Induction of DDIT4 Impairs Autophagy Through Oxidative Stress in Dry Eye

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PURPOSE. To assess how DNA damage-inducible transcript 4 (DDIT4) and autophagic flux are altered in dry eye disease and reveal the underlying mechanisms

METHODS. C57BL/6 mice were exposed to desiccating stress (subcutaneous scopolamine [0.5 mg/0.2 mL] 3 times a day, humidity < 30%) for 7 days. Primary human corneal epithelial cells and cells from a human corneal epithelial cell line were cultured under hyperosmolarity. Western blot assays and immunofluorescence staining were used to measure changes in protein expression. mRNA expression was analyzed by RT-PCR and quantitative real-time PCR. Autophagosomes were observed through electron microscopy. Cellular reactive oxygen species and mitochondrial function were detected with 2',7'-dichlorodihydrofluorescein diacetate and mitochondrial membrane potential assays. Cell Counting Kit-8 and lactate dehydrogenase assays were used to measure cell death. Apoptosis was analyzed by Annexin V-PI flow cytometry.

RESULTS. Increased expression of microtubule-associated protein 1 light chain 3 (LC3-II), sequestosome 1 (SQSTM1), and DDIT4 were observed in corneal epithelial cells in vitro and mice models of dry eye. The electron microscopy revealed large autophagic vacuoles with poorly degraded materials in human corneal epithelial cells under hyperosmolarity. In addition, we found that DDIT4 knockdown significantly suppressed the expression of LC3-II and SQSTM1 by disrupting reactive oxygen species release and restoring mitochondrial function under hyperosmolarity. Moreover, the ablation of DDIT4 effectively preserved cell viability and inhibited apoptosis.

CONCLUSIONS. Excessive reactive oxygen species release through DDIT4 induction can lead to impaired autophagy and decreased cell viability in dry eye disease.

Keywords: dry eye, autophagy, autophagic flux, DDIT4, ROS, oxidative stress

Dry eye disease (DED) is a chronic ocular surface disease and has become a common public health problem affecting approximately 5% to 35% of the population worldwide. DED patients frequently suffer ocular discomfort and thereby a deteriorating quality of life. Recent evidence suggests that increased tear film hyperosmolarity, which can induce corneal epithelium injuries through a cascade of inflammatory events, is a key mechanism underlying DED. However, the exact pathogenetic mechanism of DED is not entirely understood.

As the outermost layer of the eye, the corneal epithelium frequently encounters various environmental stresses. Previous studies have demonstrated that the overproduction of reactive oxygen species (ROS) under environmental stress plays a key role in DED development. Excessive ROS can cause severe oxidative stress and induce human corneal epithelial cell (HCEC) damage by targeting DNA, proteins, and intracellular processes. However, the ROS-related signaling pathways involved in susceptibility or resistance to cell death in DED have not been thoroughly investigated. Interestingly, one recent work suggested that oxidative stress can regulate macro-autophagy (hereafter, autophagy) in the corneal epithelium. Autophagy is a conserved process that serves to recycle defective cellular organelles and macromolecules. Autophagy occurs at a certain rate under basal conditions to maintain normal cellular homeostasis. However, the precise association between ROS and autophagy remains to be elucidated. Although cellular ROS may function as indispensable signaling molecules for autophagosome induction, their release can impair autophagy-related processes and lead to autophagosome accumulation, ultimately mediating autophagic cell death. To date, there have been few efforts to characterize the crosstalk between ROS and autophagy in DED.

Recently, researchers identified an association between a metabolic regulator, the protein DNA damage-inducible transcript 4 (DDIT4; also known as REDD1 or RPT801) and ROS production. DDIT4 has traditionally been recognized as an inhibitor of the mammalian target of rapamycin (mTOR) pathway and a regulator of cell survival and proliferation, but recent studies have found that DDIT4 ablation diminishes tissue injury caused by oxidative stimuli, such as oxygen-induced retinopathy and cigarette smoke–induced lung damage. Furthermore, it has been discovered that the overexpression of DDIT4 enhances susceptibility to oxidant-induced injuries and reduces exercise capacity. These contradictory findings suggest that additional studies are necessary to elucidate the pathophysiological roles of DDIT4.
Furthermore, the role and regulatory mechanism of DDIT4 in autophagy have not been investigated in DED.

In this study, we sought to determine whether DDIT4 is involved in DED through altering ROS and autophagy. Our results revealed increased expression of DDIT4 along with overproduction of ROS and impaired autophagy in DED. Furthermore, we found that inhibition of DDIT4 reduces excessive ROS release and enhances autophagic flux, thereby inhibiting corneal epithelial cell apoptosis in HCECs under hyperosmolarity. Our findings provide the first evidence that the DDIT4-ROS-autophagy axis is critically involved in DED development.

**Materials and Methods**

**Reagents**

 Dulbecco’s modified Eagle’s medium/F12 medium, fetal bovine serum, 0.25% trypsin-EDTA, recombinant human epidermal growth factor, and an insulin-transferrin-selenium supplement were purchased from Invitrogen/Gibco (Carlsbad, CA, USA). Six-, 24-, and 96-well culture plates and cell culture flasks were purchased from Corning (Corning, NY, USA). The ROS detection reagent 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) was purchased from Abcam (Cambridge, UK). A Cell Counting Kit-8 was purchased from Dojindo Laboratories (Kumamoto, Japan), and an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection Kit I was purchased from BD Biosciences (San Diego, CA, USA). A Pierce LDH Cytotoxicity Assay Kit was purchased from Thermo Fisher Scientific (Rockford, IL, USA). The following antibodies were used for immunofluorescence. All reagent-grade chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

**Cell Culture and Development of the In VitroHyperosmolar Stress Model**

Cells of a human SV40 immortalized corneal epithelial cell line (CRL-1135, human corneal epithelium-2 (HCE-2); ATCC, Manassas, VA, USA) were cultured on plates in a humidified atmosphere of 5% carbon dioxide (CO₂) at 37°C. Dulbecco’s modified Eagle’s medium/F12 containing 5 mg/mL insulin and 10 ng/mL human epidermal growth factor (Sigma-Aldrich, Steinheim, Germany), 10% fetal bovine serum (Thermo Fisher Scientific, HyClone, Logan, UT, USA), and 1% penicillin/streptomycin (Thermo Fisher Scientific) was used as the culture medium. Primary HCECs were obtained from corneal limbal rims after corneal transplantation at Zhongshan Ophthalmic Center (Guangzhou, China) and cultured using previously reported methods.27 The primary and immortalized HCECs were then treated for 6 to 24 hours in iso- or hyperosmolar (312 or 500 mOsm, respectively) medium; the osmolarity was achieved by adding 0 or 94 mM sodium chloride, respectively, as previously described.

**Animal Model and Treatment**

A total of 70 female C57BL/6 mice aged 8 to 10 weeks were purchased from the Guangdong Provincial Centre for Animal Research (Guangzhou, China). The mice were maintained in an environmentally controlled room that was maintained at ≤30% humidity and were exposed to a continuous air draft created by fans. The mice were given subcutaneous scopolamine hydrobromide injections (0.5 mg/0.2 mL; Sigma-Aldrich) three times per day for 7 consecutive days, as previously described, to inhibit tear secretion.28 Control mice matched for age and sex were maintained at environment of 50% to 75% relative humidity. All animal experiments complied with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Review Board of Zhongshan Ophthalmic Center (Guangzhou, China; approval ID 2017-095). The eyes of the mice were examined using a slit-lamp microscope (Carl Zeiss Meditec, Dublin, CA, USA).

In the experimental treatment groups, both eyes of each mouse received a single subconjunctival injection of a 1.5-µL solution of N-acetyl-L-cysteine (10 mM/20 mM PBS; Sigma-Aldrich). The same volume of PBS was injected in the eyes of control mice.

**Corneal Staining**

To evaluate the degree of corneal epithelial defects in the mice, 1 µL of 1% sodium fluorescein was administered to the inferior lateral conjunctival sac. The corneal epithelial integrity was graded using a cobalt blue filter under a slit-lamp microscope image system (SL-D7/DC-3/MAGENet; Topcon, Tokyo, Japan). Each cornea of the right eye was divided into four quadrants that were scored individually. The intensity of corneal staining was scored using a four-point scale (0–4): 0 = no staining; 1 = slightly punctate staining, ≤30 spots; 2 = punctate staining, >30 spots, but not diffuse; 3 = severe diffuse staining, but no positive plaque; and 4 = positive fluorescein plaque. The scores of the four areas of each mouse cornea were summed to obtain the final score, which ranged from 0 to 16.29

**Periodic Acid-Schiff (PAS) Staining**

The whole eyeball including the conjunctiva was excised and fixed in formalin. The tissues were sliced into 4-µm-thick sections and subjected to PAS staining. The number of PAS-stained cells per 100 µm was counted in each of the four different sections of each eye from the same animal, and the average of the counts for the right and left eyes was recorded as the goblet cell density.

**Cell Viability Assay**

We detected cell viability with a Cell Counting Kit-8 assay according to the manufacturer’s protocol. Briefly, HCECs were seeded in 96-well plates and exposed to a conditioned medium. Then 100 µL of a mixture of culture medium and Cell Counting Kit-8 solution was added to each well of the plate, and the plate was then incubated for 2 hours (at 37°C and 5% CO₂). The absorbance at 450 nm was measured by using a microplate reader (BioTek Instruments, Winooski, VT, USA).

**Cell Apoptosis Assay**

After 24 hours of treatment, the HCECs were rinsed and collected by centrifugation for Annexin V-FITC/PI staining. Each
pellet was resuspended in 500 µL of binding buffer. Then, 5 µL of FITC and 5 µL of PI were added to each well, and the cells were incubated for 15 minutes. The apoptotic ratios were determined by flow cytometry (BD LSRII Fortessa, San Jose, CA, USA).

ROS Activity Measurement
ROS activity was measured as described in a previous report. Briefly, HCECs were grown in conditioned medium in six-well plates. When confluent, the HCECs in the six-well plates were rinsed and incubated with 25 mM H2DCFDA at 37°C for 30 minutes. The fluorescence intensity was examined by flow cytometry. Cell fluorescence was also measured, and images were captured with a fluorescence microscope (DFC7000T; Leica, DMi8, Wetzlar, Germany).

Mitochondrial Membrane Potential (MMP) Assay
MMP was measured with a tetramethylrhodamine ethyl ester MMP assay (ab113852; Abcam). Briefly, cells were washed 3 times with PBS, incubated with tetramethylrhodamine ethyl ester for 20 minutes at 37°C, and analyzed by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 575 nm.

Lactate Dehydrogenase (LDH) Leakage
LDH release was analyzed as described previously. Briefly, after the cells were exposed to the conditioned medium, aliquots of cell-free supernatant were collected. All samples were assayed in triplicate for LDH content by a microplate reader at a wavelength of 490 nm.

RNA Interference
The HCECs were transfected at 70% confluence using Lipofectamine 3000 Transfection Reagent (Invitrogen) with short interfering RNA (siRNA) targeting DDIT4 (no: sense, 5'-CUGCU GAUGCCUAAGCGAUUGUA-3'; antisense, 5'-UUACCAACUG CUUAGCAUCAGGAC-3'; sense, 5'-CCUGCCUUCUUGG GACCCGCUUCUGU; antisense, 5'-ACGAGAAGCGGUCC AAAGGCUGG-5') or Stealth siRNA (Invitrogen) as a negative control treatment. Following the manufacturer’s instructions, each dried siRNA was dissolved in nuclease-free water to achieve 20 µM concentration. For EM, HCECs were fixed overnight at 4°C in 2.5% glutaraldehyde and 150 mM sodium cacodylate (pH 7.4). After postfixation in 1% osmium (VIII) oxide (OsO4) followed by uranyl acetate, the tissues were dehydrated in ethanol and embedded in epoxy resin. Sections (100-nm thick) were examined under a Hitachi (HF7700, Tokyo, Japan) transmission electron microscope system.

Statistical Analysis
All data are expressed as the mean ± SEM. Statistical analyses were performed using GraphPad (GraphPad Software, La Jolla, CA, USA). Student's t-test was used to compare differences between groups. One- or two-way ANOVA followed by Bonferroni's post hoc test was used for comparisons among three or more groups. P < 0.05 was considered to indicate statistical significance.

RESULTS
Autophagic Flux Is Impaired in DED
We sought to detect changes in autophagy during DED in vivo. Our mouse model of DED exhibited typical corneal epithelial defects (Fig. 1A) and a general trend of increased fluorescein-staining scores relative to those of controls (Fig. 1B). We also observed significant decreases in the number of PAS-stained goblet cells in the conjunctiva (Figs. 1C, 1D) after 7 days of ROS activity was measured as described in a previous report. 30 Briefly, HCECs were grown in conditioned medium in six-well plates. When confluent, the HCECs in the six-well plates were rinsed and incubated with 25 mM H2DCFDA at 37°C for 30 minutes. The fluorescence intensity was examined by flow cytometry. Cell fluorescence was also measured, and images were captured with a fluorescence microscope (DFC7000T; Leica, DMi8, Wetzlar, Germany).

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Western blot analysis showed that the expression of the autophagic marker microtubule-associated protein 1 light chain 3 (LC3-II) was greatly increased in mice model of DED (Fig. 1E). LC3 is a microtubule-associated protein that is processed by cleavage (producing LC3-I) and subsequent lipid conjugation (yielding LC3-II) for membrane targeting. LC3-II induction is an established marker of autophagosomes. To confirm the increase of autophagosomes in DED in vitro, we measured autophagy in immortalized HCECs exposed to hyperosmolarity. Consistent with the findings described previously, Western blot analysis revealed significant LC3-II induction in HCECs under hyperosmolarity (Fig. 2A). To verify that the results were not cell-line dependent, we determined that such increases in LC3-II induction also occurred in primary HCECs exposed to hyperosmolarity for 6 or 24 hours (Supplementary Fig. S1).

The increased levels of LC3-II and the increased numbers of autophagosomes could have been caused by either increased formation of autophagosomes or inhibited degradation through the autophagic process. We found that SQSTM1, a molecule that is mainly degraded through autophagy, was significantly accumulated both in the dry eye mouse model and in primary and immortalized HCECs under hyperosmolarity (Figs. 1E, 2A, 2B; Supplementary Fig. S1). Furthermore, the expressions of BECN1 and ATG5, two genes involved in the early stages of autophagosome formation, were not affected by hyperosmolarity treatment (Fig. 2A; Supplementary Fig. S1).

In addition, the electron microscopy revealed many large autophagic vacuoles with poorly degraded material in immortalized HCECs under hyperosmolar conditions (Fig. 2C). These results strongly suggested that autophagic flux was blocked in these cells. To further assess autophagy induction and autophagic flux, we cultured immortalized HCECs and incubated them with an inhibitor of lysosomal degradation, bafilomycin A1. The inhibition of lysosomal degradation ensured that any subsequent changes in LC3-II reflected only autophagosome formation. In the presence of lysosomal inhibitors, HCECs subjected to hyperosmolarity displayed impaired autophagy in the dry eye mouse model. The experimental mice were subjected to desiccation stress (DS) for 7 days. (A) Photographs of corneal fluorescein staining in DS mice and untreated (UT) mice after 3, 5, and 7 days. (B) Clinical evaluation of corneal fluorescein staining. (C, D) Photographs of PAS staining of the conjunctiva, showing goblet cells. Scale bar: 50 μm. The density of goblet cells in the conjunctiva was determined in DS and UT mice after 7 days. (E) Western blot results showing the changes in LC3-II and SQSTM1 expression in the corneal epithelium of DS mice relative to those in UT mice after 3, 5, and 7 days. Right: relative densitometry quantitation was monitored; 5 mice were included in each group. The data shown are representative of three or more independent experiments (mean ± SD). **P < 0.01 vs. untreated controls.
FIGURE 2. Hyperosmolarity induces impaired autophagy in HCECs. (A) Western blot results showing the changes in LC3-I/II, SQSTM1, ATG5, and beclin 1 (BECN1) expression in immortalized HCECs exposed to hyperosmotic medium (500 mOsM) relative to that in normal medium after 6 and 24 hours. Right: relative densitometry quantitation was monitored. (B) Representative fluorescence images showing the distributions of SQSTM1 puncta in immortalized HCECs exposed to hyperosmotic medium or normal medium (Ctrl) for 6 hours. Scale bar: 100 μm. (C) Transmission electron microscopy showing abnormally large autolysosomes with poorly degraded material (arrows) in immortalized HCECs subjected to hyperosmotic conditions for 6 hours. N denotes nuclei. Scale bar: 1.0 μm. (D) Immortalized HCECs were cultured in normal or hyperosmotic...
greater levels of LC3-II than did control cells, indicating that autophagy induction and autophagic flux blockade were potentially both present (Fig. 2D).

**Increased Expression of DDIT4 Regulates ROS Accumulation**

DDIT4 has been reported to be an endogenous inhibitor of mTOR that regulates cellular stress responses, which are the main pathways involved in autophagy. 

In this study, we sought to evaluate changes in DDIT4 expression in the dry eye. Western blot analysis showed time-dependent increases in DDIT4 expression along with inhibition of the mTOR pathway in both primary and immortalized HCECs subjected to hyperosmolarity (Fig. 3A; Supplementary Fig. S2). Immunofluorescence staining confirmed the induction of DDIT4 in primary HCECs under hyperosmolar condition (Fig. 3B). In the mouse DED model, increased DDIT4 expression in corneal epithelial cells was consistently detected by Western blot analysis and immunofluorescence analysis (Figs. 3C, 3D).

Previous studies have identified associations between both DED and DDIT4 upregulation with cellular ROS induction. 

Using H2DCFDA staining, we discovered that ROS levels in both primary and immortalized HCECs were greatly increased in response to hyperosmolarity (Fig. 4A and Supplementary Fig. S3). Importantly, we found that ROS levels in DDIT4-knockdown cells were significantly decreased than the corresponding levels in wild-type cells subjected to hyperosmolarity (Figs. 4B–4D). In addition, we observed significant decreases in MMP, which indicated mitochondrial dysfunction under hyperosmolar conditions, but these decreases were reversed by DDIT4 ablation (Fig. 4E). DDIT4 knockdown had little effect on the expression of mTOR, implying that decreases in ROS in the absence of DDIT4 occur in an mTOR-independent manner (Fig. 5A).

**DDIT4 Regulates Autophagy Through the ROS Pathway**

To further explore the role of DDIT4 in autophagy in DED, we investigated changes in autophagic flux after DDIT4 knockdown in immortalized HCECs under hyperosmolarity. We discovered that the levels of LC3-II and SQSTM1 (Figs. 5A, 5B). In addition, after DDIT4 knockdown, electron microscopy consistently showed significant decreases in the typical accumulation of abnormally large autolysosomes with poorly degraded cargo (Fig. 5C). Previous studies have revealed that oxidative stress can be a primary factor contributing to autophagy impairment. Therefore, to verify the important role of the DDIT4-ROS axis in autophagy under hyperosmolar condition, as evidenced by the decreases in LC3-II and SQSTM1 expressions (Fig. 5D). Furthermore, in the mouse model, subconjunctival injection of NAC protected the ocular surface in DED, yielding significant decreases of corneal epithelial defects in vivo (Fig. 5E, 5F). Taken together, these results suggest that DDIT4 is a crucial endogenous regulator of autophagy that exerts its effects by regulating ROS in DED.

**DDIT4 Knockdown Protects HCECs Under Hyperosmolarity**

HCECs are the first line of defense in the eye, and hyperosmotic stress has frequently been reported to cause cytotoxicity. Therefore, we investigated the potential effects of DDIT4 knockdown on cell fate under hyperosmolar condition. We first observed exposure of HCECs to hyperosmolarity significant reduced cell viability under in a time-dependent manner (Supplementary Fig. S4). Importantly, we observed that DDIT4 knockdown significantly preserved cell viability, and these effects were also confirmed by inhibition of elevated LDH release (Figs. 6A, 6B, 6C). Next, we sought to elucidate the precise changes in the manner of death that occur after DDIT4 knockdown. We found that under hyperosmolarity, the inhibition of DDIT4 significantly blocked apoptosis, with decreased caspase-3 cleavage observed (Fig. 6D). To quantify the number of apoptotic cells, we performed Annexin V and PI staining and flow cytometry. Our results showed that after DDIT4 knockdown, the percentage of late apoptotic cells was significantly lower than the corresponding percentage among wild-type cells (Fig. 7E).

**DISCUSSION**

Recently, increasing evidence has indicated that alterations in autophagy are involved in the pathogenesis of multiple ocular diseases. However, little is known about the changes in and evolving functions of autophagic flux in corneal disease. Our findings reveal a new mechanism in which induction of the metabolic regulator DDIT4 increases ROS levels to impair autophagy in DED.

Autophagy is commonly recognized as playing an essential role in organismal homeostasis by mediating the degradation and turnover of defective organelles within cells. Accumulating evidence suggests that the induction of autophagy promotes cell survival under multiple types of stress. However, it remains debated whether autophagy can also cause cell death. Although some studies have indicated that increases in autophagy markers occur in DED, the specific changes in autophagic flux and the underlying regulatory mechanisms remain unclear. In this study, we found increased levels of LC3-II and SQSTM1 in dry eye models both in vitro and in vivo. Using electron microscopy detection, we found that abnormal autolysosomes with poorly degraded materials were abundant in HCECs under hyperosmolarity. These results strongly suggest that corneal epithelial cells experience severe defects in autophagic flux.

ROS are key intracellular signaling molecules, but in DED, the mechanisms and targets of ROS regulation remain undefined. Our data show that in HCECs subjected to hyperosmolar conditions, ROS are overproduced, and mitochondrial function is impaired. More important, we found that NAC could reduce the expression of LC3-II and SQSTM1 in HCECs, indicating restored autophagic flux. Furthermore, in our dry eye mouse model, NAC protected the ocular surface, as
FIGURE 3. Increased expression of DDIT4 in DED both in vitro and in vivo. (A) Western blot results showing the time-dependent changes in DDIT4, mTOR, and phosphorylated mTOR (p-mTOR) expression in immortalized HCECs exposed to hyperosmotic medium (500 mOsM) relative to that in normal medium. (B) Representative fluorescence images showing the increased expression of DDIT4 in primary HCECs exposed to hyperosmolarity for 6 hours relative to that in normal medium (Ctrl). The experimental mice were subjected to DS for the indicated times (C) or for 7 days (D), whereas the control mice were left UT. (C) Western blot results showing the change in DDIT4 expression. Right: relative densitometry quantitation was monitored. (D) Representative fluorescence images showing the expression of DDIT4 in the corneal epithelium. Scale bar: 50 μm. The data shown are representative of three or more independent experiments (mean ± SD). **P < 0.01 and * P < 0.05 vs. UT controls.
evidenced by the decreased intensity of corneal staining. As a ROS scavenger, NAC has been investigated with respect to dry eye treatment. However, most studies have reported that NAC exerts its effects through the suppression of the ROS-inflammation pathway. To the best of our knowledge, this study is the first to suggest that NAC serves to protect corneal epithelium by normalizing impaired autophagic degradation in DED. However, the effects of cellular ROS levels on autophagy remain intriguing. In some cases, the intracellular burden of organelle damage induced by ROS can induce autophagy. In the present study, we observed increased expression of LC3-II, even when the HCECs were cotreated with lysosomal inhibitors under hyperosmolar conditions. Collectively, our data strongly indicate that although autophagy induction is

### Figure 4

Inhibition of DDIT4 blocks the increase in ROS and damage to the MMP under hyperosmolarity. (A) H2DCFDA fluorescence in immortalized HCECs exposed to normal medium (Ctrl) or hyperosmotic medium (500 mOsM) for the indicated times. Scale bar: 100 μm. Immortalized HCECs were transfected with DDIT4-specific siRNAs (siDDIT4-1 and siDDIT4-2) or negative control (siNC) for 24 hours. The expression of DDIT4 was determined by Western blotting (B) or quantitative PCR (C). Wild-type (WT) and DDIT4-knockdown (KD) HCECs were exposed to hyperosmotic medium (500 mOsM) or normal medium (Ctrl) for 6 hours. (D) ROS levels were measured by H2DCFDA using flow cytometry. Right: relative fluorescence units (RFU) were monitored. (E) MMP was evaluated by tetramethylrhodamine ethyl ester staining using flow cytometry. The data shown are representative of three or more independent experiments (mean ± SD). **P < 0.01 and *P < 0.05 vs. UT controls; ***P < 0.05 vs. 500 mOsM(+) DDIT4 KD(+); **P < 0.05 vs. 500 mOsM(−) DDIT4 KD(−).
Figure 5. Ablation of DDIT4 restores autophagic flux by suppressing ROS. (A) Western blot results showing the changes in LC3-I/II, SQSTM1, and p-mTOR expression in WT and DDIT4-KD immortalized HCECs following exposure to hyperosmotic medium (500 mOsM) or normal medium for 24 hours. Right: relative densitometry quantitation was monitored. (B) Representative fluorescence images showing the distributions of SQSTM1 puncta in WT and KD immortalized HCECs under hyperosmolarity (500 mOsM) or under control medium (ctrl) for 6 hours. Scale bar: 100 µm. (C) Transmission electron microscopy images showing reduced number of autolysosomes (arrows) and normal mitochondria (M) in DDIT4-KD HCECs subjected to hyperosmolarity (500 mOsM) for 6 hours. N denotes nuclei. Scale bar: 1.0 µm. (D) Immortalized HCECs were cultured in normal or...
Knockdown of DDIT4 inhibits hyperosmolarity-induced HCEC death. Immortalized HCECs were transfected with control siRNA (WT) or DDIT4 siRNA (KD) for 24 hours and then treated with 500 mOsM or normal culture medium (ctrl) for 24 hours. (A) Representative phase-contrast images were obtained. Scale bar: 100 µm. (B) Changes in cell viability were determined by Cell Counting Kit-8 assay. (C) Changes in LDH levels were monitored by LDH assay. (D) Western blot analysis results showing the expression of caspase-3 and cleaved caspase-3. (E) Flow cytometry analysis of apoptosis. The data shown are representative of three or more independent experiments (mean ± SD). *P < 0.05 vs. UT control; †P < 0.05 vs. 500 mOsM(+) DDIT4 KD(+).
increased in DED, the late stage of autophagy (lysosomal degradation) seems to be inhibited as a result of excessive ROS release. Further studies are necessary to more precisely define context-dependent changes that occur in autophagy and the underlying molecular mechanisms. Our research has clinical implications as it suggests that strategies regulating autophagy could contribute to future DED treatments.

Numerous studies have reported that DDIT4 functions as an upstream negative regulator of the mTOR pathway and is involved in various cell behaviors, such as cell proliferation, tumorigenesis, and neuronal atrophy. In addition, previous studies have revealed that DDIT4 plays a significant role in the pathogenesis of retinopathy. However, it remains unclear how the metabolic functions of DDIT4 contribute to its cellular activity in ocular surface diseases. In the present study, we discovered a marked increase in DDIT4 expression in the corneal epithelium in DED both in vitro and in vivo. In addition, it has been shown that DDIT4 affects ROS to regulate cellular responses. In this study, we observed that DDIT4 knockdown reduced ROS release and normalized mitochondrial function under hyperosmolar conditions. Moreover, we showed that the ROS-induced impairment of autophagy was markedly attenuated in DDIT4-inhibited cells. These findings strongly suggest that a DDIT4-ROS-autophagy axis exists in DED. Our present work reveals a new and specific mechanistic link between redox-dependent signaling and autophagy in DED. Interestingly, we found increases in DDIT4 expression along with mTOR suppression in HCECs under hyperosmolar conditions. However, we observed no significant changes in the mTOR pathway after DDIT4 knockdown. This finding suggests that DDIT4 might exert biological effects independently of mTOR. Future studies are needed to elucidate the specific roles of DDIT4 in corneal diseases.

Although DDIT4 plays contradictory roles in many different diseases, our current data indicate that the induction of DDIT4 promotes DED development. We discovered that DDIT4 ablation can protect cell viability and inhibit HCEC apoptosis under hyperosmolar conditions. The concept of precision medicine has been used in various ways for DED therapy. The results of our current study provide insight into potential genotype-selective agents for DED treatment. However, further studies using animal models of DED with DDIT4 knockout are necessary to clarify the specific roles of DDIT4.

Collectively, our results suggest that DDIT4-mediated activation of ROS signaling pathways can lead to autophagy deficiency. The identification of the molecular pathway described in the present study may lead to greater understanding of the pathogenesis of DED and to innovations in DED therapy.

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