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Circadian clocks regulate adenylyl cyclase activity rhythms in human RPE cells ☆

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Abstract

Genes and components of the circadian clock may represent relevant drug targets for diseases involving circadian dysfunctions. By exploiting an established cell line derived from human retinal pigment epithelium (HRPE), the cell constituting the blood-retinal barrier that is essential to maintain the visual functions of the sensorineural retina, we showed serum-shock induction of rhythmic changes in forskolin-evoked adenylyl cyclase (AC) activity. In the presence of Ca^{2+} and protein kinase A, the forskolin-induced AC activity is significantly, but not completely inhibited, suggesting the involvement of both Ca^{2+}-sensitive and Ca^{2+}-insensitive AC isoforms in the regulation of circadian rhythmicity in these cells. Semi-quantitative RT-PCR showed circadian profile in the expression of three AC isoforms, the Ca^{2+}-inhibitable AC5 and AC6 and the Ca^{2+}-insensitive AC7, and the clock genes *hPer1* and *hPer2*. Our results demonstrate for the first time circadian rhythmicity in a human cell line, identifying the isoforms involved in the circadian profile of AC activity and showing a rhythmicity of the clock gene mRNA expression in these cells. Therefore, the results reported here provide evidence for an intertwine between AC/$[Ca^{2+}]_i$ signalling pathways and *Per* genes in the HRPE circadian clockwork.

Keywords: Adenylyl cyclase; Forskolin; PKA; Circadian clock; *Per* genes; HRPE cells

The circadian clock regulates daily oscillations in a variety of biochemical, physiological, and behavioral processes. The basic molecular clockwork underlying the generation of circadian rhythms in mice consists of interwoven positive and negative transcriptional–translational feedback loops [1]. At the core of the molecular clock lie different clock genes such as *Per*, *Cry*, *Clock*, and *Bmal1* [1]. Reports about circadian clock gene expression in humans are scarce [2,3]. A recent investigation provides evidence that human *Per2* expression is acutely stimulated by light exposure [4]. The expression of circadian clock genes and their rhythmic regulation are not limited to the brain but they are widely distributed in peripheral tissues [5]. Interestingly, circadian gene expression is also present in immortalized cultured fibroblast, and it can be triggered *in vitro* by various stimuli such as a serum-shock [6,7].

☆ *Abbreviations:* $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; AC, adenylyl cyclase; ANOVA, analysis of the variance; CaM, calmodulin; cAMP, 3′,5′-cyclic adenosine monophosphate; CREB, CRE-binding protein; CREs, cAMP-responsive elements; DEPC, diethyl-pyrocarbonate; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, Ethylene glycol-bis-(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid, FBS, fetal bovine serum; FSK, forskolin; Hepes, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; HRPE, human retinal pigmented epithelium; PBS, phosphate saline buffer; *Per*, *period* gene; PKA, protein kinase A; PMSF, phenylmethylsulfonyl fluoride; PSG, penicillin–streptomycin–L-glutamine; RPE, retinal pigmented epithelium; SCN, suprachiasmatic nucleus.

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The retinal pigment epithelium (RPE) forms a monolayer at the back of the eye adjacent to the outer neural retina. The highly polarized and multifunctional RPE is essential for maintaining photoreceptor function and forms the major component of the blood-retinal barrier [8]. Many physiological responses of the RPE are mediated by membrane-bound G-protein coupled receptors which modulate the function of the cell through $[Ca^{2+}]_i$ and adenylyl cyclase (AC)/cAMP signalling pathways [9]. Among the cAMP-modulated retina-maintaining functions of the RPE, the phagocytosis of shed rod outer segment discs occurs in a circadian manner [10].

In the present study, we characterized the circadian variation of forskolin (FSK)-stimulated AC activity in a well-established human RPE cell line. Furthermore, we investigated the circadian mRNA expression of three Ca^{2+}-inhibited/insensitive AC isoforms and of two core clock genes, *hPer1* and *hPer2*. Our findings reported here provide new insights into the molecular mechanism of circadian rhythm generation using a human cultured cell line.

Materials and methods

Cell culture and serum-shock procedures. HRPE cells were cultured in DMEM supplemented with 10% FBS (Invitrogen, Milan, Italy) and 1% Penicillin–Streptomycin–L-Glutamate (PSG) (Invitrogen, Milan, Italy) at 37 °C in 5% CO_2. For extraction of cellular total RNA, cells were seeded in 24-well plates to reach confluence after about 4 days. Serum-shock was performed as previously described [6]. Briefly, at time 0, the culture medium was exchanged with serum-rich medium (DMEM + PSG, supplemented with 50% v/v horse serum; Invitrogen, Milan, Italy). After 2-h incubation at 37 °C, the serum-rich medium was replaced with pre-warmed serum-free DMEM + PSG for the rest of the duration of the experiment. At various times indicated in the Figure legends, cells were harvested in 250 μl of TRIzol reagent (Invitrogen, Milan, Italy), frozen and stored at −80 °C. RNA was later isolated according to the manufacturer's protocol. For the preparation of crude cell membranes, cells were plated in 150 cm^2 Petri-dishes and treated with serum-shock as described above. After removal of serum-free medium, cells were rinsed with 10 ml ice-cold PBS (Invitrogen, Milan, Italy) and collected by scraping in 10 ml ice-cold 10 mM Tris–HCl, pH 7.5, containing 1 mM EDTA (Sigma, Milan, Italy), 1 mM DTT (Sigma, Milan, Italy) and a cocktail of protease inhibitors (10 μM aprotinin, 10 μM leupeptin and 1 mM PMSF; Sigma, Milan, Italy). The cells were subsequently homogenized with ULTRA TURRAX (IKA-Labortechnik, Germany) and centrifuged at 18,000*g* for 30 min. The pellet obtained was resuspended in 50 mM Tris–HCl, pH 7.5, containing 5 mM $MgSO_4$ and 1 mM EGTA, and stored at −80 °C until the AC activity assay. Membrane protein content was measured by the method described by Lowry et al. [11]. Both RNA isolation and crude cell membrane preparation have been repeated in three independent experiments.

AC activity assay. AC activity was assayed as previously described, with slight modifications [12]. Briefly, AC activity was assayed in a 400 μl reaction mixture containing an "intracellular saline buffer", composed by 25 mM Hepes–Tris, pH 7.3, 140 mM KCl, 14 mM NaCl, 4 mM $MgSO_4$, and 100 nM $CaCl_2$, then 500 μM GTP, and 100 μg of membrane protein. In addition, the reaction mixture also contained 100 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma, Milan, Italy), used as an inhibitor of phosphodiesterases (PDE), and 10 μM FSK, used as an activator of AC. Where specified, 2 μM calmodulin (CaM; Sigma, Milan, Italy) was added. Membranes were pre-incubated in the above reaction mixture for 20 min at room temperature with or without 100 U/ml PKA catalytic subunit (Sigma, Milan, Italy) as reported by Chen et al. [13]. The adenylyl cyclase reaction was then initiated by the addition of 500 μM ATP (Sigma, Milan, Italy). The reaction was carried out by incubating the reaction mixture at 30 °C for 10 min, following which the reaction was stopped by placing the tubes in boiling water bath for 2 min. The tubes were centrifuged to remove membrane debris and cAMP in the clear supernatant was measured by an assay based on competition between [^{3}H]cAMP (Perkin-Elmer Life Sciences, Monza, Italy) and unlabeled cAMP in the sample for interaction with cytosolic cAMP-binding protein, prepared from bovine adrenal cortex, according to the method of Brown et al. [14]. The basal and FSK-stimulated enzyme activity was linear up to 30 min with protein concentration up to 100 μg. All samples were assayed in duplicate.

Semi-quantitative RT-PCR. Total RNA was treated with DNase I (Invitrogen, Milan, Italy) and used in oligo (dT)-primed first strand cDNA synthesis (SuperScript II RT, Invitrogen, Milan, Italy). Relative amounts of *AC5*, *AC6*, *AC7*, *Per1*, and *Per2* mRNAs were determined by semi-quantitative RT-PCR as described before [15]. Primers and PCR conditions are reported in Table 1. The PCR products were electrophoresed on an ethidium bromide-containing 1.5% agarose gel and the intensity of the cDNA bands relative to background was determined using Quantity One system (Bio-Rad, Hercules, USA). The linear range of PCR was empirically determined in initial experiments whereby PCR was set up with each primer set with different number of amplification cycles (18–40 cycles). The relationship between the intensity of the signal and the PCR cycle number was analyzed to determine the linear range for the PCR product formation. The intensities of the signals within the linear range were used for data analysis. The *AC*s amplified linearly between cycles 30 and 38 and *Per*s between cycles 28 and 34. *GAPDH* (linear amplification between 22 and 28 cycles) was used for normalization of target gene values

Table 1

Gene	Primer sequences	Primer site	Product length (bp)	T_a (°C)	*n*° of cycles	GenBank Accession No.	Ref.
AC5	Forward 5′-tccacatcaccaaggctaca-3′ Reverse 5′-cttgtccttggggtcttcaa-3′	1811–2112	302	56	32	NM_183357	[29][a]
AC6	Forward 5′-tcgggcaacactgcagtacctg-3′ Reverse 5′-cccgagtccgctgcagcttg-3′	1551–1720	170	53	32	NM_015270	[30]
AC7	Forward 5′-ctctctgtgctgatgtacgtcgag-3′ Reverse 5′-tcttgacgtagaggctgtggaagt-3′	235–820	586	53	32	NM_001114	[30]
Per1	Forward 5′-catccattcgggttacgaag-3′ Reverse 5′-ttgttgatagccagcatgag-3′	1029–1229	201	56	30	NM_002616	[29][a]
Per2	Forward 5′-ccacgagaatgaaatccgct-3′ Reverse 5′-cctcccaatgatgaaggaga-3′	837–1293	457	57	30	NM_022817	[29][a]
GAPDH	Forward 5′-gatgacatcaagaaggtggtgaagc-3′ Reverse 5′-ttcgttgtcataccaggaaatgagc-3′	766–951	186	62	24	NM_002046	[29][a]

PCRs were subjected to the following temperature cycles: 94 °C for 30″, T_a for 30″,and 72 °C for 45″. T_a, temperature of annealing.

[a] Designed by Primer3 software [29].

throughout experiments because its level did not vary significantly in function of time or serum-shock [16]. At the fixed PCR conditions, the amount of all PCR products showed a linear correlation with the amount of cDNA added to the PCR mixture; so ratios of *AC* and *Per* mRNA to *GAPDH* mRNA remain virtually constant. For an internal PCR control, we used either RNA as template (control for DNA carryover) or DEPC-treated water instead of cDNA (non-template control). Reproducibility of the amplitude (ratio of peak to trough) and phase confirmed that semi-quantitative RT-PCR was capable of detecting temporal changes of *AC* and *Per* gene expression in HRPE cells.

Data analysis. All results were expressed as means ± SEM. Treatments, sampling times, and their interaction were tested by one- or two-way ANOVA. A value of $p < 0.05$ was considered statistically significant. Bonferroni's test was applied for post hoc comparison. Data were analyzed using the software STATISTICA 5.5 (StatSoft Inc., Tulsa, USA).

Results and discussion

Several recent studies have shown that cultured mammalian cells, such as the immortalized murine fibroblasts, have the potential for periodicity [6]. Treatment of these cells with serum-rich medium has been shown to elicit the circadian expression of the clock genes *Per1* and *Per2* [6,7,16]. Interestingly, this effect is similar to the effect light has on the expression of these genes in mouse suprachiasmatic nucleus (SCN), the site where the master circadian pacemaker controlling behavioral and hormonal circadian rhythmicity resides [17]. In studies using rat-1 fibroblasts, treatment of the cells with forskolin induced the circadian expression of *Per1* and *Per2*, suggesting the involvement of AC and cAMP in the regulation of circadian rhythm [16]. Previous studies using rat heart [18] and forebrain [19] have shown circadian rhythms in AC activity, further demonstrating a role for ACs in the regulation of rhythmicity. Furthermore, a circadian rhythm in AC activity under the control of an endogenous pacemaker in an achlorophyllous ZC mutant of the algae *Euglena gracilis* has also been described [20]. However, nine AC isoforms have been identified and cloned so far which differ in their regulation by $\alpha/\beta\gamma$ subunits of the heterotrimeric G-proteins, sensitivity to Ca^{2+}, and phosphorylation [21]. It is important to identify which of these isoforms is involved in the regulation of circadian rhythm.

In the present investigation, using HRPE cell line as a model system, a direct measurement of AC activity in membranes prepared from serum-shocked cells has been performed and the isoform identity of the ACs involved in the activation of the clock genes has been established. Two-way ANOVA indicated that variation in the AC activity significantly depended on membrane treatment ($p < 0.0001$) and sampling time ($p < 0.0001$). Membranes were incubated in buffer adjusted for intracellular ionic composition, in the presence or in the absence of 100 nM Ca^{2+}, to approximate $[Ca^{2+}]_i$ conditions. Under both conditions, the inhibitor of PDE, IBMX (100 μM), was present. AC activity was significantly stimulated by FSK, a diterpene activator of ACs, which was inhibited at all times by 100 nM Ca^{2+} ($p < 0.05$–0.001, Bonferroni's test, two-way ANOVA; Fig. 1). The presence or absence of CaM (2 μM) made no difference (data not shown). This suggests that the Ca^{2+}/CaM-stimulated AC1, AC3, and AC8 [21] have no role in the regulation of rhythmicity in HRPE cells.

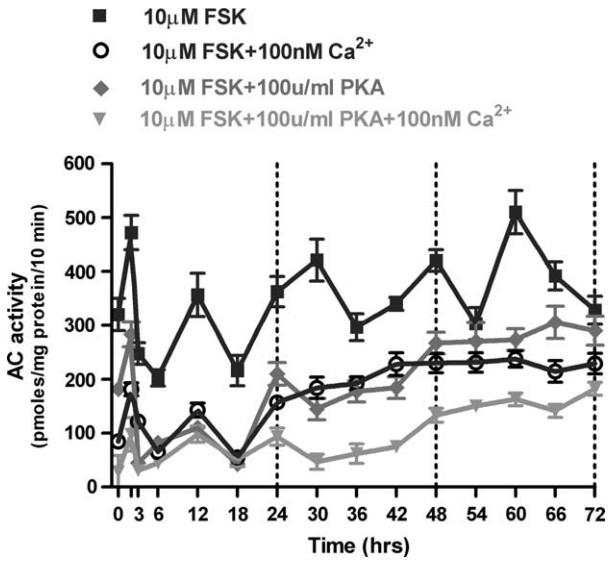

Fig. 1. Circadian patterns of AC activity in HRPE cell membranes. Membranes were prepared from cells collected at the indicated times. The 50% v/v serum-shock synchronization was done between $t = 0$ and 2 h. Data are expressed as pmoles cAMP/mg protein/10 min. Mean ± SEM at each time point obtained in three independent experiments is shown. Synthesis of cAMP was unaffected by 100 nM Ca^{2+} under unstimulated conditions (data not shown).

In the absence of Ca^{2+}, the FSK-stimulated AC activity underwent a pronounced circadian pattern up to 72 h ($p < 0.0001$, one-way ANOVA), with a period length shorter than 24 h (Fig. 1). On the other hand, in cell membranes incubated in intracellular buffer containing Ca^{2+}, which more closely reflects the intracellular environment, FSK-stimulated AC activity seems to maintain a circadian rhythm only during the initial 24 h ($p < 0.01$, Bonferroni's test, one-way ANOVA). The Ca^{2+}-insensitive AC activity in the next 24–48 h period is significantly high ($p < 0.05$, Bonferroni's test; two-way ANOVA), however, it does not exhibit a circadian profile ($p > 0.05$, Bonferroni's test, one-way ANOVA) (Fig. 1). These results suggest a role for both Ca^{2+}-sensitive and Ca^{2+}-insensitive ACs in the regulation of circadian rhythm in HRPE cells.

Since a submicromolar concentration of calcium is able to impair the circadian oscillations of FSK-evoked AC activity, we hypothesized that Ca^{2+}-inhibitable AC5 and AC6 could be the main isoforms fulfilling this circadian pattern. The involvement of AC5 and AC6 in the regulation of circadian rhythm was further confirmed using PKA. Previous studies have shown that PKA-mediated phosphorylation inhibits AC5 and AC6 activity in a feedback manner [12,22]. We measured AC activity in HRPE cell membranes following treatment with the purified catalytic subunit of PKA (Fig. 1). Membranes without PKA treatment were used as control. Pre-treatment with 100 U/ml PKA impaired oscillations of FSK-evoked AC activity in a manner similar to that seen with 100 nM Ca^{2+} ($p < 0.01$;

Bonferroni's test; two-way ANOVA). This impairment was further enhanced in the presence of 100 nM Ca^{2+} ($p < 0.001$; Bonferroni's test; two-way ANOVA; Fig. 1). Our results thus demonstrate very clearly a major and extended role for the Ca^{2+}-inhibitable AC5 and AC6 and a limited role, especially during the early stages, for Ca^{2+}-insensitive ACs. These results are similar to the results obtained by Chern et al [23] in membranes of rat striatum, who reported a circadian fluctuation in Ca^{2+}-inhibitable AC activity in these membranes.

Since it has been reported that the Ca^{2+}-insensitive *AC7* is the predominant isoform expressed in mammalian RPE [24], we next investigated the expression levels of *AC7*, and the Ca^{2+}-inhibited *AC5* and *AC6*, at the mRNA level by semi-quantitative RT-PCR. Both *AC5* and *AC6* mRNA expression showed a clear circadian oscillation from 24 h up to 72 h after serum stimulation ($p < 0.001$; one-way ANOVA), whereas *AC7* displayed a temporal fluctuation only during the first 30–36 h ($p < 0.01$; one-way ANOVA; Fig. 2). The expression of all *AC*s showed similar circadian periods which were shorter than 24 h. Thus, the expression pattern of *AC5*, *AC6* and *AC7* transcripts was similar to the circadian behavior of Ca^{2+}-sensitive and Ca^{2+}-insensitive FSK-evoked AC activity measured in HRPE cells during the 72 h following serum-shock (Fig. 1).

To further confirm the existence of circadian oscillators in the HRPE cells, we investigated the effect of serum-shock on the expression of *hPer1* and *hPer2*. Human and mouse *Period* promoters contain cAMP-responsive elements (CREs) that bind CRE-binding protein (CREB) and CREB phosphorylation is believed to be the main pathway to reset the clock by a light pulse [25,26]. We measured the levels of *hPer1* and *hPer2* by semi-quantitative RT-PCR. The results obtained show that serum-shock induced the rhythmic expression of *hPer1* and *hPer2* mRNAs in HRPE cells ($p < 0.0001$; one-way ANOVA; Fig. 3). The period length of the rhythmicity of the expression of both genes was close to the 24 h. The mRNA levels of *hPer2* showed an increase at 1 h, a decline from 6 to 18 h, and then exhibited a rhythm progressively dampened during the last 48 h in culture (Fig. 3). The levels of *hPer1* transcripts showed a similar time-dependent variation, however the expression seemed to be stronger during the later time points. In serum-shocked murine fibroblast [6,7,16], unlike what we observed in HRPE cells, *mPer1* level is first induced and then repressed. After 12 h the mRNA level rise again then assumed a strong circadian rhythmicity. Slight differences in the expression profile between human and murine *Period* genes could depend on the transcription regulatory regions in the promoter and/or on the cellular model used [27].

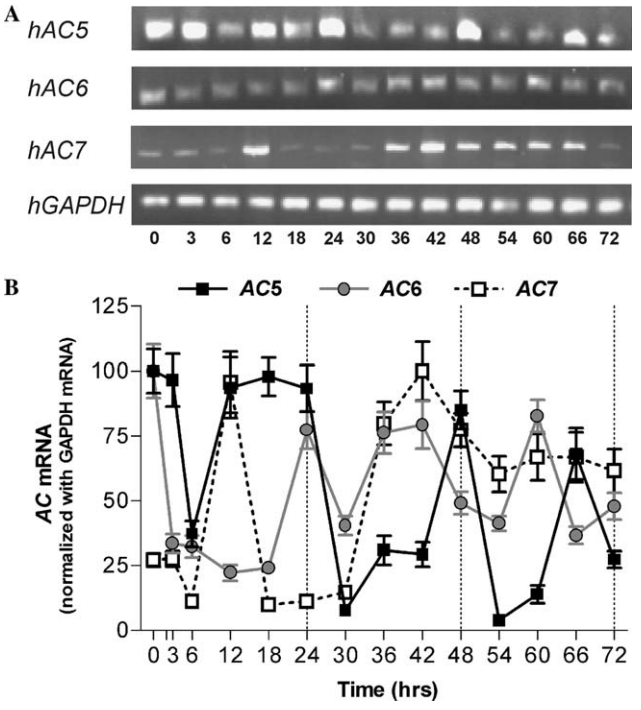

Fig. 2. Circadian gene expression of *AC5*, *AC6*, and *AC7* in HRPE cells induced by serum-shock. (A) Semi-quantitative RT-PCR analyses of *AC5*, *AC6*, and *AC7* mRNAs. HRPE cells were treated with serum-rich medium to induce serum-shock between $t = 0$ and 2 h. Total RNA was extracted at the indicated times. *GAPDH* was used as loading control. Ethidium bromide-stained gel pictures are representative of typical experimental results obtained in three independent experiments. (B) *AC* mRNA expression was normalized on the basis of *GAPDH* expression. Data of each *AC* gene are plotted against sampling times. Mean ± SEM at each time point obtained in three independent experiments is shown.

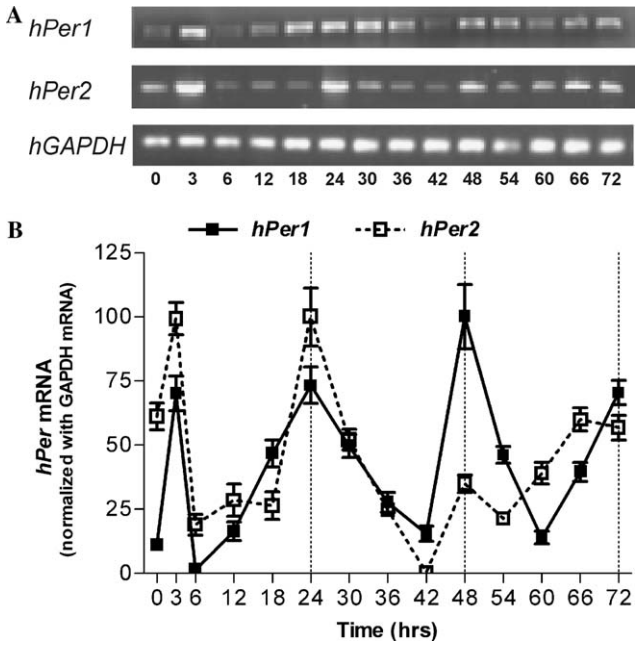

Fig. 3. Circadian gene expression of *hPer1* and *hPer2* in HRPE cells induced by serum-shock. (A) Semi-quantitative RT-PCR analysis showing the circadian expression of *hPer1* and *hPer2* mRNAs. For more details, see Fig. 2. (B) *hPer1* and *hPer2* mRNA expression was normalized on the basis of *GAPDH* expression. Data of each *Per* gene are plotted against sampling times. Mean ± SEM at each time point obtained in three independent experiments is shown.

Taken together, we report here for the first time, the existence of circadian rhythms in AC activity, both Ca^{2+}-inhibitable AC5–AC6 and Ca^{2+}-insensitive AC7, in human RPE cells. While AC5 and AC6 show extended rhythmicity, the rhythmicity of AC7 is seen only during the initial 24 h. Though Ca^{2+}-insensitive AC7 activity and expression is high during the later time points too, its circadian rhythmicity is dampened. Thus, our results show that in HRPE cells $[Ca^{2+}]_i$ differentially modulates AC activity in a rhythmic fashion, probably due to periodic changes in the expression of AC isoforms. The availability of an *in vitro* cellular system would greatly facilitate the molecular knowledge of the human RPE circadian clock, which is known to regulate, together with light, the phagocytosis of the distal tip of the photoreceptors' outer segment. Notably, impairment of the RPE circadian clockwork could play a significant role in the pathogenesis of many retinal degenerative diseases [28].

Acknowledgments

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