Award Number: W81XWH-12-1-0235

TITLE: The Tumor Suppressor Actions of the Vitamin D Receptor in Skin.

PRINCIPAL INVESTIGATOR: Daniel D. Bikle, MD, PhD

CONTRACTING ORGANIZATION:
Northern California Institute for Research and Education
San Francisco, CA 94121-1545

REPORT DATE: August 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
The epidermis of the mouse lacking the vitamin D receptor (VDR) is susceptible to chemical and UVB induced tumor formation. In previous studies we determined that the hedgehog (HH) and wnt/β-catenin pathways were activated in the skin of VDR null mice. These pathways when activated promote proliferation and inhibit differentiation, suggesting the hypothesis that they underlie the predisposition of VDR null mouse epidermis to tumor formation following chemical or UVB induced mutations. Accordingly we developed mice in which the HH pathway was either constitutively activated by deletion of patched or inactivated by deletion of sonic hedgehog (SHH). In addition we developed mice in which the wnt/β-catenin pathway was constitutively activated by deletion of exon 3 of β-catenin or inactivated by deletion of exons 2-6 of β-catenin. These were then bred with mice with the floxed VDR. Following the initial hair follicle cycle these deletions were achieved using a keratinocyte specific and tamoxifen regulated (ERT2 K14 driven cre) cre recombinase. At this point only one of these models has received the full 40 wk course of UVB, that of the VDR/β-catenin knockout, and the results did not show the expected protection. However, the other models are currently under investigation both with short term and longer term UVB exposure, with results expected within the coming year.
15. SUBJECT TERMS
epidermal tumors, keratinocytes, vitamin D receptor, sonic hedgehog, β-catenin, UVB

16. SECURITY CLASSIFICATION OF:

<table>
<thead>
<tr>
<th>a. REPORT</th>
<th>b. ABSTRACT</th>
<th>c. THIS PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>U</td>
<td>U</td>
<td>U</td>
</tr>
</tbody>
</table>

17. LIMITATION OF ABSTRACT

<table>
<thead>
<tr>
<th>18. NUMBER OF PAGES</th>
</tr>
</thead>
<tbody>
<tr>
<td>78</td>
</tr>
</tbody>
</table>

19a. NAME OF RESPONSIBLE PERSON
USAMRMC

19b. TELEPHONE NUMBER (include area code)
INTRODUCTION

Recent studies indicate that the role of vitamin D and its receptor (VDR) in protecting against the development of epidermal tumors deserves a closer look (1). The epidermis of the VDR null mouse is hyperproliferative with gross distortion of hair follicles, structures that may provide the origin for the tumors found in the skin following DMBA or UVR (2) (figure 1 in Bikle DD et al. J Ster Biochem Mol Biol 136:271-279). Two interacting pathways critical for normal hair follicle cycling, Wnt/beta-catenin(Ctnnb1) and Ptc/hedgehog (Hh), when activated abnormally also result in epidermal tumors. Thus, we considered the possibility that loss of VDR predisposes to epidermal tumor formation by activation of either or both Wnt/beta-catenin and Ptc/Hh signaling (figure 2 and 3 in Bikle DD Photochem Photobiol Sci 11: 1808 – 1816, figure 2 in Bikle DD 2013 Sunlight, Vitamin D and Skin Cancer 2nd ed). We determined that all elements of the Hh signaling pathway were upregulated in the epidermis and urimes of the VDR null mouse (3) (figure 5 in Bikle DD et al. J Ster Biochem Mol Biol 136:271-279). In addition we observed that the transcriptional activity of beta-catenin was increased in keratinocytes lacking the VDR (4). We hypothesized that the vitamin D receptor (VDR) functions as a tumor suppressor with respect to epidermal tumor formation by blocking the Wnt/beta-catenin and Ptc/Hh pathways. To test this hypothesis we set out to determine whether mice lacking VDR but with constitutively active beta-catenin show accelerated tumor formation following UVB treatment, whereas those with an inactivated beta-catenin are protected from tumor formation. Similarly we set out to determine whether mice lacking VDR but with constitutively active Hh signaling (ptch+/-) show accelerated tumor formation following UVB treatment, whereas those with an inactivated Shh are protected from tumor formation.

BODY

Task 1. Determine whether mice lacking VDR but with constitutively active beta-catenin show accelerated tumor formation following UVB treatment, whereas those with an inactivated beta-catenin are protected from tumor formation. To complete this task after obtaining ACURO approval (task 2a) we bred mice homozygous for the floxed VDR with mice homozygous for floxed beta-catenin either of exon 3 (which when deleted results in a constitutively active beta-catenin, ca beta-catenin) or of exons 2-6 resulting in a beta-catenin null when deleted (del beta-catenin) (task 1b). Deletion of the VDR and the appropriate beta-catenin exons was accomplished by breeding the double floxed mice with ER\(^\text{\text{T2}}\)-K14-cre recombinase (task 1c). This cre recombinase is expressed only in basal keratinocytes, and tamoxifen activates it enabling the gene deletions to be temporally controlled. In this study tamoxifen administraton was initiated at 4-5 wks. The
breeding produced littermates which have or lack the ER\textsuperscript{T2}-K14-cre recombinase, and the latter serve as the “wildtype” controls since they retain the functional genes. (See figure 1, in Jiang et al 2013 J Ster Biochem Molec Biol 136:229-232 for breeding details). Other controls included mice lacking only the VDR, only lacking \(\beta\)-catenin, or only expressing the activated \(\beta\)-catenin. The intent was to treat these mice acutely or up to 40wks with UVB using a dose protocol (3x/wk in escalating doses up to 400mJ/cm\(^2\)) previously reported to induce skin tumors in VDR null mice (but not controls) (task 1d). The acute (ie. one exposure to 400mJ/cm\(^2\)) exposure to UVB is intended to look for differences in cyclobutane pyrimidine dimer (CPD) formation/clearance (markers of DNA damage), hyperplasia, and activation of the \(\beta\)-catenin and Hh pathways (task 1e).

\textit{Results}. We have completed the breeding of the animals to produce the desired genotypes and are maintaining the colonies in sufficient size to perform the experiments, which are in progress. It is important first to describe the phenotype of these animal models (figure 1). As we and others have described the VDR null mouse loses hair beginning about 3 months after the VDR is deleted. Although hair loss is not as rapid in the mice in which the VDR is deleted post weaning, as in this study, compared to our previous studies in which the VDR was deleted during embryogenesis, the pattern is similar. The mice lacking \(\beta\)-catenin rapidly lose hair. Obvious hair loss can be seen within 4 wks of the tamoxifen injection in the del \(\beta\)-catenin mouse at which time the coat of the VDR null mouse looks normal (figure 1). Surprisingly, the follicles are not particularly distorted at this time point, but we have not looked yet at later time points. When both VDR and \(\beta\)-catenin are deleted, the appearance is that of the del \(\beta\)-catenin mouse at the 8 week time point (figure 1). Furthermore, at this time point neither genotype has developed the dermal cysts and grossly distorted hair follicles (utricles) that characterize the VDR null mice later on. In contrast the phenotype of the ca \(\beta\)-catenin mouse is profound with patchy hair loss, growth retardation, and on histology a predominance of large clearly dysfunctional hair follicles (figure 1). The survival of these mice is poor and precludes any long term studies. As a result we have changed our breeding strategy with this mouse so that the ca \(\beta\)-catenin is being bred as a heterozygote for the floxed exon 3 \(\beta\)-catenin gene. This has markedly improved survival and phenotype, and we are optimistic it will serve the purposes of our project.
Figure 1. Impact of breeding epidVdr-/- mice with mice expressing delCtnnb1 or caCtnnb1 in their keratinocytes. Tamoxifen was administered at week 4, and these photos and histology were obtained at 8 wks. The controls are littersmate lacking cre. The effect of deleting Vdr is not yet evident, unlike the impact of manipulating CTNNB1 levels.

At this point we have only completed the 40wk protocol of UVB radiation on the VDR null and VDR/β-catenin null mice. These results are now published (table 2 and figure 2 in Jiang et al 2013 J Ster Biochem Molec Biol 136:229-232). This publication also provides details of the breeding strategy and the efficiency of gene deletion. In this study we observed tumors in 50% of the VDR/β-catenin double null mice (figure 2 in Jiang et al 2013 J Ster Biochem Molec Biol 136:229-232). Thus we did not see the protection against tumor formation in the VDR null mouse that we expected to see. Histologic examination of these tumors is in progress. Surprisingly, the del β-catenin mice alone were more sensitive to UVB than the VDR/β-catenin double null mice with lesions in the eyes in particular that precluded our ability to complete the long term UVB radiation studies. Thus, contrary to our expectations, lack of VDR appears to protect the del β-catenin, not the other way around. This result could be explained by the increase in β-catenin expression in the absence of VDR, even in the del β-catenin mouse in which we have shown that the deletion of β-catenin is 70% in whole skin samples (figure 1 in Jiang et al 2013 J Ster Biochem Molec Biol 136:229-232), and so could be increased by VDR deletion sufficient to provide protection. This possibility is being explored. As noted above the ca β-catenin mouse when homozygous has limited survival, and we have only recently started the long term UVB protocol with the heterozygous ca β-catenin and VDR null/ca β-catenin mouse. We have not completed the assessment of the response to acute UVB exposure in these mice.
Task 2. Determine whether mice lacking VDR but with constitutively active Hh signaling (ptch null) show accelerated tumor formation following UVB treatment, whereas those with an inactivated Shh are protected from tumor formation. To complete this task we have obtained ACURO approval (task 2a), and bred mice homozygous for the floxed VDR with mice homozygous for floxed ptch1 (which will activate Hh signaling when deleted) or floxed Shh (which will inhibit Hh signaling when deleted) (task 2a). Deletion of the VDR and ptch or Shh is accomplished by breeding the double floxed mice with ER\textsuperscript{T2}-K14-cre recombinase (task 2b), and subsequently treating the mice at 4wks of age with tamoxifen that activates the cre recombinase as described in task 1 (task 2c). As in task 1, these mice are then treated acutely or for up to 40wks with UVB using a dose previously reported to induce skin tumors in VDR null mice (but not controls). The breeding produces littermates which have or lack the ER\textsuperscript{T2}-K14-cre recombinase, and the latter serve as the controls. Other controls include mice lacking only the VDR (from task 1, they will not be restudied), only lacking ptch, or only lacking Shh. The response to acute and chronic exposure to UVB will be analyzed as for task 1.

Results: We are not as far along in this task as for task 1. The initial litters of Shh null and VDR/Shh null mice had a more profound phenotype than we anticipated with poor growth, loss of hair, and on histologic exam very poor formation of hair follicles (figure 2). More recent litters have been more viable, and both long term and acute UVB exposure studies have been initiated, but we have not obtained results as of yet. Because of the large number of mice currently under study we have deferred the breeding of the floxed patched with the floxed VDR for the time being.

Figure 2. Appearance and histology of mice lacking both VDR and SHH in their epidermis. 8 wk old epidermal specific Vdr and Shh double-null mice demonstrate hair loss (prominent on ears and around eyes) and ragged ears 3 weeks after tamoxifen injection (B), versus normal hair/skin in control (A). H&E staining of the skin shows abnormal degenerating hair follicles with hyper-proliferation of sebaceous glands and epidermis (D) vs. control (C). Bar: 20x

Although not part of the original proposal, we have also observed that lack of the VDR results in the overexpression of tumorigenic long noncoding RNAs (lncRNA) and suppression of tumor suppressor lncRNAs, several of which alter the HH and β-catenin signaling pathways. Our recent
data with these observations are found in table 1 in Jiang YJ and Bikle DD 2014 J Ster Biochem Molec Biol (in press).

KEY RESEARCH ACCOMPLISHMENTS

1. First development of mice with a combined loss of VDR and β-catenin postnatally from the epidermis. Loss of β-catenin postnatally results in faster hair loss than does loss of VDR.
2. First demonstration that loss of VDR paradoxically protects β-catenin null mice from UVB damage to epithelial structures such as eyes and epidermis, whereas loss of β-catenin fails to protect VDR null mice from UVB induced tumor formation.
3. Confirmation of earlier studies that constitutive activation of β-catenin causes hyperproliferation of immature hair follicles, although this was the first demonstration that this could be induced following the developmental hair follicle cycle.
4. First demonstration that deletion of Shh postnatally produces a profound loss of hair follicles.

REPORTABLE OUTCOMES

Manuscripts and presentations:

Development of novel bioengineered mice
1. Conditional epidermal specific VDR null mouse
2. Conditional epidermal specific β-catenin null mouse
3. Conditional epidermal specific VDR/β-catenin null mouse
4. Conditional epidermal specific constitutively activated (ca) β-catenin mouse
5. Conditional epidermal specific VDR null/ca β-catenin mouse  
6. Conditional epidermal specific Shh null mouse  
7. Conditional epidermal specific VDR/Shh null mouse  
8. Conditional epidermal specific Ptch null mice

CONCLUSIONS

We have developed a number of bioengineered strains of mice that serve as models for overexpression or under expression of the HH and wnt/β-catenin pathways, to determine whether these pathways would alter the susceptibility of the VDR null mouse to UVB induced epidermal cancer. Much of the first year has been devoted to developing these mouse models, which is now achieved. In the case of the epidermal specific VDR/β-catenin null mouse, we did not find the protection we anticipated in that these mice did develop tumors over the 40wk period of UVB exposure. We have also had to modify our breeding strategy in some models in that homozygous expression of the active form of β-catenin proved excessive leading to decreased survival. However, we have initiated both long and short term UVB exposures for most of the models, and expect to complete most of the studies in the coming year.

REFERENCES

4. Bikle DD, Oda Y, and Arnaud Teichert A 2011 The Vitamin D Receptor: a Tumor Suppressor in Skin. Discovery Medicine 11:7-17

APPENDIX

Ultra violet (UV) irradiation, in particular UVB, is the single most important carcinogen for skin tumor formation. UVB induces genetic mutations and immune suppression, which lead to abnormal cell proliferation and eventually tumor formation. Previously studies from our group and others demonstrated that both global and epidermal specific VDR knock out mice are predisposed to either chemical (DMBA)- or long-term UVB-induced skin tumor formation, paralleled by an increase in β-catenin signaling. Using primary cultured human keratinocytes, we further demonstrated that 1,25(OH)2-dihydroxyvitamin D3 (1,25(OH)2D3) suppresses cyclin D1 and Gli1 which are regulated by β-catenin/TCF signaling and have a critical role in epidermal carcinogenesis. Blockage of VDR by siRNA resulted in hyperproliferation of keratinocytes, and increased expression of cyclin D1 and Gli1. In addition, we also showed that 1,25(OH)2D3/VDR directly regulates transcriptional activity of β-catenin/TCF signaling using the -catenin reporter TopGlow. Using K14 driven tamoxifen-induced cre recombinase to delete both VDR and β-catenin in keratinocytes of mice following the first hair follicle cycle, we found that ablation of epidermal specific β-catenin cannot rescue VDR null mice from UVB-induced skin tumor formation. Further study using VDR or β-catenin single null mice is necessary to compare with the data from double null mice. This article is part of a Special Issue entitled ‘Vitamin D Workshop’.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Accumulating evidence suggests that chronic sun exposure is the single most important etiological factor for the pathogenesis of non-melanoma skin cancer (NMSC), including squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) [1–4]. Epidemiological data also show an association between the development of malignant melanoma (MM) and short-term intense UV-exposure, particularly when sunburns occur in youth [5,6]. The solar UV-spectrum can be divided into several bands with different...
physical and biological properties: UVC (wavelength < 280 nm), UVB (280–310 nm) and UVC (315–400 nm). While the predominant part of the short-wave, high-energy and destructive UV-spectrum (UVC and part of UVB) can be absorbed by the ozone layer, UVA radiation penetrates deeper into the dermis and deposits 30–50% of its energy in the dermal papillae (resulting in skin aging and solar elastosis). On the other hand, the majority of UVB can be absorbed by the epidermis (resulting in skin cancer development) [7]. Both UVA and UVB radiation induce DNA damage, resulting in mutagenic photoproducts including cyclobutane pyrimidine dimer (CPD) and 6-4-photoproducts. In fact, gene mutation has been implicated for the pathogenesis of skin cancer including p53 mutations (actinic keratosis, SCC) [7], and mutations in the patched (PTCH)/sonic hedgehog pathway (BCC) [8–10]. Together, sun exposure-induced premature skin aging, sunburns, immunesuppression and activation of latent viruses, which alone or in concert with each other could contribute to photocarcinogenesis. However, the mechanism of UVB-induced skin cancer at the molecular level remains largely unknown.

In addition to the DNA damage, sunlight induces production of vitamin D whose metabolite 1α,25(OH)2D3 has significant protective effect against the development of various types of cancer [11]. Recently, studies from our group and others demonstrated that global vitamin D receptor (VDR) mice are predisposed to either chemical (DMBA) or UVB-induced skin tumor formation [12–14], indicating a role of 1α,25(OH)2D3/VDR as tumor suppressor in skin. However, the mechanism of VDR protection against chemical or UVB-induced skin tumor is not clear. 1α,25(OH)2D3 acts via binding to its corresponding receptor VDR. VDR belongs to the subfamily of nuclear hormone receptors which requires heterodimerization with RXR (retinoid X receptor) for effective DNA interaction [15]. VDR is encoded by a relatively large gene encompassing 2 promoter regions, 7 protein-coding exons and 6 un-translated exons [16,17]. It has an extensive promoter region capable of generating multiple tissue-protein-coding exons and 6 un-translated exons [16,17]. It has an extensive promoter region capable of generating multiple tissue-specific transcripts [18] In addition, VDR extends its signaling by directly or indirectly interacting with many other proteins; one such important protein is β-catenin, in which VDR and β-catenin are cross regulated via interaction between the activator function-2 (AF-2) domain of the VDR and C-terminus of β-catenin [19].

β-Catenin is a crucial component in the Wnt/β-catenin signaling pathway controlling the expression of specific target genes that regulate cell proliferation, cell fate and differentiation [20]. Furthermore, the interaction of β-catenin with VDR has been shown to contribute to at least some types of skin cancer [21]. Overexpression of β-catenin in which exon 3 is deleted or mutated leads to hyperproliferation of hair follicles eventually causing hair follicle tumors (pilomatricomas, trichofolliculomas) [22,23]. Very interestingly, deletion of VDR results in increased β-catenin activity. Since β-catenin is an oncogene for several types of cancer, we hypothesized that the predisposition of UVB-induced skin cancer is due to increased β-catenin signaling, a hypothesis that we tested by deleting both VDR and β-catenin in the skin of mice exposed to UVB.

2. Materials and methods

2.1. Animals

All animal experimentation in this study has been approved by the San Francisco VA Medical Center Animal Review Committee. Mice homozygous for floxed VDR (kindly provided by Dr. Shigeki Kato, Molecular and Cellular Biosciences, University of Yokyo, Japan, bred into the C57BL/6 background) were bred with mice expressing K14ERΔem cre recombinase (Jackson Lab), which enabled us to selectively knockout the VDR in skin using parenteral application of tamoxifen (TM). These floxedVDRKO were then crossed with mice expressing a floxed β-catenin (exon 2-6 deletion) (kindly provided by Dr. Matthias Hebrok, Diabetes Center, Department of Medicine, UCSF), to produce epidermal-specific VDR and β-catenin double knock out (DKO) mice. Genotyping was performed by PCR with different primers designed to amplify the mutant VDR or β-catenin, or wild type (WT) DNA (Table 1).

2.2. UVB irradiation and tumor monitoring

Dorsal skin was shaved with electric clippers 24 h before UVB exposure. Mice were TM injected (ip.) after weaning, and irradiated 3 times per week, with 1–2 days between treatments, and re-shaved as needed. The dorsal skin was exposed to UV irradiation from a band of eight FS-40 fluorescent lamps (Daavlin, Bryan, OH) as reported previously [12]. Briefly, mice were irradiated initially at the dose of 120 mJ/cm2. The dose was then increased to 25% per week for 5 weeks, up to 400 mJ/cm2 for 9 weeks, followed by 200 mJ/cm2 until week 40 [14]. After UVB treatment for 33 weeks, mice were examined weekly for tumor development by visual inspection and palpation. Mice bearing a skin growth 1 mm or larger, persisting for more than 7 days, were scored positive.

2.3. Epidermal preparation and RT-qPCR

Mice epidermal preparation, RNA extraction and RT-qPCR procedures were described in detail previously [24].

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floxed VDR</td>
<td>TCT GAC TCC CAC AAG TGT ACC AGG G</td>
<td>Genotype</td>
</tr>
<tr>
<td></td>
<td>ATG GAC AGG AAC ACA CAG CAT CA</td>
<td></td>
</tr>
<tr>
<td>Floxed β-catenin</td>
<td>AAG GTA GAG TCA TGA TGA AAG TGT TT</td>
<td>Genotype</td>
</tr>
<tr>
<td></td>
<td>CAT CAT GTC CTC TGT CTA TTC</td>
<td></td>
</tr>
<tr>
<td>VDR</td>
<td>ACCCTTGGTACCTTGACC</td>
<td>qPCR</td>
</tr>
<tr>
<td></td>
<td>GCAATTCTCTGTCAGGGGGGG</td>
<td></td>
</tr>
<tr>
<td>β-Catenin</td>
<td>CCCATGCCTTCAGCAGCAAGAAGAG</td>
<td>qPCR</td>
</tr>
<tr>
<td></td>
<td>CATCTACGTCCTGTCAGGGAAAAC</td>
<td></td>
</tr>
<tr>
<td>L19</td>
<td>TCACCCCTAGGAAACACGATGGGAAGGAAAG</td>
<td>qPCR</td>
</tr>
<tr>
<td></td>
<td>GGATCTCCGTGGATCCAGGATTAT</td>
<td></td>
</tr>
</tbody>
</table>

* F: forward; R: reverse.
3. Results and discussion

3.1. Generation of epidermal-specific VDR and β-catenin DKO mice

Using a Cre-loxP system, we first generated conditional mice that specifically delete both VDR and β-catenin expression in epidermis. Floxed mice in which lox P sites were inserted into the introns upstream and down stream of either exon 2 of the VDR gene (Fig. 1A), or exons 2-6 of the β-catenin gene (Fig. 1B), were bred with transgenic mice expressing Cre recombinase under the control of the keratin 14 promoter. The resulting mice homozygous floxed for both VDR and β-catenin and expressing the Cre transgene were designated as double knock out (DKO). These were compared with the control littersates that have both floxed alleles but no Cre (WT). Upon TM injection, Cre recombinase will be activated and the floxed VDR and β-catenin genes will be deleted. This was confirmed by genotyping (data not shown) and the reduction of the epidermal VDR and β-catenin mRNAs (Fig. 1, C-D). One week after TM injection, both DKO and WT mice began UVB irradiation for 40 weeks (Fig. 1E).

3.2. Increased UVB-induced skin tumor formation in DKO mice

Previously we and other groups have reported that global VDRKO mice are predisposed for chemical or UVB-induced skin tumor formation [12–14]. To examine whether the increased skin tumor formation in VDRKO mice are attributable to the increased Wnt/β-catenin signaling, we generated the conditional DKO mice lacking both VDR and β-catenin. These mice are viable and fertile; however, with time some DKO (30–40%) mice showed increase pigmentation (Fig. 2A) and developed skin lesions on nose/face even without UVB exposure (data not shown). At the end of 40 week UVB irradiation, we observed an increase in skin tumor formation in DKO mice compared with their WT littermates (Fig. 2B–D and Table 2). Of 15 WT mice, only 2 mice (13%) developed a small tumor (<2 mm), while among 8 DKO mice, 4 (50%) were found to bear at least 1 tumor, and 2 mice were found to have 2–3 tumors for each mouse (Fig. 2E). In addition, the tumors found in DKO are larger in size (2.5–4.5 mm). Histologic analysis is currently underway to examine the type of tumor.

Our results of increased UVB-induced skin tumor formation in DKO mice are surprising. Although a wealth of evidence indicates a role of β-catenin as an oncogene in hair follicle tumor formation because of its gain-of-function [23,25–27], this is the first example that lack of endogenous β-catenin can also result in tumor formation. Since global VDRKO mice are predisposed to UVB-induced skin tumor formation, and β-catenin is the co-activator of VDR signaling, it is possible that ablation of both proteins synergistically leads to photo-carcinogenesis by some unknown mechanism.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of mice</th>
<th>% with tumors</th>
<th>Tumor burden (Average)</th>
<th>Response latency (week)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>15</td>
<td>13 (2/15)</td>
<td>1</td>
<td>39–40</td>
</tr>
<tr>
<td>DKO</td>
<td>8</td>
<td>50 (4/8)</td>
<td>1.75</td>
<td>36–37</td>
</tr>
</tbody>
</table>

Fig. 1. Generation of conditional VDR and β-catenin null mice. (A and B) The gene-targeting strategy to delete VDR or β-catenin from keratinocytes by using Cre-loxP system. (C and D) Decreased expression of VDR or β-catenin mRNA was shown by RT-qPCR. (E) Method of UVB-induced skin tumor formation in K14 driven, TM-induced VDR and β-catenin null mice.
In this regard, the inhibitory effect of 1,25(OH)2-D3/VDR on Wnt/β-catenin signaling seems not to play a major role in preventing photocarcinogenesis. A thorough comparison of the outcome of long-term UVB irradiation on 3pVDR and 3pβ-catenin single KO mice will provide important comparisons.

Acknowledgments

The authors appreciate the tech help from Alicia Menendez. This study is supported by the NIH grant R01 AR055924, VA Merit Review, and DOD grant CA110338.

References

[16] L. Tremezaygues, J. Reichrath, From the bench to emerging new clinical concepts: our present understanding of the importance of the vitamin D endocrine system (VDES) for skin cancer, Dermato-Endocrinology 3 (1) (2011) 11–17.
Review

Protective role of vitamin D signaling in skin cancer formation

Daniel D. Bikle, Hashem Elalieh, JoEllen Welsh, Dennis Oh, James Cleaver, Arnaud Teichert

A R T I C L E   I N F O

Article history:
Received 29 June 2012
Received in revised form 18 September 2012
Accepted 19 September 2012

Keywords:
Vitamin D receptor
1,25-Dihydroxyvitamin D
Keratinocyte
UVR
DNA damage repair
Sonic hedgehog

A B S T R A C T

Vitamin D sufficiency is associated with protection against malignancy in a number of tissues clinically, and a strong body of evidence from animal and cell culture studies supports this protective role. Cancers in the skin differ, however, in that higher serum levels of 25OHD are associated with increased basal cell carcinomas (BCC), the most common form of epidermal malignancy. This result may be interpreted as indicating the role of UVR (spectrum 280–320) in producing vitamin D in the skin as well as causing those DNA mutations and proliferative changes that lead to epidermal malignancies. Recent animal studies have shown that mice lacking the vitamin D receptor (VDR) are predisposed to developing skin tumors either from chemical carcinogens such as 7,12-dimethylbenzanthracene (DMBA) or chronic UVR exposure. Such studies suggest that vitamin D production and subsequent signaling through the VDR in the skin may have evolved in part as a protective mechanism against UVR induced epidermal cancer formation. In this manuscript we provide evidence indicating that vitamin D signaling protects the skin from cancer formation by controlling keratinocyte proliferation and differentiation, facilitating DNA repair, and suppressing activation of the hedgehog (Hh) pathway following UVR exposure. This article is part of a Special Issue entitled ‘Vitamin D Workshop’.

Published by Elsevier Ltd.

Contents

1. Vitamin D and skin cancer .............................................................................................................................................. 271
2. Vitamin D regulation of proliferation and differentiation .................................................................................................. 272
3. Vitamin D regulation of DNA damage repair .................................................................................................................. 272
4. Vitamin D regulation of hedgehog signaling .................................................................................................................. 275
5. Summary and conclusions. ............................................................................................................................................... 277
Acknowledgements .............................................................................................................................................................. 277
References ............................................................................................................................................................................. 278

1. Vitamin D and skin cancer

Skin cancer is by far the most common malignancy. Numerous studies over the past 30 years have evaluated the potential of 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] for anticancer activity [1]. Most malignant cells express VDR including basal cell (BCC) and squamous cell (SCC) carcinomas [2,3] as well as melanomas [4]. The generally accepted potential for 1,25(OH)2D3 in the prevention and treatment of malignancy rests with its antiproliferative, prodifferentiating actions. Epidemiologic evidence linking adequate vitamin D levels to colon cancer prevention is particularly strong, although the role of vitamin D in the prevention of other cancers has also been implicated [5–9]. This epidemiologic evidence is lacking for skin cancers, however [10–12], with several studies suggesting a positive correlation between 25OHD levels and BCC [13]. Most likely the lack of epidemiologic evidence for a positive role for vitamin D in preventing skin cancer is due to the dual effect of UVB radiation (UVR) in promoting vitamin D3 and 1,25(OH)2D3 synthesis in the skin and in increasing DNA damage leading to skin cancer negating the benefit of UVR induced vitamin D production. However, a threshold of UVR exposure may exist that would meet the nutritional requirements for vitamin D production without increasing the risk for epidermal tumor formation.
Animal studies suggest that vitamin D plays a protective role in the skin with respect to carcinogenesis. Zinser et al. [14] treated VDR null mice orally with the carcinogen 7,12-dimethylbenzanthracene (DMBA), attempting to induce breast cancers, but to their initial surprise 85% of the VDR null mice developed skin tumors. The wildtype controls did not develop tumors. Other groups have confirmed these results with the topical administration of DMBA/TPA [15]. Ellison et al. [16] extended these results by demonstrating that the VDR null mice all developed tumors in response to UVR, with few tumors appearing in the wildtype controls. We have confirmed these results with our own studies [17] (Fig. 1). Surprisingly mice lacking the ability to produce 1,25(OH)2D (CYP27B1 null) did not show increased susceptibility to tumor formation following either DMBA [16] or UVR [17].

We considered three mechanisms, not necessarily independent, underlying the protective role of vitamin D in tumor formation. First, vitamin D signaling has a well established role in inhibiting proliferation and promoting differentiation of keratinocytes [18]. Thus lack of vitamin D signaling could lead to unchecked proliferation of poorly differentiated cells especially after a proliferative stimulus such as UVR. Secondly, UVR induces characteristic alterations in DNA (cyclobutane pyrimidine dimers [CPD] and 6,4-photoproducts [6,4PP]) that if not repaired lead to mutations with the potential for initiating cancer. Studies from several research groups including our own [19,20] have shown that vitamin D signaling enhances DNA repair. Finally, activation of hedgehog signaling is associated with essentially all BCC [21] and many SCC [22]. We [17] have found that mice lacking the VDR have constitutively active hedgehog signaling, and that 1,25(OH)2D can suppress this pathway in normal skin. Recent data from our laboratory supporting the role of these three mechanisms form the basis of this report.

2. Vitamin D regulation of proliferation and differentiation

The VDR null mouse shows increased proliferation and marked abnormalities in differentiation especially in the latter stages of catagen [23]. These observations in vivo were confirmed in vitro by knocking down the expression of VDR and its coactivator, DRIP205, most associated with the action of VDR in the proliferating keratinocyte [24]. The results are shown in Fig. 2 and demonstrate that lack of VDR and its coactivator DRIP205 increase proliferation, decrease apoptosis, and alter the morphology of the keratinocyte from the normal cuboidal form with tight intercellular junctions to a loosely aggregated collection of spindly shaped cells suggesting a change to a more primitive, less differentiated cell. The loss of differentiation assessed morphologically was confirmed by decreased expression of various differentiation dependent markers and functions [23–25]. When VDR null mice were exposed to one dose of UVR they showed a greater stimulation of proliferation than did their wildtype littermates (Fig. 3A), and proliferation continued to increase for at least 48 h, whereas that of the wildtype littermates reached a plateau at 24 h. This resulted in an almost 3-fold increase in epidermal thickness in the VDR null mice compared to wildtype littermates by 48 h (Fig. 3B).

3. Vitamin D regulation of DNA damage repair

The ozone layer protects us from UV wavelengths shorter than 280 nm (UVC). UV wavelengths longer than 320 nm (UVA) have
Fig. 2. Regulation of keratinocyte proliferation and differentiation by VDR and its coactivators. Epidermal keratinocytes were transfected with non-targeted siRNA for control (sicontrol), VDR (siVDR), and DRIP205 (siDRIP). VDR and DRIP expression was reduced as shown by qRTPCR (a) and western analysis (b). Cells were maintained in low calcium (0.03 mM) to keep them proliferative. Cell proliferation was assessed by BrdU incorporation ((c) and (e) BrdU) and XTT assay ((d) XTT). The BrdU incorporated cells (brown) were counted using Bioquant and expressed as % total cells (blue counter staining) (c) and (e). Keratinocyte apoptosis was evaluated by measuring DNA fragmentation using Apoptaq in situ apoptosis peroxidase detection kit (Chemicon) ((c) and (f) TUNEL staining). The brown DNA fragmented cell nuclei (black arrows) per total cells (blue counter staining) were counted. Over 5000 cells were counted in three batches of keratinocytes to make these calculations ((f) apoptosis). The hyperproliferation and decreased apoptosis were accompanied by morphologic changes from normal cuboidal epithelial cells tightly aggregated to loosely aggregated spindle shaped cells (red arrows).

been thought to have limited ability to induce the characteris-
tic mutations in DNA seen in epidermal cancers, although recent
studies indicate that UVA can cause oxidative DNA damage that is
potentially mutagenic [26]. However, the major cause of skin cancer
is attributed to UVB with a spectrum between 280 and 320 nm [27].
UVR induced DNA damage includes the formation of cyclobutane
pyrimidine dimers (CPD) and pyrimidine-(6–4)pyrimidone photo-
products (6–4PP). If these lesions are not repaired C to T or CC to TT
mutations result, the UVR “signature” lesion [28]. These mutations
are often found in p53 in BCC, SCC [29–32] and actinic keratoses, the
precursor lesion to SCC [33]. Preventing UVR induced DNA damage
from producing DNA mutations is the role of DNA damage repair
(DDR) mechanisms. DDR coordinates the response of the cell cycle
to DNA damage through mechanisms involving damage recogni-
tion, repair and signal transduction. These mechanisms include cell
cycle checkpoints to delay the cell cycle, providing time for repair or
activating senescent and apoptotic pathways. These mechanisms
vary according to cell types, species, and damaging agents. The
accuracy and tight control of DDR in normal primary cells keeps
the spontaneous mutation rate very low [34–36]. However, with
malignant transformation control of DDR is lost, and mutation rates
and copy number abnormalities increase substantially [34–38].

Nucleotide excision repair (NER) is the principal means by
which UVR damage is repaired. By removing DNA damage before
DNA replication begins NER can reduce the amount of damage
resulting in mutations that get incorporated into the DNA during
replication [39,40]. The two major processes [41] used by NER
include transcription coupled repair (TCR) involving the repair of
genes undergoing active transcription [42–46] and global genomic
repair (GGR) for the non-transcribed regions of the genome [47].
Heritable mutations in NER genes occur in several human diseases
with increased susceptibility to UVR induced epidermal malignan-
cies such as xeroderma pigmentosum (XP) and Cockayne syndrome
(CS) [47]. Identification of the genes mutated in these diseases has
assisted substantially in identifying the genes and their protein
products critical for DDR.

Keratinocytes in the epidermis of mice lacking a VDR show
markedly retarded DDR [20]. This is demonstrated by a reduced rate
of clearing CPDs and 6,4PPs following UVR whether administered
in vivo (Fig. 4A) or in vitro in epidermal sheets from VDR null mice
(Fig. 4B), and is associated with decreased survival after UVR expo-
sure. The decreased clearance of CPDs in the VDR null epidermis
represents a failure of global DNA repair rather than transcription
coupled repair in that hydroxyurea (to block DNA synthesis) did not
have a significant effect on the results in these experiments (P=0.3
(Fig. 4B). The Mason laboratory [48,49] and others [50] demon-
strated that 1,25(OH)2D3 topically applied protected the skin from
UVR induced photodamage including increased clearance of CPDs,
decreased apoptosis, increased survival, and increased expression
of p53. Moll et al. [51] observed an upregulation of two genes
important for DDR: XPC (xeroderma pigmentosum complementa-
tion group C) and DDB2 (damage-specific DNA binding protein
Fig. 3. Hyperproliferative response to UVR in VDR null epidermis. Wild-type mice exposed to 1 dose of UVB (477 mJ/cm²) showed increased proliferation ([A] PCNA staining) and epidermal hyperplasia ([B] H&E staining) up to 24 h after treatment with no further increase at 48 h. VDR null mice exposed to the same dose of UVB showed significantly more pronounced proliferation ([A]) and epidermal hyperplasia ([B]) that continued to increase at 48 h.

Adapted from Teichert et al. The Journal of Investigative Dermatology 131:2289–2297 (2011) with permission.
Fig. 4. Defective DNA damage repair in VDR null mouse epidermis following UVR. (A) Wildtype and VDR null mice were exposed to 1 dose of UVB (400 mJ/cm²) and the skin evaluated for the presence of CPDs over the subsequent 48 h by immunohistochemistry (antiCPD from Cosmo Biosciences). CPDs were completely cleared by 24 h in the wildtype mouse epidermis, but persisted through 48 h in the VDR null mouse epidermis. (B) The epidermis from 2 d old wildtype and VDR null mice was exposed to 35.4 mJ/cm² UVB, and CPDs and 6,4PPs (detected by immunoblots) measured immediately after irradiation and after 46 h. In the experiment measuring CPDs, half of the epidermal explants were treated with hydroxyurea (HU) to block DNA synthesis prior to and following irradiation. Clearance of CPDs and 6,4PPs was markedly impaired in the VDR null epidermal explants consistent with the in vivo results in A. Adapted from Oh et al. The Journal of Investigative Dermatology 132:2097–2100, 2012.

2 also known as XPE) following 1,25(OH)₂D₃ treatment. These actions of vitamin D signaling on DDR are likely to account for part of the reduced susceptibility of normal skin to UVR induced tumor formation.

4. Vitamin D regulation of hedgehog signaling

Mutations in patched 1 (Ptch1), a key component of the hedgehog signaling pathway, were discovered as the main cause of the basal cell nevus syndrome (BCNS) (Gorlin syndrome), with its high susceptibility to the development of BCCs [21,52]. Moreover, most sporadic BCCs have mutations in Ptch 1 or other alterations in Hh signaling [53]. The Ptch1+/– (Gorlin) mouse was then developed as the first practical model of murine BCCs [53], and they are quite susceptible to the development of BCC and SCC following UVR or ionizing radiation [53]. Ptch 1 is the membrane receptor for sonic hedgehog (Shh) which in the basal state inhibits the function of smoother (Smoh), also in the membrane. In the presence of Shh this inhibition of Smoh is lost resulting in the activation of a family of transcription factors Gli1, Gli2, and Gli3. These Gli factors in the basal state are maintained in the cytoplasm bound to suppressor of fused (Sufu), but with the activation of Smoh these factors are released from Sufu, enter the nucleus, and promote Hh signaling [54,55]. Mutations in Sufu have not been found in cases of BCC but are associated with medulloblastomas, an additional feature of the Gorlin syndrome [56]. Gli1 and 2 overexpression in keratinocytes increase the expression of components of the Hh pathway, the antiapoptotic factor bcl2, cyclins D1 and D2, E2F1, cdc45 while suppressing genes associated with keratinocyte differentiation including VDR [57–61]. Moreover mice overexpressing Gli1, Gli2, or Shh in their basal keratinocytes or in human skin grafts [60–63] develop BCC like lesions, and BCC overexpress these Hh pathway components [64–66]. The appearance of BCC in VDR null mice following DMBA or UVR was at first surprising since UVR, ionizing radiation, or chemical carcinogens generally induce SCC not BCC [29]. However, we
Fig. 5. Overexpression of the Hh pathway in VDR null mouse skin and tumors. (A) Shh, Ptch1, Smoh, Gli1 and Gli2 proteins as shown by the brown signal were overexpressed in the epidermis and hair follicles of VDR null mice compared to their wild-type littermates at 11 weeks after birth by immunocytochemistry. Slides were counterstained with hematoxylin (blue stain). The bar denotes 50 mm. The protein levels were quantified by western blot. The numerical value represents the average ratio of VDR null band intensity versus wild-type band intensity from three mice per group. *p < 0.05. B. Shh, Ptch1, Smoh, Gli1 and Gli2 proteins as detected by immunohistochemistry in a papilloma from a VDR null mouse treated with DMBA and in a BCC from a VDR null mouse treated with UVB. Slides were counterstained with hematoxylin (blue stain). The bar denotes 50 mm. Shh, Ptch1, Smoh, Gli1 and Gli2 protein levels were also measured by western blot in skin tumors and tumor free tissue from DMBA treated VDR null mice. The numerical value represents the mean ratio of the tumor band intensity versus tumor free tissue band intensity from three mice. *p < 0.05.

Adapted from Teichert et al. The Journal of Investigative Dermatology 131:2289–2297 (2011) with permission.

[17] (Fig. 5A) found that elements of the Hh signaling pathway are overexpressed in the epidermis and epidermal portion (utricles) of the hair follicles of adult VDR null animals. Thus we postulated that loss of 1,25(OH)2D3 and/or VDR regulation of Hh signaling is one of the causes of the increased susceptibility of the epidermis to malignant transformation. Examination of the tumors following either DMBA or UVR treatment (Fig. 5B) revealed overexpression of elements of the Hh signaling pathway compared to adjacent normal skin [17]. These observations raised the question whether vitamin D signaling in the skin regulates Hh signaling, and if so whether
the loss of such regulation predisposes animals lacking VDR to skin cancer. Indeed, we found that in epidermal sheets and full thickness explants of skin 1,25(OH)2D3 inhibits the expression of all elements of the Hh pathway (Fig. 6A), and this suppression requires the VDR (Fig. 6B). Although our results indicate that suppression of the Hh pathway occurs via the genomic actions of 1,25(OH)2D3 acting through its receptor, other studies have shown that vitamin D itself as well as its precursor 7-dehydrocholesterol can bind to and inhibit the actions of Smoh directly [67,68]. However, there remains uncertainty about the relative role of this mechanism vs that of the genomic suppression of the Hh pathway by 1,25(OH)2D and the VDR.

5. Summary and conclusions

Although clinical data regarding the protective role of vitamin D signaling in skin cancer formation remain ambiguous, the role of the VDR and its ligand in this protective function in mice is not. Lack of VDR predisposes to epidermal tumor formation. Surprisingly deletion of CYP27B1 (the 1-hydroxylase) does not by itself predispose to tumor formation with the conditions employed (DMBA, UVR in a tumor resistant mouse strain), but 1,25(OH)2D does protect against tumor formation when applied topically, and it enhances VDR regulation of proliferation, differentiation, and Hh signaling. We have explored 3 mechanisms by which vitamin D signaling might be protective against tumor formation. First we discussed the well established ability of 1,25(OH)2D and VDR to regulate keratinocyte proliferation and differentiation, noting that loss of VDR increased the proliferative response of the epidermis to UV. Second we examined the role of vitamin D signaling in DNA damage repair (DDR), and demonstrated that lack of VDR impaired this process most likely by limiting the expression of various genes involved in DDR. Third we examined the control of Hh signaling in keratinocytes by 1,25(OH)2D and VDR. Activation of Hh signaling is essentially universal in BCC formation and in most SCCs. Lack of VDR increases Hh signaling, and 1,25(OH)2D suppresses the genes involved. Thus 3 mechanisms regulated by vitamin D signaling have been investigated, and in combination are expected to provide protection in the skin against the carcinogenic actions of UV, enabling the beneficial actions of UV on vitamin D production to proceed with reduced risk.

Acknowledgements

The authors acknowledge the administrative support of Teresa Tong and Victoria Lee, the technical support of Sally Penny-packer, Stephanie Demetriou, and Katherine Ona-Vu, and the funding support from NIH RO1 AR050023 (DDB), DOD CA110338 (DDB), VA Merit Reviews (DDB and DHO), and the University of California Cancer Coordinating Committee grants (DHO, JEC).
References


Protective actions of vitamin D in UVB induced skin cancer†

Daniel D. Bikle
Departments of Medicine and Dermatology, San Francisco VA Medical Center and University of California, San Francisco, CA, USA., Fax: +415-750-2089; Tel: +415-221-4810 ext 3338

Daniel D. Bikle: daniel.bikle@ucsf.edu

Abstract
Non-melanoma skin cancers (NMSC) are the most common type of cancer, occurring at a rate of over 1 million per year in the United States. Although their metastatic potential is generally low, they can and do metastasize, especially in the immune compromised host, and their surgical treatment is often quite disfiguring. Ultraviolet radiation (UVR) as occurs with sunlight exposure is generally regarded as causal for these malignancies, but UVR is also required for vitamin D synthesis in the skin. Based on our own data and that reported in the literature, we hypothesize that the vitamin D produced in the skin serves to suppress UVR epidermal tumor formation. In this review we will first discuss the evidence supporting the conclusion that the vitamin D receptor (VDR), with or without its ligand 1,25-dihydroxyvitamin D, limits the propensity for cancer formation following UVR. We will then explore three potential mechanisms for this protection: inhibition of proliferation and stimulation of differentiation, immune regulation, and stimulation of DNA damage repair (DDR).

Introduction
Over 1 million skin cancers occur annually in the United States, 80% of which are basal cell carcinomas (BCC), 16% squamous cell carcinomas (SCC), and 4% melanomas, making skin cancer by far the most common cancer afflicting humankind.¹ Surgery is generally curative, but disfiguring and costly. Ultraviolet radiation (UVR) is the major etiologic agent for these cancers, but is also the principal means by which the body obtains vitamin D. Furthermore, the skin is capable of converting the vitamin D produced to its active metabolite 1,25(OH)₂D, and this conversion is potentiated by UVR at least in part by cytokines such as TNF-α which are increased by UVR in the epidermis. This ability of the epidermis to make its own vitamin D and 1,25(OH)₂D is likely to be of great importance for epidermal physiology and pathology. It is not at all clear, for example, whether the oral administration of vitamin D, various analogs, and/or circulating levels of 25OHD and 1,25(OH)₂D has a major impact on processes within the skin—they may or they may not. Sun avoidance may reduce one’s risk of developing skin cancer, but this practice frequently results in suboptimal levels of vitamin D in the body, not to mention the epidermis. As pointed out in the analysis by Lucas et al.,² the global disease burden due to UVR pales in comparison to the disease burden due to vitamin D deficiency, and although the latter can be prevented with vitamin D supplementation, the skin remains for most of the world’s population the major site of vitamin D availability. Most tissues have the vitamin D receptor (VDR), and several including the epidermis, are capable of producing their own 1,25(OH)₂D. Vitamin D deficiency is associated with a number of diseases including, but not limited to, osteomalacia and rickets. Increased cancer risk ranks high among these diseases, including

†Contribution to the Vitamin D update collected papers.
Correspondence to: Daniel D. Bikle, daniel.bikle@ucsf.edu.
cancer of epithelial tissues such as breast, prostate, and colon. Perhaps because UVR increases the risk at least for non-melanoma skin cancers (NMSC), the potential for vitamin D and 1,25(OH)\(_2\)D production in the epidermis to serve as protection against UVR induced epidermal carcinogenesis has received little attention. However, a low UVR dose may be protective against skin cancer via the vitamin D signaling mechanisms that will be reviewed in this article, and some epidemiologic evidence is consistent with a potential benefit of low dose UVR. For example, in the study by Armstrong and Kricker, a slight decrease in the incidence of SCC, BCC, and melanomas in 10 US populations was observed when the solar UV measurement was increased from 100 to 110, although higher levels increased the incidence. This same group, evaluating data from the Australian population, did not find a significant increase in SCC with time spent out of doors in the general population. Rosso et al., in a multicentre European study, did not find a significant increase in SCC below a threshold of 70 000 accumulated hours of sunshine, although the development of BCC had a lower threshold. Before examining the animal data supporting this hypothesis, it is useful to review vitamin D production and metabolism in the skin as mediated by UVB.

**Vitamin D metabolism in the skin**

Vitamin D\(_3\) is produced from 7-dehydrocholesterol (7-DHC) (Fig. 1). Irradiation of 7-DHC with ultraviolet B (280–320 nm) (UVR) produces pre-D\(_3\), which subsequently undergoes a temperature-dependent rearrangement of the triene structure to form D\(_3\), lumisterol, and tachysterol. This process is relatively rapid and reaches a maximum within hours.\(^6\)–\(^8\) Both the degree of epidermal pigmentation and the intensity of exposure correlate with the time required to achieve this maximal concentration of pre-D\(_3\) but do not alter the maximal level achieved. Although pre-D\(_3\) levels reach a maximum, the biologically inactive lumisterol and tachysterol accumulate with continued UV exposure. Thus, prolonged exposure to sunlight would not produce toxic amounts of D\(_3\) because of the photoconversion of pre-D\(_3\) to lumisterol and tachysterol. Melanin in the epidermis, by absorbing UVR, can also reduce the effectiveness of sunlight in producing D\(_3\) in the skin. Sunlight exposure increases melanin production, and so provides another mechanism by which excess D\(_3\) production can be prevented. The intensity of UVR is dependent on latitude and season. In Edmonton, Canada (52\(^\circ\)N), very little D\(_3\) is produced in exposed skin from mid-October to mid-April, while in San Juan (18\(^{\circ}\)N), the skin is able to produce D\(_3\) all year long.\(^9\) Clothing and sunscreen effectively prevent D\(_3\) production in the covered areas. D\(_3\) produced in the skin can be secreted from the skin by a poorly understood process, carried to the liver and other tissues for further metabolism to 25-hydroxyvitamin D (25OHD), and then to the kidney to produce the biologically active metabolite 1,25(OH)\(_2\)D by the enzyme CYP27B1. However, the keratinocyte contains the entire pathway for 1,25(OH)\(_2\)D production from vitamin D.

The production of 1,25(OH)\(_2\)D in the skin is under different regulation compared to its production by the kidney, where the parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) are the principal hormonal regulators (PTH stimulates, FGF23 inhibits). Keratinocytes respond to PTH with increased 1,25(OH)\(_2\)D production, but these cells do not have the classic PTH receptor and do not respond to cyclic AMP.\(^10\) The mechanism by which PTH stimulates 1,25(OH)\(_2\)D production in these cells remains unclear. The effect of FGF23 on keratinocyte CYP27B1 expression or function has not been reported. Furthermore, unlike the kidney, 1,25(OH)\(_2\)D does not directly affect CYP27B1 expression in keratinocytes. Rather, 1,25(OH)\(_2\)D regulates its own levels in the keratinocyte by inducing CYP24, the catabolic enzyme for 1,25(OH)\(_2\)D.\(^11\) Instead, cytokines such as tumor necrosis factor-\(\alpha\) (TNF)\(^12\) and interferon-\(\gamma\) (IFN)\(^13\) are potent inducers of CYP27b1 activity in the keratinocyte. These cytokines are activated in the skin by UVB.
Vitamin D and skin cancer

1,25(OH)₂D has been evaluated for its potential anticancer activity for approximately 25 years. Most cell types, including many cancer cells such as basal cell (BCC) and squamous cell (SCC) carcinomas, as well as melanomas, contain the vitamin D receptor (VDR). Although epidemiologic evidence supporting the importance of adequate vitamin D nutrition (including sunlight exposure) for the prevention of at least some cancers, including those of the colon, is reasonably strong, such evidence is much weaker for skin cancers. One potential complication is that UVB radiation (UVR) has the dual effect of promoting vitamin D₃ synthesis in the skin (which can be further converted to 1,25(OH)₂D₃) and increasing DNA damage leading to skin cancer. Thus, although UVR may be the most efficient means of providing the nutritional requirement for vitamin D, the advantage to the skin may be countered by the increased risk of mutagenesis if the UVR is excessive.

The potential for vitamin D signaling as protection against epidermal tumor formation received an important boost when Zinser et al. demonstrated that 85% of VDR null mice developed skin tumors within two months following 7,12-dimethyl-benzanthracene (DMBA) administration. No tumors were found in the wildtype controls. The tumors were mostly sebaceous, squamous, and follicular papillomas; however, several BCC were also observed. No SCC were reported. These results have been confirmed using topical administration of DMBA/TPA, although only papillomas were seen in the VDR null mice unlike RXRα null mice which developed both BCC and SCC. More relevant to the concept of photoprotection is the study by Ellison et al. demonstrating that VDR null mice were also more susceptible to tumor formation following UVB. These tumors included SCC. We have confirmed these results with our own studies. The appearance of BCC in at least some of these studies (including our own) is surprising since the typical malignancy induced in mouse skin by UVR, ionizing radiation, or chemical carcinogens is SCC not BCC. The appearance of BCC is characteristic of tumors formed when hedgehog (Hh) signaling is disrupted, although activation of Hh signaling (Ptch⁻/⁻ mice) also predisposes to UVR induced SCC formation. The question then becomes, through what mechanism(s) does vitamin D signaling protect against UVB induced tumor formation? We will examine three potential mechanisms and pathways within those mechanisms: regulation of proliferation and differentiation with particular attention to the hedgehog (Hh) and wnt/β-catenin pathways, immunoregulation, and DNA damage repair.

Vitamin D regulation of epidermal proliferation and differentiation

The epidermis is composed of four layers of keratinocytes at different stages of differentiation (reviewed in ref. 33). The basal layer (stratum basale, SB) rests on the basal lamina separating the dermis and epidermis. Within this layer are the stem cells. These cells proliferate, providing the cells for the upper differentiating layers. They contain an extensive keratin network comprised of keratins K5 and K14. As the cells migrate upward from this basal layer they acquire the characteristics of a fully differentiated corneocyte, which is eventually sloughed off. The layer above the basal cells is the spinous layer (stratum spinosum, SS). These cells initiate the production of the keratins K1 and K10, which are the keratins characteristic of the more differentiated layers of the epidermis. Cornified envelope precursors such as involucrin also appear in the spinous layer as does the enzyme transglutaminase K, responsible for the ε-(γ-glutamyl)lysine cross-linking of these substrates into the insoluble Cornified envelope. The granular layer, stratum granulosum (SG), lying above the spinous layer, is characterized by electron-dense keratohyalin granules containing profilaggrin and loricrin, which is a major component of the Cornified envelope. The granular layer also contains lamellar bodies—lipid-filled structures that fuse with the plasma membrane, divesting their contents into the extracellular space between the SG and stratum
corneum (SC) where the lipid contributes to the permeability barrier of skin. As the cells pass from the granular layer to the Cornified layer (SC), they undergo destruction of their organelles with further maturation of the Cornified envelope into an insoluble, highly resistant structure surrounding the keratin–filaggrin complex and linked to the extracellular lipid milieu. The outer layer of the epidermis provides not only a barrier to water loss (permeability barrier) but a barrier to invasion by infectious organisms.

As noted above, the keratinocytes of the epidermis are unique in their ability to produce vitamin D$_3$ from the precursor 7-dehydrocholesterol (7-DHC) and to convert the vitamin D produced to the active metabolite 1,25(OH)$_2$D. 1,25(OH)$_2$D increases involucrin, transglutaminase activity, loricrin, filaggrin, and Cornified envelope formation at subnanomolar concentrations, while inhibiting proliferation at concentrations above 1 nM. Much of these actions are in conjunction with or via changes in calcium responsiveness due to the ability of 1,25(OH)$_2$D to induce the calcium receptor and the phospholipase C enzymes that regulate the intracellular calcium levels critical for the differentiation process. The antiproliferative effects are accompanied by a reduction in the expression of c-myc and cyclin D1 and an increase in the cell cycle inhibitors p21$^{cip}$ and p27$^{kip}$. In addition, 1,25(OH)$_2$D and its receptor regulate the processing of the long chain glycosylceramides that are critical for permeability barrier formation and induce the receptors—toll-like receptor 2 (TLR2) and its coreceptor CD14—that initiate the innate immune response in skin. Activation of these receptors leads to the induction of CYP27B1 (the enzyme that produces 1,25(OH)$_2$D), which in turn induces calcitriol, resulting in the killing of invasive organisms. Although the most striking feature of the VDR-null mouse is the development of alopecia (also found in many patients with mutations in the VDR), referred to as hereditary vitamin D resistance, these mice also exhibit a defect in epidermal differentiation as shown by reduced levels of involucrin and loricrin and loss of keratohyalin granules. Furthermore, these mice show a reduction in the lipid content of the lamellar bodies concomitant with a reduction in glucosylceramide production and transport into the lamellar bodies leading to a defective permeability barrier. The CYP27B1 null mouse also shows a reduction in levels of the epidermal differentiation markers and altered permeability barrier with a blunted innate immune response and poor resistance to infections, but these mice do not have a defect in hair follicle cycling.

Two pathways that appear to be important for vitamin D signaling in the epidermis, with respect to the proliferation and differentiation that we believe underlie the predisposition of the VDR null mouse to tumor formation, are the Hh and wnt/β-catenin pathways.

The hedgehog (Hh) pathway (Fig. 2)—Ptch 1 is the membrane receptor for Shh. In the absence of Shh, Ptch 1 inhibits the function of another membrane protein smoothened (Smo). Shh reverses this inhibition, freeing Smo to enable the activation of a family of transcription factors Gli1, Gli2, and Gli3. Suppressor of fused (Sufu) may maintain these transcription factors in the cytoplasm and/or limit their activity in the nucleus such that the loss of Sufu leads to increased Hh signaling. Gli1 and 2 overexpression in keratinocytes can increase the expression of one another as well as Ptch 1, the anti-apoptotic factor bcl2, cyclins D1 and D2, E2F1, and cdc45 (all of which promote proliferation), while suppressing genes associated with keratinocyte differentiation such as K1, K10, involucrin, loricrin and the VDR. The appearance of BCC is characteristic of tumors formed when Hh signaling is disrupted, although activation of Hh signaling (Ptch$^{-/-}$ mice) also predisposes to UVR induced SCC formation. We have found that the epidermis and epidermal portion (utricles) of the hair follicles of adult VDR null animals overexpress elements of the Hh signaling pathway. These results suggest that one of the causes of the increased susceptibility of the epidermis
to malignant transformation is due to a loss of VDR regulation of Hh signaling in the epidermis. Furthermore, we found that 1,25(OH)\(_2\)D suppressed all elements of the Hh pathway in a dose dependent fashion that required the VDR. These results have been confirmed at least for Gli1 by others in a study demonstrating that 1,25(OH)\(_2\)D could reduce tumor growth in Pch null mice. Furthermore, the promoters of Shh and Gli1 have been found to bind to VDR, suggesting a direct genomic action on these genes. However, vitamin D may regulate this pathway not only via the genomic actions of 1,25(OH)\(_2\)D acting through its receptor, VDR, but also by direct inhibition by vitamin D independent of its receptor. The latter possibility stems from observations that vitamin D itself, as well as its precursor 7-dehydrocholesterol, can bind to and inhibit the actions of smoothened (Smo), a critical step in Hh signaling. Moreover, vitamin D may regulate this pathway not only via its receptor, VDR, but also by direct inhibition by vitamin D independent of its receptor. The latter possibility stems from observations that vitamin D itself, as well as its precursor 7-dehydrocholesterol, can bind to and inhibit the actions of smoothened (Smo), a critical step in Hh signaling. Furthermore, we have observed similar phenomena in keratinocytes. In colon cancer cells, VDR has been shown to bind to β-catenin, and reduce its transcriptional activity in a ligand dependent fashion. We have observed similar phenomena in keratinocytes. However, the suppression of β-catenin signaling by 1,25(OH)\(_2\)D does not necessarily require E-cadherin. Rather, β-catenin binds to VDR in its AF-2 domain, binding that enhances the ability of 1,25(OH)\(_2\)D to activate the transcriptional activity of the VDR, but blocks the transcriptional activity of β-catenin. Mutations in the AF-2 domain that block coactivator binding do not necessarily block β-catenin binding. Palmer et al. evaluated the interaction between VDR and β-catenin in transcriptional regulation in keratinocytes, and identified putative response elements for VDR and β-catenin/LEF in a number of genes. These interactions were either positive or negative, depending on the gene being evaluated. The hypothesis put forward is that genes in which the interaction was positive (i.e. stimulated transcription) benefited from β-catenin acting as a coactivator for VDR on VDREs, whereas in situations where the interaction was negative (i.e. suppression of transcription), VDR prevented β-catenin from binding to TCF/LEF required for transcription in those genes. We have found in keratinocytes that knockdown of VDR reduces E-cadherin expression and formation of the β-catenin/E-cadherin membrane complex, resulting in increased β-catenin transcriptional activity, whereas 1,25(OH)\(_2\)D administration has the opposite effect. This was associated with increased (with VDR knockdown) or decreased (with 1,25(OH)\(_2\)D administration) keratinocyte proliferation and cyclin D1 expression.

The wnt/β-catenin pathway (Fig. 3)—Wnt signaling via activation of β-catenin has a complex role in VDR function. Wnt ligands bind to their seven-transmembrane frizzled receptors and an LRP5 or LRP6 co-receptor leading to phosphorylation of disheveled (Dvl) resulting in disruption of the axin/APC complex and inhibition of the kinase activity of glycogen synthase kinase 3β (GSK-3β). Phosphorylation by GSK-3β of the serine(s) within exon 3 of β-catenin results in its degradation by the E3 ubiquitin ligase. Thus, wnt signaling increases the availability of β-catenin in the nucleus, where it binds to transcription factors of the T-cell factor (TCF) and lymphoid enhancer factor (LEF) families to promote the expression of genes such as cyclin D1 and c-myc important for proliferation. β-Catenin also forms part of the adherens junction complex with E-cadherin where it may play an important role in keratinocyte differentiation. Tyrosine phosphorylation of E-cadherin, as occurs after calcium administration to keratinocytes, promotes the binding of β-catenin and other catenins to the adherens junction complex making it less available for transcriptional activity. Over expression and/or activating mutations in the β-catenin pathway lead to skin tumors, in this case pilomatricomas or trichofolliculomas (hair follicle tumors), indicative of the hyperproliferative response to β-catenin in these cells. In colon cancer cells, VDR has been shown to bind to β-catenin, and reduce its transcriptional activity in a ligand dependent fashion. Furthermore, in these cells, 1,25(OH)\(_2\)D has been shown to increase E-cadherin expression, such that β-catenin is redistributed from the nucleus to the plasma membrane where it forms a complex with E-cadherin and other catenins at adherens junctions. We have observed similar phenomena in keratinocytes. However, the suppression of β-catenin signaling by 1,25(OH)\(_2\)D does not necessarily require E-cadherin. Rather, β-catenin binds to VDR in its AF-2 domain, binding that enhances the ability of 1,25(OH)\(_2\)D to activate the transcriptional activity of the VDR, but blocks the transcriptional activity of β-catenin. Mutations in the AF-2 domain that block coactivator binding do not necessarily block β-catenin binding. Palmer et al. evaluated the interaction between VDR and β-catenin in transcriptional regulation in keratinocytes, and identified putative response elements for VDR and β-catenin/LEF in a number of genes. These interactions were either positive or negative, depending on the gene being evaluated. The hypothesis put forward is that genes in which the interaction was positive (i.e. stimulated transcription) benefited from β-catenin acting as a coactivator for VDR on VDREs, whereas in situations where the interaction was negative (i.e. suppression of transcription), VDR prevented β-catenin from binding to TCF/LEF required for transcription in those genes. We have found in keratinocytes that knockdown of VDR reduces E-cadherin expression and formation of the β-catenin/E-cadherin membrane complex, resulting in increased β-catenin transcriptional activity, whereas 1,25(OH)\(_2\)D administration has the opposite effect. This was associated with increased (with VDR knockdown) or decreased (with 1,25(OH)\(_2\)D administration) keratinocyte proliferation and cyclin D1 expression,
respectively. These actions in the epidermis (and intestinal epithelium) appear to differ from those in the hair follicle in that Cianferotti et al.,\textsuperscript{79} found a reduction in the proliferation of keratinocytes in the dermal portion of the hair follicle (below the bulge) in VDR null mice, and no stimulation of proliferation when β-catenin was overexpressed in these cells, in contrast to the stimulation of proliferation in control animals. Thus, VDR/β-catenin interactions can be positive or negative, depending on the gene/cell/function being evaluated; however, in the epidermis in the absence of VDR, the unchecked activity of \textit{wnt}/β-catenin appears to be proliferative and inhibitory of differentiation.

\textbf{Vitamin D regulation of epidermal immunity}

The potential role for vitamin D and its active metabolite 1,25(OH)\textsubscript{2}D to modulate the immune response rests on the observation that VDR is found in activated dendritic cells, macrophages, and lymphocytes,\textsuperscript{80,81} that these cells produce 1,25(OH)\textsubscript{2}D (\textit{i.e.} express CYP27B1),\textsuperscript{80} and that 1,25(OH)\textsubscript{2}D regulates their proliferation and function.\textsuperscript{82} Two forms of immunity exist, adaptive and innate, and each are regulated by 1,25(OH)\textsubscript{2}D.

\textbf{Adaptive immunity}—The adaptive immune response involves the ability of T and B lymphocytes to produce cytokines and immunoglobulins, respectively, to specifically combat the source of the antigen presented to them by cells such as macrophages and dendritic cells. Vitamin D exerts an inhibitory action on the adaptive immune system. In particular, 1,25(OH)\textsubscript{2}D suppresses proliferation and immunoglobulin production and retards the differentiation of B-cell precursors into plasma cells.\textsuperscript{81} In addition, 1,25(OH)\textsubscript{2}D inhibits T-cell proliferation,\textsuperscript{83} in particular the Th1 cells capable of producing IFN-γ and IL-2 and activating macrophages\textsuperscript{84} and Th17 cells capable of producing IL-17 and IL-22.\textsuperscript{85,86} In contrast IL-4, IL-5, and IL-10 production increase,\textsuperscript{87} shifting the balance to a Th2 cell phenotype. CD4+ / CD25+ regulatory T-cells (Treg) are also increased by 1,25 (OH)\textsubscript{2}D leading to increased IL-10 production. The IL-10 so produced is one means by which Treg can block Th1 and Th17 development. At least in part, these actions on T-cell proliferation and differentiation stem from actions of 1,25(OH)\textsubscript{2}D on dendritic cells to reduce their maturation and antigen presenting capability.\textsuperscript{89} The ability of 1,25(OH)\textsubscript{2}D to suppress the adaptive immune system is beneficial for conditions in which the immune system is directed at self—\textit{i.e.} autoimmunity\textsuperscript{90} and following transplants\textsuperscript{91}—but might not be good for tumor surveillance.

\textbf{Innate immunity}—The innate immune response is the critical first line of defense against invading pathogens. The response involves the activation of toll-like receptors (TLRs) in polymorphonuclear cells (PMNs), monocytes, and macrophages as well as in a number of epithelial cells including those of the epidermis, gingiva, intestine, vagina, bladder, and lungs.\textsuperscript{92} TLRs are transmembrane pathogen-recognition receptors that interact with specific membrane patterns (PAMP) shed by infectious agents that trigger the innate immune response in the host.\textsuperscript{93} Activation of TLRs leads to the induction of antimicrobial peptides and reactive oxygen species, which kill the organism. Among these antimicrobial peptides is cathelicidin. The expression of this anti-microbial peptide is induced by 1,25(OH)\textsubscript{2}D in both myeloid and epithelial cells,\textsuperscript{94,95} cells that also express CYP27B1 and so are capable of producing the 1,25(OH)\textsubscript{2}D needed for this induction. Stimulation of TLR2 by an antimicrobial peptide in macrophages\textsuperscript{96} or stimulation of TLR2 in keratinocytes by wounding the epidermis\textsuperscript{48} results in increased CYP27B1 expression, which in the presence of adequate substrate (25OHD) stimulates cathelicidin expression. Lack of substrate (25OHD), VDR, or CYP27B1 blunts the ability of these cells to respond to a challenge with respect to cathelicidin production.\textsuperscript{48,95,96}
The major cells involved in adaptive immunity in the skin include the Langerhans cells, dendritic cells, and T-cells. The Langerhans cells are dendritic-like cells within the epidermis that when activated by invading organisms, migrate to the lymph nodes serving the skin where they present the antigens to the T-cells, initiating the adaptive immune response. Keratinocytes, on the other hand, are equipped with toll-like receptors that enable them to respond to invading organisms with elaboration of antimicrobial peptides such as cathelicidin but also a variety of cytokines that provoke an inflammatory response. UVB leads to a reduction in Langerhans cells and blunts their antigen presenting activity, but stimulates the innate immune function of keratinocytes perhaps as a consequence of UVB induced vitamin D/1,25(OH)2D production in the skin.

The potential role of altered skin immunity by UVB with respect to skin carcinogenesis was suggested by Kripke and Fisher who found that skin tumors originally induced in mice by chronic UVR grew when transplanted into other UV-irradiated mice; however, it was found that the tumors did not grow further when transplanted into non-irradiated mice. What is less clear is whether 1,25(OH)2D would enhance or protect against UVR immunosuppression. One study demonstrated that topical 1,25(OH)2D at a high concentration (1.65 μM) protected against UVR induced suppression of contact hypersensitivity to oxazolone in mouse skin; however, a study in humans from the same group showed suppression of delayed hypersensitivity (Mantoux test) by topical 1,25(OH)2D at high doses with a trend toward additive suppression even at lower doses when combined with UVR. These data are limited, but raise some concerns about the balance between innate and adaptive immunity in tumor surveillance, and how that balance is affected by vitamin D.

Vitamin D regulation of the DNA damage response

UV wavelengths shorter than 280 nm (UVC) are absorbed by the ozone layer and do not reach the earth. UV wavelengths longer than 320 nm (UVA) are capable of penetrating into the dermis, and damage DNA (e.g. 8-oxoguanine production) primarily by oxidative processes. UVA does not stimulate vitamin D production. UVB, with a spectrum range of 280–320 nm, exerts its effects primarily in the epidermis, where it causes DNA cyclobutane pyrimidine dimers (CPD) and pyrimidine (6–4)pyrimidone photoproducts (6–4PP), which if not repaired result in C to T or CC to TT mutations, the UVB “signature” lesion. This type of mutation in p53 is common (50–90%) in both BCC and SCC as well as in actinic keratoses, the precursor lesions to SCC. Preventing UVR induced DNA damage from producing DNA mutations is the role of DNA damage repair (DDR), and p53 appears to have an important role in this process. DDR involves a cascade of damage recognition, repair and signal transduction that coordinates the response of the cell cycle to DNA damage. DDR activates checkpoints that delay the cell cycle, provides time for repair, and directs damaged cells into senescent or apoptotic pathways. DDR involves a number of components, is well orchestrated, tightly controlled, and highly accurate in normal primary cells such that the spontaneous mutation rate is very low, and changes in copy number are negligible. During malignant transformation, DDR becomes less controlled, and mutation rates and copy number abnormalities increase by several orders of magnitude. Nucleotide excision repair (NER) is the principal means by which UVR damage is repaired. NER can remove DNA damage before DNA replication begins, and consequently plays a major role in reducing the amount of damage that becomes fixed as mutations during replication. The DNA damage is recognized, the DNA is unwound around the lesion, and 30 base pair portions of DNA containing the lesion are excised by endonucleases such as XPF and XPG followed by fill-in with DNA polymerases such as Polδ, Polε, and Polκ.

The NER process has two main branches distinguished by the mechanisms used for initial recognition of DNA damage: transcription coupled repair (TCR) during which DNA
polymerases stop replication at the site of the lesion until it is repaired, and global genomic repair (GGR), during which non-transcribed regions of the genome are repaired. Keratinocytes in the epidermis of mice lacking VDR are deficient in DDR as demonstrated by a reduced rate of clearing CPDs and 6-4PPs following UVB. Moreover, 1,25(OH)_2D increases CPD clearance and p53 in VDR intact mice as well as upregulation of two genes important for DDR: XPC (xeroderma pigmentosum complementation group C) and DDB2 (damage-specific DNA binding protein 2, also known as XPE). These actions of vitamin D signaling on DDR contribute to the reduced susceptibility of normal skin to UVB induced tumor formation.

Summary

UVB is critical for vitamin D production in the skin, but UVB is also the major cause of skin cancer. This article examines the question of whether the beneficial effects of vitamin D production can counter the harmful effects of carcinogenesis, and the predisposition of VDR null mice to UVB induced skin cancer suggests that it is a possibility. Three potential mechanisms for such protection were examined. The first mechanism focuses on the role of vitamin D signaling in keratinocyte proliferation and differentiation. In particular, two pathways affecting proliferation and differentiation, namely the hedgehog and wnt/β-catenin pathways, were evaluated. Mice lacking the VDR have increased expression of the hedgehog pathway and increased activation of the wnt/β-catenin pathway. 1,25(OH)_2D suppresses both pathways in cells containing the VDR. Thus, in the absence of vitamin D signaling, overexpression of the hedgehog and wnt/β-catenin pathways leads to increased proliferation and decreased differentiation associated with tumor development. The second mechanism involves the role of vitamin D signaling in the immune system of the skin. 1,25(OH)_2D/VDR promotes innate immunity but suppresses adaptive immunity. UVB likewise promotes innate immunity and suppresses adaptive immunity, perhaps in part by stimulating vitamin D/1,25-(OH)_2D production. The net effect on tumor formation with respect to protection by vitamin D signaling is unclear. The third mechanism is DNA damage repair (DDR). The epidermis of VDR null mice show impaired DDR following UVR. 1,25(OH)_2D accelerates DDR. Thus, by these mechanisms one can conclude that the skin has evolved protective mechanisms against UVR induced carcinogenesis, and that these mechanisms involve vitamin D.

Acknowledgments

This work was supported by grants from the American Institute for Cancer Research, the VA Merit Review, the Department of Defense (CA110338), and the National Institute of Health (RO1 AR050023). Much of the data referred to was generated in collaboration with Drs. Arnaud Teichert, Yuko Oda, Dennis Oh, and JoEllen Welsh. Teresa Tong and Victoria Lee provided administrative support.

References


60. Regl G, Kasper M, Schmidt H, Eichberger T, Neill GW, Philpott MP, Esterbauer H, Hauser-Kronberger C, Frischauf AM, Aberger F. Activation of the BCL2 promoter in response to...


85. Daniel C, Sartory NA, Zahn N, Radeke HH, Stein JM. Immune modulatory treatment of trinitrobenzene sulfonic acid colitis with calcitriol is associated with a change of a Th helper (Th) 1/Th17 to a Th2 and regulatory T cell profile. J Pharmacol Exp Ther. 2008; 324:23–33. [PubMed: 17911375]


Fig. 1.
Production of vitamin D and its metabolism to 1,25(OH)₂D in the keratinocyte. UVB, via a photochemical reaction, breaks open the B ring of 7-dehydrocholesterol (7-DHC) to produce pre vitamin D₃, which is subsequently converted first to 25OHD by the enzymes CYP27A1 and CYP2R1 and then to 1,25(OH)₂D by CYP27B1. Regulation of CYP27B1 is primarily by cytokines such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ).
Fig. 2.
The hedgehog signaling pathway. In the absence of Shh, Ptch 1 suppresses signaling by smoothened (Smo). Binding of Shh to Ptch 1 relieves this inhibition. Activation of Smo leads to the activation and translocation of transcription factors of the Gli family into the nucleus, with subsequent changes in gene expression.
Fig. 3.
The wnt signaling pathway. Wnts bind to their frizzled receptors (FZ) and coreceptors LRP in the membrane. This binding can be blocked by dickkopf (Dkk) or soluble frizzled related proteins (sFRP). Activation of FZ by wnt results in phosphorylation of disheveled (Dvl) which induces the disruption of the axin/APC/GSK-3β complex and recruitment of axin to the membrane. When active, this complex phosphorylates β-catenin, leading to its proteosomal degradation. However, following wnt stimulation, β-catenin is no longer degraded and can enter the nucleus, where in combination with members of the LEF/TCF family, it can induce expression of its target genes such as cyclin D1. β-Catenin also binds to the E-cadherin complex in the plasma membrane, a complex stabilized by calcium, where it may play a role in differentiation.
CHAPTER

THE VITAMIN D RECEPTOR:
A Tumor Suppressor in Skin

Daniel D. Bikle
Department of Medicine and Dermatology, VA Medical Center and University of California, San Francisco, California, USA.
Email: daniel.bikle@ucsf.edu

Abstract: Cutaneous malignancies including melanomas and non melanoma skin cancers (NMSC) are the most common types of cancer, occurring at a rate of over 1 million per year in the United States. The major cell in the epidermis, the keratinocyte, not only produces vitamin D but contains the enzymatic machinery to metabolize vitamin D to its active metabolite, 1,25(OH)2D, and expresses the receptor for this metabolite, the vitamin D receptor (VDR), allowing the cell to respond to the 1,25(OH)2D that it produces. In vitro, 1,25(OH)2D stimulates the differentiation and inhibits the proliferation of these cells and so would be expected to be tumor suppressive. However, epidemiologic evidence demonstrating a negative relationship between circulating levels of the substrate for CYP27B1, 25OHD, and the incidence of these malignancies is mixed, raising the question whether vitamin D is protective in the in vivo setting. UV radiation (UV), both UVB and UVA, as occurs with sunlight exposure is generally regarded as causal for these malignancies, but UVB is also required for vitamin D synthesis in the skin. This complicates conclusions reached from epidemiologic studies in that UVB is associated with higher 25OHD levels as well as increased incidence of cutaneous malignancies. Based on our own data and that reported in the literature we hypothesize that vitamin D signaling in the skin suppresses UVR induced epidermal tumor formation. In this chapter we will first discuss recent data regarding potential mechanisms by which vitamin D signaling suppresses tumor formation, then focus on three general mechanisms that mediate tumor suppression by VDR in the skin: inhibition of proliferation and stimulation of differentiation, immune regulation, and stimulation of DNA damage repair (DDR).
INTRODUCTION

Over 1 million skin cancers occur annually in the United States, 80% of which are basal cell carcinomas (BCC), 16% squamous cell carcinomas (SCC), and 4% melanomas, making skin cancer by far the most common cancer afflicting humankind. UV radiation (UVR) from the sun is the major etiologic agent for these cancers. The highest energy UVR, UVC (below 280 nm), does not penetrate the atmosphere. Of the solar radiation that does reach the earth 95% is UVA and 5% UVB. UVB (280–320 nm), although it does not penetrate past the epidermis, is absorbed by DNA in the epidermal cells creating characteristic mutations identified as cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6–4)pyrimidone photoproducts (6–4PP), which if not repaired result in C to T or CC to TT mutations, the UVB “signature” lesion. UV wavelengths between 320–400 nm (UVA) are capable of penetrating into the dermis, and do their DNA damage (e.g., 8 hydroxy 2’ deoxyguanosine production) primarily by oxidative processes, although at high enough dose levels UVA can produce CPDs. On the other hand UVB is required to convert 7-dehydrocholesterol levels in the skin to pre vitamin D₃, which then isomerizes to vitamin D₃. Moreover, the skin is capable of converting the vitamin D produced to its active metabolite 1,25(OH)₂D, and this conversion is potentiated by UVR at least in part by cytokines such as TNF-α which are increased by UVR in the epidermis. Both melanocytes and keratinocytes express the vitamin D receptor (VDR) and respond to 1,25(OH)₂D with reduced proliferation and increased differentiation. Sun avoidance may reduce one’s risk of developing skin cancer, but this practice generally results in suboptimal levels of vitamin D in the body. In an analysis by Lucas et al., the global disease burden due to UVR is substantially less than the disease burden due to vitamin D deficiency. Vitamin D supplementation can compensate, but the skin remains the major site of vitamin D availability for most of the world’s population. Moreover, low dose UVR may be protective against skin cancer via the vitamin D signaling mechanisms that will be reviewed in this article, and some epidemiologic evidence is consistent with a potential benefit of low dose UVR. For example, in the study by Armstrong and Kricker, a slight decrease in the incidence of SCC, BCC, and melanomas in 10 US populations was observed when the solar UV measurement was increased from 100 to 110, although higher levels increased the incidence. This same group, evaluating data from the Australian population, did not find a significant increase in SCC with time spent out of doors in the general population. Rosso et al. in a multicenter European study did not find a significant increase in SCC below a threshold of 70,000 accumulated hours of sunshine, although the development of BCC had a lower threshold. In this chapter, after a review of vitamin D metabolism and VDR function, we will examine potential mechanisms that have been proposed for vitamin D induced antitumor mechanisms in general, then focus on those mechanisms that have been shown to be operative in the epidermis.

VITAMIN D METABOLISM

Vitamin D₃ is produced from 7-dehydrocholesterol (7-DHC). Irradiation of 7-DHC with UVB produces pre vitamin D₃, which subsequently undergoes a temperature-dependent rearrangement of the triene structure to form vitamin D₃, lumisterol, and tachysterol.

T
of exposure influence the time required to achieve this maximal concentration of pre vitamin D₃. With continued UV exposure the biologically inactive lumisterol and tachysterol accumulate eliminating the risk of excessive production of vitamin D. Sunlight exposure increases melanin production, which can absorb UVB, and so provides another mechanism by which excess vitamin D₃ production can be prevented. The intensity of UVR is dependent on latitude and season. In Edmonton, Canada (52°N) very little vitamin D₃ is produced in exposed skin from mid-October to mid-April, while in San Juan (18°N) the skin is able to produce vitamin D₃ all year long. Clothing and sunscreen effectively prevent vitamin D₃ production in the covered areas. Vitamin D₃ produced in the skin can be carried to the liver and other tissues for further metabolism to 25-hydroxyvitamin D (25OHD) and then to the kidney to produce 1,25(OH)₂D by the enzyme CYP27B1. However, as noted above, the keratinocyte contains the entire pathway for 1,25(OH)₂D production from vitamin D.

The production of 1,25(OH)₂D in the skin is under quite different regulation compared with its production by the kidney. In the kidney parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23) and 1,25(OH)₂D itself are the principal hormonal regulators: PTH stimulates, whereas FGF23 and 1,25(OH)₂D inhibit 1,25(OH)₂D production. Keratinocytes respond to PTH with increased 1,25(OH)₂D production, but these cells do not have the classic PTH receptor and do not respond to cyclic AMP unlike the kidney. The effect of FGF23 on keratinocyte CYP27B1 expression or function has not been reported. Furthermore, unlike the kidney, 1,25(OH)₂D does not directly affect CYP27B1 expression in keratinocytes. Rather, 1,25(OH)₂D regulates its own levels in the keratinocyte by inducing CYP24, the catabolic enzyme for 1,25(OH)₂D. In the keratinocyte the major regulators of 1,25(OH)₂D production are cytokines such as tumor necrosis factor-α (TNF) and interferon-γ (IFN). These cytokines are activated in the skin by UVB, which of course also increases the substrate via increased vitamin D production.

VITAMIN D RECEPTOR: MECHANISM OF ACTION

The VDR is a member of the nuclear hormone receptor superfamily. These members are characterized by a highly conserved DNA binding domain characterized by two zinc fingers and a structurally conserved ligand binding domain that has at its C-terminal end the AF2 domain to which coactivator complexes bind. The ligand binding domain also serves as the region to which VDR binds to its transcriptional partners, RXR being the major one. In general ligand (ie. 1,25(OH)₂D) binding is required for the VDR/RXR heterodimer to form and bind to those regions on the DNA called vitamin D response elements (VDRE). Ligand binding also alters the structure of the VDR with major movement of helix 12 (C-terminus) into position to enclose the ligand while exposing sites on the VDR in helices 3, 5 and 12 to which coactivators bind. These coactivators can in turn recruit chromatin modifying enzymes such as histone acetyl transferases (SRC, CBP/p300, pCAF) and DNA demethylases or proteins that bridge the gap between the VDRE and the transcription machinery (Mediator complex) including TATA associated factors, TFIIb, and RNA polymerases (primarily RNA pol II). In the absence of ligand binding sites for corepressors are exposed. These corepressors recruit another set of chromatin modifying enzymes such as histone deacetylases and DNA methyl transferases. The most common VDRE is comprised of two head to tail half sites of hexanucleotides separated by 3 nucleotides, referred to as DR3 VDREs. The sequence of these DR
sites is heterogeneous, with a consensus approximated by RGKTSA where R = A or G, K = G or T, S = C or G. Moreover, many VDREs are not DR3s, although DR3s tend to have the highest affinity for VDR/RXR heterodimers.

The VDREs can be quite distant from the transcription start site of the gene being regulated, occurring in introns, between genes, and in either a 5’ or 3’ relationship to the coding region. Moreover, putative VDREs as demonstrated by techniques such as ChIP-seq, in which binding sites to the genome by VDR are identified using a combination of chromatin immunoprecipitation of the VDR to DNA followed by high throughput sequencing of those binding regions, number in the thousands, with a substantial degree of cell/tissue specificity. Most genes have several VDREs. In a review of two such ChIP-seq studies Carlberg et al. noted that the study in a lymphoblastoid line identified 2776 VDREs for 232 genes whereas the study in THP-1 monocytes identified 1820 VDREs for 638 genes. In the latter study 408 of the genes were upregulated, 230 downregulated. Most of the VDREs for the upregulated genes were within 400kbp of the transcription start site; this was less true for the downregulated genes. Only 93 of the upregulated genes had VDREs within 30kbp of the transcription start site. Moreover, only 31.7% of the VDREs were DR3s. These two studies had a mere 18% overlap of the VDREs identified, but differed not only in cell line but dose and time after 1,25(OH)2D was administered before the cells were analyzed. Earlier microarray studies had likewise demonstrated the many genes regulated by 1,25(OH)2D, and the surprising lack of consensus from one study to the next perhaps due to tissue specificity and/or differences in dose and time of 1,25(OH)2D exposure. These studies demonstrate the diversity of vitamin D regulated genes and diversity in type and location of VDREs. Such studies have revolutionized our concepts of the scope and means of vitamin D signaling, and reveal many potential mechanisms by which vitamin D signaling can regulate cancer formation. In this regard an unbiased systems biology approach mapping genetic loci underlying susceptibility to skin cancer put the VDR in the center of a complex set of networks linking regulation of barrier function, inflammation, and tumor formation.

The VDR is essential for nearly all actions of 1,25(OH)2D and its analogs. Tumors that are unresponsive to vitamin D have either lost their ability to produce 1,25(OH)2D (ie. decreased CYP27B1), increased their metabolism of 1,25(OH)2D via upregulation of CYP24A1, lost VDR transcriptional activity through post translational alterations in RXR, or decreased their VDR expression. The latter may be secondary to increased activity in tumors of inhibitors of VDR expression such as SNAIL and SLUG, increased methylation of the VDR promoter, or increased expression of miRNA125b, an inhibitor of VDR expression. In melanoma cell lines the administration of 5-aza cytidine (to inhibit DNA methyltransferase) and trichostatin (to inhibit HDAC activity) could restore responsiveness to 1,25(OH)2D by increasing VDR levels and reducing miR125b expression.

MECHANISMS OF TUMOR SUPPRESSION BY VITAMIN D: GENERAL

The demonstration that many genes and pathways are influenced by vitamin D signaling has opened up a large number of potential means by which vitamin D signaling can control tumor growth (recent reviews in refs. 37,38) (Fig. 1).
The Vitamin D Receptor

Cell Cycle Regulation

Regulation by 1,25(OH)_{2}D of the cell cycle in a number of cells, normal and malignant, has been demonstrated. This results from an upregulation of cell cycle inhibitors such as p21cip and p27kip (cyclin-dependent kinase inhibitors) and retinoblastoma like protein 2 and retinoblastoma binding protein 6 and decreased expression of cyclins and cyclin dependent kinases. In addition, 1,25(OH)_{2}D increases the interaction of FoxO proteins (tumor suppressors controlling proliferation) with VDR and FoxO regulators Sirt1 and protein phosphatase 1 that maintain FoxO in the nucleus by blocking MAPK phosphorylation. Increased c-MYC expression and activity are frequently found in cancer. c-MYC induces the expression of a number of cell cycle regulatory genes such as cyclin D2 and cdk4. 1,25(OH)_{2}D inhibits the expression of c-MYC, and c-MYC expression is increased in the skin and gut of VDR null mice. Growth Factors

1,25(OH)_{2}D and its analogs regulate a number of growth factor pathways. Insulin like growth factor (IGF) stimulated proliferation of breast and prostate cells is reduced by 1,25(OH)_{2}D via its induction of IGF-I binding protein. TGFβ2 exerts antiproliferative
actions in epithelial cells. 1,25(OH)₂D and its analogs increase the expression of TGFβ2 and TGFβ receptors in breast and prostate cancer cells, while suppressing the expression of the latent TGFβ-binding protein. GDF15 (growth differentiation factor 15) is a member of the TGFβ superfamily, and like TGFβ is antiproliferative in prostate cancer cells. Its expression is increased by 1,25(OH)₂D. Bone morphogenic proteins (BMPs) are also members of the TGFβ superfamily that have been found to be dysregulated in certain cancers. The expression of several BMPs is regulated by 1,25(OH)₂D and its analogs in a number of malignant cell lines. Wnt/β-catenin signaling will be dealt with in depth when we focus on vitamin D regulated pathways in the skin, but this pathway has been extensively studied in the colon based on the frequency of mutations in the adenomatous polyposis coli (APC) gene in colon cancer. In the canonical pathway of wnt/β-catenin signaling the APC complex that would otherwise bind and phosphorylate β-catenin, targeting it for proteosomal degradation, is inactivated, allowing β-catenin to move to the nucleus where it binds to LEF/TCF leading to transcription of genes involved with proliferation. 1,25(OH)₂D/VDR binds to β-catenin, preventing its movement into the nucleus and/or binding to LEF/TCF. Moreover, by increasing the levels of E-cadherin, which binds β-catenin in the plasma membrane, 1,25(OH)₂D can further reduce the translocation of β-catenin into the nucleus. Furthermore, 1,25(OH)₂D can suppress wnt signaling by stimulating the expression of the wnt antagonist DKK-1. Cystatin D, an inhibitor of several cysteine proteases of the cathepsin family that appear to be involved in wnt signaling, has likewise been shown to be a target gene of 1,25(OH)₂D. The induction of cystatin D and other 1,25(OH)₂D target genes such as E-cadherin appear to involve a non genomic action requiring calcium activation of RhoA-ROCK-p38MAPK-MSK in colon cancer cells. We have shown that this pathway requires the 1,25(OH)₂D induced calcium receptor in keratinocytes. These and other studies point to the interaction between calcium and vitamin D signaling in the regulation of tumor formation, an interaction that to date has received little attention.

**Apoptosis**

In addition to inhibiting proliferation, 1,25(OH)₂D promotes apoptosis in a number of malignant cell lines in part by downregulation of anti-apoptotic genes Bcl-2 and Bcl-Xl and upregulation of the proapoptotic gene GOS2 (G₀,G₁ switch gene 2). Transcripts of other pro-apoptotic genes increased by 1,25(OH)₂D include death-associated protein-3, caspase 8 apoptosis-related cysteine peptidase, and fas-associated death domain-like apoptosis regulator as well as a number of caspases. Telomerase is a mechanism that enables cancer cells to escape apoptosis. 1,25(OH)₂D suppresses telomerase expression by inducing miRNA498, a transcript in the complementary strand of CTC-360P6. Of interest is this miRNA has its own VDRE.

**Oxidative Stress**

As noted previously in the discussion of UVA induced effects on the epidermis, oxidative stress can lead to oxidative DNA damage, marked by 8 hydroxy 2′-deoxyguanosine. In VDR knockout mice, 8 hydroxy 2′-deoxyguanosine levels are increased in the colon and reduced by vitamin D supplementation in humans. 1,25(OH)₂D induces several antioxidant enzymes in cancer cells including thioredoxin reductase, superoxide dismutase, and glucose-6 phosphate dehydrogenase. The induction of genes associated
with DNA repair will be discussed at greater length when we focus on UVB damage to the epidermis, but the induction by 1,25(OH)\(_2\)D of GADD45\(\alpha\) (growth arrest and DNA-damage inducible \(\alpha\)), p53, RAD23B, PCNA, and DAP-1\(\alpha\) may all contribute to this aspect of tumor suppression by 1,25(OH)\(_2\)D/VDR.\(^{40,66,70}\)

**Prostaglandins**

Prostaglandins have been shown to stimulate cancer cell growth.\(^{71}\) 1,25(OH)\(_2\)D blocks prostaglandin signaling by inhibiting COX2 expression and that of prostaglandin receptors while increasing the expression of hydroxyprostaglandin dehydrogenase 15-NAD, the prostaglandin inactivating enzyme.\(^{53,72}\)

**Angiogenesis**

Growing tumors require a blood supply. 1,25(OH)\(_2\)D inhibits angiogenesis by blocking the expression and function of VEGF (vascular endothelial growth factor),\(^{73-75}\) and mice lacking VDR had larger and more vascular tumors when implanted with prostate cells from TRAMP mice.\(^{76}\)

**Immune System**

The immune system plays an important protective role in cancer protection\(^{77}\) as evidenced by the increased numbers of malignancies in immunosuppressed hosts including SCCs in immunosuppressed renal transplant patients.\(^{78}\) This mechanism will be dealt with in more depth when we focus on the skin, as it has not received much study in the context of tumor protection in general by 1,25(OH)\(_2\)D. In fact by stimulating innate immunity, which would promote inflammation, and suppressing adaptive immunity, which would blunt immune surveillance one might expect 1,25(OH)\(_2\)D to promote rather than block tumor development via its actions on the immune system. As such, this mechanism of action of 1,25(OH)\(_2\)D and its receptor with respect to cancer development requires further study.

**MECHANISMS OF TUMOR SUPPRESSION BY 1,25(OH)\(_2\)D/VDR IN THE EPIDERMIS**

The potential for vitamin D signaling as protection against epidermal tumor formation was demonstrated when Zinser et al.\(^{79}\) demonstrated that 85% of the VDR null mice but none of the controls developed skin tumors within two months following 7,12 dimethylbenzanthracene (DMBA) administration. These were primarily papillomas. These results have been confirmed using topical administration of DMBA/TPA.\(^{80}\) However, although only papillomas were seen in the VDR null mice, RXR\(\alpha\) null mice developed both BCC and SCC.\(^{80}\) Subsequently, Ellison et al.\(^{81}\) and our own group\(^{82}\) demonstrated that VDR null mice were also more susceptible to tumor formation following UVB, and many of the tumors were SCC and BCC. The appearance of BCC in these studies is surprising since the typical malignancy induced in mouse skin by UVR, ionizing radiation, or chemical carcinogens is SCC not BCC.\(^{83}\) Given that BCC generally result from increased hedgehog (Hh) signaling,\(^{84}\) and that lack of VDR results in BCC when \(\beta\)-catenin signaling is increased,\(^{85}\) we became interested in the relationship between
vitamin D, Hh, and β-catenin signaling in tumor suppression. The lack of a normal innate immune response in CYP27B1 null mice to wounding\textsuperscript{86} or infection\textsuperscript{87} and the increased numbers of SCC in immunocompromised patients\textsuperscript{78} suggested that disruption of the immune system might contribute to the increased susceptibility to tumor formation when vitamin D signaling was impaired. Moreover, we\textsuperscript{82} noted a reduction in clearance in CPDs following UVB exposure of the skin of VDR null mice, suggesting that disruption of DNA damage repair was playing a role in tumor susceptibility in these mice. In what follows I will examine three potential mechanisms and pathways within those mechanisms for their contribution to the role of VDR as a tumor suppressor: regulation of proliferation and differentiation with particular attention to the hedgehog (Hh) and wnt/β-catenin pathways, immunoregulation, and DNA damage repair.

**Vitamin D Regulation of Epidermal Proliferation and Differentiation**

The epidermis is composed of four layers of keratinocytes at different stages of differentiation (reviewed in ref. 10). The basal layer (stratum basale, SB) rests on the basal lamina separating the dermis and epidermis. Within this layer are the stem cells. These cells proliferate, providing the cells for the upper differentiating layers. The basal cells are characterized by keratins K5 and K14 as well as the stem cell marker K15 and integrin α6β4. As the cells migrate upward from this basal layer into the spinous layer (stratum spinosum, SS) they initiate the production of the keratins K1 and K10, the keratins characteristic of the more differentiated layers of the epidermis. Cornified envelope precursors such as involucrin also appear in the spinous layer as does the enzyme transglutaminase K, responsible for the ε-(γ-glutamyl)lysine cross-linking of these substrates into the insoluble cornified envelope. Migrating further into the granular layer (stratum granulosum, SG), lying above the spinous layer, the cells acquire the electron-dense keratohyalin granules containing profilaggrin and loricrin that give the SG its name. Loricrin is a major component of the cornified envelope. Filaggrin serves to bundle the keratin filaments, but also when proteolyzed is thought to contribute to the hydration of the outer layers. The granular layer also contains lamellar bodies—lipid, enzyme, and antimicrobial peptide filled structures that fuse with the plasma membrane, divesting their contents into the extracellular space between the SG and stratum corneum (SC). The secreted enzymes process the lipids that contribute to the permeability barrier of the epidermis in conjunction with the keratin bundles and cornified envelope. The antimicrobial peptides provide a barrier against infectious organisms in the SC.

1,25(OH)\textsubscript{2}D increases essentially every step of this differentiation process\textsuperscript{88-93} while inhibiting proliferation at least at concentrations above 1nM. These actions complement those of calcium,\textsuperscript{62} the response to which is enhanced by 1,25(OH)\textsubscript{2}D via its induction of the calcium receptor\textsuperscript{94,95} and the phospholipase C enzymes\textsuperscript{96-98} that regulate intracellular calcium and other signaling molecules critical for the differentiation process. The antiproliferative effects are accompanied by a reduction in the expression of c-myc\textsuperscript{99} and cyclin D1\textsuperscript{100} and an increase in the cell cycle inhibitors p21\textsuperscript{101} and p27\textsuperscript{102}. In addition, 1,25(OH)\textsubscript{2}D and its receptor regulate the processing of the long chain glycosylceramides that are critical for permeability barrier formation\textsuperscript{103} and induce the receptors, toll like receptor 2 (TLR2) and its coreceptor CD14, that initiate the innate immune response in skin.\textsuperscript{86} Activation of these receptors leads to the induction of CYP27B1 (the enzyme that produces 1,25(OH)\textsubscript{2}D), which in turn induces cathelicidin resulting in the killing of invasive organisms.\textsuperscript{86,102} Deletion of either VDR\textsuperscript{103,104} or CYP27B1\textsuperscript{105} results in defects
in the differentiation process leading to an abnormal barrier and increased proliferation of the epidermis with a defective innate immune response. Two pathways that appear to be important in vitamin D signaling in the epidermis with respect to proliferation and differentiation that we believe underlie the predisposition of the VDR null mouse to tumor formation are the Hh and wnt/β-catenin pathways.

**The Hedgehog (Hh) Pathway (Fig. 2).** In the skin sonic hedgehog (Shh) is the ligand for patched (Ptc) 1, a 12 transmembrane domain protein that in the absence of Shh inhibits the function of another membrane protein smoothened (Smo). Smo in turn maintains a family of transcription factors, Gli1 and Gli2 in particular, in the cytoplasm bound to Suppressor of fused (Sufu). When Shh binds to Ptc 1, the inhibition of Smo is relaxed, Gli1 and 2 are released from Sufu and move into the nucleus where they initiate transcription of a number of factors including each other as well as Ptc 1, the anti apoptotic factor bcl2, cyclins D1 and D2, E2F1, cdc45 (all of which promote proliferation), while suppressing genes associated with keratinocyte differentiation such as K1, K10, involucrin, loricrin and the VDR.

![Figure 2. Regulation of Hh and wnt/β-catenin signaling by 1,25(OH)2D/VDR.](image)

The keratinocyte expresses VDR and is capable of making its own 1,25(OH)2D3 from the vitamin D3 produced from 7-dehydrocholesterol (DHC) under the influence of UVB, as it has both CYP27A1/CYP27R1 (which convert vitamin D3 to 25OHD3) and CYP27B1 (which converts 25OHD to 1,25(OH)2D3). 1,25(OH)2D/VDR suppresses Shh and gli 1 expression, inhibiting the Hh pathway in keratinocytes. 1,25(OH)2D/VDR binds β-catenin and induces E-cadherin expression reducing the amount of β-catenin available for binding to TCF/LEF in the nucleus limiting its transcriptional activity. In combination these actions reduce the proliferative actions of Shh and β-catenin signaling in keratinocytes, limiting their ability to induce tumors in the skin.
The appearance of BCC is characteristic of tumors formed when Hh signaling is disrupted,\textsuperscript{113} although activation of Hh signaling also predisposes to UVR induced SCC formation.\textsuperscript{114} VDR null animals overexpress elements of the Hh signaling pathway in their epidermis and the epidermal portion (utricles) of the hair follicles.\textsuperscript{82} Moreover, 1,25(OH)\textsubscript{2}D suppresses the expression of all elements of the Hh pathway in a dose dependent fashion that requires the VDR\textsuperscript{82,115} and reduces tumor growth in Pich 1null mice. The promoters of Shh and Gli1 have binding sites for VD\textsuperscript{116} suggesting that the effects of 1,25(OH)\textsubscript{2}D on these genes is direct. However, vitamin D has also been shown to bind to and inhibit the actions of smoothened (Smo) directly without seeming to require further metabolism to 1,25(OH)\textsubscript{2}D.\textsuperscript{117,118}

The wnt/β-Catenin Pathway (Fig. 2). Wnt signaling via activation of β-catenin has a complex role in VDR function as discussed briefly earlier. In the canonical pathway the receptor for wnt ligands is a family of seven-transmembrane Frizzled receptors and an LRP5 or LRP6 co-receptor. When wnt binds to this complex disheveled (Dvl) is phosphorylated resulting in disruption of the axin/APC complex and inhibition of glycogen synthase kinase 3β (GSK-3β). In the basal state GSK-3β phosphorylates the serine(s) within exon 3 of β-catenin resulting in its degradation by the E3 ubiquitin ligase. Wnt signaling, by blocking this phosphorylation, increases the availability of β-catenin in the nucleus, where it binds to transcription factors of the T-cell factor (TCF) and lymphoid enhancer factor (LEF) families to promote expression of genes such as cyclin D1 and c-myc\textsuperscript{119} important for proliferation. β-catenin also forms part of the adherens junction complex with E-cadherin where it may play an important role in keratinocyte differentiation.\textsuperscript{120} Tyrosine phosphorylation of E-cadherin, as occurs after calcium administration to keratinocytes, promotes the binding of β-catenin and other catenins to the adherens junction complex\textsuperscript{120,121} making it less available for transcriptional activity. 1,25(OH)\textsubscript{2}D increases E-cadherin expression.\textsuperscript{122} Overexpression and/or activating mutations in the β-catenin pathway lead to skin tumors, in this case pilomatrixomas or trichofolliculomas (hair follicle tumors).\textsuperscript{123-125} As noted earlier VDR binds to β-catenin, and reduces the transcriptional activity of β-catenin in a 1,25(OH)\textsubscript{2}D dependent fashion.\textsuperscript{57} On the other hand binding of β-catenin to VDR in its AF-2 domain enhances the 1,25(OH)\textsubscript{2}D dependent transcriptional activity of VDR.\textsuperscript{58} Palmer et al.\textsuperscript{85} evaluated the interaction between VDR and β-catenin in transcriptional regulation in keratinocytes, and identified putative response elements for VDR and β-catenin/LEF in a number of genes. These interactions were either positive or negative, depending on the gene being evaluated. The hypothesis put forward is that genes in which the interaction was positive (ie. stimulated transcription) benefited from β-catenin acting as a coactivator for VDR on VDREs, whereas in situations where the interaction was negative (ie. suppression of transcription) VDR prevented β-catenin from binding to TCF/LEF required for transcription in those genes. We\textsuperscript{100} have found in keratinocytes that knockdown of VDR reduces E-cadherin expression and formation of the β-catenin/E-cadherin membrane complex resulting in increased β-catenin transcriptional activity, whereas 1,25(OH)\textsubscript{2}D administration has the opposite effect. This was associated with increased (with VDR knockdown) or decreased (with 1,25(OH)\textsubscript{2}D administration) keratinocyte proliferation and cyclin D1 expression. On the other hand Cianferotti et al.\textsuperscript{126} found a reduction in proliferation of keratinocytes in the dermal portion of the hair follicle (below the bulge) in VDR null mice, and no stimulation of proliferation when β-catenin was overexpressed in these cells in contrast to the stimulation of proliferation in control animals. Thus VDR/β-catenin interactions
can be positive or negative, depending on the gene/cell/function being evaluated, but in the epidermis in the absence of VDR, the unchecked activity of β-catenin appears to be proliferative and inhibitory of differentiation resulting in BCC.

**Vitamin D Regulation of Immune Function in the Skin**

VDR and CYP27B1 are found in professional immune cells, namely dendritic cells, macrophages, and lymphocytes, responsible for both innate and adaptive immune responses as well as in epithelial cells expressing the components of the innate immune response. 1,25(OH)₂D regulates the proliferation and function of these cells. Although it is not clear the extent to which dysregulated immune function contributes to cancer development in the skin, a link between inflammation and cancer susceptibility in the skin involving VDR has been established.

**Adaptive Immunity.** The adaptive immune response involves the ability of T and B lymphocytes to produce cytokines and immunoglobulins, respectively, in response to antigens presented to them by cells such as macrophages and dendritic cells. 1,25(OH)₂D suppresses the adaptive immune response by inhibiting proliferation, immunoglobulin production, and differentiation of B-cell precursors into plasma cells. 1,25(OH)₂D inhibits T cell proliferation and the differentiation of CD4 cells into Th1 cells capable of producing IFN-γ and IL-2 and activating macrophages. On the other hand, 1,25(OH)₂D stimulates IL-4, IL-5, and IL10 production by increasing CD4 cell differentiation into Th2 and regulatory T cells (Treg). The IL-10 so produced is one means by which Treg block Th1 and Th17 development. Part of these effects is mediated by the negative impact of 1,25(OH)₂D on the maturation and antigen presenting capability of dendritic cells. It is unclear if this suppression of the adaptive immune system alters tumor surveillance in the skin.

**Innate Immunity.** The innate immune response involves the activation of toll-like receptors (TLRs) that serve as transmembrane pathogen-recognition receptors detecting specific membrane patterns (PAMP) shed by a wide variety of infectious agents. Activation of TLRs leads to the induction of antimicrobial peptides and reactive oxygen species, which kill the organism. Cathelicidin is the best studied of these antimicrobial peptides. The expression of cathelicidin is induced by 1,25(OH)₂D in both myeloid and epithelial cells, cells that also express CYP27B1 and so are capable of producing 1,25(OH)₂D needed for this induction. Stimulation of TLR2 in macrophages results in increased CYP27B1 expression, which in the presence of adequate substrate induces cathelicidin expression. Lack of substrate, VDR, or CYP27B1 blunts the ability of these cells to respond with respect to cathelicidin production.

The major cells involved in adaptive immunity in the skin include the Langerhans cells, dendritic cells, and T cells. The Langerhans cells are dendritic like cells within the epidermis that when activated by invading organisms migrate to the lymph nodes serving the skin where they present the antigens to the T cells, initiating the adaptive immune response. Keratinocytes, on the other hand, are equipped with toll like receptors that enable them to respond to invading organisms with elaboration of antimicrobial peptides such as cathelicidin. However, cathelicidin also induces an inflammatory response. UVB leads to a reduction in Langerhans cells and blunts their antigen presenting activity.
but stimulates the innate immune function of keratinocytes perhaps as a consequence of UVB induced vitamin D/1,25(OH)₂D production in the skin.¹⁴⁷,¹⁴⁸

The potential role of altered skin immunity by UVB with respect to skin carcinogenesis was suggested by Kripke and Fisher.¹⁴⁹ They found that skin tumors originally induced in mice by chronic UVR, would grow when transplanted into mice that had been UV irradiated but not when transplanted into control mice. The role of 1,25(OH)₂D production in UVR immunosuppression is not clear. Topical application of high concentrations of 1,25(OH)₂D protected against UVR induced suppression of contact hypersensitivity in the mouse,¹⁵⁰ but a study in humans by the same group showed suppression of delayed hypersensitivity (Mantoux test) by topical 1,25(OH)₂D.¹⁵¹ These data are limited, but raise some concern about the balance between innate and adaptive immunity in tumor surveillance, and how that balance is affected by vitamin D.

**Vitamin D Regulation of the DNA Damage Response**

DNA damage repair (DDR) is the means by which UVR and chemical induced DNA damage is prevented from producing fixed DNA mutations.¹⁵² DDR involves a cascade of damage recognition, repair and signal transduction that coordinates the response of the cell to DNA damage. DDR activates checkpoints that delay the cell cycle, provides time for repair, and directs damaged cells into senescent or apoptotic pathways. DDR involves a number of components, is well orchestrated, tightly controlled, and highly accurate in normal primary cells such that the spontaneous mutation rate is very low, and changes in copy number are negligible.¹⁵³‑¹⁵⁵ With malignant transformation DDR becomes less controlled, and mutation rates and copy number abnormalities increase by orders of magnitude.¹⁵³,¹⁵⁴,¹⁵⁶,¹⁵⁷ Nucleotide excision repair (NER) is the principal means by which UVR damage is repaired, enabling repair before DNA replication begins. This is important as NER plays a major role in reducing the amount of damage that becomes fixed as mutations during replication.¹⁵⁸‑¹⁶⁰ During NER, the DNA damage is recognized, the DNA unwound around the lesion, and 30 base pair portions of DNA containing the lesion are excised by endonucleases such as XPF and XPG followed by fill in with DNA polymerases such as Pol δ,ε,k.

The NER process has two main branches involving different mechanisms for the initial recognition of DNA damage:¹⁶¹ transcription coupled repair (TCR) during which DNA polymerases stop replication at the site of the lesion until it is repaired,¹⁶²‑¹⁶⁶ and global genomic repair (GGR), during which non-transcribed regions of the genome are repaired.¹⁶⁷ Keratinocytes in the epidermis of mice lacking VDR are deficient in DDR as demonstrated by a reduced rate of clearing CPDs and 6,4PPs following UVB.¹⁶⁸ Moreover, 1,25(OH)₂D increases CPD clearance in VDR intact mice.¹⁶⁹,¹⁷⁰ These actions have been demonstrated with 1,25(OH)₂D analogs that are not thought to have genomic activity.¹⁶⁹ However, at least part of this enhancement of CPD clearance is due to the upregulation of two genes important for DDR: XPC (xeroderma pigmentosum complementation group C) and DDB2 (damage-specific DNA binding protein 2 also known as XPE).¹⁶⁸,¹⁷¹ Furthermore, 1,25(OH)₂D has been shown to increase the levels of p53, which could enhance apoptosis in those cells bearing excess DNA damage,¹⁷⁰ and reduce UVR induced oxidative stress contributing to the DNA damage.¹⁷⁰ As such these actions of vitamin D signaling on DDR contribute to the reduced susceptibility of normal skin to UVB induced tumor formation.
CONCLUSION

The VDR is present in nearly every cell in the body. Moreover, the enzyme, CYP27B1, required for the production of the VDR ligand, 1,25(OH)₂D, is likewise widely distributed. Because of its abundance of 7-DHC, the epidermis is unique in its capability to produce vitamin D, metabolize it to 1,25(OH)₂D, and respond to 1,25(OH)₂D in a number of ways. Recent data from microarray and ChIP-seq studies have demonstrated hundreds, perhaps thousands of genes regulated by 1,25(OH)₂D/VDR via VDREs which are located throughout the gene. The selection of the genes regulated by 1,25(OH)₂D/VDR at any one time is cell specific and most likely dose and time specific with respect to exposure to 1,25(OH)₂D. As a result of these studies, numerous pathways have been discovered by which 1,25(OH)₂D/VDR may prevent cancer. In the skin UVB is critical for vitamin D production, but UVB is also the major cause of skin cancer. This chapter examines the question of whether the beneficial effects of UVB on vitamin D production can counter the harmful effects on carcinogenesis. Epidemiologic data suggest that there may be a threshold below which UVR is not carcinogenic, a threshold that would suffice for adequate vitamin D production. Conceivably, vitamin D production at such levels of UVB exposure might even be protective. Three potential mechanisms for such protection were examined. The first mechanism focuses on the role of vitamin D signaling in keratinocyte proliferation and differentiation. In particular, two pathways affecting proliferation and differentiation, namely the hedgehog and wnt/β-catenin pathways, were evaluated. Mice lacking the VDR have increased expression of the hedgehog pathway and increased activation of the wnt/β-catenin pathway. 1,25(OH)₂D suppresses both pathways in cells containing the VDR. Overexpression of the hedgehog and wnt/β-catenin pathways lead to increased proliferation and decreased differentiation associated with tumor development. The second mechanism involves the role of vitamin D signaling in the immune system of the skin. The role of the immune system in epidermal carcinogenesis is not clear. However, in an unbiased genomic examination of pathways associated with tumor susceptibility, inflammation, keratinocyte differentiation, and tumor formation were linked through the VDR. 1,25(OH)₂D/VDR promotes innate immunity but suppresses adaptive immunity. Whether this is beneficial regarding tumor development requires further study. The third mechanism is DNA damage repair (DDR). The epidermis of VDR null mice show impaired DDR following UVR. 1,25(OH)₂D accelerates DDR by what appear to be genomic and non genomic actions. On teleologic grounds one might anticipate that the skin has developed mechanisms to protect itself from the harmful effects of UVR. Vitamin D production, metabolism, and regulation of the processes described in this chapter may play a key role in this protection.

ACKNOWLEDGMENTS

I would like to acknowledge the collaborators whose work I have referenced, namely Drs. Zhongjian Xie, Chialing Tu, Arnaud Teichert, Kumar Pillai, Yuko Oda, Yan Jiang, Dennis Oh, James Cleaver; the administrative assistance of Ms Teresa Tong and Victoria Lee; and the funding by a VA Merit Review, NIH RO1 AR050023, PO1 AR39448, and DOD CA110338
REFERENCES


102. Schaubert J, Dorschner RA, Yamasaki K, Brouha B, Gallo RL. Control of the innate epithelial antimicrobial response is cell-type specific and dependent on relevant microenvironmental stimuli. Immunology 2006; 118:509-19; PMID:16895558.


422. Luderer HF, Gori F, Demay MB. Lymphoid enhancer-binding factor-1 (LEF1) interacts with the DNA-binding domain of the vitamin D receptor. J Biol Chem 2011; 286:18444-51; PMID:21471213; http://dx.doi.org/10.1074/jbc.M110.188219.


132. Daniel C, Sartory NA, Zahn N, Radeke HH, Stein JM. Immune modulatory treatment of trinitrobenzene sulfonic acid colitis with calcitriol is associated with a change of a T helper (Th) 1/Th17 to a Th2 and regulatory T cell profile. J Pharmacol Exp Ther 2008; 324:23-33; PMID:17911375; http://dx.doi.org/10.1124/jpet.107.127209.


Title: LncRNA Profiling Reveals New Mechanism for VDR Protection against Skin Cancer Formation

Article Type: Special issue: 16th Vitamin D Workshop

Keywords: lncRNAs, profiling, keratinocytes, VDR, mice

Corresponding Author: Dr. Yan J Jiang, M.D.

Corresponding Author’s Institution: Endocrine Unit (111N), Dept. of Medicine, UCSF

First Author: Yan J Jiang, M.D.

Order of Authors: Yan J Jiang, M.D.

Abstract: Accumulating evidence strongly suggests a protective role of vitamin D signaling against chemical and UVR-induced skin cancer formation. However, the mechanism remains largely unknown. Recently, the emerging role of long, non-coding RNA (lncRNA) as a hallmark of cancer has become better appreciated. LncRNAs are mRNA-like transcripts ranging in length from 200 bases to 100 kb lacking significant open reading frames, which are involved in a broad spectrum of tumorigenic/metastatic processes. In this study we profiled 90 well-annotated mouse lncRNAs from cultured mouse keratinocytes after deleting the vitamin D receptor (VDR) ( ∼ 90%) vs. control cells using an lncRNA array analysis. We found that several well-known oncogenes, including H19, HOTTIP and Nespas, are significantly increased (6.3 ∼ 1.8 fold), whereas tumor suppressors (Kcnq1ot1, lincRNA-p21) are decreased (up to 50 ∼ 70%) in VDR deleted keratinocytes. A similar pattern of lncRNA profiling is observed in the epidermis of K14 driven, tamoxifen-regulated epidermal-specific VDR null vs. wild-type control mice, and additionally there is an increase in the expression levels of other oncogenes (mHOTAIR, Malat1 and SRA) and a decrease of other tumor suppressors (Foxn2-as, Gtl2-as, H19-as). The increased expression levels of HOTTIP and H19 were further confirmed by the real-time PCR analysis with individually designed primer sets. The major finding of this study is a novel mechanism for protection by VDR against skin cancer formation by maintaining the balance of oncogenic to tumor suppressing lncRNAs. In keratinocytes lacking VDR this balance is disturbed with increased expression of oncogenes and decreased expression of tumor suppressors, a mechanism that predisposes the VDR deficient mice to skin cancer formation. (Total Word: 261; limit: 350).

Suggested Reviewers: Moray Campbell Ph.D
Associate Professor, Department of Pharmacology & Therapeutics, Roswell Park Cancer Institute
moray.campbell@roswellpark.org

Rebecca S. Mason Ph.D
Professor, Physiology, The University of Sydney
rebecca.mason@sydney.edu.au
An excellent cancer biologist in this field
August 1, 2013

Dr. J. Adamski
Editor-In Chief
The Journal of Steroid Biochemistry and Molecular Biology

Re: Submit manuscript

Dear Dr. Adamski,

On behalf of our co-authors, I am submitting the manuscript entitled “LncRNA Profiling Reveals New Mechanism for VDR Protection against Skin Cancer Formation” as a full-length original research article. I have been represented our team, participated and presented our research as oral presentation in the 16th Vitamin D Workshop (June 11-14, 2013, San Francisco, CA). The results from this study have not been published in any other journal until the final decision from the panel of “The Journal of Steroid Biochemistry and Molecular Biology” editors.

I would like to thank you for reviewing this manuscript and looking forward to hearing from you soon.

Sincerely,

Yan (Jenny) Jiang, MD
Associate Endocrinologist
Dept. of Medicine,
University of California, San Francisco
The Endocrine Unit (111N)
Department of Veterans Medical Center,
4150 Clement Street
San Francisco, CA 94121

Tel: (415)-221-4810, x3330
Fax: (415)-750-6929
E-mail: yan9203.jiang@yahoo.com
yan.jiang@med.va.gov
Highlights

- Global VDR null mice are predisposed to either chemical- or UVR-induced skin cancer formation
- Long non-coding RNA has recently emerged as a master regulator of tumorigenesis/metastasis in various types of cancer in both humans and mice
- Following VDR deletion in cultured mouse keratinocytes *in vitro*, lncRNA-expression profiling indicates a significant increase in several onco-lncRNAs (*H19* and *HOTTIP*) paralleled with a decrease in tumor suppressors (*Kcnqlot1* and *LincRNA-p21*)
- A similar pattern of alteration in these cancer-related lncRNAs was observed in the epidermis of K14-driven, tamoxifen-regulated VDR null mice *in vivo* in a larger scale (with more lncRNAs altered), and the alterations were confirmed by real-time PCR analysis. This disruption of the balance of epigenetic regulatory networks and cellular gene expression programs may prime keratinocytes to be more susceptible to environmental stressors, eventually leading to cancer formation
Manuscript Prepared for submitting to JSBMB-Special Issue (July 31, 2013)

LncRNA Profiling Reveals New Mechanism for VDR Protection against Skin Cancer Formation

Yan J. Jiang 1*, Daniel D. Bikle*

* Endocrine Research Unit (111N), Department of Medicine, VAMC/UCSF, NCIRE

1 Corresponding author

Yan J. Jiang, M.D.
Endocrine Unit (111N)
Department of Medicine and Dermatology
VA Medical Center and University of California at San Francisco, NCIRE
4150 Clement Street
San Francisco, CA 94121

Tel: (415)-221-4810, x 3330
Fax: (415)-750-6929
E-mail: yan.jiang@med.va.gov
Abstract

Accumulating evidence strongly suggests a protective role of vitamin D signaling against chemical and UVR-induced skin cancer formation. However, the mechanism remains largely unknown. Recently, the emerging role of long, non-coding RNA (lncRNA) as a hallmark of cancer has become better appreciated. LncRNAs are mRNA-like transcripts ranging in length from 200 bases to 100 kb lacking significant open reading frames, which are involved in a broad spectrum of tumorigenic/metastatic processes. In this study we profiled 90 well-annotated mouse lncRNAs from cultured mouse keratinocytes after deleting the vitamin D receptor (VDR) (~ 90%) vs. control cells using an lncRNA array analysis. We found that several well-known oncogenes, including \textit{H19}, \textit{HOTTIP} and \textit{Nespas}, are significantly increased (6.3 ~ 1.8 fold), whereas tumor suppressors (\textit{Kcnq1ot1}, \textit{lincRNA-p21}) are decreased (up to 50~70%) in VDR deleted keratinocytes. A similar pattern of lncRNA profiling is observed in the epidermis of K14 driven, tamoxifen-regulated epidermal-specific VDR null vs. wild-type control mice, and additionally there is an increase in the expression levels of other oncogenes (\textit{mHOTAIR}, \textit{Malat1} and \textit{SRA}) and a decrease of other tumor suppressors (\textit{Foxn2-as}, \textit{Gtl2-as}, \textit{H19-as}). The increased expression levels of \textit{HOTTIP} and \textit{H19} were further confirmed by the real-time PCR analysis with individually designed primer sets. The major finding of this study is a novel mechanism for protection by VDR against skin cancer formation by maintaining the balance of oncogenic to tumor suppressing lncRNAs. In keratinocytes lacking VDR this balance is disturbed with increased expression of oncogenes and decreased expression of tumor suppressors, a mechanism that predisposes the VDR deficient mice to skin cancer formation. (Total Word: 261; limit: 350).
This article is part of a Special Issue entitled “Vitamin D Workshop”.

**Key Words:** IncRNAs, profiling, keratinocytes, VDR, mice
1. Introduction

Previously we and other groups have reported that global vitamin D receptor knock out ($VDR^{-/-}$) mice are predisposed to either chemical (DMBA) or UVB-induced skin tumor formation [1-3], indicating a role for VDR as tumor suppressor in skin. Very recently our lab has identified that aging conditional epidermal-specific $VDR^{-/-}$ mice develop spontaneous skin tumor (unpublished results), suggesting that there is a fundamental protection role of VDR against skin tumor. However, the mechanism underlying VDR protection against chemical or UVB-induced or spontaneous skin tumor formation is not completely understood.

VDR belongs to the subfamily of nuclear hormone receptors requiring heterodimerization with RXR (retinoid X receptor) for effective DNA interaction [4]. VDR is encoded by a relatively large gene encompassing 2 promoter regions, 8 protein-coding exons and 6 un-translated exons [5]. It has an extensive promoter region capable of generating multiple tissue-specific transcripts. In addition, VDR extends its signaling by directly or indirectly interacting with many other mediators; one such important molecule is steroid receptor RNA activator (SRA), in which VDR and SRC (steroid receptor coactivator) are cross regulated via interaction between the SRC-1 and SLIRP (SRA stem-loop interacting RNA binding protein) [6].

SRA belongs to the super family of long non-coding RNA (lncRNA), which have recently been discovered owing to the completion of the human genome project as well as the advancement in new technologies of deep sequencing and DNA tiling arrays. The human genome only encodes ~20,000 protein-coding gene, representing <2% of the total genome sequence, while 90% of the genome has been found to be actively transcribed
without protein coding potential [7]. These non-coding transcripts can be broadly categorized into short and long non-coding RNA. The arbitrary size delineation is at about 200 bases in length: small non-coding RNAs are less than 200 bases, including tRNAs, microRNAs, small nuclear (snoRNAs). In contrast, lncRNAs are endogenous cellular RNAs of larger than 200 bases and can even be greater than 100 kb in length [8]. LncRNAs account for 80% of the transcriptome [7]; they are spliced and contain polyadenylation signals, much like messenger RNAs [9]. LncRNAs are expressed cross mammalian genomes and have emerged as master regulators of embryonic pluripotency, differentiation, and body axis patterning, promoting developmental transitions [9, 10] and regulating histone modifications hence influencing the epigenetic programs of the transcriptome [11]. Most critically, recent studies indicate that lncRNAs function as master regulators of cancer development, by sustaining tumor cell proliferation, evading growth suppressors, enabling replicative immortality, inducing angiogenesis, and promoting invasion and metastasis [8, 12, 13]. In lieu of the emerging role of lncRNAs in tumorigenesis, we explored the potential role of lncRNAs in VDR protection against skin tumor formation by profiling the lncRNA expression using in vitro cultured mouse keratinocytes and in vivo a VDR^{-/-} mouse model.

2. Materials and methods

2.1 lncRNA profiling of cultured mouse keratinocytes

Primary mouse keratinocytes were isolated from newborn mice of floxed VDR according to the protocol reported [6]. Cells were cultured in serum-free growth medium (CnT07-BM.1, Cascade Biologics, Portland, OR) containing 0.07mM Ca^{2+} until 60~70%
confluency. For VDR deletion studies *in vitro*, cells were transduced with an adenovirus carrying the anti-sense mouse Cre recombinant cDNA (5 plaque-forming units (p.f.u)/cell) in medium containing 0.03 mM Ca$^{2+}$ for 48 hours. Control cells were transduced with an adenovirus carrying the Ad-DNR empty vector. Vectors were constructed and virus was prepared as described previously [14]. At the end of transduction, total RNA was isolated and IncRNA array analysis was performed according to the manufacturer’s instructions (System Biosciences Inc., Mountain View, CA).

### 2.2 IncRNA profiling of epidermis from conditional VDR$^{-/-}$ mice

Mice homozygous for floxed VDR were bred with mice expressing K14ER$^{tam}$ cre recombinase to selectively knockout the VDR in the keratinocytes of skin using parenteral application of tamoxifen as reported [15]. Epidermal preparation (from 8 weeks old mice; n=3–4) and RNA extraction were described previously [15], and IncRNA profiling was performed as described. All animal experimentation in this study has been approved by the San Francisco VA Medical Center Animal Review Committee.
3. Results and discussion

*The profile of lncRNA-expression reveals an increase in oncogenic lncRNAs and a decrease in tumor suppressors in both VDR deletion keratinocytes and epidermis*

To investigate the role of lncRNAs in VDR protection against skin cancer formation, we first compared the expression levels of 90 most studied mouse lncRNAs originally reported by Mattick’s group [16] in VDR deleted mouse keratinocytes versus control cells, using an lncRNA array kit in a 96-well format. The efficiency of VDR deletion by adenovirus cre transduction in these cells (> 90%) was monitored by measuring the mRNA level of VDR by RT-qPCR (data not shown). The result of the basal expression levels of lncRNAs that were significantly altered (either increase or decrease) in VDR deleted CMKs were summarized in Table 1 (right panel). Among 7 lncRNAs that up-regulated, *HOTTIP, H19* and *Nespas* are well documented oncogenes, and increased expression levels of these lncRNAs also occur in other cancers including lung, prostate, and colon [13]. Of 3 lncRNAs that decreased after VDR deletion in mouse keratinocytes, *lincRNA-p21* and *Kcnq1ot1* are two well-characterized tumor suppressors [13, 17].

To confirm the results obtained from cultured mouse keratinocytes, the epidermis was isolated from K14-driven, tamoxifen-regulated epidermal-specific VDR null mice. The floxed VDR mice from the same litter without *Cre* served as control [15]. Total RNA was isolated from the epidermis and lncRNA array profiling was performed using the same protocols as described above. The result of the basal expression levels of lncRNAs that were significantly altered in VDR null mice was summarized in Table 1 (left panel). As shown in the table, *H19, HOTTIP* and *Nespas* are significantly and consistently increased in both cultured keratinocytes and epidermis following VDR deletion. The enhancement
of H19 and HOTTIP expression level is further confirmed by real-time PCR analysis using specific primer sets recognizing individual mouse H19 and HOTTIP gene (data not shown). Additionally, more IncRNAs were found to be up-regulated (Air, HOTAIR, Malat1 and SRA; Table 1), which are all known to be oncogenic [13]. The enhancement of these oncogenes following VDR deletion may prime keratinocytes to be more sensitive to environmental stressors and eventually skin cancer formation. For instance, in humans the H19 gene is an imprinted, maternally expressed gene, and tightly linked and co-regulated with the imprinted, paternally expressed gene of insulin-like growth factor 2. The H19 gene product is not translated into protein and functions as an RNA molecule [18], which is abundantly expressed in many tissues during embryogenesis and is down regulated postnatally [19]. Interestingly, the re-expression of this “oncogenetic RNA” gene is found in adult tumors, and is essential for human tumor growth [20-22]. HOTTIP (HOXA transcript at the distal tip) is another IncRNA. It is expressed from the 5’ end of the HoxA locus and drives histone H3 lysine 4 trimethylation and gene transcription of HoxA distal genes through the recruitment of the WDR5/MLL complex [23]. In this study, we are the first to identify that H19, HOTTIP and Nespas are significantly up-regulated following VDR deletion in cultured mouse keratinocytes and epidermis. Other IncRNAs such as Dlx1as, Mistral, linc1242-LINC-Enah and recombination hot spot RNA are also increased in both keratinocytes and epidermis, but their functions are less known. AntiPeg11, Dio3os, Msx1as, PINC, and Zfas1 are also increased in epidermis; again, their functions are less known.

Of a panel of 7 IncRNAs that decreased after VDR deletion, lincRNA-p21 and Kcnq1ot1 are two well-characterized tumor suppressors [13, 17], and consistently and
significantly decreased in both keratinocytes and epidermis (Table 1). *LincRNA-p21* is a direct p53 target gene residing next to the p21 gene, which is up-regulated upon DNA damage in different tumor models [24]. *LincRNA-p21* exerts its tumor suppressor function via association with hnRNP, a well-known RNA binding protein and itself is a tumor suppressor [25]. *Kcnq1ot1* localizes in the nucleus, interacting with chromatin and also with G9a (a H3K9- and H3K27-specific histone methyltransferase) and Ezh2 (histone-lysine N-methyltransferase), resulting in cluster-wide repressive histone marks, gene silencing and DNA methylation of CpG islands. Hence it exerts its tumor suppressor effect via epigenetic gene silencing [26]. For instance, in Beckwith-Wiedemann syndrome (BWS), approximately 50% of patients show loss of DNA methylation accompanied by loss of histone H3 lysine 9 dimethylation on maternal KCNQ1OT-DMR, namely an imprinting disruption, leading to diminished expression of CDKN1C (cyclin-dependent kinase inhibitor) and subsequently cancer formation [27]. In this study, we are the first to identify that the expression levels of *lincRNA-p21* and *Kcnq1ot1* decreased following VDR deletion in mouse keratinocytes. The importance of antisense transcription has been emphasized by the widespread occurrence of antisense transcripts in the human genome: up to 5-10% of all genes have an antisense counterpart, which in most cases are associated with decreased expression of their target genes. Hence, it is not a surprise that the level of *H19-as*, the counterpart of *H19*, is decreased, which may contribute to the fold increase in H19 expression. *BGn-as* is also decreased in both keratinocytes and epidermis but its function is less understood. Other tumor suppressors including *Foxn2-as and Gil2-as* are also down-regulated in epidermis. Together, our results indicate that VDR may exert its protective function via reducing the expression
levels of oncogenic IncRNAs while upregulating tumor suppressor IncRNAs, which may explain why VDR null mice develop spontaneous skin tumors at an older age, and are prone to either UVR- or chemical-induced skin tumor formation.

Since the ultimate cause of cancer is the alteration of the balanced harmony of cellular networks and gene expression programs that maintain cellular homeostasis, our current study suggests that following VDR ablation there is an increase in the expression of oncogenic IncRNAs (H19, HOTTIP, Nespas, mHOTAIR, MALAT1, SRA), and decreased expression of tumor suppressors (kcnn1ot1, lincRNA-p21, BGn-as, H19-as, Foxn2-as, Gtl2-as) in the epidermis of VDR ablation. These fundamental alterations in the landscape of epigenetic regulatory networks of IncRNAs expression may favor keratinocyte hyper-proliferation and eventually skin tumor formation in VDR null mice.

Acknowledgements

The authors thank Dr. Chia-Ling Tu for kindly providing the adenovirus carrying Cre recombinase and the control virus Ad-NDR. We also appreciate the technical help from Ms. Alicia Menendez for animal work. This project is supported from NIH grant R01 AR055924, VA Merit Review, and DOD grant CA110338.
Table 1. The alteration of lncRNA expression levels in keratinocytes (CMK) and epidermis following VDR deletion compared to controls (WTC)

<table>
<thead>
<tr>
<th>LncRNAs</th>
<th>Epidermis* (VDR^-/-/WTC)</th>
<th>CMKs* (VDR^-/-/WTC)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Induced</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Air (antisense Igf2r RNA)</td>
<td>4.7</td>
<td>nc</td>
</tr>
<tr>
<td>AntiPeg11</td>
<td>4.1</td>
<td>nc</td>
</tr>
<tr>
<td>Dio3os</td>
<td>7.8</td>
<td>nc</td>
</tr>
<tr>
<td>Dlx1as</td>
<td><strong>3.3</strong></td>
<td><strong>22.2</strong></td>
</tr>
<tr>
<td>mHOTAIR (Hox antisense intergenic RNA)</td>
<td>4.4</td>
<td>nc</td>
</tr>
<tr>
<td>HOTITP (HOXA transcript at the distal tip)</td>
<td><strong>4.5</strong></td>
<td><strong>11.8</strong></td>
</tr>
<tr>
<td>H19</td>
<td><strong>4.8</strong></td>
<td>8.4</td>
</tr>
<tr>
<td>Nespas (Neuroendocrine secretory protein antisense)</td>
<td><strong>5.3</strong></td>
<td><strong>6.3</strong></td>
</tr>
<tr>
<td>Malat1</td>
<td>2.8</td>
<td>nc</td>
</tr>
<tr>
<td>Mistral</td>
<td><strong>5.9</strong></td>
<td>2.8</td>
</tr>
<tr>
<td>Msx1as</td>
<td>4.9</td>
<td>nc</td>
</tr>
<tr>
<td>linc1242-LINC-Enah</td>
<td><strong>4.8</strong></td>
<td><strong>2.6</strong></td>
</tr>
<tr>
<td>PINC</td>
<td>3.0</td>
<td>nc</td>
</tr>
<tr>
<td><strong>Reduced</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BGn-As</td>
<td><strong>0.17</strong></td>
<td><strong>0.3</strong></td>
</tr>
<tr>
<td>Foxn2-as</td>
<td>0.23</td>
<td>nc</td>
</tr>
<tr>
<td>Gtl2-as</td>
<td>0.18</td>
<td>nc</td>
</tr>
<tr>
<td>H19-as</td>
<td><strong>0.16</strong></td>
<td><strong>0.73</strong></td>
</tr>
<tr>
<td>Kcnq1ot1</td>
<td><strong>0.054</strong></td>
<td><strong>0.3</strong></td>
</tr>
<tr>
<td>LincRNA-p21</td>
<td>0.5</td>
<td>nc</td>
</tr>
<tr>
<td>Linc-MD1</td>
<td>0.64</td>
<td>nc</td>
</tr>
</tbody>
</table>
References


