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Vision Research 43 (2003) 3075–3079
Vision Research
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Novel targeting strategy for generating mouse models with defects in the retinoid cycle

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Received 2 June 2003; received in revised form 24 July 2003

Abstract

In addition to RDH5, other enzymes capable of oxidizing 11-*cis*-retinol are present within the retinal pigment epithelium, Müller cells and/or photoreceptors. Candidate proteins have meanwhile been identified. To study the physiological and pathological aspects of these enzymes, mice in which these genes are no longer functional are being generated. A fast-targeting strategy for the disruption of genes was developed. Generation of double and triple knockouts will aid in determining if these retinol dehydrogenases are responsible for the remaining 11-*cis*-retinol oxidation observed in RDH5 knockout animals.

Keywords: Retinoid cycle; Retinol dehydrogenase; Animal model; Retinal pathology

1. Introduction

Vision is triggered by visual pigments consisting of a protein moiety called opsin and the vitamin A-derivative 11-*cis*-retinal (for a review see McBee, Palczewski, Baehr, & Pepperberg, 2001). Phototransduction is initiated by capture of a photon and subsequent isomerization of 11-*cis*-retinal to all-*trans*-retinal. Following bleaching, the all-*trans*-retinal chromophore is released from the protein. Visual pigment regeneration requires that all-*trans*-retinal is converted back into 11-*cis*-retinal. In this process different enzymatic conversions of retinoids are needed and a number of the enzymes involved have been identified. In the best-characterized pathway, all-*trans*-retinal is reduced to all-*trans*-retinol by all-*trans*-retinol dehydrogenase(s) present in photoreceptor cells (Haeseleer & Palczewski, 2000; Rattner, Smallwood, & Nathans, 2000). All-*trans*-retinol is transported to the retinal pigment epithelium (RPE) and isomerized into 11-*cis*-retinol. Finally 11-*cis*-retinol is oxidized to 11-*cis*-retinal through the action of 11-*cis*-retinol dehydrogenases and transported back to the photoreceptor cells (Driessen et al., 1995; Simon, Hellman, Wernstedt, & Eriksson, 1995).

Recently a retinoid cycle specific for cone pigment regeneration was described (Mata, Radu, Clemmons, & Travis, 2002; Wood, 2002). It has been suggested that this second pathway involves an interplay between Müller cells and cone photoreceptors. First all-*trans*-retinal is reduced to all-*trans*-retinol in the cones. Subsequently all-*trans*-retinol is transported to the Müller cells and isomerized into 11-*cis*-retinol, which is then transported back to the cones and oxidized to 11-*cis*-retinal.

A third possible mechanism of 11-*cis*-retinal formation is the direct isomerization of all-*trans*-retinal into 11-*cis*-retinal by a retinal G-protein-coupled receptor (RGR), a retinochrome present in both Müller cells and RPE (Wolf, 2002). In vitro RGR was demonstrated to be capable of photoisomerase activity (Hao & Fong, 1999).

So far only a limited number of players in the pathways mentioned above are known. One of these is

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0042-6989/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0042-6989(03)00483-8

RDH5, an 11-*cis*-retinol dehydrogenase shown to be active in the first pathway. Mice were developed carrying a targeted deletion of this gene and it was shown that these mice are still capable of regenerating their rod visual pigment in the dark (Driessen et al., 2000; Shang et al., 2002). Hence, in addition to RDH5, other enzymes capable of oxidizing 11-*cis*-retinol need to be present within the RPE, Müller cells and/or photoreceptors. The residual retinol dehydrogenase enzyme activity in the retina was characterized (Jang et al., 2001). The characteristics concerning substrate specificity and cofactor usage of these enzymes were determined. Several candidate genes were identified (Haeseleer et al., 2002). The encoded proteins all display the correct biochemical characteristics and have unique expression patterns within the retina. The most interesting amongst these are RDH11, RDH12 and RDH15 (unpublished data). These three oxidoreductases display high levels of expression in specific retinal cell types, use NADP(H) as a cofactor and are capable of oxidizing both 11-*cis*-retinol and all-*trans*-retinol.

RDH5 knockout mice provided important information regarding the involvement of RDH5 in the retinoid cycle. To determine the relative contribution of RDH12 and RDH15 in the regeneration pathways of visual pigments, we aim to generate mice with a targeted deletion of both genes. In our targeting strategy 5′ and 3′ arms of the targeted genes were obtained by genomic PCR and cloned in a basic targeting vector, thereby eliminating the time consuming steps of screening mouse genomic DNA libraries and further characterization of isolated genomic clones. This elegant strategy will be useful for generation of mice carrying a targeted deletion in an arbitrary gene, since nowadays data from the mouse genome are available via databases and can be used to construct targeting vectors for gene inactivation.

2. Materials and methods

2.1. Construction of the basic targeting vector

In the pgem13zf(+) vector (Promega) a multiple cloning site was introduced using the phosphorylated oligomers MCS01 5′-ggccgctagcgaattcgtcgacggtaccg-gatcca-3′ and MCS02 5′-agcttggatccggtaccgtcgacgaattc-gctagc-3′. This leads to the introduction of unique recognition sites into the pgem13zf(+) vector for the following restriction enzymes (from 5′ to 3′, respectively): *Not* I, *Nhe* I, *EcoR* I, *Sal* I, *Kpn* I, *BamH* I and *Hind* III.

The herpes simplex virus thymidine kinase (HSVtk) cassette was cloned in the sense direction as a *BamH* I fragment. Subsequently the neomycin cassette was isolated as a *Xho* I fragment from Pha178pMC1neo (Stratagene) and cloned in the sense direction using the *Sal* I restriction site. Hence, the 5′ fragment of the targeted gene can either be cloned unidirectionally as a *Nhe* I/*EcoR* I fragment or random as a *Nhe* I or a *EcoR* I fragment. The 3′ fragment of the targeted gene can only be cloned as a *Kpn* I fragment. Finally the targeting vector can be linearized with either *Not* I or *Hind* III.

2.2. Construction of the RDH15 targeting vector

The 5′ and 3′ fragments of the RDH15 gene were obtained by long range PCR on genomic DNA isolated from 129/Sv mouse embryonic stem cells (ES). Primers used for amplification of the 2400 bp 5′ fragment of RDH15 were 5′-cccgctagctcctccagtggttgtaaggacaggattt-3′ and 5′-ggggctagcattatgtagccctggctgctgaaactttct-3′ (underlined are *Nhe* I restriction sites) and for the 2500 bp 3′ fragment of RDH15 5′-cccggtacccttttgagcactttaa-aatatttccactt-3′ and 5′-gggggtacccaagctacctgctttcattaaa-cggagaag-3′ (underlined are *Kpn* I restriction sites). Cycling conditions: 1 min 94 °C, 40 cycles 45 s 94 °C, 7 min 65 °C, and finally 10 min 72 °C, using rTth DNA polymerase XL (PerkinElmer Life Sciences). Both fragments were cloned in the PCRII Topo vector (Invitrogen) and isolated as a *Nhe* I or *Kpn* I fragment and subsequently cloned in the basic targeting vector.

2.3. RDH15 screening for homologous recombination

After electroporation of the *Not* I linearized RDH15 targeting vector, ES cells (129/Sv) were cultured on selective medium containing 350 μg of neomycin (G418) per ml and 0.2 μM FIAU. Appropriate targeting of the RDH15 gene was initially checked with PCR using genomic DNA isolated from ES cells cultured on gelatin coated 96 wells plates. Homologous recombination on the 5′ end was checked using the primer combination 5′-ttgtttagagaggaggttacatgggaagtg-3′ and 5′-gccccgactg-catctgcgtgtt-3′, resulting in a 2757 bp fragment. For the 3′ end we used the primer combination 5′-ctttacgg-tatcgccgctcc-3′ and 5′-tggttgaagatgaagaacctggagctcaag-3′, resulting in a 2793 bp fragment. Cycling conditions: 1 min 94 °C, 40 cycles 45 s 94 °C, 7 min 65 °C, and finally 10 min 72 °C, using rTth DNA polymerase XL (PerkinElmer Life Sciences).

To verify the ES cell lines selected with PCR, appropriate targeting was also checked with Southern blot analysis. For amplification of the 5′ probe the following primer combination was used 5′-gagccaaggaatgggcactc-tttagcaaa-3′ and 5′-ccctggtgataaggaacagagagggacttt-3′ and for the 3′ probe 5′-tccacagctgtttctgtcctgccattcat-3′ and 5′-gaccctgcatctatgcccaaactcactttt-3′, resulting in 551 and 548 bp fragments, respectively. The 5′ probe detected a 4.6 kb *BamH* I fragment derived from the targeted allele and a 3.4 kb BamH I fragment from the wild-type allele, and the 3′ probe a 3.1 and 6.4 kb *BamH*

I fragment from the targeted and wild-type alleles, respectively.

3. Results

3.1. Construction of the targeting vector

The entire murine RDH15 gene is present in clone NT_039208.1, originating from mouse chromosome 2. The positions of four exons were determined using the sequence of the murine RDH15 cDNA (Fig. 1). However, as with RDH5, the presence of a non-coding exon positioned upstream of the first coding exon cannot be excluded. The RDH5 gene from several species is composed of five exons of which the first exon is entirely non-coding (Driessen et al., 1998; Romert, Tuvendal, Tryggvason, Dencker, & Eriksson, 2000; Simon, Lagercrantz, Bajalica-Lagercrantz, & Eriksson, 1996).

The strategy for targeted disruption of the RDH15 gene requires the amplification of the 5′ and 3′ arms using genomic DNA isolated from mouse 129/Sv ES cells. Using the murine RDH15 genomic sequence, we designed primer sets in order to amplify the 5′ and 3′ arms. The primers for amplifying the 5′ arm was extended with *Nhe* I recognition sites, and primers for the 3′ arm with *Kpn* I. The size of the obtained PCR products were in good agreement with the predicted sizes (data not shown). PCR products were cloned in the PCRII Topo vector and partially sequenced to confirm that the correct genomic regions of the murine RDH15 gene were amplified and cloned. The 5′ and 3′ arms were rescued from the plasmids using *Nhe* I or *Kpn* I, respectively, and subcloned into the basic targeting vector, already containing the positive selection marker neomycin and the negative selection marker herpes simplex virus thymidine kinase (HSVtk).

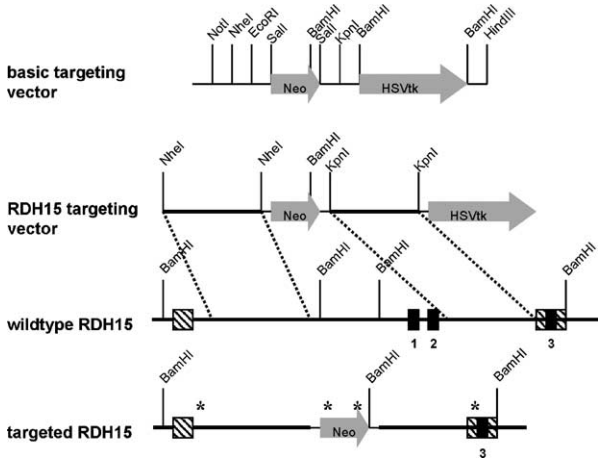

Fig. 1. Targeted disruption of the RDH15 gene. Schematic representation of the basic targeting vector, RDH15 targeting vector, organization of the wild-type and targeted RDH15 alleles. Not shown is the position of exon 4 (intron 3 is about 3.5 kb). Homologous recombination results in the replacement of exons 1 (containing the atg start codon) and 2 by a neomycin cassette (Neo). Filled boxes denote exon positions, dashed boxes denote positions of the 5′ and 3′ probe. The positions of the primers used for identification of targeted ES cell lines are marked by an asterisk.

3.2. Transfection to ES cells

The targeting construct was linearized with *Not* I and transfected to ES cells. Transfected ES cells were plated on dishes with irradiated Snl cells necessary for LIF production. After exposing the ES cells to G418 for 5–7 days, 211 clones were selected. All clones were checked for homologous recombination.

In an attempt to reduce experimental time for cloning and identification of the correct targeted ES cell lines, we decided to develop a PCR protocol to check for correct homologous recombination in RDH15 targeted ES cell lines (Fig. 2, panel A). We therefore cultured all 211 selected cell lines on gelatin-coated 96 wells plates and subsequently isolated genomic DNA. Two primer combinations were designed. A sense primer in the neomycin cassette in combination with an antisense primer downstream of the 3′ arm was used to screen for correct recombination of the 3′ arm. Four cell lines showed the presence of a 2793 bp amplified PCR product. These cell lines were subsequently used to check for correct recombination of the 5′ arm using a sense primer upstream of the 5′ arm and an antisense primer in the neomycin cassette. Cell lines 1A5 and 3A12 showed the presence of a 2757 bp PCR product.

Unfortunately, we were not able to amplify the complete targeted gene using the primers upstream of the 5′ arm and downstream of the 3′ arm. We therefore

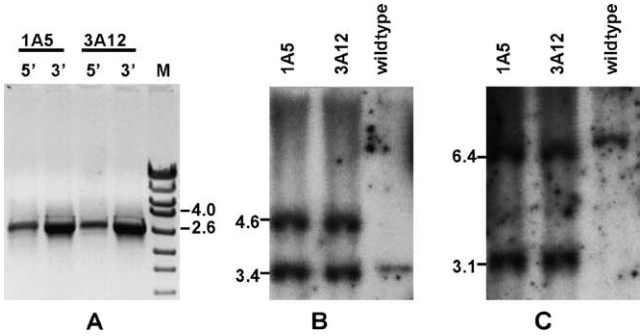

Fig. 2. Identification of RDH15 targeted cell lines using PCR (panel A) and Southern blot analysis (panels B and C). (A) For PCR analysis genomic DNA from G418-resistant ES cell lines was analysed with two primer combinations for checking the 5′ and 3′ integration events, respectively. Two positive cell lines, 1A5 and 3A12, are shown. (B) Southern blot analysis with the 5′ flanking probe of genomic DNA of cell lines 1A5 and 3A12 and wild-type 129/Sv genomic DNA digested with *BamH* I. A targeted allele is identified by a 4.6 kb fragment in addition to a 3.4 kb wild-type allele. (C) The 3′ flanking probe identified a 3.1 kb fragment for the targeted allele and a 6.4 kb fragment for the wild-type allele.

decided to check cell lines 1A5 and 3A12 for correct homologous recombination also by Southern blot analysis (Fig. 2, panels B and C). Southern blot analysis was performed using *BamH* I digested genomic DNA. Based on the sequence data from the mouse genome database we predicted a 3.4 kb fragment of the wild-type gene and a 4.6 fragment of the targeted gene upon screening the blot with the 5′ probe located upstream of the 5′ arm. The detected bands both perfectly matched the predicted size. For the 3′ probe we predicted a 6.4 and 3.1 kb fragment for the wild-type and targeted gene, respectively. The detected bands also matched those predicted sizes. These results indicated that in cell lines 1A5 and 3A12 exons 1 and 2 are replaced by a neomycin cassette, thereby confirming the results obtained by PCR. Both cell lines were used for blastocyst injection. Among the offspring, seven chimeric males (20–60% Agouti coat color) and seven chimeric females (20–60% Agouti coat color) were identified.

4. Discussion

This report describes a targeting strategy for generation of RDH15 knockout mice. This strategy will be employed also for the construction of mice with a targeted deletion of the RDH12 gene. Both oxidoreductases are thought to be candidates for playing an important role in the retinal retinoid cycle. High levels of RDH15 transcription were detected in both retina and RPE using Northern blot analysis (F. Haeseleer, unpublished data). RT-PCR using RDH15-specific primers, located in a region not conserved between retinol dehydrogenases, showed the presence of human RDH15 transcripts not only in retina and RPE but also in heart, pancreas, prostate, liver, lung, kidney and testis (J. Janssen, unpublished data). The encoding region of the mouse RDH15 transcript shows 93% identity to a retinol dehydrogenase homolog (Rdhl) cyclically expressed in the uterine epithelium of rat (Rexer & Ong, 2002). Cotransfection of RdhI and rat aldehyde dehydrogenase RALDH3 in COS cells resulted in the production of retinoic acid from retinol provided in culture. Furthermore 84% homology is found with a cDNA clone from a library of human tracheobronchial epithelial cells which was isolated as a retinoic acid responsive clone upon differential hybridization screening (Soref et al., 2001). This group provided evidence that the recombinant protein is able to metabolize all-*trans*-retinol. An identical cDNA clone was isolated by Chetyrkin and coworkers as a human microsomal 3α-hydroxysteroid dehydrogenase (Chetyrkin, Belyaeva, Gough, & Kedishvili, 2001).

Previously, RDH12 transcripts were detected in the inner segments of photoreceptors in monkey retinae using digoxigenin labelled antisense probes (Haeseleer et al., 2002). Recombinant RDH12 was found to be able to catalyze the reduction of both all-*trans*-retinal and *cis*-retinals with high efficiency.

Another candidate enzyme to play a role in the retinal retinoid metabolism is RDH11 (Haeseleer et al., 2002), originally cloned as a prostate short-chain dehydrogenase/reductase *PSDR1* (Kedishvili et al., 2002; Lin et al., 2001; Moore, Pritchard, Lin, Ferguson, & Nelson, 2002). A high level of RDH11 expression was detected in the RPE and a lower level in the Müller cells. Immunoprecipitation experiments suggested that RDH5 and RDH11 may form a larger oligomeric structure or interact with each other. RDH11 has a very similar property to retinoids to those of RDH12.

The described targeting strategy for the construction of RDH12 and RDH15 knockout mice has several advantages compared to the more conventional method, which requires screening of a mouse genomic library, construction of restriction maps, mapping of exon/intron boundaries and cloning of homologous fragments into a vector. These steps are time consuming and require several months to complete. In our method, 5′ and 3′ arms are amplified by long range PCR and subsequently cloned in a basic targeting vector already containing the necessary screening cassettes. This strategy requires only weeks and is less dependent on the availabilty of restriction sites, the only limitation being that the 5′ and 3′ arms should not contain internal recognition sites for *EcoR* I (and/or *Nhe* I), *Kpn* I, and *Not* I (or *Hind* III). This can be easily avoided by positioning the 5′ or 3′ arms in regions of the gene of interest in which these sites are not present.

For amplification of the 5′ and 3′ arms, we used rTth DNA polymerase, which might introduce mutations into the amplified products due to replication errors. This might reduce the efficiency of homologous recombination. Depending on the construct, 3–5% of the cells are found to be correctly targeted using the conventional method. For RDH15 the targeting frequency was 1%. However, the targeting frequency for RDH15 might have been higher than observed, since correctly targeted ES cell lines might have been missed due to our initial screening procedure which was based on a PCR protocol. Genomic DNA isolated from ES cell lines cultured on gelatin coated 96 wells plates, in our hands, appeared to contain a compound which negatively affects the efficiency of PCR amplification.

In conclusion, a fast targeting strategy for the disruption of the genes was developed which enables us to study physiological and pathological aspects of RDH12 and RDH15. Combining functional loss will help to determine if these retinol dehydrogenases are responsible for the remaining 11-*cis*-retinol oxidation observed in RDH5 knockout animals. It will furthermore shed light on the possibility of retinol dehydrogenases being involved in multigenic disorders.

Acknowledgements

This research was supported by NIH grants EY09339 and EY13385, a grant from Research to Prevent Blindness, Inc. (RPB) to the Department of Ophthalmology at the University of Washington, and a grant from the E.K. Bishop Foundation. KP is a RPB Senior Investigator.

References

Chetyrkin, S. V., Belyaeva, O. V., Gough, W. H., & Kedishvili, N. Y. (2001). Characterization of a novel type of human microsomal 3α-hydroxysteroid dehydrogenase: Unique tissue distribution and catalytic properties. *Journal of Biological Chemistry, 276*, 22278–22286.

Driessen, C. A. G. G., Janssen, A. P. M., Winkens, H. J., van Vugt, A. H. M., de Leeuw, A. L., & Janssen, J. J. M. (1995). Cloning and expression of a cDNA encoding bovine retinal pigment epithelial 11-*cis* retinol dehydrogenase. *Investigative Ophthalmology and Visual Science, 36*, 1988–1996.

Driessen, C. A. G. G., Winkens, H. J., Hoffmann, K., Kuhlmann, E. D., Janssen, A. P. M., Van Vugt, A. H. M., Van Hooser, J. P., Wieringa, B. E., Deutman, A. F., Palczewski, K., Ruether, K., & Janssen, J. J. M. (2000). Disruption of the 11-*cis*-retinol dehydrogenase gene leads to accumulation of *cis*-retinols and *cis*-retinyl esters. *Molecular and Cellular Biology, 20*, 4275–4287.

Driessen, C. A. G. G., Winkens, H. J., Kuhlmann, E. D., Janssen, A. P. M., van Vugt, A. H. M., Deutman, A. F., & Janssen, J. J. M. (1998). The visual cycle retinol dehydrogenase: Possible involvement in the 9-*cis* retinoic acid biosynthetic pathway. *FEBS Letters, 428*, 135–140.

Haeseleer, F., Jang, G. F., Imanishi, Y., Driessen, C. A. G. G., Matsumura, M., Nelson, P. S., & Palczewski, K. (2002). Dual-substrate specificity short chain retinol dehydrogenases from the vertebrate retina. *Journal of Biological Chemistry, 277*, 45537–45546.

Haeseleer, F., & Palczewski, K. (2000). Short-chain dehydrogenases/reductases in retina. *Methods on Enzymology, 316*, 372–383.

Hao, W., & Fong, H. K. (1999). The endogenous chromophore of retinal G proteincoupled receptor opsin from the pigment epithelium. *Journal of Biological Chemistry, 274*, 6085–6090.

Jang, G. F., Van Hooser, J. P., Kuksa, V., Li, H. J., McBee, J., Janssen, J. J. M., Driessen, C. A. G. G., & Palczewski, K. (2001). Retinoid flow in 11-*cis* retinol dehydrogenase mice. *Journal of Biological Chemistry, 276*, 32456–32465.

Kedishvili, N. Y., Chumakova, O. V., Chetyrkin, S. V., Belyaeva, O. V., Lapshina, E. A., Lin, D. W., Matsumura, M., & Nelson, P. S. (2002). Evidence that the human gene for prostate short-chain dehydrogenase/reductase (PSDR1) encodes a novel retinal reductase (RalR1). *Journal of Biological Chemistry, 277*, 28909–28915.

Lin, B., White, J. T., Ferguson, C., Wang, S., Vessella, R., Bumgarner, R., True, L. D., Hood, L., & Nelson, P. S. (2001). Prostate short-chain dehydrogenase reductase 1 (PSDR1): A new member of the short-chain steroid dehydrogenase/reductase family highly expressed in normal and neoplastic prostate epithelium. *Cancer Research, 61*, 1611–1618.

Mata, N. L., Radu, R. A., Clemmons, R. C., & Travis, G. H. (2002). Isomerization and oxidation of vitamin A in cone-dominant retinas: A novel pathway for visual-pigment regeneration in daylight. *Neuron, 36*, 69–80.

McBee, J. K., Palczewski, K., Baehr, W., & Pepperberg, D. R. (2001). Confronting complexity: The interlink of phototransduction and retinoid metabolism in the vertebrate retina. *Progress in Retinal Eye Research, 20*, 469–529.

Moore, S., Pritchard, C., Lin, B., Ferguson, C., & Nelson, P. S. (2002). Isolation and characterization of the murine prostate short-chain dehydrogenase/reductase 1 (Psdr1) gene, a new member of the short-chain steroid dehydrogenase/reductase family. *Gene, 293*, 149–160.

Rattner, A., Smallwood, P. M., & Nathans, J. (2000). Identification and characterization of all-trans-retinol dehydrogenase from photoreceptor outer segments, the visual cycle enzyme that reduces all-*trans*-retinal to all-*trans*-retinol. *Journal of Biological Chemistry, 275*, 11034–11043.

Rexer, B. N., & Ong, D. E. (2002). A novel short-chain alcohol dehydrogenase from rats with retinol dehydrogenase activity, cyclically expressed in uterine epithelium. *Biology of Reproduction, 67*, 1555–1564.

Romert, A., Tuvendal, P., Tryggvason, K., Dencker, L., & Eriksson, U. (2000). Gene structure, expression analysis, and membrane topology of RDH4. *Experimental Cell Research, 256*, 338–345.

Shang, E., Lai, K., Packer, A. I., Paik, J., Blaner, W. S., de Morais Vieira, M., Gouras, P., & Wolgemuth, D. J. (2002). Targeted disruption of the mouse *cis*-retinol dehydrogenase gene: Visual and nonvisual functions. *Journal of Lipid Research, 43*, 590–597.

Simon, A., Hellman, U., Wernstedt, C., & Eriksson, U. (1995). The retinal pigment epithelial-specific 11-*cis* retinol dehydrogenase belongs to the family of short chain alcohol dehydrogenases. *Journal of Biological Chemistry, 270*, 1107–1112.

Simon, A., Lagercrantz, J., Bajalica-Lagercrantz, S., & Eriksson, U. (1996). Primary structure of human 11-*cis* retinol dehydrogenase and organization and chromosomal localization of the corresponding gene. *Genomics, 36*, 424–430.

Soref, C. M., Di, Y. P., Hayden, L., Zhao, Y. H., Satre, M. A., & Wu, R. (2001). Characterization of a novel airway epithelial cell-specific short chain alcohol dehydrogenase/reductase gene whose expression is up-regulated by retinoids and is involved in the metabolism of retinol. *Journal of Biological Chemistry, 276*, 24194–24202.

Wolf, G. (2002). A newly discovered visual cycle necessary for vision during continuous illumination. *Nutrition Reviews, 60*, 62–65.

Wood, H. (2002). Putting the visual cycle in the shade. *Nature Reviews Neuroscience, 3*, 837.