Arch Dermatol Res (2013) 305:867–878
DOI 10.1007/s00403-013-1390-1

ORIGINAL PAPER

Control of late cornified envelope genes relevant to psoriasis risk: upregulation by 1,25-dihydroxyvitamin D_3 and plant-derived delphinidin

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Received: 8 February 2013 / Revised: 21 June 2013 / Accepted: 28 June 2013 / Published online: 10 July 2013

Abstract Psoriasis is a chronic inflammatory skin disease featuring abnormal keratinocyte proliferation and differentiation. A genetic risk factor for psoriasis (PSORS4) is a deletion of LCE3B and LCE3C genes encoding structural proteins in terminally differentiated keratinocytes. Because analogs of 1,25-dihydroxyvitamin D_3 (1,25D) are used in psoriasis treatment, we hypothesized that 1,25D acts via the vitamin D receptor (VDR) to upregulate expression of LCE 3A/3D/3E genes, potentially mitigating the absence of LCE3B/LCE3C gene products. Results in a human keratinocyte line, HaCaT, suggested that 1,25D, low affinity VDR ligands docosahexaenoic acid and curcumin, along with a novel candidate ligand, delphinidin, induce LCE transcripts as monitored by qPCR. Further experiments in primary human keratinocytes preincubated with 1.2 mM calcium indicated that 1,25D and 10 μM delphinidin upregulate all five LCE3 genes (LCE3A–E). Competition binding assays employing radiolabeled 1,25D revealed that delphinidin binds VDR weakly ($IC_{50} \approx 1$ mM). However, 20 μM delphinidin was capable of upregulating a luciferase reporter gene in a VDRE-dependent manner in a transfected keratinocyte cell line (KERTr). These results are consistent with a scenario in which delphinidin is metabolized to an active compound that then stimulates LCE3 transcription in a VDR/VDRE-dependent manner. We propose that upregulation of LCE genes may be part of the therapeutic effect of 1,25D to ameliorate psoriasis by providing sufficient LCE proteins, especially in individuals missing the LCE3B and 3C genes. Results with delphinidin further suggest that this compound or its metabolite(s) might offer an alternative to 1,25D in psoriasis therapy.

Keywords Nuclear receptors · Skin barrier · Anthocyanidin · Late cornified envelope genes

Abbreviations

1,25D	1,25-Dihydroxyvitamin D_3
VDR	Vitamin D receptor
VDRE	Vitamin D responsive element
LCE	Late cornified envelope

Introduction

Psoriasis is a chronic inflammatory skin disease characterized by abnormal keratinocyte proliferation and differentiation that affects approximately 1 % of the US population [50]. It was recognized over two decades ago that raising blood 1,25-dihydroxyvitamin D_3 (1,25D) can dramatically improve psoriasis symptoms in some patients [49]. Shortly thereafter, 1,25D analogs were developed, including calcipotriene [39], tacalcitol and maxacalcitol [55] that are now routinely used as topical agents in psoriasis treatment along with, more recently, the natural 1,25D hormone [1]. The bioactions of 1,25D are mediated by the nuclear vitamin D receptor (VDR) expressed in many tissues, including skin [47]. However, specific genes

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that are regulated by 1,25D to improve psoriasis symptoms have yet to be defined.

Genome wide searches for psoriasis susceptibility loci have implicated as many as 36 chromosomal regions [58], including 12 named linkages (PSORS1-12). One of these, the PSORS4 locus, is associated with an assembly of skin differentiation genes on human chromosome 1 [11], designated the epidermal differentiation complex (EDC) [48] (Fig. 1). A reported PSORS4 risk allele consists of a deletion encompassing two genes (Fig. 1, lower right) encoding late cornified envelope 3B and 3C (LCE3B and LCE3C) proteins [14, 19, 31, 41, 51, 54, 60, 61]. This deletion is found in 63–72 % of patients with psoriasis according to ethnicity [31, 51]. LCE3B and LCE3C belong to a cluster of five LCE3 genes that are expressed late in keratinocyte differentiation, and the protein products of these genes are crosslinked to facilitate formation of the cornified envelope [43]. Transcripts of LCE3A, -3C, -3D and -3E are upregulated in psoriasis [6, 19]. Tape stripping to induce superficial skin injury also upregulates LCE3A/3C/3D/3E expression [6]. In contrast, other LCE genes, such as LCE2B (approximately 71 kb from the PSORS4 deletion), are expressed at moderate levels in normal skin, but are downregulated in psoriasis or superficial injury [6]. Taken together, these observations suggest that LCE3 genes might play a role in skin repair [6, 20]. This study examines VDR-regulated expression of each member of the LCE3 gene cluster, along with LCE2B, which is of interest not only because of its close proximity to the LCE3 gene cluster, but also because previous experiments revealed 1,25D upregulation of this gene in a human keratinocyte (CCD-1105 KERTr) cell line [27].

The ability of VDR ligands to upregulate these genes might facilitate the normalization of keratinocyte differentiation in psoriatic lesions or skin repair after superficial injury. Further, upregulation of LCE3A/3D/3E or even LCE2B could help compensate for the lost LCE3B and 3C genes in patients with the PSORS4 deletion. Since 49–69 % of the general population (depending on ethnicity) harbors a deletion of LCE3B and LCE3C [31, 51], yet only a subset of these individuals develops psoriasis, loss of LCE3B/3C is neither necessary nor sufficient to cause psoriasis. However, the LCE3B/3C deletion may lead to an imperfect repair response, because other LCE proteins may not be present in sufficient quantity to replace adequately the missing LCE3B/3C gene products [6, 7, 19]. The resulting impaired skin barrier might then elicit a T-cell response, contributing to the inflammation and hyperproliferation seen in psoriatic lesions.

Curcumin (CM) and docosahexaenoic acid (DHA) are recently recognized VDR ligands (Fig. 2) that have been shown in previous studies to bind directly to VDR [5, 25], establishing them as bona fide VDR ligands. We therefore hypothesized that CM and DHA might also upregulate LCE genes in a manner similar to 1,25D. Finally, the present study includes delphinidin (Del), a plant-derived compound that was shown to have anti-proliferative and anti-inflammatory actions in skin [32], and has recently been shown to stimulate keratinocyte differentiation including induction of involucrin, procaspase-14 and transglutaminase-1 [12]. Its steroid-like anthocyanidin structure (Fig. 2), plus its 1,25D-like ability to induce expression of caspases [24] and Bcl2 [2], suggested that Del might be a novel VDR ligand. Herein, we provide evidence that 1,25D, DHA, CM and Del all upregulate LCE genes. Finally, we present evidence that Del can bind directly to VDR, potentially rendering it (or a metabolite) a novel low affinity VDR ligand, although other potential mechanisms for its regulation of LCE genes are possible.

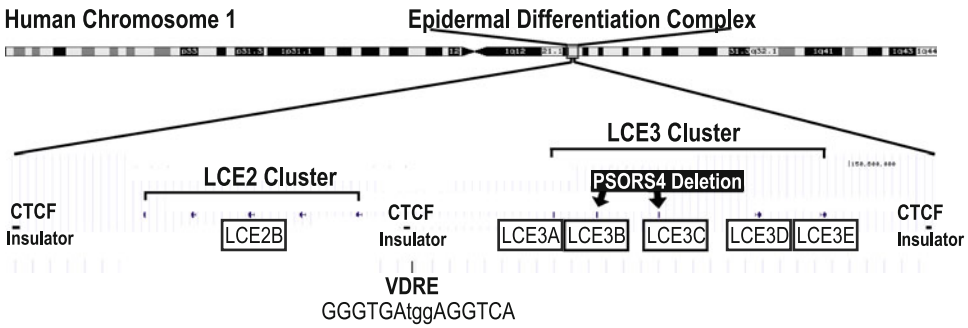

Fig. 1 Location of the PSORS4 deletion in the context of the LCE2 and LCE3 gene clusters. The PSORS4 locus for psoriasis risk has been localized to the epidermal differentiation complex (EDC) on human chromosome 1, which consists of numerous genes expressed in differentiated keratinocytes. A major proposed PSORS4 risk allele [19] contains a deletion that removes the LCE3B and LCE3C genes from the LCE3 cluster, but leaves other LCE genes intact. Both the LCE3 and the adjacent LCE2 gene clusters are bracketed by binding sites for the CTCF factor, which may serve as regulatory insulators to allow for separate transcriptional control of each LCE gene cluster [17]. A predicted VDRE is located approximately 29 kb upstream of LCE3A near the CTCF site that separates the LCE2 and LCE3 clusters

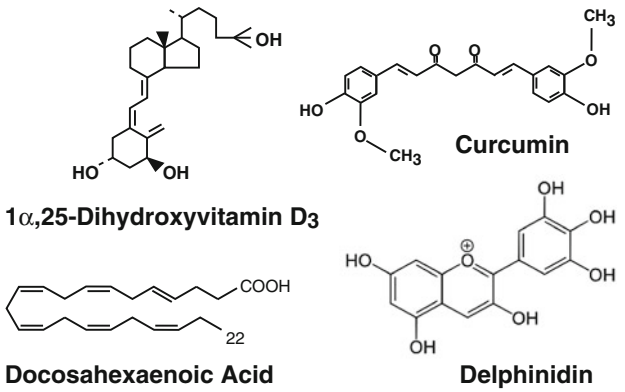

Fig. 2 Structures of known and proposed VDR ligands. The high affinity hormonal ligand, 1,25-dihydroxyvitamin D_3 (1,25D), and two recently published alternative, low affinity ligands, docosahexaenoic acid (DHA) [25] and curcumin [5] are shown along with delphinidin, an anthocyanidin with reported skin protective properties [2]. Delphinidin is proposed herein to be a low affinity VDR ligand, although it may influence gene transcription via other mechanisms

Materials and methods

Source of ligands

Crystalline 1,25D was a kind gift from Milan Uskokovic of Hoffmann-LaRoche. Delphinidin chloride, *cis*-4,7,10,13,16,19-docosahexaenoic acid and curcumin were obtained from Sigma Aldrich Corp., St. Louis, MO.

Cell lines and genotyping

The human keratinocyte cell lines HaCaT and CCD-1106 KERTr (KERTr), and the human embryonic kidney cell line HEK-293, were obtained from ATCC (Manassas, VA). HaCaT and HEK-293 were cultured in DMEM high glucose with 10 % fetal bovine serum, and KERTr cells were maintained in SFM Keratinocyte medium containing the recommended supplements (Invitrogen Corp, Carlsbad, CA) along with 5 μg/mL genticin. Culture media, fetal bovine serum, penicillin–streptomycin and genticin stocks were obtained from Gibco (Invitrogen Corp.). Human primary neonatal keratinocytes (HEKn) were purchased from Invitrogen Corp. (Carlsbad, CA) and cultured in serum-free EpiLife medium containing the recommended HKGS supplement kit reagents. Genotyping of these cell lines was as follows: genomic DNA was isolated from 4 to 5 million cells using a DNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A triple set of primers was utilized to detect the presence or absence of the LCE3B_LCE3C deletion as described by de Cid et al. [19]: LCE3CF 5′-TCACCCTGGAACTAGACCTCA; LCE3CR 5′-CTCC AACCACTTGTTCTTCTCA; and LCE3CR2D 5′-CATC CCAGGGATGCTGCATG. PCR method included 50–80 ng of DNA, 0.5 μL of an 18 μM primer stock and 5 μL of Fast Start Universal SYBR Green Master Mix (Roche Applied Science, Indianapolis, IN) in a 10-μL total volume. An ABI 2400 machine was programmed for 35 cycles of 94 C for 30 s, 60 C for 30 s and 72 C for 1 min, followed by a 72 C step for 10 min. PCR products were resolved on 3 % agarose gels. A single band at 199 bp indicates a homozygous deletion, a single band at 240 bp indicates a homozygous intact locus and the presence of both bands indicates a heterozygote [19].

Real-time PCR analysis of LCE gene expression

RNA was isolated using an Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA) from cells grown in 60 mm dishes to approximately 70 % confluence. Total RNA was assessed for purity and quantity by OD260/280 readings. First strand cDNA was synthesized using an iScript kit (Bio-Rad) and 2 μg of total RNA in a 40-μL reaction. Quantitative real-time PCR (qPCR) was performed with Fast Start Universal SYBR Green Master Mix (Roche Applied Science, Indianapolis, IN) in a System 7500 Fast thermal cycler using 2 μL of first strand DNA and 1 μL of 18 μM primer mixture in 20 μL total volume. For detection of human LCE transcripts, the primers were as follows: LCE2B, forward primer 5′-GCCAGCCCC CTCCCAAGTGT and reverse primer 5′-GGGCACTGGGG CAGGCATTT; LCE3A, forward primer 5′-GAGTC ACCACAGATGCC and reverse primer 5′-CTTGCTGA CCACTTCCC (3A primers from Ref. [6]); LCE3B, forward primer 5′-CCCAAAGAGCTCAGCACAGT and reverse primer 5′-TGCCTCTGTCACAGGAGTTG; LCE3C, forward primer 5′-AGTTGTCCCTCACCCAAGTG and reverse primer 5′-ATTGATGGGACCTGAAGTGC; LCE3D, forward primer 5′-CTCTGCACCTGGACAACTCA and reverse primer 5′-CACTTGGGTGAGGGACACTT; LCE3E, forward primer 5′-CTGATGCTGAGACAAGCGATCTT and reverse primer 5′-GATCCCCCACAGGAAAACCT (3E primers also taken from Ref. [6]). Human CYP24A1 was detected using forward primer 5′-CAGCGAACTGAACAA ATGGTCG and reverse primer 5′-TCTCTTCTCATACAAC ACGAGGCAG, and human GAPDH was amplified using forward primer 5′-TGACAACTTTGGTATCGTGGAAGG and reverse primer 5′-AGGGATGATGTTCTGGAGAGCC. Given the high similarity among LCE3 genes, the LCE2B, 3A, 3B, 3C, 3D and 3E PCR products were sequenced to confirm their specificity. Data were analyzed using the comparative Ct method, normalized to GAPDH. Fold effects were calculated relative to vehicle-treated control samples and expressed as $2^{-\Delta\Delta Ct}$ according to instructions in the Applied Biosystems software.

Competition assay to analyze VDR binding

The protocol for competition binding was as previously described [5], with modifications to the transfection and cell harvest procedures. VDR-deficient COS-7 cells (2.5×10^6 cells per 150-mm plate) were incubated overnight to attach, then transfected using ExpressIn reagent (Open Biosystems, Lafayette, CO). To 8.5 mL of serum-free medium were added 2 μg of pSG5-hVDR, 2 μg of pSG5-hRXRα and 17.5 μg of pTZ18U plasmid as carrier. This mixture was combined with an equal volume of serum-free medium containing 150 μL of ExpressIn reagent, incubated for 30 min, diluted to a total of 20 mL with serum-free medium, and added to cells for a 3-h incubation, and then 20 mL of medium containing 20 % FBS was added and the plate was incubated for 48 h. Cells were harvested by trypsinization and the cell pellet was resuspended in 1.0 mL of $KTEZ_{0.3}$ buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.3 mM zinc acetate, 0.3 M KCl) to which 5 mM dithiothreitol and mini EDTA-free protease inhibitors (Roche Applied Science, Indianapolis, IN) were freshly added. Resuspended cells were sonicated, ultracentrifuged, and the clarified supernatant was stored at −80 °C. For the competition assay, 1α,25-dihydroxy[26,27-methyl-^{3}H]cholecalciferol ([^{3}H]1,25D, 155 Ci/mmol, Perkin Elmer, Waltham, MA) was diluted to 54 Ci/mmol by drying down 36.4 μL of [^{3}H]1,25D with nitrogen and redissolving in 395.6 μL ethanol along with 4.25 μL of 1 μM unlabeled 1,25D. Each assay tube received 4–5 μL diluted [^{3}H]1,25D in a 209-μL total volume (final concentration 0.4 nM) containing 2–4 μL cell lysate, 196 μL $KTEZ_{0.3}$ + 2 and 4 μL of appropriately diluted ligands. After a 15-h incubation on ice, unbound 1,25D was removed by addition of 80 μL of dextran-coated charcoal (Sigma-Aldrich, St. Louis, MO, USA) for 15 min, followed by a 2 min microcentrifugation. Supernatants (200 μL) were combined with 4 mL of Scinti-Safe 30 % (Fisher Scientific, Pittsburgh, PA) and counted in a Beckman LS 5801 scintillation counter. Data were analyzed with Prism 4 software (GraphPad Software, San Diego, CA, USA).

Dual luciferase reporter (DLR) assay

ExpressIn (Thermo Scientific, Lafayette, CO) transfection reagent was utilized to transfect HEK-293 cells in 24-well plates (plated at 60,000 cells/well) according to the manufacturer's protocol. Briefly, each well was transfected with 2.0 μL ExpressIn Reagent, 250 ng of either empty pLUC-MCS plasmid or pLUC containing two copies of a VDRE (ttcaGGGTGAtggAGGTCAaatg) identified in the LCE3 gene cluster (Moffet and Whitfield, unpublished data—two VDRE half sites are underlined), 25 ng of pSG5-hVDR (plasmid expressing human VDR), 20 ng of pRL-null (*Renilla* luciferase reporter) and 1 μL of 100× sodium pyruvate. KERTr cells were similarly transfected utilizing 2.3 μL/well of TransIT keratinocyte reagent (Mirius Bio, Madison, WI) and the same amount of each plasmid DNA as described above for the ExpressIN transfections. After transfection, each well was treated with either 1×10^{-8} M 1,25D, various doses of delphinidin or ethanol vehicle for 20 h at 37 °C. Whole cell lysates were harvested and analyzed sequentially for Firefly luciferase and *Renilla* luciferase activity using a Dual Luciferase assay kit (Promega) and a Sirius Luminometer (Zylux Corp.) according to the manufacturers' protocols.

Results

To test whether the 1,25D, DHA, curcumin and delphinidin (Del) regulate LCE gene expression, experiments were first performed in a human keratinocyte cell line, HaCaT, treated for 24 h with each ligand, followed by RNA isolation and real-time PCR (Fig. 3). Final concentrations of 1,25D, DHA and curcumin were based on previous studies

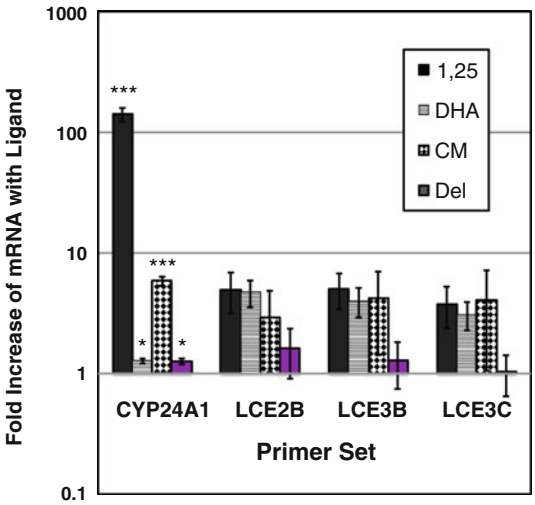

Fig. 3 Regulation of selected LCE genes in a human keratinocyte cell line, HaCaT. Cells were incubated for 24 h with ligands at a final concentration of 10 nM for 1,25D, 10 μM for docosahexaenoic acid (DHA), 5 μM for curcumin (CM) and 10 μM for delphinidin (Del). All ligands were dissolved in ethanol except for CM, which was dissolved in DMSO with a separate vehicle control. RNA isolation, first strand cDNA synthesis and real-time PCR were as described in "Materials and methods". The results shown are a composite of at least seven independent experiments. The high and low values for each treatment were discarded and averages of the remaining five values are shown as ±SEM with each sample assayed in triplicate. *Single asterisks* denote averages that are statistically significant from ethanol control, $p < 0.05$; *triple asterisks* indicate $p < 0.001$

[5, 25]. CYP24A1 was used as a positive control, since its upregulation in nearly every vitamin D target tissue [26] tends to be extremely strong and rapid due to its crucial role in detoxifying the 1,25D hormone [44]. Accordingly, treatment with 1,25D stimulated CYP24A1 transcription to an average of 141-fold over ethanol control (Fig. 3), thus confirming that VDR is present and active in HaCaT cells. Curcumin (CM) yielded a highly significant induction of CYP24A1, an average of 5.9-fold ($p = 0.0001$, $n = 6$). DHA produced a lower induction (1.3-fold), although this effect was still statistically significant ($p = 0.024$, $n = 11$). Expression of selected LCE genes suggested upregulation by VDR ligands (Fig. 3). The fold inductions of LCE genes after 24 h of 1,25D treatment were 3.8- to 5.0-fold, and DHA and CM upregulated LCE genes 2.9- to 4.7-fold. While these results reveal a trend for VDR-mediated upregulation of LCE genes, none of these effects reached statistical significance because of high variability between experiments, even though care was taken to harvest cells from 60 mm plates at equivalent confluences (approximately 70 %). After these experiments were performed, genotyping revealed that HaCaT cells are heterozygous for the deletion of LCE3B_LCE3C genes. Since these are the two genes for which primers were available, this genotype could have influenced the results since only one gene copy is present as opposed to two copies for the other LCE3 genes.

Delphinidin (Del) was also tested at a concentration of 10 μM, which was based on previous reports showing skin effects at 1–40 μM [2, 36]. Del treatment (Fig. 3) of HaCaT cultures elicited a modest but significant upregulation of CYP24A1 (1.3-fold, $p = 0.024$, $n = 7$), suggesting that Del might be a VDR ligand. Results with LCE genes showed a trend toward upregulation, but results were inconsistent, even when experiments were repeated numerous times and cultures were grown to a controlled confluence (approximately 70 %). Therefore, further experiments were performed utilizing primary human keratinocytes (HEKn) from neonatal foreskin.

Primary human keratinocytes were plated in 60 mm plates with Epilife medium containing 60 μM calcium at 30–40 % confluence such that 60–85 % confluence was reached by the time of harvest. The addition of 1,25D at concentrations up to 100 nM for 24 h elicited no changes in gross morphology, although final confluences were reduced in cultures receiving the highest dose of 100 nM 1,25D (data not shown), consistent with published reports that this concentration of 1,25D decreases proliferation of human keratinocytes [55]. In contrast, primary keratinocytes preincubated for 24 h in 1.2 mM calcium displayed a clear change in morphology, with the uniform monolayer at 60 μM calcium becoming more dense and compacted at 1.2 mM (not shown), consistent with previous reports that a shift to high calcium concentrations (>1 mM) triggers keratinocyte differentiation [9, 18, 28, 33]. No further gross morphologic changes are apparent when increasing concentrations of 1,25D are included along with the high calcium (data not shown).

Using this culture system, we assessed the ability of 1,25D to regulate LCE genes, with CYP24A1 again serving as the positive control. In order to properly interpret the results, it should be noted that the HEKn cells used in these experiments were heterozygous for the LCE3B_LCE3C deletion (data not shown), as was the case for the HaCaT cells. However, experiments in HEKn cells targeted all five LCE genes, including the LCE3A/3D/3E genes not affected by the deletion, providing a more complete picture of LCE gene regulation. Figure 4a shows results of cells in 60 μM (low) calcium that were incubated with ethanol or the indicated concentrations of 1,25D for 24 h before RNA isolation. As expected, CYP24A1 mRNA transcripts were dose-dependently enhanced by 1,25D. At this low calcium concentration, in which the primary keratinocytes are presumably relatively undifferentiated, transcripts of most LCE genes were either mildly depressed (down to 0.7-fold compared to ethanol vehicle treatment) or mildly elevated (1.2- to 2.0-fold). No dose response is noted (with the possible exception of LCE2B transcripts) and none of these effects were statistically significant in this set of four independent experiments except for the upregulation of LCE3E transcripts at 1 nM 1,25D (2.0-fold, $p = 0.009$).

After a 24-h preincubation with 1.2 mM (high) calcium (Fig. 4b), the strong effect of 1,25D on CYP24A1 expression was essentially unchanged. However, regulation of LCE transcripts became much more robust, presumably due to the greater degree of keratinocyte differentiation. Moreover, with the exception of LCE2B, the upregulation of each LCE transcript displayed dose dependence. Several of these effects reached statistical significance in this set of three independent experiments: LCE3A at 1 and 10 nM 1,25D (2.0-fold, $p = 0.0045$ and 3.1-fold, $p = 0.048$, respectively) and LCE3B at 100 nM 1,25D (1.8-fold, $p = 0.027$). The effect of 1 nM 1,25D on LCE3E transcripts approached statistical significance (2.1-fold, $p = 0.051$).

Primary human keratinocytes were also used to evaluate the ability of 5 or 10 μM Del to regulate vitamin D target genes (Fig. 5). Del significantly upregulated the CYP24A1 gene in the presence of low calcium (Fig. 5a), but showed only a trend for upregulation under high calcium conditions (Fig. 5b). Nevertheless, these results again suggest that Del is a low affinity VDR ligand, since ligand-occupied VDR is a major regulator of CYP24A1 transcription [15]. As observed with 1,25D, the effect of delphinidin on LCE gene expression in primary keratinocytes incubated in low calcium (and presumably in an undifferentiated state) is

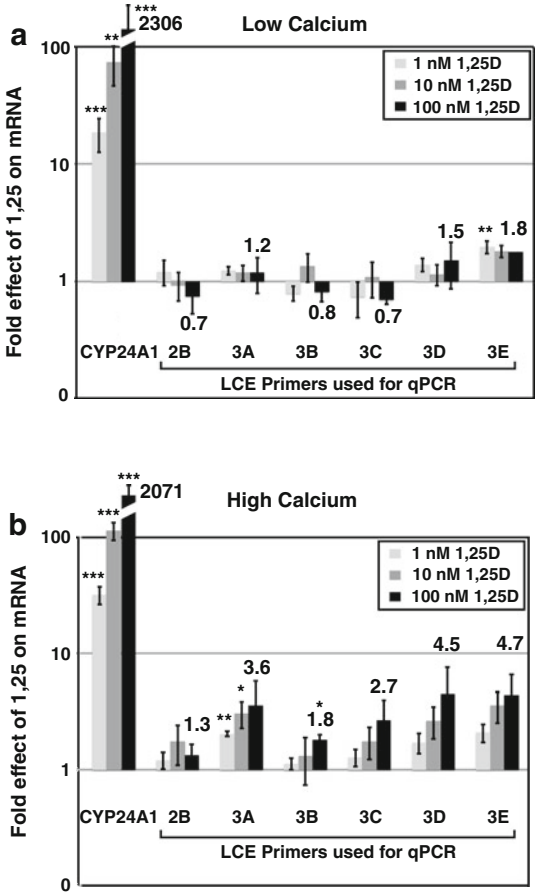

Fig. 4 Regulation of LCE genes by 1,25D in primary human keratinocytes. HEKn cells were plated at 550,000 cells per 60 mm plate, incubated overnight, then treated as follows: **a** 24 h with 1,25D (three concentrations) or ethanol vehicle without high calcium preincubation; **b** preincubation for 24 h with 1.2 mM calcium, then with 1,25D or ethanol vehicle for 24 h. RNA isolation, synthesis of first strand DNA and real-time PCR are described in "Materials and methods". Results are from three (**b**) or four (**a**) independent experiments, each in triplicate, ±SEM. *Single asterisks* denote averages that are statistically significant from ethanol control, $p < 0.05$; *double asterisks* denote $p < 0.01$; *triple asterisks* indicate $p < 0.001$

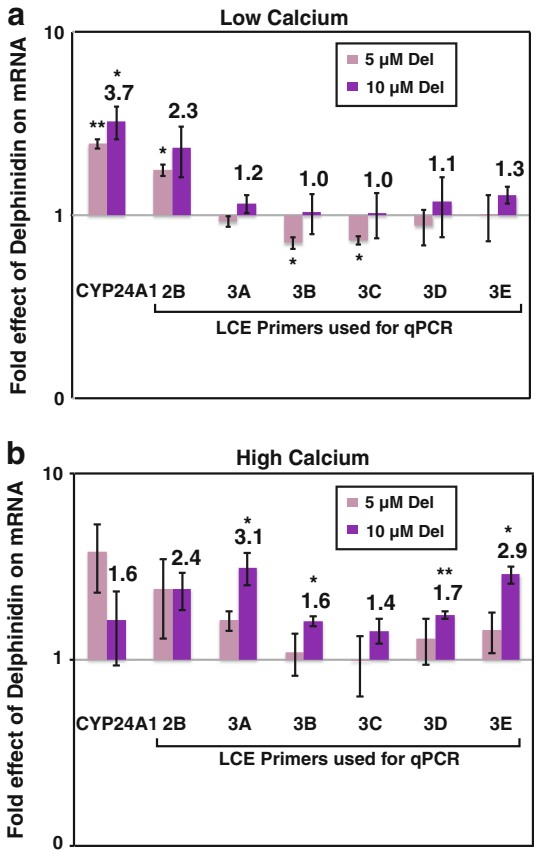

Fig. 5 Ability of delphinidin, a novel candidate VDR ligand, to regulate CYP24A1, LCE2B and all five genes in the LCE3 cluster. Cells were plated and dosed with or without 24 h of 1.2 mM calcium preincubation as described in the legend of Fig. 4, and RNA isolations, first strand cDNA synthesis and real-time PCR were performed as described in "Materials and methods". Results are from three independent experiments, each assayed in triplicate, ±SEM, except for results with 10 μM delphinidin, for which four independent experiments were carried out. *Single asterisks* denote averages that are statistically significant from ethanol control, $p < 0.05$; *double asterisks* denote $p < 0.01$

modest and in some cases inhibitory. Statistical significance was reached for reductions in transcripts for LCE3B and 3C after 5 μM Del treatment (0.71-fold, $p = 0.046$ and 0.73-fold, $p = 0.027$), and for a modest increase in LCE2B transcripts (1.8-fold, $p = 0.014$). This latter result is opposite to the results with 1,25D, in which only the cells preincubated with 1.2 mM calcium showed an increase in LCE2B transcripts (compare Figs. 4a, 5a). After a 24-h preincubation with 1.2 mM calcium, a shift from down- to upregulation was noted for the LCE3B and LCE3C genes, similar to that seen after 1,25D treatment. Dose dependence was noted for all LCE3 transcripts with the following effects by 10 μM Del being statistically significant: LCE3A (3.1-fold, $p = 0.045$), LCE3B (1.6-fold, $p = 0.029$), LCE3D (1.7-fold, $p = 0.009$) and LCE3E (2.9-fold, $p = 0.018$).

LCE mRNAs were not only upregulated by 1,25D and Del, but also by the high calcium condition. The switch from 60 μM to 1.2 mM calcium resulted in a 5.7-fold increase in LCE2B mRNA for control (ethanol treated) cultures (not shown in Figs. 4, 5), and the fold increases in LCE3 mRNAs under these conditions ranged from 2.9-fold for LCE3A up to 13.3-fold for LCE3D, rendering the latter the most highly expressed LCE gene tested relative to the GAPDH internal standard. As implied by the results in Figs. 4b and 5b, even greater increases in LCE mRNAs were seen in cultures treated with both high calcium and varying doses of either 1,25D or delphinidin. The total increases (relative to the GAPDH internal standard) in cultures treated with both high calcium and VDR ligand

ranged from fourfold for LCE3B in the presence of 10 μM Del up to over 47-fold for LCE3B and LCE3D in the presence of 100 nM 1,25D, with LCE3D again being the most highly expressed mRNA relative to the GAPDH internal standard. The latter conclusion must be interpreted in the light of the genotype of this sample of HEKn cells, in which only a single copy of the LCE3B and LCE3C genes is present. However, it can still be concluded that under the tested conditions, LCE gene expression is upregulated by both calcium (presumably via cell differentiation) and either 1,25D or delphinidin.

To examine whether Del can bind VDR directly in a fashion similar to 1,25D, radioinert Del was incubated in the presence of radiolabeled 1,25D in a competition binding assay using cell lysates containing overexpressed human VDR and RXRα (Fig. 6). As a positive control, unlabeled 1,25D competes very effectively for binding to VDR (Fig. 6, pink line), especially since unlabeled 1,25D, at 20 nM, is greatly in excess of the [^{3}H]1,25D concentration (approximately 0.4 nM) and of the K_d of 1,25D for human VDR (0.1–0.2 nM [22]). As previously published [25], DHA is capable of displacing [^{3}H]1,25D (IC_{50} at approximately log −5.1, or 0.86 μM). In contrast, the glucocorticoid receptor agonist dexamethasone (Dex, the negative control) does not compete for binding to VDR, even at concentrations up to 640 μM (approaching the limit of solubility in ethanol), consistent with its lack of appreciable affinity for VDR [5]. Del achieved approximately a 70 % inhibition of [^{3}H]1,25D binding at the maximum 1.66 mM concentration tested, indicating that Del is an ultra-low affinity VDR ligand, with an IC_{50} approaching 1 mM.

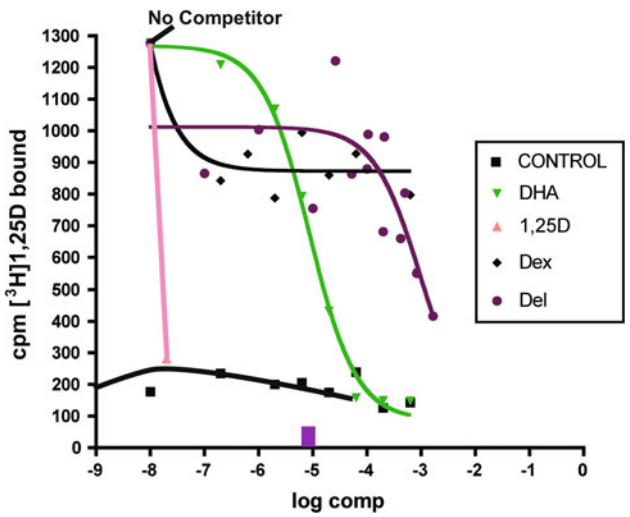

Fig. 6 Competition curves displaying the concentration range in which each ligand is able to compete for binding to VDR with approximately 0.4 nM [^{3}H] 1,25D. Dexamethasone (Dex, *upper black line* and *diamonds*) is a ligand for the glucocorticoid receptor with no appreciable binding to VDR and thus serves as a negative control. Radioinert 1,25D (*pink line* and *triangle*) serves as a high affinity ligand to show the full range of competition possible in this assay. Docosahexaenoic acid (DHA, *green line* and *triangles*) is a low-affinity VDR ligand [25]. Delphinidin (Del, *purple line* and *circles*) is able to compete with [^{3}H]1,25D, but only at very high concentrations (>100 μM), far above the 10 μM concentration that upregulates LCE3 genes by qPCR in HEKn cells (see *purple rectangle* on *X*-axis). The *lower black line* and *solid squares* show a DHA competition curve using lysates from cells that were not transfected with the VDR expression plasmid. The competition results shown here are a compilation of four similar assays performed independently

In a second test of Del as a possible VDR ligand, luciferase reporter gene constructs were utilized to determine whether Del treatment can modulate transcription of a VDRE-containing luciferase reporter gene. As depicted in Fig. 7, incubation of transfected HEK-293 cells with 10^{-8} M 1,25D induced the production of luciferase to 10.5-fold over levels seen in cells incubated with ethanol vehicle. This statistically significant induction occurs only with luciferase reporter plasmids containing two copies of a sequence upstream of the LCE3A gene corresponding to a predicted VDRE (see "Materials and methods"; Fig. 1), and not with pLUC-MCS plasmids lacking this element. In contrast, cells incubated with 20 μM Del displayed no significant effect when compared to the ethanol regardless of whether the reporter plasmid contained the VDRE sequence. Addition of Del and 1,25D yielded an effect (8.5-fold upregulation) similar to that of 1,25D alone (Fig. 7a, pair of bars to the right). Surprisingly, when this experiment was repeated in the human KERTr keratinocyte cell line, 20 μM delphinidin was able to elicit a statistically significant upregulation (2.2-fold) of luciferase expression from the VDRE-containing plasmid, similar to the 3.1-fold effect seen with 1,25D. A combination of the two ligands gave a 4.1-fold effect, which was not significantly different from either ligand alone. The conclusion from these experiments is that Del not only has dramatically different actions in keratinocytes vs. embryonic kidney cells, but also that Del is capable of acting in a VDRE-dependent manner in keratinocytes.

Discussion

The hypothesis that part of the effect of VDR ligands in the treatment of psoriasis is to upregulate LCE genes was prompted by previous studies demonstrating an increased psoriasis risk among individuals with a deletion of the LCE3B and LCE3C genes [14, 19, 31, 41, 51, 54, 60, 61], along with studies indicating that genes in the LCE3 gene cluster may play a role in skin repair [6, 19, 20]. Upregulation of LCE3 genes would therefore not only aid in skin repair, but also in upregulation of those LCE3 genes not affected by the deletion (i.e., LCE3A/3D/3E and LCE2B)

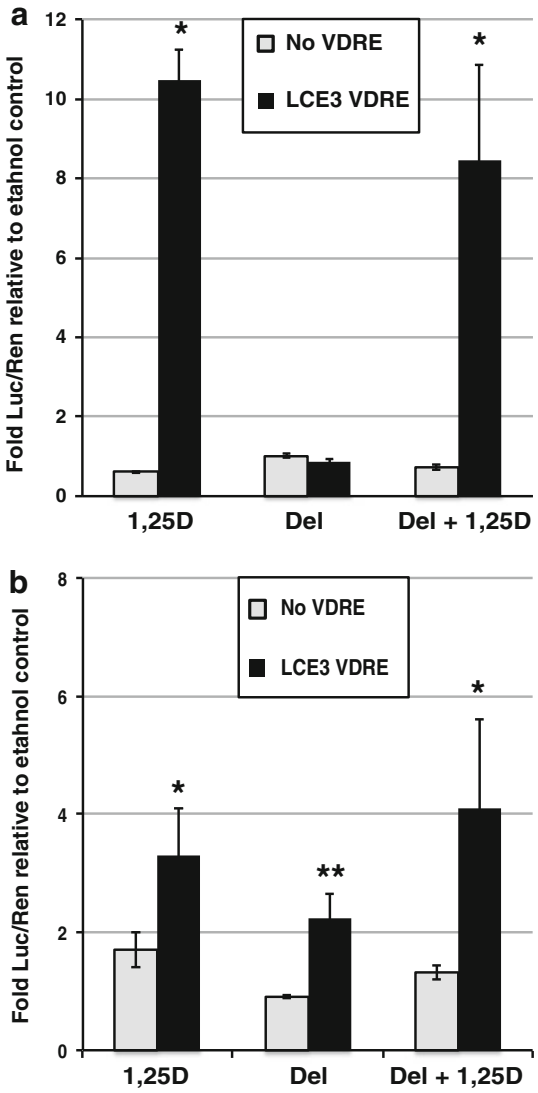

Fig. 7 Ability of delphinidin to modulate VDR/VDRE-mediated transcription of a luciferase reporter gene. **a** HEK-293 cells were cotransfected with an expression plasmid for VDR as well as either a reporter plasmid lacking a VDRE (No VDRE) or a plasmid containing two copies of a human VDRE identified approximately 29 kb upstream of the LCE3A gene (see text and Fig. 1). Cells also received a *Renilla* luciferase plasmid to control for transfection efficiency. Ligand concentrations were 10 nM for 1,25D and 20 μM for delphinidin (Del). Transcriptional activities were calculated by determining the Firefly/*Renilla* ratios for each treatment group, including an ethanol vehicle control group (not shown). The results shown here are the fold inductions for each ligand compared to the ethanol control, averaged for three independent experiments ±SEM. Results of Student's *t* test are indicated by *asterisk* denoting $p < 0.05$. **b** A similar set of three experiments performed in transfected KERTr cells. Data are expressed as described for *panel a*

that could potentially allow for more complete restoration of an effective skin barrier, thus improving the course of psoriasis treatment [53]. The initial approach was to test three known VDR ligands and one candidate ligand as inducers of LCE gene expression in a keratinocyte cell line, HaCaT. In addition to 1,25D, the known VDR ligands DHA and curcumin, along with the plant-derived potential VDR ligand, Del, yielded results indicative of an inductive effect on LCE expression, with average fold inductions ranging from 1.6- to 5.0-fold (Fig. 3). However, results obtained in HaCaT cells were inconsistent. Although the reasons for this variability are not clear, HaCaT cells represent an immortalized cell line that is routinely cultured in media containing >1 mM calcium (the medium used in the present experiments contained 1.6 mM calcium). Such high calcium concentrations are usually found in vivo only in the stratum granulosum [29] that contains terminally differentiated keratinocytes which express LCE genes [43]. However, HaCaT cells are clearly not terminally differentiated, as they are able to divide in culture indefinitely. Given these uncertainties, we elected to perform further experiments utilizing primary neonatal foreskin keratinocytes (HEKn).

Experiments with primary human keratinocytes were performed in the presence of two calcium concentrations: low calcium (60 μM) for cell maintenance and high calcium (1.2 mM) to induce differentiation, as based on earlier studies [9, 18, 28, 33]. Also, previous studies [33, 43] had demonstrated that several LCE genes, including LCE2B [43], are calcium-induced in primary human keratinocytes from neonatal foreskin, providing further evidence for the usefulness of this model system. It should be noted that there is some uncertainty in the literature as to the extent to which LCE genes are responsive to high calcium in cultured primary keratinocytes. Although Marshall et al. [43] reported that expression of mouse LCE protein (detected by a broad affinity antibody) was highly dependent on calcium, Jackson et al. [33] reported that, whereas LCE2 genes were calcium responsive in cultured human keratinocytes, transcripts from the LCE1 and LCE3 clusters were not upregulated by high calcium. In the present study, all tested LCE genes were upregulated in the high calcium condition, differing from Jackson et al. with respect to the LCE3 genes. The reasons for this discrepancy are unclear, but may be due to the specific calcium concentrations, time of exposure, or other details of the culture conditions. Nevertheless, under the present conditions, the expression of all tested LCE genes is enhanced not only by VDR ligands, but also by increasing the calcium concentration to induce differentiation.

A caveat in these studies is that complete differentiation of keratinocytes to mature corneocytes is not possible in a 2D culture system, and may require a 3D culture system and other signals besides high calcium and VDR ligands [8, 10]. Nevertheless, the present experiments with primary keratinocytes provide a window into the interplay between the differentiation state of human keratinocytes and gene regulation by liganded VDR. Regulation of CYP24A1 by

either 1,25D or Del was independent of the two tested differentiation states of the primary keratinocytes, which is in keeping with the crucial role of this enzyme in detoxifying 1,25D [44]. In contrast, LCE gene expression in response to VDR ligands was in most cases mildly repressed in cells incubated with low calcium, changing to induction when the cells were differentiated in high calcium. The fact that the upregulation appeared to be greatest with the LCE3A, 3D and 3E genes, which are the genes in the LCE3 gene cluster not affected by the PSORS4 deletion, might be at least partially explained by the fact that the HEKn cells (and also the HaCaT cells) used in the current experiments were heterozygous for the LCE3B_LCE3C deletion. Although the current conclusions are somewhat limited by the fact that we have not yet tested keratinocytes with all three PSORS4 genotypes, the group-wise upregulation of all five LCE3 genes observed in the heterozygous HEKn cells is consistent with the hypothesis that VDR ligands, including those used in psoriasis treatment, act to upregulate genes involved in skin repair to improve symptoms of psoriasis. Indeed, 1,25D has been reported to aid healing of cutaneous wounds in rats [57]. These authors speculated that fibronectin or IL-1 (acting as a growth factor) might be responsible for the wound healing effects of 1,25D; the current results suggest that upregulation of LCE3 genes might also contribute to this effect.

DHA is presumed to be acting via binding to the VDR, since its actions occur in a concentration range that corresponds to its affinity for VDR [5, 25]. The observation that the induction of CYP24A1 by DHA and by the other non-secosteroid ligands tested here is far less pronounced than the massive upregulation induced by 1,25D is not unexpected and may be due to the inability of these ligands to induce a conformation of the VDR that optimally activates CYP24A1 expression. Whether upregulation of LCE genes by curcumin and delphinidin is via a VDR/VDRE-dependent mechanism is not entirely clear. In the case of curcumin, it has been recently reported that curcumin, previously shown to activate CYP24A1 and VDR target genes p21 and TRPV6 in a VDR-dependent manner in Caco-2 cells [5] and to bind directly to VDR in a competition binding assay [25], is apparently able to activate the 1,25D-regulated human cathelicidin (CAMP) gene in a VDRE-independent manner in HaCaT cells [23], suggesting that alternative mechanisms of gene activation instead of (or in addition to) direct VDR binding are possible.

The complete scope of Del action is similarly undefined. On the one hand, the current results suggest the possibility that Del could be acting via a VDR/VDRE-dependent mechanism, with the strongest evidence for this being the results in transfected KERTr cells showing a statistically significant upregulation of a reporter gene linked to a predicted VDRE isolated from the LCE3 gene cluster (Fig. 7b). However, caution in making this conclusion is warranted in light of two other considerations. One consideration is that delphinidin does not possess an affinity for VDR in the 10–20 μM range in which the effects on LCE gene transcription are observed. The other consideration is the multitude of other signaling pathways that Del is reported to affect (see below).

The competition binding assays in which Del displaces radiolabeled 1,25D from VDR only at concentrations far above those required for activating transcription of LCE genes were performed in a cell-free assay containing very small amounts of a cell extract from COS-7 cells that had been transfected with vectors expressing VDR and RXR (see "Materials and methods"; Ref. [5]). In reconciling this result with the observation (Fig. 7b) that 20 μM Del is sufficient to activate a VDRE-linked reporter gene in transfected KERTr cells, one could hypothesize that Del is metabolized in keratinocytes, but not in HEK293 cells or COS-7 extracts, to a compound that can bind with higher affinity to VDR to activate it for transcriptional control. There are indeed reports that Del can be metabolized (or degraded) under culture conditions. In one study, delphinidin underwent spontaneous degradation at physiological pH to two products: phloroglucinol aldehyde and gallic acid [21]. In a second study, Del degradation was demonstrated within 1 h at 37 °C in common tissue culture media such as DMEM (degradation products not specified) [42]. These observations strongly suggest that addition of Del to culture media cells exposes cells not only to the parent compound, but also to degradation products that may be bioactive. However, the fact that Del is degraded in a cell-free manner does not explain the differential effects seen in KERTr vs. HEK-293 cells unless one postulates that the degradation pathway of Del is different in these two cell lines. An alternative possibility is that KERTr cells possess a different complement of VDR coactivators that allow them, but not HEK-293 cells, to respond to the active Del compound or metabolite. Further experiments will be required to explore these detailed mechanistic possibilities.

There is a growing literature on the effects of Del in skin-related systems. For example, a recent paper demonstrated that a 24-h incubation with concentrations of Del as low as 10 μM can upregulate the expression of involucrin, a marker of keratinocyte differentiation [12]. These authors concluded that delphinidin was capable of both slowing down proliferation and promoting differentiation, including the induction of involucrin, procaspase-14 and transglutaminase-1, all of which are characteristic of differentiated keratinocytes [12]. The mechanism for these effects was not explored in this report, but other studies have implicated the following non-VDR/VDRE-dependent pathways in the biological actions of delphinidin treatment: AP-1 and

NF-κB signaling [30, 32, 40], epidermal growth factor (EGF) signaling [45], cell adhesion [13], DNA repair, Bcl2-mediated cell survival, UV-induced caspase production and prostaglandin synthesis (reviewed in Ref. [3]). Specific binding targets of Del were reported to include Raf and MEK1 [36], MAPKK4, PI3 kinase and Fyn kinase [40], as well as the EGF receptor, the VEGF receptor and ERB3 [56]. The concentrations of Del eliciting these effects ranged from 1 to 200 μM, with many in the 5–40 μM range [2]. Relating these Del actions to LCE genes is not straightforward. However, it was reported that LCE3A, 3C and 3E are upregulated in reconstructed human skin by a combination of IL-1α, TNFα and IL-6 [6]. Because Del reportedly affects the MAPK and NF-κB pathways that can mediate the effects of IL-1α [4] and TNFα [34], respectively, Del could conceivably upregulate LCE genes indirectly by modifying their response to these cytokines. Since Del also targets kinases and VDR is a phosphoprotein [35], Del could also indirectly influence VDR activity via phosphorylation. Further experiments are required to determine which of these potential mechanisms might play a role in the regulation of LCE3 expression.

Finally, we cannot exclude the possibility that other genes or regulatory elements in linkage disequilibrium with the PSORS4 deletion are the actual causal factors for increased psoriasis risk. Indeed, psoriasis is clearly a multifactorial disease with components not only in keratinocytes but also in the adaptive [52] as well as innate [58] immune systems. Nevertheless, the integrity of the skin barrier remains an important factor in psoriasis pathogenesis [59].

The present data are consistent with, but do not prove, the original hypothesis that control of LCE genes may constitute at least part of the beneficial effect of 1,25D on symptoms of psoriasis. The ability of VDR ligands other than 1,25D, such as Del, curcumin and DHA, to upregulate LCE genes is significant for the following reasons. First, 1,25D and its analogs, although safe and effective for many patients, do not benefit all psoriasis patients [38]; thus, there is a need for more effective agents, especially for those patients with mild to moderate psoriasis. Indeed, although recent advances in psoriasis therapies, including biologics [37], have provided effective agents for the treatment of severe psoriasis, these newer agents are not approved for the majority of patients with mild to moderate psoriasis [16]. The improvement of topical agents to benefit such patients remains an important goal. Second, as compounds that represent entire new chemical classes of VDR ligands, curcumin, DHA and Del may have therapeutic properties very different from, and possibly superior or complementary to, 1,25D and its analogs. Non-secosteroid ligands by definition bind to VDR differently and likely induce conformations of VDR that are distinct from the 1,25D-liganded receptor. In fact, curcumin has recently been proposed to employ an alternative VDR ligand binding pocket that overlaps with the 1,25D binding domain [46]. In the case of Del, this very low affinity VDR ligand may act via a non-VDR/VDRE-mediated mechanism, thereby opening up new avenues for skin pharmacology.

The present investigation further adds to our knowledge of gene regulation by 1,25D and other nutrient-derived VDR ligands in skin, focusing on genes implicated in psoriasis risk. Studies relating to the mechanism(s) of gene regulation by 1,25D and other VDR ligands could ultimately lead to the establishment of a molecular basis for developing and testing candidate psoriasis therapies.

Acknowledgements This work was supported by National Institutes of Health grants to MRH and a grant from the Dean of the College of Medicine—Phoenix to GKW. The authors thank Eric W. Moffet for his role in the bioinformatic identification of the LCE VDRE utilized in this study.

Conflict of interest The authors state no conflict of interest.

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