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Characterization of Vitamin D Production by Human Ocular Barrier Cells

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Purpose. Vitamin D_3 is a secosteroid mainly synthesized from the conversion of the skin precursor 7-dehydrocholesterol (7DHC) to vitamin D_3 by ultraviolet (UV) B sunlight. Extrarenal synthesis of vitamin D_3 has been reported in many tissues and cells, including barrier sites. This study characterizes the expression of components of vitamin D_3 signaling in human ocular barrier cells.

Methods. Primary human scleral fibroblasts (HSF), human corneal endothelial (HCEC-12), nonpigmented ciliary body epithelial (ODM-2), and adult retinal pigment epithelial (ARPE-19) cell lines were analyzed for the expression of vitamin D receptor (VDR), the vitamin D_3 activating enzymes 1α-hydroxylase (CYP27B1), 25-hydroxylases (CYP27A1 and CYP2R1), the vitamin D_3 inactivating enzyme 24-hydroxylase (CYP24A1), and the endocytic receptors cubilin and megalin using a combination of RT-PCR, immunocytochemistry, and enzyme immunoassay (EIA).

Results. The HSF, HCEC-12, ODM-2, and ARPE-19 express mRNA and protein for all vitamin D_3 synthesizing and metabolizing components. The cell types tested, except HSF, are able to convert inactive 25-hydroxyvitamin D_3 (25[OH]D_3) into active 1,25-hydroxyviatmin D_3 (1,25[OH]$_2D_3$).

Conclusions. This novel study demonstrated that ocular barrier epithelial cells express the machinery for vitamin D_3 and can produce 1,25$(OH)_2D_3$. We suggest that vitamin D_3 might have a role in immune regulation and barrier function in ocular barrier epithelial cells.

Keywords: vitamin D3, blood–ocular barrier, epithelial cells

Vitamin D_3 is a secosteroid synthesized from the conversion of the precursor 7-dehydrocholesterol (7DHC) in the skin to vitamin D_3 by ultraviolet (UV) B sunlight. To a lesser extent, vitamin D_3 can be obtained from the diet. Sufficient levels of vitamin D_3 are required for calcium absorption and maintenance of healthy bones.[1] In the circulation, vitamin D_3 is activated by two successive hydroxylation steps. The first occurs in the liver by a cytochrome P (CYP) 450 enzyme, 25-hydroxylase (mitochondrial CYP27A1 and microsomal CYP2R1) to produce the major circulating form 25-hydroxyvitamin D_3 (25[OH]D_3). The second hydroxylation step occurs in the kidney, where 25-hydroxyviatmin D 1α-hydroxylase (CYP27B1) catalyzes the conversion of 25(OH)D_3 to its active form 1,25-dihydroxyvitamin D_3 (1,25[OH]$_2D_3$). As a feedback mechanism, 24-hydroxylase (CYP24A1), which is induced by 1,25$(OH)_2D_3$, inactivates both forms of vitamin D_3. The 1,25$(OH)_2D_3$ action is mediated via the vitamin D receptor (VDR) that heterodimerizes with retinoid X receptor (RXR), a complex that regulates gene transcription through binding to vitamin D response elements (VDRE) in the promoter region of target genes.[2]

Extrarenal synthesis of vitamin D_3 has been reported in many tissues and cells, including barrier sites. The VDR and CYP27B1 have been described in epithelial cells of skin, lung, intestine, prostate, endometrium, and breast, as well as cells of the immune system, such as macrophages and dendritic cells.[3–9] In the circulation, 25(OH)D_3 is bound to vitamin D binding protein (DBP), which facilitates its cellular uptake by multi-ligand endocytic receptors megalin/cubilin expressed on the apical surface of polarized epithelial cells in many tissues.[10]

Local production of 1,25$(OH)_2D_3$ has been shown in respiratory, bladder, and colonic epithelial cells; osteoclasts; and macrophages.[4,11–14] Of note, VDR is critical for barrier formation in human skin and the integrity of mucosal barrier in mouse intestine.[15,16] Recently, vitamin D_3 has been recognized as an immunomodulatory hormone that regulates innate and acquired immune responses.[17] One regulatory role of 1,25$(OH)_2D_3$ in the immune system (in vitro) involves the inhibition of proinflammatory cytokine production from $CD4^+$ T cells and the induction of a regulatory T cell phenotype.[18] Endogenous conversion of 25(OH)D_3 has been shown to inhibit dendritic cell antigen presentation and chemotaxis.[19] The ability to induce the production of host defense peptides (HDPs) in blood monocytes also supports an interaction between 1,25$(OH)_2D_3$ and the immune system. The HDP LL-37 (cathelicidin) and human beta defensins (hBD1-4) can kill a

Table 1. Polymerase Chain Reaction Primer Sequence and Cycle Conditions

Gene	Forward Primer 5′–3′	Reverse Primer 5′–3′	Cycles	Annealing Temperature, °C	Product, bp
VDR	CGCTCCAATGAGTCCTTCACC	GCTTCATGCTGCACTCAGGC	33	61	421
CYP27B1	CACCTGACCCACTTCCTGTT	TCTGGGACACGAGAATTTCC	35	58	302
CYP24A1	CCCACTAGCCACCTCGTACCAAC	CGTAGCCCTTCTTTGCGGTAGTC	35	60	485
CYP2R1	AGAGACCCAGAAGTGTTCCAT	GTCTTTCAGCACAGATGAGGTA	40	62	259
CYP27A1	GGCAAGTACCCAGTACGG	AGCAAATAGCTTCCAAGG	40	62	292
18S	GTTGGTGGAGCGATTTGTCT	GGCCTCACTAAACCATCCAA	20	55	400
Cubilin	GCGGCTTCACTGCTTCCTA	GAGTGATGGTGTGCCCTTGT	35	53	518
Megalin	TAAGTCAGTGCCCAACCTTT	GCGGTTGTTCCTGGAG	35	53	290

wide range of Gram-negative and Gram-positive bacteria, as well as viruses. Moreover, HDPs are immunomodulatory molecules.[20] They can act as chemotactic agents for T and dendritic cells, as well as their ability to induce T-helper 1 and 2 cytokines.[21]

Ocular immune privilege is maintained by a cluster of anatomic and physiological processes that protect the eye from sight-threatening infections and inflammatory responses.[22] The sclera is the white, opaque, fibrous support to the global structure of the eye composed of a rich extracellular matrix (ECM) of collagen and elastic fibers, together with fibroblasts that are responsible for tissue remodeling and the regeneration of the ECM.[23] On the posterior surface of the cornea, endothelial cells contribute to barrier function by mediating sodium transport, which maintains the cornea in a relatively dehydrated state, thereby preserving corneal transparency.[24] The eye consists of two key vascular barriers that afford protection: the blood–aqueous barrier and the blood–retinal barrier. The blood–aqueous barrier consists of tight junctions between vascular endothelial cells of iridociliary body blood vessels, the nonpigmented ciliary epithelial cells complexed apically with the pigmented ciliary epithelium, and the posterior iris epithelium, whereas the blood–retinal barrier is composed of tight junctions of endothelial cells in retinal vasculature and those between the retinal pigment epithelial cells.[25]

To date, very few studies have examined the presence of vitamin D_3 synthesizing and metabolizing pathways in the eye, and whether these have a role in ocular barrier cell function by the autocrine production of vitamin D_3. This study shows the expression and functionality of vitamin D_3 system in several human ocular barriers, intimating that vitamin D_3 may be important to the ocular barrier function and ocular immune privilege.

Materials and Methods

Cell Culture

All cells were grown in a humidified chamber at 37°C and 5% CO_2, and passaged by trypsinization. Penicillin (100 units/mL) and streptomycin (100 μg/mL) solution was added to all growth media (PAA Laboratories, Yeovil, UK). All cells were grown in 10% heat inactivated fetal calf serum (HIFCS) except human corneal endothelial cells, which were grown in 5% HIFCS (Biosera Ltd., Ringmer, UK).

Primary Human Scleral Fibroblasts (HSF). Primary human scleral fibroblasts were generated from redundant donated corneal scleral tissue following corneal transplant surgery. Acquisition of tissue was after approval from the local research ethics committee and adhered to the Declaration of Helsinki. The sclera was cut into 3- to 5-mm sections and layered in a T-25 cm^2 tissue culture flask (Sarstedt, Leicester, UK) for 3 to 5 days in fibro-medium (RPMI 1640 containing 1% MEM nonessential amino acid and 1% sodium pyruvate) until the fibroblasts grew out from the explant and maintained in fibro-medium (all Sigma-Aldrich, Dorset, UK).

Corneal Endothelial (HCEC-12) Cells. The HCEC-12 cell line (the German Resource Centre for Biological Material, DSMZ, Braunschweig, Germany) was grown in tissue culture flasks (Sarstedt, Leicester, UK) precoated with a mixture of laminin and chondroitin sulfate (Sigma-Aldrich) in F99 basal medium (Gibco, Invitrogen, Paisley, UK) supplemented with 20 μg/mL ascorbic acid, 20 μg/mL human recombinant insulin, and 10 ng/mL basic fibroblast growth factor (bFBF; all Sigma-Aldrich).

Adult Retinal Pigment Epithelial (ARPE-19). The ARPE-19 line, an SV40 transformed cells line, obtained from American Type Culture Collection (ATCC number: CRL-2302; American Type Culture Collection, Middlesex, UK) was cultured in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM/F12), with glutamax (Gibco, Invitrogen).

Nonpigmented Ciliary Body Epithelial (ODM-2). The ODM-2 cell line was a kind gift from Dr Coca Prados (Department of Ophthalmology and Visual Sciences, Yale University, New Haven, CT, USA). Cells were cultured in high glucose (4 g/L) DMEM (PAA Laboratories) as described previously.[26]

Reverse Transcription and PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Crawley, UK) according to manufacturer's instructions. The RNA concentration and purity were determined using Nanodrop spectrophotometer (Thermo Fisher Scientific, Loughborough, UK). Total RNA (1 μg) was reverse transcribed to cDNA using Taqman reverse transcription kit (Applied Biosystems, Warrington, UK) following manufacturer's instructions. The PCR was performed using GoTaq Flexi DNA polymerase system (Promega, Southampton, UK) in a total volume of 20 μL containing cDNA (1 μL for 18S and 2 μL for all other primers), 2 mM $MgCl_2$, ×1 GoTaq green Flexi Buffer, 0.5 U Gotaq DNA polymerase, 0.25 mM dNTP mix (Promega), 2.5 μM 18S, and 5 μM other forward and reverse primers (Alta Bioscience, Birmingham, UK). The PCR was performed using Gene Amp, PCR System 2700 (Applied Biosystems) as follows: 5 minutes at 94°C, followed by three temperature cycles of 1 minute at 94°C, annealing for 60 seconds, and 1 minute at 72°C. For cubilin and megalin, preamplification was carried out at 95°C for 5 minutes followed by cycles of denaturation at 94°C for 45 seconds, annealing for 45 seconds, and extension at 72°C for 45 seconds. All PCR reactions ended with a final extension step at 72°C for 7 minutes. Primer sequences, taken from previous publications and cycling condition were as shown in Table 1.

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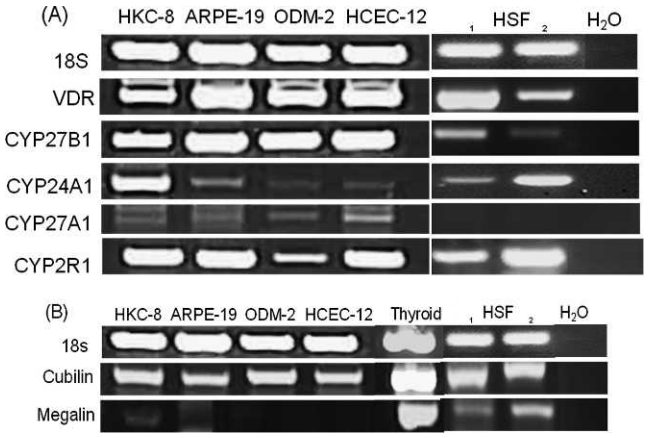

Figure 1. Ocular barrier cells express mRNA for vitamin D_3 elements. Conventional RT-PCR was carried out using total RNA extracted from ocular barrier epithelial cell lines and two primary HSF samples (1 and 2), and primers for (**A**) VDR, CYP27B1, CYP24A1, CYP27A1, CYP2R1, and for (**B**) cubilin, and megalin. Ribosomal 18S was used as an internal PCR control and water was used a negative PCR control. The HKC-8 cells were used as a positive control for vitamin D machinery, and primary thyroid cells were used as a positive control for cubilin and megalin. Results are representative of at least three independent experiments. Molecular sizes (base pairs [bp]): 18S (400), VDR (421), CYP27B1 (302), CYP24A1 (485), CYP27A1 (292), CYP2R1 (259), cubilin (518), and megalin (290), and these show the constitutive expression of vitamin D_3 synthesizing components in ocular barrier cells.

$1,25(OH)_2D_3$ Enzyme Immunoassay (EIA)

Cells were grown to confluence in 12-well culture plates. Cells were placed in serum-free medium and were either left untreated or treated with $25(OH)D_3$ (10^{-7} M) with or without pretreatment with ketoconazole (10^{-5} M), an inhibitor of CYP27B1 (both Sigma-Aldrich) for 2 hours. After 24 hours, cell culture supernatants were collected for the measurement of $1,25(OH)_2D_3$, and cells were processed for protein extraction and quantification. The $1,25(OH)_2D_3$ concentration was measured by EIA (Immunodiagnostics Systems Limited, Tyne & Wear, UK) according to the manufacturer's instructions.

Immunofluorescence

Ocular barrier cells were cultured in 8-well glass chamber slides (BD Biosciences, Oxford, UK), fixed for 20 minutes with cold methanol at 4°C, and rinsed with PBS. Cells were blocked with 5% BSA in PBS for 1 hour, then treated with mouse anti-human VDR clone H4537 (R&D Systems, Abingdon, UK), sheep anti-human CYP27B1 clone H210 (The Binding Site Ltd., Birmingham, UK), mouse anti-human CYP24A1 clone 1E1 (Novus Biologicals, Cambridge, UK), mouse anti-human mannose-6-phosphate receptors (M6P) clone 2G11 (Abcam, Cambridge, UK), or mouse anti-human trans-Golgi network 38 (TGN38) clone 2F7.1 (Novus Biologicals), respectively (1:100 in PBS blocking solution) for 1 hour at room temperature. Cells were washed several times with PBS, then incubated with FITC conjugated anti-mouse or FITC conjugated anti-sheep secondary antibody (The Binding Site Ltd.), or Texas Red (TR) conjugated anti-mouse antibody (Abcam) diluted 1:250 in blocking solution for 1 hour at room temperature. Cells were counterstained briefly with DNA-specific stain DAPI (Gibco, Invitrogen) and mounted (Vectashield; Vector Laboratories, Peterborough, UK). Staining was detected using a fluorescent microscope (AxioPlan2 Imaging; Zeiss, Cambridge, UK).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). The Kruskal-Wallis test was used, followed by Dunn's multiple comparison test to compare differences between groups. Data are expressed as mean ± SD and a P value < 0.05 was considered as statistically significant.

Results

Ocular Barrier Cells Constitutively Express mRNA for Vitamin D_3 Metabolism

Extrarenal expression of vitamin D_3 synthesizing and metabolizing components has been described in many organs. To examine the presence of these elements in ocular barriers, mRNA expression was examined by RT-PCR. The VDR was expressed strongly in all ocular cells tested (Fig. 1A). The CYP27B1 was expressed markedly by HCEC-12, ODM-2, and ARPE-19 compared to a weak expression in HSF. In contrast, CYP24A1 was expressed strongly by HSF and weakly by all other cell types. The CYP27A1 was expressed weakly in HCEC-12, ARPE-19, and ODM-2, but was undetected in HSF. The HSF, HCEC-12, and ARPE-19 highly expressed CYP2R1, whereas ODM-2 showed a weak expression. The mRNA expression of cubilin and megalin, the receptors required for the internalization of $25(OH)D_3$ through DBP, also was investigated. Cubilin was expressed strongly in all cells, while megalin was only weakly expressed in HSF (Fig. 1B). To summarize, ocular barrier cells constitutively express mRNA for vitamin D_3 metabolism.

Ocular Barrier Cells Express Proteins for Vitamin D_3 Metabolism

Immunofluorescent staining of all cell types for the different vitamin D_3 elements was performed (Fig. 2, Supplementary Figs. S1A–D). The intensity of staining varied between cell types, but all cells were positive for all vitamin D_3 proteins. The VDR showed a diffuse mild cytoplasmic and abundant speckled nuclear staining. The expression was the strongest in ODM-2 and HCEC-12, and weaker in ARPE-19 and HSF, respectively. The CYP24A1 also was present in the cytoplasm, where the signal was strong in HCEC-12 and ODM-2, and weak in HSF and ARPE-19. The CYP27B1 showed an intense cytoplasmic pattern in ODM-2 and ARPE-19, while HSF showed moderate staining. Interestingly, in HCEC-12 cells, diffuse cytoplasmic staining was present, but most of the enzyme appeared within a perinuclear vesicle.

CYP27B1 Colocalizes With MPRs and TGN38 in HCEC-12 Cells

The YP27B1 normally is located in the inner membrane of the mitochondria.[3] All the ocular cells demonstrated a staining pattern consistent with this expression, that is, cytoplasmic. The exception was HCEC-12 cells, which not only expressed CYP27B1 in the cytoplasm, but the majority of the enzyme appeared as a vesicle adjacent to the nucleus. To further identify the nature of this vesicle-like structure, HCEC-12 were stained for Mannose 6 phosphate (M6P) receptors that are known to have a role in the transport of enzymes from the trans-Golgi network (TGN) to lysosomes[27] and typically shows perinuclear localization. In addition, TGN38 protein, part of the trans-Golgi network that directs proteins to secretory vesicles, lysosomes, or plasma membrane, also was exam-

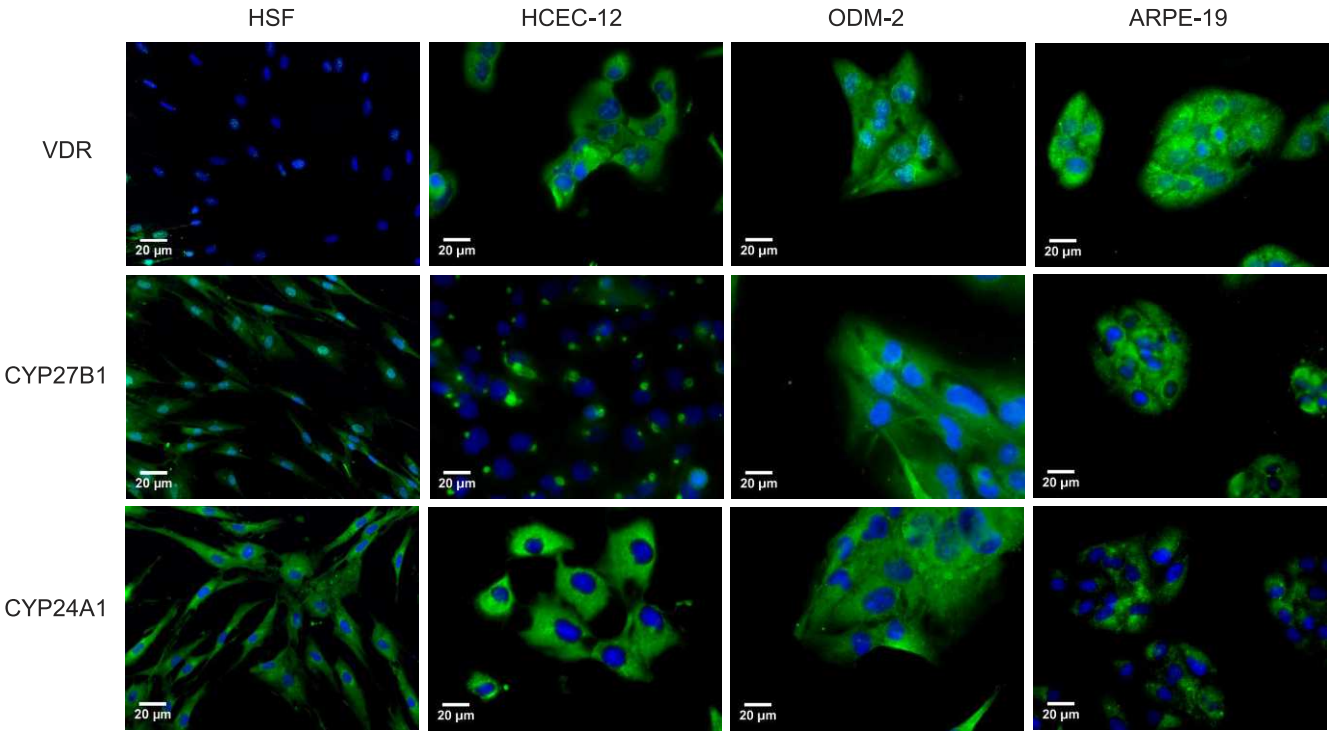

FIGURE 2. Ocular barrier cells express proteins for vitamin D_3 elements. Ocular epithelial cell lines and primary HSF cells were grown to 40% to 50% confluency in 8-well chamber slides, then fixed and processed for immunofluorescence. Cells were stained with primary mouse monoclonal anti-human VDR antibody, mouse anti-human CYP24A1, and sheep polyclonal anti-human CYP27B1 antibodies, respectively. Staining was visualized with secondary FITC (*green*) conjugated anti-mouse and anti-sheep antibodies, respectively, using fluorescence microscopy. Nuclei were stained with DAPI (*blue*). This figure shows merged images with magnification: ×20 for HSF and ×40 for the others, and these show the expression patterns of vitamin D_3 synthesizing proteins in ocular barrier cells. *Scale bars*: 20 μm.

ined.[28] The CYP27B1 colocalized to M6P receptors and TGN38 in HCEC-12 (Fig. 3A). The cytoplasmic staining, together with the perinuclear punctated presence (Fig. 3B) confirm CYP27B1 presence in the TGN in HCEC-12 cells.

Ocular Barrier Cells Can Convert Inactive Vitamin D_3 to the Active Form $1,25(OH)_2D_3$

Ocular barrier cells have the molecular components necessary to produce $1,25(OH)_2D_3$ locally. We next examined the functional capacity of CYP27B1 and its ability to convert inactive $25(OH)D_3$ (10^{-7} M) into active $1,25(OH)_2D_3$. The rate of conversion was variable among the different cells, where the highest levels were produced by HCEC-12 followed by HKC8 then ODM2, while the lowest levels were seen in ARPE-19 and none was detected in HSF (Fig. 4).

The HKC-8–positive control cells produce significant amounts of $1,25(OH)_2D_3$ (195.4 pmol/L/mg protein, $P < 0.05$). By comparison, primary HSF did not show any conversion and ARPE-19 cells showed minimal levels (8.0 and 25.0 pmol/L/mg protein, respectively). Interestingly, HCEC-12 were the most efficient of all cell types at converting the substrate into the active form of $1,25(OH)_2D_3$ (2068.1 pmol/L/mg protein, $P < 0.01$), that is, 10 times higher than HKC-8, while ODM-2 cells produced levels as high as 640.1 pmol/L/mg protein.

Substrate conversion in each cell type was inhibited by pretreatment with 10^{-5} M ketoconazole and the inhibition is statistically significant in cells that produced more than 200 pmol/L/mg protein of $1,25(OH)_2D_3$ (HCEC-12, and ODM-2).

DISCUSSION

This study showed that human ocular barrier epithelial cells and scleral fibroblasts constitutively express the receptor and the metabolic enzymes required in the vitamin D_3 pathway, and for the first time to our knowledge that all these cell types can endogenously produce $1,25(OH)_2D_3$ when treated with physiological concentrations of its precursor $25(OH)D_3$ (Table 2). These findings supported a recent report describing CYP27B1 and VDR mRNA in primary human corneal epithelium (PHCEC), while treatment with $25(OH)D_3$ or $1,25(OH)_2D_3$ enhanced barrier function as shown by decreased inulin permeability and increased transepithelial resistance in vitro.[29] In a separate study, a human corneal limbal epithelial cell line treated with UVB and 7DHC showed an increase in $1,25(OH)_2D_3$ production.[30] Previous studies also have shown that corneal epithelial cells and RPE-choroid express the molecular elements for vitamin D_3, but there were no data on $25(OH)D_3$ conversion.[31]

As stated, vitamin D_3 synthesizing components (VDR, CYP27B1, and CYP24A1) are expressed widely in many human epithelial cells. In agreement with the speckled cytoplasmic and nuclear VDR staining in ocular barrier cells and sclera fibroblasts, VDR has been described in cytoplasm and nucleus of bladder epithelium as well as skin fibroblasts.[11,32] The cytoplasmic expression of CYP27B1 in most of the ocular barrier cells is in accordance with similar expression in bladder epithelium. Conversely, the punctate perinuclear staining of CYP27B1 in HCEC-12 can be compared to the cytoplasmic localization of internalized Alexa-DBP in the perinuclear space of human breast cancer epithelial cells T-47D, where it

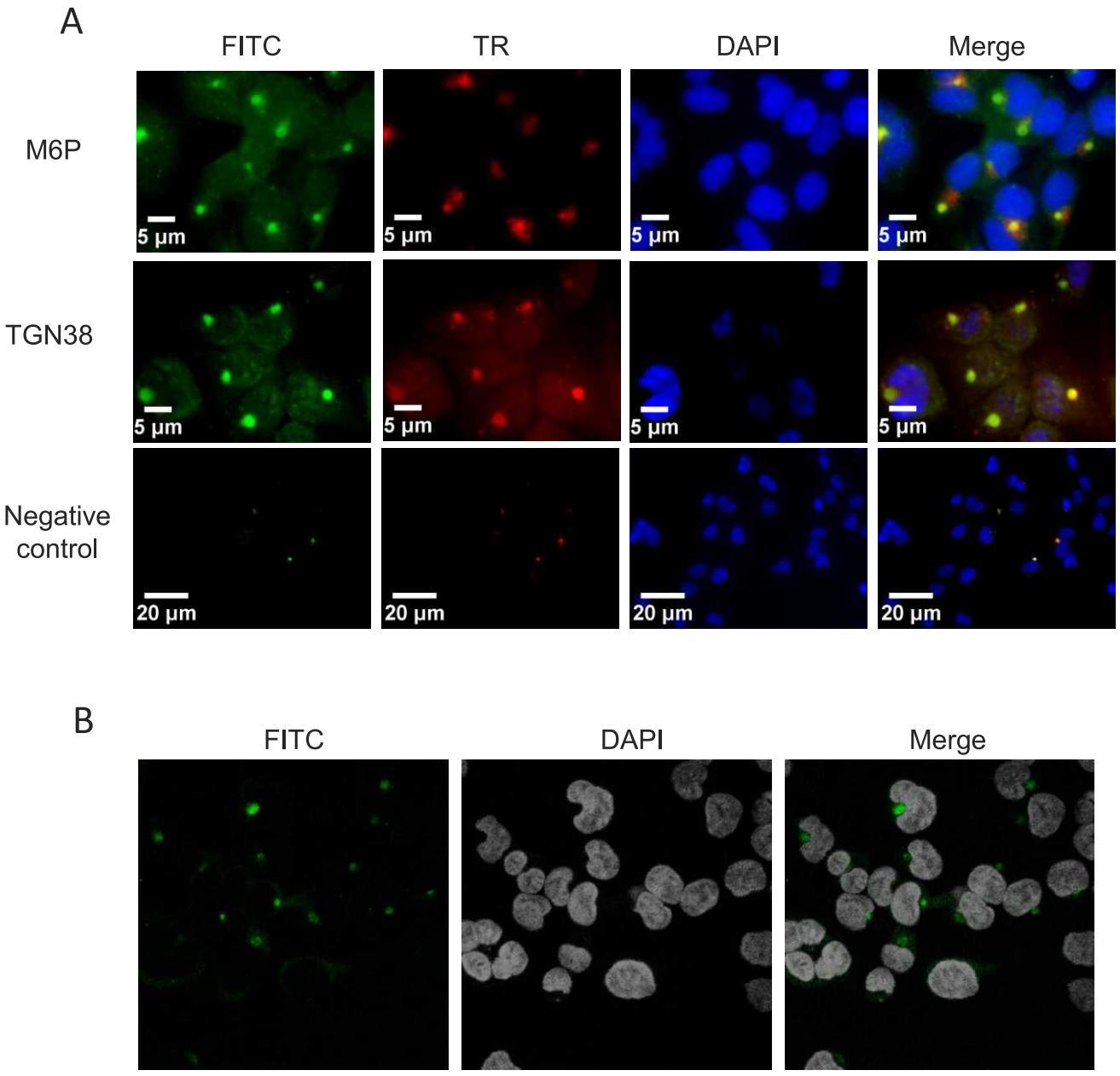

Figure 3. CYP27B1 co-localizes with M6P receptor and TGN38 in HCEC-12 . (**A**) Fluorescence microscopy images showing HCEC-12 cells grown to 40% to 50% confluency in 8-well chamber slides, then fixed and processed for immunofluorescence. Cells were stained with sheep polyclonal anti-human CYP27B1 and mouse monoclonal anti-human M6P and TGN38, respectively. Staining was visualized with FITC (*green*) and TR (*red*) conjugated anti-sheep and anti-mouse antibodies, respectively. Nuclei were stained with DAPI (*blue*). Results show colocalization of CYP27B1 with M6P (*top*) and TGN38 (*middle*). Negative control (*bottom*): cells with primary antibody omitted (*bottom*). Magnification: ×100 for M6P and TGN38 and ×40 for negative control. *Scale bars*: 5 µm for M6P and TGN38, and 20 µm for negative control. (**B**) Confocal microscopy images showing HCEC-12 cells prepared as above and were stained with primary sheep polyclonal anti-human CYP27B1 antibodies. Staining was visualized with secondary FITC (*green*) conjugated anti-sheep antibodies. Nuclei were stained with DAPI (*gray*). Magnification: ×40. Results show the perinuclear localization of CYP27B1 in HCEC-12 cells.

colocalized with lysosomes, which suggested that DBP traffics through endosomes and lysosomes.[33] The expression of CYP27B1 in TGN could be part of this enzyme's trafficking in these cells. Membrane-bound CYP450 proteins can recycle through Golgi before reaching their destined locations.[34]

Although ocular barrier cells show constitutive expression of mRNA and protein for CYP24A1, this mitochondrial enzyme is not constitutively expressed in all peripheral organs and tissues. Some cells, such as skin keratinocytes, primary respiratory epithelial cells, and colonic epithelial cell line, only express CYP24A1 in response to $25(OH)D_3$ or $1,25(OH)_2D_3$. Keratinocytes demonstrate a strong up-regulation following exposure to UVB light in the presence of 7DHC.[4,13,35] The CYP24A1 also is induced after treatment with $25(OH)D_3$ sufficient serum in macrophages.[20]

Our study showed that ocular barrier epithelial cells can convert inactive vitamin D_3 to its active form. With the exception of ARPE-19, ocular barrier cells, are able to produce significant levels of active $1,25(OH)_2D_3$ ($>0.4 \times 10^{-9}$ M) at 24 hours. This rate of conversion is comparable to that of primary

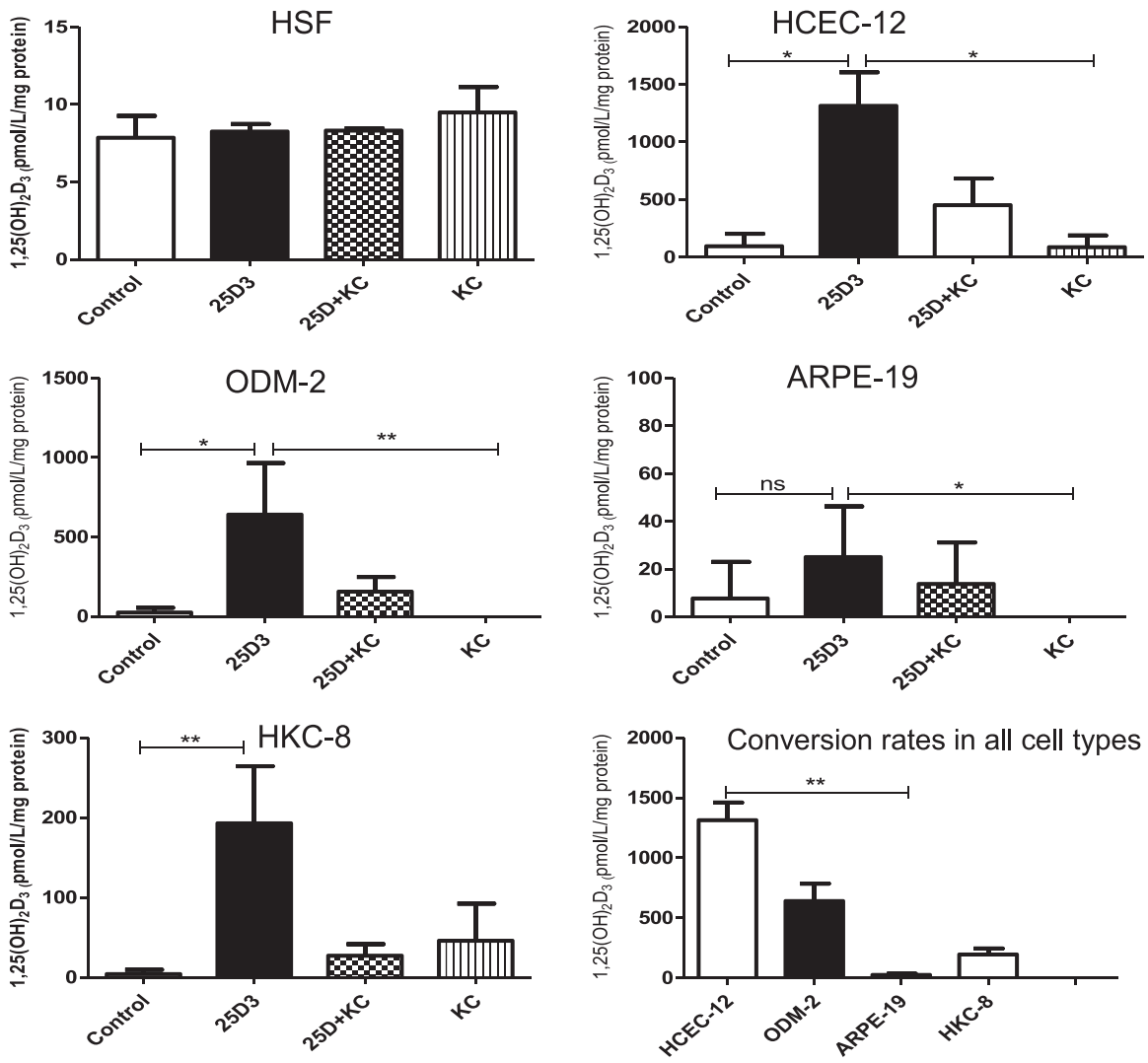

Figure 4. Ocular barrier cells can convert 25(OH)D_3 into active 1,25$(OH)_2D_3$. Ocular epithelial cells lines grown to 80% confluency in 12-well plates were either left untreated or treated with 25(OH)D_3 (10^{-7} M) for 24 hours with or without pretreatment with CYP450 inhibitor (10^{-5} M) ketoconazole (KC) for 2 hours. Cell culture supernatants were collected and 1,25$(OH)_2D_3$ was determined by enzyme immunoassay. Results were obtained from a standard curve as pmol/L, then corrected per mg of protein. The HKC-8 cells were used as a positive conversion control. Results are representative for at least three independent experiments. Graph shows mean ± SD 1,25$(OH)_2D_3$ per mg protein for each treatment. *Bottom right graph* shows conversion rates in all ocular barrier epithelial cells compared to HKC-8. Kruskal-Wallis test with Dunn's posttest were performed (*$P < 0.05$, **$P < 0.01$; ns, not significant). Results show that all ocular barrier cells except HSF convert inactive to active 1,25$(OH)_2D_3$.

Table 2. A Summary of Gene and Protein Expression for Vitamin D_3 Elements by Ocular Barrier Cells as Well as the Rate of 25D_3 Conversion

	Gene Expression							Immunofluorescence			Rate of 25D_3
Cell Line	**VDR**	**CYP27B1**	**CYP24A1**	**CYP27A1**	**CYP2R1**	**Cubilin**	**Megalin**	**VDR**	**CYP27B1**	**CYP24A1**	**Conversion**
Ocular barrier cells											
HSF	++	++	−	−	+++	+++	+	++	++	+++	−
HCEC-12	+++	+++	+	++	+++	+++	−	+++	+++	+	+++
ODM-2	+++	+++	+	+	++	+++	−	+++	+++	+	++
ARPE-19	+++	+++	++	+	+++	+++	−	++	+++	++	+
Control cells											
HKC-8	+++	+++	+++	+	+++	+++	+	ND	ND	ND	++
Thyroid	ND	ND	ND	ND	ND	+++	+++	ND	ND	ND	ND

Ocular barrier cell lines are shown with mRNA expression, immunofluorescence staining intensity, and rate of 25D_3 conversion with the highest as +++ and the lowest as +. The HKC-8 cell line was used as a positive control for the expression of vitamin D_3 pathway components and 25(OH)D_3 conversion. Thyroid mRNA was used a positive control for the expression of cubilin and megalin. ND, not done.

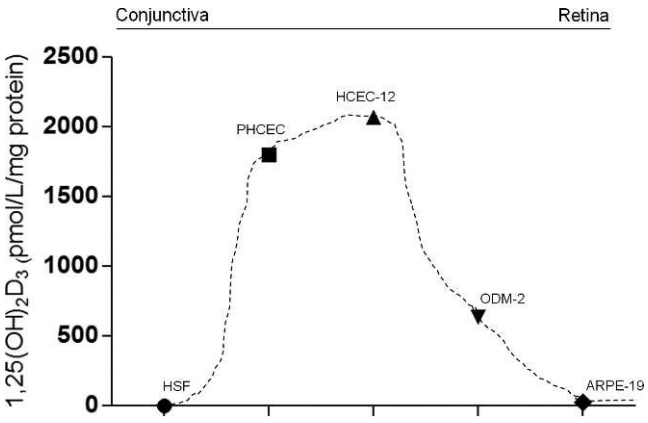

Figure 5. The rate of $25(OH)D_3$ conversion by ocular barrier cells according to their location from the outside to the inside. A schematic diagram showing $1,25(OH)_2D_3$ concentration in the different ocular barrier cells from the exterior to the interior following their anatomic location. While no conversion was seen in HSF, $25(OH)D_3$ conversion is high in the corneal epithelium (PHCEC), reaches the maximal levels in the corneal endothelium (HCEC-12), decreases in the ciliary body (ODM-2), and further in the retina (ARPE-19).

respiratory epithelial cells, bladder, and mammary epithelial cell lines.[4,11,36] These rates are much higher than those produced by human kidney cortical collecting duct (HCD) cell line.[37] As depicted in Figure 5, ocular barrier conversion is high in PHCEC (Susarla R, Wallace GR, Oswal K, et al., unpublished data, 2013), reaches a peak in corneal endothelium (HCEC-12), and starts to decrease in nonpigmented ciliary body epithelium (ODM-2), reaching the lowest levels at retinal pigmented epithelium (ARPE-19). The HSF failed to convert $25(OH)D_3$ into $1,25(OH)_2D_3$ and likewise dermal fibroblasts do not express CYP27B1, which may indicate that fibroblasts generally do not have the ability to convert $25(OH)D_3$.[38]

Ocular barrier epithelial cells appear to be efficient in producing $1,25(OH)_2D_3$ provided they have the substrate available. Despite the requirement of $25(OH)D_3$ for CYP27B1 to convert it to $1,25(OH)_2D_3$, ocular barrier cells potentially can initiate the vitamin D_3 pathway as early as the first 25-hydroxylation step via CYP27A1 and CY2R1. Extrahepatic expression of CYP27A1 and CY2R1 also has been reported previously in other tissues.[39,40] With its broad substrate specificity, CYP27A1 also is involved in cholesterol metabolism. The CYP27A1 is expressed in the retinal pigment epithelium and protects the retina from the toxic 7-ketocholesterol, a metabolite of 7-DHC as a result of photooxidation. Although CYP27A1 was expressed weakly by ocular barrier cells, the expression of microsomal CYP2R1 was considerably stronger. Moreover, the 25-hydroxylation activity of CYP2R1 is 26-fold higher than that of CYP27A1.[41] This means ocular cells may be able to synthesize vitamin D_3 directly on exposure to sunlight. However, it should be noted that the American Academy of Ophthalmology recommends protection of the eye from long hours of direct sunlight, which may contribute to cataract and cancer formation.

The expression of megalin and cubilin in ocular barrier cells indicated the importance of these receptors in vitamin D_3 metabolism. Although megalin is expressed more widely in different tissues, the expression of cubilin is more pronounced in ocular barrier cells.[10,42]

An immune privileged organ, such as the eye, requires efficient strategies that can protect it from sight-threatening infections and damaging inflammatory responses. A recent study has shown that mice injected with vitamin D_3 had reduced retinal inflammation and this was associated with a reduction of retinal macrophage activation. More importantly, these mice showed decreased levels of amyloid-beta (Aβ), a risk factor for age-related macular degeneration (AMD).[43] Decreased serum levels in humans of $25(OH)D_3$ have been correlated with increased early AMD and low visual acuity in the elderly.[44] This was investigated further by calculating UVB exposure index, which correlated with reduced neovascular AMD. Moreover, single nucleotide polymorphisms of human CYP24A1 have been shown to be linked to neovascular AMD.[31]

The ability of ocular barrier cells to locally produce large amounts of $1,25(OH)_2D_3$ may be important for their barrier function. For example, corneal endothelial cells are highly metabolic and represent an important barrier at the anterior segment of the eye, and producing large amounts of $1,25(OH)_2D_3$ may help maintain this function. More than 200 genes were significantly upregulated or downregulated in response to stimulation with $1,25(OH)_2D_3$, including several involved in the immune response.[45]

The main caveat in this work is the use of cell lines rather than primary cells. However the lines used are well-established and regarded as good correlates for freshly-isolated ocular cells in multiple experiments.

In conclusion, this study has demonstrated for the first time to our knowledge the expression of vitamin D_3 synthesizing components in ocular barrier cells, and that these cells can convert inactive $25(OH)D_3$ into active $1,25(OH)_2D_3$. Further work will show if these cells can create an environment rich in $1,25(OH)_2D_3$ and if this has an effect on ocular immune protection and immune privilege.

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