Characterization of Tetracycline-Inducible Bitransgenic 
Krt12rtTA+/tet-O-LacZ Mice

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PURPOSE. To prepare binary transgenic mouse lines that overexpress reporter genes in a corneal-epithelium–specific manner when induced by doxycycline.

METHODS. A gene-targeting construct containing an internal ribosomal entry site–reverse tetracycline transcription activator (IRES-rtTA) cassette was inserted into the Krt12 allele (keratin 12 gene) to produce a knock-in Krt12rtTA/+ mouse line through gene-targeting techniques. The Krt12rtTA/+ knock-in mice were bred with tet-O-LacZ reporter mice to obtain Krt12rtTA+/tet-O-LacZ bitransgenic mice. The expression of the LacZ gene was induced in bitransgenic mice by administration of doxycycline in the drinking water and chow.

RESULTS. Administration of doxycycline induced a 15-fold increase of β-galactosidase activity in the cornea of adult bitransgenic mice (Krt12rtTA+/tet-O-LacZ). Administration of doxycycline either to single transgenic Krt12rtTA/+ or tet-O-LacZ mice as a control did not induce overexpression of LacZ as it did in the bitransgenic mice. The induction of β-galactosidase enzyme activity by doxycycline in bitransgenic mice took place in 24 hours and reached a plateau by 2 days. Histochemical analysis also showed that β-galactosidase induction was limited to the corneal epithelium of bitransgenic mice fed doxycycline. The increased β-galactosidase activity in corneal epithelium caused by doxycycline returned to basal levels in 4 weeks after the antibiotics were omitted from the diet.

CONCLUSIONS. A binary mouse model has been successfully established that conditionally overexpresses reporter genes in corneal epithelium. This mouse model will be useful in elucidating signaling pathways of various growth factors and cytokines and gene functions in the maintenance of homeostasis and pathogenesis in the adult mouse cornea. (Invest Ophthalmol Vis Sci. 2005;46:1966–1972) DOI:10.1167/iovs.04-1464

Transgenesis by injection of customized minigenes into pronuclei of fertilized eggs has been an ideal experimental animal model to elucidate the functions of genes during embryonic development and the pathogenesis of human diseases. Furthermore, many of the transgenic animals are great resources for the production of therapeutic agents (e.g., peptide hormone, growth factors). The common strategy of transgenesis is to use a tissue- and/or cell type-specific promoter to express the gene of interest in the target tissue(s). However, the usefulness of mouse lines derived by such a conventional approach is limited in elucidating the functions of genes of interest during embryonic development and in pathogenesis in the adult, because the expression of the transgene often leads to congenital defects and/or embryonic lethality. To circumvent these difficulties, inducible transgenic systems have been developed that allow tissue-specific conditional expression of a target gene.2,3 The system was first developed by Gossen et al.,4 which constituted a tetracycline-inducible system in mammalian cells. This system includes two components: a constitutively expressed tTA (tet-Off) or rtTA (tet-On) transcription factor fusion gene, driven by a strong cytomegalovirus promoter (pCMV), and a reporter gene that is ligated to a pCMV minimum promoter and a prokaryotic tet-opener. The binding of the transcription factors tTA and rtTA to the tet-opener depend on the absence and presence of doxycycline, respectively. Thus, the reporter genes ligated to the tet-opener can be activated or inactivated in a doxycycline-dependent manner. The strategy has been used to create mouse lines in which expression of transgenes can be induced and repressed in the same doxycycline (a tetracycline derivative)-dependent manner in a temporal–spatial-specific fashion in the experimental animals.5–8

Because of the lack of a functional cornea-specific promoter, transgenesis has not been successfully used to create mouse lines that exhibit altered genetic cornea-specific functions for the study of the pathophysiology of diseased corneas. We have identified the corneal-epithelium–specific Krt12 gene, which is expressed by the stratified corneal epithelium.9,10 However, attempts to isolate and identify a functional Krt12 promoter has failed to produce a transgenic mouse line that overexpresses a reporter gene in the corneal epithelium.11 To circumvent these difficulties, we chose to use a knock-in strategy of gene targeting to modify the Krt12 allele so that it contains an internal ribosomal entry site–reverse tetracycline transcription activator (IRES-rtTA) minigene that allows the synthesis of a second protein from the bicistronic mRNA12–14 Unlike prokaryotic polycistronic mRNA, most mammalian mRNA is monocistronic—that is, one mRNA for one protein. However, many viral-derived mRNAs and certain mammalian

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mRNAs (e.g., FGF2) contain an IRES element(s) that binds to ribosome and allows a second translation initiation site for the synthesis of a second protein after the termination of protein synthesis at the stop codon of the first open reading frame. The modified Krt12^{rtTA} allele results in the synthesis of a bicistronic mRNA coding for keratin 12 and rtTA, simultaneously. The expression of rtTA is under the control of the identical control mechanism of the stop codon of the first open reading frame of keratin 12. The resultant plasmid was named pKrt12-rtTA-7.2. A 1.55-kb SalI pgk-NeoBPA DNA fragment was inserted into pKrt12-rtTA-7.2 targeting vector, to eliminate the neomycin-resistant embryonic stem (ES) cells derived from random insertion of the targeting construct (Fig. 1A). Thus, the selection efficiency of homologous recombinant ES cells was enhanced. The pKrt12-rtTA-targeting vector was linearized with Ascl and transfected into a 129S6 ES cell line via electroporation. The 129S6 ES cell line was established by the University of Cincinnati Genetically Targeted Mouse Service Core Facility. ES cell clones that stably incorporated the targeting vector were selected by growth in geneticin (225 μg/mL) for 8 days. The heterozygous recombinant ES cell clones were identified by PCR analysis using two primer pairs: K12/3372-3404 (intron 2, 5’ beyond the targeting construct), GTACTGATTACAGACATGGGGCCCATACAG; and reverse Krt12-2 (primer 3): 5’-GATCTGGGGTTGGATCTGGGTTG-3’ (Fig. 1B). Shown is the expected size of the PCR product amplified by two primer pairs, to target the wild-type and targeted knock-in alleles. Three primers were included in a single PCR mixture. PCR products were resolved by 1% agarose gel electrophoresis. (C) Western blot analysis was performed with affinity-purified and epitope-specific antibodies against keratin 12 or rtTA. Keratin 12 was detected in both wild-type and Krt12^{rtTA/rtTA} knock-in mice eyes, with and without corneas, but rtTA was detected only in the Krt12^{rtTA/rtTA} knock-in cornea.

**MATERIALS AND METHODS**

**Preparation of the Krt12-rtTA Targeting Vector and Generation of Knock-in Krt12^{rtTA/rtTA} Mice**

Conventional cloning techniques were used to prepare a Krt12-rtTA targeting vector. Briefly, a 3.7-kb mouse BamHI/StuI Krt12 genomic DNA fragment containing exons 3 to 7, introns 3 to 7, and the anterior part of exon 8, including the UAA stop codon, was cloned into a commercial vector (Bluescript SK, Stratagene, La Jolla, CA). A 1.1-kb Kpol fragment that contains the untranslated region of exon 8 was then inserted behind the 3.7-kb Krt12 genomic DNA fragment, and the resultant plasmid was named pKrt12-4.8. The rta minigene (a 1.0-kb SpeI/EagI DNA fragment) was cloned into a pIRES vector (BD-Clontech, Palo Alto, CA). A 2.4-kb EcoRI/SphI fragment, named the IRES-rtTA cassette, containing IRES, rtTA, and SV40-polyA excised from the pIRES vector was inserted into the EcoRI/EcoRV site of pKrt12-4.8, between the stop codon and untranslated region of exon 8 of Krt12. The resultant plasmid was named pKrt12-rtTA-7.2. A 1.55-kb SalI pgk-NeoBPA DNA fragment was inserted into pKrt12-rtTA-7.2 targeting vector. The resultant plasmid was designated pKrt12-rtTA-pgk-Neo8.7. Finally, a negative selection marker gene, the diphtheria toxin A fragment (pgk-DTA) cassette, was placed at the 5’ end of the targeting vector, to eliminate the neomycin-resistant embryonic stem (ES) cells derived from random insertion of the targeting construct (Fig. 1A). Thus, the selection efficiency of homologous recombinant ES cells was enhanced. The pKrt12-rtTA-targeting vector was linearized with Ascl and transfected into a 129S6 ES cell line via electroporation. The 129S6 ES cell line was established by the University of Cincinnati Genetically Targeted Mouse Service Core Facility. ES cell clones that stably incorporated the targeting vector were selected by growth in geneticin (225 μg/mL) for 8 days. The heterozygous recombinant ES cell clones were identified by PCR analysis using two primer pairs: K12/3372-3404 (intron 2, 5’ beyond the targeting construct), GTACTGATTACAGACATGGGGCCCATACAG; and reverse Krt12-2 (primer 3): 5’-GATCTGGGGTTGGATCTGGGTTG-3’ (Fig. 1B). Shown is the expected size of the PCR product amplified by two primer pairs, to target the wild-type and targeted knock-in alleles. Three primers were included in a single PCR mixture. PCR products were resolved by 1% agarose gel electrophoresis. (C) Western blot analysis was performed with affinity-purified and epitope-specific antibodies against keratin 12 or rtTA. Keratin 12 was detected in both wild-type and Krt12^{rtTA/rtTA} knock-in mice eyes, with and without corneas, but rtTA was detected only in the Krt12^{rtTA/rtTA} knock-in cornea.

**Genotyping for Krt12^{rtTA/rtTA} Knock-in Mice**

Routine, Krt12^{rtTA/rtTA} knock-in mice were identified by PCR with the following primers: forward Krt12-1 (primer 1): 5’-GTTGTGGCCGCTGGCATCCCAATC-3’; Neo 781-803 + (primer 2): 5’-CGCCCTTGGAGATTCTTCTG-3’, and reverse Krt12-2 (primer 3): 5’-GATCTGGGGTTGATCTGGGTTG-3’.
CAATGAAAGAC-3' (Fig. 1B). The tail DNA of experimental mice was subjected to PCR: denaturation at 94°C for 5 minutes, 35 cycles of amplification (30 seconds at 94°C, 30 seconds at 64°C, and 45 seconds at 72°C), followed by a 5-minute final extension step at 72°C. PCR products were analyzed by agarose gel electrophoresis.16

Animals
Animal care and use conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Cincinnati.

Krt12rtTA/+ knock-in mice were crossed to tetO-LacZ mice (FVB/N-tg; tetOR01-LacZ; Jackson Laboratory, Bar Harbor, ME), which express β-galactosidase under the control of the tet operon (tetracycline-responsive elements) via the binding of tetracycline transcription activator (tTA) and reverse tetracycline transcription activator (rtTA), in the absence and presence of doxycycline (a derivative of tetracycline), respectively.17,18 The presence of a 421-bp fragment from tetO-LacZ in the transgenic mice was verified by PCR of tail DNA with the primer pair: 5'-GGC GTG TAC GGT GGG AGG-3' and 5'-GGT GGG AAG GAG GAT CGG-3'.19

Doxycycline Treatment
Mice were subjected to systemic induction by including doxycycline in drinking water (1%) and chow (1 g/kg). Because of the light sensitivity of doxycycline, doxycycline-containing cage water was freshly prepared and replaced twice a week.

Western Blot Analysis
To isolate rtTA and keratin 12 proteins, frozen corneas and the other eye tissues without corneas were first homogenized in RIPA buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% TritonX-100, 1% sodium deoxycholate, 0.1% SDS, 1× proteinase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and 100 mM phenylmethlysulfonyl fluoride (PMSF) at 4°C. The homogenate was centrifuged at 14,000 rpm for 30 minutes. The supernatant was mixed with an equal volume of 2× SDS sample buffer, boiled for 5 minutes, and electrophoresed on an SDS-polyacrylamide gradient (4%-20%).20

For Western blot analysis, proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride (PVDF) membranes by using a semidry blotting apparatus. After they were blocked with 0.1% casein in 0.2× PBS, the membranes were incubated with rabbit anti-VP16 antibody (BD-Clontech) for rtTA and rabbit anti-K12N antibody for keratin 12 protein,21 followed by goat anti-rabbit IgG IRDye 800 conjugate (Rockland, Inc., Gilbertsville, PA). The protein bands were visualized and analyzed (Odyssey imaging system; LI-COR Biosciences, Lincoln, NE).

Wholemount X-gal Staining for β-Galactosidase Activity
Embryonic and adult tissues were subjected to wholemount histochemistry to determine LacZ expression patterns in situ. Briefly, tissues were fixed in 4% paraformaldehyde at 0.1 M PB at 4°C for 2 hours and washed three times with PBS at 4°C.22 The specimens were then incubated in a solution containing 5-bromo-4-chloro-3-indolyl-β-d-galactoside (X-gal; 1 mg/mL made from a 100-mg/mL stock in dimethylformamide; Sigma-Aldrich), 2 mM MgCl2, 0.02% NP-40, 0.01% sodium deoxycholate, 5 mM K3Fe(CN)6, and 5 mM K4Fe(CN)6 in 0.1 M PB (pH 7.5) at 37°C for 2 hours. After staining, tissues were rinsed with PBS and photographed with a stereomicroscope. The specimens were then further fixed in 4% paraformaldehyde at 4°C overnight and embedded in paraffin. Sections (5 μm) were deparaffinized, counterstained with neutral red (1%), and examined.

β-Galactosidase Enzyme Activity Assay
To measure β-galactosidase enzyme activity, the tissues were homogenized in 0.25 M Tris-HCl (pH 8.0) with 0.1% Triton X-100 at 4°C (20 mL/g tissue). Cell debris was removed by centrifugation with a microfuge at 4°C. The protein concentration was determined with a spectrophotometer at 280 nm. Fifty microliters of tissue extract was mixed with 50 μL of a solution containing 0.2 M sodium phosphate buffer (pH 7.3), 2 mM MgCl2, 100 mM β-mercaptoethanol, and 1.33 mg/mL orthonitrophenyl-β-D-galactopyranoside (ONPG, Sigma-Aldrich). The reaction mixtures were incubated for 2 hours at 37°C. The enzyme activity was determined by measuring optical density at 420 nm with a spectrophotometer and extrapolating the values to a standard curve generated from serially diluted pure β-galactosidase (Promega, Madison, WI).22

RESULTS

Generation of Krt12rtTA/+ Knock-in Mice
To achieve corneal-epithelium–specific expression of genes of interest in a doxycycline-dependent manner, we generated the Krt12rtTA/+ knock-in mouse line by using the knock-in gene-targeting strategy described in the Methods section (Fig. 1A). The germline chimeras were bred with Swiss Black females, and the genotypes of the offspring were identified by PCR, as shown in Figure 1B.

The insertion of IRES-rtTA right after the stop codon in exon 8 of Krt12 allele results in the synthesis of a bicistronic mRNA, which leads to the simultaneous production of keratin 12 and rtTA proteins (Fig. 1C).

Temporal–Spatial Expression Pattern of Keratin 12
To determine the functionality of the rtTA minigene in the modified Krt12 allele, it was imperative to characterize the temporal–spatial expression patterns of Krt12 during corneal development. We had demonstrated that keratin 12 expression is upregulated at postnatal day 4.10 In situ hybridization was used for further examination of keratin 12 mRNA expression during embryonic development. Figure 2 demonstrates that the keratin 12 mRNA was detected in the superficial layer of corneal epithelium at embryonic day (E)14.5 and peaked between E15.5 and E16.5. The observation supports the hypothesis that the two-cell-layered corneal epithelium derived from ectoderm does not differentiate to express K12 at E12.5, but at E14.5, at which time only the superficial cells of the corneal epithelium differentiate and begin to express K12. At later embryonic stages, the expression of keratin 12 decreases and remains at a low level until postnatal day 4. Immunofluorescent staining with anti-K12 antibodies confirmed the presence of keratin 12 at the superficial corneal epithelial cells at E16.5 (Fig. 2).

Induction of LacZ Expression during Corneal Development of Bitransgenic Mice
At E15.5, β-galactosidase was detected in the superficial layer of the corneal epithelium of bitransgenic Krt12rtTA/+;tetO-LacZ mouse embryos under doxycycline induction (Fig. 3). The result is consistent with the keratin 12 expression pattern during embryonic development as just shown, in that only
superficial cells of the two-cell–layered corneal epithelium expressed β-galactosidase. To determine the temporal pattern of LacZ expression on doxycycline induction in adult mice, β-galactosidase activity in the cornea was determined by wholemount histochemical staining with X-gal and corneal extracts with ONPG. Figure 4 shows wholemount X-gal staining of the eyes of 2- to 3-month-old bitransgenic and single transgenic mouse eyes with or without doxycycline induction. Bitransgenic mouse eyes exhibited an increase of β-galactosidase activity with time after continuous doxycycline induction. β-Galactosidase was detectable after 24 hours of doxycycline induction. The β-galactosidase expression levels reached a plateau within 2 days of induction, as determined by the wholemount X-gal staining pattern observed under a stereomicroscope. A similar result was observed by determining the enzyme activity of corneal extracts with ONPG. Figure 5 demonstrates that, in the absence of doxycycline, the level of β-galactosidase activity was similar to that of single transgenic mice. Within 24 hours of induction by doxycycline, there was a significant increase in enzyme activity of the bitransgenic cornea that reached a plateau in 2 days, as judged by the finding that there was no statistically significant difference among the corneas at 2, 7, and 14 days after doxycycline induction. On removal of doxycycline, a decrease in β-galactosidase activity in the cornea was observed, and the enzyme activity returned to the basal level after 28 days. To identify the cell type that expresses β-galactosidase, mouse eyes were paraffin embedded, and 5-μm sections were prepared and examined. Histologic examination by hematoxylin and eosin staining did not reveal any changes of corneal epithelium in bitransgenic mice fed doxycycline. β-Galactosidase-positive cells were observed only in the corneal epithelium (Fig. 4). In Figures 3 and 4, the corneal epithelium of bitransgenic mice exhibited a variegated pattern of X-gal-positive cells, even at the highest induction with doxycycline (at 7 days). Of note, the intensity of X-gal staining was stronger in superficial cells than in basal cells. The intensity of X-gal staining declined and returned to basal levels at 28 days after the removal of doxycycline from the drinking water and chow.
Tissue-Specific Transactivation of LacZ in Bitransgenic Mice Induced by Doxycycline

To analyze further the tissue specificity of target gene activation by doxycycline induction, the β-galactosidase activity assay was performed in different tissues of 2- to 3-month-old transgenic mice. After 2 weeks of doxycycline induction, Krt12rtTA/H11001/tet-O-LacZ bitransgenic mice expressed β-galactosidase in the cornea that was approximately 15-fold higher than levels in single transgenic and bitransgenic mice without doxycycline. The level of β-galactosidase expression in the non-K12 keratin-expressing tissues (e.g., eyeball without cornea, skin, tongue, stomach, lung, heart, liver, and kidney), regardless of doxycycline induction, was similar to the background levels found in the single transgenic animals with doxycycline (Fig. 6). Histochemical examination of X-gal staining failed to detect any increased β-galactosidase activity in other tissues (i.e., skin, tongue, stomach, lung, heart, liver, and kidney; data not shown).

**FIGURE 4.** In situ analysis of β-galactosidase enzyme activity induction by doxycycline in corneas of Krt12rtTA/+;tet-O-LacZ bitransgenic mice. Stereomicroscopic side-view (top images) of each eye after whole-mount β-galactosidase staining. Histologic examination (bottom images) of the same samples revealed that the β-galactosidase expression was restricted to corneal epithelium. Corneal epithelial cells began to express β-galactosidase in 24 hours after administration of doxycycline. The number of β-galactosidase-expressing cells in the bitransgenic mouse with doxycycline increased over the course of time. Not all corneal epithelial cells expressed β-galactosidase, even at the maximum level. BTg, bitransgenic mice; LacZ, tet-O-LacZ single transgenic mice.

**FIGURE 5.** β-Galactosidase activity in corneas of Krt12rtTA/+;tet-O-LacZ bitransgenic mice fed doxycycline. There was a significant increase in enzyme activity in the cornea within 24 hours of induction by doxycycline, which reached its peak by 7 days. There was no significant difference in induced enzyme activity in specimens after 2, 7, or 14 days of doxycycline induction. After 14 days of doxycycline exposure, the doxycycline was removed for 28 days, and the level of β-galactosidase activity in the cornea returned to the basal level, similar to that in bitransgenic mice without doxycycline induction. Each bar represents the average activity in extracts from four to six corneas ± SEM. BTg, bitransgenic mice; LacZ, tet-O-LacZ single transgenic mice.

**FIGURE 6.** Induced cornea-specific expression of LacZ in Krt12rtTA/+;tet-O-LacZ bitransgenic mice by doxycycline. β-Galactosidase activity was determined in tissue extracts from bitransgenic and tet-O-LacZ single transgenic mice. To induce transactivation, mice were fed doxycycline for 14 days. β-Galactosidase activities were determined in tissues extracts prepared from individual mouse genotypes. Some tissues (e.g., heart, liver, kidney) contained endogenous β-galactosidase. However, administration of doxycycline did not cause an increase in the enzyme's activity in such tissues. The data represent the average results in three mice ± SEM.
DISCUSSION

In the present study, by inserting the IRES-rtTA minigene cassette into the Krt12 allele via gene targeting, we established a binary doxycycline-inducible Krt12rtTA/+/tet-O-LacZ mouse model for the temporal–spatial expression of transgene under the control of the tet operator in the corneal epithelium. This binary mouse model provides an excellent tool for examining the consequences of overexpressing genes of interest involved in the maintenance of corneal homeostasis.

K12 expression was detected in the superficial cells of mouse corneal epithelium at E14.5, as shown in Figure 2 and previously by Kurpakus et al.26 β-Galactosidase-positive cells were detected at E15.5 in superficial cells of bitransgenic mouse corneal epithelium with doxycycline induction (Fig. 3). This result also demonstrates that sufficient amounts of doxycycline for induction of a tet-O reporter gene can cross the placenta, as previously reported.2,5 Thus, we can use this binary mouse model for analysis of genes during corneal development. Figures 4 and 5 also demonstrate a restricted expression pattern of β-galactosidase by doxycycline induction in adult bitransgenic mice. Furthermore, our results of β-galactosidase wholemount staining indicated that there was no detectable leaky expression of the enzyme by corneas of bitransgenic mice fed a normal diet and by those of tet-O-LacZ single transgenic mice fed doxycycline, both in utero and as adults (Figs. 3, 4). It is of interest to note that the induction of LacZ expression in bitransgenic mice was reversible, in that the removal of doxycycline from the diet led to the decline of β-galactosidase in 28 days, approximately two half-lives of epithelial cell lifespan in stratified corneal epithelium (Figs. 4, 5).24,25

Despite the success of this binary mouse model for induction of a valuable temporal–spatial expression of a reporter gene, we observed that the induced expression of a LacZ reporter gene in bitransgenic mice was not present in every cell throughout the entire corneal epithelium. In part, this observation can be explained by the possible allele-specific expression of the Krt12 gene during the differentiation of limbal stem cells.26,27 Similar allele-specific expression patterns have been observed in the expression of Cre recombinase by Krt12Cre+/- mice (Hayashi et al., unpublished observation, 2003). Thus, it is imperative to prepare Krt12rtTA/rtTA tet-O reporter mice for achieving maximum induction of tet-O reporter genes in corneal epithelium. This would be particularly important if a dominant negative mutant receptor were to be studied. It should also be cautioned that the use of the tet-O system has met with limited success because of several difficulties.28,29 Specifically with the tet-On system with rtTA, various problems have been reported, including mosaic induction due to random insertion of the transgene; no detectable transactivator expression, depending on the insertion site; and background leakiness of the reporter genes.30,31 Finally, it is possible that the expression of LacZ is epigenetically inactivated in some cells.32 Despite some limitations, our Krt12rtTA/rtTA knock-in mouse alleviated many pitfalls of producing corneal epithelium-specific transgene expression, by using an Krt12 promoter and/or other corneal-epithelium-abundant genes (e.g., aldehyde dehydrogenase and transketo- lase [TKT]), and it showed effective conditional gene expression in the corneal epithelium. We anticipate that this knock-in mouse will be a useful tool for analysis of various genes involved in corneal development or wound healing.

References


