

Efficient induction of productive Cre-mediated recombination in retinal pigment epithelium

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Purpose: To dissect gene functions in the retinal pigment epithelium (RPE), we previously generated a tetracycline-inducible RPE-specific Cre mouse line. Although this Cre mouse line was useful for several conditional gene targeting studies that were conducted by different laboratories, its potential has not been fully exploited, presumably due to a lack of knowledge or procedure for inducing Cre expression appropriately in this mouse line. The goal of the current study is to establish a procedure that will improve the reproducibility of Cre-mediated recombination in this mouse line.

Methods: Analysis of Cre expression and function was performed in double transgenic mice derived from inducible RPE-specific Cre and Cre-activatable *ROSA26 lacZ* reporter mice. A tetracycline derivative, doxycycline, was supplied to mice intravitreally to induce Cre expression. Cre expression and function were examined with reverse transcription-PCR, immunoblotting, immunostaining, and in situ enzymatic assay for β -galactosidase. Retinal integrity was examined with electroretinography and morphometry.

Results: Intravitreal Dox injection elevated Cre expression significantly and resulted in productive Cre-mediated recombination in approximately 60% of the RPE cells in this mouse line with no apparent change in retinal integrity.

Conclusions: Our results suggest that productive Cre-mediated recombination in this mouse line can be induced efficiently with intravitreal Dox delivery, with no apparent Dox or Cre toxicity. Therefore, our inducible RPE-specific Cre mice are suitable for Cre/*lox*-based gene activation and inactivation in adult RPE, which is critical to the effectiveness and suitability of this Cre mouse line in long-term studies requiring conditional gene targeting.

The RPE is a monolayer of cells between the choroidal circulation and the neural retina. In addition to serving as the outer blood-retina barrier, the RPE plays many specialized essential roles in the retina, including phagocytosis of photoreceptor outer segment discs, recycling of retinoids, and participating phototransduction (for review, see [1-4]). Loss of structural and functional integrity in the RPE is associated with many retinal and choroidal diseases, including the dry and wet forms of age-related macular degeneration and diabetic retinopathy [5-8], leading causes of blindness.

To dissect gene functions and to establish a genetic tool for conditional gene activation and inactivation in the RPE, we generated a temporal and spatial gene activation and inactivation system in the RPE using Cre/*lox* and tetracycline-inducible gene expression technologies [9-11]. In this system, an RPE-specific promoter, the promoter for *human vitelliform macular dystrophy-2 (Vmd2)* gene OMIM 607854, was used to drive the expression of reverse tetracycline-dependent transactivator (rtTA) in the RPE. In the presence of a tetracycline derivative, doxycycline (Dox), rtTA

was capable of binding the tetracycline-responsive element, which in turn, activated the expression of Cre recombinase in the RPE. This system was useful for some Cre-mediated conditional gene targeting studies that have been conducted independently by several laboratories [12,13]. However, its potential was not fully exploited, presumably due to a lack of knowledge and procedure for inducing Cre expression appropriately in this mouse line. Since intravitreal Dox delivery is likely to provide a higher level of inducer to the eye, we therefore reexamined the conditions for stringent induction of Cre-mediated recombination in inducible RPE-specific Cre mice. This report summarizes our recent effort to induce productive Cre-mediated recombination in this mouse line with intravitreal Dox injection, which will be beneficial to investigators in the field.

METHODS

Animal treatment: This study strictly followed the guidelines for the Use of Animals in Ophthalmic and Vision Research established by the Association for Research in Vision and Ophthalmology. All procedures were approved by the Institutional Animal Care and Use Committees at the University of the Oklahoma Health Sciences Center, Oklahoma Medical

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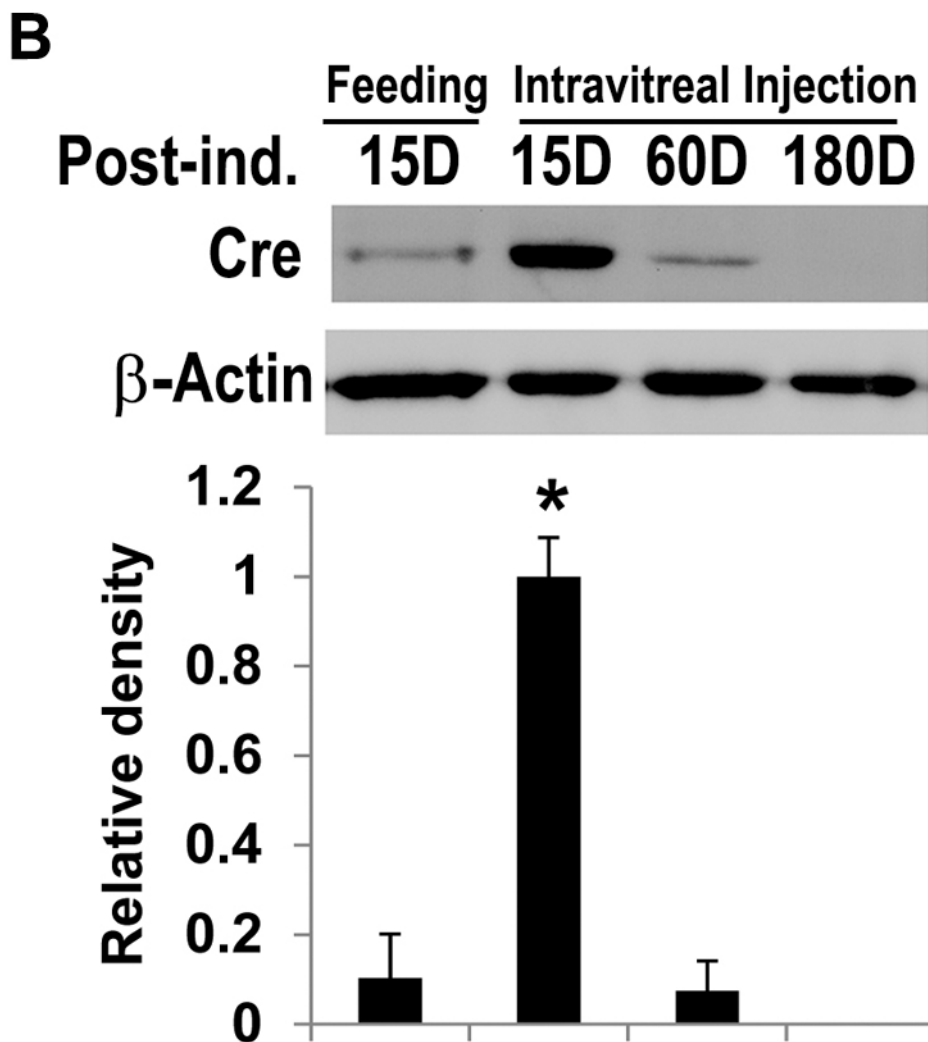
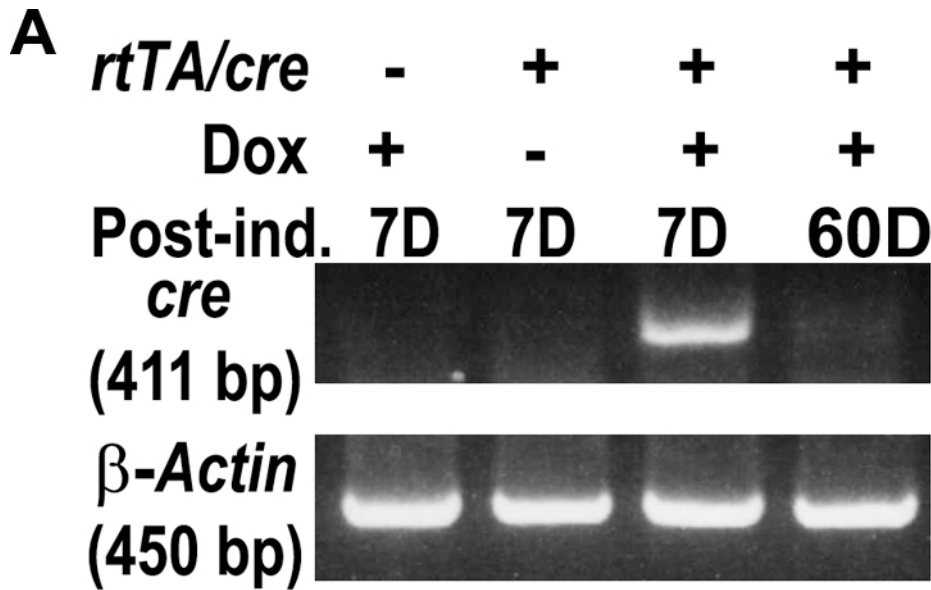


Figure 1. Induction of Cre expression in inducible RPE-specific Cre mice. **A:** *cre* mRNA induction was detected with intravitreally delivered doxycycline (Dox; 4 μ g in 1 μ l) with reverse transcription (RT)-PCR. Expression of *cre* mRNA was detected 7 days (7D) after induction and was diminished 60 days (60D) after induction. **B:** Western blot analysis shows relative amounts of Cre protein after induction by Dox feeding (daily gavage at a dose of 0.4 mg/g bodyweight for 2 days) or intravitreal Dox injection. Cre expression was robustly elevated 15 days after intravitreal injection, compared with that induced by feeding. Cre accumulation was reduced and diminished, 60 days or 180 days after intravitreal Dox induction, respectively. Error bar: standard deviation (SD); n = 3. *: p<0.01.

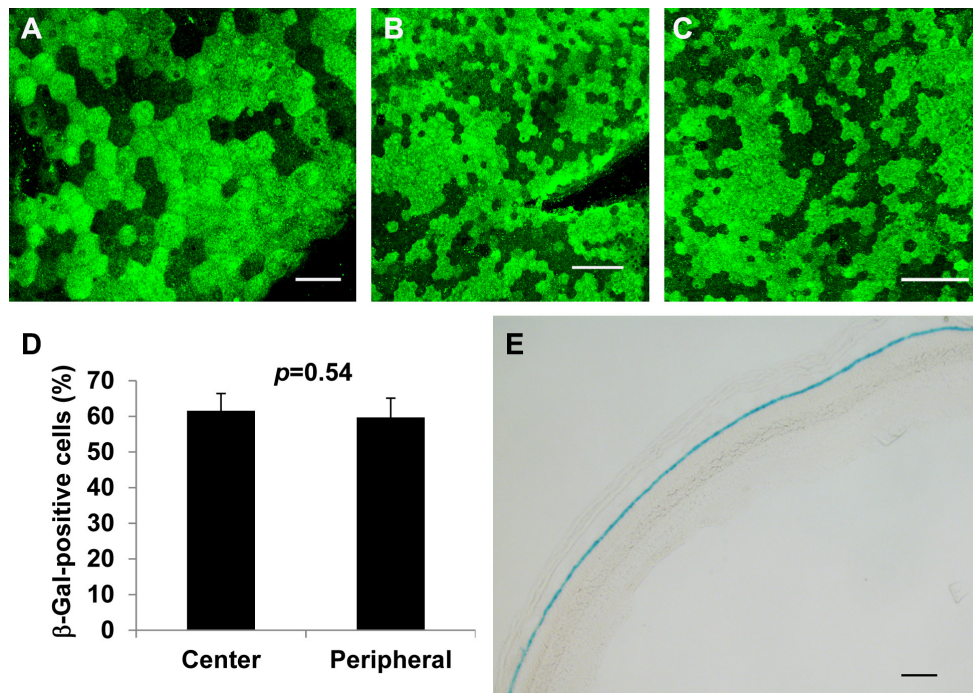


Figure 2. Analysis of productive Cre-mediated recombination by examining the frequency of β -galactosidase (β -gal)-expressing RPE cells in F1 mice derived from inducible RPE-specific Cre and *ROSA26 lacZ* reporter mice. **A–D**: Representative images and statistical analysis of immunostained RPE flatmounts for Cre-activated β -gal (green). **A**: An enlarged confocal image shows the number and frequency of β -gal-expressing RPE cells. Scale bar = 40 μ m. **B–C**: Representative images show the number and frequency of β -gal-positive cells in central (**B**) and peripheral RPE flatmounts (**C**). Scale bars = 100 nm. **D**: Statistical analysis with the Student *t* test shows that there was no significant difference in the frequency of

β -gal-positive RPE cells between the central and peripheral regions of the RPE. Error bar: standard deviation (SD), $n = 5–6$. **E**: Representative image shows homogenous Cre-activated β -gal activity in the retinal section of the Cre/ β -gal double transgenic mice. Scale bar = 100 μ m. Productive-Cre mediated recombination occurred evenly in approximately 60% of the RPE cells in inducible RPE-specific Cre mice after a single intravitreal doxycycline (Dox) injection.

Research Foundation, and Dean A. McGee Eye Institute. Double transgenic mice derived from inducible RPE-specific Cre and Cre-activatable *ROSA26 lacZ* reporter mice were used in analyzing Cre expression and function [14]. Genotyping for *rtTA*, *cre* transgene, and the Cre-activatable *lacZ* reporter gene was performed according to procedures described previously [11]. The tetracycline system inducer Dox (Sigma, St. Louis, MO) was delivered by either feeding (daily gavage feeding at a dose of 0.4 mg/g bodyweight for 2 days) or a single intravitreal injection. Intravitreal Dox (4 μ g in 1 μ l of 1X PBS formulation: 155 mM NaCl, 3 mM Na_2PO_4 , 1.06 mM KH_2PO_4 , pH 7.4) delivery was administered with a 33 gauge needle Hamilton syringe in post-weanling aged mice, after the mice were subjected to whole body and regional anaesthesia with intraperitoneally injected ketamine/xylazine (10 mg/kg-75 mg/kg body weight) and 0.5% alcaine eye drops, respectively. To prevent the induction of cataract and the disruption of the ocular structure, such as muscle and blood vessels, the needle puncture point was approximately 1 mm from the limbus, at a 45 degree angle. To avoid backflow of material, Dox was delivered at the exact preinjection site 1 min after the preinjection. Antibiotic ointment was administered to the eyes to prevent drying and infection while the

animals were recovering from the anesthesia. Animals with severe bleeding were excluded from the study.

Electroretinography: Electroretinography (ERG) was performed similarly according to our previous procedure [15,16]. Briefly, pupils were dilated with 0.5% tropicamide before the animals were kept in dark overnight. The dark-adapted mice were anesthetized with xylazine/ketamine (10 mg/kg-75 mg/kg body weight, intraperitoneal injection) and were placed on a heating pad to maintain body temperature. The corneal surface was anesthetized with proparacaine hydrochloride 1%. A Colordome Espion ERG recording system (Diagnosys, Lowell, MA) was used to measure photoreceptor function. Scotopic ERG was recorded with flashes intensities from 0.0004 to 2,000 $\text{cd}\cdot\text{s}/\text{m}^2$.

Analysis of gene expression and Cre-mediated recombination: Reverse transcription polymerase chain reaction (RT-PCR) analysis was performed according to our previous method [17]. Briefly, eyeballs were placed in liquid nitrogen immediately after dissection and were stored at -80°C . Before the RNA samples were prepared, the eye cups were dissected. RNA samples were prepared with TRIzol reagent (Life Technologies, Grand Island, NY). The first strand cDNA was made by random hexamer priming with the SuperScript

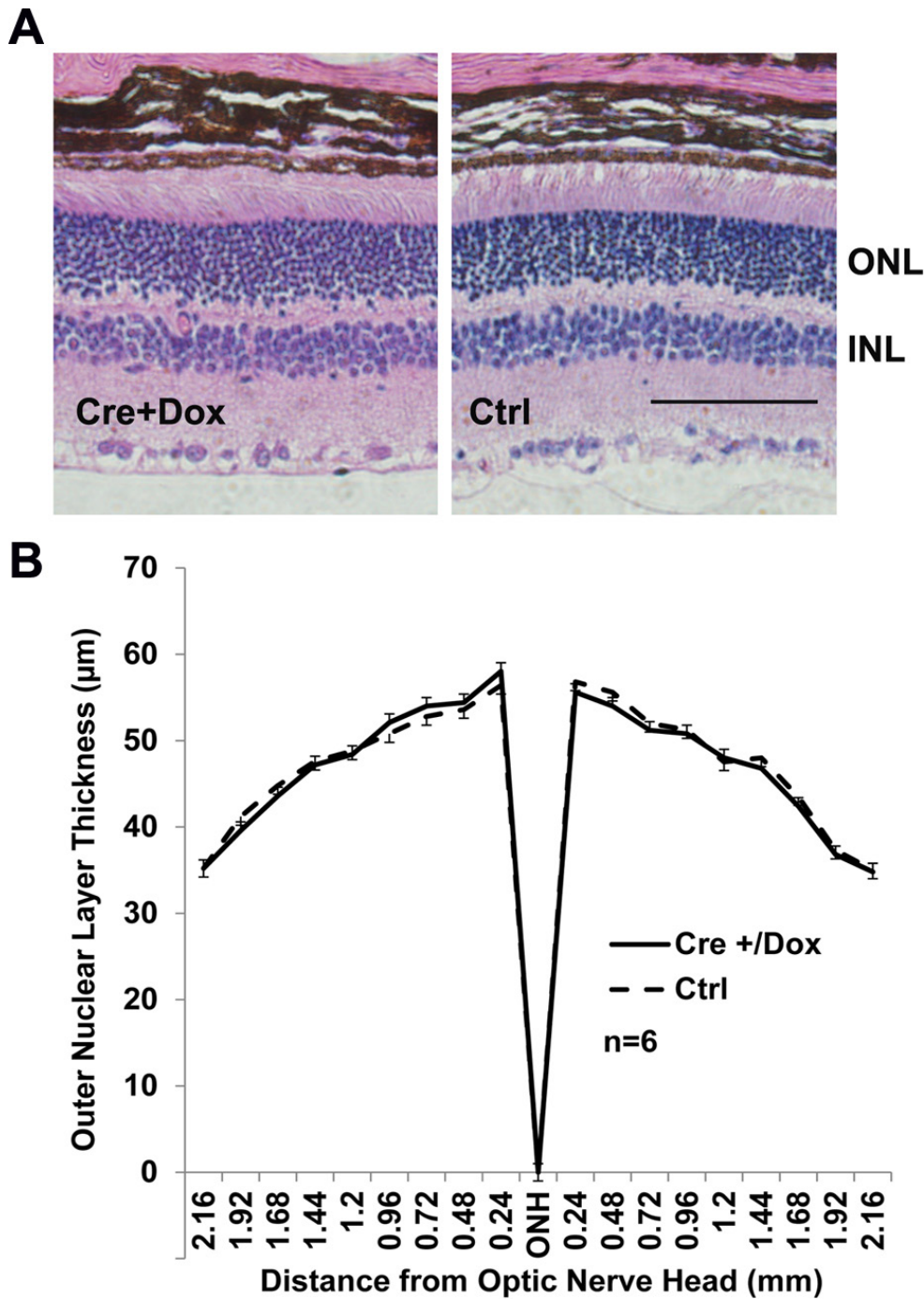


Figure 3. Retinal integrity of inducible RPE-specific Cre mice 12 months after intravitreal Dox injection (4 µg in 1 µl). **A**: Representative image of hematoxylin and eosin-stained retinal sections. Ctrl (control): age-matched wild-type littermate without Dox injection. Scale bar = 100 µm. **B**: Statistical analysis with the Student *t* test for photoreceptor outer nuclear layer (ONL) thickness. ONH: Optic nerve head. Error bar: standard deviation (SD); n = 6. No statistical significance was identified at any data point. Intravitreal Dox injection did not cause changes in retinal morphology in inducible RPE-specific Cre mice 12 months after induction.

first strand synthesis system for RT-PCR (Life Technologies) after brief treatment of RNA samples with amplification grade DNase I (Life Technologies), according to the manufacturer's instruction. The 411-bp *cre* mRNA was detected with primers (5'-AGG TGT AGA GAA GGC ACT TAG C-3') and (5'-CTA ATC GCC ATC TTC CAG CAG G-3'), and the 450 bp β-actin mRNA was detected with a primer pair (5'-GAC GAG GCG CAG AGC AAG AGA GG-3' and 5'-CTC TTT

GAT GTC ACG CAC GAT TTC-3'). The RT-PCR product was fractionated on 1.2% agarose gel, which was imaged with UVP Bioimaging System (Upland, CA).

Immunoblotting for Cre with dissected eye cups that contained RPE was performed with an antibody against Cre (Sigma), according to a previous procedure [18]. β-Galactosidase (β-gal) activity assay, which localized

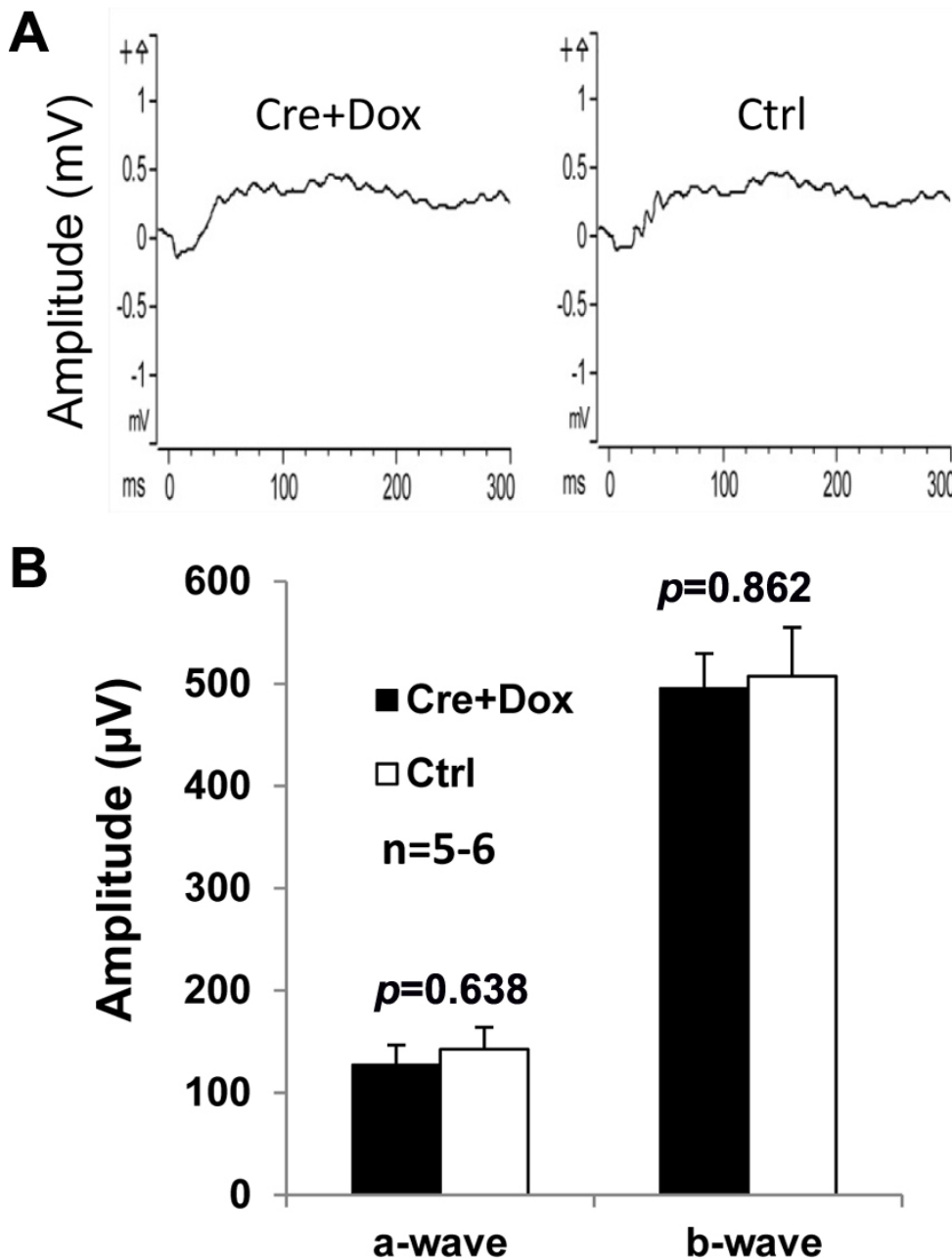


Figure 4. Retinal function in inducible RPE-specific Cre mice subjected to intravitreal Dox injection. **A:** Representative scotopic electroretinography (ERG) amplitudes (flash intensity: 200 cd·s/m²) in inducible RPE-specific Cre mice 10 days after intravitreal Dox injection (4 μ g in 1 μ l). Ctrl: no Dox control. **B:** Statistical analysis with the Student *t* test. Error bar: standard error of the mean (SEM); $p = 0.638$ (a-wave); $p = 0.862$ (b-wave). No significant change in retinal function was detected in inducible RPE-specific Cre mice 10 days after intravitreal Dox injection.

productive Cre-mediated recombination in retinal sections, was performed according to our previous procedure [11]. Immunostaining for Cre-activated- β -gal on the dissected eye cup was performed with an anti- β -gal antibody (5 Prime-3 Prime, Boulder, CO), according to our previous method [18]. Images were captured with epifluorescent or confocal microscopy. The efficiency of Cre-mediated recombination in a particular mouse was calculated by averaging the ratio of Cre-positive cells versus total RPE cells from three randomly selected areas (approximately 90° to 120° apart)

with approximately 30–50 cells in each window. The defined windows in the middle between the optic nerve and one third distance to the edge of the eye cups were deemed the center areas. Likewise, those in the middle between the edge of the flatmounts and two thirds distance to optic nerves were defined as peripheral areas.

Statistical analysis: All results were plotted as mean \pm standard deviation (SD) or standard error (SEM). Statistical comparison between two samples was performed with the Student *t* test. Statistical analysis in a group with more than two

samples was performed with one-way ANOVA. P value <0.05 was considered significant.

RESULTS

Induction of Cre expression with intravitreal Dox injection:

To determine the effect of Dox induction on Cre expression, we performed RT-PCR analysis on eye cups (containing the RPE) from inducible RPE-specific Cre mice. The *cre* mRNA was detected 7 days after intravitreal Dox injection (4 μ g in 1 μ l) and was diminished in 2 months in the inducible RPE-specific Cre mice (Figure 1A). Western blot analysis demonstrated that relatively robust Cre expression was induced, at a significantly higher level than that from induction by feeding, 15 days after intravitreal Dox injection in the eye cups of the inducible RPE-specific Cre mice (Figure 1B). The level of Cre protein was reduced dramatically 2 months after the intravitreal Dox injection, and Cre was not detectable after another 4 months (Figure 1B).

Efficiency of productive Cre-mediated recombination: To assess whether the intravitreal Dox injection-elevated Cre expression had an effect on the efficiency and reproducibility of productive Cre-mediated recombination, which is the key to the success of conditional gene activation or inactivation, we analyzed the expression of β -galactosidase (β -gal) in double transgenic mice carrying *cre* and Cre-activatable *lacZ* reporter. In ten animals (from three different litters) examined, all demonstrated homogenous Cre-activated reporter activity, as judged with the β -gal staining assay (Figure 2E). To estimate the efficiency of the inducible Cre-mediated recombination, we examined the number of β -gal-positive cells in RPE flatmounts in *cre/lacZ* double transgenic mice subjected to intravitreal Dox injection (4 μ g in 1 μ l). This information represented the number of RPE cells that had undergone productive-Cre mediated recombination. Approximately 60% of the RPE cells were β -gal-positive, with no statistical difference between the central (61.6 \pm 4.8%) and peripheral (59.7 \pm 5.4%) regions of the RPE layer (Figure 2A–D). In some patch areas, β -gal-positive cells reached 100%, suggesting almost all RPE cells in these areas had productive Cre-mediated recombination.

Retinal integrity: Since our ultimate goal in this line of work is to perform conditional gene targeting in the RPE effectively, it was necessary to determine if there was any loss of retinal integrity in the inducible RPE-specific Cre mice after the intravitreal Dox injection. The retinal morphology in inducible RPE-specific Cre mice appeared normal 12 months after intravitreal Dox injection (Figure 3A). We then further analyzed the thickness of the photoreceptor outer nuclear layer (ONL), a representation of photoreceptor integrity that

was mainly supported by healthy RPE cells. No significant difference in ONL thickness was identified in these mice (Figure 3B), suggesting that the brief Cre induction by intravitreal Dox injection was not harmful to the retina. To determine whether intravitreal Dox injection had a short-term effect on the retinal integrity, we examined retinal function in inducible RPE-specific Cre mice with ERG. Intravitreal Dox injection did not alter scotopic ERG amplitudes in inducible RPE-specific Cre mice 10 days after intravitreal Dox injection (Figure 4).

DISCUSSION

Induction of Cre expression and productive Cre-mediated recombination: Although a productive Cre-mediated recombination event requires only four Cre molecules [19], which is well below the detection level of conventional methods, it is known that the efficiency of Cre-mediated recombination at some chromosomal loci may be lower. This is likely due to the difficulty for Cre to access some site-specific recombination loci. In this scenario, a higher level of transiently expressed Cre recombinase may be desirable. As transgenic mice associated positional effect may cause variable expression of transgenes among individual animals in a particular line [20], we observed variable levels of productive Cre-mediated recombination, induced by feeding or intraperitoneal (IP) injection, in our inducible RPE-specific Cre mice (data not shown). Effectiveness in inducing Cre expression requires a sufficient amount of the inducer, Dox, which is dictated by the limitation of the blood circulation with a half-life of approximately 10 h in mice [21]. Since a single IP injection with a high dosage of Dox (50 mg/kg bodyweight) resulted in a maximal serum Dox concentration of 2 μ g/ml [21], it was unclear whether our previous approaches were optimal for inducing Cre activity. To improve the inducibility of this *Cre/lox* system, we took advantage of the intravitreal delivery system. Intravitreal injection (4 μ g in 1 μ l) elevates the average ocular Dox concentration to approximate 250-fold of the maximal level in the bloodstream delivered by feeding or IP injection (assuming the diameter of a mouse eye is 3 mm). Since feeding or IP injection is dependent on the blood circulation to deliver Dox to the eye, it is reasonable to speculate that the relative Dox concentration delivered by intravitreal injection is likely much higher than that conferred by feeding or IP injection. Indeed, intravitreal Dox injection resulted in a significant increase in Cre expression, compared with that induced by feeding (Figure 1B). As a result, we also observed approximately 60% of the RPE had undergone productive Cre-mediated recombination, with patch areas reaching 100% (Figure 2B). This result reinforced our assumption that

intravitreal Dox delivery is advantageous over feeding or IP injection, which provided a higher level of the Dox inducer for the inducible Cre/lox system in the eye of the inducible RPE-specific Cre mice. This higher level of Dox is likely to reach the required concentration threshold for the inducible system and, therefore, will reduce the variation in reproducibility of productive Cre-mediated recombination. Since Cre expression rarely reached 100% of the targeted cells in transgenic mice [22], the efficiency (approximately 60%) of intravitreal Dox induced productive Cre-mediated recombination in the inducible RPE-specific Cre mice will be suitable for most conditional gene activation or inactivation studies in general.

Retinal integrity: In this study, we performed a single intravitreal Dox injection (4 µg in 1 µl) to induce Cre expression in inducible RPE-specific *cre* mice. This procedure did not cause any apparent loss of retinal integrity (Figure 3 and Figure 4), which is in agreement with a previous result that injecting 4 µg Dox intravitreally was not harmful to the eye [23]. In our previous characterization, we did not observe any abnormality in retinal integrity 10 months after a brief Dox induction through feeding in inducible RPE-specific Cre mice [11]. This result also suggests that inserting the *cre/rtTA* transgene cassette did not cause any apparent loss of retinal integrity in this mouse line. Cre is a site-specific DNA recombinase, and Cre overexpression has been shown to cause chromosomal rearrangements in mammals [24,25]. In the mouse retina, Cre overexpression in rod photoreceptors causes retinal degeneration [26,27]. Constitutive overexpression of Cre may affect RPE integrity [28]. Since our ultimate goal in the current work is to establish a genetic system for Cre/lox-based conditional gene targeting in the RPE, an inducible approach, which triggers a burst of Cre expression transiently (Figure 1B), is likely to avoid any potential Cre-toxicity. To ascertain whether a single intravitreal Dox delivery did not result in the accumulation of a high level of Cre for a long time, we analyzed the level of Cre protein at various time points after the Dox injection. Our result suggested that 2 months after intravitreal Dox injection the Cre level is reduced to that in a Dox feeding experiment (Figure 1B), which had not caused any loss of retinal integrity in our hands previously [11]. Moreover, the fact that the Cre protein was not detectable 6 months after the intravitreal Dox injection provided more assurance for the successful use of this inducible RPE-specific Cre mouse in conditional gene targeting.

Concluding remarks: Although tremendous effort has been made by laboratories around the world to generate temporal or/and spatial gene targeting tools for the RPE [11,29-31], to our knowledge there is no “ideal” system for RPE-specific

gene targeting at this time. We therefore redefined a condition for more effective induction of productive Cre-mediated recombination in the inducible RPE-specific Cre mice. Our work provides a way for more effective use of this mouse line, beyond the knowledge of our previous publication [11]. However, other approaches capable of increasing the efficiency of productive Cre-mediated recombination may permit equal or better utilization of this mouse line. The end-users should explore these avenues that fit their individual experimental goals.

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