Inducible Podocyte-Specific Gene Expression in Transgenic Mice

TETSUYA SHIGEHARA,* CONCEPCION ZARAGOZA,* CHAGRIYA KITIYAKARA,*
HIDEKO TAKAHASHI,* HUIYAN LU,* MARCUS MOELLER,[†]
LAWRENCE B. HOLZMAN,[†] and JEFFREY B. KOPP*

*Kidney Disease Section, Metabolic Diseases Branch, National Institutes of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland; and [†]Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan.

Abstract. The podocyte plays a key role in glomerular function and glomerular disease. To facilitate studies of podocyte function, we have developed a transgenic mouse model with inducible expression in the podocyte. The tetracycline-inducible transgenic system facilitates gene expression with restricted cellular distribution and tight temporal control. Recently, Bujard and colleagues have developed a functionally improved reverse tetracycline–controlled transcriptional activator (rtTA) with substantially lower background in the off state (the absence of tetracycline) and greater inducibility in the on state (the presence of tetracycline). We used the human podocin (*NPHS2*) gene promoter to control expression of the rtTA cassette and bred these mice with a reporter mouse line that contains the cytomegalovirus minimal promoter and *tetO* promoter elements together with *LacZ*, encoding β-galactosidase. Dual transgenic mice, bearing both podocin-rtTA and *tetO-LacZ* transgenes, had no detectable expression in kidney or other organs in the absence of tetracycline. Administration of tetracycline in the drinking water was associated with podocyte expression of β-galactosidase, in a fashion that was time dependent (maximal at 1 wk) and dose-dependent (maximal at 2 mg/ml). Podocyte expression was confirmed in two ways: histochemical staining for β-galactosidase and double-immunostaining using the podocyte marker WT-1 and β-galactosidase. This transgenic system should aid future investigations of podocyte function.

Glomerular visceral epithelial cells (podocytes) are highly differentiated post-mitotic cells whose function, notably their contribution to the glomerular filtration barrier, is largely based on their complex cytoarchitecture (1,2). Podocytes have interdigitating foot processes surrounding the glomerular capillary wall. They also possess a specialized intercellular junction between those foot processes, the slit diaphragm, which plays a major role in determining the permselectivity of the glomerular filtration barrier. Glomerular diseases associated with proteinuria typically are associated with podocyte injury, manifesting as loss of the slit diaphragm, foot process effacement, and loss of permselectivity (3). Other evidence of podocyte injury includes detachment from the glomerular basement membrane and apoptosis, leading to decreased podocyte number and ultimately focal segmental glomerulosclerosis (4,5). An alternative pathway of podocyte injury involves podocyte proliferation, dedifferentiation, and ultimately collapsing glomerulopathy (6). Further evidence of the importance of podocytes in glomerular disease is the recent discovery that various podocyte gene mutations are associated with glomerular disease. These include the genes encoding Wilms tumor gene (WT-1), nephrin, podocin, and α-actinin-4 (reviewed in (7)).

Studies of podocyte function *in vivo* would be aided by the ability to up-regulate or down-regulate gene function in a cell-specific manner. Recently, specific podocyte gene expression has been demonstrated using promoter elements derived from the mouse nephrin (*NPHS1*) gene and the human podocin (*NPHS2*) gene (8,9).

The tetracycline-controlled transcriptional activation system is a powerful tool in achieving temporal control of transgene expression in mammalian systems (10). These systems are based on regulatory elements derived from the *Escherichia coli* tetracycline-resistance operon. The original tetracycline-controlled transcriptional activator (tTA) is a chimeric protein composed of the Tet repressor protein and the VP16 transcriptional activation domain. The tTA activates transcription of the target gene in the absence of tetracycline, after binding to the Tet operator sequence (*tetO*) located in the 5' region of particular target gene (Tet-off system). On the other hand, the reverse tetracycline-controlled transcriptional activator (rtTA), composed of a mutant tetracycline repressor protein and VP16, binds to *tetO* and activates transcription in the presence of tetracycline (Tet-on system, Figure 1). Recently, new rtTA versions have been developed that are more stable in eukaryotic cells, function at a lower doxycycline concentration, and cause less background expression in the absence of tetracycline (11).
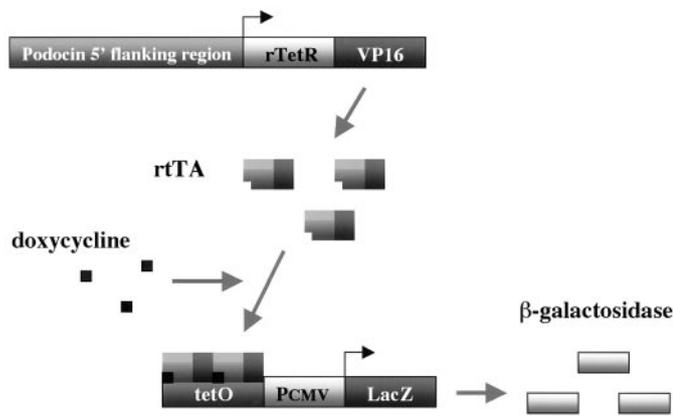
Figure 1. Reverse tetracycline-controlled transcriptional activator (rtTA) system (Tet-On system). A 2.5-kb fragment of the *NPHS2* promoter-enhancer region directs the expression of rtTA in podocytes. In the presence of tetracycline or derivative such as doxycycline, rtTA binds to tetracycline-response operon promoter element (*tetO*) and initiates transcription from the cytomegalovirus (CMV) promoter (PCMV) of the *LacZ* gene, encoding the β-galactosidase reporter gene in.

Tetracycline-controlled systems have been used to control transgene expression in mice (12–16), as reviewed recently (17). Inducible transgenic expression has several advantages over constitutive expression. First, transgene expression can be suppressed during embryogenesis to facilitate normal development, or can be activated during embryogenesis to probe developmental processes. Second, transgene expression can be set at different levels depending on tetracycline dose or can be activated for defined periods of time to allow for dose–response studies where the independent variable is either protein level or expression duration. Third, transgene expression can be coordinated with other interventions designed to induce or ameliorate renal disease. In the study presented here, we describe a tetracycline-controlled transgenic mouse model using the functionally improved rtTA under the control of the *NPHS2* promoter.

Materials and Methods
Generation of Transgenic Mice

The p2.5PodocinPE plasmid containing 2.5 kb of genomic sequence located 5' to the translation initiation codon of human *NPHS2* gene has been described previously (9). The construct, pUHrT62–1, containing the rtTA gene that is modified with humanized codon usage and the FFF minimal activator domains, was generously provided by Dr. Wolfgang Hillen, University of Erlangern, and Dr. Hermann Bujard, University of Heidelberg, Germany (11). The *NPHS2* promoter region was released from the p2.5PodocinPE plasmid by restriction digestion with *Xba*I and *Nco*I, and was blunt-ended by T4 DNA polymerase (Fermentas, Hanover, MD). The cytomegalovirus (CMV) minimal promoter was removed from the rtTA vector pUHrT62–1 by restriction digestion with *Xho*I and *Sac*II, and the remaining portion of pURrT62–1 was blunt-ended using T4 DNA polymerase. The *NPHS2* promoter was ligated into pURrT62–1 using the Quick Ligation Kit (New England Biolabs, Beverly, MA) to obtain the construct podocin-rtTA. The proper orientation of the

clones was confirmed by restriction enzyme digestion mapping. The podocin-rtTA construct was released from the plasmid vector backbone by digestion with *Sca*I and *Hin*dIII, and purified by gel electrophoresis and DNA extraction using QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA). The purified construct DNA was introduced into the pronuclei of fertilized oocytes from the FVB/N mouse (National Cancer Institute, Bethesda, MD) by microinjection using standard techniques. All animal care conformed to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and was approved by the National Institutes of Diabetes and Digestive and Kidney Diseases Animal Care and Use Committee.

Identification of Transgenic Mice

Transgenic mice were identified by Southern blotting using genomic DNA recovered from tail biopsies. Genomic DNA was isolated from the lysate of the tail of a 3-wk-old mouse using DNeasy Tissue Kit (Qiagen). Ten micrograms of the genomic DNA was digested with *Bam*HI and electrophoresed through a 1.0% agarose gel, transferred to a Nytran N membrane (Schleicher and Schuell, Keene, NH), and cross-linked with an ultraviolet Stratalinker (Stratagene, La Jolla, CA). The blots were prehybridized and hybridized in 15 ml of Hybrisol I (Intergen, Purchase, NY) at 52°C. The DNA blots were hybridized with ^{32}P-labeled cDNA probes for the human *NPHS2* promoter region.

PCR was also used for identification of transgenic mice, using the primer set Podoprobe-F: 5'CGCACTTCAGTTACTTCAGGTCCTC3' and Podoprobe-B: 5'GCTTATGCCTGATGTTGATGATGC3'. The thermocycling profile was as follows: initial denaturation 94°C for 30 s, then 30 cycles of 94°C denaturation for 30 s, 52°C annealing for 30 s, and 72°C extension for 30 s, and a final extension of 72°C for 5 min. Genomic template DNA was used in the range of 10 to 50 ng per reaction. The PCR product was detected on a standard 1% TAE-agarose gel as a 455-bp fragment. Podocin-rtTA transgenic founders were crossbred with *TetO-LacZ* mouse line (also termed RASSL mice, a generous gift of Dr. Bruce R. Conklin, University of California, San Francisco, California) (18).

β-Galactosidase Assays

Transgene expression was induced in mice by replacing normal drinking water with doxycycline-containing water supplemented with 5% sucrose to enhance palatability. Doxycycline (Sigma, St. Louis, MO) water was changed every 3 d. Quantitative assays for β-galactosidase activity in tissue homogenates were performed using a chemiluminescence assay as described by Moeller *et al.* (8) with a slight modification. Fresh tissues dissected from the mice were homogenized in lysis buffer containing 100 mM potassium phosphate (pH 7.8), 0.2% Triton X-100, 1 mM dithiothreitol, and 1 tablet/50 ml of Complete EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN). Homogenization was performed for 20 s on ice using a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA). After centrifugation at 12,500 *g* for 10 min, the supernatants were heated at 48°C for 50 min to inactivate endogenous mammalian β-galactosidase activity and centrifuged at 12,500 *g* for 5 min.

Fifty microliters of heat-inactivated homogenates were incubated for 60 min at 25°C with 300 μl of reaction buffer containing Galacton (Tropix, Bedford, MA), 100 mM sodium phosphate (pH 7.5), 1 mM MgCl$_2$, and 5% Emerald (Tropix). Light output was measured for 20 s using a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA) as an index of β-galactosidase activity. Protein concentration in the supernatants was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL) with BSA as a standard.
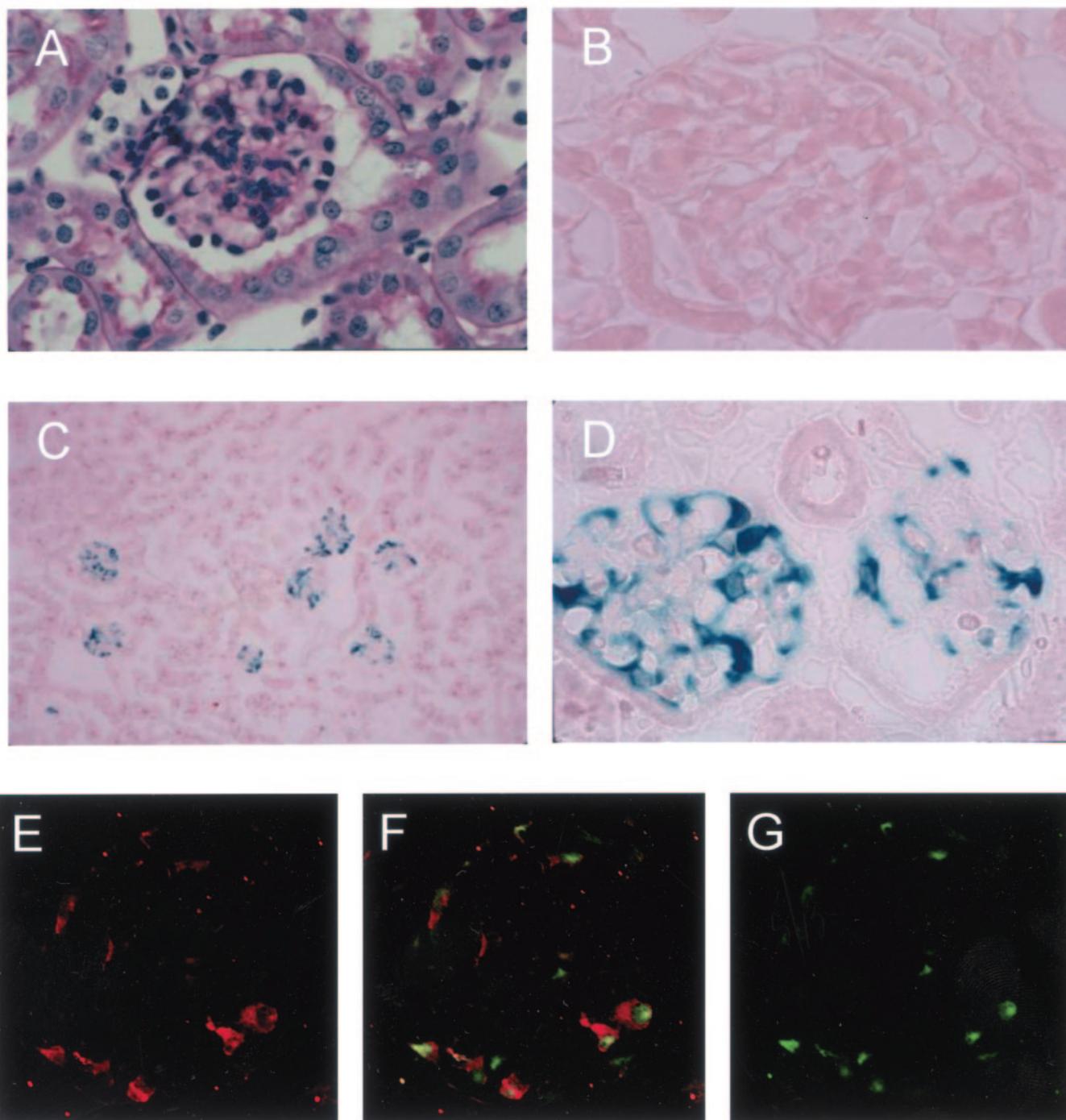
Figure 2. β-galactosidase expression in the kidney of podocin-rtTA X *tetO-LacZ* bitransgenic mice. (A) Normal appearance of kidney tissue from a bitransgenic mouse (PAS stain). (B) X-gal *in situ* staining from a bitransgenic mouse untreated with doxycycline. (C and D) β-galactosidase expression in doxycycline-treated bitransgenic mouse. Doxycycline was administered as a 2 mg/ml concentration in drinking water for 7 d. β-galactosidase was expressed predominantly in the periphery of glomeruli. (E, F, and G) Colocalization of β-galactosidase expression and WT-1. Cryosection of the kidney from the bitransgenic mouse treated with doxycycline was double-immunostained with Alexa 594-labeled anti-β-galactosidase antibody (predominantly cytoplasm, red) (E) and Alexa 488-labeled anti-WT-1 antibody (nuclei, green) (G). The two images were merged, demonstrating colocalization in podocytes (F). Magnification: ×200 (A), ×200 (B), ×40 (C), ×200 (D), ×300 (E to G).

For localization of β-galactosidase activity, tissues were fixed by intracardial perfusion of anesthetized mice using ice-cold 1.5% para-formaldehyde in PBS and 18% sucrose in PBS. The kidneys were resected, embedded in Histo Prep (Fisher, Fair Lawn, NJ), and frozen in isopentane chilled to −70°C. Cryosections were post-fixed with 4% paraformaldehyde in PBS (pH 7.8) for 5 min, and incubated overnight at 30°C in the staining solution containing 1 mg/ml 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-gal; Sigma), 5 mM

potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS (pH 7.8). The sections were counterstained with Nuclear Fast Red Staining Solution (Vector Laboratories, Burlingame, CA) for 1 min, mounted, and examined under bright-field microscopy.

Immunofluorescent Staining

Immunofluorescent staining was performed on the cryosections derived from the mice after perfusion with 1.5% paraformaldehyde in PBS and 18% sucrose in PBS. Sections were fixed with ice-cold acetone for 5 min, washed, and blocked with 10% donkey serum. Sections were incubated with goat anti-β-galactosidase polyclonal antibody (1:100 dilution; catalog number 4600–1409, Biogenesis, Kingston, NH) and rabbit anti-WT-1 polyclonal antibody (1:100 dilution; C-19 antibody, Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min. After washing, specimens were incubated with Alexa Fluor 594 conjugated donkey anti-goat IgG (1:100 dilution; Molecular Probes, Eugene, OR) and Alexa Fluor 488 conjugated goat anti-rabbit IgG (1:100 dilution; Molecular Probes). Fluorescence was examined using appropriate filters using a Leica DMR confocal microscope (Leica, Deerfield, IL) equipped with epifluorescent optics.

Statistical Analysis

Data are presented as mean ± SD.

Results

The Podocin-rtTA Transgene Expresses in a Kidney-Specific Fashion

Podocin-rtTA mice were generated using a transgene containing a 2.5-kb fragment of the human NPHS2 5' flanking region, the rtTA cassette, and SV40 3' untranslated region sequence including a polyadenylation sequence. Four founder mice were identified by Southern analysis. Two female founder mice were sterile. F1 offspring derived from mating each of two founding male podocin-rtTA line mice with wild-type FVB/N mice were crossed with heterozygous tetO-LacZ to obtain dual-heterozygous bitransgenic mice, which were identified by PCR. The structure and interaction of the podocin-rtTA and tetO-LacZ transgenes are presented in Figure 1.

Wild-type, podocin-rtTA, tetO-LacZ, and bitransgenic mice were provided with drinking water supplemented with doxycycline 2 mg/ml; control mice received plain drinking water. The gross and microscope appearance of renal tissue was normal in podocin-rtTA mice and in bitransgenic mice provided with doxycycline for up to 1 wk (Figure 2A). Tissue specificity of transgene expression was investigated by assaying β-galactosidase activity in tissue homogenates from selected organs. In mice derived from one founder line, high levels of β-galactosidase activity were detected in the kidney homogenates from doxycycline-induced bitransgenic mice, with no expression in other organs (Figure 3). In contrast, β-galactosidase activity was absent from all tissue homogenates from untreated bitransgenic mice and doxycycline-treated wild-type mice. Mice derived from the other founder podocin-rtTA mouse line did not express β-galactosidase, presumably due to transgene insertion into a transcriptionally silenced region of the genome, and these mice were not studied further.
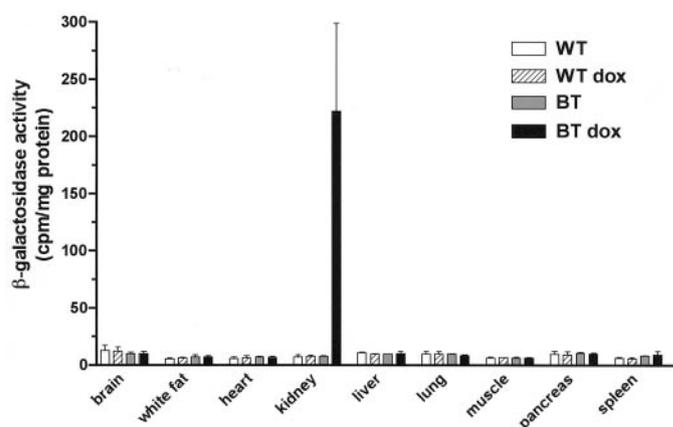

Figure 3. Kidney-specific transgene induction in podocin-rtTA X tetO-LacZ bitransgenic mice. Tissue homogenates from doxycycline-treated or untreated bitransgenic mice (BT) were tested for β-galactosidase activity by chemiluminescence and compared with values obtained from wild-type (WT) mice. Data are shown as means of counts per minute (cpm) normalized by the protein concentration of tissue homogenates derived from three animals. Transgene expression was induced with 2 mg/ml of doxycycline for 7 d.

Transgene Expression Is Detected Exclusively in Podocytes

To determine the spatial localization of LacZ expression in kidney, renal tissues were examined by X-gal staining of cryosections. β-galactosidase expression was detected in all glomeruli of specimens from podocin-rtTA X tetO-LacZ mice treated with doxycycline (Fig. 2C). Expression was most prominent in the periphery of glomeruli, in a localization most consistent with podocytes (Figure 2D). β-galactosidase activity was absent in bitransgenic mice not treated with doxycycline (Figure 2B) and mice bearing only one of the transgenes (data not shown).

Double immunofluorescence staining was performed in doxycycline-treated podocin-rtTA X tetO-LacZ mice, using Alexa Fluor 594-labeled anti-β-galactosidase antibody and Alexa Fluor 488-labeled anti-WT-1 antibody. Within the normal mature glomerulus, WT-1 is a specific podocyte marker, expressed predominantly in the nuclei (19). Superimposition of the two labels demonstrated that the cells expressing β-galactosidase (red), predominantly in the cytoplasm, also had nuclei expressing WT-1 (green) (Figure 2G). Essentially all podocytes expressed β-galactosidase.

Transgene Expression: Dose Response and Time Course

To investigate the ability to regulate transgene expression levels by the dosage of doxycycline, podocin-rtTA X tetO-LacZ bitransgenic mice received different doses of doxycycline via drinking water for 7 d. Induction of β-galactosidase activity in bitransgenic mice was detectable between 0.02 mg/ml and 5 mg/ml, and was maximal at 2 mg/ml (Figure 4A).

The time course of transgene expression level was examined after administration of doxycycline to bitransgenic mice. The
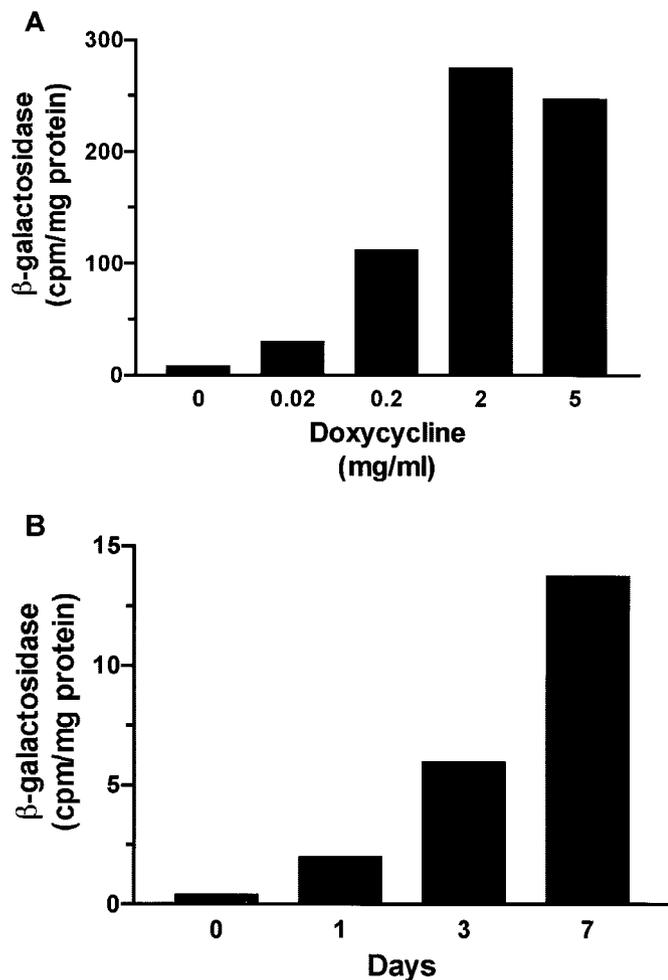
There are multiple conceivable uses for an inducible transgene system that confers podocyte-specific expression. Transgene products might include cytokines or other peptides or anti-sense RNA molecule or siRNA molecules that might either exacerbate or ameliorate podocyte injury. Transgene expression can be turned on (and off) when desired, such as at a particular stage of embryonic development or in adult life. Transgene expression can also be manipulated in tandem with other experimental maneuvers to induce renal disease (such as partial nephrectomy, administration of nephrotoxic antiserum, or therapy to increase BP) or experimental maneuvers to ameliorate renal disease (such as administration of systemic medication or gene therapy). If the transgene product is toxic to the podocyte (such as by inducing apoptosis), this model might be used to induce podocytopenia. The system could be used to express *Cre* recombinase, to allow a tissue-specific knockout in an inducible manner. In all of these approaches, the amount of the transgene product can be varied by using different doses or different duration of doxycycline therapy.

In conclusion, this transgenic model of podocyte-specific transgene expression should prove useful in studies of podocyte biology, assisting efforts to elucidate the role of the podocyte in health and disease.

Acknowledgments

Figure 4. Dose-response and time-course of *LacZ* induction. (A) Dose-response curve of β-galactosidase activity of kidney tissue in podocin-rtTA X *tetO-LacZ* bitransgenic mice. Doxycycline was administered as different concentrations as indicated for 7 d before sacrifice. Kidney lysates were analyzed for β-galactosidase activity by chemiluminescence; the results shown are the mean of tissues derived from two mice. (B) Time-course of β-galactosidase activity of kidney from bitransgenic mice induced with 2 mg/ml of doxycycline for different durations. Background activity of kidney lysates from wild-type mice was subtracted from measured activity to yield specific activity. The results shown are the mean of tissues derived from two mice.

β-galactosidase activity of kidney homogenates from bitransgenic mice was detected even 1 d after doxycycline exposure, and continued to increase up to 7 d after exposure (Figure 4B).

Discussion

In this study, we report an inducible mouse model system that permits tightly-regulated and conditional expression of a reporter transgene in the podocytes of bitransgenic mice. Transgene expression was restricted to the kidney, among nine tissues examined, and expression in kidney was restricted to the podocyte. Essentially all podocytes expressed the transgene. Transgene expression was regulated by doxycycline in a dose-dependent and time-dependent manner.

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