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Disrupted cell cycle arrest and reduced proliferation in corneal fibroblasts from GCD2 patients: A potential role for altered autophagy flux

Seung-il Choi [a,b], Shorafidinkhuja Dadakhujaev [a,b], Yong-Sun Maeng [a,b], So-yeon Ahn [a,b], Tae-im Kim [a,b], Eung Kweon Kim [a,b,c,*]

[a] Department of Ophthalmology, Severance Hospital, Yonsei University College of Medicine, Seoul, Republic of Korea
[b] Corneal Dystrophy Research Institute, Yonsei University College of Medicine, Seoul, Republic of Korea
[c] BK21 Plus Project for Medical Science and Severance Biomedical Science Institute, Yonsei University College of Medicine, Seoul, Republic of Korea

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ABSTRACT

This study investigates the role of impaired proliferation, altered cell cycle arrest, and defective autophagy flux of corneal fibroblasts in granular corneal dystrophy type 2 (GCD2) pathogenesis. The proliferation rates of homozygous (HO) GCD2 corneal fibroblasts at 72 h, 96 h, and 120 h were significantly lower (1.102 ± 0.027, 1.397 ± 0.039, and 1.527 ± 0.056, respectively) than those observed for the wild-type (WT) controls (1.441 ± 0.029, 1.758 ± 0.043, and 2.003 ± 0.046, respectively). Flow cytometry indicated a decreased G_1 cell cycle progression and the accumulation of cells in the S and G_2/M phases in GCD2 cells. These accumulations were associated with decreased levels of Cyclin A1, B1, and E1, and increased expression of p16 and p27. p21 and p53 expression was also significantly lower in GCD2 cells compared to the WT. Interestingly, treatment with the autophagy flux inhibitor, bafilomycin A_1, resulted in similarly decreased Cyclin A1, B1, D1, and p53 expression in WT fibroblasts. Furthermore, similar findings, including a decrease in Cyclin A1, B1, and D1 and an increase in p16 and p27 expression were observed in autophagy-related 7 (Atg7; known to be essential for autophagy) gene knockout cells. These data provide new insight concerning the role of autophagy in cell cycle arrest and cellular proliferation, uncovering a number of novel therapeutic possibilities for GCD2 treatment.

1. Introduction

Transforming growth factor-β (TGF-β) induced (*TGFBI*) gene-linked GCD2 is an autosomal dominant disorder caused by a point mutation in the *TGFBI* gene on chromosome 5q31. This disorder is characterized by age-dependent progressive accumulation of deposits of mutant TGF-β induced protein (TGFBIp) in the corneal epithelia and stroma, followed by interference with corneal transparency [1]. Further, *TGFBI* has also been suggested to function as an oncogene or tumor suppressor gene, depending on the type and location of the cancer [2].

The mammalian cell division cycle is traditionally divided into G_1 (gap phase 1), S (DNA synthesis), G_2 (gap phase 2), and M (mitosis) phases. Progression through each phase of the cell cycle is controlled by three sets of proteins: a co-operative family of serine/threonine protein kinases called Cyclin-dependent kinases (CDKs); CDK activation proteins known as Cyclins, which function during periods of proliferation; and CDK inhibitory proteins, which mediate cell cycle arrest in response to various antiproliferative signals. Two families of CDK inhibitory proteins have been identified in mammalian cells based on sequence homology: the CDK interacting protein/Kinase interacting protein (Cip/Kip) family, which includes p21, p27, and p57; and the inhibitors of CDK family, which includes p15, p16, p18, and p19 [3]. Importantly, both cyclins and CDK inhibitors, as well as the tumor-suppressor protein p53, which function together to drive the cell cycle, are all regulated by ubiquitin-mediated proteolysis [4]. The degradation of these cell cycle regulators is, therefore, controlled by ubiquitin–protein–ligase complexes, which target the regulators for degradation by the 26S proteasome [5,6].

Autophagy and the ubiquitin–proteasome system (UPS) pathway are the major routes for intracellular protein degradation, and ubiquitin-tagged proteins can be target substrates for

* Corresponding author at: Department of Ophthalmology, Yonsei University College of Medicine, 50 yonsei-ro, Seodaemun-gu, Seoul 120-752, Republic of Korea. Fax: +82 2 312 0541.
E-mail address: eungkkim@yuhs.ac (E.K. Kim).

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degradation via both pathways. The collaboration between the UPS and autophagy appears to be essential for protein quality control, and is required during multiple processes of the cell cycle, particularly those that involve the downregulation of critical regulatory elements. Thus, it is not surprising that selective ubiquitin-dependent degradation of cyclins, CDKs, and anaphase inhibitors occurs during several major cell cycle transitions, likely being responsible for the associated downstream gene expression changes. [5,7]. However, the function of these degradation pathways during GCD2 is largely unknown. Recently, we observed a number of morphological abnormalities in GCD2 corneal fibroblasts, including increased cell size [8], altered autophagy flux via the mammalian target of rapamycin (mTOR) pathway [9], and an accumulation of ubiquitin-bound proteins [8]. From these findings, we hypothesize that this abnormal phenotype observed for the GCD2 corneal fibroblasts might be caused by inappropriate cell cycle arrest due to the altered expression of cell-cycle regulators. Thus, we sought to investigate this theory using flow cytometry and Western blot analysis of cultured corneal fibroblasts isolated from wild-type (WT) and GCD2 patients (both heterozygous (HE) and homozygous (HO)).

2. Materials and methods

2.1. Isolation and culture of primary corneal fibroblasts

WT ($n = 3$), heterozygous (HE) ($n = 3$), and HO ($n = 3$) primary corneal fibroblasts were prepared as described previously [10]. The age, gender, and diagnosis of all GCD2 patients from which the cells were generated are listed in Supplementary Table 1. Donor confidentiality was maintained in accordance with the Declaration of Helsinki, and the study was approved by the Severance Hospital IRB Committee (CR04124), Yonsei University, Seoul, Korea. The normal human corneal fibroblast cell line was kindly provided by Dr. James Jester. WT and autophagy-related 7 (Atg7) knockout mouse embryonic fibroblasts (MEFs) were kindly provided by Dr. Masaaki Komatsu.

2.2. Proliferation assay

Cell proliferation was measured using the CellTiter 96 Aqueous One Solution Reagent Cell Proliferation Assay Kit (G3580; Promega, Madison, WI, USA) according to the manufacturer's instructions. Optical density was measured using a microplate reader (VERSAmax; Molecular Devices).

2.3. Serum starvation and treatments

WT, HE, and HO corneal fibroblasts were plated and grown in complete medium (Dulbecco's Modified Eagle Medium (DMEM; Cellgro, Herndon, VA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco/Invitrogen Corporation, Carlsbad, CA, USA)) until the colonies were 75–80% confluent. Then, the medium was changed to DMEM without serum.

2.4. Cell cycle analysis

The serum starved and non-starved primary corneal fibroblast cells were harvested, washed with 1× phosphate buffered saline (PBS), and resuspended in 500 μl of 1× PBS containing 0.1% glucose (kept at 4 °C). Immediately following resuspension, 5 ml of cold 70% ethanol was added to the cells and kept at 4 °C for 4 h after mixing. Cells were spun down 2 h prior to FACS analysis and washed with 1× PBS. Cells were then stained with propidium iodide (PI) (50 pg/ml) in the presence of 0.1 mg/ml RNase for 30 min at 37 °C. The cell cycle was analyzed using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). Mean forward scatter height (FSC-H) was determined on G1-gated cells identified by PI fluorescence (FL2-A).

To analyze the effects of autophagy and the UPS on cell cycle-related protein expression, WT corneal fibroblasts cell line were incubated with 0.1 μM bafilomycin A_1 (Sigma–Aldrich, B1793; St. Louis, MO, USA), which is known to inhibit fusion between autophagosomes and lysosomes, and/or MG132 (Sigma–Aldrich, C2211), an inhibitor of 26S proteasome function, for 8 h. Cells were analyzed for microtubule-associated protein 1B-light chain 3 (LC3) and TGFBIp levels by Western blot analysis. Crude cell lysate preparations and Western blotting were performed as described previously [10].

2.5. Statistical analysis

Data were evaluated to determine the statistically significant differences between groups and/or treatments using Student's *t*-tests or one-way analysis of variance (ANOVA), followed by the Newman–Keuls multiple comparison test. Data are expressed as the mean ± standard deviation (SD). All the data were processed using scientific graphing analysis software (Prism, version 5.0; GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Decreased proliferation rates and altered morphology of GCD2 corneal fibroblasts

The proliferation kinetics of corneal fibroblasts isolated from three different WT controls and three HO corneal fibroblasts were determined over a period of 5 days (Fig. 1A). All primary corneal fibroblasts used in these experiments had been passaged seven or eight times. As shown in Fig. 1A, the proliferation rates of the GCD2 corneal fibroblasts at 72 h, 96 h, and 120 h were significantly lower (1.102 ± 0.027, 1.397 ± 0.039, and 1.527 ± 0.056, respectively) than those observed for the WT controls (1.441 ± 0.029, 1.758 ± 0.043, and 2.003 ± 0.046, respectively) ($P < 0.005$).

Moreover, phase contrast microscopy, used to investigate the morphological differences between the WT and GCD2 corneal fibroblasts, indicates that the HO corneal fibroblasts display a number of morphological abnormalities, including increased size, which was not observed in the control cells (Fig. 1B).

3.2. Altered cell cycle distribution of GCD2 corneal fibroblasts

In a cell culture system, every cell is at different stage of the cell cycle. In order to bring all of them to the same phase, researchers typically use the synchronized cell culture system [11,12]. For cell cycle distribution analysis, we also employed the synchronized cell culture system using WT, HE, and HO corneal fibroblasts, and the samples were subsequently analyzed by flow cytometry (Fig. 1Aa–c). The proportion of HE and HO corneal fibroblasts in the G_2/M (HE: 15.85 ± 0.95%, HO: 18.36 ± 1.09%) and S (HE: 3.92 ± 0.85%, HO: 3.69 ± 0.36%) stages was significantly higher than that of WT control cells (WT: 11.33 ± 1.24% and 1.82 ± 0.13%, respectively) (Fig. 2Ba). However, the number of cells in the G_1 phase was lower for the HE (77.90 ± 2.20%) and HO (77.34 ± 2.03%) cells compared to the WT control cells (86.16 ± 1.77%) (Fig. 2Ba). Further, serum starvation for 48 h caused synchronization of the WT, HE, and HO corneal fibroblasts in the G_1 phase (HE: 78.93 ± 2.05%, HO: 80.74 ± 1.90%, and WT: 83.47 ± 1.12%), the S phase (HE: 0.74 ± 0.06%, HO: 1.35 ± 0.07%, and WT: 1.37 ± 0.09), and G_2/M phase (HE: 17.59 ± 1.01%, HO: 17.31 ± 1.14%, and WT: 15.00 ± 0.80%) (Fig. 2Bb).

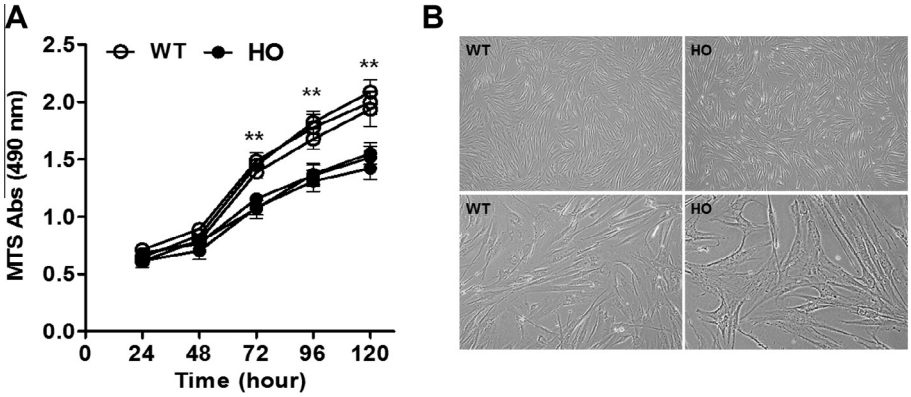

Fig. 1. Primary corneal fibroblasts isolated from GCD2 patients exhibit altered morphology and abnormal proliferation rates. (A) MTS assay for cell proliferation. At 24 h, 48 h, 72 h, 96 h, and 120 h, the proliferation rate in the HO corneal fibroblasts was significantly lower than that in the WT corneal fibroblasts (**$P < 0.005$). Experiments were done in triplicate. (B) Phase-contrast micrograph of WT and HO corneal fibroblasts. Cells were analyzed and photographed using an inverted microscope. A number of morphological abnormalities were observed for the HO corneal fibroblasts compared to the WT, and the GCD2 cells contained different-sized intracellular deposits.

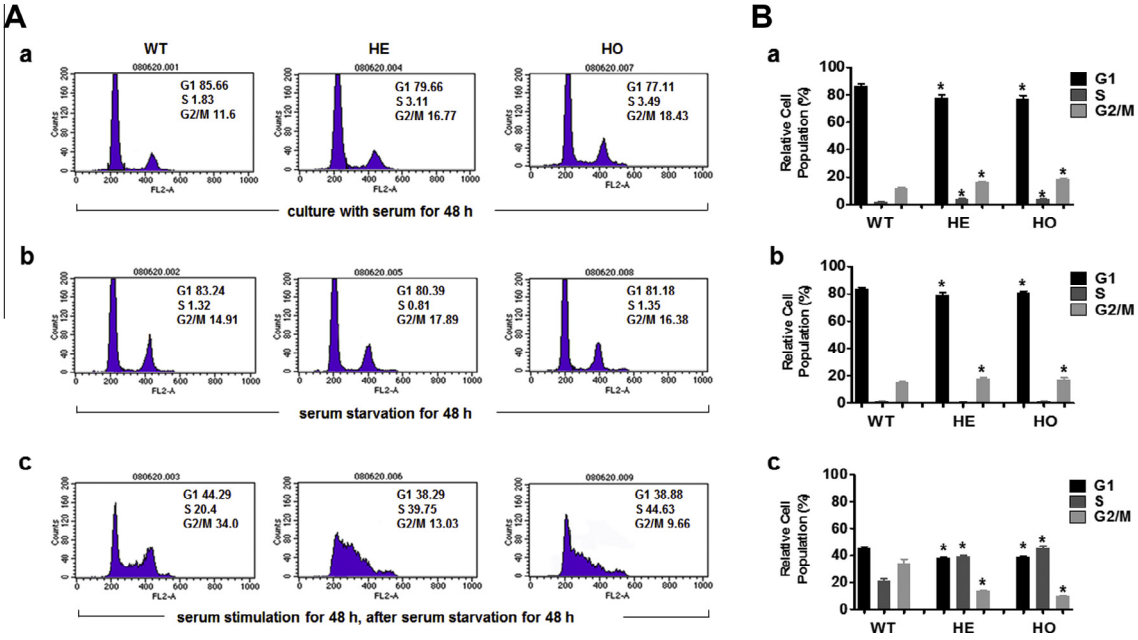

Fig. 2. Altered cell cycle arrest in the GCD2 corneal fibroblasts. (A) FACS analysis of WT, HE, and HO corneal fibroblast cells prior to serum starvation (a), after 48 h of starvation (b), and 48 h after the re-introduction of serum (c). (B) The distribution and percentage of cells in G_1, S, and G_2/M are indicated. Values represent mean ± SD for three different experiments. *$P < 0.05$ compared with the WT.

Additional incubation with serum for 48 h after serum starvation also altered the number of cells accumulated in each different cell cycle phase. Our analysis indicates that exposure of the starved HE and HO corneal fibroblasts to serum results in a statistically significant increase in S phase cells (HE: 39.35 ± 1.06% and HO: 45.02 ± 1.66%) compared to the WT control cells (21.13 ± 1.76%) (Fig. 2Bc). This increase was also accompanied by a decrease in G_1 phase cells (HE: 38.03 ± 1.08%, HO: 38.28 ± 1.58%, and WT: 45.39 ± 1.25%) (Fig. 2Bc). In contrast, the percentage of HE and HO corneal fibroblasts in the G_2/M phase decreased (HE: 13.52 ± 1.01% and HO: 9.50 ± 0.87%) when compared to the WT cells (33.87 ± 1.33%) (Fig. 2Bc).

3.3. Modulation of cell cycle-related protein expression in GCD2 corneal fibroblasts

Next, we examined the expression levels of several cell cycle activator and inhibitor proteins. The expression of Cyclin A1, Cyclin B1, Cyclin D1, Cyclin E1, CDK6, p21, and p53 was significantly lower in the HE and HO corneal fibroblasts compared to the WT control cells (Fig. 3A and B). In contrast, the expression of p16 and p27 was significantly higher in the GCD2 corneal fibroblasts (Fig. 3A–C).

The expression of these same cell cycle activators and inhibitors was also examined following serum starvation-induced synchronization. Before serum starvation, the expression of all Cyclins (A1, B1, D1, and E1) and p21 was relatively low in the HE and HO corneal fibroblasts compared to the WT control cells, while the level of p16 was significantly higher (Fig. 3A–C). Following serum starvation, the expression of Cyclin A1, B1 in the WT controls was decreased, reaching levels similar to those observed for the HE and HO corneal fibroblasts. Cyclin A1 and p21 expression still remained low, and levels of p16 were still high in the HE and HO corneal fibroblasts compared to the WT control cells. Interestingly, the levels of Cyclin E1 abruptly increased in HO corneal fibroblasts compared to WT control cells following serum starvation (Fig. 3D).

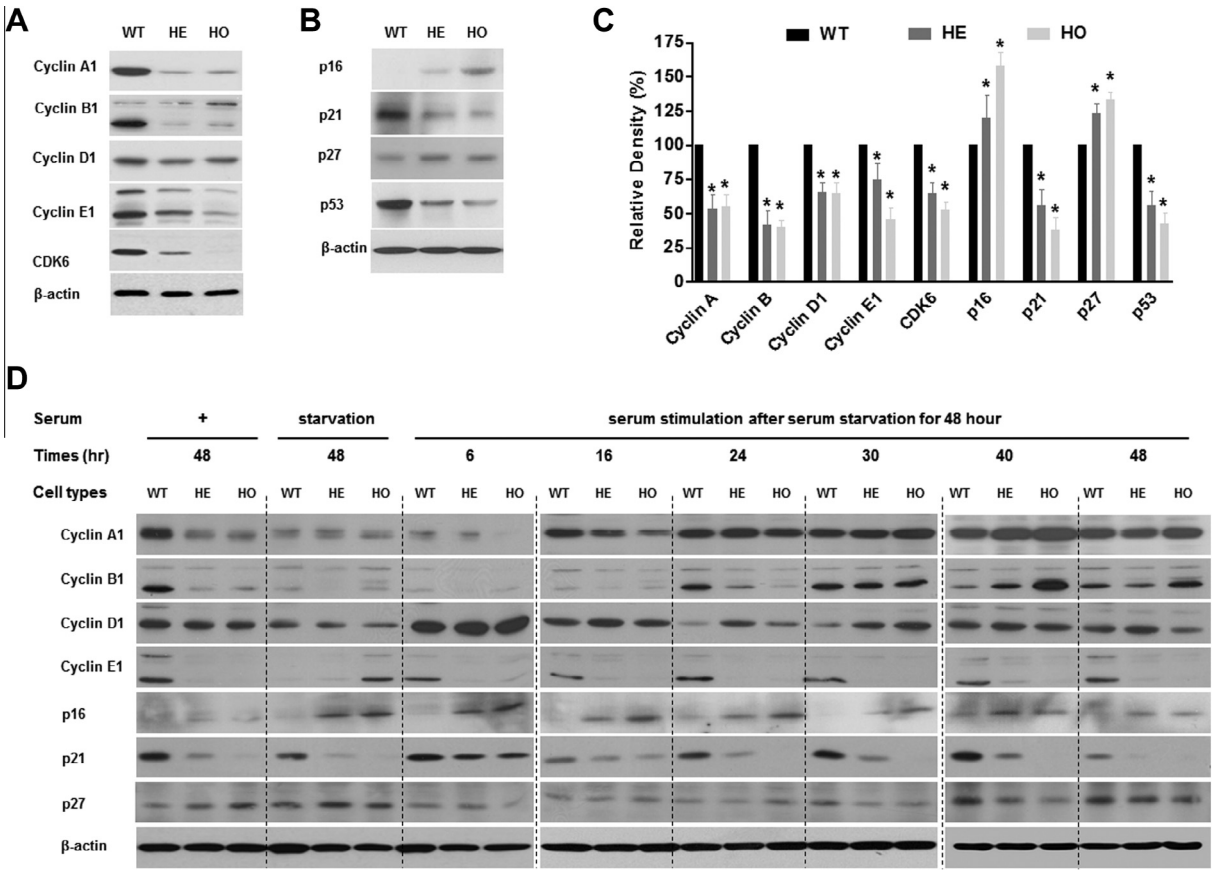

Fig. 3. Expression patterns of cell cycle-related proteins in GCD2 corneal fibroblasts. (A) Western blot analysis of Cyclin A1, Cyclin B1, Cyclin D1, Cyclin E1, and CDK6 proteins. (B) Western blot analysis of p16, p21, p27, and p53 proteins. (C) Relative abundance of these proteins after normalization with their respective β-actin protein signals. (D) Western blot analysis of these cell cycle regulators following various treatments, including the initial 48 h with serum, 48 h without serum, and variable time points (6–48 h) after the re-introduction of serum to the starved cells. The data show the mean ± SD ($n = 3$), $^{*}P < 0.05$ compared with the WT.

Notably, after serum was re-introduced to the cultures, the expression of Cyclin E1 and p21 was stable for 48 h, and remained low in the HE and HO corneal fibroblasts. The levels of Cyclin A1, on the other hand, increased during the first 16 h in the HE and HO corneal fibroblasts following the addition of serum, even though the expression of this protein remained low in the WT control cells, a trend which continued following 30 and 40 h incubations with serum. However, after incubating for 48 h with serum, Cyclin A1 expression was similar for each cell type. Levels of Cyclin B1 also increased in the HE and HO corneal fibroblasts after being incubated with serum for 30 h, but this expression was still lower than that observed for the WT control cells. Then, after being incubated with serum for 40 or 48 h, the expression of Cyclin B1 increased rapidly in the HE and HO cells, becoming significantly higher in the HE and HO corneal fibroblasts compared to the WT control cells. Further, after incubating for 6 h with serum, Cyclin D1 was expressed at a similar level in each cell type, a level that was then increased in all samples following the 30 h incubation, with higher levels being observed for the HE and HO corneal fibroblasts compared to the WT control cells. However, this high level of expression was observed to decrease approximately 40 h after the re-introduction of serum, resulting in a similar loss of expression in each cell type (Fig. 3D).

In addition to investigating the change in Cyclin expression following the re-introduction of serum in the cell culture medium, we also assessed changes in p27 and p16 expression after the serum supplement was added. As mentioned earlier, p16 expression was significantly higher under starvation conditions in the HE and HO corneal fibroblasts compared to the WT control cells, and, interestingly, this was also true following the addition of serum to the medium (Fig. 3B and D). However, the expression of p27 was lower in the HE and HO corneal fibroblasts compared to the WT control cells after only 30 h of incubation with serum (Fig. 3D).

3.4. Differential expression of cell cycle-related proteins in autophagy-defective cells

To investigate the involvement of autophagy in the observed cell cycle abnormalities, we monitored the expression of cell cycle-related proteins following autophagic flux inhibition. Interestingly, treatment with bafilomycin A_1 reduced the expression of Cyclin A1, B1, and D1 in the WT corneal fibroblasts (Fig. 4A). The expression of p53 was also decreased by bafilomycin A_1 treatment, whereas the expression of p21 was increased by bafilomycin A_1 treatment (Fig. 4A). Bafilomycin A_1 also increased LC3 II and TGFBIp, indicating that autophagosomes containing TGFBIp are accumulating as a result of inhibited autophagosome–lysosome fusion. To investigate whether the decrease in cell cycle related proteins was reversible, we treated the cells with the ubiquitin proteasome inhibitor, MG132, in the presence of bafilomycin A_1. Interestingly, MG132 treatment increased the expression of all of the investigated cell cycle-related proteins in a dose dependent manner (Fig. 4A), indicating that altering the autophagy system may induce the degradation of cell cycle related proteins through ubiquitin proteasome activation. Furthermore, we also observed

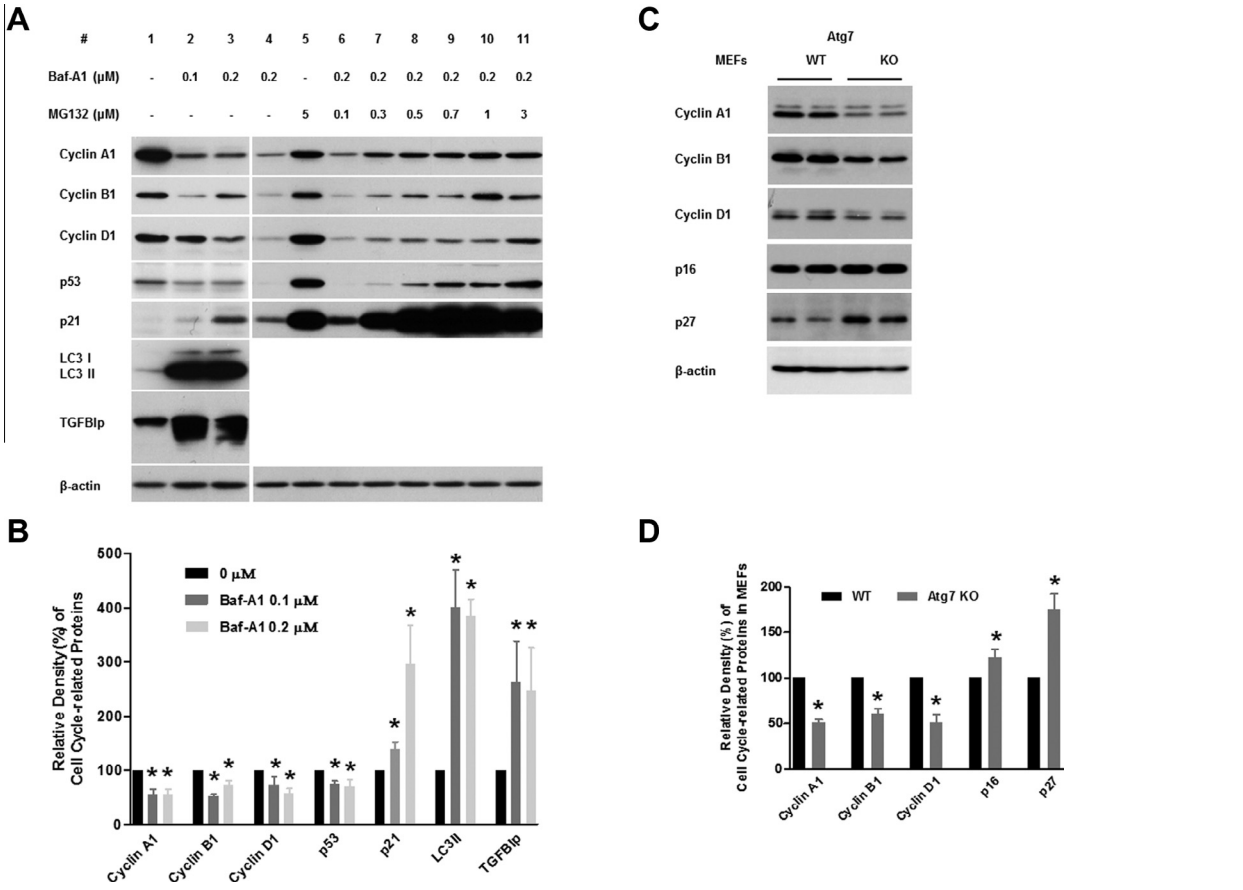

Fig. 4. Regulation of cell cycle-related proteins in autophagy-defective cells. (A) Cyclin A1, Cyclin B1, Cyclin D1, and p53 protein expression is down-regulated following 8 h of baf-A1 treatment, while p21 expression is increased. However, the addition of the ubiquitin proteasome inhibitor, MG132, prevented these baf-A1-induced changes in a dose-dependent manner. (B) Relative expression of Cyclin A1, Cyclin B1, Cyclin D1, and p53 proteins after normalization with their respective β-actin protein signals. (C) Analysis of lysate collected from Atg7 gene knockout MEFs indicates that, in comparison to the WT, the expression of Cyclins A1, B1, and D1 is reduced, while that of p16, and p27 in induced. (D) Relative expression density of Cyclin A1, Cyclin B1, Cyclin D1, p16 and p27 proteins after normalization with their respective β-actin protein signals. The data show the mean ± SD for three unique cell preparations, $^{*}P < 0.05$ compared with a non-treatment control group.

a similar decrease in Cyclin A1, B1, and D1, and increase in p16 and p27 expression (Fig. 4C and D) in an autophagy-deficient cell model (Atg7 knockout MEFs) compared to the GCD2 primary corneal fibroblasts (Fig. 3A–C).

4. Discussion

In the present study, we provide the first evidence that the pathophysiology of GCD2 involves disruption of cell cycle arrest in the affected corneal fibroblasts.

It is well known that cell cycle checkpoints are critical points of regulation during controlled cellular proliferation [13]. Our analysis shows that WT corneal fibroblasts accumulate in the G_1 phase, highlighted by the expression of p53 and p21, while GCD2 corneal fibroblasts accumulate in the G_2 phase without being properly checked at the G_1 check point, as shown by the decreased expression of p53 and p21, and were then allowed to enter the S phase. Growing evidence suggest that p53 and p21 are pivotal molecules involved in regulating the entrance of cells into the S phase [14,15]. In the absence of one of them, the rate of cellular mutation is markedly increased as cytotoxic conditions persist. It is, therefore, not surprising that lower levels of p53 and p21 in the HE cells cause more mutational damage, perturbations in cell cycle progression, and even a shift towards cell death, effects that are only exacerbated in the HO corneal fibroblasts [16]. Further, Tasdemir et al. have recently shown that cytoplasmic p53 acts as a repressor of autophagy and that the inhibition of p53 expression induces autophagy in the G_1 phase of the cell cycle [17]. Moreover, many different autophagic stimuli reduce p53 [18]. We suspect that in our primary cell culture system, the decreased expression of p53 is directly connected to the disturbances in G_1 cell cycle arrest and autophagy activation in GCD2 cells [9]. However, the defective autophagy system in GCD2 cells does not appear to eliminate mutated TGFBIp completely, resulting in the accumulation of mutated TGFBIp in these cells [8]. Furthermore, autophagy inducers significantly reduced the amounts of mutant-TGFBIp, a phenomenon which is supported in the literature [8,19]. Further studies are required in order to fully understand the function of p53 in the pathology and progression of GCD2.

Beyond the role of p53, the decision to enter S phase from G_1 is promoted by CDK/Cyclin complexes which are inhibited by CDK inhibitors, such as p16 and p27. Here, we observed an increase in p16 and p27 expression in the GCD2 cells, which likely perturbs G_1 cell cycle arrest in these corneal fibroblasts. We suspect that it is the combined loss of these CDK inhibitory proteins that results in the phenotypic abnormalities we observed in the GCD2 cells.

Although the expression of both CDK/Cyclins is primarily regulated by the UPS [20], the mTOR pathway also contributes to the regulation of these proteins [21]. mTOR is a conserved serine/threonine kinase that regulates both cell growth and cell cycle progression through its ability to integrate signals from nutrients and growth factors [22]. Recently, we have demonstrated that

autophagy is induced by reduced raptor, one of the mTOR complex 1 proteins, via the UPS in GCD2 corneal fibroblasts [9]. Further, defective autophagy flux, caused by the inhibition of autophagosome/lysosome fusion via Atg7 gene deletion resulted in similar inhibitory effects on the expression of Cyclin A1, B, D1, and p53 as that observed in the GCD2 affected cells. These changes in protein expression were also reversed with MG132. Taken together, these data indicate the possibility that insufficient and/or defective autophagy in GCD2 corneal fibroblasts may be connected to the reduced level of proliferation observed in these cells.

Corneal fibroblasts, also known as keratocytes, are the major cell type populating the stroma, being estimated to make up 9 to 17% of the cellular volume of this tissue with a density of approximately 23,000 cells/mm^3 in humans [23]. These cells are mainly involved in maintaining the extracellular matrix environment and transparency of the cornea and are responsible for secreting the collagen components [24]. Given the functional roles of keratocytes in the cornea, disturbances in the cell cycle and proliferative ability of these cells would likely affect the composition and function of the corneal stroma. However, the extent of the compositional differences between normal and GCD2 corneal stroma are still unknown. Further studies are required to study the possible correlation between the corneal stroma components/composition and mutant TGFBIp deposition.

In conclusion, we have shown that the pathophysiology of GCD2 involves a decrease in proliferation as well as altered cell cycle arrest and expression of cell cycle-associated proteins. To our knowledge, this is the first time these changes have been observed in GCD2 cells. We hope these findings will provide useful insight into the pathogeneses of this disease as well as other ocular and non-ocular diseases involving defective autophagy or abnormal cell cycle progression, such as cancer and degenerative diseases. Although this study provides a foundation for future work in this area, our understanding of GCD2 is far from complete and additional work is necessary to determine the possible role of autophagy in cellular proliferation and GCD2 treatment.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.073.

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