Distribution and localization of abnormally expressed OPTN proteins in RGC5 retinal ganglion cells and their effects on subcellular morphology

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ABSTRACT. The objectives of this study were to investigate the distributions of abnormally expressed optineurin (OPTN) proteins in retinal ganglion cells (RGC5s) of transgenic rats and their effects on subcellular morphological structures. Green fluorescent protein labeled EGFP wild-type ($OPTN_{WT}$), E50K mutant type ($OPTN_{E50K}$), and OPTN siRNA (si-OPTN) eukaryotic expression plasmids were constructed and transfected into RGC5s. Intracellular structures were labeled with organelle specific fluorescent dyes. Construct localization and cell morphologies were visualized by confocal fluorescence microscopy. $OPTN_{WT}$ was observed to be distributed as fine punctate fluorescent particles in the cytoplasm around the nucleus, along with exhibiting nuclear expression. $OPTN_{E50K}$ exhibited similar distribution but with non-uniform fluorescence particle size. si-OPTN distribution was similar to that of EGFP: uniform across the cytoplasm and nucleus. Compared with the negative control group, $OPTN_{WT}$, and $OPTN_{E50K}$ and to a lesser degree pEGFP-transfected cells

exhibited fracture and loss of myofilament proteins and mitochondrial swelling and cytoplasmic accumulation, along with abnormal lysosomal distribution and increased volume, and Golgi fragmentation. However, si-OPTN transfected cells exhibited no significant damage. Therefore, we demonstrated that the E50K mutation disrupts the uniformity of OPTN protein distribution upon exogenous overexpression. Furthermore, these results suggested that si-OPTN transfection, and thus potentially OPTN knockdown, did not impact subcellular morphology of RGC5 cells, whereas transfection, especially when combined with wild-type or mutant OPTN expression, led to substantial abnormalities in subcellular morphological structures. These findings lay a foundation for further research into the function of the OPTN protein.

Key words: OPTN; RGC5; Cell distribution; Subcellular morphology

INTRODUCTION

Glaucoma is a genetically predisposed eye disease that causes blindness (Wang, 2013). Glaucoma can be divided into many types, the most common of which is primary open-angle glaucoma (POAG) (Kwon et al., 2009). The currently accepted pathogenesis-related POAG gene is optineurin (*OPTN*), which is considered especially relevant in normal tension glaucoma (NTG) (Wang, 2013). *OPTN* is located on chromosome 10p14-15, and encodes the OPTN protein that is composed of 577 amino acids, and is expressed abundantly in retinal ganglion cells (De Marco et al., 2006; Ying and Yue, 2012). Researchers have identified four mutations in the *OPTN* gene through the investigation of glaucoma pedigrees (Rezaie et al., 2002): Glu50Lys (E50K), an AG insertion at 691692, Glu54Gln, and Met98Lys. E50K is the most commonly identified mutation in *OPTN*. Research has shown that approximately 80% of families with POAG carry this mutation, and in turn, the prevalence rate of patients with POAG in families transmitting E50K was shown to reach 18% (Aung et al., 2005). However, although the association of the *OPTN* gene with POAG has been confirmed through recent pedigree investigations, the role of *OPTN* in the pathogenesis of glaucoma has not yet been elucidated. Furthermore, the suggested distribution of the OPTN protein in cells and its effects on organelles have also been inconsistent across studies. De Marco et al. (2006) found that the localization of overexpressed OPTN was transformed from cytoplasmic to nuclear when stimulated by apoptosis, which increased the survival of NIH3T3 cells. Park et al. (2006) found that the overexpression of OPTN in retinal pigment epithelial cells and in trabecular cells could form fluorescent foci, which were suggested to induce the fragmentation of Golgi. When the E50K mutant was used, the formation of foci was enhanced and their volume was increased, as was the observed Golgi fragmentation. Therefore, researchers attributed this as induced by different cell types (Park et al., 2006) and the expression of OPTN protein (Chalasani et al., 2007). The death of retinal ganglion cells (RGCs) is the final outcome of glaucoma. Clarification of the role of OPTN in the process of RGC damage has been considered to be very important for the understanding of the pathogenesis of POAG; however, currently there are few studies regarding RGCs. Accordingly, we studied the distribution and localization of abnormally expressed OPTN protein in RGCs and its effects on subcellular morphology.

In this experiment, the eukaryotic expression plasmids containing wild-type OPTN ($OPTN_{WT}$), the E50K mutant ($OPTN_{E50K}$), and the OPTN-specific siRNA (si-OPTN) labeled by green

fluorescent protein (EGFP) were separately transfected into the rat transgenic RGC line RGC5 using Lipo2000, which was predicted to result in the abnormal expression of OPTN. Different cell organelles were labeled by organelle-specific fluorescent dyes. The distribution and localization of $OPTN_{WT}$, $OPTN_{E50K}$, and si-OPTN products in RGC5 cells were observed by confocal fluorescence microscopy and their effects on subcellular morphologies were also detected. The results from these observations might provide theoretical support for further research into the issues of cell-specific functions of OPTN.

MATERIAL AND METHODS

Material

The rat RGC5 cell line was purchased from the American Type Culture Collection cell library (Manassas, VA, USA). Cell culture related reagents were obtained from Hyclone (Logan, UT, USA) and Gibco (Gaithersburg, MD, USA). DAN polymerase and related PCR amplification kits were purchased from TaKaRa (Dalian, China). The *Xho*I, *Kpn*I, and T4DNA ligase enzymes and the reverse transcription kit were purchased from Toyobo Co. Ltd. (Osaka, Japan). Top10 competent cells, the DNA rapid purification kit, the cloning vector construction kit, and the plasmid extraction kit were purchased from Tianjen Biological Technology Co. Ltd. (Beijing, China). The Lipo2000 Reagent and the specific fluorescent dyes for organelles were purchased from Invitrogen (Carlsbad, CA, USA). The eukaryotic expression vector plasmid PEGFP-N1 was purchased from Clontech Laboratories (Mountain View, CA, USA). Thy1.1 monoclonal antibody was product of Santa Cruz Biotechnology (Dallas, TX, USA). Goat anti-mouse IgG was purchased from Zhongshan Co. (Hong Kong, China). The SABC kit was a product of booster company.

Methods

Plasmid construction and extraction

The $OPTN_{WT}$ plasmid was constructed as follows: According to the Sprague Dawley (SD) rat genomic sequence of the *OPTN* gene and its accession No. (BC086976) in the National Center for Biotechnology Information (NCBI) database, we designed primers for polymerase chain reaction (PCR) amplification with *Xho*I and *Kpn*I restriction sites added to the 5'- and 3'-ends, respectively. Primers were synthesized by Shanghai Biological Engineering Technology Co Ltd. (Shanghai, China). The cDNA sequence of the *OPTN* gene from retinal tissues of SD rats was cloned using reverse transcription PCR, and then the 1638 bp cDNA fragment was ligated to the cloning vector PGM-T, transformed into competent bacteria, and the positive clones were screened. The construct was confirmed by enzyme digestion of the expression vector pEGFP-N1 with *Xho*I and *Kpn*I sites to release the cDNA fragment, and by sequencing to ensure that no changes could be identified in the reading frame. After transformation, screening, enzyme digestion, and sequencing, the plasmid was amplified and purified. To obtain the E50K mutant of the *OPTN* gene, the mutation was created within the *OPTN* cDNA fragment by directional induction using a kit according to manufacturer instruction, and was cloned into pEGFP-N1 according to the above methods. To construct si-OPTN, we ligated the screened *OPTN* siRNA and an EGFP labeled siRNA expression vector (Guangzhou Ruibo Biological company) as previously described (Zhirong et al., 2014). All constructs were confirmed by DNA sequencing.

RGC5 identification and plasmid transfection

RGC5 cells were seeded into F12/DMEM culture medium containing 5% fetal bovine serum (FBS) and cultured on 24-well plates at 37°C and 5% CO_2, and passaged using 0.25% trypsin digestion once every 3-4 days. The cells were identified using the Thy1.1 monoclonal antibody.

Plasmids were transfected into RGC5 cells using Lipo2000. RGC5 cells were divided into six groups: controls (A), $OPTN_{WT}$ (B), $OPTN_{E50K}$ (C), pEGFP positive controls (D), si-OPTN (E), and liposome negative control (F). The procedure was as follows: plasmids were transfected according to the ratio of 3:1 of Lipo2000 (μL): plasmid (μg). F12/DMEM medium without serum was applied to cells which had been cultured for 18-24 h on 24 well plates (30-70% fusion rate) 1 h before transfection to synchronize the cells. A 1.5-μL aliquot Lipo2000 mixed with 50 μL F12/DMEM serum free medium was incubated at room temperature for 5 min, followed by addition of 500 ng plasmid mixed with 50 μL F12/DMEM serum free medium, and incubation at room temperature for 20 min. After removal of the culture liquid, the solution was slowly dropped onto the surface of the cells and transfection was allowed to proceed at high concentration exposure for 2-5 min. The mixture was complemented with 500 μL F12/DMEM without serum per well and allowed to transfect for 6 h, at which time the medium was changed to 5% FBS F12/DMEM and cultured for an additional 18 h.

The distribution and localization of $OPTN_{WT}$ and $OPTN_{E50K}$ proteins and si-OPTN tagged by green fluorescent protein in the six groups were observed in the RGC5 cells using confocal fluorescence microscopy after 24 to 48 h culture.

Morphologic and subcellular structural assessment of transfected RGC5 cells

After transfection and culture for 24 to 48 h, the organelles in RGC% cells were stained using Golgi body, mitochondria, or lysosome-specific tissue staining agents [Bodipy TR ceramide D-7540 (5 μM); MitoTracker M-7512 (250 nM); LysoTracker L-7528300 (300 nM), respectively; Invitrogen]. The actin microfilaments were fixed and permeabilized, and joined into the lipophilic fluorescein groups for 25 min [Phallotoxins B-607 (250 nM); Invitrogen]. The changes in si-OPTN cells, organelles, and sub-cellular ultrastructure were observed by confocal microscopy after transfection.

Image analysis

The results were analyzed by the Image-Pro Plus6.0 image analysis software.

RESULTS

Morphology and identification of RGC5s

RGC5 cells exhibited monolayer growth *in vitro*; the extend axons connected with each other along with the growth of cells (Krishnamoorthy et al., 2001). Features of RGC5 cells were identified using immunofluorescence and immunohistochemistry with a Thy1.1 monoclonal antibody (Figure 1A and B).

Distribution and localization of abnormally expressed OPTN proteins

The distribution and localization of EGFP-labeled $OPTN_{WT}$, $OPTN_{E50K}$, and si-OPTN in RGC5

cells were observed by confocal fluorescence microscopy. The transfection efficiencies in each group were calculated as being 17.43 ± 0.94 and 20.13 ± 1.24% after transfection for 24 and 48 h.

$OPTN_{WT}$ proteins were observed to be distributed uniformly in the cytoplasm and around the nuclei, in the form of fine punctate fluorescence particles. The $OPTN_{E50K}$ protein was also distributed in the cytoplasm, but it showed coarse punctate fluorescent particles around the nucleus. The distribution of si-OPTN in cells was similar to that of EGFP alone, which was distributed uniformly throughout the cell cytoplasm and nucleus (Figure 2A-D).

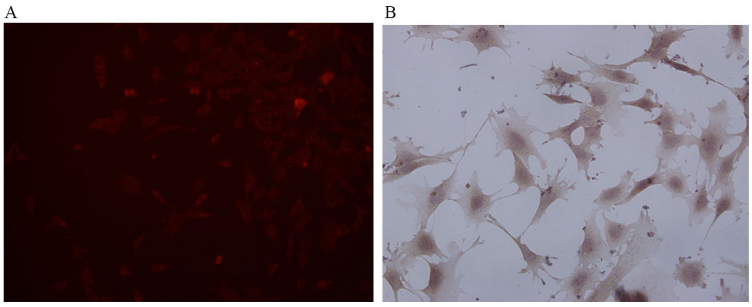

Figure 1. Identification of retinal ganglion cells (RGC5s) by immunofluorescence and immunohistochemical methods. **A.** Identification of RGC5 (200X) with immunofluorescence (Cy3 marked two anti); **B.** identification of RGC5s with immunohistochemistry. 3,3'-diaminobenzidine (DAB) staining (400X).

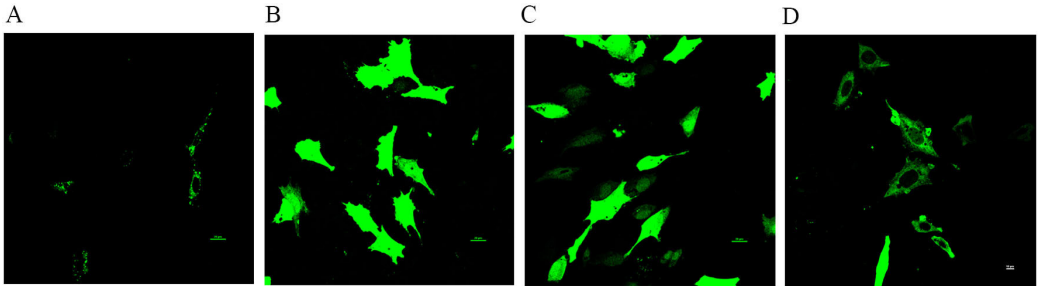

Figure 2. Distribution and localization of pEGFP, si-OPTN, $OPTN_{WT}$, and $OPTN_{E50K}$ in retinal ganglion cells (RGC5s). RGC5s were transfected with pEGFP (**A**); si-OPTN (**B**); $OPTN_{WT}$ (**C**); and $OPTN_{E50K}$ (**D**). It can be seen from the figure that the distributions of $OPTN_{WT}$ and $OPTN_{E50K}$ in the cells were around the nucleus, with $OPTN_{WT}$ visible as fine punctate fluorescent particles, whereas the $OPTN_{E50K}$ signal exhibits more coarse punctate fluorescent particles that are non-uniformly distributed. In contrast, the si-OPTN and EGFP signals are uniformly distributed throughout the cell cytoplasm and nuclei (600X).

Effects of abnormally expressed OPTN protein on RGC5 subcellular structure

Confocal imaging showed that when compared with the negative control group, the myofilament proteins of transfected cells appeared broken and even disappeared following transfection with pEGFP, $OPTN_{WT}$, or $OPTN_{E50K}$. In addition, in these cells mitochondrial swelling and accumulation on one side of the cytoplasm was observed, along with an increase in lysosomal volume. Furthermore, the Golgi bodies appeared broken, and the cap around nuclei disappeared, along with other organelle changes. However, the changes induced by $OPTN_{WT}$ and $OPTN_{E50K}$ were more extreme than those observed following pEGFP transfection: the fracture of myofilament protein curls were more common and were irregularly arranged in a small number of cells, the myofilament proteins had completely disappeared, the degree of mitochondrial swelling and total

volume was increased, the outlines were blurred, the lysosomal volume was increased to a greater degree, the function was activated, and the integrity of the Golgi apparatus was disturbed and broken to a greater extent. In contrast, compared with the negative control group, there were no obvious changes in the morphological structure of myofilament proteins, mitochondria, lysosomes, and Golgi apparatus of transfected cells in the si-OPTN group (Figures 3-6).

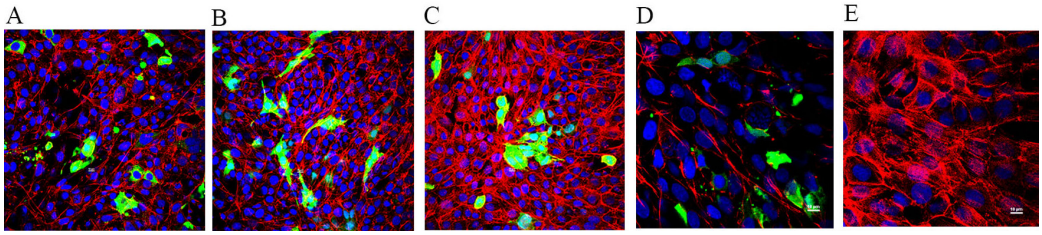

Figure 3. Effects of pEGFP, $OPTN_{WT}$, $OPTN_{E50K}$, and si-OPTN on the morphology of myofilament proteins. **A.** Negative control group; **B.** transfected pEGFP; **C.** $OPTN_{WT}$; **D.** $OPTN_{E50K}$; and **E.** si-OPTN groups. Compared with the negative control group, the myofilament proteins in the transfected cells appear fractured after transfection with pEGFP, $OPTN_{WT}$, and $OPTN_{E50K}$; the arrangement is irregular, and the numbers are decreased below those of non transfected cells. In contrast, the myofilament proteins in the transfected si-OPTN cells do not show obvious changes (600X).

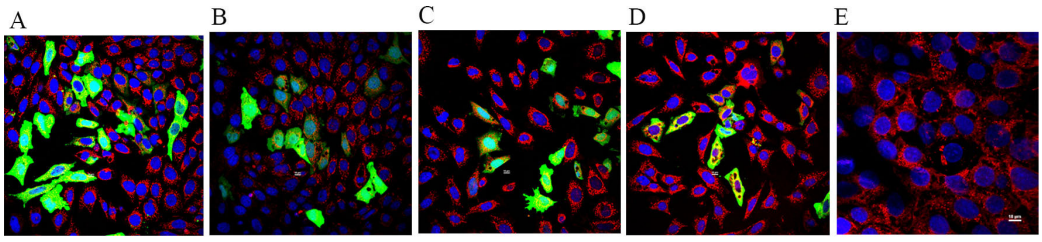

Figure 4. Effects of pEGFP, $OPTN_{WT}$, $OPTN_{E50K}$, and si-OPTN on mitochondrial morphology. Mitochondrial staining of negative control group (**A**); transfected pEGFP (**B**); $OPTN_{WT}$ (**C**); $OPTN_{E50K}$ (**D**); and si-OPTN (**E**) groups. The mitochondria are distributed perinuclearly in the negative control group, whereas the aggregation and accumulation of the mitochondria in the cytoplasm after transfection with pEGFP, $OPTN_{WT}$, or $OPTN_{E50K}$ can be observed. Similar changes in the si-OPTN group are not obvious (600X).

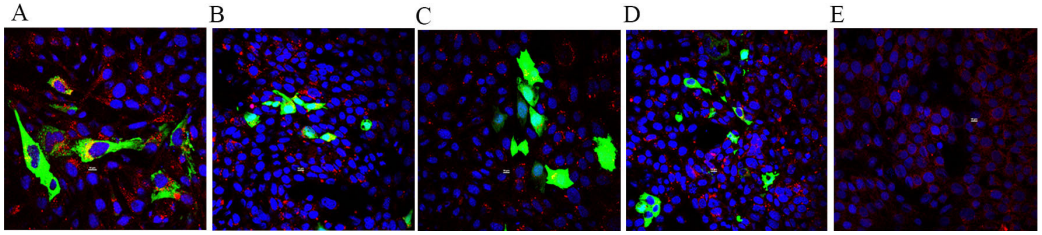

Figure 5. Effects of pEGFP, $OPTN_{WT}$, $OPTN_{E50K}$, and si-OPTN on lysosomal morphology. Lysosomal staining of negative control group (**A**); transfected pEGFP (**B**); OPTNWT (**C**); $OPTN_{E50K}$ (**D**); and si-OPTN (**E**) groups. Compared with the negative control group, not only can the numbers and volumes of the lysosomes in the transfected cells be seen to be increased after transfection with pEGFP, $OPTN_{WT}$, and $OPTN_{E50K}$, but the numbers of lysosomes in the untransfected cells are also increased, whereas the lysosomes of the si-OPTN transfected cells show no obvious changes (600X).

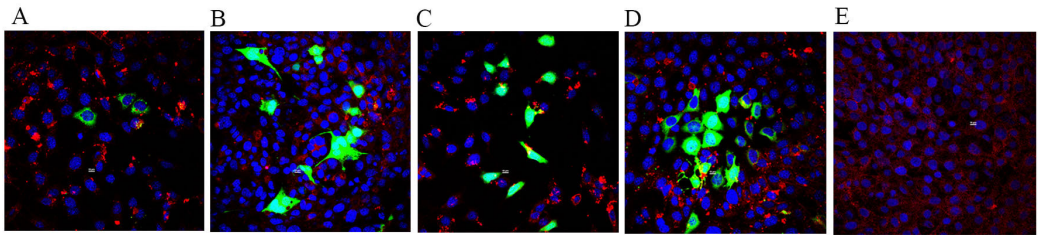

Figure 6. Effects of pEGFP, $OPTN_{WT}$, $OPTN_{E50K}$, and si-OPTN on Golgi morphology. Golgi staining of negative control group (**A**): transfected pEGFP (**B**); $OPTN_{WT}$ (**C**); $OPTN_{E50K}$ (**D**); and si-OPTN (**E**) groups. Only a few positively-stained Golgi can be observed in the negative control group. The integrity of Golgi in the transfected cells appears to have been lost following transfection with pEGFP, $OPTN_{WT}$, or $OPTN_{E50K}$, although the positive Golgi staining of whole group cells is increased, whereas the morphology of the Golgi in the si-OPTN transfected cells does not appear to have been obviously changed (600X).

DISCUSSION

The *OPTN* gene has been associated with the pathogenesis of NTG and POAG, but the mechanism of its pathogenesis has not been elucidated. One possibility is that OPTN pathogenesis might be caused by abnormal expression. To date, there have been numerous studies on the function of the OPTN protein. Studies have found that the OPTN protein can interact with transcription factor IIIA (Moreland et al., 2000) and VI (Sahlender et al., 2005), myosin Rab8 (Chibalina et al., 2008), huntingtin (del Toro et al., 2009), metabotropic glutamate receptor (Anborgh et al., 2005), and TANK protein kinase (Morton et al., 2008); and can exhibit partial co-localization with transferrin receptor (Nagabhushana et al., 2010), which has been shown to be involved in the maintenance of Golgi structural integrity (Sahlender et al., 2005). OPTN might affect the corresponding signal transmission processes through interaction with these proteins, and might also be involved in vesicular transport (Sahlender et al., 2005; del Toro et al., 2009; Nagabhushana et al., 2010). However, the results of current research have also been contradictory. Rezaie et al. (2002) and other studies have suggested that OPTN is a secreted protein, which can be secreted into the aqueous humor and is localized to the Golgi apparatus within the cells. However, Park et al. (2006) suggested that OPTN is not secreted, but is distributed in the lysosomes of cells and is not in contact with the Golgi apparatus, but rather is partially colocalized with the transferrin receptor. These conflicting results might be related to the different functions of OPTN in the different cells types utilized in the previous studies (Park et al., 2006) or to the basic expression patterns of the OPTN protein (Chalasani et al., 2007). So far, there have been few experiments regarding the mechanism of OPTN using RGCs, although the injury and death of RGCs comprises the final outcome of glaucoma. To clarify the effects of OPTN in the process of RGC pathology is very important to understand the pathogenesis of POAG. The RGC5 cell line is a good alternative to primary RGCs, as although the morphological and electrophysiological properties differ, the growth conditions, cell membrane, and intracellular material were all shown to be consistent between the two cell types (Krishnamoorthy et al., 2001). This study therefore utilized RGC5 cells, which avoids certain limitations of primary RGCs such as their shorter lifespan, lower energy requirements, and transfection inefficiency. In this study, we transfected the eukaryotic expression plasmids $OPTN_{WT}$, $OPTN_{E50K}$, and si-OPTN into RGC5 cells, which generated abnormal OPTN expression levels within the cells. We used specific fluorescent dyes to label different organelles, and observed the distribution and localization of $OPTN_{WT}$, $OPTN_{E50K}$, and si-OPTN in RGC5s by confocal fluorescence

microscopy, in addition to and their effects on subcellular morphologic structures, which might provide theoretical support for further functional studies.

Here, the results of confocal microscopic analysis showed that the $OPTN_{WT}$ protein was distributed in the cytoplasm and surrounding the nucleus, as visualized by fine punctate fluorescence particles. In contrast, whereas the $OPTN_{E50K}$ protein was also distributed in the cytoplasm, it showed a coarse punctate fluorescence particle pattern around the nuclei. The distribution of si-OPTN in cells was similar to that of EGFP: it was uniformly distributed throughout the cell cytoplasm and nucleus, consistent with previous reports (Park et al., 2006). However, the $OPTN_{WT}$ protein signals in our experiments were not all punctate. In the early transfection period, fewer punctate foci were observed and the signal was primarily uniformly distributed, whereas with prolonged transfection time, the number of foci increased. We inferred that when the number of OPTN molecules becomes excessive (namely, through increased expression), the protein density was naturally increased, increasing the likelihood of interaction and thus forming a cluster visualized as a signal focus point; this might also be the reason that the $OPTN_{E50K}$ signal was not comprised of uniformly sized coarse punctate fluorescence particles, as the conformation of the protein might have been be altered upon mutation, enhancing the binding ability, and leading to the formation of spots that were not uniform. On the other hand, the distribution of si-OPTN was uniform across the whole cell, potentially reflecting that the expressed si-OPTN siRNA had bound with the *OPTN* mRNA, and thus was distributed across the cytoplasm and the whole cell nucleus; in addition, the siRNA would have been predicted to reduce the protein levels of OPTN and concomitantly reduce the likelihood of protein-protein interaction and clustering, resulting in uniform distribution.

We also observed that the transfected cells appeared to have been injured after transfection with pEGFP, $OPTN_{WT}$, and $OPTN_{E50K}$. In these cells, the myofilament proteins were fractured and their quantities were decreased compared to untransfected cells, the mitochondria exhibited swelling and accumulated to the side of the cytoplasm, the number and volume of the lysosomes were increased, the Golgi were fragmented, and the distribution of the cap around nucleus had disappeared. Furthermore, the injury to organelles caused by transfection of $OPTN_{WT}$ and $OPTN_{E50K}$ appeared more serious, as the arrangement of the fractured microfilaments appeared irregular or even disappeared completely, the swelled mitochondria exhibited blurred outlines, even higher lysosomal numbers and volumes were observed, and greater disruption of Golgi integrity could be identified. These results were partially consistent with those of Park et al. (2006). In contrast, the morphological structure of myofilament proteins, mitochondria, lysosomes, and Golgi in the si-OPTN transfected cells exhibited no significant damaged-associated changes. By combining the results of the morphological changes of organelles with the distribution patterns of $OPTN_{WT}$, $OPTN_{E50K}$, and si-OPTN in the cells, we inferred that the homologous interaction of $OPTN_{WT}$ proteins or $OPTN_{E50K}$ proteins were enhanced by exogenous overexpression, and that they might also have interacted with the endogenous proteins of the organelles. This in turn might have affected the function and metabolism of the organelles and thus damaged the RGC5 cells. On the other hand, the effect of decreased expression of OPTN on cells might be the converse, and therefore the expression of si-OPTN resulted in no significant injury-associated changes. However, the above corollary was only determined by morphological evidence, and the specific effects of $OPTN_{WT}$, $OPTN_{E50K}$, and si-OPTN on cells and endogenous protein levels still require further research.

In conclusion, we found that exogenous $OPTN_{WT}$ and $OPTN_{E50K}$ proteins were primarily distributed in the cytoplasm of cells, and that the $OPTN_{WT}$ protein was distributed uniformly around the nucleus and could occasionally be visualized as punctate fluorescent particles, whereas the $OPTN_{E50K}$ protein was perinuclearly distributed with uneven punctate size. In contrast, the si-OPTN

was shown to be evenly distributed throughout the cytoplasm and nucleus. Furthermore, the presence of exogenous $OPTN_{WT}$ and $OPTN_{E50K}$ protein in RGC5 cells showed a damaging effect on subcellular structures, whereas si-OPTN did not yield obvious damage-associated changes.

These results suggest a preliminary understanding of the function of $OPTN_{WT}$ and $OPTN_{E50K}$ proteins and of si-OPTN expression in RGC5 cells, which might underlie the pathological effects of abnormal OPTN expression on the survival of RGC5 cells and, potentially, of RGCs in glaucoma.

Conflicts of interest

The authors declare no conflict of interest.

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