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Effects of abnormal optineurin expression on the survival of the rat retinal ganglion cell line RGC-5

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ABSTRACT. The *OPTN* gene is thought to be associated with certain types of glaucoma and the function of the protein for which it encodes, optineurin, has been extensively researched, but with contradictory results. We explored the effects of abnormal optineurin expression on the survival of the rat retinal ganglion cell line RGC-5. Plasmids expressing wild-type (WT) or E50K mutant optineurin, or *OPTN*-specific double-hairpin small interfering RNA (si-RNA), were transfected into RGC-5 cells. The effects on cell survival were monitored by observation of cell morphology and propidium iodide and Hoechst 33342 fluorescent staining, while expression of optineurin was visualized by fluorescence microscopy. Abnormal optineurin expression influenced the survival of RGCs *in vitro*, as apoptosis was induced by increased WT and E50K mutant optineurin, while a reduction in apoptosis was observed in cells transfected with *OPTN*-siRNA. Similar results were also observed in transfected cells treated with apoptotic stimuli. Overexpression

of WT and mutant E50K protein resulted in greater cell death, while downregulation decreased RGC-5 apoptosis.

Key words: *OPTN*; RGC-5; Cell survival; Apoptotic stimulus

INTRODUCTION

Glaucoma is the second leading cause of permanent blindness worldwide and is characterized by a gradual loss of vision resulting from the death of retinal ganglion cells (RGCs; Quigley, 1996). It has been suggested that a number of genetic susceptibility factors contribute to glaucoma (Wolfs et al., 1998). *OPTN*, which codes for the protein optineurin, has been implicated in normal-tension glaucoma (NTG) and primary open angle glaucoma (POAG; Rezaie et al., 2002; Sarfarazi and Rezaie, 2003). Optineurin is a cytoplasmic protein and is preferentially expressed in RGCs. Consisting of 577 amino acids, it has been reported to interact with several proteins of diverse functions, such as transcription factor IIIA (Moreland et al., 2000), myosin VI (Sahlender et al., 2005), Rab8 (Chibalina et al., 2008), huntingtin (del Toro et al., 2009), metabotropic glutamate receptor (Anborgh et al., 2005), TANK protein kinase (Morton et al., 2008), and adenovirus E3 14.7-kDa protein (Li et al., 1998). The role of optineurin in cell survival has been studied extensively, but with contradictory results. For example, De Marco et al. (2006) found that following an apoptotic stimulus, optineurin functions to increase cell survival. This is in contrast to the results obtained by Park et al. (2006), who found that overexpression of wild-type (WT) optineurin increases cell death. Some researchers have suggested that this effect may be related to cell type (Park et al., 2006) or the baseline optineurin expression of the cells under study (Chalasani et al., 2007). As this protein is highly expressed in RGCs, the rat retinal ganglion cell line RGC-5 was used to investigate the effects of its abnormal expression on cell survival.

In this study, we modified the expression of optineurin by transfecting cells with plasmids expressing the WT protein and the E50K mutant, the most common pathogenic mutation of this protein, in which glutamic acid at position 50 is replaced by lysine in the translated sequence (Sarfarazi et al., 1998; Rezaie et al., 2002; Sarfarazi and Rezaie, 2003). In other cells, *OPTN*-specific double-hairpin small interfering RNA (siRNA) was expressed. Our aim was to explore the effects of optineurin on cell survival in the rat retinal ganglion cell line RGC-5.

MATERIAL AND METHODS

Expression plasmids

The coding region of rat *OPTN* complementary DNA (cDNA) was amplified by polymerase chain reaction (PCR) using a placental cDNA library as the template. The nucleotide sequence of the cloned gene was identical to that reported in the RefSeq database (accession No. NM_145081). The PCR product was cloned in a pEGFP-N1 vector. Mutations in *OPTN* cDNA were created using a PCR-based, site-directed mutagenesis strategy to make the E50K mutant. Nucleotide sequences of mutant and WT cDNA constructs were confirmed by automated DNA sequencing, while protein expression was confirmed by fluorescence microscopy analysis of transfected cells. Expression plasmids for *OPTN*-specific double-hairpin siRNA were screened and validated using western blotting and real-time PCR.

Hoechst 33342 stain and propidium iodide (PI) were obtained from Sigma (St. Louis, MO, USA). Cell culture reagents were obtained from GE Healthcare (Waukesha, WI, USA; HyClone culture medium and fetal bovine serum) or Amresco (Solon, OH, USA; phosphate-buffered saline and trypsin solution).

Cell culture, transfections, and fluorescence imaging

RGC-5 cells were grown as monolayers in Dulbecco's modified Eagle's medium containing 5% fetal calf serum in a humidified 37°C incubator in 5% CO_2. Transient transfections were performed using column-purified plasmids (Qiagen, Valencia, CA, USA) and Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer protocol. For fluorescence imaging, RGC-5 cells were grown on coverslips and transfected with the required plasmids prior to being visualized.

Cell death assays

Quantitative analysis of dead or apoptotic cells was carried out as follows. Cells grown on coverslips were transfected with 250 ng plasmids expressing WT optineurin, the E50K mutant, or *OPTN*-specific si-RNA and were stained with Hoechst 33342 and PI at 24, 48, and 96 h after transfection.

H_2O_2 and glutamate-induced cell death assays

Cells grown on coverslips were transfected with 250 ng plasmids expressing WT optineurin, the E50K mutant, or *OPTN*-specific si-RNA. At 24 h after transfection, the cells were treated with H_2O_2 (650 μM) or glutamate (10 mM) for 1 h, before being fixed and stained with Hoechst 33342 and PI. The percentage of dead cells was quantified by determining the proportion of those stained with PI to those stained with Hoechst 33342.

Statistical analysis

Data are reported as means ± standard deviations. Statistical comparisons were performed using one-way ANOVA followed by the Student-Newman-Keuls *q*-test using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA), or the chi-square test. P values lower than 0.05 were considered to be statistically significant.

RESULTS

As the death of RGCs constitutes the primary pathological process in glaucoma, we used a useful model of these cells derived from rats, RGC-5 (Krishnamoorthy et al., 2001), to analyze the effect of abnormal optineurin expression on cell survival. RGC-5 cells grown on coverslips were transfected with plasmids expressing WT optineurin, the E50K mutant, or *OPTN*-specific si-RNA. Transfected cells were then stained with Hoechst 33342 and PI and examined by fluorescence microscopy.

Using fluorescence microscopy, transfection efficiency was found to be 17.43 ± 0.94, 20.13 ± 1.24, and 19.37 ± 0.54% at 24, 48, and 96 h after transfection, respectively. While

cells in the blank (0.74 ± 0.34%) and negative (0.96 ± 0.41%) control groups appeared normal, apoptosis was observed in the positive control, WT, E50K, and *OPTN*-siRNA groups 24 h after transfection ($P < 0.05$), as revealed by the loss of refractility, condensation of chromatin, and decrease in cell size caused by shrinkage of the cytoplasm (Figure 1). However, in the *OPTN*-siRNA group, the level of apoptosis was lower than that in the positive control group ($P < 0.05$). The rate of apoptosis increased with the length of time since transfection, i.e., it was higher at 48 h than at 24 h, with the exception of the positive control. After 96 h, apoptosis was observed in the positive control and *OPTN*-siRNA groups, but to a lesser extent than that observed in the 48-h cultures. However, the rate of apoptosis was seen to increase further in the WT optineurin and E50K groups (Table 1 and Figure 2).

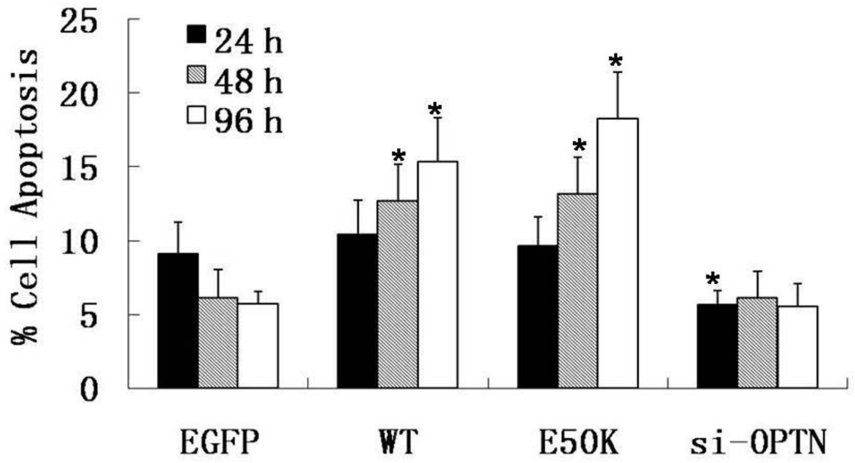

Figure 1. Apoptosis of RGC-5 cells expressing the empty EGFP vector, wild-type (WT) optineurin, E50K mutant optineurin, or *OPTN*-siRNA. *$P < 0.05$ *vs* positive control at the same time point.

Table 1. Apoptosis of RGC-5 cells expressing the empty EGFP vector, wild-type (WT) optineurin, E50K mutant optineurin, or *OPTN*-siRNA.

Group	Apoptosis (%)		
	24 h	48 h	96 h
EGFP	9.09 ± 2.17	6.11 ± 1.94	5.69 ± 0.83
WT *OPTN*	10.42 ± 2.31	10.71 ± 2.44*	15.38 ± 2.92*
E50K *OPTN*	9.62 ± 1.96	13.19 ± 2.45*	18.26 ± 3.17*
OPTN-siRNA	5.66 ± 0.94*	6.06 ± 1.83	5.56 ± 1.53

*$P < 0.05$ *vs* positive control at the same time point.

To further investigate the survival of RGCs expressing abnormal optineurin, we treated transfected cells with 10 mM glutamate. Hoechst 33342 and PI staining showed that apoptosis in the four groups had clearly increased 24 h following transfection. As the time since transfection was extended to 48 h, the apoptosis rate continued to increase, with the exception of the positive control group and those cells expressing *OPTN*-siRNA. The level of apoptosis in all four groups was higher after 96 h than after 48 h ($P < 0.05$; Table 2, Figures 3 and 4).

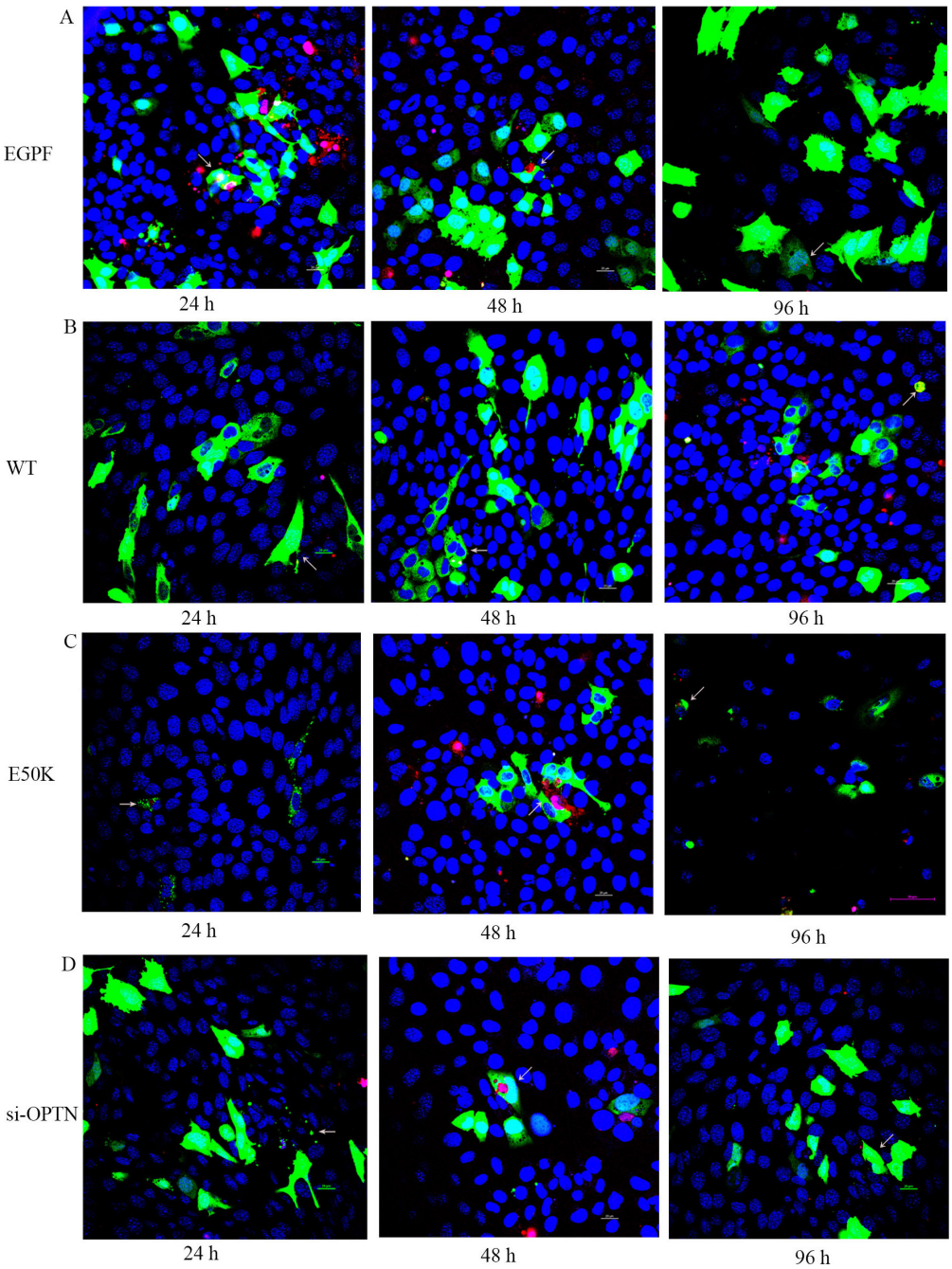

Figure 2. Laser confocal imaging of RGC-5 cells expressing the empty EGFP vector (**A**), wild-type (WT) optineurin (**B**), E50K mutant optineurin (**C**), or *OPTN*-siRNA (**D**) (transfected cells are seen in green) and stained with Hoechst 33342 (blue) and propidium iodide (red) in cells at 24, 48, and 96 h after transfection. Arrowheads indicate apoptotic and necrotic cells.

Table 2. Apoptosis of RGC-5 cells expressing the empty EGFP vector, wild-type (WT) optineurin, E50K mutant optineurin, or *OPTN*-siRNA and treated with glutamate for 1 h.

Group	Apoptosis (%)		
	24 h	48 h	96 h
EGFP	10.34 ± 3.04	6.41 ± 1.23	7.89 ± 0.98
WT *OPTN*	15.25 ± 2.39*	20.31 ± 2.51*	21.43 ± 4.93*
E50K *OPTN*	17.24 ± 5.37*	20.48 ± 2.63*	23.21 ± 2.84*
OPTN-siRNA	11.54 ± 3.36	5.74 ± 1.35	7.58 ± 1.54

*P < 0.05 *vs* positive control at the same time point.

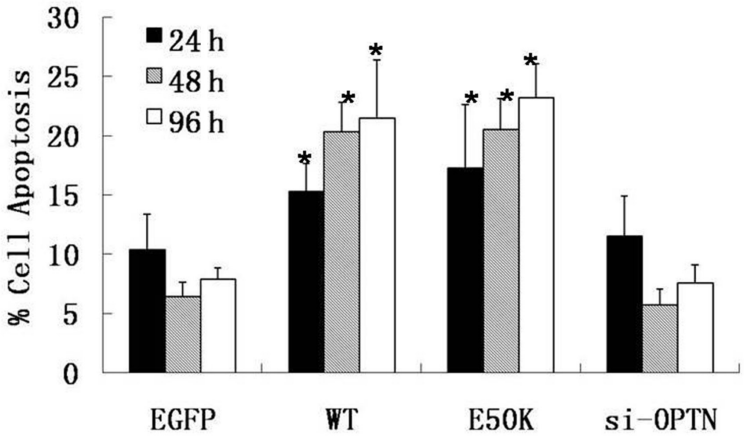

Figure 3. Apoptosis of RGC-5 cells expressing the empty EGFP vector, wild-type (WT) optineurin, E50K mutant optineurin, or *OPTN*-siRNA and treated with glutamate for 1 h. *P < 0.05 *vs* positive control at the same time point.

Cells were also treated with 650 μM H_2O_2. Compared to the negative control group (17.33 ± 4.13%), apoptosis was observed in the positive control, WT, E50K, and *OPTN*-siRNA groups 24 h after transfection (P < 0.05). However, the apoptosis rate in all four groups had decreased after 48 h. After 96 h, this rate had further decreased, with the exception of the E50K and WT optineurin groups (P < 0.05; Table 3, Figures 5 and 6).

DISCUSSION

Glaucoma is a disease causing permanent blindness and has been shown to have a hereditary component (Wolfs et al., 1998). One of the genes implicated in NTG and POAG is *OPTN*. However, little is known about the molecular mechanisms behind optineurin-associated glaucoma pathogenesis. Abnormal expression of optineurin may be associated with the onset of glaucoma, but results regarding its effect on cell survival contradict each other, possibly due to the use of different cell types or cellular baseline optineurin expression (De Marco et al., 2006; Park et al., 2006; Chalasani et al., 2007). Regardless of its pathogenesis, injury to and death of RGCs constitute the final stage of glaucoma. RGCs are the cells most affected during POAG, and the key to demonstrating the role of optineurin in the onset of this disease is the investigation of its effect on RGC survival.

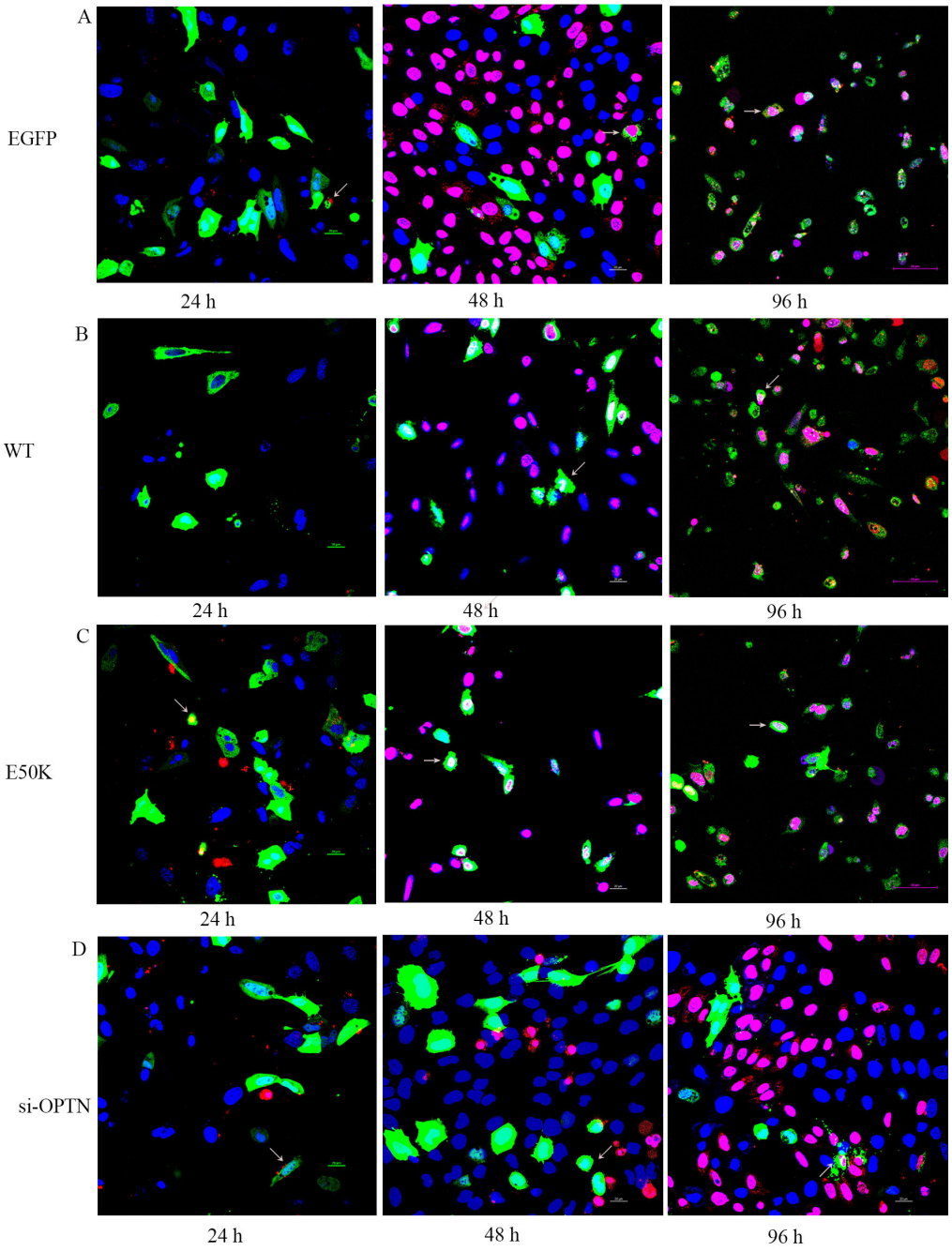

Figure 4. Laser confocal imaging of RGC-5 cells expressing the empty EGFP vector (**A**), wild-type (WT) optineurin (**B**), E50K mutant optineurin (**C**), or *OPTN*-siRNA (**D**) (transfected cells are seen in green) and stained with Hoechst 33342 (blue) and propidium iodide (red) 1 h after glutamate treatment in cells at 24, 48, and 96 h after transfection. Arrowheads indicate apoptotic and necrotic cells. Apoptosis was observed in all groups following glutamate treatment.

Table 3. Apoptosis of RGC-5 cells expressing the empty EGFP vector, wild-type (WT) optineurin, E50K mutant optineurin, or *OPTN*-siRNA and treated with H_2O_2 for 1 h.

Group	Apoptosis (%)		
	24 h	48 h	96 h
EGFP	26.42 ± 7.63	11.67 ± 2.81	9.09 ± 1.53
WT *OPTN*	22.22 ± 7.94*	9.52 ± 1.38*	25.03 ± 4.06*
E50K *OPTN*	36.28 ± 8.03*	10.81 ± 1.92	28.37 ± 4.32*
OPTN-siRNA	19.21 ± 6.37*	10.11 ± 2.04	7.36 ± 0.49

*P < 0.05 *vs* positive control at the same time point.

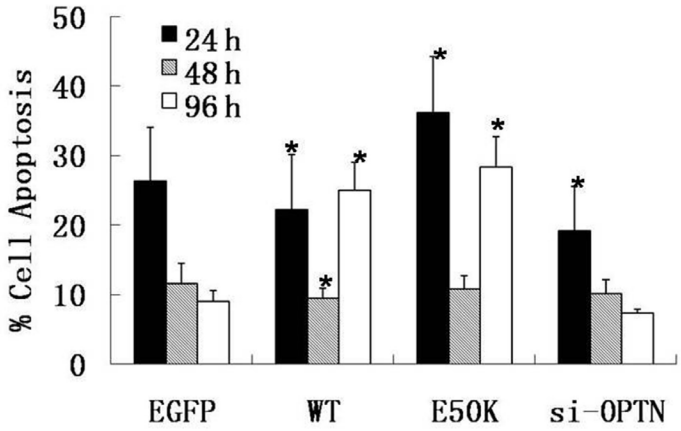

Figure 5. Apoptosis of RGC-5 cells expressing the empty EGFP vector, wild-type (WT) optineurin, E50K mutant optineurin, or *OPTN*-siRNA and treated with H_2O_2 for 1 h. *P < 0.05 *vs* positive control at the same time point.

Our results show that the degree of RGC apoptosis observed in the positive control, WT, and E50K groups increased 24 h after transfection (P < 0.05) compared to the blank (0.74 ± 0.34%) and negative (0.96 ± 0.41%) control groups. This is consistent with the results of Chalasani et al. (2007), whose report suggested that the ability of WT optineurin and the E50K mutant form to induce cell death appears to be specific to RGCs. Neither this mutant nor WT optineurin was able to induce cell death in IMR-32 (a neuronal cell line), HeLa, or COS-1 cells; differing levels of endogenous optineurin may be responsible for this phenomenon. In addition, our study shows that the effect of this protein on RGC survival was time-dependent.

Using siRNA techniques, a previous study (Rezaie and Sarfarazi, 2005) showed that downregulation of optineurin altered the morphology of the Golgi complex in normal rat kidney and HeLa cells, and increased cell apoptosis. In our investigation on RGCs, however, we found that suppression of optineurin had a cytoprotective effect in comparison to the positive control group. This discrepancy may be caused by differing baseline levels of endogenous optineurin in different cell lines.

Changes in the extent of RGC apoptosis following H_2O_2 or glutamate treatment were observed, with higher and fluctuating levels in transfected cells following an apoptotic stimulus, possibly related to the NF-κB pathway. Optineurin translocates to the nucleus after H_2O_2 stimulation, which reduces the negative regulatory effect it has on the NF-κB pathway. Thus,

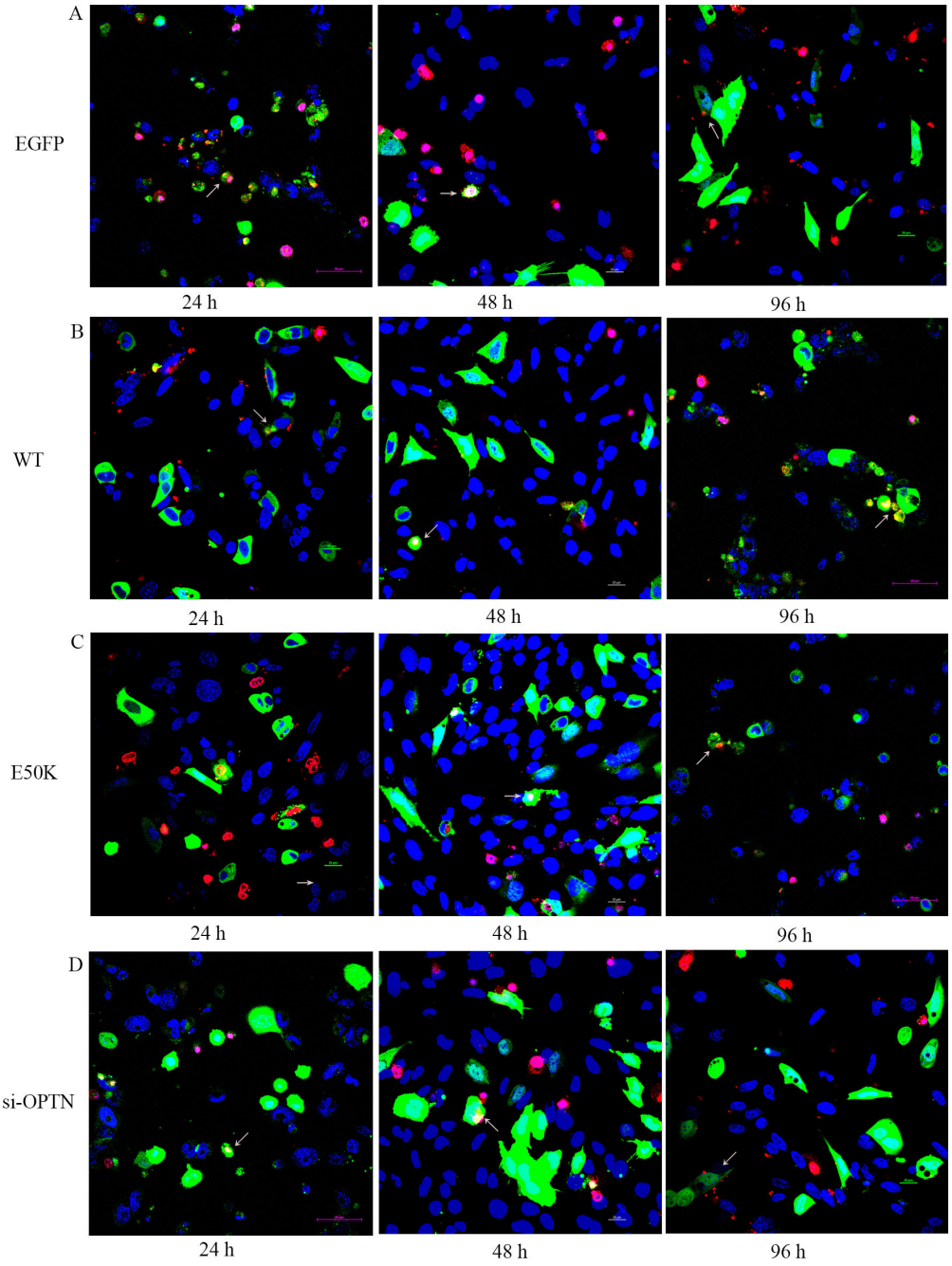

Figure 6. Laser confocal imaging of RGC-5 cells expressing the empty EGFP vector (**A**), wild-type (WT) optineurin (**B**), E50K mutant optineurin (**C**), or *OPTN*-siRNA (**D**) (transfected cells are seen in green) and stained with Hoechst 33342 (blue) and propidium iodide (red) 1 h after H_2O_2 treatment in cells at 24, 48, and 96 h after transfection. Arrowheads indicate apoptotic and necrotic cells. Apoptosis was observed in all groups following H_2O_2 treatment.

heightened expression of NF-κB increases resistance to apoptosis (De Marco et al., 2006). However, NF-κB expression increases that of optineurin, overexpression of which, combined with apoptotic stress, leads to rapid inactivation of NF-κB (Sudhakar et al., 2009).

Our study shows that overexpression of optineurin, both WT and the E50K mutant form, had a pathological effect, while its suppression afforded a degree of cytoprotection. These results may be related to oxidative stress and vesicle trafficking activities, but the exact mechanism requires further exploration.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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