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α B-crystallin is essential for the TGF- β 2-mediated epithelial to mesenchymal transition of lens epithelial cells

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Abstract

Transforming growth factor (TGF)- β 2-mediated pathways play a major role in the epithelial to mesenchymal transition (EMT) of lens epithelial cells (LECs) during secondary cataract formation, which is also known as posterior capsule opacification (PCO). Although α B-crystallin is a major protein in LEC, its role in the EMT remains unknown. In a human LEC line (FHL124), TGF- β 2 treatment resulted in changes in the EMT-associated proteins at the mRNA and protein levels. This was associated with nuclear localization of α B-crystallin, phosphorylated Smad2 (pSmad2) (S245/250/255), pSmad3 (S423/425), Smad4 and Snail and the binding of α B-crystallin to these transcription factors, all of which were reduced by the down-regulation of α B-crystallin. Expression of the functionally defective R120G mutant of α B-crystallin reduced TGF- β 2-induced EMT in LECs of α B-crystallin knockout (KO) mice. Treatment of bovine lens epithelial explants and mouse LEC with TGF- β 2 resulted in changes in the EMT-associated proteins at the mRNA and protein levels. This was accompanied by increase in phosphorylation of p44/42 mitogen-activated protein kinases (MAPK) (T202/Y204), p38 MAPK (T180/Y182), protein kinase B (Akt) (S473) and Smad2 when compared with untreated cells. These changes were significantly reduced in α B-crystallin depleted or knocked out LEC. The removal of the fibre cell mass from the lens of wild-type (WT) mice resulted in the up-regulation of EMT-associated genes in the capsule-adherent epithelial cells, which was reduced in the α B-crystallin KO mice. Together, our data show that α B-crystallin plays a central role in the TGF- β 2-induced EMT of LEC. α B-Crystallin could be targeted to prevent PCO and pathological fibrosis in other tissues.

Keywords

α B-crystallin; epithelial to mesenchymal transition; lens epithelial cells; TGF- β 2

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INTRODUCTION

Cataract formation is the most common cause of visual impairment and blindness. In Western and developed countries, cataract removal is the most frequently performed surgery in the elderly. Approximately 10 million cataract surgeries are annually performed worldwide [1]. The preferred method of cataract surgery is the removal of the fibre cell mass by a procedure known as phacoemulsification. During this procedure, a small circular portion of the anterior capsule (the capsule surrounds the entire lens) is removed, through which the emulsified fibre cell mass is aspirated, leaving behind most of the capsule for implantation of an artificial intraocular lens. Although this surgery is safe and restores vision to a large extent in most cases, posterior capsule opacification (PCO) develops in 20–30% of patients after 2–4 years [2–4].

The anterior surface of the eye lens contains a single layer of epithelial cells, which is necessary for the homeostasis of the lens. During cataract surgery, this layer of cells is almost completely removed along with most of the anterior capsule before an artificial intraocular lens is implanted. However, some cells remain stubbornly attached to the remaining anterior capsule after surgery and can undergo aberrant lens epithelial cell (LEC) proliferation and differentiation to form Elschnig's pearls, whereas other cells undergo epithelial to mesenchymal transition (EMT), possibly in response to trauma [5,6]. The EMT is characterized by the altered expression of epithelial (i.e. E-cadherin, zonula occludens and N-cadherin) and mesenchymal cell markers, i.e. alpha smooth muscle actin (α -SMA) and vimentin [7]. These changes are accompanied by the deposition of extracellular matrix proteins, including collagen and fibronectin, leading to fibrosis; in the posterior capsule, fibrosis causes wrinkling and leads to PCO, impairing the vision [8,9]. The only treatment for PCO at present is a neodymium: yttrium aluminium garnet (YAG) capsulotomy, which removes the fibrous posterior capsule to clear the visual axis. However, this procedure can lead to complications, such as retinal detachment, cystoid macular and corneal oedema and an increase in intraocular pressure [10,11]. Therefore, a better knowledge of the mechanism involved in PCO could help to design pharmacological agents to prevent it.

In the eye, transforming growth factor (TGF)- β 2 is the most abundant isoform of the TGF- β family [12,13]. TGF- β 2 has been shown to play an important role in the EMT of LECs [14,15]. During EMT, several transcription factors, such as Smad and Snail, are activated by TGF- β 2 [16]. Activation of the Smad signalling pathway via the TGF- β receptor occurs through the phosphorylation of Smad2 and 3, which hetero-oligomerize with Smad4 and translocate to the nucleus. This is accompanied by the activation of transcription regulators such as Snail and Slug, eventually leading to the expression/repression of proteins promoting transdifferentiation of cells to attain a mesenchymal phenotype [16]. TGF- β 2 also induces non-Smad signalling pathways through the activation of extracellular signal-regulated kinases (ERK), mitogen-activated protein kinases (MAPK) and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) that contribute to the EMT [17,18].

α B-Crystallin (HSPB5) is a member of the small heat shock protein family and one of the major proteins in the vertebrate lens. α B-Crystallin is a molecular chaperone and an anti-apoptotic protein; it prevents aggregation of structurally perturbed proteins and blocks

apoptosis in cells during stress. α B-Crystallin is also expressed in other tissues such as brain, heart, kidney and skeletal muscles [19]. It is up-regulated in the brain in neurodegenerative diseases, inflammation, breast cancer, retinal diseases and ischaemia/reperfusion injuries (reviewed in [20,21]). Exogenous administration of α B-crystallin has shown beneficial effects against neurological and ocular diseases (reviewed in [20,21]). Remarkably, similar beneficial effects have also been observed for a chaperone peptide derived from α B-crystallin [22,23]. However, other studies have shown that the overexpression of α B-crystallin promotes pathogenesis, as observed in basal-type breast cancer, pathological angiogenesis of the retina and pulmonary fibrosis [24–26].

Previous studies have shown that α B-crystallin promotes the EMT in hepatocellular carcinoma cells [27] and plays an important role in pulmonary fibrosis [26]. Similar fibrosis-promoting activity has been observed for Hsp27, which is a protein closely related to α B-crystallin [28]. The finding that α B-crystallin promotes fibrosis, coupled with the fact that it is one of the major proteins in LECs, prompted us to undertake the present study to investigate its role in TGF- β 2-induced EMT in LEC. In the present study, we show that α B-crystallin is integral to the EMT of LEC and that its absence or depletion reduces the EMT response by decreasing TGF- β 2-mediated signalling. We also show for the first time that α B-crystallin physically interacts with Snail and Smads during the EMT of LEC.

EXPERIMENTAL

Human lens epithelial cells (FHL124) and treatment with TGF- β 2

FHL124 cells (first developed by Dr John Reddan, Oakland University, MI) were obtained from Dr Michael Wormstone (University of East Anglia, U.K.) and cultured in minimum essential medium (MEM) containing 5% FBS and gentamicin/L-glutamate (1:100). Cells were treated with siRNA for α B-crystallin or a scrambled siRNA (100 nM) using Lipofectamine RNAiMAX transfection reagent (Life Technologies) for 24 h (GE Dharmacon), followed by human recombinant TGF- β 2 (20 ng/ml, Sigma–Aldrich) for an additional 24–48 h in serum-free medium.

Bovine lens explants and treatment with TGF- β 2

Bovine eyes were collected from a slaughterhouse, and the lenses were dissected within 8 h of enucleation of the eyes and soaked in sterile PBS containing penicillin–streptomycin (1:100) for 30 min. The lenses were then washed with PBS; a cut was made in the posterior capsule, and then the anterior capsule with the adhering LEC was peeled out and pinned on to a Petri dish with the epithelial cells facing up. The capsule was covered with MEM containing 20% FBS and gentamicin/L-glutamate (1:100). Experiments were initiated when the cells proliferated and made cell–cell junctions on the surface of the capsule (visualized by phase contrast microscope). To investigate the role of α B-crystallin in the EMT, the cells were treated with 100 nM of either scrambled or α B-crystallin siRNA (GE Dharmacon) and transfected using Lipofectamine RNAi MAX transfection reagent (Life Technologies) for 24 h, followed by treatment with human recombinant TGF- β 2 for an additional 24–48 h in serum-free medium.

Mouse lens epithelial cell culture and treatment with TGF- β 2

Lenses from wild-type (WT) or α B-crystallin knockout (KO) mice (rederived from the original stock supplied by Eric Wawrousek at the National Eye Institute) were dissected from the eyes and epithelial cells were isolated by the method previously described [29], and cultured in the medium as described above. The cells were treated with human TGF- β 2 (20 ng/ml) for 24–48 h in serum-free medium. We investigated if α B-crystallin's function has any role in the TGF- β 2-mediated response by transfecting LECs isolated from α B-crystallin KO mice with either WT or R120G α B-crystallin plasmid (the R120G human α B-crystallin plasmid was a kind gift from Dr Roy Quinlan, Durham University, U.K.). The cells were transfected for 24 h using Lipofectamine LTX and Plus Reagent (Life Technologies) as per the manufacturers' instructions and then treated with TGF- β 2 as described above.

Treatment of HeLa cells with TGF- β 2

HeLa cells were cultured in Dulbecco's Minimum Essential Medium High Glucose (DMEM-H) containing 10% FBS and penicillin–streptomycin (1:100) and treated with or without siRNA for α B-crystallin or scrambled siRNA, followed by TGF- β 2 treatment as described above.

Cell fractionation and Western blotting

Total cell lysates were prepared using the Mammalian Protein Extraction Reagent (M-PER; Thermo Scientific) that contained a protease and phosphatase inhibitor cocktail (Sigma–Aldrich) diluted 1:100. In some cases, cytosolic and nuclear fractions were prepared using the NucBuster protein extraction reagent (Millipore) containing a protease and phosphatase inhibitor cocktail (Sigma–Aldrich) diluted 1:100. The proteins obtained by these procedures (10–20 μ g) were separated on 12% SDS/PAGE and electrophoretically transferred to a nitrocellulose membrane and blocked with 5% blocking grade non-fat dry milk in Tris buffered saline and Tween-20 (TBST). The membranes were incubated overnight at 4°C with primary antibodies against the following proteins: α B-Crystallin (diluted 1:500000) (Developmental Studies Hybridoma Bank), α -SMA (Sigma–Aldrich, diluted 1:2500), β -actin (diluted 1:2000), E-Cadherin, phosphorylated and total p44/42 MAPK (T202/Y204), p38 MAPK (T180/Y182), Akt (S473), Smad2 (S245/250/255), Smad3 (S423/425), Smad4, Snail and histone (Cell Signaling Technology) (all diluted 1:1000 unless noted otherwise). Appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology) were used. The protein bands were detected using the SuperSignal West Pico or Femto Kit (Pierce Chemicals).

Co-immunoprecipitation

Cytosolic and nuclear fractions (30–50 μ g protein each) were incubated with either Smad2, 3, 4 or Snail antibodies (3–5 μ g each) or naïve rabbit IgG (Sigma–Aldrich, 4 μ g) for 5 h at 37°C on a shaker. Protein A/G Agarose (20 μ l) was added and incubated with shaking overnight at 4°C followed by centrifugation for 5 min at 1000 g. The pellet was washed with PBS and boiled with 2 $\times$ SDS sample buffer for 5 min at 95°C. The supernatant and the input (cytosolic and nuclear fractions) were then used for SDS/PAGE, followed by Western blotting using the α B-crystallin antibody (diluted 1:500,000).

Immunocytochemistry

FHL124 cells were cultured on chamber slides (Lab-Tek II) and treated with siRNA for α B-crystallin, followed by TGF- β 2, as described above. After 48 h of TGF- β 2 treatment, the cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, followed by three washes with 1 $\times$ PBS. For F-actin staining, cells were treated with 0.1% Triton X-100 in 1 $\times$ PBS for 5 min at room temperature, followed by three washes with 1 $\times$ PBS, blocked with 5% normal goat serum in PBS for 1 h, followed by incubation with Texas Red conjugated phalloidin (2.5:100 dilution, Life Technologies) at 37°C for 45 min. The cells were mounted with DAPI/Vectashield for observations of the nuclei. For α -SMA staining, cells were permeabilized with ice-cold 80% methanol in PBS for 20 min at -20°C. After blocking with 5% normal goat serum in PBS for 1 h, the cells were incubated overnight at 4°C with a monoclonal antibody for α -SMA (1:100 dilution, Sigma-Aldrich) followed by 1 h of incubation at 37°C with Texas Red conjugated goat anti-mouse IgG (1:250 dilution, Life Technologies). The cells were mounted with DAPI/Vectashield for observations of the nuclei. Images were taken with the help of fluorescence microscope (Nikon Eclipse 80i, Nikon) using Nikon NIS software.

Measurement of mRNA levels of EMT-related genes

The cells were cultured as described above, and total RNA was extracted from the cells using the RNeasy Plus Mini Kit (Qiagen). RNA was reverse transcribed to generate cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative polymerase chain reaction (qPCR) with SYBR green detection was performed using a CFX Connect Real-time PCR System (Bio-Rad Laboratories) with the set of primers listed in Table 1. In all experiments, we have shown the mRNA levels of only those markers that showed a clear up- or down-regulation upon TGF- β 2 treatment.

Lensectomy in mice

All animal experiments were performed in strict adherence to the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research and in accordance with institutional guidelines. Lensectomy in mice was performed as recently described [30] with modifications. In brief, the animals were anaesthetized and the eyes were prepped with betadine. A horizontal incision was made in the cornea and the anterior capsule with a surgical blade. The lens fibre cell mass was gently hydrodissected with Hank's balanced salt solution (BSS) and squeezed out of the eye through the corneal opening, leaving behind the lens capsule. The lens capsule was flushed with BSS, which was followed by injection of sodium hyaluronate viscoelastic material (Alcon Labs; 0.1 ml) into the empty capsular bag to prevent collapse of the bag. The cornea was sutured using 10-0 nylon suture. Betadine was applied to the site of injury immediately after surgery and once daily post-surgery. After 2 days, the animals were killed and the eyes were immediately enucleated and the suture was removed. The lens capsule was extracted using forceps and vortexed with RLT plus lysis buffer, processed according to manufacturer's instructions (Qiagen, RNA Isolation Kit) and analysed by qPCR for the EMT markers as described above.

Statistical analysis

The data are presented as the mean $\pm$ S.D. of the specific number of experiments indicated in the figure legends. The data were analysed using StatView software (version 5.0.1. SAS Institute). The statistical significance was evaluated by ANOVA followed by Fisher's protected least-significant difference test. The differences were considered significant at $P < 0.05$.

RESULTS

α B-Crystallin is required for TGF- β 2-induced EMT in human LEC

First, we investigated whether α B-crystallin is essential for TGF- β 2-induced EMT and how reduced expression of α B-crystallin would affect TGF- β 2-induced EMT in human LEC. We used siRNA for α B-crystallin to knockdown its expression. Cells treated with TGF- β 2 for 48 h showed an increase in the α B-crystallin protein content. A similar effect was not observed in the α B-crystallin siRNA-treated cells (Figure 1A). TGF- β 2-treated cells showed significantly higher mRNA levels of α -SMA, fibronectin and β 1 Integrin compared with untreated cells (Figures 1B–1D), but such effects were significantly ($P < 0.0005$) lower in α B-crystallin siRNA-treated cells than control cells. We also found a similar pattern for the α -SMA protein content (Figure 1E).

We further verified the cytoskeleton remodelling (F-actin) and EMT-associated changes by immunofluorescence. The cells without TGF- β 2 treatment showed negligible F-actin and α -SMA staining (Figures 2A1 and 2B1). However, after treatment with TGF- β 2, the cells showed much higher levels of F-actin and α -SMA staining (Figures 2A2 and 2B2). The basal levels of F-actin and α -SMA in α B-crystallin siRNA treated were comparable or slightly lower than control cells (Figures 2A3 and 2B3); after treatment with TGF- β 2 the levels of both were slightly higher (Figures 2A4 and 2B4), but appreciably lower than TGF- β 2-treated control cells.

In stressed cells, α B-crystallin translocates to nucleus [31]. To check if the up-regulation of α B-crystallin by TGF- β 2 treatment resulted in its greater nuclear translocation, we separated cytosolic and nuclear fractions of FHL124 cells. The efficacy of the fractionation was verified by sequentially immunoblotting fractions for histone and β -actin. Histone and β -actin were present only in the nuclear and cytosolic fractions respectively (Supplementary Figure S1). We found higher levels of α B-crystallin in the cytosolic fraction and its greater nuclear translocation after TGF- β 2 treatment (Figures 3A and 3B). Similar effects were absent in cells treated with α B-crystallin siRNA. TGF- β 2 treatment also resulted in greater levels of phosphorylation of Smad2, 3 and their increased translocation to nucleus compared with cells that were treated with α B-crystallin siRNA (Figures 3C–3F). Similarly, Smad4 and Snail were up-regulated and translocated into the nucleus in higher amounts in cells treated with TGF- β 2, but not in cells that were treated with α B-crystallin siRNA prior to TGF- β 2 treatment (Figures 3G–3J).

Immunoprecipitation experiments were conducted to determine the interaction of α B-crystallin with the Smads and with Snail. In these experiments, either Smad2, 3, 4 or Snail was immunoprecipitated using the appropriate antibodies and then Western blotted using an

α B-crystallin antibody. Remarkably, these experiments revealed that α B-crystallin physically interacted with Smad2, 3, 4 and Snail after TGF- β 2 treatment and was transported to the nucleus (Figure 4A). This association and transportation were drastically reduced in cells treated with α B-crystallin siRNA prior to TGF- β 2 treatment. The use of naïve rabbit IgG in the immunoprecipitation experiments ruled out non-specific reactions. α B-Crystallin was detected in the corresponding inputs of the cytosolic and nuclear fractions (Figure 4B). Taken together, the above results suggest that α B-crystallin is essential for the translocation of EMT-associated transcription factors to the nucleus and does so through a direct physical interaction with the transcription factors.

TGF- β 2-mediated EMT in bovine LEC is dependent on α B-crystallin expression

Lens explants, in which the epithelial cells are adherent to the capsule, mimic the natural environment of LECs in the eye to a certain extent. Thus, it was of interest to determine whether α B-crystallin had similar effects in lens explants as those in FHL124 cells. Unlike FHL124 cells, treatment with TGF- β 2 for 48 h did not increase the α B-crystallin levels in the explants (Figure 5A). Epithelial cells in explants treated with siRNA for 24 h showed an approximately 44% reduction in α B-crystallin protein content ($P<0.0005$) compared with the scrambled siRNA-treated cells (Figure 5B). Cells treated with TGF- β 2 showed a reduction in E-cadherin and zonula occluden-1 (ZO-1) and an increase in the fibronectin, β 1 Integrin and Snail mRNA levels (Figures 5C–5G). These changes were significantly ($P<0.0005$) suppressed in cells pretreated with the α B-crystallin siRNA. In cells treated with TGF- β 2 for 48 h, there was a concomitant decrease in E-cadherin and an increase in α -SMA protein content (Figures 5H and 5I). However, these effects were reduced in cells treated with α B-crystallin siRNA. Interestingly, the E-cadherin levels were higher in siRNA-treated cells compared with control cells (Figure 5H).

Reduced expression of α B-crystallin diminishes TGF- β 2-induced EMT in HeLa cells

Unlike in LEC, the basal levels of α B-crystallin are extremely low in HeLa cells (Figure 6A). Thus, it was of interest to determine how these cells responded to TGF- β 2 treatment. Additionally, we wanted to determine if the EMT-promoting activity of α B-crystallin is restricted to LEC or whether it is pertinent in other cells as well. Intriguingly, after TGF- β 2 treatment, there was a dramatic increase in α B-crystallin expression in these cells (Figure 6A). Pretreatment with α B-crystallin siRNA for 24 h reduced this expression by approximately 70% (Figure 6B). Cells with or without prior-treatment with α B-crystallin siRNA were treated with TGF- β 2 for 24–48 h. Cells without prior treatment with siRNA showed up-regulation in the mRNA and protein content of α -SMA (Figures 6C and 6D). However, this effect was significantly reduced ($P<0.0005$) in cells pretreated with α B-crystallin siRNA. Similarly, we found an increase in the phosphorylation of p44/42 MAPK (T202/Y204) after TGF- β 2 treatment, which was reduced in α B-crystallin siRNA-treated cells (Figure 6E). Along with the above changes, we found a large increase in the level of α B-crystallin in the nuclear fraction after TGF- β 2 treatment (Supplementary Figures S2A and S2B). Remarkably, we found higher levels of pSmad2 and Snail and their greater translocation into the nucleus (Supplementary Figures S2C–S2F). However, these changes were significantly reduced in the α B-crystallin siRNA-treated cells. Together, these results further reiterate the importance of α B-crystallin in TGF- β 2-mediated signalling during the

EMT and demonstrate that the EMT-promoting effect of α B-crystallins is also applicable in a non-lens cell.

The absence of α B-crystallin suppresses the TGF- β 2-induced EMT, signalling and nuclear translocation of transcription factors in mouse LEC

The above results support a major role for α B-crystallin in TGF- β 2-induced EMT. We sought to determine the effect in the absence of α B-crystallin. LEC from WT and α B-crystallin KO mice were isolated, cultured, treated with TGF- β 2 and fractionated into cytosolic and nuclear fractions. The efficacy of the fractionation was verified by Western blotting for histone (Supplementary Figure S3A) and β -actin (Supplementary Figure S3B). We found an increase in the α B-crystallin mRNA levels after TGF- β 2 treatment (Figure 7A). As expected, the KO cells did not express α B-crystallin and showed no differences after TGF- β 2 treatment. Interestingly, in WT cells, TGF- β 2 treatment did not alter α B-crystallin content in the cytosolic fraction (Figure 7B) but did increase it in the nuclear fraction (Figure 7C). We then measured the mRNA levels of EMT-associated genes by qPCR. TGF- β 2 treatment significantly ($P < 0.0005$) reduced the expression of ZO-1 and induced the expression of α -SMA, fibronectin and Snail in WT cells (Figures 7D–7G) ($P < 0.0005$) but not in α B-crystallin KO cells. WT cells treated with TGF- β 2 for 48 h showed an increase in the protein content of α -SMA compared with untreated cells (Figure 7H). This was significantly reduced ($P < 0.0005$) in α B-crystallin KO cells.

In WT LEC, TGF- β 2 treatment significantly increased phosphorylation of p44/42 MAPK (T202/Y204), p38 MAPK (T180/Y182) and Akt (S473) compared with untreated cells ($P < 0.0005$). However, such increases were significantly curtailed ($P < 0.0005$) in cells from the α B-crystallin KO mice (Supplementary Figures S4A–S4C). Phosphorylation of Smad2 and nuclear localization were increased after TGF- β 2 treatment in WT cells (Supplementary Figures S4D and S4E) but were significantly ($P < 0.0005$) reduced in cells from the α B-crystallin KO mice. We also observed an increase in the expression of Snail and its nuclear translocation after TGF- β 2 treatment, which were significantly inhibited in the α B-crystallin KO cells (Supplementary Figures S4F and S4G). These results further support a central role for α B-crystallin in TGF- β 2-induced EMT in LEC.

The function of α B-crystallin is required for TGF- β 2-induced EMT

Next, we determined whether the functionally active form of α B-crystallin was essential for promoting the TGF- β 2-mediated EMT of LEC. We used a functionally compromised α B-crystallin (R120G mutant) [32] in these experiments. We transfected LECs of α B-crystallin KO mice with either WT or R120G mutant plasmid (24 h). After replacing the media, the cells were cultured for an additional 24 h. Expression of WT and R120G α B-crystallin was verified by Western blotting (Figure 8A). The expression of R120G was slightly lower than the WT protein, but it was statistically insignificant. To determine the effect on TGF- β 2-mediated EMT, the cells were treated (24 h post-transfection) with TGF- β 2 for 24 h. We checked the mRNA levels of EMT-associated genes by qPCR. Cells transfected with WT plasmid showed significant increases in the mRNA levels of α -SMA, fibronectin and β 1 Integrin relative to control cells. However, in the R120G-transfected cells, those mRNA levels were lower (Figures 8B–8D). Because the basal mRNA levels of the EMT markers

were different in the WT and R120G-transfected cells, we compared the percentage increase in the mRNA levels (over control) between the two transfected cells. The mRNA levels for α -SMA were 19.04% higher in WT and 14.67% higher in R120G cells. The fibronectin levels were 170.2% higher in WT cells but only 40% higher in R120G cells and the mRNA levels of β 1 Integrin were 45.81% higher in WT and 32.15% higher in R120G-transfected cells. Although the mRNA levels of fibronectin were not statistically different between TGF- β 2-treated and untreated R120G-transfected cells, in all cases the difference in the mRNA levels between WT and R120G-transfected cells were statistically significant. These results further suggest that the functionally active α B-crystallin may be required to promote the TGF- β 2-mediated EMT of LEC.

EMT markers are up-regulated to a lesser extent in α B-crystallin KO mice relative to WT mice after lensectomy

A recent study showed up-regulation of EMT markers in lensectomized mice [30]. To test if the absence of α B-crystallin would affect such a response, we used WT and α B-crystallin KO mice, where in each animal, one eye was subjected to surgery and the other eye was used as a control. Two days after lensectomy, the animals were killed and their capsule-adherent LECs were analysed by qPCR for the EMT markers. The results are shown in Figure 9. As expected, α B-crystallin mRNA was detected only in WT cells (Figure 9A). The mRNA levels of EMT markers, α -SMA, fibronectin, β 1 Integrin and vimentin are shown in Figures 9(B)–9(E). In general, these markers were more elevated in lensectomized WT cells than in the α B-crystallin KO cells. However, we could not obtain consistent results for the down-regulated genes (ZO-1 and E-cadherin). In spite of these technical difficulties, our results reiterate an important role for α B-crystallin in the EMT of LEC.

DISCUSSION

The purpose of the present study was to determine the role of α B-crystallin in the TGF- β 2-induced EMT of LECs. It was clearly evident in different cell types used in the present study that TGF- β 2 greatly up-regulated α B-crystallin (except in bovine lens explants). A previous study also showed such up-regulation in trabecular meshwork cells and optic nerve head astrocytes [33,34]. Cells undergoing the EMT are generally resistant to apoptosis; therefore, the up-regulation of α B-crystallin in LEC during the EMT is not completely unexpected. However, the role of α B-crystallin in the context of LEC EMT was unknown until now. The up-regulation of α B-crystallin occurred along with the up-/down-regulation of EMT makers in LEC, clearly establishing an association between the expression of α B-crystallin and the EMT in LEC. Further, a reduction in α B-crystallin by siRNA treatment in the bovine LEC explants and in human LEC cultures reduced TGF- β 2-induced EMT (mRNA levels and protein expression). In addition, we established the importance of α B-crystallin in TGF- β 2-induced EMT in HeLa cells as well. Unlike LEC, HeLa cells express very little α B-crystallin under basal culture conditions but express high levels when stimulated with TGF- β 2. Depletion of α B-crystallin by siRNA treatment in these cells significantly reduced the TGF- β 2-induced EMT response, suggesting that the heightened expression of α B-crystallin is integral to the EMT response. The total absence of α B-crystallin in LEC from α B-crystallin KO mice further reduced the TGF- β 2-induced EMT response. Furthermore,

lensectomy in mice, which has been shown to trigger the EMT response in capsule-adherent epithelial cells [30], was muted in α B-crystallin KO mice when compared with WT mice. Together, these observations implicate α B-crystallin in the EMT of LEC and suggest that it is essential for PCO development after cataract surgery.

The absence of a major protein in LECs negatively affecting the TGF- β 2 response may not be completely unexpected. However, expression of the R120G mutant of α B-crystallin, with reduced chaperone and anti-apoptotic activities in LECs of α B-crystallin KO mice resulted in a weaker response to TGF- β 2 than WT protein overexpressing cells. These observations strongly suggest that the chaperone and/or anti-apoptotic activity of α B-crystallin is obligatory for the TGF- β 2-induced EMT of LEC. Elevated expression of α B-crystallin after TGF- β 2 treatment is likely to contribute to resistance to apoptosis, which is a hallmark feature of epithelial cells undergoing the EMT [35]. Another feature of EMT is the increased migratory capacity of epithelial cells, which is aided by the formation of actin-rich membrane projections called lamellipodia [36]. It has been shown that α B-crystallin localizes at the leading edges of the lamellipodia in migrating LEC [37]. Thus, the enhanced expression of α B-crystallin by TGF- β 2 could favour the above processes during the EMT of LEC.

Our results also showed a direct relationship between α B-crystallin expression and the Smad-independent pathways of the EMT. Links between the activation of p44/42MAPK, p38 MAPK and AKT signalling and TGF- β 2-induced EMT have been previously and recently established [38–40]. Crosstalk between these signalling pathways and the Smad-dependent pathway has also been reported [9]. Mechanism by which α B-crystallin activates Smad-independent signalling is not entirely clear, but analogous to our results, other studies have already established that α B-crystallin expression induces the phosphorylation of these signalling pathways [24,41,42].

Recent studies have shown a similar role for α B-crystallin in the TGF- β 2-mediated EMT response in lung and kidney epithelial cells [26] (reviewed in [21]). In addition, Hsp27, which is a closely related protein to α B-crystallin, has been shown to have pro-EMT/fibrosis properties [28,43]. One study attributed this to the inhibition of Snail ubiquitination/degradation [28]. Whether a similar inhibition of Snail degradation occurs by α B-crystallin during TGF- β 2-mediated EMT of LECs is unclear. Snail is essential for the TGF- β 2-mediated EMT of LEC [44], and Snail expression in the absence of Smad signalling is sufficient to induce TGF- β 1-induced EMT [45]. TGF- β 2-activated Snail translocates to the nucleus from the cytoplasm and reduces the expression of E-cadherin in LECs. E-Cadherin is an adherence protein present at the apical surface of LECs. Its degradation, mediated by Snail, reduces cell–cell junctions, leading to the loss of polarity of cells, a hallmark feature of EMT. What is interesting in our study is that α B-crystallin interacted with Snail and was transported to the nucleus in increasing amounts after TGF- β 2 treatment. Whether such binding of α B-crystallin is essential for Snail's translocation or its stabilization during translocation remains to be established.

During TGF- β 2-mediated EMT, pSmad2 complexes with pSmad3 and Smad4 and translocates to the nucleus. The fact that the physical interaction of α B-crystallin with these

transcription factors was observed both in the cytoplasm and nucleus in TGF- β 2-treated LEC suggests that it may be part of the Smad2, 3 and 4 complex that translocates to nucleus. The question then is whether this interaction is essential for the promotional effect of α B-crystallin on TGF- β 2 signalling during the EMT. Our results favour this possibility, as depletion of α B-crystallin resulted in a reduced or absent Smad interaction and consequently to less signalling through the TGF- β 2-mediated pathway. This raises another possibility that the interaction of α B-crystallin and Smads might promote TGF- β 2 signalling through a hitherto unknown mechanism. In this context, it is possible that the interaction of α B-crystallin with the Smads could stabilize the complex and its translocation to the nucleus. A direct effect of α B-crystallin on Smads has been recently studied; one study showed that α B-crystallin inhibits the ubiquitination of Smad4 and thereby promotes its nuclear translocation during LEC EMT [26]. Whether the α B-crystallin interactions we observed are for the purpose of protecting the Smads from ubiquitination remains to be established.

Interestingly, a recent study showed that aldose reductase (AR) is necessary, and it interacts with Smad2 during TGF- β 2-induced EMT of LEC [46]. It is possible that AR and α B-crystallin could interact with each other or with the Smad complex and promote TGF- β 2-induced EMT. Further studies are needed to verify these possibilities.

Whether α B-crystallin promotes Wnt- β -catenin signalling, which has been implicated in EMT [36,47], is also not known. A previous study suggested a direct interaction of α B-crystallin with β -catenin [48], which raises the possibility of an additional mechanism by which α B-crystallin could promote EMT.

In conclusion, our study provides convincing evidence that TGF- β 2 up-regulates α B-crystallin and that the up-regulated α B-crystallin enhances Smad-dependent and Smad-independent signalling during the EMT of LEC (Figure 10). Additionally, our study shows that α B-crystallin physically associates with Smads and Snail in both the cytoplasm and nucleus and that its functional activity is essential during TGF- β 2-mediated EMT. These data suggest that α B-crystallin could be a target to prevent or block PCO. It is likely that α B-crystallin also plays a role in EMT/fibrosis that occurs in other eye diseases, such as proliferative vitreoretinopathy, diabetic retinopathy and age-related macular degeneration (fibrotic changes in the retinal pigmented epithelial cells). Furthermore, the fact that α B-crystallin promoted the EMT of HeLa cells raises the possibility that it might be involved in the metastasis of cancer cells. Targeting α B-crystallin might provide benefits in these diseases as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Akt	protein kinase B
AR	aldose reductase
BSS	Hank's balanced salt solution
EMT	epithelial to mesenchymal transition
ERK	extracellular signal-regulated kinases
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HRP	horseradish peroxidase
KO	knockout
LEC	lens epithelial cell
MAPK	mitogen-activated protein kinases
MEM	minimum essential medium
PCO	posterior capsule opacification
qPCR	quantitative polymerase chain reaction
α-SMA	alpha smooth muscle actin
TBST	Tris buffered saline and Tween-20
TGF	transforming growth factor
WT	wild-type
YAG	yttrium aluminium garnet
ZO-1	zonula occluden-1

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Figure 1. TGF-β2-mediated EMT is inhibited by the down-regulation of αB-crystallin in a human LEC line (FHL124)

FHL124 cells were treated with siRNA for αB-crystallin for 24 h (100 nM) followed by TGF-β2 (20 ng/ml); the cells were held in this condition for 24–48 h. The αB-crystallin levels in siRNA +/- TGF-β2-treated cells and controls are shown in (A). qPCR quantification of mRNA for EMT genes α-SMA, fibronectin and β1 Integrin are shown in (B), (C) and (D) respectively. The effect of siRNA for αB-crystallin on TGF-β2-mediated induction of α-SMA protein is shown in (E). For proteins, densitometric analyses from triplicate assays (mean± S.D.) are shown in bar graphs; ** $P < 0.005$ and *** $P < 0.0005$, NS=not significant.

Figure 2. TGF- β 2-mediated cytoskeleton remodelling and the expression of α -SMA are reduced in α B-crystallin depleted cells

FHL124 cells were seeded on chamber slides, and treated with α B-crystallin siRNA and TGF- β 2 as in Figure 1. Cytoskeleton remodelling (F-actin) was detected using Texas Red conjugated phalloidin antibody (**A1–4**) at 20 $\times$ and 40 $\times$ magnification and α -SMA was detected using a monoclonal antibody against α -SMA and Texas Red-goat anti-mouse IgG (**B1–4**) at 20 $\times$ and 40 $\times$ magnification; scale bar=10 μ m.

Figure 3. Depletion of α B-crystallin reduces the Smad signalling in TGF- β 2-treated FHL124 cells
 FHL124 cells were cultured and treated with α B-crystallin siRNA and TGF- β 2 as in Figure 1. The effect of siRNA treatment on α B-crystallin in the cytosolic and nuclear fractions of TGF- β 2-treated cells is shown in (A) and (B). The cytosolic and nuclear fractions were loaded in parallel, but separately on to SDS/PAGE, and following electrophoresis transferred to a nitrocellulose membrane. The membrane was probed with an α B-crystallin antibody. The membrane was then cut into two halves; the half containing the cytoplasmic fractions was reprobed with a β -actin antibody, whereas the other half containing the nuclear fraction was reprobed with a histone H3 antibody. This figure also shows the effect of siRNA and TGF- β 2 treatment on pSmad2 (C and D), pSmad3 (E and F), Smad4 (G and H) and Snail (I and J) in the cytosolic and nuclear fractions. Densitometric analyses from triplicate assays (mean $\pm$ S.D.) are shown in bar graphs; t=total, ** P <0.005 and *** P <0.0005, NS=not significant.

Figure 4. Depletion of αB-crystallin diminishes its interaction with the Smads and Snail transcription factors in TGF-β2-treated FHL124 cells
FHL124 cells were cultured and treated with αB-crystallin siRNA and +/- TGF-β2 as in Figure 1. The cytosolic and nuclear fractions were immunoprecipitated (IP) using respective Smad or Snail antibodies and Western blotted (WB) using an αB-crystallin antibody. The effect of αB-crystallin siRNA and TGF-β2 treatment on the binding of αB-crystallin to Smad2, Smad3, Smad4 and Snail in the cytosolic and nuclear fractions is shown in (A). To rule out non-specific reactions, the fractions were immunoprecipitated using a naïve rabbit IgG and Western blotted using an αB-crystallin antibody. The αB-crystallin inputs are shown in (B).

Figure 5. TGF-β2-mediated EMT response is reduced in αB-crystallin-depleted LEC of bovine lens explants

The bovine lens capsule with adherent LEC was cultured until the cells occupied the entire surface of the capsule. The explants were then either treated with a siRNA for αB-crystallin (100 nM for 24 h) or a scrambled siRNA (control). TGF-β2 (20 ng/ml) was then added to the medium, and the explant was further treated for 24–48 h. The αB-crystallin levels in explants measured by Western blotting are shown in (A), and the effect of siRNA treatment on αB-crystallin levels is shown in (B). The mRNA levels of EMT genes in control and αB-crystallin siRNA+/- TGF-β2-treated cells were quantified by qPCR. The effect on E-cadherin and ZO-1 is shown in (C) and (D), and the effect on fibronectin, β1 Integrin and Snail is shown in (E), (F) and (G) respectively. The effect of TGF-β2 on the protein levels of E-cadherin and α-SMA is shown in (H) and (I) respectively. Data shown are the mean ± S.D. from at least three independent experiments; *** $P < 0.0005$, NS=not significant.

Figure 6. TGF- β 2 treatment up-regulates α B-crystallin and promotes α -SMA mRNA and protein synthesis in HeLa cells

HeLa cells have low basal α B-crystallin levels, which were markedly up-regulated by TGF- β 2 treatment (20 ng/ml, 48 h) (A) but were reduced in α B-crystallin siRNA-treated (24 h) cells (B). The depletion of α B-crystallin led to a lack of TGF- β 2-mediated up-regulation of α -SMA at the mRNA (C) and protein (D) levels. The phosphorylation of p44/42 MAPK was increased by TGF- β 2 in the control but not in the siRNA-treated cells (E). Densitometric analyses from triplicate assays (mean $\pm$ S.D.) are shown in bar graphs; t=total, * P <0.05 and *** P <0.0005, NS=not significant.

Figure 7. Deletion of αB-crystallin reduces the TGF-β2-mediated EMT response in mouse LEC
 Mouse LEC were isolated and cultured from WT and αB-crystallin KO mice (αB-KO). The αB-crystallin mRNA levels were measured by qPCR in TGF-β2-treated and untreated cells (A). The αB-crystallin protein levels were measured by Western blotting in the cytosolic (B) and nuclear fractions (C) of TGF-β2-treated and control cells. The mRNA levels of EMT genes were quantified by qPCR. The effects on ZO-1, α-SMA, fibronectin and Snail mRNA levels are shown in (D), (E), (F) and (G) respectively. The α-SMA protein content was determined by Western blotting (H). Densitometric analyses from triplicate assays (mean ± S.D.) are shown in bar graphs; ** $P < 0.005$ and *** $P < 0.0005$, NS=not significant.

Figure 8. Functional activity of αB-crystallin is necessary for TGF-β2-mediated EMT
LEC from a αB-crystallin KO mouse were transfected with either WT or R120G mutant αB-crystallin plasmids (24 h). Cells were then treated with or without TGF-β2 for 24 h as described in the 'Experimental' section. The protein content of αB-crystallin was verified prior to TGF-β2 treatment by Western blotting (A). The mRNA levels of EMT-associated genes, α-SMA, fibronectin and β1 Integrin, are shown in (B), (C) and (D) respectively. Densitometric analyses from triplicate assays (mean ± S.D.) for the Western blot for αB-crystallin are shown in the bar graphs; **P* < 0.05, ***P* < 0.005 and ****P* < 0.0005, NS=not significant.

Figure 9. Deletion of αB-crystallin reduces the EMT response of LEC of lensectomized mice
 One lens from WT or αB-crystallin KO mice was surgically removed, leaving behind most of the capsule with adherent LEC in the eye. The other contralateral lens was left untouched. After 2 days, the capsule with adherent LEC was removed from both eyes, the RNA was isolated and cDNA was synthesized. EMT-associated markers were measured by qPCR. αB-Crystallin expression was observed only in WT LEC (A). The EMT markers, α-SMA, fibronectin, β1 Integrin and vimentin, were all up-regulated in WT samples but to a significantly lesser extent in KO samples (B, C, D and E respectively); * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.0005$, NS=not significant.

Figure 10. Proposed mechanism by which α B-crystallin promotes TGF- β 2-induced EMT of LECs

Table 1

Primer sets used for qPCR

1	Bovine Fibronectin	FP: 5'-GGT AAC GAA GGC TCC ACT GC-3' RP: 5'-ACC AGA TTC CTC TTA TCA ACT G-3'
2	Bovine β 1 Integrin	FP: 5'-TCA CTC AAA TCC AGC CAC AG-3' RP: 5'-CAC CAA GTT TCC CAT CTC TCC A-3'
3	Bovine ZO-1	FP: 5'-CGA CCA GAT CCT CAG GGT AA-3' RP: 5'-TCC ATA GGG AGA TTC CTT CTC A-3'
4	Bovine Snail	FP: 5'-TTC TCC AGA GCT CAC TTT CCC-3' RP: 5'-GAG AGT CC AGA TGA GTC TC-3'
5	Bovine E-cadherin	FP: 5'-GTG ATA GAT GTG AAT GAA GCC C-3' RP: 5'-AAT CCG ATA CGT GAT CTT CTG-3'
6	Bovine GAPDH (glyceraldehyde 3-phosphate dehydrogenase)	FP: 5'-CAT TGA CCT TCA CTA CAT GGT-3' RP: 5'-ACC CTT CAA GTC AGC CCC AG-3'
7	Mouse α SMA	FP: 5'-TCT CTA TGC TAA CAA CGT CCT GTC A-3' RP: 5'-CCA CCG ATC CAG ACA GAG TAC TT-3'
8	Mouse Fibronectin	FP: 5'-ATG TGG ACC CCT CCT GAT AGT-3' RP: 5'-GCC CAG TGA TTT CAG CAA AGG-3'
9	Mouse β 1 Integrin	FP: 5'-TCC TTC AAT TGC TCA CCT TGT-3' RP: 5'-GCG CAC TGC TGA CTT AGG AAT-3'
10	Mouse α B-Crystallin	FP: 5'-GCT TCA CGT CCA GAT TCA CA-3' RP: 5'-GCG GTG AGC TGG GAT AAT AA-3'
11	Mouse ZO-1	FP: 5'-GCT AAG AGC ACA GCA ATG-3' RP: 5'-GCA TGT TCA ACG TTA TCC AT-3'
12	Mouse Vimentin	FP: 5'-CGG AAA GTG GAA TCC TTG CA-3' RP: 5'-CAC ATC GAT CTG GAC ATG CTG T-3'
13	Mouse Snail	FP: 5'-TCC AAA CCC ACT CGG ATG TGA AGA-3' RP: 5'-TTG GTG CTT GTG GAG CAA GGA CAT-3'
14	Mouse GAPDH	FP: 5'-TGT CAT CAT ACT TGG CAG GTT TCT-3' RP: 5'-CAT GGC CTT CCG TGT TCC TA-3'
15	Human α -SMA	FP: 5'-TTCAATGTCCCAGCCAT GTA-3' RP: 5'-GAAGGAATAGCCACGC TCAG-3'
16	Human Fibronectin	FP: 5'-CTG GAA CCG ACC GAA TAT A-3' RP: 5'-TTC TTG TCC TAC ATT CGG CGG-3'
17	Human β 1 Integrin	FP: 5'-CAAGAGAGCTGAAGAC TATCCCA-3' RP: 5'-TGAAGTCCGAAGTAAT CCTCCT-3'
18	Human GAPDH	FP: 5'-GTCAGTGGTGGACCTGA CCT-3' RP: 5'-TGCTGTAGCCAAATTC GTTG-3'