Expression of Photoreceptor-Specific Nuclear Receptor NR2E3 in Rod Photoreceptors of Fetal Human Retina

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PURPOSE. To study the physiological function of NR2E3 and possible molecular mechanisms underlying enhanced short-wavelength cone syndrome (ESCS) pathogenesis in developing human retina, and to compare its expression to that of Neural Retina Leucine zipper (NRL), a transcription factor essential for rod differentiation.

METHODS. Expression of NR2E3, a photoreceptor-specific orphan nuclear receptor, was examined in human retinas between fetal weeks (Fwk) 9 to 22 by reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridization. Both NR2E3 and NRL expression patterns were followed by immunocytochemistry. The human retina develops in a central to peripheral pattern, in which a protein may take weeks to be expressed throughout the entire retina. This allowed a detailed temporal analysis of NR2E3 and NRL expression.

RESULTS. NR2E3 expression was detected shortly after the appearance of NRL in putative immature rods on the foveal edge at Fwk 11.7. Expression of both markers was maintained in rod opsin expressing fetal photoreceptors. NR2E3 expression was not detected in either long/medium- or short-wavelength cones. Its absence from cones was also supported by the position of labeled nuclei deep in the outer nuclear layer, and by the absence of NR2E3 from the fovea.

CONCLUSIONS. A role for NR2E3 in the rod developmental pathway is suggested. The closely related expression patterns of NRL and NR2E3 supported an interactive function, where both transcription factors determine the rod fate and suppress immature rods from adopting the S-cone fate. (Invest Ophthalmol Vis Sci. 2004;45:2807–2812) DOI:10.1167/iovs.03-1317

In the vertebrate retina, rod photoreceptors function under conditions of low ambient light but provide poor visual acuity, whereas cone photoreceptors function in bright light, mediate color vision, and provide high spatial resolution. Inherited retinal dystrophies often affect the integrity and health of photoreceptors and result in progressive and significant loss of vision.1 The underlying genetic mutations, identified to date, reveal a wide range of molecules that are associated with retinal degenerations (Retina Information Network [RetNet]: http://www.sph.uth.tmc.edu/Retnet/disease.htm). Mutations in genes encoding transcription factors (e.g., CRX and NRL) that are involved in photoreceptor development, are associated with a wide array of disease phenotypes.2–7 One such inherited retinal disease is enhanced Short-wavelength Cone Syndrome (ESCS), an autosomal recessive retinal disorder, which is characterized by an increased sensitivity to short-wavelength light, color vision defects, and reduced or absent rod function.5–10 ESCS is associated with mutations in the NR2E3 gene, a photoreceptor-specific orphan nuclear receptor.11,12 Immunopathological studies of an ECS retina, taken postmortem from an 80-year-old patient, revealed a greatly increased number of short wavelength (S) cones and no detectable rods, which are presumably lost through degeneration. The number of long- and medium-wavelength (L/M) cones was reduced as well, and some ESCS cones expressed both S and L/M opsin.13 Loss of Nr2e3 function in mice is associated with a naturally occurring retinal degeneration mutant, rd7.14,15 Like ESCS patients, the rd7 mouse retina has a significantly increased number of S cones, based on S-opsin immunoreactivity and peanut agglutinin staining, suggesting that NR2E3 may function as a suppressor of S-cone differentiation.

Another transcription factor essential for photoreceptor differentiation is the Neural Retina Leucine zipper (NRL), a member of the Maf-subfamily of basic motif-leucine zipper proteins.16–18 NRL expression is detected in rod, and not cone, nuclei and precedes rod opsin expression.19 NRL interacts with the homeodomain protein CRX (cone-retinal homeobox) and other transcription factors to regulate the expression of several rod-specific genes synergistically.20–23 Missense mutations in NRL are associated with autosomal dominant retinitis pigmentosa in humans.5 In mice, targeted deletion of Nrl (Nrl−/−) results in the complete loss of rods and a marked increase in S-cones.16 This phenotype is similar to human ESCS and the rd7 mouse, yet distinct in that no rod function or rod-specific gene is expressed in the Nrl−/− mouse even during early stages of retinal development13,15 (Mears AJ, Swaroop A, unpublished data, 2003). An absence of NR2E3 transcripts in the postnatal day 10 Nrl−/− mouse retina strongly suggests that NR2E3 is downstream of NRL in the transcriptional hierarchy.16

The phenotypic similarities between the retinas of ESCS patients and the Nrl−/− and rd7 mice have led to different models to explain the anatomic and physiological changes associated with the loss of NR2E3 function.11,15,16,24 One model predicts that NRL plays a key role in rod photoreceptor development by activating NR2E3 (directly or indirectly),
which in turn suppresses the postmitotic cells from adopting an S-cone identity.\textsuperscript{10} Both genes are then required for initiation and stabilization of rod cell phenotype and for expression of appropriate rod-specific phototransduction genes. In another model, NR2E3 is suggested to block cone production since the loss of NR2E3 results in an excess of S-cones.\textsuperscript{6,12-14} A major issue in evaluating these hypotheses has been the conflicting cellular localization of NR2E3 in specific retinal cell types reported in various studies.\textsuperscript{12,25} To further investigate this issue and gain insights into its function, the expression of NR2E3 in the developing human retina was determined by in situ hybridization, and using a specific affinity-purified antibody for immunolocalization. NR2E3 expression was also compared to NRL, taking advantage of the protracted development characteristic of the human retina, which allowed a detailed temporal resolution of developmental events. This study suggested a role for NR2E3 in the rod developmental pathway and supported its proposed function in S-cone suppression.

**MATERIALS AND METHODS**

**Human Tissues**

Human eyes between fetal weeks (Fwk) 9 to 22 were obtained under approved protocols from the Fetal Tissue Program, University of Washington, or ABR, Inc., Alameda, CA. Fetal age was determined by eye size and foot length. Eyes for in situ hybridization, immunocytochemistry (ICC), and morphologic analysis were fixed unopened in 2% (1 hour) or 4% (4 to 12 hours) paraformaldehyde in 0.1 M phosphate buffered saline (PBS). Tissue was cryoprotected and serially frozen-sectioned at 12 μm. Every 10th slide was stained with cresyl violet to localize the fovea and optic disc within each retina. Only sections adjacent to or including the fovea were used for ICC and in situ hybridization analysis.

**Reverse Transcription–Polymerase Chain Reaction**

RT-PCR was performed using total retinal RNA (Fwks 9, 10, and 12; two samples at each age), as described previously\textsuperscript{15} with actin-specific primers which amplify a 539 base pair fragment between 176 to 714; forward, 5'-TGTCCTTGTCAACGAGCCAGCACAAC-3' and reverse, 5'-TCCTTAATGTCACGCACGATTTCCCG-3'. The amplified cDNA fragment was cloned into pGEX-4T-2 (Promega, Madison, WI). Glutathione-S-transferase (GST)-NR2E3 fusion protein was expressed in BL21 E. coli and purified by binding to glutathione-Sepharose beads, as described.\textsuperscript{16} The NR2E3 protein was cleaved by thrombin treatment, separated from the GST-NR2E3 fusion protein, and used for generating polyclonal antibodies in rabbit (Invtrogen, Carlsbad, CA), as described.\textsuperscript{17} The anti-NR2E3 antibody was purified by affinity chromatography.

To verify the specificity of the antibody, full-length NR2E3 cDNA was cloned into pcDNA3C (Invitrogen) expression vector and transfected into COS-1 monkey kidney cells using Fugene 6 reagent (Roche, Indianapolis, IN). After 48 hours of transfection, cell extracts were subjected to immunoblotting using established procedures.\textsuperscript{17} Control antibodies were anti-Xpress monoclonal antibody (Fig. 2A; Invitrogen) and anti-β-tubulin monoclonal antibody (Fig. 2B; Sigma, St. Louis, MO).

**RESULTS**

**Expression of NR2E3 Transcripts**

NR2E3 expression could not be detected at Fwk 10 but was observed at Fwk 12 by RT-PCR amplification from fetal eye tissue (Figs. 1A, 1B). Expression of NR2E3 began significantly later than CRX, which was detected as early as Fwk 9, and shortly after the onset of NRL when compared by RT-PCR from the same human fetal eyes\textsuperscript{20} (data not shown). Fwk 13 to 16.5 fetal retinas were processed as wholemounts for in situ hybridization to analyze the topographic pattern of NR2E3 expression. At all ages (Figs. 1C and 1D, red arrow), the foveal region, which does not contain rods and S-cones,\textsuperscript{27,32,33} was clearly devoid of NR2E3 mRNA. NR2E3 transcripts were present peripheral to the optic disc at Fwk 13.5, but expression had not yet reached the retinal edge (Fig. 1C). At Fwk 16, NR2E3 expression reached the retinal edge with lower levels of expression in the far periphery (Fig. 1D).

The cell types expressing NR2E3 mRNA were analyzed in frozen sections derived from a Fwk 15 whomlomount retina processed for in situ hybridization (Figs. 1E, 1F, and 1G). NR2E3 transcripts were absent from foveal cones (Fig. 1E), while intense staining for NR2E3 was visible near the foveal

**Immunocytochemistry**

Frozen sections from Fwks 9, 10, 11, 11.7, 12, 12.7, 14, 16, 18, and 22 retinas (10 eyes total) were incubated overnight in primary antibodies diluted in standard medium (5% Chemi blocker (Chemicon, Temecula, CA) in PBS containing 0.05% sodium azide and 0.5% Triton X-100). Primary antisera and their sources were: NR2E3 (1:500), rod opsin (1:200–400, RhO iD2; Robert S. Molday, University of British Columbia, Vancouver, BC); S opsin (1:15,000–30,000; JH455; Jeremy Nathans, Johns Hopkins University, Baltimore, MD); OS2, (1:5000; Agoston Szel, Semmelweis University, Budapest, Hungary); synaptophysin (1:500; Sigma), and NRL (1:1000–3000). Sections were washed in PBS, followed by 1 hour incubation with a mixture of anti-mouse IgG conjugated to Alexa 594 (red) and anti-rabbit IgG conjugated to Alexa 488 (green) (each 1/500; Molecular Probes, Eugene, OR) in the standard medium. Sections were imaged on a Pascal confocal microscope (Carl Zeiss, Stuttgart, Germany). Images were processed for contrast and color balance using Adobe Photoshop (Adobe systems, San Jose, CA).
Expression of NR2E3 transcripts during fetal human retinal development. (A) RT-PCR agarose gel stained with ethidium bromide from a Fwk 10 human retina. A band was only detected in lane 2, which corresponds to actin (539 bp). M indicates the DNA molecular weight marker lane. (B) RT-PCR agarose gel stained with ethidium bromide from a Fwk 12 human retina. Bands were present in lane 1 corresponding to NR2E3 (503 bp) and actin in lane 2. (C) In situ hybridization of Fwk 13 retinal wholemount shows that the fovea does not express NR2E3 (red arrow). Outside the fovea, the expression level increases and NR2E3 is expressed into the periphery, but does not reach the retinal edge. (D) In situ hybridization of Fwk 16.5 retinal wholemount shows that NR2E3 is expressed across the entire retina except in the fovea region (red arrow). (E-G) Sections from Fwk15.5 retina processed for NR2E3 in situ hybridization. (E) The fovea has a single, thin layer of cone photoreceptors forming the outer nuclear layer (ONL) with an immature inner nuclear layer (INL) separated from the ganglion cell layer (GCL) by a thin, inner plexiform layer (IPL). Foveal cone photoreceptors do not express NR2E3. (F) NR2E3 expression appears in cells on the foveal edge in a region where rods are detected. (G) At the eccentricity of the optic nerve, the outermost part of the ONL corresponding to where cone nuclei lie does not express NR2E3. There is a broad band of NR2E3 expression in the deeper ONL where rods are located. Scale bars: (C-D), 500 μm; (E-F), 15 μm.

**Expression of NR2E3 Protein**

Immunoblot analysis of the transfected COS-cell extracts expressing recombinant NR2E3 detected a specific 47 kDa band, corresponding to the Xpress-NR2E3 fusion protein as verified by the epitope specific anti-Xpress antibody (Fig. 2A). Both anti-NR2E3 and anti-Xpress antibodies detected the fusion protein in the nuclei of transfected cells (data not shown). Immunoblot analysis using the anti-NR2E3 antibody revealed a single 42 kDa protein in adult human, bovine, and mouse retina, but no signal was detected in goldfish retina (Fig. 2B).

To localize expression of NR2E3 protein, Fwk 11.7 to 22 human retina sections were stained for ICC with the anti-NR2E3 antibody. Adjacent sections were labeled with an anti-NRL antibody to determine the temporal and spatial relationship between NR2E3 and NRL expression. Similar to in situ hybridization, labeling for NR2E3 was absent from the fovea at all ages examined. NRL labeling was absent from the fovea with NRL nuclei first detected on the foveal edge between Fwk 10-11. At Fwk 11.7, only two NR2E3 positive nuclei were detected on the nasal foveal edge (Fig. 3A, arrowheads). NRL labeling was also observed on the nasal foveal edge (Fig. 3B, arrowheads) and had progressed almost to the optic nerve head by Fwk 11.7. At this and older ages, the length of retina containing the respective labels was quantified to measure the developmental progression of NR2E3 expression relative to...
NR2E3 and NRL labeling were detected in a few nuclei near the fovea at Fwk 11.7, and then both were rapidly expressed so that most of the deep nuclei in the ONL were labeled by Fwk 16. Between Fwks 11 to 14, NRL protein was present in slightly more peripheral retina than NR2E3, suggesting that NRL was expressed before NR2E3.

To further characterize cells expressing NR2E3, comparison of NR2E3 with rod and cone specific markers was made. Rod opsin was co-expressed with NR2E3 in rods at Fwk 22 near the fovea, and double-labeled rods appeared progressively in peripheral retina with increasing age (Fig. 4A). At all ages studied, when sections were double labeled with synaptophysin, an early cone marker, and NR2E3, the cone membranes including the synaptic pedicle and developing inner segment were heavily labeled for synaptophysin, but the cone nucleus, which occupied the outermost ONL, was unlabeled for NR2E3 (Fig. 4B). Previous studies showed that the human fovea lacks S cones27,33 and immature human S cone nuclei can reside at various levels in the ONL, but L/M cone nuclei form the outermost band in the ONL. To examine whether some of the deeper ONL nuclei labeled for NR2E3 could be from S cones, sections were double labeled with a monoclonal antibody to S opsin (Figs. 4C and 4D). Despite the presence of S cone nuclei deep within the band of NR2E3-positive nuclei, none were labeled (Figs. 4C and 4D). The pure cone fovea, outer ONL, and S cones contained no nuclear NR2E3 labeling, indicating that cones do not express NR2E3. Therefore, NR2E3 is specific for putative postmitotic rods and rod opsin-expressing cells in fetal human retina.

**DISCUSSION**

Nuclear receptors are key mediators of transcription in response to extrinsic or intrinsic signaling events. NR2E3 was originally identified as a photoreceptor-specific orphan nuclear receptor, based on sequence homology, putative structure, and localization to the ONL of adult mouse retina. In situ hybridization studies in adult human retina also localized NR2E3 to the ONL, but neither report clarified which type of photoreceptor(s) expressed NR2E3. Later, a splice variant of NR2E3 was independently identified in both human and mouse retina in a screen of nuclear receptors important in disease. The in situ probe used in that study showed strong expression of NR2E3 in the Müller glia and RPE in mouse and monkey, but no photoreceptor labeling was detected. This discrepancy was attributed to methodological differences in in situ hybridization protocols and the probes used. The present study demonstrated, by in situ hybridization and immunocytochemistry, the expression of NR2E3 in the fetal human retina and showed its localization specifically on rod nuclei throughout development. The full-length probe to the human NR2E3

![Immunocytochemical localization of NR2E3 (A, C, E, G) and NRL (B, D, F, H) between Fwk 11.7 and Fwk 16 fetal human outer nuclear layer (ONL).](image)

**FIGURE 3.** Immunocytochemical localization of NR2E3 (A, C, E, G) and NRL (B, D, F, H) between Fwk 11.7 and Fwk 16 fetal human outer nuclear layer (ONL). (A) NR2E3 positive cells (arrowheads) first appear in a few nuclei on the edge of the Fwk 11.7 fovea. (B) At Fwk 11.7, NRL is detected in more nuclei (arrowheads) on the foveal edge. (C–D) In Fwk 14 retina near the optic disc, there is a dramatic increase in the number of both NR2E3 positive nuclei (C) and NRL positive nuclei (D) in the deeper ONL away from the outer limiting membrane (OLM). Unlabeled cone nuclei are located between the arrowheads. (E–F) Labeling for NR2E3 (E) and NRL (F) is similar at the same point in Fwk 16 midperipheral retina. (G) The front of NR2E3 immunostaining at Fwk 16 lies within 200 μm of the retinal edge in the far periphery. (H) At Fwk 16, NRL positive cells are within 100 μm to the retinal edge. In G and H, the most peripherally labeled nucleus is indicated by an arrow. Scale bars: (A, B, G, H), 10 μm; (C–F), 20 μm.

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**TABLE 1.** Comparison of NR2E3 and NRL Percent Retinal Coverage during Fetal Human Retinal Development

Fwk, fetal week.
sequence, used in our in situ hybridization experiments, should detect all cells expressing possible splice variants of NR2E3. No evidence of NR2E3 labeling was found in either L/M or S cone nuclei, consistent with its localization in the deep ONL and absence in the fovea, or other retinal cell types. In developing human retina, NR2E3 expression began at Fwk 11.7 on the foveal edge, was present at least 1 month before rod opsin begins to be expressed in rods at the same retinal eccentricity, and was maintained in fully differentiated rods. A very early and continuing role for NR2E3 in rod cell differentiation was indicated.

Previous reports suggested different models for the role of NR2E3 in photoreceptor development. One model implicates NR2E3 in the proliferation control of the S cone photoreceptors. In the rd7 mouse, the presence of normal levels of M opsin and thyroid hormone receptor beta2 (TRβ2), which is a regulator of M-cone differentiation, transcripts suggested that their expression is not influenced by NR2E3. Since NR2E3 transcripts did not appear until E18 in the mouse, near the end of cone production and during the initiation of rod production, it was concluded that NR2E3 blocks the proliferation of cone progenitor cells.

Our data lend support to a role for NR2E3 in rod photoreceptor development. The whorls of excess photoreceptors, which are characteristic of rd7 mouse retina, did not appear during fetal development when cones are generated, but only in the postnatal retina when rods are being generated. The amount of Nr2e3 transcripts in the rd7 retina increases from E18.5 to P10.5, directly overlapping rod generation, but well after all cones are produced. The present study revealed a similar onset of NR2E3 expression in the developing human retina at Fwk 11 to 12. Extrapolation from [3H]thymidine labeling obtained in monkey retina and applied to human retinal development indicates that the first human cones should become postmitotic at Fwk 8 while the first rods are generated at Fwk 10 to 11. Therefore, it is unlikely that NR2E3 plays a role in the normal development of cones. If NR2E3 prevents cone generation, it should be expressed around the time when most human foveal cones are generated, by Fwk 10. However, there is no evidence for NR2E3 being present within the fovea at this or any later age. Expression of NR2E3 was never observed in cells which express specific cone markers including S opsin, in foveal cones, or in nuclei at the outer edge of the ONL where most cone cell bodies reside. Thus the localization of NR2E3 in fetal human retina and its temporal expression in mouse retina strongly suggested that NR2E3 acts to suppress S cone fate in a cell that already has been directed toward a rod photoreceptor fate.

The localization of NR2E3 in human rods supports a second model proposed by Mears and colleagues, which hypothesizes that rod photoreceptor identity is dependent on both Nrl and NR2E3. If either is absent or defective, rods do not develop appropriately and there is an increase in S cones. In the rd7 mouse, the presence of normal levels of M opsin and thyroid hormone receptor beta2 (TRβ2), which is a regulator of M-cone differentiation, transcripts suggested that their expression is not influenced by NR2E3. Since NR2E3 transcripts did not appear until E18 in the mouse, near the end of cone production and during the initiation of rod production, it was concluded that NR2E3 blocks the proliferation of cone progenitor cells.

Opsin expression is a late event in the life of most developing human photoreceptors. The entire central retina is free of cell division by Fwk 12, indicating that all neurons have been generated. It is not until Fwk 15 that both L/M cone opsin and rod opsin are expressed in or around the fovea, and these opsins do not reach the retinal edge until around birth. Therefore, during rod development, there is at least a month-long period between birth and rod opsin expression. The nuclear localization of NR2E3 and Nrl in rod opsin-negative cells whose nuclei lie in the inner ONL at the level of mature rods suggests that these transcription factors are expressed at a time when rods still have some plasticity or competence to

Figure 4. Double labeling of NR2E3 (green) with rod and cone markers at Fwk 22. (A) Fwk 22 midperipheral retina double labeled with rod opsin (red). The NR2E3-labeled nuclei are surrounded by rod opsin-positive membranes. (B) Double labeling with synaptophysin (red). The synaptophysin positive cones are outlined by cell membrane staining including their synaptic pedicles. Their nuclei (asterisks) clearly do not double label with NR2E3. (C) Fwk 22 midperipheral retina single labeled with NR2E3 (green). The arrows indicate unlabeled nuclei in the lower ONL. (D) Fwk 22 midperipheral retina double labeled with NR2E3 and S opsin (red). Although both S cone cell nuclei (asterisks) lie in the inner part of the ONL, their nuclei are negative for NR2E3. The arrows indicate the unlabeled nuclei observed in C. Scale bar: 10 μm.
acquire a different photoreceptor cell fate.\textsuperscript{41–45} During this period, NR2E3 and NRL guide the postmitotic precursor cells toward a rod fate and away from an S-cone fate.

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**References**


