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Melatonin induces autophagy via an mTOR-dependent pathway and enhances clearance of mutant-TGFBIp

Abstract: The hallmark of granular corneal dystrophy type 2 (GCD2) is the deposit of mutant transforming growth factor-β (TGF-β)-induced protein (TGFBIp) in the cornea. We have recently shown that there is a delay in autophagic degradation of mutant-TGFBIp via impaired autophagic flux in GCD2 corneal fibroblasts. We hypothesized that melatonin can specifically induce autophagy and consequently eliminate mutant-TGFBIp in GCD corneal fibroblasts. Our results show that melatonin activates autophagy in both wild-type (WT) and GCD2-homozygous (HO) corneal fibroblast cell lines via the mammalian target of rapamycin (mTOR)-dependent pathway. Melatonin treatment also led to increased levels of beclin 1, which is involved in autophagosome formation and maturation. Furthermore, melatonin significantly reduced the amounts of mutant- and WT-TGFBIp. Treatment with melatonin counteracted the autophagy-inhibitory effects of bafilomycin A_1, a potent inhibitor of autophagic flux, demonstrating that melatonin enhances activation of autophagy and increases degradation of TGFBIp. Cotreatment with melatonin and rapamycin, an autophagy inducer, had an additive effect on mutant-TGFBIp clearance compared to treatment with either drug alone. Treatment with the selective melatonin receptor antagonist luzindole did not block melatonin-induced autophagy. Given its ability to activate autophagy, melatonin is a potential therapeutic agent for GCD2.

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Key words: autophagy, granular corneal dystrophy type 2, LC3, melatonin, (mTOR), TGFBIp

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Introduction

Granular corneal dystrophy type 2 (GCD2, also called A-vellino corneal dystrophy) is an autosomal dominant disorder caused by a point mutation (R124H) in the transforming growth factor-β-induced gene (*TGFBI*) on chromosome 5q31 [1]. Age-dependent progressive accumulation of TGFBI protein (TGFBIp) in the corneal stroma is a hallmark of GCD2 and interferes with corneal transparency [2–4]. TGFBIp is secreted into the extracellular matrix (ECM) by various cell types [2]. This protein exhibits tumor suppressive activity [5, 6]. Recently, a study involving mice showed that TGFBIp deficiency induces cell proliferation and spontaneous tumor development [7]. The *TGFBI* gene encodes a highly conserved 683-amino acid protein with a secretory signal sequence and an Arg-Gly-Asp motif in the C-terminal region that serves as a ligand recognition site for integrins [2]. TGFBIp is a component of the ECM where it mediates cell adhesion and migration by interacting with integrins [8–10].

Autophagy involves the formation of a double membrane structure, called the autophagosome, that engulfs long-lived cytoplasmic molecules and damaged organelles, such as mitochondria, and delivers them to lysosomes for degradation and recycling [11]. Autophagy induction plays critical roles in various cellular responses such as defence against infection [12], antigen presentation [13, 14], embryogenesis [15], development [16], and metabolism [17]. In contrast to autophagy induced in response to environmental signals, basal level autophagy may have different important functions, such as cellular quality control in protein conformational disorders [18, 19]. Induction of autophagy by drugs is a promising therapeutic approach for the treatment of neurodegenerative diseases, such as Alzheimer's, Parkinson's, Huntington's, and polyglutamine diseases [20]. These diseases involve the accumulation of insoluble proteins in the cytosol of neurons. Beneficial effects of induced autophagy have also been described for other diseases associated with aggregate-prone proteins [21–23].

Melatonin is produced in the pineal gland of all vertebrates [24] and is involved in the control of various physiological functions, such as the coordination of seasonal and circadian rhythms, anti-inflammatory actions, and anticancer effects [25–29]. Melatonin and its metabolites have received much attention due to their direct free radical scavenging [30–33] and antioxidant properties [34, 35]. Numerous studies have documented that melatonin has anti-apoptotic effects on normal cells [36–38] attributable to its antioxidant properties [39]. Another anti-apoptotic mechanism of melatonin involves the inhibition of Bad translocation from the cytosol to the mitochondria by maintaining protein–protein interactions between 14-3-3β and p-Bad [40]. In mammals, some of the specific

functions of melatonin are mediated by two different subtypes of G protein-coupled receptors, MT1 and MT2 [41]. Three membrane melatonin receptor subtypes, MT1, and MT2, have been characterized [42]. Melatonin receptor subtypes are also present in the retina, cornea, ciliary body, lens, choroid, and sclera of ocular tissues [43]. MT1 is present in the endothelial cell layer, keratocytes, and the epithelial cell layer of human corneas [44]. Additionally, melatonin suppresses free radical-mediated ocular disease [45, 46]. On the basis of these findings, several clinical trials have investigated the usefulness of melatonin for treating age-related macular degeneration and glaucoma [47, 48].

We previously showed that TGFBIp is degraded by the autophagy/lysosomal degradation pathway and its degradation is delayed in GCD2 corneal fibroblasts because autophagy is impaired [49]. Furthermore, the autophagy inducer rapamycin reduced the levels of mutant-TGFBIp in GCD2 corneal fibroblasts [49]. Retention of mutant-TGFBIp in autophagosomes or autolysosomes is observed in GCD2 corneal fibroblasts because of autophagy impairment [49]. There is growing interest in the therapeutic possibilities for the treatment of GCD2 using autophagy inducers.

Melatonin was recently shown to be a regulator of autophagy [50, 51]. Here, we identify a novel role for melatonin as an autophagy inducer. Melatonin leads to enhanced clearance of mutant-TGFBIp in GCD2 corneal fibroblasts by induction of autophagy via the mTOR-dependent pathway.

Materials and methods

Materials

The lysosomal inhibitor bafilomycin A_1, dimethylsulfoxide (DMSO), rapamycin, melatonin, and 3-methyladenine (3-MA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). SuperSignal West Pico Chemiluminescent Substrate was obtained from Pierce (Rockford, IL, USA).

Isolation and culture of corneal fibroblasts

Primary corneal fibroblasts were prepared from healthy corneas obtained from the eye bank of Yonsei University Severance Hospital and from both GCD2-heterozygotic and GCD2-homozygotic (HO) patients following either penetrating or lamellar keratoplasty. Donor confidentiality was maintained according to the Declaration of Helsinki. This study was approved by the Severance Hospital IRB Committee (4-2010-0013), Yonsei University. GCD2 was diagnosed by DNA sequencing analysis of *TGFBI* gene mutations.

Corneas were washed three times with Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) containing 1,000 units/mL penicillin and 1.0 mg/mL streptomycin sulfate (Gibco BRL). Cultures and isolation of primary corneal fibroblasts were prepared as previously described with slight modifications [52]. To develop immortalized cells, primary GCD2-HO corneal fibroblasts were infected with lentiviral particles (GenTarget Inc, San Diego, CA, USA) expressing telomerase, human telomerase catalytic subunit (hTERT), and a puromycin resistance gene. Puromycin-resistant corneal fibroblasts were selected for propagation. Corneal fibroblasts were maintained in DMEM containing 10% fetal bovine serum (Gibco BRL), 1,000 units/mL penicillin, and 1.0 mg/mL streptomycin sulfate.

Treatment of corneal fibroblasts with 3-MA, bafilomycin A_1, and rapamycin

The autophagy inhibitor 3-MA was freshly dissolved in culture medium at 37°C for 1 hr before use. To analyze the effects of 3-MA on TGFBIp accumulation, corneal fibroblasts were incubated for 12 hr with 10 mM 3-MA and analyzed for TGFBIp levels by immunoblot analysis. Bafilomycin A1, which inhibits fusion between autophagosomes and lysosomes by blocking the vacuolar adenosine triphosphate (ATP) pump, was used at 0.1 μM. Rapamycin was dissolved in DMSO immediately before use.

Preparation of cell lysates and Western blot analysis

Cell lysates from cultured corneal fibroblasts were prepared in radioimmunoprecipitation assay buffer (RIPA buffer; 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris-HCl, pH 7.4) containing a protease inhibitor tablet (Complete Mini Protease Inhibitor Tablet, #1836170, Roche, Indianapolis, IN, USA). Crude cell lysates were centrifuged at $10{,}000 \times g$ for 10 min at 4°C to remove nuclear fragments and tissue debris. Total protein concentrations were determined using a BCA kit (Pierce). Total cellular proteins were electrophoresed in 10% Tris-glycine sodium dodecyl sulfate (SDS) polyacrylamide gels. Proteins were transferred onto PVDF membranes (Millipore Corp., Bedford, MA, USA). Membranes were blocked in 5% dry milk in TBS-T (0.02 M Tris and 0.15M NaCl, pH 7.5 containing 0.1% Tween 20) at room temperature for 1 hr and washed three times with TBS-T. Blots were incubated with primary antibodies to TGFBIp (0.2 μg/mL; Cat. No. AF2935; R&D Systems, Minneapolis, MN, USA), ATG5, beclin 1, and LC3 (1:1000 dilution; #2630, #3738, and #3868, Cell Signaling Technology, Beverly, MA, USA), mTOR, mTOR (S2481), mTOR (S2448), GβL, Raptor, Rictor (1:1000 dilutions; mTOR Pathway Antibody Sampler Kit #9964, Cell Signaling Technology), and β-actin (1:5,000 dilution; Cat. No. A-5441; Sigma-Aldrich). After three washes with TBS-T, blots were incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) at room temperature for 1 hr. HRP-conjugated anti-mouse IgG (1:5,000 dilution; Cat. No. NA931V; Amersham Pharmacia Biotechnology, Piscataway, NJ, USA) or anti-rabbit IgG (1:5,000 dilution; Cat. No. NA934V; Amersham Pharmacia Biotechnology) were used as secondary antibodies. Immunoblots were visualized using the ECL system (Pierce). The immunoreactive protein bands were image-scanned and optical densities of the bands were quantified using Image J software, version 1.37 (Wayne Rasband, National Institute of Health, Bethesda, MD, USA), corrected by background subtraction and normalized to the intensity of the corresponding β-actin protein bands.

RNA isolation and real-time reverse transcription PCR (RT-PCR)

For amplification of *TGFBI* and *GAPDH* mRNA, total RNA was isolated from corneal fibroblasts by extraction in TRIZOL Reagent (Invitrogen Life Technologies). We used Primer Express Software (Applied Biosystems, Foster City, CA, USA) to design the oligonucleotide primers for the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and *TGFBI*. Using Power SYBR® Green RNA-to-CT™ 1-Step kit (Applied Biosystems, Foster City, CA) StepOnePlus™ (Applied Biosystems), we measured mRNA expression in *GAPDH* and *TGFBI* genes, according to the manufacturer's instructions. The PCR conditions for all genes were as follows: 48°C for 30 min then 95°C for 10 min then 40 cycles of 95°C for 15 s and 60°C for 1 min. The results are based on cycle threshold (Ct) values. We calculated differences between the Ct values for experimental and reference genes (*GAPDH*) and graphed as a percent of each RNA to the calibrator sample. Primers used for *TGFBI* amplification were the following: TGFBI-F, 5′-CACAGTCTTTGCTCCCACAA-3′ and TGFBI-R, 5′-CTCCGCTAACCAGGATTTCA-3′. Primers used for *GAPDH* amplification were as follows: GAPDH-F, 5′-ATGGGGAAGGTGAAGGTCG-3′ and GAPDH-R, 5′-GGGGTCATTGATGGCAACAATA-3′. TGFBIp mRNA levels were normalized to levels of GAPDH. Three independent experiments were performed, and statistical analysis was carried out using the Newman–Keuls multiple comparison tests.

Immunocytochemical staining

WT and GCD2-HO corneal fibroblasts grown on culture slides (Cat. No. REF 354108; BD Falcon, Labware, Franklin Lakes, NJ, USA) were permeabilized and fixed in methanol at −20°C for 3 min. Cells were washed in PBS, blocked with 10% bovine serum albumin (Sigma-Aldrich) in PBS for 10 min and incubated overnight with primary antibodies in blocking buffer at 4°C. Cells were incubated with secondary antibodies for 1 hr at room temperature. Coverslips were mounted on the glass slides with Vectashield Mounting Medium (Vector Labs Inc., Burlingame, CA, USA). Cells were viewed under a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). The primary antibody used was polyclonal anti-LC3 (1:1000 dilution; Cell Signaling Technology, Beverly, MA) and the secondary antibody was fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG (1:200 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

Transmission electron microscopic examination of corneal fibroblasts

Primary cultured corneal fibroblasts were fixed overnight, dehydrated, and processed for electron microscopy as described previously [53]. Evaluation was performed using a transmission electron microscope (JEM1200 EX2; JEOL Ltd., Tokyo, Japan).

Statistical analysis

Data were statistically evaluated for significance ($P < 0.05$) with one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison tests. Data are expressed as means ± SD. All data were processed using GraphPad Prism version 4.0 statistical package (GraphPad Software Inc, San Diego, CA, USA).

Results

We previously demonstrated that autophagic degradation of mutant-TGFBIp is delayed in GCD2 corneal fibroblasts and an autophagy inducer enhances the clearance of mutant-TGFBIp [49]. Here, we investigated whether melatonin could specifically induce basal autophagy and eliminate delayed degradation of mutant-TGFBIp in GCD2-HO corneal fibroblasts. Upon activation of autophagy, microtubule-associated protein 1 light chain 3 (LC3 I) is converted into LC3 II. Therefore, increases in LC3 II levels can be used as a marker for autophagy induction, as the amount of LC3 II associated with autophagosome membranes correlates with the extent of autophagosome formation [54]. Western blot analysis showed that the levels of LC3 II increase significantly in both WT and GCD2-HO corneal fibroblasts treated for 12 hr with melatonin (Fig. 1A,B).

To further examine the activation of autophagy by melatonin, we performed confocal laser microscopy studies. Immunofluorescence can be used to monitor the induction of autophagy by the appearance of LC3-positive puncta that represent autophagosome formation. Corneal fibroblasts treated for 12 hr with melatonin were analyzed by confocal microscopy. The number of 100 μM melatonin-treated cells showing autophagosome formation was greater than the respective number of untreated cells (Fig. 1C). This finding correlates with the immunoblot analyses (Fig. 1A,B). To find further evidence of autophagy induction, an electron microscope (EM) was used to investigate autophagosomes in corneal fibroblasts treated for 12 hr with or without 100 μM melatonin. A marked increase in the number of autophagic vacuoles was observed in the corneal fibroblasts treated with melatonin (Fig. 1D). Together, these results suggest that melatonin-induced autophagy may help eliminate mutant-TGFBIp in GCD2 corneal fibroblasts.

We analyzed the level of TGFBIp in WT and GCD2-HO corneal fibroblasts treated for 12 hr with or without melatonin. Western blot analysis showed that melatonin reduced the levels of TGFBIp in a dose-dependent manner in WT (Fig. 2A,B) and GCD2-HO corneal fibroblasts (Fig. 2C,D). This effect on WT corneal fibroblasts was interesting, as these cells have normal autophagic function. Treatment of WT corneal fibroblasts with 100, 200, and 300 μM melatonin for 12 hr reduced TGFBIp levels by 90.9 ± 9.7%, 66.7 ± 6.1%, and 21.2 ± 2.7%, respectively, when compared to untreated fibroblasts ($P < 0.01$) (Fig. 2A,B). Melatonin treatments at the same concentrations decreased TGFBIp expression by approximately 90.9 ± 8.8%, 75.1 ± 6.7%, and 20.9 ± 3.5%,

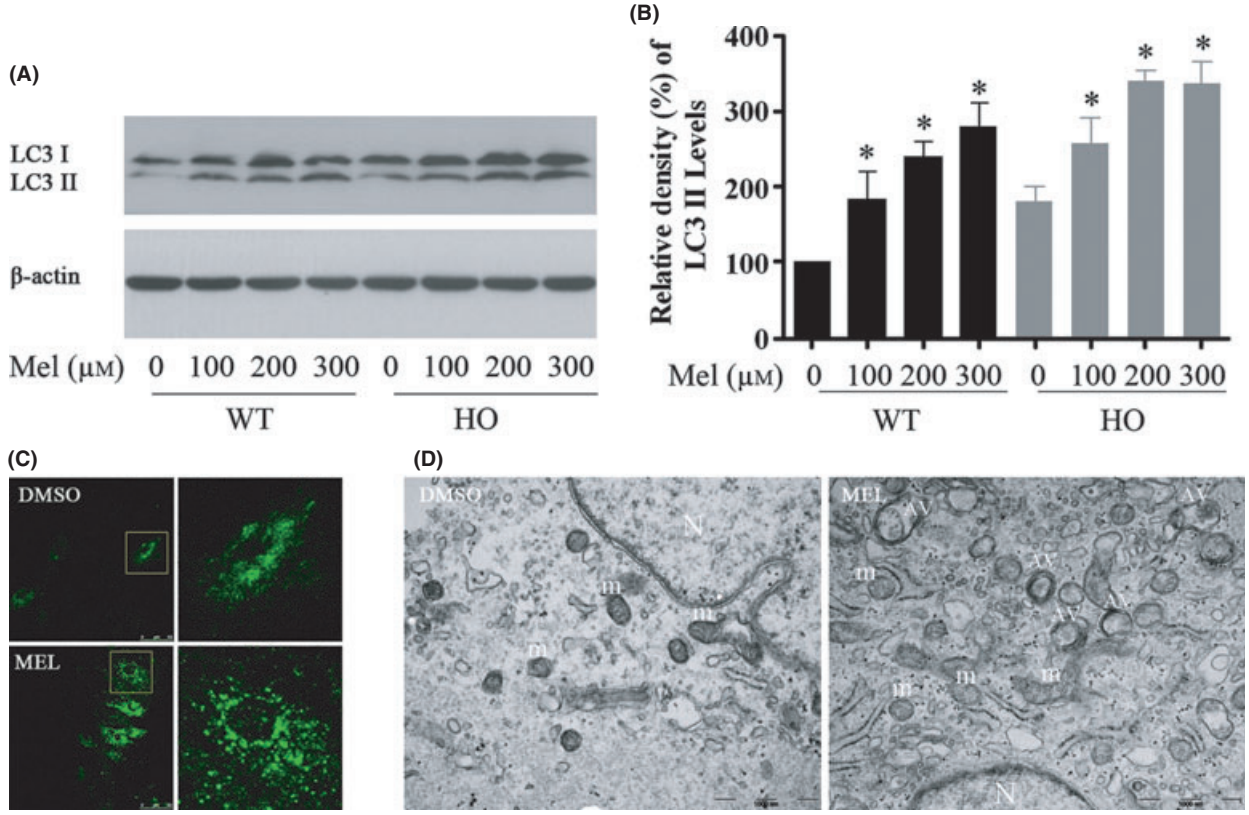

Fig. 1. Melatonin induced formation of autophagosomes in WT and GCD2-HO corneal fibroblasts. (A) Corneal fibroblasts were incubated with the indicated amounts of melatonin for 12 hr and analyzed for LC3 conversion by Western blot analysis. (B) LC3 II levels after normalization to β-actin protein signals. (C) Autophagosome formation was analyzed by fluorescence microscopy. Corneal fibroblasts were cultured for 12 hr in the absence (upper panel) or presence (lower panel) of 100 μM melatonin and stained with anti-LC3 and Alexa488-conjugated goat anti-rabbit IgG. (D) Transmission electron micrographs of corneal fibroblasts treated with 100 μM melatonin (MEL) or vehicle alone (DMSO). AV, autophagosome; N, nuclear; m, mitochondria.

respectively, in GCD2-HO corneal fibroblasts ($P < 0.01$) (Fig. 2C,D).

Because melatonin reduced the level of TGFBIp in WT corneal fibroblasts that have normal autophagic function, we investigated whether the observed reduction of TGFBIp was regulated at the transcriptional level of *TGFBI* gene. Total mRNA was isolated from cells that were either untreated or treated with melatonin for 12 hr and TGFBIp mRNA levels were measured by the real-time reverse transcription PCR (RT-PCR) (Fig. 2E). Real-time RT-PCR results showed unchanged TGFBIp mRNA levels in WT corneal fibroblasts treated with 100 and 200 μM melatonin for 12 hr, respectively (Fig. 2E). However, treatment of WT corneal fibroblasts with 300 μM melatonin for 12 hr reduced levels of TGFBIp mRNA by 74.0 $\pm$ 4.3%, when compared to untreated fibroblasts ($P < 0.01$) (Fig. 2E).

Next, we wanted to analyze whether the activation of autophagy by melatonin is responsible for the reduction of TGFBIp. For this purpose, 3-MA, an inhibitor of PI3K [55] commonly used for specific inhibition of autophagosome formation [56], and bafilomycin A_1, an inhibitor of autophagic vacuole maturation that prevents fusion between autophagosomes and lysosomes [57], were used. After a 12 hr treatment with 200 μM melatonin, GCD2-HO cell lysates were analyzed by Western blot with anti-LC3 antibodies. Quantitative analysis revealed that treatments with 200 μM melatonin significantly decreased TGFBIp levels by approximately 40.7 $\pm$ 3.5% ($P < 0.05$) (Fig. 3A, lane 2 and B, lane 2) compared with untreated cells. Thus, approximately 59.3% of TGFBIp was degraded following the addition of 200 μM melatonin. The autophagy inhibitors, 3-MA and bafilomycin A_1, increased TGFBIp levels by approximately 146.8 $\pm$ 24.9% and 276.4 $\pm$ 30.2%, respectively (Fig. 3A, lane 3 and 4, and B, lane 3 and 4). In contrast, compared with untreated cells, 3-MA treatment decreased LC3 II levels by approximately 89.6 $\pm$ 8.8% ($P < 0.05$) (Fig. 3A, lane 3 and C, lane 3), whereas bafilomycin A_1 treatment increased LC3 II levels by approximately 299.3 $\pm$ 18.3% (Fig. 3A, lane 4 and C, lane 4) ($P < 0.05$). Furthermore, TGFBIp levels were significantly decreased in cells cotreated with 200 μM melatonin and 0.1 nM bafilomycin A_1 for 12 hr (Fig. 3A, lane 5, and B, lane 5) compared to cells treated with 0.1 nM bafilomycin A_1 (Fig. 3A, lane 4, and B, lane 4). In contrast, LC3 II levels were significantly increased in cells cotreated with 200 μM melatonin and 0.1 nM bafilomycin A_1 for 12 hr (Fig. 3A, lane 5, and C, lane 5) compared to treated cells with 0.1 nM bafilomycin A_1 (Fig. 3A, lane 4, and C, lane 4).

Autophagy activation is achieved through either dephosphorylation of mTOR or activation of the beclin-1

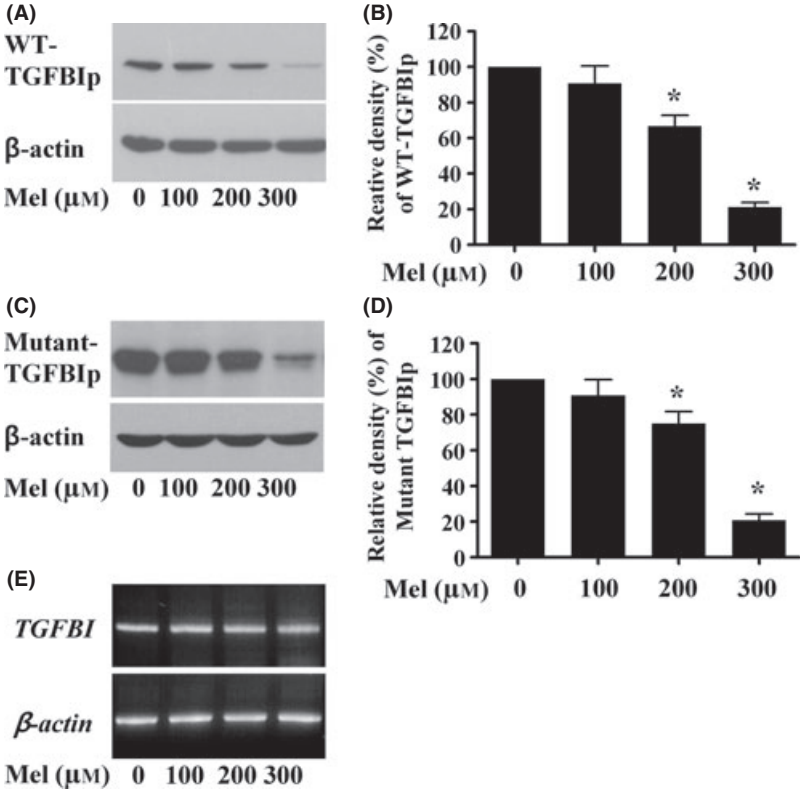

Fig. 2. Melatonin reduces TGFBIp levels in corneal fibroblasts. (A) WT corneal fibroblasts were treated with the indicated amounts of melatonin for 12 hr. TGFBIp levels were assessed by Western blot analysis. (B) Quantification of TGFBIp levels presented in (A) after normalization to β-actin protein signals. (C) GCD2-HO corneal fibroblasts were treated with the indicated amounts of melatonin for 12 hr. TGFBIp levels were assessed by Western blot analysis. (D) Quantification of mutant-TGFBIp presented in (C) after normalization to β-actin protein signals. (E) Total RNA was extracted from melatonin treated or untreated cells for 12 hr and reverse-transcribed for quantitative real-time PCR. TGFBIp mRNA levels were normalized to GAPDH mRNA and compared with untreated controls. Data represent means $\pm$ S.D. from three independent experiments. Statistical analyses were performed using one-way analysis of variance followed by the Newman–Keuls multiple comparison test. $^*P < 0.05$ with melatonin versus without melatonin.

pathway [58]. Beclin-1 is part of the Class III phosphatidylinositol 3-kinase (PI3K) complex that participates in autophagosome formation. To examine whether beclin-1 was involved in melatonin-induced autophagy, the protein levels of beclin-1 were analyzed by Western blot analysis. Beclin-1 expression was up-regulated in a dose-dependent manner following melatonin treatment (Fig. 4A and B). In contrast, the mTOR-kinase is part of two signaling complexes referred to as the mTOR complex 1 (mTORC1), which is rapamycin sensitive and contains raptor, and mTORC2, which is rapamycin insensitive and contains rictor. To understand how melatonin induces autophagy, we tested whether the activation of autophagy by melatonin was mediated via the mTOR-dependent pathway. The levels of the total and phosphorylated forms of mTOR in cells treated with or without melatonin were examined by immunoblot analysis (Fig. 4). Dose-dependent decreases in levels of phosphorylated mTOR, p-mTOR (S2448), were observed in melatonin-treated corneal fibroblasts. However, levels of other proteins were not changed (Fig. 4). These results indicate that the induction of autophagy by melatonin in corneal fibroblasts is mediated via the mTORC1 pathway.

The physiological function of melatonin could be dependent or independent of its receptor-mediated actions. To examine the involvement of melatonin receptors in melatonin-induced autophagy, luzindole was used in combination with melatonin. Cells were treated with 200 μM melatonin for 12 hr following treatment with 5 μM, 10 μM, and 15 μM luzindole for 30 min. Luzindole did not abolish the melatonin-induced increase in LC3 II levels (Fig. 5). Thus, activation of autophagy by melatonin does not directly involve receptor-mediated actions in corneal fibroblasts.

To investigate the relationship between autophagy and oxidative stress, via melatonin, we analyzed the effects of melatonin in autophagy induced by oxidative stress. We used superoxide-generating agents, parquate (PQ), because superoxide anion is the major ROS-regulating autophagy [59]. Treatment with PQ for 12 hr significantly increased levels of LC3 II in both WT and HO cells (Fig. 6A and B, lane 5 and 6). Furthermore, levels of LC3 II were more significantly increased in 50 μM PQ-treatment cells (Fig. 6A and B, lane 5 and 6) than in μM 100 melatonin-treatment cells (Fig. 6A and B, lane 3 and 4). Interestingly, while cotreatment with PQ and melatonin did not significantly increased the levels of LC3 II in WT cells (Fig. 6, A and B, lane 7), levels of LC3 II were significantly decreased by cotreatment with melatonin and PQ in HO cells (Fig. 6A and B, lane 8).

To evaluate synergistic effects of melatonin in the activation of autophagy, we cotreated cells with melatonin and rapamycin, a mTOR-kinase inhibitor that induces autophagy [60]. Cells were either untreated, treated with rapamycin or melatonin alone, or cotreated with rapamycin and melatonin for 12 hr (Fig. 7). Additional cotreatment proved synergistic effect of rapamycin and melatonin (Fig. 7A and C, lane 2 and 3). Thus, LC3 II levels was significantly higher in cells cotreated with 100 μM rapamycin and 100 μM melatonin than in cells treated with either 100 μM rapamycin or 100 μM melatonin alone (Fig. 7A, lane 2 and B, lane 2). However, levels of LC3 II and p-mTOR (S2448), which correlates with mTOR activity, were not elevated to higher levels in cotreated cells with

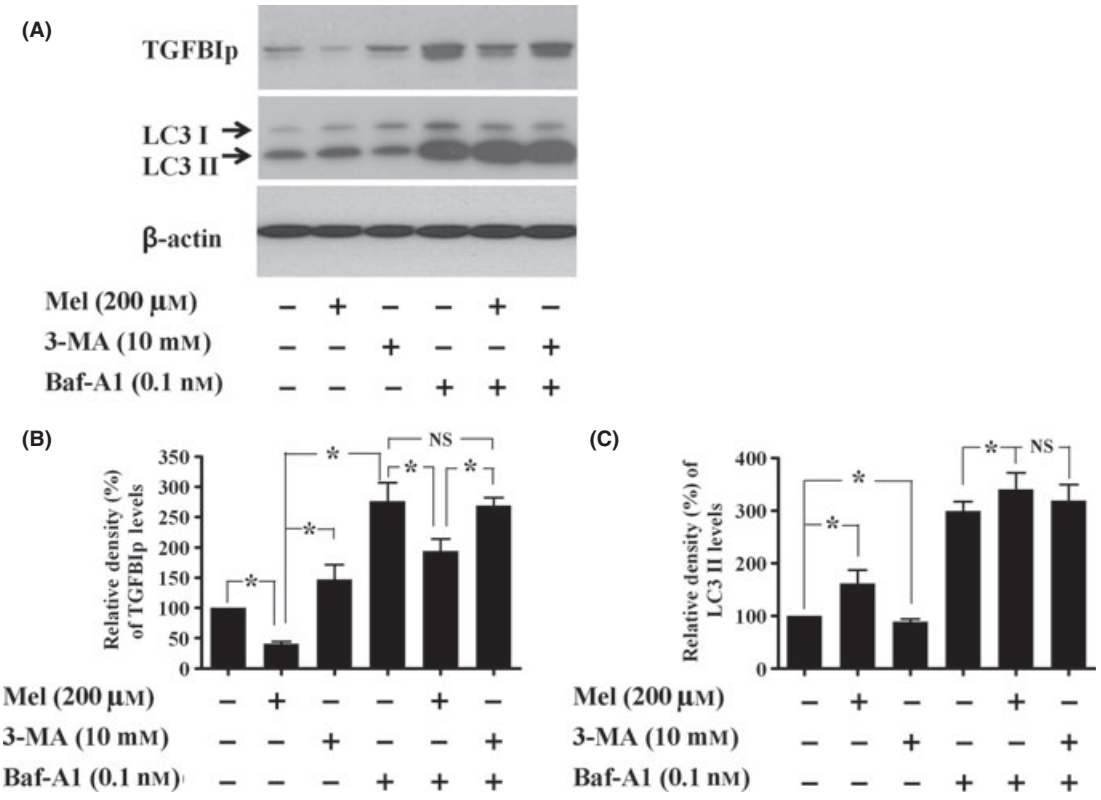

Fig. 3. Melatonin enhanced clearance of mutant-TGFBIp by autophagic degradation. (A) GCD2-HO corneal fibroblasts were untreated or treated with 200 μM melatonin, 10 mM 3-MA, 0.1 nM Baf-A1, or a combination of 200 μM melatonin with 10 mM 3-MA or 0.1 nM Baf-A1 for 12 hr and subjected to Western blot analysis using anti-TGFBIp, anti-LC3, and anti-β-actin. (B) Quantification of TGFBIp presented in (A) after normalization to β-actin protein signals. (C) Quantification of LC3 II presented in (A) after normalization to β-actin protein signals. Values represent the mean $\pm$ SD of three independent experiments. Statistical analyses were performed using one-way analysis of variance followed by the Newman–Keuls multiple comparison test. $^*P < 0.05$ with melatonin versus without melatonin.

200 μM rapamycin and 200 μM melatonin compared to co-treated cells with 100 μM rapamycin and 100 μM melatonin (Fig. 7A, lane 3, B, lane 3 and C, lane 3). In contrast, p-mTOR (S2448) levels were significantly reduced to lower levels in cotreated cells with 100 μM rapamycin and 100 μM melatonin compared to cotreated cells with 200 μM rapamycin and 200 μM melatonin (Fig. 7A and B, lane 2). In addition, essential markers of autophagy, ATG5 and becline 1 (the mammalian ATG6 homolog) were also increased in treated cells with rapamycin and melatonin (Fig. 7A, lane 2 and 3). However, there was no significant difference in the levels of belcline 1 and ATG5 between the cells treated with melatonin or rapamycin alone or cotreated, respectively.

Pathological states of many diseases are associated with oxidative stress. Oxidative stress inducting autophagy promotes either cell survival or cell death. To investigate the relationship between autophagy and oxidative stress, via melatonin, we analyzed the effects of melatonin in autophagy induced by oxidative stress. To induce oxidative stress, we used superoxide-generating agents, parquate (PQ), because superoxide anion is the major ROS-regulating autophagy [59]. Treatment with PQ for 12 hr significantly increased levels of LC3 II in both WT and HO cells (Fig. 6A and B, lane 5 and 6). Furthermore, in our hand, levels of LC3 II were more significantly increased in 50 μM PQ-treatment cells (Fig. 6, lane 5 and 6) than in μM 100 melatonin-treatment cells (Fig. 6A and B, lane 3and 4). Interestingly, while cotreatment with PQ and melatonin nonsignificantly increased the levels of LC3 II in WT cells (Fig. 6, A and B, lane 7), in HO cells, levels of LC3 II were significantly decreased by cotreatment with melatonin and PQ (Fig. 6A and B, lane 8).

Discussion

Because there is currently no pharmacological treatment for *TGFBI*-linked corneal dystrophy, development of novel therapies is necessary. One potential strategy for preventing a cytoplasmic retention of mutant-TGFBIp in GCD2 corneal fibroblasts is to enhance mutant-TGFBIp degradation. This therapeutic strategy can be achieved by activating the autophagy/lysosomal pathway. Here, melatonin treatment induced autophagy in corneal fibroblasts and this melatonin-induced autophagy effectively cleared mutant-TGFBIp without cytotoxic effects. These findings strongly suggest that melatonin application may be useful for the treatment of *TGFBI*-linked corneal dystrophies and should be further examined in future clinical trials alone or in combination with other autophagy inducers.

Growing evidence suggests that defective autophagy is involved in the pathogenesis of many diseases [61] and aging [62]. Normal cells can effectively clear misfolded proteins and damaged intracellular components [63, 64].

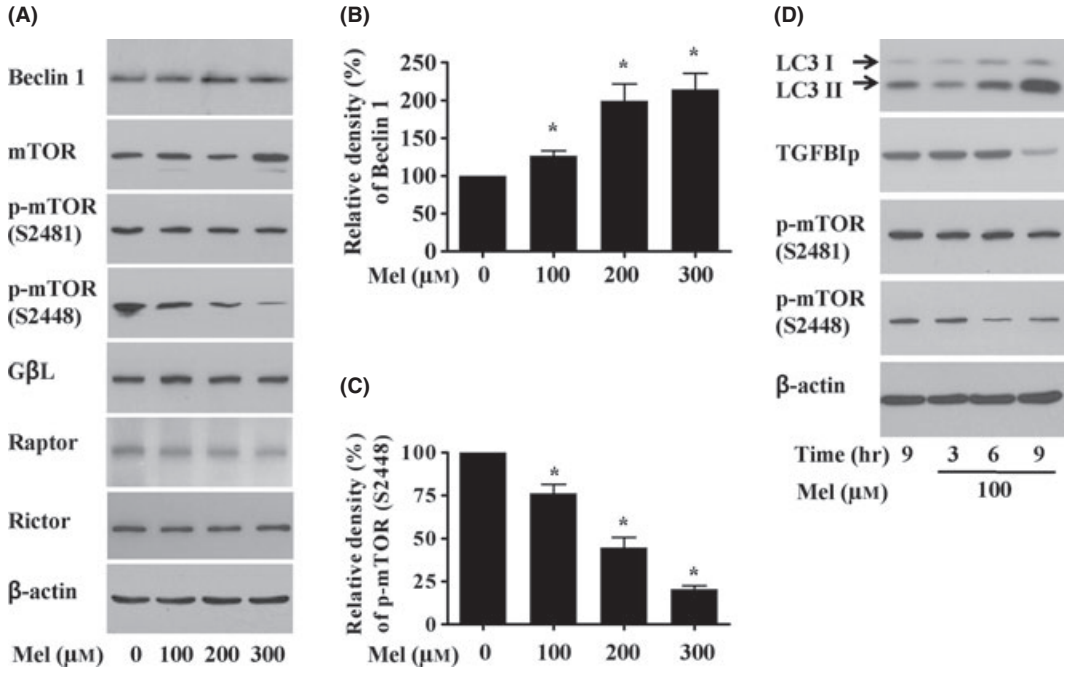

Fig. 4. Melatonin suppressed the mTOR-signaling pathway in WT corneal fibroblasts. (A) Western blot analysis of beclin-1, total mTOR, p-mTOR (S2481), p-mTOR (S2448), GβL, raptor, rictor, and β-actin from lysates of corneal fibroblasts treated with various concentrations of melatonin for 12 hr. (B) Quantification of beclin-1 expression presented in (A). (C) Quantification of p-mTOR (S2448) levels presented in (A) after normalization to β-actin protein signals. (D) Western blot analysis of LC3, total mTOR, p-mTOR (S2448), and β-actin from lysates of corneal fibroblasts treated for various times. β-actin was used as an internal control. All data were representative of three independent experiments. Statistical analyses were performed using one-way analysis of variance followed by the Newman–Keuls multiple comparison test. $*P < 0.05$.

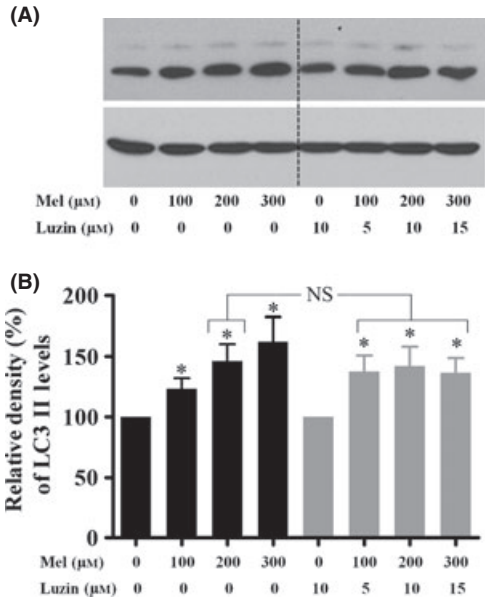

Fig. 5. Melatonin-induced autophagy was melatonin receptor-independent. WT corneal fibroblasts were preincubated for 30 min with or without 5, 10, or 15 μM luzindole (Luzin) and were exposed to 200 μM melatonin for 12 hr. (A) Western blot analysis of LC3 and β-actin with 50 μg of total protein from WT corneal fibroblasts. (B) Relative levels of LC3 after normalization to β-actin protein signals.

However, any alterations in this proteolytic system may result in the intracellular accumulation and retention of misfolded proteins or damaged organelles, causing pathological processes and disease [64].

Induction of autophagy for enhancing the clearance of disease-associated protein has been tested in several neurodegenerative diseases. For example, the induction of autophagy enhances the clearance of mutant huntingtin and α-synucleins in Huntington's and Parkinson's diseases, respectively [65]. Beneficial effects of autophagy induction with rapamycin have also been described in Charcot–Marie–Tooth type 1A and amyotrophic lateral sclerosis (ALS) [21–23]. Our previous studies indicated that mutant-TGFBIp delays degradation through the impairment of autophagy in GCD2 corneal fibroblasts [49]. Furthermore, GCD2 corneal fibroblasts are more susceptible to apoptotic cell death induced by autophagy inhibition than are WT cells [49]. Based on these results, autophagy inducers are potential therapeutic agents for *TGFBI*-linked corneal dystrophies.

Autophagy occurs at a basal rate in all eukaryotic cells [66] and is important for maintaining normal cellular homeostasis. Beclin-1 is involved in the formation of autophagosomes and autophagy initiation [67]. Reduced beclin-1 expression is associated with reduced autophagic vacuole formation [68, 69]. In contrast, increased beclin-1 expression facilitates autophagy induced by starvation [70]. In this study, beclin-1 expression was elevated in melatonin-treated corneal fibroblasts. These results suggest

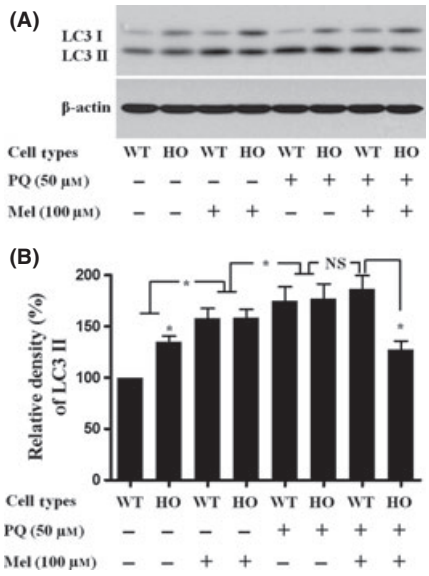

Fig. 6. Melatonin inhibits PQ-induced autophagy. Cells were treated either with melatonin, PQ or cotreated with melatonin and PQ. (A) WT and HO corneal fibroblasts were treated either with 100 μM melatonin, 50 μM PQ, or 100 μM melatonin and 50 μM PQ for 12 hr. LC3 levels were analyzed by Western blot with specific LC3 antibody. (B) The graph shows a densitometric analysis of LC3 II levels with no treatment WT corneal fibroblasts set to 100%. Quantification of LC3 II is presented (A) after normalization to β-actin protein signals. Values represent the mean $\pm$ SD of three independent experiments. Statistical analyses were performed using one-way analysis of variance followed by the Newman–Keuls multiple comparison test. $^*P < 0.05$ with melatonin versus without melatonin.

that beclin-1 is involved in triggering autophagy in melatonin-treated corneal fibroblasts.

This degradation system can also be induced through the mTOR pathway [58]. Furthermore, autophagy is an important player in cellular responses to starvation, cell survival, cell death, and cancer [27]. Rapamycin potently inhibits downstream signaling from the target of mTOR proteins. Phosphorylation of ribosomal protein S6 inhibits autophagy, and the activity of p70S6 kinase is regulated by mTOR-kinase. Autophagy is tightly regulated through the mTOR pathway [58]. mTOR is a conserved Ser/Thr protein kinase that regulates autophagy [58, 71, 72]. Therefore, inhibition of mTOR phosphorylation could induce autophagy. Correspondingly, our findings showed that melatonin inhibited the mTOR-signaling pathway by inhibiting phosphorylation of mTOR Ser2448 (Fig 4A, B). However, the detailed mechanism by which melatonin inhibits phosphorylation of mTOR Ser2448 remains unknown. Furthermore, depending on the situation, melatonin paradoxically either induces autophagy inhibition or activation [73–76]. These studies indicate that the specialized functions of autophagy are both survival-supporting [68, 77] and death-promoting [78, 79]. The roles of melatonin in autophagy may help distinguish the paradoxical roles of autophagy. Additional studies will be needed to ascertain the role of melatonin in autophagy in various physiological states.

The findings herein provide evidence that melatonin application may be useful for the treatment of *TGFBI*-linked corneal dystrophies. TGFBIp levels in GCD2-HO corneal fibroblasts were reduced following melatonin administration (Fig. 2). Autophagy was effectively induced in an additive manner when melatonin is coadministered with rapamycin (Fig. 7). The ability of melatonin-induced autophagy to clear TGFBIp provides direct evidence that retention of cytoplasmic TGFBIp could be reduced in GCD2 patients. Furthermore, 300 μM melatonin treatments reduced levels of TGFBIp mRNA (Fig. 2E). This result indicates that a large decrease in TGFBIp following 300 μM melatonin treatments may be associated with decreased levels of TGFBIp mRNA. Thus, melatonin could regulate expression of TGFBIp through down-regulated mTOR signaling, because mTOR signaling controls several cellular events, including transcription, translation, mRNA turnover, and protein stability [80]. Therefore, additional studies will focus on ascertaining whether melatonin can regulate expression of TGFBIp through mTOR signaling pathway. Nevertheless, our EM findings and the increased LC3 II and LC3 punta formation in corneal fibroblasts following melatonin treatment with or without autophagy inhibitors supports the importance of autophagy for the elimination of accumulated cytosolic TGFBIp. Together, these results demonstrate that melatonin treatment induced autophagy and activates autophagic clearance of TGFBIp in corneal fibroblasts.

We also showed that melatonin counteracted the autophagy-inhibitory effects of autophagic flux inhibitor, bafilomycin A_1 (Fig. 3). Thus, this result indicates that melatonin may promote fusion between autophagosome and lysosomes. Furthermore, to the best of our knowledge, this data are the first to demonstrate that melatonin can promote autophagic flux, although the exact mechanism is unknown.

The anti-apoptotic effects of melatonin in response to oxidative stressors are dependent on the inhibition of cytochrome *c* release and caspase-3 activation [26, 81]. Previously, we showed that oxidative stress [52] and mitochondrial dysfunction [53] are involved in the pathogenesis of GCD2. Oxidative stress may promote cellular toxicity, mitochondrial dysfunction, and ultimate apoptotic cell death. Melatonin has anti-apoptotic effects by regulating multiple apoptosis-related proteins [40, 82]. Moreover, melatonin protects against apoptotic cell death of GCD2 corneal fibroblasts induced by oxidative stress [83]. Together, these results suggest that melatonin treatment may have multifunctional benefits in *TGFBI*-linked corneal dystrophies. Given that melatonin did not induce significant death in corneal fibroblasts even at very high concentrations (300 μM) [83], it is likely that melatonin can be used as a therapeutic agent for treating GCD2 and impaired autophagy associated with other diseases.

Our data also raise the possibility of combination therapy using melatonin and rapamycin as a treatment for GCD2 (Fig. 7). Because our results show that a combination treatment consisting of lower doses of both melatonin and rapamycin is more effective than treatments consisting of higher doses of melatonin or rapamycin alone, the combination of melatonin and rapamycin is attractive as it

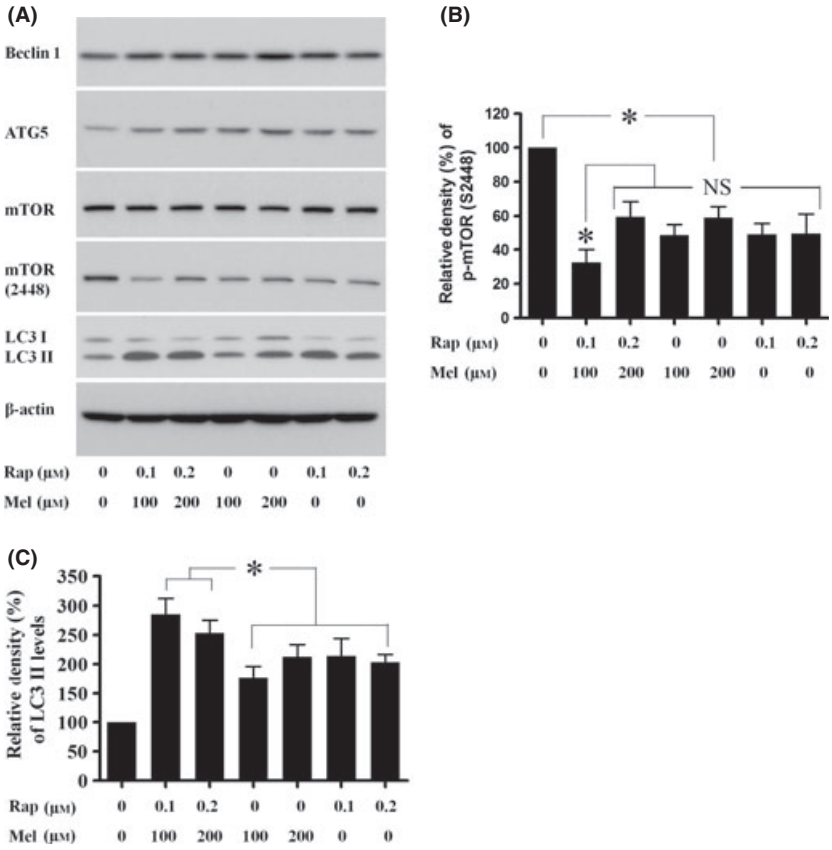

Fig. 7. Melatonin and rapamycin had an additive effect on autophagy induction. (A) Corneal fibroblasts were either untreated, treated with 0.1 μM or 0.2 μM rapamycin alone, or 100 μM or 200 μM melatonin alone, cotreated with 0.1 μM rapamycin and 100 μM melatonin or 0.2 μM rapamycin and 200 μM melatonin for 12 hr. Co-treatment of cells with rapamycin and melatonin enhanced autophagy (LC3 II) compared to treatment with either compound alone. Western blot analysis of beclin 1, ATG5, total mTOR, p-mTOR (S2448), LC3, and β-actin. (B) Quantification of p-mTOR (S2448) levels presented in (A) after normalization to β-actin protein signals. (C) Quantification of LC3 II levels presented in (A) after normalization to β-actin protein signals. Values represent the mean $\pm$ SD of three independent experiments. Statistical analyses were performed using one-way analysis of variance followed by the Newman–Keuls multiple comparison test. $^*P < 0.05$.

maximizes the benefits of autophagy activation and lessens the risk of drug-specific side effects (Fig. 7). Thus, a combination of melatonin and rapamycin can enhance autophagy, while the melatonin component can also protect corneal fibroblasts against cell death/apoptosis due to oxidative stress [83].

Chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy are three major forms of autophagy. CMA is a selective pathway for the degradation of cytosolic proteins in lysosomes. Thus, CMA targets specific cytosolic proteins that are recognized by the 73-kDa heat shock cognate protein (hsc73), which interacts with substrate protein containing the pentapeptide (a KFERQ-like motif) in the cytosol. Thereafter, chaperone-substrate protein complex interacted with the lysosome-associated membrane protein type 2A (lamp2a). Once bound to the lysosomal membrane, the substrate protein are translocated into the lysosomal lumen for degradation [84]. CMA is induced during stress conditions such as starvation, oxidative stress, or exposure to toxic compounds, in the same way macroautophagy is induced. In addition, a decline in CMA has been recently reported in aging [85] and several diseases [84]. Furthermore, under prolonged starvation, 30% of cytosolic proteins are degraded by CMA [86]. These previous reported data are considered to play pivotal role in the pathophysiology of many diseases, although the amino acid sequence of TGFBIp does not contain a KFERQ-like motif. However, the role of melatonin in CMA has not been fully investigated.

Melatonin significantly reduces ROS production and cell death caused by PQ-treatments [87]. ROS are involved in both induction of autophagosomes formation and autophagic cell death. Recently, we have demonstrated that PQ-treatment significantly increased more cell death in HO corneal fibroblasts than in WT corneal fibroblasts [83]. These data indicate that melatonin not only inhibits autophagy activation induced by oxidative stress in WT cells but also prevents oxidative stress-induced autophagic cell death in HO cells, which show defective autophagy [49] and much more susceptible to oxidative stress due to decreased catalase [52]. However, some questions about differential effects of melatonin in GCD 2 corneal fibroblasts following melatonin treatments still remain.

Although, TGFBIp is ubiquitously expressed in normal tissues, down-regulation of this gene has been found in various human tumor tissues [6]. In tumorigenesis, autophagy paradoxically promotes apoptotic cell death as a tumor suppressor while in some other cells, it prevents apoptotic cell death as an oncogenic mechanism [88, 89]. Moreover, melatonin can directly kill many different types of tumor cells [90]. Therefore, our data raise the question of whether reduced TGFBIp by activated autophagy with melatonin treatment could contribute to tumor development in corneal fibroblasts. Although this has not been fully studied, several recent reports suggest that TGFBIp inhibits tumor angiogenesis and tumor growth, as well as promoting apoptosis [91, 92]. Moreover, to the best of our knowledge, mutation of *TGFBI* gene has not yet been

identified in patients with cancer. However, whether reduced TGFBIp expression by activation of autophagy with melatonin could specifically influence on development of tumor in corneal cells has not been determined.

In conclusion, melatonin, especially in combination with rapamycin, is a good inducer of autophagy and melatonin treatment results in the effective removal of mutant-TGFBIp from GCD2-HO corneal fibroblasts. These findings may have broad applicability as melatonin-induced autophagy may also regulate the clearance of cytosolic aggregate-prone proteins associated with various forms of other diseases such as Alzheimer's, Huntington's, and Parkinson's diseases.

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Author contributions

SI Choi and EK Kim designed the experiments and wrote the article. SI Choi, KS Kim, JY Oh, JY Jin and GH Lee carried out and analyzed the experiments. EK Kim advised the experiments and assisted on all aspects of the project.

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