• Basic Research •

Inhibition of cell proliferation, migration and apoptosis in blue-light illuminated human retinal pigment epithelium cells by down-regulation of HtrA1

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Abstract

● AIM: To investigate the effect of HtrA1 on the proliferation, migration and apoptosis of human retinal pigment epithelium (RPE) cells in the light injured model, as well as the expression of the apoptosis related molecules.

● METHODS: The human RPE cell line ARPE-19 was exposed to blue light to establish the light injured model. The cells were transfected with HtrA1 siRNA to knockdown HtrA1 expression. Subsequent expression of HtrA1 was determined by real-time polymerase chain reaction (RT-PCR) and Western blot, respectively. Changes in cell proliferation, migration and apoptosis were assessed by cell counting kit-8 (CCK-8), Transwell assay and flow cytometry respectively, as well as changes in the mRNA and protein levels of Bax, Caspase-3 and Bcl-2 expression.

● RESULTS: HtrA1 was highly expressed in ARPE-19 cells after blue light irradiation. Knockdown of HtrA1 expression inhibited the proliferation, migration and apoptosis of the blue-light-irradiated ARPE-19 cells (P<0.05). Bax and Caspase-3 expression were significantly reduced both at mRNA and protein levels (P<0.05) after siRNA treatment. Bcl-2 expression significantly increased in blue-light-irradiated ARPE-19 cells after siRNA interference (P<0.05).

● CONCLUSION: Silence of HtrA1 may inhibit the proliferation, migration and apoptosis of ARPE-19 cells in light injured model. Moreover, HtrA1 suppression in blue-light-irradiated ARPE-19 cells may ameliorate cell apoptosis through down-regulation of Bax and Caspase-3, and up-regulation of Bcl-2 expression.

● KEYWORDS: HtrA1; retinal pigment epithelium; small interfering RNA

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INTRODUCTION

Age-related macular degeneration (AMD) is one of the main reason of non-reversible visual loss among old people in developed countries[1]. In United States alone, more than 7.2 million people suffer from AMD, and this figure will increase by 97% by the year 2050[2-3]. The characteristic of AMD is progressive loss of central vision as a result of degenerative and neovascular changes in central macular. The advanced stage of AMD includes two types, dry AMD and wet (neovascular) AMD. Geographic atrophy (GA) of the retinal pigment epithelium (RPE) and overlying photoreceptors are the main character of dry AMD[4]. Over 85% of cases of AMD are dry AMD, and about 20% of cases of legal blindness own to GA[5]. Wet AMD can be treated with inhibitors of vascular endothelial growth factor (VEGF)[6]. GA is not yet treatable[7]. The etiology of AMD and GA currently are largely unclear. Previous studies have demonstrated RPE plays an important role in the pathogenesis of AMD. A death or degeneration of RPE leads to damage of neurosensory retina, which contributes to the visual loss in both type of advanced AMD. RPE cells are sensitive to visible light, especially blue light. The pathological process of RPE cell induced by light is similar with retinal degenerative diseases, including AMD[8]. In addition, it has been reported that excessive light irradiation, especially blue light, can induce the apoptosis in RPE cells and trigger the onset of AMD[9-10]. Therefore, RPE cell damage induced by blue light is a suitable model for advanced AMD study.

HtrA1 is a member of the high-temperature requirement (HtrA) family, which is firstly found in SV40-transformed fibroblasts and plays a significant role in protecting cells from stress conditions such as inflammation, ischemia and cancer and so on[11]. It includes a N-terminal peptide, and a C-terminal HtrA domain[12]. HtrA1 is highly expressed in diverse tissues including skin, placenta, the female reproductive system, liver, pancreas and cardiovascular[13]. Moreover, current whole-genome studies have revealed that the HtrA1 gene is

strongly connected with sensibility to AMD and the putative gene associated with the pathogenesis of both dry and wet AMD[14-15]. HtrA1 is found up-regulated in drusen, abnormal RPE, and choroidal neovascularization in AMD eyes[16]. HtrA1 overexpression in mouse RPE shows similar choroidal vascular abnormalities in AMD[17]. Besides, HtrA1 is implicated in apoptosis and anoikis[18]. HtrA1 down-regulates TGF-β signaling and inhibits extracellular matrix proteins, which modulate cell senescence or death[19]. These findings indicate that HtrA1 play a contributory role in pathogenesis of AMD. Despite its suspected role in the AMD, the exact mechanisms of HtrA1 action are largely unknown.

In our study, we knocked down HtrA1 gene expression by small interfering RNA (siRNA) to observe cell proliferation, migration and apoptosis of RPE cell line ARPE-19 in blue light injury model, as well as the expression of the apoptosis related molecules, such as Bax, Caspase-3 and Bcl-2. Our study might reveal the effects of HtrA1on the AMD in the light injured model, which could provide a better understanding of molecular mechanism of AMD and other retinal degenerative diseases associated with HtrA1.

MATERIALS AND METHODS

Cell Culture and Transfection The human RPE cell line ARPE-19 was obtained from the China Center for Type Culture Collection (Beijing, China). Cell line was cultured in Dulbecco's modified Eagle medium (DMEM), Ham's F-12 nutrient mixture (Gibco-Invitrogen, Beijing, China) with 10% fetal bovine serum (FBS, Gibco-Invitrogen, Beijing, China), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma, Louis, MO, USA) at 37℃ in an atmosphere of 5% CO_2 with humidity. According to Cai and Yan's[20] and our previous study, ARPE-19 cells were irradiated to blue light (400 nm) at the intensity of (2000±500) lx for 6h to establish the light injured model. The ARPE-19 cells in light injured model were transiently transfected by lipofectamine (Invitrogen, Carlsbad, USA) based on the manufactures instructions. The blue-light-irradiated cells were incubated at a density of 5×10^5 cells/well in six-well plates. The cells reached a confluence of 40% after 24h incubation. Before transfection, the siRNA was mixed with 5 μL Lipofectamine 2000 and serum-free DMEM in a final concentration of 100 nmol/L and then was incubated for 20min at room temperature. The siRNA and Lipofectamine 2000 mixtures were added to the cells and incubated for 4h, and then was cultured with fresh growth medium. Cells were divided into HtrA1 siRNA group, negative control group (NC group) and blank control group (BC group). HtrA1 siRNA group and NC group were transfected with HtrA1 siRNA and control siRNA (Santa Cruz, CA, USA) respectively. The ARPE-19 cells after blue light exposure were considered as BC group. The cells were harvested 48h after transfection for further study.

Cell Counting Kit-8 Assay The transfected ARPE-19 cells of each group were seeded in 96-well culture plates at a density of 4×10^3 cells per well and incubated for 8h. Subsequently, each well was added 10 μL of cell counting kit-8 (CCK-8) (Dojindo, Japan) and then incubated at 37˚C for 4h. The optical density (OD) value of the wells was detected at a wavelength of 490 nm. The cell proliferation curve was plotted, using the absorbance at each time point. Each group was measured at 12, 24, 36, 48 and 72h post-seeding.

Transwell Chamber Assays The migration ability of cells in each group was detected using Transwell chamber system (Corning, Arizona, USA). Briefly, 1×10^5 cells per well were seeded in the upper chamber with 200 μL of serum-free medium. The bottom chamber received 600 μL of 10% FBS-containing medium. Forty-eight hours later, the migrated cells were stained by Giemsa. We counted the number of cells that migrated across the filters in 5 high-power fields per insert, and average values afterwards.

Flow Cytometry The cells were transfected and 48h later washed with ice-cold PBS for 3 times, and then resuspended in the staining buffer. After that, we incubated the cells by adding 5 μL Annexin V-FITC (R&D, New Jersey, USA) for 15min in the dark at room temperature, followed by addition of 5 μL propidium iodide (PI, R&D, New Jersey, USA) for additional 5min. Flow cytometry was performed immediately. Annexin V-positive and propidium iodide negative cells were considered as apoptotic cells.

RNA Extraction and Quantitative Reverse Transcription-polymerase Chain Reaction Total RNA was isolated from cultured cells using the Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocols. Complementary DNA (cDNA) synthesis was conducted according to the RNA PCR core kit (Invitrogen, Carlsbad, USA) protocol. β-actin was served as the internal control. The primer sequences were as follows: HtrA1, 5-AGTAAACCTGGACGGTGAAGTGATTG-3 (forward) and 5- AGAGGTCACGGGGCTAAT-3 (reverse); Bcl-2, 5-AGTAAACCTGGACGGTGAAGTGATTG-3 (forward) and 5-CCAGGTAACAAAACCCCACA-3 (reverse); Bax, 5-CAAGACCAGGGTGGTTGG-3 (forward) and 5-CACTCCCGCCACAAAGAT-3 (reverse); Caspase-3, 5-GCAGCAAACCTCAGGGAAAC-3 (forward) and 5-TGTCGGCATACTGTTTCAGCA-3 (reverse); β-actin, 5-GTCCACCGCAAATGCTTCTA-3 (forward) and 5-TGCTGTCACCTTCACCGTTC-3 (reverse). PCR reaction was performed in a 25 μL system under following conditions: Bcl-2, Bax and Caspase-3, 40 cycles of 95℃ for 15s, 58℃ for 20s, and 72 ℃ for 20s; the PCR reactive conditions of HtrA1 was: 30 cycles of 94 ℃ for 30s, 51 ℃ for 30s, and 72 ℃ for 30s. Relative quantification of gene expression was performed using 2-ΔΔCt method.

Western Blotting Analysis ARPE-19 cells were lysed with RIPA and boiled for 10min after mixed with loading buffer. Lysates were centrifuged at 12 000 g for 10min, and the protein concentration of the supernatant was determined using bicinchoninic acid (BCA) method. Equal amounts (40 μg) of protein were subjected to SDS-polyacrylamide gel electrophoresis on 12% gels. The separated proteins were then transferred to a polyvinylidencefluoride membrane (Millipore, Darmstadt, Germany). The membranes were incubated with primary antibodies against HtrA1 (1:200; R&D, New Jersey, USA), Bcl-2, Bax and Caspase-3 (1:400; CST, USA), followed by incubation with secondary antibodies (1:3000; Boster, Wuhan, China). The immunoreactive bands were developed by luminescence reagent.

Statistical Analysis All experiments were performed in triplicate independently in this research. The data are expressed as the mean±standard deviation (SD). SPSS18.0 software (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis. Student's *t*-test was used to compare the difference between means, while differences among groups were performed by analysis of one-way analysis of variance. $P<0.05$ was considered to be statistically significant.

RESULTS

Expression of HtrA1 in Blue-light-irradiated ARPE-19 Cells In order to observe the expression of HtrA1, the relative mRNA and protein levels of HtrA1 in ARPE-19 cells were detected by real-time polymerase chain reaction (RT-PCR) and Western blot. The RT-PCR and Western blot results showed that the expression for HtrA1 in ARPE-19 cells after blue light exposure were highly expressed compared to those in normal cells (Figure 1A, 1B). Besides, the expression of HtrA1 in HtrA1 siRNA group was significantly decreased than those in NC group and BC group (Figure 1C, 1D). These results showed the correlation between HtrA1 and blue light exposure in ARPE-19 cells, and HtrA1 siRNA can efficiently silence HtrA1 expression at both the levels of transcription and translation in light exposured ARPE-19 cells.

Effect of HtrA1 on the Proliferation and Migration Ability of the Blue-light-irradiated ARPE-19 Cells We use CCK-8 assay to investigate the growth ability of the blue-light-illuminated ARPE-19 cells after HtrA1 down-regulation at different time points (12, 24, 36, 48 and 72h, respectively). Compared to the NC and BC groups, the cell proliferation was significantly decreased in HtrA1 siRNA group at 36, 48 and 72h ($P<0.05$; Figure 2). The result suggested that the down-regulation of HtrA1 expression inhibits the proliferation of ARPE-19 cells in the light injured model.

Moreover, the migration of HtrA1 siRNA group in the light injured model was significantly lower than those of the NC and BC groups ($P<0.05$) (Figure 3). While the number of migrated cells between NC and BC groups were not significantly different ($P>0.05$). These results suggested that the knockdown of HtrA1 inhibits the migration ability of ARPE-19 cells in light injured model.

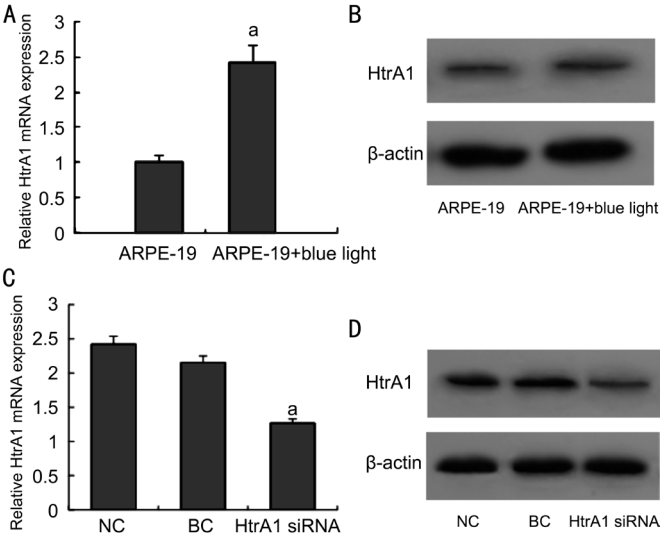

Figure 1 Expression of HtrA1 in ARPE-19 Cells A, B: The expression of mRNA and protein of HtrA1 in ARPE-19 cells were measured at 24h after blue light exposure; C, D: The expression of mRNA and protein of HtrA1 in blue-light-irradiated ARPE-19 cells were measured at 48h after siRNA transfection. Data were showed as mean±SD, $n=3$. [a]$P<0.05$ compared with NC groups.

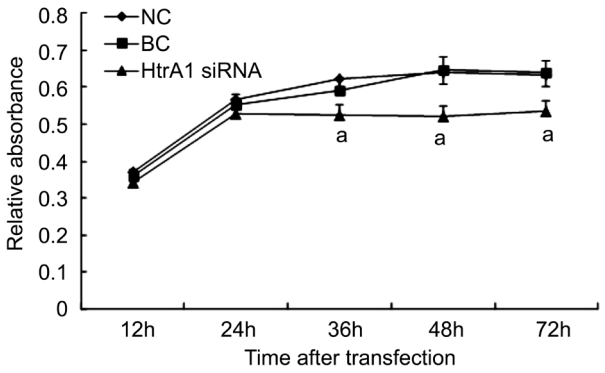

Figure 2 OD value detected of blue-light-illuminated ARPE-19 cell proliferation by CCK-8 assay at 12, 24, 36, 48 and 72h respectively Cell proliferation was significantly decreased in HtrA1 siRNA group at 36, 48 and 72h. [a]$P<0.05$ *vs* NC and BC groups.

Effect of HtrA1 on the Apoptosis of the Blue-light-irradiated ARPE-19 Cells When cells were transfected with HtrA1 siRNA, the percentage of apoptotic ARPE-19 cells induced by blue light irradiation was significantly decreased compared to those of the NC and BC groups ($P<0.05$; Figure 4B), which was present in the Annexin V-FITC results (Figure 4A). The cellular apoptosis rate was 3.24%±1.25%, 15.2%±2.38% and 17.3%±1.91% in HtrA1 siRNA, the NC and BC groups, respectively, suggesting that HtrA1 knockdown reduces cell apoptosis in the light injured model.

Detection of Cell Apoptosis-related Protein Expression in Light Injured ARPE-19 Cells After HtrA1 Suppression We compared the expression levels of Bax, Caspase-3 and Bcl-2

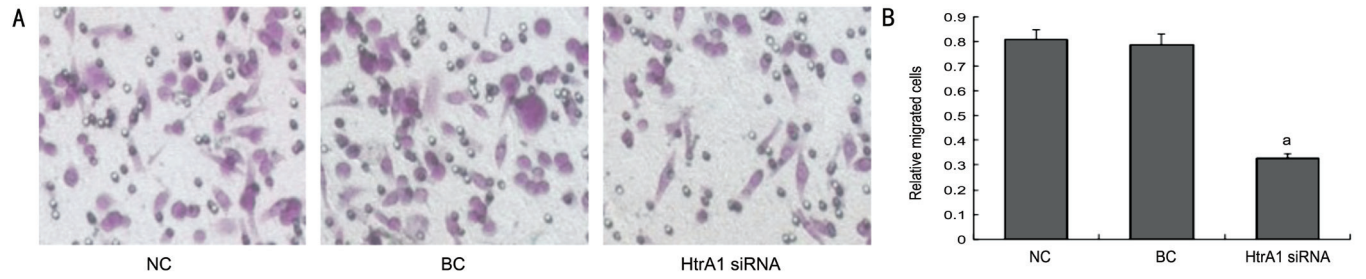

Figure 3 Migration ability of the blue-light-irradiated ARPE-19 cells detected by Transwell chamber assays at 48h after transfection A: Migration of the blue-light-illuminated ARPE-19 cells in HtrA1 siRNA, NC and BC groups (200×); B: The number of migration cells was significantly decreased by the HtrA1 siRNA transfection. [a]P<0.05 compared with control groups.

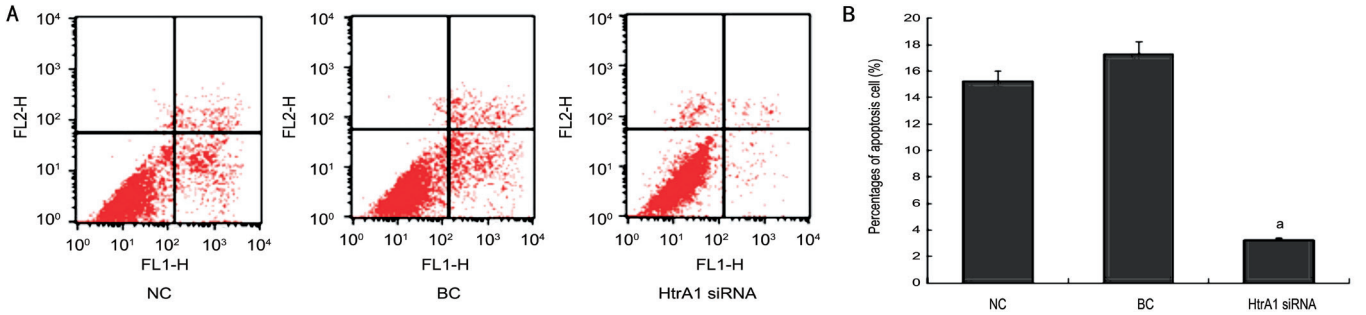

Figure 4 The apoptosis rate of blue-light-illuminated ARPE-19 cells after interfering with siRNA A: Detection of blue-light-illuminated ARPE-19 cells apoptosis by flow cytometry at 48h after transfection; B: The percentages of apoptotic cells were significantly decreased by the HtrA1 siRNA transfection compared to the NC and BC groups ([a]P<0.05).

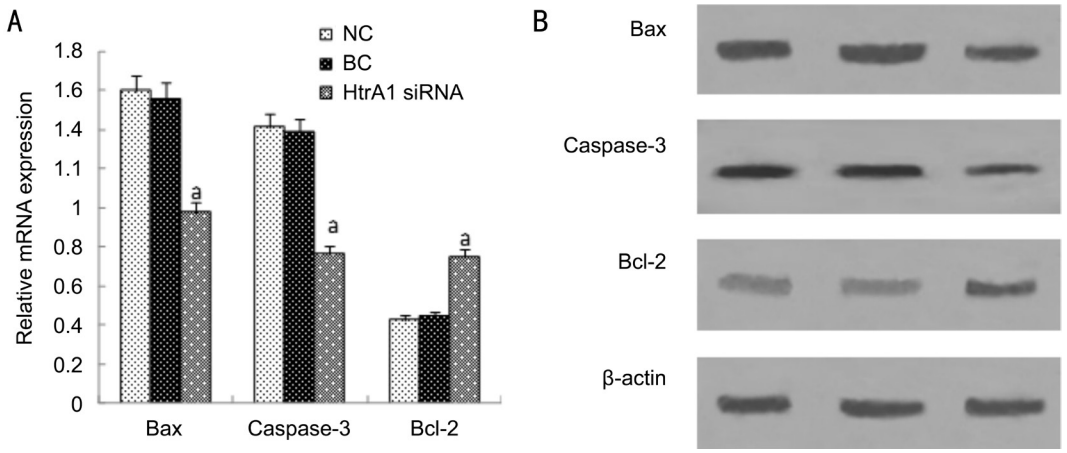

Figure 5 The expression of mRNA and protein of Bax, Caspase-3 and Bcl-2 in blue-light-irradiated ARPE-19 cells after 48h A: The mRNA expression of Bax, Caspase-3 and Bcl-2. Bax and Caspase-3 mRNA expression is reduced significantly. However, the mRNA expression of Bcl-2 was significantly increased; B: The protein expression of Bax, Caspase-3 and Bcl-2. Bax and Caspase-3 protein expression is reduced significantly. However, the protein expression of Bcl-2 was significantly increased. Data were presented as mean±SD, n=3. [a]P<0.05 *vs* NC and BC groups.

among the three groups to analyze the molecular mechanism for HtrA1 suppression on the ARPE-19 cells apoptosis in the light injured model. The relative mRNA and protein levels for Bax and Caspase-3 were significantly decreased by the suppressed HtrA1 in light injured ARPE-19 cells compared to those of the NC and BC groups (P<0.05; Figure 5). Besides, the relative mRNA and protein levels for Bcl-2 were both increased by the suppressed HtrA1 than those in controls (P<0.05; Figure 5). Briefly, the results suggest that HtrA1 siRNA transfer performed in light injured cell model may inhibit cell apoptosis, down-regulate protein expression of Bax and Caspase-3, and up-regulate that of Bcl-2.

DISCUSSION

RPE cells are important for maintaining the function of the overlying photoreceptor cells, protection of the retina from excessive light exposure, formation of blood-retinal barrier, and immune defense[21-22]. RPE is the primary pathological site of AMD. The death of RPE cells in many retinal diseases, including AMD, is a crucial event in the disease process. RPE is sensitive to blue light, which causes necrosis or apoptosis

of RPE at different doses[23-24]. A high level of exposure is found to be associated with blue light and development of AMD, especially later in life[25]. Furthermore, there was a closely relationship between extended exposure to sunlight and the 10-year incidence of early AMD and increased retinal pigment injury in Beaver Dam Eye Study[26]. The melanin pigment granules in retinal RPE cells are the main sites of light energy absorption, which are also the first location in the retina to be injured by light exposure[27]. Therefore, in this study, we established the light injured RPE model using blue light exposure for the further AMD study.

It has been suggested that HtrA1 in human vitreous humors and RPE cells increased under stress and inflammatory conditions[28]. In this study, we found that the expression of HtrA1 was increased in ARPE-19 cells after blue light exposure. Moreover, the mRNA and protein expression of HtrA1 after RNAi interference with siRNA was significantly reduced when compared with the control group. This result suggests that the siRNA is correctly designed to halt HtrA1 expression. Recent reports have shown that HtrA1 plays significant roles in the cell proliferation, invasion, and apoptosis of tumors[29-30]. It has been reported that silence of HtrA1 in ARPE-19 cells suppressed the growth and viability and HtrA1 played an important role of in AMD pathogenesis[31]. In addition, HtrA1 protected cells from cell death but promoted cell senescence under oxidative stress[32]. In order to investigate the effects of HtrA1 expression on the proliferation, migration and apoptosis of blue-light-irradiated ARPE-19 cells, we used siRNA to knockdown the expression of HtrA1. We found that down-regulation of HtrA1 expression significantly reduced the proliferation, migration ability and apoptosis of light-injured ARPE-19 cells, indicating the role of HtrA1 in the blue light injured RPE model.

Previous researches have indicated that HtrA1 is related in programmed cell death, apoptosis. HtrA1 can also trigger apoptosis in a caspase-dependent manner and in a caspase-independent manner and the serine protease activity of HtrA1 is indispensable to induce cell death[30,33]. Moreover, HtrA1-mediated apoptosis was found to be associated with activation of Caspases-3 and -7[33]. Additionally, blue light-induced apoptosis of A2E-Containing RPE is executed by Caspase-3 and regulated by Bcl-2[34]. To determine the probable mechanism involved in HtrA1 and RPE cell death in the light injured model, we evaluated the protein and transcription levels of Bax, Caspase-3 and Bcl-2. Our study showed that Bcl-2 expression was highly expressed, whereas the suppressed HtrA1 significantly decreased the expression of both Bax and Caspase-3. Therefore, we hypothesized that HtrA1 may affect the cell apoptosis of blue-light-irradiated ARPE-19 cells *via* increasing Bcl-2 and decreasing Bax and Caspase-3.

In summary, the results of our study indicate that siRNA mediated down-regulation of HtrA1 induces significant difference in proliferation, migration and apoptosis in ARPE-19 cells in the light injury model. Moreover, HtrA1 suppression in ARPE-19 cells may ameliorate cell death induced by blue light irradiation through down-regulation of Bax and Caspase-3, and up-regulation of Bcl-2 expression. In short, this study highlights the important role of HtrA1 in the pathogenesis of AMD and blue light related RPE injury.

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