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ON THE ROLE OF CLASSICAL AND NOVEL FORMS OF VITAMIN D IN MELANOMA PROGRESSION AND MANAGEMENT

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Abstract

Melanoma represents a significant clinical problem affecting a large segment of the population with a relatively high incidence and mortality rate. Ultraviolet radiation (UVR) is an important etiological factor in malignant transformation of melanocytes and melanoma development. UVB, while being a full carcinogen in melanomagenesis, is also necessary for the cutaneous production of vitamin D3 (D3). Calcitriol ($1,25(OH)_2D3$) and novel CYP11A1-derived hydroxyderivatives of D3 show anti-melanoma activities and protective properties against damage induced by UVB. The former activities include inhibitory effects on proliferation, plating efficiency and anchorage-independent growth of cultured human and rodent melanomas *in vitro*, as well as the *in vivo* inhibition of tumor growth by 20(OH)D3 after injection of human melanoma cells into

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immunodeficient mice. The literature indicates that low levels of 25(OH)D3 are associated with more advanced melanomas and reduced patient survivals, while single nucleotide polymorphisms of the vitamin D receptor or the D3 binding protein gene affect development or progression of melanoma, or disease outcome. An inverse correlation of VDR and CYP27B1 expression with melanoma progression has been found, with low or undetectable levels of these proteins being associated with poor disease outcomes. Unexpectedly, increased expression of CYP24A1 was associated with better melanoma prognosis. In addition, decreased expression of retinoic acid orphan receptors α and γ, which can also bind vitamin D3 hydroxyderivatives, showed positive association with melanoma progression and shorter disease-free and overall survival. Thus, inadequate levels of biologically active forms of D3 and disturbances in expression of the target receptors or D3 activating or inactivating enzymes, can affect melanomagenesis and disease progression. We therefore propose that inclusion of vitamin D into melanoma management should be beneficial for patients, at least as an adjuvant approach. The presence of multiple hydroxyderivatives of D3 in skin that show anti-melanoma activity in experimental models and which may act on alternative receptors, will be a future consideration when planning which forms of vitamin D to use for melanoma therapy.

Keywords

Melanoma; therapy; vitamin D; vitamin D receptor; retinoic acid orphan receptors

1. Introduction

Exposure to the highly energetic spectrum of ultraviolet radiation (UVB; λ=290–320 nm; 5% of total UVR reaching Earth surface), while representing a major risk factor for basal cell and squamous cell carcinomas (BCC and SCC) and melanomas, is also necessary for cutaneous production of vitamin D3 (D3) (Fig. 1)[1–5]. The transformation of 7-dehydrocholesterol (7DHC) to D3 after absorption of UVB energy by the unsaturated B ring represents the most fundamental reaction in photobiology [6–8], since the biologically active form of D3, 1,25$(OH)_2$D3, not only regulates body calcium homeostasis, but also displays a variety of pleiotropic effects [7, 9, 10]. Importantly, these include radioprotective and anticarcinogenic activities [3, 5, 9, 11–14]. Moreover, skin supplies >90% of the body's requirement for this prohormone.

Over 80% of non-melanoma skin cancers (NMSC) occur in sun-exposed sites, head, neck and the back of hands, attesting to a role for UVR in carcinogenesis. UVR is also considered as a full carcinogen for melanoma (Fig. 1) [4, 15–17]. UVR, depending on its wavelength, penetrates different layers of the skin affecting DNA integrity, cell and tissue homeostasis, inducing mutations, and modulating the expression of a plethora of genes including oncogenes and tumor suppressor genes [18–21]. It can also modify the expression and activity of growth factors/cytokines and their receptors, and has local and systemic immunosuppressive effects [22–27]. UVB absorbed by DNA can induce covalent bond formation between adjacent pyrimidines which leads to the production of mutagenic photoproducts such as cyclobutane pyrimidine dimers (CPD) and pyrimidine-pyrimidine adducts ((6–4)PPs)[19–21]. UVR also augments the production of reactive oxygen species

(ROS) with deleterious effects on skin, and induces tumor suppressor factor p53 as a part of the response to DNA damage [28–30]. The net effects are skin aging, solar elastosis, precancerous states such as solar keratosis, and finally skin cancers such as SCC, BCC and melanoma. Also, excessive sun damage can induce a field of cancerization, which may complicate the traditional therapy of skin cancer [31–33].

In this review, we summarize experimental and clinical data from our laboratories and the literature demonstrating that active forms of vitamin D3 show potent anti-melanoma activity in experimental models, and that defects in vitamin D signaling can contribute to melanomagenesis and tumor progression, with implications in melanoma therapy and pathological risk assessment of the disease outcome. We also discuss a hypothesis that endogenously produced active forms of D3 can attenuate or reverse UVB induced damages with net antioxidative, antimutagenic, antigenotoxic and anti-proliferative effects.

2. Activation of vitamin D

2.1 Established (canonical) pathway of vitamin D activation

Vitamin D3 is produced in the skin by the photochemical opening of the B ring of 7-dehydrocholesterol by UVR. The initial product, previtamin D3, undergoes a slow thermal isomerization to vitamin D3 at skin temperature [34]. Vitamin D3 is a prohormone and is activated by two hydroxylations, the first at C25 by CYP2R1 or CYP27A1 in the liver to produce 25(OH)D3, and the second at C1α by CYP27B1 in the kidney to produce the active hormone, 1,25$(OH)_2$D3 [34, 35]. 1,25$(OH)_2$D3 can also be produced locally in the skin with CYP2R1, CYP27A1 and CYP27B1 being expressed in skin cells [35–38]. 1,25$(OH)_2$D3 is inactivated by the action of CYP24A1, primarily in the kidney, which hydroxylates it at C24 then further oxidizes the side chain to the excretory product, calcitroic acid [39–42]. 25(OH)D3 can be metabolized by the same oxidation pathway. CYP24A1 is also expressed in the skin [35, 43].

2.2 CYP11A1-dependent (non-canonical) pathway of vitamin D activation

Over 15 years of experimental work has revealed an alternative pathway of vitamin D3 activation by CYP11A1 [44]. CYP11A1 is best known for initiating steroidogenesis where the side chain of cholesterol is hydroxylated and cleaved to produce pregnenolone (reviewed in [45, 46]. CYP11A1 can hydroxylate the side chain of vitamin D3 at C17, C20, C22 and/or C23, but no cleavage occurs. Hydroxylation at C20 is favored with 20*S*-hydroxyvitamin D3 [20(OH)D3] being the principal product. Other major products are 20,23$(OH)_2$D3, 17,20$(OH)_2$D3, 20,22$(OH)_2$D3, 17,20,23$(OH)_3$D3 and 22(OH)D3 [38, 47–51]. These secosteroids were originally identified as products of the enzyme *in vitro*. Subsequent studies where skin cells, or fragments of adrenal glands or placentae were incubated with D3 confirmed their production under *ex vivo* conditions [38]. Importantly, 22(OH)D3 and 20(OH)D3 are present in human serum in the nM range [52]. Many of the CYP11A1-derived hydroxy-secosteroids, including 20(OH)D3 and 20,23(OH)D3, are converted to their 1α-hydroxy derivatives by CYP27B1in vitro [53] and during ex vivo incubations [38] with 1,20,23$(OH)_3$D3 and 1,20$(OH)_2$D3 being present in human serum and/or the epidermis [52]. CYP27A1, CYP24A1 and CYP3A4 can act on 20(OH)D3 producing 20,24$(OH)_2$D3,

20,25$(OH)_2$D3 and/or 20,26$(OH)_2$D3 [54–56] which have also been identified in serum [52]. These dihydroxy-metabolites are good substrates for CYP27B1 which hydroxylates them at C1α [53]. CYP24A1 can also act on 20,23$(OH)_2$D3 producing 20,23,24$(OH)_3$D3 and 20,23,25$(OH)_3$D3 [55]. Thus, CYP11A1 initiates new pathways of vitamin D3 metabolism with initial products being acted on by the well characterized P450s of the canonical pathway, with 16-different hydroxyvitamin D3 metabolites being identified by NMR to date. These pathways can be regarded as activating vitamin D3 since both the primary CYP11A1-derived products and secondary products, including those produced by CYP24A1, display biological activity, as described later. Similarly, CYP11A1 can activate vitamin D2 producing 20(OH)D2, 17,20$(OH)_2$D3 and 17,20,24$(OH)_3$D3, with 20(OH)D2 being converted to 1,20$(OH)_2$D3 by CYP27B1 [57–60].

2.3. Phototransformation of Δ7- hydroxysterols into hydroxy-secosteroids

Previous studies have demonstrated that CYP11A1 can convert 7DHC to 22(OH)-7DHC and 20,22$(OH)_2$-7DHC with subsequent cleavage of the side chain producing 7-dehydropregnenolone (7DHP), *in vitro* [47, 61] and *ex-vivo* [62, 63], with some of the products detectable in the human epidermis and serum [52]. 7DHP can also be hydroxylated to Δ7-steroids by steroidogenic enzymes or have the remaining 2C side chain cleaved producing Δ7-androsta-steroids [62, 63]. In the skin, the breakage of the unsaturated B ring of these compounds by the absorption of UVB energy [6] causes their transformation into the corresponding secosteroids, with or without a full-length side chain, vitamin D-like and tachysterol-like, as well as lumisterol-like formed when the broken Bring reseals in a different stereochemical configuration to 7DHC (Fig. 2) [61, 64–69].

3. Malignant melanoma

3.1. Introduction

Cutaneous melanomas represent a challenge for patients, clinicians and researchers because of the relatively high incidence and high mortality rates, as well as the relative resistance of metastatic disease to the therapy [70–74]. For example, for 2016 in the USA 7,630 new cases of melanoma were reported for this year with an estimated 10,130 death associated with this disease [70, 71]. Therefore, this disease deserves serious attention including its prevention, early and precise diagnosis, and adequate surgical removal of the tumor which may be curable for melanoma in situ or the invasive tumor at the radial growth phase (RGP) [73, 75, 76]. The pathologist plays a crucial role in this process by providing adequate diagnosis supported by synoptic reporting of the necessary prognostic factors as well as by performing ancillary studies relating to the therapy or for predicting prognosis.

Over the last decade, there has been a significant advancement in our understanding of the immune and molecular principles regulating melanoma behavior [74, 77–81]. This led to development of new therapeutic approaches including targeted therapy using BRAF and MEK inhibitors or immunotherapy also encompassing checkpoint inhibitor antibodies against CTLA-4, PD1 and PDL-1. Unfortunately, targeted therapy retains efficacy only for a short period and the disease relapses leading to the death of patients. In addition, checkpoint immunotherapy is effective in only a certain subset of patients, has toxic side effects and is

very expensive. Thus, a new creative approach is required such as the use of natural products like vitamin D, in combination with the above strategies, to control tumor growth and to obtain a positive disease outcome [82, 83].

Such an approach appears to be supported by reports in the literature of an inverse relationship between 25(OH)D serum levels, melanoma thickness and patient survival, and polymorphisms in the genes encoding the vitamin D receptor (VDR) and the vitamin D binding protein [83–87]. This is consistent with the documented anti-cancer activity of vitamin D [9, 13, 88]. Furthermore, experiments on VDR or RXR knock-out mice have suggested a role for these receptors in the development of melanocytic tumors [89–91]. Below we summarize clinic-pathological correlations between the expression of VDR, CYP27B1, CYP24A1 and retinoic acid orphan receptors (RORs) with the progression of malignant melanoma.

3.2. Defects in vitamin signaling during melanomagenesis and melanoma progression

Clinical-based studies on melanoma patients have revealed that vitamin D plays an important role in the attenuation of melanoma development and tumor progression. Several studies show that the serum level of 25(OH)D3 is deficient in melanoma patients, and that there is a negative correlation between serum 25(OH)D concentrations and prognostic markers of melanoma such as tumor thickness [85, 92, 93], ulceration [84, 94], mitotic rate and histological type [94]. Consequently, lower 25(OH)D3 is accompanied by poorer melanoma prognosis [85, 87, 92, 93]. Recently published data also reveal that patients with melanomas formed on sun-exposed sites have higher levels of 25(OH)D than patients with melanomas on shielded-sites [95]. Some of these studies show that the 25(OH)D3/D2 concentration at the time of diagnosis is important for the prognosis of melanoma patients [84, 92, 94]. Others suggested that changes in the 25(OH)D3 concentration after diagnosis during follow-up are more important and could be a prognostic marker [96], or that both the initial 25(OH)D3 concentration and its changes during follow-up are predictors of melanoma patient prognosis [87]. Serum 25(OH)D levels can also influence the relationship between VDR polymorphisms and melanoma patient outcome [92]. This study also partly explained some inconsistencies in reports related to VDR polymorphisms and melanoma risk and outcome [83, 86, 93, 97–102].

In our studies on clinical melanocytic tumor samples we have shown that changes of VDR expression correlate with clinico-pathomorphological features. Both cytoplasmic and nuclear VDR immunostaining decreased with the development *de novo*, or progression of tumors from nevi to primary melanomas and to metastatic disease (Fig. 3). Similarly, in primary melanomas increasing tumor advancement (thickness, depth, pT stage) correlates with decreasing VDR expression [103, 104]. Moreover, the presence of other poor prognostic markers such as ulceration, higher mitotic rate, absence of tumor-infiltrating lymphocytes, nodular histological type, vertical growth phase and the presence of local and distant metastases are also accompanied by decreased VDR expression [103, 104]. Consequently, patients with low or undetectable VDR expression in melanoma cells had a higher overall stage of the disease and showed shorter overall survival (Table 1). The

statistically significant relationship between overall survival and VDR was seen for nuclear VDR but not cytoplasmic immunostaining (Table 1) [103].

Since epidemiological studies showed that melanoma patients had serum concentrations of 25(OH)D3 in the insufficient range at the time of diagnosis and/or during follow-up, we analyzed the expression of CYP27B1 in clinical samples of melanomas. Aside from kidney, CYP27B1 is widely expressed in peripheral tissues including skin [105–110]. In our study, similarly to VDR, CYP27B1 expression was higher in nevi, decreased in primary melanomas and was the lowest in metastatic lesions (Fig. 3). Also, the advancement of primary lesions (Breslow thickness, Clark's level and pT stage) correlated with decreasing CYP27B1 expression. Lack of or low CYP27B1 expression was associated with the presence of markers of poor prognosis. Melanoma at the vertical growth phase and metastasizing melanomas had lower CYP27B1 expression than in the radial growing phase and non-metastasizing tumors, respectively. In melanomas with low CYP27B1 expression the proliferative activity was also elevated. Finally, better disease-free and overall survival was seen in patients with higher tumor CYP27B1 expression (Table 1) [111].

CYP24A1 expression changed during the progression of melanocytic tumors, however, the expression pattern was different from that of VDR and CYP27B1. In nevi and primary melanomas CYP24A1 expression was elevated when compared to normal skin, with the highest expression found in nevi and early stage melanomas (Breslow thickness ≤2mm, Clark level ≤ III, pT ≤ 2) [112]. Advanced melanomas and melanoma metastases showed decreased CYP24A1 expression (Fig. 3). CYP24A1 expression decreased in melanomas developing local and distant metastases (pN1–3, pM1) and melanomas at an advanced overall stage (stage 2–3). Furthermore, CYP24A1 expression was lower in melanomas showing a more aggressive phenotype such as nodular histological type, melanomas with higher proliferative index and with ulceration. Similarly to VDR and CYP27B1, lack of or decreased CYP24A1 expression was accompanied by shorter overall and disease-free survival (Table 1) [112]

Summarizing these findings, the disruption of the expression of VDR and of the vitamin D activating enzyme, CYP27B1, is associated with melanoma development and progression. The surprising positive correlation between the $1,25(OH)_2D3$ inactivating enzyme, CYP24A1, and disease outcome could either be a reflection of activation of the VDR that would stimulate CYP24A1 expression, as expected, or it may be secondary to the recently described additional function for CYP24A1 as an activator of 20(OH)D3 [40, 54].

3.3. Retinoic orphan acid receptors (RORs) and melanoma

Vitamin D metabolites can also function as inverse agonists for the retinoic acid-related orphan receptors (RORs), RORα and RORγ, and therefore provide an alternative mechanism by which vitamin D metabolites affect melanoma development [113, 114]. Both RORα and RORγ were found to be expressed in human melanocytic tumors samples, but their expression varied and was dependent on the clinico-pathomorphological features of the tumors [113, 115]. Both RORα and RORγ expression decreased with the progression of the tumors from nevi to primary melanomas to metastases. In addition, increasing advancement of primary melanomas, as assessed by Clark's level, Breslow thickness and pT stage, was

accompanied by decreasing levels of RORα and RORγ expression. Similarly, melanomas developing metastases were also characterized by the reduced expression of RORα and RORγ [115]. The lack of or decreased expression of these receptors was also related to the presence of negative prognostic melanoma markers such as nodular histological type of melanoma, the lack of tumor-infiltrating lymphocytes, the presence of ulceration, and a high proliferation index. Immunohistochemistry showed that cytoplasmic and nuclear RORα and RORγ levels were dependent on the clinico-pathomorphological features of the melanomas. Correspondingly, expression of RORα and RORγ correlated with less advanced overall stage, better prognosis and longer disease-free and overall survival (Table 2). The relationship with overall survival (OS) and disease-free survival (DFS) was observed for both cytoplasmic and nuclear RORγ and for nuclear RORα (only for OS). This trend was also observed after adjustment for Breslow thickness (OS and DFS) and overall stage (DFS) in Cox proportional-hazards regression analysis [115].

3. Testing the anti-melanoma activity of active forms of vitamin D

3.1. Overview on canonical active forms of vitamin D

The work of Colston and colleagues originally demonstrated the anti-melanoma activity of $1,25(OH)_2D_3$ and the presence of VDR receptors in melanoma cells [116]. Shortly thereafter, the inhibitory effects of $1,24,25(OH)_3D3$ and $1,25,26(OH)_3D3$ on melanoma cells were also demonstrated [117] and production of $1,25(OH)_2D3$ and of $24,25(OH)_2D3$ by melanoma cells was shown [118]. Since then several papers have demonstrated the anti-melanoma activity of $1,25(OH)_2D_3$ and its analogs in cell culture, and the expression of VDR and vitamin D activating- and inactivating enzymes in different melanomas (for most recent reviews see [2, 83, 97, 119]). Most frequently used in these studies were human melanoma lines and rodent melanomas including murine and hamster lines. The responsiveness of melanoma cells to active forms of vitamin D was cell-type specific and dependent on the culture conditions. Interestingly, some human melanomas were not responsive to vitamin D analogs and there were conflicting results on vitamin D effects in B16 and Cloudman murine melanomas (reviewed in [83]). Furthermore, active forms of vitamin D have been shown to display protection against UVR-induced damage, including in melanocytes and against photocarcinogenesis [3, 11, 120–122]. CYP11A1-derived 20(OH)D3 also shows photoprotective properties in model systems of both cultured human skin cells (at 10^{-7} M [12] and 10^{-7} M [123]) and murine skin *in vivo* (23 or 46 pmol/cm^2), comparable to equivalent concentrations $1,25(OH)_2D3$ [12, 124]. Finally, results from Dr Indra's laboratory clearly demonstrate the protective role of VDR against UVR-induced damage in murine melanocytes *in vivo* [91].

3.2. Antimelanoma activity of CYP11A1-derived secosteroids

Novel vitamin D derivatives with a full length or short side chain, as listed in Table 3, have been tested for their *in vitro* anti-melanoma activity using established lines of human (SKMEL-188, WM35, WM1341, WM164, WM98D, SBCE2, YUROB, YUKSI and YULAC and hamster (Bomirski AbC1 and Ab) melanomas [53, 54, 59, 62, 65–67, 125–131]. Among the compounds with a complete side chain the most extensively studied are 20(OH)D2, 20(OH)D3, $20,23(OH)_2D3$ and $1,20(OH)_2D3$. They show inhibition of cell

proliferation in monolayer, inhibition of plating efficiency (colony formation in monolayer) and inhibition of anchorage-independent cell growth (ability to grow in soft agar) with potencies similar to that of 1,25(OH)$_2$D3 (Table 3). Inhibition of cell proliferation by 20(OH)D was related to arrest in the G1/G0 phase of the cell cycle and a decrease in S and G2/M phases, with no effect on subG1 (an indicator of apoptosis) [59]. Daily intraperitoneal injections of 20(OH)D3, 20,23(OH)$_2$D3 and 20(OH)D2 into rodents have shown that these secosteroids are non-calcemic at the highest doses tested, 60, 3 and 4 μg/kg, respectively [59, 132–134]. The CYP27B1-catalysed hydroxylation product, 1,20(OH)$_2$D3, also shows reduced calcemic activity in comparison to 1,25(OH)$_2$D3 [134].

It is important here to discuss the dietary supply of vitamin D to laboratory animals through commercially available chow. Vitamin D3 from the diet is easily converted to the active forms in the liver, kidney and other tissues, including malignant ones, equipped with enzymatic machinery [135]. Although the affinity of 25(OH)D3 for the VDR is approximately 500-fold less than that of 1,25(OH)$_2$D3, its presence at high concentrations may affect the outcome of *in vivo* experiments, either directly or by increased conversion to1,25(OH)$_2$D3 [136]. As shown in a preclinical study, a 5-fold rise in vitamin D3 levels in the mouse chow led to an increase of 50% and 145% in serum 25(OH)D serum levels and resulted in significant (50–60%) shrinkage of xenograft breast and prostate tumors [136]. Therefore, in order to properly establish the role of vitamin D in melanoma progression it is crucial to eliminate any external sources of vitamin D (diet and UVB exposure) to avoid compromising the interpretation of results. This should allow for better assessment of the true extent of tumor inhibition due to treatment with the secosteroids under study. According to the American Institute of Nutrition guidelines, the optimal content of vitamin D in rodent chow is 0.025 mg/kg (0.65 μmol or 1.0 IU/g) [137]. Nowadays the regular rodent chows contain 2.39 – 4.6 IU/g of vitamin D3, with the average daily chow intake of 5 g/mouse [138] (LabDiet, St. Louis, MO; Harlan Lab., Madison, WI). In mice, hypercalcemia, manifested as calcium depositions in vital organs, has been observed at a dose as low as 5.0 mg/kg for vitamin D3 and 25(OH)D3, and 0.1 μg/kg for 1,25(OH)$_2$D3 [135]. In our experiments employing 20(OH)D3 we used 30 μg/kg/day [128] (Table 3), with 60 μg/kg still being non-calcemic [133].

The lack of hypercalcemia at very high doses of 20(OH)D3 led us to test this compound more extensively. Specifically, 20(OH)D3 inhibited nuclear factor-kappa B (NF-κB) activity in human SKMEl-188 melanoma cells with concomitant reduction of melanoma proliferation [139]. It should be noted that in human melanomas, NF-κB is upregulated leading to a poor disease outcome [140, 141]. In addition, 20(OH)D3 caused inhibition of the migratory capabilities of the cells and cell-cell and cell-extracellular matrix interactions using transwell cell migration and spheroid toxicity assays [128]. Importantly, 20(OH)D3 at intraperitoneal daily doses of 30 μg/kg inhibited melanoma tumor growth in immunocompromised old female NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice kept on a vitamin D-deficient diet (TD.89123, Harlan Laboratories, Madison, WI) [128], without visible signs of toxicity. Interestingly, the total body score was higher in the 20(OH)D3 treatment group as compared to the control group (2.8 vs. 2.55) [128]. The body score (BS) scale is a 5-point practical, rapid, noninvasive methods to assess the overall health condition of an animal [142]. A score of 3 indicates the optimal health state and any deviation from

this value (above or below) indicates worsening of body condition. Precursor vitamin D3 at the same dose had no effect on tumor growth (Skobowiat et al., unpublished) indicating the requirement for vitamin D activation in order for it to exert anti-melanoma activity.

20(OH)D3 is not only further hydroxylated by CYP11A1 producing other bioactive secosteroids [49, 143] but is also hydroxylated by CYP27A1, CYP24A1 and CYP3A4 to produce 20,24$(OH)_2$D3, 20,25$(OH)_2$D3 and 20,26$(OH)_2$D3, as described above [53, 54, 56]. These are more potent and efficacious than their precursor 20(OH)D3 at inhibiting melanoma growth in soft agar [53, 54]. These secosteroids are excellent substrates for CYP27B1 [53, 126]. However, they and their 1α-hydroxyderivatives show similar antiproliferative potencies against keratinocytes [114]. Also, hydroxylation at C1α had rather minimal effect on their anti-melanoma activity [53, 54].

With respect to the mechanism of action of CYP11A1-derived vitamin D compounds with a full-length side chain, they inhibit melanoma growth in a similar manner independent of the presence or absence of hydroxyl at C1α. The involvement of VDR in this process is indicated by amplification of growth inhibition by 20(OH)D2 in melanoma cells overexpressing VDR [59]. 20(OH)D3 and its hydroxyderivatives, with or without C1α(OH), cause translocation of the VDR from the cytoplasm to the nucleus in melanoma and other skin cells with high potency, comparable to that of 1,25$(OH)_2$D3. This is consistent with their high docking scores predicted by molecular modeling, indicating high affinity for binding to the genomic site of the VDR, as reported previously [114, 143, 144]. However, their interaction with VDR-ligand binding domain using the LanthaScreen TR-FRET Vitamin D receptor Coactivator kit required the presence of the C1α(OH). Thus, for the synthetic VDRE used in this coactivator binding assay, the absence of the C1α(OH) presumably prevents the full conformational change necessary to promote binding of the coactivator. This indicates that the appropriate VDRE and complete receptor complex in the cellular environment are necessary for proper interaction of the VDR with ligands having hydroxyl groups solely on the side chain and not at C1α [114]. Therefore, we suggest that CYP11A1-derived D3 hydroxyderivatives with hydroxyl groups placed only on the side chain can act as biased agonists (the term “biased” was defined by Kenakin [145, 146] for selective activity on specific receptors), or as partial agonists (as we proposed previously [143]) on VDR in melanoma cells, selectively causing some but not all of the phenotypic effects of 1,25$(OH)_2$D3 [114, 143]. Examples of the latter are the relatively poor activation of CYP24A1 expression and the lack of calcemia by 20(OH)D3. An example of the former (similar potency and efficacy to 1,25$(OH)_2$D3) is the inhibition of melanoma cell colony formation by 20(OH)D3 [54, 128, 130]. We are in the process of further addressing these exciting observations through microarray and genomic technology, as well as crystallographic studies aimed at obtaining the structure of 20(OH)D3 bound to the VDR ligand binding domain (see also section 3.3).

As described earlier, there are alternative nuclear receptors for CYP11A1-derived secosteroids, the RORs [113, 114]. In the above context, the use of melanoma cell lines with silenced or overexpressed VDR or RORs should provide an indication of which particular receptor is involved in the regulation of selective phenotypic traits of melanoma by the CYP11A1-derived secosteroids.

Another downstream mechanism of inhibition of skin cell proliferation, including melanoma, by 20(OH)D3 and 20,23$(OH)_2$D3 is down-regulation of NF-κB activity [139, 147, 148] (Fig. 4). The mechanism of action of these secosteroids on NF-κB is very similar to that mediated by 1,25$(OH)_2$D3, i.e., stimulation of the expression of IkappaBalpha (IκBα) with subsequent sequestration of NF-κB in the cytoplasm and consequent attenuation of transcriptional and phenotypic activities [139, 147, 148] (Fig. 4). Although recent data suggest that the VDR is involved in the upstream regulation of NF-κB signaling [149, 150], the involvement of RORs cannot be entirely ruled out, since 1,25$(OH)_2$D3 may also act as an inverse agonist on RORα and RORγ [114], with the latter being placed upstream of pro-inflammatory responses [151, 152]. Of note, it has been proposed that upregulation of NF-κB can be secondary to a deregulation in upstream pathways including Ras/Raf, PI3K/Akt, and NIK that play a role in melanoma genesis [140, 141]. NF-κB, being involved in the regulation of apoptosis, angiogenesis, tumor cell invasion and tumor progression, is a druggable target for melanoma therapy [139–141, 153, 154].

Analogs of pregna- or androsta-calciferols (secosteroidal or lumisterol compounds with a short or no side chain) also show therapeutically desirable anti-melanoma activities comparable to 1,25$(OH)_2$D3 [62, 65–67, 125, 129] (Table 3). Interestingly, their antiproliferative activities, at least under certain experimental conditions (*in vitro* melanoma models) did not require translocation of VDR to the nucleus [129], consistent with previous molecular modeling predicting weak binding [144]. In addition, the pregnacalciferol compounds lack calcemic effects [155]. Thus, they may be considered as good candidates for treatment of VDR-negative melanomas, however the details of their mechanism of action require further investigation.

3. 3. Towards pre-clinical and clinical testing of novel forms of vitamin D3

Patient-derived orthotopic xenografts (PDOX) models of melanoma have been used to test their sensitivity to molecularly-targeted drugs, standard chemotherapeutics, as well as live therapeutics such as tumor-targeting bacteria [156–160]. For example, a BRAF-V600E-mutant melanoma obtained from the right chest wall of a patient was grown orthotopically in the right chest wall of nude mice to establish a PDOX model. Trametinib (TRA), a MEK inhibitor, was the only agent of the 4 tested that caused tumor regression. In contrast, another MEK inhibitor, cobimetinib (COB), could slow but not arrest growth or cause regression of the melanoma. First-line therapy with temozolomide could slow, but not arrest tumor growth or cause regression. Since the patient had a BRAF-V600E-mutant melanoma, the patient would be considered as a strong candidate for vemurafenib (VEM) as first-line therapy, since VEM targets this mutation. However, VEM was not effective in the PDOX. The PDOX model thus helped identify the very-high efficacy of TRA against the melanoma PDOX and is a promising drug for this patient. These results demonstrate the powerful precision of the PDOX model for cancer therapy, not achievable by genomic analysis alone [156–160]. Therefore, these models are the most suitable for preclinical testing the efficacy of novel non-or low-calcemic vitamin D analogs discussed above in melanoma therapy and whether high doses of vitamin D, applied either orally or parenterally, can slow tumor progression or improve efficacy of the BRAF or MEK inhibitors.

A major metabolic modification to the D3 structure in the noncanonical pathway is the hydroxylation at the C20 position to stereo-specifically form 20*S*(OH)D3. Both 20*S*(OH)D3 and its non-natural stereo-isomer 20*R*(OH)D3 can be chemically synthesized, and they show comparable potency with 1,25$(OH)_2$D3 in stimulating VDR gene expression [68, 131, 161]. Further hydroxylations to the 20(OH)D3 scaffold, performed chemically or enzymatically, produced a number of di-hydroxylated analogs such as 20*S*,23*S*$(OH)_2$D3, 20*S*,23*R*$(OH)_2$D3, 20*S*,24*S*$(OH)_2$D3, 20*S*,24*R*$(OH)_2$D3, and their 1α(OH)-tri-hydroxylated derivatives [126, 127]. While all these derivatives demonstrate similar anti-melanoma and anti-inflammatory activities, the addition of the 1α(OH) was found to significantly enhance downstream VDR transcriptional activity of *CYP24A1* and *TRPV6*, and VDR translocation to the nucleus, presumably because of the increased binding affinity/altered conformational change contributed by the 1α(OH) (discussed in section 3.2) [114, 126, 127]. Clarification of their interactions with the VDR will require obtaining crystal structures of ligand bound to the VDR-LBD, which for 20*S*(OH)D3 and 1,20*S*$(OH)_2$D3 are being generated in our laboratories (Li et al., in preparation). In addition to hydroxylation to the parental 20(OH)D3 scaffold, chemically synthesizing Gemini 20(OH)D3 analogs by adding a second identical side chain, or changing the composition of the side chain has also been explored [133, 162]. Interestingly, there were no clear correlations between the Gemini chain length or side chain composition and their VDR activation or anti-melanoma activities, suggesting high flexibility in the ligand-binding pocket of the VDR [133, 162]. The above compounds will be tested for their anti-melanoma activity in the PDOX models described above.

Most recently a pilot placebo-controlled randomized phase II trial has been started by the Australia and New Zealand Melanoma Trials Group (ANZMTG 02.09) to assess the feasibility, safety and toxicity of an oral loading dose of 500,000 IU of vitamin D, followed by an oral dose of 50,000 IU of vitamin D monthly for 2 years in patients who have been treated for cutaneous melanoma by wide excision of the primary tumor [163]. Patients with stage IIb, IIc, IIIa (N1a, N2a) or IIIb (N1a, N2a) disease were included for randomization 2:1 to vitamin D treatment or placebo. The study will determine whether vitamin D is both safe and tolerable under these conditions and whether it will prolong time to recurrence within 5 years, or improve overall survival at 5 years [163]. Although limited in patient numbers (75), this is an important step in testing vitamin D as an adjuvant agent in melanoma therapy. Results of safety will be presented at World Melanoma Congress in Brisbane Australia, October 2017.

4. Perspective and conclusion

Based on the above, it is clear that vitamin D deficiency, defects in vitamin D activation, transport, activation of corresponding receptors and downstream signaling play a role in melanoma development and progression. Aside from avoidance of solar radiation, the preventive measures could include use of high doses of precursor vitamin D or its active forms that would fulfill the definition of endogenous or natural products. In addition to the oral route of delivery of vitamin D, the active forms could be applied topically to increase their efficacy in radioprotection and/or anti-cancerogenic effects. It must be noted that the vast majority of orally delivered vitamin D will be hydroxylated in position 25 in the liver making it unavailable for biotransformation by CYP11A1, because 25(OH)D is not acted on

by this enzyme [44, 48]. Therefore, in order for vitamin D to be metabolized by the non-canonical pathway it would have to be of skin origin, or applied topically or parenterally to be metabolized in the skin or in steroidogenic tissues including adrenals, for systemic use [38, 44].

With respect to melanoma therapy or prevention of metastatic disease after removal of the primary tumor, high doses of vitamin D could be applied either orally or parenterally. Definitively, vitamin D could be included into any therapeutic regime as the adjuvant, since melanoma patients are under strict physician supervision that would identify any early signs of toxicity. This could be an economical measure to possibly amplify the therapeutic efficacy of the first line drugs [82]. As optimal melanoma therapy represents a challenge for the patients [73], testing of new naturally derived active forms of vitamin D, alone, or in combination using appropriate preclinical models may represent a dawn of a new inexpensive and less toxic therapy. Thus, while UVB is a full melanoma carcinogen, products of its activity could be used in prevention or treatment of this devastating disease.

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Highlights

- Active forms of vitamin D inhibit growth of melanomas *in vitro* and *in vivo*
- Defects in vitamin D receptors and activating enzymes affect melanomagenesis and melanoma progression
- Active forms of vitamin D warrant pre- and clinical testing against melanoma
- Vitamin D can be used as an adjuvant factor in melanoma therapy

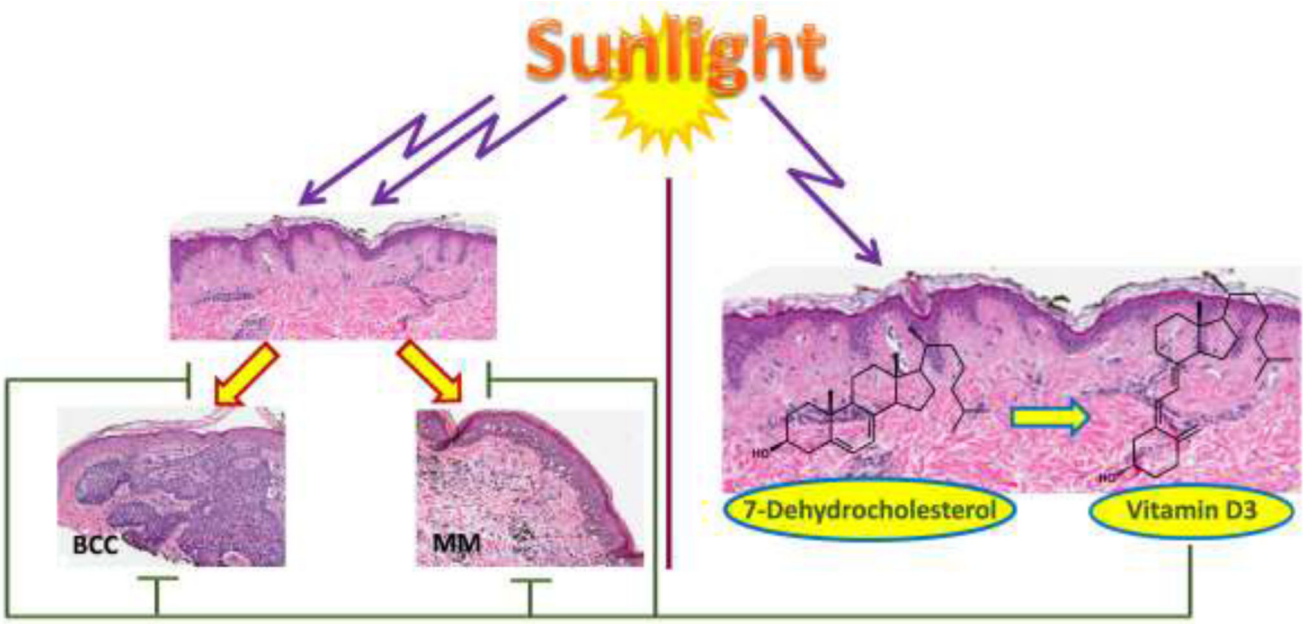

Figure 1.

Ultraviolet B acts as a double edge sword inducing skin cancer and producing vitamin D3 of which bioactive hydroxy-derivatives have photoprotective and anti-cancerogenic effects. BCC: basal cell carcinoma; MM: malignant melanoma.

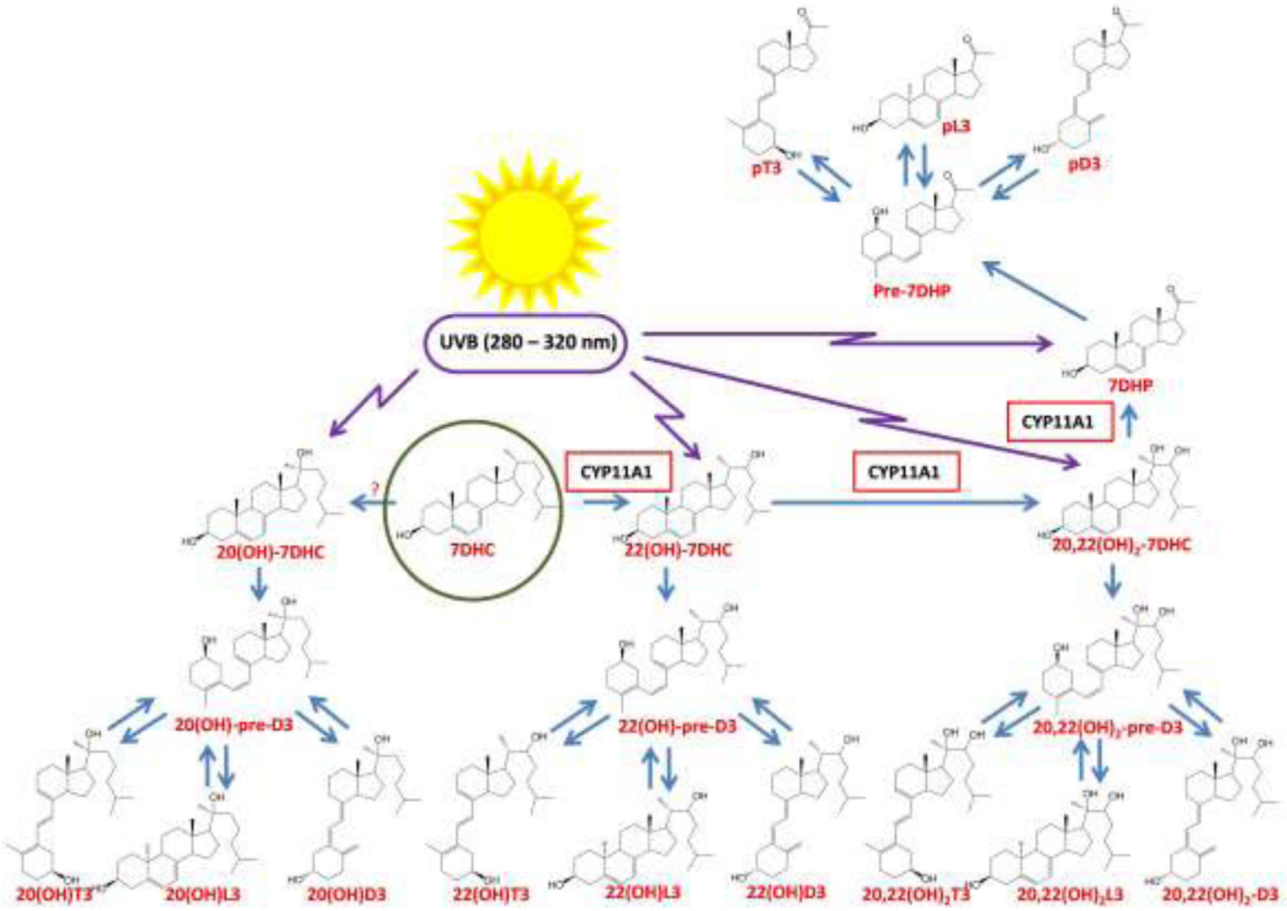

Figure 2.

UVB-induced transformation of 7DHC, its hydroxy-derivatives and 7DHP to their vitamin D-like and tachysterol-like secosteroids, as well as lumisterol-like configurations. Although the enzyme transforming 7DHC to 20(OH)7DHC must be identified there is a high probability that it is the same enzyme which transforms cholesterol to 20-hydroxycholesterol (CYP11A1 has very low activity for this). Not shown are the Δ7-products of 7DHP metabolism by steroidogenic enzymes, which are detectable in the steroidogenic tissues or serum under normal and pathological conditions [52, 62, 63, 164–166]. These Δ7-steroids can be transformed to their corresponding secosteroids after exposure to UVB [64–67, 69, 125].

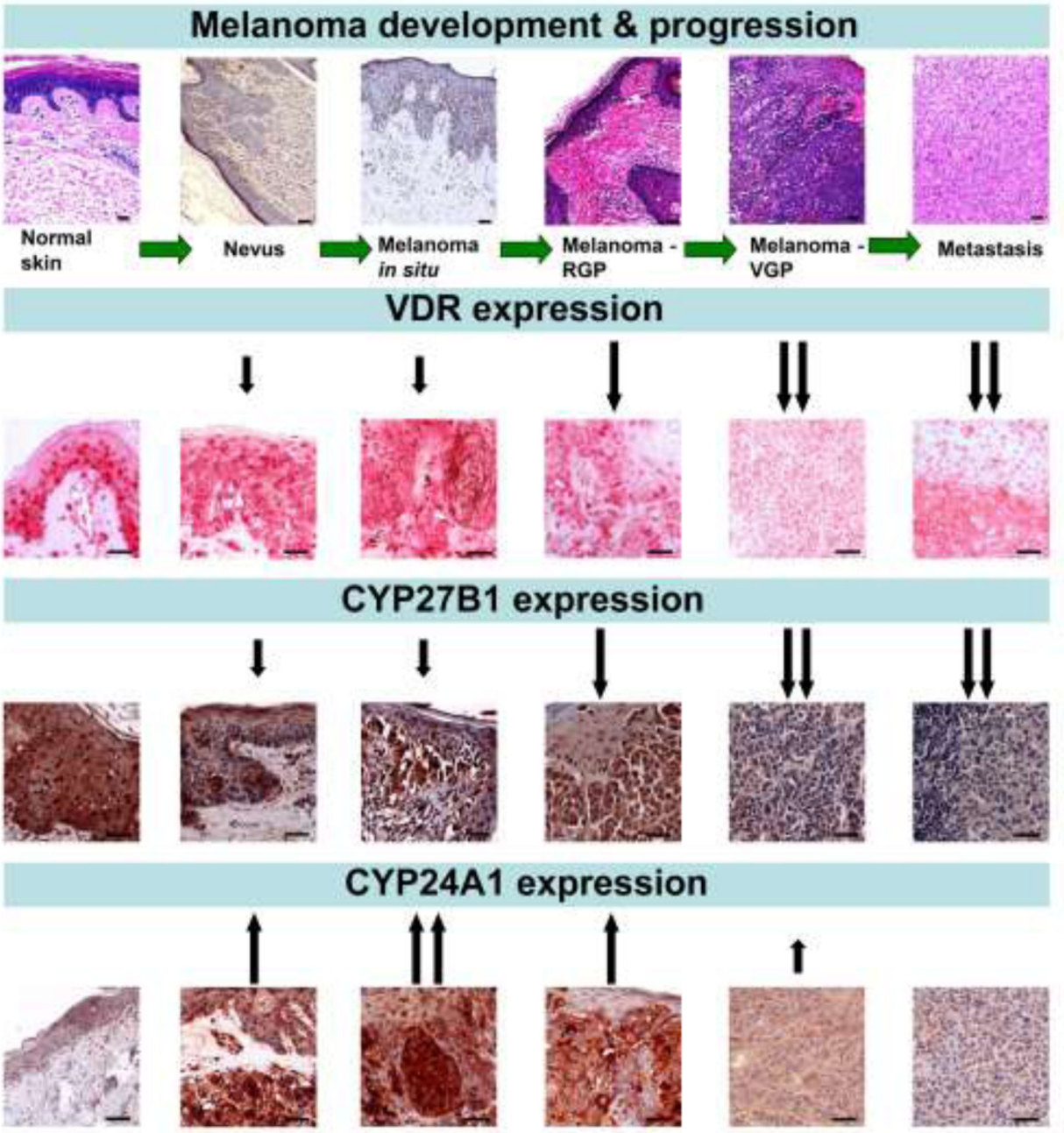

Figure 3.

Changes in VDR, CYP27B1 and CYP24A1 expression during the progression of melanocytic tumors. Upper panel presents the routine H&E sections of normal skin, junctional melanocytic nevus and melanomas at different stages of progression. VDR expression was detected using rat antibody (clone 9A7; Abcam, Cambridge, MA, USA; a dilution 1:75) and visualized with Red AP Substrate (Vector Laboratories, Burlingame, CA, USA) [104]. CYP27B1 expression was immunostained using rabbit antibody (clone H-90, Santa Cruz Biotechnology, Santa Cruz, CA, USA, a dilution of 1:75) and visualized with ImmPACT NovaRED substrate (Vector Laboratories, Burlingame, CA, USA) [111].

CYP24A1 expression was detected with mouse antibody (Abcam, Cambridge, UK, dilution 1:40) and visualized with ImmPACT NovaRED substrates (Vector Laboratories, Burlingame, CA, USA) [112]. Arrows indicate the changes of the expression in relation to normal skin, scale bars - 50 μm.

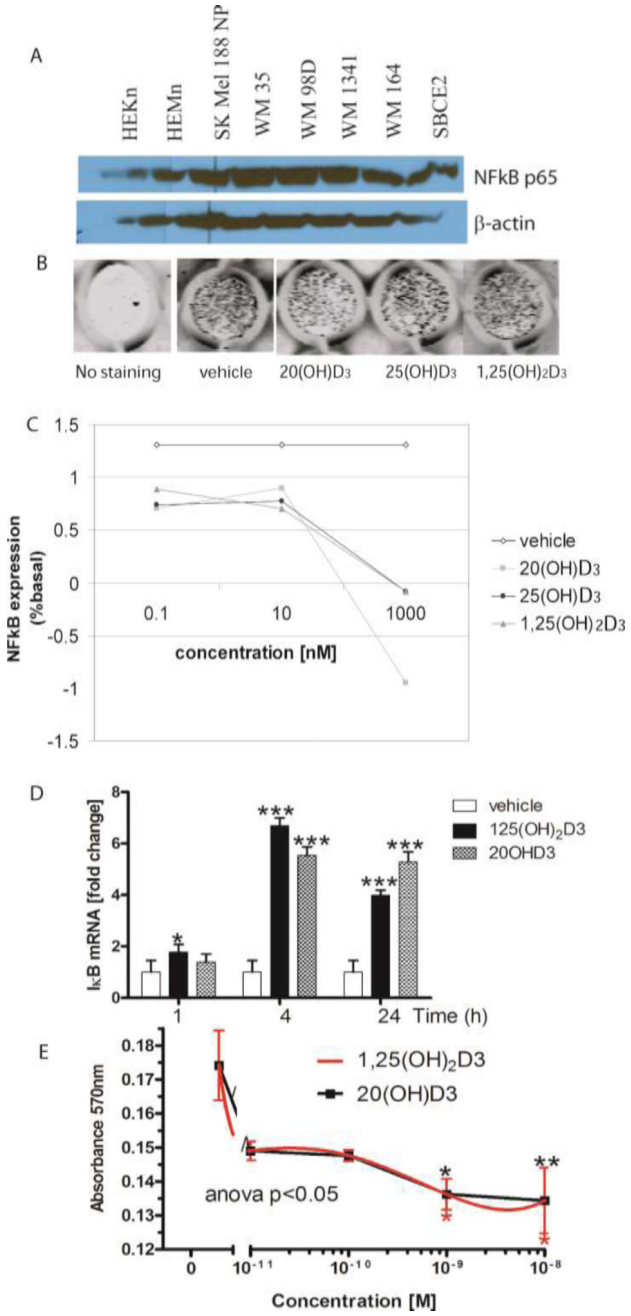

Figure 4. NF-kB as a target for bioregulation by hydroxyvitamin D3 in melanoma

A. Basal expression of NF-kB in human melanoma lines and normal epidermal melanocytes and keratinocytes. Whole cell extracts were prepared from human melanoma lines or skin cells and were subjected to Western blotting as described previously [139, 148]. B-actin served as an internal control for equal amounts of proteins loaded onto the gel.

B, C. 20(OH)D3 decreases protein expression of NF-κB in keratinocytes in a dose-dependent manner. HaCaT keratinocytes were plated in a 96-well plate and incubated for 24 h with $1,25(OH)_2D3$, 20(OH)D3 or 25(OH)D3. Cells were fixed and stained with NF-κB antibody [148], followed by incubation with secondary IR antibody. The plate was scanned

using an Odyssey infrared scanner (**B**) and data were analyzed using software for in-cell western (LI COR) (**C**).

D. Expression of the gene for IκBα (NF-kB inhibitor) is increased in human melanoma upon treatment with 1,25$(OH)_2$D3 or 20(OH)D3. SKMel-188 cells were treated with 1,25$(OH)_2$D3, 20(OH)D3 or ethanol (vehicle) for 1, 4 or 24 h. Cells were harvested for RNA isolation. cDNA was used for RT-PCR to test the expression of IκBα as described previously [139, 147, 148]. Reactions were performed using TaqMan. Cyclophillin B was used as internal control. Data were analyzed using the delta-delta CT method and presented as a fold change. Statistical analysis was performed using the t-test (*$p<0.05$; ***$p<0.001$) in comparison to vehicle.

E. 20(OH)D3 and 1,25$(OH)_2$D3 inhibit growth of human melanoma cells. SKMel-188 cells were treated with 1,25$(OH)_2$D3 or 20(OH)D3 at different concentrations (10^{-8}–10^{-11} M) for 48 h. After incubation, the MTT assay was performed and absorbance was recorded at 570 nm. $P<0.05$*, $p<0.01$**, $p<0.001$***.

Table 1

Overall survival (OS) and disease-fee survival (DFS) of melanoma patients in relation to VDR, CYP27B1 and CYP24A1 expression in primary melanomas.

Antigen	DFS time (median) [days]	OS time (median) [days]
Nuclear VDR	P>0.05	P<0.05
Absent	539	810
Present	1607	2297
Cytoplasmic VDR	P>0.05	P>0.05
Absent	539	912
Present	1607	1018
CYP27B1	P<0.001	P<0.01
Absent	471	748
Present	2520	1449
CYP24A1	P>0.05	P<0.05
Absent	459	581
Present	1607	1807

The data were extracted from [103, 104, 111, 112] P value - Log-rank (Mantel-Cox) Test

Table 2

Expression of RORα and RORγ in primary melanomas can serve as a prognostic factor for the disease outcome.

Antigen	DFS time (median) [days]	OS time (median) [days]
Nuclear RORα	P>0.05	P<0.01
Absent	780	530
Present	1607	1748
Cytoplasmic RORα	P>0.05	P>0.05
Absent	1076	972
Present	1607	1499
Nuclear RORγ	P<0.05	P<0.05
Absent	539	912
Present	1607	2297
Cytoplasmic RORγ	P<0.01	P<0.01
Absent	471	912
Present	1607	2297

The data were extracted from [115]. P value - Log-rank (Mantel-Cox) Test

OS: overall survival, DFS: disease fee survival

Table 3

Anti-melanoma activities of novel secosteroids

Compound	*in vitro* inhibitory effects	*in vivo* inhibitory effects
$20(OH)D_2$	Colony formation in monolayer [59]	ND
	Proliferation [59, 129]	
$20(OH)D_3$	Anchorage-independent growth [53, 54, 128, 130]	Tumor volume and geometric mean of tumor dimensions (NSG mice on a vitamin D-deficient diet) [128]
	Chemotactic capacity [128]	
	Colony formation in monolayer [128, 130]	
	Migratory capability, cell-ECM interactions and cell-cell interactions [128]	
	Proliferation [129, 130, 139, 167]	
$1,20(OH)_2D_3$	Colony formation in monolayer [125, 130]	ND
	Proliferation [125, 130]	
$20,23(OH)_2D_3$	Anchorage-independent growth [130]	ND
	Colony formation in monolayer [130]	
	Proliferation [125, 127, 130]	
$20,24(OH)_2D_3$	Anchorage-independent growth [54]	ND
	Proliferation [126]	
$20,25(OH)_2D_3$	Anchorage-independent growth [53, 54]	ND
$20,26(OH)_2D_3$	Anchorage-independent growth [53, 54]	ND
$1,20,23(OH)_3D_3$	Proliferation [127]	ND
$1,20,24(OH)_3D_3$	Proliferation [126]	ND
$1,20,25(OH)_3D_3$	Anchorage-independent growth [53]	ND
$1,20,26(OH)_3D_3$	Anchorage-independent growth [53]	ND
$17,20,23(OH)_3D_3$	Proliferation [125]	ND
pD_3	Anchorage-independent growth [62, 67, 129]	ND
	Proliferation [125, 129]	
21(OH)pD	Anchorage-independent growth [66]	ND
	Proliferation [66, 129]	
20(OH)pD	Anchorage-independent growth [65, 129]	ND
	Proliferation [65, 67, 125]	

The in vitro effects of the compounds tested were measured in comparison to $1,25(OH)_2D3$. The in vivo effect of $1,25(OH)_2D3$ in the experimental model described in [128] was not tested due to its toxicity at the testing dose.

Abbreviations: NSG: NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice; ND: not determined