

Prohibitin Is a Novel Target Gene of Vitamin D Involved in Its Antiproliferative Action in Breast Cancer Cells

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Abstract

Previously, we showed that *N*-methyl-*N*-nitrosourea-transformed MCF12F breast epithelial cells exhibited differential expression of several genes, including up-regulation of prohibitin and elevated sensitivity to a relatively noncalcemic vitamin D analogue, 1 α -hydroxyvitamin D₅ [1 α (OH)D₅]. In this report, we evaluated the functional significance of prohibitin in relation to the cellular response to vitamin D. The *in silico* screening for putative transcription factor binding sites identified two vitamin D receptor (VDR)/retinoid X receptor binding sites in the 1-kb promoter region of prohibitin. Prohibitin up-regulation by 1 α (OH)D₅ treatment at both transcriptional and translational levels was confirmed by real-time reverse transcription-PCR and Western blot analysis in breast cancer cells, identifying prohibitin as a vitamin D target gene. Confocal microscopic analysis showed that prohibitin was localized in the nuclei of MCF-7 cells and a portion of prohibitin was colocalized with VDR, but direct physical interaction between VDR and prohibitin in cell lysates was not detectable. In MCF-7 cells expressing tetracycline-inducible prohibitin (*Tet-On* model), the over-expression of prohibitin inhibited cell proliferation and enhanced vitamin D-induced antiproliferative activity. Knockdown of prohibitin was accompanied by increased number of cells incorporating bromodeoxyuridine in the whole population and increased cell distribution in the S phase of cell cycle. In addition, prohibitin level had no significant effect on the vitamin D-induced transactivation of *CYP24*, a VDR target gene. This is the first report to suggest that prohibitin serves as a novel vitamin D target gene, which is involved in the antiproliferative action of vitamin D without affecting *CYP24* transactivation in breast cancer cells. (Cancer Res 2006; 66(14): 7361-9)

Introduction

It is now well recognized that the active metabolite of vitamin D plays a significant role as a modulator of cell proliferation and differentiation in addition to its principal function in bone mineralization. However, its clinical use in cancer prevention and therapy is hampered due to its toxic effects at efficacious concentration. Therefore, considerable effort has been diverted toward designing and synthesizing chemical analogues of vitamin D that are less calcemic and more potent in inhibiting growth of

cancer cells. We synthesized one such less calcemic analogue, 1 α -Hydroxy-24-ethyl-cholecalciferol [1 α (OH)D₅], which has shown promising chemopreventive effect in mouse mammary organ culture system (1, 2), chemically induced rat mammary carcinogenesis and breast cancer xenograft model using athymic mice (3, 4). To this end, 1 α (OH)D₅ is being developed for phase I clinical trials for breast cancer patients. Our previous effort to identify transformation-associated genes using microarray analysis found that several genes were up-regulated in carcinogen-transformed human breast epithelial cells compared with the untransformed cells (MCF12F), including prohibitin, TCTP1, and thioredoxin (3, 5). Because it is well known that vitamin D arrests epithelial cells in G₁ phase of the cell cycle whereas prohibitin has been reported to have tumor-suppressive and antiproliferative effects, we selected prohibitin to determine if it has any functional significance in vitamin D action. The prohibitin gene encodes a protein of 275 amino acids with a molecular mass of 28 to 30 kDa. It is highly evolutionarily conserved and mapped to chromosome 17q12-21 (6). Its protein is localized into the inner membrane of the mitochondria, where it might have a role as a mitochondrial chaperon protein in a complex with BAP37 in the maintenance of mitochondrial function and protection against senescence (7-9). In addition, prohibitin also plays a regulatory role within the cell cycle, although the precise role of the protein in cell cycle regulation is not well understood (10). Earlier work considered prohibitin as a potential tumor suppressor gene because microinjection of prohibitin transcripts resulted in growth arrest in HeLa cells (11). Prohibitin has also been reported to be localized to the nuclei of breast cancer cells (12) and to mediate hormone response in prostate cancer and ovary granulosa cells (13, 14). There is evidence for the interaction of prohibitin with the cell cycle checkpoint molecules, including E2F (15), p53 (12), and pRB (16, 17), and the overexpression of prohibitin can modulate transcription of multiple genes in the transfection experiments. This suggests a potential mechanism of prohibitin function in cell cycle regulation (12, 15, 17). Recent reports indicate that prohibitin is up-regulated in tumor cells compared with normal cells (18-20). Moreover, it has been reported as one of the target genes for *c-Myc* (21, 22). The consistently higher levels of prohibitin in cancer cells may be due to the transactivation of *c-Myc* gene (8). Thus, it seems that prohibitin is involved in diverse cellular processes, including proliferation, stress (7), cell migration (23), etc., associated with its different localization in the cells; however, its nuclear function is still far from clear. Our attempt to characterize the role of prohibitin in vitamin D receptor (VDR) function led to the identification of prohibitin as a novel vitamin D target gene that is involved in the antiproliferative action of vitamin D without affecting *CYP24* transactivation in breast cancer cells.

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Materials and Methods

Vitamin D analogue. $1\alpha(\text{OH})\text{D}_3$ was synthesized according to the procedure described previously (1). $1\alpha(\text{OH})\text{D}_3$ was dissolved in ethanol, and the stock solution of 10 mmol/L $1\alpha(\text{OH})\text{D}_3$ in ethanol was stored in -80°C freezer. The appropriate controls for each experiment consisted of treatment with the vehicle (ethanol) used at a concentration $<0.01\%$ of total cell culture medium. $1,25(\text{OH})_2\text{D}_3$ was purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Cell culture. MCF-7, BT474, and MDA-MB231 cell lines were purchased from the American Type Culture Collection (Manassas, VA) and cultured as described previously (24). MCF-7 cells expressing tetracycline-inducible prohibitin (tagged by c-Myc) were grown in DMEM supplemented with 10% tetracycline-free fetal bovine serum (FBS; Clontech, Palo Alto, CA), 25 $\mu\text{g}/\text{mL}$ Zeocin (Invitrogen, Carlsbad, CA), and 0.25 $\mu\text{g}/\text{mL}$ Blastidicine (Invitrogen) as described (12). Prohibitin was induced by incubation of cells with 1 $\mu\text{g}/\text{mL}$ tetracycline or 20 ng/mL doxycycline in the MCF-7 *Tet-On* model. A day before treatment, cells were seeded and incubated for 24 hours in growth medium to allow cells to attach and the serum in the medium was reduced to 5%. Then, cells were then treated with 0.5 $\mu\text{mol}/\text{L}$ $1\alpha(\text{OH})\text{D}_3$ or 10 nmol/L $1,25(\text{OH})_2\text{D}_3$ for different times. For long treatments, medium was changed every other day.

RNA extraction and real-time reverse transcription-PCR. Total RNA extraction and reverse transcription reaction were done as described previously (24). RNA was further subjected to DNase I (Ambion, Austin, TX) digestion and purification using RNeasy Mini kit (Qiagen, Valencia, CA) before reverse transcription. Real-time PCR was done with 1 μL reverse transcription product in a MyiQ real-time PCR detection system (Bio-Rad, Hercules, CA) by using iQ SYBR Green PCR Supermix (Bio-Rad) according to the manufacturer's guidelines. The PCR cycling conditions used were as follows: 40 cycles of 15 seconds at 95°C , 15 seconds at 60°C , and 20 seconds at 72°C . Fold inductions were calculated using the formula $2^{-\Delta\Delta\text{Ct}}$, where $\Delta\Delta\text{Ct}$ is $\Delta\text{Ct}_{(\text{treatment})} - \Delta\text{Ct}_{(\text{control})}$, ΔCt is $\text{Ct}_{(\text{target gene})} - \text{Ct}_{(\text{actin})}$, and Ct is the cycle at which the threshold is crossed. The gene-specific primer pairs (and product size) for the gene analyzed here were as follows: *prohibitin*, 5'-ACCAGTAATGTGCCAGTCA-3' (forward) and 5'-TAGTCCTCC-GATGCTGGT-3' (reverse; 126 bp); *CYP24*, 5'-CTCAGCAGCCCTAGTCA-GATT-3' (forward) and 5'-ACTGTTTGCTGCTGTTTCAC-3' (reverse; 122 bp); and *β -actin*, 5'-CTCTCCAGCCTTCC-TTCCT-3' (forward) and 5'-AGCACTGTGTTGGCGTACAG-3' (reverse; 116 bp). PCR product quality was monitored using post-PCR melt curve analysis.

Cell proliferation assay. Cell proliferation was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, direct cell counting, and bromodeoxyuridine (BrdUrd) incorporation assay. MTT assay was described previously (24). Cells (1,000) per well were initially seeded in 96-well plates for MTT assay. For direct cell counting, cells were initially seeded in six-well (5,000 per well) or 12-well (2,000 per well) plates; after treatment, cells were trypsinized and cell number was determined by direct counting using Z1 Coulter Particle Counter (Beckman Coulter, Fullerton, CA) with size setting at 8 to 12 μm . For BrdUrd incorporation assay, 10 $\mu\text{mol}/\text{L}$ BrdUrd (Calbiochem, La Jolla, CA) was added to the medium 2 hours before harvesting cells. Cells were trypsinized, washed with PBS, and fixed in 1% paraformaldehyde in PBS for 15 minutes followed by incubation in PBS containing 0.2% Tween 20 for 30 minutes at 37°C . Cells were then incubated with mouse monoclonal anti-BrdUrd antibody (Calbiochem) overnight at 4°C , washed twice, and incubated with FITC-conjugated secondary antibody (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. After three washes with PBS, cells were subjected to fluorescence-activated cell sorting (FACS) analysis; a total of 20,000 events were measured per sample. BrdUrd incorporation assay is a more sensitive assay for cell proliferation, which was used to evaluate cell proliferation potential in MCF-7 cells transiently transfected with small interfering RNA (siRNA).

Fluorescent immunostaining and confocal microscopy. MCF-7 cells were grown on coverslips for 24 hours. Cells were washed in PBS and fixed in buffered formalin. Fixed cells were then washed thrice with PBS containing 0.1% Tween 20 (PBST), permeabilized in 0.2% Triton X-100/PBS

for 5 minutes, blocked with 1% bovine serum albumin in PBST for 30 minutes, and then incubated with anti-VDR rat monoclonal antibody (mAb; 1:200; NeoMarkers, Fremont, CA) for 1 hour at room temperature. Cells were washed and incubated with FITC-labeled anti-rat secondary antibody for 1 hour. Antibody incubation steps were repeated with anti-prohibitin mouse mAb (NeoMarkers) and Texas red-labeled anti-mouse secondary antibody. After staining nuclei with 4',6-diamidino-2-phenylindole (DAPI), cells were visualized with Zeiss LSM 510 (Zeiss, Thornwood, NY) confocal microscope and areas of colocalization were determined using LSM 510 software (Zeiss).

Coimmunoprecipitation and Western blot analysis. When cells grew to 50% to 70% confluence, cell lysates were prepared and subjected to either Western blot analysis as described previously (24) or coimmunoprecipitation. Mouse anti-prohibitin mAb was purchased from NeoMarkers. Rabbit polyclonal VDR antibody, c-Myc mouse mAb, and all secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For coimmunoprecipitation, cell lysates (500 μg) were incubated with VDR polyclonal antibody or c-Myc mAb in 300 μL binding buffer [50 mmol/L HEPES, 150 mmol/L NaCl, 1.5 mmol/L MgCl_2 , 1 mmol/L EDTA, 100 $\mu\text{mol}/\text{L}$ NaF, 200 $\mu\text{mol}/\text{L}$ Na_2VO_4 , proteinase inhibitor cocktail (Sigma-Aldrich), 1 mmol/L phenylmethylsulfonyl fluoride, 0.5% NP40] on a rocker platform for 3 hours at 4°C . Then, 30 μL protein A/G plus agarose (Santa Cruz Biotechnology) was added to each sample for overnight incubation. The agarose beads were washed five times with 600 μL of the same binding buffer, boiled in 20 μL SDS sample buffer, and subjected to Western blot analysis (24).

Expression plasmid and transfection. The prohibitin expression vector pcDNA3.1PHB was generated by PCR cloning using pcDNA3.1/V5-His TOPO TA Expression kit (Invitrogen). The open reading frame of prohibitin was isolated by PCR of full-length cDNA from MCF10A breast epithelial cells using primers containing start and stop codons [prohibitin, 5'-GGAAACATGGC-TGCCAAAGTG-3' (start) and 5'-GCCTCCTGAGGGC-AGCTG-3' (stop)]. Orientation and sequence of the construct were verified by direct sequencing. Transient transfection was done in the same culture medium containing 2% FBS using LipofectAMINE 2000 (Invitrogen) in 12-well plates per manufacturer's manual (24). Cells (1.5×10^5 per well) were transfected with 0.5 $\mu\text{g}/\text{well}$ pcDNA3.1 empty vector (control) or 0.53 $\mu\text{g}/\text{well}$ pcDNA3.1PHB expression vector. After 5 hours of incubation, the medium was replaced with medium containing 10 nmol/L $1,25(\text{OH})_2\text{D}_3$ or ethanol (solvent control, 1 $\mu\text{L}/10$ mL medium), and cells were incubated for an additional 24 hours before analysis.

RNA interference. siRNA against prohibitin [prohibitin siRNA, 5'-CCCAGAAACACUGUGAAAdTdT-3' (sense) and 5'-UUUCACAGUAAUUUCUGGG-dTdT-3' (antisense)] was designed to target the 3'-untranslated region at 974 to 993 bp of sequence NM_002634 and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Negative control siRNA [nonsilencing RNA, 5'-UUCUCCGAAACGUGUCA-CGUdTdT-3' (sense) and 5'-ACGUGACACGUGCGGAGAAAdTdT-3' (antisense); Qiagen] has no homology to known mammalian genes. To knock down prohibitin by RNA interference (RNAi), 10^5 cells per well were seeded in six-well plates in culture medium containing 5% FBS and incubated overnight. Prohibitin siRNA and nonsilencing siRNA were transfected using the RNAi Carrier kit (Epoch Biolabs, Sugar Land, TX). Cells were first incubated with siRNA complex (siRNA at 80 nmol/L; siRNA to RNAi Carrier, 1:6) for 6 hours in Opti-MEM (Invitrogen) containing 2% FBS, and then FBS was increased to 5%. After overnight incubation, cells were incubated in fresh culture medium containing 5% FBS for 2 days, and then cells were subjected to BrdUrd incorporation assay or cell cycle analysis (25). Prohibitin knockdown was verified at the same time using reverse transcription-PCR (RT-PCR) and Western blot analysis.

Results

Prohibitin is a vitamin D target gene. Our earlier studies showed that prohibitin could be involved in cellular response to $1\alpha(\text{OH})\text{D}_3$ in breast epithelial cells (5); therefore, a question was raised: is prohibitin a vitamin D target gene? To answer this

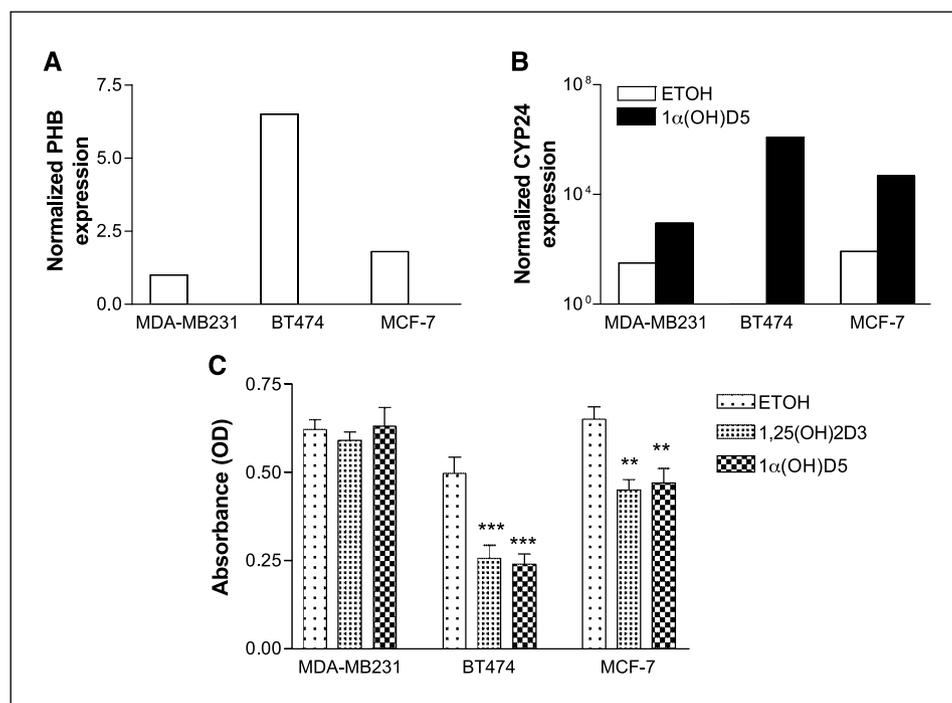


Figure 2. Correlation of prohibitin basal transcriptional sensitivity to vitamin D in breast cancer cells. *A*, basal transcriptional level of prohibitin in ER⁺ and ER⁻ breast cancer cell lines. The basal prohibitin level in MDA-MB231 cells was set as 1. *Columns*, mean of two independent experiments with duplicate analyses of each RNA sample. *B*, transcriptional induction of CYP24 by 1 α (OH)D₅ (0.5 μ mol/L) in breast cancer cell lines analyzed by real-time RT-PCR. The basal CYP24 level in BT474 cells was set as 1. CYP24 mRNA level in each sample was normalized to β -actin and the basal level of BT474 cells. *Columns*, mean of two independent experiments with duplicate analyses of each RNA sample. *C*, effects of vitamin D analogues on cell proliferation as measured by MTT assay in breast cancer cells. Cells were incubated for 4 days with 1 α (OH)D₅ (0.5 μ mol/L) and 1,25(OH)₂D₃ (100 nmol/L). ER⁺ breast cancer cells (MCF-7 and BT474) are sensitive to vitamin D treatment, whereas ER⁻ breast cancer cells (MDA-MB231) are resistant to vitamin D treatment. *Columns*, mean of three independent experiments with eight wells per treatment in each experiment; *bars*, SE. **, $P < 0.01$, compared with control; ***, $P < 0.001$, compared with control.

by 50% after 4-day treatment in BT474 cells. MCF-7 cells are responsive to 1 α (OH)D₅ treatment but less sensitive compared with BT474 cells, whereas MDA-MB231 cells were resistant to 1 α (OH)D₅ treatment. 1 α (OH)D₅ failed to inhibit cell proliferation and only slightly induced CYP24 expression in MDA-MB231 cells. These data

show that basal expression level of prohibitin could potentially contribute to cellular sensitivity to vitamin D in breast cancer cells.

Prohibitin is localized to the nuclei in MCF-7 breast cancer cells. We examined the distribution of prohibitin and VDR in MCF-7 cells by fluorescent immunostaining. MCF-7 cells are well

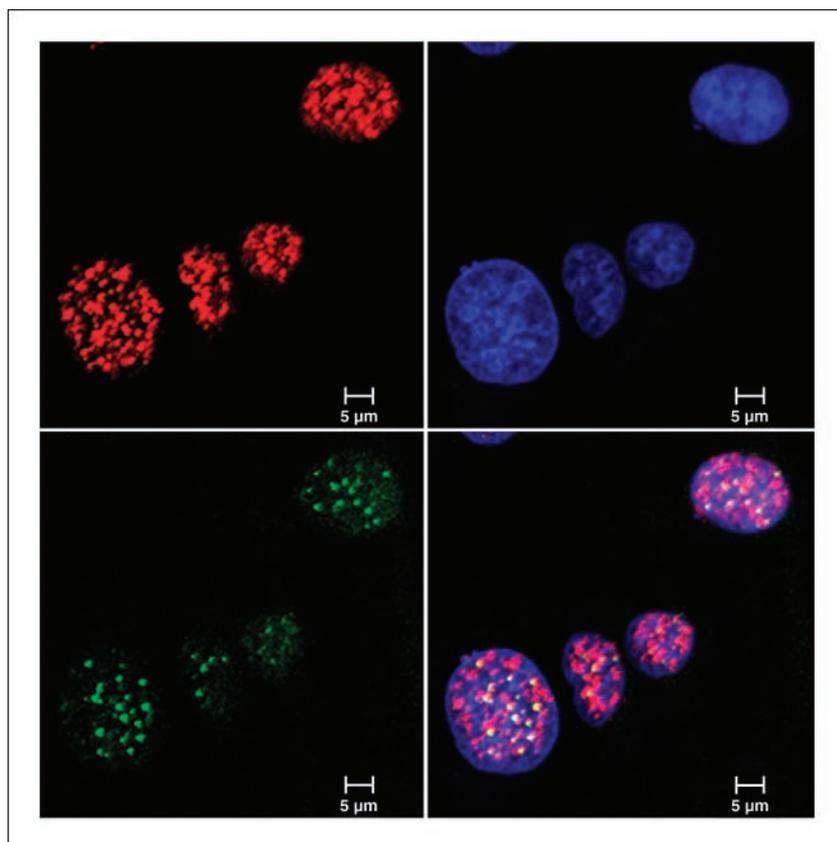


Figure 3. Localization of prohibitin and VDR in the nuclei of MCF-7 cells. Cultured MCF-7 cells were immunostained with rat anti-prohibitin (*top left*, red) and mouse anti-VDR (*bottom left*, green) mAbs. *Top right*, blue, nuclei were stained with DAPI. Fluorescent immunostaining of prohibitin (red) and VDR (green) in MCF-7 cells shows clear colocalization (*bottom right*, yellow) of VDR with a portion of prohibitin in the nuclei.

characterized, express moderate level of prohibitin (Fig. 2A), and used for cell transfection studies in our laboratory. Confocal microscopic analysis showed that prohibitin was localized in the nuclei and a portion of prohibitin was colocalized with VDR (Fig. 3). Such nuclear accumulation of prohibitin has been previously reported (11–13, 16). The colocalization of prohibitin and VDR suggested that a potential physical association between the two molecules could exist and that prohibitin could regulate VDR-mediated cellular responses.

Characterization of prohibitin expression and its role in response to vitamin D in MCF-7 prohibitin *Tet-On* model. Because prohibitin is a target gene of vitamin D and a direct physical association between prohibitin and VDR may occur, we hypothesized that prohibitin expression plays a role in response to vitamin D. To test this hypothesis, a MCF-7 cell line expressing tetracycline-inducible c-Myc-tagged prohibitin (prohibitin *Tet-On* model; ref. 11) was used. Using this model, we evaluated the effect of prohibitin overexpression on vitamin D-induced cellular response. We first confirmed the increased expression of prohibitin with tetracycline (1 $\mu\text{g}/\text{mL}$) and its derivative doxycycline (50 ng/mL), the two *Tet*-system inducers. After 24 hours of treatment, both inducers effectively induced exogenous prohibitin expression. The c-Myc-tagged prohibitin was separated with a molecular mass of ~ 34 kDa from the endogenous prohibitin (28–30 kDa; Fig. 4A). Because doxycycline is water soluble and the induction concentration is much lower than tetracycline, we further optimized the concentration of doxycycline used in this *Tet-On* system. The cells were treated with different concentrations of doxycycline, and exogenous prohibitin expression was analyzed by immunoblotting (Fig. 4B). Results showed that doxycycline

induced dose-dependent prohibitin expression with maximum induction at the concentration of 20 ng/mL. This concentration of doxycycline was therefore used for all the later experiments. Because the exogenous prohibitin is c-Myc tagged, we used anti-c-Myc antibody to confirm that the ~ 34 -kDa protein band is in fact prohibitin by immunoblotting. As shown in Fig. 4C, the doxycycline-induced ~ 34 kDa protein band was also detected by anti-c-Myc antibody, whereas in *Non-Tet-On* MCF-7 cells doxycycline treatment did not induce prohibitin expression. As expected, reprobing the membrane with anti-prohibitin antibody revealed that all endogenous and exogenous prohibitin were detected and that the anti-prohibitin antibody was specific to prohibitin without cross-reaction with other proteins (Fig. 4D). These results once again confirm the nuclear localization of prohibitin shown in Fig. 3. To detect the possible physical interaction between prohibitin and VDR, coimmunoprecipitation was done using cell lysates made from cultured MCF-7 *Tet-On* cells. As shown in Fig. 4E, after immunoprecipitating proteins with polyclonal anti-VDR and monoclonal anti-c-Myc antibodies, the precipitated complexes were subjected to Western blot analysis using anti-prohibitin antibody (Fig. 4E). The proposed physical interaction between prohibitin and VDR was not detectable (Fig. 4E, lane 4), whereas c-Myc antibody precipitated exogenous c-Myc-tagged prohibitin from cell lysates (Fig. 4E, lane 6). However, reprobing the membrane with VDR antibody once again failed to detect physical interaction between prohibitin and VDR (data not shown). Interestingly, as shown in Fig. 4E (lane 6), in addition to exogenous c-Myc prohibitin band, endogenous prohibitin (28–30 kDa) as well as multiple protein bands at 90 to 130 kDa were detected by prohibitin antibodies from c-Myc antibody-precipitated complexes.

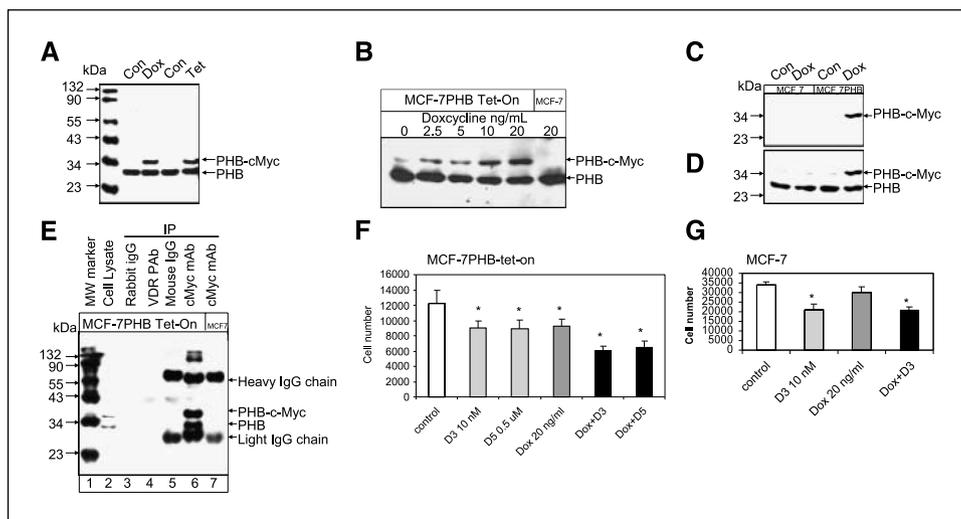


Figure 4. Characterization of prohibitin expression and cellular response to vitamin D in prohibitin *Tet-On* MCF-7 cell line. **A**, Western blot analysis of prohibitin expression in *Tet-On* MCF-7 cells expressing tetracycline-inducible c-Myc-tagged prohibitin. Both tetracycline (*Tet*; 1 $\mu\text{g}/\text{mL}$) and doxycycline (*Dox*; 50 ng/mL) induced exogenous prohibitin (~ 34 kDa) expression after 24 hours of treatment. **B**, optimization of doxycycline concentration in induction of prohibitin expression in *Tet-On* MCF-7 cells. Doxycycline (20 ng/mL) was determined to be the best concentration for this system. **C** and **D**, Western blot analyses of c-Myc-tagged prohibitin in *Tet-On* MCF-7 cells using anti-c-Myc (**C**) and anti-prohibitin (**D**) mAbs. Both antibodies specifically detect the exogenous prohibitin. **E**, immunoprecipitation (IP) of VDR (lane 4) and exogenous prohibitin (lane 6) from cell lysates made from MCF-7PHB *Tet-On* cells using anti-VDR polyclonal antibody (VDR PAb) and anti-c-Myc mAb. Prohibitin was not coprecipitated with VDR, but endogenous prohibitin was coprecipitated with exogenous c-Myc-tagged prohibitin (lane 6), indicating lack of physical interaction between VDR and prohibitin and the formation of prohibitin homomers. *Non-Tet-On* MCF-7 cells (lane 7) served as a negative control for c-Myc-tagged exogenous prohibitin. **F**, inhibition of cell proliferation and enhancement of the antiproliferative effect of vitamin D in MCF-7 cells by prohibitin overexpression. *Tet-On* MCF-7 cells were incubated with either 10 nmol/L 1,25(OH) $_2$ D $_3$ (*D3*) or 0.5 $\mu\text{mol}/\text{L}$ 1 α (OH)D $_5$ (*D5*) in the presence or absence of 20 ng/mL doxycycline for 4 days, and cell number was determined. Columns, mean of three independent experiments with triplicate wells in each experiment; bars, SE. *, $P < 0.05$, compared with the corresponding control [1,25(OH) $_2$ D $_3$ versus control; 1 α (OH)D $_5$ versus control; doxycycline versus control; doxycycline + 1,25(OH) $_2$ D $_3$ versus doxycycline or 1,25(OH) $_2$ D $_3$ only; doxycycline + 1 α (OH)D $_5$ versus doxycycline or 1 α (OH)D $_5$ only]. **G**, MCF-7 cells (*Non-Tet-On*) were grown in 12-well plates and treated with 10 nmol/L 1,25(OH) $_2$ D $_3$ in the presence or absence of doxycycline for 4 days. In *Non-Tet-On* MCF-7 cells, doxycycline at 20 ng/mL had very little effect on cell proliferation. *, $P < 0.05$, compared with the corresponding control [1,25(OH) $_2$ D $_3$ versus control; doxycycline + 1,25(OH) $_2$ D $_3$ versus doxycycline only].

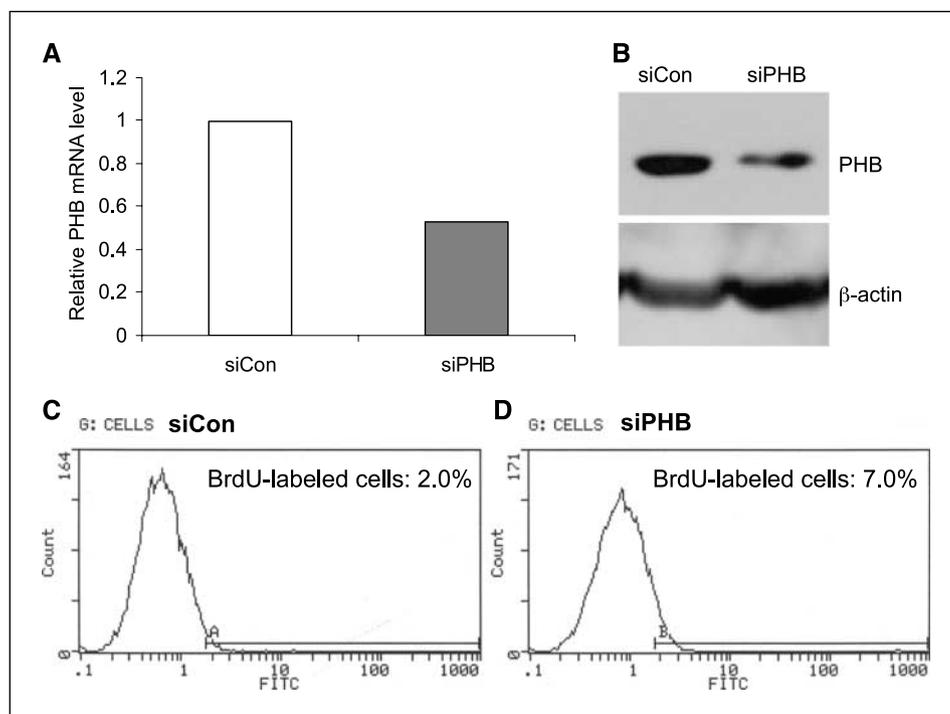


Figure 5. Correlation of silencing of prohibitin by RNAi with increased BrdUrd incorporation in MCF-7 cells. *A* and *B*, real-time RT-PCR (*A*) and Western blot (*B*) analysis of prohibitin expression after siRNA transfection, confirming knockdown of prohibitin at both mRNA and protein level. *C* and *D*, representative FACS analysis of BrdUrd incorporation in control siRNA-transfected (*siCon*; *C*) and prohibitin siRNA-transfected (*siPHB*; *D*) MCF-7 cells. Increased BrdUrd incorporation is observed after prohibitin knockdown. Experiments were repeated twice.

The nature of the protein bands at 90 to 130 kDa is not clear at this time. Coimmunoprecipitation of endogenous prohibitin showed the formation of prohibitin homomers. This is consistent with the large prohibitin spots (possibly prohibitin homomers) observed in the nuclei in Fig. 3. To test the effect of prohibitin expression on vitamin D-induced cellular response, the MCF-7 cells expressing tetracycline-induced prohibitin were treated with $1\alpha(\text{OH})_2\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ in the presence and absence of doxycycline for 4 days and the cell number in each well was determined by direct counting (Fig. 4*F* and *G*). Results showed that induction of prohibitin expression using doxycycline significantly inhibited cell proliferation, indicating that prohibitin itself has antiproliferative effect, which is supported by previous reports (11). Incubation of *Tet-On* MCF-7 cells for 4 days with 0.5 $\mu\text{mol/L}$ $1\alpha(\text{OH})_2\text{D}_3$ or 10 nmol/L $1,25(\text{OH})_2\text{D}_3$ effectively inhibited cell proliferation by $\sim 27\%$ in the absence of doxycycline, whereas vitamin D treatments in the presence of doxycycline (*Tet-On*, prohibitin is overexpressed) inhibited cell proliferation by $\sim 50\%$ in these cells. On the other hand, the incubation of *Non-Tet-On* MCF-7 cells with $1,25(\text{OH})_2\text{D}_3$ in the presence of doxycycline had no additive inhibitory effect on cell proliferation, indicating that the additive effect of vitamin D treatment is associated with the overexpression of prohibitin. These results support the notion that prohibitin is involved in the antiproliferative effect of vitamin D and at least partially contributes to cellular sensitivity to vitamin D.

Silencing of prohibitin using RNAi increased BrdUrd incorporation and cell distribution in S phase of cell cycle in MCF-7 cells. Given that prohibitin is a target gene of vitamin D and that it contributes to the antiproliferative action of vitamin D, we investigated the effect of knockdown of prohibitin by RNAi on cell proliferation in MCF-7 cells. Cell proliferation was evaluated using BrdUrd incorporation assay and cell cycle analysis. Transfection for 24 hours followed by 48 hours of incubation with regular culture medium containing 5% FBS decreased prohibitin mRNA by 50% in prohibitin siRNA-transfected cells as measured

by real-time RT-PCR (Fig. 5*A*) and was accompanied with decreased prohibitin protein expression by 80% as evaluated by Western blot analysis (Fig. 5*B*). BrdUrd incorporation assay using FACS analysis showed a significant increase (>3 -fold) in BrdUrd-labeled cells after prohibitin knockdown (Fig. 5*C* and *D*) compared with nonsilencing control siRNA-transfected cell. Cell cycle analysis of prohibitin siRNA-transfected MCF-7 cells showed increased cell distribution in S phase and decreased cell distribution in G_1 - G_0 phase of cell cycle (Table 1), confirming the results of BrdUrd incorporation assay after prohibitin is silenced by RNAi.

Prohibitin is not involved in CYP24 transactivation by vitamin D. Because prohibitin is involved in the antiproliferative action of vitamin D, we asked if prohibitin is involved in the CYP24 transactivation by vitamin D. *CYP24* is an established direct target gene of VDR and is also a very sensitive marker for vitamin D response. We initially hypothesized that prohibitin expression affects the CYP24 transcription expression if a direct physical interaction between prohibitin and VDR was present, even if it was not detectable by coimmunoprecipitation. We did real-time RT-PCR analysis of CYP24 transactivation by vitamin D in the

Table 1. Cell cycle effects of prohibitin silencing on MCF-7 cells

	G_1 - G_0	S	G_2 -M
Control siRNA	66.8 ± 2.1	31.7 ± 1.1	1.5 ± 0.4
Prohibitin siRNA	60.4 ± 1.9	36.5 ± 1.5	3.1 ± 0.7

NOTE: Cell cycle analysis of control siRNA-transfected and prohibitin siRNA-transfected MCF-7 cells. Cells were incubated with siRNA for 24 hours and then in fresh culture medium containing 5% FBS for another 48 hours. Values are mean \pm SD of triplicate samples.

Tet-On MCF-7 cells (Fig. 6A) and prohibitin siRNA-transfected MCF-7 cells (Fig. 6B). In MCF-7 *Tet-On* model, cells were pretreated with or without doxycycline for 24 hours followed by treatment with 1,25(OH)₂D₃ (10 nmol/L) for additional 24 hours. As shown in Fig. 6A, treatment with 1,25(OH)₂D₃ dramatically induced CYP24 mRNA expression as expected; however, induction of prohibitin expression by pretreatment with doxycycline did not affect CYP24 mRNA expression induced by 1,25(OH)₂D₃ (Fig. 6A). Similarly, silencing of prohibitin by siRNA in MCF-7 cells did not affect CYP24 transactivation induced by 1,25(OH)₂D₃ and 1 α (OH)D₅ (0.5 μ mol/L; Fig. 6B), indicating that CYP24 transactivation by vitamin D in *Tet-On* MCF-7 cells and prohibitin siRNA-transfected MCF-7 cells was not affected by prohibitin expression level. To further confirm this, we also transiently transfected MDA-MB231 cells expressing low level of prohibitin (Fig. 2A) with prohibitin expression vector (pcDNA3.1PHB) to overexpress prohibitin in this cell line. After transfection, cells were treated with 1,25(OH)₂D₃ for 24 hours. As shown in Fig. 6C, transient transfection caused dramatic overexpression of prohibitin as evaluated by real-time RT-PCR, but CYP24 transactivation by 1,25(OH)₂D₃ was not significantly affected, supporting the results obtained from MCF-7 cells.

Discussion

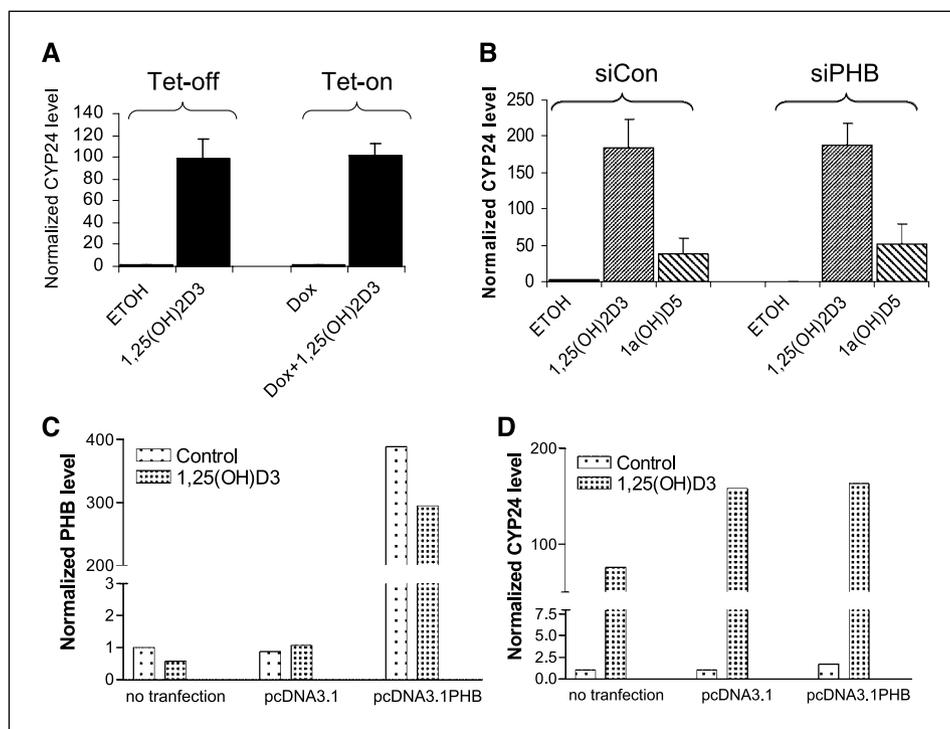
VDR signaling pathway is fundamental to chemoprevention and therapy of breast cancer using vitamin D analogues. Although the antiproliferative effects of vitamin D in breast cancer cells are well recognized, the detailed signaling pathway and target genes involved in this process still need to be identified and characterized. In this study, we identified prohibitin as a novel target gene of VDR function, which is involved in the antiproliferative action of vitamin D.

Our initial *in silico* effort identified two potential VDR/RXR binding sites in the promoter region of prohibitin. Although whether VDR directly binds to the promoter of prohibitin still

needs further experimental verification, our real-time RT-PCR and immunoblot analyses have confirmed that prohibitin is a primary vitamin D target gene. Many vitamin D target genes, including *osteopontin*, *osteocalcin*, *calbindin*, etc., which are classically involved in calcium homeostasis and bone metabolism, have been identified. In addition, a few cell cycle-regulated genes, such as *p21* and *cyclin C*, are also known to be regulated by vitamin D and related to the antiproliferative action of vitamin D. The identification of prohibitin as a novel vitamin D target gene adds a new member to the list of vitamin D-responding genes related to cell cycle control. The transcriptional and translational regulation of prohibitin by vitamin D is very similar to that of cyclin C (30, 31) in breast cancer cells. Interestingly, at mRNA level, both prohibitin and cyclin C are quickly up-regulated after vitamin D treatment with peaks at 8 and 3 hours after treatment, respectively, followed by gradual reduction in expression, whereas CYP24 induction lasts for at least 24 hours. At peak mRNA levels, both prohibitin and cyclin C are ~2-fold of their basal levels (30), whereas at protein level both are >2-fold of their basal levels (31). Such quick, transient, and significant regulation of prohibitin by vitamin D might be a sign of tight control of the expression of this gene in response to vitamin D.

Prohibitin has multiple functions ranging from a role in aging (32), epithelial cell migration (23), mitochondrial inheritance (33), and as a chaperone for the stabilization of mitochondrial proteins in yeast (9) to its role in cell cycle control, apoptosis (12), and as associated molecules in cell surface receptors in mammalian cells (34, 35). Accumulative evidence shows its nuclear function in transcription regulation. Our data show that a portion of prohibitin colocalizes with VDR in the nuclei of MCF-7 cells, and it is involved in VDR-mediated antiproliferative action of vitamin D. Prohibitin was found to interact with multiple molecules in the cells, including Bap37, Rb, p53, E2F, cRaf-1, α -actinin, and annexin A2 (36), and also form homomers with prohibitin itself (Fig. 4E). These

Figure 6. Effects of prohibitin expression on the CYP24 transactivation induced by vitamin D. **A**, real-time RT-PCR analysis of CYP24 transcription level after treatment of prohibitin *Tet-On* MCF-7 cells with 1,25(OH)₂D₃ (10 nmol/L) in the presence (*Tet-On*) or absence (*Tet-Off*) of doxycycline (20 ng/mL). *Columns*, mean of three samples with duplicate analyses of each sample; *bars*, SE. **B**, knockdown of prohibitin by RNAi in MCF-7 cells did not affect CYP24 transactivation by vitamin D. *Columns*, mean of three independent experiments done in duplicate; *bars*, SE. Cells were treated for 48 hours with 1,25(OH)₂D₃ (10 nmol/L) or 1 α (OH)D₅ (0.5 μ mol/L) after transfection of siRNA. **C** and **D**, prohibitin overexpression in MDA-MB231 cells does not affect CYP24 transactivation. MDA-MB231 cells were transiently transfected with prohibitin expression vector, and then cells were treated by 1,25(OH)₂D₃ (10 nmol/L) for 24 hours. Prohibitin overexpression (**C**) and CYP24 transactivation (**D**) were measured using real-time RT-PCR. *Columns*, mean of two independent experiments.



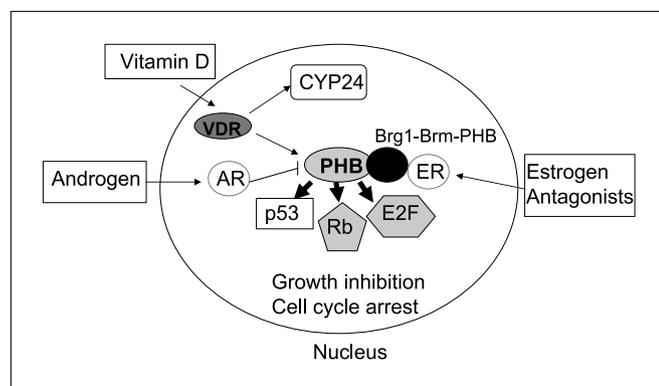


Figure 7. Schematic model to explain potential mechanism of action of nuclear prohibitin in cancer cells. Prohibitin is up-regulated by VDR-mediated action of vitamin D, which in turn inhibits cell proliferation. Action of estrogen antagonists mediated via ER also requires prohibitin for its antiproliferative action. On the other hand, prohibitin is down-regulated by androgen and in turn enhances cell proliferation. Thus, prohibitin might function as a target gene for steroid hormones and modulate the transcription by recruiting Brg-1/Brm. Prohibitin also interacts with E2F, p53, and pRb. These interactions ultimately result in the cell cycle arrest or growth inhibition. AR, androgen receptor.

results suggest that prohibitin may function as large complex homomers, heteromers, or multimers with other molecules regardless of its location. Because the primary sequence of prohibitin has predicted α -helical structures in its COOH-terminal end (37, 38), which forms the basis for interactions between prohibitin and other proteins, we initially proposed that prohibitin could physically interact with VDR and regulate VDR-mediated cellular response and placed much effort in identifying the interaction between the two molecules. However, we were not able to coprecipitate prohibitin with VDR directly from cell lysates using multiple breast epithelial cell lines and various experiment conditions, indicating that the affinity between VDR and prohibitin in the intact cells could be very low or they do not physically interact with each other in the cells. Later experiments on CYP24 transactivation by vitamin D further support this notion because CYP24 is a very sensitive marker in response to vitamin D and a direct target gene of VDR. If prohibitin physically binds to VDR, the expression level of prohibitin would affect CYP24 transactivation by vitamin D. However, the results indicated that prohibitin-mediated vitamin D response on cell proliferation was independent of CYP24 transactivation.

The detailed mechanism by which prohibitin can induce antiproliferative and cell cycle regulatory activity, although intriguing, is yet to be established. There is even considerable controversy about the function of prohibitin localized in the nuclei. Because emerging data show the diversified localization and translocation, it is not surprising to see that prohibitin is associated with multiple functions in the cells. It is believed that prohibitin can inhibit cell proliferation by directly interacting with both pRb and p53 pathways, suggesting a mechanism dependent of

the pRb and p53 pathways is involved in the antiproliferative effects of prohibitin (10). Recent evidence has shown that prohibitin recruits Brg-1 and Brm to E2F-responsive promoters and that this recruitment is required for the repression of E2F-mediated transcription by prohibitin (35, 39). Brg-1 and Brm are involved in chromatin remodeling and mediating hormone-dependent transcriptional activation by nuclear receptors (10), which could provide a common explanation for the diverse effects of nuclear prohibitin in different cells. In breast cancer cells, the depletion of prohibitin by antisense or siRNA strategies inhibits the growth-inhibitory effect of the antiestrogen 4-hydroxytamoxifen and ICI 182,780. Moreover, the transfection of a construct expressing residues 304 to 357 of E2F, which inhibits the interaction between prohibitin and E2F, blocked the antiestrogenic effect, suggesting an important role of prohibitin in the antiproliferative actions of estrogen antagonists (39). Similar mechanism may also be involved in the antiproliferative action of vitamin D. In the present study, we observed that prohibitin is up-regulated by vitamin D treatment and its level correlates with the cellular sensitivity to vitamin D. Overexpression of prohibitin enhanced the antiproliferative effect of vitamin D in MCF-7 cells, whereas knockdown of prohibitin was associated with increased proliferating cells in the whole-cell population. These results identify prohibitin as one of the important molecules involved in the antiproliferative action of vitamin D. Similarly and in consistence with our studies, in androgen-stimulated LNCap prostate cancer cells, prohibitin is down-regulated (13). Interestingly, vitamin D3 and its analogue have been reported to suppress the androgen-stimulated growth of mouse mammary carcinoma SC-3 cells (40) and block *in vitro* and *in vivo* androgen-stimulated prostate cell growth (41).

Although prohibitin is involved in the antiproliferative action of vitamin D, it is not involved in the CYP24 transactivation by vitamin D, suggesting that vitamin D could use different VDR-mediated pathways for its antiproliferative effects and CYP24 activation, respectively. Previous studies have also reported that the growth-inhibitory pathway of $1,25(\text{OH})_2\text{D}_3$ could be selectively abrogated, whereas cells remained sensitive to direct $1,25(\text{OH})_2\text{D}_3$ signaling (induction of CYP24; ref. 42). A proposed interaction of prohibitin with VDR and other steroid receptors is diagrammatically shown in Fig. 7.

This is the first report to indicate that prohibitin is involved in the antiproliferative action of vitamin D. Our results, together with the findings of others for the role of prohibitin in the action of androgen (13) and estrogen antagonists (39), suggest that prohibitin may regulate the action of steroid hormones.

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References

- Mehta RG, Moriarty RM, Mehta RR, et al. Prevention of preneoplastic mammary lesion development by a novel vitamin D analogue, 1α -hydroxyvitamin D₅. *J Natl Cancer Inst* 1997;89:212-8.
- Mehta RG. Stage-specific inhibition of mammary carcinogenesis by 1α -hydroxyvitamin D₅. *Eur J Cancer* 2004;40:2331-7.
- Hussain EA, Mehta RR, Ray R, Das Gupta TK, Mehta RG. Efficacy and mechanism of 1α -hydroxy-24-ethyl-cholecalciferol ($1\alpha(\text{OH})\text{D}_5$) in breast cancer prevention and therapy. *Recent Results Cancer Res* 2003;164:393-411.
- Mehta R, Hawthorne M, Uselding L, et al. Prevention of *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis in rats by 1α -hydroxyvitamin D₅. *J Natl Cancer Inst* 2000;92:1836-40.
- Hussain EA, Peng X, Mehta RR, Mehta RG. Growth inhibition of carcinogen-transformed MCF-12F breast epithelial cells and hormone-sensitive BT-474 breast cancer cells by 1α -hydroxyvitamin D₅. *Carcinogenesis* 2006;27:551-9.
- Sato T, Saito H, Swensen J, et al. The human prohibitin gene located on chromosome 17q21 is mutated in sporadic breast cancer. *Cancer Res* 1992;52:1643-6.
- Coates PJ, Nenulit R, McGregor A, et al. Mammalian

- prohibitin proteins respond to mitochondrial stress and decrease during cellular senescence. *Exp Cell Res* 2000; 265:262–73.
8. Nijtmans LG, Artal SM, Grivell LA, Coates PJ. The mitochondrial PHB complex: roles in mitochondrial respiratory complex assembly, ageing, and degenerative disease. *Cell Mol Life Sci* 2002;59:143–55.
 9. Nijtmans LG, de Jong L, Artal Sanz M, et al. Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins. *EMBO J* 2000;19:2444–51.
 10. Mishra S, Murphy LC, Nyomba BL, Murphy LJ. Prohibitin: a potential target for new therapeutics. *Trends Mol Med* 2005;11:192–7.
 11. Nuell MJ, Stewart DA, Walker L, et al. Prohibitin, an evolutionarily conserved intracellular protein that blocks DNA synthesis in normal fibroblasts and HeLa cells. *Mol Cell Biol* 1991;11:1372–81.
 12. Fusaro G, Dasgupta P, Rastogi S, Joshi B, Chellappan S. Prohibitin induces the transcriptional activity of p53 and is exported from the nucleus upon apoptotic signaling. *J Biol Chem* 2003;278:47853–61.
 13. Gamble SC, Odontiadis M, Waxman J, et al. Androgens target prohibitin to regulate proliferation of prostate cancer cells. *Oncogene* 2004;23:2996–3004.
 14. Thompson WE, Asselin E, Branch A, et al. Regulation of prohibitin expression during follicular development and atresia in the mammalian ovary. *Biol Reprod* 2004; 71:282–90.
 15. Wang S, Nath N, Fusaro G, Chellappan S. Rb and prohibitin target distinct regions of E2F1 for repression and respond to different upstream signals. *Mol Cell Biol* 1999;19:7447–60.
 16. Wang S, Nath N, Adlam M, Chellappan S. Prohibitin, a potential tumor suppressor, interacts with RB and regulates E2F function. *Oncogene* 1999;18:3501–10.
 17. Wang S, Fusaro G, Padmanabhan J, Chellappan S. Prohibitin co-localizes with Rb in the nucleus and recruits N-CoR and HDAC1 for transcriptional repression. *Oncogene* 2002;21:8388–96.
 18. Wang KJ, Wang RT, Zhang JZ. Identification of tumor markers using two-dimensional electrophoresis in gastric carcinoma. *World J Gastroenterol* 2004;10: 2179–83.
 19. Asamoto M, Cohen SM. Prohibitin gene is overexpressed but not mutated in rat bladder carcinomas and cell lines. *Cancer Lett* 1994;83:201–7.
 20. Shan L. cDNA microarray profiling of rat mammary gland carcinomas induced by 2-amino-1-methyl-6-phenylimidazo 4,5- β -pyridine and 7,12-dimethylbenz- α -anthracene. *Carcinogenesis* 2002;23:1561–8.
 21. Menssen A, Hermeking H. Characterization of the c-MYC-regulated transcriptome by SAGE: identification and analysis of c-MYC target genes. *Proc Natl Acad Sci U S A* 2002;99:6274–9.
 22. Haggerty TJ, Zeller KI, Osthus RC, Wonsey DR, Dang CV. A strategy for identifying transcription factor binding sites reveals two classes of genomic c-Myc target sites. *Proc Natl Acad Sci U S A* 2003;100:5313–8.
 23. Rajalingam K, Wunder C, Brinkmann V, et al. Prohibitin is required for Ras-induced Raf-MEK-ERK activation and epithelial cell migration. *Nat Cell Biol* 2005;7:837–43.
 24. Peng X, Maruo T, Cao Y, et al. A novel RAR β isoform directed by a distinct promoter P3 and mediated by retinoic acid in breast cancer cells. *Cancer Res* 2004;64: 8911–8.
 25. Peng X, Wood S, Bratescu L, Shilkaitis A, Christov K. Retinoids suppress premalignant MCF10AT but not malignant MCF10CA1a breast epithelial cells *in vivo*. Role of retinoic acid receptor β 2 expression. *Cancer Lett* 2005;222:153–63.
 26. Quandt K, Frech K, Karas H, Wingender E, Werner T. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* 1995;23:4878–84.
 27. Messegue X, Escudero R, Farré D, Nuñez O. PROMO: detection of known transcription regulatory elements using species-tailored searches. *Bioinformatics* 2002;18: 333–4.
 28. Lissos TW, Beno DW, Davis BH. 1,25-Dihydroxyvitamin D₃ activates Raf kinase and Raf perinuclear translocation via a protein kinase C-dependent pathway. *J Biol Chem* 1993;268:25132–8.
 29. Beno DW, Brady LM, Bissonnette M, Davis BH. Protein kinase C and mitogen-activated protein kinase are required for 1,25-dihydroxyvitamin D₃-stimulated Egr induction. *J Biol Chem* 1995;270:3642–7.
 30. Sinkkonen L, Malinen M, Saavalainen K, Vaisanen S, Carlberg C. Regulation of the human cyclin C gene via multiple vitamin D₃-responsive regions in its promoter. *Nucleic Acids Res* 2005;33:2440–51.
 31. Polly P, Danielsson C, Schrader M, Carlberg C. Cyclin C is a primary 1 α ,25-dihydroxyvitamin D₃ responding gene. *J Cell Biochem* 2000;77:75–81.
 32. Piper PW, Bringloe D. Loss of prohibitins, though it shortens the replicative life span of yeast cells undergoing division, does not shorten the chronological life span of G₀-arrested cells. *Mech Ageing Dev* 2002;123: 287–95.
 33. Thompson WE, Ramalho-Santos J, Sutovsky P. Ubiquitination of prohibitin in mammalian sperm mitochondria: possible roles in the regulation of mitochondrial inheritance and sperm quality control. *Biol Reprod* 2003;69:254–60.
 34. Kolonin MG, Saha PK, Chan L, Pasqualini R, Arap W. Reversal of obesity by targeted ablation of adipose tissue. *Nat Med* 2004;10:625–32.
 35. Wang S, Zhang B, Faller DV. Prohibitin requires Brg-1 and Brm for the repression of E2F and cell growth. *EMBO J* 2002;21:3019–28.
 36. Bacher S, Achatz G, Schmitz ML, Lamers MC. Prohibitin and prohibitone are contained in high-molecular weight complexes and interact with α -actinin and annexin A2. *Biochimie* 2002;84:1207–20.
 37. Chou PY, Fasman GD. Empirical predictions of protein conformation. *Annu Rev Biochem* 1978;47: 251–76.
 38. Garnier J, Osguthorpe DJ, Robson B. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J Mol Biol* 1978;120:97–120.
 39. Wang S, Zhang B, Faller DV. BRG1/BRM and prohibitin are required for growth suppression by estrogen antagonists. *EMBO J* 2004;23:2293–303.
 40. Kawata H, Kamiakito T, Takayashiki N, Tanaka A. Vitamin D₃ suppresses the androgen-stimulated growth of mouse mammary carcinoma SC-3 cells by transcriptional repression of fibroblast growth factor 8. *J Cell Physiol* 2006;207:793–9.
 41. Crescioli C, Ferruzzi P, Caporali A, et al. Inhibition of spontaneous and androgen-induced prostate growth by a nonhypercalcemic calcitriol analog. *Endocrinology* 2003;144:3046–57.
 42. Verlinden L, Eelen G, Beullens I, et al. Characterization of the condensin component Cnap1 and protein kinase Melk as novel E2F target genes down-regulated by 1,25-dihydroxyvitamin D₃. *J Biol Chem* 2005;280: 37319–30.

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