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Global gene profiling reveals novel glucocorticoid induced changes in gene expression of human lens epithelial cells

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Purpose: Prolonged use of glucocorticoids can lead to the formation of a cataract, however the mechanism is not known. We recently reported the presence of the functional glucocorticoid receptor in immortalized cultured mammalian lens epithelial cells (LECs), but the biological effect is not known. This study seeks to determine if freshly isolated human LECs respond to glucocorticoid treatment and to examine glucocorticoid induced changes in global gene expression in LECs.

Methods: Capsulorhexis specimens obtained in surgery from eyes with cataract were cultured. Primary lens cultures were transfected, in triplicate, with pGRE.Luc, which drives the expression of firefly luciferase, and treated with dexamethasone (Dex) or vehicle (Veh). RNA isolated from HLE B-3 cells, treated with Dex or Veh for 4 or 16 h in triplicate, was used to analyze global changes in gene expression by microarray hybridization. Data and cluster analyses were performed using Microarray Suite 5.0, GeneSpring 6.1, EASE, NetAffx, and SAM. Real Time PCR was used to confirm microarray data in RNA isolated from HLE B-3 cells in triplicate and a primary culture of human lens epithelial cells.

Results: Transfected primary cultures of human LECs treated with Dex demonstrated a glucocorticoid response with a greater than 4 fold increase in firefly luciferase activity over controls. Microarray data revealed that 136 genes were modulated with 4 h treatment with Dex. Of the 136 genes, 93 transcripts were upregulated and 43 were downregulated by greater than 1.5 fold. Eighty-six genes were modulated with 16 h Dex treatment. Of the 86 genes, 30 transcripts were upregulated and 56 were downregulated by greater than 1.5 fold. Microarray results were verified by Real Time PCR in both the HLE B-3 and primary cultures of lens epithelial cell.

Conclusions: The activation of a GRE reporter gene in primary cultures of human LECs demonstrates that the glucocorticoid receptor is functional in non-immortalized human lens cells. Microarray studies at 2 time periods demonstrate that glucocorticoids modulate gene expression in immortalized human LECs, reveal novel changes in gene expression, and confirm an endogenous genomic lens glucocorticoid response. This study demonstrates that primary cultures of lens epithelial cells and microarray technology can be used to determine pathways involved in a lens glucocorticoid response and lead to a better understanding of the formation of a steroid induced cataract.

Administration of glucocorticoids is an important therapeutic treatment for diseases such as rheumatoid arthritis, asthma, and various ocular diseases. It has been well established that a complication and side effect of prolonged corticosteroid therapy is the formation of posterior subcapsular cataract with the finding of nucleated epithelial cells in the posterior region of the lens [1-6]. The mechanism of cataract formation or of glucocorticoid action in the lens is not known.

Glucocorticoids (GC) are steroid hormones that play a role in numerous physiological processes, such as regulation of glucose, protein, and fat metabolism, and anti-inflammatory and immunosuppressive actions [7,8]. GCs exert their effects by a variety of different mechanisms. Classically, they exert their effects by binding to a specific intracellular receptor, the glucocorticoid receptor (GR), which acts as a ligand dependant transcription factor [9,10]. The ligand-receptor complex dimerizes, translocates to the nucleus, and binds to a cis acting element, the glucocorticoid response element (GRE), to modulate the expression of target genes.

Alternatively, GCs have been proposed to act on the lens indirectly through mechanisms involving oxidative stress and depletion of glutathione [11-15]. Another hypothesis involves a nonspecific action of GC through the covalent addition of steroids to lens proteins which results in destabilization of protein confirmation, oxidation, and cross linking of protein thiol groups [16,17]. This model may be related to studies demonstrating that the synthetic steroid dexamethasone bound α-crystalline nonspecifically in the bovine lens [18]. A membrane steroid binding protein was also recently identified in bovine lens epithelial cells [19]. Although this receptor is able to bind GC, its mRNA and protein sequence differ from the classical intracellular GR and a membrane steroid binding protein must act by non-genomic actions.

Previous studies provided evidence suggesting that the mammalian lens contained a classical intracellular GR. The bovine lens was reported to contain a GC binding protein that exhibited the characteristics of a receptor [20,21] and immu-

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nohistochemical and in situ hybridization studies demonstrated that the rat and human lens contained the GR [22,23]. Intracellular GRs are ubiquitously expressed [24,25], however, despite past evidence, the presence of the GR in the mammalian lens epithelium was questioned [26]. We reported the unquestionable presence of GR mRNA and protein by PCR and western blotting in immortalized and freshly isolated human and mouse lens epithelial cells [27]. Furthermore, we reported that the GR identified in the immortalized human (HLE B-3) and mouse (αTN4) lens epithelial cell cultures was able to activate transcription from a reporter vector containing a GRE element demonstrating a classical functional GR. The expression of the GR mRNA and protein in HLE B-3 cells is similar to that identified in freshly isolated human lens epithelial cells [27] suggesting the same GC induced GRE directed transcription would occur in a primary culture of human lens epithelial cells. However, immortalized cells differ from primary cultures. Proteome analysis and other studies revealed differences in protein expression between the HLE B-3 cell line and freshly isolated human lenses [28,29]. Immortalized cell lines are useful models to study, however, results need to be confirmed in primary cultures. It has yet to be determined if the GR identified in primary cultures of human lens epithelial cells (hLEC) is transcriptionally active.

The identification of the functional GR capable of inducing gene expression in immortalized human lens epithelial cells suggests that glucocorticoids are able to modulate the expression of target genes. Previous studies have failed to identify glucocorticoid target genes in lens epithelial cells [30]. Studies have shown changes in protein expression without demonstrating changes in gene expression [31]. Our own studies examining well known GC targets, such as IκBα and αB-crystallin, have been inconclusive (unpublished studies). GCs have a wide array of effects and play a role in a variety of cell functions that are cell type specific. The regulation of a well known target gene by GC in one cell type does not guarantee that it will be modulated in another cell type by GC treatment [32]. It may be difficult to identify specific GC targets by looking at genes individually.

Oligonucleotide microarrays have served as useful tools to monitor global gene expression changes in lens epithelial cells [33,34]. In the present report, we have utilized oligonucleotide microarrays to compare the global gene expression profiles between HLE B-3 cells treated with dexamethasone (Dex), a synthetic glucocorticoid, or vehicle (Veh) at two different time periods that demonstrate early lens GC responses and identify possible mechanisms of GC action in lens cells. Real time PCR verified the microarray results. Functional clustering of the modulated genes revealed that GCs play a role in multiple biological processes and molecular pathways. Furthermore, in this study, we demonstrated that primary cultures of human lens epithelial cells contain a transcriptionally active GR and verified that gene changes identified by microarray occurred in primary lens cells as well. These data demonstrate changes in expression of specific gene targets due to GC treatment, identify a GC response in lens epithelial cells and for the first time identify a response in primary cultures of hLECs. These novel findings conclusively identify specific glucocorticoid targets, which provide the basis for further experimentation into the action of glucocorticoids in lens epithelial cells. The broad and large number of functional categories may be related to and provide an explanation for the historical difficulty in understanding a lens glucocorticoid response.

METHODS

Tissue, cell culture, and treatment: Freshly isolated human lens epithelial cells were obtained from capsulorhexis specimens obtained from surgery or from eye bank donor eyes. Capsulorhexis specimens were placed into culture dishes. The epithelial layer (from donor lenses) was carefully separated from the fiber cells using a dissecting microscope and cultured in a dish. Cultured lens epithelial cells were maintained in phenol red free DMEM with 20% serum and passaged 1 time before treatment. Medium was replaced with medium containing a reduced amount of charcoal stripped serum 16 h before treatment. All procedures complied with the Declaration of Helsinki.

HLE B-3 cells (a gift from Dr. Usha Andley) were maintained in phenol red free MEM with 20% serum. Medium was replaced with medium containing a reduced amount of charcoal stripped serum 16 h before treatment.

Dexamethasone was purchased from Sigma (St. Louis, MO), dissolved in absolute ethanol and diluted in media according to manufacturer's protocols. Absolute ethanol served as a vehicle control. The final concentration of absolute ethanol in the samples never exceeded 0.1%.

Transfections: Plasmid pGRE.Luc (Clonetech, Palo Alto, CA) contains three copies of the GRE enhancer element fused to the TATA-like promoter region from the HSV-TK promoter and drives the expression of the firefly luciferase reporter gene. Plasmid pRL-SV40 (Promega, Madison, WI) contains the SV40 early enhancer-promoter region driving expression of the Renilla luciferase reporter gene and serves to normalize transfection efficiencies. HLE B-3 cells were seeded (in triplicate) in a 24 well plate 24 h before transfection. Primary cultures of lens epithelial cells, from capsulorhexis specimens, were seeded in triplicate in 96 well plates. Cells were co-transfected with pRL-SV40 and pGRE.Luc using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as previously reported. Transfection medium was changed after 5 h, and 16 h later cells were treated with 1 μM Dex or Veh for 24 h. The Dual Luciferase Reporter Assay System (Promega, Madison, WI) was used according to manufacturer's instructions to assay cell extracts. Luciferase activity was measured on a luminometer (Packard LumiCount, Packard Instrument Company, Downers Grove, IL).

Microarray RNA preparation, hybridization, and analysis: HLE B-3 cells (tenth passage) were seeded (in triplicate) in full serum medium and 24 h later cells were washed and medium was changed to 2% charcoal-stripped serum medium and cells were incubated for 16 h before treatment. Cells were treated with 1 μM Dex or Veh for 4 or 16 h in triplicate. All samples for a single time point were processed and analyzed at the same time.

RNA extraction was performed with RNAzol (Tel-Test, Friendswood, TX). RNAzol (1.6 ml) was added to each flask for 3 min before the homogenate was vigorously pipetted and transferred to 2 ml tubes on ice. Chloroform (160 µl) was added, shaken vigorously, and incubated on ice for 7 min. Samples were centrifuged at 12,000x g and 4 °C for 15 min and the aqueous layer was transferred to a clean tube. Isopropanol (70 µl) was added to each tube and was mixed gently by inversion before storage at -80 °C overnight. The following day the sample was centrifuged at 12,000x g for 15 min at 4 °C. The supernatant was removed and the pellet was washed with 80% ethanol (2 times) and the pellet was air dried and resuspended in 30 µl RNAse free water (Ambion, Austin, TX). Each sample was analyzed by spectrophotometry and agarose gel electrophoresis before RNA purification and clean up.

RNA was purified using the RNeasy kit (Qiagen, Valencia, CA) according to manufacturer's protocols. Concentration was determined spectrophotometrically and examined for quality on 1% agarose gels. 260/280 ratios were greater than 1.8.

Double-stranded cDNA was synthesized from total RNA using the Superscript Double Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). First strand synthesis was carried out with 5 µg total RNA, 100 pM T7-$(dT)_{24}$ primer and DEPC treated water. The sequence of the HPLC purified T7-$(dT)_{24}$ primer was 5'-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG TTT TTT TTT TTT TTT TTT TTT TTT-3' (Integrated DNA Technologies, INC, Coralville, IA). The RNA primer mixture was incubated at 70 °C for 10 min. First strand buffer and 10 mM DTT and 500 µM of each dNTP was added to the mixture and incubated at 42 °C for 2 min. Finally, 200 Units of SuperScript II Reverse Transcriptase was added and the RT reaction was carried out at 42 °C for 1 h.

Second strand cDNA synthesis was carried out by adding second strand buffer, 200 µM each dNTP, 10 Units DNA ligase, 40 Units DNA polymerase I, 2 Units RNase H and water to the first strand synthesis reaction. The mixture was incubated at 16 °C for 2 h. 10 Units T4 DNA polymerase was added to the reaction and incubated at 16 °C for 5 min. EDTA (10 µl of 0.5 M) was used to stop the reaction.

To clean up the double stranded cDNA, 1.5 ml Phase Lock Gel light tube (Eppendorf, Westbury, NY) was centrifuged for 30 s at 12,000x g to pellet the gel. Phenol:chloroform:isoamyl alcohol (25:24:1, saturated with Tris-HCL, pH 8.0, 1 mM EDTA) was added to the double stranded cDNA synthesis preparation and vortexed. The cDNA-phenol mixture was transferred to the Phase Lock Gel tube and centrifuged at 12,000x g at room temperature for 2 min. The aqueous layer was transferred to a fresh tube. NH_4Ac (0.5 volumes of 7.5 M) and 2.5 volumes of cold absolute ethanol were added to the sample, vortexed, and stored at -20 °C overnight. The next day, the mixture was centrifuged at 12,000x g for 20 min at 5 °C and the supernatant was discarded. The pellet was washed with 80% ethanol and centrifuged at 12,000x g for 5 min at 5 °C. The wash was repeated and the pellet was dried and resuspended in RNase free water and analyzed on a 1% agarose gel.

cRNA synthesis was carried out through an in vitro transcription reaction using the ENZO Bioarray HighYield RNA Transcript Labeling Kit (Affymetrix, Santa Clara, CA) according to manufacturer's protocols. cDNA was added to a mixture containing 1X HY reaction buffer, 1X biotin labeled ribonucleotides, 1X DTT, 1X RNase Inhibitor mix, 1X T7 RNA Polymerase, DEPC treated water and incubated at 37 °C for 4-5 h with gentle mixing by pipetting every 45 min. Each reaction was divided in half and half was stored away at -80 °C. The other half was purified with the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA).

RLT buffer (without β-mercaptoethanol) was added to the sample and mixed. Absolute ethanol was added and the mixture was placed into a RNeasy mini spin column and centrifuged at 8000x g for 15 s. The flow through was reapplied to the column and centrifuged at 8000x g for 15 s. The column was transferred to a fresh collection tube and buffer RPE was added before centrifugation was repeated. Filtrate was discarded and the RPE wash step was repeated. The column was placed into a new collection tube and centrifuged for 2 min at maximum speed. The column was placed into a fresh collection tube and RNAse free water was added directly to the column membrane and incubated at room temperature for 1 min. cRNA was extracted by centrifugation at 8000x g for 1 min at room temperature.

To concentrate the cRNA, 0.5 volumes of 7.5 M NH_4Ac and 2.5 volumes of cold absolute ethanol were added to the sample, mixed and stored at -20 °C overnight. The mixture was centrifuged at 12,000x g for 20 min at 5 °C and the supernatant was discarded. The pellet was washed with 80% ethanol and centrifuged at 12,000x g for 5 min at 5 °C. The wash was repeated and the pellet was dried in a Speedvac and resuspended in RNAse free water. cRNA concentration was determined by spectrophotometry and was analyzed by electrophoresis on a 1% agarose-formaldehyde-borate gel.

cRNA (15 µg) was fragmented and added to a hybridization mixture at the Center for Applied Genomics (The Public Health Research Institute, Newark, NJ.). Expression profiles were created using the HG-U133A GeneChip (Affymetrix, Santa Clara, CA), which contains 22,283 known human transcripts and ESTs coding for about 15,000 known genes. These transcripts are designed using 11-20 probe pairs consisting of 25-mer oligonucleotides. Hybridization was done overnight at 45 °C for 16 h using the GeneChip Hybridization Oven 640 (Affymetrix, Santa Clara, CA). Washing and staining (Streptavidin Phycoerythrin) was accomplished with the GeneChip Fluidics Station 400 (Affymetrix, Santa Clara, CA) using the EukGE-WS2v4 protocol. Images were acquired using the Affymetrix GeneArray scanner. Data was extracted using Affymetrix Microarray Suite 5.0.

Data analysis was performed using several different software packages including Microarray Suite 5.0 (Affymetrix, Santa Clara, CA) and GeneSpring 6.2 (Silicon Genetics, Redwood City, CA). Numeric data were extracted from DAT images and normalized using Microarray Suite. The method of normalization used was a scaling algorithm which involves

multiplying the mean intensity of each chip (not including the upper and bottom 2%) by a factor which changes the mean intensity to 500 for every chip. By scaling each chip, a direct comparison could be made between all the chips. The data were entered into the GeneSpring for further analysis. Data were filtered based on both *Detection Call* and *Signal Log Ratio* in any of the comparisons between 4 h Dex, 4 h Veh, 16 h Dex, and 16 h Veh. Comparisons made between groups had to be uniform within groups. In the case of the Affymetrix *Detection Call* algorithm, data that were declared *Absent* in all samples compared were filtered out. Samples had to be declared *Present* in at least two of three chips within a treatment group to be kept within the set. An additional filter was made based on the *Signal Log Ratio* which is the log base 2.0 of the fold change. Any comparisons which had a conditional group declared *Present* had to have at least one group with a *Signal Log Ratio* greater than ±-0.58 (a fold change greater than ±-1.5). In addition, ANOVA (assuming unequal variances) was run on the filtered list. The Benjamin-Hobbson false discovery rate was applied as a multiple correction factor.

Filtered genes identified to be differentially expressed by 1.5 fold or greater in two of three chips were analyzed for functional gene clusters using the Expression Analysis Systematic Explorer (EASE) [35], NetAffx Analysis Center [36], Significance Analysis of Microarrays (SAM) [37], and GeneSpring [38]. These programs are used to determine functional clusters by statistical representation of individual genes in specific categories relative to all genes in the same category on the array. EASE provides statistical methods for discovering enriched biological themes within gene lists and generating gene annotation tables. The NetAffx Analysis Center allows the correlation of the microarray results with the specific array design and with annotation tools. The Gene Ontology (GO) Mining Tool, used in the EASE and NetAffx Analysis Center, matches GeneChip probe sets to annotated genes within the biological process, molecular function, or cellular components to allow for biological interpretation of microarray results. GeneSpring uses data found publicly in genomics databases to build gene ontologies based on annotation information.

Real time polymerase chain reaction (RT-PCR): Capsules containing the epithelial layer from a single pair of 46-year-old donor lenses were dissected from the fiber cells and placed in DMEM containing 20% serum. HLE B-3 cells (tenth passage) were seeded (in triplicate) in phenol red free MEM containing 20% serum. Sixteen h before treatment, medium was changed to phenol red free MEM with 2% charcoal stripped serum. Cultures were treated with 1 μM Dex or Veh for 4 h.

Total RNA was isolated using RNAzol (Tel-Test, Friendswood, TX) according to manufacturer's protocols. RNA concentration was determined by spectrophotometry. Isolated RNA was then aliquoted and stored at -80 °C.

RNA was reverse transcribed using Applied Biosystems reagents on a thermocycler (GeneAmp PCR system 9700; PE Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. Briefly, 1 μg total RNA was mixed with 5 mM $MgCl_2$, 1X PCR buffer, 4 mM each dNTP, RNase Inhibitor, Oligo dT, and MuLv reverse transcriptase. The reaction was incubated at 42 °C for 60 min, 95 °C for 5 min, and held at 4 °C.

For each sample, primers for actin were used to determine the quality of the RNA. The sequences of human specific primers used in this study, along with their corresponding GenBank accession numbers and product sizes are shown in Table 1. Primers were designed by using Primer3. The human specific primers were designed to Period 1 (Per1), Delta Sleep Inducing Peptide Like Immunoreactor (DSIP), Heat Shock Protein 70 (HSP70), Protein Kinase c-AMP dependant Regulatory type 1 Alpha (PRKAR1A), Coagulation Factor II (Thrombin) Receptor (F2R), Plasminogen Activator Inhibitor-1 (PAI-1), Growth Arrest and DNA-damage-inducible protein (GADD45), Serum Glucocorticoid Regulated Kinase (SGK), Pleckstrin Homology-Like Domain, Family A (PHLDA), Immediate Early Response 3 (IER3), Nerve Growth Factor (NGF), Sodium Channel, Non-voltage-Gated 1 Alpha (SCNN1A), Cyclin D1/BCL-1 (CCND1), and Cholecystokinin (CCK). The sequence of the primers for the Monocyte Chemotactic Protein 1 (MCP-1) and Dual Specificity Phosphatase 1 (DUSP1) were previously published [39,40].

PCR was performed using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science, Indianapolis, IN), and the LightCycler 1.0 (Roche Applied Science, Indianapolis, IN). SYBR green fluoresces upon binding to the minor groove of dsDNA. Monitoring the fluorescence of the reaction in real time allows the amplification to be halted when the sample is undergoing exponential growth making quantification of small differences possible. The reaction was stopped during the log phase to allow for quantification of small differences.

The quantification of material labeled with SYBR green was analyzed by crossing point analysis, which represents the cycle number at which the sample begins exponential growth over the background noise. The data were presented in fluorescence versus cycle format in which all sample baselines were brought to a comparable level. The baselines were brought into a similar range by an arithmetic baseline adjustment in which the mean of the five lowest measured data points for each sample was subtracted from each data point. Next the exponential curve was transformed into a linear curve and a noise band was set that excluded background noise levels and established the lower limit of analysis in the exponential phase for all samples. The data were then presented in log-linear format. The number of Fit Points plotted on the exponential portion of the curve were increased to establish the upper limit of analysis and to include the maximum number of acquisition events in the crossing point assessment. The relative fold difference in exponential growth was defined as $2^{(a-b)}$ where a and b represent the crossing points of the two samples being compared. Results were normalized to actin. Specificity of PCR products was determined by melting curve analysis and visualization on 1 or 2% agarose gels stained with ethidium bromide.

RESULTS

In order to identify if primary cultures of human lens epithelial cells respond to GC treatment, primary cultures were created from explants of capsulorhexis specimens from surgery. Epithelial cells grew off the capsule and onto the plate (Figure 1). Growing explant cultures were then transfected with pGRE.Luc. Transfected cells were treated with 1 μM Dex or Veh for 24 h. Epithelial cell explants from four pairs of donor lenses were examined, in duplicate or triplicate, and, despite a large sample variation, each sample displayed greater than 4 fold increase in luciferase activity in Dex treated samples (Figure 2).

Glucocorticoid induced changes in gene expression have been reported to occur as early as 15-30 min after hormone administration [41]. In order to identify an early time point to identify a primary response through GRE mediated genes, HLE B-3 cells were transfected with pGRE.Luc and treated with 1 μM Dex or Veh over a time course of 24 h. A significant increase in luciferase activity compared to vehicle was identified between 2-4 h and this was sustained over a 24 h period (Figure 3). Although GC mediated changes in gene expression may be occurring before 4 h, a 4 h treatment time period was chosen to examine by microarray. A later time of 16 h was also examined by microarray to identify genes that may be downstream of the 4 h response to lead to a better understanding of pathways involved in the GC response.

Microarray analysis was performed on total RNA extracts from tenth passage HLE B-3 cells treated with either 1 μM Dex or Veh for 4 or 16 h (in triplicate). The complete data profiles have been deposited in NCBIs Gene Expression Omnibus (GEO; accession number GSE3040).

The scaled data generated from Microarray Suite were imported into GeneSpring for fold change analysis, filtering, and cluster analysis. To identify highly reproducible changes, data were filtered based on select criteria. To be kept within the set, transcripts had to be modulated by at least 1.5 fold in at least two of three chips within a treatment group. All of the genes listed in Table 2 passed the filtering criteria in GeneSpring. However, GeneSpring and Microarray Suite use different algorithms for generating fold change. To determine the standard error and p values by ANOVA, the data filtered by GeneSpring was analyzed in Microarray Suite. Due to the difference in algorithms, several of the genes that met the 1.5 fold criteria in GeneSpring did not meet the same criteria when analyzed in Microarray Suite 5.0 (Table 2). However, genes that fell below the 1.5 fold cutoff or had a p value of greater than or equal to 0.05 were kept within the set in order to maximize the number of genes. Lens glucocorticoid responses have been difficult to elucidate. Since the genes met the criteria in GeneSpring, they were left within the set in order to avoid the possibility of excluding potential GC lens targets. The genes included by Genespring are useful as the purpose was to find a list of potentially interesting genes that will be confirmed by real-time PCR.

Table 1. Sequences of primers for RT-PCR

Primer	Accession number	Sequence	Product size (bp)
Period 1	NM_002616	F: CTTTACCCAGGAGAAGTCCG R: CTGGATATCAGTGTCCAGGG	592
Delta Sleep Inducing Peptide	AL110191	F: TCTCCTCTGTTTCGTGAAGG R: GACAAACTGGGTCAACTTGG	358
Heat Shock Protein 70	L12723	F: AAACTGAAGGAGACAGCCG R: CACATCTCCAGAAATTTGCC	560
Protein Kinase c-AMP dependant Regulatory type 1 Alpha	NM_002734	F: GAGAACCATGGAGTCTGGC R: CTTTAACCACTGGGTTGGG	284
Coagulation Factor II Receptor	NM_001992	F: TGCAGTGCAGTATAGAATAGGC R: GCAGCTCTCAGGAATAGCC	454
Plasminogen Activator Inhibitor-1	NM_000602	F: AGTGAAGATCGAGGTGAACG R: GACCTAAAGGTCCTCCAAGG	360
Growth Arrest and DNA-damage-inducible protein	NM_001924	F: CTTATTTGTTTTTGACGGG R: GTTGAACTCACTCAGCCCC	207
Serum Glucocorticoid Regulated Kinase	NM_005627	F: GCCTTATGACAGGACTGTGG R: CTCCATGAAGTCATCCTTGG	229
Plecstrin Homology-Like Domain Family A	NM_007350	F: AAGGTTTTGAGGACTTGAGG R: TTCACCATACAAGACGATCC	235
Immediate Early Response 3	NM_003897	F: CAAAAAGAATCCGAAAAACC R: ATTAACGACGCTCTCCTTCC	221
Nerve Growth Factor	NM_002506	F: ATTGTACCACGACTCACACC R: GCCCAGGAGAGTGTAGAAGG	175
Sodium Channel Non Voltaged Gated 1 Alpha	NM_001038	F: TTCCTGTTGAGAACCTTTACCC R: CGCGGATAGAAGATGTAGGC	112
Cyclin D1	M73554	F: AGCTGTAGTGGGGTTCTAGG R: CACGCTACGCTACTGTAACC	159
Cholecystokinin	NM_000729	F: TGCCGAGGAGTATGAGTACC R: AACATTTTGTCTTCCATTTGC	239

This table lists the accession number, sequence, and product size for the primers used in the RT-PCR reactions.

Hierarchical clustering with the Pearson Correlation was performed based on signal intensities generated from Microarray Suite. A condition cluster and dendrogram were generated based on the gene list created after filtering. The condition tree clustered based on the overall expression of each chip or treatment. Concurrently, a gene tree and dendrogram were also generated on the gene list. The gene tree clustered based on the expression of each gene across treatments. The treatments clustered together and demonstrated that the triplicates were reproducible (Figure 4).

A list of 136 genes passed the criteria for the 4 h data set and 86 genes for the 16 h data set (Table 2). Of the 136 genes from the 4 h data set, 93 transcripts were upregulated and 43 were downregulated by greater than 1.5 fold. Of these, 38 genes were upregulated and 1 was downregulated by greater than 3 fold. Of the 86 genes from the 16 h data set, 30 transcripts were upregulated and 56 were downregulated by greater than

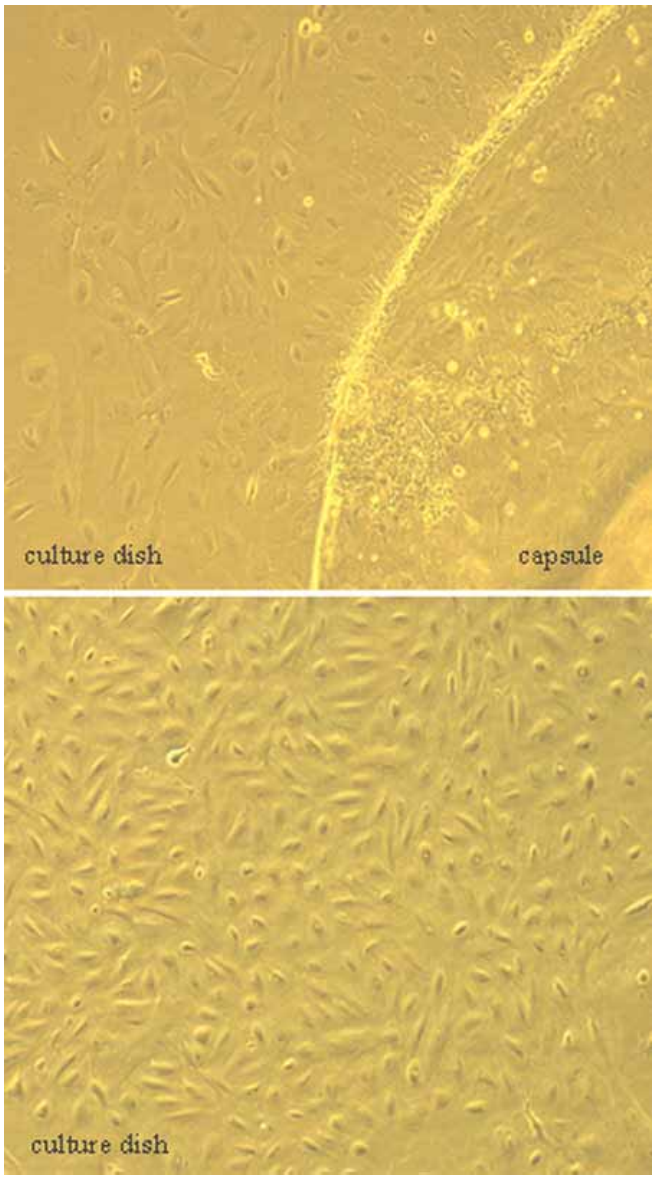

Figure 1. Primary human lens epithelial cell cultures. Capsulorhexis specimens from eyes <60 years old obtained from cataract surgery were cultured in DMEM containing 20% serum. Cells grew off the capsule and on to the culture dish. Pictures were taken by bright field phase contrast microscopy with 100x magnification.

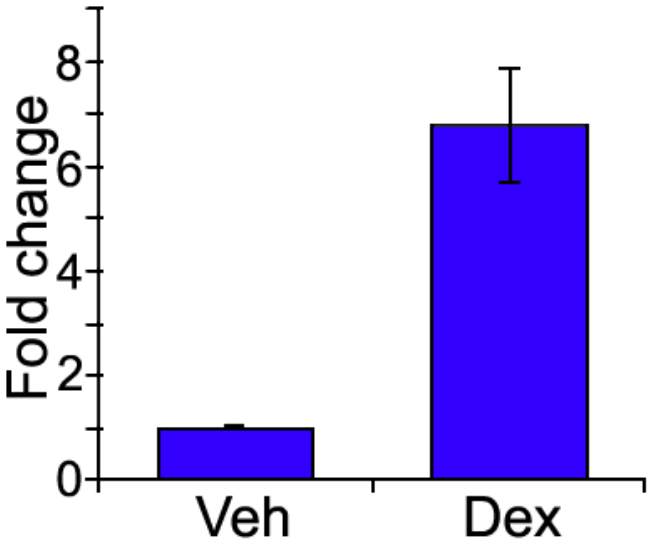

Figure 2. Transcriptionally active glucocorticoid receptor in primary cultures. Primary lens epithelial cell cultures were co-transfected with pGRE.Luc and pRL-SV40 and treated with 1 μM dexamethasone or vehicle (Veh) for 24 h before luciferase activity was measured. Data are from four different primary cultures created from capsulorhexis specimens treated in duplicate or triplicate. The error bars represent the standard error of the mean (n=11). Values were significantly different from Veh (two tail, two sample t-test assuming unequal variances; $p<0.0002$).

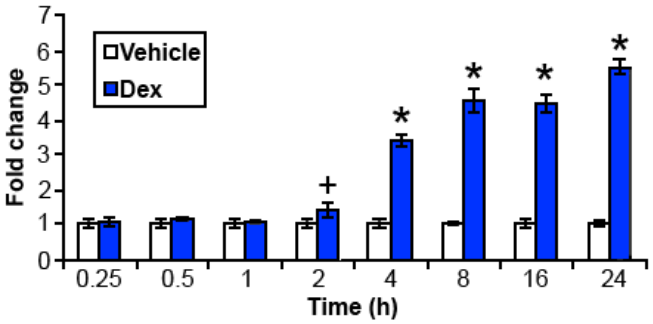

Figure 3. GRE activation in transfected HLE B-3 cells. Tenth passage HLE B-3 cells were co-transfected with pGRE.Luc and pRL-SV40 to normalize transfection efficiencies. Transfected cells were cultured in the presence or absence of dexamethasone for times indicated and luciferase activities were measured in each sample. Each time point was examined 2-5 times, each in triplicate, with similar results. Data are the mean of one experiment carried out in triplicate. The error bars represent the standard deviation (n=3). Values were significantly different (two tail, two sample t-test assuming unequal variances) from vehicle. The plus sign indicates $p<0.02$ and the asterisk indicates $p<0.00002$ when compared to the vehicle control at the same time.

Table 2. Microarray results reveal modulation of gene expression

Probe ID	Gene name	Fold change	p value	Description
Genes upregulated by 4 h Dex treatment				
202861_at	PER1	6.01 ± 1.72	0.0256	period homolog 1 (Drosophila)
208763_s_at	DSIPI	5.56 ± 0.52	0.000497	delta sleep inducing peptide immunoreactor
211015_s_at	HSPA4	4.84 ± 2.79	0.061	heat shock protein
206544_x_at	SMARCA2	4.54 ± 1.3	0.00279	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2
200605_s_at	PRKAR1A	4.37 ± 2.83	0.0858	protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)
212681_at	EPB41L3	4.28 ± 2.11	0.0437	erythrocyte membrane protein band 4.1-like 3
208671_at	TDE1L	4.16 ± 2.22	0.0464	tumor differentially expressed 2
203989_x_at	F2R	4 ± 1.66	0.00991	coagulation factor II (thrombin) receptor
217894_at	NY-REN-45	3.94 ± 1.29	0.0162	NY-REN-45 antigen
218247_s_at	LOC51320	3.89 ± 1.48	0.0303	hypothetical protein LOC51320
219166_at	C14orf104	3.81 ± 1.49	0.0595	chromosome 14 open reading frame 104
220924_s_at	SLC38A2	3.69 ± 2.54	0.064	solute carrier family 38, member 2
201417_at	SOX4	3.66 ± 1.58	0.0305	SRY box 4
204780_s_at	TNFRSF6	3.61 ± 1.13	0.018	tumor necrosis factor receptor superfamily, member 6
202948_at	IL1R1	3.56 ± 0.87	0.00245	interleukin 1 receptor, type I
213688_at	CALM1	3.54 ± 0.63	0.0129	calmodulin 1 (phosphorylase kinase, delta)
209681_at	SLC19A2	3.51 ± 1.47	0.0212	solute carrier family 19 (thiamine transporter), member 2
212526_at	TAHCCP1	3.46 ± 1.14	0.0234	spastic paraplegia 20, spartin (Troyer syndrome)
201924_at	MLLT2	3.42 ± 1.71	0.0651	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 2
209422_at	C20orf104	3.4 ± 1.02	0.00719	PHD finger protein 20
218640_s_at	PLEKHF2	3.4 ± 1.28	0.0528	phafin 2
206710_s_at	KIAA0987	3.37 ± 1.44	0.0413	erythrocyte membrane protein band 4.1-like 3
208920_at	SRI	3.37 ± 0.77	0.0121	sorcin
217870_s_at	UMP-CMPK	3.35 ± 1.58	0.0339	UMP-CMP kinase
202514_at	DLG1	3.33 ± 1.74	0.578	discs, large homolog 1 (Drosophila)
201263_at	TARS	3.22 ± 1.22	0.0222	threonyl-tRNA synthetase
212610_at	MGC14433	3.19 ± 1.55	0.0475	protein tyrosine phosphatase, nonreceptor type 11 (Noonan syndrome 1)
202611_s_at	CRSP2	3.17 ± 1.46	0.0463	cofactor required for Sp1 transcriptional activation, subunit 2, 150 kDa
213139_at	SNAI2	3.17 ± 0.74	0.00501	snail homolog 2 (Drosophila)
221841_s_at		3.17 ± 0.49	0.00421	Homo sapiens cDNA FLJ38575 fis, clone HCHON2007046.
203934_at	KDR	3.16 ± 0.38	0.000296	kinase insert domain receptor (a type III receptor tyrosine kinase)
202628_s_at	SERPINE1	3.15 ± 0.33	0.00204	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
212522_at	PDE8A	3.15 ± 0.84	0.0115	phosphodiesterase 8A
202156_s_at	CUGBP2	3.12 ± 1.25	0.0501	CUG triplet repeat, RNA binding protein 2
201921_at	GNG10	3.1 ± 0.69	0.0104	guanine nucleotide binding protein (G protein), gamma 10
202602_s_at	HTATSF1	3.08 ± 1.22	0.033	HIV TAT specific factor 1
201922_at	YR-29	3.07 ± 1.2	0.0232	hypothetical protein YR-29
204342_at	DKFZp586G0123	3.02 ± 1.14	0.0175	hypothetical protein DKFZp586G0123
201662_s_at	FACL3	2.99 ± 1.16	0.0183	acyl-CoA synthetase long-chain family member 3

212887_at	SEC23A	2.99 ± 1.45	0.0788	Sec23 homolog A (S. cerevisiae)
209595_at	GTF2F2	2.97 ± 0.67	0.0044	general transcription factor IIF, polypeptide 2, 30 kDa
214719_at	LOC283537	2.97 ± 0.36	0.0000314	hypothetical protein
209049_s_at	PRKCBP1	2.94 ± 0.65	0.00206	RACK family protein
202783_at	NNT	2.92 ± 0.82	0.0207	nicotinamide nucleotide transhydrogenase
201889_at	FAM3C	2.91 ± 0.34	0.00148	family with sequence similarity 3, member C
218566_s_at	CHORDC1	2.91 ± 0.74	0.014	cysteine and histidine-rich domain (CHORD)-containing, zinc binding protein 1
207821_s_at	PTK2	2.89 ± 0.79	0.0131	PTK2 protein tyrosine kinase 2
213605_s_at	FLJ40092	2.87 ± 0.79	0.00561	FLJ40092 protein
203486_s_at	DKFZP434A043	2.85 ± 1.13	0.0623	DKFZP434A043 protein
206385_s_at	ANK3	2.85 ± 0.99	0.0188	ankyrin 3, node of Ranvier (ankyrin G)
202430_s_at	PLSCR1	2.84 ± 0.65	0.013	phospholipid scramblase 1
218901_at	PLSCR4	2.84 ± 0.68	0.00377	phospholipid scramblase 4
208694_at	PRKDC	2.8 ± 0.9	0.0139	protein kinase, DNA-activated, catalytic polypeptide
201948_at	HUMAUANTIG	2.78 ± 0.44	0.00263	nucleolar GTPase
210466_s_at	PAI-RBP1	2.78 ± 1.07	0.0306	PAI-1 mRNA-binding protein
200066_at	IK	2.76 ± 0.75	0.0215	IK cytokine, downregulator of HLA II
203817_at	GUCY1B3	2.76 ± 0.5	0.00337	guanylate cyclase 1, soluble, beta 3
204715_at	PANX1	2.74 ± 0.32	0.00104	pannexin 1
200603_at	PRKAR1A	2.73 ± 0.91	0.0237	protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)
201075_s_at	SMARCC1	2.71 ± 0.53	0.00118	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1
201424_s_at	CUL4A	2.7 ± 0.94	0.031	cullin 4A
206542_s_at	SMARCA2	2.7 ± 0.51	0.00331	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2
208815_x_at	HSPA4	2.7 ± 0.89	0.0265	heat shock 70 kDa protein 4
209288_s_at	CDC42EP3	2.66 ± 0.92	0.0492	CDC42 effector protein (Rho GTPase binding) 3
206748_s_at	SPAG9	2.63 ± 0.52	0.0135	sperm associated antigen 9
212543_at	AIM1	2.62 ± 0.75	0.0375	absent in melanoma 1
202627_s_at	SERPINE1	2.61 ± 0.33	0.000293	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
202760_s_at	AKAP2	2.61 ± 0.94	0.0605	A kinase (PRKA) anchor protein 2
217707_x_at	SMARCA2	2.54 ± 0.32	0.011	ESTs
221776_s_at	BRD7	2.54 ± 0.48	0.00179	bromodomain containing 7
202211_at	ARFGAP3	2.5 ± 0.43	0.00187	ADP-ribosylation factor GTPase activating protein 3
221840_at	PTPRE	2.5 ± 0.37	0.0132	protein tyrosine phosphatase, receptor type, E
207761_s_at	DKFZP586A0522	2.47 ± 0.47	0.00347	DKFZP586A0522 protein
205618_at	PRRG1	2.46 ± 0.22	0.00000859	proline-rich Gla (G-carboxyglutamic acid) polypeptide 1
218330_s_at	NAV2	2.42 ± 0.48	0.0201	neuron navigator 2
201739_at	SGK	2.41 ± 0.29	0.00343	serum/glucocorticoid regulated kinase
212062_at	ATP9A	2.39 ± 0.4	0.0038	ATPase, Class II, type 9A
213327_s_at	USP12	2.36 ± 0.3	0.013	ubiquitin specific protease 12
219383_at	FLJ14213	2.36 ± 0.23	0.000528	hypothetical protein FLJ14213
212245_at	SDNSF	2.34 ± 0.41	0.00165	neural stem cell derived neuronal survival protein
203739_at	ZNF217	2.32 ± 0.32	0.0074	zinc finger protein 217
218698_at	MMRP19	2.3 ± 0.32	0.00928	likely ortholog of mouse monocyte macrophage 19
201855_s_at	KIAA0431	2.28 ± 0.56	0.0218	KIAA0431 protein
200610_s_at	NCL	2.27 ± 0.42	0.00565	nucleolin
221589_s_at	ALDH6A1	2.27 ± 0.33	0.00493	Aldehyde dehydrogenase 6 family, member A1
212412_at	PDLIM5	2.24 ± 0.51	0.0134	PDZ and LIM domain 5
220033_at		2.19 ± 0.26	0.00203	
203140_at	BCL6	2.18 ± 0.19	0.000477	B-cell CLL/lymphoma 6 (zinc finger protein 51)

211733_x_at	SCP2	2.17 ± 0.35	0.00184	sterol carrier protein 2
213341_at	FEM1C	2.17 ± 0.27	0.00224	fem-1 homolog c (C.elegans)
213507_s_at	KPNB1	2.15 ± 0.29	0.00341	karyopherin (importin) beta 1
213103_at	STARD13	2.09 ± 0.1	0.00125	START domain containing 13

Genes downregulated by 4 h Dex treatment

213070_at		-0.42 ± 2.01	0.078	Homo sapiens mRNA; cDNA DKFZp564L222 (from clone DKFZp564L222)
202500_at	DNAJB2	-1.34 ± 0.06	0.000378	DnaJ (Hsp40) homolog, subfamily B, member 2
222221_x_at	EHD1	-1.38 ± 0.14	0.000546	EH-domain containing 1
204295_at	SURF1	-1.39 ± 0.07	0.00912	surfeit 1
201234_at	ILK	-1.4 ± 0.07	0.00528	integrin-linked kinase
210075_at	LOC51257	-1.4 ± 0.09	0.000594	membrane-associated ring finger (C3HC4) 2
213789_at	EBP	-1.4 ± 0.11	0.00044	emopamil binding protein (sterol isomerase)
214892_x_at	NY-REN-24	-1.41 ± 0.1	0.000421	
34260_at	KIAA0683	-1.41 ± 0.11	0.00207	KIAA0683 gene product
211972_x_at	RPLP0	-1.43 ± 0.17	0.00339	ribosomal protein, large, P0
209445_x_at	FLJ10803	-1.44 ± 0.21	0.00173	hypothetical protein FLJ10803
211668_s_at	PLAU	-1.45 ± 0.19	0.00167	plasminogen activator urokinase
200966_x_at	ALDOA	-1.46 ± 0.1	0.00419	aldolase A, fructose-bisphosphate
202041_s_at	FIBP	-1.46 ± 0.13	0.028	fibroblast growth factor (acidic) intracellular binding protein
220326_s_at	FLJ10357	-1.46 ± 0.07	0.00287	hypothetical protein FLJ10357
221849_s_at	LOC90379	-1.46 ± 0.14	0.0076	hypothetical protein BC002926
204405_x_at	HSA9761	-1.47 ± 0.19	0.000877	putative dimethyladenosine transferase
205807_s_at	TUFT1	-1.47 ± 0.14	0.0259	tuftelin 1
201391_at	TRAP1	-1.48 ± 0.15	0.0233	heat shock protein 75
202081_at	ETR101	-1.49 ± 0.11	0.0012	immediate early protein
217994_x_at	FLJ20542	-1.49 ± 0.18	0.0365	hypothetical protein FLJ20542
204420_at	FOSL1	-1.5 ± 0.18	0.000413	FOS-like antigen 1
219170_at	FSD1	-1.5 ± 0.09	0.00335	fibronectin type 3 and SPRY domain-containing protein
219172_at	FLJ11807	-1.51 ± 0.16	0.000386	hypothetical protein FLJ11807
215498_s_at	MAP2K3	-1.52 ± 0.2	0.0143	mitogen-activated protein kinase kinase 3
218463_s_at	MUS81	-1.52 ± 0.14	0.0224	MUS81 endonuclease
219348_at	MDS032	-1.55 ± 0.21	0.0353	uncharacterized hematopoietic stem/progenitor cells protein MDS032
200035_at	HSA011916	-1.59 ± 0.28	0.00491	likely ortholog of Xenopus dullard
203234_at	UP	-1.61 ± 0.17	0.00474	uridine phosphorylase
219457_s_at	RIN3	-1.61 ± 0.2	0.00611	Ras and Rab interactor 3
217996_at	PHLDA1	-1.62 ± 0.2	0.0237	pleckstrin homology-like domain, family A, member 1
218762_at	FLJ22059	-1.66 ± 0.25	0.0312	hypothetical protein FLJ22059
205224_at	SURF2	-1.68 ± 0.21	0.00263	surfeit 2
219742_at	MGC10772	-1.72 ± 0.31	0.00581	hypothetical protein MGC10772
220134_x_at	FLJ10647	-1.74 ± 0.15	0.000309	hypothetical protein FLJ10647
214674_at	USP19	-1.78 ± 0.19	0.021	ubiquitin specific protease 19
221710_x_at	FLJ10647	-1.78 ± 0.19	0.0167	hypothetical protein FLJ10647
202996_at	POLD4	-1.8 ± 0.16	0.00359	polymerase (DNA-directed), delta 4
205561_at	FLJ12242	-1.84 ± 0.24	0.00603	hypothetical protein FLJ12242
203395_s_at	HES1	-1.97 ± 0.35	0.00353	hairy and enhancer of split 1, (Drosophila)
201631_s_at	IER3	-2.05 ± 0.44	0.0113	immediate early response 3
218105_s_at	MRPL4	-2.09 ± 0.26	0.00196	mitochondrial ribosomal protein L4
206814_at	NGFB	-2.19 ± 0.36	0.0142	nerve growth factor, beta polypeptide
216598_s_at	CCL2	-5.76 ± 1.76	0.000792	chemokine (C-C motif) ligand-2 (CCL2)

Genes upregulated by 16 h Dex treatment

203453_at	SCNN1A	11.94 ± 0.52	0.00199	sodium channel, non-voltage-gated 1 alpha
208763_s_at	DSIPI	5.97 ± 0.14	0.000153	delta sleep inducing peptide, immunoreactor
202861_at	PER1	4.59 ± 0.36	0.00831	period homolog 1 (Drosophila)

221541_at	LCRISP2	4.16 ± 0.19	0.00115	LCCL domain containing cysteine-rich secretory protein 2
201041_s_at	DUSP1	3.35 ± 0.13	6.63E-05	dual specificity phosphatase 1
209074_s_at	TU3A	3.30 ± 0.25	0.00414	TU3A protein
202859_x_at	IL8	3.30 ± 0.39	0.00825	interleukin 8
219049_at	ChGn	3.27 ± 0.26	0.00133	chondroitin beta1,4 N-acetyl galactosaminyltransferase
202627_s_at	SERPINE1	3.01 ± 0.21	0.00112	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
207761_s_at	DKFZP586A0522	2.85 ± 0.49	0.00756	DKFZP586A0522 protein
202237_at	NNMT	2.54 ± 0.23	0.00563	nicotinamide N-methyltransferase
201951_at	ALCAM	2.41 ± 0.21	0.000279	Activated leukocyte cell adhesion molecule
203962_s_at	NEBL	2.41 ± 0.37	0.000753	nebulette
212805_at	KIAA0367	2.41 ± 0.20	0.000725	KIAA0367
204326_x_at	MT1L	2.35 ± 0.20	0.0195	metallothionein 1L
36829_at	RIGUI	2.35 ± 0.21	0.00547	period homolog 1 (Drosophila)
214417_s_at	FETUB	2.26 ± 0.36	0.00735	fetuin B
209798_at	NPAT	2.19 ± 0.71	0.0135	nuclear protein, ataxia-telangiectasia locus
205730_s_at	KIAA0843	2.19 ± 0.35	0.00728	KIAA0843 protein
202887_s_at	RTP801	2.13 ± 0.11	0.0017	HIF-1 responsive RTP801
212806_at	KIAA0367	2.02 ± 0.40	0.000541	DNA-damage-inducible transcript 4
208349_at	ANKTM1	1.98 ± 0.52	0.000155	ankyrin-like with transmembrane domains 1
217738_at	PBEF	1.97 ± 0.36	0.0139	pre-B-cell colony-enhancing factor
212195_at	IL6ST	1.97 ± 0.35	0.0024	Interleukin 6 signal transducer (gp130, oncostatin M receptor)
207815_at	PF4V1	1.95 ± 0.19	0.00202	platelet factor 4 variant 1
220556_at	ATP1B4	1.54 ± 0.72	0.052	ATPase, (Na+)/K+ transporting, beta 4 polypeptide
210050_at	TPI1	1.29 ± 0.23	0.00126	
203989_x_at	F2R	1.15 ± 0.89	0.078	coagulation factor II (thrombin) receptor
218663_at	HCAP-G	1.12 ± 0.43	0.056	chromosome condensation protein G
208325_s_at	AKAP13	0.98 ± 0.46	0.076	A kinase (PRKA) anchor protein 13

Genes downregulated by 16 h Dex treatment

210253_at	HTATIP2	-1.07 ± 0.27	0.000925	HIV-1 Tat interactive protein 2, 30 kDa
216515_x_at	PTMA	-1.10 ± 0.30	0.00152	prothymosin, alpha (gene sequence 28)
201701_s_at	PGRMC2	-1.13 ± 0.82	0.000165	progesterone receptor membrane component 2
216088_s_at	PSMA7	-1.17 ± 0.17	2.82E-05	proteasome (prosome, macropain) subunit, alpha type, 7
214021_x_at	ITGB5	-1.18 ± 0.56	0.000201	ta93a09.x1 NCI_CGAP_Lu26 Homo sapiens cDNA clone IMAGE:2051608 3' similar to gb:J05633 INTEGRIN BETA-5 SUBUNIT PRECURSOR (HUMAN), mRNA sequence.
209203_s_at	BICD2	-1.19 ± 0.82	0.053	bicaudal D homolog 2 (Drosophila)
214880_x_at	CALD1	-1.19 ± 0.75	0.0193	caldesmon 1
206023_at	NMU	-1.25 ± 0.07	0.00245	neuromedin U
205818_at	DBCCR1	-1.27 ± 0.18	0.0029	deleted in bladder cancer chromosome region candidate 1
204872_at	BCE-1	-1.31 ± 0.21	0.00128	BCE-1 protein
204233_s_at	CHK	-1.32 ± 0.20	0.000575	choline kinase
210180_s_at	SFRS10	-1.34 ± 0.17	0.000305	splicing factor, arginine/serine-rich 10 (transformer 2 homolog, Drosophila)
201626_at	INSIG1	-1.36 ± 0.28	0.00584	insulin induced gene 1
203102_s_at	MGAT2	-1.36 ± 0.27	0.00444	mannosyl (alpha-1,6-)-glycoprotein beta-1,2-N-acety lglucosaminyltransferase
203706_s_at	FZD7	-1.36 ± 0.22	0.000572	frizzled homolog 7 (Drosophila)
205479_s_at	PLAU	-1.41 ± 0.20	0.0236	plasminogen activator, urokinase
215146_s_at	KIAA1043	-1.41 ± 0.32	0.000995	KIAA1043 protein
203830_at	NJMU-R1	-1.43 ± 0.36	0.00069	protein kinase Njmu-R1
215034_s_at	TM4SF1	-1.44 ± 0.10	0.000342	transmembrane 4 superfamily member 1
213256_at		-1.46 ± 0.10	0.000637	Homo sapiens clone 24707 mRNA sequence
212199_at	MGC9651	-1.47 ± 0.26	0.00802	hypothetical protein MGC9651

211297_s_at	CDK7	-1.49 ± 0.10	0.00204	cyclin-dependent kinase 7
215947_s_at	FLJ14668	-1.49 ± 0.28	0.00903	hypothetical protein FLJ14668
219375_at	CEPT1	-1.49 ± 0.33	0.0017	choline/ethanolaminephosphotransferase
200999_s_at	CKAP4	-1.53 ± 0.23	0.00433	cytoskeleton-associated protein 4
209761_s_at	SP110	-1.55 ± 0.21	0.00182	SP110 nuclear body protein
205380_at	PDZK1	-1.61 ± 0.57	0.0292	PDZ domain containing 1
205381_at	P37NB	-1.61 ± 0.33	0.0083	37 kDa leucine-rich repeat (LRR) protein
208712_at	CCND1	-1.61 ± 0.47	0.00267	cyclin D1 (PRAD1: parathyroid adenomatosis 1)
221886_at	KIAA1277	-1.65 ± 0.44	0.0181	KIAA1277
209417_s_at	IFI35	-1.68 ± 0.18	0.00831	interferon-induced protein 35
204972_at	OAS2	-1.70 ± 0.30	0.00274	2'-5'-oligoadenylate synthetase 2, 69/71 kDa
219352_at	FLJ20637	-1.70 ± 0.21	0.000935	hypothetical protein FLJ20637
204747_at	IFIT4	-1.71 ± 0.35	0.0299	interferon-induced protein with tetratricopeptide repeats 4
202458_at	SPUVE	-1.71 ± 0.13	0.00926	protease, serine, 23
217997_at	PHLDA1	-1.73 ± 0.36	0.00355	pleckstrin homology-like domain, family A, member 1
202936_s_at	SOX9	-1.73 ± 0.24	0.00157	SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)
214321_at	NOV	-1.74 ± 0.31	0.0031	nephroblastoma overexpressed gene
217996_at	PHLDA1	-1.81 ± 0.40	0.000145	pleckstrin homology-like domain, family A, member 1
201939_at	SNK	-1.84 ± 0.29	0.00175	serum-inducible kinase
202016_at	MEST	-1.84 ± 0.08	0.00222	mesoderm specific transcript homolog (mouse)
211138_s_at	KMO	-1.84 ± 0.67	0.0874	kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)
204529_s_at	TOX	-1.87 ± 0.27	0.0253	thymus high mobility group box protein TOX
218546_at	FLJ14146	-1.87 ± 0.13	0.000921	hypothetical protein FLJ14146
210550_s_at	RASGRF1	-1.90 ± 0.11	0.0015	Ras protein-specific guanine nucleotide-releasing factor 1
204457_s_at	GAS1	-1.91 ± 0.32	0.00906	growth arrest-specific 1
202207_at	ARL7	-1.95 ± 0.22	0.00415	ADP-ribosylation factor-like 7
209969_s_at	STAT1	-1.98 ± 0.19	0.00836	signal transducer and activator of transcription 1, 91 kDa
201341_at	ENC1	-2.02 ± 0.46	0.0196	ectodermal-neural cortex (with BTB-like domain)
216598_s_at	CCL2	-2.03 ± 0.27	0.00623	chemokine (C-C motif) ligand 2
205660_at	OASL	-2.11 ± 0.18	0.0183	2'-5'-oligoadenylate synthetase-like
205827_at	CCK	-2.16 ± 0.23	0.00307	cholecystokinin
216549_s_at	TBC1D22B	-2.19 ± 1.01	0.498	TBC1 domain family, member 22B
201340_s_at	ENC1	-2.24 ± 0.53	0.000999	ectodermal-neural cortex (with BTB-like domain)
204439_at	C1orf29	-2.46 ± 0.29	0.0136	chromosome 1 open reading frame 29
213797_at	cig5	-4.09 ± 0.40	0.00132	vipirin

Microarray results reveal modulation of gene expression. Microarray was performed on extracts from HLE B-3 cells treated with dexamethasone (Dex) or vehicle (Veh) in triplicate. Data were filtered to contain only those genes found on two of three chips with >1.5 fold change in between Dex and Veh treatments. In the 4 h treatment 136 genes were modulated; in the 16 h treatment, 86 genes were modulated. This table lists the Affymetrix probe ID, the gene name, fold change (±standard deviation), p value, and a description for each of the genes that passed the filtering criteria. ANOVA (assuming unequal variances) was used to calculate p values.

1.5 fold. Of these, 9 transcripts were upregulated and 1 was downregulated by greater than 3 fold. Seven genes overlapped the two data sets (Table 3). A few of the genes fell below the 1.5 fold cut off due to the different algorithms used in the two programs, Microarray Suite and GeneSpring, used for analysis of the data. Although the fold changes were small (between 1 to 3 fold), many of them were statistically significant (p less than or equal to 0.05, Table 2).

To verify the results seen in the microarray, transcripts were examined in 4 or 16 h Dex or Veh treated HLE B-3 (in triplicate) and human lens explant cultures (created from donor lenses) treated for 4 h by RT-PCR. Human lenses treated for 16 h were not examined due to lack of material. The reaction was stopped during the log phase. Although the extent to which the fold change in gene expression differed between the microarray and real time PCR results, the general trends were consistent and correlated with the microarray data providing confidence in the microarray results, yet demonstrating the need for confirmation of microarray results (Table 4). In addition to SYBR green analysis, PCR products were visu-

alized on 1 or 2% agarose gels stained with ethidium bromide (Figure 5) and these results were consistent with the RT-PCR data.

The 5' promoter region and full gene sequences of these target genes were analyzed by a signal scan program to determine if GREs were present. All of the genes examined contained putative GREs (data not shown) suggesting direct GC-GR signaling. Although no published gene sequence is available for DSIP, previously published reports indicate that DSIP is upregulated by GC treatment [42]. DSIP has also been reported to be homologous to TSC22 [43]. Our own alignment studies demonstrated that DSIP is 100% homologous to TSC22 and a signal scan of the TSC22 genomic sequence revealed putative GREs (data not shown).

To better understand what effect the change in gene expression may have on lens cells, microarray results were analyzed by functional clustering using EASE and NetAffx to identify biological and molecular pathways. EASE is an analysis tool that identifies enriched biological themes by prioritizing functional categories of genes that cluster together under a specific biological or molecular function based on the significance by determining gene sets which are statistically overrepresented. Overrepresentation is calculated based on the total number of genes assayed and annotated within each system. This is determined by the probability of seeing the number of "List Hits" (number of genes in the gene list that belong to the gene category) in the "List Total" (number of genes in the gene list) given the frequency of the "Population Hits" (number of genes in the total group of genes assayed that belong to the specific gene category) in the "Population Total" (number of genes in the total group of genes assayed that belong to any gene category) within the system. This is called the Fisher exact probability. EASE also determines an "EASE score" which is calculated by removing one gene in the category from the list and then calculating Fisher exact probability for that category [35]. The EASE score is an adjustment of the Fisher exact that strongly penalizes the significance of categories supported by few genes and negligibly penalizes categories supported by many genes. The EASE score favors higher ranked categories (lists with larger numbers of genes) compared to the Fisher exact probability [35]. The most significantly overrepresented categories that result from analysis of the gene lists are labeled "biological themes" or "molecular themes" (Figure 6, Figure 7, Table 5, and Table 6). Those categories with an EASE score of less than or equal to 0.05 are underlined. Some of the genes are unclassified, which means no annotations are available for that gene identifier under the specified parameters although an annotation for that gene may exist. The size of the pie slices and the number of genes in any one functional category do not reflect biological importance. They only reflect the results of EASE analysis and give an understanding of the biological and molecular functions in which Dex induced changes in gene expression may be involved.

Although multiple biological processes were represented, only coagulation and metabolism had an EASE score of less

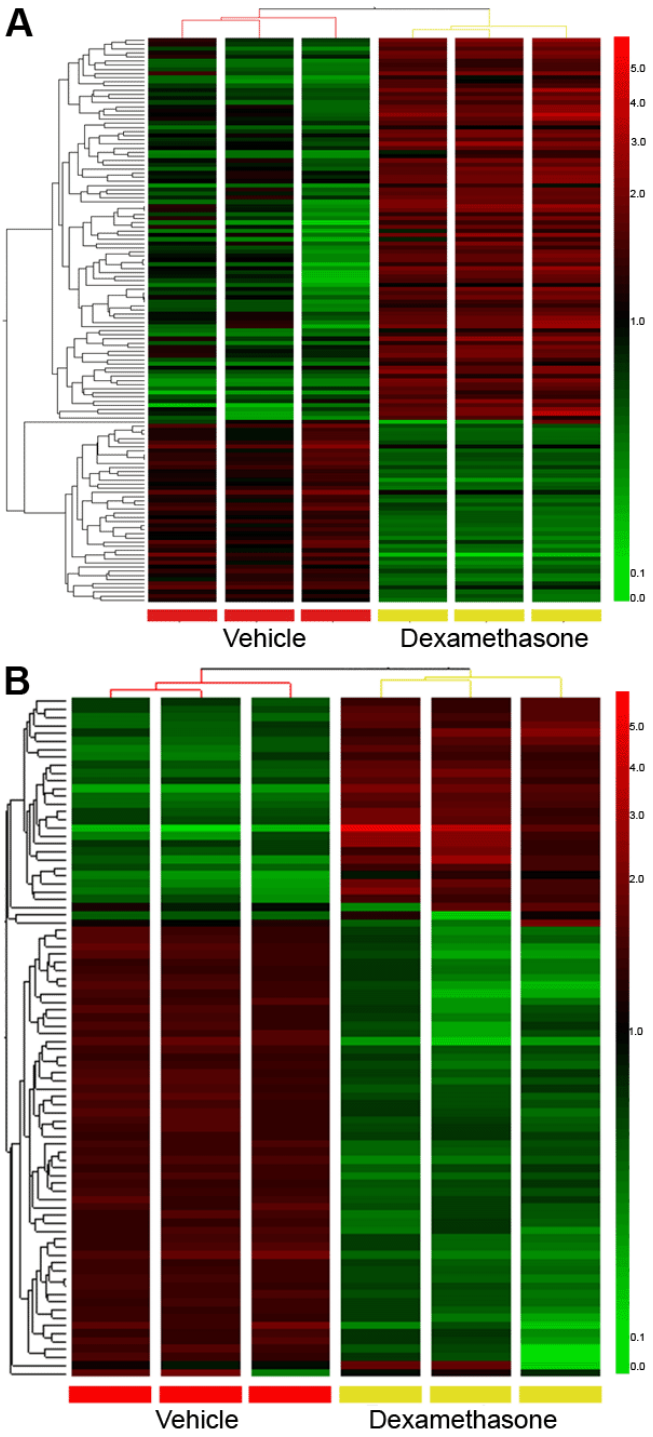

Figure 4. Clustering of microarray data based on condition and expression of genes. The filtered data (genes that change >1.5 fold compared to vehicle) were clustered based on their overall expression. They were also clustered based on their expression across the chips. The triplicates clustered together tightly with treatment and gene changes were present across at least two of three chips. Red denotes transcripts that were upregulated and green denotes transcripts that were downregulated. **A**: Clustering of 136 genes from 4 h dexamethasone (Dex) treatment of HLE B-3 cells. **B**: Clustering of 86 genes from 16 h Dex treatment of HLE B-3 cells.

Table 3. Seven genes overlap the 4 and 16 h microarray data

Probe ID	Gene name	Fold change 4 h	Fold change 16 h	Description
208763_s_at	DSIPI	5.56	5.97	delta sleep inducing peptide immunoreactor
217996_at	PHLDA1	-1.62	-1.73	pleckstrin homology-like domain, family A, member 1
216598_s_at	CCL2	-5.76	-2.03	chemokine (C-C motif) ligand-2 (CCL2)
202861_at	PER1	6.01	4.59	period homolog 1 (Drosophila)
202627_s_at	SERPINE1	3.15	3.01	serine (or cysteine) Proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
207761_s_at	DKFZP586A0522	2.47	2.85	DKFZP586A0522 protein
203989_x_at	F2R	4.00	1.15	coagulation factor II (thrombin) receptor

Seven genes overlap between the 4 h and 16 h microarray. Seven transcripts overlapped between the filtered data from the two sets of microarrays after 4 and 16 h of Dex treatment. Signal scans of the 5' promoter regions and full gene sequences revealed putative GREs. The expression of these genes over both time periods suggests continual GR-GRE signaling with GC treatment.

Table 4. RT-PCR: Confirmation of microarray findings

	Microarray		Real time		
Gene	B-3	p value	B-3	p value	Donor
4 h					
PER 1	6.01 ± 0.57	0.03	6.36 ± 0.98	0.006	3.95
DSIP	5.56 ± 0.17	0.0005	13.6 ± 0.55	0.0004	5.49
HSP70	4.84 ± 0.93	0.06	1.42 ± 0.31	0.7	1.49
PRKAR1	4.37 ± 0.94	0.09	1.51 ± 0.17	0.04	1.32
F2R	4.00 ± 0.55	0.01	1.66 ± 0.03	0.004	2.20
PAI-1	3.5 ± 0.33	0.002	6.42 ± 0.61	0.0003	1.50
GADD45	2.76 ± 0.88	0.7	1.43 ± 0.11	0.04	1.00
SGK	2.41 ± 0.097	0.003	2.86 ± 0.14	0.0004	3.60
PHLDA	-1.62 ± 0.06	0.02	-1.40 ± 0.012	0.03	-2.80
IER3	-2.05 ± 0.15	0.01	-1.77 ± 0.12	0.01	-1.69
NGF	-2.19 ± 0.12	0.01	-1.81 ± 0.007	0.005	-3.01
MCP-1	-5.76 ± 0.59	0.0008	-6.72 ± 0.037	0.03	-2.01
16 h					
SCNN1A	11.94 ± 0.17	0.002	12.04 ± 0.12	0.0001	
DSIP	5.97 ± 0.047	0.0002	9.20 ± 0.16	0.001	
PER1	4.59 ± 0.12	0.008	5.90 ± 0.42	0.02	
DUSP-1	3.35 ± 0.043	0.00007	4.40 ± 0.26	0.03	
PAI-1	3.01 ± 0.070	0.001	3.56 ± 0.33	0.04	
F2R	1.15 ± 0.30	0.08	1.55 ± 0.033	0.3	
CCND1	-1.61 ± 0.13	0.003	-1.60 ± 0.083	0.02	
PHLDA	-1.81 ± 0.13	0.0002	-1.7 ± 0.30	0.02	
CCL2	-2.03 ± 0.09	0.006	-2.3 ± 0.32	0.05	
CCK	-2.16 ± 0.076	0.003	-2.40 ± 0.23	0.02	

Fold changes (±standard error of the mean), normalized to β-actin, of genes from dexamethasone compared to vehicle treated HLE B-3 cells analyzed by microarray were verified by RT-PCR from HLE B-3 cells and RNA from a pair of treated cultured donor lenses. Data were quantitated by crossing point analysis of SYBR green fluorescence. ANOVA was used to calculate p values. Genomic DNA sequences for all the genes listed were examined and found to contain putative GREs.

than or equal to 0.05 and were significantly modulated in lens epithelial cells treated with Dex for 4 h. All the categories listed in the pie chart contained genes that were both upregulated and downregulated, except for the categories of coagulation and death, in which all the genes were upregulated. Although the categories contained genes that were both upregulated and downregulated, the majority were upregulated.

Response to stimulus, coagulation, and metabolism were significantly modulated in lens epithelial cells treated with Dex for 16 h. All the categories listed in the pie chart contained genes that were both upregulated and downregulated, except for the categories of morphogenesis and death, in which all the genes were downregulated. The categories contained relatively equal numbers of genes that were both upregulated and downregulated except for metabolism in which the majority of genes expressed were downregulated.

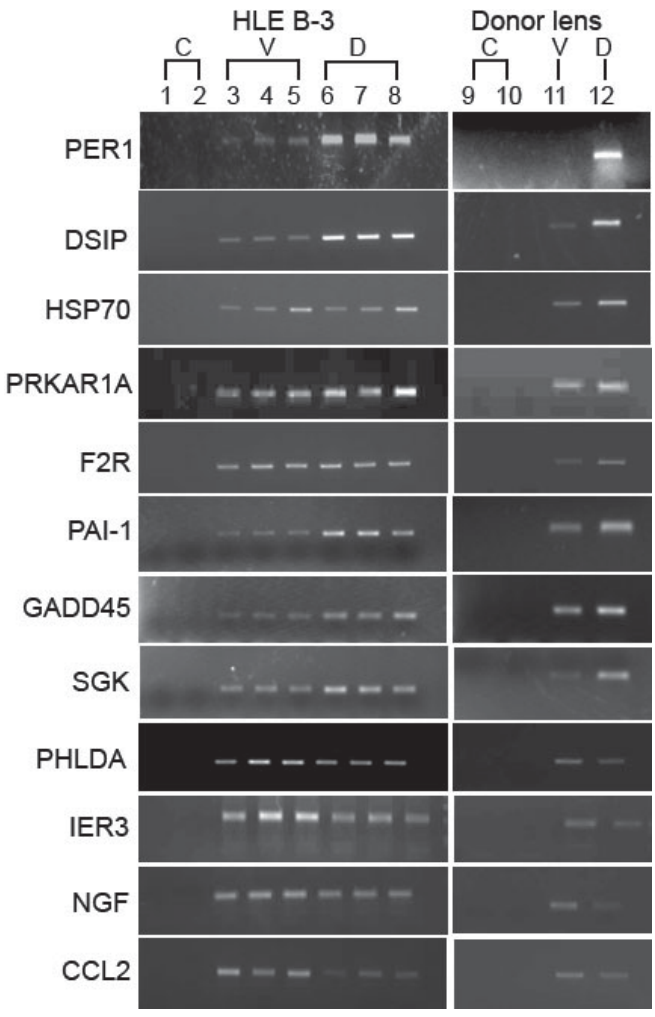

Figure 5. Agarose gel analysis of RT-PCR products in 4 h treated samples. RT-PCR products were electrophoresed on 1 or 2% agarose gels to examine product specificity. Gel analysis results were consistent with quantification of RT-PCR products by crossing point analysis (listed in Table 4). Similar results were obtained for the 16 h treated samples (data not shown). Lanes 1 and 9 are minus template negative controls (C), lanes 2 and 10 are minus reverse transcriptase negative controls (C), lanes 3, 4, 5, and 11 are vehicle (V) controls, and lanes 6, 7, 8, and 12 are treated with 1 μM dexamethasone (D).

The genes modulated with 4 h Dex treatment clustered into several molecular categories, however, none of the categories identified received an EASE score that was considered significant. Many of the categories represented contained genes that were both upregulated and downregulated, although some only contained genes that were upregulated. The molecular function category identified as significant was receptor binding in 16 h Dex treated lens cells. Once again, the categories contained both genes that were upregulated and downregulated, however, some categories only contained genes that were downregulated. Additional analysis of microarray results was performed with SAM, GeneSpring, and NetAffx (data not shown). Clustering with different programs resulted in expression of similar categories, and parallel to EASE results, the categories were very few and broad.

EASE was also used to identify pathways through the use of KEGG pathways terms associated with the differentially expressed genes. EASE analysis of 4 and 16 h modulated genes through use of KEGG pathways revealed that the phosphatidyl inositol signaling system, complement and coagulation cascades, cell cycle, integrin mediated cell adhesion, purine metabolism, and pyrimidine metabolism all contained 3-6 modulated genes that functionally clustered into these pathways. Some of the genes are unclassified in the KEGG pathways, which means no annotations are available for that gene identifier under the specified parameters designated although an annotation for that gene may exist. The size of the pie slices and the number of genes in any one category do not reflect the biological importance, only the relative number of genes with modulated expression within the pathways category. Some genes may be represented in more than one pathway (Figure 8). The transcripts from the microarray that fall into some of the different pathways are listed in Table 7.

Of the pathways represented, the phosphatidyl inositol signaling system, complement and coagulation cascades, purine metabolism, and circadian rhythm pathways contained transcripts that were upregulated from the 4 or 16 h data sets. Integrin mediated cell adhesion, pyrimidine metabolism, cell cycle, and complement and coagulation cascades contained transcripts that were downregulated from the 4 or 16 h data sets.

DISCUSSION

In the current study, we have demonstrated that primary cultures of human lens epithelial cells express a functionally active GR. Furthermore, using microarray technology, we examined the global gene expression of HLE B-3 cells treated with GC to identify GC mediated gene targets and were able to verify the results in immortalized and primary cultures of lens epithelial cells.

Previously, we identified the expression of classical GR mRNA and protein in freshly isolated human lens epithelial cells [27]. In this study, we demonstrate that the GR in primary cultures of human lens epithelial cells is able to induce transcription of a GRE mediated gene. This is similar to the

luciferase expression we previously reported in the HLE B-3 immortalized human lens epithelial cell cultures [27]. This is the first report of GR activity in primary cultures of human lens epithelial cells. These data demonstrate that primary cultures of hLECs contain a transcriptionally active GR able to induce changes in gene transcription and provide further proof that the lens GR is functionally active.

Glucocorticoids are known to elicit divergent biological outcomes and a lens glucocorticoid response has been difficult to characterize [18,26,28]. Because of the association with cataract, it is important to understand the biological significance of the GC effect in lens epithelial cells. Although GCs have been proposed to affect lens epithelial cells through a variety of mechanisms, including oxidative stress [11-15], nonspecific GC binding [16-18], and membrane steroid receptors [19], the identification of a transcriptionally active intracellular GR suggests that GCs may be modulating the transcription of target genes. To gain a better understanding of glucocorticoid induced gene changes in lens epithelial cells, microarray technology serves as a useful method to identify changes in global gene expression that can lead to identification of biological functions or signaling pathways [44,45]. A previous published report has demonstrated global changes in gene expression in lens epithelial cells after 24 and 48 h of treatment with GC, however it has not identified early glucocorticoid responses or signaling pathways involved in a GC response [34]. Understanding of a GC response requires an understanding of short term and long term effects of treatment.

GC induced changes in gene expression due to GR binding to a GRE have been reported to occur as early as 15-30 min after hormone administration but can also occur as late as 4-24 h in other cell types [41,46-48]. Although glucocorticoid effects on lens epithelial cells, such as changes in protein expression [31], the formation of a cataract [1-6], and changes in gene expression [34] have only been observed after long term treatment, a transcriptionally active receptor suggests that there may be changes in gene expression after a short treatment time. In our experiments, a significant increase in luciferase activity of Dex compared to Veh treated cells was identified as early as 2 h and this was sustained over a 24 h

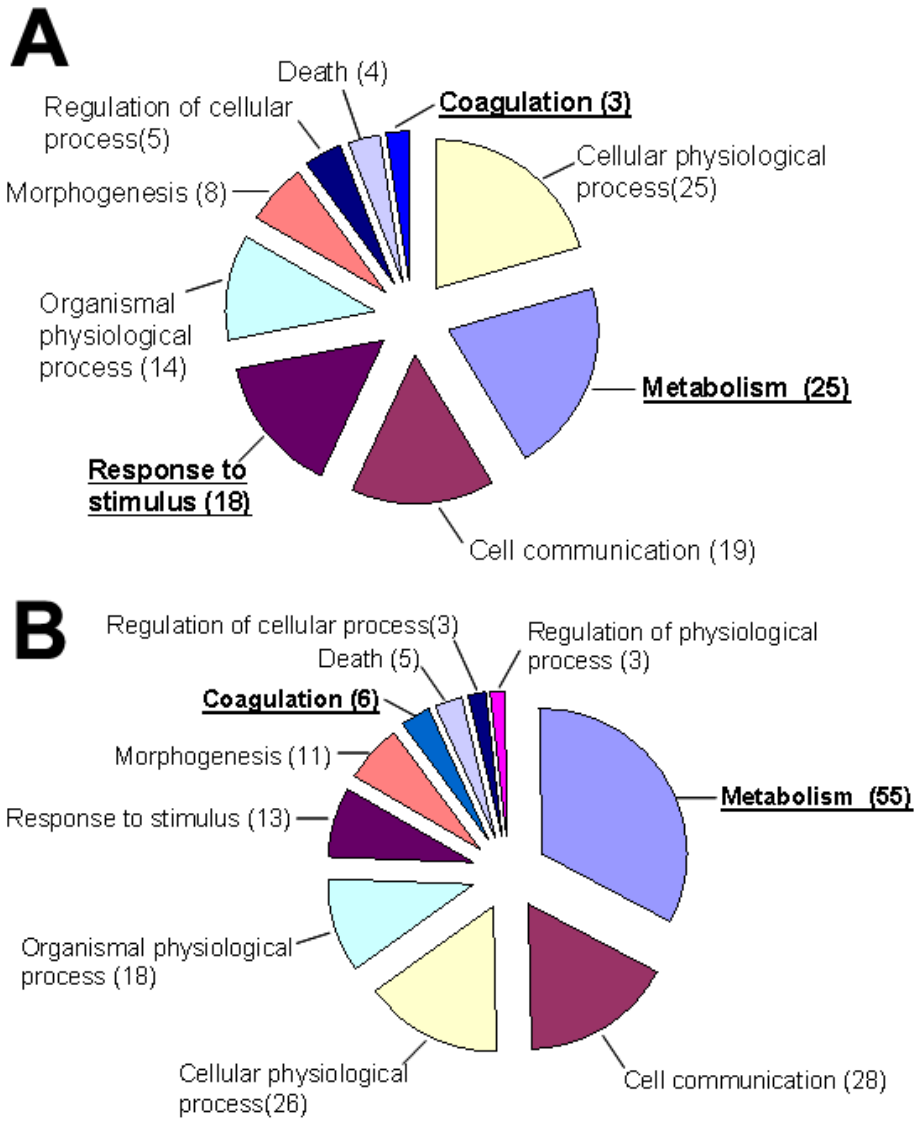

Figure 6. Cluster analysis reveals biological functions. EASE was used to statistically cluster genes that were modulated with dexamethasone (Dex) treatment compared to vehicle. The total number of genes from the filtered lists in each functional category is indicated next to the pie slice which represents the relative number of genes with modulated expression in the biological category. Some genes may be represented in more than one functional category. Not all of the genes are classified and thus do not fall into one of the functional categories represented here. Those categories with an EASE score of <0.05 are underlined in bold. **A**: Of the 136 genes from the 4 h Dex treatment, 77 were classified. **B**: Of the 86 genes from the 16 h Dex treatment, 55 were classified. Table 5 indicates the number of transcripts that were upregulated or downregulated in each of the functional categories. Although these results did not reveal a specific functional category affected by GC treatment, this is consistent with and may account for the inability of researchers, thus far, to identify a change in a biological function with GC treatment of LECs in vivo.

period. These findings demonstrate an early GC response in lens epithelial cells suggesting that GCs can play a role in gene expression as soon as 2 h. Although GC mediated changes in gene expression may be occurring as early as 2 h, at 4 h the increase in luciferase activity was more robust and was chosen for examination on microarray. This time point provides information about GC targets, however, a second treatment time of 16 h was also examined by microarray in order to better understand signaling pathways involved in a GC response.

Three biological replicates were created and analyzed for each of the treatments (Veh or Dex) at each of the time points (4 h or 16 h). The analysis process can be broken down into two parts; identification and verification of differentially expressed genes and the determination of the biological significance of groups of genes through analysis of the biological and molecular themes with respect to Gene Ontology and KEGG pathway terms associated with these genes. Although the overall expression of each of the chips was similar and the triplicates were reproducible, differences were observed. It is normal to observe differences in replicate microarrays [44]. To identify specific gene expression changes that are significantly modulated and are reproducible, in the first step of analysis the triplicates were stringently filtered. Although three biological replicates were created, genes were kept within the set if they were modulated by 1.5 fold in at least two of three chips in order to identify a greater number of genes. Based on the difficulty of demonstrating a direct effect of glucocorticoids on the lens, we predicted that glucocorticoid effects on gene expression would be few and small. The first step of analysis resulted in identifying changes in 136 transcripts after 4 h of treatment and 86 transcripts after 16 h of treatment. Only 7 transcripts overlapped the two groups, suggesting continual signaling and transcription of the 7 genes. Of the 7, the two most notable were the DSIP and the PER 1 genes which were upregulated by at least 5 fold in both data sets. It is interesting to note that of the 7 transcripts expressed at 4 and 16 h of Dex treatment in our results, it has been reported that by 48 h of Dex treatment, DSIP, PAI-1, and DKFZP586A0522 all continue to have >2 fold upregulated expression while

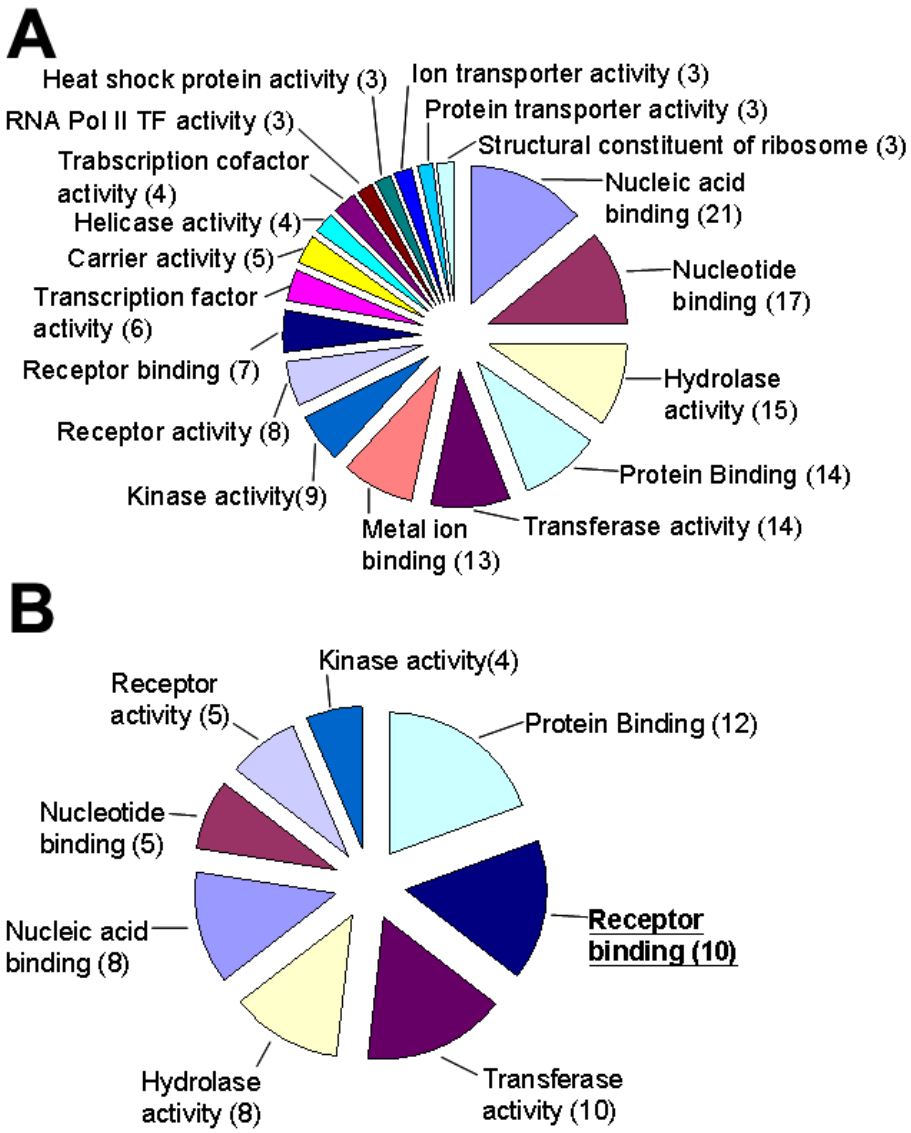

Figure 7. Cluster analysis reveals molecular functions. EASE was used to statistically cluster genes that were modulated with dexamethasone (Dex) and vehicle treatment. The total number of genes from the filtered lists in each functional category is indicated next to the pie slice which represents the relative number of genes with modulated expression in the molecular category. Some genes may be represented in more than one functional category. Not all of the genes are classified and thus do not fall into one of the functional categories represented here. Those categories with an EASE score of <0.05 are underlined. **A**: Of the 136 genes from the 4 h Dex treatment, 63 were classified. **B**: Of the 86 genes from the 16 h Dex treatment, 28 were classified. Table 6 indicates the number of transcripts that were upregulated or downregulated in each of the functional categories. Although the results did not reveal a specific functional category affected by GC treatment, is consistent with and may account for the inability of researchers, thus far, to identify a change in a molecular function with GC treatment of LECs in vivo.

Chemokine ligand and pleckstrin homology like domain are downregulated by >2 fold compared to Veh in HLE B-3 cells [34]. Period 1, which continued to exhibit nearly 5 fold expression after 16 h of Dex treatment, was not identified on the 48 h microarray [34]. Coagulation factor II receptor was immediately downregulated from 4 fold expression at 4 h to nearly 1 fold expression at 16 h. This demonstrates changes in signaling as the GC response continues over a long time period indicating the need to understand the effect of short term and long term treatment times.

Table 5. Biological functions involved in a GC response in hLECs contains both upregulated and downregulated genes

Biological Category	4 h up	4 h down	16 h up	16 h down
Metabolism	36	19	6	19
Cell communication	20	8	8	11
Cellular physiological process	17	9	11	14
Organismal physiological process	14	4	6	8
Response to Stimulus	10	3	8	10
Morphogenesis	6	5	-	6
Coagulation	5	-	-	-
Death	3	-	-	3

Each of the biological categories represented in Figure 6 were further subdivided into the expression identified by microarray.

Studies looking at changes in gene expression by microarray at early time points (2 to 8 h) after glucocorticoid treatment observed a markedly greater number of upregulated transcripts than downregulated transcripts [49,50], similar to our results. In our own studies, we observed 93 transcripts upregulated and 43 transcripts downregulated in lens epithelial cells treated with glucocorticoid for 4 h. It is interesting to note that after 16 h of treatment, 30 transcripts were upregulated while 56 were downregulated. It has been reported that after 24 h of GC treatment 57 transcripts were upregulated and 50 were downregulated and by 48 h 92 were upregulated and 42 were downregulated [34]. By 24 h, it appears that the number of up regulated and down regulated transcripts is nearly equal but by 48 h, the number of up regulated genes is markedly

Table 6. Molecular functions involved in a GC response in hLECs contains both upregulated and downregulated genes

Molecular category	4 h up	4 h down	16 h up	16 h down
Nucleic Acid Binding	16	5	-	7
Nucleotide Binding	13	4	-	5
Hydrolase Acitivity	12	3	3	5
Metal Ion Binding	12	-	-	-
Protein Binding	12	-	6	6
Receptor Activity	7	-	-	3
Transferase Activity	7	7	3	7
Kinase Activity	5	4	-	4
Transcription Factor Activity	5	-	-	-
Carrier Activity	4	-	-	-
Helicase Activity	4	-	-	-
Receptor Binding	4	3	4	6
Transcription Cofactor Activity	4	-	-	-
RNA Pol II TF Activity	3	-	-	-
Protein Transporter Activity	3	-	-	-

Each of the molecular categories represented in Figure 7 were further subdivided into the expression identified by microarray.

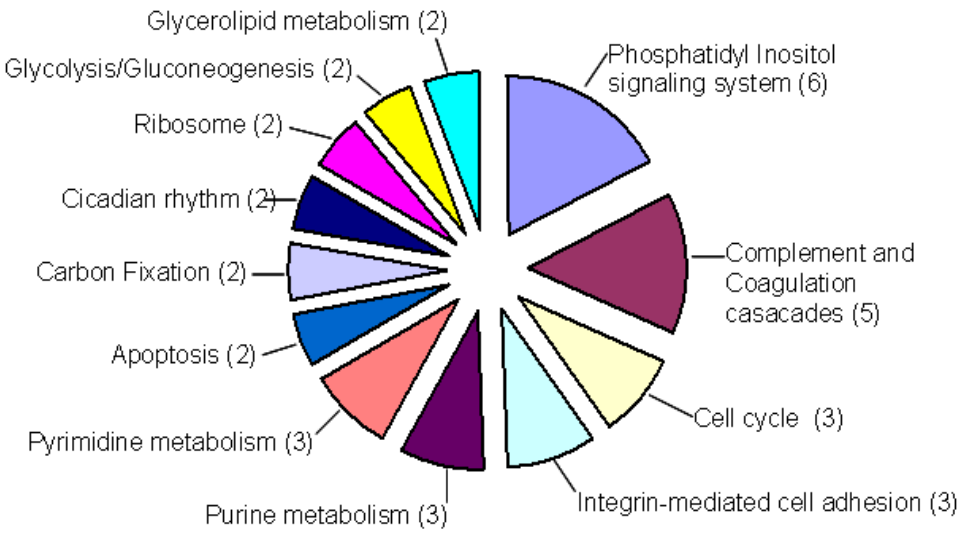

Figure 8. Pathway analysis of Dex modulated genes by EASE. EASE was used to analyze genes that were modulated with dexamethasone and vehicle treatment. The total number of genes in each pathway is indicated next to the pie slice which represents the relative number of genes with modulated expression in the pathway. Some genes may be represented in more than one pathway. Not all of the genes are classified and thus do not fall into one of the pathways represented here. Of the 215 modulated transcripts, 41 were classified. Pathway analysis by EASE reveals that Dex modulated genes play a role in important signaling pathways. Dex modulated genes may be coordinately working together to modulate a signaling pathway.

increased. Glucocorticoids appear to be modulating genes differently at early and later times.

Glucocorticoid actions at the genomic level have been reported to involve transactivation, due to the binding of the activated GR to a GRE, and transrepression, due to the binding of an activated GR to a negative GRE [51]. It is possible that the large number of upregulated transcripts at early time points could be due to positive regulation, or transactivation, of genes, and at a later time point of 16 h the large number of downregulated transcripts could be due to transrepression of genes. Glucocorticoid responses can be divided into two types, primary and secondary responses. In a primary response, the activated GR binds directly to a GRE to activate gene transcription and no new protein synthesis is needed. A secondary response involves the elapse of time during which the products of the primary response act as positive and negative transcription factors or co-factors [41]. It is possible that the large number of downregulated transcripts at 16 h could be due to a secondary glucocorticoid response with the expression of co-repressors that were upregulated at 4 h. DSIP is expressed at both the 4 and 16 h time points and has been reported to act as a transcriptional repressor inhibiting the transcription of PPAR-γ2 in adipocytes [52]. There are also examples of promoters containing GREs responding both positively and negatively to GC, depending on the conditions [53]. This may be due to differences in GR cofactor expression over time or due to different cell types and may determine whether GREs are positively or negatively regulated by GR.

Table 7. Transcripts associated with each pathway involved in a glucocorticoid response identified by EASE analysis

AFFYID	Gene name	Pathway
201041_s_at	dual specificity phosphatase 1	Phosphatidylinositol signaling system
209049_s_at	protein kinase C binding protein 1	Phosphatidylinositol signaling system
212610_at	protein tyrosine phosphatase, nonreceptor type 11 (Noonan syndrome 1)	Phosphatidylinositol signaling system
213070_at	phosphoinositide-3-kinase, class 2, alpha polypeptide	Phosphatidylinositol signaling system
213688_at	calmodulin 1 (phosphorylase kinase, delta)	Phosphatidylinositol signaling system
221840_at	protein tyrosine phosphatase, receptor type, E	Phosphatidylinositol signaling system
202627_s_at	serine/cysteine Proteinase inhibitor, plasminogen activator inhibitor type 1	Complement andcoagulation cascades
202628_s_at	serine/cysteine Proteinase inhibitor, plasminogen activator inhibitor type 1	Complement andcoagulation cascades
203989_x_at	coagulation factor II (thrombin) receptor	Complement andcoagulation cascades
205479_s_at	plasminogen activator, urokinase	Complement andcoagulation cascades
211668_s_at	plasminogen activator, urokinase	Complement andcoagulation cascades
208694_at	protein kinase, DNA-activated, catalytic polypeptide	Cell cycle
208712_at	cyclin D1	Cell cycle
211297_s_at	cyclin-dependent kinase 7	Cell cycle

The Affymetrix probe ID and gene annotation associated with the transcript that clustered into selected pathways represented in Figure 8 are listed in this table.

Several of the transcripts were analyzed by RT-PCR and revealed trends of expression similar to that seen on the microarray and thus confirmed the accuracy of the microarray results in the immortalized HLE B-3 human lens epithelial cell line and in a primary culture of human lens epithelial cells created from donor lens explants. However, although the data are statistically significant and correlate with the microarray data, the differences in fold changes seen between the microarray and RT-PCR demonstrate the need to verify microarray results after both the first and second steps of microarray analysis. Transcripts, for which genomic sequences were available, TSC22, PER1, HSP-70, PRKAR1A, F2R, PAI-1, GADD45, SGK, PHLDA, IER3, NGF, MCP-1, SCNN1A, DUSP-1, CCND1, and CCK were examined and found to contain putative GREs in the promoter regions suggesting that the modulation of these genes could be due to direct binding of the ligand bound GR to the GRE of these promoters in hLECs.

Primary cultures of LECs were created from donor lenses and capsulorhexis specimens obtained from cataract surgery. The capsulorhexis specimen was from a type of cataract not associated with prolonged glucocorticoid use, however, we do not know if LECs obtained from cataractous specimens would respond to glucocorticoid treatment differently than noncataractous specimens. Human specimens, whether they be from donor lenses or from surgical specimens, are difficult to control for because each sample will be different depending on the patient's genetics, environmental factors, and medical history. Ideally, a microarray could be repeated with freshly isolated human lens epithelial cells. However, previous reports of microarray studies with human lenses reported the use of between 12-108 samples in order to obtain sufficient material to hybridize to just one microarray chip [33,54]. Due to a limited amount of sample, we chose to verify the results through RT-PCR of RNA obtained from primary cultures of single lenses. To minimize the contribution of lens pathology, the response was measured from vehicle or dexamethasone treatment of the same specimen. We have demonstrated that primary cultures of human lens epithelial cells, from capsulorhexis specimens and donor lenses, respond to glucocorticoid treatment similarly to immortalized human lens epithelial cell cultures. Human specimens, whether they are from donor or cataractous lenses, are limited so immortalized cell cultures can be used to examine hypotheses, which can then be confirmed in primary cultures. We demonstrated that the results obtained from the HLE B-3 cell line could be confirmed by real time PCR in primary cultures of human lens epithelial cells. This is the first demonstration of a glucocorticoid induced change in gene expression in primary cultures of human lens epithelial cells.

Microarray technology through the use of simultaneous monitoring of the expression levels of thousands of genes identified gene expression changes that are small but distinct and significant, suggesting that glucocorticoids have a direct effect on lens cell function. Although a total of 215 transcripts were identified, this information alone does not provide much information about the biological response of human lens epithelial cells to GC treatment. The real understanding and discovery come from the data analysis.

EASE data analysis revealed that the genes modulated are involved in a wide array of biological and molecular processes such as coagulation, response to stimulus, and metabolism. As these categories were further subdivided, the specificity and the number of categories increased but fewer and fewer genes clustered together under a single function. It is clear that GCs modulate genes in hLECs that are involved in a wide array of processes. GCs appear to be modulating a number of genes, clearly demonstrating a glucocorticoid response, but they do not appear to be modulating a specific biological or molecular function collectively. This may account for the difficulty researchers have had in identifying a functional response to glucocorticoid treatment in lens epithelial cells and demonstrates the difficulty in understanding glucocorticoid action in LECs.

Results from the reported 24 and 48 h microarray studies also demonstrate that GC induced changes in gene expression cluster into broad categories. However, it was not reported if the number of modulated genes that clustered into any of these categories compared to the number of genes represented in the category were considered significant [34]. It is interesting to note that there is some overlap with our results in the identification of certain functional groups including transcription factor activity, cell cycle, and apoptosis.

Our analysis with EASE was confirmed through analysis of data with NetAffx, GeneSpring, and SAM. It is important to note that neither EASE nor any of the other programs used, determines the expression on the chip, nor distinguishes between upregulated and downregulated transcripts or positive or negative regulators of the selected functional category. Therefore it is important to correlate the results of the array with the functional groups and the literature database in order to understand the effect the modulation of gene expression has on a particular cell function, especially if large groups of genes cluster together under a single function.

Although the transcripts do not cluster together under a specific functional group, it does not mean they do not coordinately function together. Modulated genes may not be affecting a specific cell function, but they may be involved in signaling pathways. EASE was also used to identify pathways through the use of KEGG pathways terms associated with the differentially expressed genes. EASE analysis of 4 and 16 h modulated genes through use of KEGG pathways revealed that the phosphatidyl inositol signaling system, complement and coagulation cascades, and cell cycle contained several genes that functionally clustered into these pathways.

EASE and KEGG terms are not absolute. Although a gene may be listed as belonging to a particular pathway, careful literature searches reveal that the same gene is involved in another pathway as well. The MAP Kinase and Phosphatidyl Inositol pathways illustrate this. In this data set, EASE only identified one transcript, MEKK3 (Affy ID 218311_at) as playing a role in the MAP Kinase Pathway (data not shown). EASE identified 6 transcripts that play a role in the phosphatidyl inositol pathway and one of them is the Dual Specificity Phos-

phatase-1 (Affy ID 201041_s_at). Careful literature searches reveal that Dual Specificity Phosphatase-1 is also known as Map Kinase Phosphatase-1 [55]. Alignment of cDNA transcripts reveal that the two are 100% homologous (data not shown). Map Kinase Phosphatase-1 is an important regulator of the MAP Kinase Pathway [56]. Further examination of the microarray results and literature reveals several other modulators of the MAP Kinase pathway that were not identified by EASE, including Plasminogen Activator Urokinase which stimulates MAP Kinases [57], Serum and Glucocorticoid Regulated Kinase which inhibits an upstream activator of MAP Kinase [58], and chemokine monocyte chemoattractant protein 1/Monocyte chemoattractant protein-1 which stimulates MAPK activation [59]. It appears that glucocorticoids are modulating signaling pathways in lens cells.

The phosphatidyl inositol pathway is involved in cellular responses such as cell survival, cell proliferation, cell growth, and transformation [60,61]. The MAP Kinase pathway is involved in cell proliferation, cell differentiation, cell motility, cell survival, and apoptosis [62]. Both of these pathways are important in cell stress responses and appear to crosstalk and play roles in similar functions [63,64]. Glucocorticoid treatment of lens epithelial cells does not appear to be modulating one specific biological function, but instead appears to be playing a small role in a variety of functions. It is possible that glucocorticoids affect many cellular functions through the modulation of components and regulators of the phosphatidyl inositol and MAP Kinase pathways. Regulators of the MAP Kinase and phosphatidyl inositol pathways were reported to be modulated in the 24 and 48 h microarrays [34]. Specifically, Map Kinase Phosphatase-1, which exhibited greater than 2 fold expression on our 16 h microarray, continued to demonstrate 2 fold expression at 48 h. Like glucocorticoids, both of these pathways are involved in a multitude of cellular and molecular functions, which may be a reason that researchers have been unable to identify a specific short term glucocorticoid response. It is possible that prolonged modulation of these pathways could lead to abnormal lens epithelial cell proliferation, differentiation, motility, survival, or apoptosis, all of which have been implicated in the formation of a steroid induced cataract. HES1 (Affy ID 203395_s_at) is down regulated by nearly 2 fold in the 4 h microarray. HES1 is a transcriptional repressor that inhibits proliferation in PC12 and MCF-7 cells [65,66]. However at 16 h, cyclin D1, which is involved in proliferation in the G1 to S transition of the cell cycle, is downregulated. TNFRSF6 (AFFY ID 204780_s_at), a member of the TNF-receptor superfamily, has a >3 fold expression in the 4 h microarray and activation of the receptor is known to be involved in a wide range of responses, including cell death, cell proliferation, inflammation, and differentiation [67]. CCL2 (Affy 216598_s_at), which is downregulated in both the 4 and 16 h microarray by >2 fold has been shown to be involved in the migration of endothelial cells [68]. However, IL8, which is increased by 3 fold in the 16 h microarray, has been suggested to be involved in migration [69]. SGK (Affy ID 201739_at) was upregulated with GC treatment of LECs and appears to be involved in a glucocorticoid receptor mediated protection from apoptosis in a mammary cell line [70]. Furthermore many of these genes have connections with the MAP Kinase or phosphatidyl inositol pathways [71-76]. These seemingly diverse changes in gene expression may be due to glucocorticoid modulation of signaling pathways.

A decrease in protein expression of E cadherin and N cadherin was reported after prolonged treatment with Dex without changes in mRNA expression [31]. We did not see any changes in gene expression of the cadherins on the 4 or 16 h micorarrays. It is interesting to note that both these signaling pathways have been reported to be involved upstream and downstream of cadherin expression [77-79]. We are currently investigating the effect of glucocorticoid in hLECs on the MAP Kinase and phosphatidyl inositol pathways.

Our results presented here demonstrate conclusively that human lens epithelial cells express a transcriptionally active GR, demonstrate early GC responses, and have identified endogenous changes in gene expression that play a role in very important cell functions including cell signaling, cell communication, cell metabolism, cell proliferation, and cell death. Since glucocorticoids are used in the clinical setting for treatment of acute and chronic disorders and a negative side affect of prolonged GC use is the formation of a cataract, it is important to understand a lens GC response. The studies reveal that glucocorticoids do not appear to modulate a specific cellular function but instead modulate important biological pathways that may be involved in the lens glucocorticoid response. This will lead to a better understanding of glucocorticoid signaling in lens epithelial cells, the effect of prolonged glucocorticoid treatment and the formation of a steroid induced cataract.

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