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

Keely M. Bumsted O'Brien, Hong Cheng, Yibin Jiang, Dorothea Schulte, Anand Swaroop, and Anita E. Hendrickson

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.8–10 ESCS is associated with mutations in the NR2E3 gene, a photoreceptor-specific orphan nuclear receptor.11,12 Immunohistological studies of an ESCS 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-rod 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.10 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 development15,15 (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),

From the 1Max Planck Institute for Brain Research, Neuroanatomy Department, Frankfurt am Main, Germany; 2Department of Optometry and Vision Science, University of Auckland, Auckland, New Zealand; 3Program in Neuroscience, Departments of Ophthalmology and Visual Sciences, and 4Human Genetics, W.K. Kellogg Eye Center, University of Michigan, Ann Arbor, Michigan; and the Departments of 5Biological Structure and 7Ophthalmology, University of Washington, Seattle, Washington.

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Corresponding author: Keely M. Bumsted O'Brien, Department of Optometry and Vision Science, University of Auckland, Private Bag 92019, Auckland, New Zealand; k.bumsted@auckland.ac.nz.
which in turn suppresses the postmitotic cells from adopting an S-cone identity.\textsuperscript{16} 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 appropriate rod-specific phototransduction genes. 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) paraffin- or methanol-fixed 10\% phosphate buffered saline (PBS). Tissue was cryoprotected and serially frozen-sectioned at 12\,\mu m. 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{19} with actin-specific primers which span a 539 base pair fragment between 176 to 714: forward, 5-GTGGGCGCCCAAGCCACCA-3; and reverse, 5-GTCCTAATT/GTCACGCACGATTTCCCG-3. The NR2E3-specific primers amplified a 503 base pair fragment between 742 to 1245: forward, 5-TCCTTAAT/GTCACGCACGATTTCCCG-3; and reverse, 5-GTGGGGCGCCCCAGGCACCA-3. The NR2E3-specific primers amplified a 503 base pair fragment between 742 to 1245: forward, 5-TCCTTAAT/GTCACGCACGATTTCCCG-3; and reverse, 5-GTGGGGCGCCCCAGGCACCA-3. The NR2E3 expression could not be detected at Fwk 10 but was significantly upregulated at Fwks 11 to 12.

**NR2E3 Antibody Generation**

Full-length NR2E3 cDNA encoding the complete open reading frame was amplified from human retinal RNA by RT-PCR, using the following primers: forward, 5-AGATTCTAGAGAGAGGACACAACAG-3; and reverse, 5-ATTCTACGCTGGCGCCGGCGCAATGGTTTT-3 (initiation and stop codons are underlined). 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{17} The NR2E3 protein was cleaved by thrombin treatment, separated from the GST-NR2E3 fusion protein, and used for generating polyclonal antibodies in rabbit (Invitrogen, 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 pcDNA4C (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-fibulin monoclonal antibody (Fig. 2B; Sigma, St. Louis, MO).

**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% ChemiBlocker [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, RhoID2; Robert S. Molday, University of British Columbia, Vancouver, BC\textsuperscript{26}) S opsin (1:15,000–30,000; JH455; Jeremy Nathans, Johns Hopkins University, Baltimore, MD\textsuperscript{37,38}), OS2, (1:5000, Agoston Szel, Semmelweis University, Budapest, Hungary\textsuperscript{39}), synaptophysin (1:500; Sigma), and NRL (1:1000–3000\textsuperscript{17}). Sections were washed in PBS, followed by 1 hour incubation with a mixture of anti-mouse IgG conjugated to Alexa 594 (red) and antirabbit 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).

**In Situ Hybridization**

Whole Fwk 13 (one eye), 15.5 (two eyes), and 16 (one eye) human fetal eyes were stored frozen in 30% sucrose after fixation. After thawing, the cornea and lens were removed and the eyecup dehydrated in an ascending series of PBS containing 0.1% Tween-20 (PBT) and methyl alcohol (MeOH). The tissue was stored overnight at −20°C in absolute MeOH, and then rehydrated through a PBT/MeOH series. The retina and retina pigment epithelium (RPE) were dissected away from the sclera. The RPE was bleached as described by Hemmi and Grünert,\textsuperscript{39} with modifications.\textsuperscript{17} In situ hybridization was carried out using digoxigenin-labeled NR2E3 riboprobes, as described.\textsuperscript{37,38}

**Data Analysis**

Because the NR2E3 and NRL antibodies were both rabbit polyclonals, a direct double labeling was not possible. To compare these expression patterns, adjacent sections were labeled with NR2E3 and NRL antibodies. In each immunocytochemically-stained section, retinal landmarks such as the fovea and optic disc were identified. The sections were then systematically scanned from the retinal edge using X40 (350\,\mu m field diameter) ocular lenses. The number of X40 fields containing labeled and unlabeled cells was counted. The number of labeled fields was divided by the total number of labeled plus unlabeled fields to yield a percent coverage for NR2E3 and NRL.

**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 wholemount 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...
edge, the region where rods and S cones first appear (Fig. 1F). The inner surface of the outer nuclear layer (ONL), where rod cell bodies reside, was positive for NR2E3. Cones, which are located in the outermost part of the ONL, did not contain NR2E3 transcripts. Rod-specific expression was also observed at more peripheral locations, where the band of NR2E3 expression corresponds to the large number of putative rod nuclei in the ONL (Fig. 1G).

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...
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 (arrowbeads) 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 (arrowbeads) 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 arrowbeads. (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.

that of NRL. At Fwk 11.7, NR2E3 labeling occupied 4%, while NRL staining covered 37% of the retinal section length, indicating that the initial spread of NR2E3 expression across the retina lagged behind that of NRL (Table 1). By Fwk 12.7, the number of NR2E3 positive nuclei had increased and the labeling had progressed beyond the foveal edge, but had not yet reached the optic nerve head. NRL-positive nuclei were present slightly beyond the eccentricity of the optic disc (data not shown). At Fwk 12.7, NR2E3 covered 19% and NRL covered 65% of their respective retinal sections. At Fwk 14, the number of labeled nuclei had increased and many NR2E3- and NRL-positive nuclei were identified peripheral to the optic disc with scattered labeled nuclei present up to 700 μm (NR2E3; 87% coverage) and 350 μm (NRL; 94% coverage) from the retinal edge (Figs. 3C and 3D). All positive nuclei were in the deeper ONL, consistent with the location of rod nuclei. NR2E3 and NRL positive nuclei filled the deeper ONL across much of the retina at Fwk 16 (Figs. 3E and 3F), the age when rod opsin expression had begun in the central retina.17,19 Although antibody labeling for NR2E3 and NRL was still sparse at the Fwk 16 retinal edge, NR2E3 covered 97% and NRL 99% of the retina (Figs. 3G and 3H, arrow). The data showed that both NR2E3 and NRL 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 Fwk 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.54 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.55 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.12 In situ hybridization studies in adult human retina11 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.56 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

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

<table>
<thead>
<tr>
<th>Age (Fwk)</th>
<th>NR2E3</th>
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<tr>
<td>10</td>
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<tr>
<td>11.7</td>
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<td>22</td>
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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 E18.5 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 foveal 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 Nrl−/− mouse, no Nr2e3 transcript was detected in postnatal day 10 retina, suggesting that Nrl regulates Nr2e3 expression. In both ESCS and the rd7 mouse, NRL is expressed despite a mutated NR2E3, and an excessive number of S-opsin expressing photoreceptors are generated. 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 foveal 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.

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 opsin transcripts 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
acquire a different photoreceptor cell fate. During this period, NRE23 and NRL guide the postmitotic precursor cells toward a rod fate and away from an S-cone fate.

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