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Research Article

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CYP24A1 is overexpressed in keloid keratinocytes and its inhibition alters profibrotic gene expression

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Abstract

Background: Keloids are disfiguring, fibrotic scar-like lesions that are challenging to treat and commonly recur after therapy. A deeper understanding of the mechanisms driving keloid formation is necessary for the development of more effective therapies. Reduced vitamin D receptor (VDR) expression has been observed in keloids, implicating vitamin D signaling in keloid pathology. Vitamin D exhibits anti-proliferative and anti-inflammatory properties, suggesting it could have therapeutic utility in keloid disorder. The current study investigated vitamin D-regulated gene expression in keloid keratinocytes and the effects of inhibiting an enzyme involved in vitamin D metabolism on the phenotype of keloid-derived keratinocytes.

Methods: Normal and keloid-derived primary keratinocytes were isolated from normal skin and keloid lesions, respectively, and were cultured in the absence or presence of vitamin D. In some experiments, inhibitors of the vitamin D metabolizing enzyme CYP24A1, ketoconazole or VID400 were added in the absence or presence of vitamin D. Cellular proliferation, migration and gene expression were measured.

Results: We observed significant overexpression of *CYP24A1* mRNA in keloid versus normal keratinocytes and increased CYP24A1 protein levels in keloids versus normal skin. *CYP24A1* encodes 24 hydroxylase and is induced by vitamin D in a feedback loop that regulates vitamin D levels; thus, inhibition of CYP24A1 activity may locally increase active vitamin D levels. Ketoconazole, a non-specific cytochrome P-450 inhibitor, reduced proliferation of keloid and normal keratinocytes, but VID400, a specific CYP24A1 inhibitor, only significantly affected keloid keratinocyte proliferation. Neither inhibitor significantly reduced keratinocyte migration. The two inhibitors had different effects on vitamin D target gene expression in keratinocytes. Specifically, ketoconazole treatment reduced *CYP24A1* expression in normal and keloid keratinocytes, whereas VID400 increased *CYP24A1* expression. Both inhibitors decreased expression of profibrotic genes, including periostin and hyaluronan synthase 2, in keloid-derived cells. Combined treatment of keloid keratinocytes

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with vitamin D and ketoconazole or VID400 increased the effects of vitamin D treatment on target genes, although the effects were gene- and cell type-specific.
Conclusions: The data suggest that reduction of vitamin D inactivation with CYP24A1 inhibitors may reduce profibrotic gene expression in keloid-derived cells. Therefore, CYP24A1 inhibitors may serve as adjunctive therapies to suppress keloid-associated gene expression changes.

Key words: Keloid, Keratinocyte, Vitamin D, CYP24A1, 24-hydroxylase, Epithelial-mesenchymal transition

Highlights

- Despite reduced vitamin D receptor expression in keloid epidermis *in vivo*, keloid keratinocytes are able to respond to vitamin D stimulation *in vitro* with upregulation of target genes.
- The gene encoding CYP24A1 was observed to be upregulated in keloid-derived keratinocytes compared with normal skin-derived keratinocytes, and CYP24A1 protein was elevated in keloids compared with normal skin samples.
- Inhibition of CYP24A1 activity reduced profibrotic gene expression in keloid-derived keratinocytes.
- The results support the investigation of CYP24A1 as a potential therapeutic target for suppression of keloid pathology.

Background

Keloids are disfiguring fibroproliferative lesions that can occur in susceptible individuals following an injury to the skin [1]. Although historically considered a form of abnormal scarring, the term 'keloid disorder' is currently favored to recognize the serious and potentially systemic nature of keloid pathology [1]. Unlike hypertrophic scars—raised scars that are confined to the original wound boundary—keloids extend beyond the original wound margin and can increase in size indefinitely, contributing to reduced quality of life in affected patients [2–4]. Keloids are extremely challenging to treat, with low response rates to many current therapies and high recurrence rates after treatment [5, 6]. The relatively low effectiveness of most keloid therapies reflects our incomplete understanding of the molecular basis of keloid development. Keloids are characterized by abnormal and excessive deposition of extracellular matrix (ECM), particularly type I collagen, which may result from an imbalance of ECM production and degradation, in the context of a protracted increase in inflammation post-healing [7]. There have been a host of studies examining gene expression differences between normal uninjured skin and keloid lesions, and abnormalities of numerous signaling pathways have been identified in keloids and keloid-derived cells, including fibroblasts and keratinocytes [8–12]. Many of these abnormalities involve derangements of signaling pathways involved in tissue development and wound healing, such as transforming growth factor beta (TGF-β), JAK/STAT3 signaling and Wnt/β-catenin pathways, as well as pathways regulating inflammation [13, 14]. Effective treatment of keloids will require a deeper understanding of the molecular mechanisms driving keloid formation and identification of appropriate therapeutic targets.

Keloids are more common in populations with dark skin pigmentation, such as African Americans and others of African descent, with a 15-fold or greater increase in keloid incidence in Black versus White populations, and often greater severity of keloids in individuals of African ancestry [15–18]. Although the basis for the association of keloid risk with dark skin pigmentation has not been unequivocally determined, it likely involves a combination of genetic and environmental factors. There have been numerous genetic loci linked with keloid formation in different cohorts of patients, yet to date there is no single gene identified as the cause of keloid, and it is currently believed to be a multigenic disorder [19]. The association with dark skin pigmentation has led many researchers to investigate the potential role of vitamin D in keloid pathology. In addition to dietary sources, such as fish and fortified dairy products, vitamin D is made in the epidermis in response to ultraviolet (UV) radiation in sunlight. In individuals with dark skin, high levels of the pigment melanin shield epidermal keratinocytes from UV light, leading to lower production of vitamin D than in light skin with lower melanin levels [20, 21]. This contributes to higher rates of vitamin D deficiency in African Americans compared with the general US population [22]. Recent studies have found lower mean circulating vitamin D levels, and/or higher rates of vitamin D deficiency, in patients with keloids compared with non-keloid control patients, and an inverse correlation between mean circulating vitamin D levels and keloid severity, consistent with a role for vitamin D in keloid pathology [23–25].

Vitamin D is critical for bone health and for maintaining calcium homeostasis, but it has also been shown to have numerous extra-skeletal health benefits as well. Most of the activities of vitamin D are mediated by the vitamin D receptor (VDR), which is a member of the nuclear hormone family of transcription factors [26]. Vitamin D signaling through the VDR regulates the expression of genes involved in cellular proliferation, differentiation and inflammation, with generally anti-fibrotic and anti-inflammatory activities [27].

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Furthermore, the vitamin D/VDR signaling pathway exhibits crosstalk with many of the pathways previously implicated in keloid pathology and has been shown to regulate processes involved in wound healing, such as inflammation and ECM remodeling [28–31]. Vitamin D signaling is also important for negative regulation of the process of epithelial-mesenchymal transition (EMT) [32], which has been implicated in keloid pathology [12, 33, 34]. We previously showed that the gene expression profile of keloid keratinocytes resembles the process of EMT, including decreased expression of proteins involved in cell adhesion and increased expression of mesenchymal genes and genes involved in migration, compared with normal keratinocytes [12]. The phenotype of normal keratinocytes resembles EMT during wound healing; however, this is only a transient EMT that is normally reversed upon closure of the wound [35]. The EMT-like signature of keloid-derived cells suggests a pathological over-healing response, which persists in the context of protracted inflammation and proliferation; interventions that interrupt this over-healing may have therapeutic value for keloid suppression [33]. Consistent with this, we previously showed that reversing the abnormal overexpression of genes involved in EMT, such as hyaluronan synthase 2 (HAS), could normalize the phenotype of keloid keratinocytes [33, 36]. Hypothetically, agents that can reverse profibrotic gene expression and normalize the EMT-like phenotype of keloid keratinocytes may suppress the over-healing response and reduce keloid fibrosis.

Previous studies revealed decreased expression and reduced nuclear localization of VDR in keloid lesions compared with normal skin, further suggesting a role for vitamin D in keloid disorder [24, 37]. The current study was undertaken to investigate derangements in vitamin D signaling in keloid-derived keratinocytes. We found that *CYP24A1*, which encodes the 24-hydroxylase that metabolizes active vitamin D as part of a negative feedback mechanism to control vitamin D levels [38], is overexpressed in keloid keratinocytes compared with normal skin keratinocytes. Overexpression of *CYP24A1* has been observed in many different types of cancers, where it may contribute to reduced local levels of hormonally active vitamin D [39, 40]. Therapeutic inhibition of CYP24A1 activity has been investigated in the cancer field, as this may serve to increase the local bioavailability of vitamin D and thereby potentiate its anti-cancer effects [41, 42]. We therefore hypothesized that inhibition of CYP24A1 activity in keloid-derived cells may reduce profibrotic gene expression. This study investigated the effects of CYP24A1 inhibition on proliferation, migration and gene expression in normal and keloid-derived keratinocytes.

Methods

Tissue acquisition

All work involving human subjects was performed in accordance with the principles of the Declaration of Helsinki and with University of Cincinnati (UC) Institutional Review Board (IRB) and WCG IRB (Puyallup, WA) approval. Tissue samples were obtained from discarded, excised keloid lesions or normal skin from elective surgical procedures performed at Shriners Hospitals for Children—Cincinnati or Shriners Children's Ohio (pediatric samples) and the UC Medical Center (adult samples). Tissue samples obtained from pediatric patients (<18 years of age) were obtained with informed consent from parents or guardians and patient assent. Tissue samples from adults were collected as de-identified samples; this collection was designated as 'not human subjects research' by the UC IRB and therefore did not require informed consent. Demographic information for patients is presented in Table 1. All skin and keloid samples used for primary cell isolation were assigned a sequential identification number (ID #) upon receipt in the laboratory; the 'K' designation following the ID # indicates that the tissue sample was from a keloid (see Table 1). Note that patient race/ethnicity was self-reported: 'Black' includes all patients who self-identified as Black or African American; all patients self-identified as non-Hispanic.

Cell culture

Primary keratinocytes were isolated from skin and scar tissue samples as detailed elsewhere [43, 44]. Briefly, skin or scar samples were trimmed to remove subcutaneous fat, and dermis and epidermis were separated using Dispase II. Keratinocytes were released from epidermis using trypsin digestion and were cultured initially in collagen-coated flasks in keratinocyte growth medium consisting of MCDB-153 with 0.06 mM $CaCl_2$ supplemented with 1 ng/ml epidermal growth factor (PeproTech, Rocky Hill, NJ), 0.5 μg/ml hydrocortisone (Sigma Aldrich, St. Louis, MO), 5 μg/ml human insulin (Sigma Aldrich), 0.2% bovine pituitary extract (Hammond Cell Tech, Windsor, CA) and 1X Penicillin–Streptomycin–Fungizone (Thermo Fisher Scientific, Waltham, MA). After 5–7 days, before cells reached confluence, keratinocytes were either cryopreserved [43] or passaged into uncoated tissue culture flasks in keratinocyte growth media as described above but with 0.20 mM $CaCl_2$. Keratinocytes were used for experiments at passage 2 or 3.

For experiments involving vitamin D treatment of cells, keratinocytes were cultured in keratinocyte growth medium with active vitamin D (1α,25-Dihydroxyvitamin D3; catalog #D1530, Sigma-Aldrich, St. Louis, MO) at 10–100 nM as indicated below; vehicle-treated cells cultured under the same conditions served as controls. For experiments involving inhibitors, either ketoconazole (Sigma Aldrich), VID400 (Thermo Fisher Scientific) or vehicle (negative controls) were added at the same time as vitamin D.

Proliferation assay

Proliferation of normal and keloid keratinocytes was measured using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as detailed elsewhere [36]. Keratinocytes were inoculated into 24-well plates at 4×10^4 cells/well, and each donor strain (n = 3 normal,

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Table 1. Demographic information for samples used for keratinocyte isolation

Donor ID #	Type	Age (y)	Sex	Race/ethnicity[a]	Body site
797K	Keloid	10	Male	White	Ear
818K	Keloid	16	Male	Black	Shoulder
829K	Keloid	12	Female	Black	Ear
843K	Keloid	16	Male	White	Face
878K	Keloid	17	Male	Black	Scapula
884K	Keloid	51	Female	Black	Neck
919K	Keloid	10	Female	Black	Ear
934K	Keloid	15	Female	Black	Ear
991K	Keloid	12	Female	White	Chest
766	Normal	25	Male	Black	Abdomen
811	Normal	24	Female	Black	Breast
879	Normal	12	Female	White	Thigh
880	Normal	15	Female	White	Breast
886	Normal	17	Male	Black	Abdomen
974	Normal	15	Female	Black	Breast

[a]Race/ethnicity was self-declared; patients identifying as either Black or African American are listed here as Black

n = 4 keloid) was cultured in quadruplicate in keratinocyte growth medium plus vitamin D (0 nM, 10 nM, 50 nM or 100 nM alone, or 0 or 100 μM combined with ketoconazole or VID400), ketoconazole (0, 1, 5, 10, 20 or 30 μM alone, or 0 or 10 μM combined with vitamin D), or VID400 (0, 25, 50, 100, 200 or 400 nM alone, or 0 or 100 nM combined with vitamin D) for 24 h. The MTT assays (MP Biomedical, Santa Ana, CA) were performed according to the manufacturer's recommendations, and absorbance was measured at 570 nm. Absorbance provides an index of cellular metabolic activity, which is proportional to cell number. A mean absorbance value for each strain in each condition was calculated by averaging the quadruplicate values; the values for normal or keloid strains were then averaged to provide a mean value for normal or keloid keratinocytes in each condition.

Migration assay

An *in vitro* wound healing assay was performed as previously described [36]. Briefly, 6-well multi-well tissue culture plates (Thermo Fisher Scientific) were precoated with bovine collagen type I (PureCol® Type I Collagen; Advanced Biomatrix, Inc., Carlsbad, CA) at 5 μg/cm^2 for 1 h, and excess collagen was rinsed out and culture medium added. Normal keratinocytes (n = 3 strains) or keloid keratinocytes (n = 4 strains) were inoculated at 10^5 cells/cm^2 into collagen-coated wells and were cultured in keratinocyte growth medium without vitamin D or inhibitors for 24 h to generate confluent monolayers. Scratch wounds were created by scratching the monolayers with a sterile 200 μl pipet tip, and each well was rinsed twice prior to incubation in culture medium supplemented with 0 or 100 nM vitamin D, combined with 0 or 10 μM ketoconazole or 0 or 100 nM VID400, for 24 h. Circles were etched on the underside of each well using a 4-mm biopsy punch to mark the scratched regions, which were photographed at 0, 6 and 24 h after scratching. Image analysis was performed to quantify the open area of each microscopic field (NIS-Elements AR3.1; Nikon, Melville, NY), and these values were used to calculate percent wound closure, as previously described [36]. Two wells per condition per cell strain were cultured, and three regions per well were analyzed; the means of six scratch wounds per group were calculated to obtain a mean value for each cell strain in each condition. These values were used to calculate group means for each cell type (normal or keloid) in each condition.

Immunohistochemistry

Analysis of CYP24A1 protein localization was performed using standard immunohistochemistry techniques described in detail elsewhere [37]. Briefly, biopsies of keloid lesions or normal human skin were fixed in 10% buffered neutral formalin, and tissues were processed, embedded in paraffin and sectioned by the Histopathology Core Facility at the UC College of Medicine. Sections were deparaffinized in xylene followed by a graded alcohol series [37], and antigen retrieval was performed using Citric Acid-Based Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA). Immunohistochemistry was performed using a rabbit anti-CYP24A1 polyclonal antibody (Catalog #21582-1-AP; Proteintech Group Inc., Rosemont, IL) diluted 1:200 and incubated with sections for 16 h at 4°C. Detection was performed using the Vectastain Elite ABC Kit (Vector Laboratories, Newark, CA) with ImmPact NovaRed Peroxidase Substrate (Vector Laboratories). To control intensity of color development, all sections were incubated with the substrate for the same length of time, 60 s. Primary antibody was omitted for the negative controls. Counterstaining of nuclei was performed using Vector Hematoxylin QS (Vector Laboratories).

Analysis of gene expression

Keratinocytes were cultured in keratinocyte growth medium with 0 or 100 nM vitamin D, combined with 0 or 10 μM ketoconazole or 0 or 100 nM VID400, and cells were harvested for analysis 24 h later. Total RNA was isolated from

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Table 2. Primers used for gene expression analyses, purchased from Qiagen

Gene name	Gene symbol	Accession number	RT ^{2} PCR primer code (GeneGlobe ID)
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	NM_002046	PPH00150F-200
Vitamin D receptor	*VDR*	NM_000376	PPH02123F-200
Cytochrome P450 family 24 subfamily A member 1	*CYP24A1*	NM_000782	PPH01279A-200
Cytochrome P450 family 27 subfamily A member 1	*CYP27A1*	NM_000784	PPH01277A-200
Cytochrome P450 family 27 subfamily B member 1	*CYP27B1*	NM_000785	PPH01242A-200
Cathelicidin antimicrobial peptide	*CAMP*	NM_004345	PPH09430A-200
CD14	*CD14*	NM_000591	PPH05723A-200
Hyaluronan synthase 2	*HAS2*	NM_005328	PPH13147A-200
Frizzled 7	*FZD7*	NM_003507	PPH02420B-200
Wnt family member 5A	*WNT5A*	NM_003392	PPH02410A-200
Periostin	*POSTN*	NM_006475	PPH12343B-200
Matrix metallopeptidase 1	*MMP1*	NM_002421	PPH00120B-200
Matrix metallopeptidase 3	*MMP3*	NM_002422	PPH00235F-200

keratinocytes using the Qiagen RNeasy Mini Kit (Qiagen, Inc., Germantown, MD), and RNA was treated with DNase I (Qiagen) prior to preparation of cDNA using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher). Real-time quantitative PCR (qPCR) was performed using gene-specific primers purchased from Qiagen (Catalog No. 330001). Specific primers used, and corresponding GenBank accession numbers and GeneGlobe ID numbers, are listed in Table 2. Amplification was performed using PowerSYBR Green qPCR Mastermix (Thermo Fisher Scientific) using the StepOne Plus Real-Time PCR System (Thermo Fisher Scientific). Expression levels were referenced to *GAPDH* using the comparative $2^{-\Delta\Delta Ct}$ method [45]. In addition to performing duplicates of cell cultures for each strain and condition, each RNA sample was analyzed in triplicate by qPCR. To obtain relative gene expression levels, the means of triplicate cycle threshold (Ct) values were calculated for each sample and used to determine the mean Ct values of cell culture duplicates, and these values were used to calculate fold changes for each sample type by normalizing to mean expression in normal keratinocytes or untreated cells of the same type; the normalization scheme for each experiment is indicated in the figure legends.

Statistical analyses

Statistical analyses via *t* test (two groups), or one-way analysis of variance (ANOVA; >2 groups) with post-hoc Tukey test for pairwise comparisons, were performed using SigmaPlot 15.0 (Systat Software, San Jose, CA). Differences were considered statistically significant at $p < 0.05$. Quantitative data are plotted as means ± standard deviations.

Results

Effect of vitamin D treatment on proliferation of normal and keloid keratinocytes *in vitro*

Based on the reduced relative expression and nuclear localization of VDR in keloid lesions compared with normal skin that were previously reported [24, 37], we were interested in determining whether keloid keratinocytes can respond to vitamin D treatment in a manner similar to normal keratinocytes. First, we evaluated different concentrations of vitamin D in normal and keloid keratinocytes to measure the effects on cellular proliferation. We observed a trend toward decreased proliferation with increasing levels of vitamin D (0–100 nM) after 24 h of treatment, although the differences in proliferation were not statistically significant (Figure 1). Additionally, no statistically significant differences in proliferation were found between normal keratinocytes and keloid keratinocytes treated with vitamin D at the concentrations tested in this study (Figure 1).

Effects of vitamin D treatment on expression of vitamin D target genes

Next, we were interested in determining whether keloid-derived keratinocytes are capable of responding to vitamin D treatment with upregulation of target genes. For this analysis, we selected the known target genes cathelicidin antimicrobial peptide (*CAMP*) and *CD14*, which are involved in the innate immune response [46], and *CYP24A1*, which encodes a 24-hydroxylase that inactivates vitamin D as part of a negative feedback loop to limit vitamin D levels, thereby preventing hypercalcemia, and is considered a sensitive reporter of vitamin D activity [47, 48]. We found that vitamin D treatment of both normal and keloid keratinocytes resulted in significant upregulation of all three target genes (Figure 2). In keloid keratinocytes, vitamin D significantly induced expression of *CYP24A1, CAMP* and *CD14*, indicating that these cells are competent to respond to vitamin D treatment with similar changes in target gene expression to those observed in normal keratinocytes.

Overexpression of *CYP24A1*, encoding 24-hydroxylase, in keloid-derived keratinocytes and keloid lesions

We compared the levels of basal gene expression (non-vitamin D stimulated) between normal and keloid-derived keratinocytes for *VDR* and genes encoding enzymes involved

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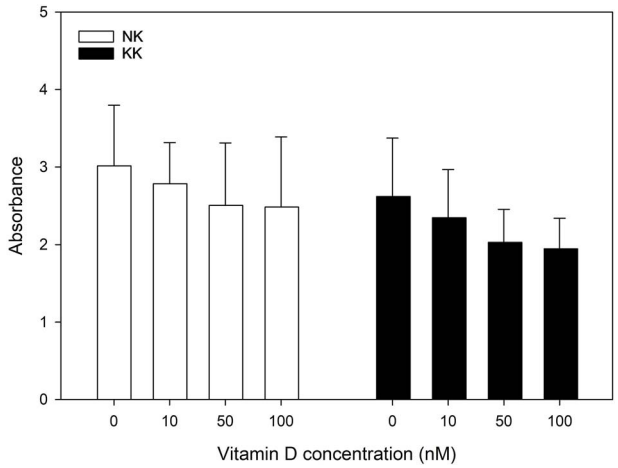

Figure 1. Proliferation of normal and keloid-derived keratinocytes treated with vitamin D. Normal keratinocytes (NK, white bars; $n = 3$ strains) and keloid keratinocytes (KK, black bars; $n = 4$ strains) were cultured in the presence of vehicle or active vitamin D (0–100 nM) for 24 h, and proliferation was measured using an MTT assay. Absorbance at 570 nm was measured, and data are plotted as means ± standard deviations. Absorbance values are proportional to cell number, with higher values reflecting greater proliferation levels. Although a trend toward decreasing proliferation with increasing vitamin D concentrations was observed, no statistically significant differences were found among different vitamin D concentrations or between normal and keloid keratinocyte groups. *MTT* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

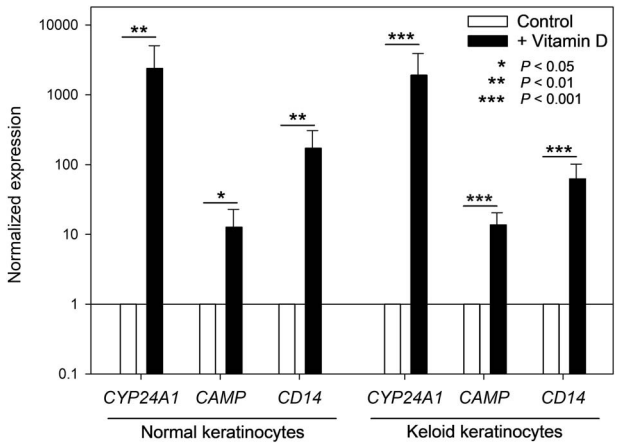

Figure 2. Vitamin D-stimulated gene expression in normal and keloid keratinocytes. Normal keratinocytes (left) and keloid-derived keratinocytes (right) respond to vitamin D treatment (100 nM for 24 h) with significant upregulation of target genes *CYP24A1,* cathelicidin antimicrobial peptide (*CAMP*), and *CD14*. Expression levels in vitamin D-treated cells (black bars) were normalized to values for untreated keratinocytes (control; white bars) of the same type. Mean values ± standard deviations are plotted ($n = 6$ for normal keratinocytes and $n = 9$ for keloid keratinocytes). Asterisks indicate statistically significant differences ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$)

in vitamin D metabolism, including *CYP27A1*, *CYP27B1* and *CYP24A1*. *CYP27A1* encodes sterol 27-hydroxylase, and *CYP27B1* encodes 25-hydroxyvitamin D3 1α-hydroxylase, both of which are involved in the production of active vitamin D. Levels of expression were variable among strains, but no significant differences in expression of *VDR*, *CYP27A1* or *CYP27B1* were observed between normal and keloid

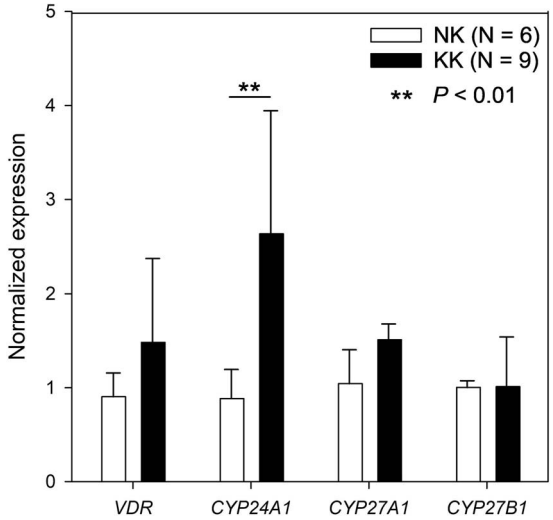

Figure 3. Expression of VDR and genes encoding enzymes involved in vitamin D metabolism in normal and keloid keratinocytes. Gene expression in normal keratinocytes (NK; $n = 6$ strains, white bars) and keloid-derived keratinocytes (KK; $n = 9$ strains, black bars) was analyzed by qPCR. Results were normalized to the mean expression in normal keratinocytes and data are plotted as means ± standard deviations. Statistical comparisons between normal and keloid keratinocytes were analyzed via *t* test; only *CYP24A1* expression was significantly different ($^{**}p < 0.01$) between normal and keloid-derived cells. *VDR* Vitamin D receptor

keratinocytes. However, expression of *CYP24A1* was found to be significantly elevated in keloid-derived keratinocytes compared with normal keratinocytes (Figure 3).

To determine whether expression of CYP24A1 is aberrantly expressed in keloid lesions, we used immunohistochemistry to assess localization of CYP24A1 protein in sections of keloids and normal human skin. In the epidermis of keloid lesions, diffuse CYP24A1-specific staining was observed as well as punctate cytoplasmic staining consistent with localization to mitochondria (Figure 4a–c). Lower levels of staining were observed in normal skin (Figure 4g–i). Although the levels of staining were variable among samples in each group, as illustrated in Figure 4, overall, the pattern indicated higher levels of staining in keloid samples compared with normal skin.

Effects of 24-hydroxylase inhibition on keratinocyte proliferation, migration and gene expression

To begin to understand the potential consequences of *CYP24A1* overexpression in keloid-derived keratinocytes, we investigated two inhibitors of CYP24A1 activity. The first, ketoconazole, is an antifungal that is also a broad inhibitor of cytochrome P450 enzymes [49], and the second, VID400, is a selective inhibitor of CYP24A1 [50]. Note that neither inhibitor directly interferes with *CYP24A1* transcription; instead, they inhibit CYP24A1 enzyme activity. We examined the effects of ketoconazole and VID400 on keratinocyte proliferation in the absence or presence of vitamin D. In the absence of vitamin D, ketoconazole inhibited proliferation of

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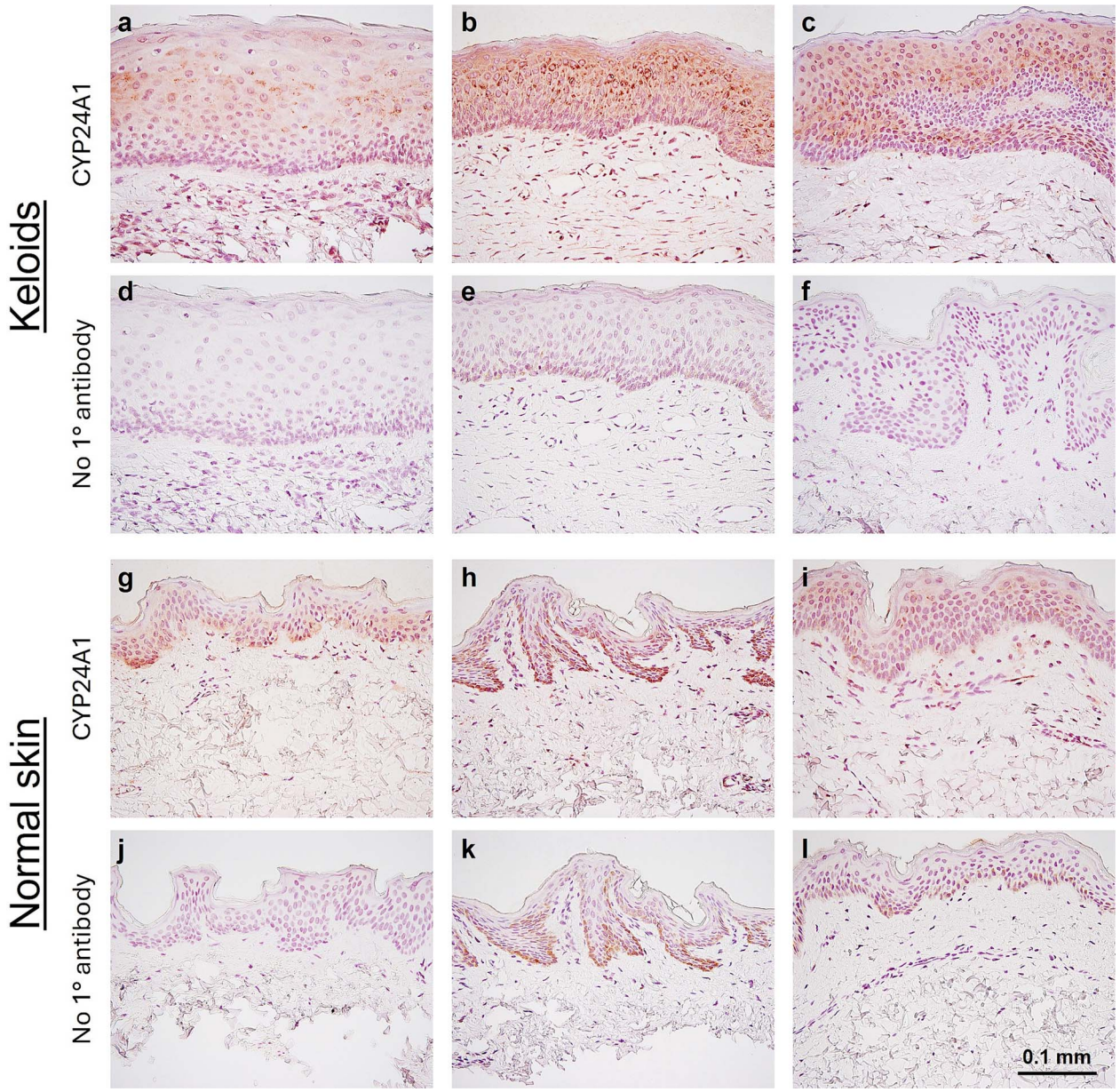

Figure 4. Expression of CYP24A1 protein in keloids and normal skin samples. Immunohistochemistry was used to localize CYP24A1 in sections of keloids (**a–c**) and normal skin (**g–i**). Negative controls were processed identically but without primary antibody (**d–f** for keloid samples; **j–l** for normal skin samples). Levels of CYP24A1 were variable among samples within each group, keloid or normal skin, but staining levels were higher overall in the keloid samples; representative sections illustrating this variability are shown. Keloid samples from donors 843K (**a**, **d**), 934K (**b**, **e**), and 991K (**c**, **f**) are shown, as are normal skin samples from donors 880 (**g**, **j**), 886 (**h**, **k**) and 974 (**i**, **l**); see Table 1 for donor demographic information. The scale bar in panel **l** (0.1 mm) is the same for all images

both normal and keloid keratinocytes, with a dose–response relationship observed at concentrations ranging from 1 to 30 μM (Figure 5a). In contrast, VID400 only significantly inhibited proliferation of keloid keratinocytes, with a dose–response relationship observed at concentrations ranging from 25 to 400 nM; although not statistically significant, a similar trend was observed in normal keratinocytes (Figure 5b). Combination of ketoconazole or VID400 with vitamin D had no significant effect on proliferation of normal keratinocytes, but ketoconazole inhibited proliferation of keloid keratinocytes in the absence or presence of vitamin D (Figure 5c). Interestingly, there were no synergistic effects on proliferation observed when either inhibitor was combined with vitamin D. No significant differences were observed between cells treated with either inhibitor alone versus that inhibitor combined with vitamin D (Figure 5c).

The effects of vitamin D with and without CYP24A1 inhibition on keratinocyte migration were analyzed using an *in vitro* wound healing assay. Neither ketoconazole nor VID400 had a significant effect on normal or keloid keratinocyte migration in the absence of vitamin D. Interestingly, vitamin D had a pro-migratory effect on normal

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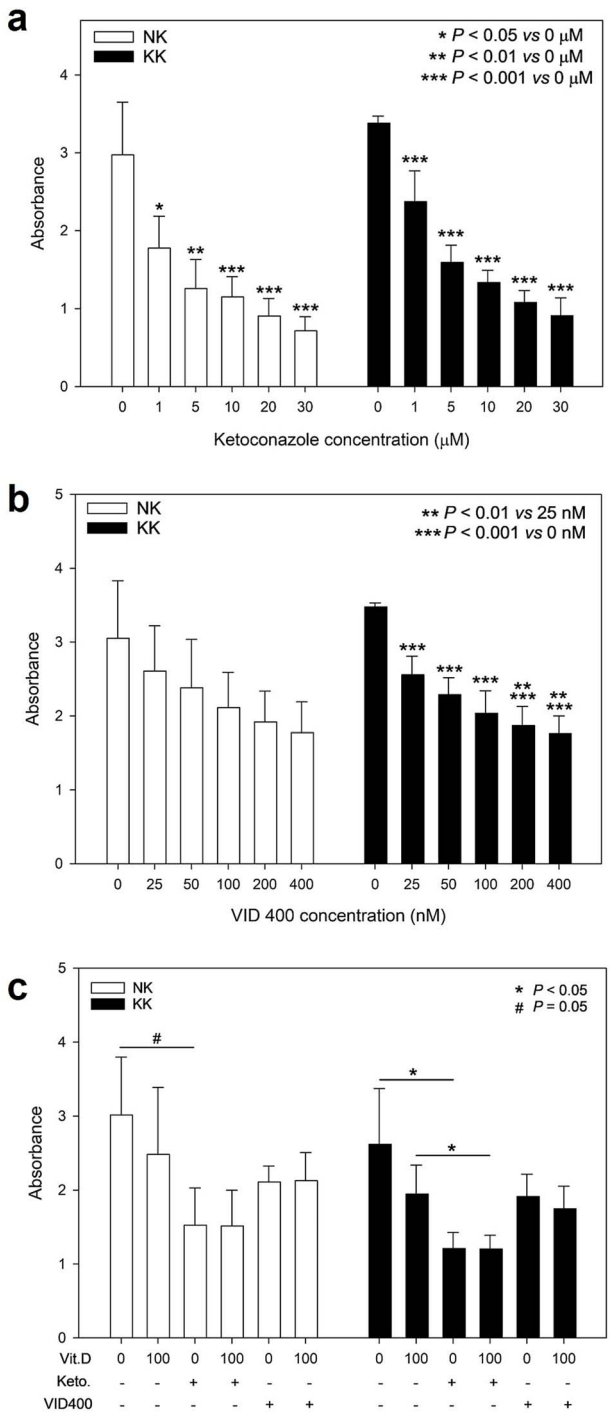

Figure 5. Effects of CYP24A1 inhibition, without or with vitamin D treatment, on keratinocyte proliferation. Normal keratinocytes (NK; white bars; $n = 3$ strains) and keloid keratinocytes (KK; black bars; $n = 4$ strains) were cultured in the absence or presence of ketoconazole, a non-specific CYP24A1 inhibitor (**a**), or VID400, a specific CYP24A1 inhibitor (**b**), with or without vitamin D (**c**), and proliferation was measured via MTT assay. Absorbance at 570 nm was measured, and data are plotted as means ± standard deviations. Statistical analyses were performed using one-way ANOVA, and pairwise comparisons were analyzed using a post hoc Tukey test. (**a**) Normal and keloid keratinocytes were cultured for 24 h with ketoconazole at concentrations ranging from 0 to 30 μM, as indicated. A statistically significant dose–response relationship was observed in both normal and keloid keratinocytes. (**b**) Normal and keloid keratinocytes were cultured for 24 h with VID400 at concentrations ranging from 0 to 400 nM, as indicated. Statistically significant differences were only observed for keloid keratinocytes. (**c**) Normal and keloid keratinocytes were treated for 24 hs with 100 nM vitamin D, ±10 μM ketoconazole or 100 nM VID400, as indicated. No statistically significant differences were observed among any of the normal keratinocyte groups, although the difference between untreated and ketoconazole-treated normal keratinocytes approached statistical significance ($^{\#}p = 0.05$). In keloid keratinocyte groups, ketoconazole treatment caused a significant reduction in keloid keratinocyte proliferation in the absence or presence of vitamin D. Statistically significant differences are indicated in each plot by asterisks ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). *ANOVA* Analysis of variance

keratinocytes, such that migration was significantly enhanced in cells treated with vitamin D + ketoconazole compared with ketoconazole alone, and vitamin D + VID400 compared with VID400 alone (Figure 6a). Migration was increased in normal keratinocytes treated with vitamin D alone compared with untreated cells, but this difference was not statistically significant (Figure 6a). No significant differences in migration were observed in keloid keratinocytes; however, this may be due to larger standard deviations, as migration rates were more variable among the different keloid keratinocyte donor strains than among the normal strains (Figure 6b).

We next examined the effects of inhibition of CYP24A1 enzyme activity on gene expression in normal and keloid keratinocytes. In addition to the *CYP24A1*-encoded 24-hydroxylase being involved in vitamin D metabolism, expression of *CYP24A1* mRNA is considered a sensitive reporter of 1,25(OH)2D3 activity [51]. In the absence of vitamin D treatment, both ketoconazole and VID400 significantly altered the expression of *CYP24A1*, although ketoconazole inhibited *CYP24A1* expression, whereas VID400 increased *CYP24A1* expression (Figure 7a). Because ketoconazole inhibits multiple CYPs, including CYP27B1 [49], it may be inhibiting the production of active vitamin D by keratinocytes, in addition to inhibiting the inactivation of vitamin D that is present. In contrast, VID400, which is specific for CYP24A1, significantly increased *CYP24A1* expression, suggesting that it increased local vitamin D levels. No synergistic effects were observed when the inhibitors were combined with vitamin D treatment. In normal keratinocytes, addition of ketoconazole with vitamin D increased *CYP24A1* expression by ~2-fold (range, 1.8–3.3X) over vitamin D treatment alone, but this change was not statistically significant due to strain-to-strain variability. In keloid keratinocytes, the increase in *CYP24A1* expression in ketoconazole + vitamin D treated cells versus vitamin D treatment alone was only 1.5-fold and was not statistically significant. Addition of VID400 to vitamin D resulted in a roughly 2.5-fold increase in *CYP24A1* compared with vitamin D alone (range, 1.8–3.3X), although this difference was not statistically significant (Figure 7a).

Expression of VDR in normal and keloid keratinocytes, in the presence or absence of vitamin D, was variably affected by the inhibitors. In normal keratinocytes, ketoconazole treatment had no significant effect on *VDR* mRNA levels, but

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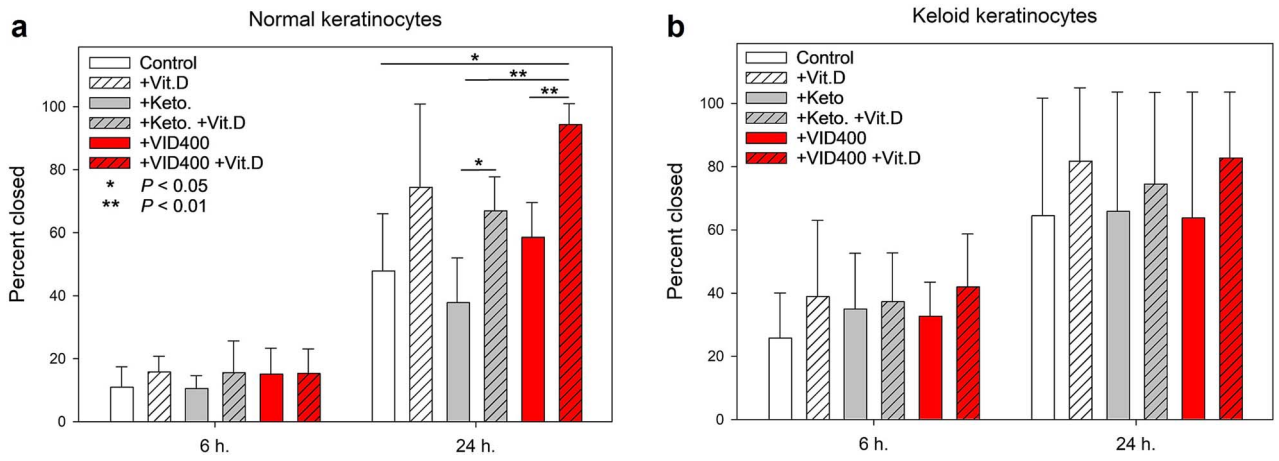

Figure 6. Effects of CYP24A1 inhibition and vitamin D on keratinocyte migration. Keratinocytes were cultured in the absence (non-patterned bars) or presence (patterned bars) of 100 nM vitamin D, and in the absence or presence of CYP24A1 inhibitors ketoconazole or VID400 (red bars). Migration was measured using an *in vitro* scratch wound assay at 6 and 24 h after wounding, and the percent of the wound closed was calculated at each time point. Data are plotted as means ± standard deviations. Statistical analyses were performed using one-way ANOVA, and pairwise comparisons were analyzed using a post hoc Tukey test. (**a**) In normal keratinocytes (n = 3 donor strains), neither inhibitor had a significant effect on cell migration at 6 or 24 h in the absence of vitamin D. Vitamin D treatment added with either ketoconazole or VID400 significantly increased migration rates at 24 h compared to either inhibitor alone. Additionally, VID400 + vitamin D significantly increased migration compared with untreated control cells. Statistically significant differences are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$). (**b**) In keloid keratinocytes, no significant differences in migration among groups were observed at either time point. However, there was greater variability in migration rates among the different keloid keratinocyte cell strains, with correspondingly large standard deviations, which may have impacted this analysis. *ANOVA* analysis of variance

treatment with VID400 in the absence of exogenous vitamin D treatment significantly induced *VDR* expression; this increase was counteracted when VID400 and vitamin D were added simultaneously to cells (Figure 7b). In keloid keratinocytes, ketoconazole treatment significantly reduced *VDR* expression, in the absence or presence of vitamin D. The effects of VID400 treatment on *VDR* expression in keloid keratinocytes were variable, and no significant differences in *VDR* gene expression due to VID400 treatment were observed (Figure 7b).

Expression of the antimicrobial peptide *CAMP*, which is highly responsive to vitamin D treatment, was not significantly affected in normal keratinocytes by treatment with either ketoconazole or VID400 (Figure 7c). Responses to treatment were dominated by the relatively strong effects of vitamin D on induction of *CAMP* expression. However, in keloid keratinocytes, ketoconazole treatment in the absence of exogenous vitamin D caused a significant reduction in *CAMP* expression (Figure 7c), suggesting a reduction of endogenous vitamin D production by ketoconazole. *CD14* expression was significantly increased upon vitamin D treatment of keloid keratinocytes; although *CD14* expression was increased over 100-fold on average in normal keratinocytes upon vitamin D treatment, the difference was not statistically significant, likely due to strain-to-strain variability in the magnitude of increase. In normal keratinocytes, ketoconazole caused a significant decrease in *CD14* expression in the absence of vitamin D, and a significant increase when combined with vitamin D treatment compared with vitamin D alone (Figure 7d). Similarly, treatment of keloid keratinocytes with ketoconazole caused a significant decrease in CD14 expression, and although combination of ketoconazole and vitamin D caused an increase in *CD14* expression compared with vitamin D alone, this difference was not statistically significant. VID400 alone did not have any significant effects on *CD14* expression in either normal or keloid keratinocytes.

Because EMT has been previously implicated in keloid pathology [12, 33, 34], we investigated the expression of genes with roles in the EMT process in normal and keloid keratinocytes. Endothelin 1 (EDN1) is a secreted peptide that is a potent vasoconstrictor and mitogen that can stimulate recruitment of immune cells in inflammation [52]. EDN1 is considered a profibrotic agent and has been shown to promote EMT in multiple different cell types, where it can contribute to cancer progression [53–55], and it was shown to be upregulated in keloids [56]. We found that vitamin D treatment significantly reduced *EDN1* expression in keloid keratinocytes but not normal keratinocytes (Figure 7e). Treatment of keloid keratinocytes with either ketoconazole or VID400 significantly decreased *EDN1* expression in the absence of vitamin D treatment. In addition, combination of either inhibitor with vitamin D significantly reduced *EDN1* expression in keloid keratinocytes compared with vitamin D treatment alone. Although similar trends were observed with inhibitor treatment of normal keratinocytes, most of the observed differences were not statistically significant, likely due to strain-to-strain variability. Ketoconazole treatment significantly reduced *EDN1* expression in normal keratinocytes, but no other significant differences were observed in this group. We also examined expression of two WNT signaling pathway genes that we previously demonstrated to be upregulated in keloid keratinocytes, *WNT5A* and the WNT receptor Frizzled 7 (*FZD7*) [12], which have also been

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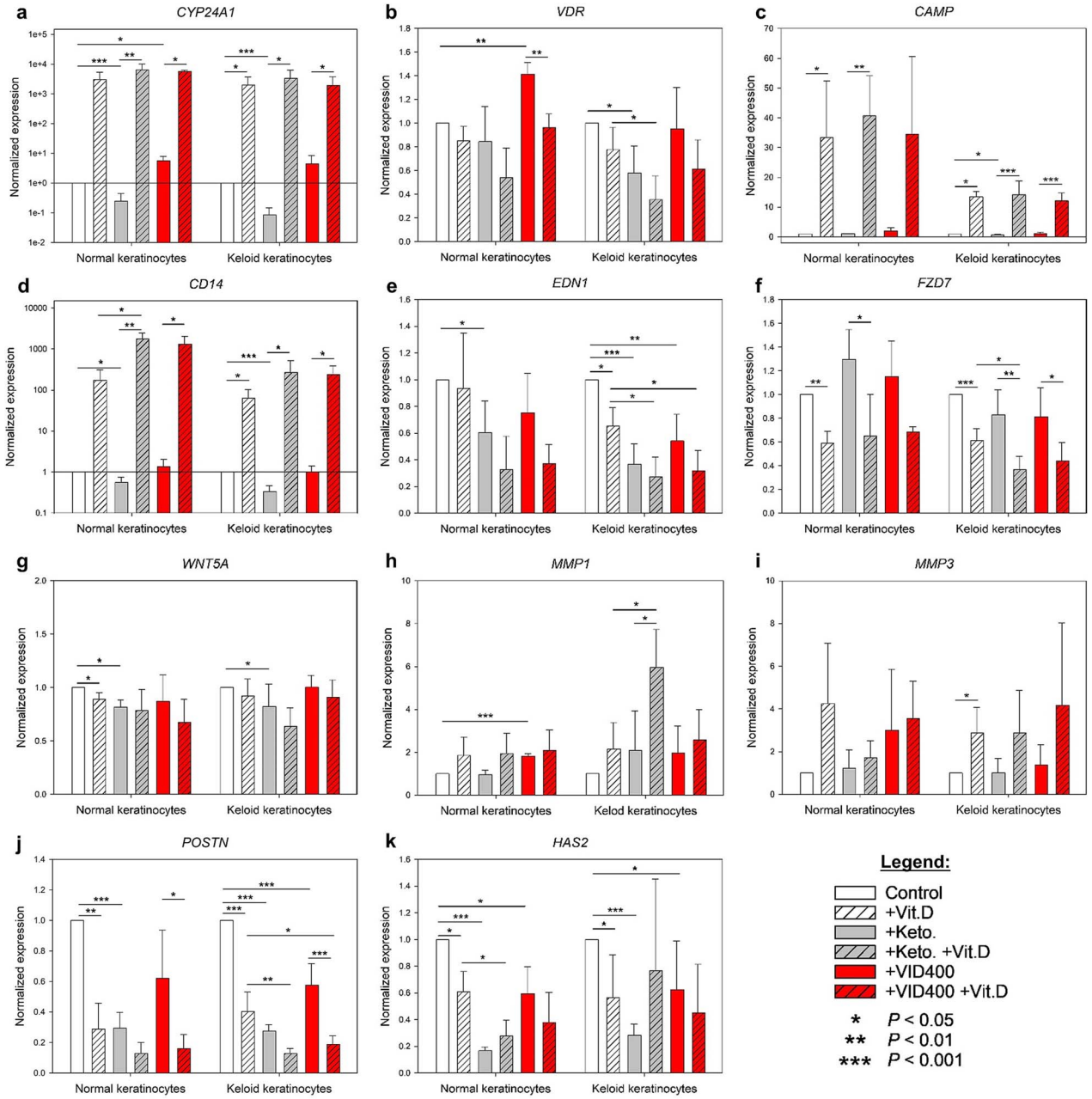

Figure 7. Gene expression in normal and keloid keratinocytes in response to vitamin D and CYP24A1 inhibition. Keratinocytes were cultured in the absence (non-patterned bars) or presence (patterned bars) of 100 nM vitamin D, and in the absence or presence of CYP24A1 inhibitors ketoconazole (gray bars) or VID400 (red bars); see legend (bottom right of figure) for details. Gene expression was analyzed by quantitative PCR, and expression levels were normalized to expression in control, untreated cells of the same type, normal or keloid, as indicated (white, open bars). Plotted are mean normalized expression levels ± standard deviations for *CYP24A1* (**a**), *VDR* (**b**), *CAMP* (**c**), *CD14* (**d**), *EDN1* (**e**), *FZD7* (**f**), *WNT5A* (**g**), *MMP1* (**h**), *MMP3* (**i**), *POSTN* (**j**) and *HAS2* (**k**). Statistical comparisons within each cell type for each gene (normal keratinocytes, left; keloid keratinocytes, right) were performed using one-way ANOVA with post hoc Tukey test, and significant differences are indicated by asterisks: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. *ANOVA* Analysis of variance, *CAMP* Cathelicidin antimicrobial peptide, *EDN1* Endothelin 1, *FZD7* Frizzled 7, *HAS2* Hyaluronan synthase 2, *MMP1* Matrix metallopeptidase 1, *MMP3* Matrix metallopeptidase 3, *POSTN* Periostin

implicated in EMT during cancer progression [57, 58]. Treatment of normal or keloid keratinocytes with vitamin D significantly reduced expression of *FZD7* (Figure 7f). Neither inhibitor had a significant effect on expression of *FZD7* in normal or keloid keratinocytes in the absence of vitamin D, but the combination of vitamin D plus ketoconazole significantly reduced *FZD7* in keloid keratinocytes compared with vitamin D treatment alone (Figure 7f). Treatment of normal keratinocytes with vitamin D resulted in a modest but statistically significant reduction in *WNT5A* expression, as did treatment with ketoconazole (Figure 7g). In keloid keratinocytes, only ketoconazole treatment caused a small but significant reduction in *WNT5A* expression (Figure 7g).

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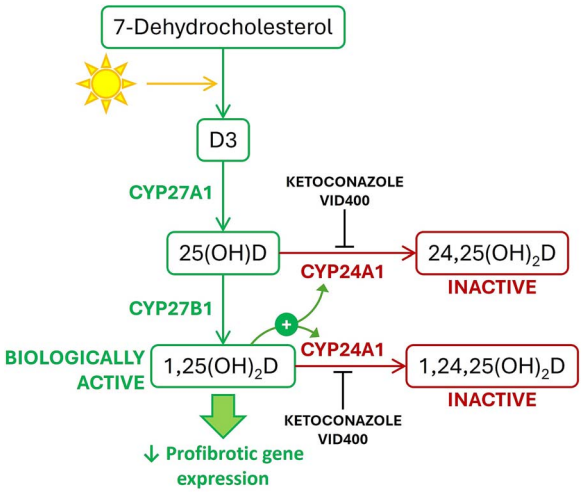

Figure 8. Production of vitamin D in the skin. Vitamin D3 (D3) is synthesized in keratinocytes from the precursor 7-dehydrocholesterol in response to ultraviolet radiation in sunlight. D3 is metabolized to 25-hydroxy vitamin D (25(OH)D), the form most commonly measured in the blood as a marker of vitamin D status, by CYP27A1. 25(OH)D is metabolized to 1,25-dihydroxyvitamin D (1,25$(OH)_2$D), the hormonally active form of vitamin D, by CYP27B1. The enzyme CYP24A1 inactivates both 25(OH)D and 1,25$(OH)_2$D. Transcription of the gene encoding CYP24A1 is induced by 1,25$(OH)_2$D, which serves as a feedback loop to modulate levels of active vitamin D. The inhibitors ketoconazole and VID400 block the activity of CYP24A1, which may lead to decreased profibrotic gene expression by decreasing inactivation of 1,25$(OH)_2$D

Next, we examined expression of matrix metalloproteinases MMP1 and MMP3, which are involved in degradation of ECM and were reported to be expressed at reduced levels in keloids and keloid-derived cells compared with normal skin and normal skin cells [12, 59]. Treatment of normal or keloid keratinocytes with vitamin D in the absence of inhibitors increased expression of *MMP1* and *MMP3*, but there was substantial variability among strains, so that only the difference in *MMP3* expression between control and vitamin D-treated keloid keratinocytes was statistically significant (Figure 7h and i). Treatment of keloid keratinocytes with ketoconazole plus vitamin D caused a significant increase in *MMP1* expression compared with vitamin D or ketoconazole alone (Figure 7h). In normal keratinocytes, VID400 significantly increased *MMP1* expression compared with untreated cells, but no other changes in MMP1 expression were statistically significant.

Finally, we examined expression of two additional profibrotic genes with roles in EMT that were previously shown to be overexpressed in keloids and keloid-derived cells, periostin (*POSTN*) and hyaluronan synthase 2 (*HAS2*) [12, 36, 60]. We found that vitamin D treatment of both normal and keloid keratinocytes significantly reduced *POSTN* expression (Figure 7j). Additionally, ketoconazole treatment in the absence of vitamin D significantly reduced *POSTN* expression in normal keratinocytes, while both ketoconazole and VID400 significantly reduced *POSTN* expression in keloid keratinocytes. Furthermore, treatment with either inhibitor together with vitamin D significantly reduced *POSTN* expression in keloid keratinocytes compared with vitamin D treatment alone (Figure 7j). Expression of *HAS2* was also significantly reduced by vitamin D treatment in both normal and keloid keratinocytes (Figure 7k). Both inhibitors, in the absence of vitamin D, reduced *HAS2* expression in normal and keloid keratinocytes compared with untreated controls (Figure 7k). *HAS2* expression in cells treated with either inhibitor plus vitamin D was variable, and only the combination of ketoconazole plus vitamin D in normal keratinocytes resulted in a significant reduction in *HAS2* expression compared with vitamin D treatment alone. A schematic diagram illustrating the effects of CYP24A1 inhibition on profibrotic gene expression is shown in Figure 8.

Discussion

Previous studies from our laboratory and others have implicated vitamin D signaling through the VDR in keloid pathology [23, 24, 37, 61]. In particular, reduced expression and nuclear localization of VDR in keloid tissues have been reported [24, 37], and reduced VDR expression was observed in peripheral blood lymphocytes of keloid patients compared with non-keloid control patients [61]. Additionally, decreased mean circulating vitamin D levels and increased rates of vitamin D deficiency have been reported in keloid patients compared with control patient cohorts [23, 24], and serum vitamin D levels were found to be inversely correlated to keloid severity, such that patients with the lowest vitamin D levels had the most severe keloid lesions [25]. Because vitamin D signaling through VDR has anti-inflammatory and anti-proliferative activities in numerous cell types and has been shown to repress EMT [32, 62, 63], it may have therapeutic value in suppression of keloid pathology. However, reduction of VDR expression levels, and/or reduction of VDR nuclear localization (which may be related to levels of vitamin D present [64–66]), may render keloid cells resistant to the therapeutic effects of vitamin D or other anti-keloid interventions. A similar phenomenon has been described in the cancer field: reduced *VDR* expression in tumor cells has been associated with cancer progression and drug resistance, while increasing *VDR* expression can reverse drug-resistance and promote vitamin D susceptibility of cancer cells [67–71].

Although correlations between circulating vitamin D levels and VDR expression have been reported in patients with keloids [24], we do not yet know whether the reduction in VDR expression observed in keloids is due to systemic vitamin D deficiency in these patients. Furthermore, it is unclear whether restoration of sufficient vitamin D levels may stimulate *VDR* mRNA expression and/or stabilize VDR protein *in vivo*. However, studies in rats suggested that epidermal *VDR* expression was reduced in vitamin D deficient animals compared with controls, and *VDR* expression could be restored by dietary vitamin D supplementation to sufficient levels [72]. Although the effects of vitamin D supplementation on *VDR* expression in keloids have not yet been investigated,

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a clinical study of patients with atopic dermatitis suggests this is feasible [73]. As with keloid disorder, an inverse correlation between vitamin D status and severity of atopic dermatitis has been reported [74]; oral supplementation with vitamin D to improve vitamin D status in atopic dermatitis patients was associated with an increase in expression of *VDR*, as well as *CAMP*, in skin lesions [73]. In addition, a study investigating topical vitamin D treatment in a small number of healthy adult patients showed that VDR protein levels in skin increased following 4 days of exposure [66]. While we do not yet know if vitamin D replacement therapy can increase expression of VDR in keloid lesions, or whether this would reduce fibrosis, the results of the current study suggest that keloid-derived keratinocytes are capable of expressing functional VDR. The data suggest that despite reduced levels of VDR protein in keloid lesions *in vivo*, keloid keratinocytes are competent to respond to vitamin D stimulation with appropriate upregulation of target genes and display responses similar to normal keratinocytes following vitamin D stimulation *in vitro*.

The results of this study indicate that ketoconazole significantly decreases proliferation of both normal and keloid keratinocytes, with a dose–response relationship observed. The inhibition of proliferation by VID400 also suggested a dose–response relationship, but this was only statistically significant in keloid keratinocytes; however, a trend for decreased proliferation with increased VID400 concentrations was observed in normal keratinocytes. This suggests the possibility that treatment of wounds with either ketoconazole or VID400 alone may inhibit wound closure, although neither inhibitor significantly inhibited keratinocyte migration in an *in vitro* scratch wound assay. For therapeutic suppression of excessive scarring, treatment could be initiated after the wound has closed to prevent possible negative effects on re-epithelialization. Interestingly, migration was increased in normal keratinocytes treated with VID400 + vitamin D compared with either VID400 alone or untreated cells, without significant impacts on proliferation, suggesting the possibility that combination of VID400 with vitamin D may accelerate wound closure. A recently published study utilizing a mouse excisional wound model found that wound closure was delayed in vitamin D-deficient mice, but was enhanced by vitamin D supplementation [75]. This same study reported enhanced *in vitro* migration of HaCaT keratinocytes, an immortalized keratinocyte cell line, treated with vitamin D, consistent with the results observed here [75]. Future *in vivo* wound healing studies will be required to fully investigate the effects of CYP24A1 inhibitors and vitamin D on wound closure.

Because ketoconazole and VID400 inhibit CYP24A1 enzyme activity (Figure 8), and therefore should block breakdown of active vitamin D in treated cells, we expected to see additive or even synergistic effects when these inhibitors were combined with exogenous vitamin D treatment of cells. However, that was not observed for many of the genes we examined. This may be due to the fact that we only tested a single, relatively high concentration of vitamin D (100 nM), which may have obscured any potential additive effects. Based on expression of *CYP24A1* mRNA as a reporter for VDR-mediated vitamin D activity [51], it appears that ketoconazole, which can inhibit enzyme activity of CYP27B1 in addition to CYP24A1 [49], may have had an overall negative effect on vitamin D levels in untreated cells, whereas VID400, which specifically inhibits CYP24A1 enzyme activity, led to increased vitamin D levels and therefore increased *CYP24A1* expression. We attempted to measure levels of active vitamin D produced in cultured keratinocytes in this study, but levels were below the limit of detection (data not shown). Future studies examining the effects of a range of vitamin D and inhibitor concentrations on gene expression are warranted.

CYP24A1 is a 24-hydroxylase and is the key enzyme that inactivates vitamin D as part of a feedback loop to regulate vitamin D levels to prevent hypercalcemia (Figure 8) [47]. By causing the degradation of hormonally active vitamin D, CYP24A1 limits vitamin D's biological activities [39]. The expression of *CYP24A1* is elevated in many different types of cancers, including the colon, lung, breast and ovary [41, 76]. Overexpression of *CYP24A1* in cancer cells is thought to reduce the bioavailability of hormonally active vitamin D, which may dampen the antiproliferative effects of vitamin D in tumors [47]. For example, in breast cancer cells, knockdown of *CYP24A1* increased susceptibility of cells to apoptosis and suppressed tumor growth in an *in vivo* model [41]. Thus, inhibitors of CYP24A1 have been investigated for anticancer activities [47]. As a downstream target and reporter of vitamin D activities in the cell [51], it may seem counterintuitive that *CYP24A1* expression levels are increased in cells with decreased expression of *VDR*, such as tumor cells, and, as suggested by the current study, in keloid-derived cells. However, *VDR* levels in tumors are often uncorrelated with *CYP24A1* levels, suggesting that *CYP24A1* overexpression in tumors is not dependent on VDR-mediated transcription [40]. Potential mechanisms for *CYP24A1* overexpression in cancers may involve gene amplification, epigenetic alterations and/or regulation by miRNA [40]. While we do not yet understand the mechanism underlying *CYP24A1* overexpression in keloid keratinocytes, the current study suggests that inhibitors of CYP24A1 activity may serve to increase local concentrations of active vitamin D in skin and may be novel candidates for suppression of keloid fibrosis.

Abnormal gene expression in keloid keratinocytes was previously described, including aberrant overexpression of genes involved in EMT, such as *EDN1*, *WNT5A*, *FZD7* and *HAS2*, and increased expression of profibrotic genes, such as *POSTN*, reflecting their profibrotic phenotype [12, 56]. Keratinocytes play a central role in regulation of fibroblast proliferation, differentiation, migration and ECM deposition, and a disruption of this paracrine regulation can result in dermal fibrosis [77]. During physiological wound healing, keratinocytes release factors that suppress ECM

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production and proliferation in fibroblasts; aberrant expression of these factors in scar keratinocytes drives profibrotic changes in fibroblasts [78–80]. For example, *in vitro* studies showed that fibroblasts co-cultured with keloid keratinocytes had increased proliferation rates and higher collagen production compared with fibroblasts cultured with normal keratinocytes [81, 82]. Thus, it is reasonable to speculate that interventions that suppress the profibrotic phenotype of keloid keratinocytes may normalize paracrine interactions between keratinocytes and fibroblasts, and may thereby serve to suppress fibroblast activation and reduce dermal fibrosis [77].

Conclusions

We found that treatment with vitamin D and/or inhibition of CYP24A1 activity in keloid keratinocytes alters gene expression to suppress genes previously found to be increased in keloid-derived cells, including profibrotic factors and genes involved in EMT, such as *EDN1*, *WNT5A*, *POSTN* and *HAS2*. Thus, treatment with vitamin D and/or inhibition of CYP24A1 activity may help to suppress fibrosis in keloid cells or, as in cancer cells, may sensitize cells to other therapeutic agents. In conclusion, our results suggest that vitamin D treatment and CYP24A1 inhibition should be further explored as potential treatments or therapeutic adjuncts for suppression of keloid development and growth.

Abbreviations

ANOVA: Analysis of variance; *CAMP*: Cathelicidin antimicrobial peptide; *CYP24A1*: Cytochrome P450 family 24 subfamily A member 1; *CYP27A1*: Cytochrome P450 family 27 subfamily B member 1; *CYP27B1*: Cytochrome P450 family 27 subfamily B member 1; ECM: extracellular matrix; EMT: epithelial-mesenchymal transition; *FZD7*: Frizzled 7; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase; *HAS2*,: Hyaluronan synthase 2; IRB: Institutional review board; *MMP1*: Matrix metalloproteinase 1; *MMP3*: Matrix metalloproteinase 3; *POSTN*: Periostin; UC: University of Cincinnati; *VDR*: Vitamin D receptor; *WNT5A*: Wnt family member 5A.

Conflicts of interest

The authors have no conflicts of interest relevant to the manuscript's content to declare.

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Authors' contributions

Jennifer M. Hahn (Conceptualization [supporting], Formal analysis [supporting], Investigation [lead], Methodology [supporting]), Kelly A. Combs (Formal analysis [supporting], Investigation [supporting], Methodology [supporting]), Caitlin M. Phillips (Investigation [supporting], Project Administration [supporting]), Petra M. Warner (Investigation [supporting], Resources [supporting]), Uzair Qazi (Investigation [supporting], Resources [supporting]), Heather M. Powell (Conceptualization [supporting], Funding acquisition [supporting], Project administration [supporting], Resources [supporting], Supervision [supporting]), Dorothy M. Supp (Conceptualization [lead], Formal analysis [lead], Funding acquisition [lead], Project administration [lead], Resources [lead], Supervision [lead]).

Ethics approval and consent to participate

Ethics approval was obtained from the University of Cincinnati IRB (Study ID# 2013-2166) and the Western-Copernicus Group (WCG) IRB (Study ID# OHI2201) for collection of pediatric tissue samples; these samples were obtained with informed consent from parents or guardians and assent from patients. Ethics approval was obtained from the University of Cincinnati IRB (Study ID# 2013-4582) for collection of adult tissue samples. This activity was designated 'not human subjects research' because samples were de-identified, thus patient consent was not required.

Consent for publication

Not applicable.

Data availability

Data supporting the results of this study can be obtained upon request from the senior author.

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