Antiproliferative Effects of $1\alpha$-OH-vitD$_3$ in Malignant Melanoma: Potential Therapeutic implications

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Early detection and surgery represent the mainstay of treatment for superficial melanoma, but for high risk lesions (Breslow’s thickness $>0.75$ mm) an effective adjuvant therapy is lacking. Vitamin D insufficiency plays a relevant role in cancer biology. The biological effects of $1\alpha$ hydroxycholecalciferol on experimental melanoma models were investigated. 105 melanoma patients were checked for 25-hydroxycholecalciferol (circulating vitamin D) serum levels. Human derived melanoma cell lines and in vivo xenografts were used for studying $1\alpha$-hydroxycholecalciferol-mediated biological effects on cell proliferation and tumor growth. 99 out of 105 (94%) melanoma patients had insufficient 25-hydroxycholecalciferol serum levels. Interestingly among the six with vitamin D in the normal range, five had a diagnosis of in situ/microinvasive melanoma. Treatment with $1\alpha$-hydroxycholecalciferol induced antiproliferative effects on melanoma cells in vitro and in vivo, modulating the expression of cell cycle key regulatory molecules. Cell cycle arrest in G1 or G2 phase was invariably observed in vitamin D treated melanoma cells. The antiproliferative activity induced by $1\alpha$-hydroxycholecalciferol in experimental melanoma models, together with the discovery of insufficient 25-hydroxycholecalciferol serum levels in melanoma patients, provide the rationale for using vitamin D in melanoma adjuvant therapy, alone or in association with other therapeutic options.

Cutaneous melanoma represents 5–7% of all skin malignancies, but it is responsible for about 75% of deaths from skin tumors. The incidence of melanoma is increasing at an alarming rate, with a lifetime risk of developing a melanoma of 1/58 for USA males and 1/25 for Australian males$^1$. Although early detection and surgery represent the mainstay of treatment for localized lesions, no effective therapy for metastatic melanoma is available so far. Furthermore patients with high risk melanomas (Breslow thickness $>0.75$ mm) are still orphans of an effective adjuvant therapy$^{2-6}$. Vitamin D, also worded D-hormone, has pleiotropic effects of relevance to cancer, which includes regulation of cell growth and differentiation, induction of apoptosis and regulation of tumor/immune-system interactions$^{7-11}$.

Interestingly a strong association between low serum levels of 25-hydroxycholecalciferol (25-OH-vitD$_3$) and increased cancer incidence and cancer-related mortality has been demonstrated in large population studies$^{12,13}$. Several clinical studies and meta-analysis of case-control and cohort studies also support this picture$^{14-16}$. Furthermore, preclinical studies indicate that active metabolites of vitamin D or their synthetic derivatives have potential anticancer activity and could be used to potentiate the anticancer effects of several cytotoxic and antiproliferative drugs$^{17-20}$. Vitamin D-mediated-biological effects require the expression of vitamin D receptor (VDR) on the target cells and the integrity of downstream effectors, among which $1\alpha$-hydroxylase ($1\alpha$-OHase) and 25-hydroxylase (25-OHase) are necessary for the synthesis of vitamin D active metabolites, whereas 24-hydroxylase (24-OHase) regulates vitamin D inactivation and catabolism (Supplementary Figure S1). Alterations of VDR receptor and/or downstream enzymatic repertoire may potentially impair vitamin D

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responsivity of the target cells\textsuperscript{21,22}. The aim of this experimental study was to analyze the biological effects induced by 1\textalpha\textsubscript{-}hydroxycholecalciferol (1\textalpha\textsubscript{-}OH-vitD\textsubscript{3}) in malignant melanoma models in vitro and in vivo. A panel of primary melanoma cell lines derived from patients with metastatic disease were found to constitutively express functional VDR, 25-OHase and 1\textalpha\textsubscript{-}OHase, indicating a potential vitamin D sensitivity. Treatment of melanoma cells with 1\textalpha\textsubscript{-}OH-vitD\textsubscript{3} strongly impaired cell proliferation in vitro and tumor growth in vivo. Moreover low serum levels of 25-OH-vitD\textsubscript{3} (10–30 ng/mL) were discovered to occur in the large majority melanoma patients (all stages) at time of first diagnosis\textsuperscript{23}.

All together these findings open a clinical relevant question on the possibility to use vitamin D or its active metabolites in melanoma adjuvant therapy, in particular for high risk lesions, for which an observational approach is currently adopted.

**Results**

**Insufficient 25-OH-vitamin-D\textsubscript{3} Serum Levels is a Common Feature of Melanoma Patients at time of first diagnosis.** Evaluation of 25-OH-vitD\textsubscript{3} serum levels was performed on 105 melanoma patients (unselected) before surgery. Surprisingly, 99 out of 105 patients (94\%) showed deficient or insufficient 25-OH-vitD\textsubscript{3} serum levels, with values ranging from 7 to 30 ng/mL. Normal serum levels of 25-OH-vitD\textsubscript{3} (30–76 ng/mL) were detected in 6 patients only (6\% of the cases), 5 of which bearing in situ or micro-invasive melanoma and one a nodular melanoma with lymph node metastasis (Supplementary Table S1 and Fig. 1a,b). Data regarding basal serum levels of 25-OH-vitD\textsubscript{3} in 101 matched (for sex and age) blood donors without evidence of neoplastic or chronic diseases were obtained from the Sant'Andrea Hospital data bank and used as comparative control. In this cohort, 55 subjects out of 101 (54\%) had 25-OH-vitD\textsubscript{3} serum levels in the normal range. The observed difference was statistically significant (p < 0.005).

The expression of vitamin D receptor (VDR) could be a critical event for vitamin D sensitivity. As demonstrated in RT-PCR the panel of melanoma cell lines considered in this study invariably expressed VDR and the complete repertoire of transcripts that is required for vitamin D activation and catabolism, namely 25-hydroxylase (gene CYP27A1), 1\textalpha\textsubscript{-}hydroxylase (gene CYP27B1) and 24-hydroxylase (gene CYP24A1) (see supplementary Figure S2A). The expression of VDR was also confirmed in western blot and immunocytochemistry for those melanoma cells used for establishing in vitro and in vivo experimental models to work with (Supplementary Figure S2B and data not shown).

The presence of VDR polymorphism was also investigated in a panel of melanoma cell lines used for experimental procedures (Supplementary Table S2). As expected, the identified polymorphisms did not impair VDR function. In fact all melanoma cell lines used in this study showed an antiproliferative response to 1\textalpha\textsubscript{-}OH-vitD\textsubscript{3} in vitro (see below).

**1\textalpha\textsubscript{-}Hydroxycholecalciferol and Vitamin D\textsubscript{3} Active Derivative Paricalcitol Inhibit Melanoma Cell Proliferation in Vitro.** Six well-characterized melanoma cell lines were used for studying the biological effects induced by 1\textalpha\textsubscript{-}OH-vitD\textsubscript{3} on melanoma growth in vitro. Cells were cultured in the presence or absence of 1\textalpha\textsubscript{-}OH-vitD\textsubscript{3} 20 ng/mL (5 \times 10^{-8} M). Two different pharmaceutical preparations of this active 1\textalpha\textsubscript{-}OH-vitD\textsubscript{3} metabolite (Diseon \textsuperscript{®} and Dediol \textsuperscript{®}) were used for these experiments. 1\textalpha\textsubscript{-}OH-vitD\textsubscript{3} consistently impaired melanoma cell proliferation in vitro and such a biological effect was measurable after 72 hrs of treatment (Fig. 2a).
Similar experiments were performed with the synthetic active derivative of vitamin D$_2$ named paricalcitol. In this case a concentration of 0.8 μg/mL was required to consistently inhibit melanoma cell proliferation in vitro (Fig. 2b). Tumorigenic melanoma cell lines VAG, IR6 and 1007 were then selected for establishing melanoma xenografts for a new set of experiments in vivo. Cells were first cultured in the presence or absence of 1α-OH-vitD$_3$ 20 ng/mL (5 × 10$^{-8}$ M) for 9 days. Once again inhibition of melanoma cell proliferation was observed after few days of 1α-OH-vitD$_3$ treatment (Fig. 3a). To better understand whether the vitamin D-dependent inhibition of melanoma cell growth was a reversible event, a longer vitamin D treatment (18 days) was considered and a proliferation assay was finally performed after 1α-OH-vitD$_3$ withdrawal. As shown in Fig. 3b cell proliferation was recoverable up to 6 days of treatment, while for longer treatment (>9 days) melanoma cells failed to be recovered in a vitamin D–free medium. This biological effect was statistically significant (p < 0.001).

Interestingly, melanoma cells exposed to vitamin-D failed to growth efficiently in vitro, but this effect was not related to cell death. Morphological changes consisting in cytosol enlargement with appearance of small dendritic processes were invariable observed in vitamin D treated melanoma cells at the end of the experiments, together with an increased expression of E-cadherin (Fig. 4a–c). The slight differences in the mobility of E-chaderin molecular species observed in western blot analysis are likely due to cell-specific post-translational modifications and/or to the occurrence of alternative splicing (expression of different isoforms) as already reported in the literature$^{24}$. These findings support the evidence that 1α-OH-vitD$_3$ impairs melanoma cell proliferation and triggers differentiation$^7$.

To deeply investigate the anti-proliferative effects induced by 1α-OH-vitD$_3$ on melanoma cells in vitro, DNA content and cell cycle distribution of 1α-OH-vitD$_3$–treated and untreated cell lines were analysed. Cytofluorimetric analysis was then performed after treatment of the cell lines with 1α-OH-vitD$_3$ 20 ng/ml (5 × 10$^{-8}$ M) at 3, 6 and 9 days.

1α-OH-vitD$_3$ induced cell cycle arrest in all melanoma cell lines, acting at different levels. IR6 melanoma cells were blocked in G1-phase (60% vs 38% of the control cells) after 72hrs of treatment. The effect was persistent at day 9 (79% vs 54%) indicating that melanoma cells were unable to recover in the presence of 1α-OH-vitD$_3$ (Fig. 5a).

Figure 2. Antiproliferative effects of 1α-hydroxycholecalciferol and vitamin-D$_3$ synthetic derivative paricalcitol on melanoma cells in vitro. Melanoma cells proliferation as determined by colorimetric tetrazolium salt assay (MTS assay) in the presence of (a) 20 ng/mL (5 × 10$^{-6}$ M) 1α-hydroxycholecalciferol (two different pharmaceutical preparations) and (b) vitamin-D$_3$ active derivative paricalcitol at concentration ranging from 80 ng/mL to 800 ng/mL. Six metastatic melanoma cell lines were used in this experiment. The assay was performed after 72 hours of vitamin-D treatment. Untreated cells (CTR) and cells incubated with ethanol (EtOH 0.1%) were used as controls. (Experiments in triplicate. S.D. is reported on the top of each column. Statistical analysis using ANOVA and Bonferroni post-hoc test: ns = not significant; *p < 0.05; **p < 0.01; ***p < 0.001).
VAG melanoma cells were blocked in G2 phase. Such a perturbation of the cell-cycle was observed after 72 hrs of treatment (32% vs 5%) but it was much more evident on day 9 (66% vs 6%) (Fig. 5a).

In 1007 melanoma cell line, α-OH-vitD3 induced cell accumulation in the proliferative compartments (S plus G2 phase) at days 6 and 9, compared to the control cells. This was associated with a loss of cells in G1 phase (55% vs 68% at day 6 and 40% vs 72% at day 9) (Fig. 5a).

The sub-G1 peak (apoptosis peak) was not observed in these experiments. Lack of apoptosis was also demonstrated by the absence of PARP-1 and procaspase-3 cleavage in western blot analysis of cell lysates from α-OH-vitD3 treated melanoma cells (data not shown and Fig. 5b). Altogether these findings, suggest that α-OH-vitD3 Induces cell-cycle perturbation and arrest of melanoma cell proliferation in G1 or G2 phase, whereas apoptosis (or cell death for toxicity) was not observed in this experimental condition.

1α-OH-vitD3 Modulates the Expression of Cell-Cycle Regulatory Molecules. To further analyze the cell-cycle perturbation induced by α-OH-vitD3 in melanoma cells, the expression of candidate cell-cycle regulatory molecules was evaluated by western blot analysis (Fig. 5b,c). Cell-cycle arrest in G1 phase, induced by 1α-OH-vitD3 in IR6 melanoma cell line correlated with an increased expression of the cyclin-dependent kinase inhibitors p21 and p27, and down-regulation of cyclin-D1. On the other side, the arrest in G2 phase observed in VAG was associated to a slight decrease in cyclin-B1 expression level (Fig. 5b,c). In 1007 melanoma
cell line $1\alpha$-OH-vitD$_3$ induced an increased expression of cyclin-A1 with slight up-regulation of p21 and p27 according to the accumulation of melanoma cells in the proliferative compartment (S plus G2 phase) (Fig. 5b,c). These findings indicate that cell cycle perturbation induced by $1\alpha$-OH-vitD$_3$ in melanoma cells involves the

**Figure 4.** $1\alpha$-hydroxycholecalciferol–mediated effects on cell morphology and differentiation.
(a) Morphological aspects of melanoma cell differentiation observed after 18 days of vitamin-D$_3$ treatment. Melanoma cells cultured in conventional vitamin-D$_3$ free medium (CRT) and medium supplemented with solvent alone (0.1% ethanol) were used as comparative controls. Note single scattered IR6, VAG and 1007 melanoma cells with enlarged cytoplasm and small dendritic processes after 18 days of vitamin-D$_3$ treatment (scale bar = 100µm). (b) E-cadherin increased expression in vitamin-D$_3$ treated cells, as evaluated in western blot analysis by using a specific mAb; The slight differences in MW of the observed bands are likely due to the expression of cell-specific isoforms of the protein. β-actin was used as loading control (figure derived from cropped gel/blot for simplification). (C) E-cadherin expression at immunohistochemical level on vitamin-D$_3$ treated and untreated representative melanoma xenografts (post-autopsy) (indirect immunoperoxidase; scale bar = 200µm).
Figure 5. Cell cycle analysis of tumorigenic melanoma cell lines with and without vitamin-D$_3$ exposure. (a) Fluorescence-activated cell sorting (FACS) of IR6, VAG and 1007 cell lines treated for 3, 6, and 9 days with (1α-OH-vitD$_3$) or without (CTR) 1α-OH-vitD$_3$, 20 ng/ml ($5 \times 10^{-8}$ M). Cell cycle perturbation and redistribution of IR6, VAG and 1007 melanoma cells in presence of vitamin-D$_3$ is demonstrated. Row data regarding cell distribution (%) in G1, S, and G2 phases are shown in detail. (b) Western blot analysis to support FACS data, showing modulation of cell-cycle key regulatory molecules expected to be specifically involved in G1 and G2 blocks, performed on total cell lysates from IR6, VAG and 1007 melanoma cells after 72 h of vitamin-D$_3$ treatment. In the bottom of panel B the lack of procaspase-3 cleavage is shown to demonstrate the absence of apoptosis in vitamin-D$_3$ treated cells. (All the experiments were performed in triplicate; figure derived from a cropped gels/blots for simplification) (c) Densitometric analysis of cyclins (D1, A,B), p27 and p21 protein bands shown in panel B. Each band intensity was first normalized to the corresponding band of internal control (β-actin). Bands obtained from vitamin-D treated and untreated cells were compared each other, normalizing those derived from untreated cells to 1. The band intensity is represented in the graph as relative densitometric unit (rdu). Data are means ± SD of the relative band intensity from three independent WB experiments. Standard deviation is shown (Statistical analysis using t-test: ns = not significant; *p < 0.05; **p < 0.01).
recruitment of regulatory molecules and pathways, which are specific for each melanoma cell line. However, the observed vitamin D-mediated effects on cell cycle, invariably converge toward an impairment of melanoma cell proliferation.

Interestingly the observed cell cycle inhibition seems to be independent by the BRAF status (see Supplementary Table S2 also).

**Long-term Systemic Administration of 1α-OH-vitD₃ Inhibits Melanoma Cell Growth in Vivo.** In vivo experimental models of human melanoma xenografts were finally used for studying the long term effects induced by 1α-OH-vitD₃ on melanoma growth. Daily administration of 1α-OH-vitD₃ (see Material and Methods section for detail) markedly inhibited melanoma growth in vivo. The biological effect observed after 42 days from melanoma cells injection was statistically significant (Fig. 6a). The antiproliferative effect of 1α-OH-vitD₃ on melanoma xenografts was finally confirmed at autopsy. The final histological evaluation of residual explanted tumours showed large areas of tumor necrosis with dystrophic calcifications in melanoma xenografts derived from vitamin D treated mice, but not in tumors explanted from control animals (Fig. 6b). A slight hypercalcemia with 5–10% body weight loss was observed in all of 1α-OH-vitD₃ treated mice at the end of the experiment but not in the control animals (data not shown). These findings might suggest the occurrence of potentially harmful side effects of long-term administration of 1α-OH-vitD₃. However, at the end of the experiment all the animals were in good health. No histological alterations in kidney, liver, lung or other organs were observed at the final histological examination (data not shown).

Interestingly, 1α-OH-vitD₃-mediated modulation of expression of cell cycle regulatory molecules, previously observed in *vitro*, was also detected in *vivo* at immunohistochemical level (Supplementary Figure S3 and data not shown). These findings further support the notion that vitamin D active metabolites may trigger inhibitory signals in melanoma cells, which impair cell proliferation and tumor growth.

**Discussion**

Vitamin D has pleiotropic effects of relevance to cancer with potential therapeutical implications in oncology. By using experimental models of human melanoma we demonstrate that 1α-OH-vitD₃ consistently impairs melanoma cell proliferation and tumor growth in *vitro* and in *vivo*. The possibility to take advantage of the vitamin D-mediated biological effects for treating melanoma patients is further supported by epidemiological and clinical evidence: i) Large observational studies show a significant association between low serum levels of 25-OH-vitD₃ and increased cancer incidence. ii) Interestingly and in line with our work hypothesis, patients with early stage non small cell lung carcinomas with normal 25-OH-vitD₃ serum levels and high vitamin D intake, had improved overall and recurrence-free survival. Preclinical data also indicate that active metabolites of vitamin D play as potential anticancer agents in different tumor models. We demonstrate here that malignant melanoma represents a potentially responsive target to vitamin D. Although mutational analysis of VDR gene, CYP27A1, CYP27B1 and CYP24A1 genes may be required before to consider a vitamin D treatment, relevant mutational events, which impair melanoma cells sensitivity to D-hormone, seem to be sporadic. The scenario of vitamin D-mediated biological effects is very complex to be analyzed in detail. Vitamin D active metabolites regulate important biological processes via genomic and non-genomic effects. Non genomic effects are in part mediated by the increase of free cytosolic calcium levels, whereas genomic effects require the binding to VDR, the predominant nuclear receptor protein expressed on target cells and tissues. VDR binds vitamin-D ligand with high affinity, resulting in heterodimerization with retinoid X receptor (RXR) and in zinc-finger-mediated binding to vitamin D responsive elements (VDREs), which modulate transcription of the target genes. As a general feature all the patients-derived melanoma cell lines we tested were sensitive to 1α-OH-vitD₃ although the occurrence of vitamin D resistant melanoma cell lines has been reported. Vitamin D mediated-arrest of cell proliferation and modulation of the expression repertoire of p21, p27, cyclin-D1 and likely of other cell-cycle key regulatory molecules, seem to be common biological events in melanoma experimental models used in this study. Interestingly this scenario has been observed also in different cell systems. It is noteworthy that the promoter regions of specific cell cycle regulatory genes possess VDREs, which modulate gene transcription in response to vitamin-D/VDR binding. This mechanism, which demonstrates a direct functional role of vitamin D in regulating the cell cycle, corroborates our findings.

By using *in vitro* experimental models we show that 1α-OH-vitD₃ induces cell cycle arrest of IR6 melanoma cells in G1 phase, whereas VAG melanoma cells were blocked in G2 phase and 1007 melanoma cells accumulate in the proliferative compartment (S plus G2 phases). Such a cell-type specific perturbation of cell cycle was recoverable up to 6 days of vitD₃ treatment, after that time melanoma cells proliferation was definitively impaired. Early VitD₃ removal by the culture medium, in fact, restored melanoma cell proliferation. This effect was quite evident in all melanoma cell lines considered in this study, with differences related to the cell-proliferation rates and/or cell-type specific biological features. A detailed analysis of modulation of cell cycle regulatory molecules in these experimental conditions represents an interesting field of research to be pursued in the near future.

Furthermore we finally demonstrate that a relatively long systemic treatment with 1α-OH-vitD₃ also inhibits melanoma growth *in vivo*.

The differences in 1α-OH-vitD₃ mediated-biological effects observed in melanoma cell lines may be explained, at least in part, by the presence of specific molecular alterations occurring in each specific tumor (i.e BRAF and KIT mutations, p53 status etc.). In each specific case, in fact, 1α-OH-vitD₃ could activate different pathways of cell response with different functional consequences on cell-cycle. Interestingly, the melanoma cell lines considered in this study included tumors bearing BRAF^V600E mutation (IR6 and FOR) and BRAF wild-type (VAG, 1007, MUL, LOJ), but the observed 1α-OH-vitD₃ mediated-biological effects on cell proliferation and tumor growth occurred independently by the BRAF status. The potential functional role of vitamin-D in triggering tumor cell
differentiation and/or senescence also deserves consideration. It is well known that normal melanocytes have a dendritic shape and that a E-chaderin plays a pivotal role in melanocyte-keratinocyte interaction in normal conditions. After neoplastic transformation this phenotype is generally lost. In the present study we observed that 1α-OH-vitD₃ induced morphological changes (dendritic processes) and E-chaderin expression in melanoma cells, suggesting a pro-differentiative activity. This interesting effect has been already observed in a different cell system. Further experiments will be carried out to deeply investigate this important issue.

Finally, although the value of normal serum level of 25-OH-vitD₃ has been recently revisited, the effective vitamin D intake for tumor prevention and therapy are not defined yet. The discovery of
insufficient 25-OH-vitD₃ serum levels (10–30 ng/mL) in almost all melanoma patients analyzed in this study, opens the question on the effects that restored serum levels of D-hormone may potentially have on the overall and recurrence-free survival and eventually on melanoma prevention. Although a direct correlation between 25-OH-vitD₃ serum levels and clinical-pathological prognostic parameters could not be found in our cohort of patients, because of the low number of cases in each specific subgroup, it is intriguing that 5 out of 6 melanoma patients with sufficient vitamin-D serum levels at time of surgery had in situ/microinvasive melanomas. Moreover, a direct correlation between 25-OH-vitD₃ serum levels and melanoma thickness has been reported in some clinical studies that fully support our work hypothesis. In conclusion this preclinical study provides the biological and clinical rationale for using vitamin D in melanoma adjuvant therapy.

What remains to be defined is if restored 25-OH-vitD₃ serum levels (30–76 ng/mL) will be sufficient to improve the overall and disease-free survival of melanoma patients with high risk of tumor progression.

The intriguing possibility to use D-hormone in association with the most recently proposed molecular targeted therapies for treating metastatic melanoma, also deserves consideration. Anti-BRAF and anti-KIT inhibitors, in fact, finally produce functional impairment of downstream molecules that regulate cell proliferation (cell cycle) and survival, some of which are direct targets of vitamin D. In this context the demonstrated vitamin D-mediated antiproliferative effects may be oncologically relevant.

Concluding, the antiproliferative activity of 1α-OH-vitD₃, in experimental melanoma models in vitro and in vivo, together with the discovery of 25-OH-vitD₃ insufficiency in almost all melanoma patients at time of surgery, provide the biological and clinical rationale for using vitamin D in melanoma adjuvant therapy, alone or in association with other therapeutic options. Long-term randomized clinical trials will be necessary for demonstrating, definitively, the real impact of vitamin D on melanoma patients' survival.

**Methods**

**Melanoma Patients, Valuation of 25-OH-vitamin-D₃ Serum Levels.** 105 melanoma patients were checked for 25-OH-vitD₃ (the active measurable vitamin D metabolite) serum levels at time of diagnosis. Patients were 43 male and 62 female, age ranging from 28 to 87 years (median age 53.7), 49 of them had high risk cutaneous melanoma (Breslow's thickness >0.75 mm).

Data regarding the basal serum levels of 25-OH-vitD₃ in 101 matched (for sex and age) healthy individuals (blood donors) from the same community, were anonymously obtained from the blood bank at Saint' Andrea Hospital and used as comparative control. The clinical features of melanoma patients object of this study are summarized in Supplementary Table S1. 25-OH-vitD₃ serum levels were determined by using the fully automated chemiluminescence method Liaison 25OHD assay system (Dia-Sorin, Saluggia VC, Italy) and classified according to the recent literature. Briefly 25-OH-vitD₃ serum levels <10 ng/mL were considered 'deficient'; >10 to 30 ng/mL 'insufficient'; and >30 to 76 ng/mL (75 to 190 nmol/L) were considered 'normal'.

**Melanoma Cell lines, phenotypic and molecular characterization.** Metastatic melanoma - derived cell lines (VAG, FOR, IR6, 1007, MAR, and LOJ) were analyzed immunohistochemically as reported below and the occurrence of VDR polymorphism was also investigated. Briefly, three major restriction fragment lengths polymorphism (RFLP) were investigated, in the exon 2 and 3′ end of the VDR gene, which may influence VDR messenger RNA stability. TaqI polymorphism is a RFLP at codon 352 in exon 9 of the VDR gene, that leads to a silent codon change from ATT to ATC, which both result in an isoleucine at codon 352.

The FokI polymorphism results in the production of a VDR protein that is three amino acids longer with an increase of transcription levels. The BsmI (GTCGTCCATGGTGAAGGA-3′ → AGCAAATAGCTTCCAAGG-3′) and CYP27B1 (1α-OHase) (TGTTTGCATTTGCTCAGA-3′ → TGTTTGCATTTGCTCAGA-3′) polymorphisms were previously described. Total RNA extraction and cDNA synthesis were previously described. PCR reactions were performed in PCR buffer (10 mM Tris-HCl pH 8.4; 500 mM KC1; 50 mM MgSO4) with 0.2 μM primers and 2U Platinum Taq DNA Polymerase (Invitrogen) in a final volume of 50 μl for 35 cycles (denaturation at 95°C for 30 seconds, annealing at 48°C–52°C, according to different pair of primers, for 30 seconds and extension at 72°C for 1 miniute). The following human oligonucleotides were used as primers: Vitamin D Receptor (VDR) for 5′-CCAGTCATCCCTGGTGAATGGTG-3′, rev 5′-ATGAGCATGATGCAGGA-3′; CYP27A1 (25-OHase) for 5′-GGCAAATGCACGAGTGAAAC-3′, rev 5′-AGCAAAATCCTGGTACAG-3′; CYP27B1 (1α-OHase) for 5′-GGTTTGACTTGGCTGAGA-3′, rev:
5′-CCGGGAGAGTACACA-3′; CYP24A1 (24-OHase) for 5′-GCAGCTATTGCGAGATT-3′, rev: 5′-ATTCCACCAGACTTGGT-3′. Integrity and equal loading of cDNA in the PCR reactions were checked by quantification of 18S mRNA levels.

Mouse monoclonal antibodies (mAbs) to p21, p27, Cyclin-D1, E-cadherin, and human VDR NR1/1 (Space Import & Export, Milan, Italy); goat polyclonal antiserum to Cyclin-B1, rabbit polyclonal antiserum to Cyclin-A1 (Space Import & Export, Milan, Italy), mouse mAbs to β-actin (Santa Cruz Biotechnology, CA, USA) and rabbit polyclonal procaspase-3 antibody sc-7148 (Santa Cruz Biotechnology) were purchased and used in immunohistochemical assays according to the manufacturer’s instruction. Immunohistochemical analysis on formalin fixed and paraffin embedded histological samples, as well as western blot analysis, were previously described57–59.

Melanoma Cell Cultures and Vitamin D Treatment in Vitro. For in vitro experiments 1α-hydroxycholecalfector (1α-OH-vitD3) (Dediol® Sanofi-Aventis SpA, Milan, Italy, or Diseon® Teva Italia Srl, Milan, Italy) was added to the culture medium at final concentration of 20 ng/mL (5 × 10−8 M). Drug concentration was selected after preliminary dose-response experiments in vitro (data not shown). Briefly, 3 × 104 cells were seeded in 6-well plate ( Falcon Labware, Oxnard, CA USA) in complete RPMI 1640 culture medium, supplemented with 2% FBS. After 24 hours 1α-OH-vitD3, or solvent alone (0.1% ethanol) were added to the culture medium. The medium was replaced every 48 hours (in both treated and control cell lines) to guarantee a constant presence of fresh vitamin D. Cells were finally collected at 3, 6, 9 and 18 days of treatment (depending by the experiment) and used as targets for proliferation assays and FACS analysis as reported below. Similar experiments were performed by using the synthetic active derivative of vitamin-D3 worded paricalcitol (Zemplar® Abbott Srl, Latina, Italy) at concentration ranging from 0.08 μg/mL to 0.8 μg/mL.77 Tumorigenic melanoma cell lines (1007, IR6, VAG) were finally selected for creating melanoma xenografts in vivo as reported below.

Determination of Melanoma Cells Growth Rate In Vitro. Melanoma cells were seeded at concentration of 3 × 104 cells/well in a six-well plate and maintained in complete RPMI 2% FBS with or without 1α-OH-vitD3, as reported above. Cell proliferation was determined by cell counting in a Burker camera after 3, 6, 9 and 18 days of vitamin D treatment, depending by the experiment.

To achieve melanoma cell cultures for 18 days, melanoma cells growing at confluence were split at appropriate time (according to the doubling time of each cell line: TdIR6 = 36 hrs, TdVAG = 48 hrs and Td1007 = 72 hrs) by using a PBS solution containing trypsin 0.05% and EDTA 0.02% (Gibco). All the experiments were performed in triplicate. In a different set of experiments the MTS assay (colorimetric tetrazolium salt assay) was also used for evaluating cell growth rate in vitro. Cells were cultured at density of 5 × 104 cells/well in flat-bottom 96-well plates. Vitamin-D treatment in vitro was performed with 1α-OH-vitD3 at final concentration of 20 ng/mL (5 × 10−8 M) or paricalcitol at concentration ranging from 0.08 μg/mL to 0.8 μg/mL. After 3 days of treatment CellTiter 96® Aqueous One Solution Reagent (Promega, Madison, WI, USA) was added to each well according to the manufacturer’s instructions. Cell viability was determined by measuring the absorbance at 490 nm using a 550 BioRad plate-reader (Bio-Rad, Hertfordshire, UK).

Cell cycle profile analysis by flow cytometry (FACS analysis). Melanoma cells adjusted to reach 60–70% confluence at the time of FACS analysis were collected after 3, 6, and 9 days of 1α-OH-vitD3 treatment, washed in phosphate buffered saline solution (PBS) pH 7.4 and fixed in 50% acetone/methanol (1:4 v/v) in PBS for 1 hour. Cells were re-suspended in a DNA staining solution containing propidium iodide (10 mg/ml concentration of 3 μg/mL) and RNAse (1.8 units/μl) for 30 minutes and finally analyzed on a FACScan (Becton Dickinson, Immunocytometry System, Mountain View, CA, USA). Twenty thousand events per sample were registered. All the experiments were performed in triplicate.

Melanoma Xenografts and 1α-OH-vitD3 Treatment in Vivo. 36 pathogen-free Balb-c nude (nu/nu) mice, 4–5-weeks-old (Charles River Breeding Laboratories, USA) and tumorigenic human melanoma cells lines 1007, IR6 e VAG were used for establishing melanoma xenografts in vivo47,48. 36 mice were organized in 9 groups in cages of 4 animals each (12 mice for each melanoma cell line) and fed with standard diet and water ad libitum. One week after subcutaneous injection of melanoma cells (5 × 106 cells/mouse) mice were treated every other day, for a total of six weeks, with 1α-hydroxycholecalciferol via gavage, according to the following protocol: Groups 1 control mice: saline solution only (200 μl/mouse); Groups 2: 1α-OH-vitD3, 4 I.U. (0.1 μg/200 μl volume/mouse); Groups 3: 1α-OH-vitD3, 12 I.U. (0.3 μg/200 μl volume/mouse). Mice in groups 2 received a total of 80 I.U. of 1α-OH-vitD3 in 42 days of treatment, whereas mice in groups 3 got 240 I.U. Tumor size was measured every 3 days and tumor weight (gm) was calculated as previously described47. Animal health conditions were monitored daily all along the experiment.

Calcium serum level was measured in each animal at the beginning and at the end of the experiment. Mice were sacrificed after 6 weeks of treatment. Explanted organs and residual tumors were processed for conventional histology and immunohistochemistry.

Statistical Analysis. In vitro experiments have been repeated 3 times and the results obtained are presented as means ± standard deviation (SD). Significant changes were assessed by using Student’s t-test for unpaired data, two-way ANOVA and Bonferroni post-hoc tests for time and concentration effects (GraphPad Prism Software, version 5.0) a p values < 0.05 were considered significant.

Ethics statement. Melanoma cell lines used in this study were established at the Immunology Laboratory, National Cancer Institute Regina Elena of Rome, according to the Institutional ethical guidelines provided by the Italian Ministry of Public Health (IMPH). In vivo animal experiments were performed at the Animal Facility of the National Cancer Institute Regina Elena of Rome, under the direct control of an independent veterinary staff.
Animal experiments were performed according to the guidelines provided by the IMPH. The study was approved by the Institutional Animal Care and Use Committee and by the multidisciplinary Board of Physicians of the Saint’ Andrea Melanoma Working Group (SAMWG).

Blood samples and tissues from humans were collected at Saint Andrea University Hospital and used anonymously in full agreement with the guidelines provided by the Institutional Review Board (Prot. CE no. 8391/2013) and Helsinki Declaration. Dermatologists from SAMWG, which have the responsibility for patients’ follow-up, collected a written informed consent from all the melanoma patients considered in this study.

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A.B.: Study concept, study coordinator and supervisor of the experimental activity. M.C., L.F., A.N.: Acquisition of clinical data and melanoma patient management. A.B., L.F., A.U., E.L.: in vitro experiments, molecular biology, cell culture, data analysis. B.B.: Cell cycle and FACS analysis, interpretation of the results. A.B., M.G.: Histopathology and immunohistochemistry. D.P.: vitamin D expert, support for in vitro and in vivo experiments. A.B.: Drafting of the manuscript. S.S., M.C., A.B.: Critical revision of the manuscript for intellectual content. L.S., M.C., L.F., A.U., A.N., M.G., E.L., L.L., B.B., D.P., S.S. and A.B.: Analysis and interpretation of the results. Final approval of the manuscript.

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