

# Nox4 Plays a Role in TGF- $\beta$ -Dependent Lens Epithelial to Mesenchymal Transition

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**PURPOSE.** Transforming growth factor- $\beta$  induces an epithelial to mesenchymal transition (EMT) in the lens, presented as an aberrant growth and differentiation of lens epithelial cells. Studies in other models of EMT have shown that TGF- $\beta$ -driven EMT is dependent on the expression of the reactive oxygen species (ROS)-producing enzyme nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase-4 (Nox4). We investigate the role of this enzyme in TGF- $\beta$ -induced lens EMT and determine whether it is required for this pathologic process.

**METHODS.** Rat lens epithelial explants were used to investigate the role of Nox4 in TGF- $\beta$ -driven lens EMT. Nox1-4 expression and localization was determined by immunolabeling and/or RT-PCR. NADPH-oxidase-produced ROS were visualized microscopically using the fluorescent probe, dihydroethidium (DHE). VAS2870, a pan-NADPH oxidase inhibitor, was used to determine the specificity of Nox4 expression and its role in ROS production, and subsequently TGF- $\beta$ -driven EMT.

**RESULTS.** We demonstrate, for the first time to our knowledge, in rat lens epithelial explants that TGF- $\beta$  treatment induces Nox4 (but not Nox1-3) expression and activity. Increased Nox4 expression was first detected at 6 to 8 hours following TGF- $\beta$  treatment and was maintained in explants up to 48 hours. At 8 hours after TGF- $\beta$  treatment, Nox4 was observed in cell nuclei, while at later stages in the EMT process (at 48 hours), Nox4 was predominately colocalized with  $\alpha$ -smooth muscle actin. The inhibition of Nox4 expression and activity using VAS2870 inhibited EMT progression.

**CONCLUSIONS.** Transforming growth factor- $\beta$  drives the expression of the ROS-producing enzyme Nox4 in rat lens epithelial cells and Nox4 inhibition can impede the EMT process.

**Keywords:** lens opacity, lens epithelium, transforming growth factor-beta

Age-related cataract (i.e., a loss of lens transparency) is the most common cause of blindness in the world. A causal link has been established between the development of cataract and reactive oxygen species (ROS), with the deletion of major cellular antioxidants leading to the development of cataract.<sup>1-3</sup> Indeed, the macromolecules of the lens (i.e., proteins, lipids, and DNA) are oxidatively damaged by these ROS in cataract.<sup>4-7</sup> This damage is thought to occur, at least in part, as a result of the levels of the cellular antioxidant defenses declining with age.<sup>8-10</sup> Routine surgery resolves cataract; however, in many cases 2 to 4 years later, a secondary cataract develops, known as posterior capsular opacification (PCO), where residual lens epithelial cells (LECs) migrate and undergo an epithelial-mesenchymal transition (EMT), as they encroach on the posterior capsule,<sup>11</sup> requiring further treatment. Developing a successful strategy to prevent the development of cataract and a treatment that offers permanent resolution of the cataract would substantially reduce health cost outlays and social benefit costs, and restore the quality of life for the aging population.

Work in our laboratory and others has focused on understanding the molecular basis of cataract development in the lens, in particular the EMT process. Specifically, it has been shown that TGF- $\beta$  induces an EMT in the lens that arises from aberrant growth and differentiation of the lens epithelial

cells.<sup>12,13</sup> Transforming growth factor- $\beta$  drives this lens EMT through Smad and ERK signaling in animal models of cataract<sup>14,15</sup> and in humans.<sup>16</sup> Recently, a link has been established between TGF- $\beta$ -driven cataract and oxidative stress,<sup>17</sup> where addition of the antioxidant glutathione (GSH) has been shown to suppress TGF- $\beta$ -induced opacification and subcapsular plaque formation.<sup>17</sup> Interestingly, studies by Dawes et al.<sup>14</sup> examined the transcriptional response of human lens epithelial cells to TGF- $\beta$  and uncovered a potential source of the TGF- $\beta$ -driven ROS production and subsequent oxidative stress. In this model, only one professional ROS producer (i.e., a direct ROS producer, not producing ROS as a side-effect of its enzymatic activity) was upregulated in response to TGF- $\beta$ , namely, NADPH oxidase 4 (Nox4). This discovery adds to the growing evidence that growth factor-dependent NADPH oxidase activity has a role in TGF- $\beta$  driven EMT of the lens and more generally in the EMT process.

Growth factors have been shown to induce NADPH oxidase activity in the lens, with PDGF stimulating Nox2 activity and ROS production in human lens epithelial cells.<sup>18</sup> However, to our knowledge this is the first time the role of Nox4 in TGF- $\beta$ -driven EMT has been investigated in the lens and we expect it to be significant given that Nox4 has been shown to have an important role in other models of TGF- $\beta$ -driven EMT.<sup>19</sup> Specifically, in breast cancer cells, TGF- $\beta$  has been shown to

signal through the Smad signaling cascade to induce Nox4 expression leading to an EMT.<sup>19</sup> Interestingly, in this model, blockade of the Smad signaling pathway inhibited Nox4 expression and abolished the development of the EMT.<sup>19</sup> Moreover, blocking Nox4 alone (either pharmacologically or by siRNA), also prevented the EMT in breast cancer cells.<sup>19</sup> Here, in experiments described, we have identified a potential source of TGF- $\beta$ -driven ROS in the lens. Specifically, we have identified that TGF- $\beta$  induces expression of Nox4 in the lens. Nox4 is an inducible enzyme, responsible for the production of the superoxide anion radical and hydrogen peroxide.<sup>20</sup> We have shown that TGF- $\beta$ -driven Nox4 protein expression leads to concomitant ROS production in the lens and now plan to identify how this contributes to the development of TGF- $\beta$ -mediated cataract. Based on these findings, we hypothesize that TGF- $\beta$ -driven Nox4-dependent oxidant production has a role in the development of an EMT in the lens and resultant cataract.

## METHODS

### Animals

Tissue was collected from postnatal-day-21 albino Wistar rats (*Rattus norvegicus*) that were euthanized by asphyxiation and subsequent cervical dislocation. All procedures were approved by the Animal Ethical Review Committee of the University of Sydney, Australia and were carried out in accordance with the National Health Medical Research Council (Australia) guidelines and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research (United States).

### Rat Explant Preparation

Lens epithelial explants were prepared as described previously<sup>21</sup> and cultured in Medium 199 with Earle's salts (Life Technologies, Waltham, MA, USA), supplemented with 50  $\mu$ g/mL L-glutamine, 50 IU/mL penicillin/50  $\mu$ g/mL streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), 2.5  $\mu$ g/mL Amphostat B (Thermo Fisher Scientific), and 0.1% BSA (Sigma-Aldrich Corp., St. Louis, MO, USA). M199 was equilibrated at 37°C, 5% CO<sub>2</sub>. To induce EMT, recombinant TGF- $\beta$ 2 was added to media in each culture dish at a working concentration ranging from 200 pg/mL up to 1 ng/mL (R&D Systems, Minneapolis, MN, USA). Before the addition of TGF- $\beta$ 2, some explants were pretreated with the pan-NADPH oxidase inhibitor VAS2870 at a working concentration of 5  $\mu$ M. The progression of EMT in live cells was observed and captured using phase-contrast microscopy (Olympus CK2, Tokyo, Japan) and a digital camera (Leica DFC-280; Leica Camera, Wetzlar, Germany). Percentage cell loss quantification was performed using the thresholding function of ImageJ (National Institutes of Health [NIH], Bethesda, MD, USA), such that cells could be distinguished from bare lens capsule.

### Immunofluorescence Analysis

At the end of the culture period, explants were fixed in 100% methanol for 45 seconds, followed by 3 consecutive 15-second rinses in PBS. Explants were blocked in 10% normal goat serum (NGS) for 1 hour at room temperature. Excess normal goat serum was removed and primary antibody was applied, diluted in 0.15% NGS/PBS supplemented with 1% BSA. Dishes were left to incubate overnight at 4°C in a humidified chamber.  $\alpha$ -Smooth muscle actin ( $\alpha$ SMA) was labeled with a specific monoclonal mouse antibody (Sigma-Aldrich Corp.), diluted 1:100. Nox4 was labeled with a specific polyclonal rabbit antibody (Santa Cruz Biotechnology, Dallas, TX, USA), diluted

1:50. E-cadherin was labeled with a specific polyclonal mouse antibody (BD Biosciences, North Ryde, NSW, Australia), diluted at 1:100.

The following day, dishes were equilibrated to room temperature and subject to 3  $\times$  5 minute washes in PBS/BSA. An appropriate secondary antibody was diluted in PBS/BSA and applied to each explant for 2 hours in dark conditions.  $\alpha$ -Smooth muscle actin and E-cadherin were detected using goat anti-mouse Alexa-Fluor 488 (Cell Signaling, Danvers, MA, USA). Nox4 was detected using an anti-rabbit whole IgG conjugated to Cy3 (Sigma-Aldrich Corp.). A dilution of 1:1000 was used for all secondary antibodies. Dishes subsequently were rinsed in PBS/BSA and a 1:2000 solution of bisbenzimidazole (Hoechst dye) diluted in PBS/BSA was applied for 3 minutes to visualize cell nuclei. Pending microscopy, explants were mounted in 10% glycerol/PBS. Immunofluorescent labeling was viewed and captured using epifluorescence microscopy (Leica-DMLB; Leica Camera), and a digital camera (MicroPublisher 3.3 RTV; Q-Imaging, Surrey, BC Canada). Where appropriate, fluorescence also was visualized and images were collected using a Zeiss LSM-5Pa confocal microscope (Carl Zeiss AG, Jena, Germany).

### RT-PCR mRNA Analysis

After appropriate treatment, lens epithelial explants were placed into a 1.5 mL centrifuge tube. Total RNA extraction was performed using a Quick-RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions. First-strand cDNA synthesis was done using 150 ng of RNA with a reverse transcription system (Bioline, Eveleigh, NSW, Australia) according to the manufacturer's instructions. Control reactions omitting reverse transcriptase were set up and later tested, confirming that genomic DNA was absent from the purified RNA template (data not shown). For PCR amplification, the MyTaq Red DNA Polymerase (Bioline) kit was used, according to manufacturer's instructions. Primers used for the detection of Nox1-4 and GAPDH mRNA were based on those previously published.<sup>22</sup> For detection of Nox1-4 mRNA, sequence-specific primers were used: Nox1: Fw: 5'-CTTCCTCACTGGCTGGGATA-3', Rev: 5'-TGACAGCATTGCGCAGGC-3'; Nox2: 5'-CCAGTGAA GATGTGTTTCAGCT-3', 5'-GCACAGCCAGTAGAAGTAGAT-3'; Nox3: 5'-GTGTCCTTCGTGGTTGCTCT-3', 5'-TCTACAGCA CAGCTCGTTC-3'; Nox4: 5'-AGTCAAACAGATGGGA-3', 5'-TGTCATATGAGTTGTT-3'; GAPDH: 5'-TGAACGGGAAGCT CACTGG-3', 5'-TCCACCACCCTGTTGCTGTA-3'. cDNA was amplified for 35 cycles: Nox1 and Nox2: 95°C for 45 seconds, 51°C for 45 seconds, and 72°C for 45 seconds; Nox1, Nox3, and GAPDH: 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds. Amplified DNA was visualized with SYBR Safe DNA Gel Stain (diluted at 1:10,000; Invitrogen, Carlsbad, CA, USA) in a 2% agarose TAE gel, run at 100 V for 20 minutes. Positive control tissues for each Nox homologue also were examined (Nox1, rat colon; Nox2 and Nox4, rat kidney). We could not validate Nox3 expression given it is predominantly expressed in inner ear.

### Staining of ROS Using Dihydroethidium (DHE)

Dihydroethidium (Life Technologies) was added to explants in each dish containing 1 mL of medium to yield a working concentration of 30  $\mu$ M. Dishes were returned to the incubator for 30 minutes. Explants then were rinsed in cold PBS (3  $\times$  15 seconds) before being mounted in 10% glycerol/PBS. In its reduced form, DHE fluoresces blue, but when exposed to ROS, including the superoxide anion radical and hydrogen peroxide, it readily reacts to form the oxidized red fluorescent DHE form<sup>23,24</sup> that can be viewed readily using epifluorescence

microscopy. Fluorescence microscopy gives an indication of the level of total ROS produced by TGF- $\beta$ 2-dependent Nox4; however, absolute quantification of specific types of ROS cannot be determined using this method.

### SDS-PAGE and Western Blotting

After appropriate treatment, lens epithelial explants were placed into a 1.5 mL tube with cold lysis buffer containing 2.5 mM EDTA, 25 mM Tris, 0.375M NaCl, 250 mM sodium orthovanadate, 10 mM sodium deoxycholate, and a protease inhibitor cocktail (complete, Mini; Roche, Basel, Switzerland). Tubes were rotated for 2 hours and protein lysates were extracted by centrifuging for 15 minutes (15,500g, 4°C). The Direct Detect spectrophotometer system (Merck Millipore, Bayswater, VIC, Australia) was used to determine the protein concentration for each lysate, according to manufacturer's instructions.

Equal amounts of protein per sample were loaded onto 10% SDS-PAGE gels for electrophoresis at 200 V for 1.5 hours and transferred onto a polyvinylidene fluoride membrane (Merck Millipore) at 100 V for 1 hour. Membranes then were incubated with a blocking solution containing 5% skim milk diluted in 0.1% Tween-20 in tris-buffered saline (TBS-T) for 1 hour at room temperature and probed overnight at 4°C with specific antibodies, appropriately diluted in blocking buffer. Rabbit monoclonal anti-Nox4 antibody (Santa-Cruz Biotechnology) was diluted at 1:200, mouse monoclonal anti- $\alpha$ -SMA (Sigma-Aldrich Corp.) was diluted at 1:2000 and mouse monoclonal anti-GAPDH (Sigma-Aldrich Corp.) was diluted at 1:5000. Membranes were then rinsed (3  $\times$  5 minutes) in TBS-T and anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (diluted at 1:5000 in TBS-T) were applied to each membrane and incubated at room temperature for 2 hours with gentle rocking. Membranes were rinsed (3  $\times$  5 minutes) with TBS-T and an equal volume of peroxidase and luminol reagent was applied immediately before imaging using the ChemiDoc MP imaging system (Bio-Rad Laboratory, Hercules, CA, USA).

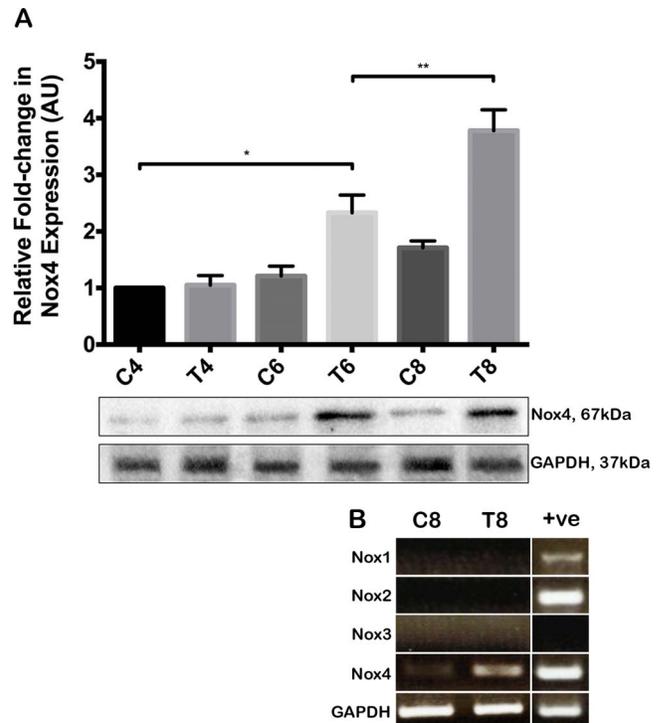
## RESULTS

### TGF- $\beta$ Induces Nox4 Expression

Nox4 expression in TGF- $\beta$ -driven lens EMT was assayed using immunolabeling of lens epithelial explant lysates. Specifically, we determined Nox4 expression using Western blotting at different time points (0, 2, 4, 6, and 8 hours). Nox4 expression was initially induced at 6 hours following treatment with TGF- $\beta$ 2 (Fig. 1), and this level was significantly different to that of the untreated control at the same time period (Fig. 1A, C6 vs. T6,  $P = 0.0392$ ). Expression of Nox4 also was significantly more pronounced at 8 hours to that observed at 6 hours (Fig. 1A, T6 vs. T8,  $P = 0.0069$ ). At 8 hours after TGF- $\beta$ 2 treatment, the level of Nox4 protein expression was approximately 2.5-fold higher to that of the matching control without TGF- $\beta$ 2 (Fig. 1A, C8 vs. T8,  $P = 0.0003$ ). This increase in Nox4 protein expression also was accompanied by an upregulation in Nox4 mRNA transcription in TGF- $\beta$ 2-treated explants compared to untreated explants at 8 hours (Fig. 1B). This was not the case for other Nox homologues, Nox1-3 (Fig. 1B).

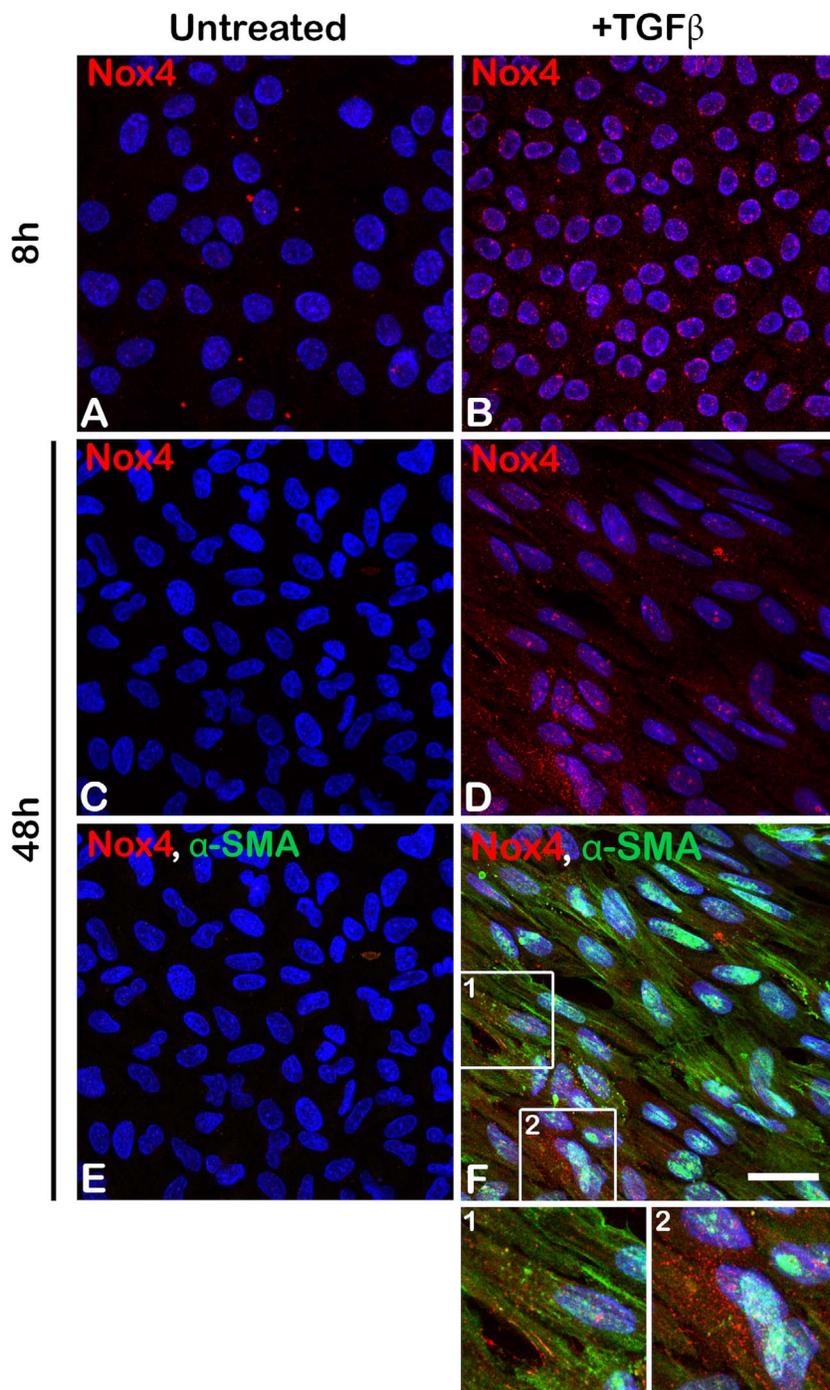
### Nox4 Exhibits Variable Subcellular Localization Patterns Over Time

Given that the expression level of Nox4 was more pronounced at 8 hours after TGF- $\beta$ 2 treatment, this time-point was used to examine the spatial localization of Nox4 in rat lens epithelial cells using immunofluorescence and



**FIGURE 1.** Transforming growth factor- $\beta$ 2 induces an increase in Nox4 expression in lens epithelial explants: Western blot. (A) Representative Western blot depicting the time-course (4, 6, and 8 hours) of Nox4 expression following TGF- $\beta$ 2 treatment (T4, T6, and T8), compared to control explants (C4, C6, and C8). Image is representative of 3 independent experiments. Above: Densitometry was performed to normalize the expression of Nox4 protein to GAPDH expression. Upon TGF- $\beta$ 2 treatment, Nox4 expression was significantly upregulated at 6 hours (T6), and these levels significantly increased by 8 hours (T8). Data are presented as mean  $\pm$  SEM. One-way ANOVA, Tukey's post hoc test (2-tailed  $t$ -test) \* $P < 0.05$  and \*\* $P < 0.01$ . (B) A representative RT-PCR of Nox1-4 mRNA transcripts after 8 hours, in the absence (C8) and presence (T8) of TGF- $\beta$ 2. Positive controls are included for Nox1, 2, and 4. For each lane, the PCR products shown correspond to the expected base pair length (Nox1, 201 base pairs [bp]; Nox2, 149 bp; Nox3, 234 bp; Nox4, 191 bp, and GAPDH, 287 bp).

confocal microscopy (Fig. 2). In untreated cells, some punctate labeling was observable, and appeared to localize throughout the cytosol (Fig. 2A). This was in contrast to the two distinct expression patterns of Nox4 seen at 8 hours following TGF- $\beta$ 2 treatment (Fig. 2B). Specifically, increased Nox4 expression was seen throughout all TGF- $\beta$ 2-treated epithelial cells at 8 hours, with more pronounced punctate labeling in the cell nuclei and perinuclear areas. Interestingly, Nox4 localization in lens epithelial cells treated for longer periods with TGF- $\beta$ 2, at 48 hours, was predominantly cytosolic (Fig. 2D), with an overall increase in punctate labeling within these areas. These cells also labeled for the myofibroblast marker,  $\alpha$ -SMA (Fig. 2E), which was first detectable by 24 hours after TGF- $\beta$ 2 treatment (data not shown). At 48 hours treatment, Nox4 appeared to colocalize with particular areas of the  $\alpha$ -SMA reactive stress fibers (see Fig. 2, inset 1), colabeled yellow. This colocalization of Nox4 and  $\alpha$ -SMA has been observed in other models of TGF- $\beta$ -driven EMT.<sup>25</sup> Interestingly, not all cells with a strong Nox4 label colabeled with  $\alpha$ -SMA (see Fig. 2, inset 2). Similar to untreated cells at 8 hours, untreated cells at 48 hours yielded negligible Nox4 expression and  $\alpha$ -SMA-positive stress fiber formation.

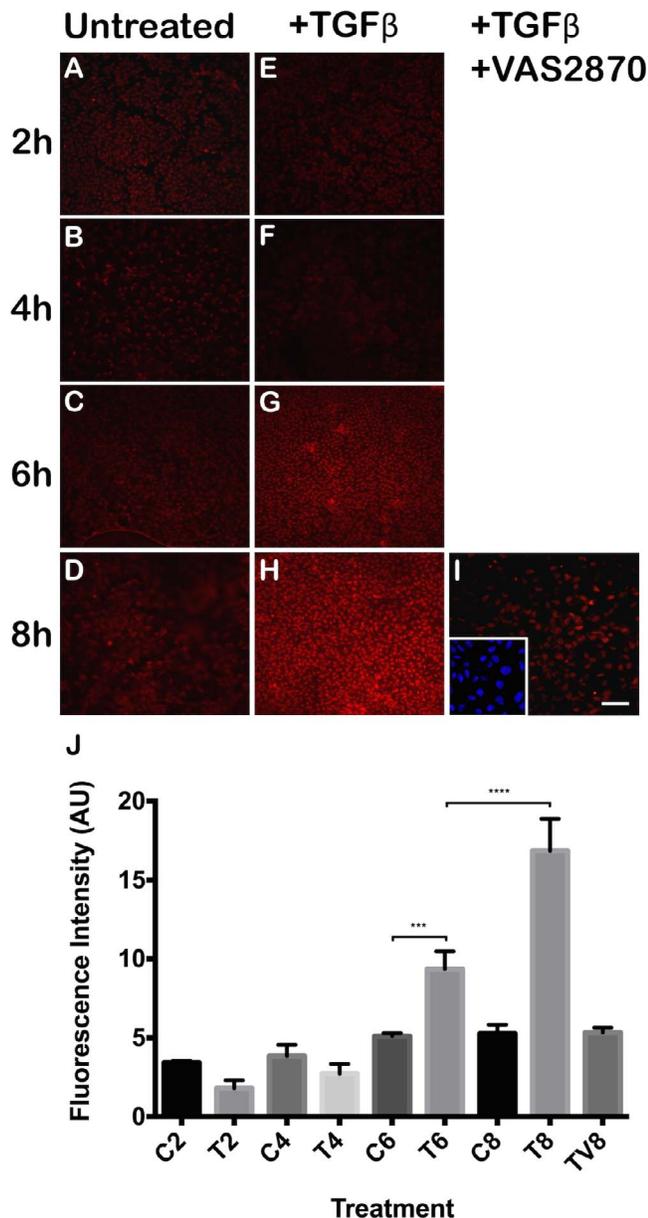


**FIGURE 2.** Transforming growth factor-β2 induces variable Nox4 localization patterns in lens epithelial cells with the progression of TGF-β induced EMT. Nox4 (red) was merged with the cell nuclear Hoechst dye (blue). Untreated explants at 8 (A) and 48 (C) hours exhibited little Nox4 expression. Explants treated with TGF-β2 (+TGF-β2) exhibited a stronger distinct punctate label by 8 hours (B) that primarily localized to the perinuclear regions of the cells. At 48 hours (D), this label became more pronounced throughout the cytosol. By 48 hours compared to control explants (E), epithelial cells treated with TGF-β2 also labeled for α-SMA (green; [F], with α-SMA labeled-stress fibres colocalizing with Nox4 (shown at higher magnification, *Inset 1*). Punctate cytosolic distribution of Nox4, independent of stress fibers, also was apparent in some cells ([F], shown at higher magnification, *Inset 2*). Images are representative of 3 independent experiments, consisting of 3 replicates per group. Scale bar: 40 and 20 μm for the *inserts*.

**TGF-β2 Induces Nox4-Derived ROS Production**

To validate the specificity of the Nox4 label seen in response to TGF-β2 treatment, we repeated our immunolabeling experiments with the pan-NADPH oxidase inhibitor VAS2870 (Fig. 3).<sup>26</sup> Inhibition of NADPH oxidase blocked Nox4 protein

expression in lens epithelial cells in response to TGF-β2 treatment (Fig. 3I, inset) when compared to TGF-β2-treated cells without the inhibitor (see Fig. 2B). We next determined whether this TGF-β2-dependent expression of Nox4 was accompanied with NADPH oxidase activity, using DHE fluorescence microscopy to assay for ROS production in lens



**FIGURE 3.** The ROS probe, DHE, was used to assess increases in ROS following TGF- $\beta$ 2 treatment. In untreated explants, basal levels of ROS were observable (A–D). Importantly, TGF- $\beta$ 2 treated explants yielded noticeable increases in ROS by 6 hours, that increased up to 8 hours (H). Treatment with VAS2870 blocked TGF- $\beta$ 2-induced ROS (I) as shown by diminished fluorescence intensity, with less ROS produced. This was in conjunction to VAS2870 also blocking Nox4 protein expression (see [I] inset). Representative images from 3 independent experiments, consisting of at least 2 replicates for each treatment group. Scale bar: 100  $\mu$ m (A–D) and 20  $\mu$ m for (inset [I]). Total fluorescence intensity also was calculated using ImageJ (J), with significant increases after 6 and 8 hours, effectively blocked by VAS2870. Data are presented as mean  $\pm$  SEM. One-way ANOVA, Tukey's post hoc test (2-tailed *t*-test) \*\*\**P* < 0.001 and \*\*\*\**P* < 0.0001.

epithelial cells. As Nox4 was first detected between 6 and 8 hours after TGF- $\beta$ 2 treatment, DHE reactivity was assayed before and at these time points. Reassuringly, Nox4 expression was accompanied by a significant increase in NADPH oxidase specific activity (DHE oxidation) in TGF- $\beta$ 2-treated lens explants (Figs. 3G, 3H) compared to the control explants not

treated with TGF- $\beta$ 2 (Figs. 3C, 3D) at 6 and 8 hours (Fig. 3J, C6 vs. T6, *P* = 0.0003; Fig. 3J, T6 vs. T8, *P* = 0.0005).

Further earlier time points that did not exhibit Nox4 expression (2 and 4 hours) did not produce a DHE label, either in the presence or absence of TGF- $\beta$ 2 (Fig. 3). In addition, when TGF- $\beta$ -driven Nox4 expression was inhibited with VAS2870 (see also Fig. 6B), ROS accumulation was abrogated (Fig. 3D).

### Nox4 Inhibition Abrogates TGF- $\beta$ 2-Induced Lens EMT

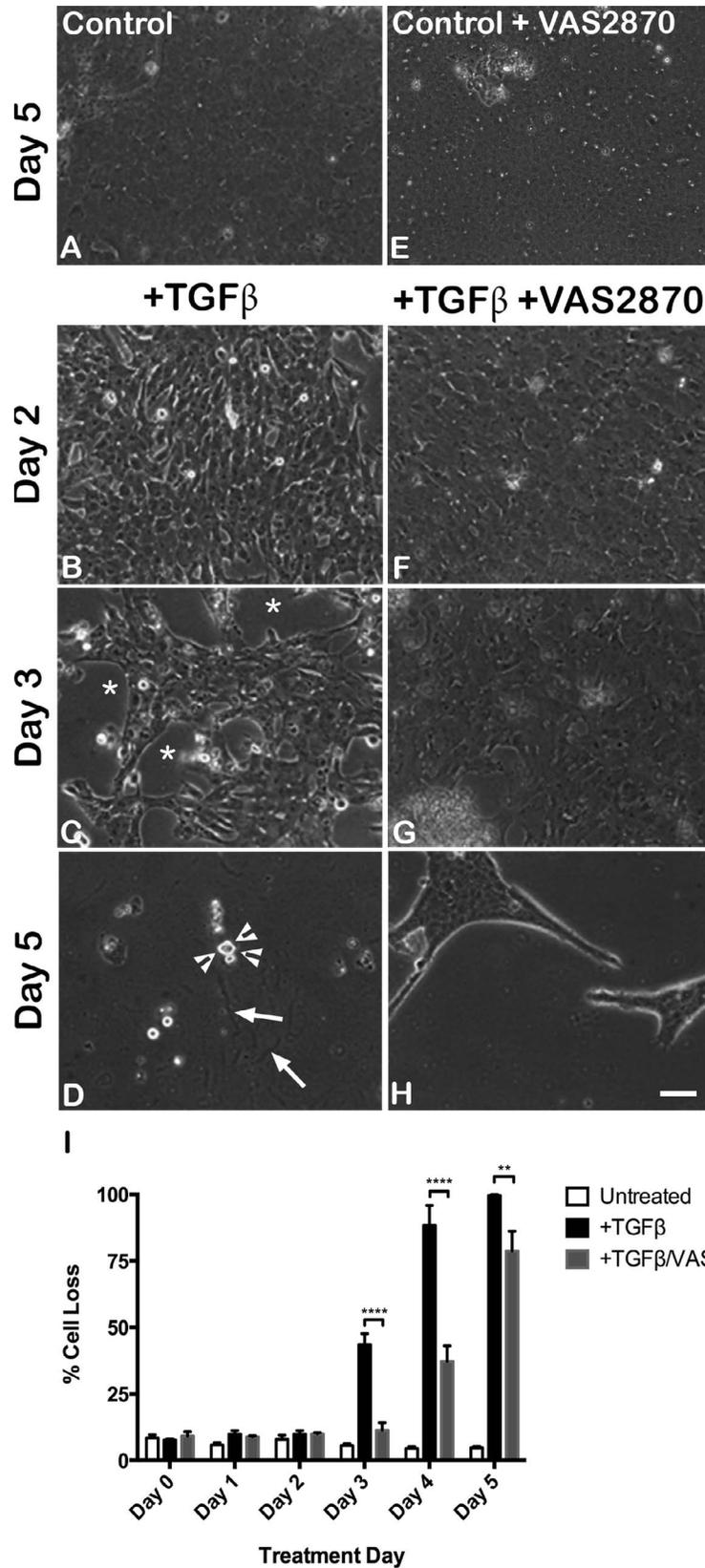
Given that we have shown Nox4 expression and activity is induced in lens epithelial cells and that we could inhibit Nox4 expression and concomitant activity with VAS2870, we next investigated the role Nox4 had in the development of TGF- $\beta$ -dependent lens EMT. The development of TGF- $\beta$ 2-induced lens EMT can be followed morphologically in lens explants using phase-contrast microscopy. In our experimental model, we observe EMT progression over 5 days. Specifically, cells begin to considerably elongate by day 2 of culture (Fig. 4B), and from day 3 exhibit progressive cell loss indicated by acellular patches (Fig. 4C, asterisk), cellular blebbing (Fig. 4D, arrowheads), and lens capsular wrinkling (Fig. 4D, arrows), with most cells lost by 5 days (Fig. 4D). Interestingly, in explants treated with TGF- $\beta$ 2 in the presence of VAS2870, cellular loss was significantly delayed by day 3 and most pronounced at 5 days after TGF- $\beta$ 2 treatment (Fig. 4I; Day 3, +TGF $\beta$  vs. +TGF $\beta$ /VAS, *P* = 0.0001 and Day 5 +TGF $\beta$  vs. +TGF $\beta$ /VAS, *P* = 0.005). Moreover, cells in these explants did not appear to undergo TGF- $\beta$ 2-dependent cellular elongation, with cells remaining on day 5 of culture, exhibiting a typical uniformly packed lens epithelial phenotype (Fig. 4H). This was confirmed by labeling for E-cadherin in these cells (Fig. 5B). VAS2870-alone treatment had no effect on E-cadherin labeling (5A). Moreover, consistent with the absence of cell elongation earlier in the culture period, indicating the absence of myofibroblastic cells that are thought to promote lens capsular wrinkling, under these conditions there was no apparent capsular wrinkling (Fig. 4H).

### Nox4 Modulates TGF- $\beta$ -Mediated $\alpha$ -SMA Expression

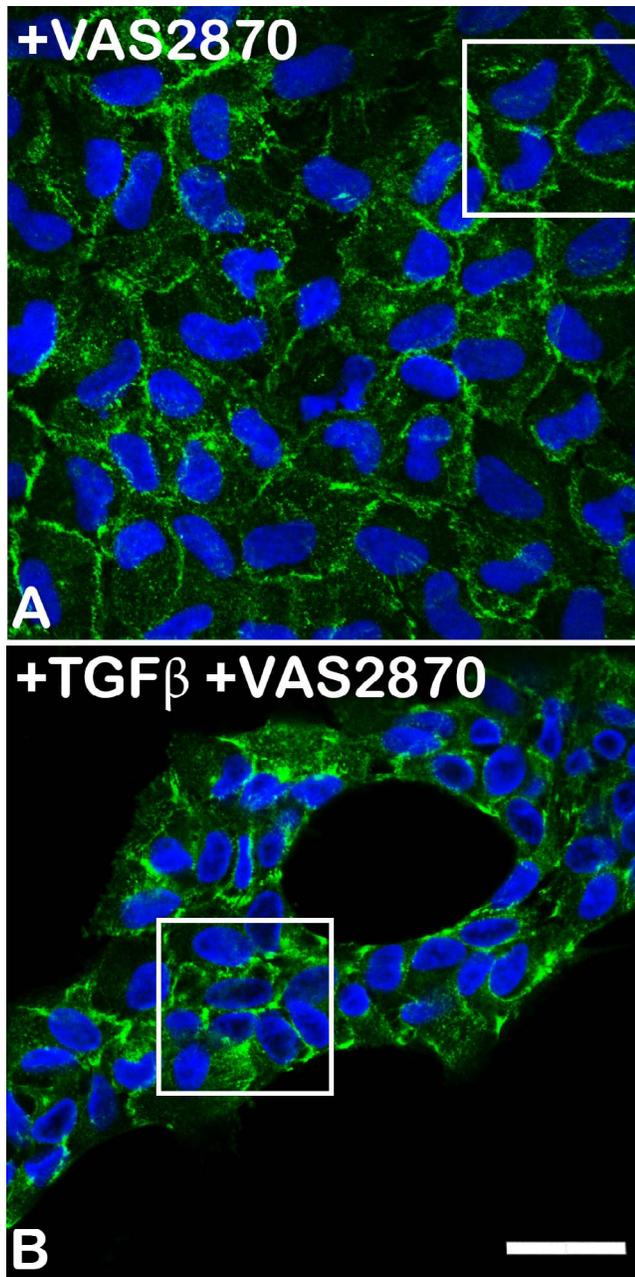
As Nox4 was previously shown (Fig. 2D) to localize with some  $\alpha$ -SMA-reactive stress fibers, it was proposed that Nox4 may have a role in  $\alpha$ -SMA accumulation. Cells treated with both VAS2870 and TGF- $\beta$ 2 yielded undetectable Nox4 expression (Figs. 6B, 6G), compared to explants treated with TGF- $\beta$ 2 alone (Figs. 6E, 6G), and in the process halted the progression of EMT, with little to no detectable labeling for  $\alpha$ -SMA expression in cells (Fig. 6G) that retained their epithelial-like appearance (see Hoechst staining, Figs. 6A, 6D).

### DISCUSSION

In the present study, for the first time, we describe the novel temporal and spatial expression of Nox4 and characterize its activity in the rat lens explant system following TGF- $\beta$  treatment. We demonstrated that Nox4 is the only Nox homologue expressed in rat lens epithelial explants, and is responsive to TGF $\beta$  consistent with other studies.<sup>14</sup> Nox4 inhibition using a pan-NADPH oxidase inhibitor slowed the progression of EMT through the promotion of cell survival. Furthermore, we also impeded the expression of the known myofibroblastic EMT marker,  $\alpha$ -SMA, and subsequent development of capsular wrinkling. The remaining/surviving cells on



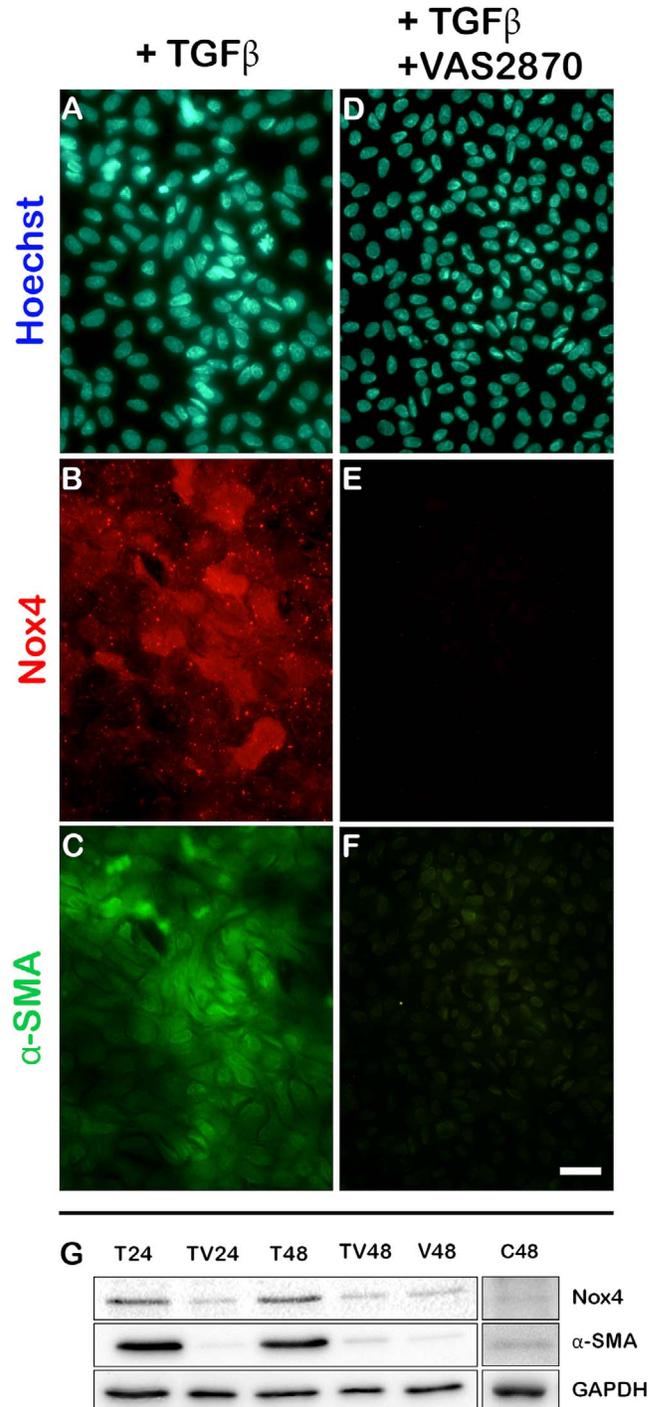
**FIGURE 4.** Suppression of TGF- $\beta$ 2-induced EMT using VAS2870. Phase contrast images of explants at days 2 (**B, F**), 3 (**C, G**), and 5 (**A, E, D, H**). Explants treated with TGF- $\beta$ 2 alone (**B–D**) underwent morphologic changes associated with EMT compared to untreated cells (**A**). Explants treated with VAS2870 and TGF- $\beta$ 2 (**F–H**) showed a delayed EMT response, with less cell elongation apparent by Day 2 (**F**), reduced cell loss by Day 3 (**G**) and the retention of epithelial-like cells by Day 5 (**H**). Note that, VAS2870 alone (**E**) had little effect on cells treated up to 5 days of culture. Images are representative of 3 independent experiments, consisting of 3 replicates per group. Scale bar: 100  $\mu$ m. (**I**) Percentage cell loss was calculated using the threshold feature of ImageJ in relation to areas of bare lens capsule, with significant cell loss in TGF- $\beta$ 2-treated explants from day 3, which was abrogated by application of VAS2870. Data are presented as mean  $\pm$  SEM. Two-way ANOVA, Tukey’s post hoc test (2-tailed *t*-test) <sup>\*\*</sup>*P* < 0.01 and <sup>\*\*\*\*</sup>*P* < 0.0001.



**FIGURE 5.** Maintenance of E-cadherin expression in cells cotreated with TGF- $\beta$ 2 and VAS2870. Explants were treated with VAS2870 (A), or cotreated with TGF- $\beta$ 2 and VAS2870 (B) and fixed at 5 days of culture. Immunolabeling for the membrane marker E-cadherin (green) was performed and nuclei were counterstained with Hoechst (blue). In cells treated only with VAS2870 (A), E-cadherin localized to the cell membrane and there was no apparent cell loss. Cotreatment with TGF- $\beta$ 2 and VAS2870 promoted increased cell survival and the maintenance of a membranous E-cadherin label (B).

the lens capsule following the Nox4 blockade retain an epithelial-like phenotype and do not progress down the EMT pathway, highlighting an important role for Nox4 in TGF- $\beta$ -dependent EMT in the lens.

Reactive oxygen species are known to have a key role in the development of cataract. Deletion of known antioxidants leads to the development of cataract. These antioxidants include the glutathione synthesis machinery,<sup>3</sup> the detoxification enzyme peroxiredoxin,<sup>1</sup> and glutaredoxins,<sup>2</sup> that are responsible for



**FIGURE 6.** VAS2870 blocks TGF- $\beta$ 2-induced Nox4 and  $\alpha$ -SMA expression. Explants cultured for 24 hours in the presence of TGF- $\beta$ 2 (A-C), or TGF- $\beta$ 2 and VAS2870 (D-F) were immunolabeled for Nox4 (B, E),  $\alpha$ -SMA (green; [C, F]) and counterstained with Hoechst dye (A, D). Compared to explants treated with TGF- $\beta$  alone (A-C), VAS2870 was shown to block TGF- $\beta$ 2-induced Nox4 (E) and  $\alpha$ -SMA (F) expression. Images are representative of 3 independent experiments, consisting of 3 replicates per group. Scale bar: represents 50  $\mu$ m. (G) Protein lysates also were collected from explants treated with and without TGF- $\beta$ 2 and VAS2870 and Western blotting demonstrated similar reductions in Nox4 and  $\alpha$ -SMA expression in the presence of VAS2870 at 24 and 48 hours. GAPDH expression remained constant regardless of treatment.

the maintenance of the thiol redox status of lens proteins, including the crystallins. Previous studies have shown the indirect role of ROS in the development of TGF- $\beta$ -dependent cataract in rats.<sup>17</sup> In these studies, addition of the antioxidant GSH was shown to strongly suppress TGF- $\beta$ -induced opacification and subcapsular plaque formation in whole rat lens cultures. Furthermore, microarray studies using TGF- $\beta$ -treated human lens cell lines highlighted that Nox4 was not only induced in response to TGF- $\beta$ , but was one of the highest induced transcripts in these cultured human lens epithelial cells compared to controls.<sup>14</sup>

While we demonstrate Nox4 to be involved in TGF- $\beta$ -dependent EMT in lens, it has previously been reported to be involved in a number of other different models of EMT. For example, in breast cancer cells, TGF- $\beta$  has been shown to induce Nox4 expression and activity (by measuring the production of ROS in the presence of a NADPH oxidase inhibitor<sup>19</sup>). Furthermore, in this earlier study, TGF- $\beta$ -dependent Nox4 expression and activity was dependent on the Smad signaling pathway, given inhibition of this pathway (via siRNA or pharmacological inhibition) inhibited Nox4 labeling/activity and abolished the development of an EMT.<sup>19</sup> Additionally, blocking NADPH oxidase activity alone (either pharmacologically or by siRNA), also prevented EMT in breast cancer cells,<sup>19</sup> highlighting that Nox4 expression and activity was responsible for the development of EMT in breast cancer cells.

It is likely that TGF- $\beta$  signals through the Smad pathway to induce Nox4 expression and activity in lens. In future studies, it would be of great interest to determine whether direct or indirect TGF- $\beta$ -Smad signaling contributes to the differential nuclear (at 8 hours) and cytosolic (48 hours) Nox4 labeling we observed here. Furthermore, Nox4 expressed at later time points in the EMT process may directly influence the actin cytoskeleton with which it is colocalized. Novel studies in the kidney have highlighted the role of Nox4 in actin cytoskeleton rearrangement,<sup>27</sup> leading to regulation of focal adhesion turnover and VSMC migration.<sup>28</sup> It is feasible that this also could occur in lens epithelial cells undergoing EMT. Members of the Rho GTPase family are strongly associated with the formation of the actin cytoskeleton. Manickam et al.<sup>29</sup> ascertained that RhoA/ROCK activation is upstream of Poldip2 and Nox4 in TGF- $\beta$  signaling in the kidney, and controls myofibroblast activation. Indeed, in the rat lens explant system we observe cell blebbing and cell loss through the activation of apoptotic pathways.<sup>28</sup> Cellular blebbing is an indirect indication of apoptosis, where cells are physically dismantled from within by modulation of the actin cytoskeleton. This process is mediated by members of the proapoptotic caspase family, where the plasma membrane is weakened allowing the cytosol to protrude from the cell.<sup>30</sup> Caspases have been shown to cleave and activate Rho kinase (ROCK I), a downstream effector of RhoA signaling.<sup>31</sup> Activation of ROCK I leads to the formation of actin stress fibers, cell contraction and dynamic membrane blebbing induced by active ROCK I.<sup>30</sup> Therefore, it is feasible that the changes seen in the TGF- $\beta$ -driven EMT in the lens may signal at least in part through the RhoA/ROCK pathway via Nox4.<sup>32</sup>

Reflective of its multiple roles in proinflammatory responses, migration, differentiation and apoptosis, Nox4-derived ROS appear enigmatic in their downstream targets. Using TGF- $\beta$ -stimulated human aortic muscle cells, Martin-Garrido et al.<sup>33</sup> found that silencing of Nox4 abolished the formation of stress fibers and  $\alpha$ -SMA accumulation in a MAPK/p38-dependent manner. This is in contrast to research using fibroblastic cells that report the c-jun N-terminal kinase (Jnk pathway) as capable of regulating  $\alpha$ -SMA accumulation<sup>34</sup> in a Nox4-dependent fashion. Together, these findings from other fibrotic models and the lens suggest that the progressive accumulation of  $\alpha$ -SMA is the result of a heterogeneous process that

converges on the  $\alpha$ -SMA promoter, and that Nox4 may act as a common mediator of these pathways. More broadly, this is indirect evidence to the notion that redox signaling is capable of serving as a nexus between parallel signaling pathways, such as Smads and MAPKs.

The present study shows for the first time to our knowledge that Nox4 has a role in TGF- $\beta$ -driven lens EMT. In particular, Nox4 appears to have a crucial role in the regulation of  $\alpha$ -SMA, potentially apoptosis, cell elongation and capsular wrinkling. The findings of this study provide new avenues for the potential treatment of cataract and the prevention of secondary cataract, given TGF- $\beta$ -driven EMT is integral to the development of some cataracts in humans.<sup>35</sup> Future studies will concentrate on expanding these findings in genetic models of TGF- $\beta$ -dependent cataract, ascertaining the upstream events crucial to Nox4 expression and delineating the specific targets of ROS during lens EMT.

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