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Matrix-bound AGEs enhance TGF β 2-mediated mesenchymal transition of lens epithelial cells via the noncanonical pathway: implications for secondary cataract formation

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Abstract

Advanced glycation end products (AGEs) are post-translational modifications formed from the reaction of reactive carbonyl compounds with amino groups in proteins. Our laboratory has previously shown that AGEs in extracellular matrix (ECM) proteins promote TGF β 2 (transforming growth factor-beta 2)-mediated epithelial-to-mesenchymal transition (EMT) of lens epithelial cells (LECs), which could play a role in fibrosis associated with posterior capsule opacification. We have also shown that α B-crystallin plays an important role in TGF β 2-mediated EMT of LECs. Here, we investigated the signaling mechanisms by which ECM-AGEs enhance TGF β 2-mediated EMT in LECs. We found that in LECs cultured on AGE-modified basement protein extract (AGE-BME), TGF β 2 treatment up-regulated the mesenchymal markers α -SMA (α -smooth muscle actin) and α B-crystallin and down-regulated the epithelial marker E-cadherin more than LECs cultured on unmodified BME and treated with TGF β 2. Using a Multiplex Assay, we found that AGE-BME significantly up-regulated the noncanonical pathway by promoting phosphorylation of ERK (extracellular signal-regulated kinases), AKT, and p38 MAPK (mitogen-activated protein kinases) during TGF β 2-mediated EMT. This EMT response was strongly suppressed by inhibition of AKT and p38 MAPK phosphorylation. The AKT inhibitor LY294002 also suppressed TGF β 2-induced up-regulation of nuclear Snail and reduced phosphorylation of GSK3 β . Inhibition of Snail expression suppressed TGF β 2-mediated α -SMA expression. α B-Crystallin was up-regulated in an AKT-dependent manner during AGE-BME/TGF β 2-mediated EMT in LECs. The absence of α B-crystallin in LECs suppressed TGF β 2-induced GSK3 β phosphorylation, resulting in lower Snail levels. Taken together, these results show that ECM-AGEs enhance the TGF β 2-mediated EMT response through activation of the AKT/Snail pathway, in which α B-crystallin plays an important role as a linker between the TGF β 2 and AGE-mediated signaling pathways.

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Author Contribution

R.H.N. and M.-H.N. conceived, designed, and analyzed all experiments. M.-H.N. performed all experiments. The authors reviewed the results and approved the final version of the manuscript.

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

Introduction

According to the National Eye Institute, the prevalence of cataracts is ~20 million (17.2%) among adults 40 years and older in the U.S.A., and this number is expected to increase to nearly 40 million by 2030 [1]. By the age of 80 years, ~70% of Americans have cataracts [1]. Many epidemiological studies have shown that diabetes is a defined risk factor for developing cataracts [2–4]. The increasing number of diabetic patients worldwide has coincided with the increasing incidence of cataracts, and diabetics develop cataracts at an earlier age than nondiabetics [5]. The lens is enclosed by a basement membrane known as a capsule, and a single layer of epithelial cells [lens epithelial cells (LECs)] is attached to the capsule on the anterior side. The remaining lens is composed of differentiated fiber cells, which are organelle-free at the center (nucleus) of the lens.

During cataract surgery, a portion of the anterior capsule is excised (capsulorhexis) to remove the cloudy lens (via a process known as phacoemulsification), and the remaining capsule is left intact to allow implantation of an intraocular lens (IOL). During this procedure, most capsule-adhering anterior epithelial cells peripheral to capsulorhexis are removed, but some cells remain attached despite the best efforts of surgeons to remove them. These residual LECs initiate a wound-healing response, which is characterized by cell proliferation and migration, with an attempt to differentiate into fiber cells [6]. Such differentiation is usually incomplete, resulting in Elschnig's pearl formation at the lens equator [7]. After cells reach the posterior capsule, they undergo epithelial–mesenchymal transition (EMT) and synthesize α -smooth muscle actin (α -SMA), resulting in contraction and wrinkle formation in the capsule. This EMT response eventually results in fibrosis, blocking the visual axis in a process known as posterior capsule opacification (PCO) or secondary cataract formation. PCO is a common post-cataract surgery complication and occurs 2–5 years after cataract surgery in ~20–40% of patients [8]. To remedy this problem, a Neodymium : yttriumaluminum-garnet (Nd : YAG) laser is routinely used, which obliterates fibrotic tissue and clears the visual axis [9]. Although this procedure is safe in the majority of cases, in some cases, it leads to complications, such as retinal tears, hemorrhages, and endophthalmitis [10,11]. Thus, efforts are underway to prevent PCO by improving the design of IOLs to prevent LEC migration to the posterior capsule and by developing inhibitors of EMT.

Several recent studies have investigated the mechanisms of PCO and have implicated cytokines and growth factors, such as transforming growth factor (TGF), fibroblast growth factor, hepatocyte growth factor, interleukins, and epithelial growth factor [12–15]. However, the major driver appears to be TGF β [16]. Three TGF β isoforms have been identified in mammals: TGF β 1, TGF β 2, and TGF β 3. In the eye, TGF β 2 is the dominant form and is mainly produced in ciliary and lens epithelia [17]. Many studies have shown that TGF β 2-mediated EMT involves a morphological change of epithelial cells from a cuboidal to an elongated spindle shape, with a decrease in expression of epithelial markers, such as E-cadherin and zonula occludin-1, and enhanced expression of mesenchymal markers, such as α -SMA and fibronectin [18].

There are two main modes of TGF β -signaling pathways. Canonical TGF β signaling is mediated by Smads [19]. In this pathway, TGF β -induced activation of the receptor leads to activation of Smad2 and Smad3 through direct C-terminal phosphorylation by Type I serine/threonine kinase receptors (T β RI) [20]. Phosphorylated Smad2 and Smad3 then form a heterotrimer with Smad4 and translocate into the nucleus, where they associate and co-operate with other DNA-binding transcription factors to activate or repress target gene transcription [19]. Noncanonical signaling is directly activated by T β RI either through phosphorylation or direct interaction with various MAP kinase pathways, such as ERK (extracellular signal-regulated kinases), JNK, and p38 MAPK (mitogen-activated protein kinases), Rho-like GTPase, and phosphatidylinositol-3-kinase/AKT [21–24]. The contribution of two pathways to EMT in other tissues also has been observed [25,26], but it is unclear for the lens.

α B-Crystallin is a major protein in the lens; it is also present in significant amounts in the heart, kidneys, and skeletal muscles [27–29]. Several previous studies have shown that α B-crystallin plays a promotional role in EMT of epithelial cells in other tissues [30–32]. We recently showed that in LECs, α B-crystallin is obligatory for TGF β 2-mediated EMT [31]. In that study, we found that α B-crystallin was essential for phosphorylation of AKT, Smad2/3, and their translocation to the nucleus. Similarly, translocation of Snail to the nucleus was also dependent on α B-crystallin. In addition, α B-crystallin bound to both p-Smad2/3 and Snail during their trans-location to the nucleus. Furthermore, the chaperone activity of α B-crystallin was found to be required for EMT promotional activity. However, whether extracellular matrix-bound advanced glycation end products (AGEs) have any effect on the α B-crystallin levels was not investigated in that study.

AGEs are post-translational modifications in proteins formed from the reaction of reactive carbonyl compounds with protein amino groups, mostly on lysine and arginine residues. AGEs have been implicated in both age- and diabetes-related eye diseases [33]. Our laboratory has previously shown that AGEs generated in basement membrane extract (BME) or lens capsules further promote TGF β 2-mediated EMT of LECs [1]. Our studies also showed that AGEs in the capsule engage RAGE, a receptor for AGEs, in LECs during the promotion of EMT [34]. However, in the latter study, after a 24 h treatment with TGF β 2, there was no change in either Smad or ERK phosphorylation during AGE-promoted EMT. This raised the possibility that AGEs may up-regulate TGF β 2 signaling pathways at an earlier time period (earlier than 24 h). The aim of this study was to investigate the mechanisms by which extracellular AGEs alter TGF β 2 signaling during EMT and to determine whether α B-crystallin has any role in these mechanisms.

Experimental

Cell culture

Human LEC-line FHL124 cells were kindly provided by Dr Michael Wormstone (School of Biological Sciences, University of East Anglia, U.K., originally from Dr John Reddan, Oakland University, MI). Cell authentication was performed using an Affymetrix gene microarray, which found 99.5% homology between FHL124 cells and native human lens epithelium in the expression pattern of 22 270 genes [35], and by analysis of short tandem

repeats [34]. FHL124 cells up to passage 20 were used in this study. FHL124 cells were cultured in MEM (Life Technologies, Carlsbad, CA) containing 5% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco, Grand Island, NY) and gentamycin/L-glutamate (1 : 100, Sigma–Aldrich, St. Louis, MO) and maintained in a humidified incubator at 37°C containing 5% CO₂.

Mouse LECs from wild-type (WT, 129Sv strain from Jackson Laboratories) or α B-crystallin knockout (KO, 129Sv strain, originally from Dr Eric Wawrousek, NEI, re-derived) mice were isolated and cultured in MEM containing 20% (v/v) heat-inactivated FBS as recently described [31]. FHL124 cells and mouse LECs were both serum-starved overnight and stimulated with TGF β 2 (Peprotech, Inc., Rocky Hill, NJ, 10 ng/ml) for the indicated times. Specific inhibitors were treated for 1 h prior to being co-treated with 10 ng/ml TGF β 2 for stimulation.

AGE modification of BME

AGE modification of BME (Cultrex® BME, Trevigen, Gaithersburg, MD) was performed as recently described [34]. Briefly, after coating in 60-mm culture dishes (Falcon, Cat#353002) with BME (50 μ g/ml), the plate was incubated at 37°C with a sterile-filtered (0.2 μ m filter) glycation mixture that consisted of 2 mM ascorbate, 25 mM D-glucose, and 250 μ M methylglyoxal (all from Sigma) in 50 mM sodium phosphate buffer for 1 week at pH 7.4. After rinsing the plate with PBS, FHL124 cells were seeded on AGE-modified (AGE-BME) or unmodified BME (BME) with MEM containing 5% FBS for 24 h.

Quantitative real-time PCR

RNA was extracted from cells using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). The quantities of isolated RNA were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Rockford, IL). RNA was reverse-transcribed to synthesize cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative PCR for analyzing mRNA gene expression was performed with iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Richmond, CA, U.S.A.) using an iCycler iQ5 Real-Time PCR Detection System (Bio-Rad). The primers used are listed in Table 1.

Western blotting

Total cell lysates were prepared using the Mammalian Protein Extraction Reagent (Thermo scientific) containing a protease and phosphatase inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, 1 : 100 dilution). Cells were separated into cytosolic and nuclear fractions using a NucBuster™ protein extraction kit (Novagen, Madison, WI) according to the manufacturer's protocol. Proteins were separated on SDS–PAGE, transferred to a nitrocellulose membrane, and blocked using 5% nonfat dry milk in TBST buffer (10 mM Tris, 150 mM NaCl, and 0.1% Tween 20, pH 7.5) for 1 h. The membranes were incubated at 4°C overnight with a primary antibody against the following proteins: α -SMA (Sigma–Aldrich, Cat#A5228), α B-crystallin (Developmental Studies Hybridoma Bank, University of Iowa, IA), E-cadherin (Cat#3195), β -actin (Cat#4970), Snail (Cat#3879), Slug (Cat#9585), zinc-finger E-box-binding (ZEB) (Cat#3396), histone H3 (Cat#9715), GSK3 β (Cat#12456), p-GSK3 β (Cell Signaling Technology, Inc., Beverly, MA) (Cat#5558), and Twist (Santa

Cruz Biotechnology, Santa Cruz, CA) (Cat#sc-81471). HRP-conjugated secondary antibodies (Cell Signaling Technology) were incubated at RT for 1 h and detected with the SuperSignal West Pico or Femto Kit (Pierce Chemicals, Rockford, IL). The band intensities were measured using Image J software (National Institutes of Health), and the results are expressed as fold change over controls.

Bead-based multiplex assays

The TGF β signaling pathway was assessed using intracellular bead-based multiplex assay kits from the EMD Millipore Corporation (Billerica, MA) according to the manufacturer's instructions. After the treatment of TGF β 2 for 1 h, cells were lysed in the provided lysis buffer and particulate matter was removed by filtration. The protein concentration in the filtered samples was measured using the BCA assay. Cell lysates (equal to 10 μ g protein) were added to the wells of a 96-well plate containing conjugated beads and incubated on a plate shaker (800 rpm) in the dark at 4°C overnight. After washing, the respective detection antibody was added to each well and the plate was incubated for 1 h at RT. The detection antibody was removed by decantation, streptavidin-R-phycoerythrin was incubated for 15 min and amplification buffer was added to each well. The beads were analyzed using the Luminex Magpix system (Luminex Corp., Austin, TX). The beads detected changes in phosphorylated Smad2 (Ser465/Ser467), Smad3 (Ser423/Ser425), ERK (Thr185/Tyr187), and AKT (Ser473), as well as the total protein levels of TGF β RII and Smad4 in cell lysates.

Transfection of siRNA for Snail

FHL124 cells were transfected with nonsilencing or Snail-specific siRNA (Qiagen) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol. On the day before transfection, cells were seeded in a six-well plate at a density of 5×10^4 cells/well. The next day, cells were transfected for 4 h with 20 nM siRNA and Opti-MEM including 7.5 μ l of Lipofectamine 2000, and the media were replaced with fresh serum-free media. After 24 h, the cells were used in experiments. The target sequences of the siRNAs were siSnail, GAGGTGTGACTAACTATGCAA, and nonsilencing siRNA, AATTCTCCGAACGTGTCACGT, as a negative control.

Statistical analysis

GraphPad Prism software version 7 (GraphPad Prism Software, Inc., San Diego, CA) was used for all statistical analyzes. Data are expressed as the means $\pm$ standard deviation (SD) of three independent experiments. We used Tukey's multiple comparison test for significant differences among treatment groups. A *P*-value of <0.05 was considered statistically significant.

Results

AGE-BME enhances the TGF β 2-induced EMT

To determine whether AGE-BME enhances TGF β 2-mediated EMT of FHL124 cells, cells were treated with 10 ng/ml TGF β 2. The α -SMA, α B-crystallin, and E-cadherin levels were compared at 24 and 48 h using qRT-PCR and Western blotting. We found that AGE-BME promoted TGF β 2-mediated up-regulation of α -SMA and α B-crystallin (Figure 1A,B).

TGFβ2 decreased the E-cadherin mRNA and protein levels by 47 and 60% compared with untreated BME control cells, respectively. The combination of AGE-BME with TGFβ2 decreased the E-cadherin mRNA and protein levels by 63 and 78% compared with the AGE-BME control, respectively. The levels of α-SMA and αB-crystallin were higher in AGE-BME than in BME, but they were not statistically significant.

AGE-BME promotes the noncanonical signaling pathway in FHL124 cells

Stimulation of Smad signaling by TGFβ is well known [20]. In response to TGFβ, Smads translocate from the cytoplasm to the nucleus and regulate target gene transcription [36]. Our data show that TGFβ2 treatment rapidly elevated the levels of the canonical pathway molecules p-Smad2 and p-Smad3, but the levels of TGFβRII and Smad4 were unchanged after 1 h (Figure 2A). However, there was no significant difference in Smad signaling between BME and AGE-BME in response to TGFβ2. In the noncanonical signaling pathway of TGFβ, the role of ERK, p38 MAPK, and PI3K (phosphoinositide 3-kinase)/AKT has been well established [22]. The addition of TGFβ2 to cells cultured on AGE-BME for 1 h increased the noncanonical signaling pathway, as indicated by an increase in the levels of p-ERK and p-AKT (Figure 2B). In the case of AKT, the increase was significantly greater than for cells cultured on BME alone. Since we used a commercial Multiplex Assay Kit that did not include p-p38 MAPK, we measured p-p38 MAPK by Western blotting in the same cell lysate used for the Multiplex Assay. The addition of TGFβ2 to cells cultured on AGE-BME for 1 h enhanced the p-p38 MAPK levels (Figure 2C).

Inhibitors of the TGFβ2-mediated noncanonical pathway suppress the EMT response

We evaluated the contribution of AGE-BME to the noncanonical TGFβ signaling pathway using specific inhibitors. FHL124 cells were treated with inhibitors of ERK1/2 (U0126, 10 μM), p38 MAPK (SB202190, 10 μM), or AKT (LY294002, 10 μM) for 1 h followed by treatment with TGFβ2 for 48 h (in the presence of inhibitor). Expression of α-SMA, αB-crystallin, and E-cadherin was determined by Western blot analysis. The inhibitors for AKT and p38 MAPK phosphorylation suppressed the expression of α-SMA and αB-crystallin in cells on AGE-BME treated with TGFβ2 (Figure 3A,B), but not from TGFβ2 alone. These results suggest that TGFβ2 induces the EMT mainly through the canonical signaling pathway, whereas AGE-BME through the noncanonical pathway. In addition, the inhibitors of ERK and AKT phosphorylation blocked the TGFβ2-mediated decrease in E-cadherin levels in cells on AGE-BME (Figure 3C).

AGE-BME promotes TGFβ2-mediated up-regulation of nuclear Snail and Slug

The levels of the EMT-inducing transcription factors Snail, Slug, Twist, and ZEB were measured. Snail and Slug were both significantly higher in the nuclear proteins of TGFβ2-treated cells on AGE-BME compared with TGFβ2-treated cells on BME (Figure 4A). However, the Twist and ZEB levels were unchanged. These results suggest that AGE-BME promotes TGFβ2-mediated EMT through the activation of Snail and Slug. To define which noncanonical TGFβ signaling pathway is associated with these transcription factors, specific inhibitors were pretreated for 1 h and cells were induced with TGFβ2 for 6 h. Blocking of the AKT pathway with LY294002 strongly suppressed the nuclear levels of Snail and Slug (Figure 4B).

Glycogen synthase kinase 3 β (GSK3 β) has been reported to enhance degradation of Snail via ubiquitination and is inactivated by phosphorylation [37]. As shown in Figure 4C, TGF β 2 increased GSK3 β phosphorylation in FHL124 cells after 1 h. The addition of TGF β 2 to cells cultured on AGE-BME for 1 h further enhanced GSK3 β phosphorylation. The addition of a specific inhibitor of AKT decreased this GSK3 β phosphorylation.

Snail is upstream of α -SMA but not α B-crystallin

To determine whether Snail is upstream of either α -SMA or α B-crystallin, cells were transfected using Snail-specific siRNA. We determined the efficiency of Snail siRNA using qRT-PCR. Snail mRNA expression was decreased by 60% (Figure 5), and both the mRNA and protein levels of α -SMA also strongly decreased. However, the level of α B-crystallin did not change. These results indicate that Snail is upstream of α -SMA, but not α B-crystallin. Interestingly, when the Snail levels were reduced, the mRNA levels, but not the protein levels, of α B-crystallin increased relative to scrambled siRNA treated LECs. The reason for this is not known.

α B-crystallin promotes GSK3 β phosphorylation and up-regulates Snail

We determined the role of α B-crystallin in Snail nuclear translocation using WT and α B-crystallin KO mouse LECs. Phosphorylation of GSK3 β was higher after treatment with TGF β 2 for 2 h in WT, resulting in higher levels of Snail. However, a similar TGF β 2 treatment in α B-crystallin KO mouse LECs did not change the p-GSK3 β levels. The Snail levels in KO mouse LECs were significantly lower than WT LECs after TGF β 2 treatment (Figure 6B,C). Together, these data indicated that α B-crystallin regulates Snail translocation via GSK3 β phosphorylation.

Discussion

The capsule secreted by the lens epithelium is the thickest basement membrane (BM) in the human body [38]. The proteins in the BM are generally long-lived and are targets for post-translational modification during aging. Our recent study showed that with aging, AGEs accumulate in the human lens capsule [1]. That study also showed that TGF β 2-mediated EMT signaling positively correlated with the AGE levels in the human lens capsule and suggested that AGEs promote TGF β 2-mediated EMT in LECs [1]. The AGE levels in AGE-BME are comparable to those in aged human lens capsules, as shown in our recent study [1]. In a subsequent study, we showed that AGEs in the capsule engage a receptor for AGEs, known as RAGE, in LECs during EMT [34]. Even though FHL124 cells are derived from a fetal lens, our recent study showed that those cells are similar to primary human LECs in their response to AGE-BME and TGF β 2 [34]. We have also recently shown that α B-crystallin is essential for TGF β 2-mediated EMT in LECs [31]. However, it is unknown whether AGE-BME promotes TGF β 2-mediated EMT in LECs through up-regulation of α B-crystallin. The purpose of this study was to investigate the mechanisms by which AGE-BME promotes TGF β 2 signaling during EMT of LECs and to determine whether α B-crystallin has a role in these mechanisms. The major findings are: (1) in early TGF β 2-mediated signaling, AGE-BME activates the noncanonical pathway; (2) AGE-BME up-regulates α B-

crystallin through activation of the AKT pathway; and (3) AGE-BME promotes TGF β 2-mediated accumulation of Snail in the nucleus through inactivation of GSK3 β .

Previous studies have shown that phosphorylation of Smad2 occurs to a maximum extent after ~2 h of TGF β 2 treatment in cells [39–41]. However, in our recent study, we found that the p-Smad2 levels remained at significantly higher levels at 24 h in cells grown on AGE-BME compared with cells grown on unmodified BME [34]. In the present study, after 1 h of TGF β 2 treatment, there was no difference in the p-Smad2/3 levels between the two groups. This indicated that the difference in the p-Smad2 levels between the two groups occurred between 1 and 24 h of TGF β 2 treatment. A similar difference was also seen for ERK phosphorylation. While a recent study showed no difference between the two groups in the p-ERK1/2 levels after 24 h of TGF β 2 treatment [1], the present study showed a significant difference after 1 h of treatment. These observations led us to investigate the mechanisms for an early increase in p-ERK1/2 and sustained up-regulation of p-Smad2 by AGEs in conjunction with TGF β 2.

We found that in the presence of AGEs, TGF β 2 activated noncanonical signaling after 1 h, while the p-Smad2 levels remained indistinguishable between TGF β 2-treated cells on BME or AGE-BME. This suggested that noncanonical signaling by TGF β 2 is triggered early by AGEs. Thus, AGE-BME exacerbated TGF β 2-mediated EMT of LECs by enhancing signaling via the noncanonical pathway.

In addition, cross-talk between the canonical and the noncanonical TGF β 2 signaling pathways can occur during TGF β 2-mediated EMT of LECs in the presence of AGEs. In fact, several studies have shown that TGF β -induced Smad signaling and ERK pathway cross-talk during EMT in other cells [26,42,43]. For example, Bakin et al. [25] showed that PI3K/AKT activation is directly involved in TGF β 1-mediated Smad2 phosphorylation during EMT of tumor cells. However, the mechanism by which TGF β activates the PI3/AKT signaling pathway is unclear. We believe, based on our results, that α B-crystallin has a role in TGF β -mediated PI3/AKT activation.

The role of α B-crystallin in EMT is becoming clearer with recent studies. Ishikawa et al. [30] reported that α B-crystallin is essential to the formation of subretinal fibrosis after EMT in retinal pigment epithelium cells. Other studies have established that α B-crystallin favors TGF β 1 signaling via Smad4 activation during fibrosis [44,45]. In our recent study, we showed that the absence of α B-crystallin results in inhibition of EMT of LECs by TGF β 2 and in lensectomized mouse lens capsule-adherent LECs [31]. In that study, we demonstrated that the chaperone activity of α B-crystallin is required and that it physically interacts with Smads and Snail during TGF β 2-mediated EMT of LECs. The lack of α B-crystallin reduced activation of ERK, p38 MAPK, and AKT signaling. In this study, we demonstrated that AGE-BME promotes TGF β 2-mediated up-regulation of α B-crystallin and that inhibition of AKT phosphorylation reduces this up-regulation.

Several transcription factors regulate EMT, including zinc-finger proteins of the Snail/Slug family, Twist, and ZEB [18]. It is established that TGF β 2-induced Smad2/3 transcription factors complex with the promoter of transcriptional regulators, such as Snail and Slug

[46,47]. We have recently shown that α B-crystallin up-regulates p-Smad2/3, Smad4, and Snail expression after TGF β 2 treatment both in the cytoplasm and in the nucleus and that α B-crystallin physically interacts with Smads and Snail [31]. The present data demonstrate that AGE-BME further up-regulates TGF β 2-induced α B-crystallin, resulting in the promotion of Snail accumulation in the nucleus. As Snail expression was stronger than Slug expression, we suspect that Snail is the primary driver of EMT by AGE-BME/TGF β 2. Inactivation of GSK3 β through phosphorylation is a well-established phenomenon during TGF β -mediated EMT [37], and inactivation of GSK3 β is directly linked to increased Snail expression and translocation to the nucleus [48,49]. Our results show that AGE-BME phosphorylates Ser9 of GSK3 β via AKT activation and results in Snail accumulation in the nucleus, supporting the idea that AKT-mediated GSK3 β phosphorylation may trigger Snail accumulation in the nucleus. Phosphorylation of GSK3 β in response to TGF β 2 was inhibited in α B-crystallin KO mouse LECs, which suggests its role in GSK3 β phosphorylation. Analogous to our results, Zhou et al. [50] found that α B-crystallin protects against retinal degeneration, in part through AKT phosphorylation and GSK3 β phosphorylation in RPE cells. Taken together, both canonical and noncanonical pathways up-regulate α B-crystallin, which is essential for the enhancement of EMT of LECs by AGE-BME.

In conclusion, our study showed that AGE-BME enhances TGF β 2-induced EMT of LECs by enhancing activation of the AKT/Snail pathway, in which α B-crystallin plays an important role as a linker between the TGF β 2- and AGE-mediated signaling pathways (Figure 7). As α B-crystallin is an important driver of EMT in LECs, we propose that the AKT/GSK3 β pathway and α B-crystallin are additional targets for the prevention of PCO.

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Abbreviations

AGEs	advanced glycation end products
BM	basement membrane
BME	basement membrane extract
ECM	extracellular matrix
EMT	epithelial-to-mesenchymal transition
ERK	extracellular signal-regulated kinases
FBS	fetal bovine serum

GSK3β	glycogen synthase kinase 3 β
IOL	intraocular lens; KO, knockout
LECs	lens epithelial cells
MAPK	mitogen-activated protein kinases
PCO	posterior capsule opacification
PI3K	phosphoinositide 3-kinase
RAGE	a receptor for AGEs
TGFβ	transforming growth factor-beta
TβRI	Type I serine/threonine kinase receptors
WT	wild type
ZEB	zinc-finger E-box-binding
α-SMA	α -smooth muscle actin

References

1. Raghavan CT, Smuda M, Smith AJ, Howell S, Smith DG, Singh A, Gupta P, Glomb MA, Wormstone IM and Nagaraj RH (2016) AGEs in human lens capsule promote the TGF β 2-mediated EMT of lens epithelial cells: implications for age-associated fibrosis. *Aging Cell* 15, 465–476 10.1111/ace.12450 [PubMed: 26853893]
2. Klein BE, Klein R and Moss SE (1985) Prevalence of cataracts in a population-based study of persons with diabetes mellitus. *Ophthalmology* 92, 1191–1196 10.1016/S0161-6420(85)33877-0 [PubMed: 4058882]
3. Klein BE, Klein R and Moss SE (1995) Incidence of cataract surgery in the Wisconsin Epidemiologic Study of diabetic retinopathy. *Am. J. Ophthalmol* 119, 295–300 10.1016/S0002-9394(14)71170-5 [PubMed: 7872389]
4. Javadi MA and Zarei-Ghanavati S (2008) Cataracts in diabetic patients: a review article. *J. Ophthalmic. Vis. Res* 3, 52–65 [PubMed: 23479523]
5. Harding JJ, Egerton M, van Heyningen R and Harding RS (1993) Diabetes, glaucoma, sex, and cataract: analysis of combined data from two case control studies. *Br. J. Ophthalmol* 77, 2–6 10.1136/bjo.77.1.2 [PubMed: 8435392]
6. de Iongh RU, Wederell E, Lovicu FJ and McAvoy JW (2005) Transforming growth factor- β -induced epithelial-mesenchymal transition in the lens: a model for cataract formation. *Cells Tissues Organs* 179, 43–55 10.1159/000084508 [PubMed: 15942192]
7. Neumayer T, Findl O, Buehl W, Sacu S, Menapace R and Georgopoulos M (2005) Long-term changes in the morphology of posterior capsule opacification. *J. Cataract Refract. Surg* 31, 2120–2128 10.1016/j.jcrs.2005.04.038 [PubMed: 16412925]
8. Awasthi N, Guo SQ and Wagner BJ (2009) Posterior capsular opacification a problem reduced but not yet eradicated. *Arch. Ophthalmol* 127, 555–562 10.1001/archophthalmol.2009.3 [PubMed: 19365040]
9. Apple DJ, Solomon KD, Tetz MR, Assia EI, Holland EY, Legler UFC, Tsai JC, Castaneda VE, Hoggatt JP and Kostick AMP (1992) Posterior capsule opacification. *Surv. Ophthalmol* 37, 73–116 10.1016/0039-6257(92)90073-3 [PubMed: 1455302]

10. Aron-Rosa D, Griesemann JC and Aron JJ (1981) Use of a pulsed neodymium Yag laser (picosecond) to open the posterior lens capsule in traumatic cataract: a preliminary report. *Ophthalmic Surg.* 12, 496–499 [PubMed: 7266992]
11. Ranta P, Tommila P and Kivelä T (2004) Retinal breaks and detachment after neodymium: YAG laser posterior capsulotomy: five-year incidence in a prospective cohort. *J. Cataract Refract. Surg.* 30, 58–66 10.1016/S0886-3350(03)00558-3 [PubMed: 14967269]
12. Wallentin N, Wickstrom K and Lundberg C (1998) Effect of cataract surgery on aqueous TGF- β and lens epithelial cell proliferation. *Invest. Ophthalmol. Vis. Sci* 39, 1410–1418 [PubMed: 9660489]
13. Meacock WR, Spalton DJ and Stanford MR (2000) Role of cytokines in the pathogenesis of posterior capsule opacification. *Br. J. Ophthalmol* 84, 332–336 10.1136/bjo.84.3.332 [PubMed: 10684849]
14. Choi J, Park SY and Joo CK (2004) Hepatocyte growth factor induces proliferation of lens epithelial cells through activation of ERK1/2 and JNK/SAPK. *Invest. Ophthalm. Vis. Sci* 45, 2696–2704 10.1167/iovs.03-1371
15. Boswell BA, Korol A, West-Mays JA and Musil LS (2017) Dual function of TGF β in lens epithelial cell fate: implications for secondary cataract. *Mol. Biol. Cell* 28, 907–921 10.1091/mbc.E16-12-0865 [PubMed: 28209733]
16. Wallentin N, Wickstrom K and Lundberg C (1998) Effect of cataract surgery on aqueous TGF- β and lens epithelial cell proliferation. *Invest. Ophthalmol. Vis. Sci* 39, 1410–1418 [PubMed: 9660489]
17. Saika S (2006) TGF β pathobiology in the eye. *Lab. Invest* 86, 106–115 10.1038/labinvest.3700375 [PubMed: 16341020]
18. Lamouille S, Xu J and Derynck R (2014) Molecular mechanisms of epithelial-mesenchymal transition. *Nat. Rev. Mol. Cell Biol* 15, 178–196 10.1038/nrm3758 [PubMed: 24556840]
19. Derynck R and Zhang YE (2003) Smad-dependent and Smad-independent pathways in TGF- β family signalling. *Nature* 425, 577–584 10.1038/nature02006 [PubMed: 14534577]
20. Moustakas A, Souchelnytskyi S and Heldin CH (2001) Smad regulation in TGF- β signal transduction. *J. Cell Sci* 114, 4359–4369 [PubMed: 11792802]
21. Wojciechowski MC, Mahmutovic L, Shu DY and Lovicu FJ (2017) ERK1/2 signaling is required for the initiation but not progression of TGF β -induced lens epithelial to mesenchymal transition (EMT). *Exp. Eye Res* 159, 98–113 10.1016/j.exer.2017.03.012 [PubMed: 28365272]
22. Zhang YE (2009) Non-Smad pathways in TGF- β signaling. *Cell Res.* 19, 128–139 10.1038/cr.2008.328 [PubMed: 19114990]
23. Dawes LJ, Sleeman MA, Anderson IK, Reddan JR and Wormstone IM (2009) TGF β /Smad4-dependent and -independent regulation of human lens epithelial cells. *Invest. Ophthalmol. Vis. Sci* 50, 5318–5327 10.1167/iovs.08-3223 [PubMed: 19516008]
24. Korol A, Taiyab A and West-Mays JA (2016) RhoA/ROCK signaling regulates TGF β -induced epithelial-mesenchymal transition of lens epithelial cells through MRTF-A. *Mol. Med* 22, 713–723 10.2119/molmed.2016.00041 [PubMed: 27704140]
25. Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL and Arteaga CL (2000) Phosphatidylinositol 3-kinase function is required for transforming growth factor β -mediated epithelial to mesenchymal transition and cell migration. *J. Biol. Chem* 275, 36803–36810 10.1074/jbc.M005912200 [PubMed: 10969078]
26. Funaba M, Zimmerman CM and Mathews LS (2002) Modulation of Smad2-mediated signaling by extracellular signal-regulated kinase. *J. Biol. Chem* 277, 41361–41368 10.1074/jbc.M204597200 [PubMed: 12193595]
27. Atomi Y, Yamada S, Strohman R and Nonomura Y (1991) α B-crystallin in skeletal muscle: purification and localization. *J. Biochem* 110, 812–822 10.1093/oxfordjournals.jbchem.a123665 [PubMed: 1783614]
28. Mitra A, Basak T, Datta K, Naskar S, Sengupta S and Sarkar S (2013) Role of α -crystallin B as a regulatory switch in modulating cardiomyocyte apoptosis by mitochondria or endoplasmic reticulum during cardiac hypertrophy and myocardial infarction. *Cell Death Dis.* 4, e582 10.1038/cddis.2013.114 [PubMed: 23559016]

29. Holcakova J, Hernychova L, Bouchal P, Brozkova K, Zaloudik J, Valik D, Nenutil R and Vojtesek B (2008) Identification of α B-crystallin, a biomarker of renal cell carcinoma by SELDI-TOF MS. *Int. J. Biol. Markers* 23, 48–53 [PubMed: 18409151]
30. Ishikawa K, Sreekumar PG, Spee C, Nazari H, Zhu D, Kannan R and Hinton DR (2016) α B-Crystallin regulates subretinal fibrosis by modulation of epithelial-mesenchymal transition. *Am. J. Pathol* 186, 859–873 10.1016/j.ajpath.2015.11.014 [PubMed: 26878210]
31. Nahomi RB, Pantcheva MB and Nagaraj RH (2016) α B-crystallin is essential for the TGF- β 2-mediated epithelial to mesenchymal transition of lens epithelial cells. *Biochem. J* 473, 1455–1469 10.1042/BCJ20160128 [PubMed: 26987815]
32. Shi C, Yang X, Bu X, Hou N and Chen P (2017) α B-crystallin promotes the invasion and metastasis of colorectal cancer via epithelial-mesenchymal transition. *Biochem. Biophys. Res. Commun* 489, 369–374 10.1016/j.bbrc.2017.05.070 [PubMed: 28506831]
33. Peppas M and Vlassara H (2005) Advanced glycation end products and diabetic complications: a general overview. *Hormones* 4, 28–37 10.14310/horm.2002.11140 [PubMed: 16574629]
34. Raghavan CT and Nagaraj RH (2016) AGE-RAGE interaction in the TGF β 2-mediated epithelial to mesenchymal transition of human lens epithelial cells. *Glycocon. J* 33, 631–643 10.1007/s10719-016-9686-y
35. Wormstone IM, Tamiya S, Eldred JA, Lazaridis K, Chantry A, Reddan JR, Anderson I and Duncan G (2004) Characterisation of TGF- β 2 signalling and function in a human lens cell line. *Exp. Eye Res* 78, 705–714 10.1016/j.exer.2003.08.006 [PubMed: 15106950]
36. Schmieder B and Hill CS (2007) TGF β -SMAD signal transduction: molecular specificity and functional flexibility. *Nat. Rev. Mol. Cell Biol* 8, 970–982 10.1038/nrm2297 [PubMed: 18000526]
37. Wang H, Wang HS, Zhou BH, Li CL, Zhang F, Wang XF, Zhang G, Bu XZ, Cai SH and Du J (2013) Epithelial-mesenchymal transition (EMT) induced by TNF- α requires AKT/GSK-3 β -mediated stabilization of snail in colorectal cancer. *PLoS ONE* 8, e56664 10.1371/journal.pone.0056664 [PubMed: 23431386]
38. Krag S and Andreassen TT (2003) Mechanical properties of the human lens capsule. *Prog. Retin. Eye Res* 22, 749–767 10.1016/S1350-9462(03)00063-6 [PubMed: 14575723]
39. Zhao BM and Hoffmann FM (2006) Inhibition of transforming growth factor- β 1-induced signaling and epithelial-to-mesenchymal transition by the Smad-binding peptide aptamer Trx-SARA. *Mol. Biol. Cell* 17, 3819–3831 10.1091/mbc.E05-10-0990 [PubMed: 16775010]
40. Kolosova I, Nethery D and Kern JA (2011) Role of Smad2/3 and p38 MAP kinase in TGF- β 1-induced epithelial-mesenchymal transition of pulmonary epithelial cells. *J. Cell Physiol* 226, 1248–1254 10.1002/jcp.22448 [PubMed: 20945383]
41. Chang KC and Petrash JM (2015) Aldose reductase mediates transforming growth factor β 2 (TGF- β 2)-induced migration and epithelial-to-mesenchymal transition of lens-derived epithelial cells. *Invest. Ophthalmol. Vis. Sci* 56, 4198–4210 10.1167/iovs.15-16557 [PubMed: 26132779]
42. Chen X, Ye S, Xiao W, Wang W, Luo L and Liu Y (2014) ERK1/2 pathway mediates epithelial-mesenchymal transition by cross-interacting with TGF β /Smad and Jagged/Notch signaling pathways in lens epithelial cells. *Int. J. Mol. Med* 33, 1664–1670 10.3892/ijmm.2014.1723 [PubMed: 24714800]
43. Li JH, Wang W, Huang XR, Oldfield M, Schmidt AM, Cooper ME and Lan HY (2004) Advanced glycation end products induce tubular epithelial-myofibroblast transition through the RAGE-ERK1/2 MAP kinase signaling pathway. *Am. J. Pathol* 164, 1389–1397 10.1016/S0002-9440(10)63225-7 [PubMed: 15039226]
44. Bellaye P-S, Burgy O, Colas J, Fabre A, Marchal-Somme J, Crestani B, Kolb M, Camus P, Garrido C and Bonniaud P (2015) Antifibrotic role of α B-crystallin inhibition in pleural and subpleural fibrosis. *Am. J. Respir. Cell Mol. Biol* 52, 244–252 10.1165/rcmb.2014-0011OC [PubMed: 25032514]
45. Bellaye P-S, Wettstein G, Burgy O, Besnard V, Joannes A, Colas J, Causse S, Marchal-Somme J, Fabre A, Crestani B, Kolb M, Gauldie J, Camus P, Garrido C and Bonniaud P (2014) The small heat-shock protein α B-crystallin is essential for the nuclear localization of Smad4: impact on pulmonary fibrosis. *J. Pathol* 232, 458–472 10.1002/path.4314 [PubMed: 24307592]

46. Peinado H, Olmeda D and Cano A (2007) Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat. Rev. Cancer* 7, 415–428 10.1038/nrc2131 [PubMed: 17508028]
47. Nieto MA (2002) The snail superfamily of zinc-finger transcription factors. *Nat. Rev. Mol. Cell Biol* 3, 155–166 10.1038/nrm757 [PubMed: 11994736]
48. Ko H, Kim HS, Kim NH, Lee SH, Kim KH, Hong SH and Yook JI (2007) Nuclear localization signals of the E-cadherin transcriptional repressor snail. *Cells Tissues Organs* 185, 66–72 10.1159/000101305 [PubMed: 17587810]
49. Kato A, Naiki-Ito A, Nakazawa T, Hayashi K, Naitoh I, Miyabe K, Shimizu S, Kondo H, Nishi Y, Yoshida M, Umemura S, Hori Y, Mori T, Tsutsumi M, Kuno T, Suzuki S, Kato H, Ohara H, Joh T and Takahashi S (2015) Chemopreventive effect of resveratrol and apocynin on pancreatic carcinogenesis via modulation of nuclear phosphorylated GSK3 β and ERK1/2. *Oncotarget* 6, 42963–42975 10.18632/oncotarget.5981 [PubMed: 26556864]
50. Zhou P, Kannan R, Spee C, Sreekumar PG, Dou G and Hinton DR (2014) Protection of retina by α B crystallin in sodium iodate induced retinal degeneration. *PLoS ONE* 9, e98275 10.1371/journal.pone.0098275 [PubMed: 24874187]

Figure 1. AGE-BME enhances TGFβ2-mediated EMT in FHL124 cells.

(A) mRNA levels of α-SMA, αB-crystallin, E-cadherin, and GAPDH were quantified by qRT-PCR. FHL124 cells were cultured on AGE-BME or BME and treated with 10 ng/ml TGFβ2 for 24 h. (B) Protein levels of α-SMA, αB-crystallin, E-cadherin, and β-actin were quantified by Western blot analysis. FHL124 cells were cultured on AGE-BME or BME and treated with 10 ng/ml TGFβ2 for 48 h. Bars represent the means ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns, not significant.

Figure 2. AGE-BME promotes MAPK phosphorylation in FHL124 cells. FHL124 cells were cultured on either AGE-BME or BME and treated with 10 ng/ml TGFβ2 for 1 h. Data obtained using intracellular bead-based multiplex assays enabled simultaneous relative quantitation of multiple phosphorylation and total pathway proteins in cell lysate. The median fluorescence intensity (MFI) was measured with the Luminex® system. The canonical (**A**) and noncanonical (**B**) TGFβ2 signaling pathways were measured. (**C**) Phosphorylation of p38 MAPK densitometric data obtained from Western blots. Bars represent the means ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, ns, not significant.

Figure 3. AGE-BME enhances TGFβ2-induced EMT of FHL124 cells through activation of MAPK pathway.

FHL124 cells cultured on either AGE-BME or BME were pretreated with MAPK-specific inhibitors for ERK1/2 (U0126, 10 μM), p38 MAPK (SB202190, 10 μM), or AKT (LY294002, 10 μM) for 1 h followed by the addition of 10 ng/ml TGFβ2 for 48 h. Expression of α-SMA (A), αB-crystallin (B), and E-cadherin (C) was determined by Western blot analysis. Values are the means ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, not significant.

Figure 4. AGE-BME activates transcription factors in TGF β 2-induced FHL124 cells. (A) Expression of Snail, Slug, Twist, and ZEB in the nuclear fraction of FHL124 cells cultured on either AGE-BME or BME was measured by Western blot analysis after 6 h TGF β 2 treatment. Nuclear proteins were normalized with histone H3. (B) Cells were pretreated with 10 μ M U0126 (U), 10 μ M SB202190 (SB), or 10 μ M LY294002 (LY) for 1 h and treated with 10 ng/ml TGF β 2 for 6 h. (C) GSK3 β phosphorylation was determined by Western blot analysis. FHL124 cells cultured on either AGE-BME or BME were pretreated with 10 μ M LY294002 for 1 h and then treated with 10 ng/ml TGF β 2 for 1 h. Bars represent the means $\pm$ SD of three independent experiments. * P < 0.05, ** P < 0.01, **** P < 0.0001, ns, not significant.

Figure 5. Snail siRNA suppresses α-SMA levels in FHL124 cells

(A) FHL124 cells cultured on BME were transfected with 20 nM Snail-specific siRNA for 48 h and treated with 10 ng/ml TGFβ2 for 24 h. mRNA levels of Snail, α-SMA, and αB-crystallin were measured by qRT-PCR. (B) Protein levels of Snail, α-SMA, and αB-crystallin were determined by Western blot after treatment with 10 ng/ml TGFβ2 for 48 h. Values are the means ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns, not significant.

Figure 6. The absence of αB-crystallin reduces phosphorylation of GSK3β and decreases Snail expression.

WT and αB-crystallin KO (αBC KO) mouse LECs were treated with or without 10 ng/ml TGFβ2 for 2 h. The levels of αB-crystallin (A), phosphorylated GSK3β (B), and Snail (C) were determined by Western blotting. Values are the means ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, ns, not significant.

Figure 7. Possible signal transduction pathways by which AGE-BME enhances TGFβ2-induced EMT of LECs.

Red arrows indicate signaling by AGE-BME that enhances the TGFβ2-induced EMT of LECs.

Table 1

Primer sequences for qRT-PCR

Gene	Primer sequence	
α-SMA	Forward	5'-TTCAATGTCCCAGCCATGTA-3'
	Reverse	5'-GAAGGAATAGCCACGCTCAG-3'
αB-crystallin	Forward	5'-CTTTGACCAGTTCTTCGGAG-3'
	Reverse	5'-CCTCAATCACATCTCCCAAC-3'
E-cadherin	Forward	5'-AGTGTCCCCCGGTATCTTCC-3'
	Reverse	5'-CAGCCGCTTTCAGATTTTCAT-3'
Snail	Forward	5'-TCTAGGCCCTGGCTGCTACAA-3'
	Reverse	5'-ACATCTGAGTGGGTCTGGAGGTG-3'
GAPDH	Forward	5'-GTCAGTGGTGGACCTGACCT-3'
	Reverse	5'-TGCTGTAGCCAAATTCGTG-3'