Abstract. Background: Melanoma cells express the nuclear vitamin D receptor (VDR), indicating that malignant melanoma represents a promising target for treatment with 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) or its analogs. We previously showed that some melanoma cell lines are resistant to the antiproliferative effects of 1,25(OH)2D3 and that 1,25(OH)2D3-sensitivity can, at least in part, be restored by co-treatment with the histone deacetylase inhibitor (HDACI) Trichostatin A (TSA) or with the DNA methyltransferase inhibitor (DNMTI), 5-azacytidine (5-Aza). This study aimed at gaining further insights into the molecular mechanisms that underlie the epigenetic modulation of 1,25(OH)2D3-sensitivity in melanoma cells. Materials and Methods: The expression of VDR mRNA, protein and two candidates of VDR microRNAs (miR-125b, miR-27b) were compared in 1,25(OH)2D3-responsive (MeWo, SK-Mel28) and -resistant (SK-Mel5, IGR) melanoma cell lines and in normal human melanocytes (NHM) using real time PCR and western blot analysis. Additionally, the effect of 1,25(OH)2D3, epigenetic modulating drugs (TSA, 5-Aza) and miR-125b antisense on the expression of VDR messenger RNA (mRNA)/protein, miR-125b and miR-27b was investigated. Results: Treatment with 1,25(OH)2D3 and/or epigenetic drugs (5-Aza, TSA) reduced the expression of miR-125b in the 1,25(OH)2D3-responsive and -resistant melanoma cell lines and in the NHM. Treatment with 1,25(OH)2D3 and/or epigenetic drugs (5-Aza, TSA) reduced the miR-27b expression in three out of four melanoma cell lines. Moreover, no difference was observed in VDR protein expression in the 1,25(OH)2D3-responsive as compared to the 1,25(OH)2D3-resistant melanoma cell lines. Transfection with miR-125b antisense did not affect the VDR mRNA/protein expression in the IGR cells. Conclusion: Responsiveness to 1,25(OH)2D3 corresponds to the expression level of VDR mRNA which in turn might be regulated by VDR microRNAs or epigenetic modulating drugs.

At present, there are no effective treatments for metastasized malignant melanoma available that can significantly reduce its mortality rates, underlining the need for the development of new therapeutic approaches for such an aggressive form of cancer (1). It is well established that the biologically active vitamin D metabolite 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) acts as a potent regulator of cellular processes such as normal cell growth, differentiation, and apoptosis of a wide variety of cancer cells including melanoma cells (2). Several molecular studies reported 1,25(OH)2D3-insensitivity in melanoma, breast, or prostate cancer cells (3;4). Until today, the molecular mechanisms behind this resistance are unclear. 1,25(OH)2D3 exerts its effects at least in part by binding with high affinity to a member of the nuclear receptor superfamily (the vitamin D receptor (VDR)).

Recently, epigenetic events have attracted more and more attention due to their involvement in cancer development and the potential of therapeutic intervention (5, 6). In our previous study, 1,25(OH)2D3-insensitivity in melanoma cell lines was overcome using epigenetic modulating drugs (3, 5). Additive effects of 30-40% growth inhibition after treatment with 1,25(OH)2D3 in combination with histone deacetylase inhibitor (HDACI), trichostatin (TSA), or DNA methyltransferase
inhibitor (DNMT1), 5-azacytidine (5-Aza) were also observed. Additionally, we previously showed a correlation between the VDR messenger RNA (mRNA) expression level and 1,25(OH)2D3-responsiveness.

In the present study, 1,25(OH)2D3-responsiveness analyzed the correlation of VDR protein level and in addition, the effect of 1,25(OH)2D3 alone and/or in combination with TSA or 5-Aza on the expression level of VDR mRNA was investigated. In the last few years, increasing evidence has been found that a large number of microRNA (miR) genes are subjected to epigenetic alterations, resulting in aberrant expression patterns upon the incidence of cancer (7). For this reason, the impact of treatment with 1,25(OH)2D3 alone and/or in combination with TSA or 5-Aza on the expression level of two VDR miR candidates (miR-125b and miR-27b) in 1,25(OH)2D3-responsive cells (MeWo and SK-Mel28) and 1,25(OH)2D3-resistant cells (SK-Mel5 and IGR) was analyzed. It was previously shown by another group that miR-27b interacts with VDR 3'UTR, identifying this miRNA as a potential VDR mRNA (8). miR-125b posttranscriptionally negatively regulates human VDR in breast cancer cells (9). In order to modulate VDR expression miR-125b antisense and the effect on transcriptional and translational VDR regulation in melanoma cell lines, was investigated.

### Materials and Methods

**Cell culture.** The human melanoma cell lines MeWo, SK-Mel-28, SK-Mel-5 and IGR (Department of Dermatology, Saarland University Hospital) were cultivated in RPMI media supplemented with 10% foetal calf serum (FCS) and 1% L-glutamine. Primary normal human melanocytes (NHM) were isolated from human foreskin and cultivated in serum free melanocyte growth medium M2 (Ready-to-use, PromoCell®, Heidelberg, Germany), after the addition of Melanocyte Growth Medium M2 supplement mix (C-39420) (PromoCell®). 1,25(OH)2D3 and TSA (Sigma-Aldrich, Taufkirchen, Germany) were dissolved in ethanol as stock solutions (4 mM and 1 mg/ml, respectively) and stored in the dark at –20°C, 5-Aza (Sigma-Aldrich) was dissolved in PBS (10 mM). Media containing varying concentrations of 1,25(OH)2D3, TSA or 5-Aza and their combinations were added to cultured cells 24 h after seeding the cells (to ensure semi confluent density) in 10 cm tissue culture dishes (Greiner Bio-One GmbH, Frickenhausen, Germany).

**Apoptosis detection.** Detection of apoptosis by FACS is based on the observation that soon after initiating apoptosis, most cell types translocate the phospholipid phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface. Once on the cell surface, PS can be easily detected by staining with a fluorescent conjugate of annexin V, a protein which has a strong natural affinity for PS.

**mRNA and miRNA isolation.** RNA isolation was performed using a RNeasy Kit (Qiagen) according to the manufacturer’s manual. The miRs were isolated using a miRNeasy Kit (Qiagen). The expression of VDR mRNA, miR-27b, and miR-125b were analyzed in the melanoma cell lines using real time PCR (LightCycler, Roche Diagnostic, Mannheim, Germany), 50 cycles.

**Real time-PCR.** RT reaction mixture was used as the template for real-time PCR, containing 2 μl of 10x VDR primers or 10x hypoxanthine guanine phosphoribosyl transferase (HPRT) primer (house keeping gene) purchased from Qiagen, (Catalogue number QT010116QT00059060). A final concentration of 1 mM MgCl2 in the miR-125b or miR-27b RT reaction mixture was used as the template for real-time PCR, containing 2 μl of 10x miR-125b primer or 10x miR-27b primer and 10x RNU6B (RNU6B is a widely used endogenous reference RNA in many mRNA quantification studies because of its small size 45 nt), purchased from Qiagen (miScript Primer Assay and miScript universal primer).

**miR-125b antisense.** The IGR melanoma cells were seeded (to ensure semi confluent density) in 6 well plate in 2 ml RPMI media supplemented with 10%, 1% L-glutamine and 1% penicillin/streptomycin. The medium was changed after 24 h and transfection of the cells with miScript miRNA-125b inhibitor, miScript inhibitor (negative control) or HiPerFect transfection reagent (mock control), (Qiagen) was carried out according to the manufacturer’s manuals. The miRNAs were isolated after 24 h from the media control, mock control, negative control, and miR-125b antisense wells.

**Western blotting.** The nuclear proteins were extracted using a NE-PER® Nuclear and Cytoplasmic Extraction Reagents Kit (ThermoFisher Scientific) and Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s manuals. Briefly the cell membrane was disrupted and the cytoplasmic contents were released then after recovering the intact nuclei from the cytoplasmic extract by centrifugation, the nuclei were lysed to yield the nuclear extract. The protein concentration was determined using a Bradford (Bio-Rad, Protein Assay, Bio-Rad Laboratories GmbH, Muenchen) protein assay and finally 40 μg of nuclear protein from each sample were used. Based on the finding that VDR protein level was relatively high in MCF7 breast cancer cell lines (10), we used this cell line as VDR protein positive control. After electrophoresis in 12.5% SDS-polyacrylamide gels, separated proteins were electrophototically transferred to a PVDF membrane. Non-specific sites were blocked by 5% non-fat dry milk in PBS for 1 h at room temperature. The membrane was incubated with the primary antibody (VDR polyclonal (C-20): sc-1008 or VDR monoclonal (D-6): sc-13133, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C or with the primary antibody nucleolin (11) as a loading control for 1 h at room temperature in PBS containing 1% non-fat dry milk, washed three times with PBS for 10 min and incubated 1 h with the respective secondary antibody in PBS containing 1% non-fat dry milk. After using FACscan flow cytometry. Apoptosis detection by FACS is based on the observation that soon after initiating apoptosis, most cell types translocate the phospholipid phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface. Once at the cell surface, PS can be easily detected by staining with a fluorescent conjugate of annexin V, a protein which has a strong natural affinity for PS.
washing, bands were visualized by chemiluminescence. The intensity of VDR band was analyzed using Bio-Rad Quantity One® 1-D analysis software in relation to the loading control gene band (nucleolin) in three independent experiments.

Results

Basal expression of VDR mRNA, miR-125b and miR-27b.

Real time RT-PCR analysis demonstrated an inversely correlated expression pattern of VDR mRNA and miR-125b in the 1,25(OH)2D3-responsive (MeWo, SK-Mel28) as compared to the 1,25(OH)2D3-resistant (IGR, SK-Mel5) melanoma cells as reported previously (3) (Figure 1). The expression level of miR-27b was relatively low in the MeWo (1,25(OH)2D3-responsive) cells as compared to the IGR (1,25(OH)2D3-resistant) cells, while the SK-Mel28 (1,25(OH)2D3-responsive) cells and SK-Mel5 (1,25(OH)2D3-resistant) cells showed similar expression levels (Figure 1).

In the NHM VDR mRNA expression level was comparable to that of the 1,25(OH)2D3-responsive melanoma cell lines (MeWo, SK-Mel28), while the VDR mRNA expression level in the 1,25(OH)2D3-resistant melanoma cell lines (SK-Mel5, IGR) was much lower.

Interestingly, the miR-125b in particular and also the miR-27b expression levels were higher both in the NHM and in the 1,25(OH)2D3-resistant melanoma cell lines as compared to the 1,25(OH)2D3-responsive cell lines (Figure 1). The 1,25(OH)2D3-responsive cell lines were characterized by a relatively high expression of VDR mRNA and a low-expression of miR-125 and miR-27b (in MeWo cells only for miR-27b).

Expression of VDR mRNA, miR-125b, and miR-27b after treatment with 1,25(OH)2D3, 5-Aza, and/or TSA. As 1,25(OH)2D3 and epigenetic modulating drugs such as TSA and 5-Aza are cytotoxic at high concentration, it was important to avoid toxic effects in our study. Using the apoptosis detection analysis kit the concentrations in which apoptotic and dead cells did not exceed 10-20% were determined. Concentrations of 10−8 M for 1,25(OH)2D3, 10 μM for 5-Aza, and 15 ng/ml for TSA showed no cytoxic effect on melanoma cell lines (data not shown) and were therefore chosen for the experiments. The treatment with 1,25(OH)2D3 alone or in combination with the epigenetic drugs increased the expression of VDR mRNA in the NHM (Figure 2a). Moreover, treatment with 1,25(OH)2D3 alone increased VDR mRNA in two out of four melanoma cell lines, but this effect was statistically insignificant (p>0.05). 5-Aza alone increased the VDR mRNA in the 1,25(OH)2D3-resistant melanoma cells (Figure 2a), but this effect was statistically insignificant. The increase in VDR mRNA expression was more pronounced in three out of the four melanoma cell lines after treatment with 1,25(OH)2D3 in combination with 5-Aza, while treatment with TSA alone or in combination with 1,25(OH)2D3 resulted in a reduced level of VDR mRNA in three out of the four melanoma cell lines. In the other 1,25(OH)2D3-resistant cell line (IGR), treatment with TSA alone or in combination with 1,25(OH)2D3 increased VDR mRNA level (Figure 2a). The treatment with 1,25(OH)2D3 alone decreased the miR-125b expression in 3 out of the 4 melanoma cell lines. The treatment with 5-Aza alone or in combination with 1,25(OH)2D3 also significantly decreased the miR-125b expression in all the melanoma cell lines also reduced the level in the NHM. In contrast, treatment with TSA alone or in combination with 1,25(OH)2D3 did not significantly affect the expression of VDR mRNA and did not affect the miR-125b (except in MeWo cells) (Figure 2b). This effect of TSA alone or combined with 1,25(OH)2D3 (decrease the VDR mRNA) might be, at least in part, the reason for losing the additive antiproliferative effect of these
Figure 2. a: Effect of treatment with 1,25(OH)\textsubscript{2}D\textsubscript{3} and/or epigenetic drugs (5-Aza, TSA) on VDR mRNA expression. NHM and melanoma cells were treated with 1,25(OH)\textsubscript{2}D\textsubscript{3} (10\textsuperscript{-8} M), 5-Aza (10 μM) and TSA (15 ng/ml) alone or in combination with 1,25(OH)\textsubscript{2}D\textsubscript{3}. RT-PCR of VDR mRNA levels was compared to vehicle-treated controls. After 24 h treatment, mRNA was isolated and experiments were performed in duplicate and repeated twice. Error bars represent the SD, *p≤0.05. b: Effect of treatment with 1,25(OH)\textsubscript{2}D\textsubscript{3} and/or epigenetic drugs (5-Aza, TSA) on m-R-125b expression. NHM and melanoma cells were treated with 1,25(OH)\textsubscript{2}D\textsubscript{3} (10\textsuperscript{-8} M), 5-Aza (10 μM), and TSA (15 ng/ml) alone or in combination with 1,25(OH)\textsubscript{2}D\textsubscript{3}. RT-PCR quantified miR-125b and compared to vehicle-treated control, after 24 h of treatment. Experiments were performed in duplicate and repeated at least twice. Error bars represent the SD, *p≤0.05. c: Effect of treatment with 1,25(OH)\textsubscript{2}D\textsubscript{3} and/or epigenetic drugs (5-Aza, TSA) on m-R-27b expression. Melanoma cells were treated with 1,25(OH)\textsubscript{2}D\textsubscript{3} (10\textsuperscript{-8} M), 5-Aza (10 μM), and TSA (15 ng/ml) alone or in combination with 1,25(OH)\textsubscript{2}D\textsubscript{3}. RT-PCR assessed miR-27b and compared to vehicle-treated control, after 24 h of treatment. Experiments were performed in duplicate and repeated at least twice. Error bars represent the SD, *p≤0.05.
two compounds in 1,25(OH)$_2$D$_3$-responsive cell lines that we reported previously (3).

The treatment with 1,25(OH)$_2$D$_3$ alone or in combination with 5-Aza or TSA significantly decreased the miR-27b expression in 3 out of the 4 melanoma cell lines (Figure 2c).

Effect of expression level of VDR. As shown by western blot in (Figure 3) VDR protein levels were nearly the same in the 1,25(OH)$_2$D$_3$-responsive cells and the 1,25(OH)$_2$D$_3$-resistant cell lines. Thus, there was no correlation between VDR protein level and 1,25(OH)$_2$D$_3$-sensitivity in these melanoma cell lines.

Among the malignant melanoma cell lines, the IGR cells (1,25(OH)$_2$D$_3$-resistant) had a relatively high miR-125b expression level, which was associated with low VDR mRNA expression. For this reason, the effect of miR-125b antisense was assessed in this cell line. The VDR protein band was not easily detected by western blot, therefore both VDR monoclonal and VDR polyclonal antibodies were used to confirm the data (9, 12). RT-PCR analysis detected a significant decrease in the miR-125b level after transfection (Figure 4). Downstream analysis by RT-PCR to investigate whether the VDR mRNA was affected by the miR-125b inhibition and by western blot to analyze the VDR protein level provided evidence that miR-125b does not regulate transcriptional or post-transcriptional VDR gene expression in the IGR melanoma cell line (Figure 5, 6).

Discussion

It is well known that 1,25(OH)$_2$D$_3$ plays a potential role in the prevention and treatment of hyper-proliferative malignancies (13). Additionally, the antiproliferative effect of HDAC inhibitors and 1,25(OH)$_2$D$_3$ converge on the interaction of the un-ligated VDR with co-repressors recruiting multi-protein complexes containing HDACs and on the stimulation of cyclin-dependent kinase inhibitor (CDKI) genes (14). Moreover, the HDAC inhibitor TSA induces the CDKIs p18 and p19, whereas the CDKIs p21 and p27 are stimulated by 1,25(OH)$_2$D$_3$. In agreement with these findings, we previously reported additive antiproliferative effects of 1,25(OH)$_2$D$_3$ and TSA in 1,25(OH)$_2$D$_3$-resistant cell lines (3, 13).
To date, more than 50 genes have been identified to be aberrantly hypermethylated during some phases of melanoma progression and metastasis (1, 5), and treatment with demethylating agents resulted in the re-expression of VDR transcripts in breast cancer cell lines, which in turn re-established sensitivity to calcitriol (1, 15). These data may, at least in part, explain the additive antiproliferative effect of 1,25(OH)2D3 in combination with 5-Aza that associated with increase in the VDR mRNA after treating the melanoma cells with this combination.

Additionally, the transcriptional regulation of miRs expression has recently been examined in tumors with epigenetic modification of DNA via methylation or HDAC, and the induction or inhibition of miRs could be achieved by the inhibition of DNA methylation or HDAC (5, 16, 17). Few studies have analyzed the role of microRNA in the development and progression of melanoma. 86% of melanoma cell lines have been reported to have an alteration in their genomic loci containing miRNA genes (18, 19). Specifically, miR-27b and miR-125b are two out of approximately 76 listed miRNAs that have repeatedly been shown to become deregulated with tumor development and progression in general and in melanoma in particular (8, 18, 20).

In the present study, a strong reduction of miR-125b and miR-27b levels we observed after treatment with 1,25(OH)2D3 in combination with 5-Aza, which in turn was associated with

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**Figure 5.** VDR mRNA expression level in IGR cells 24 h after transfection with miR-12b antisense Relative expression level: ratio between the target gene (VDR) and the corresponding housekeeping gene (HPRT). Experiments were performed in duplicate and repeated twice. Error bars represent the SD.

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**Figure 6.** Effect of transfection with miR-125b antisense on VDR protein expression in IGR cells. Western blot analysis using VDR polyclonal or monoclonal antibodies. Nucleolin used as loading control. Experiments were repeated three times and representative images are shown.
VDR mRNA induction. Based on these results, we hypothesize that vitamin D signalling is abrogated by an epigenetic mechanism and that hypermethylation is of importance for VDR mRNA, miR-125b and miR-27b regulation. Moreover, the VDR protein level did not correlate with 1,25(OH)₂D₃-sensitivity in these melanoma cell lines. Furthermore, transfection with miR-125b antisense did not regulate the VDR gene expression at the transcriptional or the translational level. It is well known that the use of miRNA inhibitors to investigate the effect of endogenously expressed miRNAs can be challenging, as the VDR gene is regulated by more than one miRNA (18, 19). Thus the miR-125b may not be specific enough it might be necessary to inhibit more than one miRNA (18, 19). Additionally, the NHM did not show a specific expression level in melanoma cells to see an effect on VDR protein expression.

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References


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