Pigment Epithelium-Derived Factor Supports Normal Development of Photoreceptor Neurons and Opsin Expression after Retinal Pigment Epithelium Removal

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Dysfunction of the retinal pigment epithelium (RPE), its loss, or separation from the underlying neural retina results in severe photoreceptor degeneration. Pigment epithelium-derived factor (PEDF) is a glycoprotein with reported neuroprotective and differentiation properties that is secreted in abundance by RPE cells. The "pooling" of PEDF within the interphotoreceptor matrix places this molecule in a prime physical location to affect the underlying neural retina. The purpose of this study was to analyze the morphogenetic activity of PEDF in a model of photoreceptor dysmorphogenesis induced by removal of the RPE. Eyes were dissected from embryonic *Xenopus laevis*, and the RPE was removed before culturing in medium containing PEDF, PEDF plus anti-PEDF antibodies, or medium alone. Control retinas were maintained with an adherent RPE. Light and electron microscopic analysis was used to examine retinal ultrastructure. Opsin was localized immunocytochemically and quantified as an index of outer segment membranous material and photoreceptor protein expression. Removal of the RPE resulted in an aberrant assembly of photoreceptor outer segments, loss of fine subcellular ultrastructure in photoreceptors, and a reduction in opsin protein levels when compared with control retinas. The addition of PEDF prevented the dysmorphic photoreceptor changes induced by RPE removal. In particular, photoreceptor ultrastructure, outer segment membrane assembly, and steady-state levels of opsin were equivalent to control conditions. Anti-PEDF antibodies completely blocked the morphogenetic activity of PEDF. These results indicate that PEDF is able to mimic the supportive role of the RPE on photoreceptors during the final stages of retinal morphogenesis.

Key words: photoreceptor; pigment epithelium-derived factor; retinal dysmorphogenesis; neuroprotection; ultrastructure; protein expression

The importance of an intact and fully functional retinal pigment epithelium (RPE) on photoreceptor development and survival has been known for many years. During development the rudimentary layers that will become the RPE and the retina are brought into close proximity upon the collapse of the optic vesicles, establishing an early relationship between these two tissues. At this developmental stage the retina is undifferentiated morphologically and photoreceptor outer segments have not yet formed, thus raising the possibility that the RPE could be a source of signals that induce or regulate photoreceptor development and outer segment elaboration. Also, the observation that outer segment development is impaired in the absence of the RPE in most species suggests that interactions between these two cell types may be of fundamental importance for the structural and functional differentiation of photoreceptors (Hollyfield and Witkovsky, 1974).

In a mature retina the photoreceptor outer segments degenerate very quickly after physical separation from the RPE, and the degree of recovery is negatively correlated with the duration of the detachment (Erickson et al., 1983; Anderson et al., 1986; Guérin et al., 1989, 1993; Lewis et al., 1991). In the Royal College of Surgeons (RCS) rat a defect in the RPE gene *Mertk* (D'Cruz et al., 2000) results in photoreceptor degeneration unless growth factors are injected into the subretinal space (Faktorovich et al., 1990) or an RPE transplant is performed (Li and Turner, 1991). In all cases the rescue extends beyond the limits of the injection or transplant, suggesting that a diffusible factor is responsible for photoreceptor rescue. Moreover, it has been demonstrated that RPE-secreted proteins positively influence photoreceptor survival (Gaur et al., 1992; Sheedlo et al., 1992, 1998). Although an abundance of data indicates that the RPE or "factors" supplied by the RPE are of paramount importance to the development and/or survival of photoreceptors, the nature of these interactions is poorly understood.

Recent findings have identified disease-causing mutations in RPE-expressed genes in several forms of retinopathies. For example, mutations cosegregating with disease manifestations have been found in the RPE65 (Gu et al., 1997; Marlhens et al., 1997) and the cellular retinaldehyde-binding protein genes (Maw et al., 1997). The gene for pigment epithelium-derived factor (PEDF) has been tightly linked to the RP13 locus on chromosome 17, implicating it as a candidate gene for this form of autosomal dominant retinitis pigmentosa (Tombran-Tink et al., 1994; Goliath et al., 1996). PEDF is an RPE-secreted glycoprotein that binds to the glycosaminoglycans of the interphotoreceptor matrix (Tombran-Tink et al., 1995; Wu et al., 1995), placing this molecule in a prime physical location to affect the underlying neural retina.

Numerous studies support the role of PEDF in neuronal development, differentiation, and survival (Taniwaki et al., 1995, 1997; Araki et al., 1998; Bilak et al., 1999; DeCoster et al., 1999). However, the direct effect of PEDF on retinal photoreceptors has not yet been evaluated, nor has the potential role of PEDF as a neuroprotective agent in retinal dysmorphogenesis been elucidated. The purpose of the present study was to evaluate the efficacy of PEDF to protect against aberrant photoreceptor development because of RPE removal. Herein we have demonstrated for the first time that exogenous PEDF specifically prevented the dysmorphic morphological and protein expression changes that are induced by

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RPE removal. In particular, photoreceptor ultrastructure, outer segment membrane assembly, and opsin protein expression were equivalent to retinas that completed morphogenesis with an attached RPE. These results strongly support the role of PEDF in retinal morphogenesis. Our results also suggest that PEDF may offer new therapeutic strategies in the treatment of retinal detachment and other degenerations that are induced by dystrophies of the RPE.

MATERIALS AND METHODS

Preparation of purified PEDF and anti-PEDF. PEDF was isolated and purified form bovine interphotoreceptor matrix by ionic exchange and gel filtration chromatography, as previously described (Tombran-Tink et al., 1991). Briefly, soluble bovine interphotoreceptor matrix was extracted from fresh bovine eyes by a gentle lavage of the eyecup after careful removal of the anterior segment and vitreous. A volume of 0.5 ml of ice-cold PBS, pH 7.4, was used in this procedure. Washes from 100 eyecups were pooled, centrifuged, and filtered using a 0.2 μM Nalgene filtration unit. Approximately 500 ml of the interphotoreceptor matrix wash was applied to a Beckman Altex TSK column equilibrated with 10 mM sodium phosphate, pH 7.2. Bound PEDF was eluted with 0.5 M NaCl at a flow rate of 8 ml/min. Absorbance was monitored at 280 and 260 nm. Fractions containing the PEDF were combined, and the protein concentration was determined by a Bio-Rad assay (Hercules, CA). The pooled fractions were concentrated and subsequently were purified by using a Sephadex G-250 column. Eluates containing PEDF were separated electrophoretically on a 10% SDS gel and stained with Coomassie blue.

Polyclonal antibodies were generated against bovine PEDF. In brief, 5 mg of the purified protein was used to inject rabbits prepared for antibody production in the Laboratory of Dr. James F. McGinnis (Dean McGee Eye Institute, Oklahoma City, OK). Three injections of ~0.5 mg were given to each rabbit over a period of 6 weeks. Serum obtained from each immunized animal was tested 2 weeks after each injection. Preimmunized serum from each animal also was analyzed. Samples containing the purified 50 kDa PEDF protein were pooled and used in Western blot analysis to determine the specificity of the polyclonal antibodies for the purified PEDF protein. Serum from rabbit 513B yielded the highest degree of specificity; therefore, this antibody was used in all of the analyses presented herein. Additional Western procedures were performed to determine the specificity of the 513B anti-PEDF antibody for *Xenopus laevis* PEDF.

Retinal culture conditions. The handling of *Xenopus laevis* was in accordance with the Declaration of Helsinki and *The Guiding Principles in the Care and Use of Animals* (DHEW Publication 80-23, National Institutes of Health). The experimental culture preparation that uses *Xenopus* embryonic eyes has been described previously (Hollyfield and Witkovsky, 1974; Lolley et al., 1977; Stiemke and Hollyfield, 1994, 1995; Jablonski et al., 1999). Adult frogs were induced to breed with injections of human chorionic gonadotropin (Sigma, St. Louis, MO). The external staging system of Nieuwkoop and Faber (1956) was used to determine retinal maturity. Embryos and isolated retinas were maintained under cyclic lighting conditions (12 hr light/dark). In all experiments the eyes were removed from embryos at stage 33/34, just as photoreceptor outer segments are beginning to be elaborated (Stiemke et al., 1994). At this stage the eye rudiments are not yet surrounded by the sclera, leaving the posterior segment coated only by the RPE layer. Taking advantage of this characteristic, we gently peeled away the overlying RPE from the neuroepithelium, leaving the underlying retina exposed to the culture medium. Intact eye rudiments without an adherent RPE were cultured in Niu-Twitty medium alone (Jacobson, 1967), Niu-Twitty containing purified PEDF, or PEDF plus anti-PEDF antibodies (513B). Control eyes were allowed to mature *in vitro* in the presence of an adherent RPE in Niu-Twitty medium alone (i.e., no PEDF was added). Eye rudiments were maintained *in vitro* for 3 d at 23°C after which they were fixed or frozen as appropriate for the subsequent analysis. With this culture protocol (removal at stage 33/34 and maintenance for 3 d at 23°C) retinas from fully intact eye rudiments have reached approximately stage 42 of the *in vivo* developmental scale, characterized by complete stratification of the retina and mature photoreceptors with well developed outer segments that express opsin and *rds*/peripherin in the proper location and amount (Hollyfield and Rayborn, 1979; Stiemke et al., 1994; Jablonski et al., 1999).

Morphological assessment. After 3 d of *in vitro* development the eye rudiments were grossly examined under a dissecting microscope for integrity and smoothness of the neuroepithelial or RPE surface. Any retina that exhibited an uneven surface or had many loose cells associated with it was discarded. For ultrastructural analysis the eyes were fixed in Tucker fixative (2% glutaraldehyde and 1% osmium tetroxide), dehydrated, and embedded in Araldite/EMbed812 (Electron Microscopy Sciences, Fort Washington, PA). To ensure that photoreceptors of equivalent stages of maturation were compared, we performed structural analyses on tissue sections taken exclusively from the posterior pole region of the retina. Sections 1 μm thick were cut, stained with toluidine blue O, and examined on a Nikon Eclipse E800 microscope equipped with Sensys Color Camera (Photometrics, Tucson, AZ). Images were collected with MetaMorph Imaging System software (Universal Imaging, West Chester, PA). For ultrastructural analyses thin sections were cut, placed on 200 mesh copper grids, and viewed on a JEOL 2000 electron microscope.

An initial series of experiments was undertaken to evaluate at the light microscopic level the ability of various concentrations of PEDF and anti-PEDF to support outer retinal morphogenesis in the absence of the RPE and to block the morphogenetic properties of PEDF, respectively. The main criterion for evaluation of the protective effect of PEDF was that of organized assembly of nascent outer segments that were elaborated in the absence of an attached RPE. The following concentrations were evaluated: 50, 100, and 500 ng/ml of PEDF and 1:500 and 1:1000 dilutions of anti-PEDF antibody. Detailed morphological and protein analyses subsequently were performed on additional retinas that were exposed to the following culture conditions: Niu-Twitty medium containing 50 ng/ml of PEDF, the empirically determined optimal concentration that allowed for elaboration of highly structured photoreceptor outer segments in the absence of the RPE; and 50 ng/ml of PEDF plus 1:500 dilution of anti-PEDF antibody, the condition that was determined experimentally to prevent the permissive effect of PEDF on photoreceptor outer segment organization. The addition of 1:500 normal rabbit serum to the medium containing 50 ng/ml of PEDF was evaluated as a control to evaluate the ability of nonimmune serum to block PEDF-induced photoreceptor cytomorphogenesis.

The degree of outer segment organization was evaluated subjectively by using a grading scale similar to that which we have used previously (Stiemke and Hollyfield, 1994, 1995). Based on our previous experience using an identical *in vitro* assay to assess the ability of various factors to promote photoreceptor cytomorphogenesis in the absence of the RPE (Stiemke and Hollyfield, 1994, 1995; Stiemke et al., 1994; Jablonski et al., 1999; Kancherla et al., 1999; Jablonski and Ervin, 2000), the structure of individual photoreceptors within a retina can range from complete absence of outer segment material or many whorls of membrane (i.e., in retinas maintained without an RPE in Niu-Twitty medium) to highly structured outer segments with a cylindrical profile and no whorls of membrane (i.e., in retinas maintained with an intact RPE in Niu-Twitty medium). It is also possible that a single retina can contain photoreceptors of varying degrees of organization. To account for the degree of variability in outer segment organization that can occur, we refined our original grading scale and expanded it to include six levels of organization. The scale used in the present study included a grade of −1 that denotes a complete absence of outer segment membranous material. Outer segments, when present, received a grade ranging from 0, which reflects a complete lack of organized structure of outer segment membranes (0% organized or only whorls of membrane are present), to 4, which describes 100% organization of all membranous material from a single photoreceptor. Each step of one grade reflects a 25% linear change in the level of organization. For each experimental condition eight contiguous photoreceptors from three individual retinas ($n = 24$) were evaluated with this scale. The total number of graded photoreceptors was 96. The grader was blinded to the experimental condition under which each graded micrograph was obtained.

Immunocytochemistry. For immunocytochemical analyses the eyes were fixed in Davidson fixative (32% ethanol, 2% formalin, and 11% acetic acid), followed by dehydration in ethanol. Eyes were embedded in Unicryl (Electron Microscopy Sciences). Sections 1 μm thick were cut from the posterior pole region of the retina and collected on microscope slides that were coated previously with Biobond (Electron Microscopy Sciences). Sections were incubated in 5% goat serum (Vector Laboratories, Burlingame, CA) in PBS, rinsed in PBS, and incubated overnight in primary antibody at 4°C [anti-opsin; B630N (Röhlich et al., 1989) at 1:2000 dilution]. Gold-conjugated goat anti-mouse secondary antibodies were applied to the tissue sections (1:50 dilution for 2 hr, ultrasmall gold particle size), followed by silver enhancement, as described by the manufacturer (Electron Microscopy Services). Controls included the absence of primary antibody. Retinal sections were viewed on a Nikon Eclipse E800 microscope equipped with Sensys Color Camera (Photometrics), and images were collected by using MetaMorph Imaging System software (Universal Imaging). Two images were collected of each retinal section: a bright-field image that shows the morphology of the tissue and another image taken with epipolarized light that shows only the immunolabeling pattern. The epipolarized image was color-enhanced and merged with the bright-field image so that the specific immunolabeling patterns could be distinguished easily. Three eyes from each experimental condition were evaluated in this series of experiments.

Quantitation of opsin. Opsin is the most abundant photoreceptor protein, composing ~80–90% of the total rod outer segment protein (Papermaster and Dreyer, 1974), thus making it an ideal candidate to assess the amount of outer segment membranous material present and overall photoreceptor protein expression levels. Opsin is localized to the lamellar portion of outer segment discs and the surrounding plasma membrane. To quantify the steady-state levels of opsin, we collected three sets of 10 eyes under each condition, ground them, and solubilized them with sodium cholate detergent (Sigma). Pooling of 10 retinas was necessary because of the small size of each individual intact eye rudiment (i.e., ~100–150 μm in diameter and ~25 μg of total protein per eye). Extracted proteins were applied in duplicate to Hybond-P membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) by using a slot blot apparatus (Bio-Rad). After solubilization and dilution the amount of total protein per slot was ~25–30

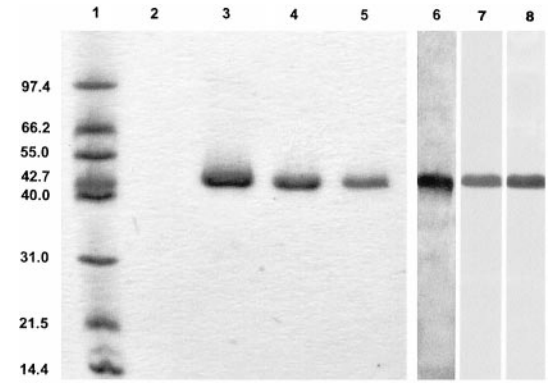

Figure 1. Migration of purified PEDF as a 50 kDa molecular weight species on 10% SDS polyacrylamide gel detected by Coomassie blue staining. Molecular weight markers (Promega midrange standards) are shown in *lane 1*. *Lane 2* is intentionally left blank. *Lanes 3, 4*, and *5* represent the purified PEDF protein at concentrations of 505, 252, and 126 ng, respectively. *Lane 6* represents the Western blot analysis showing a very specific interaction, at ~50 kDa, between 100 ng of purified PEDF and the polyclonal PEDF antibody at 1:1000 dilution. *Lanes 7* and *8*, respectively, represent anti-PEDF (1:1000 dilution) applied to *Xenopus* retinal homogenates and *Xenopus* retinal homogenates with the addition of 100 ng of purified PEDF.

$\mu g/cm^2$, which is well within the binding capacity of the Hybond-P membrane (maximum protein binding capacity of 125 $\mu g/cm^2$). Blots were blocked in Blotto (5% in PBS) for 1 hr, followed by incubation in primary antibody overnight at 4°C [anti-opsin; B630N (Röhlich et al., 1989) at 1:10,000 dilution]. The ECF Western blotting kit (Amersham Pharmacia Biotech) was used according to the manufacturer's protocols. Blots were scanned on a Storm 860 Imaging system (Molecular Dynamics, Sunnyvale, CA), and data were quantified with ImageQuant software version 1.1 (Molecular Dynamics). An evaluation of standard curves indicates that this assay methodology is able to generate a linear fit of sample dilutions over a minimum of two log units of protein concentration and also that a 50% reduction in the concentration of opsin applied to the Hybond-P membrane is equivalent to a reduction of ~45% in the level of chemiluminescent signal that is detected (data not shown). For each of the repetitions the data were normalized to the values obtained for eyes maintained with an intact RPE.

Statistical analyses. Photoreceptor outer segment grading data and steady-state opsin levels were analyzed statistically by one-way ANOVA, using SAS statistical software (SAS Institute, Cary, NC). Because graded photoreceptors were analyzed in groups of eight from three separate eyes under each experimental condition, observations within each eye could not be considered independent. To account for this, we calculated the average grading for each eye and conducted comparisons among groups only on the averages of each of the 12 eyes that were analyzed. Average steady-state opsin levels for each group of 10 eyes under the four experimental conditions were obtained as described above ($n = 12$ also in this case). Preplanned comparisons were performed between each experimental condition for a total of six comparisons for each investigated variable (i.e., photoreceptor organization and opsin steady-state levels). This is in excess of the number of comparisons allowed before an adjustment of the experimental error rate alpha is required (Sokal and Rohlf, 1995). Therefore, to minimize the risk of type I error caused by repeated comparisons, the alpha level of 0.05 was adjusted by means of the Dunn–Sidák method as described in Sokal and Rohlf (1995). In so doing, only p values < 0.009 were considered statistically significant.

RESULTS

Determination of optimal concentrations of PEDF and blocking antibody

The preparation of bovine PEDF was highly purified, yielding single Coomassie blue-stained bands of ~50 kDa after electrophoretic separation (Fig. 1, *lanes 3–5*). Western blot analysis indicated that the polyclonal anti-PEDF recognizes purified bovine PEDF (Fig. 1, *lane 6*). In addition, the anti-PEDF antibody bound to a single 50 kDa molecular species band in *Xenopus* retinal homogenates and also in *Xenopus* retinal homogenates to which purified PEDF was added before Western blot analysis (Fig. 1, *lanes 7, 8*, respectively).

Light microscopic evaluation of outer retinal structure revealed that, in the presence of an adherent RPE, outer segment membranes were highly organized and were composed of individual, stacked, flattened membranous saccules with a continuous profile (Fig. 2*a*). Retinas cultured in the absence of the RPE in Niu-Twitty medium contained photoreceptors with outer segments characterized by many whorls of membrane interspersed with packets of stacked discs (Fig. 2*b*). The addition of 50 ng/ml of PEDF to retinas maintained without an adherent RPE optimally supported the assembly of outer segments into stacked, flattened membranous saccules (Fig. 2*c*), which morphologically resembled those of photoreceptors that underwent morphogenesis in the presence of an adherent RPE cell layer (compare with Fig. 2*a*). Concentrations of PEDF >50 ng/ml did not stimulate the same level of outer segment organization in the absence of the RPE. In retinas that were exposed to 100 and 500 ng/ml of PEDF, the majority of photoreceptors had many whorls of membranous discs in and among areas of organized outer segment (Fig. 2*d,e*, respectively). Structural analysis also revealed that a 1:500 dilution of nonimmune serum was unable to block the morphogenetic properties of PEDF (Fig. 2*f*). Anti-PEDF antiserum when diluted 1:1000 was able to partially prevent the dramatic improvement in outer segment organization that was promoted by 50 ng/ml of PEDF (Fig. 2*g*). However, a 1:500 dilution of antiserum was able to block almost completely the permissive and protective effects of 50 ng/ml of PEDF (Fig. 2*h*).

Photoreceptor ultrastructure and grading of outer segment organization

Figure 3*a* shows an example of photoreceptors from retinas that were maintained with an adherent RPE. An evaluation of photoreceptor ultrastructure revealed outer segments that were composed of an array of highly organized, flattened, stacked membranous saccules that were in alignment with a corresponding inner segment. Calycal processes, budding from the inner segment, closely abutted the proximal outer segment region. The RPE was juxtaposed to the tips of the outer segments, and apical RPE processes surrounded the outer portions of the outer segments. Figure 3*b* illustrates the effects of RPE removal on photoreceptor morphology. Removal of the RPE dramatically altered the ultrastructure of the photoreceptors. After 3 d of *in vitro* maturation in the absence of the RPE the photoreceptor outer segments were grossly dysmorphic and did not comprise an orderly assemblage of membranous discs. Rather, discs of uneven lengths were elaborated, some much shorter than the width of the inner segment and others much longer. Other areas of membranous whorls were found in what would be the subretinal space. The membranous outer segments, irrespective of the conformation, often were detached from any photoreceptor inner segment.

Figure 4*a* demonstrates the organizational effects of PEDF on photoreceptor outer segments. The addition of 50 ng/ml of PEDF to eyes maintained in the absence of the RPE (Fig. 4*a*) prevented the aberrant assembly of outer segment discs that follows removal of the RPE. Nascent photoreceptor outer segments were very similar to eyes with an adherent RPE in that they were highly structured with discs of equal diameter. There were very few discontinuous membranous expanses, and individual outer segments were in line with inner segments. Multiple calycal processes surrounded, and extended beyond, the tips of the outer segments, a feature that was missing without the addition of PEDF (compare with Fig. 3*b*). Figure 4*b* exemplifies the level of photoreceptor outer segment disorganization observed after the addition of anti-PEDF antibodies. In the absence of the RPE, but in the presence of both PEDF and anti-PEDF antibody (50 ng/ml and 1:500 dilution, respectively), photoreceptor outer segments formed a staggered profile of membranes with uneven disc termination similar to that documented in eyes lacking both an RPE and the exogenous PEDF. In addition, the outer segments were detached from the corresponding inner segment.

The average organizational grade of photoreceptor outer segments under our four experimental conditions is summarized in

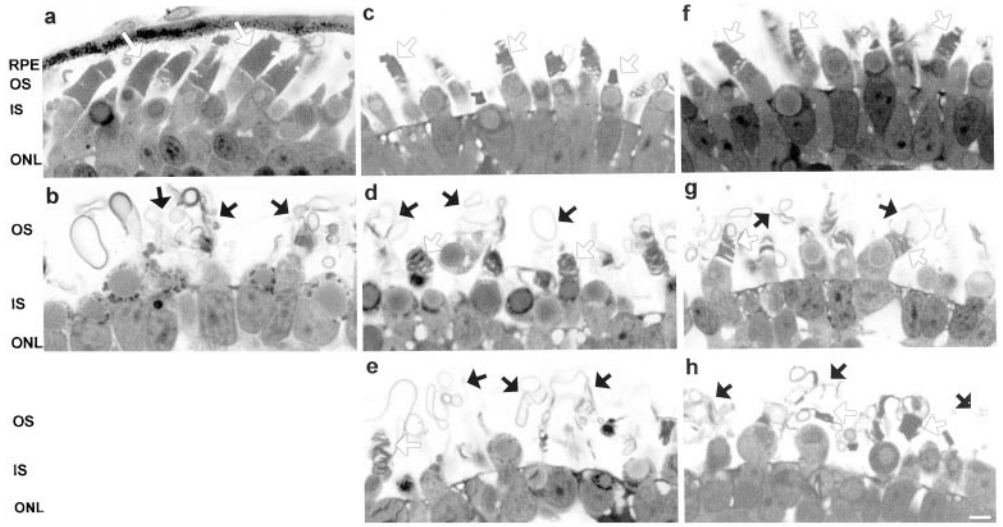

Figure 2. Outer retinal morphology under the various culture conditions used in this study. *a,* In retinas that were allowed to undergo the final stages of cytomorphogenesis *in vitro* with a normally apposed RPE, the outer segments are highly organized and tightly stacked, yielding individual profiles that are in line with individual photoreceptor inner segments. *b,* In retinas that were maintained in the absence of the RPE, photoreceptor outer segment membrane structure was markedly disorganized, with little evidence of normal disc stacking. *c,* The addition of 50 ng/ml of PEDF stimulated the proper folding of outer segment membranes. Increased concentrations of PEDF [i.e., 100 ng/ml (*d*) and 500 ng/ml (*e*)] failed to induce similar levels of outer segment organization in the absence of the RPE. Under these conditions the majority of photoreceptors had many whorls of membranous discs interspersed with areas of organized outer segments. *f,* Nonimmune serum (1:500 dilution) was unable to block the morphogenetic properties of PEDF, and most outer segments were highly structured. Although the addition of a 1:1000 dilution of anti-PEDF antiserum (*g*) partially blocked the dramatic improvement in outer segment organization that was promoted by 50 ng/ml of PEDF, a 1:500 dilution of antiserum (*h*) significantly disrupted the permissive and protective effects of 50 ng/ml of PEDF. *RPE*, Retinal pigment epithelium; *OS*, outer segment; *IS*, inner segment; *ONL*, outer nuclear layer. Organized outer segments are indicated by *white arrows*, and disorganized outer segments are marked with *black arrows*. Scale bar, 10 μm.

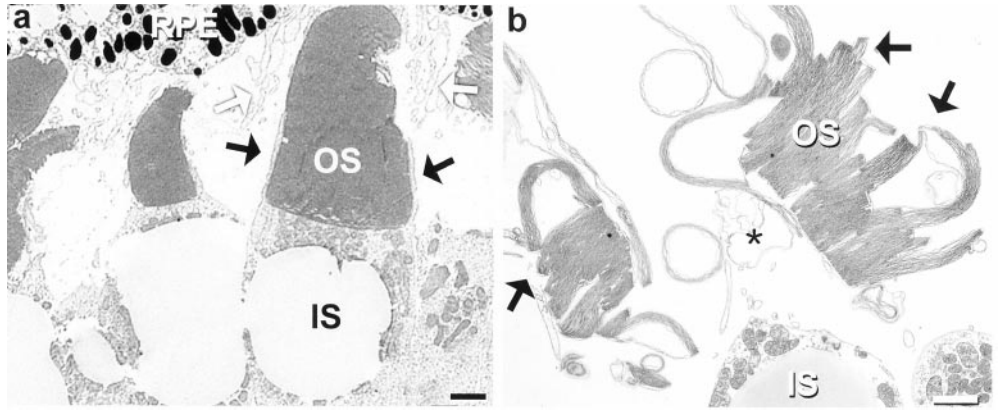

Figure 3. Ultrastructural analysis of *Xenopus laevis* retinas with and without an adherent RPE. *a,* In the presence of the RPE the photoreceptor outer segments were composed of an orderly array of stacked discs surrounded by a plasma membrane. The disc margins were in proper alignment, with all discs of a single photoreceptor being the same diameter. Calycal processes (*black arrows*) that arise from the inner segment were in close proximity to the vitreal aspect of the outer segments. The apical process of the RPE surrounded the tips of the outer segments (*white arrows*). *b,* In the absence of the RPE there was some organization of outer segment discs into flattened, stacked saccules; however, the diameter of outer segment discs differed greatly (*black arrows*), with many discs terminating prematurely. Also, areas of whorl-like membrane with minimally ordered stacked discs were present (*asterisk*). Calycal processes were not present. *OS*, Outer segment; *IS*, inner segment. Scale bar, 1 μm.

Figure 5. By one-way ANOVA the overall F test for differences among the four groups was highly significant ($F = 56.57$; $p = 0.0001$). In retinas that completed morphogenesis with an adherent RPE, the vast majority of photoreceptor outer segments was highly structured, properly folded, and contained discs of equal diameter. Use of our grading scale yielded a grade of 3.958 ± 0.200 (mean ± SD) on a scale of -1 to 4. This value indicates that nearly all of the photoreceptor outer segments that were evaluated were 100% organized. In the absence of the RPE the average grade of photoreceptor outer segment organization decreased to 1.042 ± 1.207 ($p = 0.0001$ compared with both control retinas with an adherent RPE and PEDF-treated eyes), indicating that, on average, only 25% of the outer segment material was organized into stacked, flattened membranous saccules. In the presence of 50 ng/ml of PEDF the average organizational grade was 3.042 ± 1.338, indicating that ~75% of the outer segment membranes were highly structured. This value did not differ statistically from control retinas ($p = 0.0144$), and it was significantly greater than the organizational grade of retinas that lacked PEDF stimulation or that were exposed to the anti-PEDF antibody ($p = 0.0001$ for both comparisons). The addition of anti-PEDF antibody significantly decreased the organizational grade to 0.708 ± 1.541, indicating that <20% of the outer segment material was organized ($p = 0.0001$ compared with both control retinas and those exposed to PEDF).

Immunolocalization of opsin

The patterns of opsin immunolocalization are illustrated in Figure 6. In control retinas that were allowed to mature *in vitro* with a normally apposed RPE, the opsin labeling was very heavy over the outer segments of the majority of photoreceptors. Unlabeled pho-

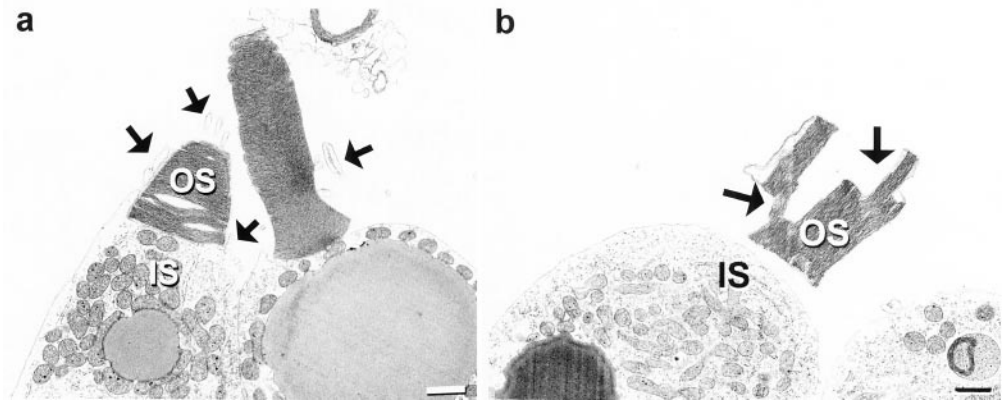

Figure 4. PEDF prevented photoreceptor degeneration after removal of the RPE. *a,* Photoreceptor outer segments were highly structured and ordered in retinas exposed to exogenous PEDF (50 ng/ml). Most discs were flattened and stacked in an orderly array and had an equal diameter. Calycal processes were present and were closely adherent to the outer segments. They extended to the tips of the outer segments, beyond their normal position (*black arrows*). *b,* Anti-PEDF (1:500 dilution) blocked the protective effect of PEDF. Photoreceptor outer segments no longer were composed of stacked membranous discs of similar diameter. The addition of both PEDF and anti-PEDF to retinas that lacked an adherent RPE resulted in premature termination of disc elongation and led to a staggered border of the membranous discs (*black arrows*). Calycal processes were not formed. *OS*, Outer segment; *IS*, inner segment. Scale bar, 1 μm.

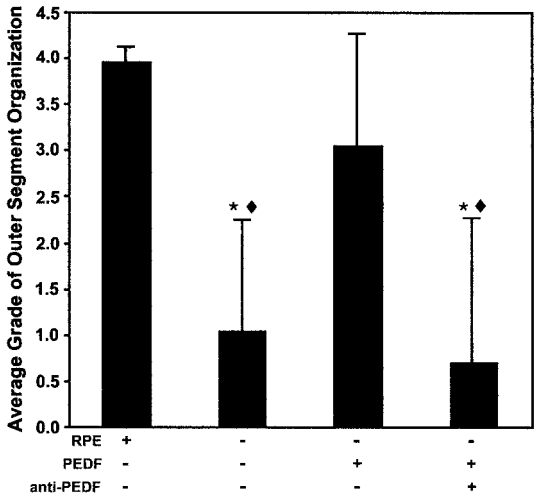

Figure 5. When allowed to complete morphogenesis in the presence of an attached RPE, the average organizational grade of individual outer segments was 3.958 ± 0.200 (mean ± SD) on a scale of −1 to 4, indicating that nearly all of the photoreceptor outer segments were 100% organized. In the absence of the RPE the average grade of photoreceptor outer segment organization decreased to 1.042 ± 1.207 (mean ± SD; $p = 0.0001$), indicating that nearly 75% of the graded outer segment material was disorganized into whorl-like or staggered profiles. In the presence of 50 ng/ml PEDF the average organizational grade was 3.042 ± 1.338 (mean ± SD), which is not significantly different from that obtained with photoreceptors that matured with an adherent RPE ($p = 0.0144$). This value indicates that ~75% of the outer segment membranes were highly structured. In the presence of anti-PEDF (1:500 dilution) the organizational grade significantly decreased to 0.708 ± 1.541(mean ± SD; $p = 0.0001$ compared with both control retinas and those exposed to PEDF), reflecting that <25% of the outer segment material was organized. The grading scale that was used is as follows: 4 = 100% organization of an individual outer segment; 3 = 75% organization of an individual outer segment; 2 = 50% organization of an individual outer segment; 1 = 25% organization of an individual outer segment; 0 = 0% organization of an individual outer segment; −1 = complete absence of outer segment membrane. Qualities of a disorganized outer segment included whorl-like and staggered profiles. $p < 0.009$ is considered statistically significant. *Significantly different from control retinas with an attached RPE; ♦, significantly different from eyes exposed to PEDF.

toreceptors are presumably cones or minor rods because of the specificity of the antibody (Stiemke et al., 1994) (Fig. 6*a*). The labeling was very dense and evenly aligned, suggestive of organized, stacked outer segment membranes. The dysmorphogenesis of nascent outer segments, induced by the removal of the RPE, also was reflected in the immunolocalization pattern of opsin (Fig. 6*b*). Opsin immunolabeling appeared to be reduced in quantity and altered in conformation. Individual outer segment profiles could not be distinguished. Patches of immunopositive label alternating with the absence of label were present distal to the inner segments, similar to the outer segment contours seen in Figures 2*b* and 3*b*. Some areas of immunolabeling were out of the plane of focus, reflecting in three dimensions the whorls of membrane rather than the tight cylinders of outer segment that are evident in the presence of the RPE. Opsin trafficking appeared to be normal in that no opsin label was detected in other areas of the cell, including the synaptic terminus.

In the absence of the RPE, 50 ng/ml of PEDF promoted the formation of well organized outer segment membranes and allowed for normal immunolabeling patterns of opsin (Fig. 6*c*). In PEDF-protected retinas opsin labeling was very similar to that of control retinas. Individual outer segment profiles with linear labeling patterns were evident, consistent with the organized outer segment membrane disc lamellae that were observed morphologically (compare with Figs. 2*c,* 4*a*). The addition of anti-PEDF (1:500 dilution) disrupted the evenly profiled opsin immunolabeling patterns that were permitted by PEDF (Fig. 6*d*). An uneven jagged labeling pattern was present, somewhat similar to that of photoreceptors induced to degenerate by removal of the RPE (compare with Fig. 6*b*).

Quantitation of opsin

Figure 7 illustrates the average levels of opsin expression detected chemifluorescently. The overall F test for differences among the four groups was highly significant ($F = 20.38$; $p = 0.0004$). In eyes undergoing degeneration induced by RPE removal, there was a significant decrease in the steady-state level of opsin to ~70% of control retinas maintained with an adherent RPE ($p = 0.0001$; Fig. 7*a,b*). The addition of 50 ng/ml of PEDF prevented the decrease in steady-state opsin levels and allowed opsin expression to reach levels that were not significantly different from retinas maintained with an intact RPE ($p = 0.3938$ compared with retinas with an intact RPE; $p = 0.0003$ compared with RPE-deprived retinas). When anti-PEDF was added to block the organizational effect of PEDF, a significant reduction in opsin steady-state expression to ~86% of control values was noted ($p = 0.0088$). This value was not significantly different from that obtained from retinas exposed to PEDF alone ($p = 0.0347$).

DISCUSSION

Although effective medical or surgical strategies have been developed to treat very highly prevalent conditions such as cataracts, glaucoma, or diabetic retinopathy, very few therapeutic options are

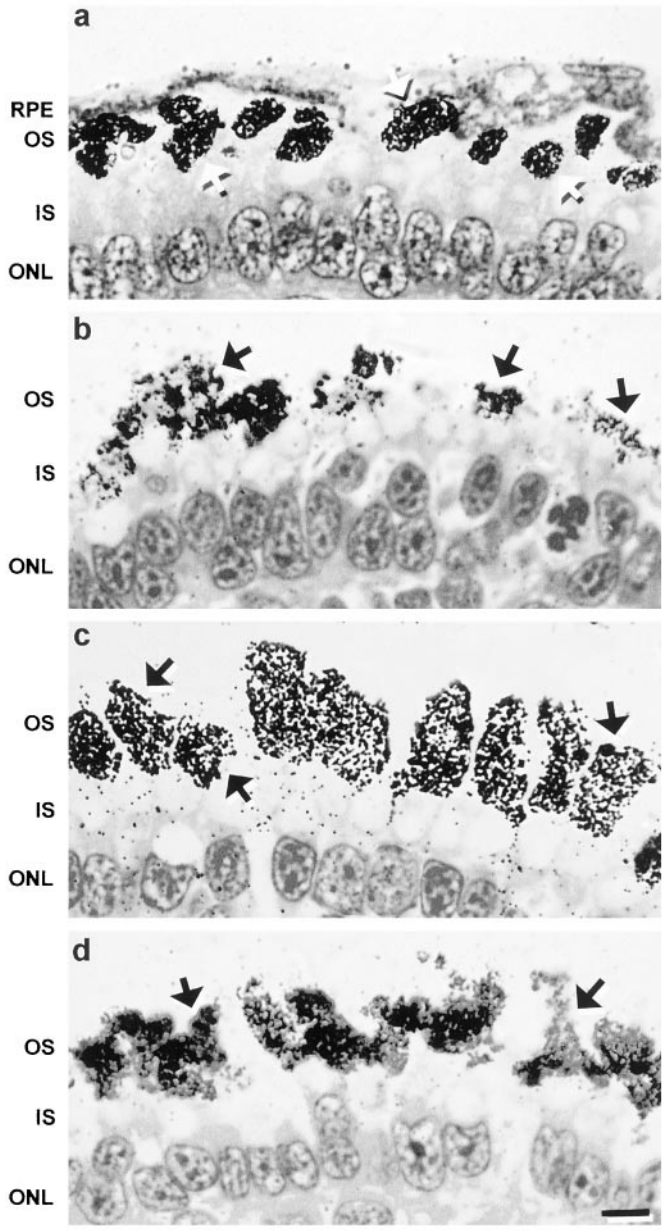

Figure 6. PEDF prevented the altered opsin immunolabeling patterns induced by the removal of the RPE. *a,* In retinas that completed morphogenesis with an adherent RPE, the opsin labeling was very heavy over photoreceptor outer segments, which was indicative of organized, stacked outer segment membranes. *b,* Removal of the RPE resulted in an altered immunolocalization pattern of opsin in which individual outer segment profiles could not be distinguished. Rather, heavy patches of label alternating with a complete lack of immunopositive label were present distal to the inner segments. *c,* In the absence of the RPE, PEDF promoted the formation of well organized outer segment membranes. Individual outer segment profiles with linear opsin immunolabeling patterns were evident, consistent with the organized outer segment membrane disc lamellae that were observed morphologically. *d,* The addition of anti-PEDF disrupted the linear and evenly profiled opsin immunolabeling patterns. Rather, an uneven jagged labeling pattern was present. *RPE*, Retinal pigment epithelium; *OS*, outer segment; *IS*, inner segment. Scale bar, 10 μm.

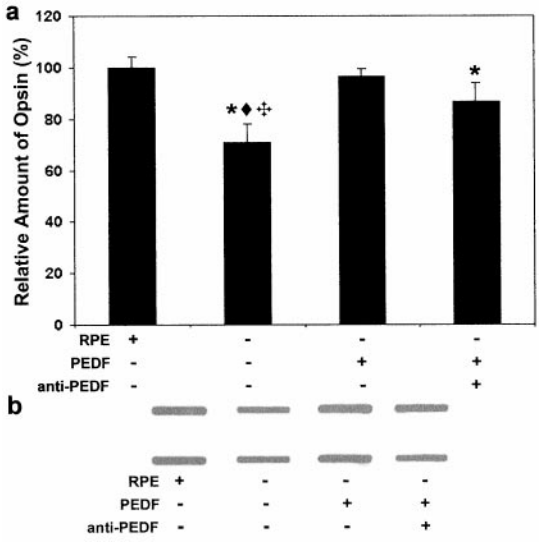

Figure 7. PEDF promoted opsin expression. *a,* Relative amounts of opsin in control, dysmorphic, and PEDF-supported photoreceptors. Values were normalized to the amounts of opsin in control retinas with an attached RPE. In the absence of the RPE the photoreceptors downregulated the expression of opsin to 70% of that synthesized in the presence of the RPE ($p = 0.0001$). PEDF prevented the downregulation induced by RPE detachment and sustained opsin expression ($p = 0.3938$ compared with control eyes; $p = 0.0003$ compared with eyes without an attached RPE). Anti-PEDF partially blocked the permissive effect of PEDF on opsin synthesis ($p = 0.0088$ compared with control eyes). $p < 0.009$ is considered statistically significant. *Significantly different from control retinas with an attached RPE; ♦, significantly different from eyes exposed to PEDF; ✣, significantly different from PEDF plus anti-PEDF antibody. *b,* Representative immunoblot illustrating the differences in opsin expression under the various experimental conditions.

available to prevent, reverse, or halt visual loss caused by retinal detachment, dysfunction linked to RPE-specific genetic mutations, or age-related macular degeneration. The evaluation of putative neuroprotective agents on animal models of retinal degeneration is fairly advanced, although the results of these studies are still somewhat controversial; therefore, direct applicability to the human condition is unclear. A landmark study by the LaVail and Steinberg groups (Faktorovich et al., 1990) was the first to indicate that growth factors normally present in the retinal microenvironment are able to rescue photoreceptors from degeneration in the RCS rat. Their work has expanded in recent years to explore the effects of multiple growth factors (or combinations thereof) on various forms of mutant rodents with inherited retinal degenerations that are similar to human mutations (LaVail et al., 1998). Their results indicate that varying degrees of rescue are achieved with some, but not all, neuroprotective agents. In addition, the effect is not universal in all mutant rodent strains. In other forms of retinal degeneration it has been documented that ciliary neurotrophic factor (Axokine) rescued photoreceptors from degeneration in the *Rdy* cat, whereas brain-derived neurotrophic factor had no effect (Chong et al., 1999). In a model of retinal detachment the brain-derived neurotrophic factor protected surviving photoreceptors and perhaps stimulated regeneration of the outer segments (Lewis et al., 1999).

PEDF is a 50 kDa glycoprotein that was isolated initially from medium conditioned by human fetal RPE cells (Tombran-Tink and Johnson, 1989), but it since has shown a wider distribution in various tissues of the eye and other parts of the CNS (Ortego et al., 1996; Tombran-Tink et al., 1996). PEDF has not, however, been demonstrated to be present in cells of the neural retina (Tombran-Tink et al., 1995). The PEDF gene shares structural and sequence homology with members of the serpin gene family, yet inhibitory activity has not been associated with its function and little homology is seen between the conserved active domain of most serpins and PEDF (Becerra et al., 1995). Morphological and biochemical changes evident in neuronal precursor cells after treatment with PEDF include extensive neurite outgrowths and the upregulation of neuron-specific enolase and neurofilament proteins. Numerous studies have documented physiological functions of PEDF in a variety of tissues, including protection of retinal neurons against hydrogen peroxide-induced

cell death (Cao et al., 1999); promotion of the survival of cultured cerebellar granule cells (Taniwaki et al., 1995); delay of photoreceptor cell death in mouse models of hereditary retinal degenerations (Cayouette et al., 1999); and protection against glutamate-induced neurotoxicity of motor neurons (Bilak et al., 1999), cerebellar granule cells (Taniwaki et al., 1997), and hippocampal neurons (DeCoster et al., 1999). In addition, PEDF differentially protects immature, but not mature, cerebellar neurons against apoptosis (Araki et al., 1998). More recently, PEDF has been demonstrated to be a major component of the ocular vitreous and aqueous humor where it has been implicated as a potent inhibitor of angiogenesis (Dawson et al., 1999).

The PEDF glycoprotein is secreted in abundance by the RPE cells into the interphotoreceptor matrix where it binds to glycosaminoglycans that fill the extracellular space between photoreceptor outer segments and the RPE (Tombran-Tink et al., 1995; Wu et al., 1995), placing this molecule in a prime physical location to affect the underlying neural retina and in particular the photoreceptors. The functional role of PEDF in retinal development and histogenesis is not fully known. Although it has been demonstrated that PEDF is synthesized early in human development (i.e., 17 weeks in gestation; Tombran-Tink et al., 1995), its precise role for its early expression is yet to be delineated. In addition, the gene for PEDF has been tightly linked to the RP13 locus on the short arm of chromosome 17 (17p13.3), implicating it as a candidate gene for one form of autosomal dominant retinitis pigmentosa (Tombran-Tink et al., 1994; Goliath et al., 1996). The disease manifestations in this retinal degeneration are reported to be consistently aggressive, with restricted visual fields and night blindness as early as four years of age (for review, see online source: Mendelian Inheritance in Man. http://www.ncbi.nlm.nih.gov/omim/). This is somewhat uncommon for an autosomal dominant disease. In retinitis pigmentosa caused by rhodopsin mutation this phenomenon is observed more commonly with mutations that disrupt molecular pathways of critical relevance to photoreceptor function and survival (Iannaccone, 1998).

In the present study we document for the first time that PEDF exerts a very powerful morphogenetic effect on photoreceptors. At the time of removal from the embryo to culture the photoreceptors had not yet completed morphogenesis. Therefore, virtually all outer segment membranous material was elaborated while in culture under all of the experimental conditions (Stiemke et al., 1994). We predict that in our experimental preparation the removal of the RPE cells eliminated the source of PEDF for retinal photoreceptor cells. The lack of PEDF stimulation affected negatively the photoreceptor development. Supplementation of the medium with exogenous PEDF (50 ng/ml) closely mimicked the presence of the RPE and supported the final stages of photoreceptor cytogenesis. PEDF permitted the assembly of photoreceptor nascent outer segment membranes into stacked, flattened membranous saccules surrounded proximally by calycal processes. In addition, it stimulated photoreceptor protein expression, as measured by means of opsin quantification.

The average level of outer segment organization in PEDF-treated retinas was significantly greater than in RPE-deprived ones and not significantly different from control retinas, although in absolute quantitative terms it was attenuated somewhat. These findings, along with the effect on opsin expression levels, are especially important on biological grounds. It is well established that the integrity of the photoreceptor outer segment is indispensable for vision to take place and that disorganization of the outer segment is associated with degeneration of that same photoreceptor cell and loss of sight. Indeed, a disruption of photoreceptor outer segments is common to both human (Green et al., 1985; Farber et al., 1987; Rodrigues et al., 1987; Birnbach et al., 1994; Li et al., 1994, 1995; Milam et al., 1998; Green, 1999) and animal models (LaVail et al., 1972, 1975, 1982, 1985; LaVail and Sidman, 1974; LaVail and Mullen, 1976a,b; Edwards and Szamier, 1977; Carter-Dawson et al., 1978; LaVail, 1979; Travis et al., 1989, 1991; Narfström et al., 1991; Pittler and Baehr, 1991; Ma et al., 1995; Cheng et al., 1997; Kohl et al., 1998; Kennedy et al., 1999; Redmond et al., 1999) of various types of retinal degenerations. Although the organization of the outer segment is critical, other studies have demonstrated that visual acuity can be preserved despite some loss of photoreceptors. Elegant psychophysical experiments by Geller et al. (1992) have demonstrated that 75% integrity of the sampling elements across degenerate arrays leaves grating visual acuity completely intact. The ability of the human eye to compensate for significant losses of photoreceptors before a measurable drop in visual acuity occurs was also noted previously by Sandberg and Berson (1983). By analogy, our results suggest that the organizational effects of PEDF on photoreceptor outer segments may allow for the preservation of an intact visual potential in treated retinas. Notwithstanding the differences between our experimental conditions and the *in vivo* state, the potential therapeutic implications of this observation are self-evident. Our results may be extended to support the plausible use of PEDF as a therapeutic option to protect against photoreceptor degeneration induced by retinal detachment in addition to various dystrophic conditions of the RPE.

In summary, our results demonstrate the critical role PEDF plays during the final stages of photoreceptor morphogenesis. We have demonstrated that PEDF prevented the dysmorphic photoreceptor changes that were induced by RPE removal. Under these experimental conditions exogenously added PEDF supported normal levels of opsin expression and permitted the proper assembly of nascent outer segment membranes in RPE-deprived, but otherwise intact, eyes from *Xenopus laevis* embryos.

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