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REVIEW

Vitamin D signaling and melanoma: role of vitamin D and its receptors in melanoma progression and management

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Ultraviolet B (UVB), in addition to having carcinogenic activity, is required for the production of vitamin D3 (D3) in the skin which supplies >90% of the body's requirement. Vitamin D is activated through hydroxylation by 25-hydroxylases (CYP2R1 or CYP27A1) and 1α-hydroxylase (CYP27B1) to produce 1,25(OH)$_2$D3, or through the action of CYP11A1 to produce mono-di- and trihydroxy-D3 products that can be further modified by CYP27B1, CYP27A1, and CYP24A1. The active forms of D3, in addition to regulating calcium metabolism, exert pleiotropic activities, which include anticarcinogenic and anti-melanoma effects in experimental models, with photoprotection against UVB-induced damage. These diverse effects are mediated through an interaction with the vitamin D receptor (VDR) and/or as most recently demonstrated through action on retinoic acid orphan receptors (ROR)α and RORγ. With respect to melanoma, low levels of 25(OH)D are associated with thicker tumors and reduced patient survival. Furthermore, single-nucleotide polymorphisms of *VDR* and the vitamin D-binding protein (*VDP*) genes affect melanomagenesis or disease outcome. Clinicopathological analyses have shown positive correlation between low or undetectable expression of VDR and/or CYP27B1 in melanoma with tumor progression and shorter overall (OS) and disease-free survival (DFS) times. Paradoxically, this correlation was reversed for CYP24A1 (inactivating 24-hydroxylase), indicating that this enzyme, while inactivating 1,25(OH)$_2$D3, can activate other forms of D3 that are products of the non-canonical pathway initiated by CYP11A1. An inverse correlation has been found between the levels of RORα and RORγ expression and melanoma progression and disease outcome. Therefore, we propose that defects in vitamin D signaling including D3 activation/inactivation, and the expression and activity of the corresponding receptors, affect melanoma progression and the outcome of the disease. The existence of multiple bioactive forms of D3 and alternative receptors affecting the behavior of melanoma should be taken into consideration when applying vitamin D management for melanoma therapy.
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VITAMIN D IN A 'NUTSHELL'

Cutaneous Vitamin D Formation and the Relationship with Skin and UV-Radiation

Ultraviolet radiation (UVR) with its highly energetic UVB wavelengths ($\lambda = 280$–320 nm) represents a major risk factor for all forms of skin cancer including malignant melanoma (Figure 1).[1] The same spectrum of solar radiation, UVB, is necessary for vitamin D production in the skin, which supplies >90% of the body's requirement for this prohormone.[2–4] The UVB energy is absorbed by the unsaturated-B ring of 7-dehydrocholesterol (7DHC) in the epidermis, which leads to its photochemical transformation to vitamin D3 (D3) or, depending on the UVB dose, to lumisterol and tachysterol (Figure 1).[5] These 'over-irradiation' products have no known classical vitamin D activity, but may contribute to protection from UV-induced DNA

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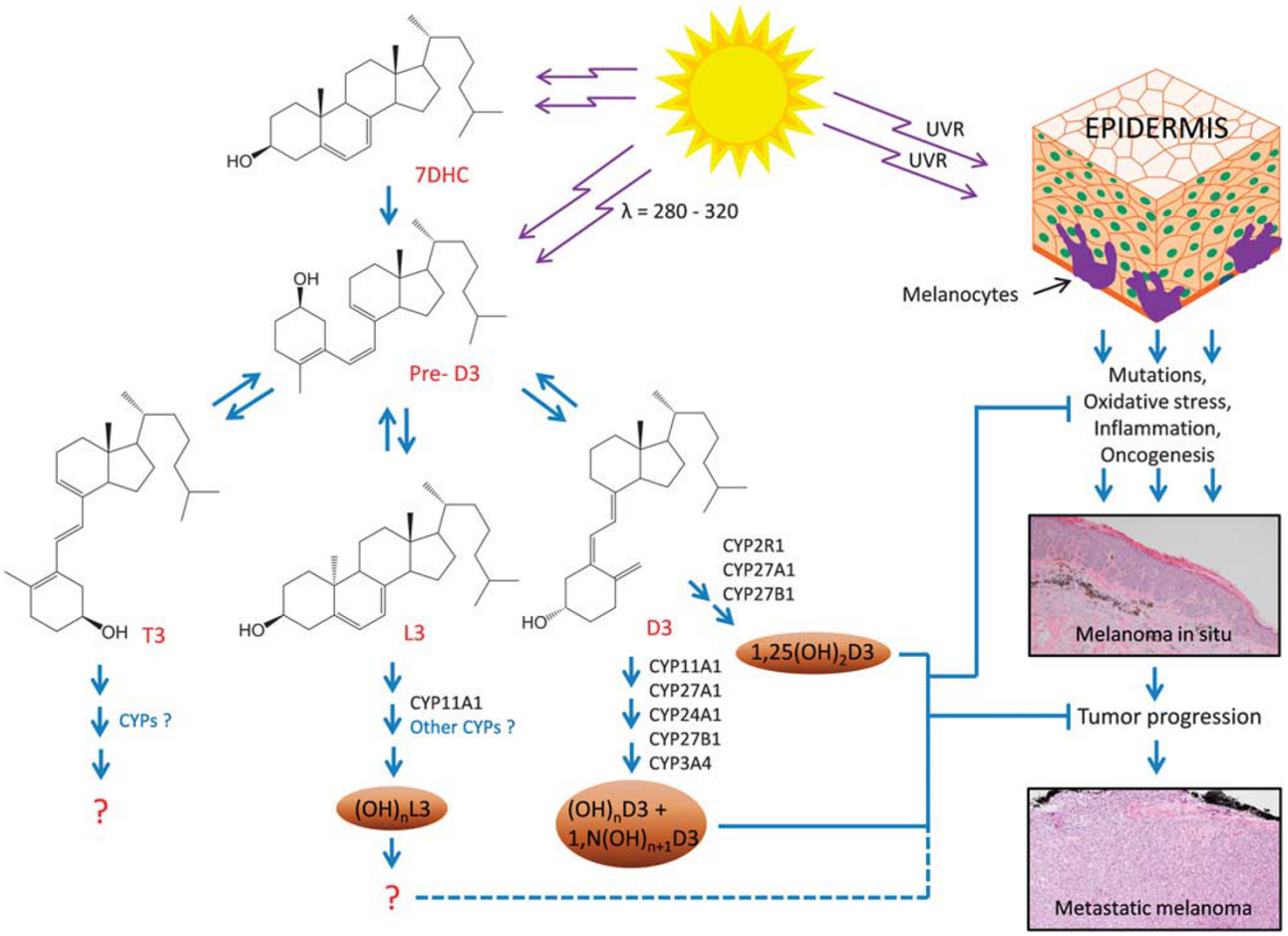

Figure 1 Ultraviolet B as a double-edged sword. UVR promotes melanomagenesis and tumor progression. However, UVB is necessary for vitamin D formation, which after activation by CYP enzymes can attenuate carcinogenesis, tumor progression and tumor growth. It is possible that hydroxylumisterols may also have similar activity as indicated by our cell culture studies (not shown). UVB, ultraviolet B; UVR, ultraviolet radiation.

damage.[6,7] The ability of UV to raise vitamin D status is determined by factors such as amounts of sun exposure and timing of that exposure,[6,7] skin surface area exposed,[7] and skin type including pigmentation level.[7,8]

How and Where the Biosynthesis of Active Forms of Vitamin D Takes Place

The liver and kidney are the major organs where the activation of D3 occurs, with liver 25-hydroxylases (CYP2R1 or CYP27A1) producing 25(OH)D3 and kidney 1α-hydroxylase (CYP27B1) converting this to 1,25-diydroxyvitamin D3 (1,25$(OH)_2$D3) (Figure 1).[2,3,9,10] Vitamin D activating enzymes are also present in extra-hepatic and extra-renal sites, including skin, so D3 can be fully activated in the epidermis.[2,11–14] 1,25$(OH)_2$D3 (calcitriol) is inactivated by CYP24A1, which initially hydroxylates at C24 then further oxidizes the side chain to produce calcitroic acid.[15–18] It carries out similar reactions on 25(OH)D3.

7DHC, the precursor to vitamin D3, is the last intermediate in cholesterol biosynthesis via the Kandutsch–Russel pathway and is UV-sensitive due to the two double bonds in the B-ring.[19] Although it had been suggested that statin drugs used for treatment of hypercholesterolemia patients may also decrease the incidence of melanoma,[20] clinical analyses do not substantiate the above hypothesis.[21,22] Thus the described anti-melanoma activity *in vitro*[23,24] may simply represent a cell culture phenomenon without a translation into clinical reality. However, it is interesting that while a decrease in 7DHC resulting from the inhibition of the cholesterol biosynthesis might be predicted to reduce vitamin D synthesis in the skin, the reverse has been found, 25(OH)D3 and 1,25 $(OH)_2$D3 levels are increased with statin treatment.[25,26] The mechanism underlying these observations is unclear.

Importance of Vitamin D in Biology, Including Cancer

The most clearly established effects of vitamin D are to help maintain calcium and phosphate homeostasis, and to optimize bone health and muscle function.[27,28] The hormonal form, 1,25$(OH)_2$D, increases active intestinal calcium (and phosphate) absorption, which helps offset obligatory calcium

losses from kidneys, gut, and skin. Severe vitamin D deficiency impairs bone mineralization, resulting in rickets (in children) and osteomalacia in adults. Vitamin D deficiency is an independent predictor of falls in the elderly, and circulating 25OHD3 levels <60–75 nmol/l have been associated with lower extremity muscle weakness and impaired balance, and accelerated losses in muscle mass, strength, and physical function.[28] Several meta-analyses report that giving vitamin D, usually with calcium, to vitamin D deficient individuals reduces falls, fractures and overall mortality.[29–33] Associations between vitamin D deficiency and poor outcomes of a variety of diseases including various types of malignancies (eg, colon-, skin-, and breast cancer), autoimmune diseases, infectious diseases, and cardiovascular diseases have been generally reported in a large number of studies.[3,34]

Novel Pathways of Vitamin D Activation

Alternative pathways of vitamin D activation have been described recently.[19] They are initiated by the action of CYP11A1 on the side chain of vitamin D with the preferred initial site of hydroxylation being at C20. The major pathways are: D3 → 20(OH)D3+22(OH)D3+17(OH)D3 → (OH)nD3 and D2 → 20(OH)D2 → 17,20$(OH)_2$D2 → 17,20,24$(OH)_3$D2+ 1,20$(OH)_2$D2.[35–47] CYP11A1 is the rate-limiting enzyme of steroidogenesis, where its role is the conversion of cholesterol to pregnenolone.[48,49] 20(OH)D3 and other intermediates of the pathway can be further hydroxylated by CYP27A1, CYP24A1, CYP3A4, and CYP27B1, producing potentially more than 21 hydroxymetabolites (reviewed in refs 19,50). Many of these, including 20(OH)D3 and 22(OH)D3, are produced in tissues expressing CYP11A1 (Figure 1), and they are present in human epidermis and serum.[44,51,52] They are also biologically active (reviewed in refs 50,53,54).

In addition, CYP11A1 can hydroxylate 7DHC (pro-vitamin D3) at C22 and C20 and then catalyze the oxidative cleavage of the bond between C20 and C22 to produce 7-dehydropregnenolone (7DHP),[55,56] which can further be hydroxylated to Δ7-steroids.[57–61] Since Δ7-steroids are detectable in the serum and epidermis,[52,57–61] they can potentially be transformed to the corresponding vitamin D analogs with a short side chain (pregnacalciferols (pD)) or no side chain (androgen-like (aD)) or their pregnalumisterol (pL) counterparts after exposure of skin to UVB.[56,62–65] The pD and aD compounds are also biologically active.[63–67] Moreover, CYP11A1 can metabolize lumisterol to several hydroxylumisterol (L) derivatives and pL[68] (Figure 1).

MECHANISM OF ACTION OF VITAMIN D

The pleiotropic activities of active forms of vitamin D are mediated through interaction with the vitamin D receptor (VDR) also known as nuclear receptor subfamily 1, group I, member 1 (NR1/1),[69–76] a member of the nuclear receptor superfamily.[77] Genomic activities of vitamin D are initiated when the VDR-ligand complex forms a heterodimer with the retinoid receptor, RXR, in the cytoplasm which then translocates to the nucleus, where it binds to vitamin D responsive elements in target genes and recruits either coactivators or corepressors to regulate transcription.[71,72,78] The VDR is expressed in all organs and almost all cells of the body, where it regulates a variety of their functions in addition to the regulation of calcium metabolism.[70,71,73] Approximately 3% of the mammalian genome is regulated, directly and/or indirectly by signaling secondary to activation of the VDR.[73] The VDR is also widely expressed in skin[79] and regulates various functions, including barrier, secretory, adnexal and immune functions, and protecting against UV-induced damage.[73,80–88] Thus the skin is not only a source of active vitamin D3 but is also a target of its activity.

The VDR also contains an alternative 1,25$(OH)_2$D3-binding A-pocket occupation of which can induce rapid non-genomic responses at the membrane level, independent from its action as a nuclear receptor.[76,89–91] Furthermore, 1,25D3-membrane-associated, rapid response steroid-binding protein (1,25D3-MARRS, PDIA3) has been identified as an alternative membrane bound receptor for active forms of D3 that can regulate some phenotypic functions.[92,93] Finally, some active hydroxylated forms of D3 can act on the retinoic acid-related orphan receptors (RORs) α and γ as inverse agonists.[94–96] Thus, in addition to the well-established mechanism of activation by binding of active forms of vitamin D to the genomic site of the VDR, there are non-genomic membrane-associated sites of action (A-VDR and 1,25D3-MARRS) as well as other nuclear receptor targets comprising ROR-α and -γ.[95] The regulatory targets for pD and aD secosteroids or lumisterol compounds remain to be identified.

ANTICARCINOGENIC PROPERTIES OF VITAMIN D: AN OVERVIEW

Population-based studies that originally started in 1980 by Garland and Garland,[97] proposed that insufficient levels of vitamin D in the serum increase the risk and incidence of human cancers and decreases survival.[98–106] The results of epidemiological studies have been supported by animal-based reports showing an increased cancer risk in VDR-deficient animals,[107] and reduced cancer incidence and tumor shrinkage when treated with vitamin D.[9,108–110] Moreover, malignant cells, including melanoma, express VDR and respond to the pleiotropic activities of 1,25$(OH)_2$D3.[108,111] Subsequently, molecular analysis identified a number of genes and signaling pathways located downstream of the VDR.[3,4,69] Active forms of D3 can enhance superoxide dismutase (SOD) 1 and 2 activities, upregulate expression of GADD45, p53 and others, all in order to protect against oxidative DNA damages (reviewed in ref. 69). In addition, 1,25$(OH)_2$D3, acting through the VDR, inhibits cell proliferation both in normal and malignant cells.[112–115] Similarly, newly discovered vitamin D derivatives, such as 20(OH)D3, 20(OH)D2, 1,20 $(OH)_2$D3, and 20,23$(OH)_2$D3, show VDR-mediated anti-proliferative properties comparable to those of 1,25$(OH)_2$

D3,[39,41,116–120] while, at the same time showing less calcemic effects.[39,54,117]

The proliferation of cells is regulated via complex, tissue-dependent signaling pathways with active forms of vitamin D affecting the expression of growth factors and proteins controlling the cell cycle. Regulation of the cyclin-dependent kinase (CDK) inhibitors, p21 and p27, by 1,25 $(OH)_2$D3 induces cell cycle arrest.[121–123] Also, active forms of vitamin D increase the expression of IGF-binding protein 3, thereby inhibiting the IGF-1- and IGF-2-stimulated cell proliferation[124–126] and decreases telomerase reverse transcriptase (TERT), which leads to an attenuation of telomerase activity and cell division (reviewed in ref. 127). 1,25$(OH)_2$D3 is important both in early and late stages of cancer development and progression regulating the expression of TGFβ. By increasing the expression of TGFβ, active forms of vitamin D enhance growth inhibition.[128–130] On the other hand, 1,25$(OH)_2$D3 attenuates the invasion and migration induced by TGF-β1/β2, in addition to inhibiting the epithelial–mesenchymal transition (EMT) and inhibiting the secretion of MMP-2 and MMP-9.[131] These mechanisms, and enhanced expression of E-cadherin, a well-known tumor protein suppressing the invasive phenotype of cancer cells, by vitamin D, are able to decrease the metastatic potential of cancer cells treated with 1,25$(OH)_2$D3.[132,133]

Active forms of vitamin D, both in normal and malignant cells, stimulate differentiation, maturation and senescence.[39,116,118,134,135] These processes are regulated by cell-specific mechanisms and involve inhibition of hedgehog, β-catenin, NFκB, and PI3K signaling pathways (refs 134,136,137 reviewed in refs 127,138). Apoptosis is also regulated by vitamin D. 1,25$(OH)_2$D3-induced programmed cell death is mainly due to downregulation of the anti-apoptotic proteins Bcl-2 and Bcl-X_L, upregulation of pro-apoptotic BAX, GOS2, DAP-3, FADD, and caspases (reviewed in refs 69,127,139). In addition, to control and modulation of proliferation, apoptosis, and differentiation/maturation, a variety of other tissue functions, important in tumor initiation, development and progression, are regulated by active forms of vitamin D. Consistently, vitamin D has been shown to act as an anticancer drug by inhibiting angiogenesis.[140,141] As illustrated by cell- and animal-based studies, inhibition of IL-8-mediated angiogenesis, a reduction in endothelial cell proliferation, and a downregulation of vascular endothelial growth factor (VEGF), including hypoxia-induced VEGF expression, is mediated through vitamin D.[140,142,143] Autophagy represents a double-edged sword that has an essential role in cell survival, but at the same time it causes cell death when apoptotic pathways are inactive. In tumors, autophagy is activated following anticancer treatment (reviewed in ref. 144) and 1,25$(OH)_2$D3-induced death of malignant cells includes this apoptosis-independent pathway through upregulation of beclin-1 (autophagy-related gene, the mammalian ortholog of yeast Atg6 protein). Similar autophagy-related cancer cell death induction is also shown by the vitamin analog, EB1089.[145–148] Vitamin D-mediated cell death by autophagy is enhanced when p19 is lost and attenuated by loss of p27.[149] Detailed analysis of autophagy in cancer cells revealed that 1,25$(OH)_2$D3 switches the mode of autophagy from cytoprotective to cytotoxicity, sensitizing cells to antitumor treatment.[145,150] Another autophagic mediator, mTOR, can be suppressed by vitamin D-regulated mTOR inhibitors.[151] These very recent discoveries concerning 1,25$(OH)_2$D3 and autophagy implicate a novel potential therapeutic approach for melanoma therapy through targeting autophagy with active forms of vitamin D.[152]

The 1,25$(OH)_2$D3-regulated anti-inflammatory effect is primarily mediated by inhibition of prostaglandin (PG) signaling. 1,25$(OH)_2$D3 regulates the PG pathway by suppressing cyclooxygenase 2 (COX-2), by increasing expression of the catabolic enzyme 15-hydroxyprostaglandin dehydrogenase, and by reducing expression of prostaglandin receptors (reviewed in ref. 153). Promoting the expression of mitogen-activated protein kinase phosphatase-5 (*MKP5*), which prevents phosphorylation and activation of the stress kinase p38, results in attenuation of the production of pro-inflammatory cytokines such as IL-6. The anti-inflammatory effect of 1,25$(OH)_2$D3 has been shown in normal cells.[154] The nuclear factor kappa B (NFκB) signaling pathway is also regulated by 1,25$(OH)_2$D3, and shows both pro- or anti-inflammatory properties since 1,25$(OH)_2$D3 regulates both the phosphorylation of the inhibitor I kappaB alpha (IκBα) via Akt kinase and increases IκBα synthesis.[155] 1,25$(OH)_2$D3 inhibits NFκB signaling by preventing the translocation of the p65 subunit to the nucleus, thereby attenuating NFκB-mediated IL-8 transcriptional activity.[143]

Vitamin D effects on inflammation (important in cancer development) are not limited to just regulation of anti- and pro-inflammatory factors, but also involve the regulation of cells of the immune system. This regulation is complex and multidirectional (reviewed in ref. 69). The major immune cells targeted by 1,25$(OH)_2$D3 are T helper (Th2) lymphocytes. 1,25$(OH)_2$D3 downregulates Th1 cytokines, upregulates Th2 cytokines by inhibiting production of the pro-inflammatory cytokines such as IL-17, TNF, IL-1, IFN-γ, and IL-2, while at the same time promoting production of the anti-inflammatory cytokines such as IL-4 and IL-10. Immune cells that are the target for 1,25$(OH)_2$D3 and essential for cancer prevention are the monocytes/macrophages, dendritic cells, and regulatory T cells (Treg). Vitamin D is able to modulate the function of immune cells since both the VDR and CYP27B1 are expressed by these cells (reviewed in refs 69,156). As is mentioned above, the anticancer action of vitamin D is complex and multi-dimensional, resulting from its pleiotropic properties, and involves modulation of cancer cell function, modification of the cancer microenvironment, alteration of the immune response, and others.

VITAMIN D AND MELANOMA: CLINICAL IMPLICATIONS

Relationship between UVR and Melanoma

The relationship between sunlight exposure and melanoma is not direct, since high levels of intermittent UV exposure seem to be more related to development of melanoma in susceptible individuals rather than high continuous exposure, as seen in outdoor workers.[157] UV exposure produces DNA damage and immune suppression, both of which contribute to melanoma development.[158,159] Inadequately repaired DNA damage produced by both UVB and UVA, along with UV-induced immune suppression are involved in the pathogenesis of melanoma, particularly on sun-exposed skin.[160,161] Inadequately repaired DNA damage in melanocytes may lead to mutations or amplifications of genes involved in a variety of growth and survival pathways, such as *BRAF*, *Kit* and *cyclin D1*.[162] Melanocytes differ from keratinocytes in that they have reduced proliferation and apparently reduced capacity to repair DNA, but may be more resistant to apoptosis, despite significant DNA damage. These biological differences may help to explain the different patterns of sun exposure associated with melanoma in comparison with squamous cell carcinoma.[160,163]

Malignant Melanoma: An Overview

Malignant melanoma, affecting large segments of the population with a relatively high incidence rate (estimated new cases and deaths in 2016 in the USA are 7630 and 10 130, respectively) compared with other cancers and a high mortality rate,[164,165] represents a significant clinical problem. Melanoma encompasses, respectively, 6% and 3% of all new cancer cases in the USA in 2016 for males and females, excluding non-melanoma skin cancers (NMSC) of epithelial origin.[165]

The most efficient methods of melanoma management involve prevention, early diagnosis and surgical excision of lesional skin when the disease is localized to the skin.[166,167] An impressive advancement in new therapeutic approaches including targeting molecular pathways for advanced melanomas (stages III and IV diseases) or modulations of immune responses, have been made.[168–173] Unfortunately, the utilities of these strategies are somewhat limited because of adverse effects, financial costs and inherent or acquired tumor resistance mechanisms leading to recurrent disease and death of the patient (discussed in refs 173–175). Therefore, defining new regulatory targets and compounds that are nontoxic, economical with relatively limited side effects, is needed. Examples of such targets and such compounds are linked with vitamin D signaling as indicated by the anticancer activity of active forms of vitamin D and the information listed below.

Polymorphism of Vitamin D-related Genes and Melanoma

Low levels of 25(OH)D3 or 25(OH)D2 are associated with histologically thicker tumors and reduced melanoma survival,[176–182] which can also be connected with a polymorphism of the gene encoding the vitamin D-binding protein (*VDBP*).[183,184] There is also evidence that single-nucleotide polymorphisms (SNPs) of the *VDR* gene may affect melanomagenesis or disease outcome.[178,182,185–189] The role of the VDR in melanomagenesis is further illustrated by experimental models of melanoma induction where silencing of VDR or its partner RXR resulted in development of melanocytic tumors after chemically or UVB-induced carcinogenesis.[190–192] This is consistent with the fundamental role of the VDR in protection against skin carcinogenesis.[1,72,80,82,193–196]

Although a number of *VDR* gene polymorphisms have been identified and some of them can modify the risk and disease outcome (reviewed in refs 197,198), the published reports present some contradictory data related to VDR polymorphisms in melanomas and their association with disease outcome.[185,186,199,200] The most studied *VDR* polymorphisms are *Fok*I, *Bsm*I, *Taq*I, and *Cdx*2. In some studies the *Bsm*I A allele was associated with improved melanoma survival but increased melanoma risk,[185,186] while other reports showed reduced melanoma risk[178] or poor prognosis of patients with low serum vitamin D levels.[104] Another polymorphism, the *Fok*I T allele was associated with increased melanoma risk,[178] and the *Taq*I 't'(C) allele with a protective role in melanoma-specific survival.[185] The observed discrepancies could be in part explained by the study of Newton-Bishop *et al*,[104] showing that the effect of specific polymorphisms on survival of melanoma patients was associated with the serum level of vitamin D. Also, although *VDR* SNPs rs7299460, rs3782905, rs2239182, rs12370156, rs2238140, rs7305032, rs1544410 (*Bsm*I), and rs731236 (*Taq*I) showed a statistically significant trend ($P<0.05$) for association with melanoma-specific survival in multivariate analysis, none of them was significantly associated with Breslow thickness, ulceration or mitotic rate.[185] The authors of this study proposed that the *VDR* gene may influence survival from melanoma, although the mechanism by which VDR exerts its effect may not be driven by tumor aggressiveness, but influenced by the host environment.[185]

Negative Correlation between VDR Expression and Melanoma Progression

Since the VDR is a critical mediator of the biological action of active forms of D3 and the reduction of its expression abrogates anti-tumorigenic activity (reviewed in ref. 69), clinicopathological studies have been performed on a cohort of patients monitored by the Oncology Center in Bydgoszcz to establish a relationship between VDR expression and progression of disease in melanoma patients[201,202] (see Figure 1 for an overview). VDR expression decreased in advanced cutaneous melanomas and their metastases and this decrease was associated with the vertical growth phase. The lowest and/or lack of VDR expression was associated with highest Breslow thickness, Clark level and the highest melanoma stage. Also, the ulcerated melanomas, with lack

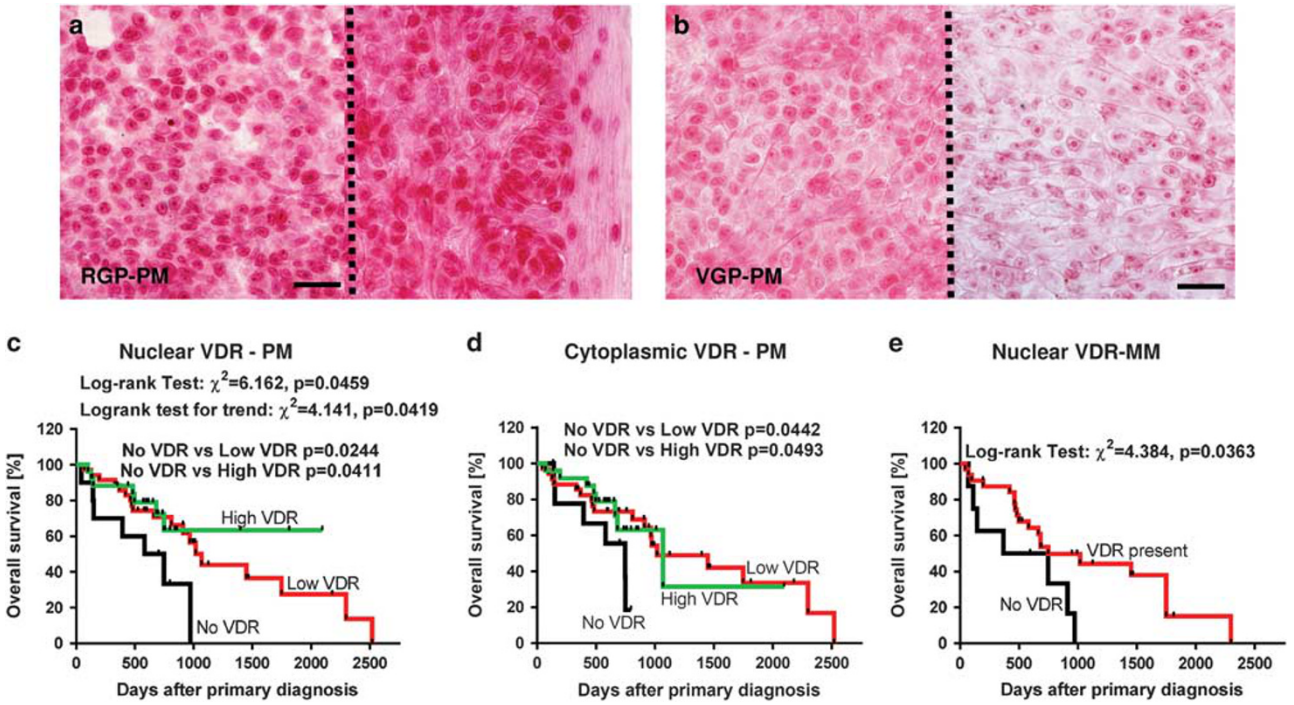

Figure 2 Correlation between VDR expression and melanoma survival. The representative VDR immunostaining in primary melanomas in RGP (**a**; two cases separated with dotted line) and VGP (**b**; two cases separated with dotted line) are shown. Dependence of OS on nuclear (**c**) and cytoplasmic (**d**) immunostaining VDR in RGP and VGP of primary melanomas, respectively, and on nuclear VDR in melanoma metastases (**e**). VDR-positive cells are visualized with Red AP Substrate; scale bars, 50 μm. The OS graphs (slightly modified) are reproduced with permission from ref. 201 (**c**–**e**). Vitamin D receptor was immunodetected with rat antibody (clone 9A7; Abcam, Cambridge, MA, USA; a dilution 1:75) and visualized with Red AP Substrate (Vector Laboratories, Burlingame, CA, USA). VDR immunostaining was scored as negative (0), weak (1), moderate (2), and strong (3), based on staining intensity. For analysis melanomas assessed as having moderate and strong VDR immunostaining were classified as high VDR, weak as low VDR, and negative as no VDR. Overall survival was calculated as the time between surgical treatment and diagnosis of primary melanoma and the time of death. Survival analysis was performed using Mantel–Cox (Log-rank) test and Log-rank test for trend. Scale bars, 50 μm. MM, metastatic melanomas; OS, overall survival; PM, primary melanomas; RGP, radial growth phase; VDR, vitamin D receptor; VGP, vertical growth phase.

or non-brisk tumor infiltrating lymphocytes and nodular type were characterized by lower VDR expression. Correspondingly, longer overall and disease-free survival (DFS) was accompanied by higher VDR expression both in primary and metastatic melanomas (Figure 2).[201,202]

As mentioned above, NFκB is an important regulator of inflammation and cancer development,[203,204] including melanoma, in which it has an important role in maintaining the malignant behavior.[205–207] We have shown that biologically active forms of vitamin D not only induce the VDR translocation to the nucleus and inhibit melanoma proliferation[39] but also that this process is associated with the downregulation of the NFκB pathway via inhibition of the nuclear translocation of the p65 NFκB subunit, its accumulation in the cytoplasm and inhibition of NFκB binding to DNA.[120] Furthermore, this process differs in nonpigmented and pigmented cells.[120] Nonpigmented cultured melanoma cells and nonpigmented and slightly pigmented human cutaneous melanomas show predominantly nuclear localization of the p65 NFκB subunit compared with highly pigmented melanomas.[120] Interestingly, the nonpigmented melanoma cells, showing higher nuclear VDR and NFκB expression, are more susceptible to vitamin D-mediated downregulation of NFκB activity and inhibition of proliferation than pigmented melanoma cells.[120] Combined analysis of clinical melanoma samples reported in refs 120,201,202 showed positive correlation between the higher percentage of NFκB-positive melanoma cells and the higher nuclear immunostaining of VDR ($r=0.35$, $P=0.001$) and percentage of the Ki-67-positive melanoma cells ($r=0.20$, $P=0.039$). This suggests a complex interaction between ligand-activated nuclear anti-melanoma activity of the VDR, connected at least in part with inhibition of NFκB, in a context-dependent manner. Paradoxically, less differentiated amelanotic melanoma cells expressing NFκB with higher proliferative potential are a better target for the anti-melanoma activity of vitamin D than the more differentiated melanotic cells.[120] One of possible explanations for this phenomenon would be a communication between the hypoxia-inducible factor-1α (HIF-1α) and VDR and NFκB signaling pathways, since induction of melanogenesis dramatically stimulates nuclear expression of HIF-1α.[208] The mechanism of these interactions deserve further careful studies, since recent reports have shown a negative effect of melanin content on melanoma

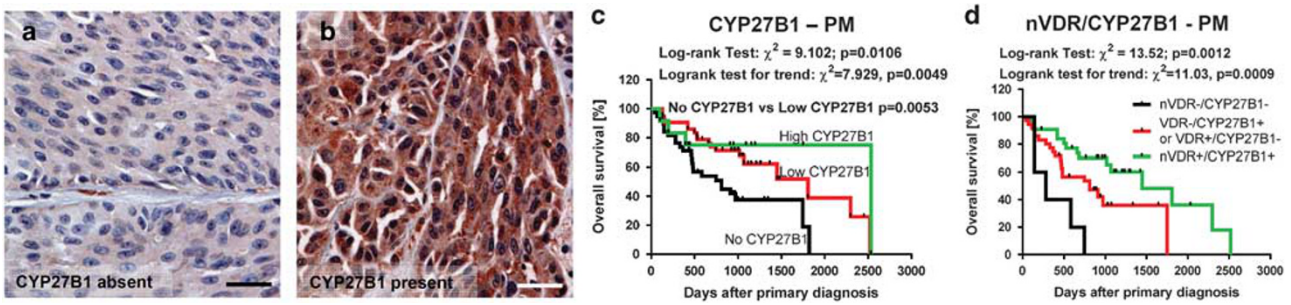

Figure 3 Correlation between CYP27B1 expression and melanoma survival. The immunostaining of melanomas classified as CYP27B1-negative (**a**; VGP) and CYP27B1- positive (**b**; RGP) are shown. Dependence of overall survival on immunostaining of CYP27B1 (**c**) and concomitant nuclear VDR immunostaining (nVDR) and CYP27B1 (**d**) in RGP of primary melanomas are also shown. The graph **c** (slightly modified) is reproduced with permission from ref. 223. CYP27B1 was detected with 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). CYP27B1 immunostaining was scored semiquantitatively, as follows: SQ = mean (IR × SI)/100, where IR represents the percentage of immunoreactive cells and SI is the staining intensity as negative (0), weak (1), moderate (2), or strong (3) and melanoma cases were stratified according to SQ-score as follows: no CYP27B1 = 0.0–0.99, low CYP27B1 = 1.0–1.99, high CYP27B1 = 2.0–3.0. Overall survival was calculated as time between surgical treatment and diagnosis of primary melanoma and the time of death. Survival analysis was performed using Mantel–Cox (Log-rank) test and Log-rank test for trend. nVDR immunostaining assessment was presented above and melanomas showing low or high nuclear VDR immunostaining were classified as 'nVDR+' and melanomas showing lack of VDR as 'nVDR-'. Melanomas showing low or high CYP27B1 immunostaining were classified as 'CYP27B1+' and melanomas showing lack of CYP27B1 as 'CYP27B1-'. Scale bars, 50 μm. PM, primary melanomas; RGP, radial growth phase; VDR, vitamin D receptor; VGP, vertical growth phase.

outcome in stages III and IV disease,[209] or the outcome of radiotherapy.[210] This is consistent with a double-edge sword role for melanogenesis in the behavior of melanoma cells.[211–220]

Negative Correlation between CYP27B1 Expression and Melanoma Progression

Besides kidney, CYP27B1 is also expressed by skin cells,[11,12,221,222] including melanoma cells.[222] In human cutaneous melanomas a significant reduction of CYP2B1 expression *vs* normal skin was observed.[223] The expression pattern in a variety of clinical samples of cutaneous melanomas was again similar to VDR, with the lowest CYP27B1 level being observed in more aggressive and more advanced melanomas (vertical growing melanomas, Clark levels III–V and Breslow thickness >2.0 mm and metastasizing melanomas; Figure 3a and b). Melanoma cells localized in deeper layers of skin (reticular dermis) were characterized by lower CYP27B1 expression when compared with papillary dermis. A high proliferation index and ulceration of melanomas were accompanied by a decreased CYP27B1 level. Consequently, a lack of or reduced expression of CYP27B1 in melanoma cells was associated with both shorter overall and disease-free survival of melanoma patients (Figure 3c).[223] This effect was even more evident when analysis was performed for OS of patients that were both negative for nuclear VDR and negative for CYP27B1 expression (Figure 3d). The reduction of *CYP27B1* expression in melanoma cells was also seen in a series (8 out of 11) of melanoma cell lines *vs* both normal melanocytes and keratinocytes.[223] In addition, similar to the VDR, both cultured melanoma cells and clinical samples of melanoma, showed an inverse correlation between the CYP27B1 level and high melanin content.[223]

What is the Role of CYP24A1 in the Regulation of Melanoma Behavior?

The best known physiological function of CYP24A1 is inactivating $1,25(OH)_2D3$ and maintaining vitamin D homeostasis via a negative feedback loop.[61,119,224] Since elevated levels of CYP24A1 have been observed in some cancers,[225] it has been proposed that inhibition of CYP24A1 activity represents a realistic molecular target for cancer therapy (reviewed in refs 69,127,226). However, this theory may not fully apply towards melanoma treatment. Specifically, CYP24A1 hydroxylates 20(OH)D3), producing several dihydroxy-derivatives including 20,24-dihydroxyvitamin D3 (20,24$(OH)_2D3$) and 20,25-dihydroxyvitamin D3 ($20,25(OH)_2D3$) which show enhanced anti-melanoma activity *in vitro*.[119] In addition, our studies on CYP24A1 expression in human cutaneous melanomas, showed the highest CYP24A1 levels in melanocytic nevi and early stage melanomas.[227] CYP24A1 expression decreased with the melanoma progression as defined by Breslow thickness, Clark levels, pT, pN, pM, and overall stage (Figure 4a and b).[227] Decreased CYP24A1 expression was also associated with poor prognostic factors including nodular type, high mitotic index, presence of ulceration, and necrosis.[227] Finally, reduced CYP24A1 expression was related with shorter overall and disease-free survival of melanoma patients (Figure 4c).[227] Again, melanoma patients that were positive for both CYP24A1 and nuclear VDR had significantly the best probability of survival (Figure 4d). Reduced CYP24A1 gene expression was also found in 12 out of 13 melanoma cell lines in comparison to normal melanocytes.[227] It is possible that these unexpected

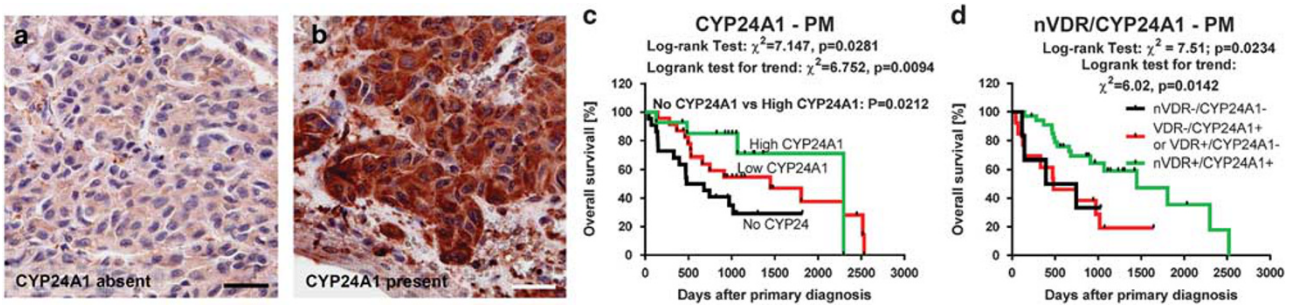

Figure 4 The relationship between CYP24A1 expression, VDR and melanoma survival. The immunostaining of melanomas classified as CYP24A1-negative (**a**; VGP) and CYP24A1-positive (**b**; RGP) are shown. Dependence of overall survival on immunostaining of CYP24A1 (**c**) and concomitant nuclear VDR immunostaining (nVDR) and CYP24A1 (**d**) in RGP of primary melanomas are also shown. CYP2A1 was detected with mouse antibody (Abcam, Cambridge, UK, dilution 1:40) and visualized with ImmPACT NovaRED substrates (Vector Laboratories, Burlingame, CA, USA). CYP24A1 immunostaining was scored semiquantitatively, as follows: SQ = mean (IR × SI)/100, where IR represents the percentage of immunoreactive cells and SI is the staining intensity as negative (0), weak (1), moderate (2) or strong (3) and melanoma patients were stratified according to the SQ-score as follows: SQ 0.0–1.0 = no CYP24A1, SQ 1.1–2.0 = low CYP24A1, SQ 2.1–3.0 = high CYP24A1. Overall survival was calculated as the time between surgical treatment and diagnosis of primary melanoma and the time of death. Survival analysis was performed using Mantel–Cox (Log-rank) test and Log-rank test for trend. nVDR immunostaining assessment was presented above and melanomas showing low or high nuclear VDR immunostaining were classified as 'nVDR+' and melanomas showing lack of VDR as 'nVDR-'. Melanomas showing low or high CYP24A1 immunostaining were classified as 'CYP24A1+' and melanomas showing lack of CYP24A1as 'CYP24A1-'. Scale bars, 50 μm. RGP, radial growth phase; VDR, vitamin D receptor; VGP, vertical growth phase.

findings can be explained by the recently described CYP24A1 ability to generate new potent vitamin D hydroxy derivatives with anti-melanoma activity.[119] In addition, expression of CYP24A1 had, in contrast to VDR and CYP27B1, a positive correlation with melanin pigmentation both in melanoma samples and cultured melanoma cells.[227] Therefore, we propose that the role of CYP24A1 in progression of melanocytic tumors may be complex, because it is involved not only in the inactivation of $1,25(OH)_2D3$, but also in the metabolism of 20(OH)D3 to its more active forms that are prone to 1α-hydroxylation.

Special Considerations on the Role of Vitamin D Signaling in Melanomagenesis and Melanoma Progression

Both the information from the literature and our own data summarized above clearly show that the attenuation of vitamin D signaling at the local and systemic levels affects melanoma progression and the natural history of the disease including overall survival. Specifically, serum levels of 25(OH) D3, SNPs in *VDR* and *VDBP* will indicate global defects affecting both systemic and local responses aimed at cancer prevention or inhibition. In addition, loss of VDR, CYP27B1 and CYP24A1 antigens is associated with negative pathological prognostic factors and with shorter OS and DFS. Re-analysis of the data reported in refs 201,202,223,227 also showed positive correlation between nuclear expression of VDR and expression of CYP24A1 ($r = 0.32$, $P = 0.01$), but not with CYP27B1, and positive correlation between CYP27B1 and CYP24A1 expression ($r = 0.22$, $P = 0.045$). This is probably related to an upregulation of CYP24A1 by CYP27B1-produced $1,25(OH)_2D3$ acting on the VDR. With respect to CYP27B1 and CYP24A1 SNPs, adequate information on their relationship with melanomagenesis is missing; however, it is possible as described for other tumors.[228–230] Unfortunately, to the best of our knowledge there is a lack of information on the association between SNPs of *CYP11A1* and melanoma, and with cancer in general. Also, there is a lack of information on alternative splicing of *VDR*, *CYP27B1*, *CYP24A1*, and *CYP11A1* gene transcripts and the role the resulting proteins may have in melanomagenesis. It is worthy of mention that in another signaling system (corticotropin releasing hormone receptors that regulate melanogenesis and have anti-melanoma activity[231–233]), alternative splicing can produce a variety of protein products with different and sometimes opposite functions.[234–236] In additionally, findings that vitamin D signaling and its antitumor activity can be affected by melanin content are intriguing, but consistent with the hypothesis that inhibition of melanin synthesis could sensitize melanoma cells to antitumor treatment and improve survival.[175,210,211,213]

VITAMIN D AND EXPERIMENTAL MODELS OF MELANOMA

Anti-melanoma Activity of Classical Forms of Vitamin D

In 1974, Oikawa and Nakayasu described for the first time the effects of cholecalciferol and ergocalciferol on melanoma, reporting induction of melanoma pigmentation in culture,[237] an effect that could not be reproduced by other investigators (reviewed in ref. 213). However, it was Colston and colleagues, who first observed the inhibition of melanoma cell growth by $1,25(OH)_2D3$ and the presence of the VDR in cultured melanoma cells, as well as in tumor tissue.[111] These two findings, confirmed by other groups, opened the door for the testing of various natural and synthetic secosteroids as potential candidates for melanoma treatment (see refs 197,238 for recent reviews). Two years after Colston's

discovery, Frampton and collaborators, showed that two known metabolites of 1,25(OH)$_2$D3, namely: 1,24,25(OH)$_3$D3 and 1,25,26(OH)$_3$D3 also effectively suppressed the proliferation of malignant melanoma MM96 cells.[239] This observation was supported by the description of vitamin D metabolism in melanoma cells.[240,241] Further studies showed that vitamin D inhibited the growth of several melanoma cell lines, including: human: A375,[242] ME18,[243] MeWo,[244–246] RPMI 7951,[247,248] SK Mel 28,[247,249,250] SKMEL-188, WM35 and WM1341;[39,40,64,118,119,242,251] mouse B16;[67,252,253] and hamster Bomirski melanomas.[67,118] Furthermore, 1,25(OH)$_2$D3 inhibited anchorage-independent growth and plating efficiency of human SKMEL-188, hamster AbC1,[39,64,118] and murine B16 (ref. 253) melanomas. Interestingly, Reichrath's group and many others noticed that some melanoma lines did not respond to classic vitamin D analogs.[246,250] Vitamin D-resistant melanomas in some experimental conditions included human Mel-Juso,[250] SK Mel 5,[246,250,254] SK Mel 25, and IGR,[250] as well as in murine melanomas S91,[255] and B16.[256] For the mouse B16 melanoma cell line it should be noted that the diverse effects of 1,25(OH)$_2$D3 were observed by several groups listed above and this phenomenon was linked to different experimental setups and the use of different subclones of B16 (see ref. 197 for discussion).

Although some authors reported melanoma cell-type dependent pro-apoptotic activity of 1,25(OH)$_2$D3,[244] others have failed to find such a correlation[243] despite of a significant reduction in melanoma proliferation.[245,257] It has been reported that 1,25(OH)$_2$D3 induces apoptosis of WM1341 melanoma, but not MeWo cells.[244] Therefore, it is possible that the pro-apoptotic activity of 1,25(OH)$_2$D3 depends on cell-type-specific factors as recently described in the gastric cancer cell line HGC-27 (see recent review ref. 69). Finally, 1,25(OH)$_2$D3 protects normal human primary melanocytes from apoptosis.[258]

There is still ongoing debate concerning the influence of vitamin D on melanin production.[213,255,259] An early study by Oikawa,[237] was supported by reports from different laboratories, that induction of pigmentation occurred through tyrosinase activation.[253,260] However, other reports concerning 1,25(OH)$_2$D3 (ref. 255) as well as a studies with the vitamin D precursor (7DHC)[237,255] and other vitamin D metabolites, including 25(OH)D3, 1(OH)D3, and 24R,25(OH)$_2$D3 (ref. 253) showed no effect on pigmentation.

The major problem in treatment of malignant melanomas is the high metastatic rate and multidrug resistance. 1,25(OH)$_2$D3 was found to inhibit invasiveness, cell adhesion to the extracellular matrix and type IV collagenase activity of B16 cells; however, it did not influence cell migration.[256] The sensitivity of melanoma to 1,25(OH)$_2$D3 seems to correlate with stimulation of expression of genes encoding enzymes that metabolize this hormone. Thus, in melanomas responding to the inhibitory effects of 1,25(OH)$_2$D3, rapid overexpression of the 24-hydroxylase gene (*CYP24A1*) was observed, which coincided with decreased expression of the *CYP27B1* gene.[67,246,250] Also, several studies indicated elevated expression of VDR in melanomas subjected to vitamin D treatment.[67,250,261] This effect appeared to be cell line specific.[246,249] Taken together, the responsiveness of melanoma cells to 1,25(OH)$_2$D3 strongly depends on VDR expression and its transcriptional activity as shown by Harant *et al*[262] explaining the higher sensitivity of RPMI 7951 cells[247] to 1,25(OH)$_2$D3, effects also substantiated by our studies.[39]

Anti-melanoma properties of natural metabolites of vitamin D have also been tested in animal models of melanoma. The inhibition of solid tumor growth by 1,25(OH)$_2$D3 was observed for VDR-expressing COLO 239F cells derived from a malignant melanoma, but not for the receptor-negative RPMI 7932 melanoma cell line.[263] Interestingly, while 1,25(OH)$_2$D3 did not inhibit exponential tumor growth in mice inoculated with mice B16 melanoma, both spontaneous or experimental pulmonary metastasis were inhibited by 1,25(OH)$_2$D3.[256] Also, Albert and coworkers found that 1(OH)D2 effectively decreased growth of pigmented ocular tumor in the Tyr-Tag transgenic mouse.[264]

Anti-Melanoma Activity of New Forms of Vitamin D

As mentioned in section "Novel Pathways of Vitamin D Activation," novel vitamin D hydroxy derivatives and pD and aD secosterids show antiproliferative, pro-differentiation, anticancer, anti-inflammatory and antifibrotic activities that depend on the target cell-type.[39,57,63–66,116,117,120,135,265–271] Hydroxy derivatives of vitamin D3 with a full-length (8C) side chain of which the best characterized so far are 20(OH)D3, 20(OH)D2 and 20,23(OH)$_2$D3, are nontoxic and noncalcemic in rodents at pharmacological doses (3-4 μg/kg (reviewed in ref. 53). Recent tests performed on mice showed a lack of calcemia by 20(OH)D3 at extremely high doses of 30–60 μg/kg administered to mice daily,[268,272] indicating its potential to be used therapeutically.

20(OH)D3, 20(OH)D2 and 20,23(OH)$_2$D3 were extensively tested *in vitro* for their activities on melanocytes and melanoma cells (reviewed in ref. 53). Specifically, they inhibited melanocyte and human and hamster melanoma proliferation, and melanoma colony formation in monolayer and soft agar (anchorage-independent growth) with a potency similar or better than 1,25(OH)$_2$D3, while having no effects on melanogenesis or dendrite formation.[39,67,118,120,271] These effects on melanoma were significantly higher than those exerted by 25(OH)D3. The *in vitro* anti-melanoma effect of 20(OH)D3 was associated with inhibition of NF$\kappa\beta$ activity in a human melanoma line.[120] Similar anti-melanoma activities were exerted by their 1α-hydroxy analogs. Interestingly, 1,25(OH)$_2$D3 and 1,20(OH)$_2$D3 inhibited dendrite formation in normal melanocytes, while derivatives without a 1α-hydroxyl group had no effect on melanocyte morphology. The addition of a 1α-hydroxyl group potentiated the antiproliferative effect against melanocytes but not melanoma cells.[118] The anti-melanoma effect of 20(OH)D3 was also demonstrated in

some clones of B16 melanoma, however, with lower potency than that for 1,25(OH)$_2$D3.[67]

Since melanogenesis can affect behavior of normal and malignant melanocytes,[159,208,211–214,273] the effect of melanogenesis on responsiveness to vitamin D analogs was tested. Induction of melanin pigmentation attenuated the responsiveness of human melanoma cells to 20(OH)D3 and 1,25(OH)$_2$D3, which was associated with a decrease in VDR expression.[120] This was consistent with lower expression of VDR in pigmented melanomas as measured by immunocytochemistry.[201,202] However, moderate melanogenic activity amplified the anti-melanoma effect of 1,25(OH)$_2$D3 and 20(OH)D2 in the F10 clone of B16 melanoma, with 20(OH)D3 having the opposite effect.[67] Interestingly, pigmentation attenuated 1,25(OH)$_2$D3-induced translocation of the VDR to the nucleus and hyperpigmentation of B16 melanoma cells was associated with a decrease in the expression of the *VDR* and *RXR* genes.[67]

We also performed tests on products of CYP27A1 and CYP24A1 action on 20(OH)D3, namely 20,24(OH)$_2$D3, 20,25(OH)$_2$D3 and 20,26(OH)$_2$D3 and their 1α-hydroxy derivatives (products of CYP27B1 action).[40,119] These secosteroids showed stronger inhibition of colony formation by human melanoma cells than the parent 20(OH)D3, with addition of a 1α-hydroxyl group having either small stimulatory or attenuating effects.[40,119] The potencies of the hydroxy derivatives of 20(OH)D3 are consistent with their docking scores predicted using the crystal structure of the ligand-binding domain of G-VDR.[53] Most recently, routes of total chemical synthesis of 20(OH)D3 and some of its dihydroxy-derivatives were established and the products show anti-melanoma activity in *in vitro* assays.[274,275]

Although the physiological significance of short side-chain Δ7-steroids and secosteroids is still not fully understood, *in vitro* experiments showed that they have antiproliferative activity in several cellular models, including human leukemias, human epidermal keratinocytes and melanoma cells,[64,67,117,238,276] and also display antifibrotic activity.[66,238] Importantly, vitamin D analogs with a shorter pD or aD side chain possess proven or predicted low calcemic activity,[277–279] confirmed further for 17,20*S*(OH)$_2$pD and 17,20*R*(OH)$_2$pD compounds.[66] The anti-melanoma activities of various vitamin D analogs with a shortened side chain (pD and aD) were recently reviewed.[53,197] pD and its precursor, 7DHP, effectively attenuated the growth of human SKMEL-188 and hamster AbC1 melanoma cell lines in soft agar.[57,280] In addition, several hydroxy derivatives of secosteroids with a short side chain were produced by UVB irradiation of the corresponding 5–7 dienes.[63,64,251,281] Two of the products, 21(OH)pD and 3β,21-dihydroxy-9β,10α-pregna-5,7-dien-20-one (21(OH)pL), showed comparable potency to that of 1,25(OH)$_2$D3 for the inhibition of growth of human SKMEL-188 melanoma cells.[64] In contrast to 1,25(OH)$_2$D3 and 20(OH)D3, the short side-chain secosteroids 20(OH)pD and pD had minimal or no effect on the proliferation of normal primary melanocytes,[238] with inhibition only seen for the melanotic but not amelanotic immortalized human melanocyte line (PIG1).[57] Nevertheless, their anti-melanoma efficacy was found to be lower in comparison to hydroxylated analogs of vitamin D$_3$ with a full-length side chain (25(OH)D3, 20(OH)D3, 20,23(OH)$_2$D3 and 1,25(OH)$_2$D3).[238] Furthermore, 20(OH)pD treatment of melanoma cells resulted in inhibition of proliferation but not cell death, while 1,25(OH)$_2$D3 showed antiproliferative and cytotoxic activities.[238] Overall, short side-chain secosteroids, such as pD, 21(OH)pD, 17α,20R(OH)$_2$pD and its lumisterol-like isomer (17α,20S (OH)$_2$pL) were shown to inhibit colony formation of SKMEL-188 melanoma cells *in vitro* with at least equal potency to that of 1,25(OH)$_2$D3.[63,64] Also, 20(OH)pD and its lumisterol-like isomer 20(OH)pL, as well as 21(OH)pD, showed anti-melanoma activities against human SKMEL--188, hamster Ab and AbC1 melanoma lines and the mouse B16-clone F10.[63,64,67] Similarly, another steroidal derivative with a 5,7-diene moiety, 17-COOH-7DA (3β-hydroxyandrosta-5,7-diene-17β-carboxylic acid), was found to be more potent than 1,25(OH)$_2$D3 in inhibiting proliferation, colony formation and DNA synthesis by human SKMEL-188, WM35, WM1341 and hamster AbC1 melanoma cell lines.[251] In addition, anti-melanoma activity of several pD and aD secosteroids, including (5*Z*,7*E*)- 9,10-secoandrosta-5,7,10(19)-trien-3β-ol were confirmed by examining anchorage-independent growth of SKMEL-188 melanoma cells.[280]

Recently, we speculated that a high concentration of reactive oxygen species (ROS) produced during melanogenesis may have an impact on melanoma biology.[211] Interestingly, the short side chain secosteroid, 21(OH)pD, similar to 1,25(OH)$_2$D3, was found to aggravate the effect of the model ROS molecule, H_2O_2, on human immortalized HaCaT keratinocytes.[276] Induction of pigmentation in the SKMEL-188 melanoma line sensitized cells towards treatment with the short side chain lumisterol derivative, 21(OH)pL, while the anti-melanoma potency of the parental compound, 21(OH)7DHP, and its vitamin D-like derivative 21(OH)pD, was not affected by pigmentation.[64] In addition, the antiproliferative activities of pD and pL compounds were affected by active melanogenesis in Ab hamster and B16-F10 mouse melanoma cells.[67]

MELANOMA MANAGEMENT: WHAT IS NEW UNDER THE SUN?

Targeting Retinoic Acid Orphan Receptors (RORs) in Melanoma Therapy

Recent studies have demonstrated that certain hydroxylated vitamin D derivatives can function as inverse agonists for both RORα and RORγ and as a consequence are able to modulate the physiological and molecular processes regulated by these receptors.[94] This includes regulation of embryonic development, several immune functions and circadian rhythm, as well as lipid and glucose homeostasis.[282,283] In

addition, RORs have been implicated in the control of several pathologies, including several (auto)immune diseases, metabolic syndrome, and cancer. The first link between loss of RORγ function and cancer was observed in RORγ-deficient mice, which develop thymic T-cell lymphomas that rapidly metastasize to other tissues.[284] Subsequent studies showed that RORα/γ expression correlates inversely with tumorigenesis and positively with cancer survival outcomes.

We recently reported that there is an inverse correlation between the level of RORα and RORγ expression and melanoma progression.[285] These studies on a series of melanoma biopsy specimens showed that the expression of RORα and RORγ decreased with melanoma progression being lowest in most advanced melanomas (Breslow thickness >2 mm, Clark level >2, pT3-4, stages III–IV, cases that developed metastases) and in melanoma metastases.[285] The presence of markers of poor prognosis (ulceration, absent TILs, nodular type, vertical growth phase) was accompanied by the lowest RORα and RORγ levels. In addition, RORα and RORγ expression was inversely correlated with high melanin content, and this result was confirmed in melanoma cells with inducible melanogenesis. Similarly to the VDR, CYP27B1 and CYP24A1, and a lack of or low level of RORα or RORγ correlated to shorter overall and diseases-free survival.[285]

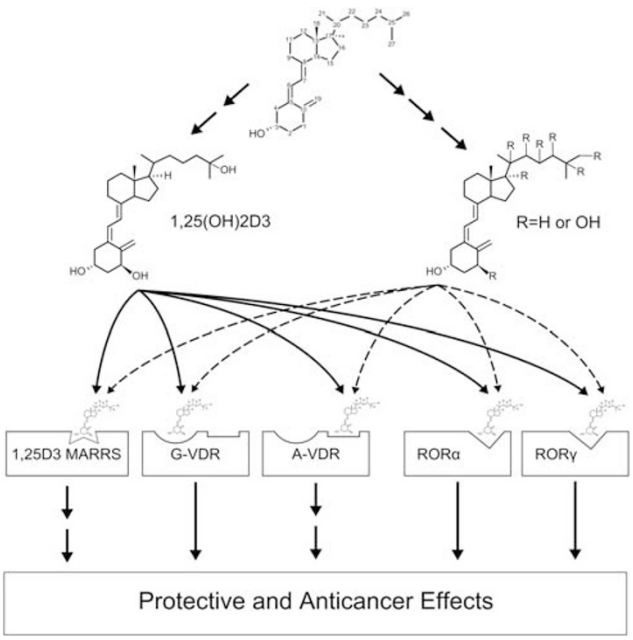

Figure 5 1,25(OH)$_2$D3 and other active forms of hydroxyvitamin D3 can exert protective and anticancerogeneic effects by interaction with the VDR and/or alternative nuclear or membrane bound receptors. In addition to the classical pathway producing 1,25(OH)$_2$D3, activation of D3 via combined action of CYP11A1, CYP27A1, CYP27A1 and CYP27B1 produce several hydroxy derivatives that can interact with the VDR, RORα, RORγ and 125D3MARRS, depending on the ligand structure. 1,25(OH)$_2$D3 can also interact with these receptors in addition to its action on the VDR. ROR, retinoic acid orphan receptor; VDR, vitamin D receptor.

The above observations are consistent with reports on the level of RORα/γ expression in several other cancers, including breast cancer, non-small cell lung carcinoma, and hepatocellular carcinoma and studies showing a positive association with prognosis.[286–290] These antitumor effects of RORs have contributed to decreased cell proliferation as well as inhibition of the EMT.[286,289,291] The inverse correlation between RORα/γ expression and tumorigenesis suggests that agonists might inhibit tumor growth and progression and provide a promising new strategy for anticancer therapy.

A recent study showed that growth of melanoma cells is considerably reduced in RORγ-deficient bone marrow chimeric mice.[292] Deficiency in RORγ leads to decreased Th17 differentiation and IL-17 levels, but increased IL-9 production.[293–295] This suppression of tumor growth in this model has been attributed to increased expression of IL-9, which has been reported to promote antitumor immunity.[296] In this case, inverse agonists/antagonists, such as hydroxylated vitamin D derivatives, might inhibit tumor growth and progression by promoting antitumor immunity and provide an alternative therapeutic strategy. However, ROR antagonists, by acting directly on tumor cells, might potentially promote tumor progression by repressing ROR activity. Inversely, ROR agonists might inhibit tumor growth, however, they may reduce antitumor immunity.

Potential use of Novel Nocalcemic Derivatives of Vitamin D

Despite anti-melanoma activities of 1,25(OH)$_2$D3 described above, a major barrier for its use at pharmacological doses is its toxicity secondary to calcemic activity.[3,9,69,71,127,297] Although there are more than 3000 chemically synthesized analogs of D3 with low-calcemic effects that target VDR, none of them have entered clinical or preclinical trials in melanoma, and none of them has entered the clinic as a general anticancer drug. A possible limitation on man-made analogs could relate to their relative toxicity *vs* the optimal antitumor serum concentrations necessary for anticancer activity, their relative resistance to metabolism and high target selectivity. In contrast, D3 of either cutaneous or nutritional source can be metabolized *in vivo* to a large number of hydroxy derivatives with multiple regulatory targets, potentially resulting in protective and anticancer effects (Figures 1 and 5).

In this context, intermediates and products of an alternative pathway of D3 metabolism initiated by CYP11A1 and producing 20(OH)D3 as the major product as well as other hydroxy derivatives (OH)$_n$D3, represent attractive alternatives for D3-based anti-melanoma therapy.[53,175,298] It should be noted that these metabolites are detectable in the human body, with 20(OH)D3 being present in human serum in the nM range.[44,52,54] The CYP11A1-derived secosteroids also display biological potency equal to or higher than that of classical 1,25(OH)$_2$D3, with antiproliferative, antitumor, and anti-inflammatory activities on melanoma

cells.[39,67,94,118,120,272] The presence of CYP11A1-derived hydroxymetabolites in the human serum suggests that they have hormonal function. Furthermore, 20(OH)D3 is noncalcemic and nontoxic at pharmacological doses (30–60 μg/kg), which are >100 times higher than toxic doses of 1,25$(OH)_2$D3 or its precursor, 25(OH)D3.[117,268,272] These novel vitamin D hydroxy derivatives could be used as an adjuvant (supplement) to already established melanoma therapies, because of their low toxicity and endogenous origin. Their local metabolism may actually increase their anti-melanoma potency,[40] with an increased spectrum of regulatory targets (Figure 5). Finally, secosteroids with a short side chain may also serve as excellent candidates for adjuvants in melanoma therapy, because they also lack calcemic activity and some of them are of endogenous origin.[63,64,66,67,238,279] One caveat on short side chain secosteroids is their immediate target (receptor) for bioregulation is unclear.[19,50,238]

Vitamin D as an Adjuvant in Melanoma Management: An Australian Clinical Trial

In view of the observed relationship between vitamin D status and Breslow thickness and outcomes in melanoma patients,[104,177,178,299] the next question is whether supplementation with vitamin D at an early stage after diagnosis is safe and improves outcomes in patients with melanoma. Randomized clinical trials to examine these questions are now underway in Belgium, Italy and Australia, though none have reported outcomes as yet. 'Vitamin D supplementation in cutaneous malignant melanoma outcome;' (VIDMe), Clinical Trials.gov Identifier NCT01748448), is a Phase 3 RCT based in Belgium. Melanoma patients, stage Ib to III, will be given oral vitamin D, 100 000 IU per month or placebo, for a maximum of 3.5 years or until relapse occurs. The primary outcome is relapse-free survival. The MelaViD trial in Italy[300] is an RCT, with resected stage II melanoma patients randomized to 100 000 IU every 50 days or so (approx. 2000 IU/day). The primary outcome is disease-free survival. The Mel-D trial, a phase II RCT conducted by the Australia and New Zealand Melanoma Trials Group,[301] involves randomization of 75 patients with stage IIb, c or stage IIIa, b melanoma within 9 weeks of wide excision of the primary, in a ratio of 2:1 to active treatment or placebo. These patients are at high risk of recurrence. Active treatment is an oral loading dose of 500 000 IU of vitamin D3 followed by an oral dose of 50 000 IU monthly for 2 years. The aim of the loading dose is to rapidly increase serum 25(OH)D concentrations as early as possible in these patients, to mimic, as far as possible, the effect of high vitamin D status at diagnosis. Primary outcomes are dose sufficiency, adherence to medication and safety, with secondary outcome of progression-free survival. To date, there have been few safety concerns, despite the large loading dose.

CONCLUDING REMARKS AND PERSPECTIVE

In summary, it is becoming unquestionable that defects in vitamin D signaling that include systemic or local defects in vitamin D activation and inactivation, and in expression and signaling through the corresponding receptors, can affect melanomagenesis, tumor progression, and outcome of the disease (Figure 1). It should also be emphasized that there is more than one form of active vitamin D besides 1,25$(OH)_2$D3) and more than one receptor target besides the VDR, which can potentially affect the behavior of melanoma cells and the outcome of the disease or its therapy (Figure 5). From an anatomic pathology point of view, changes in the expression of VDR, CYP27B1, CYP24A1, or RORs could serve as promising markers of melanoma prognosis, or as excellent reference points when considering vitamin D therapy or pharmacological targeting of the VDR or RORs in melanoma patients. With respect to prevention, testing for SNPs in *VDR* and perhaps in *RORs, CYP27B1* and *CYP24A1*, may identify subgroups of patients that are at particular risk of developing melanoma. In terms of clinical pathology, measurement of serum 25(OH)D3 and other D3 metabolites including CYP11A1-derived hydroxy derivatives, should represent standard practice since vitamin D deficiency may affect progression of the disease. While it has to be acknowledged that the use of chemically synthesized vitamin D analogs targeting the VDR has not been successful in cancer therapy to date, nutritional and perhaps parenteral application of vitamin D may represent an excellent adjuvant strategy in cancer management, including melanoma. Furthermore, the discovery of new active hydroxy derivatives of vitamin D that are noncalcemic and produced in the human body may represent a dawn for their use in cancer management.

In conclusion, while UVB wavelengths of solar radiation can serve as an etiological factor in melanomagenesis, it must be acknowledged that it is also necessary for vitamin D formation that can not only act as a protector against UVR, but also has a role in attenuating carcinogenesis and tumor progression.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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