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Growth of the postnatal rat retina in vitro: Quantitative RT-PCR analyses of mRNA expression for photoreceptor proteins

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Purpose: To investigate whether previously reported changes in protein expression of middle and long (M/L) and short (S) wavelength cone opsin pigments in cultured retina are correlated with changes in their gene expression. Additionally, to elucidate the importance of a functional retinal pigment epithelium for the development of photoreceptor outer segments.

Methods: Neonatal rat retinas were maintained in culture for 11 days and either fixed in 4% paraformaldehyde for immunohistochemistry or prepared for RNA extraction, reverse transcription polymerase chain reaction (RT-PCR), and quantitative RT-PCR. S-cone and M/L-cone photoreceptors as well as rod photoreceptors were immunohistochemically identified using specific antibodies. Peanut agglutinin (PNA)-lectin histochemistry was used to identify interphotoreceptor matrix associated with cone photoreceptors. Immunolabeling for ED-1 and RPE65 was performed in combination with PNA-lectin staining to examine interactions between photoreceptor cells and the retinal pigment epithelium. Relative estimates of mRNA expression levels for M/L-opsin, S-opsin, recoverin, and rhodopsin in normal and cultured retina were determined by using quantitative RT-PCR.

Results: Strong immunolabeling for recoverin and rhodopsin accumulated in outer segments as well as photoreceptor somata in vitro. Cultured and normal retinas showed similar relative expression levels of recoverin and rhodopsin mRNA. In cultured rat retina, the density of S-cones was high and M/L-cones could not be immunohistochemically detected. However, M/L-cone photoreceptor mRNA was detectable, but at a fourfold lower level in cultured than in vivo retinas. The S-cone photoreceptor mRNA level was almost twofold lower than in vivo. Retinal pigment epithelium cells in cultured specimens showed no RPE65 immunolabeling, but expressed immunolabeling for ED-1 indicating phagocytic activity of these cells in vitro.

Conclusions: We assume that the high density of S-cones and virtually no M/L-cones seen in in vitro retinas might represent an immature stage with numerous S-cones and suppressed transdifferentiation into M/L-cone phenotype. A nonfunctional relationship between photoreceptor cells and a dysfunctional retinal pigment epithelium may have severe consequences for the development of outer segments.

Photoreceptor cells are highly specialized sensory cells with elaborate outer segments containing visual pigments that initiate the visual process after light absorption. Rod photoreceptors mediate dark vision and cone photoreceptors mediate light vision. In nocturnal animals like rats, rods greatly outnumber cones [1]. During the course of evolution, different types of visual pigments with different spectral sensitivities have evolved. Mammals have in general two types of cones with visual pigments tuned to middle and long wavelengths (M/L opsin) and short wavelengths (S-opsin) pigments. In the adult rat retina, M/L-cones vastly outnumber S-cones, which comprise only 0.05% of all photoreceptor cells [1,2].

Rat cone photoreceptors are born early during development, and the immunohistochemical expression of cone opsins is detected during the first postnatal week [3]. S-cone and M/L-cone phenotype appear in sequence, starting with S-cones during the first postnatal week. Szél and colleagues [3] found that S-cone density in the early postnatal rat retina initially is large but reduces in size as the M/L-cones differentiate. From lineage studies of retinal precursor, it is anticipated that cell-cell interactions and environmental factors are important as regulators of cell differentiation and determination [4,5]. Local environmental factors and cell-cell interactions during development may control the ratio of cone photoreceptor subtypes and their spatial distribution. Reports of increased ratio of S-cones to other cones come from organotypic retinal cultures [6], transplantation of embryonic retina into adult host [7], and a nuclear receptor gene mutation (enhanced S-cone syndrome) [8].

In this study, we investigated photoreceptor development in explant cultures with the objective to determine the neurochemical and morphological differentiation of these cells in vitro. Under culture conditions, the morphological differentiation of photoreceptor cells results in dysmorphic outer segments, although some phototransduction-related proteins and opsins are expressed in these structures. This indicates that photoreceptor cells differentiate similarly to the situation in vivo. However, the immunohistochemical expression of S-cone and M/L-opsin shows that the ratio of cone photoreceptors is changed and does not correspond to the in vivo pattern [6,9].

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METHODS

Animals: Postnatal pigmented rats (B& K Universal, Sollentuna, Sweden) were used and the first day of birth was designated as postnatal day zero (P0). Experiments and animal care were according to the ARVO convention for ophthalmologic animal experimentation and according to approved national guidelines for animal care.

Tissue culture: Freshly enucleated eyes of postnatal day 3 rats were immersed in cold CO_2-independent medium (Gibco, Paisley, UK) and rinsed. After incubation with 0.5 mg/ml Proteinase K (Sigma, St. Louis, MO) for 18-19 min at 37 °C, the retinas were dissected free of surrounding ocular tissue and the hyaloid vessels were removed. Retinas with pigmented epithelium were explanted with the vitreal side up on Millicell® PCF 3.0 mm culture plate insert (Millipore, Bedford, MA), and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum and maintained at 37 °C with 95% humidity and 5% CO_2. A cocktail containing L-glutamine, penicillin and streptomycin (Sigma) was added and the medium was changed every second day. Specimens of postnatal day 3 were cultured for 11 days to a stage that corresponded to postnatal day 14 (P14). Tissues from litter-matched specimens of P14 constituted controls.

Tissue handling: Inserts with retinal tissue were immersed in fixative consisting of 4% paraformaldehyde in 0.1 M Sorensen's phosphate buffer (PB) pH 7.2 for 4 h at 4 °C. Several rinses in PB followed whereupon the specimens were transferred from 10 to 20% sucrose in 0.1 M PB, and embedded for cryo-sectioning.

Immunohistochemistry and lectin histochemistry: Subsets of cone photoreceptors containing S-cone or M/L-cone opsin were identified using specific monoclonal antibodies (both kindly provided by Dr. A. Szél, Semmelweis University, Budapest, Hungary) used at 1:10000 dilution. Rod photoreceptor cells were identified with a mouse antibody against rhodopsin (generously provided by Dr. R. Molday, University of British Colombia, Vancouver, Canada) diluted 1:400. Photoreceptor cells were also identified with a rabbit antibody directed against the calcium-binding protein recoverin, used at 1:10000 dilution (kindly provided by Dr A. Dizhoor, Wayne State University School of Medicine, Detroit, MI). A monoclonal antibody directed against ED-1 (Serotec, Oxford, UK), diluted 1:500, was used to label phagocytic microglia and macrophage-like cells. The presence of functional pigment epithelium was identified using a monoclonal antibody against RPE65 (kindly provided by Dr. D. Thompson, Kellogg Eye Center, University of Michigan, Ann Arbor, MI) at 1:400 dilution.

For immunohistochemical staining, the sections were preincubated with phosphate buffered saline containing 0.25% Triton X (PBST) and 1% bovine serum albumin for about 15 min at room temperature. Primary antibodies were then applied over night, and subsequently detected using FITC or Texas Red conjugated goat anti-mouse IgG Fab or donkey anti-rabbit IgG Fab (1:200; Jackson Laboratories, West Grove, PA). The secondary antibodies were applied for 45 min at room temperature. After washing in PBST, the sections were coverslipped with anti-fade mounting medium. Both the primary and secondary antibodies were diluted in PBST containing 1% bovine serum albumin (BSA). Control experiments were processed, in which phosphate buffered saline was substituted for the primary antibody.

The extracellular matrix surrounding cone outer segments was labeled using FITC-conjugated peanut agglutinin (PNA; Sigma). The lectin was diluted 1:50 in PBS containing 1% BSA, centrifuged at 10,000 rpm for 10 min and applied for 40 min at room temperature. Anti-fade mounting medium was used as above.

Some sections were double stained for PNA and ED-1 or RPE65. The antibody was applied over night and detected with a Texas Red-conjugated goat anti-mouse IgG Fab. After thorough washes, FITC-conjugated PNA was applied as above. All antibodies and the lectin were diluted in PBS containing 1% BSA.

Microscopic handling: Micrographs of immunolabeled sections were captured using a Nikon E400 microscope equipped with an Optronix DEI digital camera and appropriate filter settings for FITC and Texas Red. Images were sharpened and brightness adjusted in Adobe Photoshop®.

RNA extraction and reverse transcription: For PCR, retinal tissue was obtained from four independent culture experiments in order to compensate for inter-animal variations. In each experiment, cultured or control retinas from littermates were pooled and homogenized in TRIzol solution (GibcoBRL, Paisley, UK). Total RNA was extracted using MiniPreps according to the manufacturer's instruction (Qiagen, Valencia, CA). First strand cDNA was reverse transcribed from 2 ug total RNA using oligo-dT and MuLV transcriptase for 15 min at 42 °C, followed by a 5 min incubation at 95 °C (RNA PCR Core kit protocol; Applied Biosystems; Foster City, CA).

Quantitative PCR: All data from quantitative PCR reactions were collected using an ABI Prism® 7700 Sequence Detector (Applied Biosystems) on quadruplicates of the different cDNA samples according to the manufacturer's general instructions. Quantitative PCR was performed using

Table 1. Primers used in this study

Protein	Primers (forward and reverse)	Size	GenBank
Recoverin	5'-CTGAAGGAGTGTCCTAGT-3' 5'-TACGTCGTAGAGCGAGAA-3	228 bp	NM_080901
Rhodopsin	5'-TACATGTTCGTGGTCCACTT-3 5'-TTGGAGCCCTGGTGGGTAAA-3	230 bp	Z46957
S-cone opsin	5'-TCGCCAGCTGTCATGGATAC-3' 5'-GCCCAAGAAGGCCTCCAG-3'	64 bp	AF051163
M/L-cone opsin	5'-TCTGCAAGCCCTTTGGCAATGT-3' 5'-ACGATGATGCTGAGTGGGAAGA-3'	254 bp	AH006946
GAPDH	5'-GTCATCATCTCCGCCCCTT-3' 5'-TTTCTCGTGGTTCACACCCA-3'	63 bp	AF106860

Target-specific primers for recoverin, rhodopsin, S-cone and M/L-cone opsin and GAPDH.

SYBR® Green PCR Master Mix (Applied Biosystems) [10]. Primers for GAPDH, M/L-cone and S-cone opsins, as well as recoverin and rhodopsin (Table 1) were designed from GenBank database sequences using the Primer Express version 1.5 software (Applied Biosystems). Reactions were performed in a 25 µl volume with primers, nucleotides, Taq DNA polymerase and buffer. A typical protocol took 2 h to complete and included a 10 min denaturation step followed by 40 cycles with a 95 °C denaturation step for 15 s, and 60 °C annealing/extension step for 60 s.

The values corresponding to the PCR cycle number monitored in real time were used to determine the threshold cycle (C_t). The threshold was set within the range where fluorescence is increasing exponentially and C_t has a linear relationship with the logarithm of the copy number. As a control for variations in the quality of mRNA between the samples, the gene expression of GAPDH was analyzed in parallel. To confirm amplification specificity of the PCR for each primer pair used, a melting curve analysis was performed.

Data analysis: Known dilutions of one cDNA sample were used to construct a standard curve for M/L-cone opsin, S-cone opsin, recoverin, rhodopsin (targets), and the internal standard GAPDH. The generated standard curves were compared and showed that target and GAPDH mRNAs were amplified with the same efficiency. Since we were not interested in comparing differences in the absolute mRNA copy levels, the PCR data were used to determine relative differences in template copy numbers by differences in threshold cycle (C_t). Expression levels of the different targets (M/L-cone opsin, S-cone opsin, recoverin, and rhodopsin) were normalized to those of the housekeeping gene GAPDH. Normalized C_t ($C_{t,normalized}=C_{t,target}-C_{t,GAPDH}$) was determined for each sample of cultured and normal retina. Differences in the C_t value for each target were evaluated by comparisons between results from cultured and normal specimens and expressed as mean C_t values (±standard error of the mean). Unpaired t-tests were conducted to determine whether differences in C_t values were significant, using a significance level of α=0.05.

RESULTS

Morphology: In the cultures of 11 days duration, the outer nuclear layer (ONL) was seen to consist of several rows of photoreceptor nuclei. In each case the number of cell rows in the ONL of cultured specimens was reduced compared to the number of cell rows in the ONL of P14 litter-mates (Figure 1A,B). In general, photoreceptor outer segments (OS) were

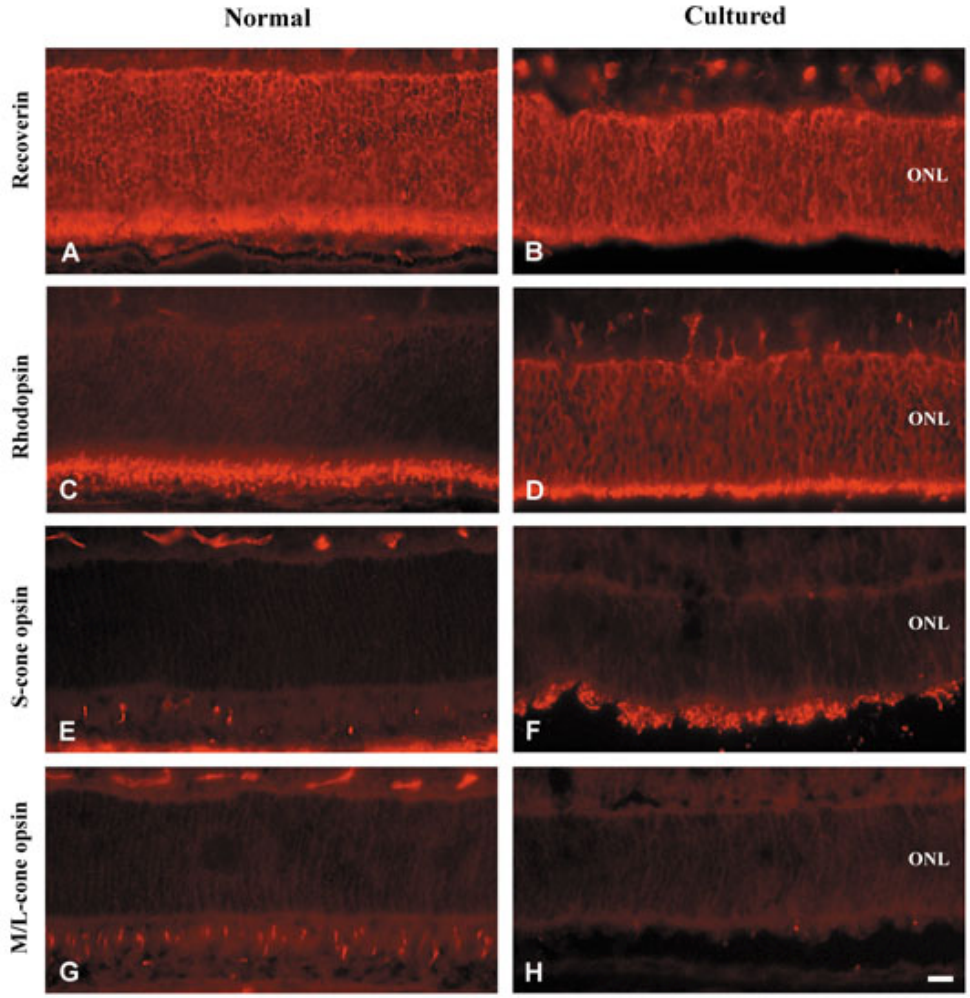

Figure 1. Immunolabeling of in vivo and cultured retinas. Immunofluorescent labeling of photoreceptor cells and outer segments in normal P14 retina and cultured retinas. Photoreceptor cells in P14 (**A**) and 11 days in vitro (**B**) retina are immunolabeled for recoverin. Note that the outer nuclear layer (ONL) of P14 retina is considerably thicker. Immunolabeling for rhodopsin accumulates in photoreceptor outer segments at P14 (**C**). In cultured retina, rhodopsin expression is present in the outer segments and intense labeling is also evident in the entire ONL and in cell somata located at the vitreal aspect of this layer (**D**). Compared to the P14 retina (**E**), numerous cones are immunolabeled for S-cone opsin in the 11 days in vitro retina (**F**). The density of cone photoreceptor cells immunolabeled for M/L-opsin is much higher in the P14 (**G**) than in the 11 days in vitro retina (**H**). Scale bar represents 15 µm.

very short in cultured retinas. Long outer segments appeared to develop in two specific areas: rosette formations and areas separated from the retinal pigment epithelium (RPE). The patterns of immunofluorescence obtained with an antibody against recoverin demonstrated distinct labeling in both rods and cone photoreceptors in explant cultures of 11 days duration and P14 littermates (Figure 1B). All photoreceptor cell somata showed very high levels of recoverin immunoreactivity throughout the ONL, and in the proximal part of the outer segments. Although the ONL displayed a reduced height in cultured specimens, the labeling patterns for recoverin in cultured and normal P14 retinas were clearly comparable (Figure 1A,B).

Rod differentiation: Comparisons between retina kept in culture to P14 and litter-matched P14 retinas of the same age showed some changes of rhodopsin immunolabeling (Figure 1C,D). There was an increased intensity of cytoplasmic labeling in cell somata in the ONL of cultured specimens, but variations were observed within this cell layer. Although the rod outer segments appeared degenerated and very short, they were intensely immunolabeled for rhodopsin (Figure 1D). As shown in Figure 1D, there was a consistent increase of rhodopsin labeling within the outer plexiform layer (OPL) of cultured retinas. Also, an increased density of misplaced rhodopsin labeled photoreceptor cells was evident in the inner nuclear layer (INL; Figure 1D). The INL normally houses the cell somata of retinal interneurons, although scattered rod photoreceptors are normally present in the INL during early postnatal development [11].

Cone differentiation: Peanut agglutinin (PNA) lectin, which binds to the plasma membrane and the adjacent extracellular matrix of cone photoreceptors [12], was used to identify cones. In the retinas cultured to postnatal day 14, PNA revealed small rounded structures corresponding to cone outer segment matrix sheaths (Figure 2B). The number of PNA-positive cone outer segment sheaths appeared to approach the ones observed in P14 retinas. With minor exceptions, practically all cone outer segments appeared short and disrupted in cultured retinas compared to those in P14 controls. Long outer segments with intense PNA-labeling could be discerned in retinal folds and in rosette formations. The os-2 and cos-1 antibodies discriminate between blue and red/green visual pigments in several species, and allow identification of S-sensitive and M/L-sensitive cones, respectively [13]. In the postnatal day 14 retina, M/L-cones were seen to far outnumber S-cones. Considerable changes in the labeling patterns were apparent with both antibodies in cultures of 11 days duration. In the case of S-cone opsin, there was a considerable increase of labeled outer segments (Figure 1E,F). The cone outer segments were short and disrupted, but numerous S-opsin immunolabeled outer segments could be discerned in the cultured retinas (Figure 1E,F). Distinct S-cone opsin labeling was also evident in long outer segments that developed inside rosette formations. The antibody to M/L-cone opsin did not produce any significant labeling of cone outer segments in cultured retinas (Figure 1G,H).

Activation of retinal pigment epithelium: During development, as well as in the adult retina photoreceptor, outer segments (OS) are in close apposition to the RPE (Figure 2A). As OS are renewed, and old discs in the apical part of the OS are shed and eventually phagocytosed by RPE cells [14]. The RPE65 protein is normally expressed in the RPE by P14 (Figure 2A) and is essential in the vitamin A metabolism [15-17]. As illustrated in Figure 2, no immunolabeling for RPE65 protein could be detected in pigmented RPE cells of cultured specimens (Figure 2B,C). Within a few days in vitro, proliferative

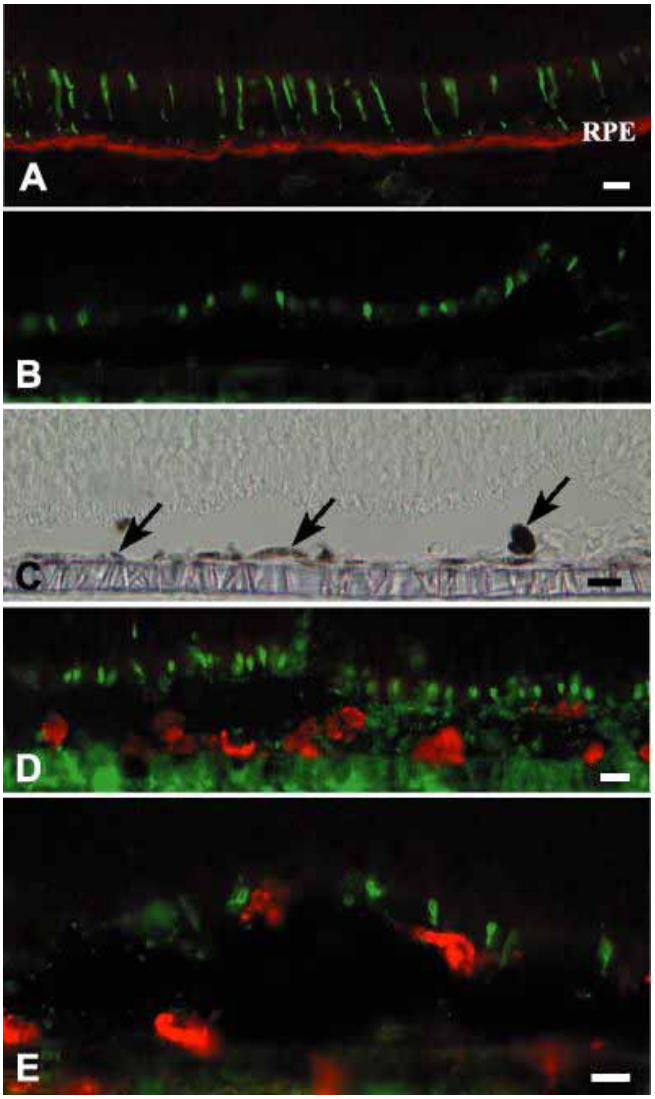

Figure 2. Photoreceptor outer segments and retinal pigment epithelium. (**A**) PNA-positive (green) cone matrix sheaths are closely associated with the retinal pigment epithelium (RPE; red) in normal P14 retina. The RPE is immunolabeled for RPE65. (**B**) In cultured retinas, the cone matrix sheaths are rudimentary but distinctly PNA-positive. Note the absence of immunolabeling for RPE65 in the RPE. (**C**) Light microscopy of the same field as shown in **B**, showing the presence of several RPE cells with distinct pigmentation (arrows). (**D**) Labeling with anti-ED1 (red) shows the presence of activated macrophage-like cells in the RPE-layer. Cone matrix is labeled for PNA (green). (**E**) High magnification of PNA-labeled (green) cone matrix sheaths approached by ED1-positive (red) activated macrophage-like cells. Scale bars represent 15 μm in **A**-**D** and 10 μm in **E**.

changes were apparent in the RPE (data not shown). In cultures of 11 days duration, numerous RPE cells were transformed into macrophage-like cells and/or phagocytic microglial cells. In general, activated cells had acquired an amoeboid morphology and were distinctly immunolabeled for ED-1 (Figure 2D,E). Immunolabeling for ED-1 was also expressed in migrating cells that could be observed at different levels of the subretinal space as well as in folds and in complete rosette formations. Thus, changes in morphology, tissue distribution, and altered expression of immunological markers indicate that RPE-cells are dysfunctional and several are transformed into phagocytic and activated microglia in vitro.

mRNA expression: Melt curve analysis of the amplification products demonstrated that each of the primer pairs constructed to the targets (S-cone and M/L-cone opsin as well as recoverin and rhodopsin) and GAPDH mRNA amplified a single product (Figure 3). Quantitative estimates of the relative abundance of S-cone and M/L-cone opsin, recoverin,and rhodopsin mRNAs were determined using quantitative PCR and calculated from differences in threshold cycle (C_t). The threshold (C_t) is set within the range where SYBR Green fluorescence show an exponential increase for each target (Figure 4) and GAPDH (data not shown) in cultured and normal retinas. Also, standard curves for the targets and GAPDH were linear over a dilution range spanning over two orders of magnitude. Thus, a linear standard curve of the target mRNAs and the endogenous reference (GAPDH) could routinely be generated (Figure 5).

The normalized C_t values for recoverin and rhodopsin were reproducible in different samples of cultured and normal retinas. Analysis of 2 separate cultures and P14 litter-mates revealed mean C_t values for recoverin 3.50±0.98 in cultured

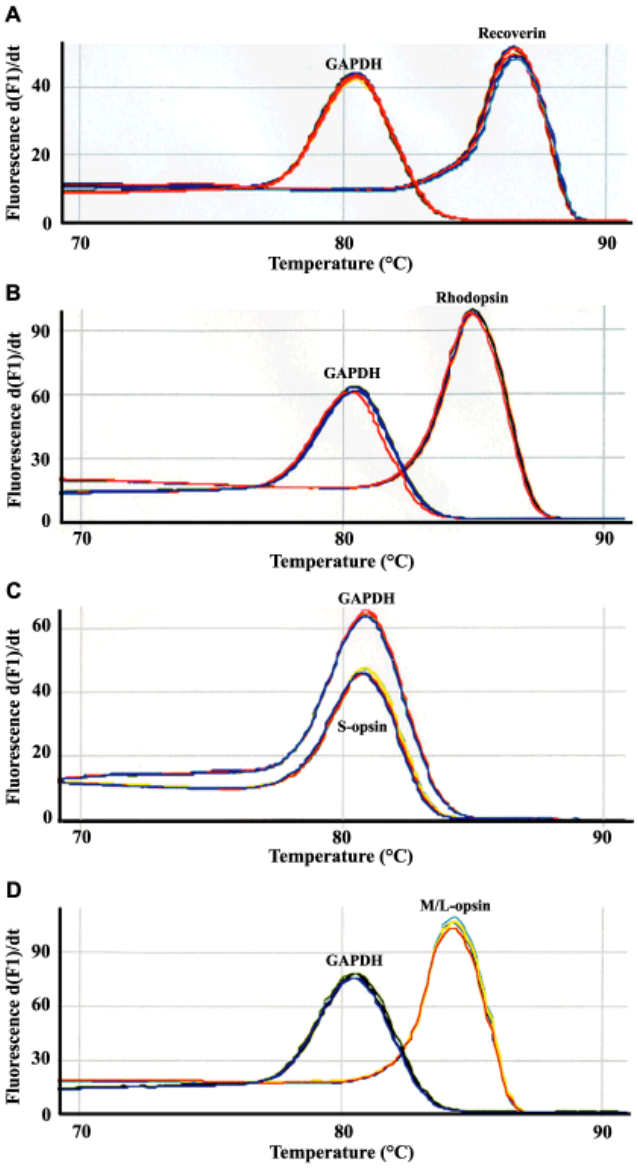

Figure 3. Specificity of amplification reactions. Melt curve analysis of the amplification reactions in the real-time PCR. Melting curves of recoverin (**A**), rhodopsin (**B**), S-cone opsin (**C**) and M/L-cone opsin (**D**) in cultured and control retinas. Each analysis also shows a melt curve analysis of each target amplified with GAPDH in cultured and control retinas. The peaks in the first derivate plot indicate the presence of a specific melting product for each target and GAPDH.

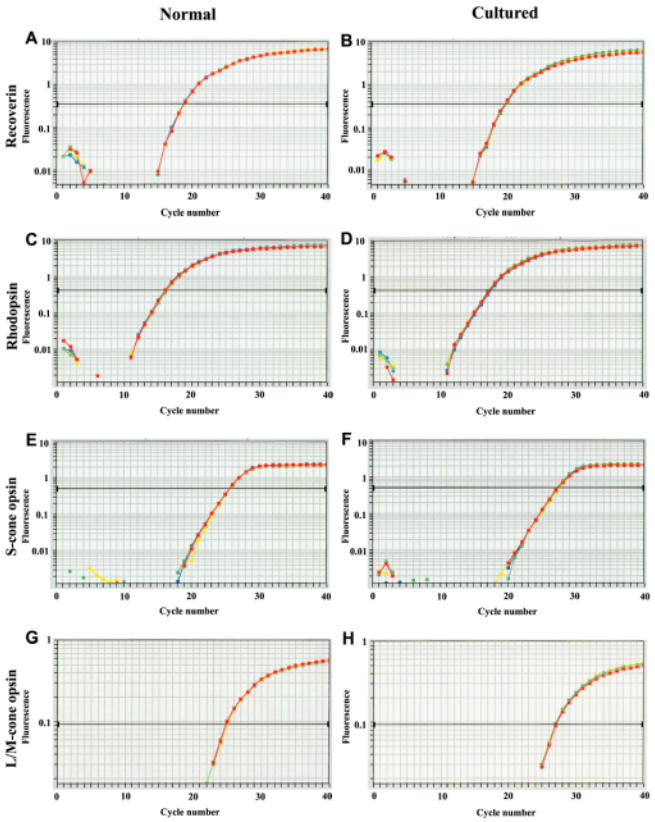

Figure 4. Accumulation of RT-PCR products. Representative accumulation curves showing the increase of SYBR green fluorescence with increasing cycle number, for the targets recoverin (**A**,**B**), rhodopsin (**C**,**D**), S-opsin (**E**,**F**) and M/L-opsin (**G**,**H**) in cultured and control retinas are presented. SYBR green fluorescence is shown on a logarithmic scale.

retinas and 3.43±0.99 for normal P14 retinas. The relative difference was 0.07 and p=0.963 (n=6). The mean C_t value for rhodopsin in cultured retinas was 1.18±0.48 and 2.26±0.51 for normal P14 retinas. The relative difference was 1.08 and p=0.16 (n=6). Thus, the relative mRNA levels for recoverin and rhodopsin did not vary significantly between cultured and normal retinas.

Also, the C_t values of the targets S-cone and M/L-cone opsin normalized to values of the endogenous reference (GAPDH) were reproducible in different samples of cultured and normal P14 retinas. The mean C_t value for S-cone opsin in cultured retinas was 8.67±0.32 and 7.42±0.21 for normal P14 retinas. The relative difference was 1.26 and p=0.0038 (n=11). Analysis of four separate cultures and normal littermates revealed mean C_t (±standard error of the mean) values for M/L-cone opsin 6.38±0.37 in cultured retinas and 4.32±0.38 for P14 retinas. The relative difference between the samples was 2.06 and p=0.0044 (n=5). These values show that the relative threshold for M/L- and S-opsin is higher in cultured retinas. Thus, the relative mRNA levels for S-cone and M/L-cone opsins are about twofold and fourfold higher, respectively, in normal P14 retinas than in culture.

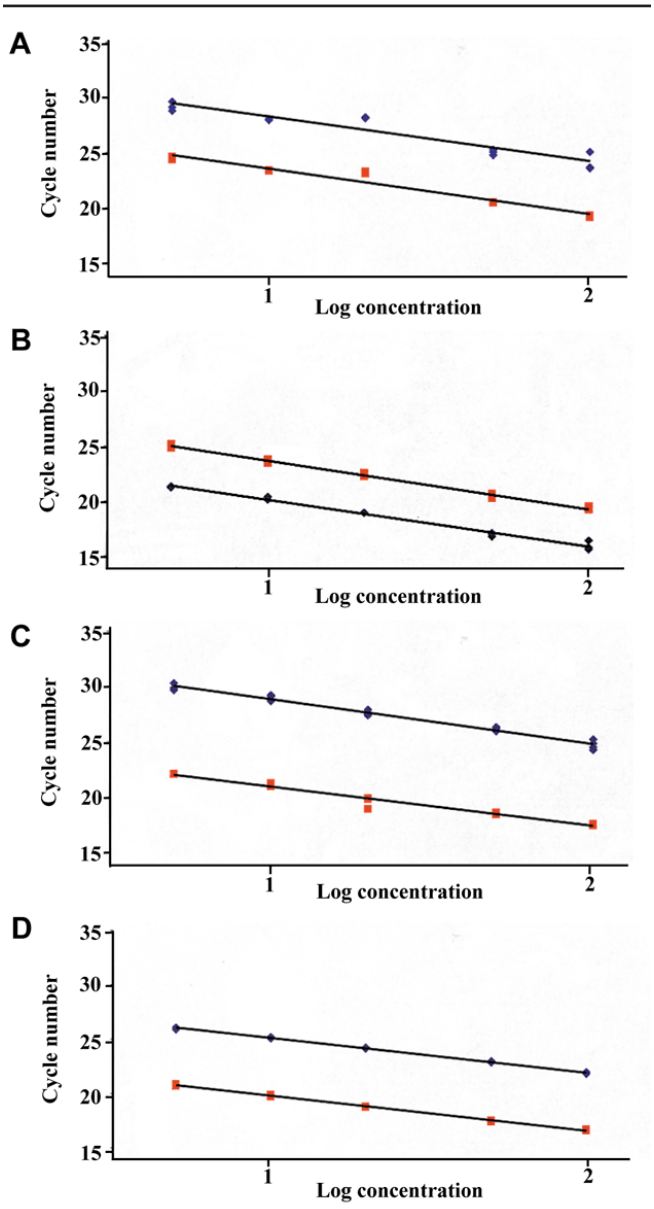

Figure 5. Amplification of dilution series. RT-PCR amplification of mRNA for recoverin (**A**), rhodopsin (**B**), S-cone opsin (**C**) and M/L-cone opsin (**D**) in dilutions of cDNA samples. The examples show linear standard curves for GAPDH (red) amplified in parallel with each target (blue). Data points from triplicate measurements are given.

DISCUSSION

The data presented in this and previous studies [6,18] demonstrate that rod and cone photoreceptor cells develop in cultured retinas and that different photoreceptor proteins and RNA are synthetised. Retinal explants of neonatal rat retina maintained for 11 days in culture show that photoreceptor outer segment morphogenesis is initiated, but the appropriate development of outer segments and the assembly of well-organized discs fail [6,19,20]. Although outer segment development is severely impaired, lectin binding with PNA indicates that cone photoreceptors in the rat retina retain their capability to generate components of their interphotoreceptor matrix in vitro. The expression of recoverin and different opsin proteins in the outer segment of normal retina and in the degenerating and much shorter outer segments in cultured retina, indicates that photoreceptor cell differentiation occurs in vitro. However, the ONL is reduced in vitro, but the non-significant differences in relative mRNA levels of recoverin and rhodopsin indicate that cultured and normal retinas are comparable from a developmental point of view. However, the isolation procedure of neonatal retinas for explant culture involves the death of photoreceptor cells [21], a feature that may contribute to the reduction of the ONL in vitro.

We examined and compared the relative expression levels of M/L-cone and S-cone opsin as well as recoverin and rhodopsin relative to GAPDH using SYBR Green I [10]. As illustrated by the linear relationship between the log of template concentration and cycle number, this approach gives reproducible results in all experiments. The sensitivity of SYBR Green dye seems to be reliable and allows comparisons of the expression of several different genes without high costs. In order to generate comparable data, we observed that it is important that similar amounts of total RNA are used to produce first strand cDNA.

In studies of retinal detachment to detect the pattern of mRNA expression for rhodopsin and cone opsins in surviving photoreceptors, it was concluded that the expression of rhodopsin mRNA is not influenced by detachment and cone opsin mRNA decreased [22]. We have made similar cellular responses in rod photoreceptor cells that are comparable to the situation in detached retina [22]. Our data show unchanged expression levels of rhodopsin mRNA and distinct accumulation of rhodopsin immunoreactivity in rod photoreceptor cell bodies. As in vitro, experimental detachment studies show a rapid degeneration of outer segments [23] and accumulation of rhodopsin immunolabeling throughout the plasma mem-

brane of rod photoreceptor cells [24,25]. This can be explained by a continued biosynthesis but impaired trafficking of rhodopsin into the rudimentary outer segment. Second, the stress induced by experimental detachment also causes an apoptotic cell death in the photoreceptor population [26], a feature recently shown in the ONL of retinas kept for 11 days in culture [21]. Finally, detachment studies show that surviving cones decrease both their protein and their mRNA expressions for S-opsin and M/L-opsin [22,25,27]. Similarly, our PCR data showed that the relative expression levels of S-cone and M/L-opsin mRNA were significantly lower in cultured retina. On the cell basis, this down-regulation could reflect the presence of fewer S-cones, which is not the case for cultured specimens. Immunohistochemistry showed that the density of S-cones in cultured retinas by far outnumber the density of S-cones in litter-mates. We assume that the reduction of S-cone mRNA in cultured retinas corresponds to an altered gene expression during the culture period. Our data of cultured retinas also revealed that the mRNA expression for M/L-cone approached the one for S-cone opsin mRNA. However, the density of M/L cones detected by immunohistochemistry was very low in cultured retinas. Thus, judging from the cones in cultured retina may display a mechanism for surviving stress as cones in detached retina [25].

The presence of dysmorphic outer segments is a common photoreceptor cell response in cultured retinas [19,28]. Although photoreceptor rescue is possible in retina with inherited degenerations, supplementation of neurotrophic factors does not promote the development of normal outer segments [28]. One possible obstacle that hampers the development of outer segments in vitro, may be the presence of activated and migrating RPE cells. The ED-1 immunolabeling revealed that numerous cells in the RPE layer show phagocytic activity and subsets of them are migrating into rosette formations. The cultured retina is deprived of several blood-carried factors, which are important for the proper development of retinal cells. For instance, retinoic acid and transforming growth factor-β are known to possess anti-proliferative effects on the RPE [29], indicating that sufficient inhibition of RPE proliferation does not occur in vitro. It is likely that the entire RPE-layer appears dysfunctional and is not able to supply the developing outer segments (see also [18,20]). However, a certain amount of phagocytosis of outer segment debris may occur by the activated ED-1 positive cells, especially in restricted parts of rosette formations. Functional interactions between photoreceptor cells, the RPE and the interphotoreceptor matrix are essential for the maintenance and formation of outer segments [30,31]. The absence of immunolabeling for RPE65 in RPE cells demonstrated in this study is also known from primary cultures of RPE cells [16]. Thus, a dysfunctional RPE and thereby disrupted vitamin A metabolism [17] may have severe consequences for the developing photoreceptor outer segments in vitro. Indeed, the RPE cells have been shown to reduce the immunohistochemical expression in photoreceptor cells in cultured chicken retina [32]. Interestingly, the presence of PNA-labeling suggests that the presence of a cone interphotoreceptor matrix in vitro. However, the important adhesion between the neural retina and the RPE [33], and the firm attachment to the RPE appears to be lost during the culture conditions.

A major aim for undertaking this study was to obtain quantitative information about cone opsin mRNAs in cultured retina. Our data extends previous observations of differences in the immunohistochemical expression of S-cone and M/L-cone opsin in cultured retina [6,9] to not include changes at the mRNA level. The relative expression levels of S and M/L mRNAs were almost twofold and fourfold lower in cultured retina. Yet the immunohistochemical data show that M/L-opsin expression decreased to undetectable levels and high density of S-cones [6]. The underlying mechanisms for these features are currently unknown. One plausible explanation may be that the developmental change in cone phenotype described by Szél and colleagues [3] never starts or is delayed in vitro. In the normal retina, cone differentiation is delayed for several days after the cells are post-mitotic and immunoreactivity for S-opsin appear at postnatal day 5 [3,34]. As the retinas are explanted before the detection of S-cone immunoreactivity, S-cone differentiation appears to proceed to the immature stage in the absence of exogenous factors. However, the absence of such factors could be involved in unknown regulatory mechanism(s) and explain the presence of immature S-cones and suppressed differentiation into the M/L-cone phenotype.

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