Alterations in gene expression induced by cyclic mechanical stress in trabecular meshwork cells

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Purpose: To investigate the changes in gene expression induced by cyclic mechanical stress (CMS) in trabecular meshwork (TM) cells.

Methods: Human TM cultures from three donors were plated on type I collagen-coated flexible silicone bottom plates and subjected to 15% stretching, one cycle per second for 6 h. Non-stressed parallel cultures were incubated under the same conditions in the absence of CMS. Total RNA from each culture was amplified (1 round of amplification) and hybridized to Operon Human Oligo Arrays version 3.0 (35 K probes). Differences in gene expression induced by CMS were analyzed using Genespring 7.2. quantitative polymerase chain reaction (Q-PCR) was used to confirm changes in the expressions of 12 selected genes. The effects of chemical inhibitors for p38, ERK (extracellular signal-regulated kinase), JNK (Jun N-terminal kinase), PKA (protein kinase A), PI3K (phosphoinositide 3-kinase), and P2 (purinergic 2) receptors on the induction of MMP3 (matrix metalloproteinase 3), HSP70 (heat shock protein 70), ECSM1 (endothelial cell specific molecule 1), BMP2 (bone morphogenetic protein 2), VEGFC (vascular endothelial growth factor C), and IL-8 (interleukin 8) were evaluated in porcine TM cells subjected to the same regime of CMS as that used in human cells.

Results: CMS induced extensive gene expression changes (664 genes, p≤0.05) twofold or higher in cultured TM cells. Many of these changes were related to extracellular matrix (ECM) synthesis and remodeling including the upregulation of two metalloproteinases (MMP3 and MMP10). Cytoskeleton and cell adhesion genes were also affected by CMS as well as genes known to be involved in cellular protection against stress including several members of the HSP70 family. Inhibition of PI3K/AKT and P2 receptors pathways significantly reduced the induction of MMP3 and IL-8 whereas the inhibition of the PKA/cAMP pathway decreased ECSM1 and BMP2.

Conclusions: CMS activated many genes that could influence the aqueous humor outflow facility, specifically genes involved in ECM synthesis and remodeling (e.g. MMPs), cytoskeletal organization, and cell adhesion. Induction of MMP3 has the potential to increase the aqueous humor outflow facility and could be part of a homeostatic mechanism involved in the maintenance of normal intraocular pressure (IOP) levels. Other observed changes are more likely to be related to general cellular responses to stress (e.g., HSP70, ECSM1, and BMP2). Although these latter changes may initially help to repair mechanical damage, some of them such as the induction of BMP2 could eventually increase tissue rigidity and compromise the ability of the TM to maintain normal levels of outflow resistance.

The trabecular meshwork (TM) and Schlemm’s canal form the major conventional route for aqueous outflow from the anterior chamber of the human eye. The TM is also the site of the abnormal increase in outflow resistance that leads to elevated intraocular pressure (IOP) in glaucoma [1-4]. Similar to other tissues in the body, the TM is subjected to mechanical forces that can exert important effects on the normal physiology of the tissue as well as contribute to pathological alterations [5,6].

Several studies have demonstrated that the TM responds to the stretch produced by a static increase in IOP by altering both its morphology and patterns of gene expression [7-10]. Such changes in gene expression have been proposed to play a role in restoring normal levels of IOP through homeostatic influences on aqueous humor outflow facility [7,9].

However, mechanical stress in the TM in vivo does not only result from simple static changes in IOP. In vivo, the TM is constantly subjected to transient spikes of IOP such as those associated with systole of the cardiac cycle, blinking, and eye movement [1,11]. In particular, the cardiac cycle leads to oscillations of IOP potentially in the order of 2.7 mmHg, which then produces cycles of TM tissue stretching and relaxing [11].

In several cell types, cyclic regimes of mechanical stress are known to exert different effects from static stretching [5]. Therefore, it should be expected that cyclic mechanical stimulation of TM cells might elicit different responses from those observed after static stretching.

Surprisingly, Ramos and Stamer [12] recently reported that cyclic IOP in perfused anterior segments of human and porcine eyes resulted in a significant decrease in outflow facility. These changes in outflow facility were not associated with detectable damage to the cells or structures of the outflow pathway, suggesting that it may result from active cellular responses to the cyclic mechanical stimulus.
A better characterization of the cellular responses to cyclic mechanical stress (CMS) in the TM is needed to understand the influences of the biomechanical environment on the physiologic function of the conventional outflow pathway.

To gain insight into these mechanisms, we investigated the changes in gene expression induced by cyclic mechanical stress in cultured TM cells using gene microarrays. We also analyzed the potential involvement of several regulatory pathways on these observed changes in gene expression.

**METHODS**

**Cell cultures:** Within 48 h post mortem, human trabecular meshwork (HTM) cell cultures were obtained from cadaver eyes that did not have any history of eye disease [13]. Tissues were manipulated in accordance with the Declaration of Helsinki. Three HTM primary cell lines (from ages 14, 16, and 25 year old eyes) were used in these experiments. Porcine TM (pTM) cells were generated from fresh pig eyes using the same protocol. Cell cultures were maintained at 37 °C in 5% CO2 in media (low glucose Dulbecco’s Modified Eagle Medium [DMEM] with L-glutamine, 110 mg/ml sodium pyruvate, 10% fetal bovine serum, 100 µM non-essential amino acids, 100 units/ml penicillin, 100 µg/ml streptomycin sulfate, and 0.25 µg/ml amphotericin B). All the reagents were obtained from Invitrogen Corporation (Carlsbad, CA).

**RNA isolation and quantitative polymerase chain reaction:** HTM and pTM primary cell cultures were washed with phosphate buffered saline (PBS) and immediately submerged in RNA-later (Ambion Inc., Austin, TX). Total RNA was isolated using an RNeasy kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions and then treated with DNase. RNA yields were measured using RiboGreen fluorescent dye (Molecular Probes, Eugene, OR). First strand cDNA was synthesized from total RNA (1 µg) by reverse transcription using oligodT and Superscript II reverse transcriptase (Invitrogen Corporation) according to the manufacturer’s instructions. Quantitative polymerase chain reactions (Q-PCR) were performed in a 20 µl mixture that contained 1 µl of the cDNA preparation and 1X iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), using the following PCR parameters: 95 °C for 5 min followed by 50 cycles of 95 °C for 15 s, 65 °C for 15 s, and 72 °C for 15 s. The fluorescence threshold value (Ct) was calculated using the iCycle system software (Bio-Rad, Hercules, CA). The absence of nonspecific products was confirmed by both the analysis of the melt curves and by electrophoresis in 3% Super Agarose gels.

**β-Actin** (ACTB) was used as an internal standard of mRNA expression. This gene was selected as a control because it did not show any significant difference in expression in the array analysis. The primers used for Q-PCR amplification are shown in Table 1.

**Table 1. Primers used for Q-PCR amplification.**

| Gene                         | Forward                              | Reverse                              |
|------------------------------|--------------------------------------|--------------------------------------|
| **Human genes**              |                                      |                                      |
| β-Actin                      | 5′-CCTCGCCCTTTGCGCAATCGC-3′          | 5′-GCCGAGCGGTGTTGCGACG-3′            |
| HSP70                        | 5′-ACAGGAGCAAGTGAAAGTCT-3′           | 5′-TTCATGAAATCATCTTCGCA-3′           |
| MMP10                        | 5′-TGACATCAAGCAGAACTGAGC-3′          | 5′-TGTGGGTAGAGGAAGACG-3′             |
| ECSM1                        | 5′-TTTCTCTCAGGAGCAGTAGAC-3′          | 5′-GGACGATCTCCTTTCGCAA-3′            |
| MMP3                         | 5′-GGGAGAGGATTAACTGAGATG-3′          | 5′-ATTTCTATGAGGACCGAGG-3′            |
| Regulator of G protein 20    | 5′-GAAGATCAGACAGCCCAAAAT-3′          | 5′-GGCGTGTACCTTCGCAAGG-3′            |
| BMP2                         | 5′-GGACGCTTCTTCAATGAGAC-3′           | 5′-ACCACGTGACATTGTTGAGA-3′           |
| Hyaluronan synthase          | 5′-CACGACAGACAGCAGTGAGGAC-3′         | 5′-TCCAAAGAGTGAGTGTCAAA-3′           |
| ECMV1                        | 5′-GGATGCTGAGGAGTAGACTCA-3′          | 5′-TCTACAGCTTCTGTTTGG-3′             |
| JunB proto-oncogene          | 5′-CGAGTCAGCAGAAATAGGAC-3′           | 5′-GCTGCTGAGGATGGTCAA-3′             |
| Nuclear receptor subfamily 4A| 5′-GAAAGCTGAGATGCCCCTGATC-3′         | 5′-ATGGTGCCGCTAGTGAACCTC-3′          |
| Basic transcription element  | 5′-GGCTGCTGAGGAAAAGTCTATGAG-3′       | 5′-CCGTCATGTCTGACTGC-3′              |
| Early growth response 3      | 5′-GGTGAAGCCCTGGCTGTGTTA-3′          | 5′-ACATGATTTCAGAGCGGATG-3′           |
| **Porcine genes**            |                                      |                                      |
| β-Actin                      | 5′-AAGATCAAGATCAGGCCGCTC-3′          | 5′-TGGAATGACAACTAAGTCGGCTC-3′        |
| HSP70                        | 5′-CCACAAAGTGATCAGGTGGGA-3′          | 5′-GGGTGGCCCCTTGGCTGTC-3′            |
| MMP3                         | 5′-TTTCTCTGAGAGCAGTAGAC-3′           | 5′-TGAAGAGAGACCCAGGAAGAATG-3′        |
| ECSM1                        | 5′-TTTCTCTCAGGAGCAGTAGAC-3′          | 5′-TGCAGCTGACAGTCTGCTG-3′            |
| BMP2                         | 5′-ACCAACCTGTTTGCAAAAGG-3′           | 5′-GTTCCCACCAGGAGGATATT-3′           |
| VEGFC                        | 5′-TTTCTCTTCTGAGCAGTAGAC-3′          | 5′-CACACGATGTTGGGAAAAGT-3′           |
| IL8                          | 5′-AAACACTTGGCTGGTCTTCCTG-3′         | 5′-ATTTATGACAGCCATGCAAGAA-3′         |

List of all primers used in this study to validate the microarray data by Q-PCR. β-Actin was used as the normalizing control gene for all comparisons.

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**Cyclic mechanical stress application in cell culture:** HTM (passage 3) and pTM (passage 4) cultures were plated on type I collagen-coated flexible silicone bottom plates (Flexcell, Hillsborough, NC). One day after confluence, culture medium was switched to serum-free DMEM 3 h before cyclic mechanical stress. Cells were stressed for 6 h (15% stretching, 1 cycle/s), using the computer-controlled, vacuum-operated FX-3000 Flexercell Strain Unit (Flexcell). A frequency of 1
cycle/s was selected to mimic cardiac frequency. Since it is difficult to estimate the amount of stress to which TM cells are subjected in vivo but IOP can change as much as 15 mmHg in 10 s [11], we selected 15% stretching because at 1 cycle/s, it was high enough to induce detectable changes in gene expression while exerting only minimal effects on cell survival for both porcine and human TM cells. Control cells were cultured under the same conditions, but no mechanical force was applied.

**Gene microarray and data analysis:** Total RNA from three HTM cell cultures stressed or held static were amplified (one round amplification) and hybridized to Operon Human Oligo Arrays version 3.0 (35 K probes; Eurofins Operon, Huntsville, AL) at the Duke University Microarray facility (Durham, NC). The Human Genome Oligo Set Version 3.0 represents 24,650 genes and 37,123 gene transcripts. Raw data was normalized and analyzed using GeneSpring 7.2 (Silicon Genetics, Wilmington, DE). Genes were filtered to their intensities in the control channel (control used was Universal Reference Human RNA from Stratagene, Huntsville, AL). Raw data values below 100 were considered unreliable. Intensity-dependent normalization was performed per spot and per chip (LOWESS, locally weighted scatterplot smoothing). An ANOVA test was performed (p values≤0.05 were considered significant) for genes differentially expressed using the Benjamin and Hochberg False Discovery Rate correction test.

**Cell viability assay:** Cell viability was assayed after 6 h of CMS by measuring lactate dehydrogenase released to the culture media as a result of plasma membrane damage. The Cito Tox 96® Non-Radioactive Cytotoxicity assay (Promega, Madison, WI) was used to carry out the measurement following manufacturer’s instructions.

**Inhibitors:** Chemical inhibitors for p38 (SB203580), mitogen-activated protein kinase kinase and extracellular regulated kinase (MEK/ERK; PD98059), Janus kinase protein (JNK; SP600125), protein kinase A (PKA; H89), phosphoinositide kinase (MEK/ERK; PD98059), Janus kinase protein (JNK; SP600125), protein kinase A (PKA; H89), phosphoinositide kinase (PI3K; wortmannin), and P2 receptors (suramin) were all from Sigma (St Louis, MO). The inhibitors were used at a concentration of 10 μM 1 h before and during the stretching.

**RESULTS**

**Changes in gene expression after mechanical stress in human trabecular meshwork cells:** HTM cells were subjected to mechanical stress for 6 h to evaluate the effects of CMS on the gene expression profile. Gene array analysis showed statistically significant (p≤0.05) 2.0 fold or higher changes in 664 gene transcripts. Out of these 664 genes, 349 were upregulated and 315 were downregulated. The genes most highly upregulated or downregulated after CMS are shown in Table 2.

CMS induced changes in the expression of genes that are known to be involved in cell protection as well as genes that because of their known function could potentially influence aqueous humor outflow facility. Specifically, CMS affected the expression of genes involved in cellular stress (Table 3) and extracellular matrix (ECM) synthesis and remodeling (Table 4) as well as genes known to affect cytoskeleton and cell adhesion (Table 4 and Table 5).

Numerous stress defense and cell defense genes showed changes in expression. This group included 14 heat shock and heat shock related genes (Table 3). Heat shock protein 70 B (HSP70B') showed the largest upregulation, and other members of the HSP70 family also showed high levels of induction. In addition, several metallothioneins (1A, 1B, 1E, 1G, 1H, and 2A), angiopoietin-like 4, and cluster of differentiation (CD) antigens were among the upregulated genes.

Several genes coding for proteins that can affect the ECM exhibited expression changes after CMS. Among these, the upregulation of two metalloproteinases, MMP3 and MMP10, with levels of induction of 29 and 26 fold, respectively, was particularly noticeable. Various proteoglycan genes showed either downregulation or upregulation after CMS. The most upregulated was the endothelial cell specific molecule-1 (ECM1 or endocan). Genes associated with proteoglycan synthesis and degradation such as hyaluronan synthase and several sulfotransferases were also upregulated. Important ECM structural components such as collagens and laminin showed a decrease in expression. Other significant changes related to the ECM included the upregulation of growth factors (fibroblast growth factor [FGF] acidic and vascular endothelial growth factor C [VEGFC]), bone morphogenetic proteins (2 and 6), and plasminogen activator tissue (Table 4).

CMS altered the expression of many cytoskeleton and cell adhesion genes. The most upregulated cytoskeleton gene was an actin binding protein that is also known to be induced by oxidative stress (ectodermal-neural cortex). The most downregulated gene was Arg/Abl-interacting protein (ArgBP2), which belongs to a family that regulates both cell adhesion and cytoskeletal organization (Table 5). Among the genes involved in cell adhesion, particularly evident was the increase in expression of integrin beta 6 (Table 6).

The original array data files are available at the Gene Expression Omnibus (GEO) under accession number GSE14768.

**Validation of microarray results by quantitative polymerase chain reaction:** Twelve genes upregulated or downregulated by more than twofold expression were further analyzed by quantitative PCR in HTM cells. Although, the precise fold change values observed by Q-PCR were different from those in the gene arrays, the results were in general agreement with the arrays for all the analyzed genes (Table 7). In addition, six upregulated genes (MMP3, interleukin 8 [IL8], VEGFC,
| Genes upregulated and downregulated in HTM cells after CMS | Fold | p value | GenBank accession number |
|--------------------------------------------------------|------|---------|-------------------------|
| Heat shock 70 kDa protein 6 (HSP70B')                  | 489.4| 0.0206  | NM_002155               |
| Protein phosphatase 1, subunit 14C                      | 66.58| 2.84E-05| NM_030949               |
| Integrin, beta 6                                        | 59.16| 0.000405| NM_000888               |
| Regulator of G-protein signaling 20                     | 53.81| 0.000828| NM_003702               |
| Chromosome 9 open reading frame 26 (NF-HEV)            | 40.98| 5.60E-05| NM_033439               |
| Endothelial cell-specific molecule 1                    | 33.41| 0.000613| NM_007036               |
| Matrix metalloproteinase 3 (stromelysin 1)             | 29.78| 1.13E-05| NM_002422               |
| Matrix metalloproteinase 10 (stromelysin 2)            | 26.72| 0.000597| NM_002425               |
| Nm23-phosphorylated unknown substrate                  | 23.27| 1.77E-05| NM_032873               |
| Bone morphogenic protein 2                             | 20.07| 0.000553| NM_001200               |
| Neuronal protein                                        | 19.73| 0.0002   | NM_013259               |
| Hypothetical protein FLJ23657                          | 17.56| 0.00366  | AK074237                |
| T-cell activation kelch repeat protein                  | 17.12| 0.00134  | NM_023255               |
| Parathyroid hormone-like hormone                       | 16.73| 0.0055   | NM_002820               |
| Hypothetical protein FLJ12604                          | 15.98| 0.00264  | NM_024621               |
| Kruppel-like factor 7 (ubiquitous)                     | 15.62| 0.00214  | BC012919                |
| Fibroblast growth factor 1 (acidic)                    | 13.75| 0.00479  | NM_000800               |
| Hypothetical protein FLJ13391                          | 12.28| 5.88E-05 | NM_032181               |
| Galanin                                                | 10.68| 0.00155  | NM_019973               |
| ATPase family, AAA domain containing 3B                | 10.64| 1.38E-05 | NM_031921               |
| Pentaxin-related gene                                  | 9.761| 0.00016  | NM_002852               |
| Polymerase (RNA) III polypeptide D                     | 9.255| 4.25E-06 | NM_001772               |
| Metallothionein 1G                                     | 8.385| 0.0134   | BC020757                |
| Hypothetical protein MGC11324                          | 8.315| 0.00094  | NM_032717               |
| Heat shock 70 kDa protein 1B                            | 8.117| 0.000324 | NM_005546               |
| Potassium voltage-gated channel, member 4              | 7.964| 6.90E-05 | NM_004978               |
| Nucleoside phosphorylase                               | 7.604| 7.38E-05 | NM_000270               |
| Protein phosphatase 1, subunit 15A                     | 7.104| 0.000387 | NM_032717               |
| Regulator of G-protein signaling 17                     | 6.969| 0.000364 | NM_012419               |
| Carbohydrate (chondroitin 4) sulfortransferase 11      | 6.801| 0.00106  | NM_018413               |
| ADP-ribosylation factor-like 7                         | 6.475| 0.000369 | NM_005737               |
| Dickkopf homolog 1 (Xenopus laevis)                    | 6.464| 0.00163  | NM_012422               |
| Solute carrier family 17, member 1                     | 6.442| 0.00049  | NM_005074               |
| BTB (POZ) domain containing 11                         | 6.193| 5.74E-05 | NM_152322               |
| Thymic stromal lymphopoietin                           | 6.189| 0.00254  | NM_033035               |
| Mitogen-activated protein kinase kinase 4              | 6.099| 0.00378  | NM_003010               |
| Heat shock 105 kDa 110 kDa protein 1                   | 5.997| 0.000789 | NM_006644               |
| Vascular endothelial growth factor C                   | 5.917| 7.05E-05 | NM_005429               |
| GTase activating Rap/RanGAP domain-like 4              | 5.888| 0.00123  | AB028962                |
| Iduronidase, alpha-L-                                  | 5.829| 7.76E-05 | NM_000203               |
| T-box 3 (ulnar mammary syndrome)                       | 5.765| 6.16E-05 | NM_016569               |
| Hyaluronan synthase 2                                  | 5.388| 0.00474  | NM_005328               |
| Heparan sulfate 3-O-sulfortransferase 1                | 5.377| 0.00618  | NM_005114               |
| Bone morphogenic protein 6                             | 5.079| 0.00223  | NM_001718               |
| Myosin X                                               | 5.073| 3.50E-05 | AB018342                |
| Integrin, alpha 2                                      | 5.06 | 0.0112   | NM_002203               |
| Basic transcription element binding protein 1          | -13.88| 0.0022  | NM_001206               |
| Contactin 3 (plasmacytoma associated)                  | -13.41| 0.00967 | AB040929                |
| Nuclear receptor subfamily 4, group A, member 2        | -13.28| 0.00121 | NM_006186               |
| Nuclear receptor subfamily 4, group A, member 1        | -12.58| 0.00702 | NM_173158               |
| Chromosome 20 open reading frame 129                  | -10.39| 0.000593| NM_032919               |
| Distal-less homeo box 2                                | -9.82 | 2.50E-05| NM_004405               |
| Neuroepitope Y receptor Y1                             | -9.366| 0.00977| NM_000909               |
| Early growth response 3                                | -8.314| 0.000761| NM_004430               |
| FB1 murine osteosarcoma viral oncogene homolog B       | -8.036| 0.000207| NM_006732               |
| Chromosome 2 open reading frame 11                     | -7.839| 0.00422| NM_146629               |
| B-cell CLL/Lymphoma 3                                  | -7.259| 0.00165| NM_005178               |
| Chromosome 6 open reading frame 111                   | -7.021| 0.00265| NM_032870               |
| Tumor necrosis factor superfamily, member 10          | -6.621| 0.000944| NM_003810               |
| F-box protein 32                                       | -6.561| 0.00181| NM_058229               |
| Nuclear receptor subfamily 4, group A, member 3        | -6.488| 0.00476| NM_173200               |
| Solute carrier family 40, member 1                     | -6.414| 0.000916| NM_014585               |
| Phosphodiesterase 5A, cGMP-specific                    | -6.261| 0.00289| NM_001083               |
| Zinc finger protein 36, C3H type-like-2                | -5.684| 0.000817| NM_006887               |
| Myeloid/lymphoid or mixed-lineage leukemia             | -5.466| 0.00544| NM_004529               |
| KIAA1199                                               | -5.371| 0.00384| AB030205                |
| TGFB inducible early growth response                   | -5.246| 0.00796| NM_005655               |

Columns show the gene name, the fold expression change determined by microarray analysis, the calculated p value, where p<0.05 was considered to be statistically significant, and the GeneBank accession number.
**HSP70**, bone morphogenetic protein 2 (BMP2), and ECSM1 were validated in pTM cells to further analyze the effect of inhibitors on gene expression during mechanical stress.

**Cell viability:** After CMS (6 h -15% stretching, one cycle/s), both HTM and pTM cells showed only a small decrease in viability (5% and 7%, respectively) when compared to cells in the same conditions without stress.

### Table 3. CMS-induced changes in HTM cells stress/defense genes.

| Stress induced genes up and down regulated in HTM cells | Fold  | p value | GenBank accession number |
|--------------------------------------------------------|-------|---------|-------------------------|
| Heat shock 70 kDa protein 6 (HSP70B') | 489.4 | 0.0206 | NM_002155 |
| Pentaxin-related gene, rapidly induced by IL-1 beta | 9.761 | 0.00016 | NM_002852 |
| Metallothionein 1G | 8.385 | 0.0134 | BC020757 |
| Heat shock 70 kDa protein 1B | 8.117 | 0.000324 | NM_005346 |
| Protein phosphatase 1, regulatory (inhibitor) subunit 15A | 7.104 | 0.000387 | NM_004530 |
| Heat shock 105 kDa/110 kDa protein 1 | 5.997 | 0.000789 | NM_006644 |
| Heat shock 70 kDa protein 8 | 4.551 | 0.00105 | NM_006597 |
| Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa) | 4.268 | 0.0309 | NM_005347 |
| Serum/gluocorticoid regulated kinase | 4.265 | 0.00277 | NM_005627 |
| Phosphoprotein with glycosphingolipid-enriched microdomains | 4.079 | 0.000293 | NM_018440 |
| DnaJ (Hsp40) homolog, subfamily A, member 1 | 3.838 | 0.00456 | NM_001539 |
| DnaJ (Hsp40) homolog, subfamily B, member 1 | 3.79 | 0.000307 | NM_006145 |
| Metallothionein 1B (functional) | 3.286 | 0.00116 | NM_005947 |
| BCL2-associated athanogene 3 | 3.18 | 0.00513 | NM_004281 |
| Metallothionein 2A | 3.164 | 0.0026 | NM_005953 |
| similar to *Escherichia coli* DnaJ, but lacks a J-domain | 3.108 | 0.000794 | AF395440 |
| UL16 binding protein 2 | 3.104 | 0.0157 | NM_025217 |
| Metallothionein 1H | 3.086 | 0.00131 | NM_005951 |
| Stress-induced-phosphoprotein 1 | 3.016 | 0.000709 | NM_006819 |
| Metallothionein 1E (functional) | 2.92 | 0.0407 | AF395759 |
| Protein phosphatase 1, regulatory subunit 10 | 2.789 | 0.0343 | NM_002714 |
| Angiopoetin-like 4 | 2.753 | 0.00858 | NM_139314 |
| DnaJ (Hsp40) homolog, subfamily B, member 4 | 2.742 | 0.0108 | NM_007034 |
| Heat shock 90 kDa protein 1, alpha-like 3 | 2.616 | 0.00257 | M30627 |
| Metallothionein 1A (functional) | 2.546 | 0.00823 | BC029475 |
| Heat shock 90 kDa protein 1, alpha | 2.446 | 0.00395 | NM_005348 |
| Chaperonin containing TCP1, subunit 6A (zeta 1) | 2.33 | 0.00148 | NM_001539 |
| Heat shock 90 kDa protein 1, beta | 2.306 | 1.09E-05 | NM_007355 |
| Fas (TNFRSF6)-associated via death domain | 2.285 | 0.0025 | NM_003824 |
| Heat shock 70 kDa protein 9B (mortalin-2) | 2.248 | 0.00138 | NM_004134 |
| Px19-like protein | 2.137 | 0.00876 | NM_013237 |
| AHA1 | 2.133 | 0.00863 | NM_012111 |
| Nuclear receptor subfamily 4, group A, member 2 | -1.28 | 0.000121 | NM_006186 |
| Tumor necrosis factor (ligand) superfamily, member 10 | -6.621 | 0.00094 | NM_005381 |
| Angiopoetin-like factor | -4.358 | 0.0103 | NM_000146 |
| synonyms: GI10P1, IFI56, IFI-56, IFNAI1, RNMT61, GARG-16; Hypoxia-inducible protein 2 | -3.888 | 0.00272 | NM_001548 |
| Interferon regulatory factor 1 | -3.185 | 0.00155 | NM_002198 |
| Chemokine-like receptor 1 | -3.145 | 0.00408 | NM_000472 |
| Bradykinin receptor B2 | -2.916 | 0.00242 | NM_000623 |
| Histone deacetylase | -2.88 | 0.00044 | NM_005474 |
| Calcium modulating ligand | -2.562 | 4.70E-05 | NM_001745 |
| Interferon gamma receptor 2 (interferon gamma transducer 1) | -2.546 | 0.000401 | NM_005534 |
| Zinc finger protein 179 | -2.425 | 0.0108 | NM_007148 |
| Heat shock transcription factor 2 | -2.42 | 6.74E-07 | NM_004506 |
| Oxidation resistance | -2.373 | 0.0148 | BC032170 |
| Nuclear factor, interleukin 3 regulated | -2.166 | 0.000751 | NM_005384 |
| Heat shock 27 kDa protein 2 | -2.125 | 0.0199 | NM_001541 |
| Collagen, type IV, alpha 3 binding protein | -2.124 | 0.00719 | NM_005713 |
| Prostaglandin-endoperoxide synthase 2 | -2.008 | 0.0142 | NM_000963 |

**Effect of inhibitors on gene expression during mechanical stress:** Chemical inhibitors were used to analyze the effect of the MAP kinase pathways (p38, ERK, JNK), phosphoinositide 3-kinase (PI3) pathway, PKA pathway, and P2Y receptor signaling pathway on gene expression changes induced by mechanical stress. We evaluated the effects of these inhibitors upon the induction of some ECM regulator/
modifier genes (MMP3, BMP2, and ECSM1), the most upregulated stress response gene on the array (HSP70B'), one inflammatory response gene (IL8), and one growth factor gene (VEGFC).

The inhibition of the MAP kinase pathways (p38, JNK, and ERK) resulted in the CMS-mediated induction of the heat shock protein 70 (HSP70), but this effect was only statistically significant for the inhibition of ERK. The induction of IL8 was decreased with the JNK inhibitor. ERK and JNK inhibitors decreased the level of induction of IL8 and MMP3, respectively. PKA pathway inhibition reduced the induction of IL8, ECSM1, BMP2, and VEGFC. Inhibition of the PI3 pathway affected the induction of MMP3 and IL8. Treatment with suramin also resulted in the induction of HSP70 (Figure 1A-F).

**DISCUSSION**

Our results document that CMS induced an intricate response in cultured TM cells with extensive gene expression changes. Some of these changes could participate in outflow regulator mechanisms and could be part of a homeostatic mechanism to maintain normal IOP levels. Others probably serve to maintain cellular integrity from mechanical stress.

Although some changes such as the induction of MMP3 have been reported in other models of mechanical stress [7], the overall pattern of gene expression associated with cyclic mechanical stress was clearly different from that reported in similar experiments using static stress. One factor that could potentially lead to such differences is the cell type used in the experiments. While Vittal et al. [9] used porcine TM cells, our study was conducted using human TM cells. However, Q-PCR analyses of six genes upregulated in human cells were also upregulated in porcine cells, suggesting that the responses
in these two species may be similar. Therefore, it appears more likely that the type of stress and the specific experimental conditions could play a more relevant role in our observed results.

Some of the more prominent changes in gene expression induced by cyclic mechanical stress affected genes involved in cellular protection against different types of stress. In particular, these changes included upregulation of the HSP70 family. This family of HSPs comprises several highly evolutionarily conserved proteins with different levels of inducibility in response to metabolic stress that are known to provide cytoprotection to cells, making them resistant to otherwise lethal levels of stress. HSP70 proteins maintain cell survival through the regulation of multiple steps within apoptotic pathways (i.e. stress activated protein kinase [SAPK] and JNK), and they are also believed to regulate key upstream mediators of apoptosis including oxidative stress and Ca²⁺ overload. The HSP70 family member exhibiting higher levels of mRNA induction in our model was HSP70B⁺, which is characterized by tight regulation and high inducibility. This protein is transiently induced in response to stress and then rapidly degraded by the proteasome system [14].

The observed upregulation of genes such as ECSM1 (endocan) and VEGFC could potentially be associated with the mitogenic effects that mechanical stress produces in some other cell types [15]. ECSM1 is a dermatan sulfate proteoglycan that promotes mitogenic activity through interaction with hepatocyte growth factor/scatter during embryonic development and tissue regeneration [16]. The VEGF family of proteins can also exert mitogenic responses and is implicated in embryogenesis and tissue regeneration [17,18]. VEGFC has been specifically determined to be required for the development of the vascular and lymphatic systems [19]. The activation of mitogenic responses by CMS in the TM together with the induction of protective mechanisms (e.g., HSP70) could help to explain the observation that in organ culture, the TM did not show a net cell loss but rather an increase in cellularity after CMS [12].

Cyclic mechanical stress induced changes in a large number of genes that are known to affect the outflow facility such as those influencing the composition of the ECM, cellular cytoskeleton, and cell adhesion. However, some of these changes might be expected to exert contradictory effects in outflow facility. For instance, while the relatively large induction observed in MMP3 would be expected to increase aqueous humor outflow facility [20], the upregulation of BMP2 would be more likely to decrease outflow facility [21]. The induction of some MMPs by mechanical stress has been hypothesized to be part of a homeostatic response aimed

### Table 5. CMS-induced changes in the expression of cytoskeleton-related genes.

| Gene                                                                 | Fold   | p value   | GenBank accession number |
|---------------------------------------------------------------------|--------|-----------|--------------------------|
| Ectodermal-neural cortex (with BTB-like domain)                     | 4.998  | 0.0015    | NM_003633                |
| Tubulin, beta, 4                                                    | 4.489  | 0.00178   | NM_006086                |
| Myosin 1XB                                                          | 3.572  | 0.000919  | NM_004145                |
| Actin related protein 2/3 complex, subunit 5-like                   | 3.519  | 4.30E-05  | NM_030978                |
| Leucine rich repeat (in FLII) interacting protein 1                 | 3.016  | 0.00186   | NM_004735                |
| Syndecan 1                                                          | 2.802  | 0.00156   | NM_002997                |
| Chromosome 16 open reading frame 31                                 | 2.672  | 0.00977   | NM_152330                |
| Kinesin family member 21A                                           | 2.588  | 0.000145  | AK000059                 |
| Tubulin, beta polypeptide                                           | 2.388  | 0.00293   | NM_001069                |
| Leucine rich repeat (in FLII) interacting protein 1                 | 2.212  | 0.00923   | NM_004735                |
| Filamin C, gamma (actin binding protein 280)                        | 2.205  | 0.00172   | NM_001458                |
| Paxillin                                                            | 2.168  | 0.00754   | NM_002859                |
| Molecule interacting with Rab13                                     | 2.153  | 0.00962   | AB051455                 |
| Tropomyosin 3                                                       | 2.126  | 0.0175    | AK092712                 |
| Tubulin beta MGC4083                                                | 2.119  | 0.00802   | NM_032525                |
| Tubulin alpha 6                                                     | 2.073  | 0.000692  | NM_032704                |
| Arg/Abl-interacting protein ArgBP2                                  | -4.37  | 0.000424  | NM_021069                |
| Ankyrin repeat, family A (RFXANK-like), 2                           | -3.24  | 0.0035    | NM_023039                |
| Protein kinase C and casein kinase substrate in neurons 3           | -2.913 | 0.00147   | NM_016223                |
| A kinase (PRKA) anchor protein (yotiao) 9                           | -2.604 | 0.00434   | NM_147185                |
| Syntrophin, beta 2                                                  | -2.415 | 0.00777   | NM_006750                |
| Profilin 2                                                          | -2.058 | 0.00371   | NM_002628                |
| Downregulated in ovarian cancer 1                                   | -2.036 | 0.0402    | NM_014890                |
at increasing outflow facility after an increase in IOP [10]. On the other hand, BMP2 have been shown to increase ECM deposition, and BMP2 activity in HTM cells has been proposed to contribute to outflow resistance by the induction of osteogenic factors during aging and glaucoma [21-23].

In our model, CMS elicits responses that could potentially increase outflow facility together with other responses that are associated with the need to maintain tissue integrity in the presence of mechanical forces. While the first set of responses may help to prevent abnormal elevations of IOP, the second could potentially contribute to increased rigidity of the TM over time and lead to increased outflow resistance. A similar combination of homeostatic and pathogenic effects induced by mechanical stress have been well documented in other tissues including the vascular system [24-26].

To gain insight into the regulatory mechanisms governing the observed responses to CMS, we analyzed the effects of the inhibition of several regulatory pathways on the induction of six relevant genes upregulated after CMS (HSP70B","gene1","ECSM1","MMP3","Hyaluronan Synthase","BMP2","Reg. G protein 20","VEGFC","MMP10","NRFFa4a1","EGR3","BTEbp-1","JunB protooncogene"") in TM cells.

| Gene                        | Q–PCR fold | p value | Array fold | p value |
|-----------------------------|------------|---------|------------|---------|
| HSP70B'                     | 200.81     | 6.35E-05| 489.4      | 0.0206  |
| ECSM1                       | 70.92      | 1.07E-04| 33.41      | 0.000613|
| MMP3                        | 44.67      | 1.32E-04| 29.78      | 1.13E-05|
| Hyaluronan Synthase         | 16.57      | 4.85E-04| 5.38       | 0.00474 |
| BMP2                        | 38.87      | 7.98E-05| 20.07      | 0.000553|
| Reg. G protein 20           | 30.76      | 3.33E-05| 53.81      | 0.000828|
| VEGFC                       | 4.65       | 0.00167 | 5.92       | 7.05E-05|
| MMP10                       | 255.31     | 8.90E-05| 26.77      | 0.0138  |
| NRFFa4a1                    | -258.07    | 5.35E-04| -12.58     | 0.000121|
| EGR3                        | -47.5      | 5.99E-05| -8.31      | 0.000761|
| BTEbp-1                     | -52.14     | 1.18E-04| -13.88     | 0.0022  |
| JunB protooncogene          | -41.66     | 1.18E-04| -4.67      | 0.00183 |

Genes whose expression changes after CMS was validated by Q-PCR. Columns show gene name, fold of expression change after CMS determined by Q-PCR analysis, the p-value for Q-PCR analysis calculated using the ANOVA test for the differences in means between the normalized CT values in stretched cells versus non-stretched controls, fold expression change determined by microarray analysis, and their corresponding p-value.
VEGFC, MMP3, BMP2, ECSM1, and IL8). Since the activation of inflammatory cytokines has been previously reported as a potentially important factor associated with CMS [13,27,28], we also evaluated the effects of these inhibitors on the expression of IL-8, which was the inflammatory cytokine most upregulated in our model.

MAPK and cytokines have previously been reported to be affected by mechanical stress and to induce MMPs in the TM [8,13,29,30]. Our model supports the involvement of ERK in the induction of IL8 and JNK in the induction of MMP3. These two pathways may also have an inhibitory effect in the induction of HSP70. However, general MAPK inhibitors do not provide information about specific isoforms of the different MAPKs, which may play different roles. More specific analysis will be necessary to clarify the role of the MAPK isoforms in the responses to CMS in the TM. The role of PI3K/AKT and P2 receptors in the induction of MMP3 and IL8 but not in other responses associated with tissue damage and regeneration, (e.g., ECSM1, VEGFC, and BMP2) suggests that PI3K/AKT and P2 receptors could potentially play a contributing role to homeostatic responses aimed at lowering IOP. Protective responses aimed at preventing tissue damage and regeneration such as the induction of ECSM1 and BMP2 could potentially be induced by the PKA/cAMP pathway.

All together, our results show that TM cells exposed to CMS manifest extensive changes in gene expression. Some of these changes, such as the upregulation of MMP3, have the
potential to increase outflow facility and could be part of an homeostatic mechanism involved in the maintenance of normal IOP levels. Other changes are more likely to be related to protective responses aimed at preventing cell and tissue damage (e.g., HSP70, ECSM1, and BMP2). Our results also show that several regulatory pathways may contribute to the diverse responses induced by CMS. The relative contribution of each of these pathways to the gene expression changes induced by mechanical stress may depend on the specific experimental model, which could help explain the variation in results obtained using different models. It is also possible that most that in vitro models may not reflect accurately the balance between responses associated with tissue protection and those involved in the modulation of outflow facility.

In conclusion, the effects of CMS on TM cells seem to include a complex set of responses. While some of these responses may contribute to an increase in outflow facility, others that are perhaps aimed at preserving tissue integrity from mechanical damage may have opposite effects on outflow facility. Since the TM is subjected to CMS in vivo, elucidating the mechanisms that protect the cells against mechanical damage and those induced in outflow facility homeostasis may provide important insight into both normal and pathophysiological outflow function.

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REFERENCES
1. Johnstone MA. The aqueous outflow system as a mechanical pump: evidence from examination of tissue and aqueous movement in human and non-human primates. J Glaucoma 2004; 13:421-38. [PMID: 15354083]
2. Johnstone MA. Pressure-dependent changes in nuclei and the process origins of the endothelial cells lining Schlemm's canal. Invest Ophthalmol Vis Sci 1979; 18:44-51. [PMID: 103860]
3. Hashimoto JM, Epstein DL. Influence of intraocular pressure on aqueous outflow facility in enucleated eyes of different mammals. Invest Ophthalmol Vis Sci 1980; 19:1483-9. [PMID: 6777330]
4. Davies PF, Tripathi SC. Mechanical stress mechanisms and the cell. An endothelial paradigm. Circ Res 1993; 72:239-45. [PMID: 8418981]
5. Wang JH, Thampatty BP. An introductory review of cell mechanobiology. Biomech Model Mechanobiol 2006; 5:1-16. [PMID: 16489478]
6. Chien S, Li S, Shyy YJ. Effects of mechanical forces on signal transduction and gene expression in endothelial cells. Hypertension 1998; 31:162-9. [PMID: 9453297]
7. Gonzalez P, Epstein DL, Borras T. Genes upregulated in the human trabecular meshwork in response to elevated intraocular pressure. Invest Ophthalmol Vis Sci 2000; 41:352-61. [PMID: 10670462]
8. Tumminia SJ, Mitton KP, Arora J, Zelenka P, Epstein DL, Russell P. Mechanical stretch alters the actin cytoskeletal network and signal transduction in human trabecular meshwork cells. Invest Ophthalmol Vis Sci 1998; 39:1361-71. [PMID: 9660484]
9. Vittal V, Rose A, Gregory KE, Kelley MJ, Acott TS. Changes in gene expression by trabecular meshwork cells in response to mechanical stretching. Invest Ophthalmol Vis Sci 2005; 46:2857-68. [PMID: 16043860]
10. Bradley JM, Kelley MJ, Zhu X, Anderssohn AM, Alexander JP, Acott TS. Effects of mechanical stretching on trabecular matrix metalloproteinases. Invest Ophthalmol Vis Sci 2001; 42:1505-13. [PMID: 11381054]
11. Coleman DJ, Trokel S. Direct-recorded intraocular pressure variations in a human subject. Arch Ophthalmol 1969; 82:637-40. [PMID: 5357713]
12. Ramos RF, Stamer WD. Effects of cyclic intraocular pressure on conventional outflow facility. Invest Ophthalmol Vis Sci 2008; 49:275-81. [PMID: 18172103]
13. Liton PB, Luna C, Bodman M, Hong A, Epstein DL, Gonzalez P. Induction of IL-6 expression by mechanical stress in the trabecular meshwork. Biochem Biophys Res Commun 2005; 337:1229-36. [PMID: 16229816]
14. Noonan EJ, Place RF, Giardina C, Hightower LE. Hsp70B' regulation and function. Cell Stress Chaperones 2007; 12:393-402. [PMID: 18229458]
15. Kook SH, Lee HJ, Chung WT, Hwang IH, Lee SA, Kim BS, Lee JC. Cyclic mechanical stretch stimulates the proliferation of C2C12 myoblasts and inhibits their differentiation via prolonged activation of p38 MAPK. Mol Cells 2008; 25:479-86. [PMID: 18443411]
16. Bechard D, Gentina T, Delehedde M, Scherpereel A, Lyon M, Aumercier M, Vazeux R, Richet C, Degand P, Jude B, Janin A, Fernig DG, Tonnel AB, Lassalle P. Endocan P. Endocan is a novel chondroitin sulfate/dermatan sulfate proteoglycan that promotes hepatocyte growth factor/scatter factor mitogenic activity. J Biol Chem 2001; 276:48341-9. [PMID: 11590178]
17. Saaristo A, Tammela T, Farkkila A, Karkkainen M, Suominen E, Yla-Herttuala S, Alitalo K. Vascular endothelial growth factor-C accelerates diabetic wound healing. Am J Pathol 2006; 169:1080-7. [PMID: 16936280]
18. Karpanen T, Wirzenius M, Makinen T, Veikkola T, Haisma HJ, Achen MG, Stacker SA, Pytowski B, Yla-Herttuala S, Alitalo K. Lymphangiogenic growth factor responsiveness is modulated by postnatal lymphatic vessel maturation. Am J Pathol 2006; 169:708-18. [PMID: 16877368]
19. Ribatti D. Transgenic mouse models of angiogenesis and lymphangiogenesis. Int Rev Cell Mol Biol 2008; 266:1-35. [PMID: 18544491]
20. Bradley JM, Vranka J, Colvis CM, Conger DM, Alexander JP, Fisk AS, Samples JR, Acott TS. Effect of matrix metalloproteinases activity on outflow in perfused human organ culture. Invest Ophthalmol Vis Sci 1998; 39:2649-58. [PMID: 9856774]
21. Xue W, Comes N, Borras T. Presence of an established calcification marker in trabecular meshwork tissue of glaucoma donors. Invest Ophthalmol Vis Sci 2007; 48:3184-94. [PMID: 17591888]
23. Seko Y, Azuma N, Takahashi Y, Makino H, Morito T, Muneta T, Matsumoto K, Saito H, Sekiya I, Umezawa A. Human sclera maintains common characteristics with cartilage throughout evolution. PLoS One 2008; 3:e3709. [PMID: 19002264]

24. Haga JH, Li YS, Chien S. Molecular basis of the effects of mechanical stretch on vascular smooth muscle cells. J Biomech 2007; 40:947-60. [PMID: 16867303]

25. Lehoux S, Castier Y, Tedgui A. Molecular mechanisms of the vascular responses to haemodynamic forces. J Intern Med 2006; 259:381-92. [PMID: 16594906]

26. Rath B, Nam J, Knobloch TJ, Lannutti JJ, Agarwal S. Compressive forces induce osteogenic gene expression in calvarial osteoblasts. J Biomech 2008; 41:1095-103. [PMID: 18191137]

27. Harada M, Osuga Y, Hirotta Y, Koga K, Morimoto C, Hirata T, Yoshino O, Tsutsumi O, Yano T, Taketani Y. Mechanical stretch stimulates interleukin-8 production in endometrial stromal cells: possible implications in endometrium-related events. J Clin Endocrinol Metab 2005; 90:1144-8. [PMID: 15585560]

28. Ning Q, Wang X. Role of Rel A and IkappaB of nuclear factor kappaB in the release of interleukin-8 by cyclic mechanical strain in human alveolar type II epithelial cells A549. Respirology 2007; 12:792-8. [PMID: 17986105]

29. Kelley MJ, Rose AY, Song K, Chen Y, Bradley JM, Rookhuizen D, Acott TS. Synergism of TNF and IL-1 in the induction of matrix metalloproteinase-3 in trabecular meshwork. Invest Ophthalmol Vis Sci 2007; 48:2634-43. [PMID: 17525194]

30. Kelley MJ, Rose A, Song K, Lystrup B, Samples JW, Acott TS. p38 MAP kinase pathway and stromelysin regulation in trabecular meshwork cells. Invest Ophthalmol Vis Sci 2007; 48:3126-37. [PMID: 17591882]