# Vitamin D, Calcidiol and Calcitriol Regulate Vitamin D Metabolizing Enzymes in Cervical and Ovarian Cancer Cells

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**Abstract.** *Background: The vitamin D metabolizing enzymes 25-, 1α- and 24-hydroxylase are expressed in malignant cells of the cervix and the ovaries. The aim of this study was to obtain further information about the regulation of the aforementioned enzymes by vitamin D, calcidiol and calcitriol in cervical and ovarian cancer. Materials and Methods: The human cervical adenocarcinoma cell line HeLa and the human ovarian adenocarcinoma cell line OVCAR-3 were incubated with vitamin D, calcidiol and calcitriol. The influence of vitamin D and its metabolites on the expression of 25-, 1α- and 24-hydroxylase was assessed by real-time RT-PCR. Results: Calcitriol significantly increased the 24-hydroxylase mRNA levels in HeLa and OVCAR-3 cells. The expression of 25- and 1α-hydroxylase was not regulated in a statistically significant manner. Conclusion: These results suggest that in HeLa as well as OVCAR-3 cell lines, the metabolism of vitamin D is regulated via the expression of the catabolizing 24-hydroxylase.*

Cervical and ovarian cancers are severe diseases. In 2008, approximately 529,000 new cases of cervical and 225,000 new cases of ovarian cancer were registered worldwide. At the same time, approximately 274,000 and 140,000 women died from these malignancies, respectively (1). Several studies have indicated that sunlight exposure, which leads to elevated vitamin D plasma levels, is negatively correlated with the risk and mortality of various malignancies, such as breast, colon, prostate as well as cervical and ovarian cancer (2, 3).

Vitamin $D_3$ can be obtained from the diet or from endogenous synthesis due to ultraviolet radiation. The secosteroid hormone 1,25-dihydroxyvitamin D (calcitriol) is the biologically most active metabolite of vitamin D, possessing a high potency in elevating serum calcium and phosphate levels (4). The genomic effects of calcitriol are mediated *via* its interaction with the specific nuclear vitamin D receptor (VDR) (5). Two enzymes are principally involved in the synthesis of calcitriol. Initially, vitamin D is hydroxylated by the hepatic 25-hydroxylase (25-OHase) and consequently converted to 25-hydroxyvitamin D (calcidiol). Subsequent hydroxylation by the renal 1α-hydroxylase (1α-OHase) yields the formation of calcitriol. In target cells, both calcidiol and calcitriol are catabolised by the 24-hydroxylase (24-OHase) and therefore become functionally inactivated. The 1α- and 24-OHase are regulated in a reciprocal manner (6).

In addition to its well-known role in the endocrine regulation of serum calcium and phosphate levels, a strong body of evidence indicates that calcitriol holds further biological functions. This theory is supported by the fact that numerous studies revealed the expression of the VDR and of the key enzymes 1α- as well as 24-OHase in various cell types, which are not involved in calcium and phosphate homeostasis, including cells of the cervix and the ovaries (7). In contrast to calcitriol synthesised in the kidney, extra-renally synthesized calcitriol is presumed to act exclusively in an auto- and/or paracrine fashion under physiological conditions (8, 9). The expression of the VDR and of the key hydroxylases has also been demonstrated in different types of cancer cells, such as breast, colon, prostate, cervical and ovarian cancer cells (7, 10, 11). Various studies have elucidated the anti-proliferative, differentiating and pro-apoptotic effects of calcitriol on a wide range of malignant cells. Moreover, calcitriol is assumed to lower the rate of metastasis as well as to inhibit angiogenesis (12). Nevertheless, the *in vivo* use of calcitriol is definitely limited due to its calcemic side-effects (13). Conceivably, calcidiol, which is intracellularly hydroxylated to the active metabolite calcitriol, has a stronger potential to be used in clinical practice because of its lower calcemic effects. In search for preventive as well as curative pharmaceuticals, several *in vitro* studies have been performed

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Figure 1. *PCR analysis of the mRNA expression of the VDR in HeLa and OVCAR-3 cells. Ctrl A: control A (water instead of cDNA), Ctrl B: control B (cDNA synthesis without reverse transcriptase), M: marker.*

in order to compare the antiproliferative effects of calcitriol and calcidiol. While Schwartz *et al*. (14) and Barreto *et al*. (15) demonstrated equivalent anti-proliferative effects of these vitamin D metabolites, other studies partly did not confirm any anti-proliferative activity of either calcidiol or calcitriol, although the investigated cells were capable of expressing the VDR and 1α-OHase (16-18).

The present study analysed the influence of vitamin D and its metabolites on the expression of the vitamin D metabolising enzymes 25-, 1α- and 24-OHase in HeLa and OVCAR-3 cancer cells.

## Materials and Methods

*Cell culture*. The human cervical adenocarcinoma cell line HeLa and the human ovarian adenocarcinoma cell line OVCAR-3 (ATCC/LGC Standards, Wesel, Germany) were grown in RPMI-1640 medium with 25 mM HEPES and 2 mM L-glutamine (PAA Laboratories, Cölbe, Germany) at 37˚C in a 5% $CO_2$ in air atmosphere. The medium for HeLa cells was supplemented with 10% foetal bovine serum (FBS; PAA Laboratories). The OVCAR-3 cells medium was supplemented with 20% FBS and 0.01 mg/ml insulin (Sigma-Aldrich, Seelze, Germany).

*Stimulation with vitamin D, calcidiol and calcitriol*. For the stimulation with vitamin D, calcidiol and calcitriol, the cells were plated on 94-mm cell culture dishes (Greiner, Frickenhausen, Germany) for three days. Medium was then exchanged with medium containing vitamin D, calcidiol or calcitriol (Sigma-Aldrich) at concentrations of 1 nM and 100 nM. All substances were dissolved as 1 mM stock solutions in 100% ethanol. As controls, cells were treated with the diluent ethanol in a concentration corresponding to the ethanol concentration in the 100 nM treatment groups. Incubation times were 24, 48, 72 and 96 h.

*Isolation of total RNA and cDNA synthesis*. After the indicated periods of time, total RNA was extracted with TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. The purity and the amount of the RNA were examined spectrophotometrically.

For first-strand cDNA synthesis, 2 μg of total RNA were diluted in RNase-free water and reverse transcribed by using 250 ng random primers, 1 μl M-MLV reverse transcriptase (200 U/μl), 1 μl dNTP mix (10 mM) and 1 μl RNaseOUT (40 U/μl) (Invitrogen) according to the manufacturer's instructions. Controls did not contain reverse transcriptase.

*Polymerase chain reaction and gel electrophoresis*. For polymerase chain reaction (PCR) the following primer pair was used: VDR forward 5'-CCA GTT CGT GTG AAT GAT GG-3', reverse 5'GTC GTC CAT GGT GAA GGA-3' (Metabion, Martinsried, Germany). One μl of cDNA was amplified using Taq DNA polymerase (5000 U/ml; New England Biolabs, Frankfurt am Main, Germany). MCF-7 cells were used as VDR-positive control. Negative controls were included by omitting cDNA (Ctrl A) or by utilizing products from cDNA synthesis without reverse transcriptase (Ctrl B). Thermal cycling conditions were 40 cycles at 94˚C for 30 s, 60˚C for 30 s, 72˚C for 30 s and a final extension at 72˚C for 10 min. PCR was performed in a PTC-200 thermal cycler (MJ Research, Waltham, MA, USA). PCR products were separated on a 1.5% ethidium bromide-stained agarose gel, visualised by ultraviolet light and captured on Polaroid films.

*Real-time PCR*. In order to quantify the expression levels of 25-, 1α- and 24-OHase mRNA real-time PCR was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) in an Opticon 2 real-time PCR engine (Bio-Rad, Munich, Germany). All experiments were repeated in triplicate. Each real-time PCR was performed in duplicate and hypoxanthine-guanine phosphoribosyl transferase (HPRT) was used for normalization of the expression levels of the target genes. For absolute quantification, a standard curve was used in tenfold serial dilutions (50-5×$10^6$ molecules), prepared from reverse transcription-PCR products of the corresponding target genes. Primers: 25-OHase forward 5'-GGC AAG TAC CCA GTA CGG-3', reverse 5'-AGC AAA TAG CTT CCA AGG-3'; 1α-OHase forward 5'-TGT TTG CAT TTG CTC AGA-3', reverse 5'-CCG GGA GAG CTC ATA CAG-3'; 24-OHase forward 5'-GCA GCC TAG TGC AGA TTT-3', reverse 5'-ATT CAC CCA GAA CTG TTG-3'; HPRT forward 5'-CCT GGC GTC GTG ATT AGT GAT-3', reverse 5'-CCA GCA GGT CAG CAA AGA ATT TA-3' (Metabion). Two μl of cDNA were added to a 25 μl reaction in a 96-well PCR plate (Sarstedt, Nümbrecht, Germany) and real-time PCR was performed according to the following protocol: 44 cycles (25-OHase), 49 cycles (1α- and 24-OHase) and 39 cycles (HPRT) of denaturation at 95˚C for 15 s, annealing at 57.3˚C (25-, 1α- and 24-OHase) and at 60˚C (HPRT) for 15 s and extension at 72˚C for 15 s. After the corresponding number of cycles, the specificity of the PCR products was verified by melting curve analysis. For data analysis, the expression levels were calculated from standard curve values, which were generated in parallel in each run. Subsequently, the expression levels of the target genes were normalized to the expression levels of the housekeeping gene *HPRT*. All normalised data were calculated as the n-fold change of expression of the treated samples in relation to the controls.

*Statistical analysis*. The statistical analysis of the real-time PCR results was carried out using a two-way ANOVA followed by a Bonferroni *post-hoc* test. Data are expressed as means±standard

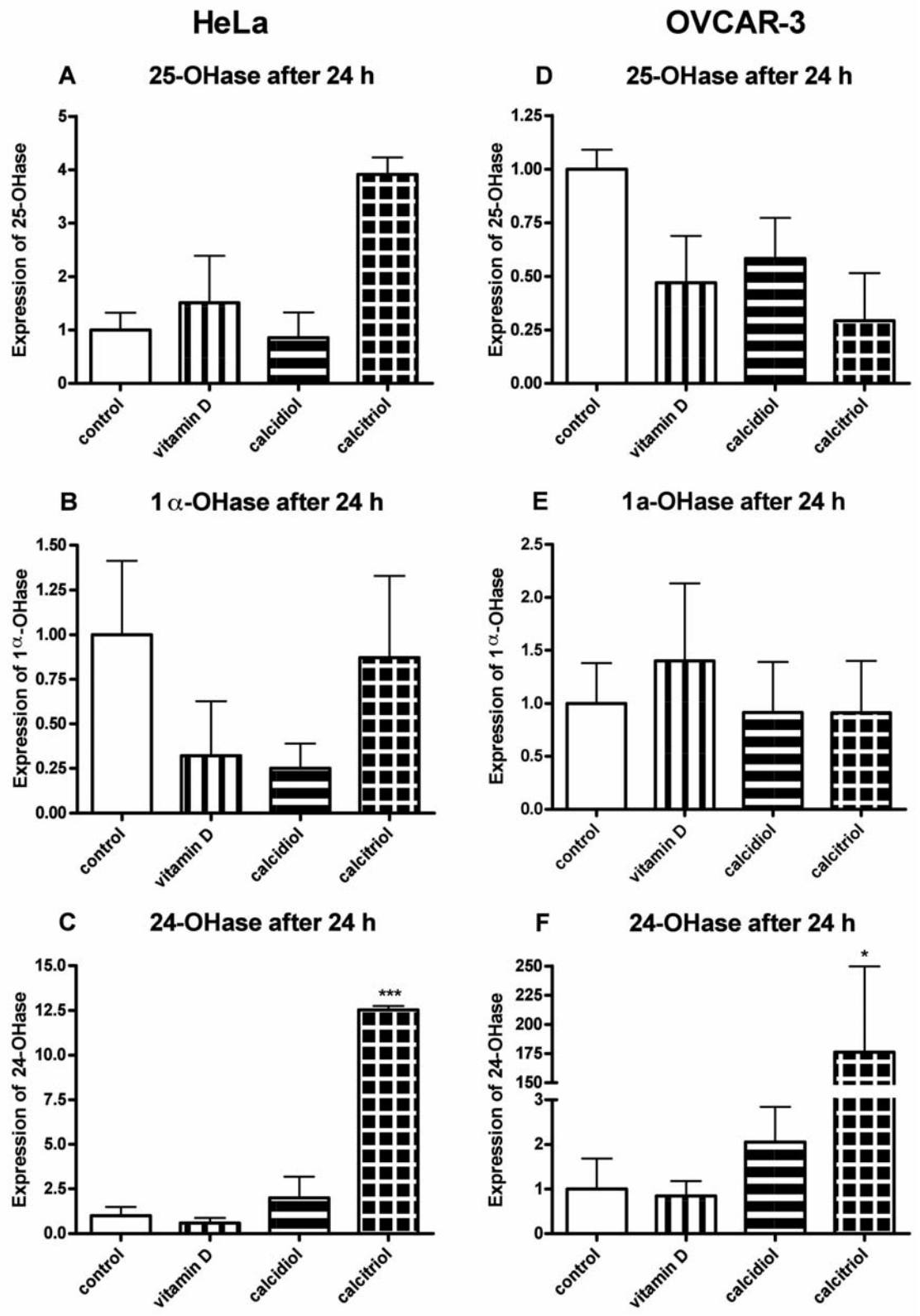


Figure 2. *Real-time PCR analysis of the influence of the different treatments at a concentration of $10^{-7}$ mol/l on the mRNA expression of 25- (A, D), 1α- (B, E) and 24-OHase (C, F) in HeLa cells (A, B and C) and OVCAR-3 cells (D, E and F) after 24 h. The indicated expressions were calculated as the ratio of the expression levels in the treatment to the control group. The data are expressed as means±SEM (*p<0.05 vs. control, ***p<0.001 vs. control; n=3).*

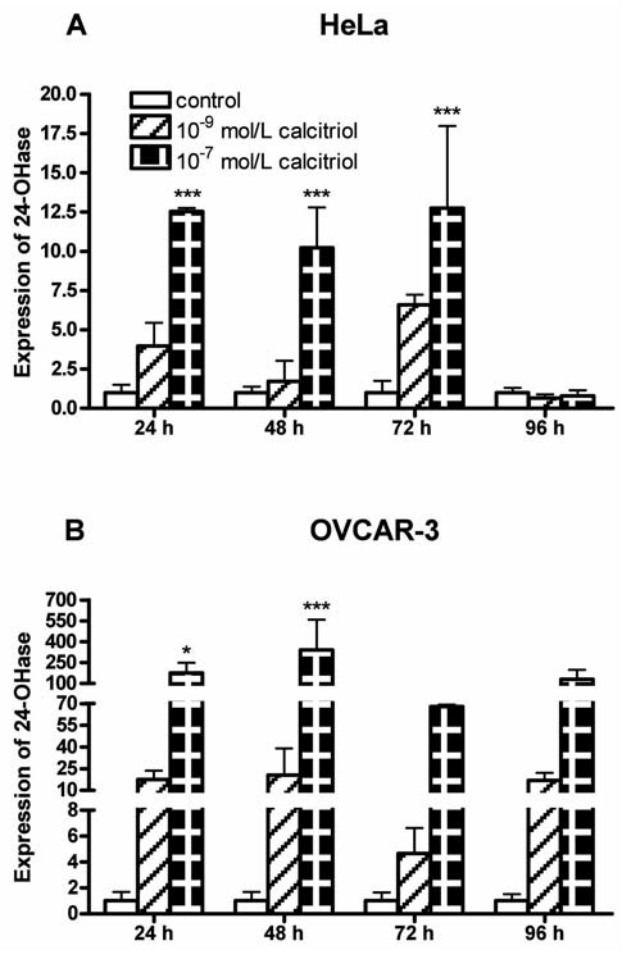


Figure 3. *Real-time PCR analysis of the influence of $10^{-9}$ mol/l and $10^{-7}$ mol/l calcitriol on the mRNA expression of 24-OHase in HeLa cells (A) and OVCAR-3 cells (B) after 24h, 48h, 72h and 96 h. The indicated expressions are calculated as the ratio of the expression levels in the treatment to the control group. The data are expressed as means±SEM (*p<0.05 vs. control, ***p<0.001 vs. control; n=3).*

error of the mean (SEM) obtained from duplicate wells of three independent experiments. All data were calculated with GraphPad Prism 4 (GraphPad Software, Version 4.00, San Diego, CA, USA).

## Results

The expression of the VDR in the investigated cell lines is a necessary condition in order to be able to observe a response of these cells to vitamin D and its metabolites. As Figure 1 shows, HeLa and OVCAR-3 cells expressed the VDR.

With regard to 25-OHase, neither cell line was statistically significantly affected by any of the different treatments after the indicated periods of time (Figure 2). Moreover, none of the treatments statistically significantly altered the mRNA expression of 1α-OHase in HeLa and OVCAR-3 cells at any of the investigated time points (Figure 2). In HeLa cells, the expression of 24-OHase mRNA was statistically significantly increased by 13-, 10- and 13-fold after 24, 48 and 72 h of incubation with 100 nM calcitriol, respectively ($p<0.001$) (Figure 3).

In OVCAR-3 cells, the treatment with 100 nM calcitriol led to a 176-fold increase ($p<0.05$) of 24-OHase mRNA expression after 24 h (Figure 3). After 48 h of incubation with 100 nM calcitriol, 24-OHase expression was 341-fold higher ($p<0.001$) than in the control (Figure 3). The same treatment caused a tendency to an increased 24-OHase mRNA expression after 72 h and 96 h; however, this trend was not statistically significant (Figure 3). Moreover, 1 nM calcitriol and 100 nM calcidiol showed a tendency to an increased expression of 24-OHase in both cell lines; however this tendency was not statistically significant (Figures 2, 3).

## Discussion

The present study demonstrated the expression of the VDR and of the main vitamin D metabolizing enzymes, *i.e.* 25-, 1α- and 24-OHase, in the human cervical cancer cell line HeLa and the human ovarian cancer cell line OVCAR-3. This pattern of expression indicated that HeLa as well as OVCAR-3 cells are capable of metabolizing vitamin D and its derivates autonomously and also of controlling growth and differentiation in an auto- and/or paracrine fashion. Beyond that, the expression levels of the various hydroxylases were shown to be influenced by calcitriol and its precursors. As the most striking effect of the present study, there was a statistically significant up-regulation of the expression levels of 24-OHase by 100 nM calcitriol in both cell lines.

Hence, the present findings join a growing list of results from other studies that have demonstrated calcitriol to be a potent inductor of 24-OHase gene expression and activity in malignant cells, such as breast, colon and prostate cancer cells (10, 19-23). Christopherson *et al.* (24) described an increased 24-OHase activity in cervical as well as ovarian cancer cells under treatment with calcitriol. It was recently hypothesized that neoplastic cells may abrogate the anti-proliferative effects of calcitriol by a dysregulated increase of 24-OHase expression in comparison to their benign counterparts. In fact, various studies disclosed an increased expression of 24-OHase in malignant cells, such as breast, cervical, ovarian and prostate cancer cells (7, 25-27). Thus, in breast cancer cells, 24-OHase has been found to be a potential oncogene (28). Accordingly, surveys analysing different strains of prostate cancer cells revealed an inverse correlation between the anti-proliferative effects of calcitriol and its ability to induce 24-OHase gene expression (17, 29).

The most pronounced result in the present study was the 341-fold induction of 24-OHase expression by 100 nM calcitriol in OVCAR-3 cells. Nevertheless, 100 nM calcitriol

is well-known to elicit anti-proliferative effects in OVCAR-3 cells (30-32). Although this may be explained by the applied dose of calcitriol, which may have potential toxic effects, further studies are needed to clarify whether the elevated mRNA levels of 24-OHase can be verified at the protein level and whether these proteins are functionally active. Conceivably, alternative splicing as well as processes of post-translational modification may lead to the formation of functionally inactive enzymes. Splice variants of 24-OHase have already been detected in breast as well as prostate cancer cells (33, 34). Intriguingly, Fischer *et al*. (25) demonstrated the expression of potential splice variants of the 24-OHase in tissue derived from ovarian cancer samples. Further research is needed to elucidate the existence of 24-OHase splice variants in HeLa and OVCAR-3 cells. Such splice variants may be the reason for the discrepancy between the high induction of 24-OHase by calcitriol observed in OVCAR-3 cells and the anti-proliferative effects of calcitriol on these cells described in other studies.

In the future, vitamin D and its derivates may be considered as useful agents for the prevention and therapy of cervical and ovarian carcinomas.

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