Transgenic mice expressing Cre-recombinase specifically in M- or S-cone photoreceptors

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ABSTRACT

Purpose: To establish lines of transgenic mice that express Cre-recombinase in M- or S-cone photoreceptors for generating cone photoreceptor-specific (conditional) mutants.

Methods: 5.0 kb of 5'-upstream sequence of the mouse red/green (M) opsin gene or 0.5 kb of the mouse blue (S) opsin gene was cloned into a Cre-expression plasmid. Transgenic mice were generated and characterized, and appropriate lines were established. The Cre-transgenic mice were crossed with ROSA26-*lacZ* mice (containing floxed β-galactosidase gene) and analyzed to determine Cre-recombinase activity.

Results: Immunofluorescence study showed successful targeting of Cre-recombinase expression to cone photoreceptors. Double staining with anti-Cre antibody and anti-M- or anti-S-opsin antibody revealed specificity of Cre expression in M-opsin and/or S-opsin positive photoreceptors. Mating with ROSA26-*lacZ* mice demonstrated that Cre-recombinase was functionally active in M- or S-cones.

Conclusions: We have established lines of transgenic mice that specifically express functional Cre-recombinase in M- or S-cones. Since mutations in several widely-expressed genes lead to photoreceptor degeneration, these transgenic mice should be valuable in generating conditional mutants to specifically investigate the function of various genes in cone photoreceptors.

INTRODUCTION

The retina of mammals has two kinds of photoreceptors, rods and cones. Rods contain the visual pigment rhodopsin and are responsible for vision under conditions of low ambient light. Cones bestow high visual acuity under bright light conditions, and their different subtypes (each with unique visual pigments) constitute the basis of color vision. Rods dominate the mammalian retina; and cones represent only 3-5% of all photoreceptors in rodent and primate retina[1,2]. Hence, despite the importance of cone function in vision, cone biology is greatly under-explored.

Defects in photoreceptor development and function are the major cause of inherited retinal degenerative diseases, which constitute a clinically and genetically heterogeneous group (RetNet; http://www.sph.uth.tmc.edu/Retnet/home.htm). In many instances, these diseases result from mutations in retina-specific genes; however, mutations in several widely-expressed genes have also been identified in retinal disorders[3-15]. For example, the majority of X-linked RP patients have mutations in RPGR or RP2, two ubiquitous proteins of unknown function[3-11]. One approach to delineate mechanisms of disease pathogenesis involves the generation and characterization of animal (particularly mouse) models. Gene targeting using homologous recombination (gene knockout strategy) offers a unique opportunity to produce mouse models of human disease[16]. Investigations of knockouts in mice have

revealed significant insights into gene function during retinal differentiation and disease[17-22]. However, conventional techniques are generally not sufficient to evaluate cell type specific function of widely-expressed genes. Embryonic lethality is often observed in mice harboring two copies of targeted non-functional alleles (or one copy on X chromosome in males). In addition, the germ-line incorporation of the mutation may result in gene inactivation in all cells, leading to secondary, non-cell autonomous phenotypes that may be difficult to distinguish from cell autonomous phenotypes. Moreover, complex systemic changes due to a targeted mutation may obscure more subtle phenotypes of the retina.

The Cre/loxP recombination system offers an opportunity to introduce the mutations in a tissue-specific or inducible fashion[16,23-25] (Figure 1A). In mice, this approach has been successfully used to accomplish both cell-type restricted activation of transgenes (TG) and generation of cell type restricted null alleles by the deletion of loxP flanked (floxed) gene segments[16,23-25]. Several lines of transgenic mice expressing Cre-recombinase in specific cell types (see http://www.mshri.on.ca/nagy/), including ocular tissues, have been established[26-32]. As a prelude to cone photoreceptor specific gene targeting, we have generated transgenic mouse lines that express Cre-recombinase under the control of mouse red/green (M) or blue (S) pigment gene promoters. These Cre-transgenic mouse lines will be invaluable for studying the *in vivo*

function of genes in cone photoreceptors using a conditional gene targeting strategy.

MATERIALS AND METHODS

Materials

All reagents, buffers or enzymes were purchased from Invitrogen (Carlsbad, CA), New England BioLabs (Beverly, MA), or Sigma (St. Louis, MO).

Transgene vectors and generation of transgenic mice

We generated promoter-less pCI vector (pCIpl) by excision of CMV promoter from pCI expression vector (Promega, Madison, WI) by *Msc*I and *Nhe*I endonuclease digestion, followed by blunt-ending and self-religation. Promoter-less Cre vector (pCICre) was generated by insertion of a 1.1 kb *Mlu*I fragment from pMCCre[23] (a generous gift of Dr. K. Rajewsky) into *Mlu*I site of pCIpl. We then amplified mouse red/green (GenBank accession # S44742) or blue (GenBank accession # L27831) pigment gene promoter region by PCR using genomic DNA from R1 mouse strain. The primers used were: Forward 5'-CTAGCTAGCATACCTTGAAACCCACA-3' and Reverse 5'-CGCCTCGAGGCTGTAGAAAACTG-3' for mouse red/green pigment gene (mRGP) promoter; and Forward 5'-GGCAGGATGCAGTTGTTTCT-3' and Reverse 5'-TCCCGCTTGGGATGCCCT-3' for mouse blue pigment gene (mBP) promoter. The 5.0 kb PCR product of mRGP promoter and the 500 bp of mBP promoter were

subcloned into pGEM-T Easy (Promega, Madison, WI), digested by *Eco*RI, and then cloned into pCICre vector. We excised mRGP promoter driving Cre-recombinase or mBP promoter driving Cre-expression cassette from recombinant plasmids by *Nhe*I and *Nae*I digestion or *Xho*I and *Nae*I digestion, respectively (Figure 1C). After purification with NucleoSpin^{TM} (Clontech, Palo Alto, CA), each fragment was injected into pronuclei of (C57BL/6 X SJL) F2 mouse eggs, which were implanted into pseudo-pregnant foster mothers using standard techniques. Transgenic founder mice and their progeny were identified by PCR using the following primers: Forward RGPF: 5'-AATGGGAACAGTGGTGTGTG-3'; BPF: 5'-AGGAGGGTGCTGTAGGGAAG-3'; Reverse (CreR): 5'-GAACGAACCTGGTCGAAATC-3'. Southern blot analysis of *Bam*HI- or *Hinc*II-digested genomic DNA or dot blot analysis was performed by hybridization with 1.1 kb Cre gene probe, excised by *Mlu*I from pMCCre, and copy numbers were estimated. Founders were bred to C57BL/6 mice to generate F1 progeny.

The research reported here was performed in accordance with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the institutional review committee.

Immunohistochemical analysis of Cre-recombinase and β-galactosidase

Mouse eyes were fixed for 1 to 2 hr with fresh 4% paraformaldehyde in 100 mM

phosphate buffer, pH 7.2, and immersed overnight at 4°C in 20% sucrose in phosphate-buffered saline (PBS). Next day, the eyecups were infiltrated in a solution containing 2:1 ratio of 20% sucrose/PBS and O.C.T. Compound (Tissue-Tek; Sakura Finetek, Torrance, CA), embedded in 100% O.C.T., and frozen. 10 or 25 μm thick sections were cut and mounted on silanized slides, dried and kept in freezer at –80^{o}C until use. Polyclonal rabbit (at 1:500 dilution) or mouse (at 1:500 dilution) monoclonal anti-Cre antibody (Covance, Madison, WI) was used as primary antibody. For double staining, rabbit polyclonal M-opsin, or S-opsin antibody (at 1:500 dilution) was used[21]. Secondary antibodies were Alexa Fluor488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) and cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) antibodies.

To evaluate the activity of Cre-recombinase, Cre-mice were mated to ROSA26-*lacZ* mice[33], in which β-galactosidase gene is knocked-in. In the resulting mice, cells with functional Cre-recombinase should express β-galactosidase driven by ROSA26 promoter (Figure 1B). X-gal staining was done by the following protocol[34]. Eyes were pre-fixed by immersion in 4% paraformaldehyde / PBS for 2 hr, then washed in cold PBS and incubated in PBS containing 1 mg/ml of X-gal (5-bromo-4-chloro-3-indolyl-β- D-galactopyranoside), 0.01% sodium deoxycholate, 0.02% NP40, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6$ overnight at 37^{o}C.

Each sample was embedded and sectioned, and then observed by conventional microscopy.

RESULTS

Generation of transgenic mice

Eight founder animals of RGP-Cre, and seven of BP-Cre were identified by PCR and Southern blot analysis. Each founder was bred to C57BL/6 mice to generate F1 progeny. Four of the eight lines of RGP-Cre and two of the seven lines of BP-Cre showed transgene expression. Typical results of genomic Southern blotting with *Hinc*II digestion are shown in Figure 1D. Genomic PCR generated the expected 768 bp band for RGP-Cre transgenic mouse and a 600 bp product for BP-Cre (Figure 1E). Transgene inheritance was confirmed through more than five generations.

Immunostaining of Cre and M- or S-opsin

The expression of Cre-recombinase protein was examined by immunohistochemical methods. Both polyclonal and monoclonal anti-Cre antibodies revealed similar cone-specific staining. The retinae of RGP-Cre and BP-Cre transgenic mice show the inferior to superior gradient distribution of Cre-positive cells, which is similar to the distribution of normal M- or S-cone photoreceptors[35] (Figure 2A, 3A). Higher background is consistently detected in BP-Cre mouse retina compared to

RGP-Cre due to differences in expression level. M- or S-cone specificity was examined by double staining with anti-Cre antibody and either M- or S-opsin antibody. In RGP-Cre transgenic mice, Cre and M-opsin immunostaining overlapped completely, whereas S-opsin staining demonstrated a partial overlap (Figure 2B, 2C). In BP-Cre transgenic mice, Cre and S-opsin immunostaining was completely overlapping, and M-opsin staining had a partially overlap (Figure 3B, 3C). This is consistent with previous findings that in mouse many cone photoreceptors express both M-opsin and S-opsin[36].

X-gal staining

To examine the enzymatic activity of Cre-recombinase in cone cells, Cre-transgenic mice were mated with ROSA26-*lacZ* mice, and the litters were analyzed. In both RGP-Cre and BP-Cre mouse retina, cone photoreceptors are stained in blue demonstrating functional Cre-recombinase. Several cells in the retinal ganglion cell (RGC) layer and a few dispersed cells in the inner nuclear layer (INL) are also positive (Figure 2D, 3D).

DISCUSSION

Diseases exhibiting clinical phenotypes of the retina can result from mutations in genes that are expressed in multiple tissues and cell types. The conditional gene inactivation or activation strategy using Cre/loxP system offers an attractive method to

investigate gene function and/or disease pathogenesis focusing on cell-type specific effects. One excellent example of this is the elucidation of KIF3A function in photoreceptors[27]. Our goal in this study was to create Cre-recombinase expressing mouse lines that could be used to conditionally knockout (or activate) the expression of loxP-modified genes in only M- or S-cones in order to understand their specific function in cone photoreceptors. Here, we describe the successful generation of RGP-Cre and BP-Cre transgenic mice, where Cre-recombinase is under the transcriptional control of the mouse red/green (M) or blue (S) opsin gene promoter, respectively.

Our data supports previous studies showing that 6.5 kb upstream region of the human red/green visual pigment gene can direct transgene expression specifically to M-cone photoreceptors[37,38], and that 500 bp upstream region of the human blue pigment gene can target expression to S-cone photoreceptors[39,40]. The human S-opsin promoter also directed the reporter gene expression to bipolar cells in addition to S-cones[39,40], though the 6.4 kb upstream region of mouse S-opsin promoter was specific for S-cones[41]. In our studies, the mouse 500 bp promoter directed reporter gene expression primarily to S-cones; hence, this sequence in mouse but not human promoter may include the minimal S-cone specific element. The detection of Cre-transgene expression in S-opsin positive cells of RGP-Cre and M-opsin positive cells of BP-Cre mouse retina is consistent with a previous study that demonstrated

co-expression of both M- and S-opsins in many cone photoreceptors in mice[36].

Retina from both RGP-Cre and BP-Cre transgenic mice show *lacZ* expression in a subset of cells in the ganglion cell layer, even though Cre-immunoreactivity can not be observed. This discrepancy may result from the difference in expression levels and the sensitivity of detection. Even low level of Cre expression, undetectable by immunofluorescence, may excise the floxed region and activate β-galactosidase expression. Interestingly, a recent study demonstrated the existence of a subpopulation of photoreceptor and cone bipolar cells displaced to the ganglion cell layer[42]. We have not characterized X-gal positive cells in RGC layer in Cre-transgenic mice as yet, but these cells may represent displaced photoreceptors and/or cone bipolars. Alternatively, these cells may indicate residual background staining as a previous report implied[43]. Whatever the cause, the small amount of ectopic expression should not affect the usefulness of the Cre-transgenic mice.

The Cre-transgenic mouse lines, reported here, will permit specific deletions of loxP harboring genes in M- or S-cone photoreceptors, overcoming problems encountered in conventional gene-knockout techniques. Several examples of the potential use of these Cre-transgenic mice can be illustrated. Mutations in the *RPGR* and *RP2* genes are the primary cause of X-linked retinitis pigmentosa, a relatively severe form of retinal degenerative disease[3-11]. While mouse Rp2-knockout has not yet

been generated, some of the human *RPGR* mutations[6,7] and the only published mouse Rpgr knockout model[22] demonstrate cone photoreceptor degeneration. Conditional disruption of Rp2 and Rpgr in cone photoreceptors will therefore provide significant new insights into their role in cone biology. Similarly, many transcription factors, including several homeodomain proteins and nuclear receptors, exert a major influence on retinal development. The RGP-Cre and BP-Cre mice that we have generated should be valuable for delineating the specific function of these and other widely-expressed proteins in cone photoreceptors.

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FIGURE LEGENDS

Figure 1. Cre/loxP strategy and the transgene constructs.

(A) Schematic representation of the Cre/loxP system (see ref. Weden et al.[16] for a review). Cre-recombinase activity introduced in a specific cell excises the region flanked by loxP sites.

(B) Schematic representation of ROSA26-*lacZ* Cre reporter system. Cre-recombinase excises stuffer sequences, inducing β-galactosidase gene expression.

(C) BP-Cre and RGP-Cre transgenes. *White box*: 500bp of blue pigment gene promoter and 5.0 kb of red and green pigment gene promoter; *black box*: the Cre-coding region; *gray box*: simian virus (SV) 40 polyadenylation signal; *Arrows*: PCR primers used; H: *Hinc*II sites.

(D) Representative results of genotyping by Southern blotting. *Hinc*II digests at two adjacent sites in the transgene constructs. The 2 kb band in BP-Cre and the 7 kb band in RGP-Cre represent the transgene fragment. Lane M contains 1 kb ladder.

(E) Representative results of genotyping by PCR. 768 bp product is obtained for RGP-Cre and 600 bp for BP-Cre. Lane M indicates 100 bp ladder.

Figure 2.

(A) Immunofluorescence of Cre-immunoreactivity against rabbit anti-Cre polyclonal antibody in RGP-Cre transgenic mouse retina. Insets show immunostaining at higher

magnification. RGC, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

(B, C) Double staining with mouse anti-Cre monoclonal antibody and rabbit anti-mouse-S-opsin or anti- mouse-M-opsin polyclonal antibody.

(D) X-gal staining of retina from ROSA26-*lacZ* and RGP-Cre double transgenic mice. *Arrow heads* show blue staining of cone photoreceptors.

All scale bars = 50 μm

Figure 3.

(A) Immunofluorescence of Cre-immunoreactivity against rabbit anti-Cre polyclonal antibody in BP-Cre transgenic mouse retina. Background is higher than Figure 2A due to low signal intensity. Insets show immunostaining at higher magnification. RGC, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

(B, C) Double staining with mouse anti-Cre monoclonal antibody and rabbit anti-mouse-S-opsin or anti- mouse-M-opsin polyclonal antibody

(D) X-gal staining of retina from ROSA26-*lacZ* and BP-Cre double transgenic mice. *Arrow heads* show blue staining of cone photoreceptors.

All scale bars = 50 μm

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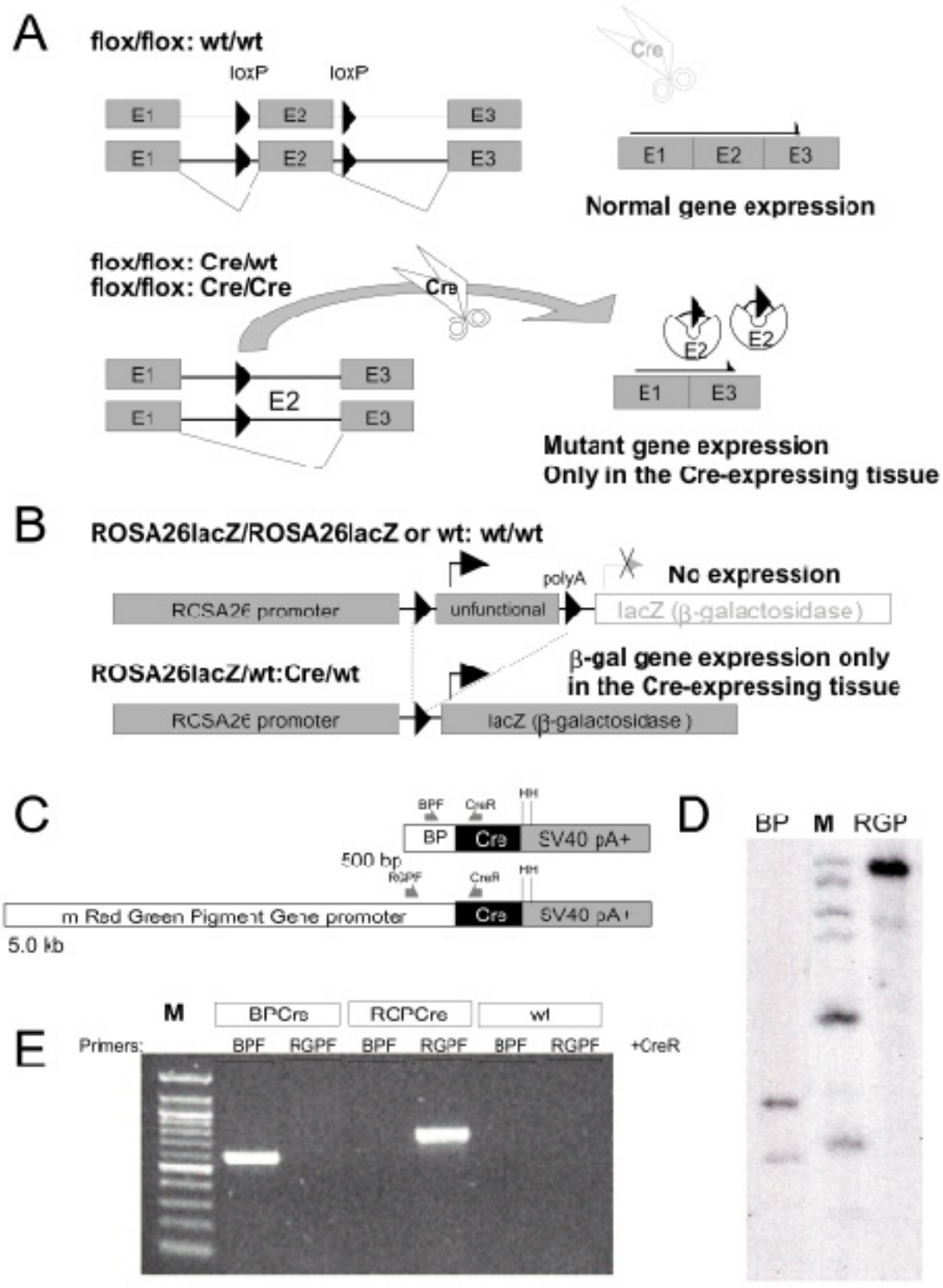
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Figure 1

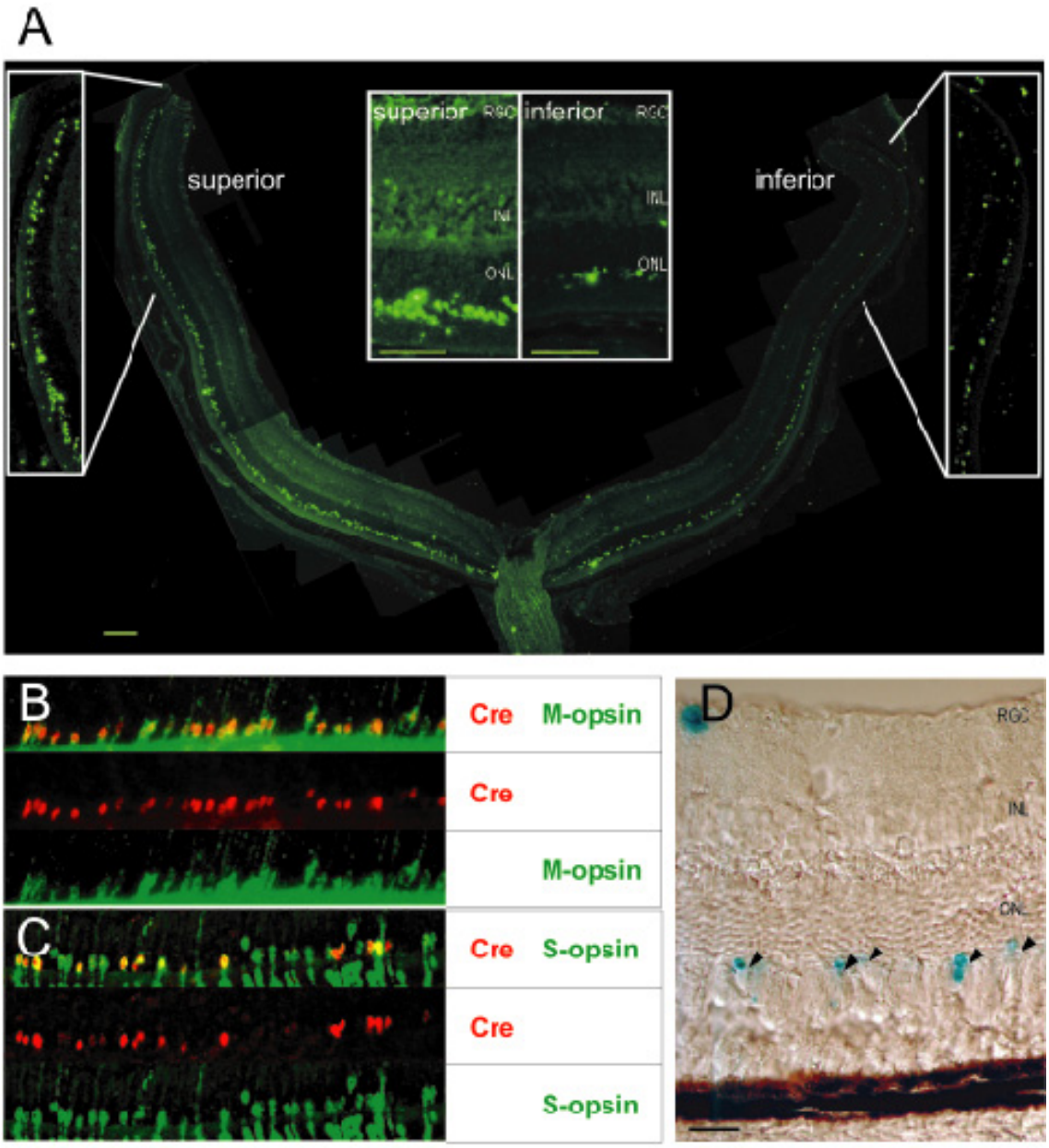
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Figure 2.

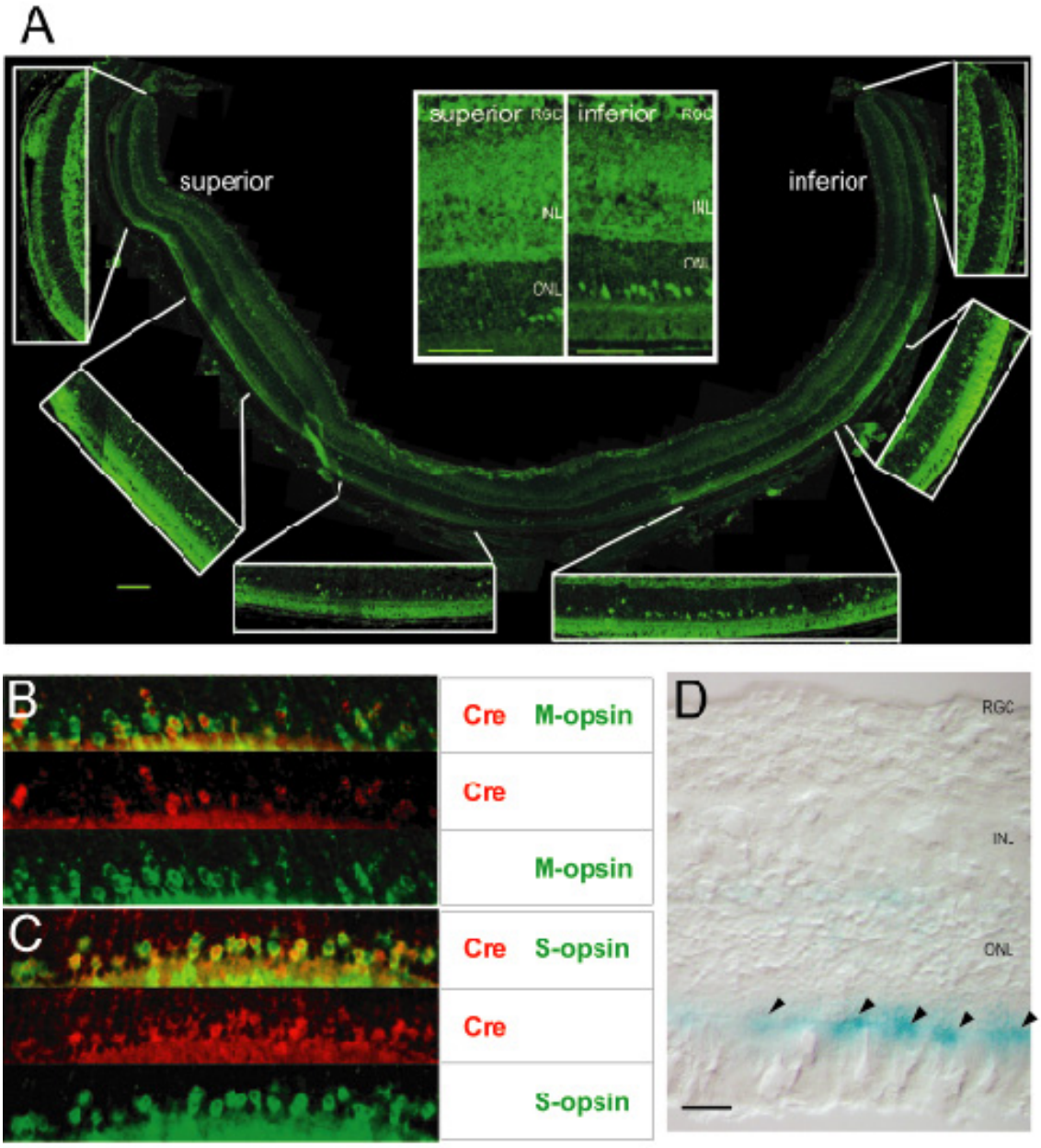
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Figure 3.