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Expression of photoreceptor-associated molecules during human fetal eye development

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Purpose: A characteristic feature of the human retina is the early differentiation of foveal cells followed by a central to peripheral wave of maturation. This can obscure the true onset of differentiation when regions other that the fovea are sampled, or when methods based on whole retina or whole eye tissue are employed, such as reverse transcription-polymerase chain technique (RT-PCR). In order to assess the suitability of RT-PCR based approaches during human retinal development and to gain insight into the developmental progression of photoreceptor differentiation and maturation in the human, we analyzed the expression of several photoreceptor-associated genes by immunocytochemical labeling (ICC) of the foveal region as well as by RT-PCR of total RNA from whole fetal eyes from different developmental stages.
Methods: Expression of phosphodiesterase beta (PDEB), interphotoreceptor binding protein (IRBP), tubby-like protein (TULP), short wavelength specific (S) opsin, long and medium wavelength specific (L/M) opsin, rod opsin and the transcription factors Crx and Nrl were assessed by RT-PCR from total RNA prepared from snap frozen intact human fetal eyes ranging from fetal week 9 (Fwk 9) to Fwk 18. ICC labeling was performed in a large number of eyes within an age group for IRBP, TULP, Nrl, S opsin, L/M opsin and rod opsin on frozen sections that included the fovea centralis.
Results: All ICC markers appeared first in or around the fovea. We detected PDEB and Crx expression as early as Fwk 10, by RT-PCR. TULP and IRBP were first observed with ICC in a small number of foveal cones at Fwk 9, although the first transcripts were not detected until Fwk 12. Nrl-positive nuclei appeared around the fovea by Fwk 11 and S opsin-positive cones by Fwk 12. L/M opsin-positive cones and rod opsin-positive rods were first detected between Fwk 15-16. In general, ICC labeling in the fovea was present for most genes up to 2 weeks before the corresponding transcripts could be successfully amplified by RT-PCR from whole eye tissue.
Conclusions: Our results indicate that in order to pinpoint exactly when and where a molecule appears, ICC labeling of the fovea is a more reliable indicator. RT-PCR was prone to underestimate the exact onset of expression of the molecules tested, yet it faithfully recapitulated the sequence in which they appeared. In addition, our data show that in the human fetal retina, Crx and Nrl are both expressed when the first rod photoreceptors are being generated. This agrees well with previous in vitro results suggesting a synergistic action of both proteins during differentiation of human rod photoreceptors.

Studying the spatial and temporal expression of molecules important for photoreceptor differentiation can lead to valuable insights concerning phenotypic fate choice. In the human retina, the anatomical and visual axis is centered on the fovea, which is characterized as a pit is located on the center of gaze that is overlain with the highest density of cone photoreceptors. The human retina contains on average 4.6 million cones with a foveal density around 200,000 cones/mm^2 [1]. Cone density decreases 100-fold into the far periphery. Throughout most of the human retina, there are four photoreceptor types, long (L), medium (M) and short (S) wavelength-selective cones plus rods, which outnumber cones by a ratio of 20:1 across most of the retina. However, in the central 100 μm of the fovea, S cones are absent [2,3] and rods are absent from the central 300 μm [1]. In human retina, therefore, photoreceptor phenotypic choice not only involves specifying four different opsin types, but requires a topographic mapping of each type.

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During retinal development, differentiation events occur first in the fovea and then proceed in a central to peripheral wave. In the *Macaca* monkey retina, a well-studied model of the human retina, the first ganglion cells become postmitotic at fetal day (Fd) 33 and cones at Fd 36, corresponding to human fetal week (Fwk) 7-8 [4]. All layers and most cell types can be identified within the human fovea by Fwk 11 [5-7]. Development continues progressively across the human retina for many months with adult retinal layers and many photoreceptor markers not present in the far periphery until near birth [8]. Therefore, human retinal development has an early onset, but a protracted developmental program, making it an excellent system to study the developmental progression of differentiation and maturation of various retinal cell types. However, this marked spatial and temporal developmental gradient can also lead to erroneous conclusions when (a) regions outside the fovea, rather than the fovea itself, are analyzed by either immunocytochemical labeling (ICC) or in situ hybridization (ISH) or (b) when reverse transcription RT-PCR approaches based on whole retina or even whole eye tissue are performed.

We undertook a comparison of data obtained using ICC and RT-PCR amplification of whole human fetal eye tissue in order to determine to what extent these two standard methods are suited to analyze onset of expression of various photoreceptor associated proteins. We found that, while RT-PCR was prone to underestimate the exact onset of expression of the molecules tested, it faithfully recapitulated the sequence in which they appeared. In order to pinpoint exactly when and where a molecule appears, ICC labeling of the fovea needs to be performed. Our data from this combined approach indicate that in the human fetal retina, transcription factors Crx and Nrl are both expressed when the first rod photoreceptors are being generated on the foveal edge. This supports previous reports which showed synergistic action of both transcription factors during differentiation of human rod photoreceptors [9], but contradicts a recent RT-PCR study on human retinal development which found a later expression of both molecules during early rod genesis [10].

METHODS

Human eyes between Fwk 9-19 were obtained under approved protocols from the Human Embryology Laboratory at the University of Washington or Advanced Bioscience Resources, Inc. (Santa Rosa, CA). Age was determined by eye size, foot length, or crown rump length. In each age group, one eye from the same fetus was fixed for ICC and the other processed for RT-PCR. These eyes were fixed unopened in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) overnight while additional eyes were fixed in 2% paraformaldehyde for 1-2 h. Additional eyes within an age group were used for ICC and were processed as indicated previously. Tissue was cryoprotected and serially frozen sectioned at 12 µm. Every tenth slide was stained with cresyl violet to localize the fovea and optic disc within each retina. Only sections including the fovea were used for ICC.

RT-PCR: Whole eyes were snap frozen in liquid nitrogen and total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA). The RNA was treated with 2 U DNase (Invitrogen) for 15 min at 37 °C to remove possible genomic contamination. For each reaction, 0.5 µg of RNA was amplified by the SuperScript one step RT-PCR System (Invitrogen; 40 cycles) using the primer sets listed in Table 1. The band for each molecule was purified, cloned using the TOPO TA cloning kit (Invitrogen) and sequence verified.

Immunocytochemistry: Frozen sections were incubated overnight in a mixture of primary antibodies diluted in standard medium (5% Chemiblocker, Chemicon, Temecula, CA) in PBS containing 0.05% azide and 0.5% Triton-X100 (Sigma Chemical, Munich, Germany). Primary antibodies and their sources were: rod opsin (1:200; monoclonal Rho4D2; R. Molday [11]), S opsin (1: 15,000; polyclonal JH455; J. Nathans [3]), L/M opsin (1:5000; polyclonal, J. Saari [3]), TULP (1:1000; rat polyclonal, P. Nashina [12]), Nrl (1:1000; polyclonal, A. Swaroop [13]), and IRBP (1:5000; polyclonal, G. Chader [14]). Sections were washed in PBS, followed by a 1 h incubation with a mixture of anti-mouse or anti-rat IgG conjugated to Alexa 594 (red) and anti-rabbit IgG conjugated to Alexa 488 (green; both from Molecular Probes, Eugene, OR) in the standard dilution media. Sections were imaged in a fluorescence microscope or a Zeiss Pascal confocal microscope. Images were processed for contrast and color balance using Adobe Photoshop (Adobe systems, San Jose, CA).

RESULTS

We performed ICC and RT-PCR on paired eyes from the same fetus. ICC was done on additional retinas from fetuses of known age. Cresyl violet stained sections from an eye typical of each age group are shown to illustrate the progression of morphological development across the retina.

Fetal weeks 9-10: The fovea at Fwk 10 (Figure 1A) contained a single layer of cone photoreceptors in the outer nuclear layer (ONL) adjacent to the heavily pigmented retinal pigment epithelium (RPE). The cones were separated from an immature inner nuclear layer (INL) by a very narrow outer plexiform layer (OPL). A thin inner plexiform layer (IPL) separated off the thick ganglion cell layer (GCL). Only 2 mm peripheral, near the optic disc (Figure 1B), the Fwk 10 retina was much less mature and resembled the remainder of the retina. The peripheral retina was divided into a darkly stained and densely packed outer neuroblastic layer (ONbL) and a more loosely packed and more lightly stained inner neuroblastic layer (INbL). The IPL was not yet formed at this eccentricity.

TULP positive cones were detected at Fwk 9, but only within the fovea (Figure 1C; arrowheads). IRBP was also detected in the fovea, and most foveal cones were double labeled with IRBP and TULP (Figure 1D, arrowheads). There was no ICC staining for any of the other markers examined

Table 1. Nucleotide sequences of the RT-PCR primers

Primer	5' 3'
Actin-F	GTGGGGCGCCCCAGGCACCA
Actin-R	TCCTTAATGTCACGCACGATTTCCCG
Nrl-F	GTGCCTCCTTCACCCACC
Nrl-R	CAGACATCGAGACCAGCG
IPRB-F	GAGAATTCTCCGTGCTGCTCTGTGG
IRBP-R	CATCTCGAGTCCTGTGCAGTGCCGG
TULP-F	GTCTACGCCAGGTTCCTCAG
TULP-R	TTCAGGGCTTTCTTGTCAGG
S opsin8-F*	GCCACACTGCGCTACAAAAAG
S opsin9-R*	TGAAGCAGAAGATGAAGAGGAACC
L/M opsin2-F*	GGATCACAGGTCTCTGGTCTCTGG
L/M opsin7-R*	TGGCAGCAGCAAAGCATGC
Crx1-F	GAGAATTCGCGTATATGAACCCGGGG
Crx1-R	CATCTCGAGCATGGCATAGGGGGCGG
PDEB-F**	AGGAGACCCTGAACATCTACC
PDEB-R**	ATGAAGCCCACTTGCAGC
Rod opsin-F	CATCGAGCGGTACGTGGTGGTGTG
Rod opsin-R	GCCGCAGCAGATGGTGGTGAGC

The forward (F) and reverse (R) primers are identified by their suffix. Primers marked with an asterisk ("*") are from Hagstrom et al. [30]; those marked with a double asterisk ("**") are from Bibb et al. [10].

(data not shown). Figure 2 shows the RT-PCR results for total RNA of an age matched whole eye. We were only able to detect actin (lane 1), crx (lane 2; red arrow) and PDEB (lane 3; white arrow), but not TULP or IRBP. As there was no antibody to Crx available to us for this study, ICC analysis of Crx protein could not be performed.

Fetal weeks 11-13: At Fwk 12, the foveal region contained a single layer of cone photoreceptors and an INL that had horizontal cells, bipolar cells, and amacrine cells. The IPL was clearly defined and the GCL was several cell layers thick (Figure 3A). At the eccentricity of the disc, the retina had begun to differentiate (Figure 3B). A row of cones could be identified at the outer border of the ONbL adjacent to the RPE, and a thin IPL separated the OnbL from the differentiating GCL. The nerve fiber layer (NFL) was obvious on the inner border of the GCL close to the optic disc.

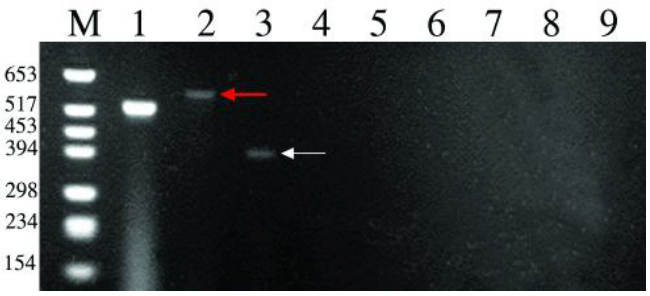

Figure 2. Expression of actin, Crx, and PDEB in the Fwk 10 human eye. A reverse transcription polymerase chain reaction (RT-PCR) agarose gel stained with ethidium brominde from a Fwk 10 human retina. Bands were only detected in lanes 1-3 which correspond to actin, Crx (red arrow) and PDEB (white arrow), respectively. M indicates the DNA molecular weight marker. 1, actin; 2, Crx; 3, PDEB; 4, IRBP; 5, TULP; 6, Nrl; 7, S opsin; 8, L/M opsin and 9, Rod opsin.

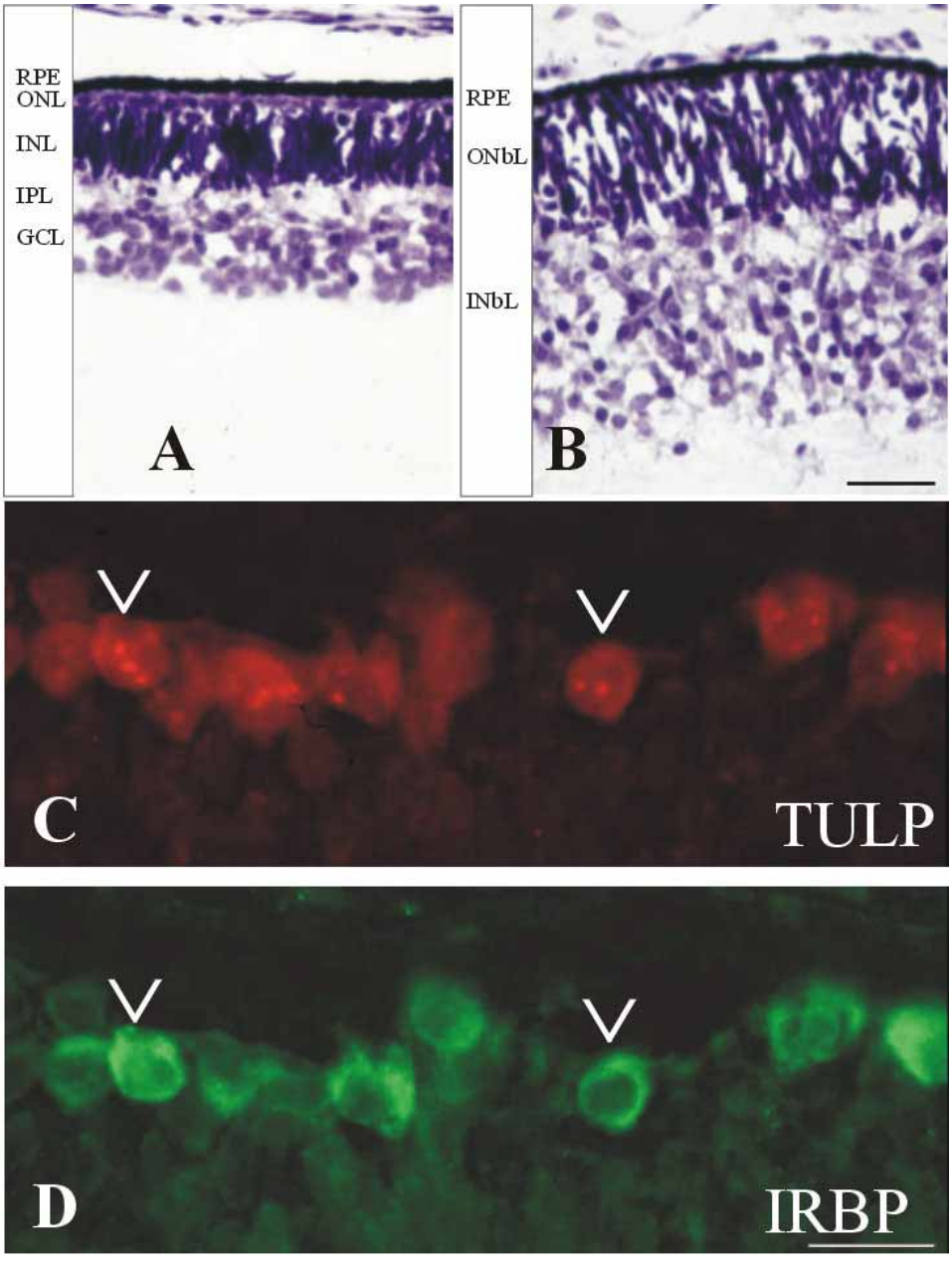

Figure 1. Human retina at Fwk 9-10. **A**,**B**: Cresyl violet stained frozen sections of a Fwk 10 retina showing the fovea (**A**) and the retina near the optic disc (**B**). The fovea has a single thin layer of cone photoreceptors forming the outer nuclear (ONL) adjacent to the pigment retina pigment epithelium (RPE). An immature inner nuclear layer (INL) is separated from the ganglion cell layer (GCL) by a thin inner plexiform layer (INL). **B**: Retina near the optic disc is thicker and is divided into the outer neuroblastic layer (ONbL) and inner neuroblastic layer (INbL). **C**,**D**: The same section from the Fwk 9 fovea showing immunocytochemical labeling for TULP (**C**) and IRBP (**D**). Most of the cones are double labeled (arrowheads). The scale bars represent 25 μm in **A** and **B**; in **C** and **D** they represent 10 μm.

Between Fwk 10-11, we observed the first Nrl labeled nuclei on the foveal edge (data not shown). By Fwk 13, nuclear Nrl staining extended as far as 3.6 mm into the periphery (Figure 3C; arrows). S opsin was detected in scattered cones around the fovea at Fwk 11 (Figure 3D), and labeling extended to the optic disc by Fwk 13. The RT-PCR amplification products of the corresponding photoreceptor-associated genes from whole eye tissue of a 12 week old specimen are shown in Figure 4. In addition to actin (lane 1), crx (lane 2), and PDEB (lane 3), IRBP (lane 4), TULP (lane 5) and Nrl (lane 6; arrow) could now be detected. However, we were not able to amplify S opsin message under the conditions used (lane 7), even though a small number of S opsin-positive cells were clearly visible around the fovea by Fwk 11 (Figure 3D).

Fetal weeks 14-16: At Fwk 14, the foveal layers (Figure 5A) had changed little compared to Fwk 12, with the exception that cones in the ONL were larger and the OPL (arrow) was more prominent. At the eccentricity of the disc (Figure 5B), the retina had three clear nuclear layers and two synaptic layers. A single layer of cone photoreceptors and 1 to 2 layers of rods could be identified morphologically in the ONL adjacent to the RPE. In the nasal retina, about 5 mm peripheral to the optic disc (Figure 5C), the retina was much less differentiated. Here the IPL and GCL were present, but the outer retina was still mainly composed of undifferentiated neurons and progenitor cells.

By Fwk 15, L/M opsin (Figure 5D; green) and rod opsin (Figure 5D,E; red) antibodies labeled cones and rods in and around the foveal region. S opsin-positive cones were also present well beyond the optic disc (data not shown). The cell

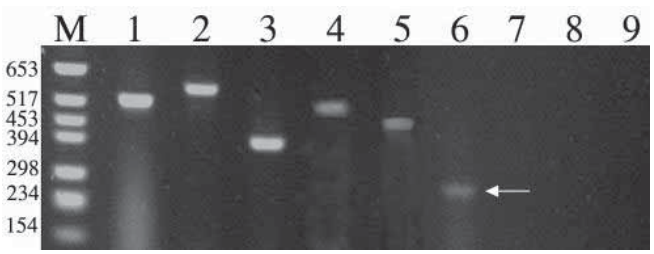

Figure 4. Expression of actin, Crx, PDEB, IRBP, TULP, and Nrl in the Fwk 12 human eye. An RT-PCR agarose gel stained with ethidium brominde from a Fwk 12 human retina. Bands were present in Lanes 1-3 corresponding to actin, Crx, PDEB, and were detected for the first time for IRBP (lane 4), TULP (lane 5) and Nrl (lane 6). M indicates the DNA molecular weight marker. Lanes 7 (S opsin), 8 (L/M opsin), and 9 (Rod opsin) have no detectable bands.

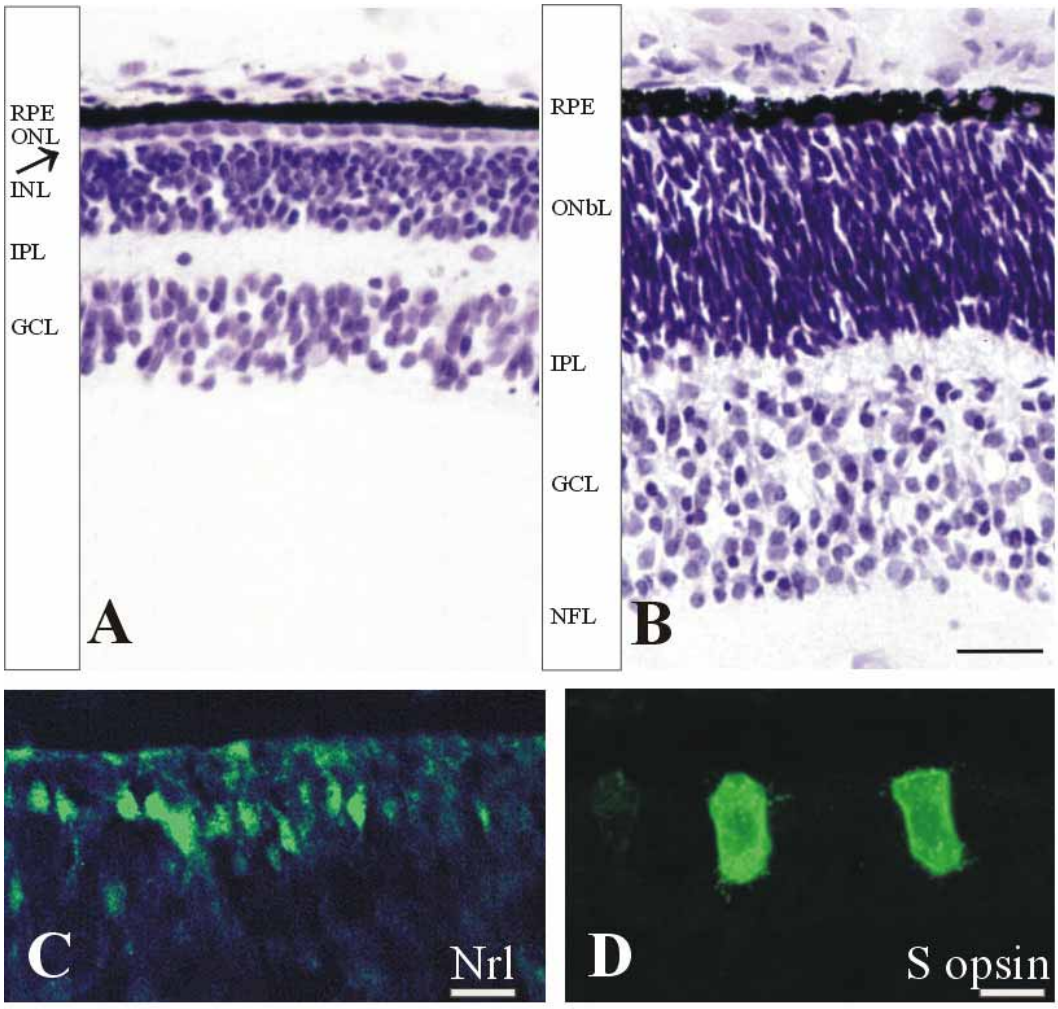

Figure 3. Human retina at Fwk 11-14. **A**,**B**: Cresyl violet stained sections from a Fwk 12 retina showing the fovea (**A**) and the retina near the optic disc (**B**). Foveal morphology (**A**) has changed relatively little except that the OPL is more apparent (arrow). At the optic disc (**B**), a thin IPL now separates off the GCL and a nerve fiber layer (NFL) is present. **C**: Nrl-positive nuclei (green) present at the peripheral edge of Nrl expression, which is 3.6 mm from the fovea, at Fwk 13. These nuclei form a row under the unstained cones. **D**: S opsin (green) is detected in a subset of cones across the entire central retina by Fwk 14. The scale bars represent 25 μm in **A** and **B**, 15 μm in **C**, and 10 μm in **D**.

membranes and cytoplasm of many foveal and some parafoveal cones (Figure 5D) labeled for L/M opsin. These L/M cones also had short, heavily-labeled outer segments (arrow; Figure 5D). Scattered rods, easily identifiable by a membrane labeled for rod opsin, were found nested in between the parafoveal cones (Figure 5D; red). The nuclei of all rods in this region labeled with Nrl (Figure 5E, green), but only a few rods were expressing rod opsin (Figure 5E, red). Nrl-positive nuclei were numerous and extended well into the periphery (data not shown), indicating that Nrl expression significantly preceded rod opsin expression.

At this stage in development, all the photoreceptor-associated genes analyzed were readily detected by RT-PCR (Figure 6).

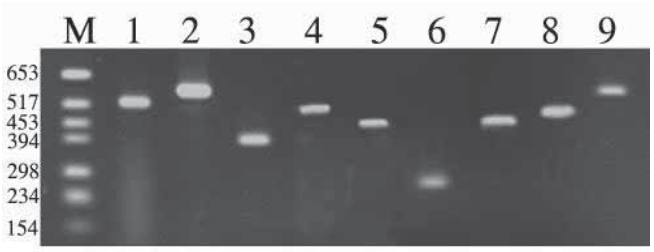

Figure 6. Expression of photoreceptor-associated molecules in the Fwk 16 human retina. An RT-PCR agarose gel stained with ethidium brominde from a Fwk 16 human retina. All photoreceptor-associated genes tested by PCR were expressed at Fwk 16 with bands now present for S opsin (lane 7), L/M (lane 8), and Rod opsin (lane 9).

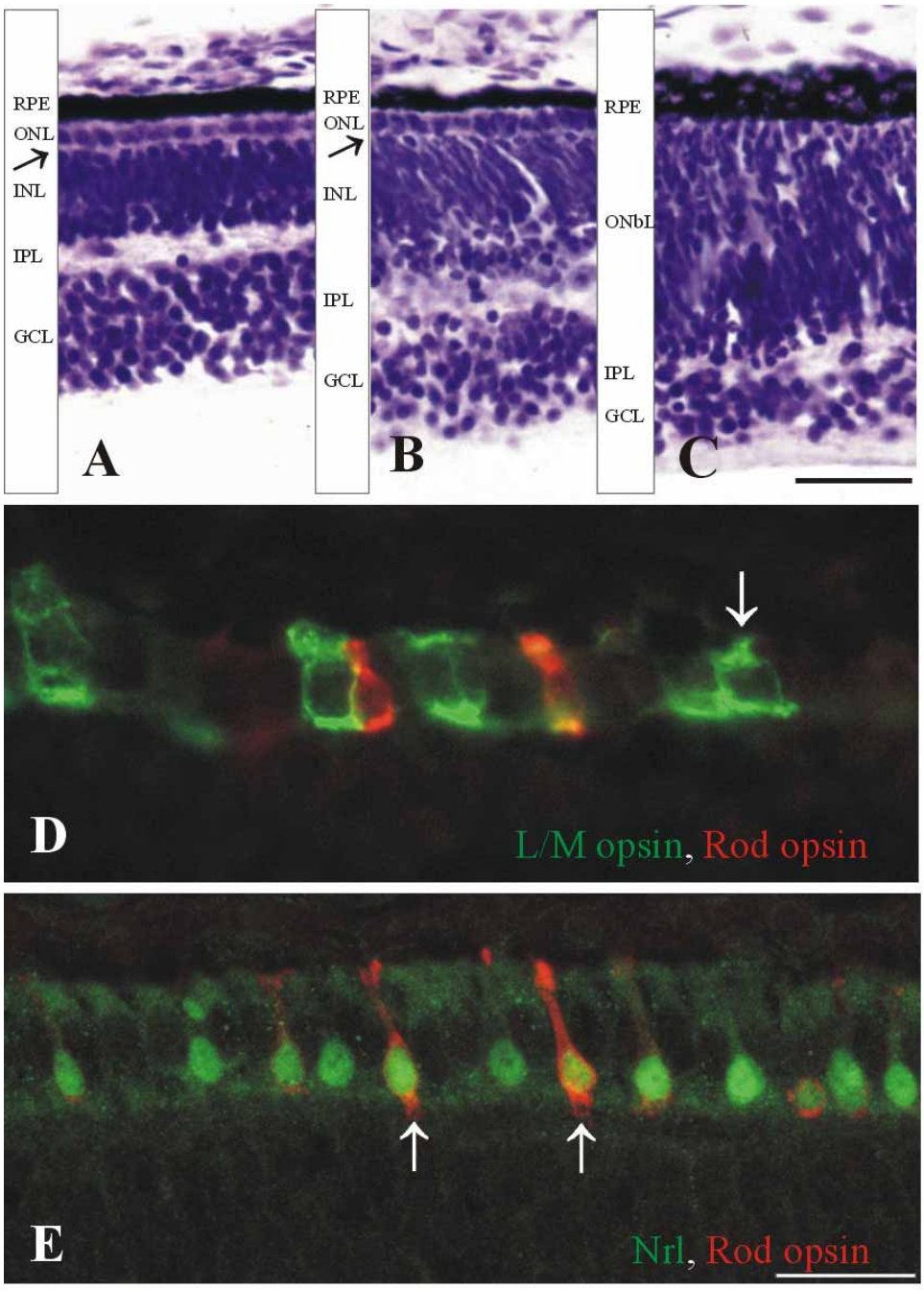

Figure 5. Human retina at Fwk 14-16. **A**,**B**,**C**: Cresyl violet stained frozen sections from a Fwk 14 retina. The foveal region (**A**) has more mature cones in the ONL and the OPL (arrow) is more apparent. **B**: At the eccentricity of the disc, a thin ONL and think INL are present with a thin OPL separating them (arrow). **C**: In the peripheral nasal retina, a thin IPL separates the GCL from a thick OnbL. **D**: A section on the edge of the Fwk 16 fovea is double labeled for L/M opsin (green) and Rod opsin (red). The arrow indicates a heavily labeled cone outer segment. **E**: Section on the foveal edge at Fwk 16 double labeled for Nrl (green) and Rod opsin (red). All rods have a Nrl-labeled nucleus (green), but only a few express Rod opsin (arrows). The scale bars represent 25 μm in **A** through **C**, and 12 μm in **D** and **E**.

Fetal weeks 17-19: The foveal region at Fwk 19 (Figure 7A) consisted of the same layers as shown at Fwk 14, but cones were further elongated and the OPL was thicker. The inner retina within the fovea still contained many layers of cell nuclei, as foveal pit formation will not begin until after mid-gestation [15]. Near the optic disc (Figure 7B), the ONL was composed of a single layer of cones overlying multiple layers of rods, which was a major change from Fwk 15 (Figure 5). The other retinal layers were distinct and there was a thick NFL at this eccentricity. In the nasal periphery (Figure 7C), the IPL was much thicker and the beginning of the OPL (arrow) was marked by a lighter gap dividing the ONbL. Cones were recognizable adjacent to the RPE.

Immature S-cones, which were morphologically similar to the earliest central S-cones detected at Fwk 11, were found in the far retinal periphery by Fwk 19 (Figure 7D). These cones labeled for TULP and IRBP (data not shown). Nrl was expressed by rods almost to the retinal edge (Figure 7E), but neither L/M opsin nor rod opsin was detected peripheral to the optic disc. The RT-PCR data for all molecules examined was similar to that observed at Fwk 16. Table 2 summarizes and compares data gathered by RT-PCR with those obtained by ICC.

DISCUSSION

Early differentiation and marker expression in the human fovea: Most photoreceptor-associated molecules analyzed in this study appeared first in or directly around the fovea. Crx was detected in the youngest eye by RT-PCR, but since there was no antibody available to us for testing, the developmental pattern of Crx could not be determined. In more peripheral retina, molecules were expressed after a significant delay and many markers were not present until near birth [8]. The fovea between Fwk 9-11 is less than 1 mm across, has not yet undergone the characteristic pit formation, but is clearly developmentally distinct from nearby retinal areas. This is strikingly demonstrated by comparing the completely different morphology in fovea and the nasal retina 2 mm away in Figure 1 and Figure 2. Thus, fetal retinal anatomy is a major consideration in analyzing human developmental sequences. The data presented in this paper as well as other studies show that cells in the human fovea also precociously express photoreceptor-associated proteins [3,8,12,16]. Notably, this precocious maturation of a small number of central cells does not occur in most other mammals, which has to be taken into account when respective comparisons are drawn.

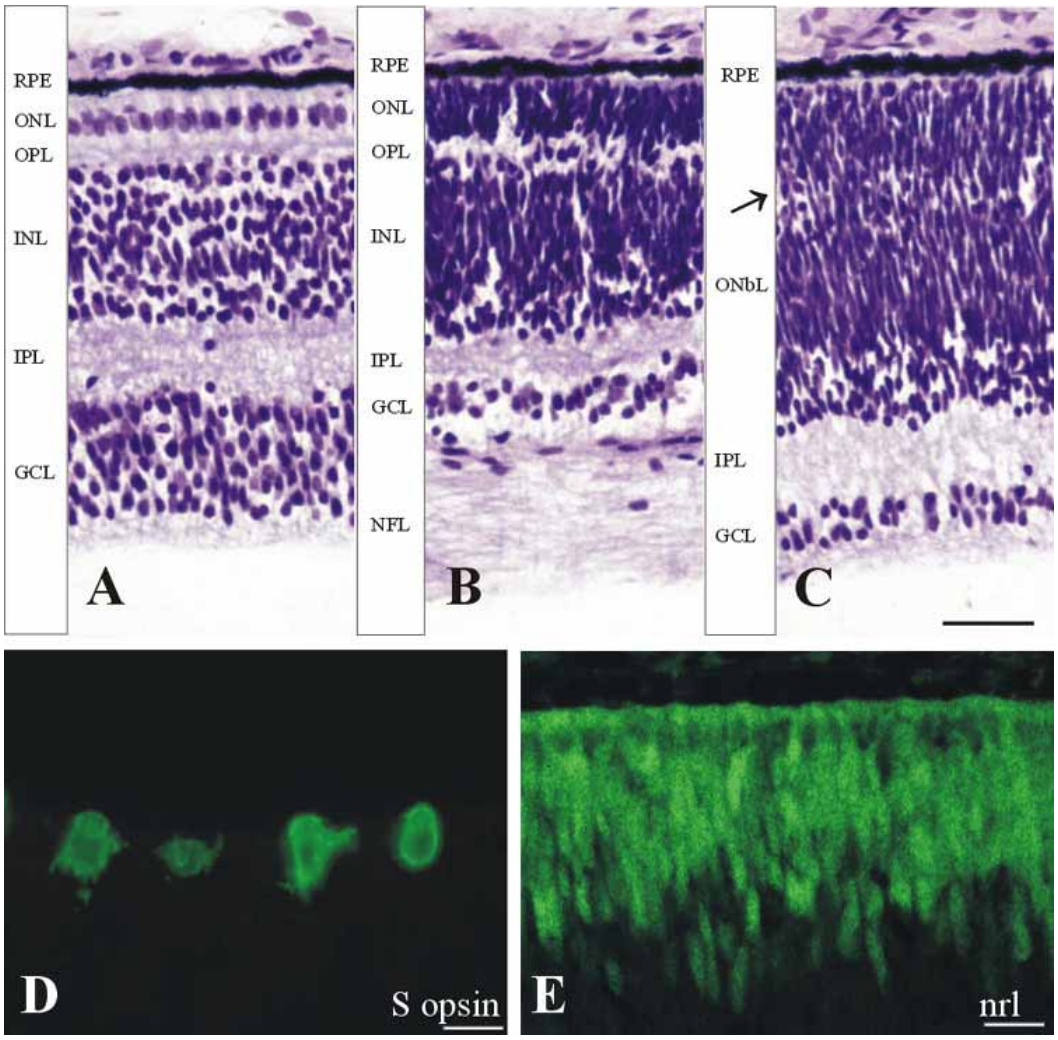

Figure 7. Human retina at fetal weeks 18-19. Cresyl violet stained frozen sections from a retina at Fwk 18 (**A**,**B**,**C**). All layers in the fovea (**A**) and optic disc (**B**) are more mature. The ONL at the optic disc is much thicker due to the addition of rods (**C**). In the periphery, a thick IPL is now present and a thin discontinuous OPL (arrow) is dividing the OnbL into ONL and INL. **D**,**E**: ICC labeled S cones (**D**) and Nrl-labeled rod nuclei (**E**) are present near the retina edge in the outer retina, despite its immature appearance. The scale bars represent 25 μm in **A** through **C**, 12 μm in **D**, and 8 μm in **E**.

We demonstrate here that, depending upon the method used to detect photoreceptor-associated gene products in the human retina, very different time points for onset of particular differentiation programs may be obtained. When the fovea is analyzed by ICC, several markers of photoreceptor differentiation and maturation were detected up to two weeks before the corresponding transcripts could be amplified by RT-PCR. For instance, cone specific labeling for IRBP and TULP, as well as Nrl labeling of rod nuclei were clearly present in and around the fovea by Fwk 10-11 (Table 2), but transcripts for these were not detected by RT-PCR until 2 weeks later. By Fwk 11-12, S opsin-labeled cones were clearly visible in the vicinity of the fovea (Figure 3D), but S opsin transcripts could not be amplified until significantly later. In contrast, L/M opsin and rod opsin were detected simultaneously by both methods, even when only a relatively few cells were labeled by ICC. If onset of opsin expression would have been assessed only by RT-PCR, all three opsins would have been estimated to appear around Fwk 16. The reason for this discrepancy most likely reflects fundamental differences in both methods: ICC can detect the onset of gene expression in a small number of cells in a distinct spatial manner, while RT-PCR samples globally from all cells included into the RNA pool. If whole, snap frozen eyes are used for RNA isolation, which is often the case for human tissue, the RNA pool even includes all ocular tissues rather than being specific for the neural retina. As our ICC analysis demonstrates, onset of expression of all molecules tested started in a small number of cells in or around the fovea, thus in a tiny fraction of the total tissue sampled by RT-PCR. It is therefore likely that the amount of mRNA expressed in this small number of cells is too low to be detected by conventional RT-PCR. In addition, the success of any RT-PCR amplification depends on the relative abundance of each transcript in the cells expressing it. This has especially to be taken into account when the expression of photoreceptor-associated genes is analyzed, as it is generally accepted that there are large differences in the abundance of these transcripts. This may account for the fact that expression of Rod opsin in rod photoreceptors was detected simultaneously at Fwk 16 by RT-PCR and ICC, while RT-PCR failed to detect expression of the transcription factor Nrl before Fwk 12 although the first Nrl-positive cells appeared in the central retina at least one to two weeks earlier.

Table 2. Summary of immunocytochemical (ICC) and RT-PCR data

	Fwk 9-10		Fwk 11-13		Fwk 14-16		Fwk 17-19	
	ICC	PCR	ICC	PCR	ICC	PCR	ICC	PCR
Actin	N/A	+	N/A	+	N/A	+	N/A	+
CRX	N/A	+	N/A	+	N/A	+	N/A	+
PDEB	N/A	+	N/A	+	N/A	+	N/A	+
IRBP	+	-	+	+	+	+	+	+
TULP	+	-	+	+	+	+	+	+
NRL	+	-	+	+	+	+	+	+
S opsin	-	-	+	-	+	+	+	+
L/M opsin	-	-	-	-	+	+	+	+
Rod opsin	-	-	-	-	+	+	+	+

At Fwk 9-10, ICC detected the expression of IRBP and TULP, while the corresponding PCR results were negative. Actin, Crx, and PDEB were detected at Fwk 10 by RT-PCR. By Fwk 12, ICC demonstrated labeling for Nrl and S opsin. RT-PCR at Fwk 12 detected TULP, IRBP, Nrl, and Crx, but not S opsin. The ICC and PCR data were consistent after Fwk 14 when all molecules investigated were expressed at high levels. In the table, "N/A" indicates not tested, "+" indicates expression was detected, and "-" indicates expression was not detected.

These results show that in order to pinpoint the spatial and temporal pattern of expression, it is essential to perform ICC or in situ hybridization analysis. For primates, it also is necessary to identify and label sections through the fovea, the developmentally most mature region of the human fetal retina. This is in striking contrast to the mouse, where photoreceptor associated genes are expressed in a narrow time window and almost simultaneously over much of the retina. RT-PCR techniques still have advantages with perhaps the most obvious being that a number of different ages can be screened simultaneously for a particular gene product to detect a temporal sequence. Moreover, multiple transcripts can be screened in parallel from the same sample, providing a direct comparison from a small amount of tissue.

Temporal and spatial expression pattern of Nrl and Crx: The cone-rod homeodomain protein Crx, an Otx-like transcription factor, plays a major role in photoreceptor development and maintenance in the vertebrate retina [17-19]. Mutations in this gene have been associated with cone-rod dystrophy II and Leber's congenital amaurosis [18,20-23]. Moreover, targeted disruption of the gene in mice caused attenuated circadian entrainment as well as retinopathy. Photoreceptor outer segments did not elongate in these animals and expression of many photoreceptor-associated genes was diminished [24]. Nrl acts synergistically with Crx in regulating rod opsin expression [9,13,25,26]. Human mutations in Nrl are associated with autosomal dominant retinitis pigmentosa [27,28] and deletion of Nrl in the mouse resulted in complete loss of rod function and rod proteins accompanied by an abnormally large number of cones containing S opsin [29]. A previous report analyzing Crx and Nrl expression by RT-PCR in the human retina detected Crx expression beginning at Fwk 10.5, but failed to detect Nrl expression even at Fwk 13.5 [10]. This finding argues against a synergistic action of both transcription factors during rod differentiation in the human. In contrast, we analyzed Nrl expression by ICC as well as RT-PCR from Fwk 10-18 and detected the first Nrl-positive nuclei at the foveal edge between Fwk 10-11. This corresponds well to the first appearance of immature rods in this region (Figure 5D,E). Positive RT-PCR amplification products were reproducibly obtained in tissue 12 weeks of gestational age or older (Figure 6). Because expression of Nrl started in a few isolated cells on the foveal edge, it is not surprising that amplification of Nrl transcripts from whole eye tissue might not detect expression in such a small number of cells. RT-PCR was positive at Fwk 12 when the number of Nrl-expressing cells had increased markedly (Figure 5E, green).

Crx expression, as assayed by RT-PCR, was readily detected in our earliest sample at Fwk 10 (Figure 2), which corresponds well to reports from a similar PCR based approach [10]. It has been proposed that Crx may be subject to post-transcriptional regulation, as Crx protein was not detected in fetal human retina until Fwk 15 [10]. Although we can not formally rule out this possibility, based on the data presented here, it is more likely that isolated Crx positive cells are indeed already present in or around the fovea at this age or even slightly earlier, which would have gone unnoticed in sections that did not include the fovea centralis. Our data, therefore, suggest that Crx and Nrl are both expressed during the time when the first rod photoreceptors are generated in the human retina, which agrees well with the in vitro data of a physical and functional interaction of both proteins.

Taken together, the data presented in this paper indicate that the comparative use of two common methods for studying gene expression development, RT-PCR and ICC, provides both a more accurate and a more comprehensive view of the molecular and histochemical maturation sequence of human photoreceptors. Because of the early expression of developmentally important molecules in the fovea followed by a protracted wave of expression toward the periphery, the localization of these molecules is important for understanding possible interactions that function to generate specific cell types and topography.

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