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Targeted expression of Cre recombinase to cone photoreceptors in transgenic mice

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Purpose: Disruption of widely expressed essential genes in mice often leads to embryonic or neonatal lethality. To circumvent this problem and dissect gene functions in the cone photoreceptors, we elected to generate cone photoreceptor specific *cre* transgenic mice.

Methods: Transgenic mice expressing Cre recombinase directed by the human red/green pigment (HRGP) gene promoter were generated. Candidate Cre-expressing lines were identified with RT-PCR. Cre-expressing mice were characterized with immunocytochemical assays and functional studies using a Cre activatable *lacZ* reporter mouse strain (R26R). Cone distribution was determined by immunohistochemistry and retinal function was measured by electroretinography (ERG) on six month old HRGP-*cre* mice.

Results: RT-PCR analysis suggested that several transgenic lines expressed *cre* mRNA in the retina. β-Galactosidase staining on retinal flat mounts and sections from F1 mice derived from the HRGP-*cre* and R26R reporter mice suggested that two mouse strains were capable of carrying out efficient Cre mediated recombination in cone photoreceptors. Immunocytochemical staining of retinal sections demonstrated that Cre expression was localized to cone photoreceptors. Cone distribution and cone ERG analysis suggested that cone photoreceptors were normal in adult HRGP-*cre* mice.

Conclusions: We have generated transgenic mice that efficiently express Cre recombinase in cone photoreceptor cells. The adult transgenic mice have normal cone distribution and function. They can be used in conditional knockout experiments for gene function studies in cone photoreceptors.

Gene targeting with homologous recombination in murine embryonic stem (ES) cells has provided many insights into gene function and the pathophysiology of human diseases [1,2]. However, disruption of essential genes often causes embryonic or early postnatal lethality and obscures the particular role of genes in a target tissue or in adults [3,4]. To circumvent this problem, a conditional gene targeting strategy using the Cre/*lox* site specific DNA recombination system has become a method of choice to disrupt genes in a temporal and spatial fashion [5,6]. In Cre-*lox* based conditional knockout strategy, a *loxP* flanked gene or gene segment is removed at a particular time or in a target tissue by the Cre recombinase expressed temporally or spatially (for review see [7]). This strategy has been successfully used in dissecting gene function for kinesin-II in rod photoreceptors [6] and RXR-alpha in the RPE [8]. This strategy also permits dissection of the roles of multifunctional genes in a particular tissue/cell type [6,9]. Cone photoreceptors are responsible for color/bright light vision and mutations that cause loss of function in cone photoreceptors in humans are particularly devastating. Since cone photoreceptors only constitute a small percentage of retinal cells in rodents, it is technically more challenging to dissect the function of genes expressed in cone photoreceptors. Disruption of gene function in cone photoreceptors specifically and efficiently can provide clear cut information on the pathophysiology of ocular disease related genes. As a first step towards dissecting gene function in cone photoreceptors, we decided to generate cone specific Cre mice using a transgenic strategy. This report describes the generation and characterization of the transgenic *cre* mice.

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METHODS

The use of animals 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 cone photoreceptor specific cre *transgene:* The recombinant plasmid carrying human red/green pigment promoter controlled *cre* transgene was constructed from plasmids pBS185 and pJHN60 [10,11]. Briefly, the human red/green pigment promoter (HRGP), a 6.3 kb *Nco*I fragment on pJHN60, was inserted in front of *cre* in the pBS185. The resulting plasmid pLE109 contained *Not*I restriction sites flanking the transgene that carries the cone photoreceptor specific promoter (HRGP), a translationally optimized *cre*, 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 of transgenic mice: The plasmid DNA carrying the transgene was prepared and purified using the cesium chloride double centrifugation procedure. The purified plas-

mid DNA was digested with restriction enzyme *Not*I to isolate the transgene (Figure 1). The DNA was then fractionated on agarose gel and purified with Qiagen gel extraction kit (Valencia, CA). The zygote injection was performed using FVB/N background mice at the Microinjection Core at the Oklahoma Medical Research Foundation. PCR detection of the *cre* transgene was performed on mouse tail DNA according to the conditions described by Le and Sauer [7] with primer a (5'-AGG TGT AGA GAA GGC ACT TAG C-3') and b (5'-CTA ATC GCC ATC TTC CAG CAG G-3'). Transgenic founder mice were further confirmed to carry the transgene with Southern hybridization using a 0.5 kb *Bam*HI-*Nco*I *cre* DNA fragment from pLE109 (described above). PCR detection of the Cre activatable *LacZ* reporter gene in R26R mice was performed according to the conditions described by Le et al. [12] with primers c (5'-GAG TTG CGT GAC TAC CTA CGG-3') and d (5'-GGC TTC ATC CAC CAC ATA CAG G-3').

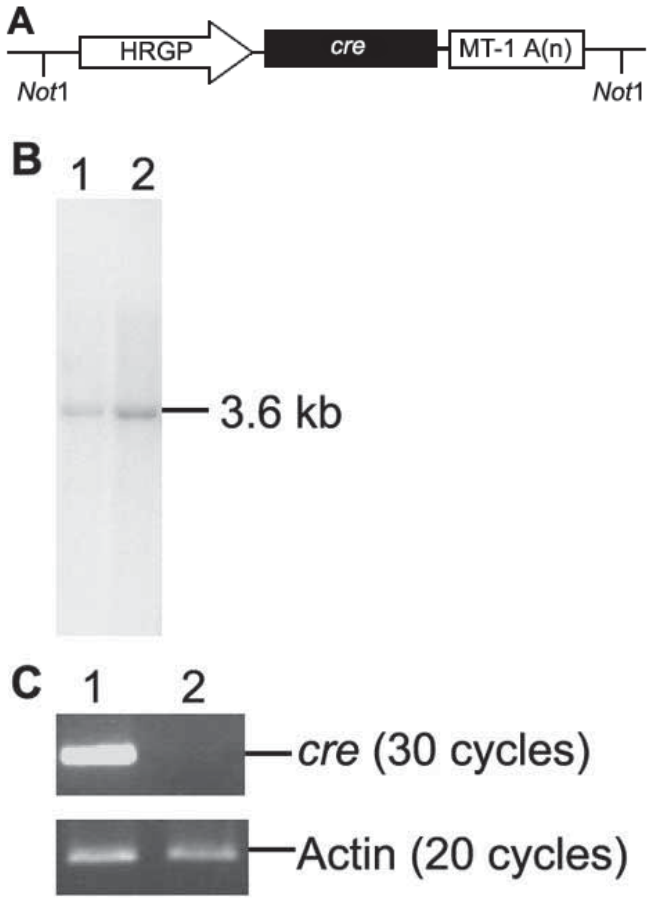

Figure 1. Generation of HRGP-*cre* mice. **A**: Schematic drawing of the transgenic construct. HRGP labels the human red/green pigment promoter, *cre* labels the coding region of Cre recombinase gene, and MT-1 A(n) labels mouse metallothionein (MT-I) polyadenylation signal. **B**: Southern blot analysis of *Nco*I digested genomic DNA from two (1 and 2) representative transgenic founders. A 0.5 kb *Bam*HI-*Nco*I *cre* DNA segment of the transgenic construct was used as probe. **C**: RT-PCR detection of Cre expression in two representative HRGP-*cre* transgenic founder mice.

RT-PCR analysis: RT-PCR analysis was performed similarly as described previously [12]. Briefly, Mouse tissues were placed in liquid nitrogen immediately after dissection from euthanized mice, stored at -80 °C, and used in making total RNA with Trizol reagent (Invitrogen, Carlsbad, CA). First strand cDNA was made by oligo dT priming using the SuperScript first strand cDNA synthesis system for RT-PCR (Invitrogen). To prevent trace amount of DNA contamination, RNA samples were treated with amplification grade DNase I (Invitrogen) before reverse transcription. Typically, 2 μg of RNA was used in each 20 μl reverse transcription reaction. Of the resulting cDNA, 2 μl (corresponding to 0.2 μg of original RNA) was used in a subsequent 25 μl PCR reaction. The cDNA was used in the PCR reaction according to conditions described above using the primer pairs a and b (described above), c and d (described above) and e (5'-GAC GAG GCG CAG AGC AAG AGA GG-3') and f (5'-CTC TTT GAT GTC ACG CAC GAT TTC-3') to detect the 411 bp *cre*, the 495 bp Cre activated reporter gene *LacZ*, and 450 bp β-actin transcripts, respectively.

β-Galactosidase staining: The function of Cre in transgenic mice was assayed with β-galactosidase staining assays using the retinas of double transgenic F1 mice derived from HRGP-*cre* and Cre activatable *LacZ* reporter mice [13]. Briefly, genotyped mice were euthanized and a dye was injected at the superior pole of the cornea to maintain orientation. The eyes were removed and fixed for 10 min in PBS containing 2% formaldehyde. The lens and vitreous were then removed and the eyecup was replaced in fixation buffer for 10 more min. The eyecups were then washed twice (10 min each) with 1X PBS and incubated overnight at room temperature in X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) staining solution (1 mg/ml X-gal, 6 mM potassium ferricyanide, 6 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.02% NP-40, and 0.01% sodium deoxycholate in 1X PBS). After staining, sclera and choroid of one eye were dissected from the retina and the β-galactosidase staining in the flat mount retinas was observed under a dissecting microscope. The remaining eyecup was washed three times with 1X PBS, postfixed overnight in fixative at 4 °C, embedded in paraffin, cut into 5 μm sections, and observed under a microscope. To evaluate ectopic expression of Cre, brain, heart, liver, skeletal muscle, and spleen were obtained and placed in 12 well plates, sliced sagittally with a razor blade to facilitate penetration, rinsed with 5 ml of 1X PBS, and fixed for 15 min at room temperature. Tissues were rinsed twice with 1X PBS-0.02% NP-40 and stained overnight at 25 °C in X-gal staining solution. After staining, organs were washed three times with PBS (10 min each) and postfixed overnight at 4 °C. The expression of β-galactosidase was observed under a dissecting microscope.

Immunohistochemistry: Eyes were enucleated, fixed with 2% paraformaldehyde in PBS, and embedded in OCT media. Cryosections (10 μm thick) were cut and placed on glass slides. For immunostaining with anti-Cre polyclonal antibody (Novagen, San Diego, CA), anti-M-opsin polyclonal antibody (Chemicon, Temecula, CA), and peanut agglutinin (Vector

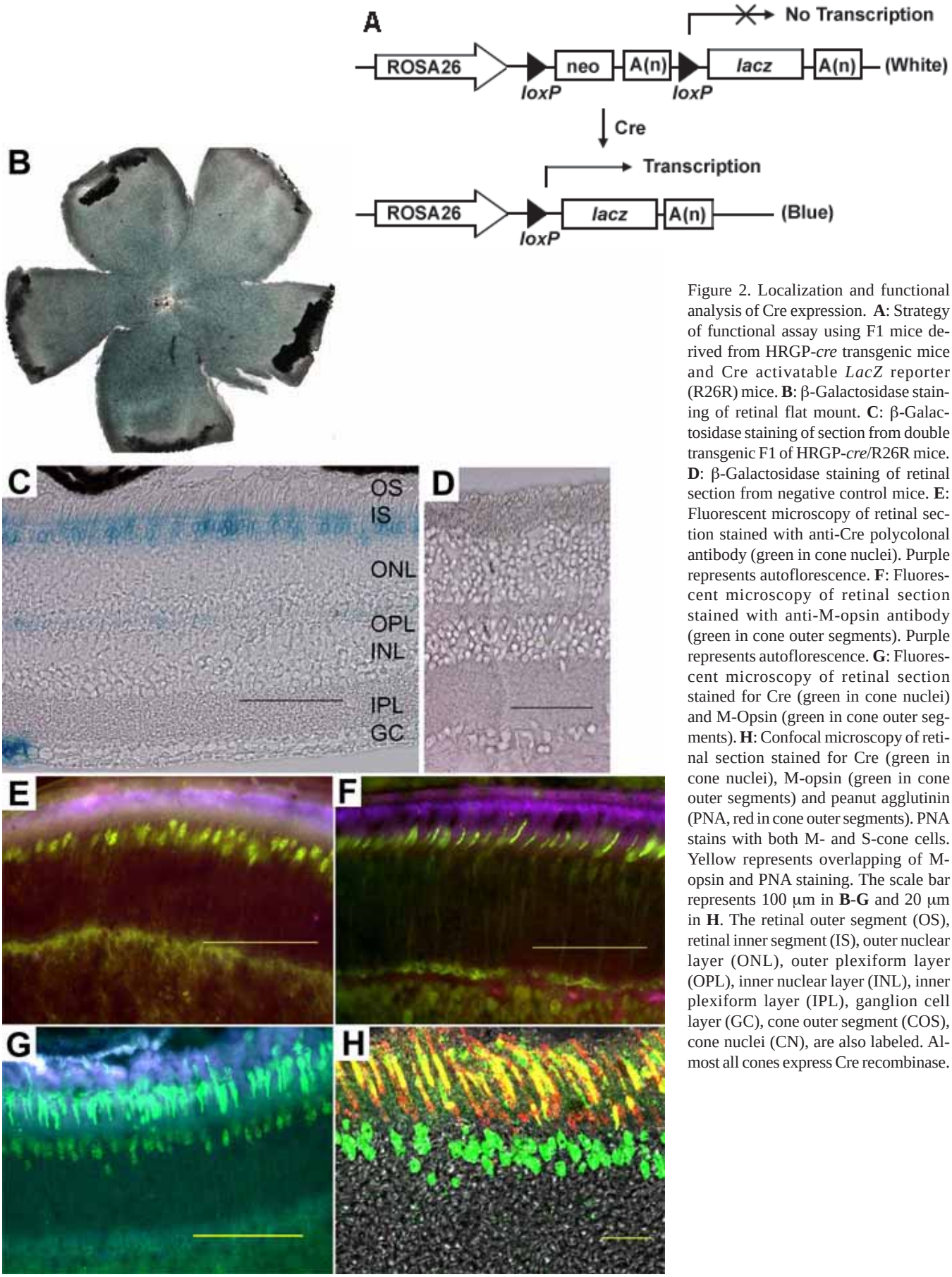

Figure 2. Localization and functional analysis of Cre expression. **A**: Strategy of functional assay using F1 mice derived from HRGP-*cre* transgenic mice and Cre activatable *LacZ* reporter (R26R) mice. **B**: β-Galactosidase staining of retinal flat mount. **C**: β-Galactosidase staining of section from double transgenic F1 of HRGP-*cre*/R26R mice. **D**: β-Galactosidase staining of retinal section from negative control mice. **E**: Fluorescent microscopy of retinal section stained with anti-Cre polycolonal antibody (green in cone nuclei). Purple represents autoflorescence. **F**: Fluorescent microscopy of retinal section stained with anti-M-opsin antibody (green in cone outer segments). Purple represents autoflorescence. **G**: Fluorescent microscopy of retinal section stained for Cre (green in cone nuclei) and M-Opsin (green in cone outer segments). **H**: Confocal microscopy of retinal section stained for Cre (green in cone nuclei), M-opsin (green in cone outer segments) and peanut agglutinin (PNA, red in cone outer segments). PNA stains with both M- and S-cone cells. Yellow represents overlapping of M-opsin and PNA staining. The scale bar represents 100 μm in **B**-**G** and 20 μm in **H**. The retinal outer segment (OS), retinal inner segment (IS), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GC), cone outer segment (COS), cone nuclei (CN), are also labeled. Almost all cones express Cre recombinase.

Laboratories, Burlingame, CA), sections were blocked with 10% horse serum in PBS, incubated with primary antibody at 4 °C overnight, washed with PBS three times, incubated with corresponding fluorescent chromophore (Alexa 488 or Alexa 568) conjugated secondary antibody (Molecular Probe, Eugene, OR) for 1 h at room temperature, washed with PBS for three times, and cover slipped. For lectin staining of the whole retina, the posterior part of the eye, including sclera, choroid and RPE, were carefully removed. The remaining part of the eye containing the whole retina with lens and cornea were fixed with 2% paraformaldehyde in PBS, washed with 0.5% $NaBH_4$ for 5 min at room temperature to diminish the autoflorescence [14], blocked overnight with blocking buffer (3% IgG free BSA and 5% normal goat serum in washing buffer) at 4 °C with gentle shaking, stained with TRITC labeled triticum vulgaris lectin (product number L5266; Sigma, St. Louis, MO) for 2 h at room temperature, and washed three times with 0.2% Triton X-100. Following the removal of the cornea and the lens, the lectin stained retina was flat mounted on the slide.

Electroretinography (ERG): For assessment of the visual function of the transgenic mice, both scotopic and photopic ERG was measured with a UTAS-E 3000 ERG system (LKC technologies, Inc., Gaithersburg, MD) according to the conditions described by Xu et al. [15]. Briefly, mice were dark adapted overnight and anesthetized with an intramuscular (IM) injection of 85 mg/kg ketamine and 14 mg/kg xylazine. After pupil dilation with 2.5% phenylephrine, mice were placed on a heating pad to maintain body temperature at 37 °C. Responses were differentially amplified, averaged, and stored. For the assessment of rod photoreceptor function (scotopic ERG), a strobe flash stimulus was presented to the dark adapted, dilated eyes in a Ganzfeld with a 138 cd x sec/mm^2 flash intensity. The amplitude of the a-wave was measured from the prestimulus baseline to the a-wave trough. The amplitude of the b-wave was measured from the trough of the a-wave to the peak of the b-wave. For the evaluation of cone function (photopic ERG), a strobe flash stimulus was presented to 5 min light adapted, dilated eyes in a Ganzfeld with a 79 cd x sec/mm^2 flash intensity. The amplitude of the cone b-wave was measured from the trough of the a-wave to the peak of the b-wave.

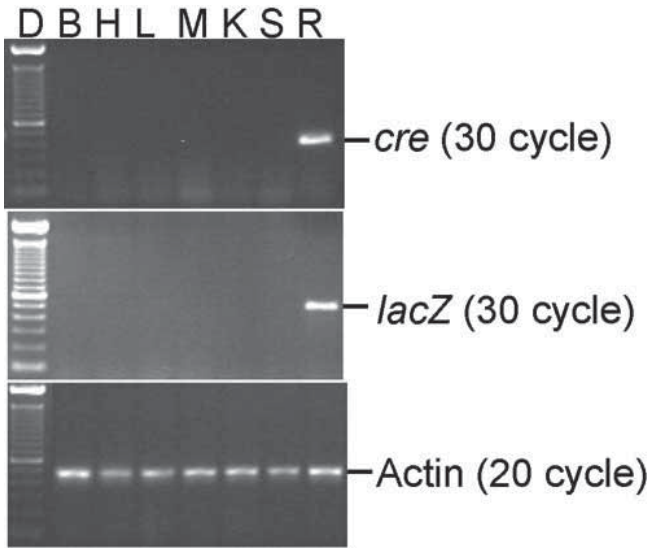

Figure 3. Analysis of Cre expression in representative tissues. Semi-quantitative RT-PCR analysis of Cre expression in brain (B), heart (H), liver (L), muscle (M), kidney (K), spleen (S), and the retina control (R). The lane labeled "D" has a 100 bp DNA size marker. The expected RT-PCR products for cre (411 bp), Cre activated reporter gene *LacZ* (495 bp), and Actin (450 bp) were identified on the gel. No apparent ecotopic Cre expression was identified.

RESULTS & DISCUSSION

Generation of cone specific Cre transgenic mice: PCR analysis of genomic DNA of the 143 mice obtained from zygote injection identified 31 positive mice carrying the *cre* transgene. The presence of the transgene was confirmed by Southern blot analysis of *Nco*I digested genomic DNA as shown in Figure 1B. Of the 31 positive mice carrying the *cre*,

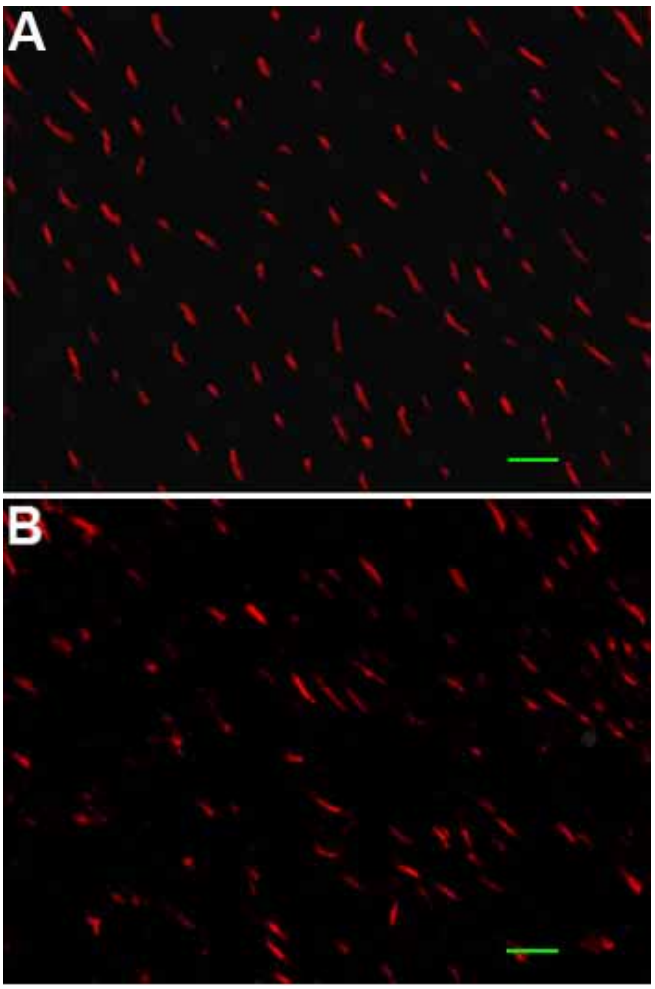

Figure 4. Cone distribution in HRGP-*cre* mice. Lectin staining showing cone photoreceptor distribution in 6 month old transgenic (**A**) and wild type littermate (**B**). The scale bars represent 16 μm.

25 had germline transmission. No apparent abnormalities in size, morphology, or behavior were observed in these mice, suggesting that the transgene insertion did not cause any detrimental mutation. Semi-quantitative RT-PCR analysis (Figure 1C) of *cre* expression in retinas from 10 to 15 day old transgenic mice suggested that 12 lines expressed *cre* mRNA in the retina. These mouse strains were capable of transmitting the transgene through germline in a Mendelian fashion and were characterized further.

Localization and functional analysis of Cre expression: To perform Cre functional assay and localize Cre expression, we took advantage of an existing Cre activatable *LacZ* reporter mouse line R26R [13]. This mouse strain carries a *loxP* flanked *neo* cassette that prevents the expression of *LacZ* reporter gene (Figure 2A). Upon Cre mediated recombination that removes the *neo* cassette, the *LacZ* reporter gene is expressed under the control of the generalized ROSA26 promoter. The cells expressing Cre recombinase are blue after β-galactosidase staining. Our preliminary studies using double transgenic F1 mice derived from R26R and EIIa-*cre* mice [10], a strain capable of deleting *loxP* flanked DNA in all cell types, suggested that ROSA26 promoter was able to direct *LacZ* reporter gene expression in all retinal cells (data not shown). Thus, we used F1 mice derived from R26R (C57B6) and HRGP-*cre* (FVB/

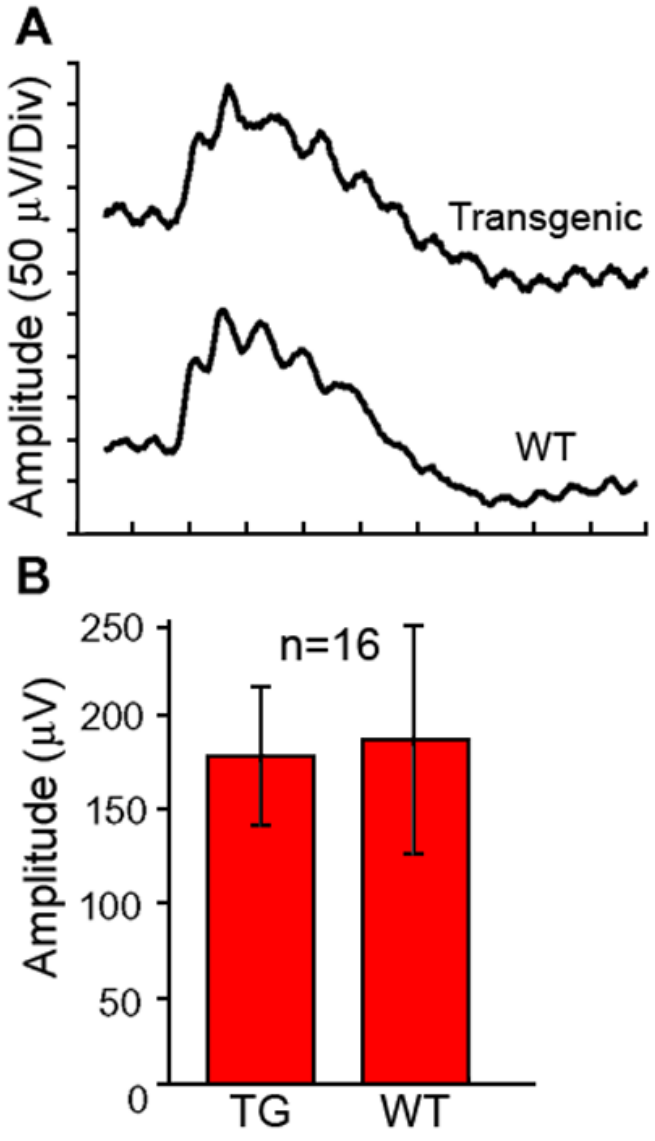

Figure 5. Cone Function in 6 month old HRGP-*cre* mice. **A**: Representative photopic ERG of transgenic mouse and wild type (WT) littermate. Amplitude is indicated as difference between baseline and the peak. **B**: Average b-wave amplitude from 16 eyes of the transgenic mice (TG) and wild type (WT) littermates. Error bars represent standard deviation. Cone function in 6 month old HRGP-*cre* mice was apparently undamaged.

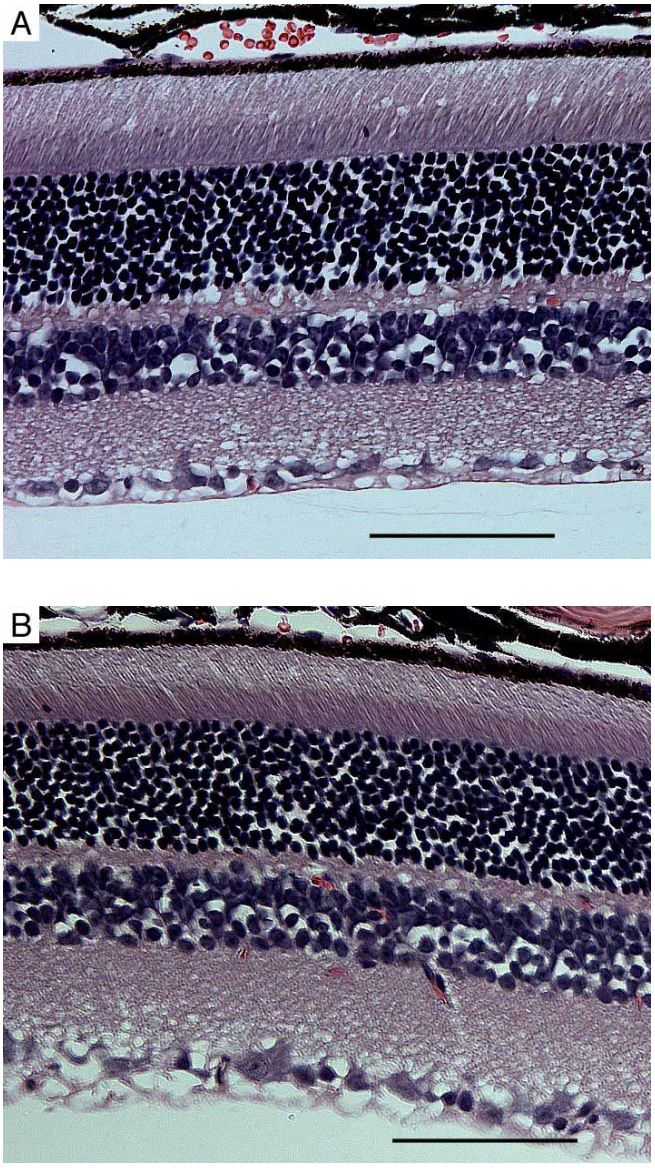

Figure 6. Retinal morphology in 6 month old HRGP-*cre* mice. Hematoxylin and eosin stained retinal section in 6 month old transgenic mouse (**A**) and wild type (WT) littermate (**B**). The scale bars represent 20 µm. No apparent abnormality was observed in 6 month old HRGP-*cre* mice.

N) mice throughout this study (unless otherwise indicated). Initial characterization using 6 to 8 week old F1 double transgenic mice showed that 10 of the Cre-expressing candidate strains, identified with RT-PCR analysis previously, had only limited amount of Cre activities, and β-galactosidase staining was punctuated. These strains were not characterized further. Two Cre-expressing candidate strains had efficient Cre expression in the retina, as shown in the X-gal stained retinal flat mount (Figure 2B). X-gal staining was strong near the center of the retina which is consistent with the cone distribution in mice [16]. Sections of X-gal stained retina showed that the β-galactosidase activity was localized to the presumptive cone photoreceptor cells (Figure 2C). The expression in the inner nuclear is consistent with the transgenic pattern conferred by the promoter of the human red-green pigment gene [11,17].

A small number of cells in the ganglion cell layer were positive with X-gal staining, although we have not observed any Cre expression in immunohistological staining. This result supports the observation of Akimoto et al. [18] that mouse M-opsin promoter conferred similar specificity in transgenic mice. Since a productive Cre mediated recombination is permanent and requires only a limited number of Cre molecules [19], the expression of Cre in low amounts or a brief expression of Cre at certain developmental stages may account for the discrepancy between Cre function and Cre immunoreactivity. The population of the X-gal staining cells in the retinal ganglion cell layer was very low; therefore, it is very unlikely that these cells will cause any dramatic effect in phenotype observation in cone photoreceptor specific gene knockout studies.

To determine Cre expression in developing retinas, β-galactosidase staining was also performed on neonatal double transgenic mice. Spotty β-galactosidase staining was observed in P10 retina but not P7 retina (data not shown), suggesting that detectable Cre mediated recombination occurred after P7 but before P10. This result is consistent with the finding that M-opsin mRNA is expressed at P10 [20]. Interestingly, β-galactosidase staining never reached the strong level shown in Figure 2 until the mice until about P30.

To further confirm the expression of Cre in cone photoreceptor cells, immunohistological staining was performed on retinal sections. Immunohistological staining with a polycolonal anti-Cre antibody showed that Cre was exclusively localized to the nucleus of cone photoreceptor cells (Figure 2E), consistent with our earlier observation that Cre carried a nuclear localization signal [21,22]. Double labeling for Cre and M-opsin showed that cells expressing M-opsin in cone outer segments also have Cre staining in their nuclei (Figure 2G), suggesting that almost all M-opsin-expressing cells expressed Cre recombinase. Mice only express M- and S-cone opsins. However, most cone cells express both M- and S-opsins and only a small percentage of cone cells express a single type of pigment [23]. Immunohistological examination of retinal sections labeled for Cre, M-opsin, and PNA suggested that almost all cone photoreceptors were M-opsin and Cre positive in our transgenic mice (Figure 2H).

The level of ectopic Cre expression was examined in the following representative tissues: brain, heart, liver, muscle, kidney, and spleen. Since Cre can be expressed anytime during development and may not be active at the time of assay, semi-quantitative RT-PCR was performed to detect *cre* mRNA and Cre activatable product *LacZ* mRNA, using tissues obtained from F1 mice of HRGP-*cre*/R26R. Our results showed that no detectable *cre* mRNA or *LacZ* mRNA was present in tissues examined (Figure 3). Tissues from F1 mice of HRGP-*cre*/R26R mice were also stained with X-gal. As discussed above, EIIa-*cre* mice [10] are capable of deleting *loxP* flanked DNA in all cell types. Thus, positive control tissues were obtained from double transgenic F1 of EIIa-*cre*/R26R mice that have the exact same genetic background as that of HRGP-*cre*/R26R mice. Tissues from Cre negative littermate mice were used as negative controls. No detectable X-gal staining was observed in brain, heart, muscle and kidney of the HRGP-*cre*/R26R mice. Since mouse liver and spleen have endogenous β-galactosidase activity, liver and spleen of the negative control mice were slightly blue after X-gal staining. In comparison with the tissues from the negative control mice, no detectable β-galactosidase staining beyond control level was observed in the liver and spleen of the of HRGP-*cre*/R26R mice (data not shown). We have not observed any apparent ectopic expression in HRGP-*cre* mice; thus, conditional knockout of essential genes with these mice is not likely to cause any detrimental effect in non-ocular tissues.

Distribution and function of cone photoreceptors: Cre is a DNA recombinase, and there is evidence suggesting that over-expression of Cre in retinal cells may cause retinal degeneration [24,25], presumably due to undesired recombination that causes chromosomal rearrangement [26,27]. To determine the usefulness of our cone specific Cre transgenic mice in gene functional studies, it is necessary to determine if any toxicity resulted from over-expression of Cre. The integrity of cone photoreceptors was analyzed by examining the distribution of cones in six month old F1 HRGP-*cre*/R26R mice. Fluorescent microscopic analysis of lectin stained retinas indicated that there were no significant differences in distribution and density of cone photoreceptor cells between the transgenic mice and wild type littermates (Figure 4), suggesting that there was no cone photoreceptor degeneration. To confirm that the cone function was normal, ERG analysis was performed on six month old F1 HRGP-*cre*/R26R mice. The photopic ERG suggested that there were no significant differences between the transgenic mice and the wild type littermates, as shown in Figure 5. In addition, two ten month old mice also had normal photopic ERGs (data not shown). The scotopic ERG data demonstrated that our transgenic mice had normal rod function (data not shown), suggesting that the insertion of the transgene did not cause any change in retinal function. Finally, hematoxylin and eosin staining of retinal sections from six month old F1 HRGP-*cre*/R26R mice suggested that the retinas of the transgenic mice were normal (Figure 6). In short, the adult HRGP-*cre* mice have normal cone

photoreceptors and are suitable for gene function studies in cone photoreceptors.

In summary, to study the function of essential genes expressed in multiple tissues, we have generated cone specific Cre mice that are efficient in carrying out Cre mediated gene activation and inactivation. During the preparation of this manuscript, Akimoto et al. [18] published their results on the generation and characterization of mouse M- or S-opsin promoter controlled *cre* transgenic mice. Our HRGP-*cre* mice showed a similar expression pattern as the mouse M-opsin promoter controlled *cre* mice. However, the following points are worth noting. Our studies provide additional information suggesting that cone function and distribution are normal in adult HRGP-*cre* mice. In light of the observation that overexpressing Cre causes DNA damage in mammalian cells [26], chromosome rearrangement in spermatids [27], and retinal degeneration in rod photoreceptors [24,25], our present information is crucial if the transgenic mice are to be used in gene function analysis. In addition, we have provided information regarding the onset of Cre expression in cone photoreceptors and ecotopic Cre expression in non-ocular tissues, which will be very useful in designing conditional gene expression studies.

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