



## Technical Brief

# Mouse opsin promoter-directed Cre recombinase expression in transgenic mice

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**Purpose:** Gene inactivation with homologous recombination in mice is a widely used tool to study gene function. However, many proteins play essential roles in a number of tissues and germline gene inactivation often results in embryonic lethality. To overcome this limitation and to dissect the functions of essential genes beyond embryonic development, we generated mouse rod opsin promoter-controlled *cre* transgenic mice with a goal of obtaining transgenic lines with a range of Cre activity in rod photoreceptors.

**Methods:** Transgenic mice expressing Cre recombinase directed by a long or short mouse opsin promoter were generated. Candidate Cre-expressing lines were identified with RT-PCR and Western blot analysis. Potentially useful Cre-expressing lines were characterized further with immunohistochemistry, PCR, and functional analysis using a Cre-activatable *lacZ* reporter mouse strain (R26R) to determine temporal and spatial patterns of Cre expression. Retinal function and morphology in these mouse lines were analyzed with electroretinography (ERG) and light microscopy of hematoxylin and eosin stained retinal sections.

**Results:** Transgenic mice expressing Cre in rod photoreceptors were generated. Characterization of candidate photoreceptor-specific Cre mice using immunohistochemistry and functional assays demonstrated that an efficient Cre-mediated recombination occurred in rod photoreceptor cells in one mouse line and a mosaic Cre-mediated recombination occurred in rod photoreceptors and rod bipolar cells in another mouse line. Further analysis of these mice with ERG and morphological examination suggested that the retinas of eight-month-old adults were normal.

**Conclusions:** We have generated transgenic mice expressing Cre recombinase in rod photoreceptors. One transgenic mouse line was capable of carrying out efficient Cre-mediated recombination in rod photoreceptors. Another transgenic mouse line was capable of carrying out mosaic Cre-mediated recombination in rod photoreceptors and bipolar cells across the whole retina. These mice will be useful tools for *Cre/lox*-based gene activation and inactivation, as well as genetic mosaics, in rod photoreceptors and rod bipolar cells.

Gene targeting with homologous recombination in murine embryonic stem (ES) cells has generated much information in elucidating gene function and pathophysiology of human diseases. However, disruption of essential genes that are involved in multiple tissues often causes embryonic and neonatal lethality. This problem obscures the particular role of genes in a target tissue or in the adult, and can be overcome by using a conditional gene disruption strategy that only disrupts a gene of interest in a target tissue or at a particular time. Owing to its efficiency, the *Cre/lox* recombination system has become a widely used approach in conditional gene expression. The Cre recombinase of bacteriophage P1 is a 38 kDa protein that catalyzes site-specific DNA recombination between the target 34 bp *loxP* sites and is proficient in performing recombination in mice [1,2]. The *Cre/lox*-based gene disruption is based

on the site-specific recombination between adjacent *loxP* sites that results in the deletion of the intervening sequence. This strategy often requires two genetic components: a target mouse carrying a *loxP*-flanked gene to be disrupted and a transgenic mouse that expresses Cre under the control of a temporal and/or spatial promoter. When these two components are combined in one mouse, the *loxP*-flanked gene will be deleted in Cre-expressing cells in a temporal or spatial fashion [3,4].

Rods make up 97% of the photoreceptors in mice [5] and the rod-dominant mouse retina is an ideal model for biochemical, morphological, and phenotypic characterization of essential gene functions in the retina. We are interested in the roles of phosphoinositide signaling, glucose transport and metabolism, and neuro-protection in the retina. Establishment of transgenic lines that express Cre with a range of expression levels and recombination efficiencies in rod photoreceptors is a key to the successful dissection of the roles of essential genes in the retina. This report describes the generation and characterization of a short (0.2 kb) mouse opsin promoter-controlled *cre* (SMOP-*cre*) mouse line and a long (4.1 kb) mouse opsin-

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promoter controlled *cre* (LMOP-*cre*) mouse line. These mice are available for noncommercial research and can be obtained by contacting the corresponding author.

## METHODS

The use of animals in this study conformed to the guidelines established by the ARVO statement for the "Use of Animals in Ophthalmic and Vision Research" and was approved by the Institutional Animal Care and Use Committees of the University of Oklahoma Health Sciences Center, the Dean A. McGee Eye Institute, and the Oklahoma Medical Research Foundation.

**Construction of a photoreceptor-specific *cre* transgene:** The recombinant plasmid pLE103 carrying a short (0.2 kb) opsin promoter-controlled *cre* transgene (SMOP-*cre*) was constructed from plasmids pBS185 [2] and pMOPS [6]. The recombinant plasmid pLE111 carrying a long (4.1 kb) opsin promoter-controlled *cre* transgene (LMOP-*cre*) was constructed from pBS185 [2] and pRG3 [7]. Briefly, the 0.2-kb mouse opsin promoter on pMOPS was inserted in front of *cre* in the pBS185 to generate SMOP-*cre* plasmid pLE103 (Figure 1A). The 4.1-kb mouse opsin promoter on pRG3 was inserted in front of *cre* in the pBS185 to generate LMOP-*cre* plasmid pLE111 (Figure 1A). Both plasmids contained *Hind*III restriction sites flanking the transgene that carries the short or the long mouse opsin promoter, a translationally optimized *cre* [8], and an intron containing mouse metallothionein (MT-I) polyadenylation signal (Figure 1A). Junctions between different parts of the transgene were confirmed by DNA sequencing.

**Generation and genotyping of transgenic mice:** Cesium chloride double centrifugation was used to prepare the plasmid DNA carrying the transgene. The purified plasmid DNA was digested with the restriction enzyme *Hind*III to isolate the transgene (Figure 1A). The digested DNA was fractionated on an agarose gel and purified with Qiagen gel extraction kit (Valencia, CA). The purified DNA was used in zygote injection with FVB/N background mice at the Oklahoma Medical Research Foundation Microinjection Core Facility. PCR diagnostic for *cre* transgene was performed according to the procedures described by Le and Sauer [4] using primers (a) 5'-AGG TGT AGA GAA GGC ACT TAG C-3' and (b) 5'-CTA ATC GCC ATC TTC CAG CAG G-3' to detect a 411 bp product. Further confirmation of the presence of the transgene was performed with Southern hybridization using a 0.5-kb *cre* DNA fragment, according to an established procedure [9]. PCR detection of the Cre-activatable *lacZ* reporter gene in R26R mice was performed according to an established procedure [10] with primers (c) 5'-GAG TTG CGT GAC TAC CTA CGG-3' and (d) 5'-GGC TTC ATC CAC CAC ATA CAG G-3' to detect a 495 bp product. PCR detection of Cre-mediated *lacZ* reporter gene activation in the R26R mouse genomic DNA was performed using primers (e) 5'-AAA GTC GCT CTG AGT TGT TAT-3' and (f) 5'-CAT TCG CCA TTC AGG CTG CG-3' to detect a 710 bp product (25 to 30 cycles at 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 60 s). PCR diagnostic for an internal DNA control,  $\beta$ -actin, was performed according to an es-

tablished procedure [10] using primers (g) 5'-GAC GAG GCG CAG AGC AAG AGA GG-3' and (h) 5'-CTC TTT GAT GTC ACG CAC GAT TTC-3' to detect a 450 bp product.

**Reverse transcriptase polymerase chain reaction analysis:** RT-PCR analysis was performed similarly according to a documented procedure [10] using primer pairs (a) and (b), and (g) and (h) (described above) to detect a 411 bp *cre* and 450 bp  $\beta$ -actin transcripts, respectively.

**$\beta$ -Galactosidase assay:** The function of Cre in transgenic mice was analyzed with  $\beta$ -galactosidase ( $\beta$ -gal) staining assays using the retinas of F1 double transgenic mice derived from Cre and Cre-activatable *lacZ* reporter mice [11]. The  $\beta$ -gal assay was carried out on a retinal whole mount using an established method [12]. For  $\beta$ -gal assays on frozen retinal sections, genotyped mice were euthanized and a permanent dye was injected at the superior pole of the cornea to maintain orientation. The dissected eyes were fixed in 2% paraformaldehyde and 0.25% glutaraldehyde in phosphate-buffered saline (PBS) at 4 °C for 1 h. After the removal of lens and vitreous, the remaining part of the eyes was cryoprotected, mounted in OCT medium, and cut at 10  $\mu$ m on a cryostat. The sections were briefly fixed in 0.5% glutaraldehyde for 5 min, washed with PBS 3 times, and incubated overnight at room temperature in X-gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside) staining solution (1 mg/ml X-gal, 6 mM potassium ferricyanide, 6 mM potassium ferrocyanide, 2 mM MgCl<sub>2</sub>, 0.02% NP-40, and 0.01% sodium deoxycholate in 1X PBS). Sections were then cover-slipped and observed under a microscope.

**Immunohistochemistry and Western blot analysis:** For immunohistochemistry (IHC), frozen retinal sections were prepared as described previously [12]. The anti-Cre polyclonal antibody (Novagen, San Diego CA) was diluted 1:200 for IHC and 1:2500 for Western blotting. The anti-protein kinase C- $\alpha$  monoclonal antibody (Sigma, St. Louis, MO) was diluted 1:500 for IHC. The anti- $\beta$ -galactosidase polyclonal antibody (5 Prime-3 Prime, Boulder CO) was diluted 1:100 for IHC. The biotinylated peanut agglutinin (Vector Laboratories, Burlingame, CA) was diluted 1:500 for IHC. The anti- $\beta$ -actin monoclonal antibody (Affinity Bioreagents, Golden, CO) was diluted 1:1000 for Western blotting. The procedures for primary and secondary antibody incubation followed the conditions described previously [12].

**Quantification of Cre-expressing cells:** Quantification of Cre-expressing cells was performed by counting the number of Cre-positive or Cre-negative nuclei in confocal images of anti-Cre antibody stained retinal sections. To ascertain the accuracy of the assay, three confocal images in peripheral and central areas of a retina were used and each image contained approximately 500 or more rod nuclei. At least three mice in each group were used in the calculation. The ratio of Cre-positive cells was expressed as the percentage $\pm$ SD.

**Electroretinography and retinal morphology:** Overnight dark-adapted mice were used for scotopic and photopic electroretinography (ERG) with a UTAS-E 3000 ERG system (LKC technologies, Inc., Gaithersburg, MD), according to the conditions described previously [6,12,13]. For scotopic ERG, a strobe flash stimulus was presented to the overnight, dark-

adapted, dilated eyes in a Ganzfeld with a 138 cd x s/m<sup>2</sup> flash intensity. For photopic ERG, a strobe flash stimulus was presented to 5-min, light-adapted, dilated eyes in a Ganzfeld with a 79 cd x s/m<sup>2</sup> flash intensity. For retinal morphology, the eyes were dissected after euthanization and fixed at room temperature overnight in Perfix (4% paraformaldehyde, 20% isopropanol, 2% trichloroacetic acid, 2% zinc chloride), paraffin-embedded, sectioned (5 μm thickness), and stained with hematoxylin and eosin. The thickness of the outer nuclear layer (ONL) was measured at a distance of 240 μm from the optic nerve to the inferior and superior ora serrata. Differences were assessed with Student's t-test. p<0.05 was considered significant. All results requiring statistical analysis were expressed as the mean±SD.

**RESULTS & DISCUSSION**

*Generation of opsin promoter-controlled cre transgenic mice:* Previous studies have shown that a 4.1-kb or 0.2-kb mouse opsin promoter was capable of directing transgenic expression in rod photoreceptors [6,7]. However, indirect comparison of different lengths of mouse or bovine opsin promoters in transgenic studies suggested that the longer opsin promoter is likely to confer a more uniform transgenic expression in rod photoreceptors, while the short opsin promoter is likely to achieve a mosaic expression pattern [7,14]. With a goal of obtaining a range of Cre activity in the rod photoreceptors of mice, we generated transgenic mice with the 4.1-kb and the 0.2-kb mouse opsin promoters to direct Cre expression (Fig-

ure 1A). The 4.1-kb long mouse opsin promoter-controlled *cre* (LMOP-*cre*) and 0.2-kb short mouse opsin promoter-controlled *cre* (SMOP-*cre*) constructs were used in generating transgenic mice. The transgenic mice were identified by PCR analysis and confirmed with Southern blot hybridization (data not shown). The *cre*-positive mice were mated to obtain germline transmitted mice. All germline transmitted mice appeared normal in size, morphology, and behavior, and were screened for *cre* expression with reverse transcription (RT)-PCR using 7 to 15-day-old retinas (Figure 1B). Mice expressing *cre* mRNA were subjected to functional analysis using an existing Cre-activatable *lacZ* reporter mouse line R26R in C57B6 background [11]. Since the F1 double transgenic Cre/R26R mice did not have inherited retinal degeneration, all further characterization was performed using this type of mice. Our preliminary characterization of all candidate lines with the F1 double transgenic Cre/R26R mice indicated that one LMOP-*cre* (LMOPC1) and one SMOP-*cre* (SMOPC1) transgenic line had productive Cre-mediated *lacZ* reporter expression across the retina, as well as normal retinal morphology and function (data not shown, see detailed discussion below). These mice were characterized further. Cre expression at the protein level in these mice was analyzed by Western blotting using retinal homogenates from F1 double transgenic Cre/R26R mice, as shown in Figure 1C.

*Localization and functional analysis of Cre expression:* As discussed earlier, an existing Cre-activatable *lacZ* reporter mouse line R26R [11] was utilized to identify potentially use-

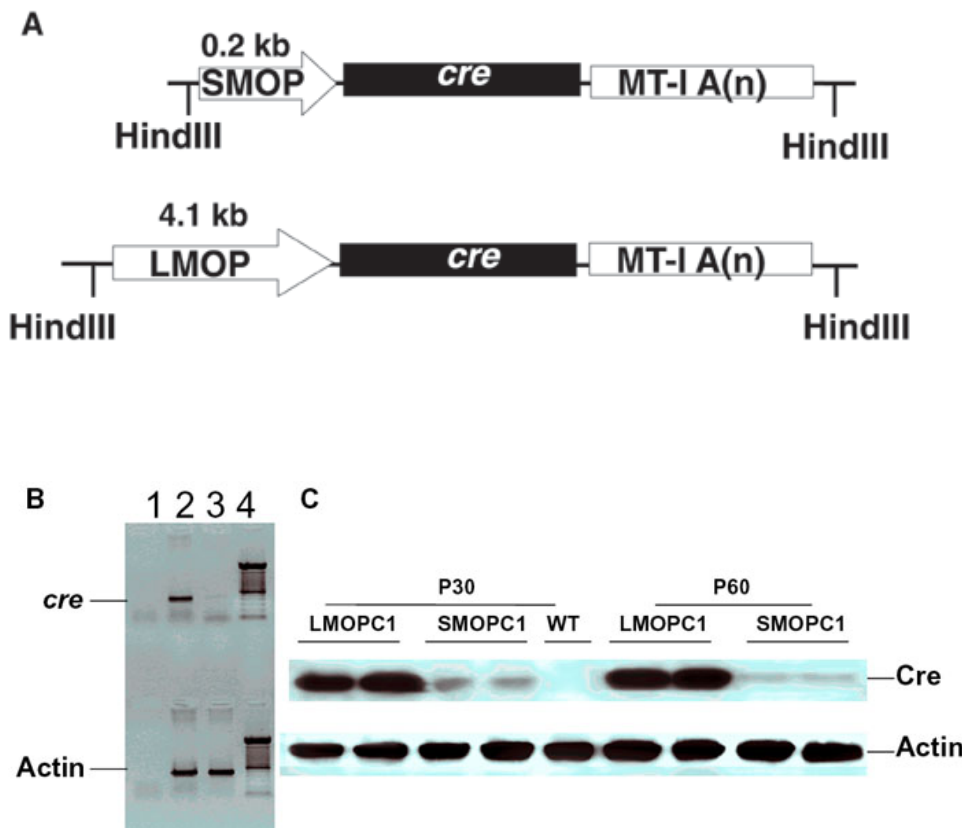


Figure 1. Generation of mouse opsin promoter-controlled *cre* mice. **A:** Schematic drawing of transgenic constructs. SMOP represents the 0.2-kb short mouse opsin promoter, LMOP represents the 4.1-kb long mouse opsin promoter, *cre* represents the coding region of Cre recombinase gene, and MT-1 A(n) represents mouse metallothionein polyadenylation signal. **B:** Inverted gel image of a representative RT-PCR analysis of *cre* expression in mouse retinas. Lanes 1 to 4: label negative control (-RT) of a *cre* positive mouse (1), RT-PCR of a *cre*-positive mouse (2), RT-PCR of a *cre*-negative mouse (3), and 100 bp DNA marker (4). **C:** Comparison of Cre expression between LMOPC1 and SMOPC1 mice with Western blot. Homogenized retinal proteins (30 μg) from two different mice were used for each group. LMOPC1 mice produced more Cre than SMOPC1 mice. No significant change in Cre concentration was observed from P30 to P60 in either the LMOPC1 mice or the SMOPC1 mice.



ful mouse lines. The R26R reporter mice carry a *loxP*-flanked transcriptional “STOP” sequence that prevents the transcription of the *lacZ* gene (Figure 2A). In the double transgenic *cre/lacZ* mice, this transcriptional “STOP” sequence can be removed only in cells expressing Cre. The  $\beta$ -galactosidase ( $\beta$ -gal) will then be expressed under the generalized promoter ROSA26. Thus, the Cre-expressing cells will produce a blue color using  $\beta$ -gal staining assay.

In the F1 double transgenic LMOPC1/R26R mice, a weak  $\beta$ -gal reporter expression was detected at postnatal day 7 (P7). Expression increased gradually until approximately six weeks of age when no further change was detected. Strong  $\beta$ -gal expression was localized to the outer nuclear layer where photoreceptors are located (Figure 2B,D,F). Immunohistochemistry (IHC) analysis of Cre expression in the retinal sections with an anti-Cre antibody suggested that Cre was mainly localized within the perinuclear cytoplasm and the membrane of the rod photoreceptor nuclei (Figure 3A), which supports our earlier observation that Cre carried a eukaryotic nuclear localization signal [15]. Confocal microscopic analysis of the retinal sections stained for Cre with an anti-Cre antibody and the cone photoreceptor marker stained with peanut agglutinin (PNA) showed that PNA-positive cones were not Cre positive (Figure 3D), suggesting that Cre was exclusively local-

ized to the rod photoreceptors. The onset of Cre expression in the LMOPC1 mice was also determined by PCR analysis diagnostic for Cre-mediated activation of *lacZ* reporter gene with the genomic DNA isolated from the F1 double transgenic retinas of LMOPC1/R26R mice. The PCR analysis suggested that Cre-mediated recombination was detected at P7 (Figure 4A), when a weak  $\beta$ -gal stain was observed in the retinal sections.

In the F1 double transgenic SMOPC1/R26R mice, a weak Cre-activated  $\beta$ -gal reporter expression was detected at P11 when Cre-mediated recombination at the DNA level was observed (Figure 4A). Expression increased gradually until approximately eight weeks of age, when no additional change was detected (Figure 2C,E,G). The expression was localized to the presumptive photoreceptor cells and a subset of cells in the inner nuclear layer across the whole retina (Figure 2E,G). However, the intensity of the  $\beta$ -gal staining in the rod photoreceptors from SMOPC1/R26R mice was lower than in the rod photoreceptors obtained from LMOPC1/R26R mice in a side-by-side comparison of age-matched retinal sections (Figure 2D-G), suggesting that the efficiency of Cre-mediated recombination in the SMOPC1 mouse line was relatively lower than in the LMOPC1 mouse line. This was indeed reflected in the lower level of immunoreactivity measured in a side-by-side assay with the same anti-Cre antibody used to localize

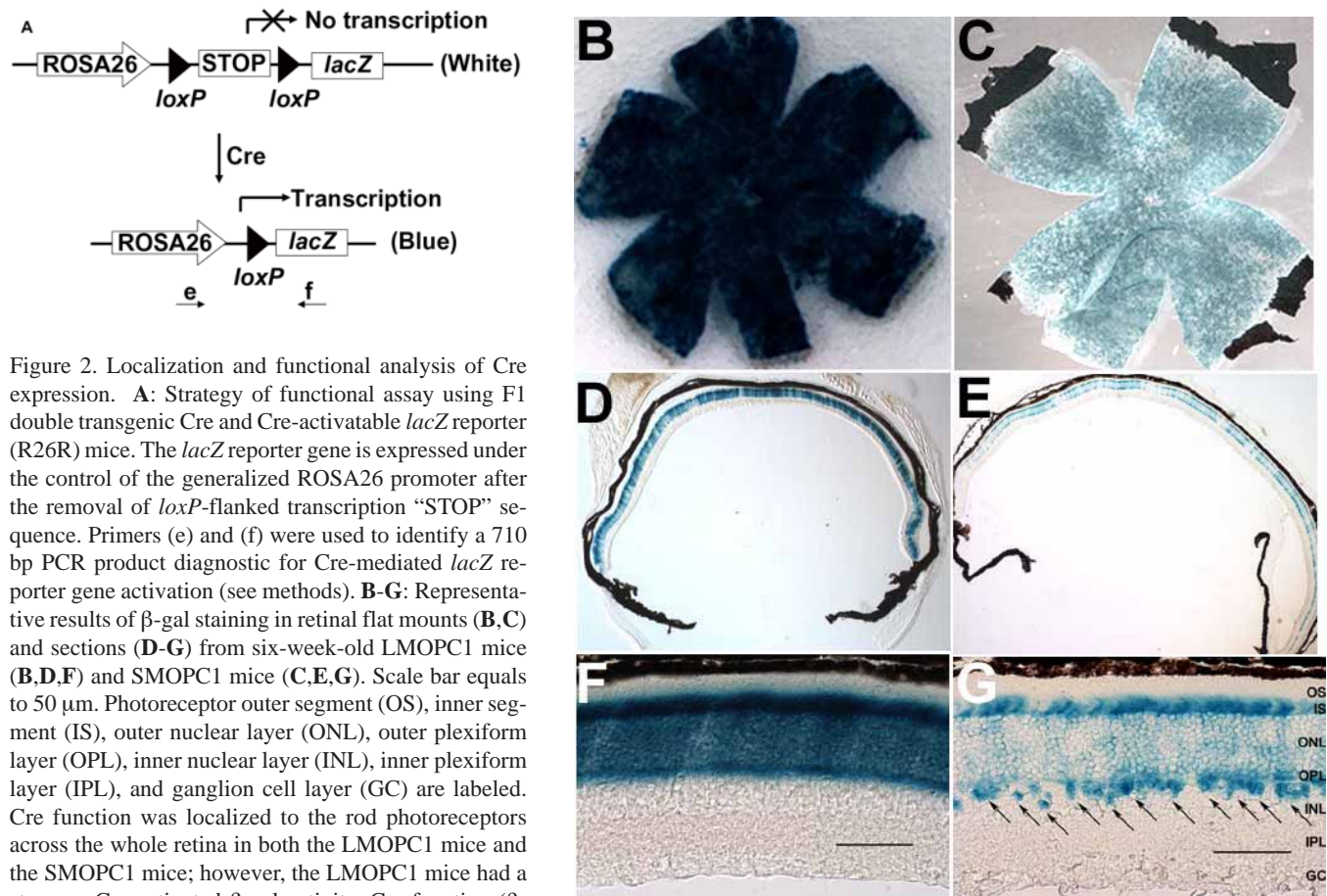


Figure 2. Localization and functional analysis of Cre expression. **A:** Strategy of functional assay using F1 double transgenic Cre and Cre-activatable *lacZ* reporter (R26R) mice. The *lacZ* reporter gene is expressed under the control of the generalized ROSA26 promoter after the removal of *loxP*-flanked transcriptional “STOP” sequence. Primers (e) and (f) were used to identify a 710 bp PCR product diagnostic for Cre-mediated *lacZ* reporter gene activation (see methods). **B-G:** Representative results of  $\beta$ -gal staining in retinal flat mounts (**B,C**) and sections (**D-G**) from six-week-old LMOPC1 mice (**B,D,F**) and SMOPC1 mice (**C,E,G**). Scale bar equals to 50  $\mu$ m. Photoreceptor outer segment (OS), inner segment (IS), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GC) are labeled. Cre function was localized to the rod photoreceptors across the whole retina in both the LMOPC1 mice and the SMOPC1 mice; however, the LMOPC1 mice had a stronger Cre-activated  $\beta$ -gal activity. Cre function ( $\beta$ -gal) was also localized to the INL region (**G**, arrows) in the SMOPC1 mice.

Cre expression in the LMOPC1 mice (Figure 3). Nevertheless, the Cre-expression was mainly localized within the perinuclear cytoplasm and the membrane of the rod photoreceptor nuclei, indicating that Cre was expressed in the rod photoreceptors of the SMOPC1 mice. However, our IHC assay did not detect any anti-Cre immunoreactivity in the inner nuclear layer of the SMOPC1 mice, suggesting that a low level of Cre was present in the  $\beta$ -gal-positive stained cells in this region, or Cre could be expressed early in the precursor cells that ultimately give rise to the bipolar cells.

To localize Cre-activatable *lacZ* expression in the inner nuclear layer, we performed a combination of  $\beta$ -gal staining and immunostaining on retinal sections with anti-synaptophysin and anti-calbindin antibodies, markers for photoreceptor terminals in the outer plexiform layer and horizontal cells [16,17]. The  $\beta$ -gal stained cells were clearly in the inner nuclear layer and were not the terminals of photoreceptors. In addition,  $\beta$ -gal staining did not co-localized with calbindin-positive horizontal cells (data not shown). Double labeling retinal sections with anti- $\beta$ -galactosidase and anti-protein kinase C (PKC) antibodies demonstrated that  $\beta$ -gal and PKC, a rod bipolar marker [18], co-localized in the inner nuclear layer (Figure 5), indicating that Cre activated the *lacZ* reporter in rod bipolar cells (white arrows in Figure 5E-G). This result suggested that Cre was expressed in rod bipolar cells in the SMOPC1 mice at a level sufficient for productive

Cre-mediated recombination. An alternative approach to localize Cre expression in the INL of SMOPC1 could be to use Cre-activatable fluorescent reporter mice, such as *Z/EG* reporter mice [19], in conjunction with immunostaining. The *Z/EG* reporter mice could be beneficial in precise localization of Cre expression in transgenic mice.

Although we only characterized one line from this transgenic construct and thus it is not possible to conclude whether the expression in the rod bipolar cells was caused by regulatory elements on the 0.2-kb opsin promoter or a positional effect in transgenics, the following scenario could account for the observed expression pattern in SMOPC1 mice. It was reported that the human blue cone promoter was capable of directing transgene expression in a subset of bipolar cells [20,21]. In these studies, transgenic expression in the bipolar cells is likely controlled by the interaction of the cis-acting element with transcription factors, such as OTXs or CRX, that are capable of directing photoreceptor- and bipolar cell-specific gene expression under normal conditions [22-26]. The loss of up-stream repressive element(s) could cause the 0.2-kb opsin promoter to direct the transgene expression in bipolar cells.

Both LMOPC1 and SMOPC1 mice were capable of performing productive Cre-mediated recombination. However, the expression of both Cre and Cre-activatable  $\beta$ -galactosidase in the SMOPC1/R26R mice was lower than that in the

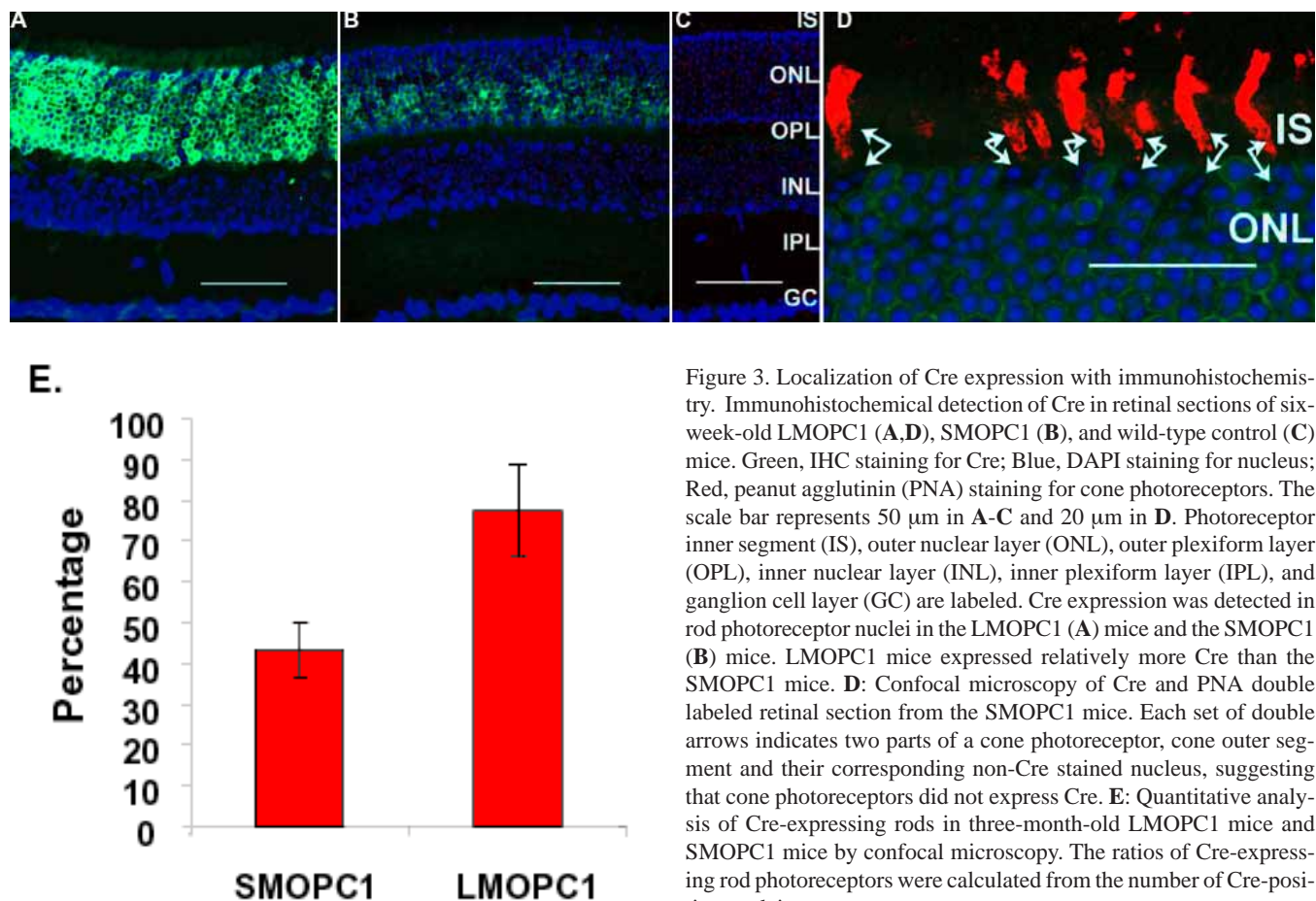


Figure 3. Localization of Cre expression with immunohistochemistry. Immunohistochemical detection of Cre in retinal sections of six-week-old LMOPC1 (A,D), SMOPC1 (B), and wild-type control (C) mice. Green, IHC staining for Cre; Blue, DAPI staining for nucleus; Red, peanut agglutinin (PNA) staining for cone photoreceptors. The scale bar represents 50  $\mu$ m in A-C and 20  $\mu$ m in D. Photoreceptor inner segment (IS), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GC) are labeled. Cre expression was detected in rod photoreceptor nuclei in the LMOPC1 (A) mice and the SMOPC1 (B) mice. D: Confocal microscopy of Cre and PNA double labeled retinal section from the SMOPC1 mice. Each set of double arrows indicates two parts of a cone photoreceptor, cone outer segment and their corresponding non-Cre stained nucleus, suggesting that cone photoreceptors did not express Cre. E: Quantitative analysis of Cre-expressing rods in three-month-old LMOPC1 mice and SMOPC1 mice by confocal microscopy. The ratios of Cre-expressing rod photoreceptors were calculated from the number of Cre-positive nuclei.

age-matched LMOPC1/R26R mice, suggesting that SMOPC1 mice produced relatively less Cre than LMOPC1 mice. These results were supported by the observation that Cre-mediated recombination in the SMOPC1/R26R mice was detected at P11 (Figure 4A), four days later than in the LMOPC1/R26R mice. These observations also correlated with a level of Cre protein demonstrated by Western blot analysis of retinal homogenates (Figure 1C). In our hands, SMOPC1 mice consistently showed a lower level of Cre protein (Figure 1C). Our results also suggested that from P30 to P60, there were no significant increases in the amounts of retinal Cre protein in both LMOPC1 and SMOPC1 mice (Figure 1C).

To determine the ratio of Cre-positive photoreceptors in LMOPC1 and SMOPC1 mice, a direct assay with quantitative confocal microscopy (as shown in Figure 3D) was performed by calculating the percentage of Cre-positive rod nuclei in the anti-Cre antibody-stained retinal sections from three-month-old mice. Our results showed that there were no significant changes in the ratio of Cre-positive photoreceptors between the peripheral and the central retina in each mouse and 77.5% of LMOPC1 rods and 43.3% of SMOPC1 rods had detectable Cre expression (Figure 3E). However, no apparent increases in the ratio of Cre-positive rods were observed in six-month-old mice (data not shown), suggesting that the

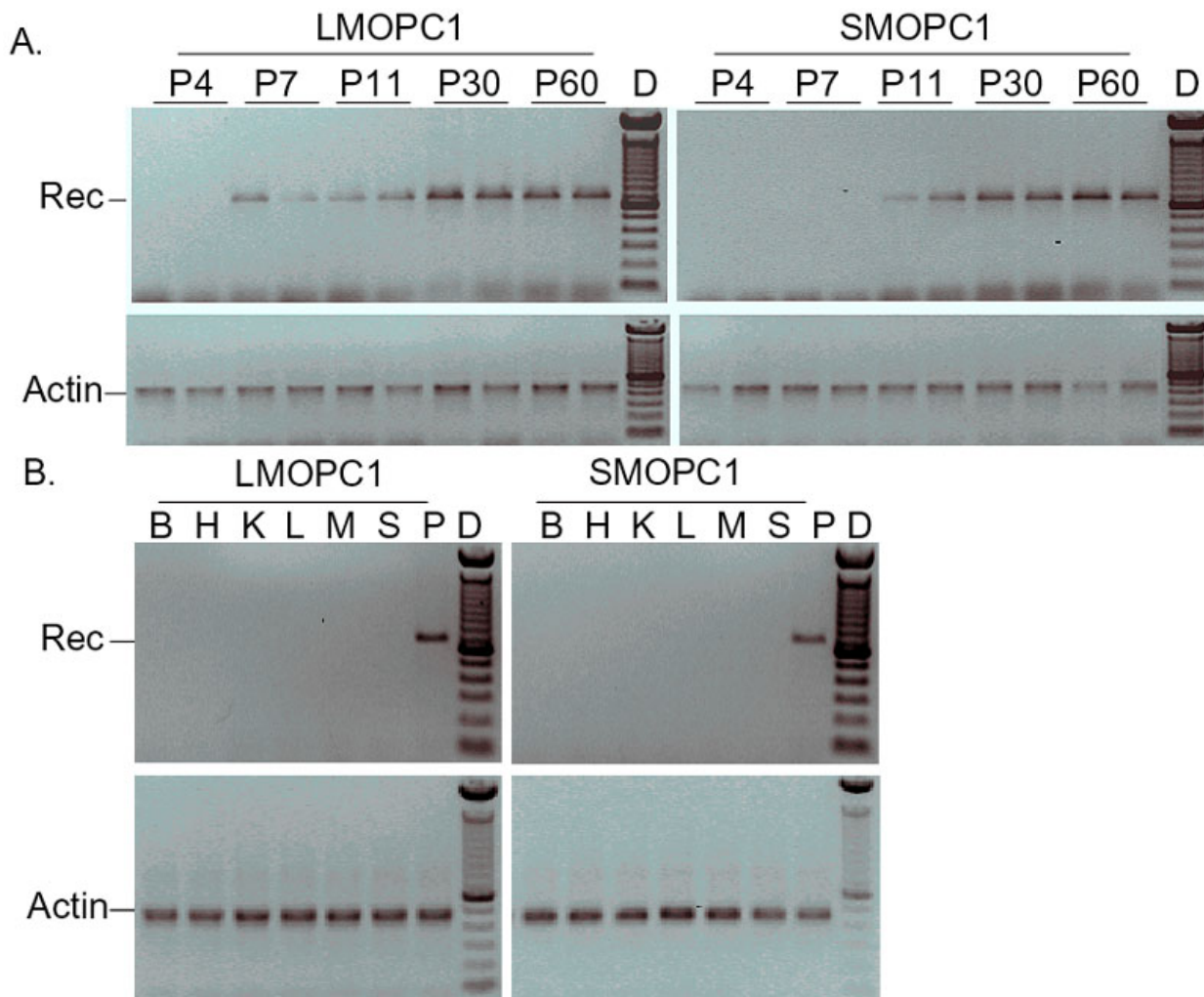


Figure 4. PCR analysis of Cre-mediated recombination. **A:** Time course of Cre-mediated activation of *lacZ* reporter gene at the DNA level using retinal DNA from P4 to P60 from the F1 double transgenic Cre/R26R mice. Retinal DNA from two different mice was used in PCR analysis. Top, inverted gel image of PCR (25 cycles) reaction diagnostic for a 710 bp Cre-mediated recombination in retinal genomic DNA of the F1 double transgenic Cre/R26R mice. Bottom, inverted gel image of PCR (20 cycles) reaction diagnostic for a 450 bp product in  $\beta$ -actin gene (internal DNA control). Lane D is a 100 bp DNA marker. Cre-mediated recombination was detected at P7 in the LMOPC1 mice and at P11 in the SMOPC1 mice. **B:** PCR analysis of ectopic Cre expression in representative tissues from both the LMOPC1 mice and the SMOPC1 mice. Lane B is brain, lane H is heart, lane K is kidney, lane L is liver, lane M is muscle, lane S is spleen; lane P is positive control DNA, and lane D is a 100 bp DNA marker. No apparent Cre-mediated recombination was identified in representative tissues from either the LMOPC1 mice or the SMOPC1 mice.



number of Cre-expressing cells was relatively stable after early development. Since a successful Cre-mediated recombination requires only four Cre recombinase molecules [27] and immunohistochemistry is not likely to detect cells expressing a very low level of Cre, the ratios of Cre-expressing rods in these mice were likely a conservative estimation.

**Ectopic expression of Cre:** The level of ectopic Cre expression was examined in the following representative tissues: brain, heart, liver, muscle, kidney, and spleen in both two-month-old F1 double transgenic LMOPC1/R26R mice and two-month-old F1 double transgenic SMOPC1/R26R mice. PCR analysis diagnostic for Cre-mediated recombination in the R26R mouse genomic DNA suggested that there was no apparently productive Cre-mediated recombination in these representative tissues in either the LMOPC1 mice or the SMOPC1 mice (Figure 4B). This result was also supported by the observation that no detectable Cre-mediated *lacZ* reporter expression was observed beyond the negative control level in these representative tissues from either the F1 double transgenic LMOPC1/R26R mice or the F1 double transgenic

SMOPC1/R26R mice (data not shown). Therefore, the LMOPC1 mice and the SMOPC1 mice are not likely to cause any phenotypic changes in non-ocular tissues in conditional gene knockout studies.

**Integrity of rod photoreceptors:** Several lines of evidences suggest that over-expression of Cre could cause unwanted chromosomal rearrangement in mammals [28,29]. Over-expression of Cre in photoreceptors may induce retinal degeneration and the transgenic mice may not be suitable for gene function studies. This concern was supported by previous observations that over-expression of Cre in rod photoreceptors correlated with retinal degeneration (data not shown) [30]. Thus, the rod photoreceptor integrity was a major criterion in our characterization of potentially useful Cre mice. ERG and morphologic analysis for LMOPC1 and SMOPC1 mice were performed using eight-month-old F1 double transgenic Cre/R26R mice. The scotopic and photopic ERG analysis suggested that both LMOPC1 mice and SMOPC1 mice had normal rod and cone function when they were eight-month-old (Figure 6). Hematoxylin and eosin staining of the retinal sections from

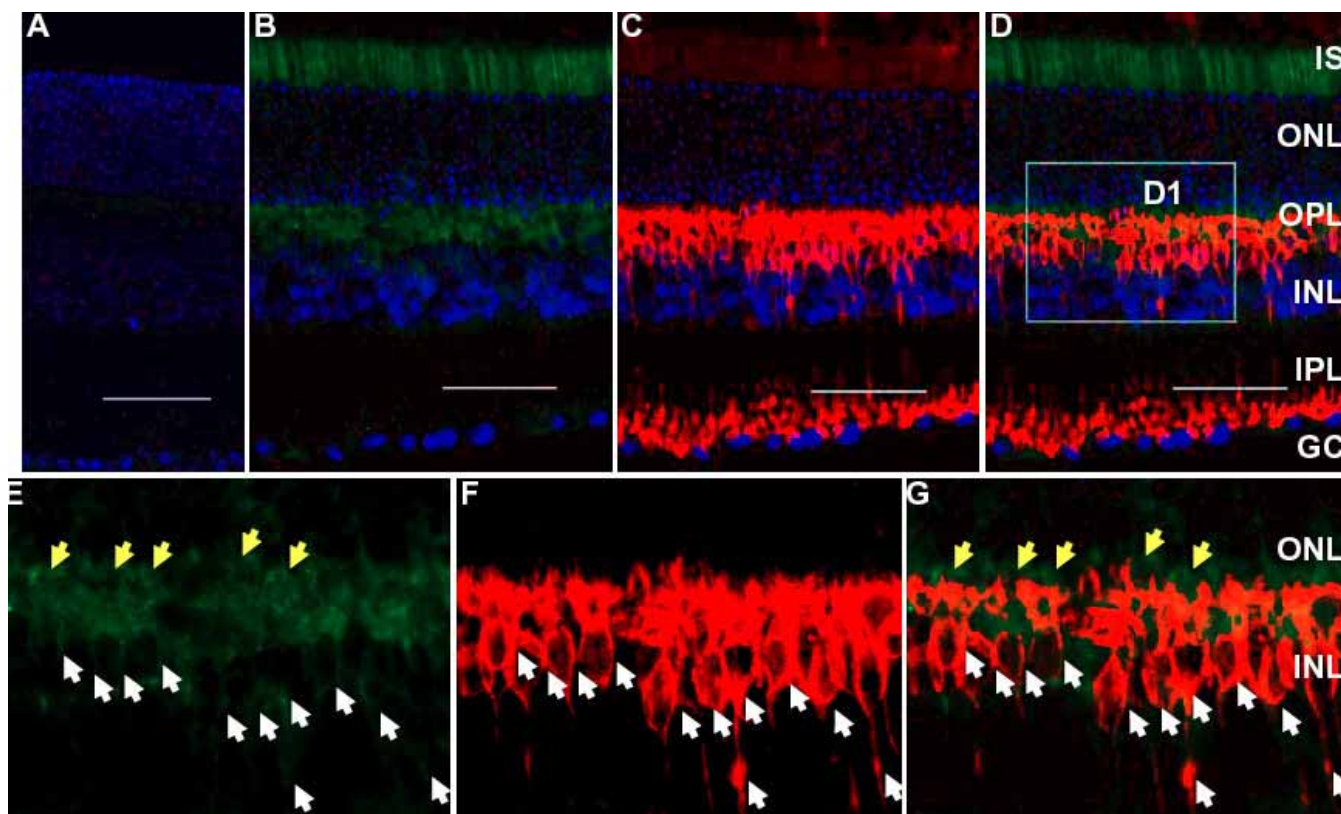


Figure 5. Localization of Cre expression in the rod bipolar cells. Immunohistochemical detection of Cre-activated  $\beta$ -gal expression in the inner nuclear layer of six-week-old SMOPC1 mice. **A,B:** Representative immunohistochemistry (IHC) staining with a polyclonal anti- $\beta$ -gal (green) antibody using retinal sections from a wild-type littermate and a SMOPC1/R26R double transgenic mouse, respectively. **C:** IHC staining of the identical section used in Panel **B** with a monoclonal anti-protein kinase C (PKC) antibody (red) for rod bipolar cells. **D:** Merged image of Panels **B** and **C**. Blue is DAPI nuclear staining. **E-G:** Enlarged images (identical area as D1) corresponding to **B-D**, respectively, without showing DAPI staining. The scale bar represents 50  $\mu$ m. Photoreceptor inner segment (IS), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GC) are labeled. In addition to the strong  $\beta$ -gal staining in the photoreceptor inner segment,  $\beta$ -gal staining was also observed in the photoreceptor terminals (yellow arrows) and the INL (white arrows). The Cre-activated  $\beta$ -gal in the INL of the SMOPC1 mice was co-localized to the rod bipolar cells (white arrows).

eight-month-old F1 double transgenic Cre/R26R mice showed that Cre expression did not cause retinal degeneration and that the retinas of both the LMOPC1 transgenic mice and the SMOPC1 transgenic mice were normal (Figure 7). Since Western blot analysis of Cre expression with retinal homogenates showed that there was no apparent increase in Cre protein accumulation in either the LMOPC1 mice or the SMOPC1 mice from P30 to P60 (Figure 1C), the effect of Cre toxicity is not likely to increase over time in the adult mice. Therefore, these mice are potentially useful for gene function studies.

**Summary:** To dissect gene function in rod photoreceptors, several laboratories have made tremendous efforts to generate and characterize rod-specific *cre* mice [30-32]. A common goal of these studies was to generate a resource of rod-specific Cre mouse lines with a range of expression levels to suit the requirement for each individual studies. At this time, there are two published potentially useful rod photoreceptor-specific Cre mouse lines [31,32]. Regarding any possible re-

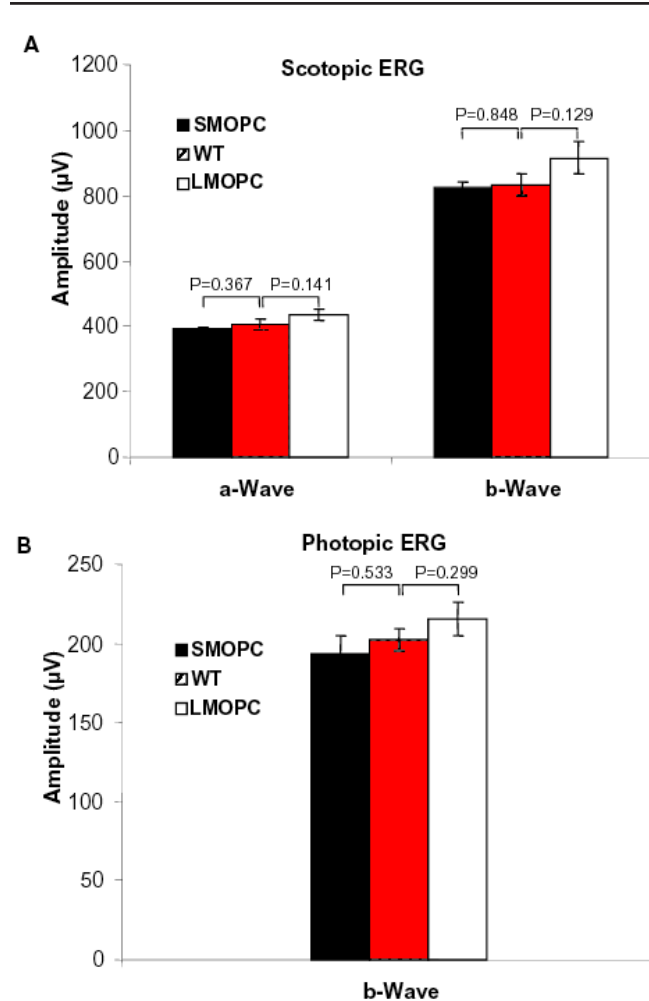


Figure 6. Photoreceptor function in transgenic *cre* mice. Comparison of average scotopic (A) and photopic (B) ERG among eight-month-old LMOPC1, SMOPC1, and wild-type (WT) littermate mice. At least 16 eyes were used for each group. No significant change in the rod or the cone functions was observed in either the LMOPC1 mice or the SMOPC1 mice.

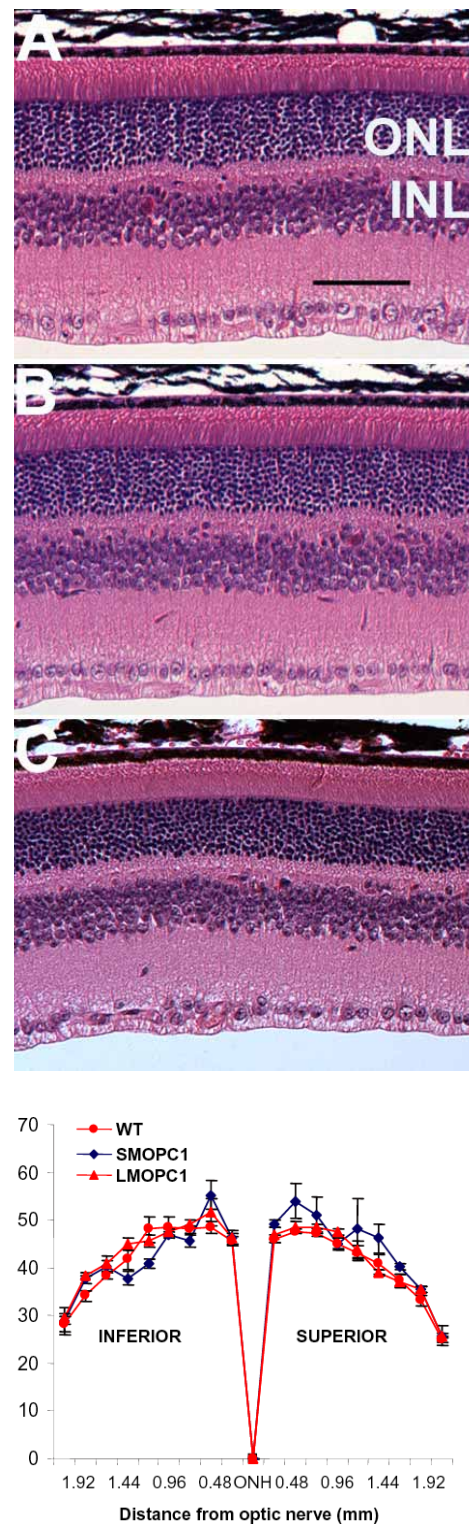


Figure 7. Retinal morphology of adult transgenic *cre* mice. Hematoxylin and eosin stained retinal sections from eight-month-old wild-type (WT; A), LMOPC1 (B), and SMOPC1 (C) mice. The scale bar represents 20 µm. Outer nuclear layer (ONL) and inner nuclear layer (INL) were labeled for orientation. D: ONL thickness along the vertical meridian of eight-month-old transgenic *cre* mice. ONH: optical nerve head. The ONL thickness was obtained from at least eight eyes for each group. Retinal morphology was normal in both eight-month-old SMOPC1 mice and eight-month-old LMOPC1 mice.



dundancy among these Cre-expressing animals, having multiple Cre lines is definitely beneficial, as no two Cre-expressing lines are the same, and subtle variations in the expression of Cre is useful in understanding the function of a floxed gene. Thus, there is indeed great importance and necessity for several Cre mouse lines. Here we contribute two new novel and useful mouse lines expressing Cre in rod photoreceptors. Mice have 20 chromosomal pairs, thus there is a chance of 5% that a *cre* transgene and a floxed gene of interests are located on the same chromosome. Our LMOPC1 mice expressed a detectable level of Cre in at least 77.5% of the rods and thus can be utilized for studies requiring efficient Cre-mediated gene activation or inactivation in rod photoreceptors (Figure 3E). The SMOPC1 mice expressed a detectable level of Cre in 43.3% of the rods across the whole retina. The mosaic Cre-mediated recombination in the SMOPC1 mice (Figure 2E,G, Figure 3E) could result in a less detrimental phenotype in mice and may provide an advantage for some gene knockout studies. Owing to the relatively little effort and a high reproducibility in generating desired phenotypes, genetic mosaics have become a method of choice to investigate cell lineages, patterns of growth and gene function, and to provide a means to circumvent the challenge in phenotypic analysis of mice generated with an all-or-none genetic approach [33]. Since the conditional gene knockout of *Bcl-x* with SMOPC1 mice resulted in detectable functional changes [34], the level of Cre expression in SMOPC1 mice is sufficient for photoreceptor gene inactivation. In addition, the amount of Cre protein present in the SMOPC1 mouse retina was much lower than in the LMOPC1 mouse retina (Figure 1C), the long-term Cre toxicity, if any, should be much lower in the SMOPC1 mouse line. Finally, the SMOPC1 mice expressed Cre in rod photoreceptors and rod bipolar cells; therefore, this novel mouse strain may also be useful for dissecting the function of genes that are involved in multiple neuronal processes in the rod photoreceptors and/or rod bipolar cells.

An additional advantage of having more than one characterized rod-specific *cre* mouse line is that it will circumvent a situation where disruption of a particular gene is located on the same chromosome of the *cre* transgene insertion. For conditional gene knockout studies, a low cost experiment to determine whether a floxed gene is located on the same chromosome of *cre* transgene insertion is to simply breed Cre mice with the mice carrying a floxed gene for two generations. The genotyping results from F2 mice will tell if the floxed gene is on the same chromosome of *cre* transgene insertion.

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#### REFERENCES

1. Sauer B, Henderson N. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci U S A* 1988; 85:5166-70.
2. Lakso M, Sauer B, Mosinger B Jr, Lee EJ, Manning RW, Yu SH, Mulder KL, Westphal H. Targeted oncogene activation by site-specific recombination in transgenic mice. *Proc Natl Acad Sci U S A* 1992; 89:6232-6.
3. Le Y, Sauer B. Conditional gene knockout using Cre recombinase. *Mol Biotechnol* 2001; 17:269-75.
4. Le Y, Sauer B. Conditional gene knockout using cre recombinase. *Methods Mol Biol* 2000; 136:477-85.
5. Jeon CJ, Strettoi E, Masland RH. The major cell populations of the mouse retina. *J Neurosci* 1998; 18:8936-46.
6. Quiambao AB, Peachey NS, Mangini NJ, Rohlich P, Hollyfield JG, al-Ubaidi MR. A 221-bp fragment of the mouse opsin promoter directs expression specifically to the rod photoreceptors of transgenic mice. *Vis Neurosci* 1997; 14:617-25.
7. Lem J, Applebury ML, Falk JD, Flannery JG, Simon MI. Tissue-specific and developmental regulation of rod opsin chimeric genes in transgenic mice. *Neuron* 1991; 6:201-10.
8. Kozak M. Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J Biol Chem* 1991; 266:19867-70.
9. Le Y, Dobson MJ. Stabilization of yeast artificial chromosome clones in a *rad54-3* recombination-deficient host strain. *Nucleic Acids Res* 1997; 25:1248-53.
10. Le Y, Gagneten S, Larson T, Santha E, Dobi A, v Agoston D, Sauer B. Far-upstream elements are dispensable for tissue-specific proenkephalin expression using a Cre-mediated knock-in strategy. *J Neurochem* 2003; 84:689-97.
11. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 1999; 21:70-1.
12. Le YZ, Ash JD, Al-Ubaidi MR, Chen Y, Ma JX, Anderson RE. Targeted expression of Cre recombinase to cone photoreceptors in transgenic mice. *Mol Vis* 2004; 10:1011-8.
13. Xu X, Quiambao AB, Roveri L, Pardue MT, Marx JL, Rohlich P, Peachey NS, Al-Ubaidi MR. Degeneration of cone photoreceptors induced by expression of the *Mas1* protooncogene. *Exp Neurol* 2000; 163:207-19.
14. Zack DJ, Bennett J, Wang Y, Davenport C, Klaunberg B, Gearhart J, Nathans J. Unusual topography of bovine rhodopsin promoter-lacZ fusion gene expression in transgenic mouse retinas. *Neuron* 1991; 6:187-99.
15. Le Y, Gagneten S, Tombaccini D, Bethke B, Sauer B. Nuclear targeting determinants of the phage P1 cre DNA recombinase. *Nucleic Acids Res* 1999; 27:4703-9.
16. Wiedenmann B, Franke WW. Identification and localization of synaptophysin, an integral membrane glycoprotein of Mr 38,000 characteristic of presynaptic vesicles. *Cell* 1985; 41:1017-28.
17. Peichl L, Gonzalez-Soriano J. Morphological types of horizontal cell in rodent retinae: a comparison of rat, mouse, gerbil, and guinea pig. *Vis Neurosci* 1994; 11:501-17.
18. Vaquero CF, Velasco A, de la Villa P. Protein kinase C localization in the synaptic terminal of rod bipolar cells. *Neuroreport* 1996; 7:2176-80.
19. Novak A, Guo C, Yang W, Nagy A, Lobe CG. Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis* 2000; 28:147-55.

20. Chen J, Tucker CL, Woodford B, Szel A, Lem J, Gianella-Borradori A, Simon MI, Bogenmann E. The human blue opsin promoter directs transgene expression in short-wave cones and bipolar cells in the mouse retina. *Proc Natl Acad Sci U S A* 1994; 91:2611-5.
21. Chiu MI, Nathans J. Blue cones and cone bipolar cells share transcriptional specificity as determined by expression of human blue visual pigment-derived transgenes. *J Neurosci* 1994; 14:3426-36.
22. Baas D, Bumsted KM, Martinez JA, Vaccarino FM, Wikler KC, Barnstable CJ. The subcellular localization of Otx2 is cell-type specific and developmentally regulated in the mouse retina. *Brain Res Mol Brain Res* 2000; 78:26-37.
23. Liu Y, Shen Y, Rest JS, Raymond PA, Zack DJ. Isolation and characterization of a zebrafish homologue of the cone rod homeobox gene. *Invest Ophthalmol Vis Sci* 2001; 42:481-7.
24. Boatright JH, Borst DE, Peoples JW, Bruno J, Edwards CL, Si JS, Nickerson JM. A major cis activator of the IRBP gene contains CRX-binding and Ret-1/PCE-I elements. *Mol Vis* 1997; 3:15.
25. Chen S, Wang QL, Nie Z, Sun H, Lennon G, Copeland NG, Gilbert DJ, Jenkins NA, Zack DJ. Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. *Neuron* 1997; 19:1017-30.
26. Furukawa T, Morrow EM, Cepko CL. Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. *Cell* 1997; 91:531-41.
27. Guo F, Gopaul DN, van Duyne GD. Structure of Cre recombinase complexed with DNA in a site-specific recombination synapse. *Nature* 1997; 389:40-6.
28. Schmidt EE, Taylor DS, Prigge JR, Barnett S, Capecchi MR. Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. *Proc Natl Acad Sci U S A* 2000; 97:13702-7.
29. Loonstra A, Vooijs M, Beverloo HB, Allak BA, van Drunen E, Kanaar R, Berns A, Jonkers J. Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. *Proc Natl Acad Sci U S A* 2001; 98:9209-14.
30. Feiner L, Navaratnarajah M, Pierce EA. Expression of Cre recombinase in photoreceptors leads to retinal degeneration. 2004 ARVO Annual Meeting; April 25-29; Fort Lauderdale (FL).
31. Li S, Chen D, Sauve Y, McCandless J, Chen YJ, Chen CK. Rhodopsin-iCre transgenic mouse line for Cre-mediated rod-specific gene targeting. *Genesis* 2005; 41:73-80.
32. Marszalek JR, Liu X, Roberts EA, Chui D, Marth JD, Williams DS, Goldstein LS. Genetic evidence for selective transport of opsin and arrestin by kinesin-II in mammalian photoreceptors. *Cell* 2000; 102:175-87.
33. Collinson JM, Hill RE, West JD. Analysis of mouse eye development with chimeras and mosaics. *Int J Dev Biol* 2004; 48:793-804.
34. Zheng L, Anderson RE, Le Y. Loss of BCL-XL causes increased rod photoreceptor susceptibility to bright light stress. *Invest Ophthalmol Vis Sci*. In press 2006.