Transcription Factor SOX9 Plays a Key Role in the Regulation of Visual Cycle Gene Expression in the Retinal Pigment Epithelium

Received for publication, February 6, 2014 Published, JBC Papers in Press, March 14, 2014, DOI 10.1074/jbc.M114.556738

Tomohiro Masuda †1, Karl Wahlin ‡1, Jun Wan †1, Jianfei Hu, Julien Maruotti †1, Xue Yang †, Jared Iacovelli †2, Natalie Wolkow †, Ralf Kist †, Joshua L. Dunaief †, Jiang Qian †, Donald J. Zack ‡1,*, and Noriko Esumi ‡13

From the †Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, Maryland 21231, the ‡F. M. Kirby Center for Molecular Ophthalmology, Scheie Eye Institute, University of Pennsylvania, Philadelphia, Pennsylvania 19104, the §Center for Oral Health Research, School of Dental Sciences, Newcastle University, Newcastle upon Tyne NE2 4BW, United Kingdom, the ¶Departments of Neuroscience, Molecular Biology and Genetics, and Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21231, and the **Institut de la Vision, Université Pierre et Marie Curie, 75012 Paris, France

† These authors contributed equally to this work.
* This work was supported, in whole or in part, by National Institutes of Health Grants EY016398 (to N. E.) and EY009769 (to D. J. Z.) and National Institutes of Health Core Grant EY001765 (to the Wilmer Eye Institute). This work was also supported by Foundation Fighting Blindness, by an award from the Wilmer Pooled Professor Research Fund, by unrestricted funds from Research to Prevent Blindness, Inc., and by gifts from Mr. and Mrs. Robert and Clarice Smith and the Guerrieri Family Foundation.
‡ This article contains supplemental Tables S1–S4.
§ These abbreviations used are: RPE, retinal pigment epithelium; LRAT, lecithin retinol acyltransferase; RGR, retinal G protein-coupled receptor; MIF, microphthalmia-associated transcription factor; RDH, retinol dehydrogenase; RLBP1, retinaldehyde binding protein 1; RBP1, retinol binding protein 1, cellular; SOX, sex-determining region Y box; miRNA, microRNA; hRPE, human retinal pigment epithelium; qPCR, quantitative PCR; H5, hypersensitivity; TSS, transcription start site; OTX2, orthodenticle homeobox 2; Otx2, orthodenticle homolog 2; cko, conditional knockout.

Significance: Understanding visual cycle gene regulation may have implications for treating retinal degenerative diseases.

Background: The visual cycle is an enzymatic cascade that regenerates the visual chromophore. The retinal pigment epithelium (RPE) performs specialized functions to support retinal photoreceptors, including regeneration of the visual chromophore. Enzymes and carrier proteins in the visual cycle function sequentially to regenerate and continuously supply 11-cis-retinal to retinal photoreceptor cells. However, it is unknown how the expression of the visual cycle genes is coordinated at the transcriptional level. Here, we show that the proximal upstream regions of six visual cycle genes contain chromatin-accessible sex-determining region Y box (SOX) binding sites, that SOX9 and LIM homeobox 2 (LHX2) are coexpressed in the nuclei of mature RPE cells, and that SOX9 acts synergistically with LHX2 to activate the promoters of the visual cycle genes is coordinated at the transcriptional level. Here, we show that the proximal upstream regions of six visual cycle genes contain chromatin-accessible sex-determining region Y box (SOX) binding sites, that SOX9 and LIM homeobox 2 (LHX2) are coexpressed in the nuclei of mature RPE cells, and that SOX9 acts synergistically with LHX2 to activate the promoters of the visual cycle genes.

Results: The visual cycle is an enzymatic cascade that regenerates the visual chromophore. Enzymes and carrier proteins in the visual cycle function sequentially to regenerate and continuously supply 11-cis-retinal to retinal photoreceptor cells. However, it is unknown how the expression of the visual cycle genes is coordinated at the transcriptional level. Here, we show that the proximal upstream regions of six visual cycle genes contain chromatin-accessible sex-determining region Y box (SOX) binding sites, that SOX9 and LIM homeobox 2 (LHX2) are coexpressed in the nuclei of mature RPE cells, and that SOX9 acts synergistically with LHX2 to activate the promoters of the visual cycle genes. Conditional inactivation of Sox9 in mouse RPE results in reduced expression of several visual cycle genes, most dramatically Rpe65 and Rgr. Furthermore, bioinformatic analysis predicts that multiple common microRNAs (miRNAs) regulate visual cycle genes, and cotransfection of miRNA mimics with luciferase reporter constructs validated some of the predicted miRNAs. These results implicate SOX9 as a key regulator of visual cycle genes, reveal for the first time the functional role of LHX2 in the RPE, and suggest the possible regulation of visual cycle genes by common miRNAs.

The retinal pigment epithelium (RPE) is essential for supporting the survival and function of retinal photoreceptor cells. One of the key functions of the RPE is enzymatic regeneration of the photoreceptor visual chromophore (11-cis-retinal) through the visual cycle, a cyclical pathway consisting of a series of enzymatic reactions in which each step converts retinoid intermediates into a substrate for the next step. Illustrating the importance of the visual cycle, mutations affecting nearly every step of this pathway can cause retinal degeneration (2). Major RPE-expressed components of the visual cycle are three enzymes (RPE65, LRAT, and RDH5), two retinoid carrier proteins (RLBP1 and RBP1), and a modulator (RGR), which work sequentially to regenerate and continuously supply 11-cis-retinal to retinal photoreceptors. Although efficient functioning of the visual cycle requires coordinated expression of its various components and some progress has been made in defining the cis-regulatory elements involved in transcriptional regulation of individual visual cycle genes such as Rpe65 and Rlbp1 (3–7), only limited studies have addressed the regulatory mechanisms that coordinate expression across the visual cycle genes.
Rapid down-regulation of multiple visual cycle genes under different stress conditions has been reported. After exposure of albino mice to bright light (light damage), transcripts for all known visual cycle proteins in the RPE decreased within 24 h (8). A similar down-regulation of visual cycle genes was observed after retinal detachment created in mice (8). Injection of sodium iodate (NaIO₃), an oxidizing agent known to induce selective RPE cell death followed by retinal degeneration, resulted in down-regulation of RPE65, LRAT, and RLBP1 within 2 days (9, 10). These findings support the idea that pan-down-regulation of visual cycle genes may be a general response of RPE cells to stress (8). However, the regulatory mechanisms leading to such pan-down-regulation are still largely unknown.

Among the key transcription factors required for RPE specification and differentiation are microphthalmia-associated transcription factor (MITF) (11) and orthodenticle homeobox 2 (OTX2) (12). Disruption of either gene or their upstream regulator β-catenin in mouse RPE results in transdifferentiation of the RPE into neural retina-like tissues (12–14). The critical role of OTX2 in RPE development is well documented (12, 15). Its importance in maintaining adult RPE functions has also been reported recently (16). In our previous studies, we identified sex-determining region Y box 9 (SOX9) as a key regulator that activates the human BEST1 promoter in the RPE through interaction with MITF and OTX2 (17). SOX transcription factors are important regulators of organogenesis and promote the assembly of transcriptional complexes by facilitating interaction between distant binding sites (18). In the retina, SOX9 is expressed in retinal progenitor cells before birth, but its expression becomes restricted to Müller glia postnatally (19, 20). Conditional inactivation of SOX9 in mouse retina revealed an essential role for SOX9 in the differentiation and/or survival of postnatal Müller glial cells (20). In mouse RPE, SOX9 protein is strongly and continuously detected from early embryonic to adult stages (20).

LHX2, a member of the LIM homeobox family (21), plays an essential role in early eye development, particularly in the transition of the optic vesicle into the optic cup (22, 23). In the retina, Lhx2 expression is detected in the optic vesicle as early as embryonic day 8.5 and throughout the neural retina before birth, but it becomes largely restricted to Müller glia postnatally (22, 24). This sequence of expression is notably similar to that of Sox9, and both genes are expressed predominantly in Müller glia in the mature retina (20, 22, 25). In the RPE, however, the expression and function of LHX2 have not been described. Of interest, Sox9 has been identified as a direct target of LHX2 in mouse skin keratinocytes, suggesting a possible direct link between the two factors (26).

In addition to transcriptional control, there are important controls at the posttranscriptional level. For example, growing evidence suggests that microRNAs (miRNAs) play critical roles in various biological processes, including cellular responses to stress by controlling gene expression through mRNA degradation or translational inhibition (27–29). MicroRNAs are 19- to 22-nucleotide, non-coding RNAs and bind to short sequences in the 3′ UTR of target mRNAs that are complementary to nucleotides 2–7 of the miRNAs (“seed”). Of interest, RPE65 was predicted as a target of a subset of miRNAs that were down-regulated during RPE differentiation of human ES cells, suggesting the possible role of miRNAs in regulating RPE65 during RPE development (30).

In this study, we have begun to elucidate the mechanisms regulating the possible coordination of visual cycle gene expression with a focus on RPE65, RLBP1, and RGR. Here we describe a core transcriptional network of SOX9, OTX2, and LHX2 that controls the expression of multiple visual cycle genes. We report that Sox9 inactivation in mature mouse RPE significantly decreases the expression of several visual cycle genes. We also show that LHX2 is highly expressed in mature RPE and that the expression of SOX9 and LHX2 proteins overlaps in the nuclei of Müller glia and RPE cells in adult mice. Furthermore, we show that the 3′ UTRs of the visual cycle genes share binding sites for common miRNAs, suggesting that visual cycle genes may also be coordinately regulated at the posttranscriptional level.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Promoter-luciferase constructs were made with DNA fragments containing human RPE65 −703 to +51 (RPE65 −703/+51), RLBP1 −520 to +59 (RLBP1 −520/+59), and RGR −565 to +36 bp (RGR −565/+36). These fragments were generated by PCR using human genomic DNA as a template with the primers listed in [supplemental Table S1](#) and cloned into pGL2-Basic (Promega, Madison, WI).

Expression vectors for human SOX9, OTX2, and MITF have been made previously (17, 31, 32). To construct an expression vector for human LHX2, cDNA was generated by RT-PCR using RNA from M1 human RPE primary cells (17) with the primers listed in [supplemental Table S1](#) and inserted downstream of the CMV promoter in the pcDNA3.1/Myc-His(−) B vector (Invitrogen).

To construct luciferase-3′ UTR vectors, DNA fragments of the 3′ UTR of human RPE65, RLBP1, RGR, SOX9, OTX2, and LHX2 were generated by PCR using human genomic DNA as a template with the primer pairs listed in [supplemental Table S1](#) and cloned downstream of the coding region of the firefly luciferase gene in the pmirGLO Dual-Luciferase vector (Promega). Five miR-137 sites are present in the 3′ UTR of RPE65, designated sites 1–5 from 5′ to 3′. For RLBP1, a single miR-137 site is found. Mutated 3′ UTR fragments were generated by PCR using the wild-type constructs as a template, with long primers containing a mutated miRNA site in the middle (GCAA to CGTT) and primers located at each end of the 3′ UTR (supplemental Table S1). The mutated sites in RPE65 were designated m1-m5 and m in RLBP1. Mutated DNA fragments were inserted into pmirGLO in the same manner as described above.

**Generation of Sox9 Conditional Knockout in Mouse RPE**—All mice were treated in accordance with the Federal Guide for the Care and Use of Laboratory Animals. To conditionally inactivate Sox9 in RPE cells, Sox9<sup>lox</sup> mice (33) were mated with BEST1-cre mice in which Cre recombinase is driven by the human BEST1 promoter (34). Both mouse lines were established on the C57BL/6J background by backcrossing for more than eight generations. In BEST1-cre mice, Cre expression is detected in the RPE from postnatal day 10 onward (34). Mouse genotyping was performed by PCR of tail DNA with the primers listed in [supplemental Table S1](#).
**SOX9 Regulates Visual Cycle Gene Expression**

**Cell Culture**—The human RPE cell line D407 was cultured as reported previously (35). HEK293 cells (36) were cultured as recommended by the ATCC. Human fetal RPE (hRPE) cells (ScienCell, Carlsbad, CA) were plated at 5000 cells/cm² and cultured on Matrigel (BD Biosciences) in epithelial cell medium (ScienCell) (37). The medium was changed every other day, and hRPE cells were harvested after 2 months.

**Immunohistochemistry**—The expression of SOX9, RPE65, and RLBP1 proteins was analyzed with one eye of 4-week-old, wild-type, heterozygous Sox9<sup>ko/+</sup>, and homozygous Sox9<sup>ko/ko</sup> mice by immunohistochemistry. Mouse eyes were fixed in 1% paraformaldehyde in 0.1 M phosphate buffer at room temperature for 1 h, and immunohistochemistry of frozen eye sections was performed as described previously (38). Primary antibodies were anti-SOX9 (1:200, catalog no. AB5535, rabbit polyclonal, Millipore, Billerica, MA), anti-RPE65 (1:1000, catalog no. ab13826, mouse monoclonal, Abcam, Cambridge, MA), and anti-cellular retinaldehyde binding protein (CRALBP) (1:250, catalog no. ab15051, mouse monoclonal, Abcam). Secondary antibodies were Alexa Fluor 488-conjugated anti-rabbit IgG or anti-mouse IgG (1:500, Invitrogen). To obtain clear signals in the RPE, pigment was bleached following a published protocol (39). The sections were mounted in Vectashield mounting medium with DAPI (Vector Laboratory, Burlingame, CA), and images were acquired using a Zeiss 510 confocal microscope.

Sox9 and LHX2 double label immunohistochemistry was performed as described previously (38) with eye sections of 1-month-old BALB/cJ mice. Primary antibodies, anti-SOX9 (1:1000, catalog no. AB5535, Millipore) and anti-LHX2 (1:500, catalog no. sc-19344, goat polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA) were visualized with Alexa Fluor-conjugated anti-rabbit or anti-goat secondary antibodies. Hoechst nuclear counterstain was used to identify the RPE, and images were acquired using a Zeiss 710 confocal microscope. Immunostaining of hRPE cells was performed with anti-RPE65 antibody (1:100, catalog no. ab13826, Abcam) as described previously (40).

**RT Quantitative PCR (RT-qPCR)**—The expression of Sox9 was analyzed by RT-qPCR with the contralateral eye of the 4-week-old mice used for the immunohistochemistry described above. Mouse eyes were dissected to remove the cornea and lens, the retina was peeled off, and the eye cup, including the RPE, choroid, and sclera, was collected for RNA extraction using an RNeasy mini kit (Qiagen, Valencia, CA). RT-qPCR was performed as described previously (31, 32). We analyzed the mRNA levels of Sox9, controls Gapdh and Rplp0, selected genes expressed in the RPE, including six visual cycle genes (Rpe65, Lrat, Rhlbp1, Rgr, Rdh5, and Rbp1), and cre (supplemental Table S1). On the basis of threshold cycle values, the mRNA level of each gene was normalized by the average of the levels of Gapdh and Rplp0, and relative expression was calculated as the ratio to the level in wild-type mice (presented as 1). Relative expression of cre was presented as normalized values.

To analyze the expression of transcription factors in human tissues and cells, we used total RNA prepared previously from human RPE, retina, and culture cells (D407 and ARPE19 RPE cell lines and M1 RPE primary cells) as well as total RNA from five human tissues (brain, liver, kidney, spleen, and testes; Clontech, Mountain View, CA) (17). The mRNA levels of Sox9, LHX2, and control GAPDH (supplemental Table S1) were analyzed by RT-qPCR as described above. The expression levels of Rpe65, RLBP1, RGR, and control GAPDH in hRPE cells were analyzed by RT-qPCR in the same manner as described above (supplemental Table S1).

**DNase I Hypersensitivity (DNase I HS) Assay**—DNase I HS assays were performed with RPE cell nuclei from 20 bovine eyes following the published procedures (41) with modifications (32). The nuclei were divided into 10 tubes and digested by a gradient of DNase I (0, 1, 2, 4, 8, 16, 32, 64, 128, and 256 units). Genomic DNA was purified by phenol-chloroform extraction and ethanol precipitation. The resuspended DNA samples were analyzed by qPCR using 30 ng of DNA with primers amplifying 100- to 150-bp fragments at ~100-bp intervals in the region near the transcription start site (TSS) to 1 kb upstream of Rpe65, RLBP1, RGR, RDH5, and RBP1 as well as near the TSS of control ALB and RHO (supplemental Table S2). For Lrat, qPCR was performed with primers amplifying 100- to 150-bp fragments at ~200-bp intervals in the region near the TSS to 2 kb upstream (supplemental Table S2). The relative amount of PCR template in each sample was calculated as the ratio to the amount of PCR template in the undigested sample (presented as 1). Experiments were repeated three times independently, and each sample was analyzed by qPCR in duplicate.

**Prediction of SOX Binding Sites**—MatInspector (42) was used with the MatBase database (Genomatix, Munich, Germany) to search binding sites of transcription factors in the 2-kb region upstream of the TSS of human visual cycle genes (Rpe65, Lrat, RLBP1, RGR, RDH5, and RBP1). These upstream regions were also inspected manually. Sequences of the 2-kb region of human, mouse, and bovine were compared for each gene using Vector NTI (Invitrogen), and conservation was determined manually on the basis of the arrangement of the predicted SOX binding sites in humans.

**Plasmid Transfection**—To analyze the effect of transcription factors on visual cycle gene promoters, cotransfection was performed using Dual-Luciferase assays as described previously (17, 32). All plasmid transfections were carried out with D407 cells in 12-well plates using Lipofectamine Plus (Invitrogen). For each well, plasmid DNA included 0.5 μg of a promoter-luciferase construct, a total of 1.1 μg of expression vectors, and 5 ng of pRL-TK containing the Renilla luciferase gene (Promega). In combinations of transcription factors, 0.1, 0.5, and 0.5 μg of expression vectors were used for Sox9, Otx2, and Lhx2, respectively, and the total amount was adjusted to 1.1 μg by adding empty cDNA3.1/Myc-His(-). As a promoter-luciferase vector, pGL2 constructs containing human Rpe65—703/+51, RLBP1—520/+59, and RGR—565/+36 were used. Cell lysates were analyzed 48 h after transfection using the Dual-Luciferase reporter system (Promega). Firefly luciferase activity was normalized by Renilla luciferase activity, and relative luciferase activity was calculated as the ratio of the normalized luciferase activity with the expression vectors to that with empty pCDNA3.1. Cotransfection was performed three to four times independently in duplicate each time.

**ChIP—ChIP** with bovine RPE was performed as described previously (17, 32). Antibodies used were anti-SOX9 (catalog no. AB5535, Millipore), anti-OTX2 (catalog no. ab21990, AB5535, Millipore), and anti-RPE65 (catalog no. ab13826, Abcam) as described previously (40).
Abcam), anti-LHX2 (catalog no. sc-19344, Santa Cruz Biotechnology), anti-SOX10 (catalog no. sc-17342, Santa Cruz Biotechnology), and anti-SOX10 (catalog no. ab25978, Abcam). The antibodies for SOX9, OTX2, and SOX10 were used in ChIP in our previous studies (17, 32). The antibody for LH2X was used in ChIP with mouse keratinocytes (26), and its specificity was confirmed (43). ChIP precipitates and diluted input (1:50) were analyzed by qPCR with primers at different genomic locations of RPE65, RLBP1, and RGR (supplemental Table S2). Relative enrichment was calculated as the ratio of the amount of PCR template in ChIP samples to that in diluted input. ChIP was performed four times, and each sample was analyzed in duplicate.

ChIP with hRPE cells was performed in the same manner as described above for ChIP with bovine RPE, except that only antibodies for SOX9 (catalog no. AB5535), OTX2 (catalog no. ab21990), and SOX10 (catalog no. sc-17342) were used. ChIP precipitates and diluted input (1:100) were analyzed by qPCR with primers for human RPE65, RLBP1, and RGR (supplemental Table S2). Because of the limited amount of available hRPE cells, ChIP was performed twice, and each sample was analyzed in triplicate.

Bioinformatic Prediction of miRNAs—First, we integrated two databases downloaded from miRBase and microRNA.org. We selected genes of interest from these databases containing a total of 720 human miRNAs and 25,367 target genes. Then, we calculated the number of overlapped predicted miRNAs between a set of genes and a reference gene (marked in boldface) at the far left in each figure. The overlap percentage is defined as overlap (%). We set the number of miRNAs predicted for gene i, NOverlap. The overlap percentage (a dotted line in figures) was calculated as the ratio of the number of miRNAs predicted for a reference gene to the total number of miRNAs in our database (n = 720).

Transfection with miRNA Mimics—The effect of miRNAs on target mRNAs was analyzed by cotransfection of miRNA mimics with luciferase-3’ UTR constructs. In these experiments, 0.1 μg of pmirGLO construct, either empty or with 3’ UTR fragments, and 33 nm of mirVana miRNA mimics (Invitrogen) were transfected into HEK293 cells in 24-well plates using Lipofectamine 2000 (Invitrogen). The 3’ UTR of human RPE65, RLBP1, RGR, SOX9, OTX2, and LH2X was tested with miRNA mimics for miR-18a, miR-19a, miR-25, miR-32, miR-92a, miR-124, miR-137, miR-340, miR-363, and negative control #1. The 3’ UTRs of RPE65 and RLBP1 containing mutated miR-137 and miR-25 sites were also analyzed in the same manner. Luciferase activity was analyzed 36 h after transfection using the Dual-Luciferase reporter system (Promega), and firefly luciferase activity was normalized by Renilla luciferase activity. To assess the effect of miRNA mimics, double normalization was used to nullify any effect of miRNA mimics on pmirGLO itself. First, initial relative luciferase activity was calculated as the ratio of the normalized luciferase activity with miRNA mimics to that without mimics (miRNA +/− ratio) for each construct. Then, final relative luciferase activity was calculated as the ratio of the initial relative luciferase activity for pmirGLO containing 3’ UTR inserts to that for empty pmirGLO (3’ UTR +/− ratio). Cotransfection was repeated three to four times independently, on different days, in duplicate each time.

Statistical Analysis—Unpaired Student’s t test was used for statistical analysis.

RESULTS

The Upstream Regions of Visual Cycle Genes Contain Chromatin-accessible SOX Binding Sites—Examination of the DNA sequences upstream of six human visual cycle genes (RPE65, LRAT, RLBP1, RGR, RDH5, and RBP1) revealed that they all contain one or more putative SOX binding sites in the 2-kb region upstream of the TSS (Fig. 1A and supplemental Table S3). In LRAT and RDHS, single sites are arranged in tandem. Many of the identified sites are conserved among human, mouse, and bovine. We also found that RPE65, RLBP1, RGR, and RBP1 contain at least one consensus OTX site in the proximity of the identified SOX sites. LRAT and RDHS also contain OTX sites, but they are distant from the SOX sites (Fig. 1A).

To test whether the predicted SOX binding sites are located in accessible chromatin areas near visual cycle gene promoters, we performed DNase I HS site mapping in the 1-kb upstream regions of RPE65, RLBP1, RGR, RDHS, and RBP1 as well as in the 2-kb upstream of LRAT (Fig. 1B). In some genomic locations, we could not obtain adequate primers even after several attempts, and such locations are marked by an interruption of the x axis in Fig. 1B. Therefore, DNase I HS profiles in such locations and the downstream region from the TSS are not proportional to the genomic distance. RPE65 and RGR exhibited DNase I HS sites at −500 to +100 kb and −400 to +100 kb, respectively. In contrast, RLBP1, RDHS, and RBP1 showed two separate DNase I HS sites within the 1-kb upstream region: −200 to +100 kb and −1 kb to −800 kb, around the TSS and −600 to −300 bp, and −200 to +100 bp and −800 to −700 bp, respectively. LRAT showed two DNase I HS sites in the 2-kb upstream region, at −200 to +100 bp and −1.8 to −1.6 kb, and the latter corresponded to the location of some of the possible SOX binding sites in the bovine. The results indicate that these visual cycle genes share the presence of chromatin-accessible SOX binding sites in RPE cells in vivo, suggesting that one or more SOX proteins may be involved in coordinately regulating the expression of visual cycle genes and that the putative SOX regulatory protein(s) act through interaction with an OTX family member.

SOX9 and LH2X Are Coexpressed in the Nuclei of Mature RPE Cells—To identify putative SOX and OTX family members involved in regulating visual cycle gene expression, we chose to initially study SOX9 and OTX2 on the basis of their previously defined physical and functional interaction in regulating BEST1 in the RPE (17). As additional candidate factors for analysis, we first chose MITF because of its important role in RPE development and its demonstrated ability to physically and functionally interact with SOX9 and OTX2 (17). Secondly, we chose LH2X, a LIM homeodomain protein, because its binding motif is similar to that of OTX2 and its expression pattern in Müller glia is similar to that of SOX9 (20, 22, 24). We first compared the expression of SOX9 and LH2X in human adult tissues and cultured cells, including human RPE primary cells, by RT–qPCR. The tissue distribution of LH2X was overall similar to that of SOX9, with the highest expression in RPE cells, except that
LHX2 expression was low in the testis and remained similar in RPE tissue and primary cells (Fig. 2A). Next, we analyzed the expression of SOX9 and LHX2 proteins by immunohistochemistry (Fig. 2B). Both SOX9 and LHX2 were expressed in Müller glia spanning the retina and in the RPE. They both demonstrated strong nuclear signals in RPE cells, whereas LHX2 also showed a weaker and more diffuse staining pattern in RPE cytoplasm.

**SOX9, OTX2, and LHX2 Synergistically Activate RPE65, RLBP1, and RGR Promoters**—To test the effects of the selected four candidate transcription factors, we generated promoter-luciferase constructs for RPE65, RLBP1, and RGR. On the basis of the high sequence homology of their proximal upstream region between human and bovine, we used human promoter fragments corresponding to the bovine genomic sequences containing DNase I HS site (Fig. 1B). The RPE65 −703 to +51 bp region also corresponds to the mouse Rpe65 promoter reported to drive RPE-specific expression (3). Promoter-luciferase constructs were transfected with various combinations of expression vectors for the candidate factors. Because our initial analysis showed that MITF had no effect on these promoters (data not shown), we focused on SOX9, OTX2, and LHX2. A low dose of SOX9, used to avoid a nonspecific effect, modestly activated all three promoters, whereas OTX2 activated the RPE65 and RLBP1 promoters (Fig. 2C). When SOX9 and OTX2 were combined, they synergistically activated the promoter of RPE65 (**p*/H11005 = 0.019) and RLBP1 (**p*/H11005 = 0.029) compared with OTX2 alone. Overall activation profiles of the RPE65 and RLBP1 promoters were strikingly similar (Fig. 2C). In contrast, although LHX2 did not activate either promoter by itself, its combination with SOX9 led to synergistic activation of the RGR promoter compared with SOX9 alone (**p*/H11005 = 0.011). Of interest, OTX2 and LHX2 inhibited the activity of each other on the RGR and RLBP1 promoters, respectively, possibly because of...
SOX9 Regulates Visual Cycle Gene Expression

FIGURE 2. Coexpression of SOX9 and LHX2 in RPE cells and synergistic activation of visual cycle gene promoters by SOX9, OTX2, and LHX2. A, expression of SOX9 and LHX2 in human tissues and cells. Total RNAs from human culture cells (D407 and ARPE19 RPE cell lines and M1 RPE primary cells) and seven human tissues, including RPE and retina, were analyzed by RT-qPCR. The mRNA level of SOX9 and LHX2 was normalized by that of GAPDH and presented as relative expression. The values are mean ± S.E. (error bars) of PCR replicates. B, SOX9 and LHX2 are coexpressed in the nuclei of mouse RPE. Double label immunohistochemistry was performed on eye sections of BALB/cJ mice. Images of immunostaining are shown. a, SOX9. b, LHX2. c, merged SOX9 (red) and LHX2 (green). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. C, SOX9, OTX2, and LHX2 synergistically activate RPE65, RLBP1, and RGR promoters. Promoter-luciferase constructs containing the proximal promoter of human RPE65 (left panel), RLBP1 (center panel), and RGR (right panel) indicated in Fig. 1 were transfected with various combinations of expression vectors for SOX9, OTX2, and LHX2 or empty pcDNA3.1 (pcDNA). The total amount of expression vectors was adjusted by empty pcDNA3.1, and pRL-TK containing the Renilla luciferase gene was used for normalization. Relative luciferase activity was calculated as the ratio of the normalized (firefly/Renilla) luciferase activity with expression vectors to that with empty pcDNA3.1 (defined as 1). Data are mean ± S.E. (error bars) of three to four independent experiments. *, p < 0.05; **, p < 0.01.

binding competition. These results suggest that visual cycle gene expression is finely tuned by transcriptional complexes containing different combinations of SOX9, OTX2, and LHX2. SOX9, OTX2, and LHX2 Bind to Visual Cycle Gene Promoters in RPE Cells in Vivo—To test whether SOX9, OTX2, and LHX2 bind to visual cycle gene promoters in vivo, we performed ChIP with bovine RPE tissue. Four independent ChIP experiments using the anti-SOX9 or anti-OTX2 antibody consistently yielded a peak of relative enrichment near the TSS of RPE65, RLBP1, and RGR, with no enrichment at the upstream or downstream regions (Fig. 3). ChIP with the anti-LHX2 antibody showed a peak of relative enrichment near the TSS of RPE65 and RGR but not RLBP1. As control, ChIP with two anti-SOX10 antibodies that had been successfully used in ChIP with other tissues (44, 45) did not show any peak of enrichment, confirming the specificity of SOX9 binding (Fig. 3). These results indicate that SOX9, OTX2, and LHX2 indeed bind to the promoter region of all or some of the three visual cycle genes in RPE cells.

Next, we wanted to test whether these transcription factors also bind to visual cycle gene promoters in human RPE. Because of the difficulty in obtaining fresh human donor eyes in sufficient quantity, we sought an alternative source of human RPE cells. On the basis of reported findings and our own experience that hRPE cells can proliferate and then differentiate in culture to exhibit many characteristics of mature RPE cells (37, 40, 46), we decided to use hRPE cells. After 2 months in culture, hRPE cells showed a cobblestone-like morphology (Fig. 4A, a) and began to express RPE65 protein (Fig. 4A, b). Analyses by RT-qPCR showed that the expression levels of RPE65, RLBP1, and RGR in the hRPE cells were higher than or at least comparable with those in RPE primary cells derived from human mature RPE. However, it should be noted that the expression levels of these genes in the two types of RPE cells are still substantially lower than those in human adult RPE tissues (47). Because of the limited amount of available hRPE cells, we performed ChIP only for SOX9, OTX2, and control SOX10. We obtained a peak of relative enrichment near the TSS of RPE65, RLBP1, and RGR with the anti-SOX9 and anti-OTX2 antibodies but not with the anti-SOX10 antibody (Fig. 4B), which was consistent with the ChIP results with bovine RPE described above (Fig. 3).

SOX9 Regulates Visual Cycle Gene Expression in Vivo—To further explore our hypothesis that SOX9 might play a role in coordinating the expression of visual cycle genes in the RPE, we generated a conditional knockout (cko) of Sox9 in the RPE by mating Sox9flox mice (33) with BEST1-cre mice (34). First, we analyzed Sox9 expression in wild-type, heterozygous Sox9<sup>cko/+</sup> (BEST1-cre;Sox9<sup>flox/+</sup>), and homozygous Sox9<sup>cko/cko</sup> (BEST1-cre;Sox9<sup>flox/flox</sup>, described as Sox9 cko) mice at 4 weeks using immunohistochemistry of one eye and RT-qPCR of the other eye. We chose 4 weeks of age to achieve a balance between minimizing secondary effects of Sox9 inactivation and yet allowing sufficient time to achieve Sox9 ablation in the majority of Sox9-flabeled and - floxated cells.
of RPE cells. We found previously that BEST1-cre mice are mosaic, showing Cre expression in 50–90% of RPE cells, with Cre protein being first detectable at postnatal day 10 and reaching a plateau at postnatal day 28 (34). By immunohistochemistry, SOX9 protein was strongly expressed in the nuclei of RPE cells of wild-type mice but was undetectable in the majority of RPE cells in the mosaic, showing Cre expression in 50–90% of RPE cells, with Cre protein being first detectable at postnatal day 10 and reaching a plateau at postnatal day 28 (34). By immunohistochemistry, SOX9 protein was strongly expressed in the nuclei of RPE cells of wild-type mice but was undetectable in the majority of RPE cells of Sox9cko mice (Fig. 5A). At 4 weeks, we did not observe obvious morphological abnormalities in the retina and RPE of Sox9cko mice. RT-qPCR analysis showed that Sox9 mRNA levels were reduced to 35% in the RPE of Sox9cko mice compared with the levels in wild-type mice (Fig. 5B), confirming the mosaic Cre-mediated recombination profile of BEST1-cre mice observed previously (34).

To assess the consequences of Sox9 inactivation, we tested the expression of visual cycle genes and other selected RPE-related genes by RT-qPCR. Most notably, Rpe65 and Rgr showed substantially decreased expression in Sox9cko mice, with only 7.8% and 9.3% of the levels in wild-type mice, respectively. Other visual cycle genes also showed significantly reduced expression in Sox9cko mice. Lrat, Rlbp1, Rdh5, and Rbp1 were expressed at levels of 35, 41, 56, and 61% of the levels of wild-type mice, respectively (Fig. 5B). In contrast, some genes showed increased expression. For example, Otx2, Lhx2, and Tyr showed 1.4-, 2.5-, and 3.0-fold higher expression, respectively, relative to wild-type mice. Other genes, such as Mitf, Best1, Tyrp1, and Dct, did not show significant changes compared with wild-type mice. The expression of Ptgds, a known Sox9 target in the testis (48), was also decreased by 60% in the RPE of Sox9cko mice, whereas the mRNA level of the control genes, Gapdh and ribosomal protein large P0 (Rplp0), was unchanged (Fig. 5B). By immunohistochemistry, we observed that Rpe65 and RLBP1 (also known as CRALBP) proteins are undetectable in Sox9-ABLATED RPE (Fig. 6). These results demonstrate that Sox9 is involved in regulating the expression of at least six visual cycle genes in vivo.

Bioinformatic Analyses Predict Multiple Common Regulatory miRNAs for Visual Cycle Genes—Next, we wanted to test whether visual cycle genes also share common regulatory mechanisms at the posttranscriptional level. On the basis of the reported rapid down-regulation of multiple visual cycle genes, we hypothesized that visual cycle genes may be targets of common miRNAs that have been shown to be able to coordinate the
SOX9 Regulates Visual Cycle Gene Expression

FIGURE 5. SOX9 regulates visual cycle gene expression in vivo. A, immunohistochemistry of retinal sections of wild-type mice (a and b) and Sox9 cko mice (c and d). Images show immunostaining for SOX9 (a and c) and merged SOX9 staining and DAPI (b and d). Higher magnification images show wild-type RPE (e) and Sox9 cko RPE (f) for SOX9, DAPI, and merged SOX9 and DAPI. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. B, expression of visual cycle genes decreased in the RPE of Sox9 cko mice. The mRNA levels of selected genes were analyzed by RT-qPCR using the opposite eyes of the 4-week-old mice as used for immunohistochemistry. The mRNA level of each gene was normalized by an average of the levels of Gapdh and Rplp0, and relative expression was calculated as the ratio to the level in wild-type mice (defined as 1). Relative expression of cre is shown as normalized values. Three eyes were analyzed for each genotype, and results are presented as mean ± S.E. (error bars). *, p < 0.05; **, p < 0.01; ***, p < 0.001.

FIGURE 6. RPE65 and RLBP1 proteins are undetectable in Sox9-ablated RPE cells. A–H, immunohistochemistry of retinal sections of wild-type mice (A–D) and Sox9 cko mice (E–H). Images of immunostaining show RPE65 (A and B), merged RPE65 and differential interference contrast (DIC) (B and F), RLBP1 (C and G), and merged RLBP1 and DIC (D and H). I–J, higher magnification of immunohistochemistry of wild-type RPE (I) and Sox9 cko RPE (J). Images of immunostaining show RPE65, DAPI, and merged RPE65 and DAPI (top panels) and RLBP1, DAPI, and merged RLBP1 and DAPI (bottom panels). Anti-mouse IgG antibodies used as secondary antibody stained the Bruch’s membrane and choroid, serving as markers for the RPE layer and signal intensity. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

expression (29). Using bioinformatics, we identified potential miRNAs that could bind to the 3’ UTR of visual cycle genes and other selected genes (supplemental Table S4). For the six visual cycle genes and three pigment-related genes, we calculated the number and percentage of miRNAs that overlapped with the ones predicted for RPE65 (Fig. 7A). RLBP1 and LRAT shared significantly more miRNAs with RPE65 than the expected overlap by random chance (marked by a dotted line). The percentage of overlap of miRNAs for RDH5 and TYRP1 was much lower but still statistically significant. Of the three visual cycle genes focused on in this study, RPE65 and RLBP1 share more miRNAs with each other than with RGR (Fig. 7B). Among the three genes encoding visual cycle enzymes, RPE65 and LRAT share more miRNAs with each other than with RDH5. The pigment-related genes TYR, TYRP1, and DCT share much fewer miRNAs. We also compared miRNAs predicted for the three transcription factors involved in visual cycle gene regulation. SOX9 and OTX2 share significantly more miRNAs with each other than with LHX2 (Fig. 7B). Interestingly, MITF, a critical factor for RPE identity, and CRX, a member of the OTX family that plays important roles in photoreceptors but is also expressed in the RPE (32), share many miRNAs with SOX9 (Fig. 7C). The percentage of overlap for NR2E3 and NRL, factors important for photoreceptor development, and MYOD1 and MYOG, factors important for muscle development, remained low or below the expected level, suggesting that miRNA sites may also be conserved among genes sharing similar function and/or expression patterns. These results suggest that RPE65, LRAT, and RLBP1 can be regulated by common miRNAs.
Validation of Predicted Regulatory miRNAs for Visual Cycle Genes—To validate the predicted miRNAs, we chose miR-25, miR-32, miR-92a, miR-137, miR-340, and miR-363, which were predicted to bind two or more of RPE65, RLBP1, and RGR, and miR-18a, miR-19a, and miR-124, which were predicted to bind two or more of SOX9, OTX2, and LHX2 (supplemental Table S4). By cotransfection with miRNA mimics, we analyzed the effect of the selected miRNAs on the 3′ UTR-luciferase constructs. Although the first set of miRNAs were all predicted for RPE65 and RLBP1, only miR-137 repressed the 3′ UTR constructs of both genes, and miR-25, miR-32, miR-92a, and miR-363 repressed only the RPE65 3′ UTR construct (Fig. 8A). Although miR-340 was predicted for RPE65, RLBP1, SOX9, OTX2, and LHX2, it did not show any effect on either construct (Fig. 8, A and B). As expected from the literature (49, 50), we confirmed that both SOX9 and LHX2 are targets of miR-124 (Fig. 8B). We also observed a marginal reduction in luciferase activity of the RGR 3′ UTR construct with miR-92a (p = 0.017) and miR-340 (p = 0.033) (Fig. 8A).

Next, we tested the sequence specificity of repression by miR-137 and miR-25 using mutated 3′ UTR constructs. The seeds of miR-137 (UAUUGC) and miR-25 (AUUGCA) are similar, with one nucleotide sliding. Allowing a single nucleotide mismatch in the seed, RPE65 contains five sites for miR-137 and three sites for miR-25, whereas RLBP1 contains one site for both miR-137 and miR-25 (Fig. 8C). Cotransfection of the 3′ UTR-luciferase constructs with mutated sites (GCAA to CGTT) and miRNA mimics showed that mutation of sites 4 (m4) and 5 (m5) in RPE65 abolished or reduced repression by miR-137 and that mutation of the site (m) in RLBP1 completely prevented repression by miR-137, suggesting that these sites are functional (Fig. 8D). Cotransfection of the mutated 3′ UTR constructs also revealed that site 5 in RPE65 is essential for repression by miR-25. The most effective sites, RPE65 site 4 and RLBP1 site for miR-137 and RPE65 site 5 for miR-25, matched to miRNA nucleotides 1–7 (seed + first) or nucleotides 2–8 (seed + eighth) (Fig. 8C), supporting the finding that matching at additional nucleotide(s) beyond seed pairing increases site efficacy (51). We also tested the sequence specificity of repression by miR-32, miR-92a, and miR-363 using cotransfection with the mutated 3′ UTR constructs. Site 5 was the most effective site for these miRNAs, which match at nucleotides 2–8 with site 5. However, the effect of mutation (RPE65 m1-m5) was slightly different with each miRNA (Fig. 8, C and D), supporting the reported findings that additional features beyond the seed contribute (52). These results indicate that some of the predicted miRNA sites are functional and that RPE65 and RLBP1 share functional sites for miR-137.

DISCUSSION

We have described a core transcriptional network regulating visual cycle genes as a group of genes that encode proteins functioning in the same pathway. Because previous work on the regulation of visual cycle genes studied only individual genes (3–7), this is the first report that describes their regulation from a systematic perspective. We found that SOX9 is a key player in the regulation, directly or indirectly, of multiple visual cycle genes, rendering the ability for coordination of their expression.

Down-regulation of visual cycle genes has been reported in various conditions, including light damage (8), retinal detachment (8), NaO₃-induced oxidative damage (9, 10), subretinal injection of amyloid-β (1–42) (53), intravitreal injection of leukemia inhibitory factor (LIF) (54), and conditional inactivation of mitochondrial transcription factor A (Tfam) in mouse RPE (10). The gp130/STAT3 pathway is required for the effect of LIF (54). Mammalian target of rapamycin (mTOR) is crucial in the RPE response to Tfam ablation and NaO₃-induced oxidative damage (10). Our findings that Sox9 ablation in the RPE resulted in down-regulation of six visual cycle genes add SOX9 deficiency to the growing list of conditions that lead to down-regulation of multiple visual cycle genes. Whether these conditions converge upon the common regulatory mechanism controlling the final output of expression of visual cycle proteins remains to be elucidated.
FIGURE 8. Validation of predicted regulatory miRNAs for visual cycle genes. A, transfection of miRNA mimics with the 3′ UTR of visual cycle genes. Luciferase constructs containing the 3′ UTR of RPE65, RLBP1, and AGR or empty pmirGLO were transfected into HEK293 cells with miRNA mimics or negative control #1 (NC #1). To nullify any effect of miRNA mimics on pmirGLO itself, double normalization was used. First, initial relative luciferase activity was calculated as the ratio of the normalized (firefly/Renilla) luciferase activity with miRNA mimics to that without mimics (miRNA/H11001/H11002 ratio). Then, final relative luciferase activity was calculated as the ratio of the initial relative luciferase activity for pmirGLO with 3′UTR fragments to that for empty pmirGLO (3′UTR/H11001/H11002 ratio). Data are mean ± S.E. (error bars). B, transfection of miRNA mimics with the 3′ UTR of transcription factor genes. Cotransfection was performed as described in A, except that luciferase constructs containing the 3′ UTR of SOX9, OTX2, and LHx2 were used. C, predicted sites of miRNAs in human RPE65 and RLBP1. In the 3′ UTR, five sites for miR-137 (sites 1–5, black bar) and three sites for miR-25 (sites 3–5, gray bar) were predicted for RPE65, and a single site for both miR-137 and miR-25 was predicted for RLBP1. A perfect match (wide bar) and a single nucleotide mismatch (narrow bar) with seed (nucleotides 2–7) are shown differentially. Mutated miRNA sites (GCAA to CGTT) are designated m1-m5 for sites 1–5 in RPE65 and m for the single site in RLBP1. Alignment of miR-137 and miR-25 with RPE65 sites 4 and 5 and RLBP1 site as well as miR-32, miR-92a, and miR-363 with RPE65 sites 4 and 5 is shown. The seed of each miRNA is marked in boldface. D, transfection of mutated 3′ UTR constructs with miRNA mimics. Cotransfection was performed in the same manner as in A, except that pmirGLO constructs with wild-type and mutated miRNA sites in RPE65 and RLBP1 were used. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
The role of transcription factors in the RPE has been studied most extensively in development. For example, the crucial role of MITF (11) and OTX2 (12) in RPE development is well documented. The finding that some transcription factors that are critical in RPE development are continuously expressed into mature stages raises the question as to what roles they play in adult RPE. It has been reported recently that tissue-specific inactivation of Otx2 in the mature retina and RPE led to abnormalities in the RPE, such as reduction of the number and size of melanosomes and a loss of RPE contacts with photoreceptor outer segments followed by retinal degeneration, revealing the essential role of OTX2 in maintaining the integrity of the adult RPE (16, 55). OTX2 coordinates RPE-specific functions, such as retinoid metabolism and melanogenesis, and directly regulates several visual cycle-related genes, including Rldhs (16). Although the authors did not report Rpe65 and Rlbp1 as direct targets of OTX2, we found that RPE65 and RLBP1 are also direct targets of OTX2 in this study. The discrepancy in the two studies may be due to differences in experimental approaches, ChIP antibodies, and species analyzed (mouse versus human/bovine). We found that SOX9 plays a key role in regulating BEST1 (17) and visual cycle genes, including RPE65 (this study), both of which are important for RPE mature functions and also widely used as RPE differentiation markers. Although the role of SOX9 in RPE development remains to be defined, SOX9 plays an important role in adult RPE cells by regulating genes crucial for their function.

Our study shows that LHX2 is coexpressed with SOX9 in mature Müller glia and RPE cells. Importantly, at least two visual cycle genes, RLBP1 and RGR, are also expressed in Müller glia and RPE cells (56, 57). In fact, our transfection and ChIP results show that SOX9 regulates both RLBP1 and RGR, whereas LHX2 regulates RGR in the RPE. Of interest, re-expression of LHX2 in eye-committed progenitor cells in the Lhx2−/− forebrain induced pigmented foci containing RPE-like cells expressing Lhx2, Mitf, and Pax6 (58), suggesting a possible role of LHX2 in RPE development.

Our study revealed the complex combinatorial regulation of visual cycle genes that involves the interaction of at least three transcription factors, SOX9, OTX2, and LHX2. Although the proximal upstream regions of the six visual cycle genes we studied all contain chromatin-accessible SOX binding sites and consensus OTX sites, the promoter regions of RPE65, RGR, and RLBP1 have a particularly homologous arrangement of these sites. Of the three promoters analyzed in this study, however, SOX9 acts synergistically with OTX2 to activate the RPE65 and RLBP1 promoters but acts synergistically with LHX2 to activate the RGR promoter. Interestingly, our ChIP results with both bovine RPE and hrRPE cells show that SOX9 and OTX2 bind to all of the three promoters, including the RGR promoter. On the basis of our previous finding that SOX9 and OTX2 physically and functionally interact on the BEST1 promoter in the RPE (17), it is not surprising that OTX2 is also detected at the RGR promoter even though it does not activate this promoter. The colocalization of OTX2 can be due to either direct binding to the promoter or indirect binding as part of a protein complex through interaction with SOX9 or other factors. In contrast, ChIP with bovine RPE shows that LHX2 binds to the RPE65 and RGR promoters but not to the RLBP1 promoter. There are multiple reasons that could explain this difference in binding patterns between OTX2 and LHX2, including different preferences of each factor for the detailed binding site arrangement, differences in binding affinity of the sites in each promoter, different expression levels of each factor, and the existence of binding motifs for other factors that interact with them. A recent genome-wide analysis using large scale mass spectrometry has found that an unusually large number of trans-acting factors colocalize at the so-called “HOT regions” that are frequently associated with promoters (59). Thus, it is likely that many more factors that bind, directly or indirectly, to the visual cycle gene promoters will be identified in coming years, and as a result we will appreciate increasing complexity in the visual cycle gene regulatory landscape.

It has been demonstrated that miRNAs can act both as a switch and as a fine-tuner of gene expression and as important modulators of gene expression during stress responses (27, 29, 60). We show that RPE visual cycle genes, particularly RPE65, LRAT, and RLBP1, share significantly more predicted regulatory miRNAs than expected by random chance. To exert rapid down-regulation of multiple visual cycle proteins, miRNA-mediated regulation is one plausible mechanism. In addition, although Rpe65 mRNA is detected as early as embryonic day 18, its protein becomes detectable at postnatal day 4, suggesting posttranscriptional regulation of RPE65 (61, 62). For this delay in RPE65 protein expression during development, miRNA-mediated regulation could also be a mechanism. Of interest and possible relevance, bioinformatic analyses predicted RPE65 as a target of miRNAs that were down-regulated during differentiation of human ES cells to RPE cells (30). Among the miRNAs analyzed in our study, miR-137 targets not only RPE65 and RLBP1 but also MITF in human melanoma cells (63), suggesting complex interactions of genes important for the RPE through miRNAs. Another set of miRNAs analyzed is from the miR-17/92 family of miRNA clusters, which consists of the miR-17/92 cluster and its two paralogs, the miR-106b/25 and miR-106a/363 clusters that emerged by gene duplication (64, 65). They are polycistronic miRNA clusters harboring four seed families, and miR-92a, miR-25, and miR-363 belong to the same seed family. We found that RPE65 is a target of these miRNAs and miR-32 that share the seed. In the study referred to above, at least six miRNAs in this miRNA cluster family were down-regulated during RPE differentiation of human ES cells (30). Importantly, the miR-17/92 cluster is regulated by MYC, the E2F family, and STAT3 and can be induced by these regulators under some conditions (64, 66).

On the basis of our results, we propose a core regulatory network for visual cycle gene expression in the RPE (Fig. 9). SOX9 is a major hub at the transcriptional level, and OTX2 is also a key player in the network. From the literature, we added additional possible connections into the network. The direct relations reported in the RPE are regulation of Mitf and Otx2 by β-catenin (14) and regulation of Rldh5 by OTX2 (16). The direct relations reported in non-RPE cells are regulation of MITF by SOX9 in human skin melanocytes (67) and regulation of Sox9 by Lhx2 in mouse skin keratinocytes (26) (Fig. 9). The regulatory relations in non-RPE cells need to be tested in the RPE.
SOX9 Regulates Visual Cycle Gene Expression

However, we add these connections as a reference because SOX9, MITF, and LHX2 are all expressed in RPE cells and because regulation in other tissues indicates the existence of regulatory elements, rendering the potential for links in the RPE. Beyond regulatory relations, we included our previous finding of the physical and functional interaction of SOX9, OTX2, and MITF in the RPE (17). Because Rdh5 is down-regulated in Sox9-ablated RPE (this study) and Otx2-ablated RPE (16), both factors are necessary for normal expression of Rdh5, possibly by cooperative function of a SOX9-OTX2 complex on the Rdh5 promoter. We also added experimentally validated miRNAs to the network (Fig. 9). Of the three genes analyzed in this study, RPE65 and RLBP1 are positioned closer to each other than to RGR in the network by sharing transcriptional regulation by SOX9 and OTX2 as well as functional sites for miR-137. This network functions dynamically, depending on conditions.

There are several additional changes in Sox9cko mice that drew our attention. First, we did not observe decreased Sox9 expression in heterozygous Sox9<sup>cko/+</sup> mice. Because cre-mediated recombination clearly occurred, this suggests compensatory up-regulation from the remaining wild-type Sox9 allele and could indicate the existence of robust autoregulation and/or feed-forward loops for Sox9 to maintain its level in RPE cells, as has been described in other tissues (68, 69). Secondly, mouse Best1 expression was not down-regulated in Sox9cko mice, whereas human BEST1 is a target of SOX9. This can be explained by the use of alternative promoters in human and mouse. Thirdly, Lhx2 expression was significantly up-regulated in Sox9-abloated RPE, suggesting that SOX9 might repress Lhx2 in vivo. However, our results also show that, although SOX9 mRNA levels were lower in human RPE primary cells than RPE tissue, LHX2 expression was similar in both cells, resulting in a higher LHX2/SOX9 ratio in RPE primary cells as seen in Sox9-ablated RPE. Lastly, Tyr expression was up-regulated in Sox9cko mice. Again, we found that TYR expression in human RPE primary cells was significantly higher than that in RPE tissue. Thus, Sox9-ablated RPE cells show similar molecular characteristics as RPE primary cells. Because RPE primary cells are more similar to fetal RPE in gene expression, including MITF isoform profiles (40, 47), Sox9-deficient RPE cells may be dedifferentiated to some extent. Indeed, RPE65 expression is substantially lower in RPE primary cells than in RPE tissue (47). Accordingly, SOX9 appears to be necessary for maintaining the mature state of adult RPE cells.

In summary, this study provides the first systematic look at the regulation of visual cycle genes in the RPE and proposes a core regulatory network in which SOX9 plays a key role. This study also presents the first evidence for the functional role of LHX2 in mature RPE.

REFERENCES

1. Strauss, O. (2005) The retinal pigment epithelium in visual function. Physiol. Rev. 85, 845–881
2. Travis, G. H., Golczak, M., Moise, A. R., and Palczewski, K. (2007) Diseases caused by defects in the visual cycle: retinoids as potential therapeutic agents. Annu. Rev. Pharmacol. Toxicol. 47, 469–512
3. Boulanger, A., Liu, S., Henninggaard, A. A., Yu, S., and Redmond, T. M. (2000) The upstream region of the Rpe65 gene confers retinal pigment epithelium-specific expression in vivo and in vitro and contains critical octamer and E-box binding sites. J. Biol. Chem. 275, 31274–31282
4. Kennedy, B. N., Goldflam, S., Chang, M. A., Campochiaro, P., Davis, A. A., Zack, D. J., and Crabb, J. W. (1999) Transcriptional regulation of cellular retinaldehyde-binding protein in the retinal pigment epithelium. A role for the photoreceptor consensus element. J. Biol. Chem. 273, 5591–5598
5. Nicoletti, A., Kawase, K., and Thompson, D. A. (1998) Promoter analysis of RPE65, the gene encoding a 61-kDa retinal pigment epithelium-specific protein. Invest. Ophthalmol. Vis. Sci. 39, 637–644
6. Vázquez-Chona, F. R., Clark, A. M., and Levine, E. M. (2009) Rbp1 promoter drives robust Muller glial GFP expression in transgenic mice. Invest. Ophthalmol. Vis. Sci. 50, 3996–4003
7. Vogel, J. S., Bullen, E. C., Teygong, C. L., and Howard, E. W. (2007) Identification of the RLBP1 gene promoter. Invest. Ophthalmol. Vis. Sci. 48, 3872–3877
8. Rattner, A., Toulabi, L., Williams, J., Yu, H., and Nathans, J. (2008) The genomic response of the retinal pigment epithelium to light damage and retinal detachment. J. Neurosci. 28, 9880–9889
9. Kim, J. W., Kang, K. H., Burrola, P., Mak, T. W., and Lemke, G. (2008) Retinal degeneration triggered by inactivation of PTEN in the retinal pigment epithelium. Genes Dev. 22, 3147–3157
10. Zhao, C., Yasumura, D., Li, X., Matthes, M., Lloyd, M., Nielsen, G., Ahern, K., Snyder, M., Bok, D., Dunaiet, L. J., LaVail, M. M., and Volkath, D. (2011) mTOR-mediated dedifferentiation of the retinal pigment epithelium initiates photoreceptor degeneration in mice. J. Clin. Investig. 121, 369–383
11. Bharti, K., Nguyen, M. T., Skuntz, S., Bertuzzi, S., and Arnheiter, H. (2006) The other pigment cell: specification and development of the pigmented

5 N. Esumi, unpublished results.
epithelium of the vertebrate eye. Pigment Cell Res. 19, 380–394
30. Hu, G., Huang, K., Yu, J., Gopalakrishna-Pillai, S., Kong, J., Xu, H., Liu, Z.,
31. Esumi, N., Kachi, S., Campochiaro, P. A., and Zack, D. J. (2007) VMD2
32. Esumi, N., Kachi, S., Hackler, L., Jr., Masuda, T., Yang, Z., Campochiaro,
33. Kist, R., Schrewe, H., Balling, R., and Scherer, G. (2002) Conditional inac-
34. Iacovelli, J., Zhao, C., Wolkow, N., Veldman, P., Gollomp, K., Ojha, P.,
35. Davis, A. A., Bernstein, P. S., Bok, D., Turner, J., Nachtigal, M., and Hunt,
36. Wilhelm, D., Hiramatsu, R., Mizusaki, H., Widjaja, L., Combes, A. N.,
37. Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977) Characteris-
38. Mardaryev, A. N., Meier, N., Poterlowicz, K., Sharov, A. A., Sharova, T. Y.,
39. Bhutto, I. A., Kim, S. Y., McLeod, D. S., Merges, C., Fukai, N., Olsen, B. R.,
40. Westenskow, P., Piccolo, S., and Fuhrmann, S. (2009) SOX9 Regulates Visual
41. Hobert, O., and Westphal, H. (2000) Functions of LIM-homeobox genes.
42. Porter, F. D., Drago, J., Xu, Y., Cheema, S. S., Wassif, C., Huang, S. P., Lee,
43. Yun, S., Sajoib, Y., Hirokawa, K. E., Kopinke, D., Murtaugh, L. C., Monuki,
44. Jang, S. W., and Svaren, J. (2009) Induction of myelin protein zero by early
45. LeBlanc, S. E., Ward, R. M., and Svaren, J. (2007) Neuropathy-associated
46. Muto, A., Iida, A., Satoh, S., and Watanabe, S. (2009) The group E Sox
47. Poché, R. A., Furuta, Y., Chaboissier, M. C., Schedl, A., and Behringer, R. R.
48. Wilhelm, D., Hiramatsu, R., Mizusaki, H., Widjaja, L., Combes, A. N.,
49. Kanai, Y., and Koopman, P. (2007) SOX9 regulates prostaglandin D syn-
50. Davis, A. A., Bernstein, P. S., Bok, D., Turner, J., Nachtigal, M., and Hunt,
51. Muto, A., Iida, A., Satoh, S., and Watanabe, S. (2009) The group E Sox
52. Bartel, D. P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and
53. Bartel, D. P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and
54. Porter, F. D., Drago, J., Xu, Y., Cheema, S. S., Wassif, C., Huang, S. P., Lee,
55. Hobert, O., and Westphal, H. (2000) Functions of LIM-homeobox genes.
56. Muto, A., Iida, A., Satoh, S., and Watanabe, S. (2009) The group E Sox
57. Bartel, D. P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and
58. Porter, F. D., Drago, J., Xu, Y., Cheema, S. S., Wassif, C., Huang, S. P., Lee,
59. Hobert, O., and Westphal, H. (2000) Functions of LIM-homeobox genes.
60. Muto, A., Iida, A., Satoh, S., and Watanabe, S. (2009) The group E Sox
61. Bartel, D. P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and
62. Porter, F. D., Drago, J., Xu, Y., Cheema, S. S., Wassif, C., Huang, S. P., Lee,
63. Hobert, O., and Westphal, H. (2000) Functions of LIM-homeobox genes.
64. Muto, A., Iida, A., Satoh, S., and Watanabe, S. (2009) The group E Sox
65. Bartel, D. P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and
66. Porter, F. D., Drago, J., Xu, Y., Cheema, S. S., Wassif, C., Huang, S. P., Lee,
67. Hobert, O., and Westphal, H. (2000) Functions of LIM-homeobox genes.
68. Muto, A., Iida, A., Satoh, S., and Watanabe, S. (2009) The group E Sox
69. Bartel, D. P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and
70. Porter, F. D., Drago, J., Xu, Y., Cheema, S. S., Wassif, C., Huang, S. P., Lee,
SOX9 Regulates Visual Cycle Gene Expression

tanabe, D., Kondo, M., Yamashita, T., and Furukawa, T. (2011) miR-124a is required for hippocampal axogenesis and retinal cone survival through Lhx2 suppression. Nat. Neurosci. 14, 1125–1134

51. Lewis, B. P., Burge, C. B., and Bartel, D. P. (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120, 15–20

52. Grimson, A., Farh, K. K., Johnston, W. K., Garrett-Engele, P., Lim, L. P., and Bartel, D. P. (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol. Cell 27, 91–105

53. Bruban, J., Glotin, A. L., Dinet, V., Chalour, N., Sennlaub, F., Jonet, L., An, N., Faussat, A. M., and Mascarelli, F. (2009) Amyloid-β(1–42) alters structure and function of retinal pigmented epithelial cells. Aging Cell 8, 162–177

54. Chucair-Elliott, A. J., Elliott, M. H., Wang, J., Moiseyev, G. P., Ma, J. X., Béby, F., Housset, M., Fossat, N., Le Greneur, C., Flamant, F., Godement, F., and Redmond, T. M. (2010) Otx2 gene deletion in adult mouse retina induces rapid RPE dystrophy and slow photoreceptor degeneration. PLoS ONE 5, e11673

55. Bunt-Milam, A. H., and Saari, J. C. (1983) Immunocytochemical localization of two retinoid-binding proteins in vertebrate retina. J. Cell Biol. 97, 703–712

56. Jiang, M., Pandey, S., and Fong, H. K. (1993) An opsin homologue in the retina and pigment epithelium. Invest. Ophthalmol. Vis. Sci. 34, 3669–3678

57. Hägglund, A. C., Dahl, L., and Carlsson, L. (2011) Lhx2 is required for patterning and expansion of a distinct progenitor cell population committed to eye development. PLoS ONE 6, e23387

58. Xie, D., Boyle, A. P., Wu, L., Zhai, J., Kawli, T., and Snyder, M. (2013) Dynamic trans-acting factor colocalization in human cells. Cell 155, 713–724

59. Mukherji, S., Ebert, M. S., Zheng, G. X., Tsang, J. S., Sharp, P. A., and van Oudenaarden, A. (2011) MicroRNAs can generate thresholds in target gene expression. Nat. Genet. 43, 854–859

60. Hamel, C. P., Tsoulou, E., Harris, E., Pfeffer, B. A., Hooks, J. J., Detrick, B., and Redmond, T. M. (1993) A developmentally regulated microsomal protein specific for the pigment epithelium of the vertebrate retina. J. Neuropathol. Exp. Neurol. 52, 141–425

61. Manès, G., Leduq, R., Kucharzczak, J., Pagès, A., Schmitt-Bernard, C. F., and Hamel, C. P. (1998) Rat messenger RNA for the retinal pigment epithelium-specific protein RPE65 gradually accumulates in two weeks from late embryonic days. FEBS Lett. 423, 133–137

62. Bemis, L. T., Chen, R., Amato, C. M., Classen, E. H., Robinson, S. E., Coffey, D. G., Erickson, P. F., Sheehan, Y. G., and Robinson, W. A. (2008) MicroRNA-137 targets microphthalmia-associated transcription factor in melanoma cell lines. Cancer Res. 68, 1362–1368

63. Mendell, J. T. (2008) miRiad roles for the miR-17–92 cluster in development and disease. Cell 133, 217–222

64. Ventura, A., Young, A. G., Winslow, M. M., Lintault, L., Meissner, A., Erkeland, S. I., Newman, J., Bronson, R. T., Crowley, D., Stone, J. R., Jae-nisch, R., Sharp, P. A., and Jacks, T. (2008) Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. Cell 132, 857–866

65. Brock, M., Trenkmann, M., Gay, R. E., Michel, B. A., Gay, S., Fischler, M., Ulrich, S., Speich, R., and Huber, L. C. (2009) Interleukin-6 modulates the expression of the bone morphogenetic protein receptor type II through a novel STAT3-microRNA cluster 17/92 pathway. Circ. Res. 104, 1184–1191

66. Passeron, T., Valencia, J. C., Bertolotto, C., Hoashi, T., Le Pape, E., Taka-hashi, K., Ballotti, R., and Hearing, V. J. (2007) SOX9 is a key player in ultraviolet B-induced melanocyte differentiation and pigmentation. Proc. Natl. Acad. Sci. U.S.A. 104, 13984–13989

67. Mead, T. J., Wang, Q., Bhattachar, P., Dy, P., Afelik, S., Jensen, J., and Lefebvre, V. (2013) A far-upstream (-70 kb) enhancer mediates Sox9 auto-regulation in somatic tissues during development and adult regeneration. Nucleic Acids Res. 41, 4459–4469

68. Moniot, B., Declosmenil, F., Barrionuevo, F., Scherer, G., Aritake, K., Malki, S., Marzi, L., Cohen-Solal, A., Georg, L, Klattig, J., Englert, C., Kim, Y., Capel, B., Eguchi, N., Urade, Y., Boizet-Bonhoure, B., and Poulat, F. (2009) The PGD2 pathway, independently of FGF9, amplifies SOX9 activity in Sertoli cells during male sexual differentiation. Development 136, 1813–1821

69. Dong, C., Wang, H., Xue, L., Dong, Y., Yang, L., Fan, R., Yu, X., Tian, X., Ma, S., and Smith, G. W. (2012) Coat color determination by miR-137 mediated down-regulation of microphthalmia-associated transcription factor in a mouse model. RNA 18, 1679–1686