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Progranulin Insufficiency Affects Lysosomal Homeostasis in Retinal Pigment Epithelium

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Abstract. *Background: Homozygous loss-of-function progranulin gene (GRN) mutation carriers develop adult-onset neuronal ceroid lipofuscinosis (NCL), a lysosomal storage disease. Clinically, NCL patients display retinal degeneration and visual dysfunction. However, there is little information about the effects of progranulin dysfunction on lysosomal function of the retinal pigment epithelium (RPE). Materials and Methods: We performed RNA interference knock down of progranulin in primary human RPE (hRPE) cells and observed RPE function and lysosomal activity. Results: Progranulin localized to the lysosome in RPE cells. Loss of progranulin did not affect the biogenesis of lysosomes in RPE cells, while it was necessary for the activation of lysosomal proteases. Furthermore, progranulin deficiency decreased cell viability and disrupted the cell-cell junctions. Conclusion: Our results demonstrate that progranulin insufficiency disturbs lysosomal activity and physiological functions in RPE cells.*

Progranulin is an evolutionarily conserved glycoprotein, which is highly expressed in numerous tissues, including epithelial cells, neuronal tissue, and immunocytes (1-3). Progranulin has various biological functions, including anti-inflammation, cell survival, tumorigenesis, and proteolysis (4). Homozygous loss-of-function progranulin gene *(GRN)* mutation carriers completely lose progranulin expression and develop adult-onset neuronal ceroid lipofuscinosis (NCL), a lysosomal storage disease (5, 6). Gene mutations associated with different NCL types are mostly related to the process of lysosomal degradation, indicating that progranulin is also involved in lysosomal function (7). Accumulating evidence indicating that progranulin is indispensable for proper lysosomal function is as follows: Extracellular progranulin undergoes endocytosis and intracellular trafficking to reach the lysosomes (8, 9). Progranulin expression facilitates lysosomal acidification in HEK293T and SH-SY5Y cells (10) while its deficiency leads to lysosomal dysfunction in microglia of *GRN*-knockout mice (11, 12). In addition, loss of progranulin also affects the biogenesis of lysosomes via the activation of transcription factor EB (TFEB) in neurons and microglia (10, 13). These reports support the role of progranulin in regulating the formation and function of lysosomes.

Clinically, NCL patients display retinal degeneration and visual dysfunction, which is accompanied by the accumulation of auto fluorescent aggregates in the retina (14, 15). In addition, there are some available transgenic mouse models for most NCLs, and these models show characteristic retinal phenotypes (16-20). Regarding progranulin, some mouse models with progranulin deficiency have been generated, and retinal degeneration and accumulation of auto fluorescent materials have been observed in these mice (21-23). Our previous studies demonstrated photoreceptor degeneration and abnormal astrogliosis in the retinas of progranulin-deficient mice (24, 25). These reports suggest that progranulin may be indispensable for maintaining retinal homeostasis.

The retinal pigment epithelium (RPE) is a polarized epithelial monolayer located between the neuronal retina and the choriocapillaris. RPE cells participate in visual reception through the regeneration of visual pigments. In addition, the major roles of the RPE monolayer include regulation of nutrient transportation from the choriocapillaris into the neural retina and disposal of metabolic waste from photoreceptors (26, 27). The phagocytosis-lysosome pathway plays an essential role in RPE cells in digesting the rod and cone outer segments of photoreceptors and maintaining retinal homeostasis (28). In our previous study, we demonstrated that the addition of recombinant progranulin stimulates phagocytosis of RPE cells (29). However, there is little information about the effects of progranulin dysfunction or other NCL-related mutations on the lysosomal function of RPE cells.

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Thus, the purpose of the present study was to determine whether progranulin deficiency has any effect on the lysosomal function of RPE cells. In this study, we performed RNA interference knockdown of progranulin in primary human RPE (hRPE) cells and observed RPE function and lysosomal activity.

Materials and Methods

Cell cultures. hRPE cells were obtained from Lonza (Morristown, NJ, USA, Cat# 00194987). The hRPE cells were grown in RtEGM Retinal Pigment Epithelial Cell Growth Medium BulletKit™ (Lonza, Cat# 00195409) containing 2% fetal bovine serum (FBS), gentamicin sulfate/amphotericin -1000, L-glutamine, and basic fibroblast growth factor, according to the manufacturer's protocol. Cultures were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. The cells were passaged every two days. Subconfluent monolayers of hRPE cells from passages 3 to 4 were used in the experiments.

siRNA transfection. To reduce the expression level of human progranulin in hRPE cells, three small interfering RNA (siRNA) sequences targeting *GRN* (Thermo Fisher Scientific, Waltham, MA, USA, Cat# 1299003) were used. The following sequences of siRNA were used: sequence #1, 5'-GCAGACGGGCGAUCCUGCUUCCAAA-3' (sense) and 5'-UUUGGAAGCAGGAUCGCCCGUCUGC-3' (antisense); sequence #2, 5'-GAUGUGAAAUGUGACAUGGAGGUGA-3' (sense) and 5'-UCACCUCCAUGUCACAUUUCACAUC-3' (antisense); sequence #3, 5'-AGAUCGUGGCUGGACUGGAGAAGAU-3' (sense) and 5'-AUCUUCUCCAGUCCAGCCACGAUCU-3' (antisense).

Stealth RNAi siRNA Negative Control (Thermo Fisher Scientific, Cat# 12935200) was used as a negative control for RNA interference.

To evaluate the effect of *GRN* siRNA knockdown, the cells were cultured in 96- or 24-well plates containing culture medium at 37˚C for 24 h. Then, the medium was changed to antibiotic-free culture medium and the siRNAs (20 nmol) were transfected into the cells using Lipofectamine® RNAiMAX Reagent (Thermo Fisher Scientific, Cat# 13778-150) and Opti-MEM® (Thermo Fisher Scientific, Cat# 31985070). After 48 h of exposure, the medium was replaced with fresh culture medium.

Lysosomal activity assay. To evaluate the lysosomal activity in RPE cells, LysoTracker™ Red DND-99 (Thermo Fisher Scientific, Cat# L7528) and DQ™ Red BSA (Thermo Fisher Scientific, Cat# D12051) were dissolved in PBS (500 nM and 100 μg/mL) and stored at 4˚C. The cells were incubated with 500 nM LysoTracker™ Red DND-99 or 10 μg/mL LysoTracker™ Red DND-99, for 30 min or 12 h at 37˚C. Hoechst 33342 solution (1:1,000; Thermo Fisher Scientific, Cat# H3570) was added 15 min before imaging. Images were taken with All-in-One Fluorescent Microscope (BZ-X710, Keyence, Osaka, Japan). The obtained images were analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunocytochemistry. hRPE cells were fixed with 2.67% paraformaldehyde at room temperature for 30 min. After fixing, the cells were incubated with PBS containing 0.2% Triton X-100 (Bio-Rad Laboratories, Cat# 1610407) for 30 min, followed by 1 h in blocking buffer [PBS containing 1% bovine serum albumin (Nacalai Tesque, Kyoto, Japan, Cat# 01863-06)]. The samples were incubated with primary antibodies overnight at 4˚C. After three washes with PBS, the cells stained with secondary antibodies and Hoechst 33342 (1:1,000; Thermo Fisher Scientific, Cat# H3570) in PBS, for 1 h. The following antibodies were used: goat polyclonal anti-progranulin (1:200; R&D Systems, Minneapolis, MN, USA, Cat# AF2420), rabbit polyclonal anti-LAMP2 (1:200; Proteintech, Rosemont, IL, USA, Cat# 66301-1-Ig), rabbit polyclonal anti-occludin (1:200; ATLAS Antibodies, Stockholm, Sweden, Cat# A75354), mouse monoclonal anti-vimentin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA, Cat# sc-6260), Alexa Fluor® 488 donkey anti-rabbit IgG (1:1000; Thermo Fisher Scientific, Cat# A21206), Alexa Fluor® 546 donkey anti-mouse IgG (1:1000; Thermo Fisher Scientific, Cat# A10036), and Alexa Fluor® 647 donkey anti-goat IgG (1:1000; Jackson ImmunoResearch, West Grove, PA, USA, Cat# 705-605-147). Images were acquired with All-in-One Fluorescent Microscope (BZ-X710, Keyence, Osaka, Japan).

Western blot analysis. For western blotting, the cells were cultured in 24-well plates. Briefly, the cells were lysed with radioimmunoprecipitation assay buffer (Sigma-Aldrich, St. Louis, MO, USA, Cat# R0278) containing protease inhibitor and phosphatase inhibitor cocktails. The cell lysate was centrifuged at 12,000 × *g* for 20 min at 4˚C, and the protein concentration in the supernatants was measured using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Cat# 23225). The sample buffer solution (FUJIFILM Wako, Osaka, Japan, Cat# 196-16142) was added to the supernatants (supernatant:sample buffer solution=3:1) and then boiled for 5 min.

The protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, in 5-20% gradient gels (SuperSep™ Ace; FUJIFILM Wako, Cat# 194-15021), and then transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA, USA, Cat# IPVH00010). The membranes were incubated with the following primary antibodies overnight at 4˚C: goat polyclonal anti-progranulin (1:500; R&D Systems, Cat# AF2420), rabbit polyclonal anti-occludin (1:500; ATLAS Antibodies, Stockholm, Sweden, Cat# A75354), rat monoclonal anti-LAMP1 (1:500; Abcam, Cambridge, UK, Cat# ab25245), goat polyclonal anti-cathepsin D (1:500; R&D Systems, Cat# AF1029), rabbit anti-LAMP2 (1:500; Proteintech, Cat# 66301-1-Ig), and mouse monoclonal anti-β-actin (1:2,000; Sigma-Aldrich, Cat# A2228). After incubation with the primary antibodies, the membranes were incubated with the following antibodies for 1 h at room temperature: horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:2,000; Thermo Fisher Scientific, Cat# 32460), goat anti-mouse IgG (1:2,000; Thermo Fisher Scientific, Cat# 32430), and rabbit anti-goat IgG (1:2,000; Thermo Fisher Scientific, Cat# 31402). The bands were visualized using ImmunoStar® LD (FUJIFILM Wako, Cat# 290-69904) and then measured using the Amersham Imager 680 (Cytiva, Marlborough, MA, USA).

Cell viability assay. Cellular viability assay was conducted using the Cell Counting Kit 8 (Dojindo Molecular Technologies, Kumamoto, Japan, Cat# 343-07623). The cells were seeded in 96-well plates at a density of 10,000 cells/well. After 48, 72, and 96 h of siRNA treatment,10 μl of CCK-8 solution was added to each well. The plates were incubated for 1 h at 37˚C, and then the absorbance was measured at 450 nm with a reference wavelength of 650 nm using the Varioskan Flash 2.4 microplate reader (Thermo Fisher Scientific).

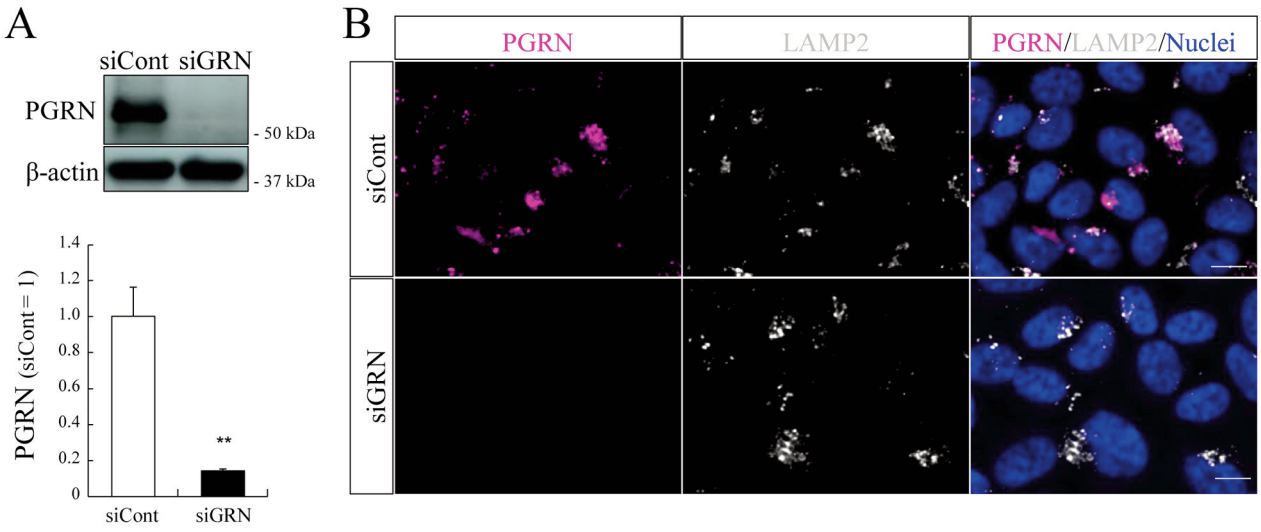

Figure 1. *Expression and location of progranulin in human RPE cells. (A) Immunoblotting of cell lysates, obtained from hRPE cells, transfected with either negative control RNA (siControl) or siRNA against progranulin (siGRN). The quantification of progranulin signal was normalized against β-actin. n=4, mean±standard error of the mean (S.E.M.). **p<0.01 vs. siControl using Welch's t-test (B) Immunostaining for progranulin (magenta) and LAMP2 (white) in siControl- or siGRN-treated cells. Nuclei are stained with Hoechst 33342 (blue). Scale bar=10 μm.*

Statistical analyses. Data are expressed as the mean±standard error of the mean (S.E.M.) of at least four independent wells. Two data sets were compared using a two-tailed Welch's *t*-test. *p*-Values <0.05 were considered statistically significant. All statistical tests were conducted using IBM® SPSS® Statistics 24 (IBM, Armonk, NY, USA).

Results

Progranulin located in lysosome of human RPE cells. To elucidate the expression and location of progranulin in RPE cells, we conducted western blotting and immunostaining after RNA interference with siRNA against progranulin. First, we checked that the efficiency of progranulin knockdown contained the #1, #2, or #3 sequences (data not shown). We chose sequence #1, which showed the highest efficiency of progranulin knockdown (Figure 1A). Next, the location of progranulin in RPE cell culture was examined by fluorescent immunostaining. Progranulin is a lysosome-resident protein found in several cell types (30). Therefore, we conducted co-immunostaining of progranulin and lysosome-associated membrane protein 2 (LAMP2). In the siControl-treated group, progranulin co-localized with LAMP2, while the signal of progranulin was not detected in siGRN-treated cells (Figure 1B). In contrast, the expression pattern of LAMP2 was not different between the siControl- and siGRN-treated groups (Figure 1B).

Progranulin-deficiency induces the disruption of the lysosomal function in RPE cells. To evaluate lysosomal activity in progranulin-silenced RPE cells, lysosomal staining with two fluorescent dyes was performed. LysoTracker™ Red DND-99 is a fluorescent acidotropic probe for labeling and tracking acidic organelles in live cells (31). The fluorescence intensity of LysoTracker dye in the progranulin-silenced hRPE cells was significantly lower than that in the siRNA negative control treated-cells (Figure 2A, B). DQ™ Red BSA is a fluorogenic substrate for lysosomal proteases that emits strong fluorescence after being disassembled in lysosomes (32). Consistent with the result of LysoTracker dye, the brightness of DQ red dye decreased in siGRN-treated RPE cells compared to control cells (Figure 2C, D).

Progranulin disfunction does not affect the expression level of lysosome-related proteins. In nerve cells and immune cells, including microglia and macrophages, it has been reported that the loss of progranulin leads to overexpression of lysosome-related proteins (10, 33). To evaluate the impact of progranulin deficiency on the expression of lysosomal proteins in RPE cells, the expression levels of lysosome-associated membrane proteins (LAMP1 and LAMP2) and lysosomal aspartyl protease (pro- and mature-cathepsin D) were compared between siGRN-and siControl-treated hRPE cells by western blotting. The expression levels of both LAMP1 and LAMP2 were unaffected by progranulin knockdown (Figure 3A-C). Similarly, the expression levels of pro- and mature-cathepsin D did not change between siControl- and siGRN-treated RPE cells (Figure 3A, D, E).

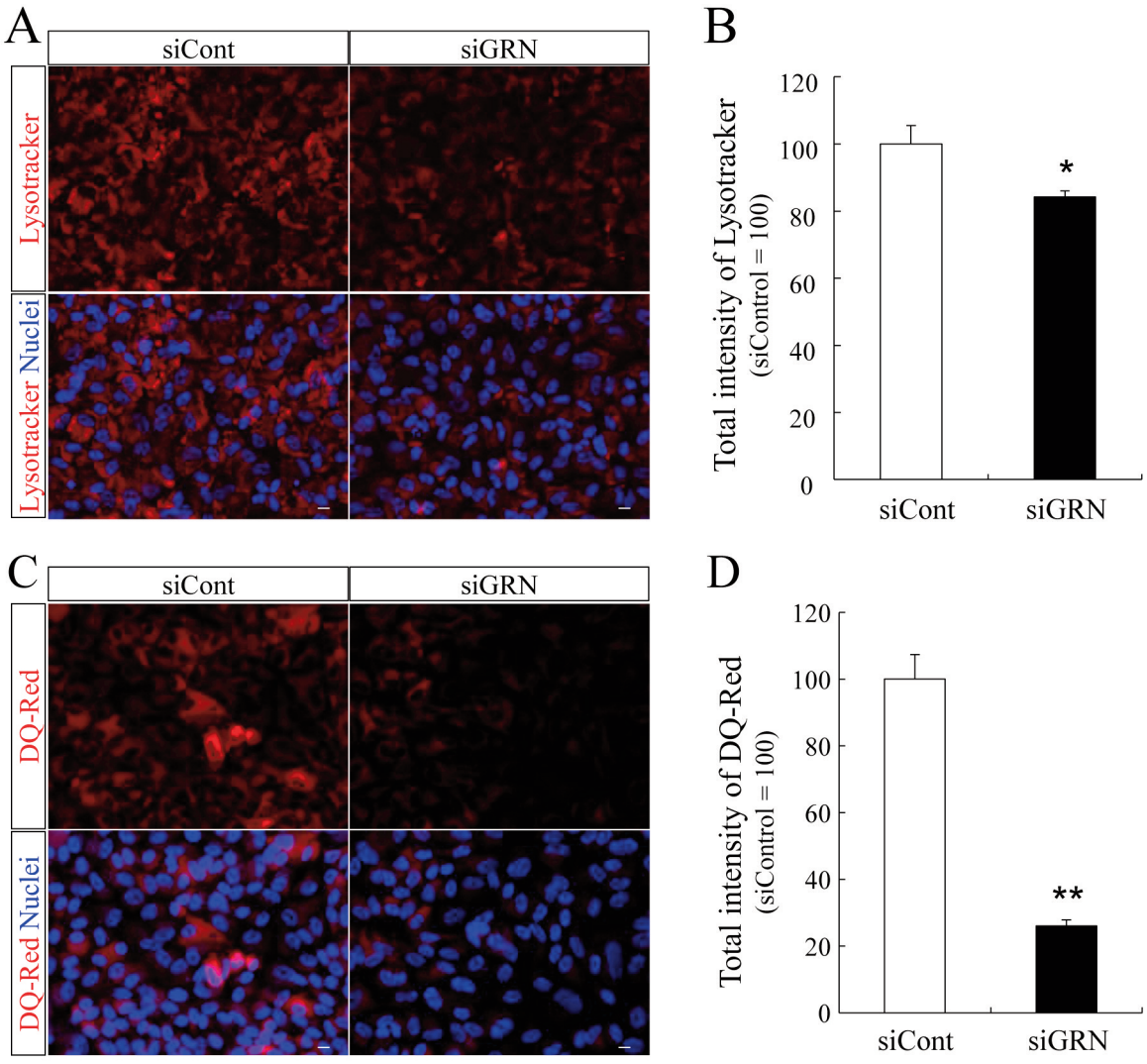

Figure 2. *Effects of progranulin-deficiency on lysosomal function of hRPE cells. (A) Representative images of LysoTracker™ Red DND-99 staining (red) and nuclei staining (Hoechst 33342, Blue) of hRPE cells. (B) The fluorescent intensity of LysoTracker™ Red DND-99 in hRPE cells, transfected with negative control RNA or siGRN. (C) Representative images of DQ-Red staining (red) and nuclei staining (Hoechst 33342, Blue) of hRPE cells. (D) Quantitative analysis of the fluorescence intensity of DQ-Red+ cells, transfected with negative control RNA or siGRN. n=6, mean±S.E.M. *p<0.05, **p<0.01 vs. siControl using Welch's t-test. Scale bars=10 μm.*

Progranulin-deficiency induces the disfunction of RPE cells. To examine the effects of progranulin silencing on hRPE cell function, we determined cell viability. The cell viability of siGRN-treated hRPE cells decreased in a time-dependent manner compared to that of the siRNA negative control-treated cells (Figure 4A). Next, we evaluated the expression pattern of occludin, a typical tight junction protein, in progranulin -silenced RPE cells. The expression level of occludin in siGRN-treated hRPE cells was significantly lower than that in siControl-treated cells (Figure 4B). In addition, progranulin knockdown disrupted occludin integrity, accompanied by changes in the expression pattern of vimentin, a cytoskeletal protein (Figure 4C).

Discussion

In the current study, we reported that progranulin localized to the lysosome in RPE cells. Additionally, loss of progranulin did not affect the biogenesis of lysosomes in RPE cells, while it was necessary for the activation of lysosomal proteases. Progranulin deficiency decreased cell viability and disrupted the cell-cell junctions. These results

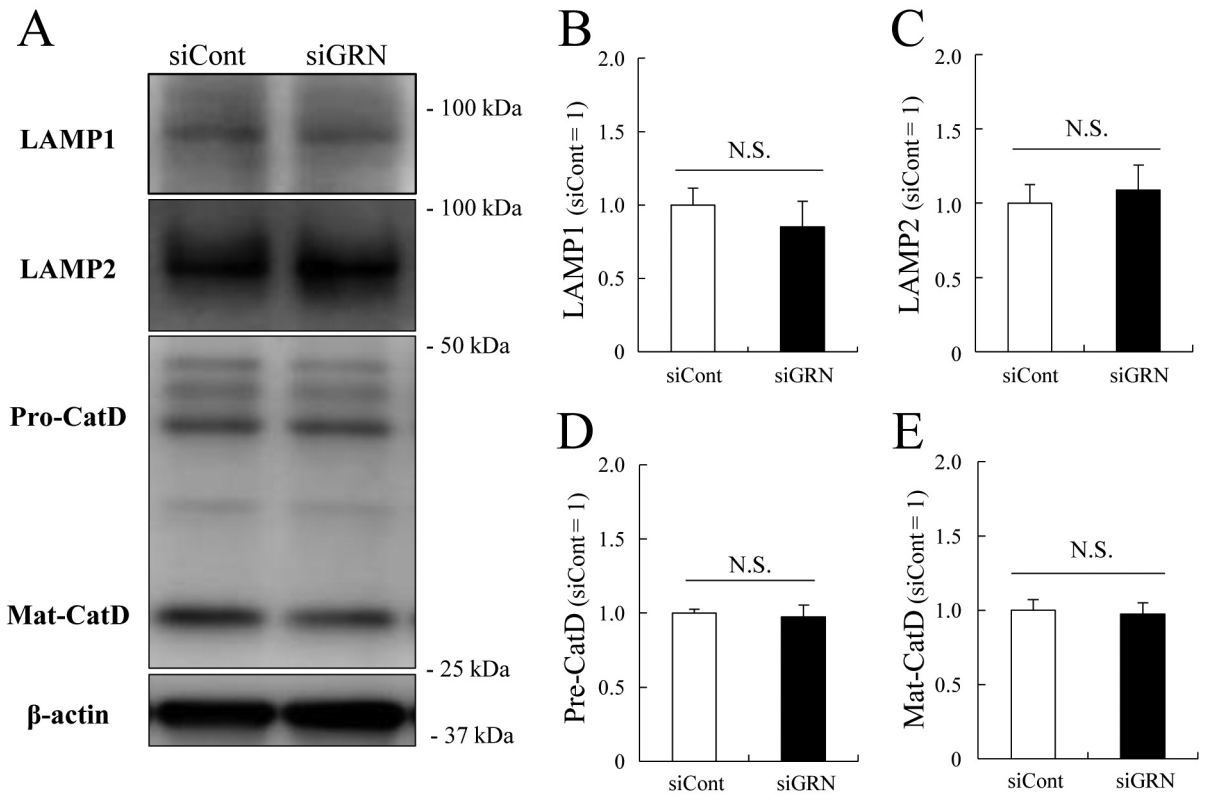

Figure 3. *Expression level of lysosomal-related protein in progranulin-deficient hRPE cells. (A) Immunoblotting of LAMP1, LAMP2, CatD, and β-actin in hRPE cell lysates. (B-E) The expression level of LAMP1 (B), LAMP2 (C), pro-CatD (D) and mat-CatD (E) normalized by β-actin is shown as the fold-change to siControl-treated hRPE cells. n=4, mean±S.E.M.*

are the first to demonstrate that progranulin dysfunction affects cell function and lysosomal activity in RPE cells.

Progranulin is secreted from various types of cells, including neurons, microglia, macrophages, fibroblasts, and cancer cells (3). In addition, progranulin directly or indirectly binds to trafficking receptors, sortilin, or cation-independent mannose 6-phosphate receptor (CI-M6PR) and is carried to the lysosome (8, 30). In the retinal cross section of wild-type mice, high levels of progranulin expression were observed in the RPE layer as well as in the neuronal retina (24, 34). However, there was no evidence of the intracellular localization and function of progranulin in RPE cells. We showed that endogenous progranulin in RPE cells was co-localized with lysosomal membrane proteins (Figure 1). This result suggests that progranulin functions as a lysosome-related protein in RPE cells, which is similar to its role in other tissues.

Lysosomes are the major digestive organelles in the RPE cells. They have over 60 acidic hydrolases in their vesicles, each capable of degrading specific substrates (35). Most lysosomal enzymes in the RPE are known to work in a narrow pH range in the acidic environment of the lysosomal vesicle (36). The normal functioning of lysosomes is necessary to degrade both extracellular (photoreceptor outer segment) and intracellular (autophagy) materials (28). In a previous study, we demonstrated that the addition of progranulin promoted the phagocytic activity of PRE cells (29). It was also reported that the expression of progranulin facilitates acidification of lysosomal vesicles in some cultured cells (10). In the current study, our results showed that the acidity of intracellular vesicles and lysosomal activity were attenuated in progranulin-silenced RPE cells (Figure 2). These findings indicate that progranulin plays an important role in maintaining the acidity of lysosomes in RPE cells. Lysosomal acidification is mainly regulated by vacuolar-type H^+-ATPase (V-ATPase), which transports protons across the membrane (37). To date, there is no evidence of a direct interaction between progranulin and V-ATPase. Further studies are needed to unravel the mechanism by which progranulin maintains lysosomal acidity in RPE cells.

Progranulin insufficiency has been reported to promote the biogenesis of lysosomal proteins in some cultured cells (10, 33). Additionally, lysosomes were enlarged and increased in number in progranulin-deficient mouse cortical neurons (38).

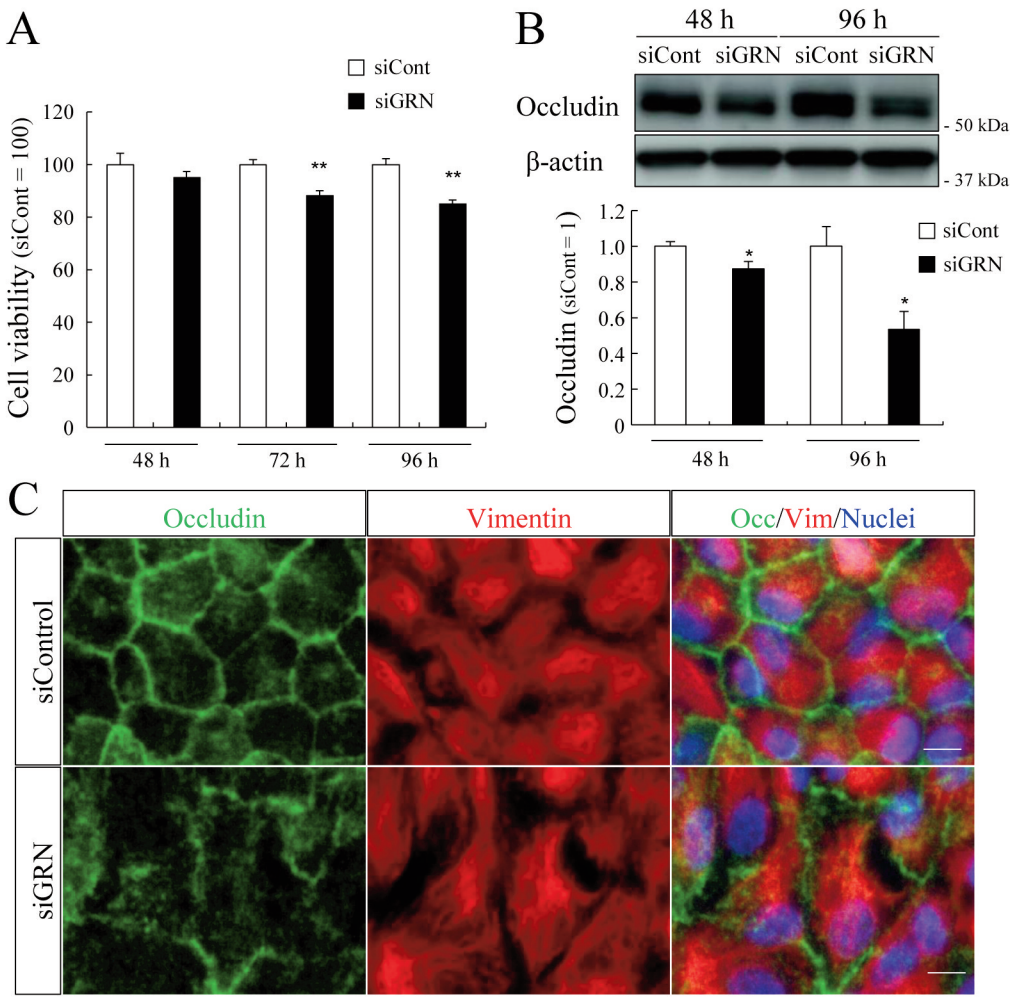

Figure 4. *Effects of progranulin-deficiency on cell viability and tight junction of hRPE cells. (A) Quantification of cell viability in hRPE cells exposed to negative control RNA or siGRN at the indicated time point. n=6, mean±S.E.M. **p<0.01 vs. siControl using Welch's t-test. (B) The expression level of occludin normalized by β-actin is shown as the fold-change to siControl-treated hRPE cell. n=4, mean±S.E.M. *p<0.05 vs. siControl using Welch's t-test. (C) Immunostaining of hRPE cells with anti-occludin (green) and anti-vimentin (red) antibodies. Nuclei were stained with Hoechst 33342 (blue). Scale bars=10 μm.*

However, in the current study, transient progranulin knockdown did not affect the expression level of lysosome proteins in RPE cells (Figure 3). Consistent with the results of the current study, the expression of lysosomal proteins in the RPE-choroid complex was not affected in PGRN knockout mouse (34). Lysosomal dysfunction may result from dysregulation of any of the myriad of proteins required for maintaining lysosomal homeostasis. However, in each case, the disease phenotype and affected tissues can differ (28). The mechanisms by which lysosomal function is regulated in the RPE may be unique. Additionally, some studies suggested that there are some modifier genes which regulate phenotypes of progranulin deficiency. Grn and Tmem106b double knockout mice showed a significant increase of lysosomal proteins in neuron and microglia as compared to wildtype and Grn single knockout mice (39). Nemo-like kinase regulated the expression level of progranulin via lysosomal degradation in microglia, but not through neurons (40). These modifiers might affect the level of lysosome biogenesis in progranulin deficient RPE cells.

Lysosomal dysfunction affects the physiological functions of the RPE. Treatment with the lysosomal alkalizers bafilomycin A1 and chloroquine increases vulnerability to oxidative stress in ARPE-19 cells (41). Likewise, progranulin-deficient RPE cells showed decreased cell viability (Figure 4). In addition, the expression level and distribution of occludin were affected by transient loss of progranulin (Figure 4). These results suggest that progranulin might play an important role in maintaining the normal function of RPE cells.

In conclusion, our results demonstrate that progranulin insufficiency disturbs lysosomal activity and physiological functions in RPE cells. These findings might help to disentangle the pathological basis of NCL and other retinal degenerative diseases.

Conflicts of Interest

The Authors have no conflicts of interest to disclose in regard to this study.

Authors' Contributions

KT, SN, MS, and HH contributed to the conception and design of the study. KT did the acquisition, analysis, and interpretation of data. KT and MS drafted the work and critically revised it. KT, SN, MS, and HH approved the final version to be published. KT, SN, MS, and HH agreed on all aspects of the work.

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