

# Altered Stability of mRNAs Associated with Glaucoma Progression in Human Trabecular Meshwork Cells Following Oxidative Stress

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**PURPOSE.** The goals of this study were to determine if oxidative stress on human trabecular meshwork (HTM) cells influences the stability of key mRNAs containing AU rich elements (AREs) known to be associated with glaucoma progression, and if the presence of topographic cue alters the stability of these mRNAs.

**METHODS.** HTM cells were treated with 300  $\mu$ M hydrogen peroxide ( $H_2O_2$ ) for 1 hour in the presence of 5  $\mu$ g/mL actinomycin D and compared with untreated cells. The selected mRNAs (IL-6, IL-8, myocilin, SPARC [secreted protein, acidic and rich in cysteine], matrix metalloproteinase [MMP]-3, and MMP-9) from the cells were analyzed by using relative quantitative PCR. Immunohistochemistry for Hu antigen R (HuR) was performed in addition to Western blots of HuR. HTM cells were also grown on topographically patterned surfaces, and IL-6 mRNA was analyzed by quantitative PCR.

**RESULTS.**  $H_2O_2$  increased IL-6 mRNA stability 0.145 (0.095–0.27) to 0.345 (0.2–0.48) (normalized ratio, median [interquartile range]) ( $n = 5$ ), while IL-8 mRNA was increased from 0.565 (0.408–0.6) to 0.775 (0.486–0.873) ( $n = 5$ ). These differences were statistically significant ( $P = 0.0313$ , for both IL-6 and IL-8; Wilcoxon signed-rank test). The mRNAs of myocilin, SPARC, and MMP-3, which do not have AREs, were more stable after actinomycin D treatment and were not altered with oxidation. Western blot and immunohistochemistry demonstrated that  $H_2O_2$  treatment induces the translocation of HuR from the nucleus to the cytoplasm. Nanopatterned surfaces did not alter IL-6 mRNA stability.

**CONCLUSIONS.** Oxidative stress stabilizes IL-6 and IL-8 mRNAs significantly. The decay of certain mRNAs associated with

glaucoma may be altered in the trabecular meshwork of glaucoma patients. (*Invest Ophthalmol Vis Sci.* 2012;53:1734–1741) DOI:10.1167/iovs.12-7938

Glaucoma is a major cause of blindness throughout the world. Despite the devastating consequences of this disease, the exact mechanisms leading to the onset and progression of glaucoma remain unknown. However, it is known that human trabecular meshwork (HTM) cells play a central role in the regulation of intraocular pressure (IOP) under both normal and disease conditions.

The antioxidative capacity of the aqueous humor of individuals with glaucoma is decreased compared with aqueous humor from normal individuals,<sup>1</sup> and this decrease in antioxidative capacity has been experimentally verified in a rabbit model of glaucoma.<sup>2</sup> In addition, increased oxidative stress in calf eyes resulted in a decrease in outflow facility.<sup>3</sup> Reactive oxygen species (ROS) were implicated in studies demonstrating the amount of oxidative DNA damage in the HTM paralleled IOP increases and visual field defects in patients with glaucoma.<sup>4–6</sup> Oxidative stress is present in glaucoma, and certain cytokines, specifically IL-6 and IL-8, have been shown to be increased in porcine trabecular meshwork (TM) cells as a result of oxidative challenge.<sup>7</sup> The concentration of IL-8 is elevated in the aqueous humor of primary open angle glaucoma (POAG) patients.<sup>8,9</sup> There is no direct evidence that IL-6 level is also elevated in POAG patients; however, IL-6 has been reported to be up-regulated in response to experimentally elevated IOP in human donor eyes.<sup>10</sup> In addition, the IL-6 level in the aqueous humor has been reported to be elevated in pseudoexfoliation syndrome<sup>11</sup> and in patients with neovascular glaucoma.<sup>12</sup> It is unclear why these cytokines are elevated, but the mRNAs for these cytokines have an AU-rich element (ARE) on the 3' untranslated regions (UTRs) that targets the mRNAs for rapid degradation.<sup>13,14</sup> Hu antigen R (HuR) is a member of the Hu family of proteins and binds to AREs.<sup>14–16</sup> HuR has been shown to stabilize mRNAs with AREs. HuR shuttles from the nucleus to the cytoplasm with these mRNAs, thereby protecting the mRNA associated with them from degradation.<sup>14</sup> At least 57 mRNAs, including IL-6, IL-8, and matrix metalloproteinase [MMP]-9, have been reported to be stabilized by HuR.<sup>17–21</sup> The increased levels of certain cytokines in patients with glaucoma suggest the normal mRNA degradation pathway for these cytokines in HTM cells is altered with disease.

Work in our laboratory has established that biophysical cues of nano to submicron topographic features play a fundamental role in determining HTM cellular behavior and gene expression.<sup>22</sup> Recent studies employing topographically patterned substrates consisting of anisotropically ordered grooves and ridges in the submicron range (mimicking fibers present in

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basement membrane) dramatically altered cell behaviors and protein expression in HTM cells. However, the influence of topographic cues on the stabilization of cytokine mRNAs has never been investigated for any cell type.

The purpose of this study was to determine if the oxidative stress on HTM cells associated with glaucoma influences the stability of key ARE-containing mRNAs. Since topography has been shown to influence a number of HTM cell behaviors, experiments were also conducted to explore the effects of this ubiquitous biophysical cue on the mRNA stabilization of mRNAs for cytokines reported to be upregulated in glaucoma. In addition, we examined the mRNAs of SPARC (secreted protein, acidic and rich in cysteine), myocilin, and MMP-3, which are known to be altered in glaucoma; however, their mRNAs lack AREs.

## MATERIALS AND METHODS

### Cell Cultures

HTM cells were obtained from corneal buttons that were deemed unsuitable for transplant (aged from 18 to 70 years; from the Heartland Lions Eye Bank, St. Louis, MO), as previously described.<sup>23</sup> All work was conducted according to the tenets of the Declaration of Helsinki. HTM cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 1:1 medium (HyClone, Logan, UT) with 10% fetal bovine serum and antibiotics. All HTM cells were used before the sixth passage. All cells were maintained in a 95% air/5% CO<sub>2</sub> atmosphere at 37°C.

To introduce oxidative stress, cells were washed with serum-free medium and subsequently incubated with serum-free medium for 2 hours followed by 1 hour incubation with 300  $\mu$ M H<sub>2</sub>O<sub>2</sub> (Fisher Scientific, Fair Lawn, NJ). In control cultures, the medium without H<sub>2</sub>O<sub>2</sub> was changed at the same time points.

### Real-Time PCR

The mRNA levels for IL-6, IL-8, MMP-9, MMP-3, SPARC, and myocilin were analyzed by real-time PCR. We cultured 300,000 to 500,000 cells in 6-cm dishes. Cells were processed routinely for analysis by PCR 3 days after plating.

For oxidative stress, HTM cells were washed with serum-free medium and subsequently incubated with serum-free medium for 1.5 hours. Then, the cells were treated with 5  $\mu$ g/mL actinomycin D that prevents additional mRNA synthesis ( $t = -30$  min) so that effects of H<sub>2</sub>O<sub>2</sub> on mRNA stability could be investigated independently from new mRNA transcription. After 30 min, cells were treated with or without 300  $\mu$ M hydrogen peroxide ( $t = 0$ ) and incubated for 1 hour ( $t = 1$  hour). The mRNA was extracted at  $t = -30$  min and  $t = 1$  hour with RNeasy kits according to the manufacturer's protocol (Qiagen, Valencia, CA).

The amount of mRNA was measured with the NanoDrop Spectrophotometer (ND-1000, Thermo Scientific, Rockford, IL), and 70 ng was used with the OneStep kit for real-time PCR (TaqMan; Applied Biosystems, Inc., [ABI] Foster City, CA). Individual reactions with the real-time PCR machine (StepOne; ABI) were performed with a total volume of 10  $\mu$ L. The reverse transcription reaction was performed for 30 minutes at 50°C followed by PCR enzyme activation for 10 minutes at 95°C. Forty cycles of 60°C for 1 minute followed by 95°C for 15 seconds were performed. GAPDH served as the reference RNA. We obtained the following probes from ABI: GAPDH (Mm99999905\_m1), 18S (Hs99999901\_s1), IL-6 (Hs00985639\_m1), IL-8 (Hs01567912\_g1), SPARC (Hs00277762\_m1), MMP-3 (Hs00968308\_m1), MMP-9 (Hs00234579\_m1), and myocilin (Hs00165345\_m1). The samples were done in triplicate and then normalized to the mean of  $t = -30$  minute samples.

### Immunofluorescence

HTM cells were plated on glass-bottom dishes treated with FNC coating mix (Athena Enzyme Systems, Baltimore, MD). After plating, the cells were allowed to attach to the dishes in normal media overnight. Next the cells were incubated for 2 hours in serum-free media and then treated with 300  $\mu$ M H<sub>2</sub>O<sub>2</sub> in serum-free medium for an additional 1 hour. The cells were fixed in 4% paraformaldehyde for 20 minutes. After washing with PBS, the cells were permeabilized with 0.5% Triton X-100 for 10 minutes. Then, the cells were blocked with 1% BSA in PBS for 30 minutes and washed again. The cells were incubated with anti-HuR antibody (sc-5261; 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature and secondary antibody (1:400 dilution; Alexa Fluor 488, Molecular Probe, Eugene, OR). Alexa Fluor 567 phalloidin (1:200 dilution; Molecular Probe) and 4',6'-diamino-2-phenylindole (DAPI; Molecular Probe) were used for fluorescent staining of the actin filaments and the cell nucleus, respectively. Images were obtained with the LSM 5 Pascal/Axiovert 200M microscope (Carl Zeiss Microscopy Japan, Tokyo, Japan).

### Western Blotting

HTM cells were cultured in T-75 flasks incubated for 2 hours in serum-free media after which they were treated with or without 300  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 1 hour in serum-free medium. The cells were then lysed with NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL) containing protease inhibitor cocktail (1:100 dilution; Pierce). Protein concentration was assessed using the BCA protein assay. Samples of 20  $\mu$ g were electrophoresed on a 10% polyacrylamide gel (Invitrogen, Carlsbad, CA) and the protein was transferred to nitrocellulose membrane. The membrane then was blocked in SuperBlock blocking buffer (Thermo Scientific) for 1 hour. The primary antibodies (anti-HuR diluted 1:1000; anti-lamin B1, ab16048, diluted 1:4000, Abcam, Cambridge, MA) or the antibody to GAPDH conjugated with horse radish peroxidase (diluted 1:20,000, ab9482, Abcam) were allowed to react for 1 hour at room temperature. Following washes in PBS containing 0.1% Tween 20 (PBST), we incubated the membrane with secondary antibody for 1 hour and washed again in PBST. A chemiluminescent detection kit (Immobilon Western; Millipore, Billerica, MA) was used to detect bands. The banding pattern was taken with the ImageQuant 350 (GE Healthcare, Piscataway, NJ) and quantified with accompanying software (ImageQuant TL7.0; GE Healthcare). Background signal was subtracted by the software. The HuR bands intensities were normalized for the GAPDH or lamin B1 loading controls, and then the ratios of oxidatively stressed sample to control sample were calculated for both cytoplasmic and nuclear fractions.

### Topographically Patterned Substrates

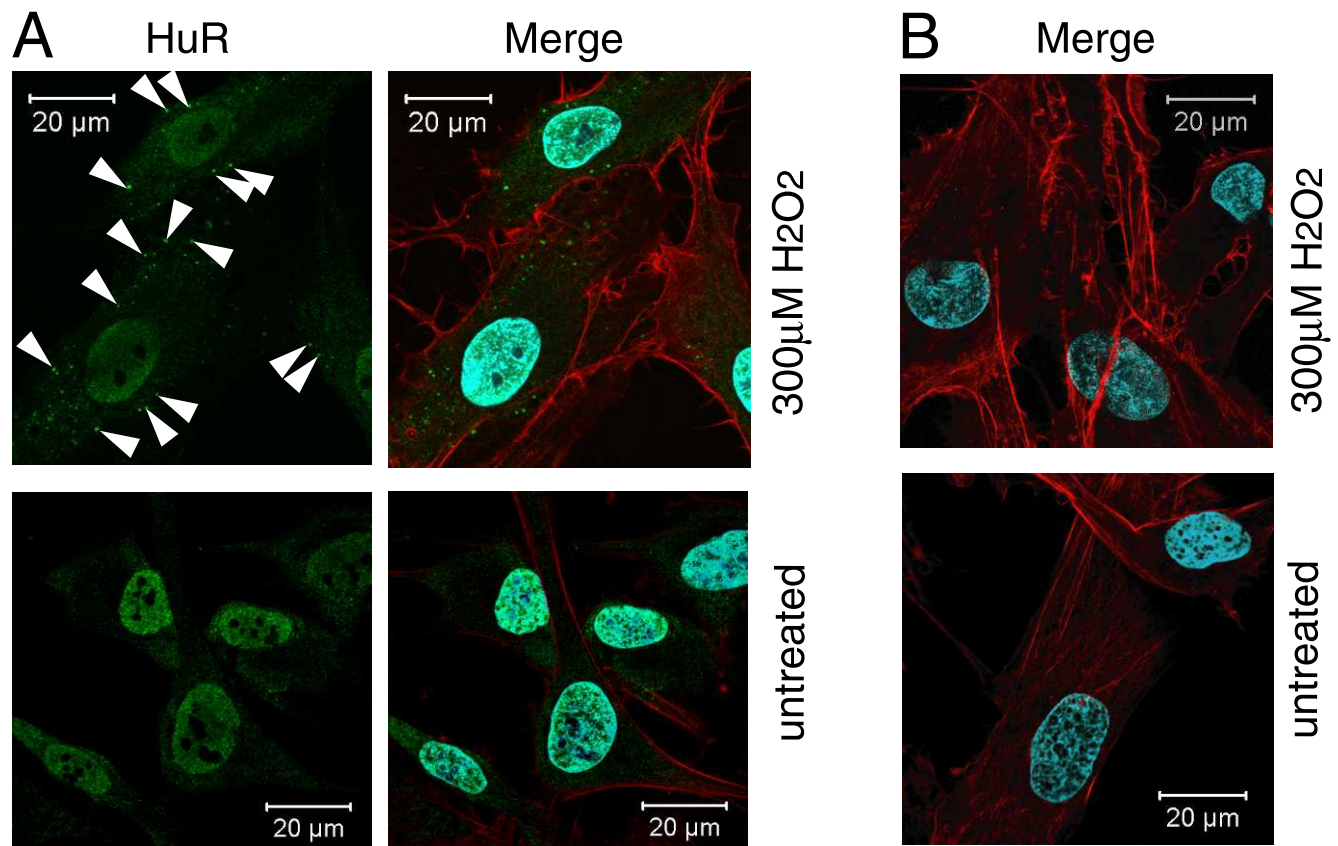
The topographically patterned and planar control substrates using optical adhesive (NOA 81; Norland Products Inc., Cranbury, NJ) were fabricated as previously reported.<sup>24</sup> Three-square-centimeter nano-grooved surfaces of 1400-nm pitch (700 nm equal ridge and groove widths with grooves having a 300-nm depth) were used. The substrates were coated with FNC coating mix prior to cell seeding.

### Statistical Analysis

The Wilcoxon signed-rank test was used to compare the mRNA stability with or without H<sub>2</sub>O<sub>2</sub> treatment. For Western blots, a two-sided *t*-test was used. The null hypothesis was that there would be no difference of the ratio of stressed sample to control sample. A *P* value of <0.05 was considered statistically significant. The statistical package used was JMP version 8.0 (SAS Institute, Cary, NC).

## RESULTS

H<sub>2</sub>O<sub>2</sub> induced translocation of HuR from the nucleus to the cytoplasm in primary HTM cells. Localization of HuR was



**FIGURE 1.** Subcellular localization of HuR in HTM cells. (A) HuR (green) was visualized by laser confocal microscopy on cultured cells that were either left untreated or treated with 300  $\mu$ M  $H_2O_2$  (1 hour). DAPI (blue) staining served to visualize the nucleus and phalloidin (red) staining visualized actin in the cytoplasm. Note the distinct overlap of DAPI and HuR signals; while  $H_2O_2$ -treated cells also exhibited abundant nuclear HuR, the treatment caused an increase in the cytoplasmic HuR (arrow heads). (B) Negative control without primary antibody to HuR showed no reaction with the secondary antibody.

detected faintly in the cytoplasm of untreated cells by laser confocal microscopy but was abundantly present in the nucleus. However, HuR in the cytoplasm increased following  $H_2O_2$  treatment, and abundant HuR signal was still seen in the nucleus after  $H_2O_2$  treatment (Fig. 1A). Negative control staining without anti-HuR antibody showed no reaction with the secondary antibody (Fig. 1B).

Protein lysates from the cytoplasm and the nucleus of HTM cells of four donors were analyzed by Western blot. Intensity analyses of the Western blots showed that HuR in the cytoplasm increased following  $H_2O_2$  treatment consistent with the immunofluorescent data. The analysis of HuR showed that it was increased 42% ( $n = 4$ , average ratio 1.42, 95% confidential interval 1.04–1.81,  $P = 0.04$ ,  $t$ -test) in the cytoplasm of the  $H_2O_2$ -treated cells compared to the control cells (Fig. 2). In contrast, there was no statistical difference in HuR band intensities in the nucleus ( $n = 4$ , average ratio 1.26, 95% confidential interval 0.66–1.86,  $P = 0.26$ ,  $t$ -test) (Fig. 2).

To select a proper internal control gene for real-time PCR analysis, we compared the 18S rRNA to the GAPDH mRNA expression. Samples from two out of three different individuals showed that GAPDH was less variable than the 18S after actinomycin D and  $H_2O_2$  treatment (Fig. 3). Therefore, GAPDH mRNA was used as an internal control gene in all experiments. As already stated, use of actinomycin D allowed us to follow mRNA stabilization without new transcription. Cell cultures derived from five different individuals were analyzed ( $n = 5$ ) for IL-6 mRNA stability. Exposure to  $H_2O_2$  increased IL-6 mRNA compared to controls. One hour after treatment, the normal-

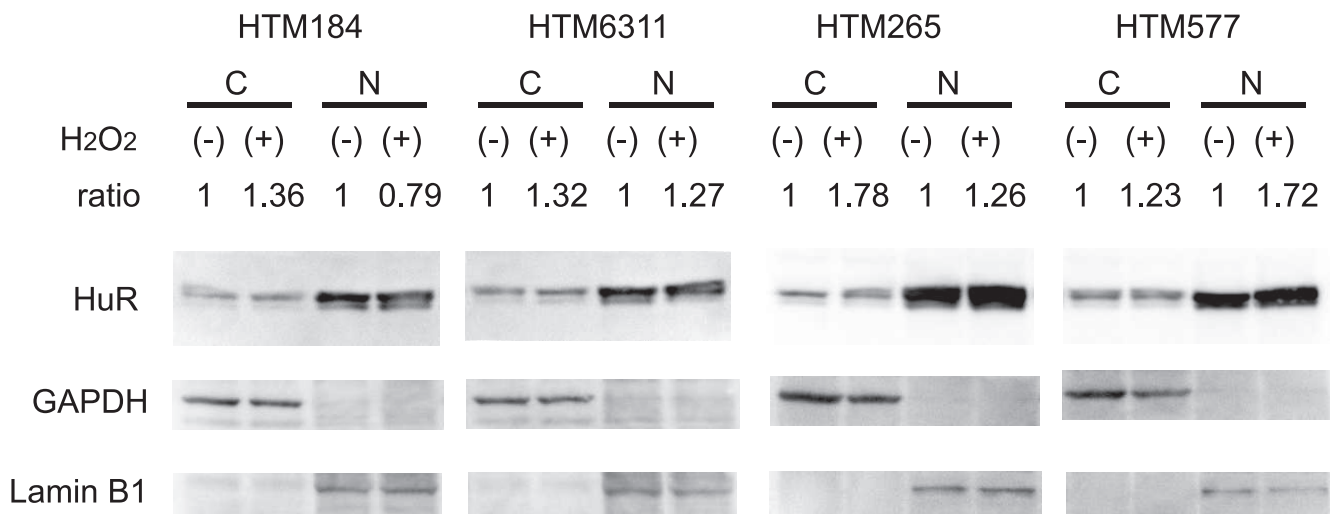
ized values for the remaining IL-6 were 0.145 (0.095–0.27) (normalized ratio, median [interquartile range]) for the control cultures and 0.345 (0.2–0.48) for the cultures treated with  $H_2O_2$  (Fig. 4A). Similarly IL-8 mRNA increased after  $H_2O_2$  treatment. In control cultures, the normalized value of IL-8 mRNA was 0.565 (0.408–0.6) compared with the value 0.775 (0.486–0.873) with  $H_2O_2$  treatment ( $n = 5$ ; Fig. 4B). Both increases were statistically significant ( $P = 0.0313$ , both for IL-6 and IL-8; Wilcoxon signed-rank test).

The change of myocilin, SPARC, and MMP-3 mRNA levels after  $H_2O_2$  treatment varied in each individual ( $n = 5$ ), but the differences were not statistically significant (for myocilin; without  $H_2O_2$ , 0.98 [0.875–1.22]; with  $H_2O_2$ , 1.07 [0.883–1.18];  $P = 0.6563$ ) (for SPARC; without  $H_2O_2$ , 0.75 [0.71–0.913]; with  $H_2O_2$ , 0.755 [0.71–0.868];  $P = 0.75$ ) (for MMP-3; without  $H_2O_2$ , 1.275 [0.908–1.41]; with  $H_2O_2$ , 1.125 [0.868–1.27];  $P = 0.2813$ ) (Fig. 4C–E).

MMP-9, which also has AREs in its 3' UTR, did not vary much in the two samples in which it could be detected. The amounts of MMP-9 mRNA were low with Ct values around 35 cycles. MMP-9 mRNA were undetectable in three out of five samples (Fig. 4F).

To determine if topographic cues in the biomimetic range would influence this mRNA stabilization, HTM cells from two different individuals were analyzed for IL-6 mRNA stability when cultured on 1400-nm pitch surfaces. Stabilization of the mRNA was similar to the planar substrates. One representative result from a 26-year-old donor is shown in Figure 5.





**FIGURE 2.** Increased cytoplasmic HuR after exposure to oxidative stress. HTM cells were harvested 1 hour after incubation with or without 300  $\mu$ M H<sub>2</sub>O<sub>2</sub>. Cytoplasmic and nuclear lysates were prepared, and HuR levels were determined by Western blotting. GAPDH and lamin B1 show definitive fractionation of the cytoplasm (C) and nucleus (N). GAPDH and lamin B1 were used as loading controls for cytoplasmic and nuclear samples, respectively. Cytoplasmic and nuclear HuR and were normalized for GAPDH or lamin B1 and the intensities of oxidatively stressed samples were compared with control samples. Cytoplasmic HuR was increased after exposure to H<sub>2</sub>O<sub>2</sub> (average ratio 1.42, 95% confidential interval 1.04–1.81,  $P = 0.04$ , Student's  $t$ -test). In contrast, there was no statistical difference in nuclear HuR ( $n = 4$ , average ratio 1.26, 95% confidential interval 0.66–1.86,  $P = 0.26$ , Student's  $t$ -test).

## DISCUSSION

This is the first report to demonstrate HuR is present in HTM cells and that with oxidative stress, the HuR protein moves from the nucleus to the cytoplasm of the HTM cells. In a previous report, oxidative stress simulated by H<sub>2</sub>O<sub>2</sub> exposure induced IL-6 and IL-8 in porcine TM cells.<sup>7</sup> Our results demonstrate oxidative stress increased the levels of IL-6 and IL-8 mRNA in HTM cells as well. The observed increase in mRNA induced by oxidative stress suggests that the dynamics of mRNA degradation pathways for these mediators may be altered in the HTM cells by glaucoma-associated oxidative stress. HuR is known to stabilize mRNAs with AREs during

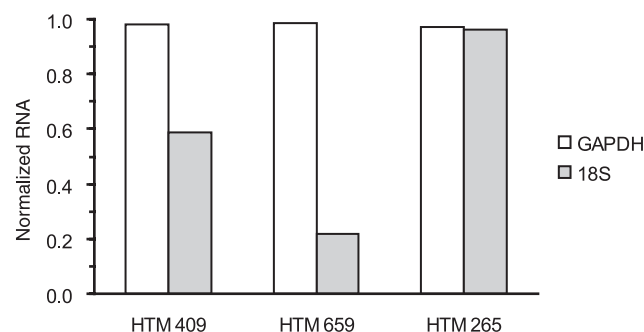
H<sub>2</sub>O<sub>2</sub> treatment in a variety of cell types.<sup>25–28</sup> The altered stabilization of mRNA in other cell types suggests HuR has a similar role for certain cytokines in HTM cells.

HuR has been reported to shuttle from the nucleus to the cytoplasm.<sup>14</sup> By Western blots, we found HuR to increase in the cytoplasm after oxidative stress. However, increased levels of HuR in the cytoplasm were not as large in the primary HTM cells compared with changes previously reported in cell lines. Moreover, the abundance of HuR in the nucleus in the HTM cells may have made it difficult to detect mild to moderate decreases of HuR in the nucleus in our four samples.

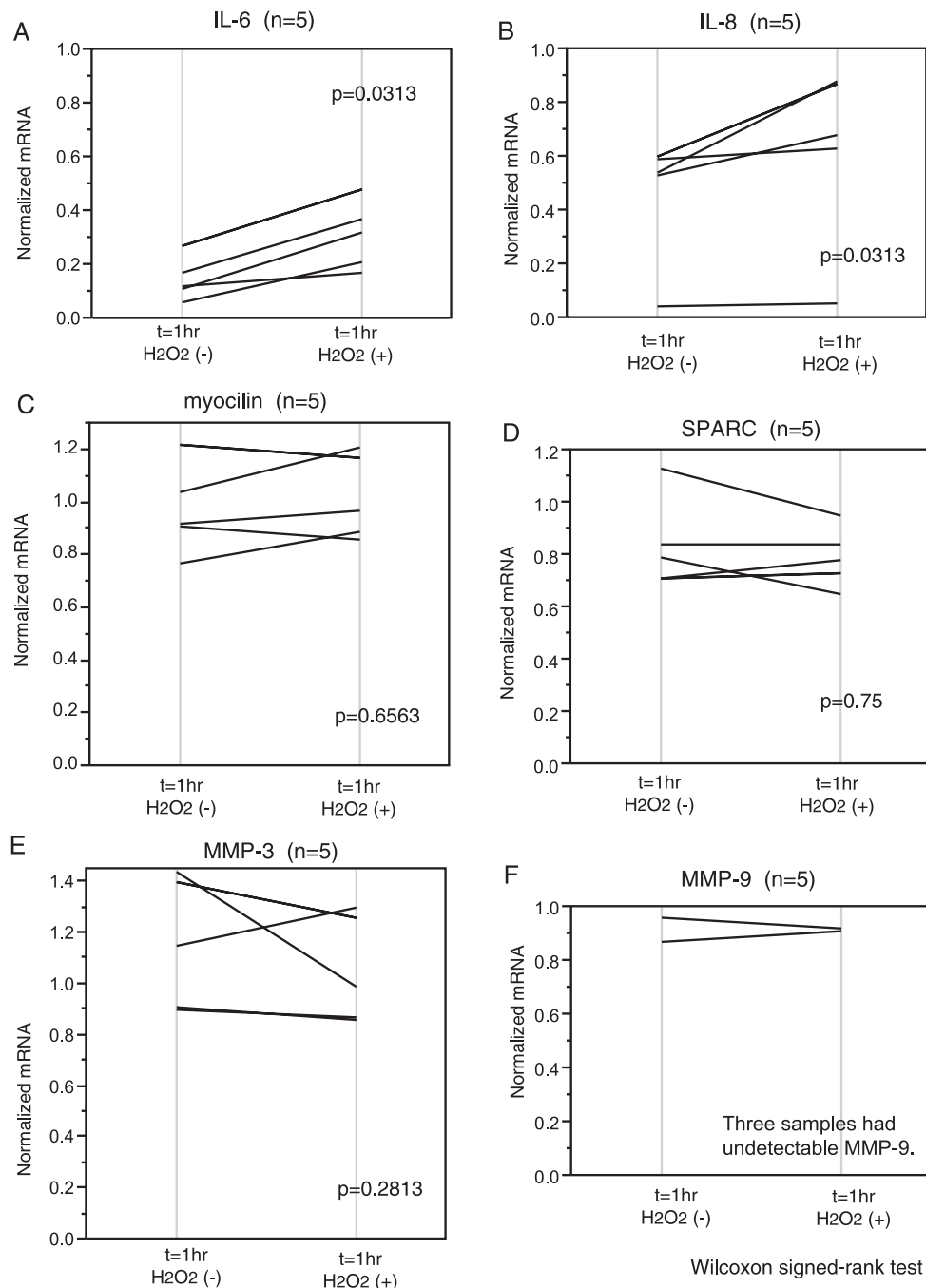
We selected the inflammatory mediators IL-6 and IL-8 for investigation because an increased expression of these inflammatory markers in the outflow pathways has been linked to glaucoma. IL-6 level is elevated in the aqueous humor in pseudoexfoliation syndrome<sup>11</sup> and neovascular glaucoma,<sup>12</sup> though a direct link with POAG has not been made. It has been reported that increasing IOP in ex vivo perfused human anterior segments increased IL-6 mRNAs<sup>10</sup> and that induced IL-6 increased outflow facility in perfused anterior segments of porcine eyes.<sup>29</sup> In aggregate, our findings and previous reports suggest a scenario whereby an increase in oxidative stress associated with glaucoma would lead to increased HuR in the cytoplasm thereby protecting IL-6 mRNA and a concomitant increase in protein expression with the associated beneficial effect on IOP control.

The concentration of IL-8 is elevated in the aqueous humor of glaucoma patients,<sup>8,9</sup> and HTM cells have been shown to secrete IL-8.<sup>30</sup> It has also been reported that IL-8 modulates the permeability of the Schlemm's canal endothelial cells.<sup>31</sup> These reports suggest that IL-8 plays an important role in the outflow pathway. As with IL-6, it may be that the increased levels of these cytokines are a means by which the HTM cells attempt to ameliorate the change in outflow resistance associated with glaucoma.

A consensus does not exist regarding the relationship between these inflammatory mediators and the onset/progression of glaucoma. While an increase in these cytokines can have beneficial effects on outflow, it is also possible that chronic activation of these inflammatory mediators could



**FIGURE 3.** Determination of endogenous control for mRNA stabilization. To select a proper internal control gene for real-time PCR analysis, we compared 18S rRNA to GAPDH mRNA expression in our experiments. Cell cultures from three different individuals were analyzed. HTM cells were treated with 5  $\mu$ g/mL actinomycin D to suppress transcription ( $t = -30$  minutes). After 30 minutes, cells were treated with 300  $\mu$ M hydrogen peroxide ( $t = 0$ ) and incubated for 1 hour ( $t = 1$  hour). They were harvested at  $t = -30$  minutes and  $t = 1$  hour. The same amount of total cellular RNA was used to standardize the samples. Each real-time PCR amplification was run in triplicate. Data are shown as mean and are normalized to  $t = -30$  minutes. GAPDH RNA appeared more consistent than 18S RNA. Therefore, we used GAPDH mRNA as an internal control in subsequent experiments.

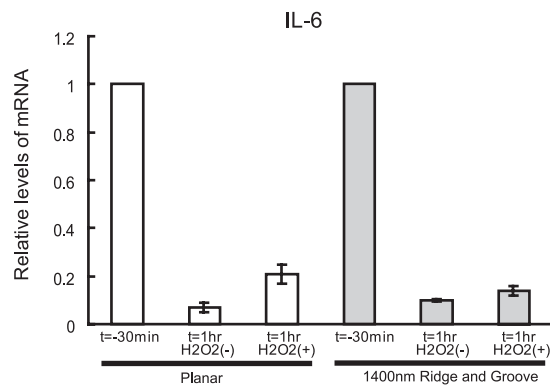


**FIGURE 4.** Stability change of mRNA with H<sub>2</sub>O<sub>2</sub> treatment in HTM cells. HTM cells were treated with 5  $\mu$ g/mL actinomycin D to suppress transcription ( $t = -30$  minutes). After 30 minutes, cells were treated with or without 300  $\mu$ M H<sub>2</sub>O<sub>2</sub> ( $t = 0$ ) and incubated for 1 hour ( $t = 1$  hour). They were harvested at  $t = -30$  min and  $t = 1$  hour. Each real-time PCR amplification was run in triplicate. Data are shown as mean of the triplicate and normalized to  $t = -30$  minutes. H<sub>2</sub>O<sub>2</sub> increased IL-6 (A) and IL-8 (B) mRNA stability. The change of myocilin (C), SPARC (D), and MMP-3 (E) mRNA stability varied with each individual but was not significantly different between the treated and untreated cells. MMP-9 (F) did not vary much in the two samples.

induce pathologic changes in the TM contributing to the progression of glaucoma. IL-6 and IL-8 have been implicated in induction of cellular senescence,<sup>32</sup> known to occur in the outflow pathway in glaucoma.<sup>33</sup> Thus while these two cytokines may have valuable effects in the short term, their continued presence may ultimately have deleterious effects to the HTM cells.

MMP-3, MMP-9, SPARC, and myocilin were investigated because they have been associated with the meshwork and

changes in the amounts of these proteins are associated with glaucoma. Many MMPs have been detected in TM cells.<sup>34</sup> MMP-3 has been reported to be up-regulated in response to a variety of stimuli,<sup>35–40</sup> and to be increased in aqueous samples from pseudoexfoliation eyes.<sup>41</sup> A mixture of MMPs including MMP-3 and MMP-9 increased outflow facility in human anterior segment organ culture.<sup>42</sup> In addition, MMP-9 has multiple AUUA motifs in the 3' UTR. SPARC is distributed throughout the TM<sup>43</sup> and has been demonstrated in aqueous humor of



**FIGURE 5.** Stability of mRNA for IL-6 with H<sub>2</sub>O<sub>2</sub> treatment in HTM cells grown on 1400-nm pitch topography compared with planar surfaces. The result of one representative culture of two, obtained from a 26-year-old donor, is shown. HTM cells were treated with 5  $\mu$ g/mL actinomycin D to suppress transcription ( $t = -30$  minutes). Data are shown as mean  $\pm$  standard deviation of the triplicate and normalized to  $t = -30$  minutes of planar or topographic surfaces. No difference in stabilization of mRNA was noted between the cells on topography and planar substrates. Although nanopatterned surfaces influence a number of cellular behaviors, nanopatterned surfaces did not alter IL-6 mRNA stability. This result suggests topographic cues do not affect ARE stabilization by HuR.

humans.<sup>44</sup> SPARC-null mice had lower IOP values compared to wild-type mice.<sup>45</sup> Myocilin was the first gene to be associated with glaucoma.<sup>46</sup> Although a consensus has not been reached regarding the physiological function of myocilin, it has been linked to elevations in IOP.<sup>47,48</sup> Thus each of these proteins may be modulated by glaucoma associated oxidative stress, but only MMP-9 has AREs that would bind to HuR.

The expression of the mRNAs for inflammatory cytokines is normally tightly controlled as a result of having the AREs on their 3' UTRs to ensure a short half-life. This is consistent with our results. Even with oxidative stress, the mRNAs of IL-6 and IL-8 were decreased compared with SPARC, myocilin, and the MMPs at the 1-hour time point. These data suggest that IL-6 and IL-8 mRNAs have a rapid turnover in the HTM cells. The observed lack of effect on stability of matricellular proteins, myocilin, SPARC, and MMP-3 mRNAs that do not have AREs and their high levels of expression during the course of our experiments indicate H<sub>2</sub>O<sub>2</sub> treatment does not influence their mRNA stability.

Although HuR has been reported to stabilize rat MMP-9 mRNA,<sup>49</sup> we could not detect any stabilization of HTM-associated MMP-9 mRNA despite the documented increase in cytosolic HuR associated with oxidative stress. MMP-9 mRNA expression in our experiments was minimal and near the limits of detection with quantitative PCR. This low level of expression may have prevented us from detecting any stability difference in MMP-9. Oh et al.<sup>40</sup> reported an inability to detect MMP-9 mRNA in several samples of fresh HTM, and our findings would be consistent with their results.

This mRNA stabilization might also participate in the induction of inflammatory mediators by mechanical stress as well. Liton et al.<sup>50</sup> observed that p38 mitogen-activated protein kinase (MAPK) contributes to increased IL-6 with mechanical stress on HTM cells. They suggested that p38 promoted IL-6 expression through mRNA stabilization. The p38 MAPK pathway leads to the inactivation of mRNA decay-promoting proteins such as TTP,<sup>51-53</sup> and also regulates HuR's nuclear-cytoplasmic shuttling.<sup>25,54</sup>

Topographic cues have been shown to modulate many cellular behaviors, including proliferation, adhesion, and

migration.<sup>55-58</sup> Moreover, increased mRNA levels of myocilin and versican were observed in HTM cells grown on topographically patterned substrates.<sup>22</sup> Growth on 1400-nm pitch topographically patterned substrates did not alter the stability of mRNAs for IL-6 in our experiments compared with planar substrates. These results suggest topographic features do not influence the mRNA stabilities in HTM cells explored in our experiments; the impact of this important and ubiquitous biophysical cue on the stability of other mRNAs remains unexplored. Thus, while biomimetic topographic cues profoundly influence multiple characteristics of HTM cells, our results suggest that the mRNA stabilization of specific mRNAs investigated are not affected by this biophysical cue.

In conclusion, oxidative stress stabilized IL-6 and IL-8 mRNA in HTM cells. Our data suggests that the decay of certain mRNAs associated with glaucoma is altered in the TM of glaucoma patients in a manner consistent with the presence of AREs and HuR stabilization. While induction of both of these cytokines would appear to be useful in counteracting the effects of high IOP, their continued presence may ultimately have deleterious effects on HTM cells. As a consequence of the oxidative stress associated with glaucoma, the stabilization of these cytokines may actually contribute to the progression of the disease.

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