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Functionality of unliganded VDR in breast cancer cells: repressive action on CYP24 basal transcription

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Abstract

It is well-established that CYP24, an immediate target gene of VDR is upregulated by VDR ligands. This study is focused on the functional role of unliganded VDR by investigating the correlation between the expression of VDR protein and basal mRNA levels of CYP24 in breast cancer cell lines. Analyses of multiple breast cancer cell lines demonstrated an inverse correlation between VDR protein expression and CYP24 mRNA expression levels; while in the presence of ligand, VDR protein level was positively correlated with CYP24 expression. In MCF-7 cells, VDR was mainly distributed in the nuclei in the absence of ligand. VDR overexpression in MCF-7 cells and MDA-MB231 cells decreased CYP24 mRNA expression levels and CYP24 promoter activity. Conversely, knockdown of VDR using siRNA techniques in MCF-7 and T47D cells significantly increased CYP24 mRNA expression. We also found that overexpression of VDR with a polymorphic site (FokI-FF) at its AF-1 domain, which makes VDR shorter by three amino acids, failed to repress CYP24 promoter activity. This report provides conclusive evidence for the repressive action of unliganded VDR on the expression of its target gene CYP24 and the importance of an intact VDR AF-1 domain for its repressive action.

Keywords

Vitamin D receptor; CYP24; Breast cancer cells; Unliganded VDR

Introduction

Vitamin D receptor (VDR) is a ligand-dependent transcription factor that targets multiple genes involved in calcium metabolism, cell proliferation, differentiation, and apoptosis. $1,25(\text{OH})_2\text{D}_3$ is the natural ligand of VDR and most of the regulatory role of VDR is determined in the presence of $1,25(\text{OH})_2\text{D}_3$ or vitamin D analogs. On the other hand, recently, it has been recognized that VDR protein independent of ligand may have a functional significance [1–3]. For example, unliganded VDR was reported to stimulate the prolactin promoter in the presence of Ets-1, behaving as a constitutive activator [4]. It has been proposed that unliganded VDR interacts with retinoid X receptor (RXR), which is essential for ligand-independent functions of VDR and its nuclear accumulation [5,6].

In the absence of their specific ligands, thyroid hormone receptor (TR) and retinoic acid receptor (RAR) can bind to RXR as heterodimers and repress basal transcription of target genes [7–9]. The classical VDR pathway is believed to be initiated upon vitamin D_3 binding to its receptor. Through conformational changes, VDR dimerizes with itself and/or its partners, such as RXR, which then binds to specific vitamin D responsive elements (VDREs) in a target gene promoter region, leading to the activation of gene transcription [10]. Like other nuclear hormone receptors, VDR protein displays a modular structure comprising of A/B, C, D, and E regions exhibiting different degrees of evolutionary conservations [11,12] except that the A/B region of VDR consists of only 20 amino acids, and deletion of these residues was reported not to affect VDR function [13].

The 25-hydroxyvitamin D_3 -24-hydroxylase gene (CYP24) is one of the major direct target genes of VDR involved in vitamin D catabolism. Its promoter region contains multiple VDREs, and is very responsive to vitamin D treatment, and therefore is a sensitive marker for VDR function in the presence of ligand [14]. During the characterization of VDR function, in the absence of ligand, we found that the basal levels of CYP24 mRNA expression in breast cancer cells were inversely correlated with the VDR protein levels. In this report, we provide evidence for the repressive functional role of unliganded VDR on its target gene CYP24, demonstrating another novel function of unliganded VDR in regulating its target gene expression.

Materials and methods

Cell culture

MCF-7, T47D, BT474, MDA-MB435, and MDA-MB231 cell lines were purchased from American Type Tissue Collection (Manassas, VA) and maintained in MEM containing 10% FBS as described previously [15]. Medium was switched to MEM containing 5% charcoal-stripped FBS 24 h before the initiation of experiments. $1\alpha(\text{OH})\text{D}_5$ was prepared as described previously [16].

RNA extraction and RT-PCR

Total RNA extraction, RT, and real-time PCR reactions were performed as described previously [16]. The gene specific primer pairs (and product size) for real time PCR were as follows: CYP24 gene forward 5'-CTCAGCAGCC TAGTGCAGATT-3' and reverse 5'-ACTGTTTGCTG-TC GTTTCCAC-3' (122 bp), β -actin forward 5'-CTCTTCCA GCCTTCCT-TCCT-3' and reverse 5'-AGCACTGTGTTG GCGTACAG-3' (116 bp). PCR product quality was monitored using post-PCR melt curve analysis. The cycling conditions used for real time PCR were: 40 cycles of 15 s at 95°C, 15 s at 60°C, and 20 s at 72°C. CYP24 primer pairs for conventional PCR were: forward 5'-ATCCCCAAGTGCAACAAAAG-3', reverse 5'-TTC CTTCTCCTGAAGCCAAC-3' (for mature mRNA) and forward 5'-TCAAGAAACAGCACGACACC-3', reverse 5'-

GGTAGCCTTCTTTGCGGTAG-3' (for both spliced and unspliced CYP24 RNA). The conventional PCR cycling conditions for CYP24 were 35 cycles of 20 s at 95°C, 20 s at 60°C, and 30 s at 72°C.

Western blot, immunostaining, and confocal microscopy

Cell lysates were prepared at different time points and subjected to western blot analysis [16]. Procedures for immunostaining and confocal microscopy were described previously [16]. VDR monoclonal antibody (Vit. Rec Ab-1) for western blot and immunostaining was purchased from NeoMarkers (Fremont, CA).

Plasmid construction

The VDR expression vector pcDNA3.1VDR was generated by PCR-cloning using a pcDNA3.1/V5-His TOPO TA Expression Kit (Invitrogen, Carlsbad, CA) as described previously [15]. pcDNA3.1VDRFF ("f" indicates the presence of the FokI restriction site and the first start codon (ATG), "F" indicates the absence of the FokI site and the first ATG) expression vector was generated from pcDNA3.1VDR expression vector (correspondingly, this vector is shown in some figures as VDRff) through point mutation using QuickChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). RXR α expression vector (pcDNA3.1RXR α) was also generated by PCR-cloning using a pcDNA3.1/V5-His TOPO TA Expression Kit. RNA was isolated from T47D cells for cDNA synthesis and RXR α PCR-cloning. In order to make CYP24 promoter– luciferase reporter plasmid, approximately 400 bp 5' flanking region (–296/+109 relative to the transcription start site) of CYP24 was isolated by PCR from genomic DNA extracted from MDA-MB435 breast cancer cells and cloned to the Kpn/Bgl II sites of the promoterless PGL3 basic vector (Promega, Madison, WI) [17].

Transient transfection of VDR and siRNA

MCF-7 cells were transfected with 2 μ g VDR expression vector (pcDNA3.1VDR) or pcDNA3.1 empty vector) using Lipofectomine 2000 (Invitrogen) per the manufacturer's instruction in OPTI-MEM (Invitrogen) containing 2% FBS. After overnight incubation of lipofectomine–DNA complex, cells from each culture dish were divided into two dishes with same amount of cells in each dish and cultured in regular medium containing 5% charcoal-stripped FBS for 24 h. The experiment was then terminated and cells from one dish were subjected to protein extraction and western blot analysis, cells from the other dish were subjected to RNA extraction and quantitative RT–PCR analysis. The same transfection protocol and incubation time were used for siRNA transfection in MCF-7 and T47D cells [16]. siVDR was purchased from Dharmacon (D-003448-01-0010, Lafayette, CO). Treatment of cells with 150 nM siVDR effectively decreased VDR protein expression. Co-transfection of VDR expression vectors and CYP24 promoter construct was conducted in MCF-7 cells and MDA-MB231 cells in the same condition as described previously [17]. Luciferase activity was measured at 24 h after transfection [17].

Stable transfection of VDR in MDA-MB231 and MCF-7 cells

Nearly confluent MDA-MB231 cells grown in 10 cm dish were transfected with empty pcDNA3.1 or pcDNA3.1VDR (10 μ g expression vector/dish) using Lipofectomine 2000 (Invitrogen) [15]. After 5 h incubation, transfected cells were incubated in fresh medium containing 10% FBS for 24 h, then cells were subjected to selection with G418 (1 mg/ml). After 3-weeks of selection, all clones resistant to G418 selection were pooled together to generate two cell lines: MB231_{vector} and MB231_{VDR}. These cell lines were used to examine the basal level of CYP24 and VDR protein side by side under the same culture condition. In order to decrease the background of endogenous VDR in MCF-7 cells, multiple single cell

clones were generated through cell dilution in 96-well plates, the single cell clones were expanded and VDR expression was examined in these clones. The clone expressing the lowest VDR protein was selected for stable transfection of empty pcDNA3.1 and pcDNA3.1VDR(ff). After G418 selection, single cell clones were expanded to generate 2 cell lines: MCF-7 vector and MCF-7VDRff. These cell lines were used for CYP24 promoter activity assay and PCR analysis.

Results

VDR protein levels negatively correlated with CYP24 basal mRNA expression in the absence of ligand in breast cancer cells

In order to determine whether CYP24 basal transcriptional levels correlated with VDR protein levels in the absence of ligand binding, we first used multiple breast cancer cell lines including MDA-MB231 (ER⁻), MDA-MB435 (ER⁻), MCF-7 (ER⁺), T47D (ER⁺), and BT474 (ER⁺) to evaluate the CYP24 basal mRNA expression levels and VDR protein expression. As shown in Fig. 1a, in both ER-negative and ER-positive cell lines, CYP24 mRNA expression levels were inversely correlated with their VDR protein expression levels. Overall, ER-negative cells expressed lower or non-detectable VDR protein and higher CYP24 mRNA levels in comparison to ER-positive cells, although MCF-7 cells expressed VDR protein at a lower level in comparison to T47D and BT474 cells. BT474 cells expressed the highest level of VDR protein and the lowest level of CYP24 mRNA, whereas MDA-MB231 expressed the lowest level of VDR protein and the highest level of CYP24 mRNA, which was ~38-fold higher than that of BT474 cells. In contrast to this, in the presence of the ligand, 24 h treatment with $1\alpha(\text{OH})\text{D}_5$ (a vitamin D analog) significantly upregulated VDR protein and dramatically induced CYP24 transcription in both ER-negative and ER-positive breast cancer cells (Fig. 1b). Induction of VDR by $1\alpha(\text{OH})\text{D}_5$ in MDA-MB231 cells was not detectable due to low endogenous levels of VDR in these cells. qRT-PCR analysis of VDR mRNA levels in these cell lines demonstrated that the basal VDR mRNA expression levels were correlated with their VDR protein expression levels, but $1\alpha(\text{OH})\text{D}_5$ treatment did not increase the VDR mRNA expression (data not shown), suggesting that $1\alpha(\text{OH})\text{D}_5$ could stabilize the VDR protein. These data demonstrate the differential function of VDR, which depends on whether or not the VDR is bound to the vitamin D ligand.

Since unliganded VDR appeared to inhibit basal CYP24 mRNA expression, we hypothesized that VDR binds to the promoter of CYP24 to inhibit its transcription. We expected that in the absence of ligand, at least a portion of VDR could be localized in the nuclei of cells. Immuno- fluorescent staining was therefore performed in MCF-7 cells to determine the localization of VDR. As shown in Fig. 1c, in the absence of ligand, VDR (green) was mainly distributed in the nuclei of MCF-7 cells and multiple VDR molecules appeared to stay together. Nuclear localization of VDR in absence of ligand was also observed in T47D cells (data not shown). Nuclear localization of VDR demonstrates that unliganded VDR could play its functional role through the classic nuclear receptor pathway.

Effect of VDR overexpression on CYP24 mRNA expression and promoter activity in the absence of ligand

Since VDR appeared to suppress CYP24 mRNA expression levels, we selected MCF-7 and MDA-MB231 cells that express low levels of VDR for VDR transfection. This was to assess whether VDR overexpression in these cell lines affects CYP24 basal mRNA expression. As shown in Fig. 2a, transient transfection of VDR in MCF-7 cells caused overexpression of VDR protein (Fig. 2a, lower panels) and greatly increased the VDR mRNA expression (data not shown). VDR protein overexpression in MCF-7 cells was accompanied by ~30%

decrease in CYP24 mRNA expression. Similarly, MDA-MB231 cells stably overexpressing VDR also consistently showed ~25% downregulation of CYP24 mRNA expression (Fig. 2b). In addition, co-transfection of the CYP24 promoter reporter construct and VDR expression vector in MCF-7 cells and MDA-MB231 cells also showed that VDR overexpression decreased CYP24 promoter activity by 30% ($P < 0.05$, $n = 3$) and 28% ($P < 0.05$, $n = 3$), respectively (Fig. 2c, d). These data demonstrate that unliganded VDR represses CYP24 basal mRNA expression in both ER-positive and ER-negative breast cancer cells by directly acting on the CYP24 promoter.

Effect of decreased VDR on CYP24 mRNA expression in the absence of ligand

In order to confirm the repressive effect of unliganded VDR on CYP24 expression, we further decreased VDR expression using siRNA techniques to observe how CYP24 expression is affected. As shown in Fig. 3a and b, in both MCF-7 and T47D cells, decreased expression of VDR protein by introducing siVDR resulted in significant upregulation of CYP24 mRNA expression. The VDR knock-down increased CYP24 expression by 2-fold ($P < 0.05$, $n = 3$) in MCF-7 cell and 4.7-fold ($P < 0.01$, $n = 3$) in T47D cells. It was noted that since CYP24 basal mRNA level was low and VDR protein level was high in T47D cells, upregulation of CYP24 was easily observed by decreasing VDR protein expression in T47D cells. In support of this, we also observed that 4 h treatment of MCF-7 and T47D cells with the protein synthesis inhibitor cycloheximide (1 μ M) consistently inhibited VDR expression, but upregulated CYP24 mRNA expression (data not shown).

Effect of VDR FokI polymorphism on CYP24 expression

Traditionally, the AF-1 domain of nuclear receptors in the A/B region functions in a ligand-independent fashion to activate or repress target gene transcription. In order to further determine whether the AF-1 domain plays a role in repressing CYP24 expression by unliganded VDR, we introduced a polymorphic FokI site (FF) in the A/B region of the VDR construct, which makes the A/B region shorter by three amino acids. Figure 4a shows that transient transfection of VDRff (wild-type) and VDRFF in MCF-7 cells differentially affected CYP24 promoter activity: VDRff overexpression inhibited CYP24 promoter activity by ~30% in MCF-7 cells, while VDRFF overexpression did not inhibit CYP24 promoter activity in a statistically significant manner. In MDA-MB231 cells, a similar pattern of CYP24 promoter activity was observed (Fig. 4b). VDR-transfected MCF-7 and MDA-MB231 cells showed comparable polymorphic VDR protein expressions in both cell lines, with a slightly higher expression of the F-VDR variant in comparison to the expression of the f-VDR variant in the transfected MCF-7 cells (data not shown). Consistently, the same pattern of endogenous CYP24 mRNA expression was also observed in MCF-7 and MDA-MB231 cells transfected with polymorphic VDR constructs (data not shown). Since the intact VDR (f-VDR) seemed important for its repressive effect, MCF-7 cells with a low level of VDR were selected and stably transfected with pcDNA3.1VDRff to overexpress VDR with an intact AF-1 domain (Fig. 4c, lower panel) to create a significant difference in VDR expression. qRT-PCR analysis showed that a 60% decrease of endogenous CYP24 mRNA expression in MCF-7VDRff cells in comparison with MCF-7 vector control (data not shown). A CYP24 promoter reporter assay in these two cell lines showed that VDRff overexpression decreased CYP24 promoter activity by 50% ($P < 0.001$, $n = 4$). These results demonstrated that the AF-1 domain contributes to the repressive effect of unliganded VDR and that the polymorphic change in AF-1 domain of VDR affects its ligand-independent repressive function.

The transcriptional suppressive effect of VDR is known to be attributed to its DNA binding and subsequent recruitment of receptor co-repressors and the DNA binding of VDR requires its heterodimerization with RXR. Therefore, we cotransfected the RXR α expression vector

(pcDNA3.1RXR α) with FokI polymorphic VDR variants (VDRff and VDRFF) and an empty vector in MCF-7 cells to determine whether RXR α overexpression enhances the suppressive effect of unliganded VDR in the CYP24 reporter assay. We found that RXR α cotransfection did not enhance the suppressive effect of unliganded VDR on CYP24 promoter activity (data not shown), suggesting that the heterodimerization of RXR α and VDR may not play a key role in the suppressive action of unliganded VDR.

We recently observed that CYP24 splicing is regulated by 1,25(OH) $_2$ D $_3$ (Peng and Mehta, unpublished data), we therefore examined whether unliganded VDR affects CYP24 splicing. As shown in Fig. 4d, VDRff overexpression in MCF-7 cells decreased the expression of both spliced and unspliced CYP24 RNA without significantly affecting the ratio (spliced vs. unspliced), suggesting that unliganded VDR mainly represses CYP24 at the transcription level, which is consistent with the results of the promoter reporter assay (Fig. 4a).

Discussion

Members of the nuclear receptor family including thyroid receptors, RARs and VDR bind to regulatory elements of the target genes as heterodimers with RXR, both in the absence and presence of their cognate ligands [18]. In the absence of ligands, these receptors including VDR recruit the nuclear co-repressors, NCoR and SMRT, which act as platforms for multi-protein complexes that mediate transcriptional repression via histone deacetylation [19,20]. More recently, the functional significance of unliganded steroid receptors including estrogen receptor, progesterone receptor, and androgen receptor have been reported [21–23], suggesting a differential function for the liganded and unliganded steroid hormone receptors. In this study, we assessed the expression of VDR protein and CYP24 mRNA using multiple breast cancer cell lines to demonstrate whether the liganded and unliganded VDR differentially regulate CYP24, a VDR-target gene, specifically. This report provides convincing evidence for the repressive action of unliganded VDR on CYP24 mRNA expression.

In general, ER-positive breast cancer cells are responsive to vitamin D, whereas ER-negative cells are less responsive [15]. Our study shows that in the absence of the ligand, in both ER-negative and ER-positive cells, VDR levels were inversely correlated with CYP24 basal expression, which is opposite to the action of ligand associated-VDR (Fig. 1b). The inverse correlation between VDR protein level and CYP24 basal expression levels was confirmed in five cell lines. Moreover, the localization of unliganded VDR in the nuclei suggests a functional role of VDR in MCF-7 cells.

Results derived from both transient and stable transfection experiments demonstrated that VDR overexpression repressed CYP24 basal transcription, while knock-down of VDR protein significantly increased CYP24 expression. On the other hand, the inhibition was approximately 30% in comparison to that of empty vector transfected cells. These results suggested that although other factors might be involved in CYP24 basal expression, unliganded VDR is clearly involved in maintaining a low level of basal CYP24 expression in breast cancer cells.

Vitamin D receptor (VDR) polymorphism has been epidemiologically associated with various diseases including breast cancer (29), however, experimental evidence regarding the functional significance of the FokI site at the N-terminal domain of VDR has been contradictory [13,24–26]. In one report, deletion of 20 amino acids at the N-terminus of VDR was reported not to affect VDR function (13). However, all these reported results were obtained in the presence of ligand, and no data are available for the functional significance of the VDR FokI polymorphism in the absence of ligand. Results described in this report, for

the first time, show that the FokI polymorphism can affect the function of unliganded VDR. The f-VDR is more active in repressing CYP24 basal transcription, whereas the polymorphic F-variant is inactive in repressing CYP24 basal transcription. Our findings are also consistent with the established concept that the AF-1 domain of a nuclear receptor is responsible for ligand-independent function; although the AF-1 domain of VDR is shorter in comparison with other nuclear receptors, it functions in a similar way. Our results also provide new experimental evidence for the association of VDR polymorphism with breast cancer. It is important to note that the VDR FokI polymorphism causes minor alteration in VDR function, which might not always be detected in an insensitive system, but accumulation of minor alterations can cause significant changes.

The effect of unliganded VDR on the basal level of its target genes could be a common phenomenon. Our preliminary research has shown that p21, another VDR-target gene was downregulated by knock-down of VDR in MCF-7 cells (data not shown). In the absence of the ligand, VDR associates via co-repressor proteins, such as NcoR1 and SMRT/NcoR2, with HDACs [19,27]. This complex represses gene transcription by stabilizing DNA-histone contact and closing chromatin structure. It should be noted that the formation of VDR-CYP24 promoter VDRE complex in the absence of ligand in MCF-7 cells has been reported previously [28], which is consistent with our observation. Interestingly, MCF-7 cells express the polymorphic F-VDR variant [29], suggesting that Fok I polymorphism does not affect the nuclear translocation of VDR.

Vitamin D receptor (VDR) was previously shown to repress basal transcription and exert dominant negative activity on triiodothyronine-mediated transcriptional activity [30] and repression of basal transcription by VDR was reported to require two receptor interaction domains in RIP13Δ1 [31]. However, most previous studies used VDR-target gene promoter constructs and transfection experiments to achieve this conclusion. In the present study, we directly examined the transcriptional expression of an endogenous target gene of VDR in different cells. The differential action of liganded and unliganded VDR on its target gene CYP24 suggests an important role of VDR in vitamin D catabolism. In the absence of the ligand, VDR represses CYP24 expression to prevent the degradation of a low amount of 1,25(OH)₂D₃ to 1,24,25 (OH)₃D₃. However, in the presence of 1,25(OH)₂D₃, the cells need to have a mechanism to reduce vitamin D-toxicity. Therefore, in the presence of 1,25(OH)₂D₃, the liganded VDR can quickly induce CYP24 expression to enhance catabolism of 1,25(OH)₂D₃ and prevent vitamin D-toxicity.

Our data provide consistent and direct experimental evidence for the repressive action of unliganded VDR on its target gene transcription and the important role of an intact VDR AF-1 domain in the repressive action, implicating the biological significance of VDR FokI polymorphism.

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Abbreviations

VDR	Vitamin D receptor
RT-PCR	Reverse transcription–polymerase chain reaction
TR	Thyroid hormone receptor

RAR	Retinoic acid receptor
RXR	Retinoid X receptor
AF-1	Activation function-1

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Fig. 1.

Differential regulation of CYP24 mRNA expression by VDR in the absence (**a**) and presence of ligand (**b**) and VDR nuclear localization in MCF-7 cells (**c**). **a** Protein levels are inversely correlated to CYP24 basal mRNA expression in the absence of the ligand. *Upper panel*: qRT-PCR analysis of CYP24 mRNA expression in different breast cancer cells. CYP24 basal mRNA levels in the BT474 samples were set as one after normalization to β -actin. CYP24 mRNA level in each sample was normalized to β -actin and the basal CYP24 level of BT474 cells. Duplicate real-time PCR analyses were carried out for each RT sample. The results are expressed as a mean $\pm$ SEM of three experiments. *Lower panel*: VDR protein expression in different breast cancer cell lines, β -actin served as a loading control. **b** VDR protein levels positively correlated with CYP24 induction in the presence of the ligand. Cells were treated with $0.5 \mu\text{M}$ $1\alpha(\text{OH})\text{D}_3$ for 24 h and analyzed for CYP24 transcription by RT-PCR (*upper panel*) and VDR protein by western blot (*lower panel*). **c** Immunofluorescent staining of VDR in MCF-7 cells in the absence of ligand. *Upper panel*: nuclei stained with DAPI; *lower panel*: unliganded VDR (green) is localized in the nuclei of MCF-7 cells.

Fig. 2.

Effect of VDR overexpression on CYP24 expression and promoter activity in breast cancer cell lines. **a** MCF-7 cells were transiently transfected with VDR expression vector, CYP24 mRNA (*upper panel*) and VDR protein (*lower panel*) were analyzed at 48 h after transfection. **b** CYP24 basal transcriptional levels inversely correlated with VDR protein levels in MDA-MB231 cells stably transfected with VDR expression vector. All the results are expressed as a mean $\pm$ SEM of three samples obtained from three independent experiments. A two-tailed student's *t*-test was performed to determine any significant differences between control and VDR-transfected cells (* $P < 0.05$, ** $P < 0.01$). **c** and **d** Overexpression of VDR repressed CYP24 promoter activity in both MCF-7 and MDA-MB231 cells MCF-7 (**c**) and MDA-MB231 cells (**d**) were co-transfected with VDR expression vectors or empty vector (pcDNA3.1) and CYP24 promoter-luciferase reporter construct. At 24 h after transfection, CYP24 promoter activity was evaluated by luciferase assay. Data are expressed as a mean $\pm$ SEM of three-independent experiments with at least triplicate wells for each transfection. * $P < 0.05$ in comparison to control

Fig. 3.

Decreased VDR protein expression was accompanied by increased CYP24 expression in breast cancer cells. MCF-7 (a) and T47D (b) cells were transfected with siVDR or si-Control. CYP24 mRNA expression (*upper panel*) and VDR protein level (*lower panel*) were examined 36 h after transfection. Results are expressed as a mean $\pm$ SEM of three samples obtained from two independent experiments. * $P < 0.05$, ** $P < 0.01$ in comparison to control

Fig. 4.

Effect of VDR FokI polymorphism on CYP24 promoter activity and CYP24 mRNA expression. **a** and **b** MCF-7 and MDA-MB231 cells were co-transfected with VDR expression vectors (containing the FokI site, VDRff, and VDRFF, respectively) or empty vector (pcDNA3.1) and CYP24 promoter reporter construct. CYP24 promoter activity was evaluated 24 h after transfection. Data shown are representatives of two independent experiments. Bar, mean $\pm$ SEM of four samples; ** $P < 0.01$, * $P < 0.05$ in comparison to vector control. **c** *Upper panel*: CYP24 promoter activity in MCF-7 cells stably transfected with VDRff. Bar, mean $\pm$ SEM of three independent experiments, *** $P < 0.001$, $n = 3$; *lower panel*: VDR expression in the MCF-7 cells expressing empty vector and VDRff. β -actin served as a loading control. **d** RT-PCR analysis of CYP24 expression of both unspliced and spliced forms. qRT-PCR analysis of β -actin demonstrated equal loading for the two lanes (data not shown). *M* maker, *vector* MCF-7 cells stably expressing empty vector, *VDRff* MCF-7 cells stably expressing VDR with an intact AF-1 domain