Efficient In Vivo Doxycycline and Cre Recombinase–Mediated Inducible Transgene Activation in the Murine Trabecular Meshwork

Pu Liu,[1,2] *Xueyao Fu,*[1,2] *and Randy L. Johnson*[1,2]

Purpose. To generate new mouse lines that facilitate inducible gene activation in the murine trabecular meshwork in vivo.

Methods. Two expression cassettes were knocked into the 3′-UTR of the *Myocilin* (*Myoc*) locus, an abundantly expressed extracellular matrix protein produced by cells of the trabecular meshwork. The first cassette directs expression of an inducible form of Cre recombinase, CreER(T2), which is activated by tamoxifen administration under the control of endogenous Myoc regulatory elements. The second cassette contains a reverse tetracycline transactivator, rtTA(M2), which directs the expression of tetracycline-operator transgenes on exposure of animals to doxycycline (Dox). These lines were crossed to *GFP* and *lacZ* reporter mice to assay for tamoxifen or Dox-induced transgene expression.

Results. Both the *Myoc-CreER(T2)* and the *Myoc-rtTA(M2)* lines were capable of directing efficient and inducible expression of transgenes in the murine trabecular meshwork in vivo. In addition, activation of transgenes by *Myoc-rtTA(M2)* was reversible with loss of transgene expression after Dox withdrawal. Examination of multiple tissues demonstrates efficient transgene activation in the trabecular meshwork, with additional sites of transgene activation including cells in the retina, sclera, lung, kidney, and abundant activation in the neocortex and hippocampus.

Conclusions. Two new mouse lines have been generated that allow for efficient and inducible transgene activation in the murine trabecular meshwork in vivo. (*Invest Ophthalmol Vis Sci.* 2011;52:969–974) DOI:10.1167/iovs.09-5052

The trabecular meshwork controls the major outflow pathway of aqueous humor from the anterior chamber.[1] If the trabecular meshwork is blocked or not functioning properly, elevated intraocular pressure (IOP) ensues, leading to high-tension glaucoma. Although the role of the trabecular meshwork in regulating intraocular pressure has been well studied, molecular mechanisms that contribute to increased outflow resistance are only beginning to be revealed.

One contributing factor to these recent advances is the use of the mouse as a model system for in vivo glaucoma studies.[2] The laboratory mouse has several advantages for the generation and use of glaucoma research models. The anatomic structure of the murine anterior segment and aqueous humor dynamics mirrors that of the human,[3,4] and tools for noninvasive measurement of intraocular pressure have been modified for use in the mouse.[1,5–9] Many inbred lines differ with respect to resting IOP levels,[10,11] and these differences have been used to isolate genes that affect intraocular pressure in both mice and humans.[12,13]

As additional glaucoma genes are identified through combined mouse and human genetic studies, there is a growing need for tools to manipulate gene expression in the trabecular meshwork in vivo in mouse models. Here we report the generation of reagents to activate gene expression in an inducible manner in mouse trabecular meshwork cells. Two knock-in lines of mice were made that express either a tamoxifen-inducible form of Cre recombinase (CreER(T2))[14,15] or a reverse tetracycline transactivator (rtTA(M2))[16] under the control of endogenous Myoc regulatory elements. Both these lines are effective in mediating inducible expression of conditional transgenes with either tamoxifen or doxycycline (Dox), respectively. These new reagents will allow a greater range of genetic experiments that can be carried out in the mouse trabecular meshwork in vivo and enhance the usefulness of the laboratory mouse as a model organism for glaucoma research.

Materials and Methods

Targeting Constructs and Generation of *Myoc-CreER(T2)* and *Myoc-rtTA(M2)* Knock-in Mouse Line

The targeting strategy for *Myoc* knock-in lines were illustrated in Figure 1A. Briefly, a 4-kb *Kpn*I/*Hin*dIII fragment (5′ homologous arm of *Myoc*) and a 2.5-kb *Hin*dIII/*Bam*HI fragment (3′ homologous arm of *Myoc*), together with *MC1TK* cassette, were subcloned into *pCOND-TK*. Then a *Pac*I/*Not*I fragment containing *IRES-rtTA(M2)pA-PGKNeoloxPA* or *IRES-CreER(T2)pA-PGKneo-loxPA* was inserted into the site between the 5′ and 3′ homologous arms. Linearized targeting vector was electroporated into 129/B6 hybrid embryonic stem cell line RJ2.2 (available on request). Targeted clones were screened by Southern hybridization using 3′ probes. Wild-type and targeted alleles were distinguished by an *Xba*I fragment length difference. Correctly targeted clones were sent to Genetic Engineered Mouse Facility (University of Texas MD Anderson Cancer Center, Houston, TX) for blastocyst injection.

Male chimeras were mated with CD1 females. Their progeny underwent genotyping for the presence of *rtTA* or *Cre* by PCR using the following primer sets: *rtTA* forward, 5′-gacgacaaggaaactcgctc-3′; *rtTA* reverse, 5′-caaaatcgtcaagagcgtca-3′; *Cre* forward, 5′-tccaatttactgaccgtacaccaa-3′; *Cre* reverse, 5′-cctgatcctggcaatttcggcta-3′. All studies involving animals were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animal protocols were approved by University of Texas MD Anderson Cancer Center Animal Care and Use Committee.

From the [1]Department of Biochemistry and Molecular Biology, University of Texas, MD Anderson Cancer Center, Houston, Texas; and the [2]Program in Genes and Development, Graduate School of Biomedical Sciences, University of Texas Health Sciences Center, Houston, Texas.

Supported by National Eye Institute Grants EY123113 (RLJ) and EY011930 and EY010608 (WHK) and the Robert A. Welch Foundation Grant G-0010 (WHK).

Submitted for publication December 14, 2009; revised June 24 and August 23, 2010; accepted September 1, 2010.

Disclosure: **P. Liu**, None; **X. Fu**, None; **R.L. Johnson**, None

Corresponding author: Randy L. Johnson, Department of Biochemistry and Molecular Biology, University of Texas, MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030; rljohnso@mdanderson.org.

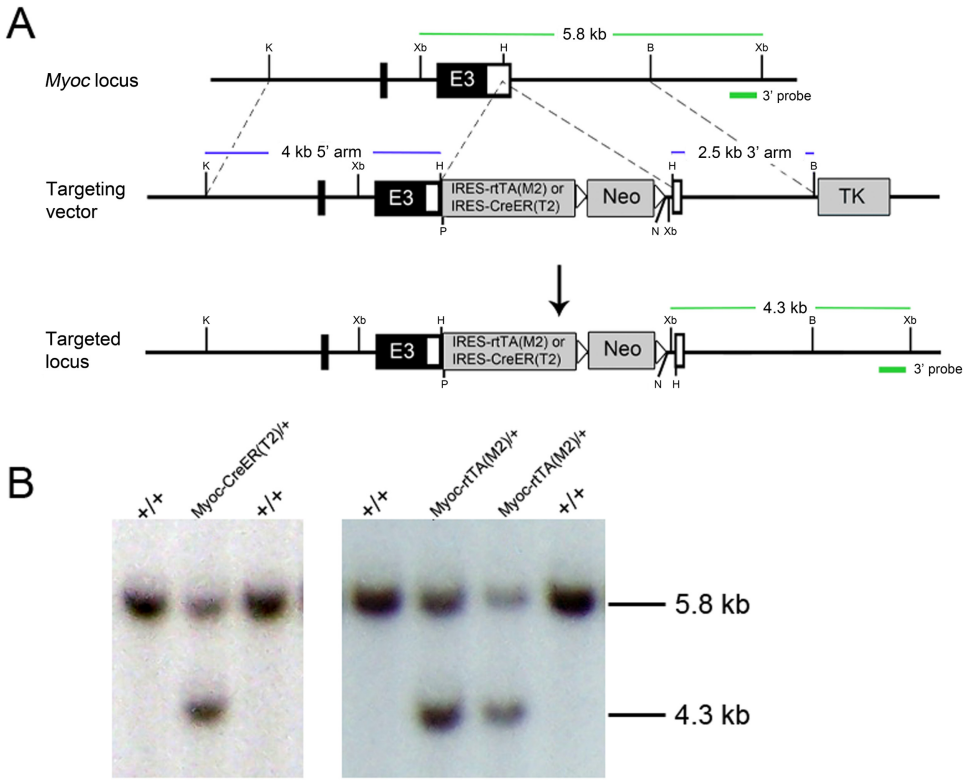

FIGURE 1. Generation of *Myoc-rtTA(M2)* and *Myoc-CreER(T2)* knock-in alleles. (**A**) Strategy for targeting the *Myoc* locus. Insertion of *IRES-rtTA(M2)* or *IRES-CreER(T2)* cassette (the *Pac*I/*Not*I fragment) into the *Hin*dIII site of *Myoc* 3′-UTR results a 5.8-kb *Xba*I fragment from the wild-type allele and a 4.3-kb fragment from the targeted allele (*green lines*). Location of the 3′ external probe used for mini-Southern blot analysis (*green bar*). 5′ and 3′ homologous arms (*blue lines*). E3, exon 3; K, *Kpn*I; Xb, *Xba*I; H, *Hin*dIII; B, *Bam*HI; P, *Pac*I; N, *Not*I. (**B**) Mini-Southern results. Using the 3′ external probe, wild-type allele (*top band*) is distinguished from targeted allele (*bottom band*).

Dox and Tamoxifen Induction

Dox was administered by inclusion in drinking water (2 mg/mL). Because of the sensitivity of Dox to light, drinking water containing Dox was replaced every 3 days. Tamoxifen was prepared at a concentration of 10 mg/mL in sunflower oil. It was intraperitoneally injected at 6 mg/40 g body weight for 3 consecutive days. Eyeballs were harvested approximately 4 weeks after injection. At least 8 eyeballs/4 animals were analyzed for each time point.

Whole Mount X-gal Staining and X-gal Staining on Frozen Sections

Adult eyes were enucleated, fixed in X-gal fixative (0.2% glutaraldehyde, 2% paraformaldehyde (PFA), 5 mM EGTA, pH 8.0, 2 mM $MgCl_2$ in 0.1 M phosphate buffer, pH 7.3) for 15 minutes, rinsed in rinse buffer (0.1% sodium deoxycholate, 0.2% Nonidet P-40, 2 mM $MgCl_2$ in 0.1 M phosphate buffer, pH 7.3) three times, and stained in staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/mL X-gal in rinse buffer) overnight, protected from light. Stained eyes were briefly washed in PBS and postfixed in 4% PFA.

For staining on frozen sections, 4% PFA-fixed tissues were rinsed, cryoprotected in 30% sucrose, and embedded in optimum temperature cutting compound (OCT; Sakura Finetek USA, Torrance, CA). Sections were cut at 15 μm and subject to X-gal staining to assay for β-gal activity. Stained slides were counterstained with nuclear fast red (Vector Laboratories, Burlingame, CA).

Immunofluorescence

Adult eyeballs were fixed and embedded in OCT. Frozen sections were cut at 20 μm and subjected to immunostaining using standard methods. Briefly, slides were washed in PBST (phosphate-buffered saline, 0.1% Triton) to remove OCT, blocked with 2% BSA, and incubated with primary antibody (rabbit GFP, 1:1000; Molecular Probes, Invitrogen, Carlsbad, CA); mouse Pax6 (1:400; Developmental Studies Hybridoma Bank; University of Iowa, Iowa City, IA); goat Pou4f2 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), and species-specific secondary antibody (1:400; Molecular Probes, Invitrogen). Slides were counterstained with propidium iodide and mounted (Aqua-Mount; Fisher Scientific, Pittsburgh, PA). Fluorescence was visualized on a confocal system (FV500; Olympus, Tokyo, Japan).

RESULTS

Generation of *Myoc-CreER(T2)* and *Myoc-rtTA(M2)* Knock-in Lines

To express CreER(T2) recombinase and rtTA(M2) in the trabecular meshwork in vivo, we designed a strategy to couple expression of these proteins to regulatory elements of the *Myoc* locus. Myoc is an extracellular matrix protein abundantly expressed in the murine trabecular meshwork and expressed to a lesser degree in the sclera, iris, and retina.[17–20] It is mutated in human open angle glaucoma.[21] We have previously shown that Myoc is dispensable for murine development and that *Myoc* null mice exhibit normal IOP and ocular histopathology.[22] Hence, integration of expression cassettes for *CreER(T2)* and *rtTA(M2)* at the *Myoc* locus is an attractive strategy to generate mice capable of specific inducible gene expression in the trabecular meshwork. We chose to insert these expression cassettes into the 3′-UTR of *Myoc* together with an internal ribosome entry site (IRES) that results in bicistronic expression of Myoc and either CreER(T2) or rtTA(M2) from a single combined transcript. The strategy for generation of these mice is shown in Figure 1A.

Efficiency of Tamoxifen and Dox-Induced Transgene Expression in the Trabecular Meshwork of *Myoc-CreER(T2)* and *Myoc-rtTA(M2)* Knock-in Mice

To test the effectiveness of transgene induction, we first generated *Myoc-CreER(T2);ROSA26-LSL-LacZ* double heterozygotes. The *ROSA26-LSL-LacZ* allele (Fig. 2A) contains a latent *Escherichia coli* β-*galactosidase* gene inserted at the *ROSA26*

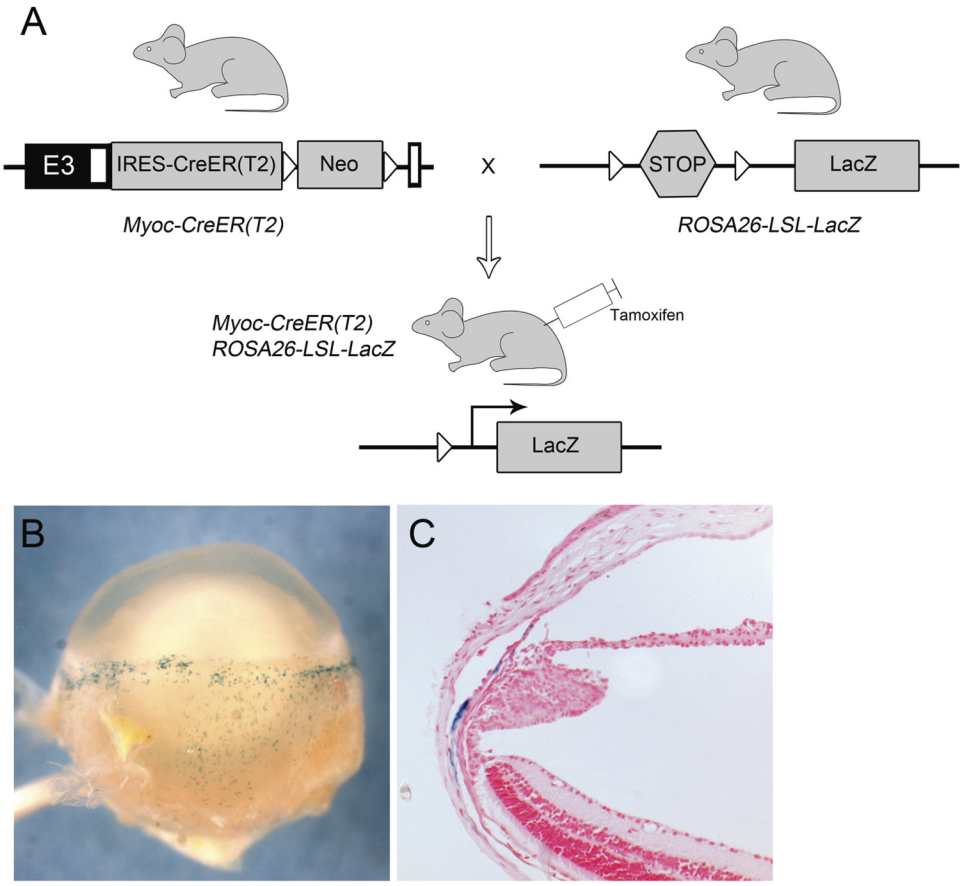

FIGURE 2. Tamoxifen-induced LacZ expression in *Myoc-CreER(T2);R26-LSL-LacZ* adult eye tissues. (**A**) Crossing scheme to generate bigenic mice. Mosaic X-gal staining in the trabecular meshwork and sclera is shown in whole mount (**B**) and in sections (**C**).

locus on mouse chromosome 6. The *ROSA26* locus encodes a nonessential, noncoding transcript that is widely expressed in embryonic and adult tissues.[23] The *LSL* (*loxP*-stop-*loxP*) element, placed between the *ROSA26* promoter and the *lacZ* coding region, contains multiple stop codons and polyadenylation signals. These are removed on the action of Cre that recombines the stop cassette and allows constitutive lacZ expression, which can be detected by a simple histochemical stain with X-gal. To achieve recombination and lacZ expression, we injected *Myoc-CreER(T2);ROSA26-LSL-LacZ* double heterozygotes with a dose of 6 mg/40 g body weight tamoxifen and assayed mice for β-galactosidase activity 4 weeks after injection. Mosaic staining was observed in whole mount specimens localized to trabecular meshwork tissue (Fig. 2C). Lower levels of mosaic activity were also observed in the sclera. Hence, *Myoc-CreER(T2)* mice are able to express transgenes in a tamoxifen-inducible manner in the trabecular meshwork, though at a mosaic level.

To test the ability of Dox to induce transgene expression in *Myoc-rtTA(M2)* knock-in mice, we first generated bigenic *Myoc-rtTA(M2);tet-O-GFP* double heterozygotes. *Tet-O-GFP* mice contain a *tet-O-GFP* transgene that is not detectably expressed.[24] However, when combined with a reverse tetracycline transactivator, expression can be achieved with Dox administration. Dox, a tetracycline analog, binds to the reverse tetracycline transactivator protein, inducing the ability of the transactivator to bind to tetracycline-responsive elements (TREs) contained in the *tet-O* portion of the *tet-O-GFP* transgene. Feeding *Myoc-rtTA(M2);tet-O-GFP* bigenic mice ad libitum with 2 mg/mL Dox in their drinking water resulted in efficient induction of GFP expression in the trabecular meshwork that increased with time of Dox exposure (Figs. 3A–D). Minimal GFP expression was observed in the absence of Dox (Fig. 3E). Lower levels of GFP activity were detected in the sclera, and scattered GFP-positive cells were observed in the retina (Fig. 3F, Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.09-5052/-/DCSupplemental). Hence, *Myoc-rtTA(M2)* knock-in mice are effective in Dox-induced expression of TRE-regulated transgenes in trabecular meshwork tissues in vivo.

To further extend the usefulness of *Myoc-rtTA(M2)* knock-in mice, we used a triple-transgenic system in which the *Myoc-rtTA(M2)* allele is combined with *tet-O-Cre* and *ROSA26-LSL-LacZ* transgenes. The design of this experiment is to use Dox to induce trabecular meshwork–restricted expression of Cre recombinase that then can act to recombine the stop cassette at the *ROSA26-LSL-LacZ* locus, thereby resulting in LacZ expression. To that end, *Myoc-rtTA(M2);tet-O-Cre; ROSA26-LSL-LacZ* triple heterozygous mice were fed Dox in their drinking water for up to 4 weeks, and ocular tissues were stained for LacZ activity. Abundant and localized LacZ activity was seen in angle tissues and increased with time of exposure, indicating efficient expression in the trabecular meshwork (Figs. 4A–C, 4E). Hence, this system is efficient for Dox-mediated Cre-induced activation of *LSL* transgenes in the murine trabecular meshwork.

An additional advantage of Dox-regulated systems is the ability to silence transgene expression by removal of Dox from the drinking water. To test whether *Myoc-rtTA(M2)* mice are useful for both Dox-mediated transgene activation and for transgene silencing after Dox removal, we treated *Myoc-rtTA(M2);tet-O-GFP* double heterozygotes with Dox for 30 days, removed Dox, and assayed for GFP expression 4, 12, and 16 days later. Although GFP is a relatively stable protein, diminished expression is observed within 4 days; this expression progresses so that by 16 days, few GFP-expressing cells

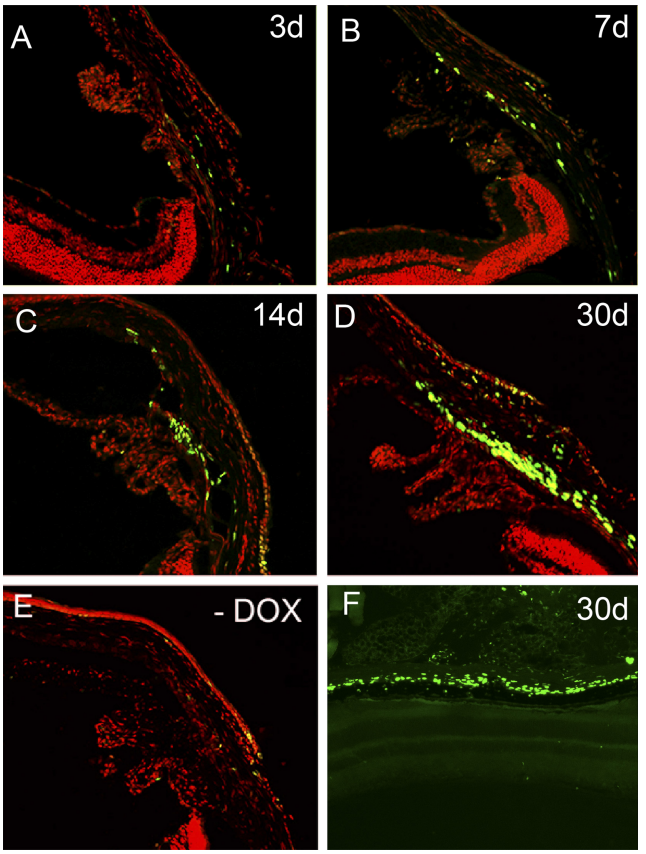

FIGURE 3. Timed induction of GFP expression by Dox in the trabecular meshwork and sclera of *Myoc-rtTA(M2);tet-O-GFP* transgenic mice. Eyeballs were sectioned and immunostained for GFP. GFP level in the trabecular meshwork is increased with time of treatment (**A–D**) and peaks at 1 month (**D**), at which time almost all trabecular cells express GFP. No GFP-positive cells are detected in bigenic eyes without Dox induction (**E**). GFP is also detected in the sclera after 30 days of Dox treatment (**F**).

remain (Fig. 5). Hence, *Myoc-rtTA(M2)* mice are useful for Dox-induced transgene activation and for transgene repression after Dox removal.

Although *Myoc* is known to be expressed in the trabecular meshwork of the mouse, expression has also been reported in other ocular and nonocular tissues, including the retina, iris, brain, skeletal muscle, kidney, and lung.[17,25] To establish whether *Myoc-rtTA(M2)* mice also have expression of rtTA(M2) in tissues other than the trabecular meshwork, we examined tissues from *Myoc-rtTA(M2); tet-O-Cre;ROSA26-LSL-LacZ* mice for LacZ activity after 28 days of Dox induction. In addition to positive staining in the trabecular meshwork, we observed a smaller number of positive cells in the sclera and scattered positive cells in the retinal ganglion cell layer, the inner nuclear layer, kidney, lung, and skeletal muscle (Fig. 4). Abundant LacZ activity was also detected in the brain, with prominent staining in the neocortex and hippocampus (Figs. 4G, 4H). However, within the eye, most staining was observed in the trabecular meshwork and sclera; minimal staining was found in the retina and iris, and none was found in the cornea. Hence, with the exception of the brain, *Myoc-rtTA(M2)* directs transgene activation largely in the trabecular meshwork.

DISCUSSION

In this report, we characterize two new knock-in lines at the *Myoc* locus that were designed to facilitate inducible transgene

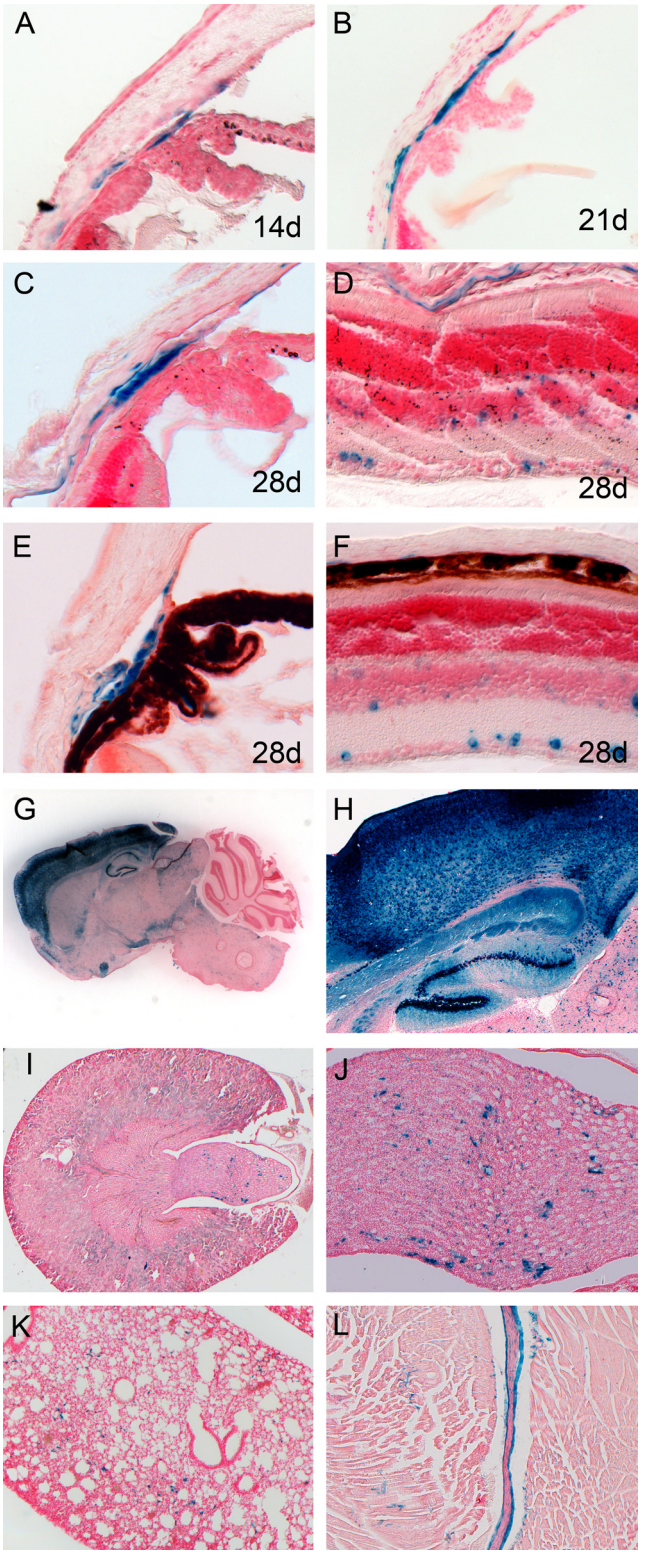

FIGURE 4. Expression of Myoc in ocular and nonocular tissues of *Myoc-rtTA(M2);tet-O-Cre;ROSA26-LSL-LacZ* mice 2 to 4 weeks after Dox treatment. (**A–C**, **E**) Robust lacZ staining was observed in the trabecular meshwork of albino and pigmented eyes. (**D**, **F**) Scattered lacZ-positive cells were found in retinal ganglion cell layer, inner nuclear layer, and sclera. In nonocular tissues, lacZ expression was detected in neocortex and hippocampus of brain (**G**, **H**), kidney (**I**, **J**), lung (**K**), and skeletal muscle (**L**).

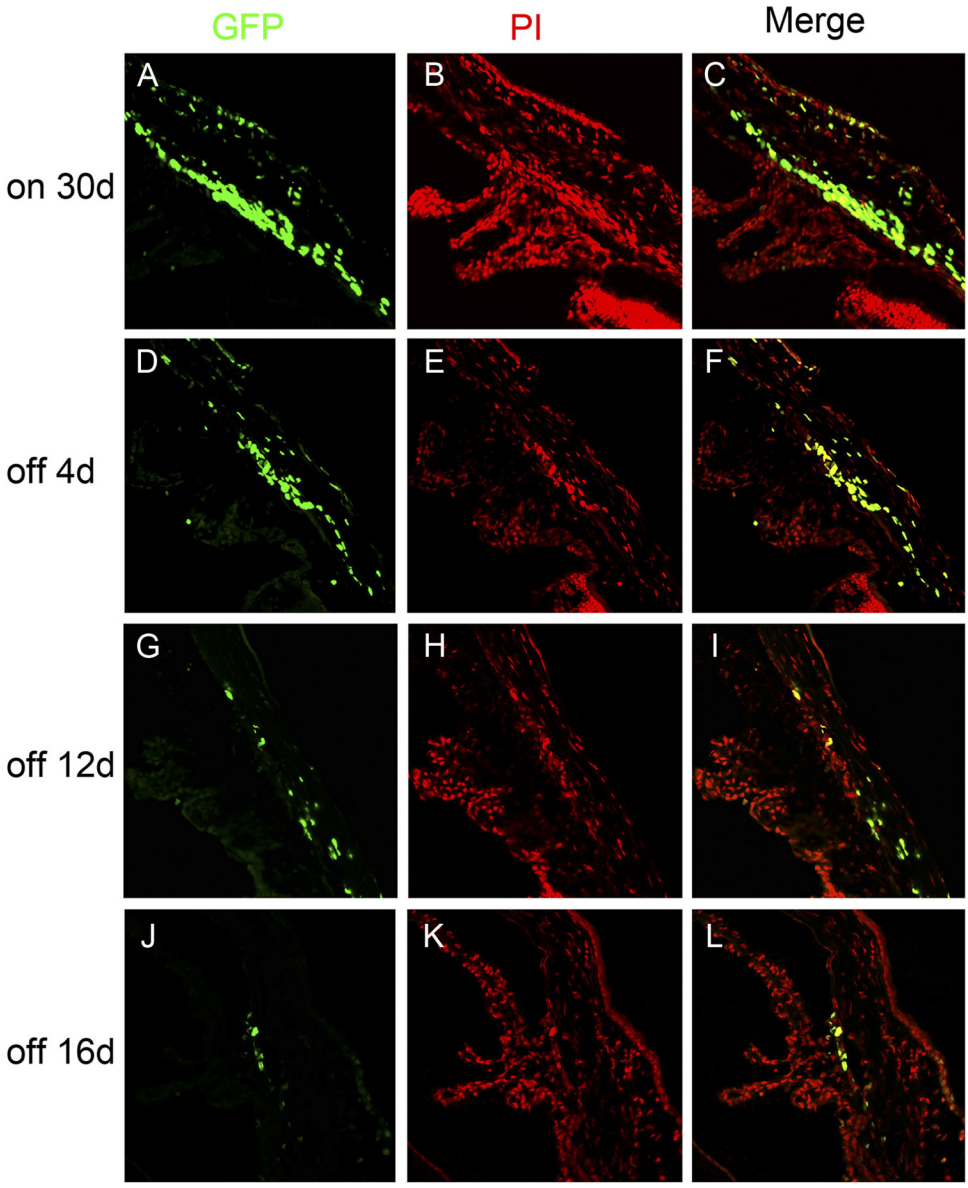

FIGURE 5. Reversible GFP expression after removal of Dox. Dox was removed after 1 month of treatment (**A–C**), and GFP activity decreased rapidly (**D–L**). Twelve days after removal, approximately one-fourth of trabecular cells expressed GFP (**G–I**). By 16 days after treatment (**J–L**), the level of GFP was significantly reduced to basal levels in most cells.

activation in the trabecular meshwork. Both the tamoxifen-inducible *Myoc-CreER(T2)* and Dox-inducible *Myoc-rtTA(M2)* lines direct efficient activation of Cre and tet-O–regulated transgenes in the murine trabecular meshwork. In the case of the *Myoc-rtTA(M2)* mice, we have further demonstrated that induction is reversible, thereby facilitating experimental designs that require activation and deactivation of transgenes in vivo. Whereas *Myoc-rtTA(M2)* and likely *Myoc-CreER(T2)* mice exhibit activity outside the trabecular meshwork, this activity is minimal with the exception of the central nervous system. Although we have focused in this study on the induction of *GFP* and *LacZ* reporter transgenes, it is likely that these new *Myoc* knock-in lines will be equally useful for the activation of other transgenes for the study of trabecular meshwork function and to generate new models of high-tension glaucoma in mice. Furthermore, both *Myoc-CreER(T2)* and *Myoc-rtTA(M2)* lines can be applied to tamoxifen- or Dox-inducible Cre recombinase activity, thereby allowing for inducible gene inactivation when combined with floxed alleles. Taken together, our results demonstrate the feasibility of inducible gene activation in vivo in the murine trabecular meshwork, and our novel enabling reagents significantly expand the tools available for manipulating gene expression in the trabecular meshwork, thereby expanding the usefulness of the mouse as a genetic model for glaucoma research.

Acknowledgments

The authors thank Jan Parker-Thornburg and Jennifer Alana for blastocyst injections, and James Meadows and Naomi Iwai for sharing tet-O reporter mouse strains.

References

1. Gould DB, Smith RS, John SW. Anterior segment development relevant to glaucoma. *Int J Dev Biol.* 2004;48(8–9):1015–1029.
2. McKinnon SJ, Schlamp CL, Nickells RW. Mouse models of retinal ganglion cell death and glaucoma. *Exp Eye Res.* 2009;88(4):816–824.
3. Smith RS, Zabaleta A, Savinova OV, John SW. The mouse anterior chamber angle and trabecular meshwork develop without cell death. *BMC Dev Biol.* 2001;1:3.
4. Aihara M, Lindsey JD, Weinreb RN. Aqueous humor dynamics in mice. *Invest Ophthalmol Vis Sci.* 2003;44(12):5168–5173.

5. Dalke C, Pleyer U, Graw J. On the use of Tono-Pen XL for the measurement of intraocular pressure in mice. *Exp Eye Res.* 2005; 80(2):295–296.
6. Nissirios N, Goldblum D, Rohrer K, Mittag T, Danias J. Noninvasive determination of intraocular pressure (IOP) in nonsedated mice of 5 different inbred strains. *J Glaucoma.* 2007;16(1):57–61.
7. Pang IH, Wang WH, Millar JC, Clark AF. Measurement of mouse intraocular pressure with the Tono-Pen. *Exp Eye Res.* 2005;81(3): 359–360.
8. Pease ME, Hammond JC, Quigley HA. Manometric calibration and comparison of TonoLab and TonoPen tonometers in rats with experimental glaucoma and in normal mice. *J Glaucoma.* 2006; 15(6):512–519.
9. Reitsamer HA, Kiel JW, Harrison JM, Ransom NL, McKinnon SJ. TonoPen measurement of intraocular pressure in mice. *Exp Eye Res.* 2004;78(4):799–804.
10. John SW, Hagaman JR, MacTaggart TE, Peng L, Smithes O. Intraocular pressure in inbred mouse strains. *Invest Ophthalmol Vis Sci.* 1997;38(1):249–253.
11. Savinova OV, Sugiyama F, Martin JE, et al. Intraocular pressure in genetically distinct mice: an update and strain survey. *BMC Genet.* 2001;2:12.
12. Libby RT, Smith RS, Savinova OV, et al. Modification of ocular defects in mouse developmental glaucoma models by tyrosinase. *Science.* 2003;299(5612):1578–1581.
13. Chang B, Smith RS, Hawes NL, et al. Interacting loci cause severe iris atrophy and glaucoma in DBA/2J mice. *Nat Genet.* 1999;21(4): 405–409.
14. Feil R, Brocard J, Mascrez B, LeMeur M, Metzger D, Chambon P. Ligand-activated site-specific recombination in mice. *Proc Natl Acad Sci U S A.* 1996;93(20):10887–10890.
15. Indra AK, Warot X, Brocard J, et al. Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. *Nucleic Acids Res.* 1999; 27(22):4324–4327.
16. Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proc Natl Acad Sci U S A.* 2000;97(14):7963–7968.
17. Tomarev SI, Tamm, ER, Chang B. Characterization of the mouse Myoc/Tigr gene. *Biochem Biophys Res Commun.* 1998;245(3): 887–893.
18. Abderrahim H, Jaramillo-Babb VL, Zhou Z, Vollrath D. Characterization of the murine TIGR/myocilin gene. *Mamm Genome.* 1998; 9(8):673–675.
19. Takahashi H, Noda S, Imamura Y, et al. Mouse myocilin (Myoc) gene expression in ocular tissues. *Biochem Biophys Res Commun.* 1998;248(1):104–109.
20. Fingert JH, Ying L, Swiderski RE, et al. Characterization and comparison of the human and mouse GLC1A glaucoma genes. *Genome Res.* 1998;8(4):377–384.
21. Stone EM, Fingert JH, Alward WL, et al. Identification of a gene that causes primary open angle glaucoma. *Science.* 1997;275(5300): 668–670.
22. Kim BS, Savinova OV, Reedy MV, et al. Targeted disruption of the myocilin gene (*Myoc*) suggests that human glaucoma-causing mutations are gain of function. *Mol Cell Biol.* 2001;21(22):7707–7713.
23. Soriano, P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet.* 1999;21(1):70–71.
24. Tumbar T, Guasch G, Greco V, et al. Defining the epithelial stem cell niche in skin. *Science.* 2004;303(5656):359–363.
25. Swiderski RE, Ying L, Cassell MD, Alward WL, Stone EM, Sheffield VC. Expression pattern and in situ localization of the mouse homologue of the human MYOC (*GLC1A*) gene in adult brain. *Brain Res Mol Brain Res.* 1999;68(1–2):64–72.