

Research Article

BAC-*Dkk3*-EGFP Transgenic Mouse: An *In Vivo* Analytical Tool for *Dkk3* Expression

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Dickkopf (DKK) family proteins are secreted modulators of the Wnt signaling pathway and are capable of regulating the development of many organs and tissues. We previously identified *Dkk3* to be a molecule predominantly expressed in the mouse embryonic retina. However, which cell expresses *Dkk3* in the developing and mature mouse retina remains to be elucidated. To examine the precise expression of the *Dkk3* protein, we generated BAC-*Dkk3*-EGFP transgenic mice that express EGFP integrated into the *Dkk3* gene in a BAC plasmid. Expression analysis using the BAC-*Dkk3*-EGFP transgenic mice revealed that *Dkk3* is expressed in retinal progenitor cells (RPCs) at embryonic stages and in Müller glial cells in the adult retina. Since Müller glial cells may play a potential role in retinal regeneration, BAC-*Dkk3*-EGFP mice could be useful for retinal regeneration studies.

1. Introduction

In vertebrates, *Dickkopf* (*Dkk*) family genes encode four secreted proteins (*Dkk1*–*4*) and a unique *Dkk3*-related protein, Soggy or Dkk-like1 (*Dkk1l*) [1]. *Dkks* are potent inhibitors of β -catenin stabilization in the Wnt signaling pathway and suppress the expression of a variety of canonical Wnt/ β -catenin signaling target genes [2]. *Dkk1*, *Dkk2*, and *Dkk4* inhibit the Wnt signaling pathway through both their high-affinity binding to the Wnt coreceptors *Lrp-5/6* [3–5] and blockade of the signaling cascade downstream of Wnt receptor *Frizzled*. In contrast, *Dkk3* appears to inhibit the Wnt signaling pathway by directly binding to β -catenin through a complex with a β -transducin repeat-containing protein, β TrCP [6].

We previously reported identifying the *Dkk3* gene as a molecule that is predominantly expressed in mouse retinal progenitor cells [7]. *Dkk3* expression is observed during vertebrate development in various organs [1, 8–11]. In our previous report, we used *in situ* hybridization to show that mouse *Dkk3* mRNAs were detectable in the entire neuroblastic layer (NBL) of the retina at embryonic stages,

and then in the inner nuclear layer (INL) at postnatal stages [7]. Moreover, we generated a BAC-*Dkk3*-*Cre* transgenic mouse, which utilizes a bacterial artificial chromosome (BAC) expressing *Cre* recombinase inserted into the *Dkk3* gene locus [7]. This transgenic mouse line is a powerful tool for ablating a gene of interest in mitotic retinal progenitor cells (RPCs) [12–14]. The expression pattern and the possible function of *Dkk3* suggest that this molecule exerts a role in proliferation, cell fate determination, and/or maintenance of RPCs during mouse development. In addition, *Dkk3* expression continues into the adult stage. However, it is still unclear what types of differentiated cells express *Dkk3* in the developing retina.

Here, we report the generation of a BAC-*Dkk3*-EGFP mouse line, which expresses the EGFP gene under the control of *Dkk3* regulatory elements. This mouse enables us to investigate the expression pattern of *Dkk3* in the developing retina in detail. In the current study, we found that EGFP is specifically and strongly expressed in RPCs at embryonic stages, and in Müller glial cells and a subset of amacrine cells at mature stages.

2. Materials and Methods

2.1. Construction and Generation of the BAC-Dkk3-EGFP Transgenic Mouse. A BAC clone (clone ID: RP23-12M6) containing the entire *Dkk3* gene was purchased from Children's Hospital Oakland Research Institute. According to the NCBI database (available at <http://www.ncbi.nlm.nih.gov/>), this BAC clone also contained a fragment of ubiquitin-specific peptidase 47 (*Usp47*), a microtubule-associated monoxygenase called calponin, and a LIM domain-containing gene (*Mical2*). However, neither the transcription initiation site nor the start codon of *Usp47* is included in the BAC clone, but it does contain the 5' untranslated region of *Mical2*. Two homology arms (5' arm, 486 bp; 3' arm, 453 bp) from exon 1 of the *Dkk3* gene were amplified by PCR from the BAC clone DNA and cloned into a T-easy vector (Promega). The PCR primers used were 5'-GGCGCGCCTATGTGCGCTGTCTAGGGACTT-3' and 5'-CCCGGGGCAAGCTGGATCTGGTCACGACCGG-3' for the 5' homology arm and 5'-TTAATTAATTGGGTGAGC-GGTGGTCATCGTC-3' and 5'-GGCCGGCCAGACTC-AATCCCTGCTGGAACA-3' for the 3' homology arm. The two homologous arms were inserted into both sides of the enhanced green fluorescent protein-*polyA* (*EGFP-pA*) cassette in the modified pLD53 SCA-Cre-B shuttle vector [15]. The *EGFP-pA* cassette was introduced into the 5'UTR of the first exon of the *Dkk3* gene by homologous recombination (Figure 1(a)) [15]. PCR and sequencing were used to confirm the correct insertion of the *EGFP* gene into the *Dkk3* locus. The entire *Dkk3-EGFP* transgene was purified by dialysis on a filter (Millipore, VSWP02500) floating in an injection buffer (10 mM Tris, pH 7.5; 0.1 mM EDTA; 100 mM NaCl). The purified construct was injected as circular DNA into the pronuclei of fertilized one-cell eggs of *B6C3F1* mice then implanted into pseudopregnant foster mothers (ICR, Japan SLC Inc). All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the procedures were approved by the Institutional Safety Committee on Recombinant DNA Experiments and the Animal Research Committee of Osaka Bioscience Institute.

2.2. Genotyping. PCR genotyping for the *Dkk3-EGFP* transgene was performed under the following conditions: 94°C for 2 min, then 94°C, 1 min 60°C, 1 min; 72°C, 1 min for a total of 35 cycles and followed by 72°C for 10 min (primers: 5'-CAGACCATACTAGTTTGGCAGTAC-3' and 5'-GCAGCTTGCCGGTGGTGCAGATGAACT-3'). The primers amplify a fragment of the *Dkk3-EGFP* transgene.

2.3. Northern Blot Analysis. We extracted total RNAs (retina: 10 µg, other tissues: 20 µg) from adult *BAC-Dkk3-EGFP* transgenic mice using the TRIzol reagent (Invitrogen). Northern blot hybridization was performed as described previously [7]. A full-length cDNA of *EGFP* was used as a radiolabeled probe for hybridization.

2.4. Immunostaining. Mouse eye cups were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for

immunohistochemistry. The samples were cryoprotected, embedded, frozen, and sectioned into 20 µm thick sections. We preincubated them with blocking solution (4% normal donkey serum and 0.1% Triton X-100 in PBS) for 30 min. Then, we incubated the sections with primary antibodies at 4°C overnight, rinsed them with blocking solution, and incubated them with secondary antibodies for 30 min. The sections were coverslipped with gelvatol after rinsing with 0.1% Triton X-100 in PBS. We used the following primary antibodies: rhodopsin (LSL, LB-5597; rabbit polyclonal; 1:10000), S-opsin (Chemicon, AB5407; rabbit polyclonal; 1:1000), M-opsin (Oriental Bioservice; rabbit polyclonal; 1:1000), S100β (Sigma, S-2532; mouse monoclonal; 1:1000), SOX9 (Chemicon, AB5535; rabbit polyclonal; 1:750), PAX6 (DSHB, P3U1; mouse monoclonal; 1:200), and CHX10 (MBL; rabbit polyclonal; 1:100) [16]. We used Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, no. 711-165-152; 1:400) and anti-mouse IgG (Jackson ImmunoResearch Laboratories, no. 711-165-150; 1:400) secondary antibodies. DAPI was applied to stain nuclei (Sigma). We also used rhodamine-labeled Peanut Agglutinin (PNA, Vector Laboratories, RL-1072; 1:200). Confocal images were acquired with a Zeiss LSM 700 inverted confocal microscope.

3. Results

3.1. Generation of BAC-Dkk3-EGFP Mouse. To generate a *Dkk3* regulatory elements-driven, EGFP-expressing mouse, we took advantage of a bacterial artificial chromosome (BAC) transgenic mouse, carrying BACs modified using homologous recombination in bacteria [15, 17, 18]. We designed a BAC construct with the *EGFP* cDNA placed in the 5'UTR of the *Dkk3* gene by homologous recombination (Figure 1(a)). To verify the tissue-specific EGFP expression, total RNA from the *BAC-Dkk3-EGFP* mouse retina and other tissues at the adult stage was analyzed by Northern blotting. A single transcript was detected in the retina and cerebrum, but no significant signal was observed in other tissues examined (Figure 1(b)). *Dkk3* expression was strongly detected in the retina and cerebrum by Northern blot analysis using wild-type adult mouse tissues (Figure 1(c)). Since the EGFP expression level was extremely high in the retina, we even observed the EGFP expression as "green eyes" under the room light at the adult stage after Mydrin-M ophthalmic solution 0.4% (Santen, no. 084162243) was administrated (Figures 1(d) and 1(e)).

3.2. The EGFP Expression Patterns in the Developing Retina of the BAC-Dkk3-EGFP Mouse. In the *BAC-Dkk3-EGFP* mouse embryo, we observed an EGFP expression in the eye, heart, and spinal cord at embryonic day 9.5 (E9.5) (Figures 2(a) and 2(b)). An EGFP signal was weakly detected in the palate and heart at E16.5 (Figures 2(c) and 2(d)). In our previous study, *Dkk3* mRNA expression was detected in the entire NBL of the embryonic retina and in the INL in the postnatal retina using *in situ* hybridization [7]. To confirm this result, we investigated EGFP expression in the developing retina of the *BAC-Dkk3-EGFP* mouse.

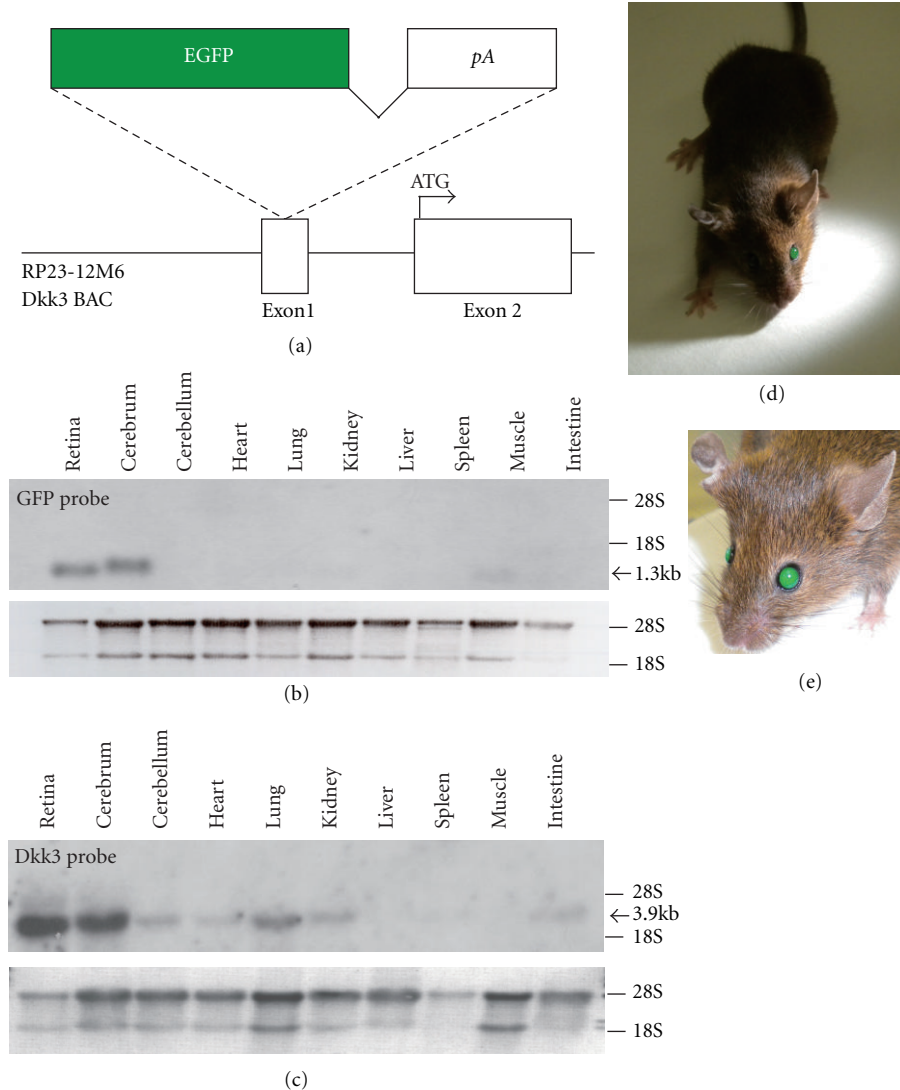


FIGURE 1: Strategy for preparing the *Dkk3-EGFP* transgene and Northern hybridization analysis of EGFP expression in adult mouse organs. (a) A schema of the modified BAC integrated with the *EGFP-pA* cassette into the first exon of the mouse *Dkk3* gene. (b) Northern hybridization analysis of total RNA (retina, 10 μ g; other tissues, 20 μ g) isolated from adult organs in the *BAC-Dkk3-EGFP* mouse. The hybridization signal was obtained with the EGFP probe. The lower panel shows ethidium bromide staining of the RNA. (c) Northern blot analysis using a *Dkk3* probe for wild-type mouse tissues. (d, e) Photographs of the adult *BAC-Dkk3-EGFP* mouse, which displays “green eyes” even under the room light because of the intense expression of EGFP in the retina.

From E15.5 to postnatal day 0 (P0), EGFP was detected in most mitotic and undifferentiated RPCs throughout the NBL (Figures 2(e)–2(g)). At P3, EGFP expression was limited to RPCs, and differentiated retinal cells were not labeled by EGFP (Figure 2(h)). At P6, EGFP was expressed in Müller glia-like cells, which exhibit long glial fibers and localize their cell bodies in the INL (Figure 2(i)). At P14, only Müller glial cells in the INL expressed EGFP, and we observed a large number of glial fibers extending into the surrounding retinal layers (Figure 2(j)).

3.3. Cell Types Expressing EGFP in the Adult *BAC-Dkk3-EGFP* Mouse Retina. It remains to be clarified which cell types express *Dkk3* in the adult mouse retina. To examine

cell types expressing EGFP, we performed immunostaining using antibodies against several photoreceptor cell-specific markers (Figure 3). Using antibodies against rhodopsin (rod photoreceptors), PNA, S-opsin, and M-opsin (cone photoreceptors), we did not detect an EGFP signal significantly overlapping with any photoreceptor cell-specific markers (Figures 3(a)–3(l)). Then, we immunostained the adult retina with another set of retinal cell-type antibodies, including antibodies against S100 β , SOX9 (Müller glial cells), PAX6 (amacrine cells), and CHX10 (bipolar cells) (Figure 4). We observed an EGFP signal overlapping with that of S100 β in the Müller glial fibers extending into other retinal layers (Figures 4(a)–4(c)). Moreover, cell bodies of the Müller glial cells immunostained with SOX9 antibody were

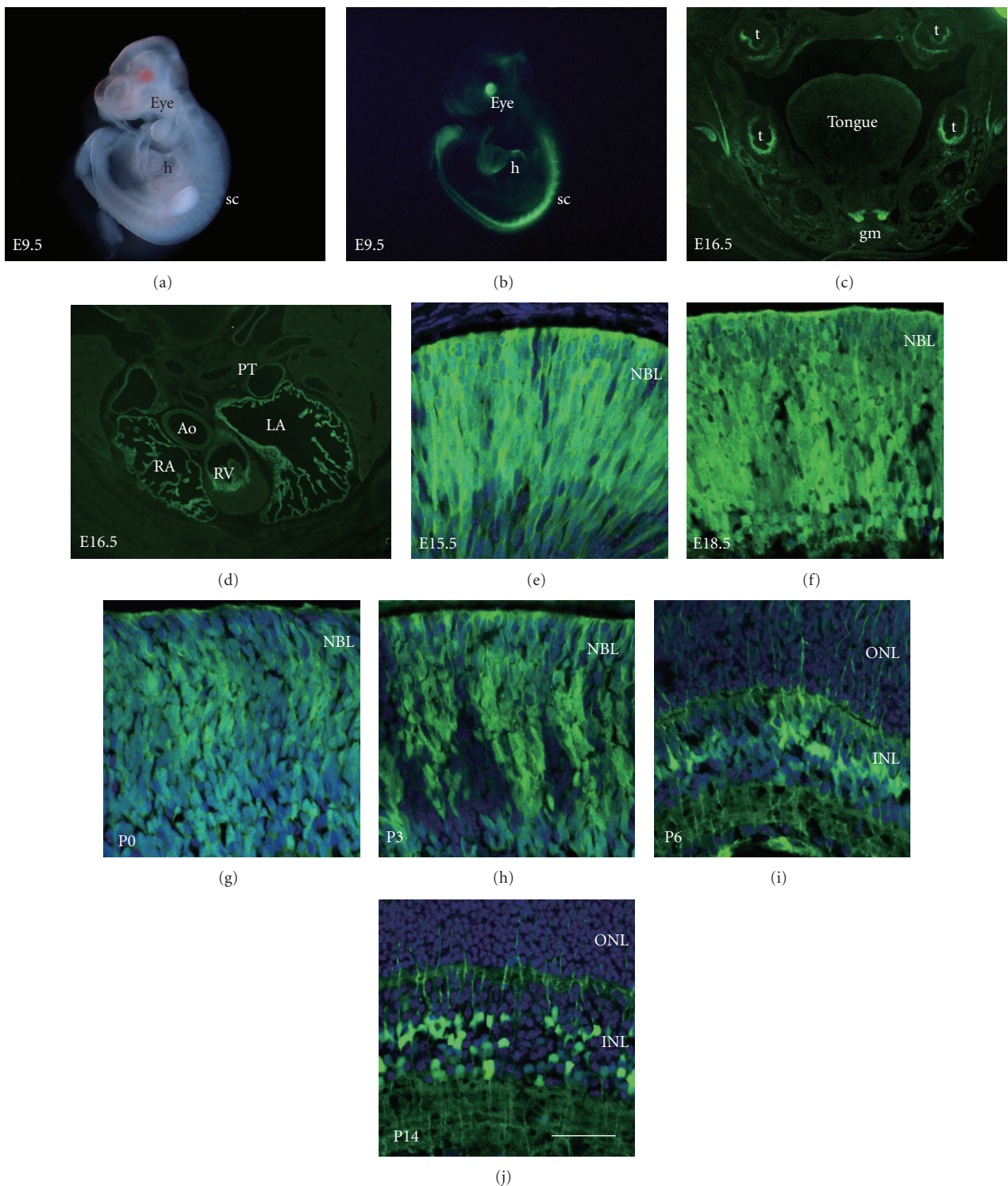


FIGURE 2: (a, b) Photographs of the *BAC-DKK3-EGFP* mouse embryo at E9.5. The embryo was visualized under bright-field (a) and fluorescence (b). h, heart; sc, spinal cord. (c) A coronal section through the palatal region of the *BAC-Dkk3-EGFP* mouse at E16.5. t, tooth; gm, genioglossus muscle. (d) A coronal section through the heart of the *BAC-Dkk3-EGFP* mouse at E16.5. PT, pulmonary trunk; Ao, Aorta; LA, left atrium; RA, right atrium; RV, right ventricle. (e–j) EGFP expression in the *BAC-Dkk3-EGFP* mouse retina. EGFP expression was detected at E15.5 (e), E18.5 (f), P0 (g), P3 (h), P6 (i), and P14 (j). Nuclei were counterstained with DAPI. NBL, neuroblastic layer; ONL, outer nuclear layer; INL, inner nuclear layer. Scale bar represents 50 μm .

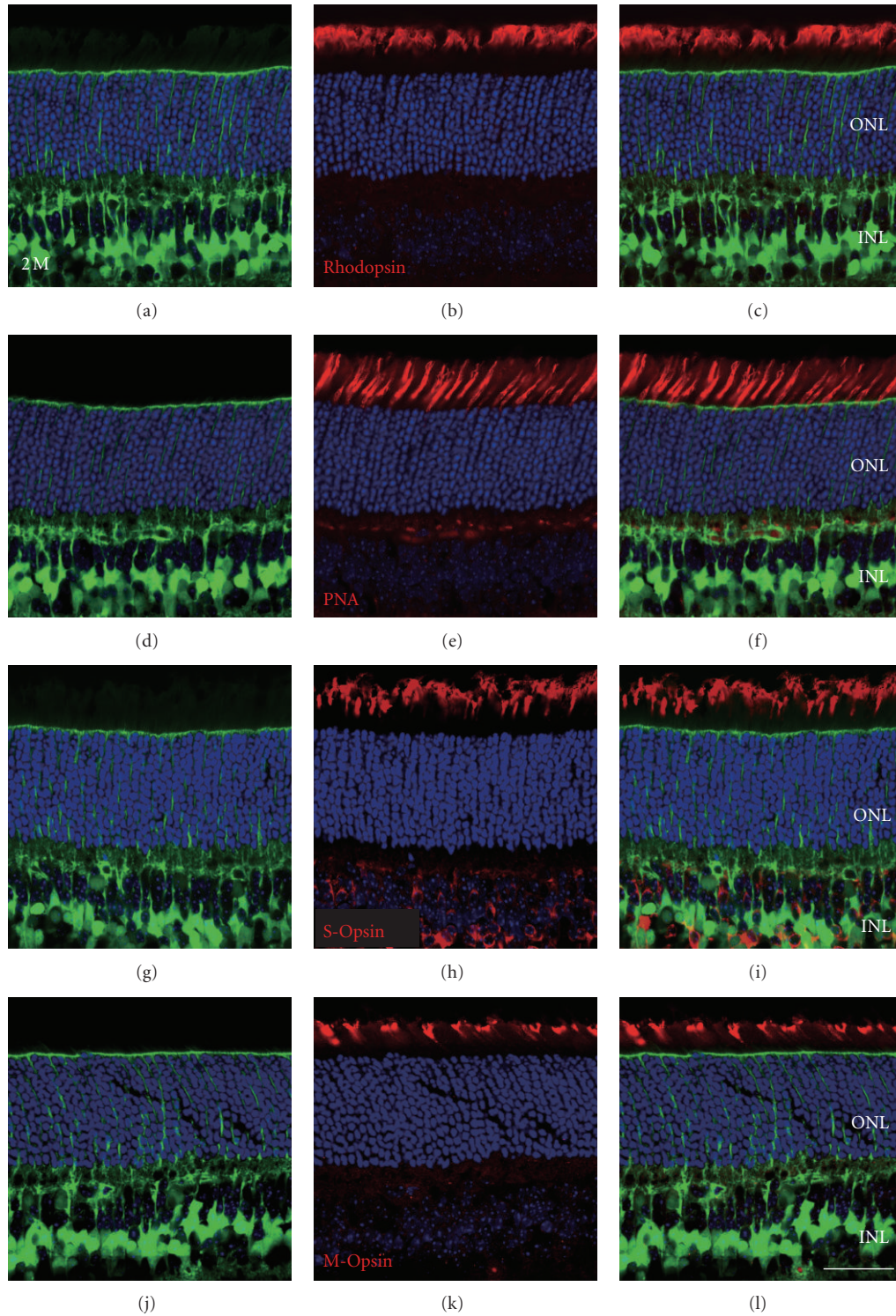


FIGURE 3: (a–l) Immunostaining of the retina of the *BAC-Dkk3-EGFP* mouse with antibodies against photoreceptor cell-specific markers. Immunostaining with photoreceptor cell-specific markers was performed with primary antibodies against rhodopsin (rod photoreceptors; (a–c)), PNA (cone photoreceptors; (d–f)), S-opsin (cone photoreceptors; (g–i)), and M-opsin (cone photoreceptors; (j–l)). Nuclei were counterstained with DAPI. Scale bar represents 50 μm .

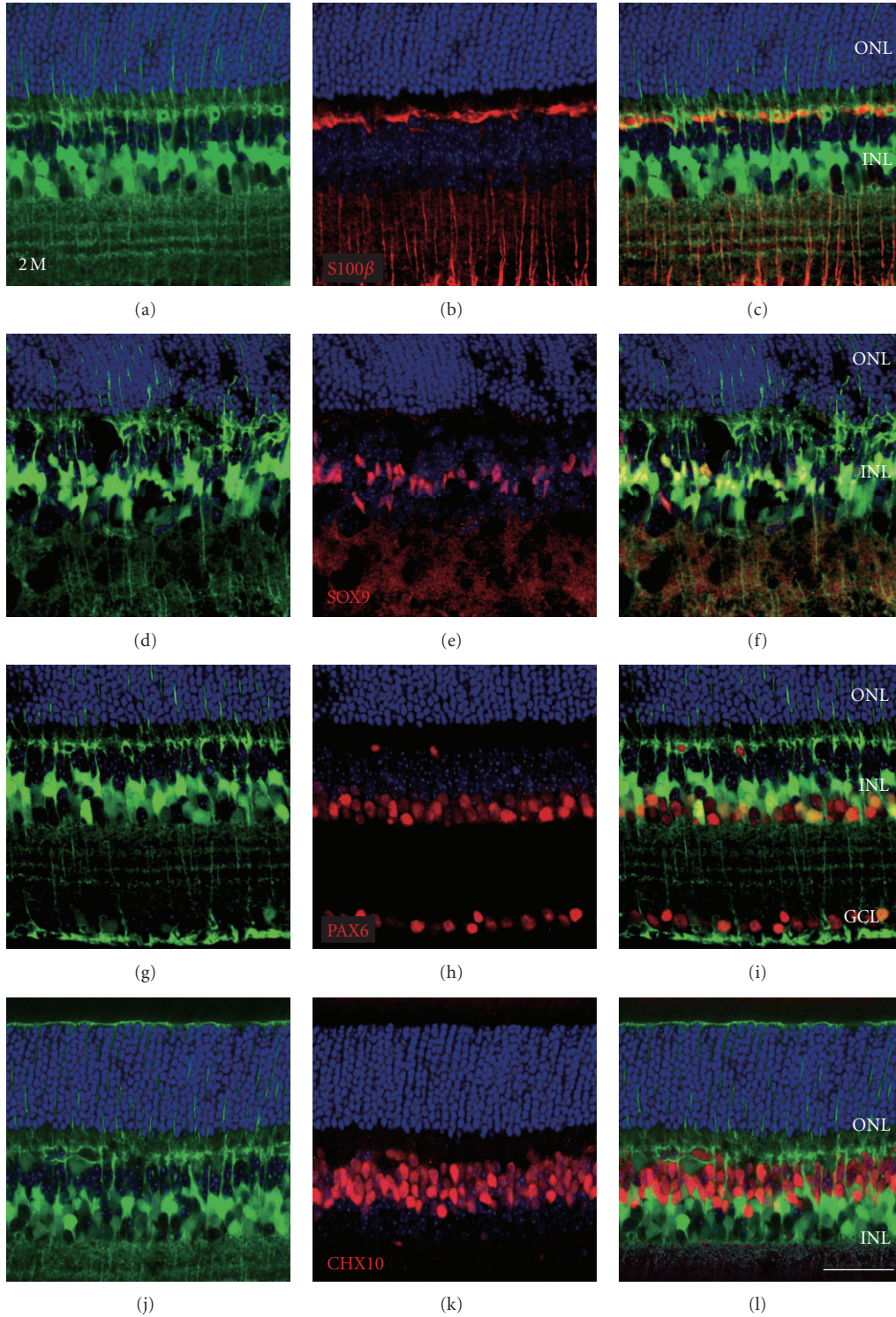


FIGURE 4: (a–l) Immunostaining on the retina of the BAC-Dkk3-EGFP mouse with antibodies against retinal cell type-specific markers. Immunostaining of retinal cell-type-specific markers was performed with primary antibodies against S100 β (Müller glia; (a–c)), SOX9 (Müller glia; (d–f)), PAX6 (amacrine cells; (g–i)), and CHX10 (bipolar cells; (j–l)). Nuclei were counterstained with DAPI. Scale bar represents 50 μ m.

positive for EGFP (Figures 4(d)–4(f)). We also found that a small number of amacrine cells express EGFP in the INL (Figures 4(g)–4(i)). In contrast, bipolar cells were easily distinguished from EGFP-expressing cells (Figures 4(j)–4(l)).

4. Discussion

Dkk3 was reported to be expressed in RPCs at embryonic stages and INL cells at postnatal stages, as detected by *in situ* hybridization [7, 10]. However, the precise identification of the cell types expressing *Dkk3* remains to be elucidated. In the current study, we generated a *BAC-Dkk3-EGFP* transgenic mouse in which *Dkk3*-expressing cells were labeled by EGFP and easily distinguished from *Dkk3*-nonexpressing cells (Figures 3 and 4). We showed that almost all RPCs in embryonic retinas and the Müller glial cells in postnatal retinas were labeled by EGFP (Figures 2, 3, and 4), indicating that the *BAC-Dkk3-EGFP* mouse is a useful tool to specifically identify the Müller glial cells *in vivo*.

Recently, growing evidence suggests that the Müller glial cells appear to contain a similar potential to RPCs in cases of acute injury to the retina [19, 20]. In response to excitotoxic damage elicited by N-methyl-D-aspartate (NMDA), a large number of Müller glial cells undergo dedifferentiation, reentering the cell cycle, and upregulating transcription factors expressed in RPCs including *Pax6*, *Chx10* [21], *Six3* [22], *Sox2* [23, 24], and *Sox9* [23, 25]. These reports suggest that the Müller glial cells are a potential source of neurons in retinal regeneration in the adult retina [21]. Thus, further studies of the Müller glial cells may lead to development of new therapies for retinal damage. The *BAC-Dkk3-EGFP* mouse may give us clues to address the molecular and cellular nature of the Müller glial cells.

Abnormal overactivation of Wnt signaling is a major feature of various human cancers. A large number of studies have focused on the Wnt signaling pathway to develop a novel cancer therapy [26–28]. Notably, *Dkk3* has been identified as a downregulated gene in many human cancer cell lines [29, 30]. Moreover, *Dkk3* exhibits an ability to suppress cancer cell growth due to its inhibitory function in the Wnt signaling pathway, suggesting that *Dkk3* can be a potential clinical target in cancer therapies [2, 30]. The *BAC-Dkk3-EGFP* mouse may thus be useful for the development of innovative cancer therapies as well as that in the Müller glial cell studies, because *Dkk3* expression can be easily detected by the intense EGFP signals.

5. Conclusions

In the current study, we generated the *BAC-Dkk3-EGFP* transgenic mouse in which *Dkk3*-expressing cells were strongly labeled by EGFP. By analyzing the developing retina of this mouse, we showed that almost all RPCs express EGFP at embryonic stages and the Müller glial cells at postnatal stages. Because of the important function of the Müller glial cells in retinal regeneration, the *BAC-Dkk3-EGFP* mouse is a valuable tool for developing regenerative therapies of the retina. Furthermore, since *Dkk3* is an

attractive tool for anticancer drug development, the *BAC-Dkk3-EGFP* mouse may contribute to the advancement of novel cancer therapies.

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