Influence of Vitamin D on Corneal Epithelial Cell Desmosomes and Hemidesmosomes

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Purpose. We have observed noticably weak epithelial attachment in vitamin D receptor knockout mice (VDR KO) undergoing epithelial debridement. We hypothesized that VDR KO negatively affects corneal epithelial cell desmosomes and/or hemidesmosomes.

Methods. Transcript levels of desmosome and hemidesmosome proteins in VDR KO corneas were assessed by qPCR. Western blotting and immunochemistry were used to detect proteins in cultured cells exposed to $1,25(OH)_2D3$ and $24R,25(OH)_2D3$.

Results. VDR KO resulted in decreased corneal desmosomal desmoglein 1 (DSG1) and desmocollin 2 (DSC2) mRNA, and hemidesmosomal plectin mRNA. DSG1 and plectin protein expression were reduced in VDR KO corneas. DSG1 protein expression increased in VDR wild types (VDR WT) and VDR KO mouse primary epithelial cells (MPCEC) treated with $1,25(OH)_2D3$ and $24R,25(OH)_2D3$. $24R,25(OH)_2D3$ treatment resulted in increased plectin and integrin β4 levels in VDR WT MPCEC, and decreased levels in VDR KO MPCEC. Treatment of human corneal epithelial cells (HCEC) with $1,25(OH)_2D3$ and $24R,25(OH)_2D3$ resulted in increased DSC2 and DSG1 protein expression. Plectin and integrin β4 were only increased in $24R,25(OH)_2D3$ treated HCEC.

Conclusions. VDR KO results in reduced desmosomal and hemidesmosomal mRNA and protein levels. $1,25(OH)_2D3$ and $24R,25(OH)_2D3$ increased DSG1 protein in all cells tested. For hemidesmosome proteins, $24R,25(OH)_2D3$ increased plectin and integrin β4 protein expression in VDR WT and HCEC, with decreased expression in VDR KO MPCEC. Thus, vitamin D3 is involved in desmosome and hemidesmosome junction formation/regulation, and their decreased expression likely contributes to the loosely adherent corneal epithelium in VDR KO mice. Our data indicate the presence of a VDR-independent pathway.

Keywords: cornea epithelium, vitamin D, hemidesmosome, desmosome

Cell-to-cell adhesion and adhesion to the basement membrane stabilizes cell morphology and position, which is critical for proper cell proliferation, differentiation, and migration.[1–3] Defects in cell adhesion give rise to a wide range of diseases, such as respiratory and urinary tract infections and pemphigus.[4,5] An understanding of cell adhesion is critical to understanding basic biological processes, such as development and wound healing.[6,7] Adherens junctions are responsible, in part, for regulating and maintaining cell adhesions. Desmosomes, comprising primarily proteins in the cadherin family, initiate and maintain cell–cell adhesion.[8,9] Hemidesmosomes, comprising integrins and other linker-type proteins, mediate cell-to-matrix adhesion, connecting the basal surface of epithelial cells to the basement membrane.[10–13]

Desmosomes are constituted in part by desmogleins (DSG) and desmocollins (DSC), which are transmembrane proteins belonging to the cadherin superfamily.[14] The human genome encodes three DSC (DSC1–DSC3) and four DSG (DSG1–DSG4) proteins. DSC2 and DSG2 are expressed widely in all desmosome-forming tissues, whereas other desmosomal cadherins are expressed specifically in stratified epithelia with graded, overlapping patterns.[7,15,16] Desmosomes are abundant in suprabasal cells of stratified squamous epithelia, such as skin and tonsil, and different cell–cell junctional complexes exist at different depths of these layers.[17] Corneal epithelium consists of superficial cells, middle wing cells, and basal cells. Desmosomes are known to be present throughout the corneal epithelial cell layers, particularly between the interdigitating cell borders of wing cells.[18]

Hemidesmosomes mediate cell-substrate and in some cases cell–cell adhesion throughout the body, including the corneal epithelium.[19] Integrins are common to hemidesmosomes, and are heterodimers comprising α and β subunits, each of which contain a large, extracellular domain responsible for ligand binding, a single transmembrane domain, and a cytoplasmic domain. A variety of integrin heterodimers are expressed in the corneal epithelium.[17,20] Hemidesmosomes, present in the basal cell layer, provide structural stability and help resist shearing forces in the corneal epithelium.[17,20,21] Plectin, another protein component present in hemidesmosomes, is an integrator molecule with the ability to interact with integrins and other diverse cytoskeletal elements.[22,23]

Vitamin D is a hydrophobic vitamin with many physiologic activities. The traditional pathway of vitamin D activation and metabolism is for the inactive precursor to be released from the skin after UV-B stimulation, followed by hepatic hydroxylation to 25 hydroxyvitamin D (25[OH]D3), the major circulating form of vitamin D. 25(OH)D3 is then hydroxylated by 1α-hydroxylase

(CYP27B1)[24] in the kidney to its active form, 1,25-dihydroxyvitamin D (1,25[OH]$_2$D3). 1,25(OH)$_2$D3 activates the specific intranuclear, vitamin D receptor (VDR) in target cells.[25] Membrane-associated protein disulfide isomerase family A member 3 (Pdia3) has been shown to be a secondary receptor for 1,25(OH)$_2$D3, with each receptor separately activating its own downstream mediators.[26,27] 25(OH)D3 also can be hydroxylated by 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) to produce 24R,25-dihydroxyvitamin D3 (24R,25[OH]$_2$D3). In addition to this traditional vitamin D metabolic pathway, vitamin D activation has also been demonstrated at the tissue and cell level. Our lab demonstrated the presence of the vitamin D metabolic enzymes in corneal epithelial cells as well as the ability of the corneal epithelium to activate vitamin D.[28–30]

Vitamin D has been demonstrated to regulate a wide range of physiologic and pathologic processes, including cell growth, migration, immune response modulation, and differentiation.[29,31,32] Our previous work demonstrated that 1,25(OH)$_2$D3 influences the function of corneal epithelial cell gap junctions and tight junctions.[33,34] In addition, we have observed weak epithelial attachment in vitamin D receptor knockout mice (VDR KO) undergoing epithelial debridement for wound healing studies (unpublished observation). Only a few studies have linked vitamin D with adhesion molecule regulation. Microarray data demonstrated that VDR deletion decreased epithelial adherens junction signaling in epidermis, and E-cadherin (CDH1) and DSG1 were decreased in the epidermis of VDR knockout mice and in a VDR/calcium-sensing receptor double knockout mouse.[31,35] Conversely, vascular cell adhesion molecule-1 was found to be significantly abolished after incubation of human cardiac endothelial cells (ECs) with 1,25(OH)$_2$D3.[36]

We have observed in previous and ongoing corneal epithelail wound healing studies that the corneal epithelium is noticiably easier to remove from VDR KO mice than from VRD WT mice (unpublished observation). Based on this observation, we hypothesize that vitamin D regulates adherens junctions in the corneal epithelium and that VDR KO negatively affects corneal epithelial cell adherens junctions.

Methods

Materials

1,25(OH)$_2$D3 and 24R,25(OH)$_2$D3 were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Antibodies for DSG1, DSC2, and integrin β4 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Plectin and integrin α6 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Prestained protein markers were obtained from Bio-Rad (Hercules, CA, USA). Polyvinylidene difluoride (PVDF) membrane and the enhanced chemiluminescence (ECL) detection system were obtained from Bio-Rad.

Human Corneal Epithelial Cell Line

The immortalized human corneal epithelial cell line (HCEC) has been previously described.[29,30,37] While standard short-tandem repeat (STR)-based validation of this line is not possible due to lack of availability of the original source cells, we do routinely check the cells for the presence of keratin K12 and keratin K3 mRNA by PCR. All cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with fetal bovine serum (3%), 1% insulin-transferrin-selinium (ITS; BD Biosciences, Bedford, MA, USA), and 40 μg/mL gentamicin (Life Technologies, Grand Island, NY, USA). Cells were subpassaged using trypsin (Sigma, Ann Arbor, MI, USA) digestion, seeded in 35-mm dishes (Fisher Scientific, Breinigsville, PA, USA), and cultured in a humidified incubator at 37°C with 5% CO_2. Culture medium was replaced every 2 days. HCEC and all cultured cells in this study were found to be mycoplasma negative (Mycoplasma Detection Kit; R&D Systems Inc, Minneapolis, MN, USA)

Mouse Primary Corneal Epithelial Cells (MPCEC)

VDR WT and VDR KO mice were obtained and bred from the Jackson Labs (Strain: B6.129S4-Vdr^{tm1Mbd}; Bar Harbor, ME, USA). All animal studies were approved by the University Institutional Animal Care and Use Committee, and animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Primary corneal epithelial cell cultures were established using a modification of the established explant culture method.[38,39] Briefly, eyes were enucleated, and the cornea was washed with Ca^{++}-free PBS (pH 7.2). Each cornea was cut in half and placed in a 35-mm dish (Fisher Scientific) with the epithelial side up. One and half milliliters of DMEM with 10% serum containing 40 μg/mL gentamicin, 1% ITS, and 100 ng/mL cholera toxin (LIST Biological Laboratories, Inc., Campbell, CA, USA) was added and the tissue was cultured in a humidified incubator at 37°C with 5% CO2. Culture medium was replaced every 2 days. Cells were passged using 0.25% trypsin (Fisher Scientific), and subculturing in DMEM with 3% serum containing 40 μg/mL gentamicin, 1% ITS, and 100 ng/mL cholera toxin.

Real-Time PCR

Total RNA was obtained from mouse epithelial cells. Real-time PCR was used to quantify DSC2, DSG1, integrin α6, integrin β4, and plectin mRNA levels. RT-PCR primers were generated from the Primer Bank database[40,41] using National Center for Biotechnology Information (NCBI) sequence identification numbers (NM_013505, NM_181682, NM_001079818, NM_133663, NP_035247 for mouse DSC2, DSG1, integrin α6, integrin β4, and plectin respectively). The primers for mouse TATA box binding protein (TBP; NM_013684) were generated from the Universal Probe Library of Roche Life Sciences. Primers are listed in Table 1.

mRNA was isolated and cDNA was synthesized using the Bio-Rad RT-PCR system. First-strand synthesis was done at 42°C for 60 minutes, and inactivated at 85°C for 5 minutes. Equal amounts of cDNA were applied for PCR amplification in triplicate using the Bio-Rad system and SYBR probes. Amplification was performed at 95°C for 10 minutes, followed by 40 cycles at 95°C for 10 seconds and 62°C for 30 seconds. Quantitative values were obtained from the quantification cycle value (C_q), which is the point where a significant increase of fluorescence is first detected. TBP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal RNA controls, and each sample was normalized on the basis of its gene content (ΔC_q). The formula $2^{-(\Delta\Delta Cq)}$ was used to analyze the results.

Transmission Electron Microscopy

Ten-week-old VDR WT and VDR KO mice were used for transmission electron microscopy (TEM; JOEL 2000; JOEL Ltd., Tokyo, Japan). Eyes were enucleated from euthanized mice and fixed using 4% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M cacodylate. Fixed eyes were embedded into resin blocks for sectioning. Images (×15,000) were taken of the epithelium and Bowman's membrane to identify hemidesmosomes.

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Table 1. Summary of the Suggested Primers Pair Sets

Gene	Direction	Primer Sequence	Amplicon Size	Temperature
Mus musculus Desmocollin 2	Forward	5′-ATGGCGGCTGTGGGATCTAT-3′	77	62°C
	Reverse	5′-GCAAGGATCGCAAGGGTCAA-3′		
Mus musculus Desmoglein 1	Forward	5′-GCAGTGGTGGTAATCGTGACC-3′	142	62°C
	Reverse	5′-GGATTTTGCCTACCGGGAGTG-3′		
Mus musculus Integrin α6	Forward	5′-TGCAGAGGGCGAACAGAAC-3′	175	62°C
	Reverse	5′-GCACACGTCACCACTTTGC-3′		
Mus musculus Integrin β4	Forward	5′-ACTCCATGTCTGACGATCTGG-3′	124	62°C
	Reverse	5′-GGGACGCTGACTTTGTCCAC-3′		
Mus musculus Plectin	Forward	5′-GCGGAGGAACAGTTGCAGAA-3′	139	62°C
	Reverse	5′-GCCCCTTGTACTCATTCAGTTG-3′		
Mus musculus TBP	Forward	5′-GGCGGTTTGGCTAGGTTT-3′	83	62°C
	Reverse	5′-GGGTTATCTTCACACACCATGA-3′		

Immunofluorescence Studies

Eyes were enucleated from euthanized mice and prepared for cryosectioning. Eyes were flash-frozen in liquid nitrogen and embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek, Torrance, CA, USA). Ten-micrometer thick cryosections were fixed for 10 minutes in 4% paraformaldehyde (4% PFA) and blocked with 10% goat serum in 0.1% Triton X-100/PBS for 1 hour at room temperature. Cryosections were incubated with primary antibodies (anti-DSC2, anti-DSG1, anti-integrin α6, anti-integrin β4, and anti-plectin) followed by incubation with secondary antibodies. Cryosections were examined using a Zeiss LSM 780 upright laser-scanning confocal microscope (Carl Zeiss Microscopy, White Plains, NY, USA).

Protein Extraction and Western Blot Analysis

Protein was isolated from confluent HCEC, VDR WT, and VDR KO MPCEC cells grown on 35-mm dishes, and from freshly isolated epithelial cells scraped from the corneas of three mice using a Gill corneal knife (Bausch + Lomb Storz Ophthalmic Instruments, Rochester, NY, USA). After washing cells with PBS, they were exposed to lysis buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 0.02% N3Na, 100 μg/mL phenylmethylsulfonyl fluoride, 1% NP-40, 50 mM NaF, 2 mM EDTA, and protease inhibitor; Sigma). Cell lysates were collected and Western blotting was performed as previously described.[30] Blots were labeled with DSG1, DSC2, integrin β4, integrin α6, and plectin antibodies at a dilution of 1:1000. Membranes were washed and then incubated with anti–rabbit horseradish peroxidase-conjugated secondary antibody (1:3000). Detection was performed using the enhanced chemiluminescence method (Pierce Biotechnology, Rockford, IL, USA).

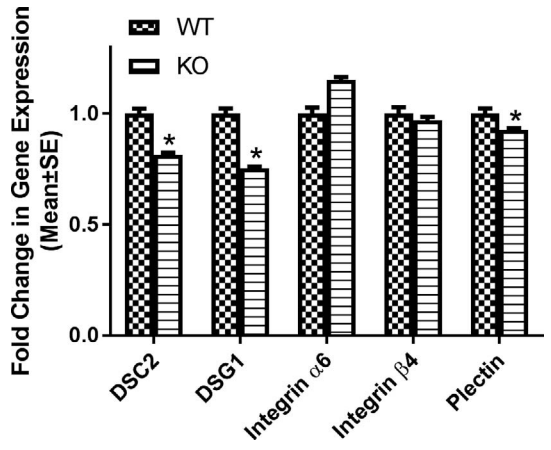

Figure 1. DSC2, DSG1, and plectin mRNA levels were significantly decreased in VDR KO mouse corneal epithelium. Integrin subunit α6 and β4 mRNA levels were not changed (*$P < 0.05$, $n = 3$).

Corneal Epithelial Cells Treated With Vitamin D

$1,25(OH)_2D3$ and $24R,25(OH)_2D3$ were dissolved in dimethyl sulfoxide (DMSO). Corneal epithelial cells were treated with $1,25(OH)_2D3$ (10 nM) or $24R,25(OH)_2D3$ (100 nM) for 24 hours. Control groups were treated only with DMSO.

Statistical Analysis

All data are provided as the mean ± SE of at least three experiments. Data was analyzed using the unpaired Student's *t*-test only comparing experimental groups against controls. $P < 0.05$ was considered statistically significant.

Results

Effects of VDR KO on Desmosome and Hemidesmosome mRNA Expression

Corneal epithelial cells from VDR WT and KO mice were collected, and transcript levels of desmosome and hemidesmosome proteins were assessed by qPCR. Figure 1 demonstrates that mRNA levels of the desmosome proteins DSG1 and DSC2 were significantly reduced in VDR KO versus WT mice ($P < 0.05$). In addition, the mRNA level of the hemidesmosome crosslinker protein plectin was significantly reduced in VDR KO mouse corneal epithelium ($P < 0.05$). There were no significant differences in the mRNA levels of the hemidesmosome proteins integrin α6 or integrin β4. Table 2 summarizes these results as well as all results from this study.

Transmission Electron Microscopy

TEM images showed decreased hemidesmosomes in VDR KO mouse corneal basal epithelium and Bowman's membrane (Fig. 2). Desmosomes were not examined due to the difficulty in positively identifying them compared to other lateral membrane junction subtypes.

Effects of VDR Knockout on Desmosome Protein, DSG1, and DSC2 in the Mouse Cornea

We examined the expression of desmosomal molecules DSG1 and DSC2 in VDR WT and KO mouse corneas using

Table 2. Effects of $1,25(OH)_2D3$ and $24R,25(OH)_2D3$ Treatment on Desmosomal/Hemidesmosomal Components Compared With Untreated Cells

Cell and VitD3 Junction Proteins	VDR WT MPCEC		VDR KO MPCEC		HCEC	
	$1,25(OH)_2D3$	$24R,25(OH)_2D3$	$1,25(OH)_2D3$	$24R,25(OH)_2D3$	$1,25(OH)_2D3$	$24R,25(OH)_2D3$
DSG1	+	+	+	+	+	+
Integrin β4	NC	+	−	−	NC	+
Plectin	NC	+	NC	−	NC	+
DSC2					+	+
Integrin α6					NC	NC

+, significantly increased; −, significantly decreased; NC, no change.

immunostaining and Western blotting. Expression of DSG1 and DSC2 was clearly detectable throughout all epithelial layers (Figs. 3a, 3d). Figure 3a shows that DSG1 in VDR KO mice was decreased in corneal wing and basal epithelial cells. DSG1 protein labelling and expression was decreased in VDR KO mouse cornea epithelium compared with WT corneas (Figs. 3b, 3c). DSC2 protein expression was not affected by VDR KO (Figs. 3e, 3f).

Effects of VDR Knockout on Hemidesmosome Protein Plectin, Integrin β4, and Integrin α6 in the Mouse Cornea

Plectin (Fig. 4a), integrin β4 (Fig. 4d), and integrin α6 (Fig. 4e) expression were, as expected for hemidesmosome proteins, confined primarily to the basal cell/Bowman's membrane interface. Plectin protein labelling and expression were decreased in VDR KO mouse cornea epithelium compared with WT corneas (Figs. 4a–c). There were no significant differences in integrin α6, β4 protein expression in VDR KO versus WT mice (Western data shown in Supplementary data Fig. S1).

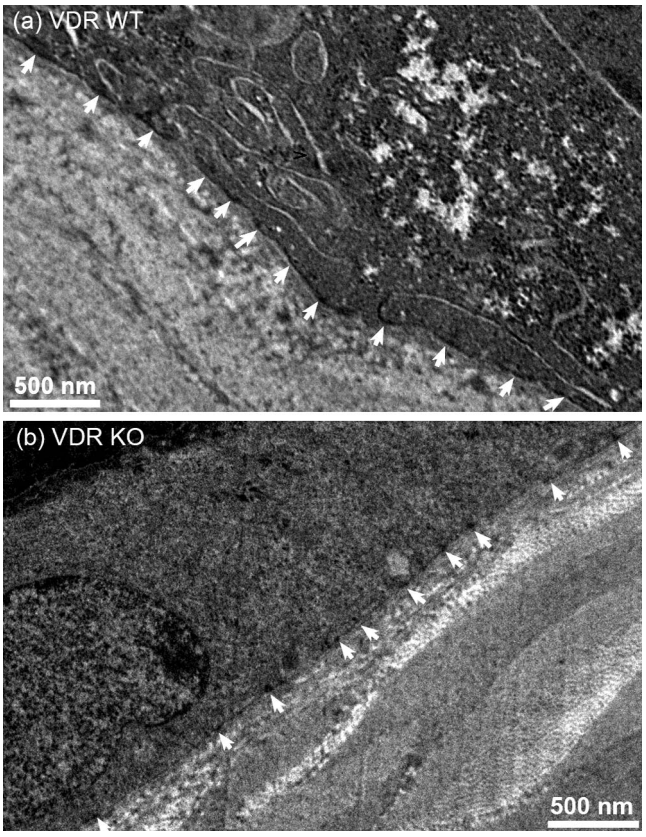

Figure 2. Representative TEM micrographs of control and VDR KO mouse basal epithelium. VDR WT corneas (**a**) had well-defined, organized hemidesmosomes (*arrows*) on the basal surface of corneal basal epithelial cells. In contrast, hemidesmosomes in VDR KO corneas (**b**) were smaller and fewer in number.

Effects of Vitamin D3 on DSG1 Protein Expression in VDR WT and VDR KO MPCEC

DSG1 protein expression was significantly increased in VDR WT MPCEC treated with $1,25(OH)_2D3$ and $24R,25(OH)_2D3$ ($P < 0.05$; Figs. 5a, 5b). DSG1 protein expression was also

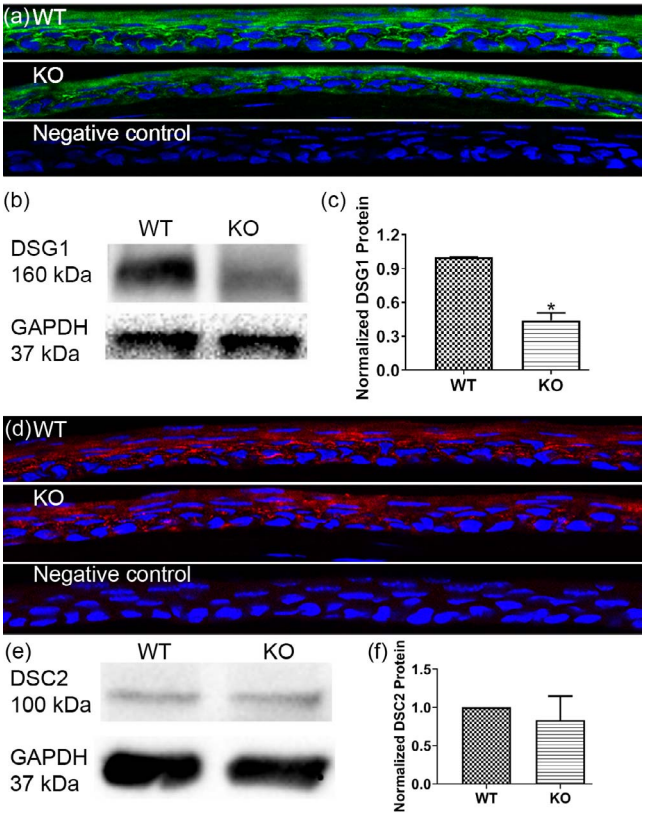

Figure 3. Effect of VDR KO on desmosomal DSG1 and DSC2 localization and protein expression. Representative DSG1 (**a**) and DSC2 (**d**) immunostaining in WT and KO mouse corneal epithelium (DSG1, *green*; DSC2, *red*; nuclear dapi staining, *blue*) demonstrate descreased DSG1 protein expression in the wing cells. Western blot data (**b**, **c**) from mouse corneal tissue confirms this result (*t*-test, $\bar{x} \pm$ SE, $^*P < 0.05$, $n = 3$). DSC2 expression was not affected. Uncropped blots and PVDF membrane images shown in Supplementary Figures S2 and S3.

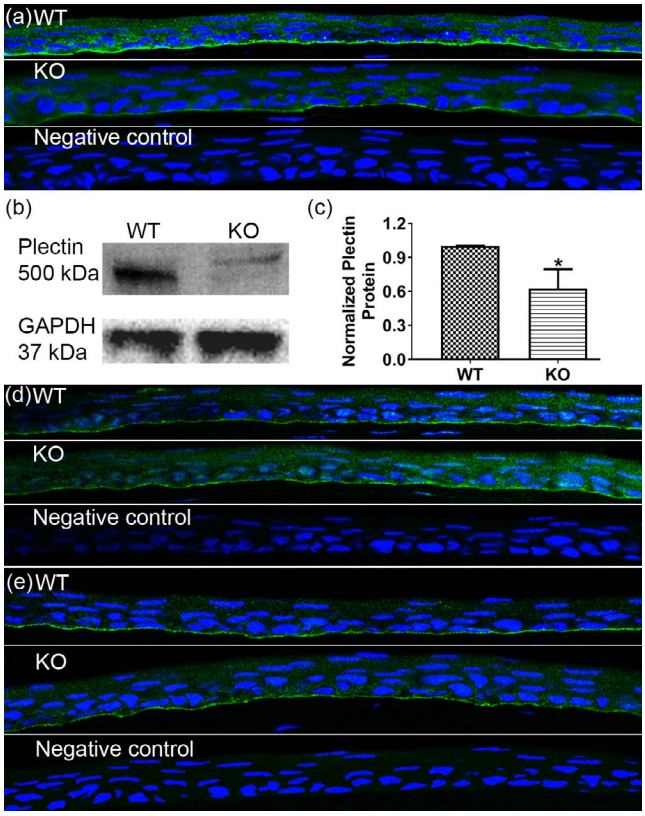

Figure 4. Effect of VDR KO on hemidesmosomal plectin, integrin β4, and integrin α6 localization and protein expression. Representative plectin (**a**), integrin β4 (**d**), and integrin α6 (**e**) immunostaining in WT and KO mouse corneal epithelium (plectin, integrin β4, and integrin α6, *green*; nuclear dapi staining, *blue*) demonstrate descreased plectin protein expression in the basal cells. Western blot data (**b**, **c**) from mouse corneal tissue confirms this result (*t*-test, $\bar{x} \pm$ SE, $^*P < 0.05$, $n = 3$). Uncropped blot shown in Supplementary Figure S4.

increased in VDR KO MPCEC treated with 1,25$(OH)_2$D3 and 24R,25$(OH)_2$D3 (Figs. 5c, 5d).

Effects of Vitamin D3 on Hemidesmosome Protein Plectins and Integrin β4 in VDR WT and VDR KO MPCEC

Plectin and integrin β4 protein expression levels were significantly increased in VDR WT MPCEC treated with 24R,25$(OH)_2$D3 ($P < 0.05$; Figs. 6a, 6b, 7a, 7b). There were no significant changes in plectin or integrin β4 protein expression in VDR WT MPCEC treated with 1,25$(OH)_2$D3. However, plectin expression levels were significantly reduced in VDR KO MPCEC treated with 24R,25$(OH)_2$D3 ($P < 0.05$), with no change after 1,25$(OH)_2$D3 treatment (Figs. 6c, 6d). Integrin β4 protein expression was significantly reduced ($P < 0.05$) in VDR KO MPCEC treated with 1,25$(OH)_2$D3 and 24R,25$(OH)_2$D3 (Figs. 7c, 7d).

Effects of Vitamin D3 on Adhension Protein Expression in HCEC

Expression of the desmosome proteins DSG1 (Figs. 8a, 8b) and DSC2 (Figs. 8c, 8d) was significantly increased ($P < 0.05$) in HCEC cultured with 1,25$(OH)_2$D3 and 24R,25$(OH)_2$D3. There was no significant increase in expression of the hemidesmosome proteins plectin (Fig. 9a) or integrin β4 (Fig. 9b) in HCEC cultured with 1,25$(OH)_2$D3. However, plectin and integrin β4 expression was increased in HCEC treated with 24R,25$(OH)_2$D3 ($P < 0.05$). Integrin α6 protein expression was unchanged in HCEC treated with 1,25$(OH)_2$D3 or 24R,25$(OH)_2$D3 (Fig. 9c).

Discussion

This study is the first in any tissue type to specifically examine the influence of vitamin D on desmosomes and in hemidesmosomes in a static epithelium. We initiated the study following our observation when performing corneal epithelial wound healing experiements, that the epithelium was much easier to remove from VDR KO mice than from WT mice. Proper expression of cell adhesion components is essential for corneal epithelial structural integrity.[42] Specific desmosome proteins expressed in corneas have been shown to be species specific. In bovine cornea, the desmosome proteins DSC2 and DSG2 were detected in corneal epithelium, while DSC1, DSC3, DSG1, and DSG3 were absent.[43] In rat epithelium, DSG1 and DSG2 were detected.[44] In human corneal epithelial cells, DSG1[42] and DSG3[45] were found. Meanwhile, in mice, DSG1, DSG2, DSC1, DSC2, and DSC3 were all detected in the corneal epithelium.[42,46] For hemidesmosome proteins, a variety of integrin heterodimers are expressed in the corneal epithelium,[17,20] many of which have been linked to the adherence of epithelial cells to the stroma.[47–49] Integrin subunits α6 and β4 were first detected in mouse and rabbit corneal epithelium.[50,51] Integrin subunits β1, β4, β5, α2, α3, α5, α6, and αV were detected in rat corneal epithelium.[20] Integrin subunits α3, α6, β1, and β4 were detected in human corneal epithelium.[52,53] In previous studies, plectin was found to be enriched in corneal (and skin) basal epithelial cells.[54] Plectin was found to interact with integrin α6/β4 in migrating epithelial cells[55] and with integrin α5/β6 to promote corneal wound healing.[56,57] In the current study, we focused on the desmosome proteins DSG1 and DSC2, and hemidesmosome proteins plectin and integrin subunits α6 and β4.

In previous studies, vitamin D deficiency was found to be associated with both poor wound healing and cell junction abnormalities.[58,59] Our lab found that 10-week-old VDR KO mouse corneas showed decreased occludin and ZO-1 expression compared with WT, along with a decreased epithelial wound healing rate.[33] Studies by Oda et al.[31,35] have found that VDR ablation reduces DSG1 expression in the leading edge of healing cutaneous wounds. VDR KO mice have also recently been shown to have decreased lung expression of ZO-1, occludin, and the adherens junction protein VE-cadherin.[60]

The current study found decreased desmosome formation as observed using TEM in VDR KO mice. DSG1 mRNA and protein expression were significantly reduced in the VDR KO mouse corneal epithelium, and immunohistochemistry revealed reduced DSG1 in the basal epithelial cells of these mice. For hemidesmosomes, we found that plectin mRNA and protein expression were significantly decreased in VDR KO mouse corneal epithelium, while integrin levels were not affected. We conclude that VDR KO attenuates cell–cell adhesion and cell–matrix interactions in cornea not only through the reduction of the desmosomal protein DSG1, but also by inducing abnormal hemidesmosomal function through the reduction of plectin expression.

Oral vitamin D supplementation was found to improve wound healing of patients with diabetic foot ulcers compared with placebo.[61] Vitamin D and calcium signaling in keratinocytes have been found to be required for the normal regenerative response of the skin to wounding.[31,35] A previous study from our laboratory determined that 24R,25$(OH)_2$D3

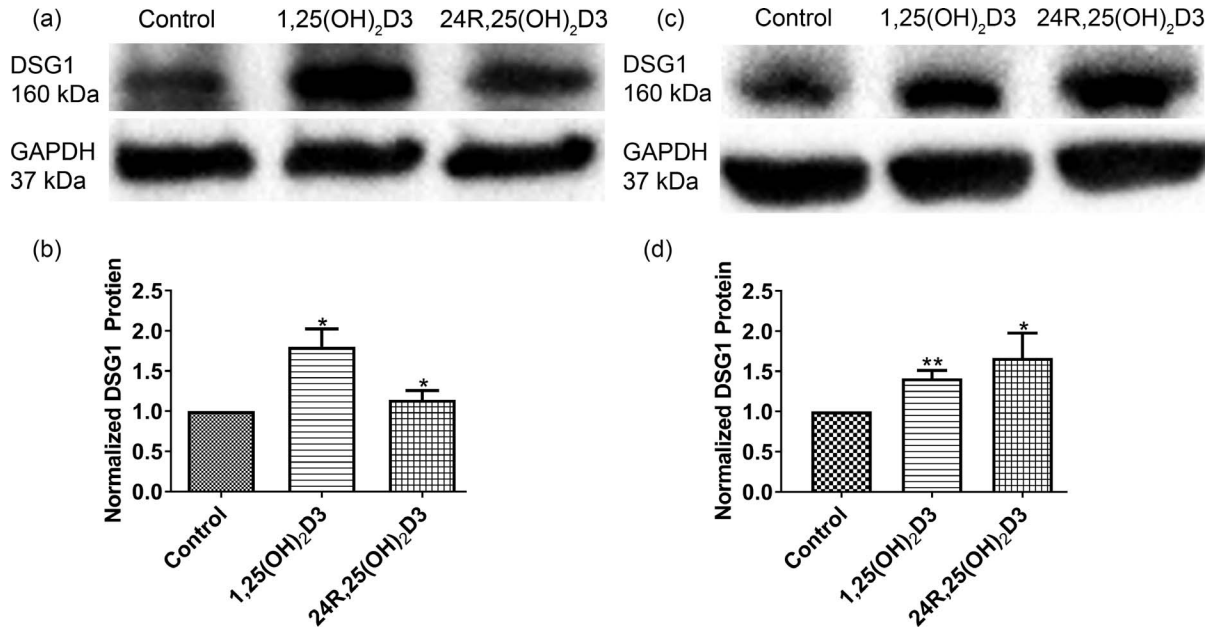

Figure 5. Desmosomal molecular DSG1 protein expression in WT and VDR KO MPCEC treated with $1,25(OH)_2D3$ and $24R,25(OH)_2D3$. Representative Western blot (**a**) and blot densities (**b**) demonstrating increased DSG1 protein expression in VDR WT MPCEC treated with $1,25(OH)_2D3$ and $24R,25(OH)_2D3$ (*t*-test, $\bar{x} \pm$ SE, $^*P < 0.05$, $n = 3$). DSG1 protein expression (**c**, **d**) was also increased in VDR KO MPCEC treated with $1,25(OH)_2D3$ and $24R,25(OH)_2D3$ (*t*-test, $\bar{x} \pm$ SE, $^*P < 0.05$, $n = 4$). Uncropped blots and PVDF membrane images shown in Supplementary Figures S5 to S6.

increases corneal epithelial cell migration and proliferation.[29] Moreover, it has been demonstrated that the remodeling of desmosomes is critical for cell proliferation, differentiation, migration.[17,62] In the current study, we have determined that both $1,25(OH)_2D3$ and $24R,25(OH)_2D3$ increase DSG1 protein levels in mouse and human corneal epithelium. DSC2 protein levels were also increased in human corneal epithelium treated with $1,25(OH)_2D3$ and $24R,25(OH)_2D3$. We also determined that $24R,25(OH)_2D3$ increased the expression of the hemidesmosomal proteins integrin β4 and plectin in mouse and human corneal epithelium. These results, along with our initial observations that VDR KO mice have easily removable epithelium, indicate that vitamin D plays a role in adhesion junction formation and maintenance in the cornea.

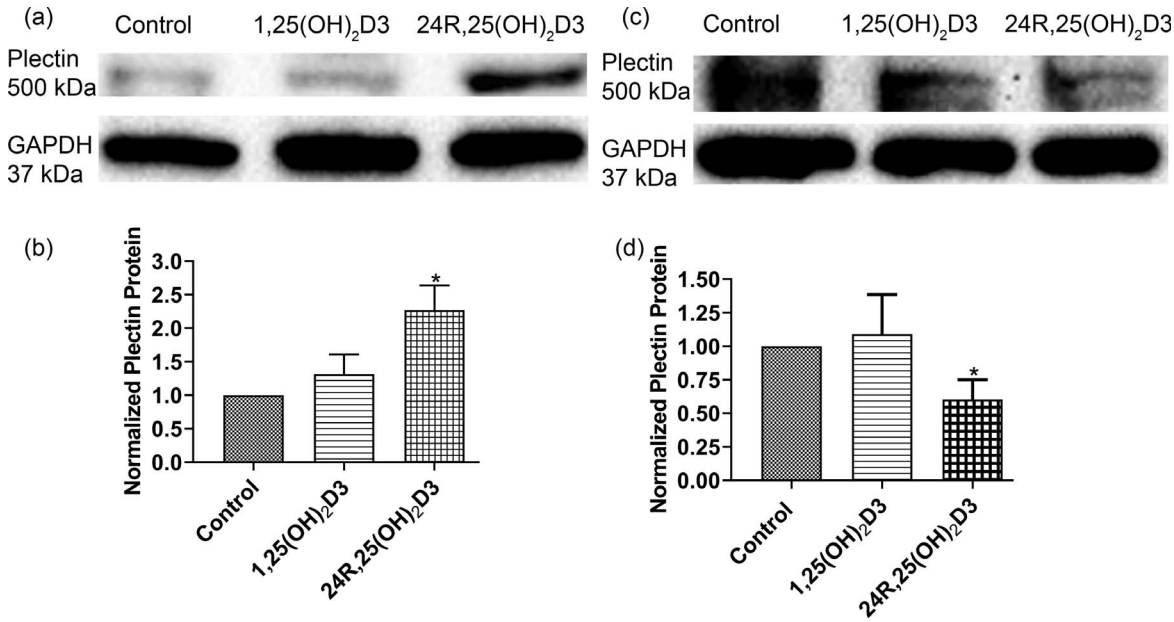

Figure 6. Hemidesmosomal plectin protein expression in WT and VDR KO MPCEC treated with $1,25(OH)_2D3$ and $24R,25(OH)_2D3$. Representative VDR WT MPCEC Western blot (**a**) and blot densities (**b**) demonstrating increased plectin protein expression in cells treated with $24R,25(OH)_2D3$ (*t*-test, $\bar{x} \pm$ SE, $^*P < 0.05$, $n = 5$). Plectin protein expression in VDR KO MPCEC (**c**, **d**) was decreased following treatment with $24R,25(OH)_2D3$ (*t*-test, $\bar{x} \pm$ SE, $^*P < 0.05$, $n = 3$). There was no change in plectin protein expression in WT or VDR KO MPCEC treated with $1,25(OH)_2D3$. Uncropped blots and PVDF membrane images shown in Supplementary Figures S7 to S8.

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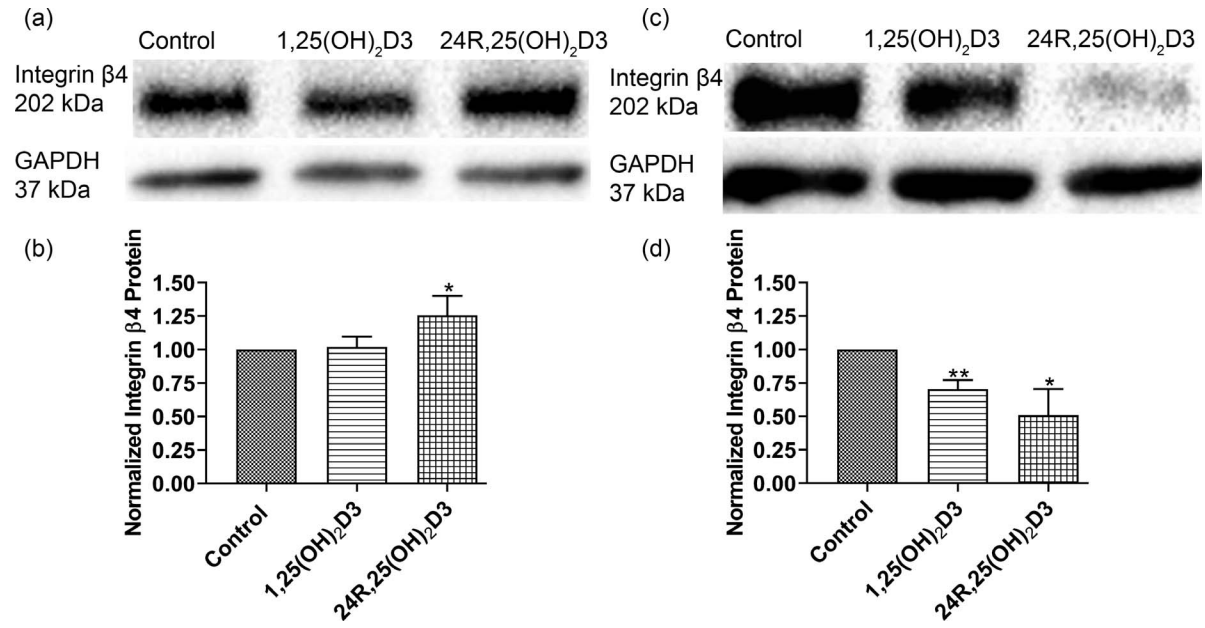

Figure 7. Hemidesmosomal integrin β4 protein expression in WT and VDR KO MPCEC treated with $1,25(OH)_2D3$ and $24R,25(OH)_2D3$. Representative VDR WT MPCEC Western blot (**a**) and blot densities (**b**) demonstrating increased integrin β4 protein expression in cells treated with $24R,25(OH)_2D3$ (*t*-test, $\bar{x} \pm$ SE, $^*P < 0.05$, $n = 5$). VDR KO MPCEC integrin β4 protein expression (**c**, **d**) was decreased in cells treated with $1,25(OH)_2D3$ and $24R,25(OH)_2D3$ (*t*-test, $\bar{x} \pm$ SE, $^*P < 0.05$, $n = 4$). There was no change in integrin β4 protein expression in WT MPCEC treated with $1,25(OH)_2D3$. Uncropped blots and PVDF membrane images shown in Supplementary Figure S9.

It is generally believed that $24,25(OH)_2D3$ is an inactive form of vitamin D3. Previous work from our laboratory demonstrated that $24R,25(OH)_2D3$ stimulates both HCEC cell proliferation and migration, which are crucial for corneal epithelial wound healing.[29] In the current study, $24R,25(OH)_2D3$ was found to increase DSG1, integrin β4, and plectin protein levels in HCEC and VDR WT MPCEC. Thus, it is apparent that $24R,25(OH)_2D3$ is active in and beneficial to the anterior segment of the eye.

We found that the desmosomal protein DSG1 was significantly elevated in VDR KO MPCEC treated with $1,25(OH)_2D3$. Moreover, $24R,25(OH)_2D3$ was found to increase DSG1 and decrease integrin β4 and plectin levels in VDR KO MPCEC. There was no change of integrin β4 or plectin expression in mouse or human corneal epithelium, but the integrin β4 protein level was decreased in VDR KO MPCEC treated with $1,25(OH)_2D3$. In our previous study, $1,25(OH)_2D3$ and $24R,25(OH)_2D3$ were both found to promote CYP24A1 and CYP27B1 protein expression in VDR KO MPCEC.[29] Our data indicate that both VDR and VDR-independent pathways are involved in the regulation of $1,25(OH)_2D3$- and

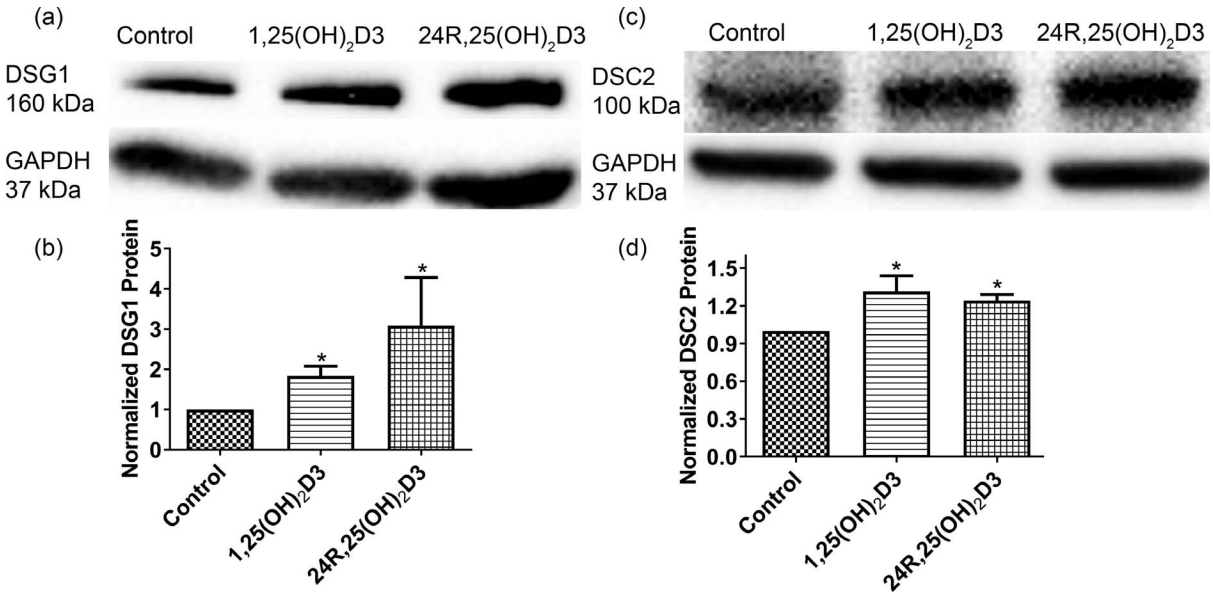

Figure 8. Desmosomal DSG1 and DSC2 protein expression in HCEC treated with $1,25(OH)_2D3$ and $24R,25(OH)_2D3$. Representative Western blots and blot densities demonstrating increased DSG1 (**a**, **b**) and DSC2 (**c**, **d**) protein expression in HCEC treated with $1,25(OH)_2D3$ and $24R,25(OH)_2D3$ (*t*-test, $\bar{x} \pm$ SE, $^*P < 0.05$, $n = 3$). Uncropped blots shown in Supplementary Figure S10.

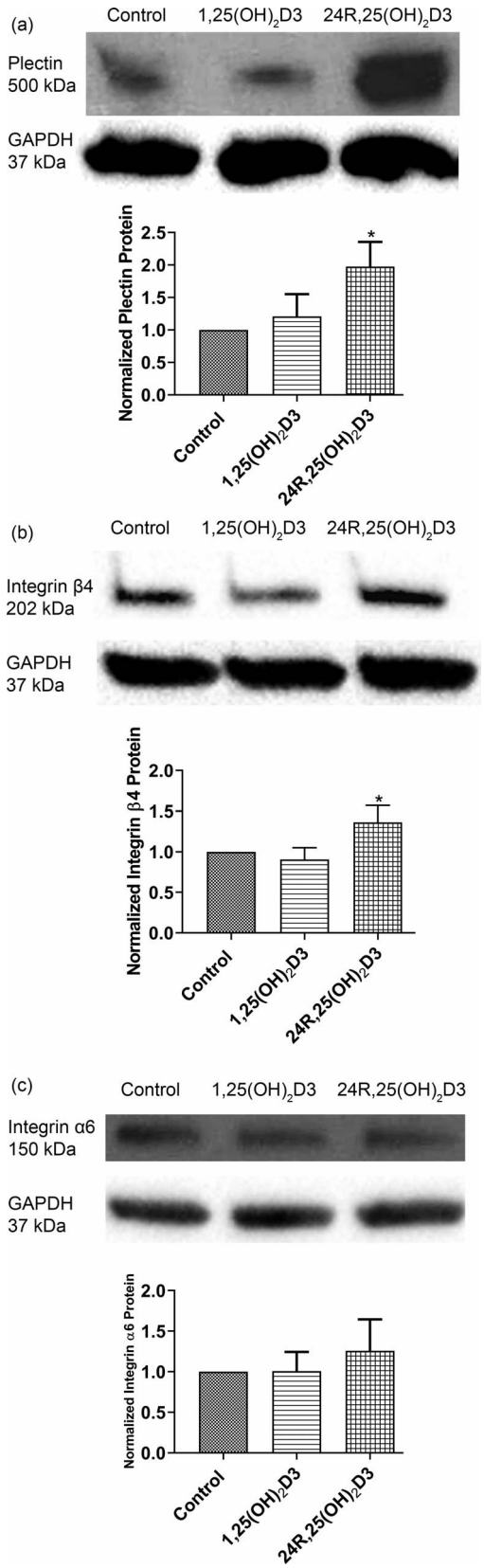

Figure 9. Hemidesmosomal plectin, integrin β4, and integrin α6 protein expression in HCEC treated with $1,25(OH)_2D3$ and $24R,25(OH)_2D3$. Representative Western blots and blot densities demonstrating increased plectin (**a**) and integrin β4 (**b**) protein expression in HCEC treated with $24R,25(OH)_2D3$. There was no change in plectin or integrin β4 protein expression in HCEC treated with $1,25(OH)_2D3$ and no change in integrin α6 (**c**) protein expression in HCEC treated with $1,25(OH)_2D3$ or $24R,25(OH)_2D3$ (*t*-test, $\bar{x} \pm$ SE, $^*P < 0.05$, $n = 6$). Uncropped blots shown in Supplementary Figure S11.

$24R,25(OH)_2D3$-induced corneal epithelial desmosomal and hemidesmosomal protein expression, along with the regulation of the expression of additional corneal proteins.

In conclusion, adherens junction proteins are affected by VDR KO. Poorly formed desmosomes and hemidesmosomes likely contribute to the easily removed corneal epithelium in VDR KO mice. Both $1,25(OH)_2D3$ and $24R,25(OH)_2D3$ appear to be involved in desmosome/hemidesmosome formation/regulation. Moreover, because both $1,25(OH)_2D3$ and $24R,25(OH)_2D3$ affect adherens protein expression levels in VDR KO mouse corneal epithelium, it is likely that vitamin D signaling related to adherens junctions is occuring through both VDR-dependent and -independent pathways. The results from this study indicate that severe vitamin D deficiency may lead to adherens junction alterations in the cornea. While patients with diseases linked to vitamin D deficiency (e.g., rickets, osteomalacia) do not typically present with primary ophthalmic defects linked to their disease state, these adherens alterations could lead to exaserbation of otherwise unassociated ophthalimc pathologies, such as recurrent errosions, diabetic keratopathy, or surgical complications.

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