Effect of Vitamin D Receptor Knockout on Cornea Epithelium Wound Healing and Tight Junctions

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Purpose. Our laboratory previously determined that vitamin D3, the vitamin D receptor (VDR), and 1α hydroxylase are present and active in the eye. In this study, we examined the effects of VDR knockout on wound healing, the tight junction–associated proteins occludin and ZO-1, and tight junction numbers in mouse corneas.

Methods. Epithelial wounds (2-mm) were made with an agar brush on 4-week-old and 10-week-old wild-type, heterozygous, and VDR knockout mouse corneas. Mice were on a normal or high lactose, Ca^{2+}, and PO_4^- diet. Wound-healing area was measured over time. Real-time PCR was used to quantify occludin and ZO-1 message expression. Western blot was used for protein expression. Transmission electron microscopy was used to examine corneal epithelium and endothelium tight junctions. Immunofluorescence was used to examine epithelial ZO-1 distribution.

Results. Results showed a decreased healing rate in 10-week-old VDR knockout mice compared with wild-types. Vitamin D receptor knockout mice on the special diet had no difference in healing rate compared with wild-types. Real-time PCR showed decreased expression of occludin and ZO-1 in 10-week-old VDR knockout mice compared with wild-types. Western blot of 10-week-old knockout mouse corneas showed decreased occludin expression compared with wild-types. Transmission electron microscopy showed a significant difference in tight junction numbers in VDR knockouts versus wild-types. Immunofluorescence showed a change in ZO-1 distribution among genotypes.

Conclusions. Vitamin D receptor knockout affects mouse corneal epithelium wound healing and tight junction integrity.

Keywords: wound healing, tight junctions, vitamin D, corneal epithelium

The corneal epithelium has unique structural and physiologic properties that allow it to fulfill its functions in the eye. One function of the stratified corneal epithelium is that it forms a protective barrier to the external environment. Adherence junctions, desmosomes, and tight junctions form the corneal epithelial barrier.[1,2] Tight junctions have diverse roles, ranging from the control of ion, water, and molecule movement across intercellular junctions to providing a physical seal across the epithelial sheet and assisting in the maintenance of cell polarity.[3] The tight junction complex is composed of different proteins: zonula occludens (ZO), occludin, and claudin, among others. Occludin is a major protein component of the tight junction complex and it is widely studied due to its role in barrier function control. Zonula occludens-1 is a tight junction–associated protein that works as occludin's binding partner and their interaction forms one of many tight junction–associated protein complexes.[4] If these junction proteins are disrupted by external factors, such as pathogens or calcium removal, or if they are physically damaged, there is loss of barrier function that can lead to a number of corneal pathologies ranging from epithelial defects to infections and corneal melting.[1,5,6]

Vitamin D is known to be produced in the skin and is hydroxylated in the liver and kidneys for further use by the gut epithelium to increase the uptake of dietary calcium. Recent research has shown several other actions of vitamin D in tissues such as skin, smooth muscle, breast, and most recently our group discovered the presence of vitamin D, vitamin D receptor (VDR), and 1α hydroxylase in the eye. In that study, we cultured and supplemented human cornea epithelial cells with different concentrations of 1α,25(OH) D3 or 25(OH) D3 and determined that supplementation with these metabolites enhanced corneal barrier function. Results showed an increase in transepithelial resistance and a decreased FITC-inulin flux after the addition of either 1α,25(OH) D3 or 25(OH) D3. Also, increased expression of occludin was measured by Western blot following the addition of 1α,25 (OH) D3 or 25(OH) D3.[7] These data indicate that vitamin D supplementation likely has an influence on the differentiation state of the epithelium and the degree of in vitro tight junction integrity. As a result of these previous studies, we chose to study wound healing and tight junction structure in VDR knockout mice. Specifically, we hypothesized that VDR knockout affects wound healing, tight junction–associated proteins (occludin and ZO-1), and microscopic tight junction structural appearance.

Methods

Ethics Statement

Wild-type (+/+), heterozygous (+/–), and VDR knockout (–/–) C57BL/6 mice were bred from the Jackson Laboratories (strain: B6.129S4-Vdr<tm1Mbd>/J; Bar Harbor, ME, USA). All animal studies were approved by the University of Tennessee Health Science Center Institutional Animal Care and Use Committee (IACUC), and animals were treated according to the ARVO statement for the Use of Animals in Ophthalmic and Visual Research.

Wound Healing

The first group was composed of 4-week-old +/+, +/–, and –/– mice. The second group was composed of 10-week-old +/+, +/–, and –/– mice plus –/– mice on a special diet (20% lactose, 2.0% Ca^{2+}, 1.5% PO_4^-) previously shown to alleviate many of the VDR –/– phenotypical features.[8–10] Before the wound-healing procedure, mice were weighed to adjust anesthetic dosage (ketamine 100 mg/kg plus xylazine 10 mg/kg). Topical proparacaine hydrochloride 0.5% was also used. After anesthesia, a 2-mm epithelial wound was made with an agar brush. To monitor wound size, a drop of fluorescein sodium 0.25% with proparacaine hydrochloride 0.5% was applied to the wounded eye. An antibiotic ointment (bacitracin, neomycin, polymixin) and 0.05% buprenorphine (intramuscular) were used after every procedure. Wounds were photographed with a slit lamp at 0, 5, 24, and 29 hours or until the wounds were healed. Wounds were traced using ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) software, and wound areas were measured at each time point. Wound areas were normalized to percentage values and analyzed by ANOVA at each time point. Healing rates were calculated using both raw and normalized data by linear regression and comparisons were made between genotypes using each data set.

Real-Time PCR

Ten-week-old +/+, +/–, and –/– mice were used for real-time PCR analysis. Total RNA was obtained from mouse corneal epithelium. Real-time PCR was used to quantify occludin and ZO-1 mRNA levels; 2 μg total RNA was used for synthesis of cDNA using the ThermoScript RT-PCR system (Applied Biosystems, Foster City, CA, USA). First-strand synthesis was done at 25°C for 10 minutes, then 50°C for 50 minutes, and finally inactivated at 85°C for 5 minutes. Equal amounts of cDNA were applied for PCR amplification in quadruplicate at a final volume of 25 μL 2X RT2 Real-Time SYBR Green master mix (Qiagen, Germantown, MD, USA). Amplification was performed at 95°C for 10 minutes, followed by 40 cycles at 94°C for 15 seconds and 60°C for 60 seconds. Quantitative values were obtained from the threshold cycle value (C_t), which is the point where a significant increase of fluorescence is first detected. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal RNA control, and each sample was normalized on the basis of its GAPDH gene content (ΔC_t). The $2^{-\Delta\Delta Ct}$ method was used to analyze the results.

Western Blot

Ten mouse corneas were used from each genotype to measure expression of occludin in +/+, +/–, –/–, and –/– mice on the special diet. Tissue was collected and stored in a protease inhibitor solution at –80°C. Corneas were freeze-dried with liquid nitrogen and crushed. Lysis buffer D at 95°C (0.3% SDS, 10 mM Tris/HCL, 10 μM sodium orthovanadate, 100 μM sodium fluoride, and protease inhibitor) was added to the tissue, which was collected for further processing. The mixture was sonicated five times in ice water followed by incubation at 95°C for 10 minutes, and centrifuged at 18187*g* (4°C) for 10 minutes followed by supernatant recollection. Sample protein concentration was measured using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA). Equal amounts of protein (20 μg) were loaded onto an 8% gel and separated by SDS-PAGE. Mouse anti-occludin, (Zymed; Invitrogen, Carlsbad, CA, USA) and a horseradish peroxidase conjugated goat anti-mouse secondary antibody was used to enhance detection. For loading controls, membranes were stripped and re-probed with B-actin antibody (CP01; Calbiochem, Billerica, MA, USA). Western blots were digitally photographed and blot density was determined by Fiji (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

Transmission Electron Microscopy

Ten-week-old +/+ and –/– mice were used for transmission electron microscopy (TEM) (JOEL 2000, JOEL Ltd., Tokyo, Japan). After euthanasia, eye enucleation, and fixation (4% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M cacodylate), eyes were embedded into resin blocks for sectioning. Thin sections were cut to obtain tissue closest to the center of the cornea. Random images at ×15,000 were taken of the epithelium and endothelium to identify tight junctions. After collection, Fiji (Wayne Rasband, National Institutes of Health) was used to process the images manually to identify and count tight junctions.

Immunohistochemistry

VDR +/+, +/–, and –/– mice had their epithelium wounded as described above and mice were euthanized 24 hours after wounding. Eyes were enucleated and embedded in Tissue-Tek optimum cutting temperature compound (Sakura Finetek, USA, Torrance, CA, USA) and frozen on dry ice surrounded with 95% ETOH. Coronal sections were cut at 30 μm using a Microm HM505E Cryostat (Microm International GmbH, Walldorf, Germany). The sections were mounted on superfrost-plus slides (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) and stored at –80°C until ready to stain.

Corneal sections were fixed with 0.5% formaldehyde at room temperature (RT) for 20 minutes, washed twice in PBS, and treated with blocking buffer (10% normal goat serum, 1% BSA, 0.5% Triton X-100 in PBS) for 3 hours at RT. Sections were then incubated with anti-ZO-1 antibody (1:250 dilution; Invitrogen) in blocking buffer at RT. Following overnight incubation, sections were washed twice in PBS and exposed to fluorescein-labeled anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame CA, USA) for 2 hours at RT at a 1:200 dilution. Slides were then washed twice in PBS before immersion in coverslip mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories). Immunostained sections were imaged along the wound margin using a Zeiss LSM 510 confocal microscope equipped with LSM 510 software (Zeiss, Oberkochen, Germany). All images were obtained using a Plan-Apochromat 63 × /1.4 oil differential interference contrast objective, scan frame size 1024 × 1024 pixels, pixel time 1.6 μs, pinhole set at 98 μm, and gain of 508. Fluorescence was excited with the argon laser at 488 nm at 17% power and emission was detected between 505 and 550 nm.

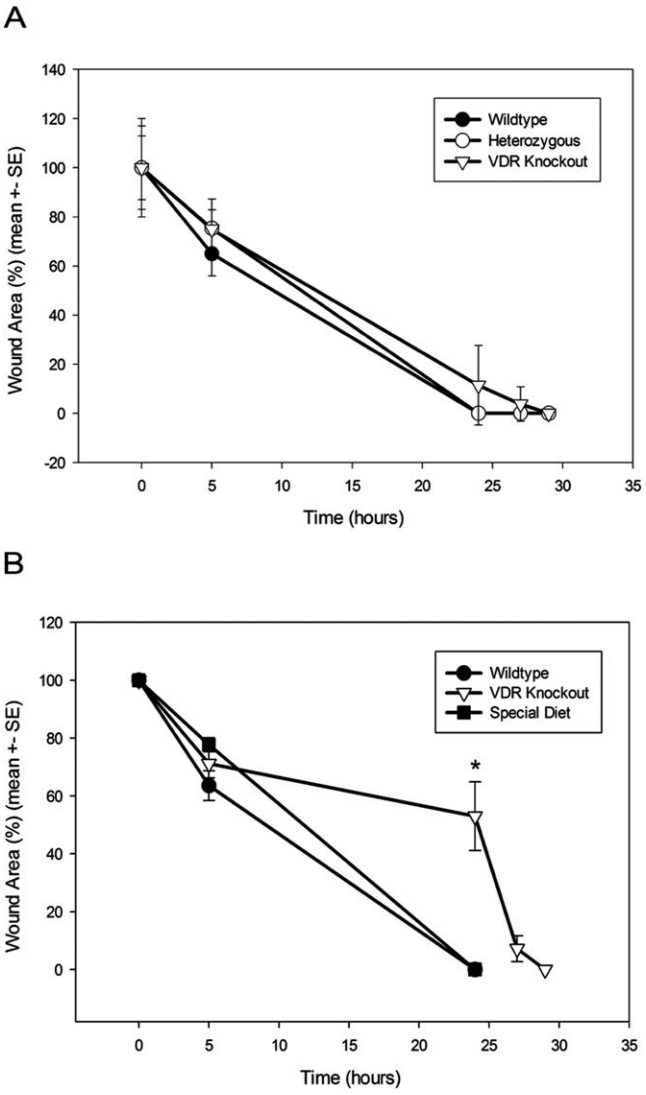

Figure 1. (**A**) Four-week-old mice wound healing versus time. There is no significant difference in wound healing among groups. (**B**) Normalized wound-healing data. Cornea lesion area was normalized to 100% at time 0 and areas at each subsequent time point were adjusted accordingly. The knockout mouse wound-healing rate is significantly different ($P < 0.01$) from wild-types and VDR knockouts on the special diet. The *asterisk* indicates a significant difference in knockout versus wild-type and VDR knockout mice on special diet ($P < 0.01$).

Statistical Analysis

GraphPad 5.0 (GraphPad Software, La Jolla, CA, USA) and SigmaPlot (Systat Software, Inc. [SSI], San Jose, CA, USA) were used to analyze data. ANOVA and post hoc Tukey's test were used to analyze real-time PCR data. Independent *t*-test was used for TEM data. Analysis of wound-healing data was performed as mentioned previously.

Results

Wound Healing

Four-week-old VDR –/– mice showed no significant differences in healing rates at any given time point as compared with +/+ and +/– groups (Fig. 1A). The wound closure rate (slope) for VDR –/– mice was significantly slower than controls in 10-week-old mice. A rate of –0.053 mm/h was measured in VDR –/– mice versus a rate of –0.095 mm/h in controls ($P < 0.05$) (Fig. 1B). In addition, VDR –/– mice took 26 hours or more to heal completely, whereas all +/+ and +/– wounds closed in less than 24 hours. Wound-healing rates between VDR –/– mice fed the special diet versus +/+ mice were not significantly different (–0.097 vs. –0.095 mm/h, respectively). Unlike –/– mice, wounds of VDR –/– mice on the special diet were completely healed before 24 hours. Analysis of variance showed a significant difference in wound area at the 24-hour time point in –/– mice compared with +/+, +/–, and –/– mice on the special diet ($P < 0.01$). There was no significant difference in wound-closure rates between +/+ and +/– mice (data not shown). Normalized data comparisons yielded the same significant differences with a stronger *P* value in the +/+ versus –/– mouse comparison ($P < 0.01$).

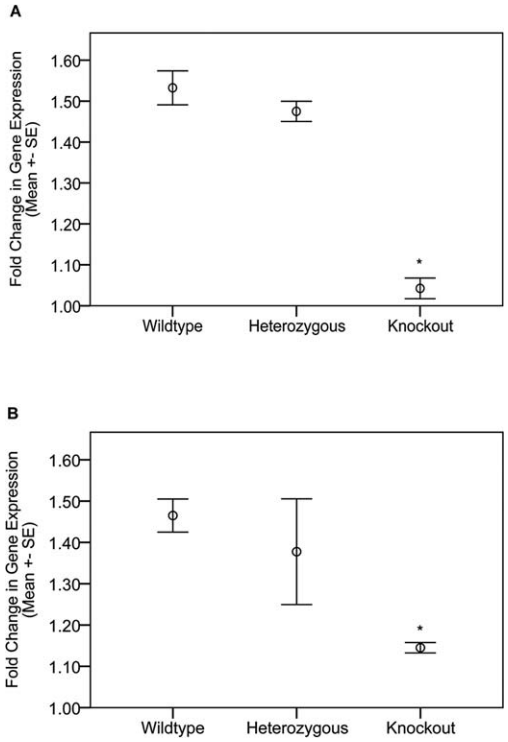

Figure 2. Fold change of corneal occludin and ZO-1 gene expression. The $2^{-\Delta\Delta Ct}$ method was used to graph the results. (**A**) There is a significant decrease (*) in occludin mRNA expression in knockout mice. (**B**) Messenger RNA expression of ZO-1 is decreased (*) in knockout mice compared with wild-type, but not with heterozygous mice ($n = 4$).

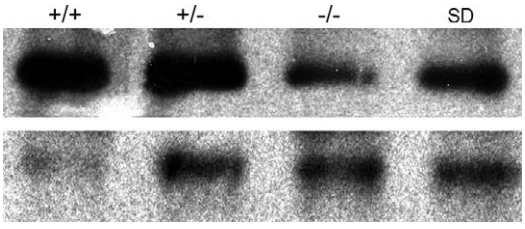

Figure 3. Western blot of occludin expression in 10-week-old mice. The β-actin loading controls are shown below the occluding blots. Note the decreased expression in knockout mice and the recovery of protein expression in VDR knockout mice on the special diet (SD).

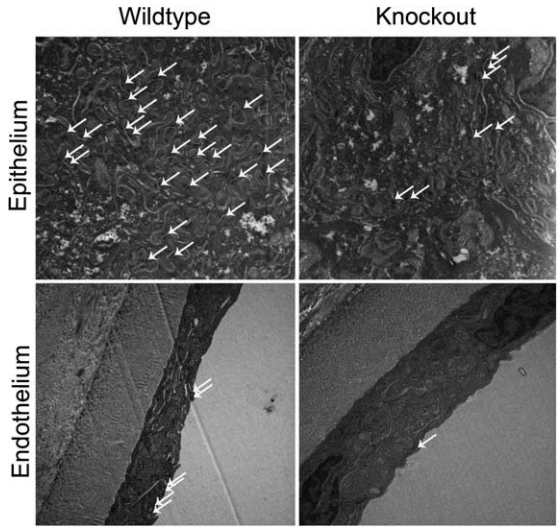

Figure 4. Representative TEM image of a 10-week-old mouse cornea (×15,000 magnification). *Arrows* point to tight junctions. *Upper row* shows the epithelium of +/+ versus −/− mice. *Lower row* shows endothelium of +/+ versus −/− mice. Note the reduced number of tight junctions between −/− cells as compared with +/+ cells.

Real-Time PCR

Real-time PCR showed a decreased quantity of occludin and ZO-1 message in 10-week-old VDR −/− mice. Tukey's test showed significantly lower occludin mRNA in VDR −/− versus +/+ mice and between VDR −/− and +/− mice ($P < 0.01$) (Fig. 2). There was no significant difference between +/+ and +/− mouse occludin mRNA according to Tukey's post hoc test. Differences between ZO-1 +/+ and −/− mouse mRNA approached significance ($P = 0.05$). There was no significant difference between VDR −/− and +/− mouse ZO-1 mRNA levels.

Western Blot

Western blotting for occludin showed a decrease in −/− mouse protein expression as compared with wild-types. The −/− mice on the special diet recovered some protein expression (Fig. 3).

Transmission Electron Microscopy

Transmission electron microscopy images showed a decrease in visible tight junction numbers in the epithelium and endothelium of VDR −/− mouse corneas versus +/+ mouse corneas. For epithelium, VDR −/− and +/+ mice had respective mean ± SEs for the number of tight junctions of 11.02 ± 0.50 vs. 17.52 ± 0.56. For corneal endothelium VDR −/− mice had a mean ± SE of 1.52 ± 0.21 compared with 2.83 ± 0.19 for +/+ mice (Fig. 4). Student's *t*-test confirmed that there was a significant difference between the number of tight junction structures in VDR −/− versus +/+ mice in both the corneal epithelium and endothelium ($P < 0.01$).

Immunofluorescence

Confocal microscopy images of ZO-1 staining of −/− mouse epithelium following 24 hours of wound healing showed lower intensity and multiple breaks in the staining pattern along the perimeter of the cell membranes when compared with +/+ mouse epithelium (Fig. 5). Epithelium from +/− epithelial cells also showed some disrupted ZO-1 staining.

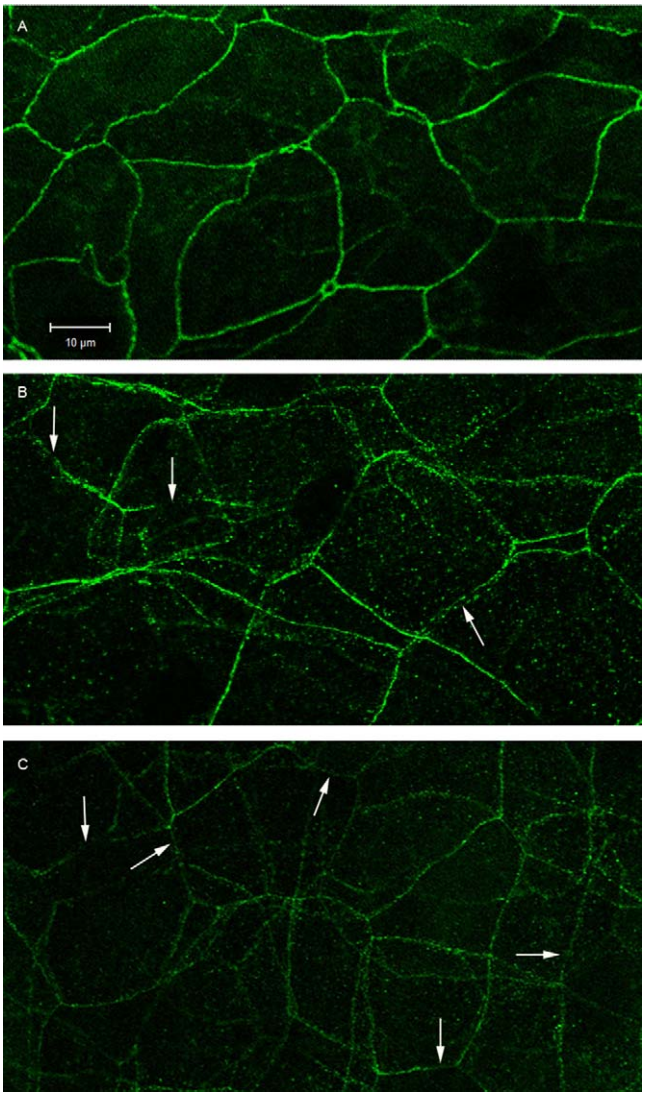

Figure 5. Representative ZO-1 immunohistochemistry results of 10-week-old mice 24 h after epithelial wounding. Confocal images of (**A**) wild-type, (**B**) heterozygous, and (**C**) homozygous mouse corneal epithelium coronal sections. Images were taken at the wound margin. *Arrows* indicate breaks in ZO-1 staining in +/− and −/− mice; −/− mice also showed lower-intensity ZO-1 staining.

Discussion

Vitamin D is well known for its calcium homeostasis activity in conjunction with parathyroid hormone, but there is also research demonstrating that vitamin D has other physiologic actions in the body. There are many nonclassical functions of vitamin D that are being studied in different organs and in disease prevention, including skin,[11–18] immune-modulation,[19–22] the cardiovascular system,[23–26] and cancer prevention.[27–38] Although most vitamin D availability in humans is through the initial conversion of 7-dehydrocholesterol to previtamin D in the skin via UV-B exposure from the sun, to date there are but a few published studies examining the physiological functions of vitamin D in the only organ other than the skin that is exposed directly to sunlight: the eye. Most of the previous vitamin D and VDR research studies in the eye have focused on the immune response.[39,40] It has been determined that vitamin D suppresses inflammation by inhibiting Langerhans cell migration and neovascularization

in mice.[20] We have previously shown that human corneal limbal epithelial cells supplemented with 7-dehydrocholesterol can produce 25(OH) D3 when exposed to UV-B. Also, in the same study we found increased concentrations of 25(OH) D3 and 24R,25(OH) D3 in tear fluid and aqueous humor of rabbits whose diet was supplemented with vitamin D.[41]

Previously published studies from our laboratory have demonstrated the presence of VDR, 1α hydroxylase, and vitamin D metabolites in the eye.[41] We have also shown that vitamin D supplementation to human corneal epithelial cells enhances corneal barrier function, likely through increased expression of the tight junction–associated protein occludin, as was shown by Western blot analysis.[7,19,42] The decreased occludin message and protein expression in VDR −/− mice compared with wild-types further demonstrates the link between vitamin D and occludin expression. Occludin is a major tight junction–associated protein in the corneal epithelium and is needed for proper wound healing and cell migration.[41] A decreased expression of occludin can lead to decreased rates in wound healing, decreased transepithelial resistance, and increased permeability.[43–45] Recent research has shown that occludin knockout in MDCK cells blocks wound closure, the microtubule organization center, and cell protrusion.[46] In a control versus occludin knockout MDCK cell wound model, it was determined that 90% of the wound was closed in control cells compared with 28.6% ± 6.0% in occludin knockout cells 16 hours after wounding.[47,48] Occludin also has been shown to regulate the organization of actin, making it essential for proper microtubule network organization during wound healing.[49]

Zonula occludens-1 is one of the members of the membrane-associated guanylate kinase (MAGUK) homolog family. It has been shown that ZO-1 binds with occludin at its N-terminal half.[4] In another study, MDCK II cells were selectively depleted of ZO proteins. MDCK II ZO −/− cells showed a reduced localization of occludin, increased paracellular permeability, and expansion of the apical actomyosin contractile array at the apical junction complex.[50,51] Further studies showed that ZO-1 is needed for proper tight junction assembly, cellular permeability, and regulation of the contractile array.[52] In the current study, we show that 10-week-old VDR −/− mice have a decreased expression of occludin and ZO-1 mRNA as compared with +/+ mice. There was no significant difference between −/− and +/− due to a wide range in the SE. Transmission electron microscopy revealed a decreased number of tight junctions and immunofluorescence showed a lower intensity and multiple breaks of the staining pattern throughout the cellular membrane in 10-week-old VDR −/− and +/− versus +/+ mice.

In general, the VDR −/− mouse phenotype is characterized by alopecia, hypocalcaemia, hypophosphatemia, hyperparathyroidism, and growth retardation. These phenotype characteristics are typically not apparent until 21 days of life.[10] In the current study, wound healing in 4-week-old VDR −/− mice was not significantly different from +/+ mice. On the other hand, 10-week-old VDR −/− mice had slower healing rates than +/+ and +/− mice, with a significant difference at 24 hours. Knockout mice showed a steady plateau followed by rapid wound healing after 24 hours. Some wound-healing images show stromal fluorescein staining below the advancing epithelium (data not shown). We hypothesize that this staining under the advancing epithelium is a result of the defective expression of tight junction barrier proteins related to the lack of VDR, as shown by Western blot and immunofluorescence.

Calcium is an essential mineral throughout the body, and in the corneal epithelium it plays a role in the response to wound healing.[53] During wound healing there are calcium wave currents that propagate from the injured site to neighboring cells. Calcium waves stimulate epithelial cell proliferation.[17,54,55] Also, intracellular calcium is used by calcium-dependent E-cadherin–mediated cell-cell adhesion to initialize signaling for intercellular tight junction assembly.[2,56,57] This process is critical for proper cell migration to the wound area. Calcium is an important ion that is necessary for tight junction maintenance and cell proliferation. It is likely that hypocalcemia induced by VDR knockout impairs tight junction reformation and the corneal epithelium wound-healing response. The use of a high-lactose, Ca^{2+}, and PO_4^- diet has been previously described in VDR −/− mice. It has been shown that diet supplementation of these mice with high concentrations of lactose, Ca^{2+}, and PO_4^- restores calcium, phosphate, and parathyroid hormone serum levels.[8–10] A goblet cell mucin packaging study in VDR-ablated mice found that −/− mice fed the special diet recovered their ability to package mucin similar to +/+ mice. It was concluded that calcium plays a role in mucin packaging on goblet cells.[8] In the current study, administration of the special diet high in lactose, Ca^{2+}, and PO_4^- to VDR −/− mice resulted in normalization of occludin expression. In addition, in contrast to VDR −/− mice fed a normal diet, VDR −/− mice fed the special diet showed no significant difference in wound-healing rates when compared with +/+ mice. These mice also healed in less than 24 hours, which was the same as +/+ mice and quicker than −/− mice fed the normal diet. Calcium, phosphate, and lactose replenishment by the special diet not only alleviated the phenotypic features of the VDR −/− mice but also normalized wound healing.

It is well established that during wound healing, cells can migrate vertically (basal to apical) and horizontally (periphery to center). Limbal stem cells play a major role in horizontal migration and centripetal reepithelialization during wound closure.[58] The absence of limbal stem cells results in defective and delayed wound healing.[59] Vitamin A, through its receptors retinoid x receptor α (RXR) and retinoic acid receptor γ, stimulates the differentiation of limbal stem cells into transient amplifying cells for further epithelial differentiation.[60] These receptors also play a significant role in regulating tight junction proteins and barrier functions.[61] The vitamin D receptor may play a role in the differentiation of limbal stem cells given that it also binds to the RXR receptor. Vitamin D has been shown to induce differentiation of other epithelial stem cells as well as cancer cells.[11,17,28] There is strong evidence that vitamin D deficiency affects corneal barrier function and wound healing through altered expression of tight junction–associated proteins and epithelial cell differentiation.

In conclusion, this study demonstrates that VDR −/− mice have decreased mRNA expression of the tight junction–associated proteins occludin and ZO-1 and a significant delay in wound healing compared with +/+ mice. Also, VDR −/− mice on special diet (high lactose, Ca^{2+}, PO_4^-) recovered the expression of occludin and wound-healing rates similar to +/+ mice. Occludin and ZO-1 are important components of the barrier function complexes and defective expression likely leads to a wound-healing delay in VDR −/− mice. Hypocalcemia affects migration of epithelial cells and expression of tight junction proteins. When these mice are fed the special diet they recovered their capacity to heal wounds with results similar to +/+ mice. Vitamin D and dietary minerals are important factors for maintaining corneal epithelial integrity and function during wound healing.

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