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Role of local bioactivation of vitamin D by CYP27A1 and CYP2R1 in the control of cell growth in normal endometrium and endometrial carcinoma

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Vitamin D (VD) deficiency has been suggested as a risk factor for cancer. One recognized mechanism is that the low-serum 25-hydroxyvitamin D (25(OH)D) of VD deficiency reduces intratumoral 25(OH)D conversion to 1α,25-dihydroxyvitamin D (1,25D, the hormonal form of VD), compromising 1,25D-VD receptor (VDR) antitumoral actions. Reduced tumoral VDR and increased CYP24A1, the enzyme that degrades 1,25D and 25(OH)D, further worsen cancer progression. Importantly, in cells expressing CYP27A1 and/or CYP2R1, which convert inert VD into 25(OH)D, low-serum VD may reduce intratumoral 25(OH)D synthesis thereby compromising VDR antitumoral actions because 25(OH)D can activate the VDR directly and enhance 1,25D-VDR action. Therefore, this study examined whether abnormal endometrial expression of CYP27A1 and/or CYP2R1 may impair VDR-antiproliferative properties in endometrial carcinoma (EC). Immunohistochemical analysis of tissue microarrays of normal human endometrium (NE; $n = 60$) and EC ($n = 157$) showed the expected lower VDR expression in EC ($P = 0.0002$). Instead, CYP24A1 expression was lower in EC compared with NE, while CYP27A1 and CYP2R1 expressions were higher ($P = 0.0002$; $P = 0.03$). Furthermore, in NE and EC, CYP2R1 and CYP27A1 expression correlated directly with nuclear VDR levels, an indicator of ligand-induced VDR activation, and inversely with the proliferation marker Ki67. Accordingly, in the endometrioid carcinoma cell lines IK, RL95/2 and HEC1-A, which express VDR, CYP27A1, and CYP2R1, VD efficaciously reduced cell viability and colony number, with a time course that paralleled actual increases in both intracellular 25(OH)D and nuclear VDR levels. Thus, VD may protect from EC progression in part through increased intratumoral 25(OH)D production by CYP27A1 and CYP2R1 for autocrine/paracrine enhancement of 1,25D-VDR-antiproliferative actions.

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Endometrial carcinoma (EC) results from neoplastic transformation of normal endometrium (NE)[1] leading to two main clinicopathological variants: endometrioid carcinoma (EEC) and non-endometrioid carcinomas (NEEC). EECs are estrogen-related tumors, frequently well differentiated, and developing mostly in peri- and postmenopausal women. Low grades (1 and 2) tumors are usually confined to the uterus (stage I) while grade 3 EECs are less frequent, with a higher tendency for extrauterine spread. NEEC, either serous or clear cell (CC) carcinomas, occur in older women, are estrogen-unrelated tumors, with aggressive behavior and frequent extrauterine spread to the peritoneum or lymph nodes.[2,3]

One risk factor for human cancer that can be safely modified is vitamin D (VD) deficiency/insufficiency.[4] Indeed,

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preclinical and clinical studies demonstrated that 1,25-dihydroxyvitamin D (1,25D, the most active endogenous VD metabolite and a potent steroid hormone) and/or its less calcemic synthetic analogs are efficacious, preventive, and therapeutic anticancer agents.[5–8] However, cholecalciferol (VD_3) and its homolog in plants, ergocalciferol (VD_2), are inactive compounds that require bioactivation to exert their biological actions. In mammals (Figure 1), the inactive cholecalciferol, synthesized from its precursor, 7-dehydrocholesterol through skin exposure to UVB light, is first 25-hydroxylated mainly in the liver by either mitochondrial CYP27A1 or microsomal CYP2R1. In humans, CYP2R1 is the most critical VD-25-hydroxylase, as mutations in this enzyme, but not in CYP27A1, cause VD insuficiency,[9] defined as serum 25-hydroxyvitamin D (25(OH)D) below 20 ng/ml.[10,11] The final 1α-hydroxylation of 25(OH)D to produce 1,25D is catalyzed by mitochondrial CYP27B1, mainly in the kidney. 1,25D biological actions require 1,25D binding to the cytosolic VD receptor (VDR), which translocates to the nucleus and acts as a transcriptional regulator of the expression of more than 200 genes controlling normal and cancer cell growth, differentiation, DNA repair, apoptosis, angiogenesis, and metastatic potential.[12–14] The endometrium is a target of 1,25D/VDR antitumoral actions: in the EEC cell lines, IK, HEC-1A, and RL-95/2, 1,25D treatment induces differentiation, growth arrest,[15] and apoptosis.[16] Importantly, the increased risk for colorectal,[17] breast,[18] and prostate[8,19] cancer in VD-deficient individuals is not the result of defective renal 1,25D synthesis because serum 1,25D, mostly of renal origin, remains normal until serum 25(OH)D levels decrease below 4 ng/ml.[20] Instead, during VD deficiency, low serum 25(OH)D limits 1,25D production by numerous non renal cells expressing CYP27B1[21] thus impairing local VDR antiproliferative actions.

Normal human endometrial cells express CYP27B1,[22] whose increases during pregnancy result in higher serum 1,25D to meet the enhanced calcium requirements.[23,24] However, in the course of human cancer, several mechanisms worsen the defective local 1,25D synthesis and VDR antitumoral actions induced by VD deficiency aggravating tumor progression. These include: (a) reductions in VDR and CYP27B1 expression; (b) the appearance of CYP27B1 splicing variants,[25] and (c) increases in CYP24A1, the enzyme that degrades both 1,25D and its precursor 25(OH)D.[26–28] Indeed, the VDR-null mice has higher propensity for premalignant lesions in the descending colon than heterozygous VDR+/− or wild-type mice.[29] Also, increases in CYP24A1 were associated to poor prognosis in high-grade colonic,[30] pulmonary, ovarian,[31] and breast tumors.[32]

A novel mechanism may also link VD deficiency with higher cancer risk: defective local VD activation to 25(OH)D in cells expressing CYP27A1 or CYP2R1, as breast and prostate cells,[12] because locally produced 25(OH)D can activate the VDR directly,[33,34] and also enhance 1,25D/VDR growth inhibition, as in prostate cancer cell lines. The former has been conclusively demonstrated in cells from the CYP27B1-null mice, unable to convert 25(OH)D to 1,25D,

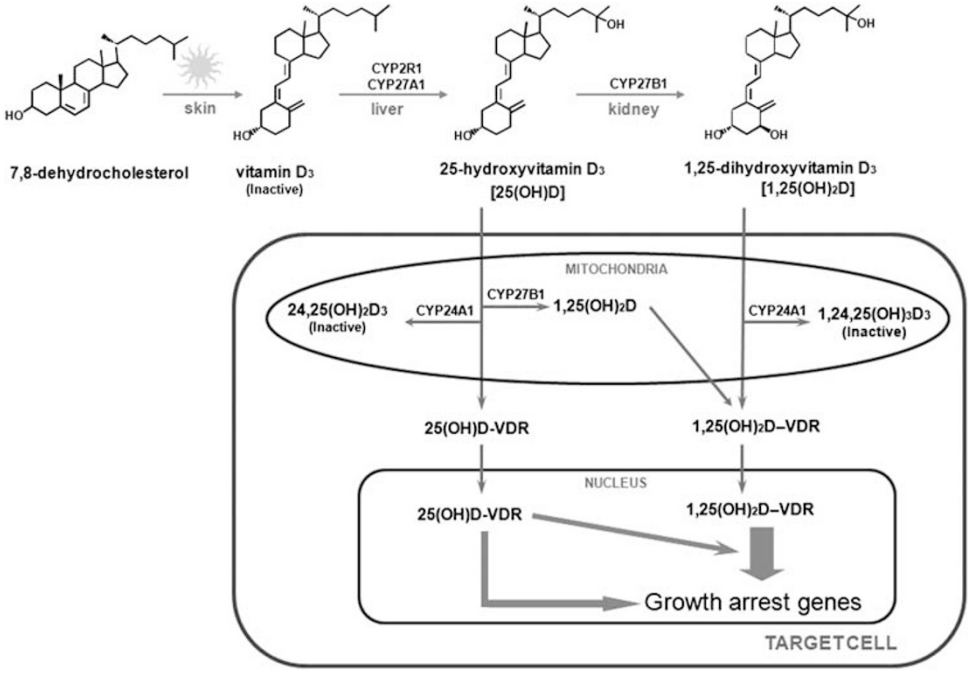

Figure 1 VD synthesis, systemic and cell-specific bioactivation, catabolism, and ligand-specific VDR activation for antiproliferative actions of distinct efficacy. (See text for details).

and also in various cell types using either specific inhibition of the 1-hydroxylation of 25(OH)D,[33,35] or VD metabolites that cannot be 1-hydroxylated.[36] However, the potency of 25(OH)D-bound VDR is at least 100-fold lower than that of 1,25D-bound VDR. Because EC cells express CYP27A1,[37] this study examined whether a defective endometrial conversion of VD_3 to 25(OH)D may contribute to alter 1,25D/VDR control of normal and cancerous endometrial growth. To this end, CYP27A1 and CYP2R1 expression was examined in proliferative *vs* secretory NE, and also in EC using human tissue microarrays (TMAs), and correlated with nuclear VDR, a marker of VDR activation, with the cell proliferation marker Ki67, and with physiological, prognostic, and clinicopathological data. The accuracy of TMA analyses in reflecting the ability of EC cells to convert VD into 25(OH)D to induce VDR translocation to the nucleus and growth arrest was directly assessed using VD treatment of IK, HEC-1A, and RL-95/2 cells, which derive from EEC, the prevalent EC in our population, after demonstrating that these 1,25D-responsive cells also express CYP27A1 and CYP2R1.

MATERIALS AND METHODS

Tissue samples were obtained from Hospital Universitari Arnau de Vilanova de Lleida and Hospital de Sant Pau, Barcelona, Spain. A specific informed consent was obtained from each patient, and the study was approved by the local Ethical Committee. Material included 60 samples of NE tissue (age ranging from 25 to 55, mean = 43) and 157 samples of EC (age range from 41 to 86, mean = 66), which were fixed in formalin and embedded in paraffin. Overall, the series of 157 EC included 47 EEC grade I, 58 EEC grade 2, 26 EEC grade 3, 15 serous carcinomas (SC), 4 CC carcinomas, and 7 carcinosarcomas. A total of 108 tumors were stage I, 15 were stage II, 22 were stage III, and 1 was stage IV. Staging information was incomplete in 11 cases.

Tissue Microarrays

Three TMAs were constructed using a Tissue Arrayer device (Beecher Instrument, MD). The first TMA was constructed from 60 paraffin-embedded samples of NE in different phases of the endometrial cycle (20 proliferative and 40 secretory).

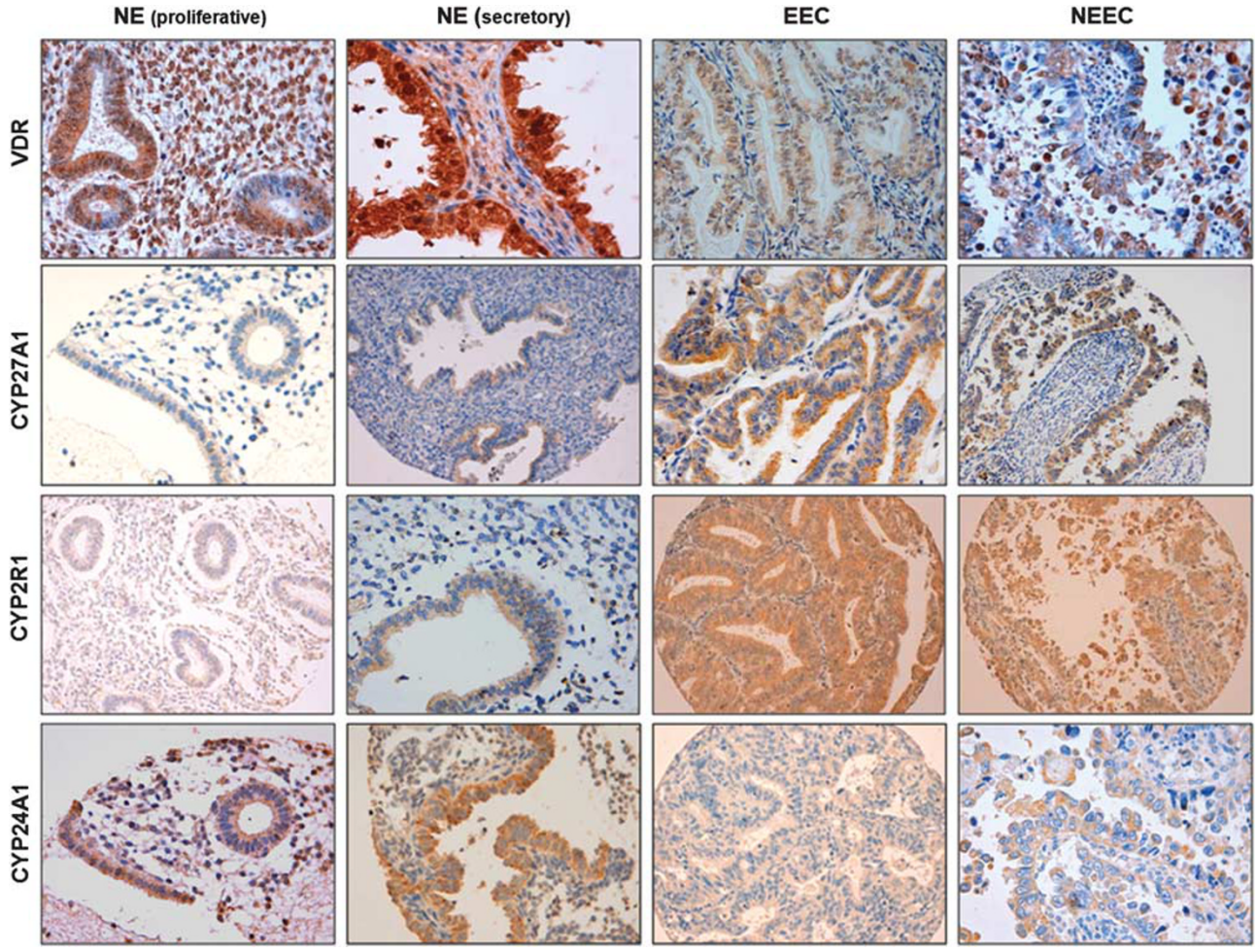

Figure 2 Distinct expression of VDR and VD-metabolizing enzymes in normal and cancerous endometrium. Representative immunostaining of VDR, CYP27A1, CYP2R1, and CYP24A1 in proliferative and secretory NE, and in EEC and NEEC.

The second TMA was composed of 95 EC and a third TMA containing 62 specimens from EC and their adjacent NE was also included in the study. All the samples were histologically reviewed and representative areas were marked in the corresponding paraffin blocks. Two selected cylinders (0.6 mm diameter) from two different areas were included in each EC case.

Immunohistochemical Study

Sample sections of 3 μm were blocked for endogenous peroxidase and incubated with primary antibodies: VDR (1:1000), CYP27A1 (1:500), CYP2R1 (1:300) (Abcam). CYP24A1 (1:100, Sigma), and Ki67 (Ready To Use (RTU), DAKO). The reaction was visualized with the EnVision FLEX Detection Kit (DAKO). Sections were counterstained with haematoxylin. Immunohistochemical results were evaluated by two pathologists following uniform preestablished criteria. Staining intensity and % positive cells were graded semi-quantitatively. Histological scores were obtained from each sample as follows: histoscore $= 1\times$ (% light staining) $+ 2\times$ (% moderate staining) $+ 3\times$ (% strong staining), which ranged from 0 (no immunoreaction) to 300 (maximum immunoreactivity). The reliability of such scores for interpretation of immunohistochemical staining in EC TMAs has been shown previously.[38] All statistical comparisons used the average histoscore from the two different tumor cylinders for each specimen included in the TMAs.

Statistical Analysis

The levels of VDR, CYP27A1, CYP2R1, and CYP24A1 are described by their respective range, mean, and s.d. For each biomarker, we assessed differences between endometrial cycle

Table 1A Statistical analysis of nuclear VDR immunoexpression levels in NE and EC

	n	min	max	Mean	s.d.	Fold change	*P*-value
Normal samples							
All	55	0.0	290.0	142.5	90.9		
Proliferative	14	0.0	135.0	54.3	46.2	1.00	
Secretory	41	10.0	290.0	171.9	82.9	**3.17**	**0.00002**
Normal vs tumor samples							
All	192	0.0	290.0	105.7	77.2		
Normal	55	0.0	290.0	142.5	90.9	1.00	
Tumor	137	0.0	270.0	90.7	65.5	**0.64**	**0.0003**
Tumor samples							
All	137[a]	0.0	270.0	90.7	65.5		
NEEC	22	30.0	250.0	131.8	71.8	1.00	
EEC	114	0	270	82.4	61.7	**1.60**	**0.002**
Grade 1	39	15	250	87.6	57.0	1.0	
Grade 2	52	0	250	91.2	62.1	1.0	
Grade 3	46	0	270	92.8	76.5	1.0	
					Increasing trend *P*-value	0.71	
					Global *P*-value	0.93	
Stage I	77	0	270	81.49	60.7	1.0	
Stage II	23	0	250.0	77.61	57.1	1.0	
Stage III	15	0	210.0	92.53	60.2	1.2	
Stage IV	1	20	20.0	20.00		0.2	
					Trend *P*-value	0.91	
					Global *P*-value	0.64	

Abbreviations: min and max, minimum and maximum values; *n*, number of patients; s.d., standard deviation; VDR, vitamin D receptor.
Fold change denotes the fold change in biomarker expression relative to the mean value for the first category. *P*-values from a Mann–Whitney, Kruskal–Wallis (global *P*-value), or trend tests. Values are bolded when statistically different.
[a]Denotes one unclassified specimen of EC.

Table 1B Statistical analysis of cytosolic VDR immunoexpression levels in NE and EC

	n	min	max	Mean	s.d.	Fold change	*P*-value
Normal samples							
All	48	10	290	119.9	70.3		
Proliferative	14	10	130	76.1	34.7	1.00	
Secretory	34	40	290	137.9	73.7	**1.81**	**0.006**
Normal vs tumor samples							
All	185	0	290	115.7	61.7		
Normal	48	10	290	119.9	70.3	1.00	
Tumor	137	0	285	114.2	58.5	0.95	0.89
Tumor samples							
All	137[a]	0	285	114.2	58.5		
NEEC	22	20	210	108.7	46.6	1.00	
EEC	114	0	285	114.5	60.3	1.05	0.71
Grade 1	38	30	270	115.1	59.7	1.00	
Grade 2	52	0	270	114.2	55.3	0.99	
Grade 3	46	10	285	111.4	61.3	0.97	
				Decreasing trend *P*-value	0.77		
					Global *P*-value	0.17	
Stage I	77	0	285	119.9	63.2	1.00	
Stage II	23	25	240	109.3	58.6	0.91	
Stage III	15	38	210	109.4	45.2	0.91	
Stage IV	1	30	30	30	NA	0.25	
				Decreasing trend *P*-value	0.22		
					Global *P*-value	0.42	

Abbreviations: EC, endometrial cancer; EEC, endometrioid carcinoma; min and max, minimum and maximum values; *n*, number of patients; NEEC, non-endometrioid carcinomas; s.d., standard deviation; VDR, vitamin D receptor.
Fold change denotes the fold change in biomarker expression relative to the mean value for the first category. *P*-values from a Mann–Whitney, Kruskal–Wallis (global *P*-value), or trend tests. Values are bolded when statistically different.
[a]Denotes one unclassified specimen of EC.

phases in normal samples, between NE and EC samples and finally, among EC samples (EEC *vs* NEEC, FIGO grade and stage). Fold changes measured the relative change between different conditions. The non-parametric Mann–Whitney test measured the statistical significance of the differences in biomarker immunoexpression. In addition, a Kruskal–Wallis test analyzed global differences between more than two conditions, to disregard normality assumptions, usually compromised by small sample sizes. All EC cases that met the inclusion criteria were studied. Therefore, differences in sample size among subgroups reflect the frequency of a particular tumor type in our patient population. A trend test was also conducted when suitable. The Pearson correlation coefficient and the non-parametric Spearman correlation coefficient were used to evaluate potential relationships between nuclear and cytoplasmic expression levels of a biomarker, or between histoscores for pairs of biomarkers. Values reported are the most conservative results. All analyses were performed using the R statistical package. Statistical significance was set at a threshold of 0.05.

Cell Lines and Culture Conditions

Three EC cell lines from EEC, Ishikawa 3-H-12(IK), RL-95/2, and HEC-1-A were used (ATCC, Manassas, USA). Cells were grown in Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, USA), 1 mmol/l HEPES, 1 mmol/l sodium pyruvate, 2 mmol/l L-glutamine (Sigma), and 1% of penicillin/

Table 2 Statistical analysis of Ki67 immunoexpression levels in NE and EC

	n	min	max	Mean	s.d.	Fold change	*P*-value
Normal samples							
All	47	0	70	14.9	22		
Proliferative	14	5	60	26.6	22.3	1.00	
Secretory	33	0	70	9.8	20.2	**0.37**	**0.0004**
Normal vs tumor samples							
All	181	0	95	25.2	24.7		
Normal	47	0	70	14.9	22	1.00	
Tumor	134	0	95	28.8	24.6	**1.93**	**<0.00001**
Tumor samples							
All	134[a]	0	95	28.8	24.6		
NEEC	21	0	95	41.3	23.4	1.00	
EEC	112	2	95	26.6	24.3	**0.64**	**0.004**
Grade 1	41	3	60	18.2	15.9	1.00	
Grade 2	50	2	90	27.1	25.6	1.49	
Grade 3	42	0	95	41.7	25.3	2.29	
				Increasing trend *P*-value	**0.00004**		
					Global *P*-value	**<0.00001**	
Stage I	81	3	95	29.9		1.00	
Stage II	24	0	90	31.1		1.04	
Stage III	14	10	85	40.4		1.35	
Stage IV	0						
				Increasing trend *P*-value	0.19		
					Global *P*-value	0.19	

Abbreviations: EC, endometrial cancer; EEC, endometrioid carcinoma; min and max, minimum and maximum values; *n*, number of patients; NE, normal endometrium; NEEC, non-endometrioid carcinomas; s.d., standard deviation.
Fold change denotes the fold change in biomarker expression relative to the mean value for the first category. *P*-values from a Mann–Whitney, Kruskal–Wallis (global *P*-value) or trend tests. Values are bolded when statistically different.
[a]Denotes one unclassified specimen of EC.

streptomycin (Sigma) at 37°C with saturating humidity and 5% CO_2.

Chemical Reagents

VD_3 (cholecalciferol) was obtained from Sigma. VD_3 stock (4.5 M in 100% ethanol) was accepted qualitatively when the ratio of the absorbances at 265 and 228 nm was higher than 1.6. The concentration was calculated from the ratio of the absorbance at 265 nm and the VD_3 molar extinction coefficient (18,200). 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was from Sigma.

Cell Viability Assays

EC cell viability after VD_3 treatment, at doses from 2 to 10 μM for 24, 48, or 72 h was measured using MTT assays following the manufacturer's instructions, and quantified by measuring the absorbances at 595 and 620 nm using a spectrophotometer (Bio-Rad, Richmond, USA).

Clonogenic Assay

EC cells were seeded onto 6-well plates at a concentration of IK: 10^4 cells, RL-95/2, and HEC-1-A: 2×10^4 cells per 1.5 ml of media. Cells were treated with vehicle or VD_3 (2–10 μM) for 48 h. Clonogenic capacity was obtained as previously described in Bergada *et al.*[39]

Western Blot Analysis

Nuclear and cytoplasmic extracts were obtained with the NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, USA). Protein concentrations were determined

Table 3 Statistical analysis of CYP27A1 immunoexpression levels in NE and EC

	n	min	max	mean	s.d.	Fold change	*P*-value
Normal samples							
All	52	0.00	40.00	6.9	11.3		
Proliferative	13	0.00	40.00	3.9	11.2	1.0	
Secretory	39	0.00	40.00	8.0	11.2	2.1	0.06
Normal vs tumor samples							
All	184	0.00	190.00	56.7	51.2		
Normal	52	0.00	40.00	6.9	11.3	1.0	
Tumor	132	0.00	190.00	76.1	47.5	**11.0**	**<0.00001**
Tumor samples							
All	132	0.00	190.00	76.1	47.5		
NEEC	21	0	160	59.3	47.7	1.0	
EEC	111	0.00	190.00	79.7	46.9	1.3	0.08
Grade 1	36	0	190	84	53.1	1.0	
Grade 2	52	0	155	81	46.1	1.0	0.81
Grade 3	44	0	160	65	43.0	0.8	0.1
					Decreasing trend *P*-value	0.07	
Stage I	75	0	190	76.4	49.63	1.0	
Stage II	23	5	170.00	89.3	45.50	1.2	0.27
Stage III	13	5	103.00	39.5	35.67	**0.4**	**0.03**
Stage IV	1	110	110.00	110.0	—	2.8	—
						Trend *P*-value	0.19
					Global *P*-value	**0.02**	

Abbreviations: EC, endometrial cancer; EEC, endometrioid carcinoma; min and max, minimum and maximum values; *n*, number of patients; NE, normal endometrium; NEEC, non-endometrioid carcinomas; s.d., standard deviation.
Fold change denotes the fold change in biomarker expression relative to the mean value for the first category. *P*-values from a Mann-Whitney, Kruskal Wallis (global *P*-value) or trend tests. Values are bolded when statistically different.

with the Protein Assay Kit (Bio-Rad). Equal amount of protein was resolved by 10% SDS–PAGE, and electroblotted onto PVDF membrane (Millipore, Bedford, USA). After probing overnight at 4°C with primary antibodies: VDR (Millipore), lactate dehydrogenase (LDH, Rockland Immunochemicals, Gilbertsville, USA), Histone 1 (H1; Santa Cruz Biotechnology, Santa Cruz, USA) or Tubulin (Sigma), membranes were incubated with secondary antibodies for 1 h, and visualized using the ECL Advance western blotting detection system (Amersham, Little Chalfont, UK).

Measurements of VD Conversion to 25(OH)D by the Immortalized EC/IK Cells

The amount of 25(OH)D synthesized from VD_3 in IK cells that remained within the cell was measured using the Chemiluminescence-Immunoassay for 25(OH)Din the Liaison XL Analyzer (DiaSorin). Briefly, 2.5×10^{-6} IK cells were seeded in 100-mm plates and exposed to vehicle (0.1% ethanol) or 10 μM VD_3 for 0, 12, 24 or 48 h. Media was then removed, and attached cells were washed with PBS. Intracellular VD metabolites were obtained by acetonitrile extraction as previously described.[40] Samples were spun down at 2500 r.p.m. for 10 min to obtain the supernatants containing VD metabolites and dried for 2 h under vacuum. The remaining solution was subjected to a 2-step liquid–liquid extraction with ethyl acetate, as described in Ding *et al.*[41] The pooled upper phases were dried under nitrogen and intracellular 25(OH)D was measured by the Liason Assay.

Immunofluorescent Quantification of Nuclear VDR Translocation

After the indicated treatment with VD_3, IK cells were washed with PBS and fixed for 10 min with 4% paraformaldehyde

Table 4 Statistical analysis of CYP2R1 immunoexpression levels in NE and EC

	n	min	max	mean	s.d.	Fold change	*P*-value
Normal samples							
All	27	5	180	113.5	44.1		
Proliferative	4	5	60	30.0	23.5	1	
Secretory	23	80	180	128.0	17.1	**4.3**	**0.001**
Normal vs tumor samples							
All	155	5	240	141.0	52.5		
Normal	27	5	180	113.5	44.1	1	
Tumor	128	20	240	146.8	52.4	**1.3**	**0.005**
Tumor samples							
All	128	20	240	146.8	52.4		
NEEC	21	50	240	158.4	55.3	1	
EEC	107	20	230	144.0	51.7	0.9	0.24
Grade 1	36	30	220	134.47	49.5	1	
Grade 2	48	20	220	147.06	53.6	1.1	0.22
Grade 3	44	50	240	155.26	52.5	1.2	0.08
					Increasing trend *P*-value	0.07	
					Global *P*-value	0.20	
Stage I	71	20	230	149.2	52.14	1	
Stage II	23	30	207.5	130.4	51.40	0.9	0.10
Stage III	13	50	240	144.6	63.52	1.0	**0.89**
Stage IV	1	110	100	110	—	—	
					Trend *P*-value	0.31	
					Global *P*-value	0.46	

Abbreviations: EC, endometrial cancer; EEC, endometrioid carcinoma; min and max, minimum and maximum values; *n*, number of patients; NE, normal endometrium; NEEC, non-endometrioid carcinomas; s.d., standard deviation.
Fold change denotes the fold change in biomarker expression relative to the mean value for the first category. *P*-values from a Mann–Whitney, Kruskal–Wallis (global *P*-value), or trend tests. Values are bolded when statistically different.

and permeabilized with 0.2% Triton for 6 min. After 1 h blocking (0.2% BSA), cells were incubated overnight at 4°C with VDR antibody (1:50, Millipore) and then washed with PBS and incubated with labeled secondary antibody (1:300, Alexa Fluor 594, Invitrogen) for 1 h. Nuclei were stained with Hoechst (Sigma). Cells were counted under microscope (Leica Microsystems) and for quantification, a minimum of 300 cells were analyzed for each time point.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was obtained with the RNeasy Mini kit (Qiagen, Venlo, Netherland) in IK cell line and with the RNeasy FFPE (Qiagen) in paraffin sections of NE and EC. Three tumor samples were selected from secretory and proliferative NE and from EC based on their histoscores close to the media for CYP27A1 of their respective sample group. Primers to measure mRNA levels of *CYP27A1* (Hs.PT.56a.14491853), *CYP2R1* (Hs.PT.56a.39660677), and *GUSB* (Hs.PT.53a.3993263), were obtained from the PrimeTime Mini qPCR Assay-on-demand (Integrated DNA Technologies, Madrid, Spain). Quantitative reverse transcriptase polymerase chain reaction analysis was performed in triplicate with the ABI Prism 7900 Sequence Detector System (Applied Biosystems). Relative mRNA expression levels were calculated by the comparative cycle threshold (C_t) method using the C_t values obtained for *GUSB*as internal references.

RESULTS

VD Local Control of Proliferation Rates in the Cycling NE

1,25D-VDR-antiproliferative actions require appropriate VDR levels and a normal translocation of cytosolic VDR to the nucleus, a process induced by the binding of an activating

Table 5 Statistical analysis of CYP24A1 immunoexpression levels in NE and EC

	n	min	max	mean	s.d.	Fold change	*P*-value
Normal samples							
All	53	15	295	131.6	84.3		
Proliferative	14	15	220	64.3	49.3	1.00	
Secretory	39	30	295	155.8	81.4	**2.42**	**0.00006**
Normal vs tumor samples							
All	177	10	295	101.8	62.6		
Normal	53	15	295	131.6	84.3	1.00	
Tumor	124	10	210	89.2	45.6	**0.68**	**0.01**
Tumor samples							
All	124	10	210	89.2	45.6		
NEEC	19	25	190	114.2	44.8	1.00	
EEC	105	10	210	84.2	44.3	**0.74**	**0.008**
Grade 1	36	10	200	86.4	39.1	1.0	
Grade 2	47	15	180	80.0	45.7	0.9	0.42
Grade 3	41	10	210	101.0	48.8	1.3	0.059
					Increasing trend *P*-value	0.14	
Stage I	67	10	210	92.04	46.7	1.0	
Stage II	22	15	170	79.77	41.5	0.9	0.25
Stage III	14	25	190	105.00	50.5	0.7	0.17
Stage IV	1	120	120	120.00			
					Increasing trend *P*-value	0.56	

Abbreviations: EC, endometrial cancer; EEC, endometrioid carcinoma; min and max, minimum and maximum values; *n*, number of patients; NE, normal endometrium; NEEC, non-endometrioid carcinomas; s.d., standard deviation.
Fold change denotes the fold change in biomarker expression relative to the mean value for the first category. *P*-values from a Mann–Whitney, Kruskal–Wallis (global *P*-value), or trend tests. Values are bolded when statistically different.

ligand.[42] In NE from premenopausal women (average age = 43), cytoplasmic and nuclear VDR expression was heterogeneous and significantly decreased in the proliferative endometrium compared with the secretory phase (Figure 2; Tables 1A and 1B). As expected, Ki67 Hscores accurately reflected the changes in proliferation rates (Table 2).

The expression of the two main 25-hydroxylases for VD activation to 25(OH)D also changed in the cycling NE. The proliferative NE showed very mild or absent cytoplasmic CYP27A1 staining, which doubled in the secretory phase, although with no statistical significance ($P = 0.06$) (Table 3; Figure 2). The glandular epithelium also showed a mild and possibly artifactual CYP27A1 nuclear staining.

CYP2R1 immunoexpression was also cytosolic and low in endometrial glands during the proliferative phase with no stromal staining, and increased by 4.3-fold in the secretory endometrial glands (Table 4), with a very low staining in endometrial stroma.

Systemically, the 1,25D-VDR complex induces CYP24A1 to degrade circulating excess of 1,25D or its precursor 25(OH)D in order to avoid VD toxicity.[42] In NE, the higher VDR HScore in epithelial cells of the secretory NE coincided with higher cytosolic CYP24A1 expression (Table 5, Figure 2).

More significantly, Table 6 shows that nuclear VDR levels correlated inversely with the marker of cell proliferation Ki67. Furthermore, two significant correlations were found between CYP27A1 and CYP2R1 expression, one direct with nuclear VDR expression, a marker of VDR activation, and one inverse with Ki67, which support a physiological role for local 25(OH)D production in VDR antiproliferative actions throughout the endometrial cycle. Furthermore, not only CYP24A1 expression correlated directly and strongly with nuclear VDR expression, but also with the cytosolic expression of both CYP27A1 and CYP2R1, thus supporting a role for both 25(OH)D-producing enzymes in VDR activation of CYP24A1 expression.

Taken together, these results demonstrate that the NE is capable of full activation of inert VD to 1,25D. As CYP27B1 expression remains unchanged throughout the endometrial cycle,[22] these results also suggest that the changes in 25(OH)D synthesis along the endometrial cycle may be stronger regulators of VDR control of proliferation rates and CYP24A1 expression than local 1,25D synthesis.

Abnormalities in VD Local Control of Proliferation Rates in EC

Similar to other human cancers, Table 1A and Figure 2 show that EC samples elicited a significantly decreased nuclear VDR expression compared with NE. Also, EEC had less nuclear VDR than NEEC, with no significant differences among histological grades or pathological stages. VDR was also observed in the cytoplasm of EC cells with a quite heterogeneous expression pattern (mean cytoplasm Hscore: 114; range: 0–285; see Table 1B). Different from nuclear VDR levels, average cytosolic VDR did not differ between EC and NE. This finding and the lack of the correlation between cytosolic and nuclear VDR of NE suggest that, in EC, a defective VDR translocation to the nucleus may contribute to impaired VDR-antiproliferative actions.

Interestingly, also different from the proliferative NE, both 25-hydroxylases were significantly enhanced in EC. CYP27A1 expression was 11-fold higher than in NE ($P=0.0000$) (Table 3; Figure 2). EECs elicited a 30% higher CYP27A1 expression compared with NEECs, although nonstatistically significant ($P=0.08$). Also, there was only a mild, although not significant, trend for decreased CYP27A1 immunostaining in EC according to FIGO grades or with the severity in pathologic stages that only reached statistical significance for stage III tumors ($P=0.003$). Importantly, CYP27A1 expression in EC samples correlated significantly and inversely with Ki67 expression ($r=-0.26$; $P=0.004$; Table 6).

EC also showed a cytoplasmic and heterogeneous CYP2R1 immunoexpression, 1.3-fold higher than in NE in any of the phases of the endometrial cycle ($P=0.005$; Table 4). No statistical differences were demonstrated in CYP2R1 expression among EC, with histological type, FIGO grade or pathological stage (Table 4). Importantly, CYP2R1 correlated positively with nuclear VDR immunoexpression ($r=0.19$, $P=0.04$). To evaluate whether quantitative PCR could be a better tool to assess actual expression of 25-hydroxylases in EC, three representative samples of EC were selected, based on their Hscore for CYP27A1 close to the average of this group, that is 11-fold higher than in NE (Table 2). Intriguingly, mRNA levels of *CYP27A1* and *CYP2R1* were very similar (*CYP27A1*: 1.49 ± 2.56 *vs* *CYP2R1*: 1.74 ± 2.89) despite their remarkable differences in average increases in protein expression.

Interestingly, in EC, CYP24A1 expression was significantly lower than in NE with a granular and heterogeneous expression pattern (Figure 2), with levels 1.4 higher in NEECs compared with EECs, and with the highest expression in FIGO grade 3 ECs. No differences in CYP24A1 expression occurred among different pathological stages, but again, stage III and IV tumors showed the highest levels of CYP24A1 immunostaining (Table 5). Furthermore, different from NE, there was no correlation between CYP24A1 and nuclear VDR expression, or with CYP27A1 or CYP2R1, suggesting a distinct, if any, ligand-activated VDR induction of CYP24A1 in EC.

Next, studies in EEC cell lines examined the actual role of enhanced CYP27A1 and CYP2R1 expression in the activation of VDR antiproliferative actions by locally produced 25(OH)D.

Functional Relevance of CYP27A1 and CYP2R1 Expression in VDR Antiproliferative Actions

These *in vitro* studies were conducted exclusively in EC cell lines, because DNA profiling analysis revealed that the two normal, immortalized endometrial cell lines HES and hTERT EEC were HeLa cervical carcinoma and MCF7 breast cancer cells, respectively.[43] First, we examined whether the EC cell lines IK, HEC-1A, and RL-95/2 reported to respond to 1,25D with growth inhibition,[15,16] expressed CYP27A1 and CYP2R1. Upon immunohistochemical staining confirming the expression of both 25-hydroxylases in the three cell lines (Figure 3), dose response and time course studies examined the potential anti-proliferative effects of the local bioactivation of VD_3 to 25(OH)D. Cells were treated with concentrations of VD_3 ranging from 0 to 10 μmol/l for 24, 48, or 72 h. The results of MTT assays depicted in Figure 4a show

Table 6 Correlation analysis between pairs of biomarkers in NE and EC

	Normal samples		Tumor samples	
Pair	*r*	*P*-value	*r*	*P*-value
KI67 *vs* VDR.nuc	**−0.73**	**<0.00001**	−0.08	0.36
VDR.nuc *vs* CYP24	**0.55**	**0.0001**	−0.1	0.25
VDR.nuc *vs* VDR.cito	**0.69**	**<0.00001**	0.06	0.49
KI67 *vs* CYP271A.cito	−0.29	0.07	**−0.26**	**0.003**
KI67 *vs* CYP271A.nuc	0.07	0.67	−0.02	0.84
KI67 *vs* CYP2R1	**−0.57**	**0.004**	−0.02	0.80
KI67 *vs* CYP24	**−0.51**	**0.0005**	0.03	0.78
VDR.nuc *vs* CYP271A.cito	**0.35**	**0.02**	−0.09	0.29
VDR.nuc *vs* CYP271A.nuc	**−0.36**	**0.01**	**0.18**	**0.04**
VDR.nuc *vs* CYP2R1	**0.56**	**0.004**	**0.19**	**0.04**
CYP24 *vs* CYP271A.cito	**0.36**	**0.009**	−0.04	0.69
CYP24 *vs* CYP271A.nuc	**−0.38**	**0.006**	−0.05	0.56
CYP24 *vs* CYP2R1	**0.66**	**0.0005**	0.11	0.25

Abbreviations: EC, endometrial cancer; VDR, vitamin D receptor.
r and *P*-values indicate correlation coefficient and statistical significance from Spearman non-parametric analysis. Values are bolded when statistically different.

that all three cell lines reduced viability in response to VD_3 in a dose- and time-dependent manner. Maximal reduction of viability post treatment of 62% (IK), 52% (RL-95-2), and 55% (Hec-1A) occurred by 72 h of treatment with 10 μM VD_3. The lack of a significant reduction in viable cells upon a 24 h exposure to 10 μM VD_3 suggests that VD is not directly responsible for the reduced survival. The time delay in the response to VD indicates that the conversion of VD to 25(OH)D is an essential step for the observed cell viability effect.

Figure 4b shows that VD_3 treatment (0–10 μmol/l for 48 h) caused a dose-dependent decrease in the capacity of all three EC cell lines to form colonies.

These three cell lines are derived from EEC, but only IK cells are well differentiated as most of the EEC samples in our TMAs, and also elicited a higher response to VD. Therefore, time course studies were conducted in IK cells to evaluate the contribution of intracellular 25(OH)D synthesis and VDR translocation to the nucleus to VD antiproliferative actions. While in two independent experiments, there was no detectable intracellular 25(OH)D produced by 7 million IK cells exposed to 10 μM VD_3 for 0, 12 or 24 h, an average 1.24 ng of 25(OH)D were synthesized in 48 h. Figure 5 shows marked increases in nuclear VDR staining in IK cells only if exposed to VD_3 for at least 24 or 48 h. This VDR translocation was confirmed through western blot analysis of changes in

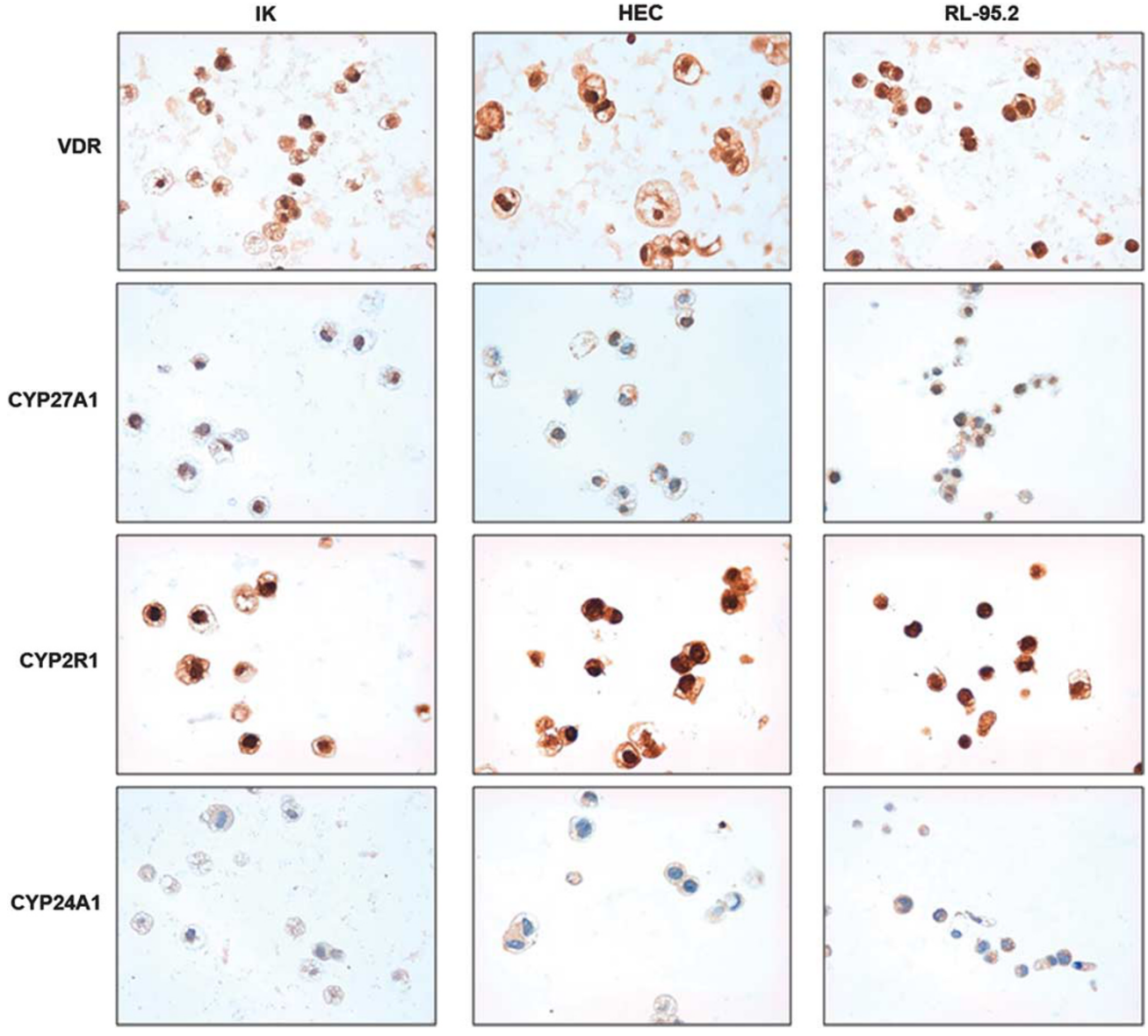

Figure 3 Expression of VDR and VD-metabolizing enzymes in immortalized EC cell lines. Representative immunostaining of VDR, CYP27A1, CYP2R1, and CYP24A1 in the 1,25D-responsive EEC cell lines, IK, RL-95/2, and HEC-1A cells. Magnification $\times 60$.

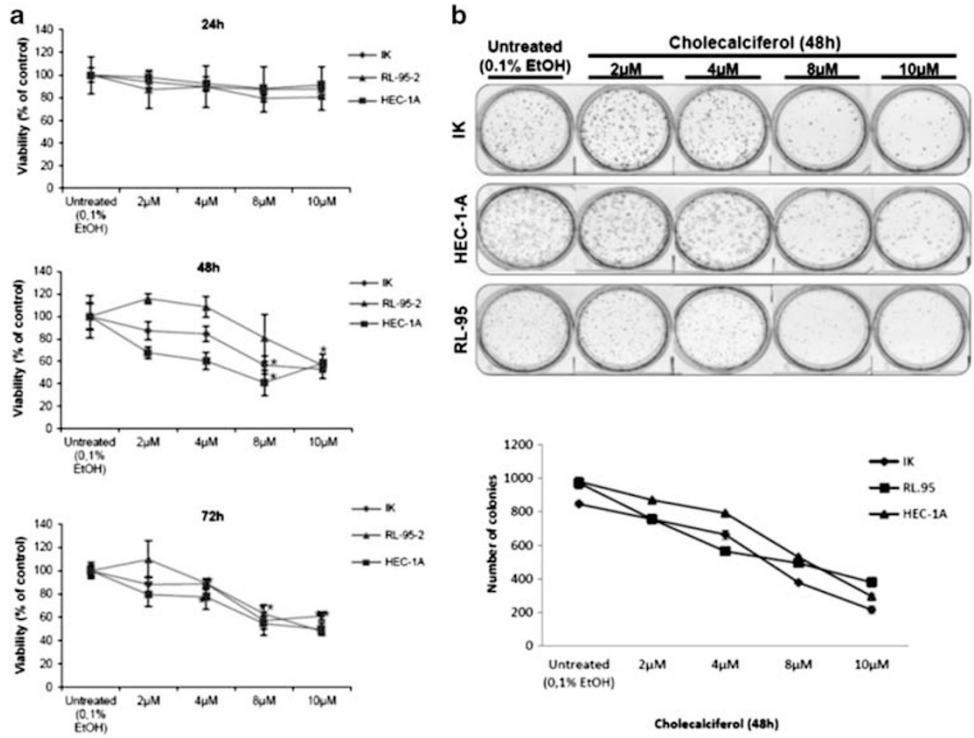

Figure 4 Cholecalciferol (VD_3) treatment is sufficient to suppress the growth in EC cell lines. (**a**) Dose-dependent reductions in cell viability (MTT assay) of IK, RL-95/2, and HEC-1A cells treated for 24 (upper graph), 48 (middle graph), or 72 h (lower graph) with vehicle (0.1% ethanol (EtOH)) or the indicated doses of cholecalciferol (VD_3). Results are expressed as percent decrease from control values (untreated cells) (*$P<0.05$; **$P<0.01$). (**b**) Dose-dependent decreases in colony formation 14 days after seeding by IK, RL-95, and HEC-1A cells (1×10^3) treated with either vehicle (0.1% EtOH) or cholecalciferol (VD_3), at the specified concentrations for 48 h. Top: representative images of colony number; bottom: mean and s.d. of the quantification of colony number (*$P<0.05$).

nuclear and cytosolic VDR content with VD treatment. Maximal VDR translocation to the nucleus occurred after 48 h exposure to VD_3, the time required for detectable levels of locally produced 25(OH)D. Lack of tritiated-cholecalciferol and of a specific antibody to measure CYP27B1 Hscores have impeded a direct assessment of both the rates of local conversion of 25(OH)D to 1,25D and of 25(OH)D-1,25D synergy in EC cell lines.

DISCUSSION

This work constitutes the first demonstration of an enhancement of CYP27A1 and CYP2R1 expression in the secretory phase of the cycling NE and also in EC for autocrine/paracrine activation of VD to 25(OH)D for VDR-antiproliferative actions. This provides new mechanistic understanding for VD supplementation strategies that efficaciously prevent/attenuate endometrial disorders associated with impaired VDR actions, including EC growth.

In NE, the secretory tissue elicited higher CYP27A1 and CYP2R1 expression than the proliferative tissue, that concurred with lower proliferation rates, as measured by Ki67 Hscores, and which correlated directly with elevations in nuclear VDR and with CYP24A1 induction. The latter is a classical ligand-activated VDR induction of gene transcription. The delineated changes in 25-hydroxylating capacity along the endometrial cycle, while CYP27B1 expression remains constant,[22] suggest that local 25(OH)D synthesis could be more relevant than 1,25D production for VDR control of NE cyclic growth. Therefore, customizing VD supplementation strategies throughout the endometrial cycle to achieve an appropriate local 25(OH)D production should improve 1,25D/VDR autocrine/paracrine regulation of genes controlling Th1/Th2 responses toward tolerance[37,44] in endometriosis, or the increases in the HOXA10 gene,[45,46] required to correct abnormalities in fertility due to embryo implantations defects. The direct correlation existing between the increases in 25-hydroxylating enzymes and CYP24A1 induction, the enzyme degrading the excess of active VD metabolites, also suggest that the NE mimics the systemic VD endocrine system in its tight self-regulation for protection against VD toxicity.[42]

In EC, CYP27A1 and CYP2R1 expression were 11- and 1.3-fold higher than in NE, and did not decrease in more advanced stages. These sustained increases in 25-hydroxylating capacity with EC progression may partially compensate for the reductions in nuclear VDR, as suggested by the direct correlations between the Hscores for CYP27A1 and CYP2R1 with enhanced nuclear VDR and reduced Ki67.

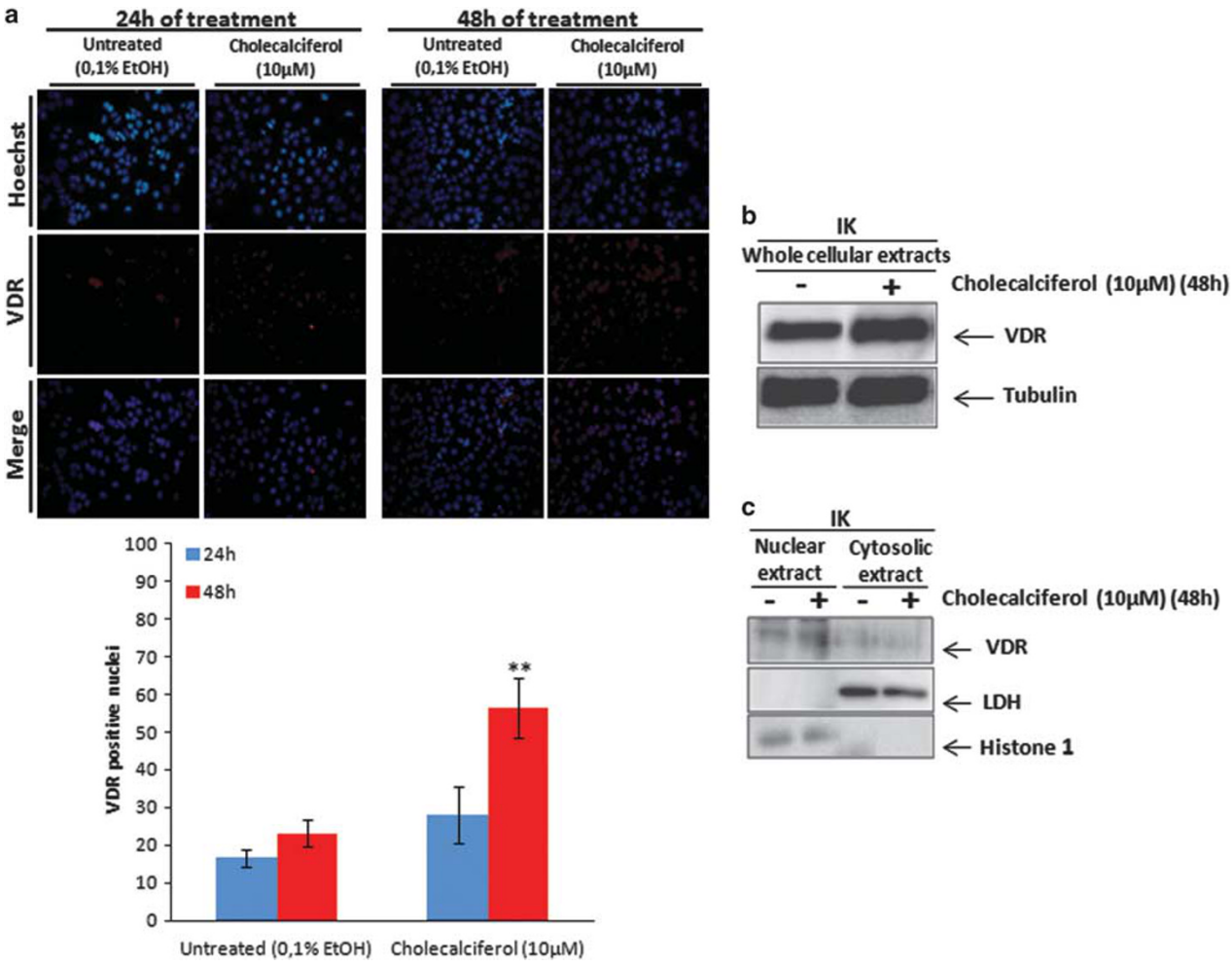

Figure 5 VDR activation by locally produced 25(OH)Din IK cells. Nuclear VDR content in IK cells (1×10^3) treated with vehicle (0.1% EtOH) or with cholecalciferol (VD_3) 10 μM for 24 or 48 h as assessed by: (**a**) Immunofluorescence staining (magnification $\times 20$) and colocalization with Hoescht nuclei staining (top panels) with quantification of number of VDR-positive nuclei (bottom); and (**b**) Representative western blot analysis of VDR expression in whole-cell extracts, or in nuclear and cytosolic fractions (**c**) using 20 μg of total protein from IK cells treated for 48 h as indicated in panel **a**. Tubulin, LDH, and Histone 1 were used as specific markers of whole-cell extracts, cytosolic and nuclear fractions, respectively. ** indicates $P<0.05$ from *t* test analysis of two independent experiments with triplicate determinations per condition.

Importantly, the maintenance of enhanced 25-hydroxylating capacity with EC progression could also compensate for reductions in 1-hydroxylating capacity. indeed, CYP27B1 was shown to increase only at early stages of nonendometrial cancer development, but decrease in more advanced stages.[10] The indirect evidence from TMAs data of a role for local 25(OH)D production to arrest growth in human EC was supported by the efficacy of treatment of EC cell lines with inactive VD_3 for 48 h in suppressing growth and colony formation. Furthermore, the time course for the antiproliferative effects of inactive VD_3 was parallel to actual increases in 25(OH)D synthesis by IK cells, causing maximal increases in nuclear VDR expression. As CYP2R1 has a higher physiological relevance to maintain normal VD status,[9] the mild 30% increase in its expression in EC could be relevant in attenuating intratumorally the impact of systemic VD deficiency on EC progression. Thus, in EC, the simple correction of VD deficiency could help increase intratumoral levels of 25(OH)D and nuclear VDR transactivating potency through 25(OH)D synergy with the impaired 1,25D/VDR-driven growth arrest caused by VDR and/or CYP27B1 reductions. If the observed low conversion rates of VD_3 into 25(OH)D in IK cells occur in other cancer cell types, they may partially explain why only high daily doses of VD_3 effectively reduce cancer risk,[11] including EC.[47]

This study also demonstrates that CYP24A1 cytoplasmic expression was lower in EC than in NE suggesting a minor role, if any, for enhanced catabolism of VD metabolites in the early progression of EC in VD-deficient individuals. Similar findings were reported in other hormonal-regulated neoplasms. In prostate cancer[48] and in MCF-7 breast tumor cells,[49] CYP24A1 mRNA expression is downregulated in malignant lesions compared with adjacent histologically benign lesions. Herein, only the more aggressive types of

EC, NEEC, grade 3 and stage III and IV tumors exhibited the highest CYP24A1 expression concurring with low nuclear VDR, thus supporting the association between the induction of CYP24A1 with poor prognosis reported for human esophagus and colon carcinoma.[50,51] Clearly, in VD-deficient patients with advanced stages of EC, the induction of CYP24A1 expression could aggravate local 25(OH)D or 1,25D deficiency and the already defective VDR activation of antitumoral actions due to low VDR.

Our study underscores several advantages in improving VD_3 supplementation strategies over exclusive interventions with the potent hormone 1,25D, or its analogs,in attenuating EC progression. Not only 1,25D inhibits endometrial CYP27B1 activity at physiological levels,[52] but also suppresses CYP2R1 expression in fibroblasts and prostate cancer cells.[53] Thus, 1,25D treatment could simultaneously compromise endometrial 1,25D production and aggravate systemic VD deficiency. Furthermore, as 25(OH)D synergyzes with 1,25D in the growth arrest induced by VDR activation[33] and has a 25-fold lower affinity for CYP24A1 than 1,25D, our findings also suggest that appropriate VD_3 supplementation could help reduce the doses of 1,25D or related compounds for the prevention and treatment of EC in low VDR-expressing tumors through several mechanisms, namely, reducing the induction of CYP24A1; increasing the activities of intratumoral CYP27A1, CYP2R1, and perhaps, synergizing with CYP27B1 activity for VDR activation. Undoubtedly, prospective clinical trials with examination of CYP27A1, CYP2R1, and CYP24A1 and VDR expression at diagnosis are necessary to obtain evidence-based recommendations for VD_3 supplementation strategies effective in attenuating EC progression and of the accuracy and sensitivity of these markers of endometrial VD metabolism in predicting clinical outcomes.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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