Sections were incubated overnight with the primary antibody in ChemMate Antibody Diluent (DakoCytomation, Glostrup, Denmark). The used primary antibodies were anti-CRE polyclonal antibody (Stratagene, La Jolla, CA; 1:1000), anti-human placental AP polyclonal and monoclonal antibodies (Biomeda, Foster City, CA; 1:50), anti–WT-1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:50), and anti-synaptopodin mAb (Progen Biotechnik GmbH, Heidelberg, Germany; 1:200). After washing, the sections were incubated with affinity-purified TRITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA; 1:200) and/or affinity-purified FITC-conjugated rabbit anti-mouse IgG (DakoCytomation; 1:200) in ChemMate Antibody Diluent for 30 min and mounted with Vectashield Mounting Medium for fluorescence (Vector Laboratories Inc., Burlingame, CA).

Table 2. List of tissues of JRC-CRE × Z/AP mice tested by AP substrate staining[a]

Tissue	Dox−	Dox+
Kidney	Neg	Pos
Heart	Neg	Neg
Liver	Neg	Neg
Pancreas	Neg	Neg
Lung	Neg	Neg
GI tract, small intestine	Neg	Neg
GI tract, large intestine	Neg	Neg
Testis	Neg	Neg
Ovary	Neg	Neg
Muscle tissue	Neg	Neg
Skin	Neg	Neg
Mesenteric lymph node	Neg	Neg
Spleen	Neg	Neg
Blood	Neg	Neg
CNS, cortex	Neg	Neg
CNS, cerebellum	Neg	Neg
CNS, olfactory bulb	Neg	Neg

[a]AP, alkaline phosphatase; CNS, central nervous system; GI, gastrointestinal.

Results

JRC-CRE Mice

Before pronuclear injection, the transgene was tested *in vitro* in A293 cell culture, and Cre recombinase expression was localized within the nuclei and was tightly regulated by doxycycline administration (data provided as supplemental data; Supplemental Figures 1 and 2). Sixteen of 122 offspring were identified to carry the transgene after oocyte injections. Founders were bred with C57Bl/6 wild-type mice. All founders transmitted the transgene to their offspring. All founders and

their F1 offspring were fertile, and the phenotype was normal by gross morphology and by light microscopy of the kidneys (data not shown). After a 2-wk administration period of doxycycline, the phenotype of JRC-CRE litters remained normal, and no difference in the body weight of the mice compared with wild-type controls was observed (Table 1). The behavioral observations were a modification of the Irwin procedure (18).

Cre recombinase expression was tested in tissues from all founder lines by using AP substrate staining (Table 2). Three of 16 founder lines showed Cre recombinase expression in podocytes also without doxycycline administration. Two of 16 founder lines expressed Cre recombinase also in heart tissue after 2 wk of doxycycline administration. For the further experiments, one founder line was selected on the basis of the high Cre recombinase expression level in podocytes and lack of leakiness without doxycycline and in other tissues in preliminary experiments (data not shown).

Doxycycline-Inducible Expression of Cre Recombinase mRNA

Cre recombinase mRNA expression was studied in the kidneys of JRC-CRE–positive mice with or without doxycycline administration by reverse transcription–PCR. Doxycycline administration was started at the age of 8 to 10 wk. Expression of Cre recombinase mRNA was observed only after 2 wk of doxycycline treatment (Figure 2).

Expression of Cre Recombinase Protein in Adult Mice

To determine the spatial localization of Cre recombinase protein expression in kidney, we examined renal tissues by Cre antibody immunofluorescence staining of cryosections in JRC-CRE mice. Expression of Cre recombinase was prominent in the periphery of glomeruli, in localization consistent with podocytes (Figure 3, D and F). Cre recombinase protein expression was absent in wild-type mice (Figure 3, A and B) and in JRC-CRE–positive mice that were not treated with doxycycline (Figure 3, C and E). Cre recombinase protein expression was not found in other observed tissues (heart, liver, and pancreas) in JRC-CRE mice with or without doxycycline administration (Figure 4).

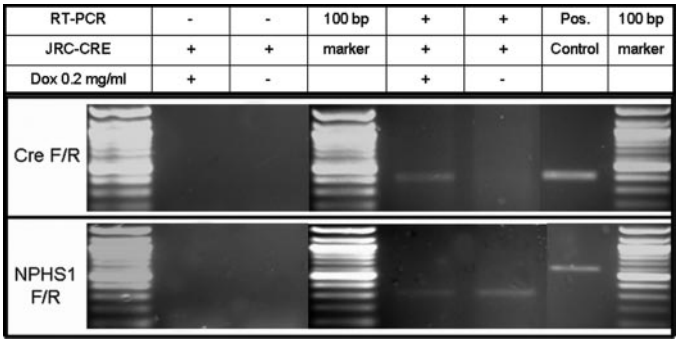

Figure 2. Reverse transcription–PCR of Cre-specific mRNA of JRC-CRE mouse kidneys. RNA was isolated after 2 wk of doxycycline (0.2 mg/ml) administration from JRC-CRE–positive mouse kidneys. Genomic DNA that was isolated from JRC-CRE mouse tails was used as positive control. Expected fragment size for Cre recombinase genomic and cDNA was 420 bp for both. For NPHS1, genomic DNA fragment size was 570 bp and for cDNA was 317 bp.

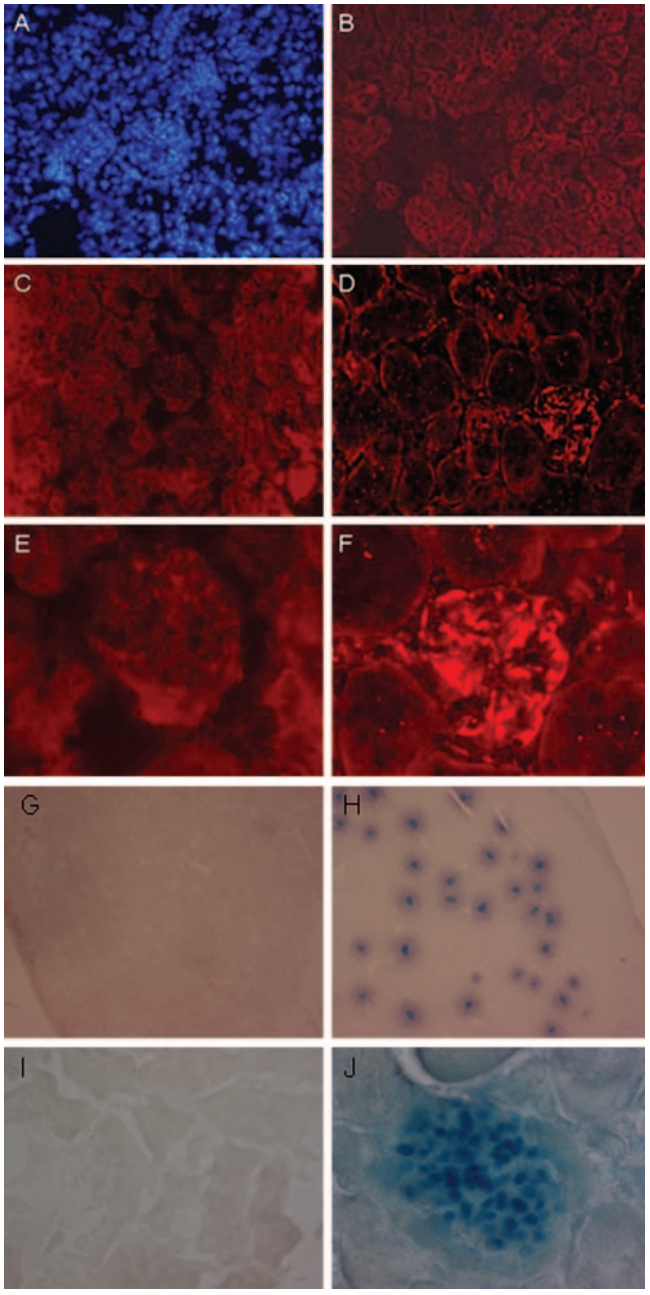

Figure 3. Immunofluorescence staining in JRC-CRE mouse kidneys. (A) Nuclear staining by DAPI in wild-type control mouse. Cre recombinase protein expression in wild-type control mouse (B), JRC-CRE mouse without doxycycline treatment (C), doxycycline-treated JRC-CRE mouse (D), JRC-CRE mouse without doxycycline treatment (E), and doxycycline-treated JRC-CRE mouse (F). (G through J) Alkaline phosphatase substrate staining in JRC-CRE × Z/AP without doxycycline treatment (G and I) and in doxycycline-treated JRC-CRE × Z/AP mouse kidneys (H and J). Magnification, ×200 in A to D; ×40 in G and H; and ×600 in E, F, I, and J.

Functional Cre Recombinase Activity

To test Cre recombinase functionality (capability to catalyze recombination between two loxP sites), we performed cross-

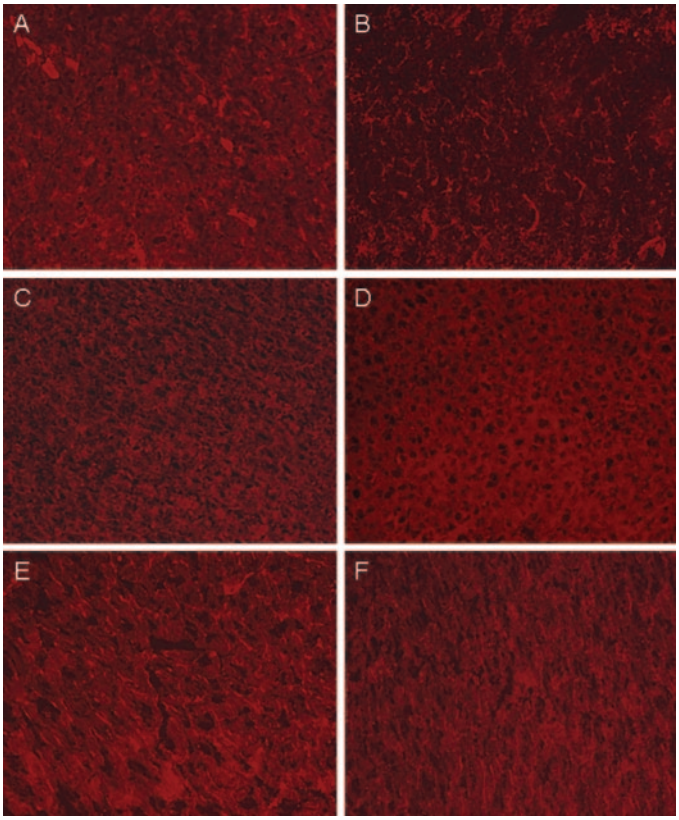

Figure 4. Immunofluorescence staining in JRC-CRE mouse tissues. Cre recombinase protein expression in pancreas without doxycycline treatment (A), pancreas after doxycycline (B), liver without doxycycline treatment (C), liver after doxycycline treatment (D), heart without doxycycline treatment (E), and heart after doxycycline treatment (F). Magnification, ×200.

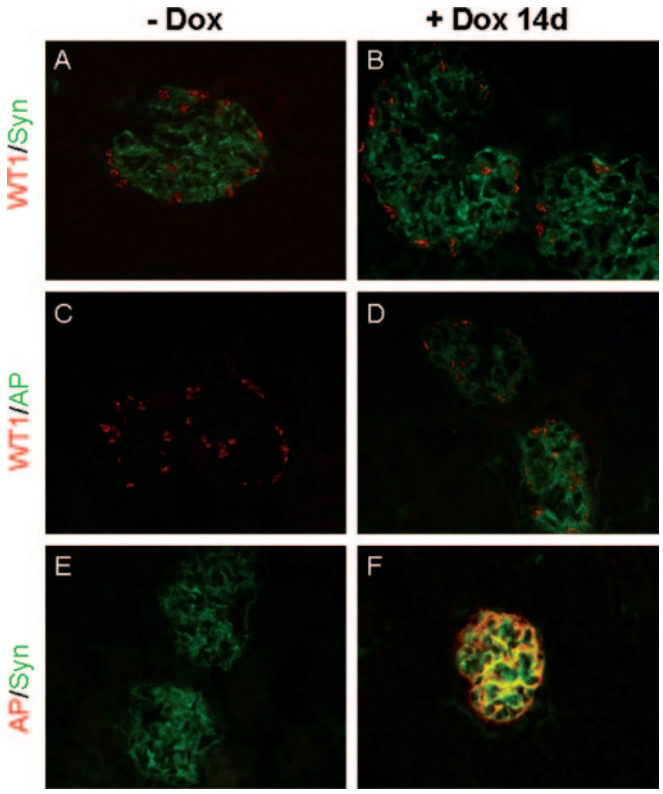

Figure 5. Immunofluorescence stainings in JRC-CRE × Z/AP mouse kidneys. (A, C, and E) Double stainings in mice without doxycycline treatment. (B, D, and F) Double stainings in mice after doxycycline (0.2 mg/ml) treatment. Magnification, ×400.

breeding with Z/AP reporter mouse line. Kidney sections from double-transgenic mice with or without 2 wk of doxycycline administration were analyzed by AP substrate staining. AP expression was found in the podocytes only after the doxycycline treatment (Figure 3, G through J). Wild-type control specimen did not show any staining. Other tissues of the double transgenic mice that were studied did not show any staining measured by AP substrate (Table 2; Supplemental Figure 3) or immunofluorescence assay (Supplemental Figure 4).

Localization of AP Protein in the Glomeruli

To determine the localization of human placental AP protein expression, we examined the kidney tissues with several antibodies and immunofluorescence microscopy in JRC-CRE × Z/AP mice that were treated with doxycycline. Staining of AP co-localized with synaptopodin protein but not with WT-1 staining (Figure 5). A total of 100 glomeruli from several sections were counted. All glomeruli that were positive for synaptopodin also showed high expression of AP in doxycycline-treated double-transgenic mice. AP protein expression was not observed in double-transgenic mice without doxycycline (Figure 5) or in wild-type controls (data not shown). Nevertheless, it cannot be claimed that all podocytes express Cre recombinase upon induction, but our data suggest that the percentage is very substantial.

Discussion

Here we describe a transgenic mouse line with tightly regulated inducible expression of the Cre recombinase exclusively in the kidney podocytes in the adult mouse. This viable and fertile transgenic mouse line was generated by injection of oocytes with a single DNA construct that contained a podocyte-specific promoter (NPHS2) that drives the expression of rtTA M2 and a Cre recombinase gene whose expression is initiated by rtTA in the presence of doxycycline.

There are several advantages to using a single inducible transgene vector that confers site-specific expression. First, the cell type specificity can be selected easily by a single cloning step that exchanges the one desired cell type–specific promoter for the other. Moreover, any gene of interest can be made inducible and cell type specific by putting it in the place of the Cre recombinase gene. Second, the single construct gives superior speed and economy, because no multiple rounds of oocyte injections and further breeding steps are needed. Third, the transgene expression can be turned off as desired, giving invaluable targeting, *e.g.*, to a particular stage of embryonic development or in adult life, thus overcoming the limitations of embryonic lethality or redundancy.

Because of perinatal lethality, conventional KO mouse mod-

els often fail to reveal the detailed biologic function and dynamics of specific proteins within the adult, fully functional kidney. In case of nephrin, the structure–function relationship is relatively well described, whereas several key questions concerning gene regulation *in vivo* still remain unresolved. Site-specific recombinases (*e.g.*, Cre-loxP system) are excellent tools to establish tissue-specific gene deletions in the adult mouse. The cell type specificity depends on the expression characteristics of the promoters that drive the expression of the recombinase gene. Recently, two podocyte-specific Cre recombinase mouse lines were generated. In the first line, Cre recombinase expression is under the control of the NPHS1 (nephrin) promoter (20) and in the other line under NPHS2 promoter (17). Moeller *et al.* (21) used the Cre-loxP system to tag podocytes in a murine model of crescentic glomerulonephritis and was able to show that cells within the glomerular crescents are derived from the podocyte cell lineage. One drawback with these two models is that Cre recombinase is already expressed at early developmental stages of the embryo. Subsequently, the phenotype after gene silencing during embryogenesis is often lethal.

Thus, inducible cell type–specific Cre recombinase mouse lines will be needed to silence genes that are expressed in podocytes in the adult animal. Inducible gene deletion has been used for many mouse tissues, including the liver (22,23), heart (24,25), and, *e.g.*, for molecules such as prostaglandin receptors (26). Previously, inducible Cre recombinase mouse lines have been generated, particularly one specific for the kidney collecting ducts cells (27). Bugeon *et al.* (28) developed a mouse line in which a transgene encoding the mutated estrogen receptor–Cre recombinase fusion protein was introduced into the mouse genome. Animals were crossed with Z/AP reporter mice, and after injection of the inducer drug tamoxifen, Cre fusion protein translocates to the nuclei of podocytes, where it becomes active and mediates recombination of DNA carrying loxP target sequences. These animals provide for the first time a tool for silencing genes in podocytes of adult animals, but complete tissue-specific activity was not achieved (28). Furthermore, doxycycline-controlled podocin-specific β-galactosidase expression has been reported under control of NPHS2 promoter (29). Still, no kidney-specific inducible gene deletion has been published.

Conclusion

This novel doxycycline-inducible Cre recombinase mouse line will be useful for conditional deletion of essential podocyte proteins and study of their functions in detail in adult mice. This is important for further diagnostic and pharmacologic developmental platforms. Furthermore, the binary construct can serve as an invaluable tool to overexpress particular podocyte-specific proteins and to tightly control inducible gene deletion in other tissues by changing cell type–specific promoters, as a concept already shown by Utomo *et al.* (15) with the original construct.

Acknowledgments

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