



Role of Vitamin D₃ in Modulation of Δ Np63 α Expression during UVB Induced Tumor Formation in SKH-1 Mice

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Abstract

Δ Np63 α , a proto-oncogene, is up-regulated in non-melanoma skin cancers and directly regulates the expression of both Vitamin D receptor (VDR) and phosphatase and tensin homologue deleted on chromosome ten (PTEN). Since Δ Np63 α has been shown to inhibit cell invasion via regulation of VDR, we wanted to determine whether dietary Vitamin D₃ protected against UVB induced tumor formation in SKH-1 mice, a model for squamous cell carcinoma development. We examined whether there was a correlation between dietary Vitamin D₃ and Δ Np63 α , VDR or PTEN expression *in vivo* in SKH-1 mice chronically exposed to UVB radiation and fed chow containing increasing concentrations of dietary Vitamin D₃. Although we observed differential effects of the Vitamin D₃ diet on Δ Np63 α and VDR expression in chronically irradiated normal mouse skin as well as UVB induced tumors, Vitamin D₃ had little effect on PTEN expression *in vivo*. While low-grade papillomas in mice exposed to UV and fed normal chow displayed increased levels of Δ Np63 α , expression of both Δ Np63 α and VDR was reduced in invasive tumors. Interestingly, in mice fed high Vitamin D₃ chow, elevated levels of Δ Np63 α were observed in both local and invasive tumors but not in normal skin suggesting that oral supplementation with Vitamin D₃ may increase the proliferative potential of skin tumors by increasing Δ Np63 α levels.

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Introduction

1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) has been investigated as an adjuvant to anti-cancer therapies. Upon binding to Vitamin D Receptor (VDR), 1,25(OH)₂D₃ induces expression of genes involved in apoptosis, differentiation and growth suppression while down regulating expression of genes that are involved in proliferation (reviewed in [1]). Keratinocytes synthesize 7-dehydrocholesterol, which is then converted to cholecalciferol by exposure to ultraviolet B (UVB) light between 280–320 nm. Intriguingly, these wavelengths of UVB are also the primary cause of skin cancer. Unlike keratinocytes, no other cell types can produce 1,25(OH)₂D₃ from 7-dehydrocholesterol and must rely on the sequential transport of cholecalciferol to the liver and kidneys to produce 25-hydroxyvitamin D₃ and 1,25(OH)₂D₃, respectively. Due to the relative instability of 1,25(OH)₂D₃, dietary supplements commonly consist of cholecalciferol, also referred to as Vitamin D₃ and rely on the conversion to 1,25(OH)₂D₃ by the liver and kidneys.

Severe Vitamin D₃ deficiency, measured by serum 25-hydroxyvitamin D levels, or deletion of the VDR gene is associated with increased cancer risk [2,3]. Although topical application of 1,25(OH)₂D₃ reduced UVB-induced tumor burden in the SKH-

1 mouse model of squamous cell carcinoma [4], protective effects of dietary Vitamin D₃ against the development of skin cancer has not been examined. This is an important study due to recent reports highlighting the frequency of Vitamin D₃ deficiency, and its association with a myriad of disease states which has led to an increase in Vitamin D₃ supplement intake by the general public [5].

On a cellular level, 1,25(OH)₂D₃, a downstream metabolite of Vitamin D₃, exerts its biological function by binding the transcription factor VDR to control the expression of target genes. We have previously demonstrated that p63 inhibits cell invasion by directly regulating VDR and that both VDR and p63 are needed to inhibit cell invasion [6,7]. The transcription factor p63 is essential for normal epidermal stratification and the proliferative potential of the epithelial stem cells [8,9]. The *Tp63* gene can form several isoforms with contrasting functions, using alternate promoters and 3' splicing. The TA isoforms (TAp63 α , TAp63 β and TAp63 γ) have a full-length N-Terminal transactivation domain, whereas the Δ N isoforms (Δ Np63 α , Δ Np63 β and Δ Np63 γ) have a unique truncated transactivation domain [10]. Our laboratory as well other researchers have previously shown that Δ Np63 α is the only detectable p63 isoform expressed in the epidermis, specifically found in the proliferative

basal layer [7,11–15]. $\Delta Np63\alpha$ is overexpressed in squamous cell carcinomas (SCC) and basal cell carcinomas (BCC) [11–13,16,17]. Contrary to its known roles in promoting epidermal differentiation, VDR levels, much like $\Delta Np63\alpha$, are also elevated in BCC and SCC [18,19]. Through its ability to induce VDR, $\Delta Np63\alpha$ could enhance 1,25(OH)₂D₃ signaling in non-melanoma skin cancers.

In cell culture systems, 1,25(OH)₂D₃ seems to have paradoxical pro-growth and pro-apoptotic functions. 1,25(OH)₂D₃ can prevent apoptosis of UV-irradiated keratinocytes in culture through the stabilization of $\Delta Np63\alpha$ [20], or promote apoptosis through increased expression of the tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) [21]. We have demonstrated that $\Delta Np63\alpha$ negatively regulates PTEN expression and localization in keratinocytes to maintain normal growth rates. Moreover, the ratio of $\Delta Np63\alpha$ to PTEN expression is significantly perturbed in human non-melanoma skin cancers [15].

In this study, we sought to delineate whether dietary Vitamin D₃ offered any protection against UVB induced tumor formation and whether it preferentially induced expression of $\Delta Np63\alpha$, VDR, or PTEN *in vivo*. We fed SKH-1 hairless mice chow containing increasing concentrations of Vitamin D₃ (cholecalciferol) and chronically exposed them to UVB light modeling the process of UV induced skin carcinogenesis in humans [14]. It has been shown that development of skin tumors in the SKH-1 hairless mice resemble UV induced squamous cell carcinomas in humans both morphologically as well at the molecular level [22].

Our results demonstrated that dietary Vitamin D₃ offered no protection from UVB induced tumor formation and in fact increased tumor size at the highest dose tested. We observed differential effects of Vitamin D₃ diet on $\Delta Np63\alpha$ and VDR but not PTEN expression in chronically irradiated, but otherwise normal skin and in UVB induced tumors.

Results

Effects of dietary Vitamin D₃ on epidermal structure

To investigate the effect of increasing dietary Vitamin D₃ on epidermal biology we first measured the skin thickness in SKH-1 hairless mice exposed to chronic UVB irradiation. Dietary Vitamin D₃ alone did not alter the epidermal thickness of unirradiated mice at any dose tested indicating that dietary Vitamin D₃ alone is insufficient to change epidermal proliferation. Chronic UVB exposure significantly increased epidermal thickness in all mice (Figure 1a & b). Interestingly, animals fed chow with higher concentrations of dietary Vitamin D₃ displayed increased epidermal thickness in response to chronic UVB as compared to a standard (3 IU) diet (Figure 1a & b). To assess changes in proliferation, non-tumor dorsal skin sections were stained for Ki67. As shown in Figure 1c regardless of Vitamin D₃ diet there was an increase in Ki67-positive cells in irradiated skin when compared to un-irradiated skin from SKH-1 mice.

Epidermal thickness is mediated by changes in keratinocyte proliferation and differentiation, both of which are regulated by VDR and $\Delta Np63\alpha$. 1,25(OH)₂D₃ has also been shown to stabilize both VDR and $\Delta Np63\alpha$ [20,23]. To determine whether the increase in epidermal thickness caused by increased dietary Vitamin D₃ was the result of enhanced VDR or $\Delta Np63\alpha$ expression, we stained skin tissues from UVB irradiated or control SKH-1 mice fed varying doses of dietary Vitamin D₃ for VDR, $\Delta Np63\alpha$ and their common transcriptional target PTEN. Since, $\Delta Np63\alpha$ is the only detectable p63 isoform found in the epidermis we used a pan p63 antibody to detect $\Delta Np63\alpha$ expression levels in the skin tissues [7,11–15]. In unirradiated skin, increasing

concentrations of dietary Vitamin D₃ had little effect on the expression of VDR (Figure 2a, quantitated in lower panel). Lower doses of dietary Vitamin D₃ significantly increased VDR expression in chronically UVB irradiated skin as compared to unirradiated skin (Figure 2a). Interestingly, the increase in VDR was not observed with higher concentrations of dietary Vitamin D₃ in irradiated skin and in fact VDR was significantly down regulated in mice fed 1000 IU of Vitamin D₃ diet compared to irradiated mice fed the standard (3 IU) diet (Figure 2a).

Similarly, Vitamin D₃ diet did not drastically alter $\Delta Np63\alpha$ expression in unirradiated skin (Figure 2b, quantitated in lower panel). In mice fed a standard diet of Vitamin D₃, chronic exposure to UVB led to a significant increase in $\Delta Np63\alpha$ expression in the epidermis as compared to unirradiated mice (Figure 2b). Contrary to previous reports in cultured keratinocytes treated with calcitriol and exposed to acute UV radiation [20], increasing concentrations of dietary Vitamin D₃ led to a reduction in the $\Delta Np63\alpha$ expression in response to chronic UVB exposure (Figure 2b).

Epidermal growth is also regulated by the tumor suppressor PTEN, which inhibits cell proliferation [24,25]. Interestingly, increasing concentrations of dietary Vitamin D₃ (25 and 1000 IU) significantly decreased PTEN expression in the epidermis of unirradiated mice as compared to mice fed a standard 3 IU Vitamin D₃ diet (Figure 2c, quantitated in lower panel). Chronic exposure to UVB significantly reduced the expression of PTEN in the epidermis compared to unirradiated mice (Figure 2c). Increasing dietary Vitamin D₃ in UVB irradiated mice did not further reduce PTEN levels.

Dietary Vitamin D₃ trends toward increased UVB-induced tumor development

We next wanted to determine whether dietary Vitamin D₃ affects tumor formation, specifically tumor size and grade, in response to chronic UVB exposure. Representative images of the histology of the normal skin, papilloma, micro-invasive squamous cell carcinoma (MiSCC) and SCC are shown in Figure S1, as described previously [22]. Although increasing the amount of Vitamin D₃ in the diet trended toward an increase in the average tumor area (Figure S2a) it was not statistically significant. Moreover, mice fed higher doses of dietary Vitamin D₃ displayed a higher frequency of fully invasive squamous cell carcinomas (SCC) as compared to mice fed a standard diet (Figure S2b), but again this trend was not statistically significant. The increase in SCC in mice fed 1000 IU VD₃ did not alter the frequency of papillomas, but rather correlated with a decrease in MiSCC as compared to the mice fed standard diet, suggesting that higher dietary Vitamin D₃ may enhance tumor progression rather than tumor initiation (Figure S2b).

Dietary Vitamin D₃ differentially affects proteins involved in epidermal maintenance during tumor progression

VDR has been shown to inhibit cell invasion [7], a hallmark of tumor progression, and yet it has also been reported to be elevated in BCC and SCC [18,19]. To determine whether there is a correlation between VDR expression, Vitamin D₃ diet, and tumor grade, we determined VDR intensity in tumors of each grade from mice fed increasing doses of dietary Vitamin D₃. VDR expression was significantly reduced in papillomas when compared to normal epidermal tissue regardless of dietary levels of Vitamin D₃ (Figure 3). VDR levels were also significantly reduced in MiSCC and SCC as compared to normal epidermal tissue for all doses of dietary Vitamin D₃ tested. Interestingly, VDR expression was

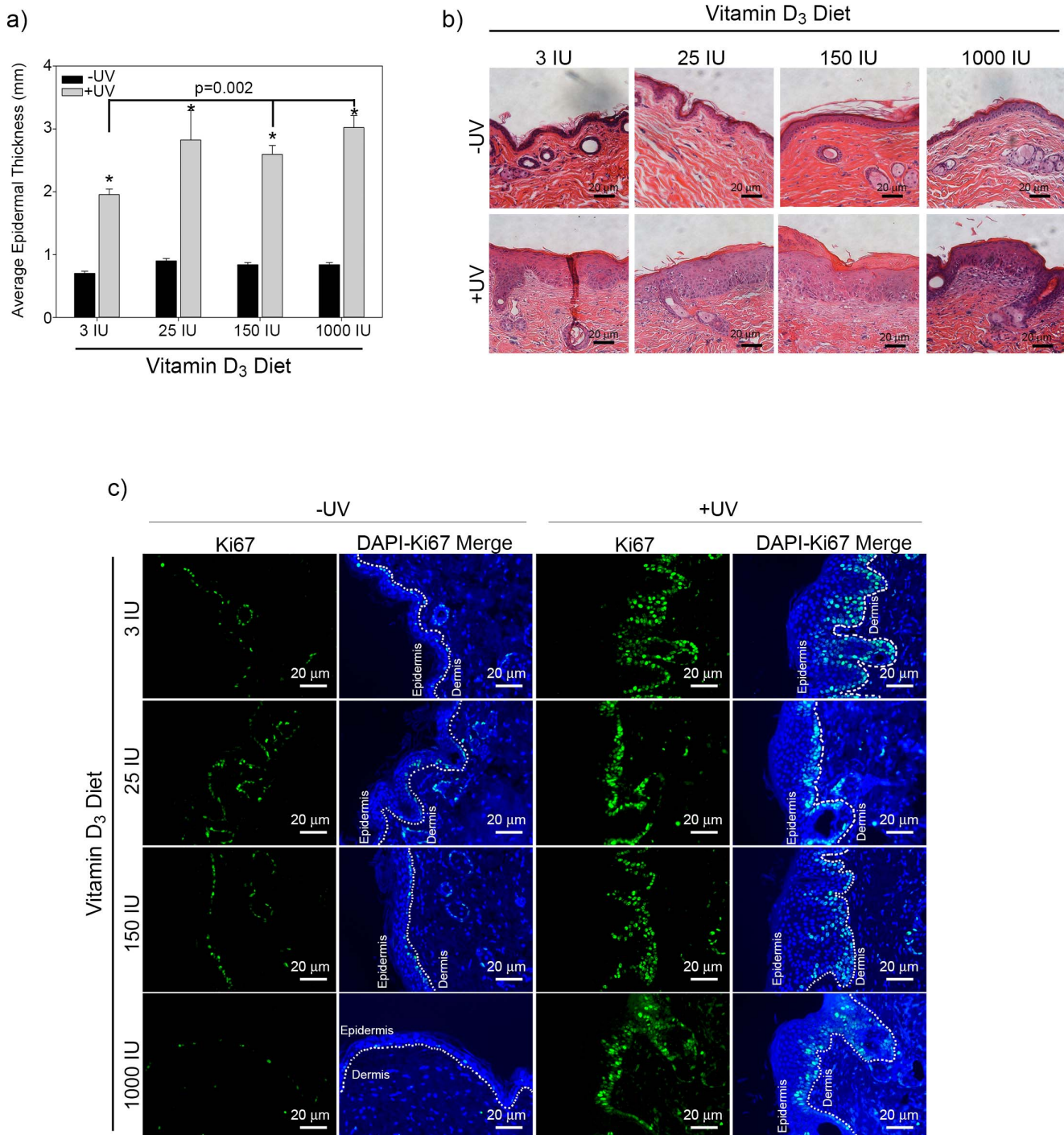


Figure 1. Dietary Vitamin D₃ does not significantly alter epidermal thickening induced in response to UVB irradiation. (a) Male SKH-1 mice were fed diets with increasing concentrations of Vitamin D₃ and irradiated thrice weekly for 25 weeks with UVB. The epidermal thickness from UV irradiated and unirradiated control mice are plotted. Error bars represent s.e.m. n = 15 UV exposed and n = 10 control unirradiated mice per treatment condition. (b) Representative images of irradiated and unirradiated skin after Haematoxylin and Eosin (H&E) staining. Photos from SKH-1 mice fed standard or increasing concentration of vitamin D₃ chow were taken at a 10x magnification, scale bar = 20 μm. (c) Ki67 staining in normal skin from irradiated and unirradiated skin obtained from mice fed diets with increasing concentration of Vitamin D₃ as indicated. Ki67 images were taken at a 20x magnification, scale bar = 20 μm. doi:10.1371/journal.pone.0107052.g001

significantly reduced in SCCs formed in mice fed a 1000 IU Vitamin D₃ diet when compared to SCCs formed in mice fed a standard diet. The lack of VDR, which has tumor suppressive functions [3], in SCCs from mice fed 1000 IU Vitamin D₃ diet

(Figure 3b) may explain the trend toward increased frequency of SCC in animals on this diet (Figure S1b).

ΔNp63α, known to increase the proliferation of epidermal keratinocytes, was significantly down regulated in normal epider-

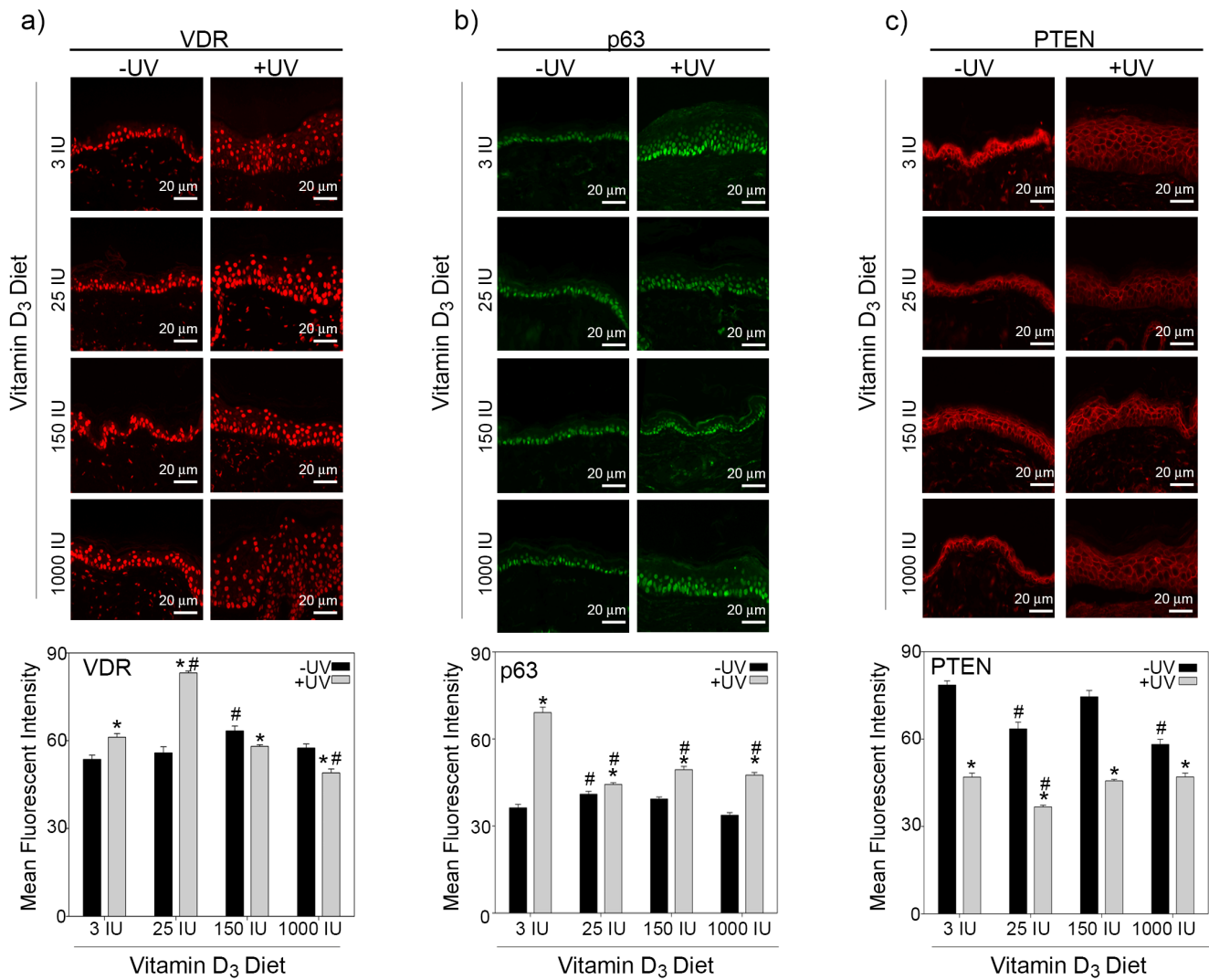


Figure 2. Dietary Vitamin D₃ differentially affects VDR, ΔNp63α and PTEN levels in response to UVB. Top panels show representative images of (a) VDR, (b) ΔNp63α, or (c) PTEN staining in normal skin from irradiated and unirradiated skin obtained from mice fed diets with increasing concentration of Vitamin D₃ as indicated were taken at a 20x magnification, scale bar = 20 μm. Quantitation of (a) VDR, (b) ΔNp63α, or (c) PTEN staining from three animals per treatment condition is plotted in the lower panels. Y-axis represents the mean fluorescent intensity, normalized to background, in arbitrary units. Error bars represent standard error of mean. * = p ≤ 0.05 compared to unirradiated skin; # = p ≤ 0.05 compared to respective unirradiated or irradiated skin from mice fed 3 IU Vitamin D₃. doi:10.1371/journal.pone.0107052.g002

mal tissue at all doses of dietary Vitamin D₃ when compared to mice fed a standard diet (Figure 4). Similar to VDR, ΔNp63α expression was also increased in a dose dependent manner in papillomas fed increasing doses of vitamin D₃ chow. However, unlike VDR, ΔNp63α expression levels were also increased in both MiSCCs and SCCs (Figure 4b) with increasing doses of Vitamin D₃ diet. Interestingly, papillomas and MiSCC from mice on the higher dietary Vitamin D₃ (150 IU and 1000 IU) expressed significantly more ΔNp63α than normal epidermal tissue from mice of the same diet (Figure 5b). Loss of p63 has been associated with increased cell invasion in urothelial and bladder cancers [26,27]. Our results also demonstrated a significant reduction in ΔNp63α expression in SCCs compared to MiSCC and normal epidermal tissues from mice fed a standard diet (Figure 4b). However, SCCs from mice fed increasing concentrations of Vitamin D₃ diet exhibited a dose dependent increase in ΔNp63α expression levels suggesting that dietary Vitamin D₃ enhances the

proliferative nature of SCC by preventing the down regulation of ΔNp63α (Figure 4b).

To investigate if dietary Vitamin D₃ leads to a reduction in the expression of tumor suppressor PTEN, we measured the expression of PTEN by immunofluorescence in normal skin and tumors from UVB irradiated mice fed each of the Vitamin D₃ diets. Increasing the concentration of Vitamin D₃ in the diet did not have consistent trends on the expression of PTEN between tumor types (Figure 5). Consistent with previous reports [28], PTEN was significantly reduced in UVB induced SCC compared to normal skin independent of the Vitamin D₃ diet (Figure 5), suggesting that dietary Vitamin D₃ does not increase the tumor size or burden by augmenting UVB mediated degradation of PTEN.

We have previously demonstrated that the ratio of ΔNp63α to PTEN is critical for mediating keratinocyte proliferation and that this ratio is significantly perturbed in human BCC and SCC [15].

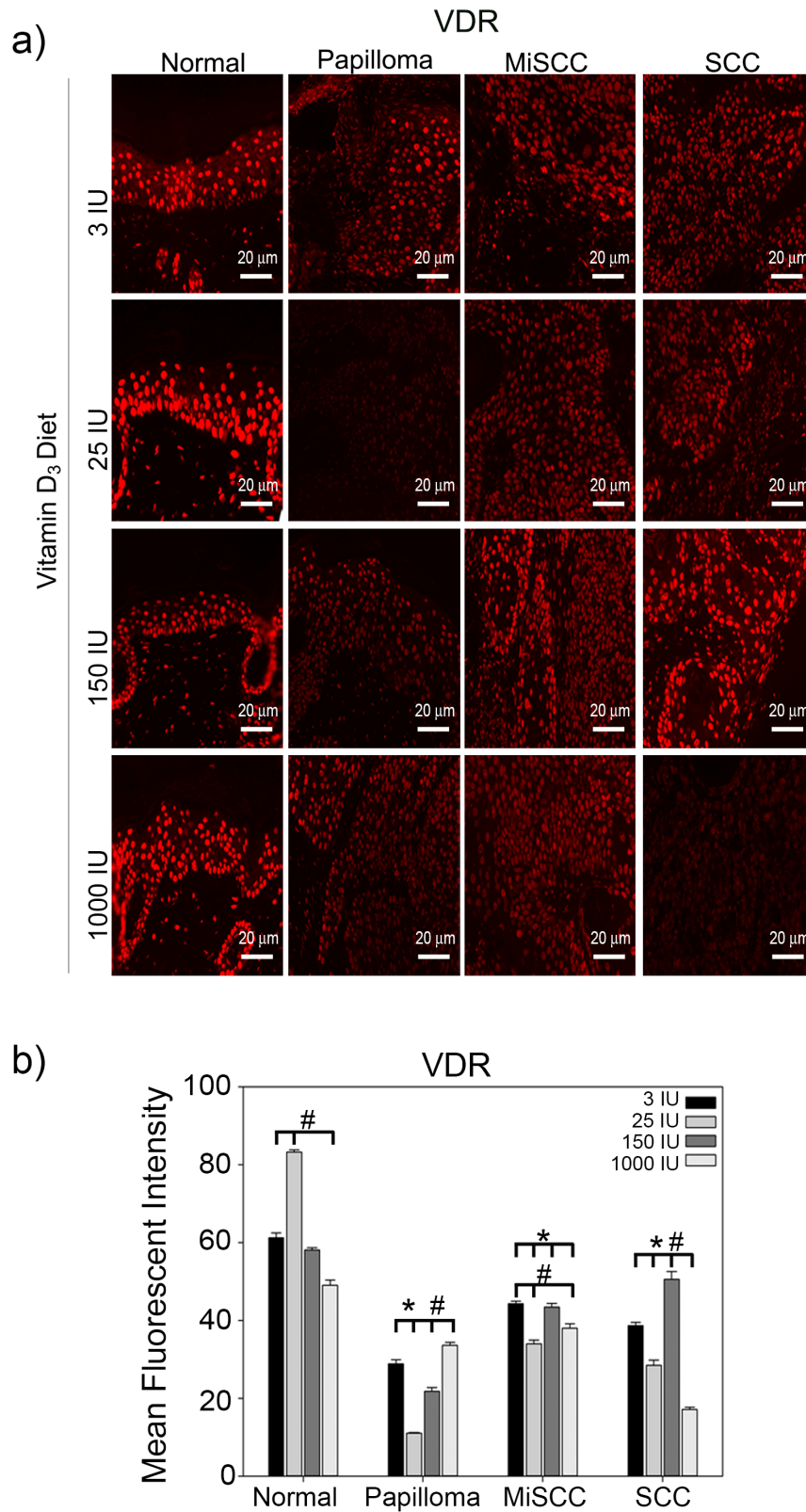


Figure 3. Effects of dietary Vitamin D₃ on VDR expression during tumor progression. (a) Top panels show representative images taken at a 20x magnification, scale bar = 20 μm of normal skin, benign papillomas, MiSCC and squamous cell carcinoma (SCC) from mice fed diets of increasing concentrations of Vitamin D₃ stained for VDR. (b) Quantitation of VDR levels from three animals per treatment condition is plotted. Y-axis represents the mean fluorescent intensity, normalized to background, in arbitrary units. Error bars represent s.e.m. * = p ≤ 0.05 compared to normal skin from the same diet; # = p ≤ 0.05 compared to tissue of same tumor grade from mice fed 3 IU Vitamin D₃. doi:10.1371/journal.pone.0107052.g003

To determine if perturbation of the balance between Δ Np63 α and PTEN by dietary Vitamin D₃ was contributing to the increase in tumor size and SCC frequency, we calculated the ratio of Δ Np63 α to PTEN fluorescence intensity in normal skin and tumors from UVB irradiated mice fed each of the Vitamin D₃ diets. Mice fed a diet of 1000 IU Vitamin D₃ displayed consistently higher ratios of Δ Np63 α to PTEN, indicative of an increased proliferation potential, in all tumor types as compared to normal skin (Figure 6). Taken together, these studies suggest that increased dietary Vitamin D₃ may enhance UVB induced tumor formation and progression, at least at supra-physiologic doses, by decreasing the expression of VDR while increasing the Δ Np63 α to PTEN ratio.

Discussion

1,25(OH)₂D₃ has been investigated as an adjuvant to anti-cancer therapies because of its growth suppressive and pro-differentiation properties. Although the association of Vitamin D₃ consumption and serum 25-hydroxyvitamin D with the prevention of a wide range of cancers has been widely studied [29], evidence supporting the role of 1,25(OH)₂D₃ in protecting against skin cancer is often conflicting [30–32]. In this study we demonstrate that increased consumption of dietary Vitamin D₃ in the SKH-1 mouse model of squamous cell carcinoma does not protect against UVB-induced tumor formation (Figure S1). Moreover, supra-physiologic levels (1000 IU) of dietary Vitamin D₃ may actually promote epidermal proliferation and tumor formation as evidenced by increased epidermal thickness and Ki67 staining (Figure 1) and dose-dependent trends toward larger, more aggressive tumor development (Figure S2).

The enhanced proliferation and tumor development in UVB irradiated mice fed 1000 IU Vitamin D₃ may be related to the stabilization of the Δ Np63 α (Figure 4), which is often overexpressed in human non-melanoma skin cancers [11–13,16,17]. Numerous models of acute UVB irradiation have demonstrated that Δ Np63 α must be down regulated to allow for apoptosis in the epidermis [33–35]. It has been previously shown that ablation of the basal layer cells of the interfollicular epidermis comprising of mutant p53 and p63-positive cells led to a significant delay in the onset of tumor formation in SKH-1 mice, suggesting that Δ Np63 α likely contributed to tumor formation [36]. Our studies show that,

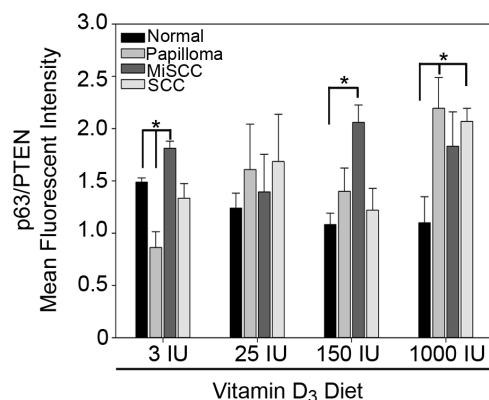


Figure 6. Dietary Vitamin D₃ alters the ratio of Δ Np63 α to PTEN during tumor progression. The average ratio of Δ Np63 α fluorescence intensity to PTEN fluorescence intensity from normal skin, benign papillomas, MiSCC, and SCC from mice fed diets of increasing concentrations of Vitamin D₃ as indicated is plotted. Error bars represent standard error of mean from three animals per treatment condition. * = $p \leq 0.05$ compared to unirradiated skin. doi:10.1371/journal.pone.0107052.g006

unlike acute UVB exposure, Δ Np63 α levels were significantly higher in chronically UVB irradiated skin (Figure 2b) potentially predisposing skin to tumor development. While we did not observe an increase in Δ Np63 α levels in response to increased dietary Vitamin D₃ in normal skin, we found that dietary Vitamin D₃ was able to limit the down regulation of Δ Np63 α during tumor progression (Figure 4). The sustained expression of Δ Np63 α by dietary Vitamin D₃ could contribute to the proliferation and expansion of UVB induced tumors.

Interestingly, the increase in Δ Np63 α expression did not correlate with increased expression of VDR, a direct transcriptional target of p63 (Figures 3–4) [6]. This suggests that dietary Vitamin D₃, at least in the context of concomitant UVB irradiation, may enhance the oncogenic properties of Δ Np63 α by increasing the ratio of Δ Np63 α to PTEN (Figure 6), rather than altering its tumor suppressive attributes, namely induction of VDR.

Unlike previous studies conducted in 1,25(OH)₂D₃ deficient rats, we did not observe an increase in epidermal VDR expression in response to increased dietary Vitamin D₃ (Figures 2a and 3) [37]. This could be attributed to the inherent differences between rats and SKH-1 mice and/or the differences in experimental approach. In the studies conducted by Zineb et al., VDR expression was measured in Wistar rats that were kept in the dark, preventing the cutaneous production of 1,25(OH)₂D₃, and fed a diet lacking Vitamin D₃ to induce 1,25(OH)₂D₃ deficiency before re-supplementation of dietary Vitamin D₃ [37]. To better mimic the environmental conditions experienced by humans, our studies utilized a hairless mouse strain chronically exposed to UVB without inducing 1,25(OH)₂D₃ deficiency prior to dietary Vitamin D₃ supplementation. It is important to note that while UVB is the most common cause of non-melanoma skin cancers and its use as a carcinogen is most physiologically relevant, the ability of keratinocytes in the epidermis to generate 1,25(OH)₂D₃ in response to UVB can confound the interpretation of how dietary Vitamin D₃ affects tumor formation.

Our results suggest that increased dietary Vitamin D₃ may enhance UVB induced tumor formation and progression (Figure S2) by decreasing the expression of VDR in the epidermis (Figure 3) while increasing Δ Np63 α (Figure 4). The deleterious effects of dietary Vitamin D₃ observed in this study are consistent with previous epidemiological studies showing that the risk for non-melanoma skin cancers was positively correlated with increasing serum 25-hydroxyvitamin D levels [30]. The U.S. Preventive Services Task Force has reported that there is insufficient data to support Vitamin D₃ supplementation as a cancer prevention method [38]. However, more efficient delivery of 1,25(OH)₂D₃ to keratinocytes may also be critical to generating protective rather than deleterious effects with regard to UVB induced skin cancer.

A study by Dixon *et al.* demonstrated that topical application of 1,25(OH)₂D₃ led to a reduction in the development and size of UV-induced tumors in the SKH-1 mouse model of squamous cell carcinoma [4]. In contrast to our data obtained with dietary Vitamin D₃ (Figure S2), topical 1,25(OH)₂D₃ led to a reduction in the incidence and progression of UV induced tumors [4]. Aside from choice and route of delivery of vitamin D, there were differences in the light source, UV exposure protocol, and sex of mice used in our study compared to the topical calcitriol study. Exposure of keratinocytes to UVB compared to solar simulated light can alter signaling pathways in the skin [39,40]. Additionally, our lab has demonstrated significant differences in the response to UV light between the sexes [41] and also in response to treatment [42]. Topical application of the active Vitamin D₃ metabolite

1,25(OH)₂D₃ allows for direct activation of VDR and its downstream effects in the skin. In contrast, the dietary Vitamin D₃ used in our study, must be absorbed by the intestines, converted by liver and the kidney to 1,25(OH)₂D₃ and shuttled back through the blood stream to the tumor site where it has to reach critical levels to inhibit tumor progression.

Xenograft mice models of breast cancer have shown that dietary vitamin D₃ inhibited tumor formation in breast fat pad, metastases to the lungs and reduced tumor size [43]. In this study they observed that mice fed diets of up to 5000 IU/kg dietary vitamin D₃ had elevated 25(OH)D₃ serum levels but no hypercalcemia as evidenced by lack of increased calcium levels in serum. [43]. Moreover, mice fed 5000 IU/kg of dietary vitamin D₃ showed a reduction in the number and size of breast tumors. Differences in the effects of dietary Vitamin D₃ supplementation in the two studies may be attributed to a 5 fold higher dose used in the breast cancer xenograft model when compared to the 1000 IU/kg used in our study as well as the tumor type being studied.

The current studies did not specifically examine the role of interfollicular vs follicular cells and Vitamin D₃ supplementation in SCC formation. However, it has previously been shown that while removal of the interfollicular epidermis by abrasion in CD-1 haired mice decreased the quantity of papilloma developed by half, it did not delay or stop the development of papillomas [44]. Similarly, CO₂ laser ablation of the interfollicular epidermis of hairless mice did not delay or stop the development of tumors, suggesting that a pool of cells deep in the hair follicle might be responsible for the SCC development [45]. UV-induced ablation of the epidermal basal layer in hairless mice further showed SCC originated from the interfollicular epidermis which was being repopulated from the hair follicle [36]. These studies suggest that the decrease in hair follicles in our hairless mice, observed as they age, did not impact tumor development in our study.

These studies demonstrate the complexity of Vitamin D₃ supplementation and suggest the necessity for additional studies to determine whether dietary Vitamin D₃ or topical 1,25(OH)₂D₃ are viable therapeutic options since the application of 1,25(OH)₂D₃ to un-irradiated normal hairless mouse skin results in dose and time dependent increases in mitosis and hyperplasia [46]. Taken together these studies demonstrate that Vitamin D₃ may have differing effects depending on the target organ and mode of delivery. In the case of non-melanoma skin cancers it may be detrimental at high levels because of its ability to stabilize ΔNp63α levels and increase, rather than prevent, UVB induced tumors.

Materials and Methods

Animal Treatments

Male SKH-1 hairless mice were obtained from Charles River Laboratories (Wilmington MA). Male SKH-1 mice were housed in the vivarium at The Ohio State University according to the requirements established by the American Association for Accreditation of Laboratory Animal Care. The Ohio State University Institutional Animal Care and Use Committee approved all procedures before the initiation of any studies (Protocol Number: 2010A00000083) and all efforts were made to minimize suffering. Four week old animals were assigned to different diets consisting of either standard chow with only 3 IU/kg Vitamin D₃ (8640 Teklad 22/5 Diet, Harlan Laboratories, Madison, WI), or AIN93G diet modified to contain 25 IU/kg, 150 IU/kg, or 1000 IU/kg Vitamin D₃ (Research Diets, New Brunswick, NJ). The concentrations of Vitamin D₃, in the form of cholecalciferol, was based on the study by Fleet *et al.*

demonstrating that the dietary Vitamin D₃ concentrations needed for modeling human borderline deficiency (25–40 nmol/L) average (50–60 nmol/L) and optimal (80–100 nmol/L) serum 25-hydroxyvitamin D concentrations as defined by NRC are 25–50, 100, and 400 IU Vitamin D₃/kg diet in growing rodents [47]. Twenty-five mice were assigned to each diet. Fifteen mice per diet were dorsally exposed to 2240 J/m² UVB, previously determined to be to one minimal erythemic dose, 3 times weekly for a total of 25 weeks. UVB dose was calculated using a UVX radiometer and UVB sensor (UVP, Upland, CA) and delivered using Philips TL 40W/12 RS SLV UVB broadband bulbs emitting 290–315 nm UVB light (American Ultraviolet Company, Lebanon, as previously described [48]). Ten mice per diet served as age matched, unirradiated controls. All mice were sacrificed by CO₂ inhalation.

Quantitation of epidermal thickness

Epidermal morphology was analyzed using the Accustain trichrome stain (Masson) kit according to manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). Epidermal thickness was measured using ImageJ software at a magnification of 10x in all tissue samples. Dorsal skin morphology was examined using H&E staining and visualized/imaged using a Leica CTR 6000 Microscope (Leica Microsystems, Wetzlar, Germany) and ImagePro 6.2 software (Media Cybernetics, Bethesda, MD).

Tumor development and grade

Neoplastic lesions located on the dorsal skin measuring greater than 1 mm in size were counted and measured (length × width). Tumors were measured using digital calipers throughout the duration of the study. Tumor grade was determined from hematoxylin and eosin (H&E)-stained sections of tumors isolated from UVB irradiated mice graded in a blinded manner by a board certified veterinary pathologist as previously described [48]. Briefly, papillomas were exophytic tumors (tumors that grow outward from the originating epithelium) that showed no invasion of the stroma [22]. MiSCCs were distinguished by the depth of penetration into the dermis [22]. Only tumors that invaded the panniculus carnosus were classified as fully invasive SCCs [22]. Average tumor percentages were calculated using the total number of graded tumors per treatment group.

Antibodies

PTEN, VDR, Ki67 and p63 antibodies were used to conduct immunofluorescence staining. Pan p63 (clone: 4A4) used to detect ΔNp63α, VDR (clone: 9A7) and PTEN (#9552) antibodies were purchased from (Santa Cruz, CA, USA), (Thermo-Scientific, Fremont, CA) and Cell Signaling (Danvers, MA, USA) respectively. Ki67 (clone: SP6) antibody was purchased from abcam (Cambridge, MA, USA).

Immunofluorescence

Tumors excised from dorsal skin as well as non-tumor dorsal skin were formalin fixed, paraffin-embedded and stained for p63, VDR and PTEN as previously described [7,15]. Ki67 staining was performed analogous to previously described staining of p63 [7,15]. For detection of VDR, paraffin was removed by four 10 minute washes in Histo-Clear (National Diagnostics, Atlanta, GA) and rehydrated in graded series of alcohols with a final wash in distilled water. After rehydration slides were incubated at 37°C for 20 minutes at 60°C in 2 N HCl. Slides were neutralized with 3 washes of 0.1 M sodium borate buffer (pH 8.5), followed by three washes in PBS. Tissues were blocked for 3 hours with 5% normal goat serum followed by overnight incubation with anti-VDR at

4°C (clone 9–A7, Thermo-Scientific, Fremont, CA). Excess primary antibody was removed with three consecutive washes in PBS followed by incubation with AlexaFluor 568 goat anti-rat antibody for 1 hour at room temperature. Excess secondary was removed with three consecutive 5 min washes in PBS prior to mounting with Vecta-Shield plus DAPI Mounting Media (Vector Laboratories, Burlingame, CA). Cells were visualized and imaged using a Leica CTR 6000 Microscope (Leica Microsystems, Wetzlar, Germany) and ImagePro 6.2 software (Media Cybernetics, Bethesda, MD). Mean fluorescence intensity for each tissue sample was calculated using ImagePro 6.2 software after normalization for background intensity. Multiple measurements (at least 5), all of the same size, were taken of the epidermal tissue for each tissue sample. Average mean fluorescence intensity was calculated as previously described [15].

Statistics

Differences in mean fluorescence intensities were analyzed by one-way ANOVA followed by pairwise multiple comparison testing (Tukey test method, SigmaPlot 12, Dundas Software).

Supporting Information

Figure S1 SKH-1 mice skin following UVB induced tumor development. SKH-1 mice fed chow with increasing

concentration of Vitamin D₃ were irradiated thrice weekly for 25 weeks with UVB. Tumor excised from dorsal skin as well as non-tumor (normal) dorsal skin were formalin fixed, paraffin embedded and subjected to H&E staining. Representative images of a normal skin, papilloma, MiSCC and SCC were taken at a 20x magnification. Scale bar = 20 μm.

(PSD)

Figure S2 Effect of dietary Vitamin D₃ on tumor development. (a) The average tumor area per mouse is plotted after 25 weeks of thrice weekly irradiation in mice fed diets with increasing amounts of Vitamin D₃. Error bars represent s.e.m. (b) The distribution of premalignant papillomas and malignant microinvasive squamous cell carcinomas (MiSCC) and malignant SCC is plotted. Error bars represent s.e.m.; n = 15 mice per treatment condition.

(PSD)

Author Contributions

Conceived and designed the experiments: TMO KLT MPK NTH. Performed the experiments: NTH GHGM ARH. Analyzed the data: NTH GHGM MKL. Contributed to the writing of the manuscript: NTH MKL MPK.

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