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Increased O-GlcNAc causes disrupted lens fiber cell differentiation and cataracts

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ABSTRACT

Diminished proteolytic functionality in the lens may cause cataracts. We have reported that O-GlcNAc is an endogenous inhibitor of the proteasome. We hypothesize that in the lens there is a cause-and-effect relationship between proteasome inhibition by O-GlcNAc, and cataract formation. To demonstrate this, we established novel transgenic mouse models to over-express a dominant-negative form of O-GlcNAcase, GK-NCOAT, in the lens. Expression of GK-NCOAT suppresses removal of O-GlcNAc from proteins, resulting in increased levels of O-GlcNAc in the lenses of our transgenic mice, along with decreased proteasome function. We observed that transgenic mice developed markedly larger cataracts than controls and lens fiber cell denucleation was inhibited. Our study suggests that increased O-GlcNAc in the lens could lead to cataract formation and attenuation of lens fiber cell denucleation by inhibition of proteasome function. These findings may explain why cataract formation is a common complication of diabetes since O-GlcNAc is derived from glucose.

Introduction

Cataracts are the leading cause of blindness worldwide. Studies of cataractous lenses have suggested that the accumulation of damaged lens proteins, arising from long-term exposure to oxidative species and ultraviolet light, is the cause of lens opacity [1–3]. The aggregation of damaged proteins forms inclusions in the lens, obstructing the passage of light, leading to subsequent cataract formation. Thus, it is believed that the failure to remove these proteins proteolytically results in cataract formation. The job of eliminating unwanted proteins in all cells in a controlled way is done by the proteasome, so it is not surprising to find that proteasome function is less active in cataractous lenses [4,5].

O-linked β-N-acetylglucosamine (O-GlcNAc) modification is a ubiquitous post-translational modification consisting of the monosaccharide N-acetylglucosamine (GlcNAc) linked to the side chain hydroxyls of either serine or threonine residues of many cytoplasmic and nuclear proteins [6,7]. O-GlcNAcylation is a cyclical and highly dynamic process. The addition of the GlcNAc to protein serine or threonine residues is catalyzed by O-GlcNAc transferase (OGT) [8,9], and the enzymatic removal of these sugars is achieved by O-GlcNAcase (OGN, OGA, or NCOAT) [10,11]. We previously reported that proteasome function is reversibly inhibited by O-GlcNAc modification [12]. This loss of function of purified 26S proteasome can be initiated with OGT, and conversely, reversed by NCOAT. O-GlcNAc modification of the Rpt2 ATPase of the 19S regulatory cap probably accounts for the observed inhibition of proteasome activity [12].

Cataract is a common complication of diabetes and onset occurs earlier in diabetic patients than is seen in the normal population. However, there is no clear mechanism to explain this phenomenon. Studies have shown that hyperglycemia and hyperinsulinemia are associated with increased O-GlcNAc modification levels in the cell [13–16]. It has also been shown that lens epithelial cells and lens fiber cells have a fully functional UPS [17,18]. Taken together, the evidence indicates that in lens cells, the increased O-GlcNAc modification levels resulting from diabetes, might lead to cataract formation through proteasome inhibition.

In this paper, we report that conditions promoting an increase in O-GlcNAc modification of lens proteins, result in enhanced cataract formation and inhibition of lens fiber cell denucleation. We demonstrated this *in vivo* by using a transgenic mouse model to express a dominant-negative version of NCOAT (GK-NCOAT) [11] in the mouse lens. We used the gamma-F-crystallin promoter [19] to direct expression of the GK-NCOAT gene to lens fiber cells in a tetracycline-inducible, bi-transgenic mouse system [20,21]. Activation of the GK-NCOAT transgene with administration of

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doxycycline, increased the level of O-GlcNAc modification in the lens concomitant with the inhibition of proteasome activity. We hypothesize that this increase in O-GlcNAc modification in the lens leads to cataract formation through inhibition of proteasome function and that the impaired proteasome activity may inhibit lens fiber cell denucleation. This observation provides a biochemical mechanism explaining cataract formation in diabetes and possibly may have more general implications in the pathology of age-related diseases.

Materials and methods

Generation of transgenic mice. To generate the gamma-F-crystallin-rtTA transgene, the gamma-F-crystallin promoter bases 777–1640 were synthesized by PCR using oligonucleotides; 5′-CATGGT ACCGTCGACGAATTCAGCATCCAGAACAGTCC-3′, and 5′-CATGGATC CGGCTGGTGTTGGCAGGTCAGATGG-3′ and using mouse liver genomic DNA as template. We cloned this PCR product into a previously characterized transgene cassette, upstream of the rtTA sequence (See Supplemental Fig. 1s). The final 3.6 kb transgene containing the gamma-F-crystallin promoter and rtTA sequences was linearized (KpnI), purified and microinjected into BL/6 × SJL mouse zygotes at a concentration of 2 ng/ml at the University of Alabama at Birmingham Transgenic Animal Facility. A total of three founders were identified by PCR, using a sense primer in the crystalline gene, 5′-CTTCCATTCTTTCAGCAGTAC-3′, and an antisense rtTA primer, 5′-TGAATGTTAGGACTGTTGCA-3′. Line 1 was identified as the most promising transgenic line to use in the current study (See Supplemental Fig. 2s). The "chimera" TRE-GK-NCOAT transgenic mouse line has been described previously [20]. The GFP-GK-NCOAT was constructed by inserting the GFP fragment upstream of the NCOAT sequence (See Supplemental Fig. 1s). The 5.6-kb ClaI-XhoI fragment containing the entire transgene cassette was used to generate the TRE-GK-NCOAT transgenic line, resulting in two founder transgenic lines that were identified by pcr; NCOAT sense, 5′- CCTTGCCAACTTCCCTTCTCTG-3′, and antisense sequence to SV40, 5′-GTCCTTGGGGTCTTCTACCTTTCTC-3′. Doxycycline was administered to mice via a grain-based rodent diet containing doxycycline (6 gm/KG) (Cat. No. F4096, Bio-Serv, Frenchtown, NJ). The animal care and procedures were approved by the Animal Resources Program of the University of Alabama at Birmingham (IACUC approval number and Animal Protocol No. 070606612).

Western blot analysis. Lenses were homogenized in cold lysis buffer containing 50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.25% deoxycholate, pH 8.0, and the protein supernatant were collected after centrifugation. Protein concentration was determined by using a colorimetric protein assay (Bio-Rad Laboratories, Hercules, CA). Lens homogenates containing equal amounts of 100 μg protein were separated on 10–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gels followed by transfer to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences, Piscataway, NJ). Membranes were blocked with 5% milk powder in TBST for 1 h and then incubated with primary antibodies overnight at 4 °C. The dilution ratios for anti-GFP (Sigma–Aldrich), anti-NCOAT (generous gift from Dr. Gerald Hart), RL2, and anti-beta-actin (Sigma–Aldrich) antibodies were 1:1000. The membranes were washed three times in TBST and incubated with goat anti-mouse or rabbit horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature, followed by three TBST washings. The blots were visualized with enhanced chemiluminescence (ECL) solution as described by the manufacturer (Amersham Biosciences, Amersham Biosciences, Piscataway, NJ).

Cataract formation examination. Eyes were removed from mice and lenses were dissected. The lenses were immediately put in PBS in 6 cm tissue culture dishes and images were captured using a camera linked to a microscope. The cataract area and lens area were measured by image analysis using SigmaScan Pro5 software

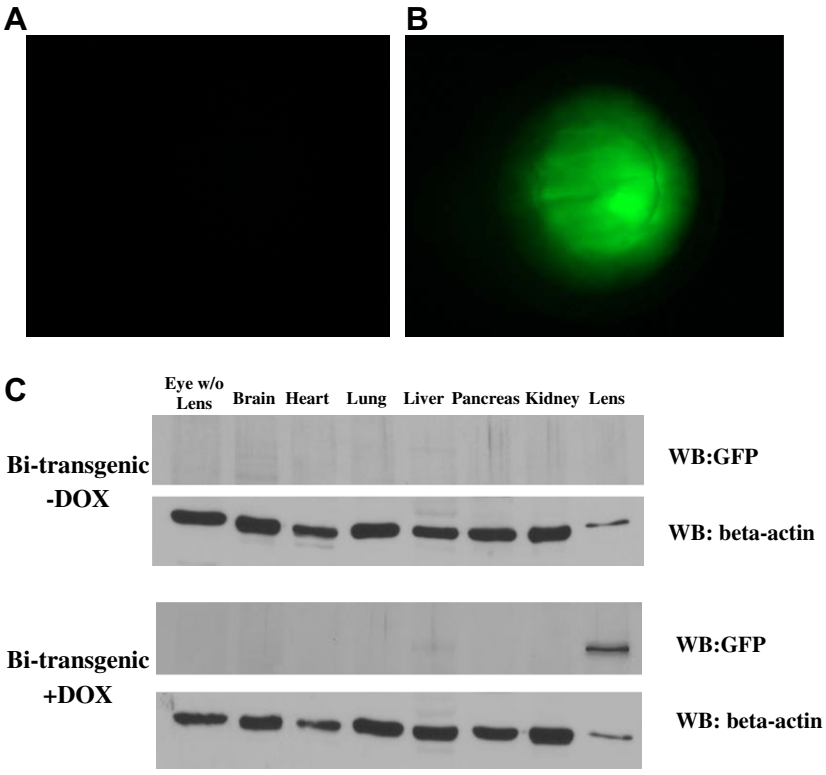

Fig. 1. Induced GFP-GK-NCOAT expression in lenses of bi-transgenic mice. GFP fluorescence in enucleated lenses of bi-transgenic mice, doxycycline-fed (B) or not (A). (C) Western blot analysis of tissues from bi-transgenic mice without, or with doxycycline using anti-GFP and β-actin antibodies.

(SPSS Science, Chicago, IL). For slit-lamp examination, eye pupils were dilated with atropine (Atropine Sulphate, 1 drop, Alcon Laboratories, USA) *in vivo* and then the lenses were inspected using a slit-lamp microscope and images were captured.

Proteasome activity assay. Proteasome activity analysis was conducted following the method described previously [12]. Two microliters of the fluorogenic substrate, suc-LLVY-AMC (100 μM, Sigma–Aldrich) was added to 50 μl lens homogenate containing 30 μg proteins, from bi-transgenic mice with or without administration of doxycycline, and were incubated at 37 °C for 90 min. The reaction was then stopped by adding 900 μl of 1% SDS. The fluorescence intensity was measured (excitation 360 nm, emission 450 nm) using a Turner Qantech Digital Fluorometer in triplicate.

Lens histology. Eyes were dissected from mice and fixed overnight in 10% neutral buffered formalin. The eyes were then washed three times with PBS, dehydrated in increasing concentrations of ethanol and embedded in paraffin. Sections were cut at 5 μm, dewaxed with xylene, rehydrated with decreasing concentrations of ethanol and stained with hematoxylin and eosin.

Results and discussion

The gamma-F-crystallin-rtTA transgenic mice were bred with the TRE-GFP-GK-NCOAT (target transgene) mice, enabling the doxycyclin-inducible expression of GFP-GK-NCOAT in the lenses of the off-spring. The GFP tag was included as a fusion protein to aid in the identification of the GK-NCOAT, since at the time the construct was made, no reliable anti-O-GlcNAcase antibody was available. As we previously reported, the GK-NCOAT splice variant lacks O-GlcNAcase activity but retains binding activity, so can act as a dominant-negative form of NCOAT, resulting in a general increase in the level of cytoplasmic and nuclear protein O-GlcNAc modification [11,20,21]. We have used TRE-GK-NCOAT (minus GFP) previously in cultured cells and also in mammary tissue [20,21], effectively increasing O-GlcNAc levels. GFP-GK-NCOAT expression in the lens was induced in pregnant mice by administration of doxycycline at embryonic day 10, the newborn mice were sacrificed at two weeks of age. In this and subsequent experiments, bi-transgenic mice with doxycycline administration will be

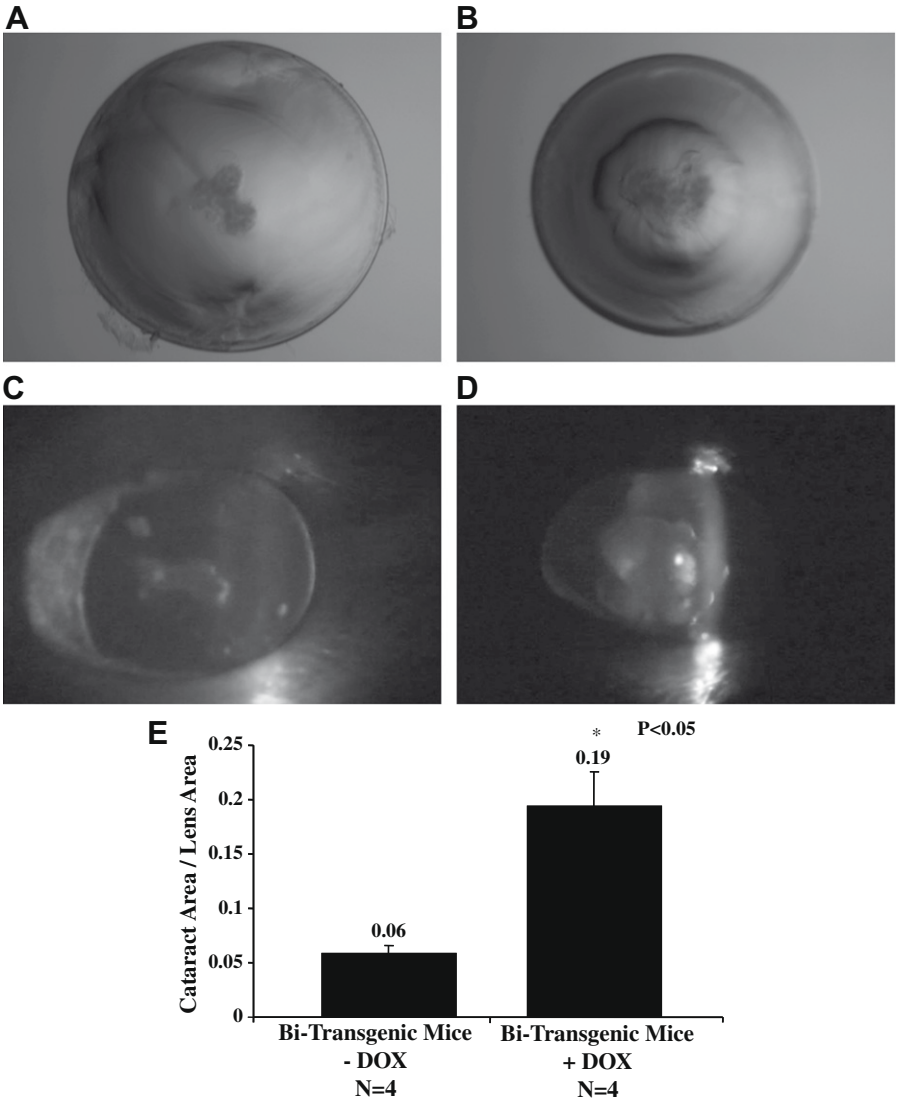

Fig. 2. Cataract formation in bi-transgenic mice. (A,B) Lenses were removed from the eyes of 2-week-old doxycycline treated (B) or untreated (A) bi-transgenic mice, placed in PBS, and photographed. Cataract area and lens area of these lenses were measured using SigmaScan Pro5 software and the ratio of cataract area to lens area was compared (E). (C,D) Cataracts in live bi-transgenic mice were also examined by slit-lamp, shown here in mice without doxycycline administration (C) and with doxycycline treatment (D).

the experimental animals, whereas the bi-transgenic mice without doxycycline administration will serve as control animals. The isolated lenses were placed in phosphate buffered saline (PBS) and GFP fluorescence detected. As shown, the lens from the bi-transgenic mouse with doxycycline administration indicated strong GFP expression (Fig. 1B), compared to the control mice (Fig. 1A). The lens-specific expression of GFP-GK-NCOAT was confirmed by Western blot analysis using anti-GFP antibody (Fig. 1C). GFP, and presumably GK-NCOAT, was highly expressed in only lens tissue and only when induced with doxycycline.

Intact lenses of 2-week-old bi-transgenic mice that had been treated with doxycycline at E10 were extracted and immediately immersed in PBS and photographed. Cataracts were well defined in the bi-transgenic mice with doxycycline administration (Fig. 2B). Although the untreated control bi-transgenic mice also developed cataracts, these were very diffuse and small in comparison (Fig. 2A). Cataracts were not observed in either the single transgenic TRE-GFP-GK-NCOAT mice, or the wild-type mice. We believe that the formation of cataracts in the un-induced bi-transgenic mice was likely due to the rtTA, or possibly due to a slight "leakiness" of the tet-inducible transgenes. The rtTA is a fusion protein, composed of the reverse tetracycline repressor of *Escherichia coli* and the transcription activation domain of viral protein 16 of herpes simplex virus, and with doxycycline induction, acts as the "trans-activator" to induce transgene expression. It has been reported that expression of rtTA in pulmonary epithelial cells under the control of the rat Clara cell secretory protein promoter in transgenic mice, resulted in an emphysema phenotype [22]. In the current study, the mechanism is unclear, but it is possible that the expression of rtTA may lead to injury of the lens. Another po-

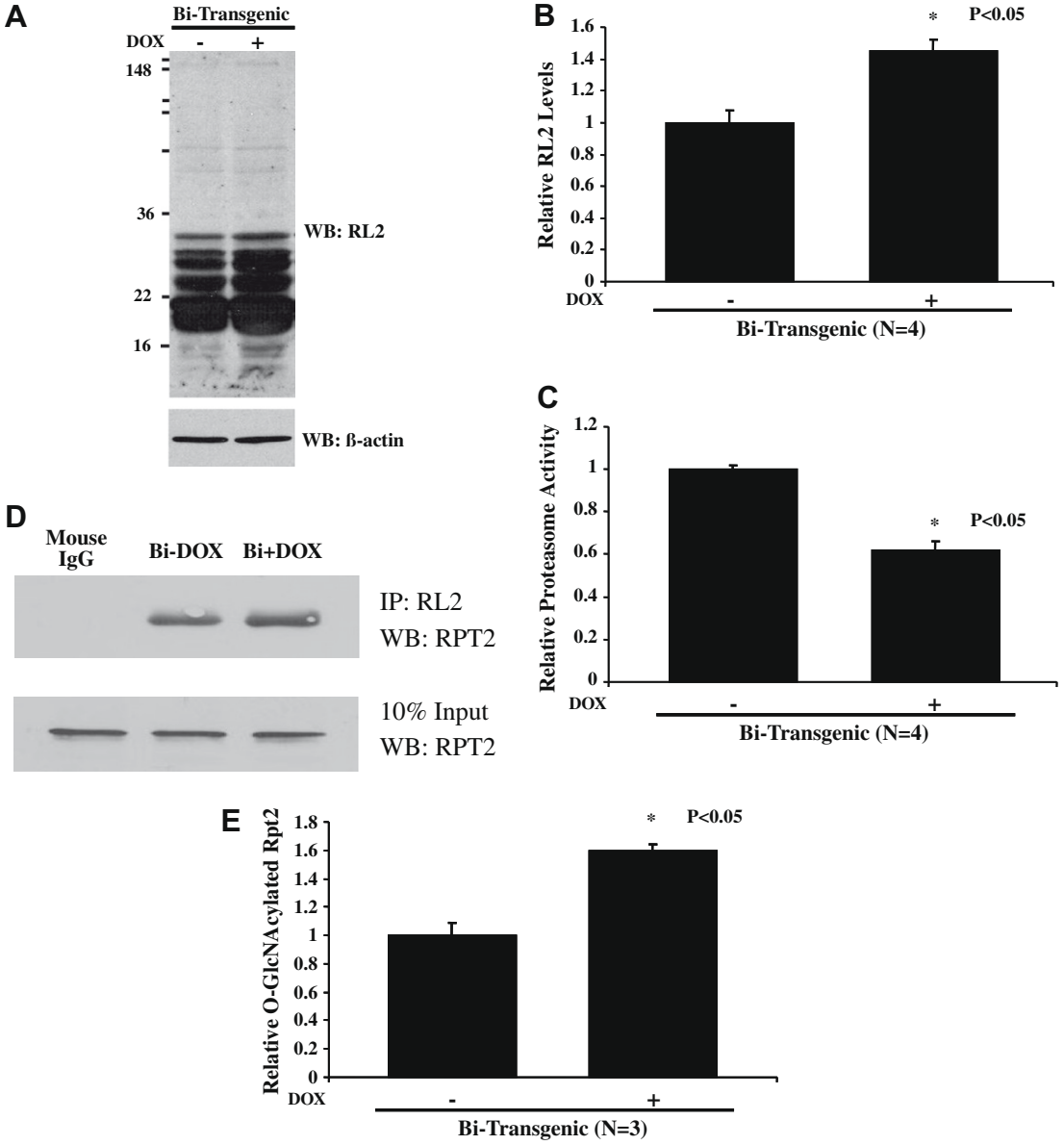

Fig. 3. Increased O-GlcNAc modification levels cause inhibition of proteasome function in the lenses of bi-transgenic mice with administration of doxycycline. (A) Western blot analysis of lenses from mice treated with doxycycline, probed with the anti-O-GlcNAc antibody RL2. (B) The corresponding densitometry relative to β-actin. (C) Proteasome activity in the lenses of doxycycline-treated bi-transgenic mice was assayed using the fluorogenic peptide substrate, Suc-LLVY-AMC. Spectroscopic data are normalized at 100% for the control mice and the Student's *t*-test was used for the analysis of *P* values. (D) RL2 immunoprecipitation was performed on lens extracts and blotted with anti-Rpt2 antibody. (E) Bar graph showing densitometry of the immunoblots where data are presented as a ratio of intensities of O-GlcNAcylated Rpt2/input Rpt2. All densitometry in this figure was determined using Image J.

tential mechanism is that the integration site of the gamma-F-crystallin-rtTA transgenic construct into the murine genome may alter the expression of a gene that may lead to cataract formation. According to our data, among the three founder lines generated of the gamma-F-crystallin-rtTA mice, two lines showing high rtTA expression levels developed small cataracts, suggesting that cataract formation is not caused by disruption at a specific gene locus.

Cataract formation in transgenic mice was also examined by slit-lamp. Mouse eye pupils were dilated with atropine and lenses were inspected by slit-lamp. Nuclear cataracts were detected and the cataracts in bi-transgenic mice after doxycycline administration (Fig. 2D) were much more prominent than those untreated (Fig. 2C). Quantization of cataract infiltration revealed an average 3-fold increase in cataract surface area in the lenses of doxycycline-treated mice verses untreated ones (Fig. 2E). The above results indicate that lens-specific over-expression of a dominant-negative form of NCOAT, GK-NCOAT, gives rise to increased cataract formation.

Based on our previous studies on over-expression of GK-NCOAT [20,21], we expected to see an increase in protein-O-GlcNAc levels in lens tissues. Indeed, we showed by Western blot analysis utilizing RL2 antibody [23], an increase in O-GlcNAc-modified proteins in the lenses of the bi-transgenic mice that had been treated with doxycycline (Fig. 3A). Densitometric analysis of immunoblots from 3 separate experiments indicated a 1.45-fold increase in O-GlcNAc levels in the lenses of the DOX-treated bi-transgenic mice (Fig. 3B).

To examine whether the proteasome activity was affected in the lenses of bi-transgenic mice with increased O-GlcNAc levels, the proteasome chymotrypsin-like peptidase activity was measured using the fluorogenic peptide substrate, suc-LLVY-AMC [12]. The data showed that proteasome activity was reduced 40% in the lenses of DOX-treated bi-transgenic mice compared to control mice (Fig. 3C). These results, taken together with the previously reported correlative data of proteasome dysfunction and cataract formation [4,5], suggest a mechanism explaining the advanced cataract induction seen in these transgenic mice may occur via the reduction of proteasome function due to high O-GlcNAc levels. Data providing the link between increased O-GlcNAc levels and loss of proteasome activity originate from our previous studies where we showed that O-GlcNAc modification of the Rpt2 subunit of the proteasome 19S cap can inhibit proteasome function [12]. To confirm that the increased O-GlcNAc levels translate to increased O-GlcNAc modification of Rpt2 in the lenses of the bi-transgenic mice, RL2 immunoprecipitation was performed and blotted with Rpt2 antibody. The Rpt2 subunit of the proteasome showed a 1.6-fold increase in O-GlcNAcylation in the lenses of the DOX-treated bi-transgenic mice (Fig. 3D and E).

We observed inhibition of lens fiber cell denucleation in DOX-treated bi-transgenic mice. The entire lens is made of two cell types, lens epithelial cells, and lens fiber cells. The lens epithelial cells normally form a monolayer covering the anterior surface of the lens, whereas the transparent lens fiber cells occupy the bulk of the lens. Lens epithelial cells are induced to differentiate into lens fiber cells at the transitional zone, after which point the transitioning cells lose their nuclei and other intracellular organelles to form mature fiber cells at the lens core. Denucleation is an essential process for lens maturation, providing a scatter-free light path. Previous studies haves shown that this carefully regulated maturation of lens fiber cells may be disrupted by events such as oxidative damage or proteasome dysfunction, causing a thickening of the anterior epithelial cell layer and a delay in denucleation [24]. The lenses of 2-week-old mice were examined for cellular differentiation. As shown in the histological sections of the DOX-treated bi-transgenic mice, fiber cell nuclei can be found extending into the central area of the lens (Fig. 4B and D), compared to the control mice (Fig. 4A and C). Interestingly, the lenses of the doxycycline-

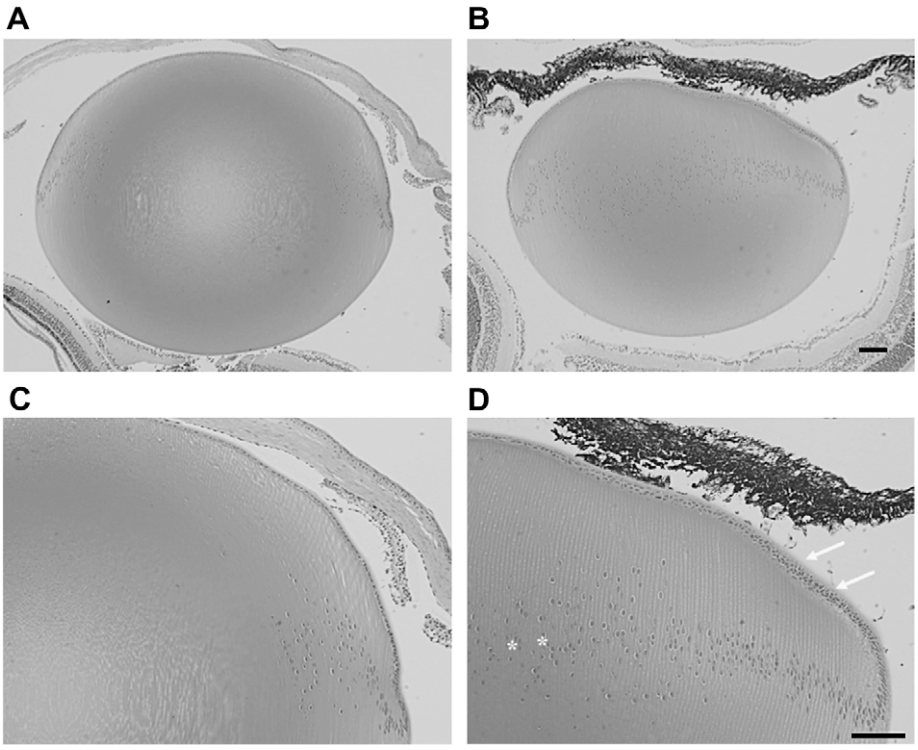

Fig. 4. Inhibition of lens fiber cell denucleation in bi-transgenic mice with administration of doxycycline. Eyes of bi-transgenic mice without administration of doxycycline (A,C) and bi-transgenic mice with administration of doxycycline (B,D) were removed at 2 weeks of age, sectioned and stained with Hematoxylin and Eosin. Higher magnification images of (A) and (B) are shown in (C) and (D), respectively. The lens fiber cell denucleation inhibition is identified by the asterisks and multiple epithelial cell layers are indicated by the arrows (D). The scale bars represent 100 μm.

treated bi-transgenic mice were smaller than the control untreated bi-transgenic mice. The observed delay in denucleation indicated that lens fiber cell differentiation and maturation were disrupted in mice expressing GK-NCOAT, presumably by increasing the O-GlcNAc modification levels in the lenses. We also observed that lens anterior epithelial cells formed multiple layers (Fig. 4D) and we speculate that the multiple layering, or hyperplasia, of lens epithelial cells might be responsible for the disruption and/or attenuation of lens fiber cell differentiation. Although somewhat variable, this phenomenon was detected in the majority of doxycycline-treated bi-transgenic mice (data not shown). However, none of the control mice showed this abnormal hyperplasia of the epithelia. It has been reported that the subunits of the 19S regulatory cap and the 20S core particle of the proteasome were present in both cytoplasm and nuclei of lens epithelial cells, but were restricted to the nuclei of differentiating fiber cells [25]. Consequently, it has been proposed that proteasome may play a role in denucleation during lens fiber cell differentiation. Our *in vivo* data would indicate that decreased proteasome activity (Fig. 3C) could retard denucleation of lens fiber cells in mice with elevated O-GlcNAc modification levels. Furthermore, we have shown that the proteasome ATPase subunit, Rpt2, has increased O-GlcNAc modification in the transgenic lenses, linking loss of proteasome function with elevated O-GlcNAc levels.

Our results provide a novel link between the increased O-GlcNAc modification level and cataract formation. This work might explain why those patients with diabetes have a predisposition for developing cataracts earlier than non-diabetic subjects because the substrate for O-GlcNAc modification, UDP-O-GlcNAc, derives from glucose. It is widely accepted that cataracts are caused by protein aggregation that may be due to the diminished ability to degrade damaged proteins. We have previously shown that O-GlcNAc modification is an endogenous inhibitor of the proteasome [12], by inhibiting the ATPase activity of the proteasome 19S cap and thus the peptidase function of the proteasome. In this report, we were able to demonstrate consistency with this finding by showing that increased O-GlcNAc modification levels of the lens proteins of transgenic mice also have decreased proteasome activity. This attenuation of activity leads to the development of more prominent cataracts compared to controls. Our data makes a strong case that the O-GlcNAc modification is involved in cataract formation through proteasome inhibition.

The age-related accumulation of damaged and aggregated proteins will form inclusions in the lens, obstructing the light passage through the lens and causing cataracts. This characteristic of inclusion bodies is shared in many degenerative diseases including neuro-degeneration found in Alzheimer's and Parkinson's disease [26]. Thus, the pathogenesis of these inclusions could have implications beyond cataract formation. In this report, we conclude that the increased O-GlcNAc modification may lead to cataract formation through the inhibition of proteasome function. This result may explain why cataract is a common complication in diabetes. Moreover, this study implies that O-GlcNAc modification might be involved in the pathogenesis of diseases associated with the accumulation of damaged and aggregated proteins. We speculate that the pathways regulating O-GlcNAc modification levels may represent novel therapeutic targets in the prevention and treatment of these diseases.

Acknowledgments

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We would like to dedicate this manuscript to Dr. Jeffrey E. Kudlow as a small example of his philosophy, insight, and mentorship spanning 3 decades.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.06.132.

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