Vitamin D Induces Global Gene Transcription in Human Corneal Epithelial Cells: Implications for Corneal Inflammation

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PURPOSE. Our previous studies show that human corneal epithelial cells (HCEC) have a functional vitamin D receptor (VDR) and respond to vitamin D by dampening TLR-induced inflammation. Here, we further examined the timing of the cytokine response to combined vitamin D–TLR treatment and used genome-wide microarray analysis to examine the effect of vitamin D on corneal gene expression.

METHODS. Telomerase-immortalized HCEC (hTCEpi) were stimulated with polyinosinic-polycytidylic acid (poly[I:C]) and 1,25-dihydroxyvitamin D3 (1,25D3) for 2 to 24 hours and interleukin (IL)-8 expression was examined by quantitative (q)PCR and ELISA. Telomerase-immortalized HCEC and SV40-HCEC were treated with 1,25D3 and used in genome-wide microarray analysis. Expression of target genes was validated using qPCR in both cell lines and primary HCEC. For confirmation of IκBα protein, hTCEpi were treated with 1,25D3 for 24 hours and cell lysates used in an ELISA.

RESULTS. Treatment with 1,25D3 increased poly(I:C)-induced IL-8 mRNA and protein expression after 2 to 6 hours. However, when cells were pretreated with 1,25D3 for 24 hours, 1,25D3 decreased cytokine expression. For microarray analysis, 308 genes were differentially expressed by 1,25D3 treatment in hTCEpi, and 69 genes in SV40s. Quantitative (q)PCR confirmed the vitamin D–mediated upregulation of target genes, including nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α (IκBα). In addition to increased transcript levels, IκBα protein was increased by 28% following 24 hours of vitamin D treatment.

CONCLUSIONS. Microarray analysis demonstrates that vitamin D regulates numerous genes in HCEC and influences TLR signaling through upregulation of IκBα. These findings are important in dissecting the role of vitamin D at the ocular surface and highlight the need for further research into the functions of vitamin D and its influence on corneal gene expression.

Keywords: vitamin D, cornea, inflammation, toll-like receptors, microarray

The cornea is the anterior covering of the eye that serves to protect the underlying tissues from damage. Along with physical protection from injury, the cornea also defends against infection. Toll-like receptors (TLRs) are pattern-recognition receptors which stimulate an innate immune response upon contact with pathogen-associated molecular patterns. The inflammation resulting from TLR activation leads to recruitment of immune cells, enhanced cytokine, and chemokine production, and initiation of an adaptive immune response to rid the cornea of infection. These inflammatory events triggered by TLRs are dependent on intracellular signaling pathways which lead to the activation of nuclear factor κB (NF-κB), activator protein-1 (AP-1), signal transducer and activator of transcription (STAT), and interferon regulatory factor (IRF) transcription factors. In particular, NF-κB is recognized as the key regulator of inflammatory signaling, enhancing proinflammatory cytokine levels and also influencing cell survival. In addition to acting in a paracrine fashion to influence surrounding cells, proinflammatory cytokines produced by TLR signaling also act in an autocrine feedback loop to further enhance cytokine production and the cellular stress response. Mediators such as interleukin 1 β (IL-1β) and tumor necrosis factor (TNF), for example, are produced by TLR activation and in turn lead to increased NF-κB activation and activation of the mitogen-activated protein kinases (MAPK) pathway.

Inflammatory signals must be regulated and kept in check in order to prevent tissue damage. This is especially true in the cornea, where damage can result in loss of transparency, essential for vision. Toll-like receptors are present at the ocular surface, protecting against infection, but have also been implicated in the pathogenesis of dry eye disease (DED), an inflammatory condition that affects millions of individuals in the United States. Patients with DED experience ocular pain, discomfort, burning, and dryness that can result in visual
transcriptional regulator.18 The vitamin D receptor is expressed out the eye. transcriptional regulation still needs to be evaluated through- metalloproteinases.19 Therefore, vitamin D has the potential to inflammation. Vitamin D (1,25-dihydroxyvitamin D3) has been inflammatory therapeutics that limit aberrant ocular surface important area of research is the development of new anti- cornea.19–22 Vitamin D is able to regulate the innate immune are also present in human corneal epithelial cells (HCECs), enzymes that activate vitamin D are implicated in general ocular health and has been shown to be protective during various ocular diseases and pathologies, such as macular degeneration, uveitis, and myopia. However, its transcriptional regulation still needs to be evaluated throughout the eye.

Vitamin D acts through its receptor, the VDR, a member of the nuclear hormone receptor family which acts as a potent transcriptional regulator.18 The vitamin D receptor is expressed in the human cornea and the enzymes that activate vitamin D are also present in human corneal epithelial cells (HCECs), suggesting that vitamin D has a functional role in the cornea.19–22 Vitamin D is able to regulate the innate immune response to infection and inflammation through interactions with TLR signaling23–27 and enhances host protection through antimicrobial peptide production. 28–31 Our previous studies occur during a viral infection, resulting in NF-κB activation. For the nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α (IκBα) study, hTCEpi were pretreated with 10 μM Bay 11-7082 (Invitrogen, Carlsbad, CA, USA) for 30 minutes before poly(I:C) addition for 6 hours.

**RNA Isolation and Quantitative Real-Time PCR**

We extracted total RNA from cultured cells using TRizol reagent (Life Technologies, Grand Island, NY, USA) and a purification and isolation kit (RNeasy kits with DNase I treatment; Qiagen, Valencia, CA, USA). Concentration of RNA was measured with a spectrophotometer (Nanodrop 2000; Thermo Fisher Scientific, Wilmington, DE, USA) and cDNA was transcribed with a cDNA synthesis kit (AffinityScript; Agilent Technologies, Santa Clara, CA, USA). Quantitative PCR was performed using a master mix (Brilliant II SYBR Green QPCR; Agilent Technologies) and intron-spanning primers (Table 1). Untreated samples served as the calibrator for relative quantity determination and all samples were normalized to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

**Sample Preparation and Microarray Analyses**

We treated hTCEpi and SV40 immortalized (SV40) HCECs, respectively.22–33 Human donor corneas were received from Saving Sight eye bank (St. Louis, MO, USA) and primary HCEC isolated following overnight corneal digestion with protease (dispase II; Roche Diagnostics, Indianapolis, IN, USA).34 The optimal range of circulating serum vitamin D with protease (dispase II; Roche Diagnostics, Indianapolis, IN, USA).35,36 Therefore, cells were treated with a physiologically relevant concentration of vitamin D for these studies, 10^{-7} M (100 nM, 1,25D3) is between 30 and 80 ng/mL (75–200 nM). 35,36 Therefore, cells were treated with a physiologically relevant concentration of vitamin D for these studies, 10^{-7} M (100 nM, 1,25D3) is between 30 and 80 ng/mL (75–200 nM). However, its transcriptional regulation still needs to be evaluated throughout the eye.

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**Sample Preparation and Microarray Analyses**

We treated hTCEpi and SV40 cell lines with 1,25D3 (10^{-7} M) or vehicle (0.01% ethanol/PBS) for 6 hours and RNA was collected as above. Biological replicates and dye-swapped samples were used in a genome-wide microarray analysis performed using
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RESULTS

Vitamin D Affects Poly(I:C)-Induced IL-8 Expression in a Time-Dependent Manner

Previous studies have indicated that vitamin D plays a role in dampening proinflammatory cytokine production in HCEC. In these studies, treatment with vitamin D for 24 hours significantly decreased the expression of IL-8, IL-1β, IL-6, TNFα, and CCL20 induced by TLR3 agonist poly(I:C), a potent activator of inflammatory signaling. However, early activation of NF-κB (after 2 hours of treatment) was not affected by vitamin D. Therefore, here we further explored the effect of 1,25D3 on poly(I:C)-mediated cytokine production, focusing on early time points, to evaluate the initial effect of vitamin D. Costimulation with poly(I:C) and 1,25D3 for 2 to 6 hours showed a significant increase in IL-8 production above the expression induced by poly(I:C) alone (Fig. 1). This increase was significant at the mRNA level after 2 hours, with a jump from a 64-fold increase in IL-8 expression with poly(I:C) to a 155-fold increase when 1,25D3 was added (Fig. 1A; P < 0.01). Concentrations of IL-8 in cell supernatants were also increased at 4 and 6 hours of combined treatment, yielding an increase of approximately 50 pg/mL above poly(I:C) alone (Fig. 1B; P < 0.05). However, at the 6-hour time point, IL-8 mRNA expression with combined treatment was no different than with poly(I:C) alone, suggesting that at this time, vitamin D starts to lower IL-8 expression. When we pretreated hTCEpi...
with 1,25D3 24 hours before poly(I:C) stimulation, there was a significant reduction in IL-8 expression (50% at 6 hours; \( P < 0.05 \), Fig. 1).

We next examined the effect of vitamin D when administered after poly(I:C) treatment. When hTCEpi were stimulated with poly(I:C) for 4 hours and then treated with 1,25D3 for an additional 24 hours, there was an \(~80\%\) decrease in IL-8 expression (Fig. 2A). There was a similar reduction in IL-8 expression when cells were pretreated with poly(I:C) and then washed before 1,25D3 addition, indicating that vitamin D does not physically interfere with poly(I:C), disrupting its ability to initiate signaling events (Fig. 2B; \( P < 0.05 \)). These results suggest that vitamin D treatment is able to attenuate IL-8 levels even when poly(I:C) is administered first. However, during the immediate response to poly(I:C), vitamin D cotreatment increases IL-8 expression (Fig. 1).

**Vitamin D Regulates Multiple Genes in Corneal Epithelial Cells**

In order to further examine the influence of vitamin D on corneal epithelial cell gene expression and its influence on inflammatory events, genome-wide microarray analysis was performed. First, a time course of vitamin D treatment was performed, examining the expression of several genes known to be regulated by 1,25D3 to determine an optimal time point for analysis. The hydroxylase CYP24A1, antimicrobial peptide LL-37, and lipopolysaccharide coreceptor CD14 all exhibited maximum transcript levels 6 hours after vitamin D treatment in hTCEpi (Fig. 3). Therefore, for the gene array, cells were treated with 1,25D3 for 6 hours and gene expression was compared to vehicle-treated control cells. To determine the optimal concentration, primary HCEC were treated with physiologically relevant concentrations of vitamin D and LL-37 gene expression determined. Based on these data, \( 10^{-7} \) M (40 ng/mL) 1,25D3 was chosen for this study (Supplementary Fig. S1). Two corneal epithelial cell lines, hTCEpi and SV40 HCEC, were used in the microarray comparison to determine vitamin D–induced gene regulation.

We identified 308 genes in hTCEpi as differentially expressed at 6 hours of vitamin D treatment; while in SV40s, 69 genes were changed. Of these 69 genes, 24 genes (35%) were changed in both cell lines (Fig. 4, Table 2). In both cell lines, the majority of regulated genes were upregulated (77% in hTCEpi and 70% in SV40) with the lowest \( P \) value in both cell lines was

**Figure 2.** Vitamin D reduces IL-8 expression after poly(I:C) pretreatment. We stimulated hTCEpi with 1 \( \mu \)g/mL poly(I:C) for 4 hours and \( 10^{-7} \) M 1,25D3 was added without washing (A) or with washing to remove poly(I:C) (B), before culturing for an additional 24 hours. We collected RNA from cell lysates for qPCR analysis of IL-8 expression. Data represent the mean \( \pm \) SEM of three independent experiments. *\( P < 0.05 \).

**Figure 3.** Time course analysis of CYP24A1, LL-37, and CD14 expression after vitamin D treatment. We stimulated hTCEpi with \( 10^{-7} \) M 1,25D3 or vehicle control (0.01% ethanol/PBS) for the indicated times and RNA was collected for qPCR analyses. Data represent the mean \( \pm \) SEM of three to four independent experiments. Statistical analysis was by ANOVA with Bonferroni’s test for multiple comparisons. *\( P < 0.05 \). **\( P = 0.01 \). ***\( P = 0.001 \).
upregulation of the CYP24A1 gene, the cytochrome p450 enzyme that inactivates 25D_3 and 1,25D_3 through 24-hydroxylation.

In order to classify the 24 genes that were commonly regulated by 1,25D_3 in hTCEpi and SV40 cells, enriched biological functions were identified using DAVID software. The largest process influenced by vitamin D was intracellular signaling ($P = 0.0002$), which included genes involved in both protein kinase cascades and GTPase-mediated signal transduction. Interestingly, several genes were identified that play a role in negatively regulating signal transduction, including insulin.

### Table 2. Genes That Are Regulated by Vitamin D Treatment in Both hTCEpi and SV40 Cell Lines

| Gene Symbol | Gene Name                                               | hTCEpi P Value | SV40 P Value |
|-------------|---------------------------------------------------------|----------------|--------------|
| **Upregulated** |                                               |                |              |
| CYP24A1     | 1,25-dihydroxyvitamin D(3) 24-hydroxylase              | $2.86 \times 10^{-11}$ | $4.50 \times 10^{-11}$ |
| IL1RL1      | Interleukin 1 receptor-like 1; ST2                     | $1.65 \times 10^{-5}$ | $2.50 \times 10^{-5}$ |
| GEM         | GTP-binding protein GEM                                | $8.85 \times 10^{-8}$ | $3.25 \times 10^{-6}$ |
| SEMA6D      | Semaphorin-6D                                          | $9.48 \times 10^{-8}$ | $1.47 \times 10^{-5}$ |
| TXNRD1      | Thioredoxin reductase 1                                | $5.51 \times 10^{-7}$ | $0.00387$    |
| CLEC1       | Cardiotrophin-like cytokine factor 1                   | $1.25 \times 10^{-6}$ | $4.66 \times 10^{-5}$ |
| SERPINB1    | Serpin peptidase inhibitor                             | $2.38 \times 10^{-6}$ | $0.00017$    |
| EFTUD1      | Elongation factor Tu GTP binding domain 1              | $3.42 \times 10^{-6}$ | $8.95 \times 10^{-6}$ |
| DUSP10      | Dual specificity phosphatase 10                        | $7.88 \times 10^{-6}$ | $0.000274$   |
| MAFB         | V-maf fibrosarcoma oncogene homolog B                  | $8.86 \times 10^{-6}$ | $0.00254$    |
| ZNF114       | Zinc finger protein 114                                | $9.04 \times 10^{-6}$ | $0.000645$   |
| TMEM40       | Transmembrane protein 40                               | $1.46 \times 10^{-5}$ | $0.001698$   |
| KLK6         | Kallikrein-related peptidase 6                         | $4.14 \times 10^{-5}$ | $1.34 \times 10^{-6}$ |
| RHOF         | Ras homolog family member F                            | $5.87 \times 10^{-5}$ | $0.003357$   |
| NET1         | Neuroepithelial cell transforming 1                    | $5.95 \times 10^{-5}$ | $0.003553$   |
| RNF149       | E3 ubiquitin-protein ligase ring finger protein         | $0.000114$      | $0.001842$   |
| IGFBP3       | Insulin-like growth factor binding protein 3           | $0.000423$      | $0.000598$   |
| Q8NHV5-2     | (uncharacterized)                                      | $0.000585$      | $0.001842$   |
| G6PD         | Glucose-6-phosphate dehydrogenase                      | $0.000785$      | $0.004424$   |
| CREG2        | Cellular repressor of E1A-stimulated genes 2           | $0.000823$      | $4.42 \times 10^{-5}$ |
| RASD2        | GTP-binding protein rhes                               | $0.00086$       | $0.002113$   |
| PGM2L1       | Phosphoglucomutase 2-like 1                            | $0.001226$      | $0.003013$   |
| NFKBIA       | Nuclear factor of k light polypeptide gene enhancer in B-cells inhibitor, k | $0.0013760$     | $0.0046$     |

| **Downregulated** |                  |
|-------------------|                  |
| HIST1H4j          | Histone cluster 1, H4j | $0.000651$ | $0.004772$ |

Genes are listed in order of increasing $P$ value for hTCEpi cells, determined with the empirical Bayes $t$ test using R statistical software.
like growth factor binding protein 3 (IGFBP3), interleukin 1 receptor-like 1 (IL1RL1 or ST2), and NFKBIA (IκBα). Importantly, when classifying regulated genes by molecular function and cellular compartment, calcium-binding proteins and calcium channel activity were enriched, known areas of vitamin D influence. In addition, the biological function termed “response to vitamin D” was significantly enriched ($P = 0.0002$).

We found hTCEpi cells had a larger number of genes differentially expressed upon vitamin D treatment, indicative of being more responsive. Upon examination of VDR expression, hTCEpi had higher relative receptor expression, possibly explaining the increased responsiveness (data not shown). Thus, this cell line was specifically examined for enriched biological processes among the regulated genes (Table 3). Similar to the gene set in common with SV40s, the largest process influenced by vitamin D was intracellular signaling, which included 47 genes ($P = 2.10 \times 10^{-8}$). Vitamin D-regulated genes were also highly enriched among the processes cell proliferation, apoptosis, wounding, and transcription.

Microarray analyses are useful as a guide to identify changes in the transcriptome based on cell type or treatment. However, changes discovered from arrays must be validated with a complementary technology. Therefore, six genes identified as regulated in both cell lines were examined by real-time quantitative (q)PCR analyses (Fig. 5). These results confirmed the array data. It is also clear that the amplitude of the response, while significant in both cell lines, was higher in hTCEpi cells, explaining the larger number of genes detected by microarray for this cell line. Further, expression of these genes was evaluated in primary HCEC, from two different donors. The pattern of vitamin D regulation was the same as in the cell lines (Fig. 6), confirming the in vivo relevance of the experimental setup.

Vitamin D Attenuates Poly(I:C) Inflammatory Signaling Through Upregulation of NF-κB Inhibitor IκBα

In order to identify how vitamin D could be influencing TLR inflammatory pathways, enriched pathways were examined for genes involved in both TLR signaling and MAPK signaling (Table 4). Engagement of TLR results in an inflammatory
cascade that activates members of the MAPK and NF-κB pathways, leading to the production of proinflammatory cytokines and proteins involved in the cellular stress response as well as cell survival.

Vitamin D treatment significantly upregulated NFKBIA (IκBα) in both hTCEpi and SV40, as well as in primary HCEC (Table 3, Figs. 5, 6). Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α is a negative regulator of NF-κB signaling. It sequesters NF-κB in the cytoplasm, preventing nuclear translocation and transcriptional effects. In addition to increased IκBα transcript levels, protein expression of IκBα was also increased by 28% following 24 hours of vitamin D treatment (Fig. 7). Thus, vitamin D treatment increases IκBα, potentially inhibiting NF-κB signaling.

Upon TLR activation, signaling events lead to IκBα phosphorylation, which releases NF-κB and results in IκBα degradation. Therefore, we decided to investigate if IκBα was important in poly(I:C)-induced IL-8 expression. When phosphorylation of IκBα was inhibited by an irreversible inhibitor of IκBα phosphorylation—Bay 11-7082—there was a significant 41% decrease in IL-8 production upon poly(I:C) stimulation (Fig. 8A). This demonstrates that the NF-κB pathway is activated through IκBα phosphorylation and this is important for poly(I:C)-mediated cytokine production. Further, 24-hour vitamin D pretreatment significantly increased the expression of total IκBα protein in hTCEpi treated with poly(I:C) (Fig. 8B), suggesting that vitamin D could potentially attenuate poly(I:C) inflammatory signaling via upregulation of the NF-κB inhibitor IκBα.

**Discussion**

Our prior studies have indicated that vitamin D is able to attenuate TLR-induced inflammatory signals in corneal epithelial cells. In the current study, we explored how vitamin D treatment affects genome-wide gene regulation and TLR3-induced IL-8 expression in these cells, further examining the influence of vitamin D on corneal inflammation. Our findings demonstrate that while direct vitamin D treatment at early time points (6 hours) enhances poly(I:C)-induced IL-8 levels, 24-hour vitamin D pretreatment significantly attenuates this pathway. Microarray findings show that vitamin D regulates multiple genes in HCEC, including genes involved in MAPK and NF-κB pathways, important signaling pathways in TLR-induced inflammation.

The time-course data indicate that the ability of vitamin D to suppress cytokine production is a delayed effect. In the case of inflammation, this could be beneficial: Cytokine production and inflammatory events such as neutrophil infiltration following TLR ligation aid in the resolution of infection or injury. During pathogenic challenge, the inflammatory process is necessary for the ultimate elimination of microbial threat. However, too much inflammation is harmful and a dampening of the response is necessary to restore tissue homeostasis following the insult, preventing chronic inflammation and tissue damage. Therefore, the initial effect of vitamin D to

**Table 4. Genes Regulated by Vitamin D Involved in MAPK Signaling and TLR Signaling Pathways**

| Gene Symbol | Gene Name |
|-------------|-----------|
| DUSP1       | Dual specificity phosphatase 1 |
| DUSP10      | Dual specificity phosphatase 10 |
| DUSP4       | Dual specificity phosphatase 4 |
| DUSP5       | Dual specificity phosphatase 5 |
| GADD45A     | Growth arrest and DNA-damage-inducible, α |
| IL1B        | Interleukin 1, β |
| MAPK13      | Mitogen-activated protein kinase 13 |
| MAP2K6      | Mitogen-activated protein kinase 6 |
| PDGFA       | Platelet-derived growth factor α polyepitope |
| TGFB2       | Transforming growth factor, β 2 |
| FOS         | V-fos FBj murine osteosarcoma viral oncogene homolog |

![Figure 7](image) Protein IκBα increases with vitamin D treatment. We treated hTCEpi with $10^{-7} M$ 1,25D$_3$ for 24 hours and total IκBα protein expression was measured in cell lysates by ELISA. Data represent the mean ± SEM of 5 independent experiments. Statistical analysis was by Student’s t-test. *$P < 0.05$. 

![Figure 6](image) Confirmation of microarray results in primary HCEC. Primary HCECs were isolated from donor corneas and cultures were treated with $10^{-7} M$ 1,25D$_3$ for 6 hours. We collected RNA from cell lysates for qPCR analyses. Graphs reflect data from one of two corneal donors and data are mean ± SEM of triplicate values (24A1, cytochrome P450 hydroxylase CYP24A1).
increase IL-8 expression could help to jumpstart inflammatory events and then its delayed action may suppress ongoing, potentially harmful cytokine production. Vitamin D was also able to decrease IL-8 levels when the cells were pretreated with poly(I:C), further indicating that it is able to suppress inflammatory signals after their initiation. This is an important finding considering a potential therapeutic use of vitamin D for treatment of ongoing inflammatory conditions.

Nuclear factor κB is a transcription factor that regulates the expression of inflammatory genes. It is activated upon TLR ligation as well as by cytokine signaling. Following TLR activation, signaling events result in phosphorylation of IκBα, which results in its targeting for degradation, hence allowing NF-κB to translocate to the nucleus and activate transcription.4

Our microarray analysis identified IκBα as a target gene regulated by vitamin D in both hTCEpi and SV40 cell lines, which was confirmed in primary HCEC as well. During poly(I:C) stimulation, pretreatment with vitamin D also raised IκBα levels, thereby increasing the cell’s capacity for inhibiting NF-κB activation during an inflammatory stimulus. Other studies have demonstrated that increasing IκBα transcription and translation decreases NF-κB translocation to the nucleus.44 In human peritoneal macrophages, vitamin D suppressed NF-κB activation through increasing IκBα expression, resulting in reduced TLR-induced TNFα production.45 In addition, basal levels of IκBα were lower in cells from mice lacking VDR, increasing the amount of NF-κB in the nucleus of resting cells.46 Therefore, we suggest that the vitamin D–mediated attenuation of poly(I:C)-induced IL-8 expression in corneal cells is due at least in part from its upregulation of IκBα expression.

Another candidate pathway for the influence of vitamin D on cytokine production is the MAPK family of signaling kinases. Multiple dual specificity protein phosphatase (DUSP) genes were upregulated by vitamin D during poly(I:C) stimulation, as highlighted by the microarray data and confirmed by qPCR (DUSP1, 4, and 5; Table 4, Supplementary Fig. S2A). Dual specificity protein phosphatases negatively regulate signal transduction through dephosphorylating threonine and tyrosine residues on members of the MAPK pathway, rendering them inactive.47 Dual specificity protein phosphatases preferentially regulate p38 and JNK (c-jun NH2-terminal kinases), the stress-activated MAPKs, leading to downregulation of cytokines and proinflammatory genes.48 The anti-inflammatory effects of glucocorticoids have been associated with DUSP expression49 and mice deficient in DUSP have increased levels of cytokines.50 In particular, we found that DUSP10 expression was increased in hTCEpi, SV40, and primary HCEC (Supplementary Fig. S2A). Dual specificity protein phosphatase 10, also called MKP5, dampens inflammatory signals through its actions on p38, which has been shown to induce cytokine production.50–53 However, in our study, despite increasing DUSP expression, vitamin D did not decrease p38 phosphorylation. Instead, there was an increase in p-p38 with vitamin D (Supplementary Fig. S2B). Investigating this apparent paradox further, we found evidence that p38 can have dual functions, having both pro- and anti-inflammatory roles depending on the cell type and stimulus involved. Activation of p38 has in fact been shown to increase DUSP expression, limiting the cycle of inflammation.54 The influence of vitamin D on these pathways needs to be further dissected in order to determine if increased DUSP expression modulates inflammation through p38.

The potential for vitamin D to modulate inflammatory signaling pathways has important clinical relevance in the cornea, not only during infectious keratitis, as previously mentioned, but also for chronic ocular surface inflammation, such as occurs in DED. Dry eye disease is a multifactorial condition that is a significant public health concern, affecting millions of individuals each year in the Unites States alone, causing ocular irritation, burning, pain, and dryness.7,9,10 Inflammation is involved in the pathogenesis of DED and there have been many studies showing that there is an increase in tear, corneal, and conjunctival proinflammatory cytokines, chemokines, and matrix metalloproteinases during disease.55–58 Therefore, anti-inflammatory therapeutics have shown promise in the treatment of DED.59–61 Specifically, MAPK signaling pathways have been shown to be activated during experimental dry eye, with an increase in JNK, ERK, and p38 phosphorylation in the cornea and conjunctiva during desiccating stress.59,62 The ability of vitamin D to modulate these inflammatory signaling pathways make it a good
therapeutic candidate to evaluate in the treatment of DED and other ocular inflammatory disorders.

In examining the microarray results, we found that the response to vitamin D between the two HCEC cell lines was very different. Baseline levels of gene expression in hTCEpi and SV40 have not been compared and warrant further investigation, based on this differential response. Transcriptome analysis should be performed to compare hTCEpi and SV40 cells with primary HCEC, to determine which cell line most closely resembles nonimmortalized corneal epithelial cells. Our data suggest that gene expression changes in hTCEpi more closely resemble the response of primary cells to vitamin D, based on relative fold changes. However, a separate microarray analysis would be interesting to directly compare similarities and differences among primary cultured HCEC and cell line gene expression. These results would be important to consider when choosing a cell line for in vitro studies.

In summary, we show that vitamin D has a strong influence on gene expression in HCECs. This regulation modulates inflammatory cytokine production, in a time-dependent manner. We propose that the vitamin D-mediated upregulation of IL1R1, GADD45, TRAF4, TGFB2, and MAP2K6, and will be interesting to investigate further.

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