Influence of Vitamin D on Corneal Epithelial Cell Desmosomes and Hemidesmosomes

Xiaowen Lu\textsuperscript{1} and Mitchell A. Watsky\textsuperscript{1,2}

\textsuperscript{1}Department of Cellular Biology and Anatomy, Medical College of Georgia, Augusta University, Augusta, Georgia, United States
\textsuperscript{2}The Graduate School, Augusta University, Augusta, Georgia, United States

Correspondence: Mitchell A. Watsky, Department of Cell Biology & Anatomy, Medical College of Georgia, Augusta University, 1120 15th Street, C2-2201, Augusta, GA 30912, USA; mwatsky@augusta.edu.

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Cell-to-cell adhesion and adhesion to the basement membrane stabilize cell morphology and position, which is critical for proper cell proliferation, differentiation, and migration.\textsuperscript{1–3} Defects in cell adhesion give rise to a wide range of diseases, such as respiratory and urinary tract infections and pemphigus.\textsuperscript{4,5} An understanding of cell adhesion is critical to understanding basic biological processes, such as development and wound healing.\textsuperscript{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.\textsuperscript{8,9} Hemidesmosomes, comprising integrins and other linkertype proteins, mediate cell-to-matrix adhesion, connecting the basal surface of epithelial cells to the basement membrane.\textsuperscript{10–15}

Desmosomes are constituted in part by desmogleins (DSG) and desmocollins (DSC), which are transmembrane proteins belonging to the cadherin superfamily.\textsuperscript{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.\textsuperscript{2,15,16} 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.\textsuperscript{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.\textsuperscript{18}

Hemidesmosomes mediate cell-substrate and in some cases cell-cell adhesion throughout the body, including the corneal epithelium.\textsuperscript{19} Integrins are common to hemidesmosomes, and are heterodimers comprising $\alpha$ and $\beta$ 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.\textsuperscript{17,20} Hemidesmosomes, present in the basal cell layer, provide structural stability and help resist shearing forces in the corneal epithelium.\textsuperscript{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.\textsuperscript{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]D$_3$), the major circulating form of vitamin D. 25(OH)D$_3$ is then hydroxylated by 1$\alpha$-hydroxylase
Vitamin D on Corneal Epithelium Desmosomes and Hemidesmosomes

**METHODS**

**Materials**

1,25(OH)2D3 and 24,25(OH)2D3 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 from Bio-Rad (Hercules, CA, USA). Polyvinylidene difluoride (Danvers, MA, USA). Prestained protein markers were obtained from Biotechnology, Inc. (Santa Cruz, CA, USA). Plectin and integrin α6 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

**Human Corneal Epithelial Cell Line**

The immortalized human corneal epithelial cell line (HCEC) has been previously described. 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-selenium (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% CO2. 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<sup>+/mtdb</sup>; 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. Briefly, eyes were enucleated, and the cornea was washed with Ca<sup>2+</sup>-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 cholaer 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 passaged using 0.25% trypsin (Fisher Scientific), and subculturing in DMEM with 3% serum containing 40 μg/mL gentamicin, 1% ITS, and 100 ng/mL cholaer 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<sup>10,47</sup> using National Center for Biotechnology Information (NCBI) sequence identification numbers (NM_013505, NM_181682, NM_0010797818, NM_135663, 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 (Cq), 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 (ΔCq). The formula 2<sup>−ΔΔCq</sup> 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.
**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% NaN3, 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.50 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).

**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...

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**Table 1. Summary of the Suggested Primers Pair Sets**

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<th>Temperature</th>
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<tr>
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<td>Forward</td>
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<tr>
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<td>139</td>
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<tr>
<td>Mus musculus Plectin</td>
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<td>83</td>
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<tr>
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<td>Forward</td>
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</tr>
</tbody>
</table>

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**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).
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).

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

| 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 | VDR WT MPCEC | VDR KO MPCEC | HCEC |
| Junction Proteins | 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 | NC | NC | + |
| Plectin | NC | + | NC | NC | NC | NC |
| DSC2 | + | + | + | + | + | + |
| Integrin α6 | NC | NC | NC | NC | NC | NC |

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

**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.

**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 decreased DSG1 protein expression in the wing cells. Western blot data (b, c) from mouse corneal tissue confirms this result (t-test, x ± SE, *P < 0.05, n = 3). DSC2 expression was not affected. Uncropped blots and PVDF membrane images shown in Supplementary Figures S2 and S3.
Effects of Vitamin D3 on Hemidesmosome Protein Plectin 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)2D3 (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)2D3. However, plectin expression levels were significantly reduced in VDR KO MPCEC treated with 24R,25(OH)2D3 (P < 0.05), with no change after 1,25(OH)2D3 treatment (Figs. 6c, 6d). Integrin β4 protein expression was significantly reduced (P < 0.05) in VDR KO MPCEC treated with 1,25(OH)2D3 and 24R,25(OH)2D3 (Figs. 7c, 7d).

Effects of Vitamin D3 on Adhesion 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)2D3 and 24R,25(OH)2D3. 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)2D3. However, plectin and integrin β4 expression was increased in HCEC treated with 24R,25(OH)2D3 (P < 0.05). Integrin α6 protein expression was unchanged in HCEC treated with 1,25(OH)2D3 or 24R,25(OH)2D3 (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 experiments, 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. Specific desmosomal proteins expressed in corneas have been shown to be species specific. In bovine cornea, the desmosomal proteins DSC2 and DSG2 were detected in corneal epithelium, while DSC1, DSC3, DSG1, and DSG3 were absent. In rat epithelium, DSG1 and DSG2 were detected. In human corneal epithelial cells, DSG1 and DSG3 were found. Meanwhile, in mice, DSG1, DSC2, DSC1, DSC2, and DSC3 were all detected in the corneal epithelium. For hemidesmosomal proteins, a variety of integrin heterodimers are expressed in the corneal epithelium, many of which have been linked to the adherence of epithelial cells to the stroma. Integrin subunits were first detected in mouse and rabbit corneal epithelium. Integrin subunits α6, β4 were detected in rat corneal epithelium. Integrin subunits α5, α4, β1, and β4 were detected in human corneal epithelium. In previous studies, plectin was found to be enriched in corneal (and skin) basal epithelial cells. Plectin was found to interact with integrin α6/β4 in migrating epithelial cells and with integrin α5/β6 to promote corneal wound healing. In the current study, we focused on the differentiation of the desmosomal proteins DSG1 and DSC2, and hemidesmosomal 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. Oral vitamin D supplementation was found to improve wound healing of patients with diabetic foot ulcers compared with placebo. Vitamin D and calcium signaling in keratinocytes have been found to be required for the normal regenerative response of the skin to wounding. A previous study from our laboratory determined that 24R,25(OH)2D3...
increases corneal epithelial cell migration and proliferation. Moreover, it has been demonstrated that the remodeling of desmosomes is critical for cell proliferation, differentiation, migration. In the current study, we have determined that both 1,25(OH)_{2}D_{3} and 24R,25(OH)_{2}D_{3} 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)_{2}D_{3} and 24R,25(OH)_{2}D_{3}. We also determined that 24R,25(OH)_{2}D_{3} 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 5.** Desmosomal molecular DSG1 protein expression in WT and VDR KO MPCEC treated with 1,25(OH)_{2}D_{3} and 24R,25(OH)_{2}D_{3}. Representative Western blot (a) and blot densities (b) demonstrating increased DSG1 protein expression in VDR WT MPCEC treated with 1,25(OH)_{2}D_{3} and 24R,25(OH)_{2}D_{3} (t-test, x ± SE, *P < 0.05, n = 3). DSG1 protein expression (c, d) was also increased in VDR KO MPCEC treated with 1,25(OH)_{2}D_{3} and 24R,25(OH)_{2}D_{3} (t-test, x ± SE, *P < 0.05, n = 4). Uncropped blots and PVDF membrane images shown in Supplementary Figures S5 to S6.

**Figure 6.** Hemidesmosomal plectin protein expression in WT and VDR KO MPCEC treated with 1,25(OH)_{2}D_{3} and 24R,25(OH)_{2}D_{3}. Representative VDR WT MPCEC Western blot (a) and blot densities (b) demonstrating increased plectin protein expression in cells treated with 24R,25(OH)_{2}D_{3} (t-test, x ± SE, *P < 0.05, n = 5). Plectin protein expression in VDR KO MPCEC (c, d) was decreased following treatment with 24R,25(OH)_{2}D_{3} (t-test, x ± 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)_{2}D_{3}. Uncropped blots and PVDF membrane images shown in Supplementary Figures S7 to S8.
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. In the current study, 24R,25(OH)2D3 was found to increase DSG1, integrin \( \beta 4 \), 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 \( \beta 4 \) and plectin levels in VDR KO MPCEC. There was no change of integrin \( \beta 4 \) or plectin expression in mouse or human corneal epithelium, but the integrin \( \beta 4 \) 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. Our data indicate that both VDR and VDR-independent pathways are involved in the regulation of 1,25(OH)2D3- and 24R,25(OH)2D3-induced changes in desmosomal and hemidesmosomal proteins.

**Figure 7.** Hemidesmosomal integrin \( \beta 4 \) 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 \( \beta 4 \) protein expression in cells treated with 24R,25(OH)2D3 (t-test, \( x \pm SE, *P < 0.05, n = 5 \)). VDR KO MPCEC integrin \( \beta 4 \) protein expression (c, d) was decreased in cells treated with 1,25(OH)2D3 and 24R,25(OH)2D3 (t-test, \( x \pm SE, *P < 0.05, n = 4 \)). There was no change of integrin \( \beta 4 \) protein expression in WT MPCEC treated with 1,25(OH)2D3. Uncropped blots and PVDF membrane images shown in Supplementary Figure S9.

**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, \( x \pm SE, *P < 0.05, n = 5 \)). Uncropped blots shown in Supplementary Figure S10.
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 occurring 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 exacerbation of otherwise unassociated ophthalmic pathologies, such as recurrent erosions, diabetic keratopathy, or surgical complications.

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