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## Molecular Mechanism of Vitamin D Action and its Implications in Ovarian Cancer Prevention and Therapy

Feng Jiang  
*University of South Florida*

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Molecular Mechanism of Vitamin D Action and  
its Implications in Ovarian Cancer Prevention and Therapy

by

Feng Jiang

A dissertation submitted in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy  
Department of Pathology  
College of Medicine  
University of South Florida

Major Professor: Wenlong Bai, Ph.D.  
Santo V. Nicosia, M.D.  
Richard Jove, Ph.D.  
John C.M. Tsibris, Ph.D.  
Jin Q. Cheng, M.D., Ph.D.  
Douglas W. Cress, Ph.D.

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ABSTRACT

1,25-dihydroxyvitamin D<sub>3</sub> (1,25VD), the active form of vitamin D (VD), suppresses the growth of numerous human cancer cell lines by inhibiting cell cycle progression and inducing cell death. Genes that mediate each of these activities remain largely unidentified and there are no preclinical data for 1,25VD analogues in ovarian cancer (OCa). We hypothesize that 1,25VD and its analogues inhibit the development of OCa. In this study, we demonstrated, (a) 1,25VD causes cell cycle arrest at the G<sub>1</sub>/S and G<sub>2</sub>/M transition and induces apoptosis in OCa cells. (b) We also found that *gadd45* is one of primary target genes for 1,25VD-mediated G<sub>2</sub>/M arrest. A direct repeat 3 (DR3) vitamin D response element (VDRE) is identified in the fourth exon of *gadd45*. This exonic VDRE forms a complex with the vitamin D receptor (VDR)/retinoid X receptor (RXR) heterodimer *in vitro* and mediates the induction of reporter activity by 1,25VD *in vivo*. VDR is recruited in a ligand-dependent manner to the exonic enhancer but not to the *gadd45* promoter regions. In OCa cells expressing GADD45 anti-sense cDNA or GADD45-null mouse embryo fibroblasts, 1,25VD fails to induce G<sub>2</sub>/M arrest, suggesting that G<sub>2</sub>/M arrest induced by 1,25VD is mediated through GADD45. Further study showed that GADD45 mediates the effect of 1,25VD by decreasing *cdc2* kinase activity. (c) hTERT, the catalytic subunit of telomerase, is identified as a primary target for

1,25VD. 1,25VD decreases telomerase activity and hTERT mRNA expression. The down-regulation of hTERT mRNA is due to decreased mRNA stability by 1,25VD, rather than decreased transcription of hTERT through VDRE. Clones stably transfected with hTERT showed higher telomerase activity and longer telomere length than parental cells. Moreover, hTERT clones resist 1,25VD-induced apoptosis and growth inhibition. In contrast to parental cells which do not recover from prolonged treatment with 1,25VD, hTERT clones re-grew rapidly after 1,25VD withdrawal. (d) We demonstrated that the 1,25VD analogue EB1089 inhibits OCa cells *in vitro* and OCa xenograft *in vivo* without inducing hypercalcemia. We also demonstrated precursors for epithelial OCa express VDR and human primary ovarian surface epithelial cells respond to 1,25VD. Taken together, these results strongly suggest that 1,25VD analogues may be effective in the chemoprevention and chemotherapy of OCa.

## INTRODUCTION

### **Nuclear receptor superfamily**

The nuclear receptor superfamily consists of two classes of genes, nuclear hormone receptors and orphan receptors, which represent one of the most abundant classes of transcriptional regulators in animals (Robinson-Rechavi, 2003). It affects a wide variety of functions, including homeostasis, reproductive development, fatty acid metabolism and detoxification of foreign substances. The cognate ligands have been identified for nuclear hormone receptors, but for orphan receptors, the physiologically relevant ligand(s) remain unknown. The discovery of ligands for orphan receptors and additional receptors has become a very active research field. It has now been almost 20 years since the isolation of cDNAs encoding the glucocorticoid, estrogen and thyroid receptors (Hollenberg, 1985; Green, 1986; Weinberger, 1986), which were the first several receptors identified. Novel signaling pathways controlled by nuclear receptors have opened up new aspects of nuclear receptors action with respect to homeostasis, reproduction, development and metabolism in various organisms.

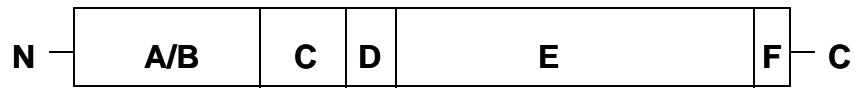
Nuclear receptors form a superfamily of phylogenetically related proteins, with 21 genes in the complete genome of the fly *Drosophila melanogaster*, 48 in humans and, unexpectedly, more than 270 genes in the nematode worm *Caenorhabditis elegans* (nuclear receptor nomenclature committee, 1999).

Nuclear hormone receptors are a class of molecules that function as both signal transducers and transcription factors. The hormonal ligands for these receptors are hydrophobic molecules such as the sex steroids (androgens, estrogens, progesterone), glucocorticoids, thyroid hormones, mineralocorticoids, 1,25VD and retinoids (all-trans retinoic acid and 9-cis retinoic acid) etc. Other ligands, such as ecdysone, oxysterols, bile acids, leukotrienes and prostaglandins have been recently identified and characterized to have receptors that are structurally related to steroid receptor hormones and appear to act through similar mechanisms as the steroid receptors. Unlike the water-soluble peptide hormones and growth factors, which bind to cell surface receptors, the fat-soluble steroid hormones can pass through the lipid bilayer of the cell membrane and interact with their cognate receptors in the cell. These lipophilic hormones are potent regulators of development, cell differentiation, and organ physiology.

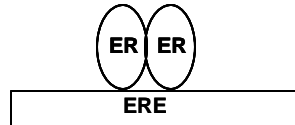
### **Classification of nuclear receptors**

The nuclear receptor superfamily can be broadly divided into four classes (class I, II, III and IV), as proposed by Manglesdorf *et al.* (1995) based on their dimerization and DNA-binding properties (Fig. 1).

Class I receptors include the known classical steroid hormone receptors, which function as ligand-induced homodimers and bind to palindromic response element. Class I receptors include receptors for steroids such as estrogens (ER), progesterones (PR), glucocorticoids (GR), mineralocorticoids (MR), and androgens (AR). These molecules have widespread effects on the development and control of the reproductive system (Beato, 1995; Cole, 1995; Couse, 1998 and 1999).

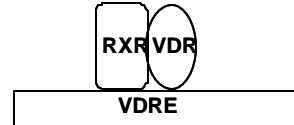


**Class I :  
Steroid receptors**



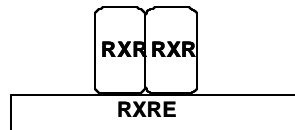
**ER** estrogen  
**PR** progesterone  
**AR** androgen  
**GR** Glucocorticoid  
**MR** mineralocorticoid

**Class II :  
RXR heterodimers**



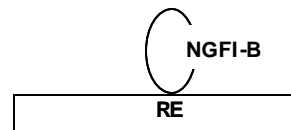
**T<sub>3</sub>R** thyroid hormone  
**RAR** all-trans RA  
**VDR** 1,25VD  
**PPAR** fatty acids  
**EcR** ecdysone  
**FXR** bile acids  
**CAR** androstane  
**LXR** oxysterol

**Class III :  
Dimeric Orphan Receptors**



**RXR** (9-cis RA)  
**COUP**  
**HNF-4**  
**TR2**  
**TLX**  
**GCNF**

**Class IV:  
Monomeric Orphan Receptors**



**NGFI-B**  
**ELP/SF-1**  
**Rev-erb**  
**ERR**  
**ROR**

**Fig. 1. Structural and functional organization of nuclear receptors.**

**Fig. 1. Structural and functional organization of nuclear receptors.** The six domains (A-F) of nuclear receptors comprise regions of conserved function and sequences. All of nuclear receptors contain a central DNA binding domain (DBD), region C, which is the most highly conserved domain and includes two zinc finger modules. A ligand binding domain (LBD, region E) is located in the C-terminal half of the receptor, which contains activation function 2 (AF-2). Between the DBD and LBD is a hinge region (region D) that has variable length. In the amino terminus, the variable N-terminal region (A/B) contains transcriptional activation function 1 (AF-1). Some receptors also contain the F trail, the function of which is poorly understood.



Steroid receptors are believed to be synthesized in cytoplasm from single messenger RNAs (mRNAs). In contrast to other nuclear receptors, unliganded steroid receptors are associated with a large multi-protein complex of chaperones, including Hsp90 and the immunophilin Hsp56, which maintain the receptors in an inactive but conformation. When complexed with Hsp90, through amino acid residues at the receptor's C-terminus, steroid receptors are unable to bind DNA (Pratt, 1993).

The ER has two subtypes: ER $\alpha$  and ER $\beta$ . Considerable divergence between ER $\alpha$  and ER $\beta$  is apparent in the N-terminus (18 % homology). ER $\alpha$  is the predominant subtype expressed in the major female organs such as ovary, uterus, vagina, mammary gland and certain areas of central nervous system especially in the hypothalamus. ER $\beta$  exhibits a more limited expression patterns and is primarily detected in the ovary, prostate, testis, spleen, lung, hypothalamus and thymus (Couse, 1999; Kuiper, 1997). The differential distribution of each receptor in a certain tissues may reflect their distinct receptor functions.

The PR is unique among steroid receptors in that it is composed of two naturally occurring hormone-binding forms of proteins, A and B. The A isoform is an N-terminally truncated version of the full-length B isoform. The molecular weights of the human receptors as deduced from cDNA sequence are 98K and 86K, respectively. The A and B forms of the progesterone receptor differ in their ability to activate target genes and are regulated differently in various types of cells (Conneely, 2002).

The glucocorticoid receptor was the first transcription factor to be isolated and studied in detail (Muller, 1991). There are 3 isoforms of GR,  $\alpha$ ,  $\beta$  and  $\gamma$ . GR $\alpha$  modulates the expression of glucocorticoid-responsive genes by binding to a specific glucocorticoid

response element (GRE) DNA sequence. In contrast, GR $\beta$  and GR $\gamma$  inhibit the effects of hormone-activated GR $\alpha$  (Bamberger, 1995; Ray, 1996; Rivers, 1999), suggesting that GR $\beta$  and GR $\gamma$  may be a physiologically and pathophysiologically relevant endogenous inhibitor of glucocorticoid action and may participate in defining the sensitivity of tissues to glucocorticoids.

The MR is a 107 kD protein. MR was cloned by Arriza *et al.* (1987) and plays important roles in regulation of hydroelectrolytic homeostasis. MR activation by aldosterone raises renal salt reabsorption by increasing the activity of the epithelial sodium channel in the distal nephron. Although the distal nephron is recognized as the major site of action of mineralocorticoids, expression of MR in hippocampus, heart, and endothelium has suggested extrarenal activity (Le Menuet, 2000).

The AR gene is more than 90 kb long and codes for a 76 kD protein. AR mediates the biological action of androgens, principally testosterone and 5 alpha-dihydrotestosterone, that play critical roles in the development and growth of the male reproductive and nonreproductive systems. AR is found in a variety of tissues and changes throughout development, aging and malignant transformation (Chang, 1988; Lubahn, 1988).

Class II receptors heterodimerize with the receptor for 9-*cis* retinoic acid (RXR) and characteristically bind to direct repeats. Exclusive of the classical steroid hormones, this group includes almost all other known ligand-dependent receptors. The list of RXR-dimer partners includes receptors for thyroid hormone (TR), vitamin D (VDR), retinoic acid (RAR), 9-*cis* retinoic acid (RXR), and ecdysone (EcR). These receptors are necessary for normal development, differentiation or organ morphogenesis (Kastner,

1995; Sambrook,1994). Moreover, its heterodimeric partner, RXRs, has been thought to exert multiple functions via their partner. The detection of an abnormal phenotype may raise the question as to which of these pathways has been affected.

The class II receptors also include former orphan receptors for which endogenous ligands have been recently discovered, such as receptors for polyunsaturated long-chain fatty acids and their metabolites (peroxisome proliferator-activated receptors, PPARs), oxysterols (LXRs), bile acids (FXR), xenobiotics (xenobiotic receptor/pregnane X receptor, SXR/PXR and constitutive androstane receptor, CAR). These receptors have been “adopted” when they were shown to bind a physiological ligand (Chawla, 2001, Giguere, 1999, Blumberg B, 1998) and are now in the class II receptor category due to their ability to form heterodimers with RXR. Many of these “adopted” orphan receptors function as lipid sensors that respond to cellular lipid levels and elicit gene expression changes to ultimately protect cells from lipid overload.

Three peroxisome proliferator-activated receptors (PPARs) isoforms have been characterized: PPAR $\alpha$ ,  $\beta/\delta$  and  $\gamma$ . PPARs play a critical role in lipid and glucose homeostasis. Lately they have been shown to interfere with different steps of the inflammatory response by modulating the expression of chemokines, chemokine receptors and adhesion molecules in endothelial cells, smooth muscle cells, monocytes/macrophages and T cells (Blanquart, 2003).

LXR are commonly known as cholesterol sensors that respond to elevated cholesterol concentrations. Oxysterols are natural ligands for LXR. LXR is abundantly expressed in the liver and other tissues that are associated with lipid metabolism (Peet, 1998). Of particular interests are two xenobiotic receptors, SXR/PXR (human steroid

xenobiotic receptor/pregnane X receptor) and CAR (constitutive androstane receptor), which regulate the metabolic cascade of toxic endogenous lipids to protect the body from foreign chemicals. Whereas CAR mediates the response to phenobarbital-like inducers, SXR/PXR respond to many prescription drugs, steroids and toxic bile acids. These receptors seem to increase the clearance of foreign chemicals and provide an important feedforward loop for the xenosensors to initiate another round of signaling (Chawla, 2001; Xie, 2001).

Collectively, unlike the 'classical nuclear hormone receptors' of which ligands were well known before the receptors were cloned and the role of which was well established in reproduction and homeostasis via feedback regulation, aforementioned lipid sensors establish a new role for nuclear receptors in activating feedforward metabolic cascades that maintain lipid homeostasis by governing the transcription of genes involved in lipid metabolism, storage, transport and elimination.

Most of the orphan receptors fall into the class III and IV categories. They represent a diverse and ancient component of the nuclear receptor superfamily, being found in nearly all animal species examined (Blumberg, 1998). Class III receptors, such as RXR, bind to direct repeats as homodimers. RXR is involved in the transduction of retinoid signaling pathway and it is activated *in vitro* by the vitamin A metabolite 9-cis retinoic acid but little is known of the natural activators of RXR. Although RXR forms ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) can function as homodimers, they also serve as the dimerization partner of other nuclear receptors including RAR, TR, VDR, PPARs, LXR, FXR. Thus, as a heterodimerization partner, RXR is involved in the regulation of multiple cellular pathways. Heterodimers PPAR-RXR, LXR-RXR, and FXR-RXR are permissive because

both heterodimeric partners can bind their cognate ligands and induce transcription. Ligand binding by RXR has been demonstrated in the context of RAR and TR heterodimers, suggesting the possibility of permissiveness for RXR-RAR and TR-RXR heterodimers. Recently, allosteric modification of RXR by liganded VDR has been demonstrated. RXR acquires the holoreceptor conformation in the absence of its cognate ligand and gains the ability to recruit coactivators. These results provide evidence for RXR as a functionally active, nonsilent partner in 1,25VD-mediated RXR-VDR-dependent gene expression (Bettoun, 2003).

Class IV receptors typically bind to extended core sites as monomers, such as RevTR $\alpha$  (EAR1) and NGFI- $\beta$  which recognize the AGGTCA sequence without repetition.

Although structurally related to the known receptors, no physiological ligands are known for these orphan receptors that are more numerous than receptors with known ligands (Blumberg, 1998). Taken together with the future identification of their definite ligand, orphan receptors will also provide an opportunity to identify potentially new functions in physiology.

### **Domain structure of nuclear receptors**

Despite their evolutionary and functional differences, members of the nuclear receptor superfamily generally share a similar structural organization. Typical nuclear receptor consists of multiple functionally distinct domains, including domains involved in DNA or ligand binding, dimerization, and transcriptional activation. Once they have bound their ligands, dimerized and achieved high-affinity association with specific

responsive elements in DNA, their transactivation domains function together at the molecular level, resulting in gene activation (Issa, 1998; Kumar, 1999).

The N-terminus of the nuclear receptor, A/B domain, has ligand-independent transactivation activity, termed activation function 1 (AF-1). The sequence and length of the A/B domain are highly variable among receptors (Weinberger, 1985). In addition, this region is the most frequent site of alternative splicing and secondary start sites, also contains a variety of kinase recognition sequences. It is thought that A/B domain may be responsible for the receptor-, species-, and cell type-specific effects, as well as promoter context-dependent properties of nuclear receptor transactivation.

Nuclear receptors are characterized by a central DNA-binding domain (DBD), which targets the receptor to specific DNA sequences known as hormone response elements. DBD, which is the most conserved region among various members of the superfamily, sets the nuclear receptors apart from other DNA-binding proteins. There are nine cysteine residues within the DBD that are strictly conserved throughout the superfamily of receptor proteins. The first eight cysteines in the N-terminus coordinate two zinc atoms to form the so-called zinc finger DNA-binding motifs that are responsible for high-affinity interaction with specific DNA sequences in the genomic region of hormone target genes. The first zinc finger contains the P (proximal)-box region, an alpha helix that is responsible for high affinity recognition of the “core half site” of the response element. Located within the second zinc finger, D (distal)-box, an alpha helix, determines the spacing between half-sites and mediates receptor dimerization. The second zinc finger contains a six-residue region, referred to as the T-box, which has been suggested to form a dimerization interface for the interaction with the RXR DBD.

Adjacent to the DBD is the D or hinge domain. This region has an poorly defined function. The D domain appears to allow for conformational changes in the protein structure following ligand binding. Also, this region may contain nuclear localization signals and protein–protein interaction sites.

The C-terminal half of the receptor encompasses the ligand-binding domain (LBD), which possesses the essential property of hormone recognition and ensures both specificity and selectivity of the physiologic response. Although this domain is highly variable among family members, all nuclear receptors share a common structure of 10 to 13 alpha helices organized around a hydrophobic binding pocket. A ligand-dependent activation function (AF2) is located at the extreme C-terminus (helix-12). LBD also contains nuclear localization signals, dimerization motifs interaction surfaces for heat shock proteins, coregulators and for other transcription factors. The AF-2 domain provides an interactive surface for transcriptional corepressors and coactivators which links nuclear receptor activity with the preinitiation complex (PIC).

Nuclear receptors act in three steps: repression, derepression and transcriptional activation (MacDonald, 2001). Repression is characteristic of the apo (unliganded)-nuclear receptor, which recruits a corepressor complex that possesses histone deacetylase activity (HDAC). Derepression occurs following ligand binding, which dissociates this complex and recruits a first coactivator complex that has histone acetyltransferase (HAT) activity, resulting in chromatin condensation. In the third step, the HAT complex dissociates and a second coactivator complex is assembled (TRAP/DRIP/ARC), which is able to establish an interaction with the basal transcription machinery leading to transcriptional activation of the target gene.

Nuclear receptors may or may not contain a final domain at the C-terminus of the E domain, the F domain. The sequence of the F domain is extremely variable among different receptors and their function is largely unknown.

### **Regulation of vitamin D receptor**

#### **VD and VDR**

VD was discovered nearly a century ago as the nutrient that prevented rickets, a devastating skeletal disease characterized by undermineralized bones (Brown, 1999). VD is a lipophilic hormone essential for normal bone structure and to maintain serum calcium levels. VD acts in concert with parathyroid hormone (PTH) to tightly regulate the concentration of serum calcium and phosphate, thereby assuring proper skeletal mineralization. In addition to its classic function in mineral homeostasis, the present concept of VD had been broadened to that of a hormone involved in a complex system that regulates proliferation and differentiation of a variety of cell types and tissues (Sutton, 2003) (Fig. 2).

1,25VD is the biologically active form of VD. In the skin, 7-dehydrocholesterol is photo converted to pre-VD upon exposure to ultraviolet light, is then isomerized to VD. Subsequently, VD is metabolized in the liver to 25-hydroxy-VD<sub>3</sub> and, primarily in the kidney, to 1,25VD. The major source of VD is through sunlight (UV-B)-induced photobiosynthesis in the skin instead of food consumption (Sutton, 2003) (Fig. 2).

VD action is mediated through the VDR, a member of class II nuclear receptors. VD-dependent stabilization of the VDR protein appears to occur in virtually all cell types. VDR heterodimerizes predominantly with RXR. Although VDR also forms



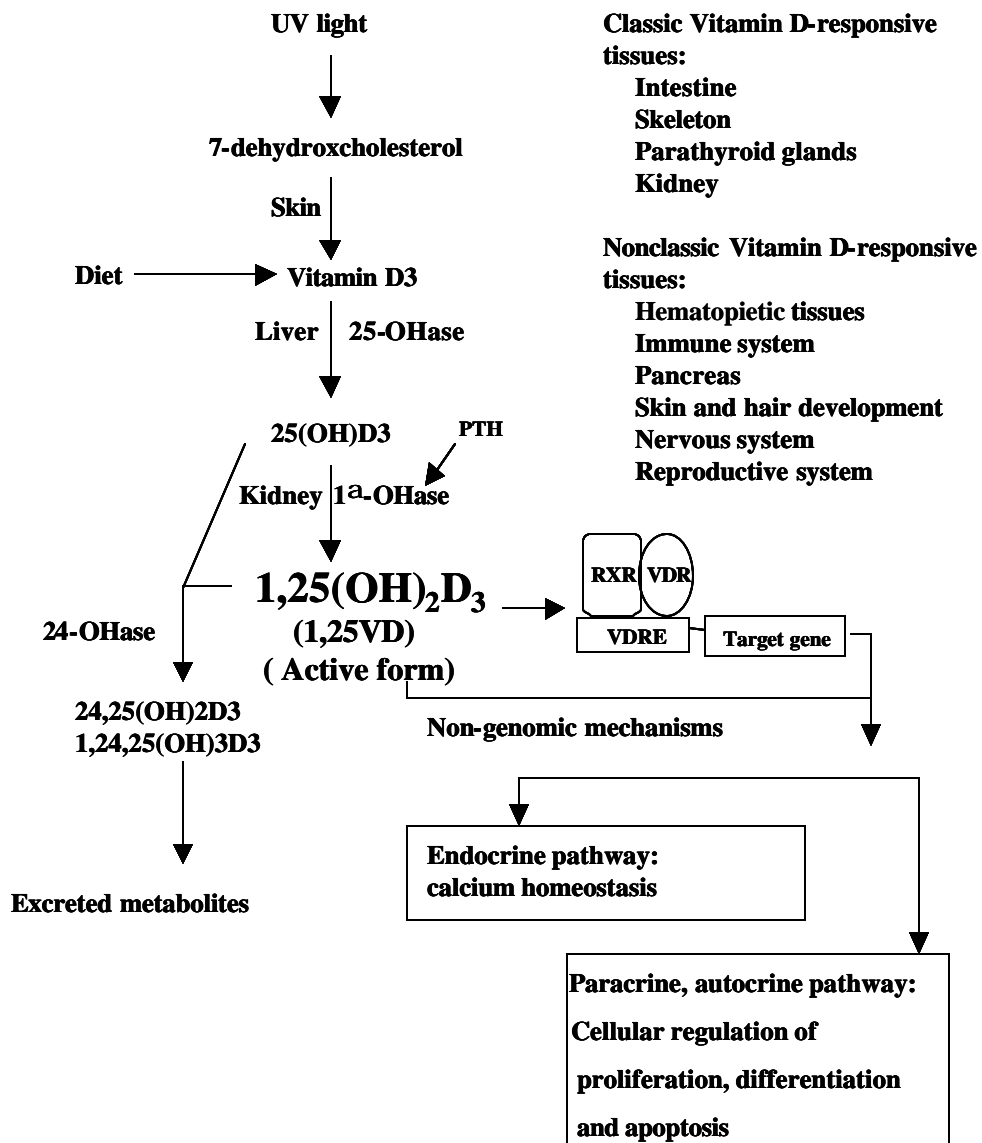


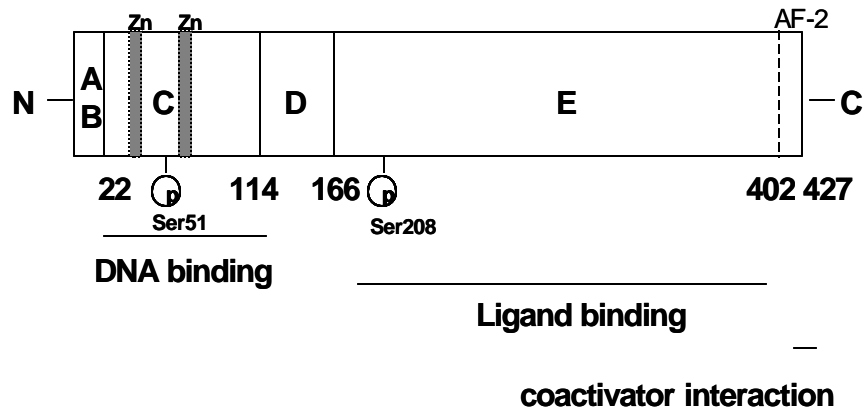
Fig. 2. Metabolism and biological response of Vitamin D.

**Fig. 2. Metabolism and biological response of Vitamin D.** Bioactive 1,25VD is generated by sequential hydroxylations of its precursor Vitamin D<sub>3</sub> in the liver and kidney. PTH (parathyroid hormone) stimulates 1 $\alpha$ -OHase expression in the kidney and promotes calcium mobilization from the bone and reabsorption from the kidney. 1,25VD, in turn, induces calcium absorption in the intestine and calcium release from the skeleton. The action of 1,25VD is mediated through VDR or non-genomic pathway. The distinct endocrine and paracrine/autocrine roles of 1,25VD are emphasized. The classic and nonclassic Vitamin D-responsive tissues are also listed.

homodimers, these homodimers may not be transcriptionally active (Nishikawa, 1994; Issa, 1998).

The human VDR (hVDR) gene has been localized to chromosome 12q13-14. The hVDR cDNA was cloned from a human jejunal poly(A)<sup>+</sup> RNA library using avian VDR cDNA probe. hVDR gene contains 11 exons that span more than 75kb (Miyamoto, 1997). The noncoding 5-prime end of the VDR gene includes exons 1A, 1B, and 1C, while its translated product is encoded by 8 additional exons (2 to 9). Three mRNA isoforms are produced as a result of the differential splicing of exons 1B and 1C. A 4.6 kb human transcript, contains a 1281 nucleotide open reading frame that codes for the full length VDR protein of 427 amino acids. The hVDR coding sequence is highly homologous to the avian, amphibian, mouse and rat sequences, particularly in the highly conserved nine cysteine residues of the DBD. Mammalian forms of VDR protein range in molecular weight between 52-60 by biochemical analysis, although the calculated molecular weight deduced from the amino acids sequence is 48.3 kD. The promoter of hVDR is GC-rich and does not contain an apparent TATA box, but has multiple Sp-1 recognition sites and an array of putative binding sites for transcription factors.

VDR shares structural homology with other nuclear receptors. However, AF1 is absent in the short A/B region at the N-terminus in VDR since removal of the A/B domain does not affect ligand binding, DNA binding or transactivation (Fig. 3). Two highly conserved zinc finger DNA binding motifs constitute the DBD, which also contains the nuclear localization signal. The D domain or hinge region regulates the receptor's flexibility for conformational changes. Most of the natural mutations found in human VDR are located in the zinc finger region, resulting in defective DNA binding and



**Fig. 3. Molecular structure of the VDR.** The DNA binding domain contains two Zinc finger structures. The ligand binding domain contains putative heterodimerization interfaces. Also shown are two phosphorylation sites at Ser-51 and Ser-208. A TFIIB-interactive interface and a coactivator binding domain are also shown.

the most severe clinical phenotype of VD resistance (Haussler, 1998). The VDR LBD contains nine heptad repeats that form hydrophobic surfaces thought to act as dimerization interfaces.

### **Coregulators of VDR**

In response to VD activation, VDR recruits multiple co-activators, including members of the p160 SRC family (Gill, 1998) and CBP/p300 family (Castillo, 1999) that either have or can recruit histone acetyl transferase activity. These coactivators are essential for the formation of the initial transcription complex with RNA polymerase II. Recently, another coactivator complex has been identified as DRIP/TRAP complex (Rachez, 1999) that has no HAT activity and serves as a mediator between the VDR and RNA polymerase II complex (Pol II). DRIP/TRAP and SRC/p160 exist as distinct complexes but act cooperatively, as suggested in recent studies where both complexes were shown to act during the early stages of keratinocyte differentiation. In the later stages of differentiation, DRIP/TRAP levels decrease and SRC/p160 assumes a predominant role (Oda, 2003). In addition to SRC and DRIP, NcoA-62/ski-interacting protein (skip) can augment VDR transcriptional activity (Baudino, 1998; MacDonald, 2001). Skip lacks LXXLL motifs, selectively associates with the VDR-RXR through the LBD, but through a domain that is distinct from the Helix3-Helix5/Helix12 interaction surface. In contrast to coactivators, corepressors, NCoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptors) have been found to associate with ligand unbound VDR, TR and RAR to repress ligand-induced transactivation functions.

Recently, Kitagawa *et al.* (2003) identified a multiprotein complex (WSTF Including Nucleosome Assembly Complex, WINAC); This is a novel ATP-dependent chromatin remodeling complex that directly interacts with VDR through the Williams syndrome transcription factor (WSTF). WINAC, like other SWI/SNF and ISWI/complexes, can reorganize the chromatin structure either by opening it to allow transcription or compacting it to repress gene expression. Manipulation of WSTF expression levels established that it is essential for VDR activity by either stimulating transcription from promoters regulated by positive response elements or repressing transcription from promoters containing negative response elements. Overexpression of WSTF can restore the impaired recruitment of VDR to VD-regulated promoters in fibroblasts obtained from Williams syndrome patients. This suggests that WINAC dysfunction contributes to Williams syndrome. This discovery has shed new light on the molecular mechanisms by which VDR controls gene expression with unexpected clinical implications (Belandia, 2003).

### **Phosphorylation of VDR**

Hormone induced phosphorylation has been found in most nuclear receptors and may be involved in the regulation of receptor function. VDR contains two phosphorylation sites through which its activity can be modulated. Casein kinase positively regulates VDR activity while protein kinase C or protein kinase A negatively regulate it (Hsieh, 1991; Jurutka, 1996). VDR is phosphorylated by PKC at Ser-51 *in vitro* and *in vivo*, resulting in retardation of specific interactions of VDR with VDREs. Ser-208 is the preferred site phosphorylated by casein kinase II (CKII). Phosphorylation of Ser-51 or Ser-208 is not essential for VDR function but may reflect kinase specific

inputs that can be either positive or negative. The functional consequence of phosphorylation on VDR activity depend on both the cellular context and the signal transduction pathway or specific kinase involved. VDR phosphorylation represent a mechanism by which signals from the cell membrane in response to growth factor stimulation can modulate nuclear hormone receptor function.

### **VDRE and VDR**

VDR forms heterodimers with RXR. Isoforms of the RXR ( $\alpha, \beta, \gamma$ ) serve as dimeric partners for VDR. In most cases, the VDR/RXR heterodimer binds VD response elements (VDREs) to mediate the biological activities of VD through transcriptional activation or repression of target genes containing VDREs in their regulatory sequence. Almost all VDREs were identified in the promoter region of VD-regulated genes (Issa, 1998).

Although there is considerable variations between natural VDREs, a consensus DR3 type VDRE can be defined as a direct repeat of two six-base half elements of the sequence, separated by a spacer of three nucleotides. The VDRE sequence directs the VDR-RXR heterodimer where RXR binds the 5' half site and VDR occupies the 3' half site (Haussler, 1998). The orientation and spacing of the response elements directs the polarity of heterodimer binding. The fifth nucleotide position of the 3' half site is thought to be necessary for heterodimer binding. Although VDR-RXR complexes that interact with DR3 VDREs predominate the transcriptional response to 1,25VD, alternative routes appear to be possible both with respect of dimmer formation and target gene VDRE structure (direct repeat 6, DR6 and inverted repeat 9, IR9). The RXR dimerization surfaces on VDR are located in the first zinc finger and in a structural motif designated as

heptad repeats in the LBD. Asn37 in the P-box of the first zinc finger, Lys91 and Glu92 situated in the T-box COOH terminal of the second zinc finger, and two of the heptad repeats within the LBD are critical in determining selective association between the VDR and its protein partner, RXR. Heterodimerization of the ligand-activated VDR with RXR induces a VDR conformation that is essential for VDR transactivating function (Brown, 1999). VDR homodimers showed binding activity for DR6 type elements in both ligand-dependent and independent manner, while the functional significance of a VDR homodimer is yet unclear.

Several negatively regulated VDREs were also identified in some genes such as, avian and human PTH (Liu, 1996) and protein kinase A (PKA) inhibitor (Rowland-Goldsmith, 1999). Interestingly, by changing the two 3'-terminal bases GT of the avian PTH VDRE to the consensus CA, the VDRE reversed from a negative to a positive VDRE (Koszewski, 1999).

### **Nongenomic effect of VD**

Most of the biological actions of VD are thought to occur through the nuclear VDR-mediated expression of target genes. Because these responses require transcription and translation of target genes, they are typically delayed by at least 30 min. However, more rapid effects (within seconds to minutes) in response to steroid hormones are also observed. For example, 1,25VD can stimulate the rapid formation of second messengers including ceramides, cAMP, inositols, calcium and to activate a variety of protein kinases such as protein kinase C, protein kinase A, raf, MAPK, and src kinases family (Gniadecki, 1996; Gniadecki, 1998; Marcinkowska, 1997). Although many *in vitro* studies have shown rapid effects by 1,25VD and numerous other steroids, the field is



hindered by the inability to identify the putative membrane receptors that trigger these nongenomic effects. The study in VDR-knock out (VDRKO) mice showed that nongenomic effects of VD in osteoblasts are abrogated in the absence of nuclear VDR, suggesting that some nongenomic response require a functional nuclear VDR (Erben, 2002). VDRKO mice are important tools to decipher the molecular requirements of classical VDR that mediate the genomic and nongenomic effects of 1,25VD *in vivo*.

### **Biological actions of VD**

The genomic and nongenomic actions of VD produce a multitude of responses. VDR has been found in classical VD target organs such as the intestine, bone, kidney, and the parathyroid glands as well as a host of target tissues not involved in calcium homeostasis, such as skin, muscle, pancreas, reproductive organs, and hematopoietic, immune and nervous systems (Berger, 1988; Clemens, 1988). The diversity and the growing number of VD-regulated genes are a reflection of the pleiotropic effects of 1,25VD on calcium and phosphate homeostasis, bone turnover, and proliferation and differentiation of a wide variety of cells, including keratinocytes, cancer cells and immune cells. Therefore the diversity and importance of VDR function goes far beyond mineral metabolism. Moreover, locally produced 1,25VD may serve as a paracrine modulator of cell growth and differentiation.

To investigate the functional role of VDR, Yoshizawa *et al.* (1997) generated mice deficient in VDR. VDRKO mice are viable and develop normally until the weaning period irrespective of reduced expression of VD target genes. Perhaps, the higher calcium content of murine milk, compared to human milk, keeps serum calcium normal, assuring normal growth of VDR-null mutant mice before weaning. Not surprisingly, after

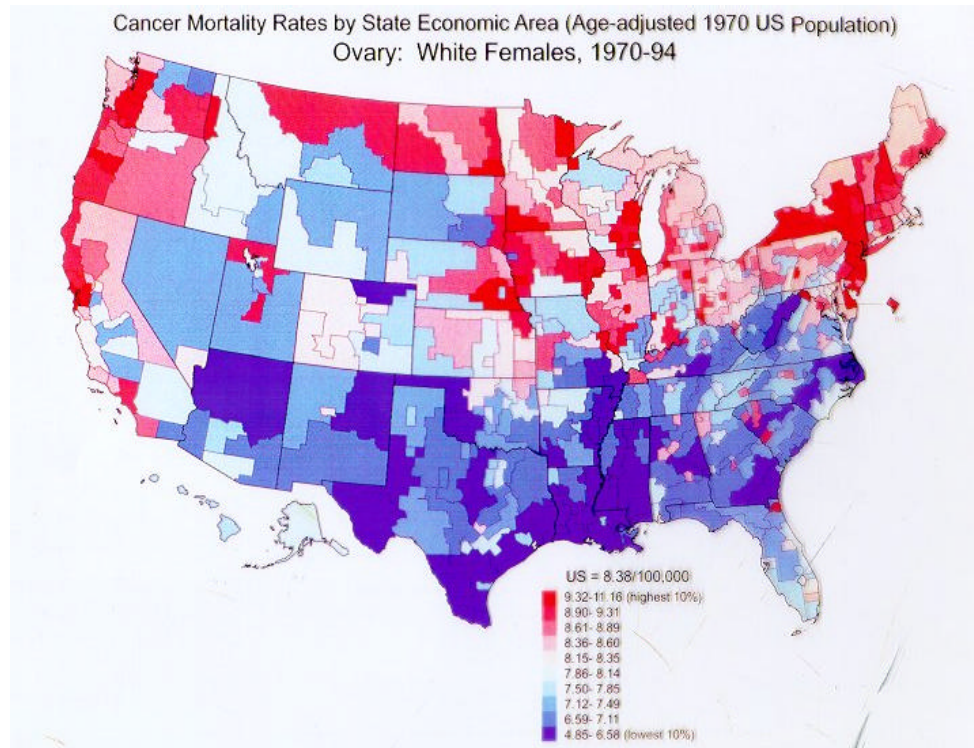
weaning, VDRKO mice displayed typical features of hereditary vitamin-D-resistant rickets (HVDRR), a rare genetic disorder caused by mutations in the VDR genes, with symptoms such as severe bone formation, hypocalcemia and alopecia. Although uterine hypoplasia, infertility and early lethality are not pronounced in patients with VD-dependent rickets type II, possibly because of therapy with calcium supplements, most VDR-null mutant mice died within 15 weeks after birth; uterine hypoplasia with impaired folliculogenesis were observed. Uterine hypoplasia was caused by impaired estrogen synthesis in the mutant ovaries. However, the uterus of these animals responded normally to administration of estrogen. Male reproductive organs appeared normal in VDRKO mice. The fact that VDRKO mouse not only exhibits all features of human rickets, but also has marked growth retardation after weaning and uterine hypoplasia, implicates a role for VDR and calcium homeostasis during reproductive development and growth.

Classical target tissues of VD related to calcium homeostatis are the intestines (calcium and phosphate absorption), kidney (phosphate and calcium reabsorption), parathyroid glands (suppression of PTH) and bone (osteoclastogenesis, osteoblasts and mineralization). Target genes in these organs with prominent VDR in their promoters are the calcium binding proteins calbindin D9K (intestine), calbindin D28K (kidney and other tissues), the non-collagenous bone-specific matrix protein osteocalcin and osteopontin (extracellular matrix protein in bone and other tissues) (Haussler, 1998). Serum 1,25VD is held constant in the normal state and is strictly regulated in response to factors controlling calcium homeostasis. 1,25 VD regulates its own metabolism and biosynthesis by stimulation of 24-hydroxylase activity and inhibition of  $1\alpha$ -hydroxylase (Sutton, 2003).

In addition to its role in calcium homeostasis and bone metabolism, 1,25VD exhibits anti-inflammatory and immunosuppressive properties (Bouillon, 1995; Issa, 1998). 1,25VD interacts with mature monocytes and macrophages, enhancing their immune function and improving host defense against both bacterial infection and tumor cell growth. In contrast to the stimulatory effects of the hormone on monocytes and macrophages, the principal action of 1,25VD in lymphocytes is as an immunosuppressive agent. It does so by decreasing both the rate of proliferation and the activity of T cells and B cells. 1,25VD and its analogues may prove beneficial as potential therapeutics in autoimmune diseases, such as psoriasis, multiple sclerosis, rheumatoid arthritis, diabetes and in transplantation.

#### **Effect of vitamin D in cancer**

Epidemiological data suggest that low VD levels increase the risk and mortality (Fig. 4) of prostate, breast and colon cancers (Waterhouse, 1976; Devasa, 1999). 1,25VD modulates cellular proliferation and differentiation of both normal and malignant cells. 1,25VD and its synthetic analogues inhibit carcinogenesis in mouse skin (Chida, 1985; Webb, 1988), decrease the size of transplanted sarcomas, reduce lung metastasis in mice (Sato, 1982), suppress the growth of human colonic, prostate and pancreatic cancer xenografts *in vivo* (Eisman, 1987; Blutt, 2000; Colston, 1997), increase cell differentiation and decrease proliferation of leukemia (Dodd, 1983), breast (James, 1994) and prostate cancer (Blutt, 1997) cells. Studies in cancer cell lines have shown that 1,25VD causes cancer cells to accumulate in the G1 phase of the cell cycle (Blutt, 1997), in the G2 phase (Eisman, 1989) or undergo apoptosis (Blutt, 2000; Simboli-Campbell, 1996). The list of VD-induced proteins (Segaert, 1998) that pertain to cell growth and



**Fig. 4. Ovarian cancer mortality rates in US, 1970-1994** (Devasa DJ et al. 1999, Atlas of cancer mortality in the United States. NIH publication No. 99-4564: pp 226-230).

differentiation is rapidly growing with HoxA10 (homeobox protein causing G1 arrest), Mad1 (differentiation-related transcriptional repressor), TNF $\alpha$ , insulin-like growth factor binding proteins 3 and 5, apolipoprotein D, tumor suppressors BRCA1 and E-cadherin. VDREs of numerous VD-responsive genes have been identified and characterized. Some VDREs are simple direct repeats while others are complex with often overlapping multimeric structures. The induction of DNA protein kinase links 1,25VD to DNA repair and cancer chemoprevention mechanisms.

1,25VD and its synthetic analogues appear to exert their growth-inhibitory effects via regulation of cell cycle progression. Typically, treatment of cells with 1,25VD causes cell arrest in the G<sub>1</sub> phase, resulting in a decreased number of cells in S phase, and increase in G<sub>0</sub>/G<sub>1</sub>. This change is associated with alterations in the expression of cell cycle regulators. Although a number of genes involved in cell cycle control, apoptosis and cell differentiation have been identified, the exact mechanisms underlying the growth regulatory actions of 1,25VD and its analogues have not been completely defined.

In prostate model systems, VD has significant anti-tumor activity *in vitro* and *in vivo* (Blutt, 1997 and 2000; Johnson, 2002). The effect of 1,25VD is associated with an increase in cell cycle arrest, apoptosis, differentiation and modulation of growth factor receptors. VD induces G<sub>0</sub>/G<sub>1</sub> arrest and modulates cyclin dependent kinase inhibitors, p21 and p27. VD induces PARP cleavage, increases bax/bcl-2 ratio, reduces levels of p-MAPKs, and p-AKT, induces caspase-dependent MEK cleavage and up-regulation of MEKK-1, increases annexin V binding, which are markers of the apoptosis pathway (Johnson, 2002). Microarray studies in prostate cancer cells reveal many biologically relevant molecular targets of 1,25VD. For example, in LNCaP cells, 1,25VD causes

growth arrest through the induction of insulin-like growth factor binding protein-3 (IGFBP3). These results provide a starting point for additional investigations to fully elucidate the mechanism of 1,25VD action in the prostate. 1,25VD can also significantly increase the efficacy of drug-mediated cytotoxicity. Phase I and II trials of 1,25VD either alone or in combination with carboplatin, paclitaxel or dexamethasone have been initiated in patients with prostate cancer. Data from these studies indicate that high-dose 1,25VD is feasible on an intermittent schedule, and provides proof of the concept that 1,25VD or its analogs are clinically effective (Johnson, 2002).

1,25VD inhibits the proliferation and induces the differentiation of normal and leukemic myeloid cells into monocytes (Abe, 1981). 1,25VD regulates numerous genes such as c-fos, c-myc, IL-1, IL-6, TNF $\alpha$ , p21, p27 and Mad1, a pro-differentiating gene. These findings suggest that 1,25VD action on leukemic cells involves cell cycle control and differentiation. The response of hematopoietic cells to 1,25VD treatment depends on the cell type, differentiation state and dose of 1,25VD used. In most cell types, growth inhibition and maturation accompany each other. Both antiproliferative and differentiating effects of 1,25VD suggest a therapeutic role for the drug in hematological malignancies.

In keratinocytes (Kitano, 1991), colonic adenocarcinoma cell lines (Scaglione-Sewell, 2000) and squamous cancer cells (Hager, 2001), 1,25VD also blocks cell cycle progression in the G1 phase, preceded by the induction of the cyclin-dependent kinase inhibitors p21 and p27. This induction involves direct activation of p21 promoter through VDRE, as well as indirect mechanisms involving induction of growth inhibitory cytokine TGF $\beta$ 1 (transforming growth factor beta 1) and its type II receptor. In squamous cancer

cells, increased levels of CDK inhibitors prevent phosphorylation of the retinoblastoma protein and subsequent release of transcription factors of the E2F family. Therefore, upregulation of S-phase-related genes, such as the proto-oncogene c-myc, is inhibited. Non-classical positive VDREs were also demonstrated in the promoter of c-fos and phospholipase C  $\gamma$ 1, that both play a role in growth regulatory pathways. In human osteoblastic cells, cell cycle arrest at G0-G1 by a VD analogue is accompanied by hypophosphorylation of Rb followed by strong inhibition of Cdk2 activity (Maenpaa, 2001). These effects also correlated with increased level of p27, decreased level of Cdk2 and cyclin E, but p21 and cyclinD1 were not affected. G2/M arrested by VD or a VD analogue was also detected in Keratinocytes (Kobayashi, 1998).

1,25VD has also been shown to induce apoptosis in breast (Welsh, 1994), prostate (Hsieh, 1997), colon (Diaz, 2000) and glioma (Baudet, 1996) cell lines. 1,25VD and its analogues induce apoptosis in MCF-7 breast cancer cells; induction of apoptosis was associated with upregulation of p53 and Bax. 1,25VD and its analogues are also capable of inducing apoptosis in T47-D breast cancer cells, which possess a mutated p53. It appears that induction of apoptosis is independent of p53 status (Mathiasen, 1999). This study suggests that sensitivity to 1,25VD-mediated apoptosis may be determined by the relative expression or subcellular distribution of pro- and anti-apoptotic members of the bcl-2 family rather than activation of any known caspase (Mathiasen, 1999).

Upregulation of apoptosis-related proteins such as clusterin, cathepsin B and TGF $\beta$  has been reported in MCF-7 cells undergoing apoptosis in response to 1,25VD and its analogues (James, 1996). In prostate cancer cells, 1,25VD-induced decreases in the levels of antiapoptotic proteins Bcl-2, Bcl-XL, and Mcl-1, BAG1L, XIAP, cIAP1, and

cIAP2 (without altering proapoptotic Bax and Bak) in association with increases in apoptosis (Guzey, 2002). Moreover, induction of apoptosis by 1,25VD was suppressed by overexpressing Bcl-2, a known blocker of cytochrome c release (Blutt, 2000; Guzey, 2002). At this time the molecular mechanisms by which 1,25VD may induce apoptosis are not fully understood.

### **Vitamin D analogue in cancer prevention and therapy**

Since the potential use of 1,25VD in the treatment or prevention of cancers is limited by the tendency of 1,25VD to cause hypercalcemia, recent research has focused on the development of analogues with less calcemic and/or greater antineoplastic activity than 1,25VD. Numerous VD analogues have been synthesized and many of them have modifications in the C-17 side chain of VD.

Among them, Seocalcitol (EB1089) is the analog that has been widely administered to patients in Europe in a Phase I trial resulting in stabilization of the disease in patients with advanced breast and colorectal cancer (Gulliford, 1998). EB1089 contains a conjugated double bond system and is approximately 50 times more potent than 1,25VD *in vitro*, while the actions of EB1089 in calcium metabolism *in vivo* are markedly reduced. Phase II trials with hepatomas showed reduction in tumor dimensions in patients with an advanced bulky, solid tumor (Dalhoff, 2003). Their studies suggest that 1,25VD or its analogue have an effect in the treatment of multiple human cancers.

Either 1,25VD or EB1089 in combination with 9-cis RA act cooperatively to inhibit growth of breast, prostate, ovarian or small cell lung cancer cells (James, 1995; Guzey, 1998). 9-cis RA is a metabolite of vitamin A that has potent influences on cell differentiation, proliferation, homeostasis, and development. 1,25VD acts synergistically



with dexamethasone to suppress growth of breast, OCa and prostate cell lines (Johnson, 2002; Saunders, 1995). These findings have important implications for the use of retinoids or dexamethasone with 1,25VD in cancer therapy. 1,25VD treatment enhances the sensitivity of prostate cancer cells to a number of anti-cancer drugs, such as paclitaxel (Taxol) and cisplatin (cis-diamminodichloroplatinum), indicating that VD compounds may be useful if used in combination with conventional chemotherapy (Johnson, 2002).

### **Vitamin D and ovarian cancer (OCa)**

Similar to breast and prostate cancers, OCa mortality and incident rates are lower in countries within 20 degrees of the equator (Waterhouse, 1976) where there are high amounts of sunlight. In the US women between the ages of 45-54 living in the North have 5 times the OCa mortality rate than women living in Southern states (Devasa, 1999; Waterhouse, 1987). The inverse correlation between sunlight exposure and OCa mortality indicates that in American women decreased synthesis of 1,25VD may contribute to OCa initiation and/or progression (Lefkowitz, 1994).

OCa has the worst prognosis and remains the most challenging among gynecological malignancies, since it is not diagnosed at an early stage and has severely progressed when diagnosed. Although surgical dissection of tumors and intense chemotherapy is routinely used to treat OCa, severe drug resistance results in only 20-30% survival rates. Poor response of advanced OCa to current treatments necessitates the development of novel therapeutic strategies to fight this deadly disease. In recent years studies have been initiated to develop synthetic VD analogs as therapeutic agents for a variety of human cancers including breast, prostate and colon cancers, while the similar studies in ovary are very limited.

VDR has been found in both normal and cancerous human ovarian cells by immunohistochemistry; 83.3% of normal surface epithelium shows weak to moderate VDR immunoreactivity. Moderate to strong nuclear immunoreactivity for VDR was detected in almost all ovarian carcinomas tested (Villena-Heinsen, 2002). VDR was also found in rat ovaries by immunohistochemistry (Johnson, 1996) and in hen ovaries by ligand-binding assays (Dokoh, 1983), indicating that ovarian cells can respond to VD. VDR expression in gynecologic neoplasms, including OCa (Ahonen, 2000; Saunders, 1992; Villena-Heinsen, 2002), has also been described, indicating that VD could be an effective agent in OCa treatment and/or chemoprevention. The presence of VDR was demonstrated in the ovary and the VD-induced decrease in cell number in CHO and OVCAR3 was also described. Specifically, Ahonen *et al.* (2000) showed that a 9-day treatment of OVCAR3 cells with 100 nM 1,25VD resulted in 73% inhibition of growth. However, the mechanism of VD action in OCa cells, as well as the potential of 1,25VD and its analogues in OCa treatment remains unknown.

### **GADD45 and ovarian cancer**

The growth arrest and DNA damage-inducible (GADD) gene GADD45 codes for a multifunctional protein (19 kD) that binds numerous proteins and plays a role in cell cycle progression as well as the maintenance of genomic stability. Both genotoxic (i.e., UVR, IR, cisplatin, and adriamycin) and nongenotoxic stresses (i.e., apoptotic and/or growth-inhibitory cytokines, serum starvation) induce GADD45 activation. GADD45 $\alpha$  (GADD45) belongs to the GADD45 family which also contains GADD45 beta and gamma. GADD45a is the only member that is induced by p53; the p53 binding site has

been identified in intron 3 (Hollander, 1993). p53-independent induction of GADD45 may also be achieved depending on the insult.

GADD45 interacts with the products of two other p53-regulated genes, *p21* and *PCNA* (proliferating cell nuclear antigen) (Vairapandi, 1996; Smith, 1994). *PCNA* impedes Gadd45-mediated negative growth control (Vairapandi, 2000). Zhan *et al.* (1999) have shown that Gadd45, through its association with *cdc2*, appears to disrupt interactions between *cdc2* and Cyclin B1 and thus may induce arrest at G<sub>2</sub>/M.

GADD45-null mice have increased sensitivity to dimethylbenzanthracene (DMBA)-induced carcinogenesis (Hollander, 2001). It is worth to note that in *Gadd45a*-null mice, DMBA induces a dramatic increase in female ovarian tumors compared to the wild type (Hollander, 2001). Therefore, GADD45 may protect the ovary from carcinogenesis, while the role of GADD45 in OCa is not well defined. Although the upregulation of GADD45 by 1,25VD has been found in squamous cancer cells (Akutsu, 2001), the mechanism of the upregulation and the role of GADD45 in 1,25VD induced growth inhibition remain unknown.

### **Telomerase and ovarian cancer**

The ends of chromosomes, the telomeres, are subject to progressive shortening in normal somatic cells, leading ultimately to irreversible growth arrest. In contrast, telomeres in all cancer cells are stabilized in length and effectively immortalized by the enzyme telomerase, which catalyzes the synthesis of telomeric DNA repeats. Telomerase is a ribonucleoprotein complex that is made up of three components: 1. RNA template-human telomerase RNA (hTR) contains a sequence complementary to the telomeric TTAGGG repeat; Telomeres act as protective caps, stabilizing the chromosomes by

preventing their degradation and aberrant recombination during cell division. 2. Protein component -human telomerase associated protein 1- (hTEP1). 3. Catalytic subunit, known as hTERT, which is a type of reverse transcriptase able to synthesize TTAGGG repeats from the RNA template. The cellular activity of telomerase is determined by the presence or absence of hTERT. All human somatic cells constitutively express hTR (Cech, 2004; Newbold, 2002).

Telomerase is responsible for the replication of chromosome end structures and is strongly upregulated in most human cancers. RT-PCR analysis revealed that hTR and TP1 mRNA were expressed in more than 80% of OCa and even in normal ovaries. There was a significant correlation of telomerase activity with hTERT mRNA expression but not with TP1 or hTR. Repression of telomerase activity is associated with hTERT mRNA, but the expression of hTR and TP1 remained unchanged (Park, 1999; Kyo, 1999). The rate-limiting step for telomerase activity seems to be the expression of the hTERT gene. The precise mechanism of how hTERT is regulated has not been elucidated yet.

It has been shown that OCa cells containing wild type hTERT readily produce tumors while cells with dominant negative hTERT failed to form tumor in nude mice. This study confirms the hypothesis that inhibition of telomerase will decrease tumorigenicity of OCa cells *in vivo* and supports the concept that hTERT is a potential site for anti-cancer drug design for OCa (White, 2001). The protective effect of telomerase from apoptosis has been proposed for human fibroblasts and neuron cells (Ren, 2001; Gorbunova, 2002; Fu, 2000). A study of epidermoid tumor cells indicated that telomerase inhibition in cells with short telomeres leads to chromosomal damage,

which in turn triggers apoptotic cell death (Zhang, 1999). These data indicated that inhibition of telomerase activity is a potential approach for the treatment of human malignancy. Until now, little is known about the mechanism and significance of telomerase repression by VD in solid tumors including OCa. We observed growth inhibition by VD in OCa cells. Consequently, the mechanism and role of telomerase in VD induced growth inhibition was investigated in OCa cells.

## **STUDY OBJECTIVES**

We hypothesize that 1,25VD through VDR-mediated gene regulation inhibits the development of OCa; We further hypothesize that 1,25VD and its synthetic analogues might be used for OCa prevention and therapy. Based on hypothesis, the study objectives are: 1. Determine the biological responses of OCa cells to 1,25VD treatment; 2. Examine molecular mechanism of 1,25VD action by identifying target genes which mediate the different biological response; 3. Test the antitumor activity of 1,25VD analogues in vitro and in vivo.

## **RESULTS**

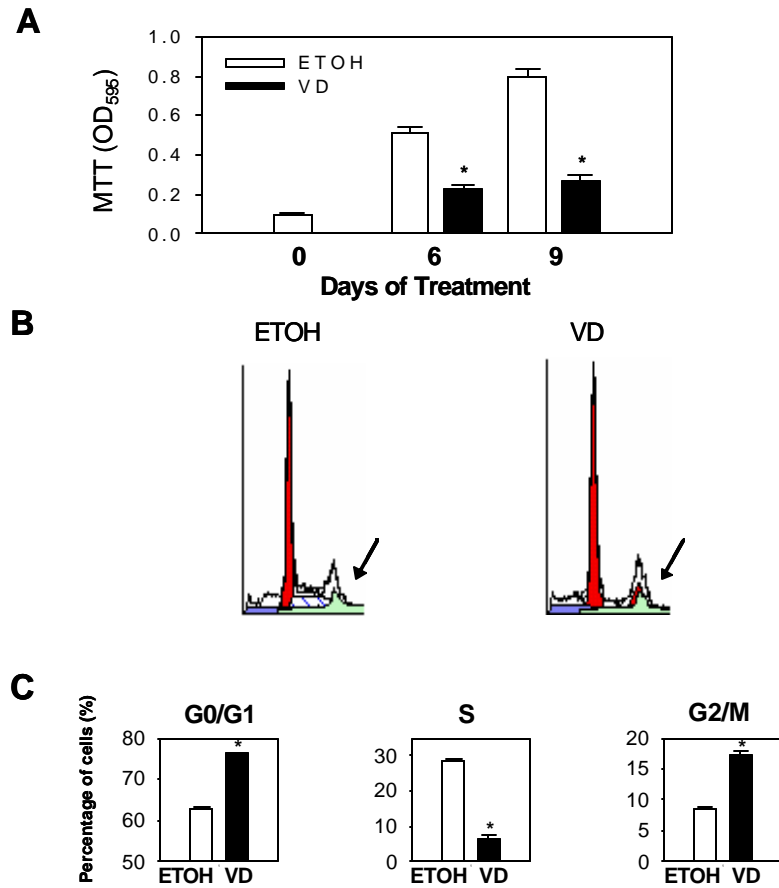
The work presented here provides evidence that GADD45 and telomerase are regulated by 1,25VD and defines a mechanism for 1,25VD in OCa cells. Our studies identify GADD45 and hTERT as important mediators of the tumor-suppressing activity of 1,25VD in OCa cells. Our study also provides preclinical data indicating the effectiveness of synthetic 1,25VD analogues in OCa prevention and therapy.

### **Effect of 1,25VD in OCa cells**

#### **1. 1,25VD suppresses OCa cell growth and induces cell cycle arrest at G1/S and G2/M**

To better understand the molecular mechanism of 1,25VD action in OCa cells, we tested the response of OCa cells to 1,25VD in proliferation assays. Cells were treated with vehicle (ethanol, ETOH) or  $10^{-7}$  M 1,25VD for 6 or 9 days and cell growth was determined by MTT assays. As shown in Figure 5, panel A, OVCAR3 cell growth decreased in the presence of  $10^{-7}$  M 1,25VD in a time-dependent manner, confirming the sensitivity of OVCAR3 to VD (Saunders, 1992). Since cell growth was not significantly affected by 1,25VD at concentrations of  $10^{-8}$  M or lower (data not shown), it appears that there is a threshold to the action of 1,25VD.

To determine the mechanism underlying the 1,25VD-induced growth suppression, OVCAR3 cells were treated with vehicle or  $10^{-7}$  M 1,25VD for 9 days and analyzed by



**Fig. 5. 1,25VD inhibits OCa cell growth and induces cell cycle arrest at G1/S and G2/M checkpoints.**



**Fig. 5. 1,25VD inhibits OCa cell growth and induces cell cycle arrest at G1/S and G2/M checkpoints.** (A) Suppression of cell growth by 1,25VD. OVCAR3 cells were plated in 96 wells and treated with  $10^{-7}$  M 1,25VD (VD) or ethanol (ETOH) as a vehicle. Cell numbers were determined by the MTT assay. Eight samples were analyzed for each data point, and the data were reproduced three times, \*P<0.01 (versus ETOH treatment). (B) Induction of cell cycle arrest at G1/S and G2/M checkpoints by 1,25VD. OVCAR3 cells were treated with ETOH or  $10^{-7}$  M 1,25VD for 9 days. Treated cells were subjected to flow cytometry analysis. Data were reproduced three times. The cell cycle profile of a representative experiment is shown. G2/M peak (red color) is indicated by the arrow. (C) Bar graphs show the average percentages of cells at G0/G1, S and G2/M in OVCAR3 cells treated with 1,25VD or ETOH for 9 days, \* P<0.05 (versus ETOH treatment).

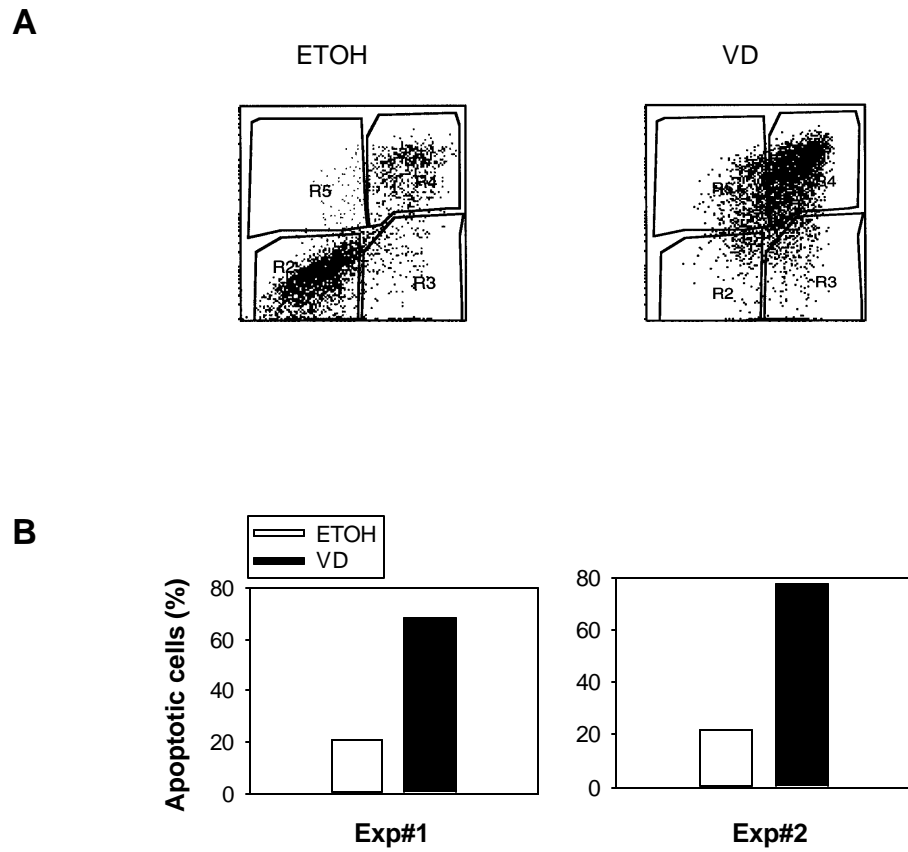
flow cytometry. Figure 5, panel B shows that 1,25VD decreased the percentage of cells in the S phase, which was accompanied by an accumulation of cells in G0/G1 and G2/M. This suggests that 1,25VD causes cell cycle arrest at both G1/S and G2/M checkpoints. The increase in G0/G1 was estimated as 13% while the percentage of cells at G2/M increased by 8% (Fig. 5C), suggesting that cell cycle arrest at both checkpoints contributed roughly equally to the growth-suppressing activity of 1,25VD. Contrary to drugs used in conventional cancer chemotherapy, inhibition of cancer cell growth by 1,25VD is a chronic process, explaining why the effect on the cell cycle is modest and requires treatment for a longer time.

## **2. 1,25VD induces apoptosis in OCa cells**

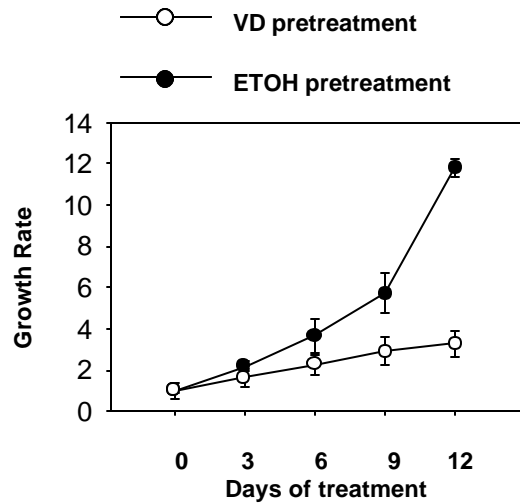
To determine whether 1,25VD treatment induces apoptosis in OVCAR3 cells, OVCAR3 cells were treated with vehicle or 1,25VD for 9 days and fragmented DNA was detected by the Annexin V apoptosis assay kit. Apoptosis was induced to more than 60% in OVCAR3 cells after 9 days treatment of 1,25VD, as shown in Figure 6.

## **3. 1,25VD-treated OVCAR3 cells recover slowly after 1,25VD withdrawal.**

To determine whether changes induced by 1,25VD are sustainable, we tested whether growth inhibition of OVCAR3 cells by 1,25VD can be reversed by removal of the hormone. OVCAR3 cells are treated with 1,25VD for 9 days, and then 1,25VD was removed and cells were plated in 96 well plates with fresh medium. Cells were subsequently grown for an additional 12 days in the absence of the hormone. Consistent with studies in prostate cancer cells (Blutt, 2000), Figure 7 demonstrated that OVCAR3 cells pretreated with 1,25VD recover slowly from the treatment when



**Fig. 6. 1,25VD induces apoptosis in OCa cells.** (A) OVCAR3 cells were treated with ETOH or  $10^{-7}$  M 1,25VD (VD) for 9 days. Apoptosis index was determined by DNA fragmentation assay and a representative profile is shown. (B) Bar graphs show percentage of apoptotic cells in two experiments.



**Fig. 7. OCa cells recover slowly after 1,25VD treatment.** OVCAR3 cells were pretreated with  $10^{-7}$  M 1,25VD (VD) or ETOH for 9 days. Recovery of treated cells was assessed at indicated times after plating pretreated cells in 96 well plates. Cell numbers were determined with the MTT assay. Eight samples were analyzed for each data point, and the data were reproduced three times.

1,25VD is removed from the medium compared with vehicle treatment. The poor recovery of OVCAR3 cells from 1,25VD treatment further confirmed that extensive growth inhibition was induced by 1,25VD.

### **G2/M arrest by 1,25VD in OCa cells is mediated through the induction of GADD45 via an exonic enhancer**

To identify the genes that mediate the inhibitory effects of 1,25VD on cell cycle progression, a microarray analysis was performed to screen for genes that are differentially expressed in OCa cells treated with vehicle vs.  $10^{-7}$  M 1,25VD. OVCAR3 cells treated with 1,25VD showed a significant induction of the 1,25VD-dependent 24-hydroxylase gene, a gene is known to be upregulated by 1,25VD. In OVCAR3 cells, 1,25VD regulated genes encoded growth factors/modulators, cytokines, kinases and transcription factors. Some of them were implicated in cell cycle regulation and apoptosis (data not shown).

GADD45 is a nuclear protein with well-known roles in G2 control and it has been shown to be upregulated by 1,25VD in our microarray analysis. In order to explore the molecular mechanism of 1,25VD-induced G2/M arrest, the regulation of GADD45 was investigated in OCa cells.

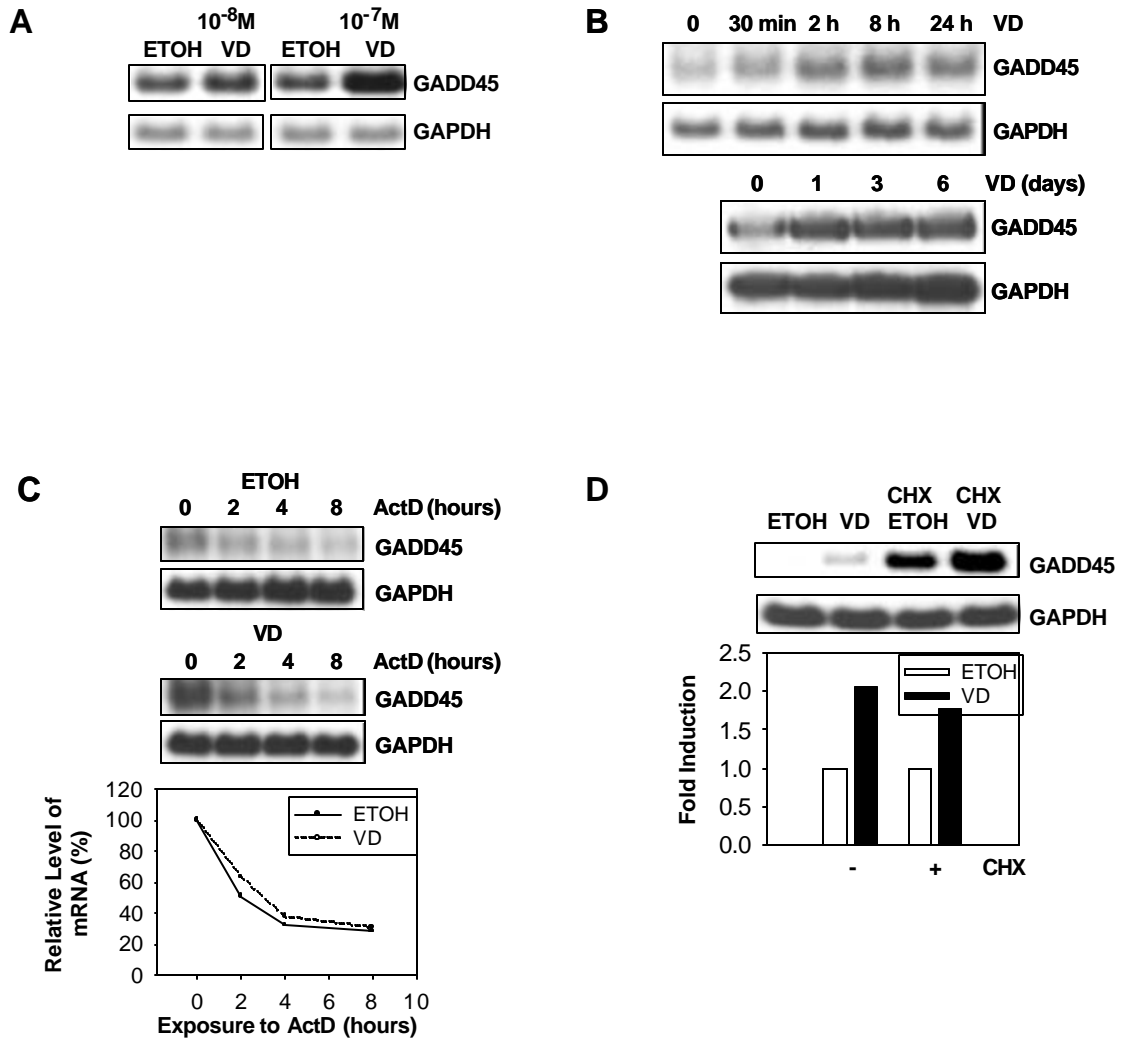
#### **1. GADD45 is a primary and immediate early response gene for 1,25VD in OCa cells**

Among the many differentially expressed genes from the microarray experiment, GADD45 was one of the genes that were upregulated by 1,25VD (data not shown). Since GADD45 has a well-established role in cell cycle control (Wang, 1999; Zhan, 1999) in ovarian tumorigenesis (Hollander, 2001), we used Northern blots to confirm GADD45

regulation by 1,25VD. Compared to cells treated with vehicle,  $10^{-7}$  M 1,25VD significantly increased GADD45 mRNA levels;  $10^{-8}$  M 1,25VD caused a barely detectable increase (Fig. 8A), showing that 1,25VD induction of GADD45 mRNA is dose-dependent. The induction was detectable as early as 2 hours following 1,25VD treatment with maximum induction detected at 8 hours (Fig. 8B), suggesting that GADD45 is an immediate early responsive gene for 1,25VD. Treatment for times longer than 8 hours maintained the induction but did not further enhance it. mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were not affected by 1,25VD, showing some specificity for the effect of 1,25VD on GADD45. The stability of GADD45 mRNA, as measured in the presence of actinomycin D, an inhibitor of RNA synthesis, was not different between cells treated with vehicle and 1,25VD (Fig. 8C), suggesting that the regulation of GADD45 by 1,25VD is transcriptional. Although the fold-induction of GADD45 was decreased (Fig. 8D), it persisted in the presence of an inhibitor of protein synthesis, cycloheximide. This shows that GADD45 induction by 1,25VD does not require new protein synthesis, thus identifying GADD45 as a primary 1,25VD target gene in OCa cells. This is consistent with the data from squamous carcinoma cells (Prudencio, 2001; Akutsu, 2001).

## **2. A novel VDRE in the 3' untranslated region of GADD45 mRNA mediates the transcriptional up-regulation of GADD45 by 1,25VD**

To define the specific DNA elements that mediate the induction of GADD45 by 1,25VD, the genomic sequence of GADD45 was examined for the presence of putative VDREs. Based on similarity to the consensus VDRE sequence (Toell, 2000)



**Fig. 8.** 1,25VD increases transcription of GADD45 mRNA in OCa cells.

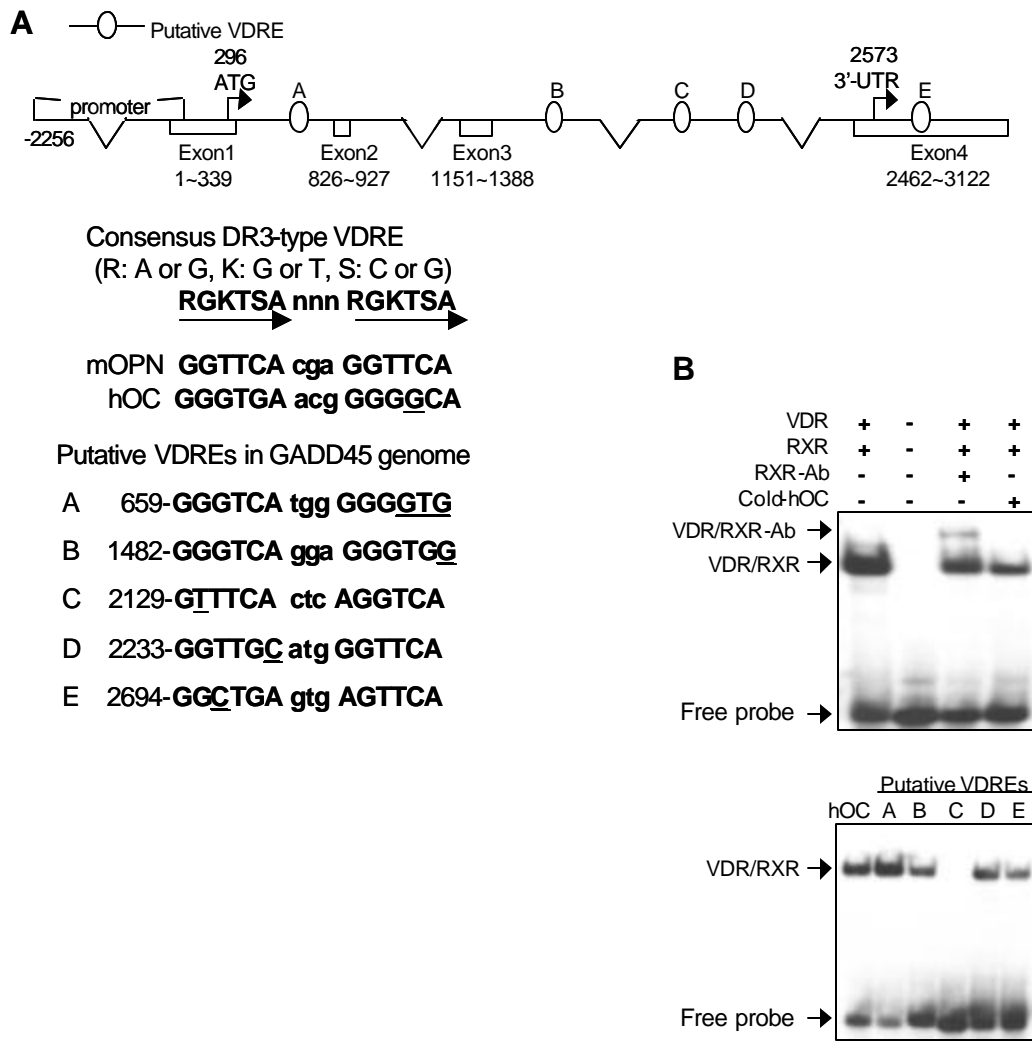
**Fig. 8. 1,25VD increases transcription of GADD45 mRNA in OCa cells.** (A) Dose-dependent induction of GADD45 mRNA by 1,25VD. Total RNA was isolated from OVCAR3 cells treated with ETOH or 1,25VD (VD) at indicated dosages for 24 h. 20 µg RNA was used for Northern blot analysis with radio-labeled GADD45 and GAPDH probes. (B) Time course of the induction of GADD45 mRNA by 1,25VD. OVCAR3 cells were treated with ETOH or  $10^{-7}$  M 1,25VD for the indicated times. Total RNA was isolated and Northern blot was performed as in panel A. (C) Lack of 1,25VD effect on GADD45 mRNA stability. OVCAR3 cells were treated with ETOH or  $10^{-7}$  M 1,25VD for 24 h. The cells were washed and subsequently treated with 5 µg/ml actinomycin D (ActD) for the indicated times. Northern blot analysis was performed as in panel A. The signals on the Northern blots were quantified using Scion image Beta 4.02 software. The GADD45 signal was normalized with the corresponding GAPDH signal and presented in the graph as percentage of the GADD45 mRNA level at time 0. (D) Effect of cycloheximide (CHX) on the induction of GADD45 mRNA by 1,25VD. OVCAR3 cells were exposed to ETOH or  $10^{-7}$  M 1,25VD for 24 h in the absence or presence of 25 µM CHX. Northern blot was performed as in panel A. The GADD45 signal was quantified and normalized with the corresponding GAPDH signal as in panel C and presented in the bar graph as fold induction.



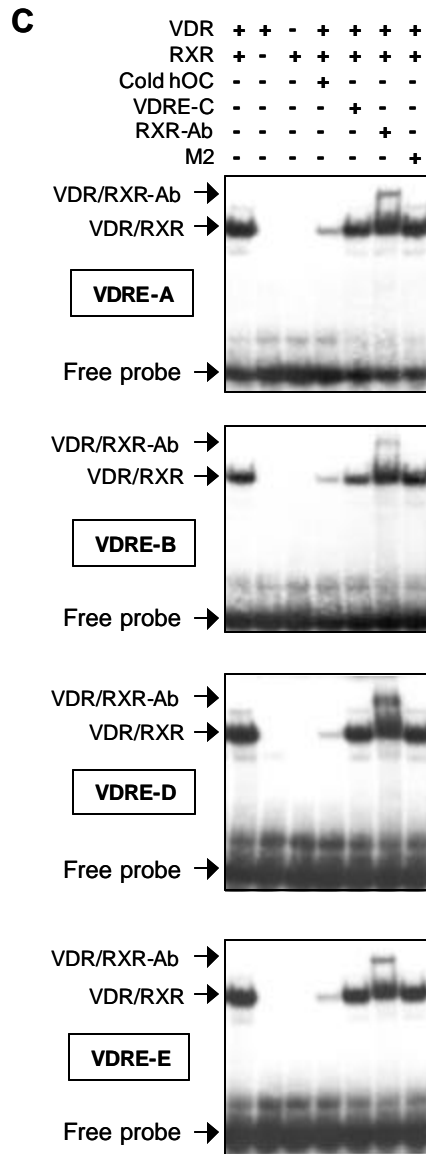
and the VDRE sequence of known VD target genes, such as osteopontin (OPN) (Noda, 1990) and osteocalcin (OC) (Morrison, 1989), five putative DR3-type VDREs were identified (Fig. 9A). Four were in introns, one in exon 4 but none in the 5' promoter region.

To test which of the five putative VDREs bind the receptors, gel mobility shift assays (EMSAs) were performed with recombinant VDR and RXR proteins. As shown in Figure 9, panel B, our conditions allow the detection of a specific VDR/RXR complex with the OC VDRE. The complex is up-shifted by an RXR antibody and decreased by excess cold OC probe (upper panel). Under the same conditions, all putative VDREs except VDRE-C bound RXR/VDR with an affinity comparable to OC VDRE (Fig. 9B, lower panel). The binding is specific for the VDR/RXR heterodimer since neither RXR nor VDR alone formed a detectable complex with the VDRE probes. The VDREs were displaced from the complexes with an excess amount of cold OC VDRE but were not affected by the putative VDRE-C that did not bind VDR/RXR (Fig. 9C). This shows that the binding is specific. The RXR antibody up-shifted the complex while a non-related antibody did not, confirming the presence of RXR in the complex.

To determine whether VDREs binding the receptors in OVCAR3 cells mediate the induction of GADD45 by 1,25VD, a reporter gene was constructed with a 2.6 kb genomic DNA fragment of GADD45 containing all the putative VDREs located upstream of SV40 promoter and the cDNA of firefly luciferase gene (Fig. 10A). In OVCAR3 cells transiently transfected with this reporter, 1,25VD induced the luciferase activity in a dose (Fig. 10B) and time dependent manner (Fig. 10C).

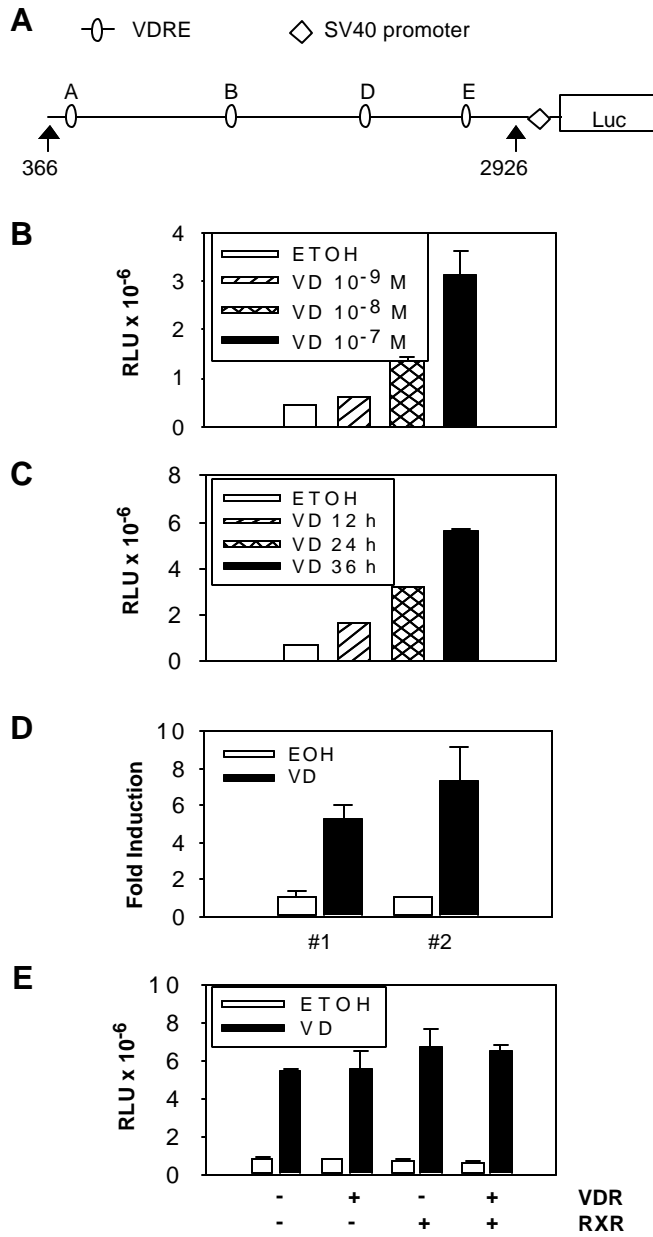


**Fig. 9. Multiple putative VDREs are present in GADD45 genome, which interact with VDR/RXR *in vitro*.**



**Fig. 9. Multiple putative VDREs are present in GADD45 genome, which interact with VDR/RXR *in vitro*.**

**Fig. 9. Multiple putative VDREs are present in GADD45 genome, which interact with VDR/RXR *in vitro*.** (A) Schematic representation of the human GADD45 genome and the position of the putative VDREs. The sequences of consensus DR3, mouse OPN (mOPN) VDRE, human OC (hOC) VDRE and the five putative GADD45 VDREs are listed. Hexameric VDRE half sites are shown in bold capital letters. The 3-bp space is shown in small letters. Deviations from the consensus sequence RGKTSA are underlined. (B) *In vitro* interaction of VDR/RXR heterodimer with putative GADD45 VDREs. EMSAs were performed in the presence of  $10^{-7}$  M 1,25VD using hOC (upper panel) or putative GADD45 (lower panel) VDRE probes. Pre-incubation with 2  $\mu$ g anti-RXR $\beta$  (RXR-Ab) or 100-fold molar excess of cold hOC VDRE (Cold-hOC) was performed for the super-shift and competition experiments, respectively. (C) Specificity of the interaction between VDR/RXR heterodimer and the putative GADD45 VDREs. EMSAs were performed as in panel B. Specificity of the interaction was demonstrated by competition with 100-fold molar excess of unlabeled hOC VDRE oligos as a specific competitor and the lack of competition with VDRE-C oligos as nonspecific competitor. 2  $\mu$ g anti-Flag M2 monoclonal antibody was used as a non-specific antibody control for the super-shifting with anti-RXR antibody.



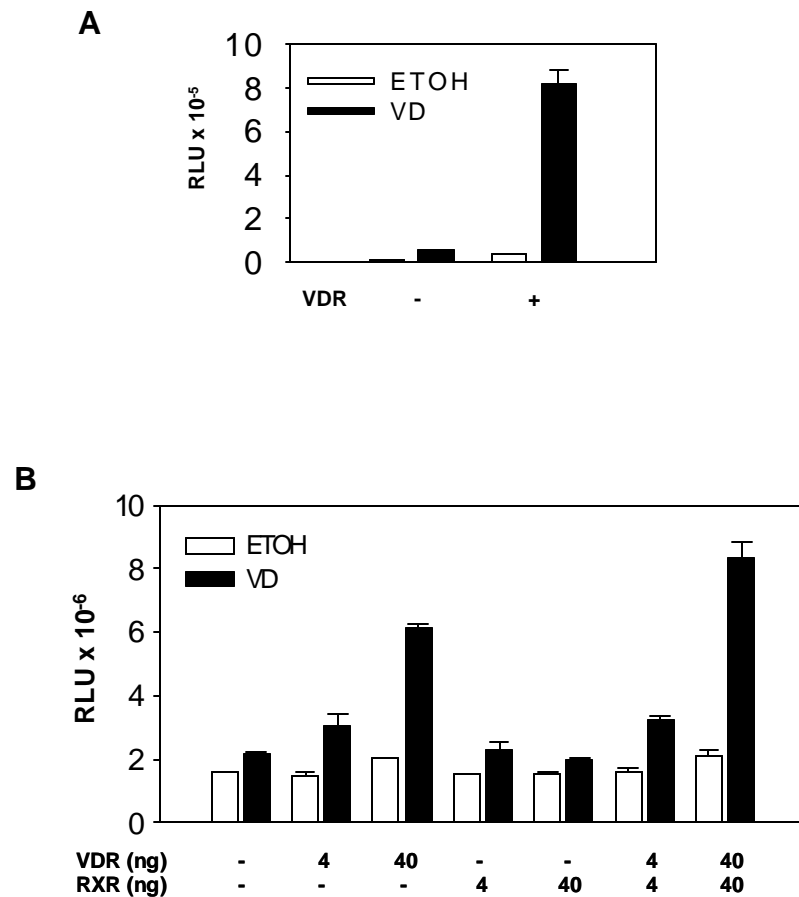
**Fig. 10. 1,25VD induces GADD45 reporter activity through endogenous receptors in OCa cells.**

**Fig. 10. 1,25VD induces GADD45 reporter activity through endogenous receptors in OCa cells.** (A) Schematic representation of GADDLuc construct. (B) Dose-dependent induction of GADD45 VDRE reporter activity by 1,25VD. OVCAR3 cells were transfected with 0.2  $\mu\text{g}$  GADDLuc, 0.05  $\mu\text{g}$  pCMVgal, 0.05  $\mu\text{g}$  p91023B-VDR and 0.05  $\mu\text{g}$  pCMX-RXR $\beta$  and treated with ETOH or 1,25VD at the indicated concentrations. Luciferase activity was determined and normalized with cognate  $\beta$ -gal activity. Each data point was analyzed in duplicate and reproduced three times. (C) Time-dependent induction of GADDLuc luciferase activity by 1,25VD. OVCAR3 cells were transfected as in panel A and treated with ETOH or  $10^{-7}$  M 1,25VD for the indicated times. Luciferase activity was determined as in panel B. (D) Induction of reporter activity by 1,25VD in cells stably transfected with the GADDLuc. OVCAR3 cells stably transfected with GADD45 reporter were transfected with 0.05  $\mu\text{g}$  pCMVgal, 0.05  $\mu\text{g}$  p91023B-VDR and 0.05  $\mu\text{g}$  pCMX-RXR $\beta$  and treated with ETOH or  $10^{-7}$  M 1,25VD for 36 h. Luciferase activity from two stable clones was determined and shown as fold induction. (E) Endogenous VDR/RXR in OVCAR3 cells is sufficient for 1,25VD induction of GADD45 reporter. OVCAR3 cells were transfected with GADDLuc and pCMVgal with or without p91023B-VDR (VDR) and pCMX-RXR $\beta$  (RXR). The total amount of plasmid DNA was balanced with empty vectors. Luciferase activity was determined as in panel B.

This induction was also detected in OVCAR3 cells in which the reporter was stably integrated into the genome (Fig. 10D), showing that it is not an artifact of the transient transfection. Expression of additional VDR, RXR or both did not further increase the induction in OVCAR3 cells (Fig. 10E), suggesting that endogenous RXR and VDR in OVCAR3 cells are sufficient.

To test whether 1,25VD regulates the GADD45 reporter through VDR, the activation of the reporter was tested in HeLa cells that lack functional VDR. As shown in Figure 11, panel A, 1,25VD did not induce the activity of a known VDR reporter, p23, that was constructed with the promoter of 24-hydroxylase (Arbour, 1998). The induction of the p23 reporter was restored by the ectopic expression of VDR in HeLa cells. Similar to the p23 reporter, 1,25VD did not cause measurable induction of luciferase activity in the GADD45 reporter in HeLa cells (Fig. 11B). Co-transfection with VDR restored the 1,25VD induction in a manner that is dependent on the dose of the transfected receptor (Fig. 11B). Transfection with RXR alone did not affect reporter activity but its co-transfection with VDR enhanced the induction compared to cells transfected with VDR alone. Similar to the results with OVCAR3 cells, 1,25VD induced reporter activity in HeLa cells in a dose and time dependent manner after receptor transfection (data not shown). These experiments demonstrate that the regulation of the reporter is VDR-dependent and involves the RXR.

To determine which of the putative VDREs is functional, OVCAR3 cells were transfected with reporter constructs where VDREs, either individually or in



**Fig. 11. Induction of GADD45 reporter activity by 1,25VD is VDR-dependent. (A)**

The lack of functional VDR in HeLa cells. Cells were transfected with 0.2  $\mu$ g p23 and pCMVgal with or without p91023B-VDR. Transfected cells were treated and luciferase activity was determined as in Fig. 10B. **(B)** VDR-dependent induction of GADD45

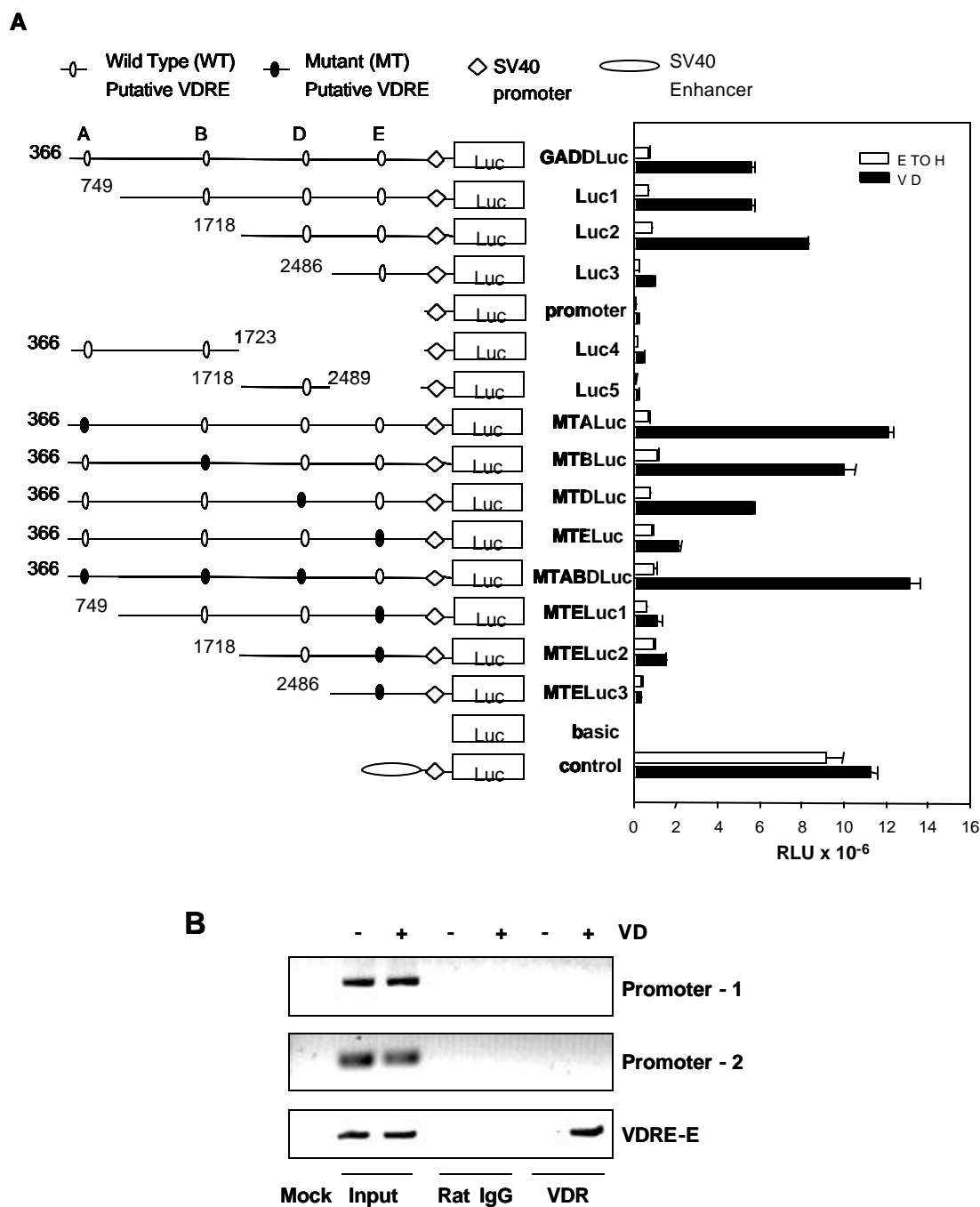
reporter activity by 1,25VD. HeLa cells were transfected with GADDLuc and pCMVgal with the indicated amounts of p91023B-VDR, pCMX-RXR $\beta$  or both. The total amount of plasmid DNA was balanced with empty vectors. Luciferase activity was determined as in Fig. 10B.



combination, were deleted or mutated by altering key nucleotides known to be essential for receptor interaction. As shown in Figure 12, panel A, deletion of the VDRE-A or VDRE-A plus VDRE-B region did not affect 1,25VD induction. Deletion into the VDRE-D region caused a significant decrease while further deletion into the VDRE-E region eliminated 1,25VD induction. These analyses suggest that regions around VDRE-D and VDRE-E are essential for 1,25VD regulation of GADD45.

Site-directed mutation in VDRE-E eliminated the induction, while single or multiple mutations of key nucleotides in the intronic VDREs had no effect. This suggests that only VDRE-E is essential for the induction. This conclusion is consistent with the lack of VD induction in mutant reporters containing only VDRE-D or both VDRE-A and VDRE-B (Fig. 12A). It is also consistent with the induction of the reporter that contains only VDRE-E (Luc3) and with the loss of this induction by site-directed mutation of VDRE-E (MTELuc3) (Fig. 12A).

Individual mutation of the first two VDREs or combined mutation of all first three VDREs actually increased VD induction, indicating that some VDREs may function in a negative fashion. The deletion of the VDRE-D region, not the mutation of the VDRE-D sequence, caused a decrease in VD induction suggesting that DNA elements in the VDRE-D region for transcription factors other than VDR may cooperate with VDR/RXR binding to VDRE-E to mediate the up-regulation of GADD45. The lack of a VD effect on the activity of pGL3-basic, pGL3-promoter and the pGL3-control vectors showed that the regulation of the reporters by 1,25VD is specific to the GADD45 sequence.



**Fig. 12.** The VDRE in the fourth exon of GADD45 genome is the functional VDRE that mediates the transcriptional induction of GADD45 by 1,25VD in OCa cells.

**Fig. 12. The VDRE in the fourth exon of GADD45 genome is the functional VDRE that mediates the transcriptional induction of GADD45 by 1,25VD in OCa cells.** (A) Mutational analysis of the GADD45 reporter. OVCAR3 cells were transfected with 0.2  $\mu\text{g}$  of the reporter constructs together with pCMVgal, p91023B-VDR and pCMX-RXR $\beta$  and treated with ETOH or  $10^{-7}$  M 1,25VD for 36 h. Luciferase activity was determined as in Fig. 10B and shown on the right. Schematic representation of the different reporter constructs is shown on the left. pGL3-basic, pGL3-promoter and pGL3-control vectors were used as controls. (B) ChIP assays. Soluble chromatin was prepared from OVCAR3 cells treated with ETOH or  $10^{-7}$  M 1,25VD for 60 min. ChIP assays were performed with control (rat IgG) or anti-VDR antibody. The mock control is performed with immunoprecipitates from buffer that contains no soluble chromatin from OVCAR3 cells.

To demonstrate that the VDRE-E interacts with VDR *in vivo*, OVCAR3 cells were treated with or without 1,25VD and ChIP assays were performed with anti-VDR antibodies (Fig. 12B). From soluble chromatin prepared from OVCAR3 cells treated with 1,25VD, the anti-VDR antibody precipitated GADD45 DNA fragments containing the VDRE-E but not the promoter regions. The specificity of the ChIP assay was demonstrated by the lack of VDRE-E DNA in the mock as well as in the immunoprecipitates of rat IgG control. The data shows that VDR is recruited to the VDRE-E *in vivo*. More importantly, the recruitment of VDR to the response element is apparently ligand-dependent since the anti-VDR antibody did not precipitate GADD45 DNA fragments from soluble chromatin prepared from cells treated with the vehicle.

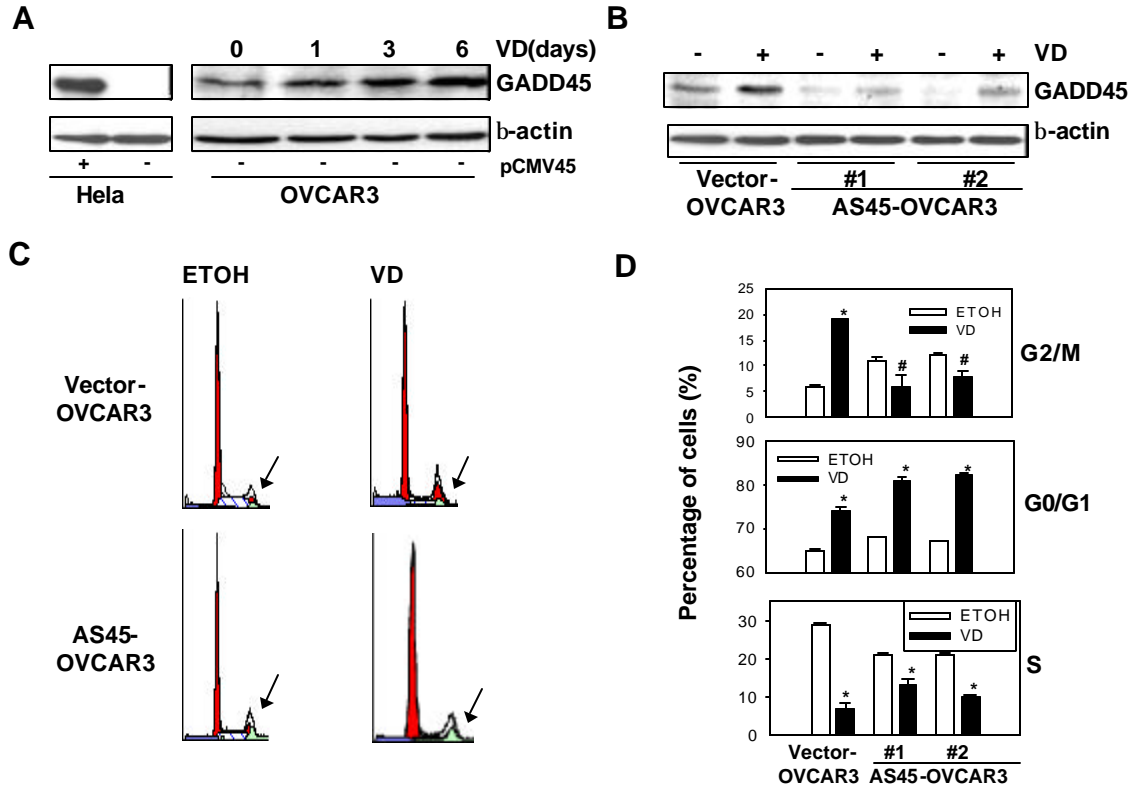
### **3. Upregulation of GADD45 protein is required for the hormone-induced cell cycle arrest at the G2/M but not the G1/S checkpoint**

Our studies have established GADD45 as one of the immediate early response genes for 1,25VD, but questions remain whether regulation at the RNA level extends to the protein level and whether GADD45 mediates the growth-suppressing activity of 1,25VD in OCa cells. To address these questions, OVCAR3 cells were treated with 1,25VD for up to 6 days and GADD45 protein expression was examined by immunoblotting. As shown in Figure 13, panel A, 1,25VD increased the level of GADD45 protein in a time dependent manner in OVCAR3 cells whereas the level of  $\beta$ -actin remained constant during treatment. Compared to the data on GADD45 mRNA (Fig. 8B), 1,25VD-induced accumulation of GADD45 protein is a much slower process. This suggests the presence of additional regulations for GADD45 expression at post-

transcriptional steps. The slower accumulation of GADD45 protein in response to 1,25VD may explain why the hormonal effect on the cell cycle requires longer treatment.

To test whether GADD45 mediates the inhibitory effect of 1,25VD on OCa cell cycle progression, OVCAR3 cells were stably transfected with an expression vector containing GADD45 cDNA in the anti-sense orientation (Zhan, 1994) or an empty vector. Immunoblot analysis of GADD45 was used to select stable clones with significantly reduced level of GADD45 protein by the anti-sense GADD45 as compared to the control clones (Fig. 13B). Although the anti-sense GADD45 did not completely eliminate GADD45 protein (Fig. 13B), it decreased the expression of GADD45 protein in the presence of 1,25VD to a level lower than basal level of control clones (Fig. 13B). In other words, the anti-sense clones represent a functional “knock out” of GADD45 in terms of VD induction.

Flow cytometry showed that a decrease of GADD45 protein in the anti-sense clones was associated with an increase of the proportion of cells in G2/M phase and a decrease of those in S phase (Figs. 13C and 13D). In the control clones, 1,25VD decreased the percentage of cells in S phase and increased the cells in G0/G1 and G2/M, showing that the hormone induced a similar cell cycle arrest at both G1/S and G2/M transitions similar to that in the parental OVCAR3 cells (Figs.5B and 5C). In the anti-sense clones, 1,25VD-induced G2/M accumulation was blocked whereas 1,25VD-induced decrease in S phase and increase in G0/G1 still occurred (Figs. 13C and 13D). The data strongly suggest that GADD45 mediates the



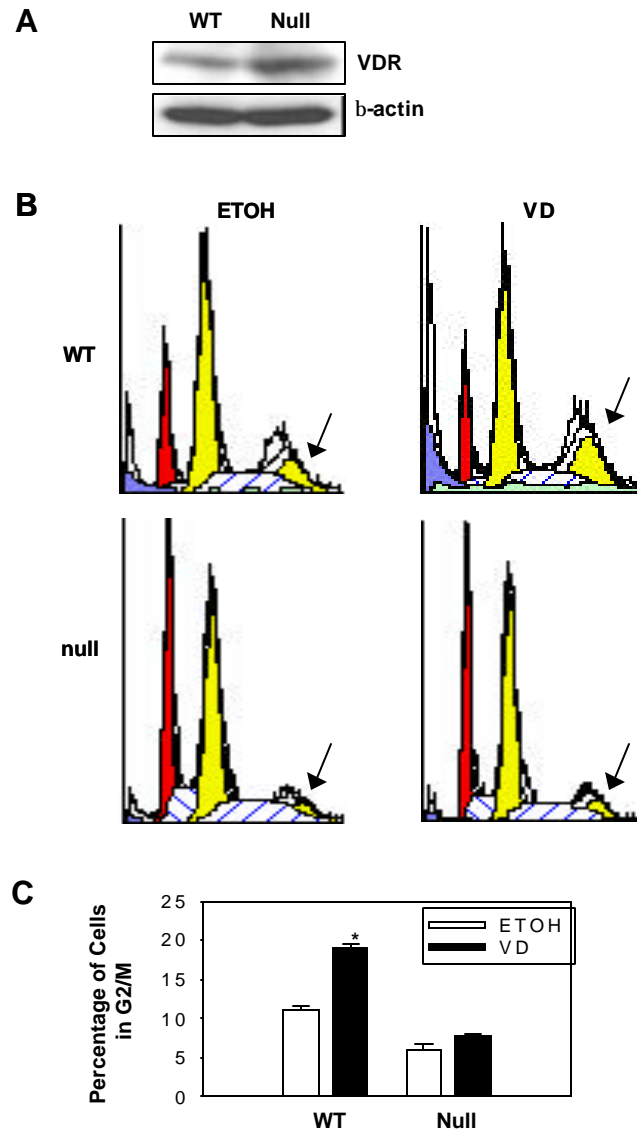
**Fig. 13. Anti-sense GADD45 blocks 1,25VD-induced cell cycle arrest at G2/M, but not G1/S checkpoint.**

**Fig. 13. Anti-sense GADD45 blocks 1,25VD-induced cell cycle arrest at G2/M, but not G1/S checkpoint.** (A) Induction of GADD45 protein expression by 1,25VD in VD-sensitive human ovarian cancer cells. OVCAR3 cells were treated with ETOH or  $10^{-7}$  M 1,25VD for indicated times and the level of GADD45 protein was analyzed by immunoblotting. HeLa cells transfected with pCMV45 plasmid was included as a positive control.  $\beta$ -Actin was used to show the equal amount of total protein is present in each lane. (B) Suppression of GADD45 protein expression by stable expression of GADD45 anti-sense cDNA. OVCAR3 cells stably transfected with control vector (Vector-OVCAR3) or the anti-sense cDNA of GADD45 (AS45-OVCAR3) were treated with ETOH or  $10^{-7}$  M 1,25VD for 24 h. The level of GADD45 and  $\beta$ -actin protein was determined as (A). (C) Abrogation of 1,25VD-induced G2/M arrest in AS45-OVCAR3 clones. Vector-OVCAR3 and AS45-OVCAR3 clones were treated with ETOH or  $10^{-7}$  M 1,25VD for 9 days. Cell cycle distribution was determined by flow cytometry. Three independent experiments were performed and the profile of a representative experiment is shown. G2/M peak is indicated by arrow. (D) Bar graphs show percentage of cells at G2/M, G0/G1, and S phases. Each data point was analyzed in duplicate, \* $P < 0.05$ ; # $P > 0.05$  (versus ETOH treatment).

inhibitory effect of 1,25VD on the G2/M transition in OVCAR3 cells. Similar to the data in colon cancer cells (Wang, 1999), the two untreated anti-sense GADD45 clones had slightly increased G0/G1 and G2/M fractions and slightly lower S fractions, compared with vector-control OVCAR3 cells.

To firmly establish GADD45 as the mediator for the 1,25VD-induced cell cycle arrest at the G2/M transition, we tested the effect of 1,25VD on the cell cycle progression of mouse embryo fibroblasts (MEFs) established from either wild type or GADD45-null mice (Hollander, 1999). Both MEFs expressed similar levels of VDR protein as determined on immunoblots (Fig. 14A). It is known that the MEFs from this strain of mice are a mixture of diploid and tetraploid cells (Hollander, 1999). This complicates the flow cytometry analysis of diploid cells, largely due to the inability to distinguish diploid cells at G2/M from tetraploid cells at G0/G1 phases. Therefore, we compared the changes induced by 1,25VD in the cell cycle distribution of tetraploid cells. As shown in Figure 14, panels B and C, 1,25VD induced a consistent increase in the percentage of cells in G2/M and a decrease of that in S phase of wild type MEFs, although the magnitude of the response was less than in OVCAR3 cells. In GADD45-null MEFs, no induction of G2/M arrest by 1,25VD was observed, confirming the conclusion reached in OVCAR3 cells with the anti-sense approach that GADD45 is required for 1,25VD-induced cell cycle arrest at the G2/M transition. In the wild type and GADD45-null MEFs, 1,25VD did not induce G1/S arrest (data not shown). Since G1/S arrest by 1,25VD was observed in the MEFs derived from another strain of mice (unpublished data for a separate

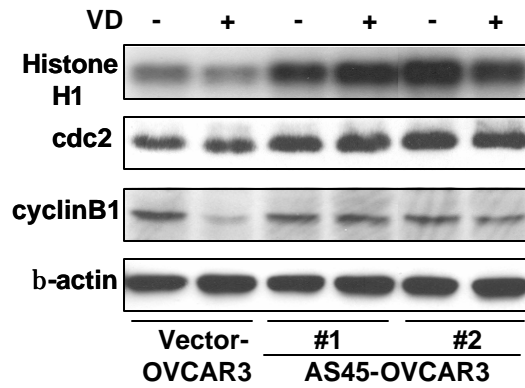




**Fig. 14. 1,25VD induces G2/M arrest in wild type but not in GADD45-null MEFs.**

**Fig. 14. 1,25VD induces G2/M arrest in wild type but not in GADD45-null MEFs.**

(A) Expression of VDR protein in wild type (WT) and GADD45-null MEFs. The level of VDR protein was determined by immunoblotting with anti-VDR antibody and equal loading was shown by immunoblotting with anti- $\beta$ -actin antibody. (B) Induction of G2/M arrest in WT but not GADD45-null MEFs by 1,25VD. WT and GADD45-null MEFs were treated with ETOH or  $10^{-7}$  M 1,25VD for 9 days. The cells were subjected to flow cytometry analysis. Three independent experiments were performed and the profile of a representative experiment is shown. The G2/M peak for tetraploid cells is indicated by the arrow. (C) Bar graph show the percentage of tetraploid cells at G2/M. Each data point was analyzed in duplicate, \*P<0.01 (versus ETOH treatment).



**Fig. 15. 1,25VD decreases cdc2 kinase activity in OVCAR3 transfected with control vector but not in cells stably transfected with the anti-sense cDNA of GADD45.** Vector-OVCAR3 and AS45-OVCAR3 cells were treated with ETOH or  $10^{-7}$  M 1,25VD (VD) for 9 days. Cellular extracts were immunoprecipitated with anti-cyclin B1 antibody. The activity of cdc2 was assayed using Histone H1 as a substrate. The level of cdc2 and cyclin B1 protein was determined by immunoblotting.  $\beta$ -Actin blot was included to show the equal loading.

study in our lab), the response to 1,25VD appears to vary among MEFs from different strains.

#### **4. GADD45 mediates 1,25VD-induced decrease in cdc2 kinase activity in OCa cells**

It is well established that the G2/M transition of mammalian cells is controlled by the M phase promoting factor, a heterodimeric complex between cdc2 and cyclin B. Studies in recent years suggest that GADD45 may directly regulate the activation of cdc2 kinase (Zhan, 1999). Therefore, the effect of 1,25VD on cdc2 kinase activity in control and GADD45 anti-sense clones was measured by *in vitro* immunocomplex kinase assays. As shown in Figure 15, 1,25VD decreased the kinase activity of cdc2 in the control clone. In the GADD45 anti-sense clones, there is an increase in cdc2 kinase activity (Fig. 15), presumably due to the decrease in the level of GADD45 protein. More importantly, 1,25VD did not decrease the activity to a level below the basal activity of control clones. Immunoblot analysis showed that 1,25VD decreased the level of cyclin B protein and that the decrease was not observed in the anti-sense clones (Fig. 15), suggesting that GADD45 mediates the VD effect on cdc2 activity by regulating the cyclin B level in OCa cells. The data clearly suggest that G2/M arrest induced by 1,25VD in OCa cells is mediated through GADD45 and the subsequent decrease in cdc2 kinase activity.

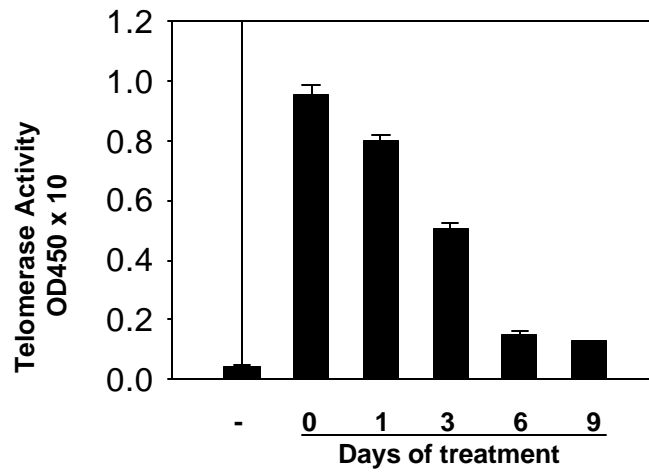
Collectively, our data demonstrated that G2/M arrest by 1,25VD in OCa was mediated through the induction of GADD45 via an exonic enhancer. Next, to further demonstrate the mechanism of 1,25VD induced growth inhibition, we investigated the regulation of telomerase by 1,25VD.

## **1,25VD-induced apoptosis is mediated by destabilization of hTERT mRNA and a decrease in telomerase activity**

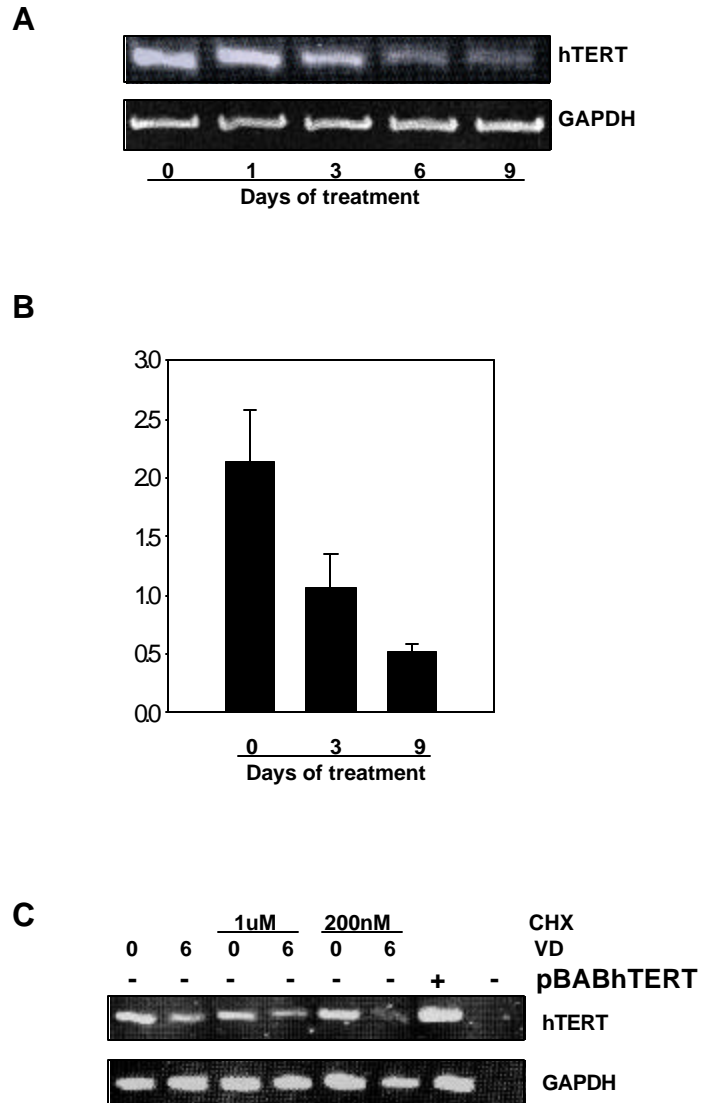
Telomerase activity is increased in most human cancers. It has been shown that 1,25VD reduced telomerase activity in HL-60 human acute myeloblastic leukemia cells correlated with 1,25VD-induced differentiation (Xu, 1996). In an effort to understand the mechanism underlying 1,25VD induced apoptosis, our study provides evidence that telomerase is decreased by 1,25VD and its decrease is responsible for the apoptosis induced by 1,25VD.

### **1. 1,25VD down-regulates telomerase activity in OCa cells**

Cells undergoing apoptosis are associated with the decrease in telomerase activity (Saretzki, 2003). As shown in Figure 6, apoptosis was induced more than 3-fold in OVCAR3 cells by 1,25VD. To fully understand the role of 1,25VD in OCa cells and determine telomerase function in 1,25VD-induced apoptosis, telomerase activity was determined in OVCAR3 cells using PCR-based telomeric repeat amplification protocol (PCR-TRAP) assay. OVCAR3 cells constitutively expressed significant telomerase activity and 1,25VD decreased telomerase activity in a time-dependent manner (Fig. 16). PCR-TRAP assay performed with various amounts of protein indicated that 200 ng of protein extract provided a quantitative and reproducible assay for telomerase activity on OVCAR3 cells (data not shown). The activity was not detectable when the extract was heated to 65°C to abolish the enzymatic activity of protein components of telomerase. These results confirmed the specificity of the telomerase signal measured in this assay. Within 3 days of 1,25VD treatment, telomerase activity remained ~ 50% of the control, whereas at day 6 and day 9



**Fig. 16. 1,25VD down-regulates telomerase activity in OCa cells.** The OVCAR3 cells were treated with  $10^{-7}$  M 1,25VD for the indicated times. The protein was extracted and subjected to the PCR-TRAP assay, as described in Materials and Methods



**Fig. 17. 1,25VD down-regulates hTERT mRNA expression in OCa cells.**

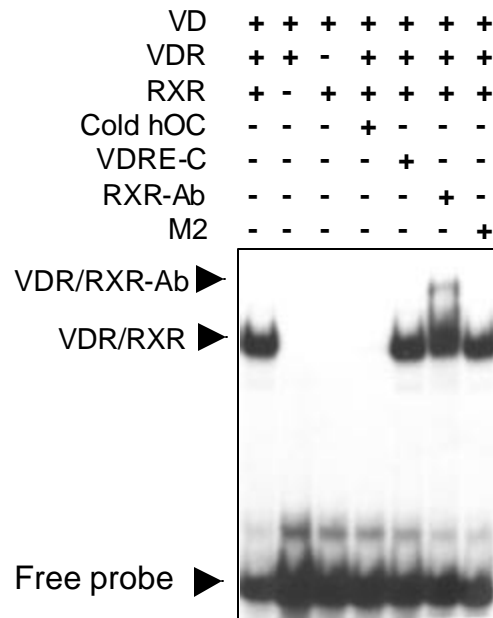
**Fig. 17. 1,25VD down-regulates hTERT mRNA expression in OCa cells.** Dose-dependent inhibition of hTERT mRNA by 1,25VD. Total RNA was isolated from OVCAR3 cells treated with ETOH or  $10^{-7}$  M 1,25VD for indicated times. 2  $\mu$ g RNA was used for RT-PCR analysis (**A**) and Real-time PCR analysis (**B**) as described in Materials and Methods. (**C**) Effect of cycloheximide (CHX) on the inhibition of hTERT mRNA by 1,25VD. OVCAR3 cells were exposed to ETOH or  $10^{-7}$  M 1,25VD for 6 days in the absence or presence of CHX at the indicated concentrations. RT-PCR was performed as shown on panel A. RNA purified from pBABhTERT transfected OVCAR3 cells as positive control. RT reaction without reverse transcriptase was the negative control.



telomerase activity fell to only 15% of the control cell. Combined with data in Figure 5, these experiments showed that inhibition of telomerase activity by 1,25VD was correlated with the growth inhibition and apoptosis induced by 1,25VD. These data supported that 1,25VD induced apoptosis was associated with decreased telomerase activity.

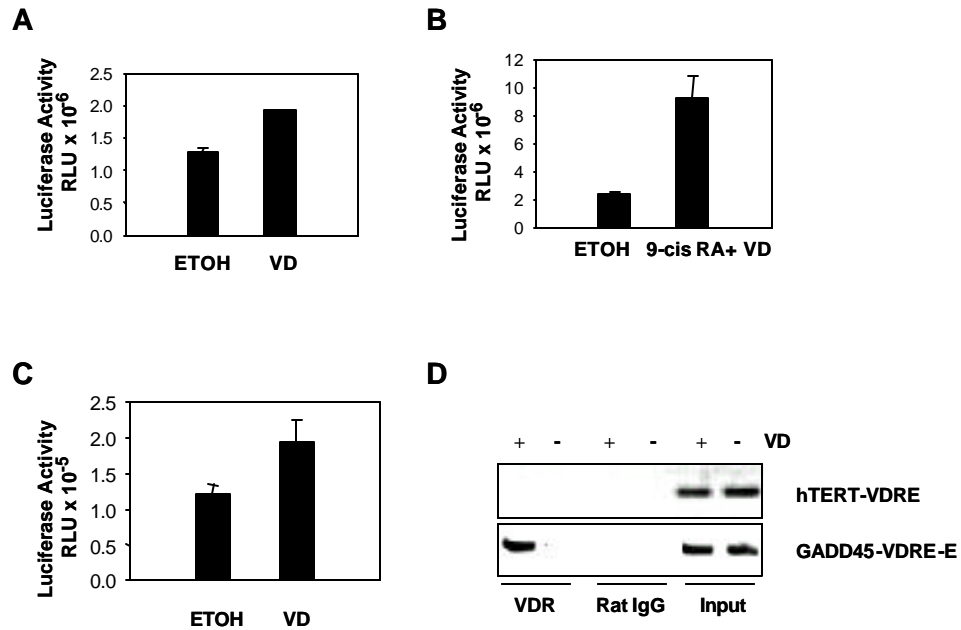
## **2. hTERT is a VD-targeted gene in OCa cells**

To investigate the mechanism of VD action on telomerase, RT-PCR was used to determine the mRNA level of hTERT. Compared to cells treated with vehicle,  $10^{-7}$  M 1,25VD decreased mRNA levels of hTERT in a time-dependent manner (Fig. 17A). Consistent with decreased telomerase activity by 1,25VD, inhibition began after 1 day of 1,25VD treatment, with further decrease detected after treatment for 3, 6 and 9 days (Fig. 17A). The mRNA levels of GAPDH were not affected by 1,25VD, showing some specificity for 1,25VD. In order to quantitatively determine the decrease of hTERT mRNA, real-time PCR was performed using total RNA purified from OVCAR3 cells treated with vehicle or  $10^{-7}$  M 1,25VD. The results confirmed the decrease of hTERT mRNA after 3 days of 1,25VD treatment and further decrease after 9 days of treatment (Fig. 17B). These data indicate that the mechanism of telomerase down-regulation involves the change in the mRNA of hTERT. The down-regulation of hTERT mRNA by 1,25VD persisted in the presence of cycloheximide, an inhibitor of protein synthesis (Fig. 17C), suggesting that hTERT inhibition by 1,25VD does not require new protein synthesis, and that hTERT is a primary responsive gene for 1,25VD in OCa cells.



**Fig. 18. Putative hTERT VDRE specifically binds to VDR/RXR heterodimer.**

EMSAs were performed in the presence of  $10^{-7}$  M 1,25VD using putative hTERT VDRE probes. Pre-incubation with 2  $\mu$ g anti-RXRb (RXR-Ab) or 100-fold molar excess of cold hOC VDRE (Cold-hOC) was performed for the super-shift and competition experiments, respectively. Specificity of the interaction was demonstrated by competition with 100-fold molar excess of unlabeled hOC VDRE oligos as a specific competitor and the lack of competition with Gadd45 VDRE-C oligos as nonspecific competitor. 2  $\mu$ g anti-Flag M2 monoclonal antibody was used as a non-specific antibody control for the super-shifting with anti-RXR antibody.



**Fig. 19. The putative VDRE is not a functional VDRE in OVCAR3 cells.** The OVCAR3 cells were transfected with 0.2  $\mu\text{g}$  pGL3-3328Luc (**A**, **B**) together with 0.1  $\mu\text{g}$  CMVbgal and 0.1  $\mu\text{g}$  VDR. The transfected cells were treated with  $10^{-7}$  M 1,25VD,  $10^{-6}$  M 9-cis RA or ETOH as indicated. Luciferase activity was determined and normalized by bgal activity. (**C**) 3328Luc was stably transfected into OVCAR3 cells. The 3328Luc-OVCAR3 cells were treated with  $10^{-7}$  M 1,25VD for 6 days and luciferase activity was determined. (**D**) ChIP assays. Soluble chromatin was prepared from OVCAR3 cells treated with ETOH or  $10^{-7}$  M 1,25VD for 60 min. ChIP assays were performed with control (rat IgG) or anti-VDR antibody.

One putative DR3-type VDRE was identified in the hTERT promoter in a recent report (Ikeda, 2003). To determine if this putative VDRE is functional in 1,25VD-mediated inhibition of telomerase activity in OCa cells, EMSAs were performed and the results showed that this VDRE bound recombinant VDR and RXR proteins (Fig. 18). The binding is specific for the VDR/RXR heterodimer since neither VDR nor RXR alone formed a detectable complex with the VDRE probes. The VDRE was displaced from the complexes with an excess amount of cold hOC VDRE but was not affected by the non-specific sequence VDRE-C. VDRE-C has been shown not to bind VDR/RXR in our previous study (Fig. 9B).

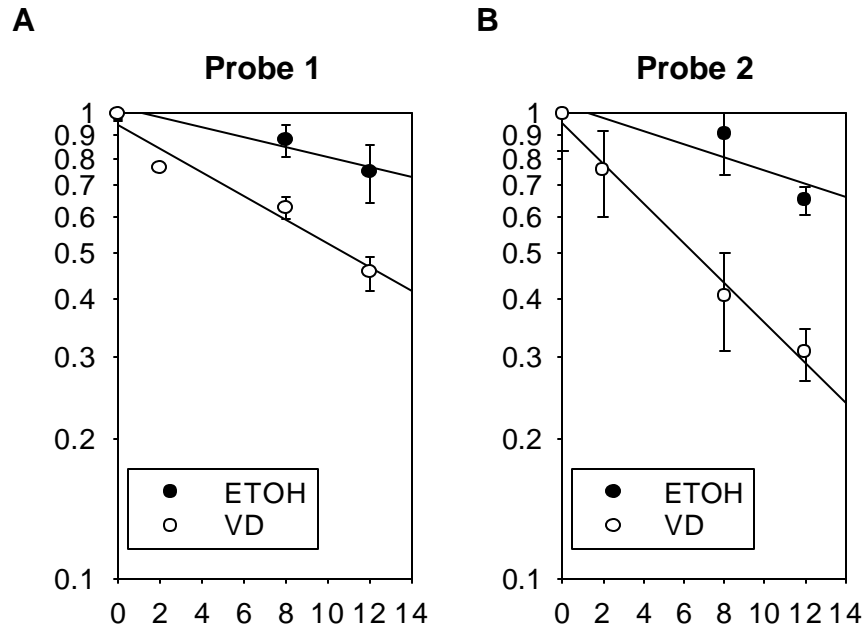
To determine whether binding the receptors to VDRE *in vitro* mediates the down-regulation of hTERT by 1,25VD in OVCAR3 cells, a reporter gene with a 3.3 kb promoter region of hTERT containing the putative VDRE located upstream of the cDNA of firefly luciferase gene was analyzed. In OVCAR3 cells transiently transfected with this reporter, 1,25VD did not decrease the luciferase activity (Fig. 19A). This is consistent with the study in prostate cancer cells showing that this VDRE-mediated inhibition of telomerase activity required 1,25VD and 9-cis RA, not 1,25VD alone (Ikeda, 2003). However, in OVCAR3 cells the combination of 1,25VD and 9-cis RA did not decrease the activity of telomerase reporter (Fig.19B). There is also no detectable decrease in reporter activity by prolonging the treatment of 1,25VD to 6 days in OVCAR3 cells in which the reporter was stably integrated into the genome (Fig. 19C).

To find out if the putative VDRE interacts with VDR *in vivo*, ChIP assays were performed with anti-VDR antibodies. In the soluble chromatin prepared from

OVCAR3 cells treated with 1,25VD, the anti-VDR antibody precipitated the GADD45 exonic region containing the functional VDRE (Fig. 12B), but could not precipitate hTERT promoter fragments containing the putative VDRE (Fig. 19D). These data indicate that VDR is not recruited to the putative VDRE *in vivo*. It is concluded that in OVCAR3 cells the putative VDRE in hTERT promoter is not a functional VDRE.

### **3. The stability of hTERT mRNA is decreased by 1,25VD**

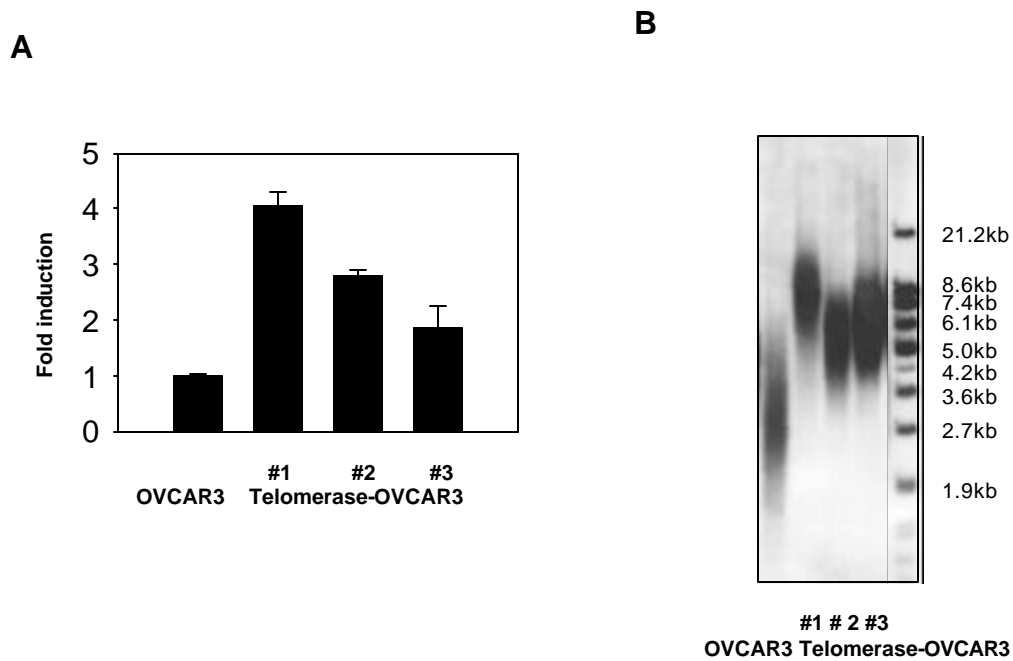
The down-regulation of hTERT mRNA after 1,25VD treatment could be due to the short half-life of the hTERT message. To examine whether 1,25VD-mediated changes in hTERT mRNA stability contribute to its decreased expression, we performed Real-time PCR analysis using RNA from actinomycin D-treated cells. After OVCAR3 cells were treated for 3 days with 1,25VD or vehicle, transcription was inhibited by adding actinomycin D. At different time intervals, total RNA was isolated and hTERT mRNA was determined by Real-time PCR analysis. As shown in Figure 20 A, the level of hTERT mRNA, after normalization with GAPDH, decreased within approximately 10 hr in 1,25VD-treated cells, whereas hTERT mRNA from vehicle-treated cells was comparatively stable over this period of time. The half-life of hTERT mRNA in 1,25VD treated and control cells is approximately 10 hr and 29 hr, respectively. The same results were obtained using a second probe and primers designed for another region of hTERT cDNA (Fig. 20B). The rate of degradation of the hTERT mRNA was increased by the addition of 1,25VD, which demonstrates that the decrease of hTERT mRNA by 1,25VD is due to its decreased stability.



**Fig. 20. The stability of hTERT mRNA is decreased by 1,25VD.** OVCAR3 cells were treated with  $10^{-7}$  M 1,25VD (VD) or ETOH for 3 days, followed by treatment with 5  $\mu\text{g/ml}$  actinomycin D. Total RNA was extracted at 0, 2, 8, 12 hrs and subjected to Real-time PCR using probe 1 (**A**) and probe 2 (**B**), as described in Materials and Methods.

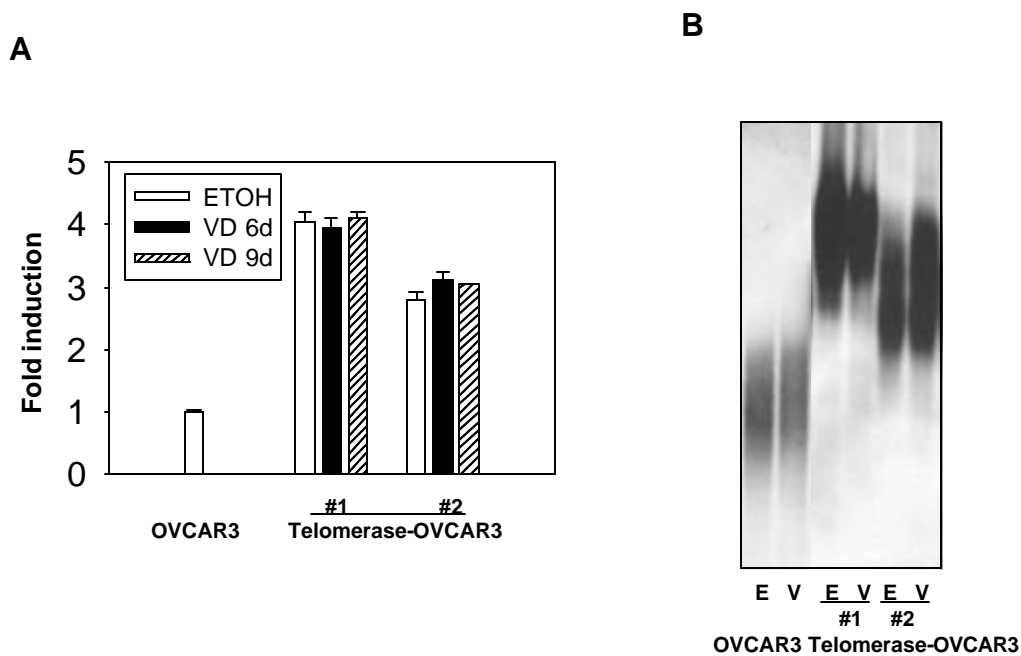
#### **4. Telomerase stably-transfected cells have increased telomerase activity and prolonged telomere length.**

Ectopic expression of hTERT in telomerase-negative human fibroblasts and endothelial cells resulted in substantial telomerase activity and telomere maintenance. Furthermore, the ectopic expression of hTERT circumvented senescence and enabled these cells to be immortalized, i.e, proliferate indefinitely in culture (Hahn, 1999). To determine the role of telomerase in 1,25VD-induced growth inhibition in OVCAR3 cells, we tested whether the overexpression of telomerase was sufficient to abolish 1,25VD-induced growth inhibition. hTERT cDNA, under the control of a viral promoter insensitive to 1,25VD, was stably transfected into OVCAR3 cells. As shown in Figure 21A, the telomerase-OVCAR3 cell lines express 2-4 folds higher levels of telomerase activity than parental OVCAR3 cells. Telomere length in OVCAR3 cells is ~3 kb as shown in Figure 21B. This is consistent with results from previous studies showing that telomere terminal restriction fragments (TRF) are ~3 kb in ovarian carcinoma cells (Villa, 2000; Braunstein, 2001). It has been known that the majority of human cancers have much shorter telomeres than the corresponding normal tissues; in many cases approaching that is associated with crisis in normal cells (2-4 kb) (Wynford-Thomas, 1999; Liu, 1999). Telomerase plays an important role in maintaining stable telomere length. Telomere length in telomerase-OVCAR3 clones was prolonged to 6-10 kb, as shown in Figure 21B. The ectopic expression of hTERT in OVCAR3 cells dramatically increased telomerase activity and prolonged telomere length.



**Fig. 21. Telomerase-stably transfected cells have increased telomerase activity and prolonged telomere length.** (A) Proteins were extracted from OVCAR3 cells and telomerase-OVCAR3 cells and subjected to PCR-TRAP assay, as described in Materials and Methods. (B) Genomic DNA extracted from OVCAR3 and Telomerase-OVCAR3 were used to measure telomere length. Southern blot hybridization was performed as described in Materials and Methods.





**Fig. 22. Overexpression of telomerase blocks 1,25VD-induced down-regulation of telomerase activity.** (A) OVCAR3 cells and telomerase clones were treated with ETOH or  $10^{-7}$  M 1,25VD for 6 days (6 d) or 9 days (9 d). The protein was extracted and subjected to PCR-TRAP assay, as described in Materials and Methods. (B) Genomic DNA extracted from OVCAR3 and Telomerase-OVCAR3 treated with  $10^{-7}$  M 1,25VD (V) or ETOH (E) for 9 days to measure telomere length. Southern blot hybridization was performed, as described in Materials and Methods.

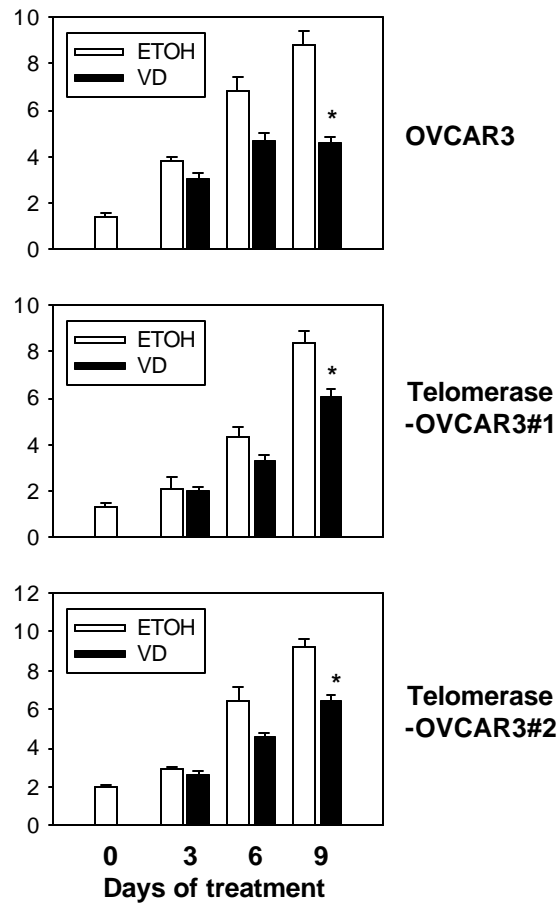
In contrast to parental cells, no dramatic decrease of telomerase activity or telomere length was observed in telomerase-OVCAR3 cells after treatment with 1,25VD for 6 or 9 days compared with vehicle treatment (Fig. 22 A and B). Cells with ectopic hTERT expression maintain high telomerase activity after 1,25VD treatment compared with parental OVCAR3 cells (Fig. 22A). These results demonstrate that ectopic expression of hTERT is sufficient to protect 1,25VD-inhibition of telomerase activity. Therefore, these cell lines can be used to determine whether telomerase plays any role with respect to 1,25VD.

Although 1,25VD dramatically decreased telomerase activity, no remarkable loss of telomeric repeats was detected after 9 days of treatment with 1,25VD (Fig. 22B). No decrease in telomere length could be due to the fact that cells undergo only a few doublings in 9 days, the potential base pair loss in 9 days are estimated to be ~200 bp, the loss of telomeric repeats may not be measurable by conventional Southern Blotting. A recent study demonstrated that telomere dysfunction rather than the mean telomere length was modulated when glutathione (GSH) levels influenced the c-myc-dependent cell death (Biroccio, 2003). Telomerase and telomere structure are dynamically regulated in normal human cells and disruption of telomerase activity alters the maintenance of the 3' single-stranded telomeric overhang without changing the rate of overall telomere shortening (Masutomi, 2003), suggesting that modulating telomere integrity maybe essential regardless of telomere length. It is highly possible that although maintaining telomere length is important, 1,25VD down-regulated telomerase activity could regulate OCa cell growth through modulation of telomere integrity.

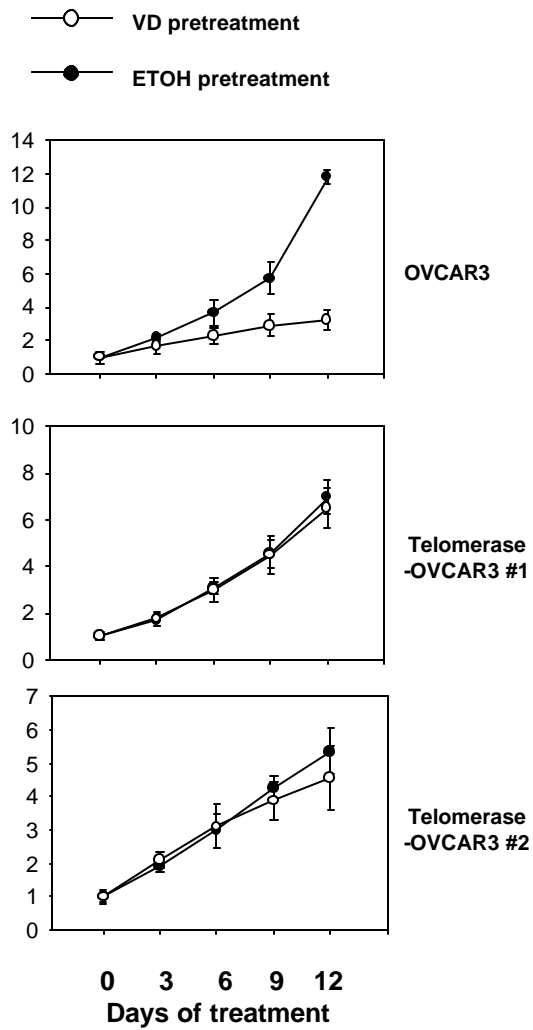
## **5. Overexpression of telomerase partially blocks 1,25VD-induced apoptosis and increases the ability to recover after 1,25VD withdrawal.**

To address the question if telomerase participates role in growth-suppressing activity of 1,25VD in OCa cells, we compared the response of telomerase-OVCAR3 clones to 1,25VD to that of parental OVCAR3 cells. The effect of 1,25VD on cell growth as well as the ability to recover from treatment after removal of the hormone was investigated. Similar to parental cells, growth of telomerase-OVCAR3 cells (Fig. 23) were also inhibited by 1,25VD, but to a lesser extent. Cells overexpressing hTERT keep growing in the presence of 1,25VD while the growth of parental cells halted after 6 days treatment with 1,25VD. Telomerase clones are more resistant to 1,25VD-induced growth inhibition. Additionally, after 9 days of 1,25VD treatment, the recovery of telomerase-OVCAR3 cells was rapid compared to the recovery of the parental cell line (Fig. 24). Our observation suggests that ectopic expression of hTERT in OCa cells protects about 50% of 1,25VD reduced growth, although other mechanisms independent of telomerase may also exist.

Since overexpression of telomerase altered cell response to 1,25VD in the recovery assay, we next assessed whether alterations in apoptotic index occurred. Parental and telomerase-OVCAR3 cells were treated with 1,25VD and then analyzed by flow cytometry. As shown in Figure 25, in contrast to the massive apoptosis (70%) induced in parental cells after 9 days treatment with 1,25VD, telomerase clones showed fewer apoptotic cells (<40%). The ectopic expression of hTERT partially rescued long-term 1,25VD treated OVCAR3 cells from cell death. This may contribute to



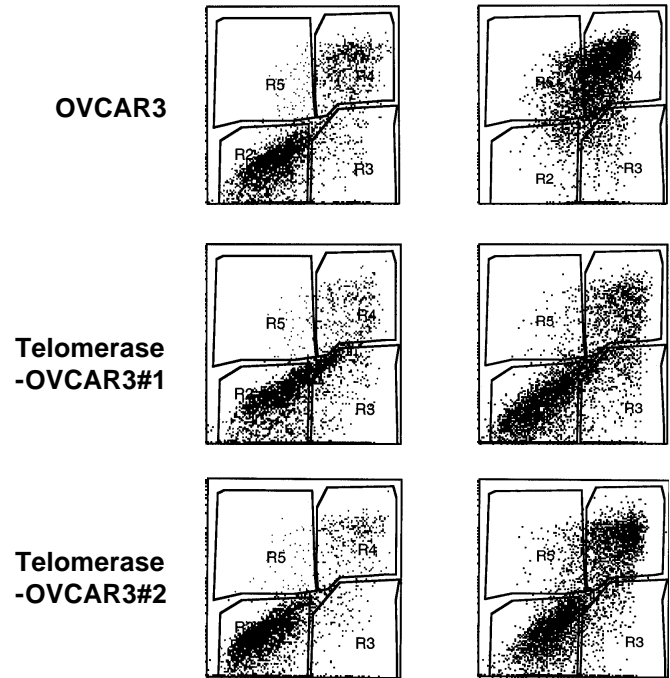
**Fig. 23. Telomerase-OVCAR3 cells are less sensitive to growth inhibition by 1,25VD.** OVCAR3 and telomerase-OVCAR3 cells were plated in 96 wells and treated with  $10^{-7}$  M 1,25VD (VD) or ETOH as a vehicle. Cell numbers were determined with the MTT assays. Eight samples were analyzed for each data point. \* $P < 0.05$  (versus ETOH treatment).



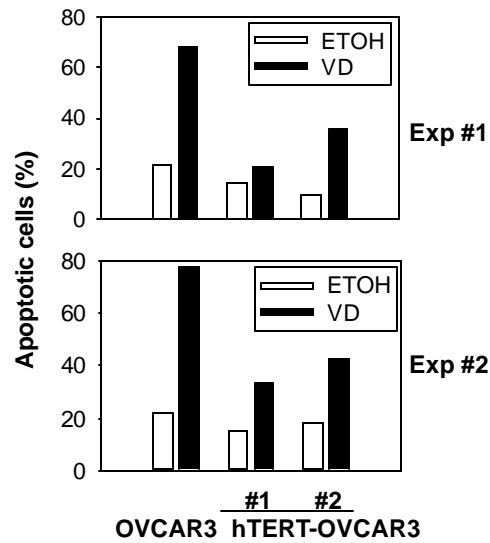
**Fig. 24. Telomerase-OVCAR3 cells recover quickly from 1,25VD treatment.**

OVCAR3 and Telomerase-OVCAR3 cells were pretreated with  $10^{-7}$  M 1,25VD or ETOH for 9 days. Recovery of pretreated cells was assessed by plating pretreated cells in 96 well plates. Cell numbers were determined with the MTT assay. Eight samples were analyzed for each data point.

**A**



**B**



**Fig. 25. Overexpression of telomerase partially blocks 1,25VD-induced apoptosis.**

**Fig. 25. Overexpression of telomerase partially blocks 1,25VD-induced apoptosis.**

(A) OVCAR3 and Telomerase-OVCAR3 clones were treated with ETOH or  $10^{-7}$  M 1,25VD for 9 days. Apoptotic index was determined by flow cytometry and a representative profile is presented. (B) Bar graphs show percentage of apoptotic cells in two experiments.

the reduced growth inhibition of telomerase clones by 1,25VD treatment (Fig. 23).

Collectively, these studies show that in OVCAR3 cells down-regulation of telomerase is correlated to 1,25VD-induced growth inhibition and apoptosis. The enhancement of telomerase function in OVCAR3 cells, as measured by telomerase activity, allows the cells to recover from 1,25VD treatment and rescues them from 1,25VD-induced apoptosis. Telomerase activity remains a key parameter that determines long-term cell survival in OCa cells. This work supports our hypothesis that telomerase inhibition by 1,25VD may serve as an effective tool to eliminate OCa cells that have short telomeres.

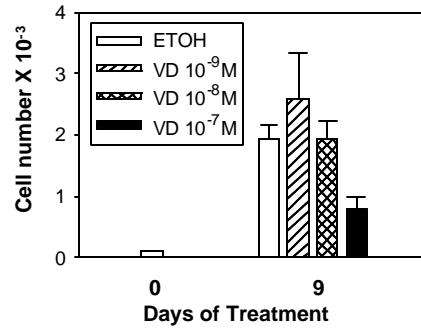
**EB1089 is more potent than 1,25VD in suppressing OCa cell growth *in vitro* and in nude mice.**

The effective concentration of 1,25VD ( $10^{-7}$  M) is pharmacological, which is expected to induce hypercalcemia *in vivo*. EB1089, a 1,25VD analogue, is less calcemic and was shown to be more effective against breast (Colston, 1992), pancreatic (Colston, 1997), colon (Akhter, 1996) and prostate (Blutt, 2000) cancers. To test whether EB1089 can be used for long-term treatment and/or chemoprevention of OCa, our study investigated VDR expression in human ovarian tissues, the growth of OVCAR3 cells and tumor xenografts in nude mice.

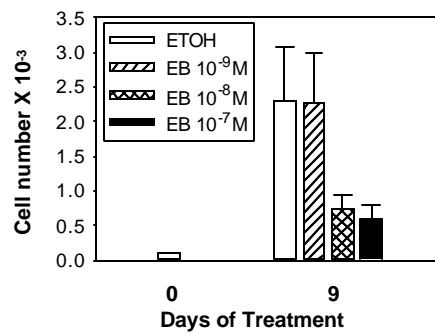
In MTT assays, EB1089 is effective against OCa cells at concentrations 10 times lower than 1,25VD, making it a promising candidate for *in vivo* treatment of OCa (Fig. 26 A and B). As expected, EB1089 induced GADD45 reporter activity effectively



**A**

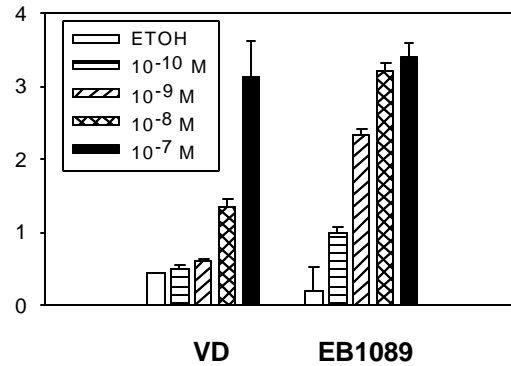


**B**



**Fig. 26. EB1089 is more potent than 1,25VD in suppressing OCa cell growth.**

OVCAR3 cells were plated in 96 well plates and treated 1,25VD (VD) (A) or EB1089 (EB) (B) at the indicated concentrations. Cell numbers were determined with MTT assay. Eight samples were analyzed for each data point, and the data were reproduced three times.



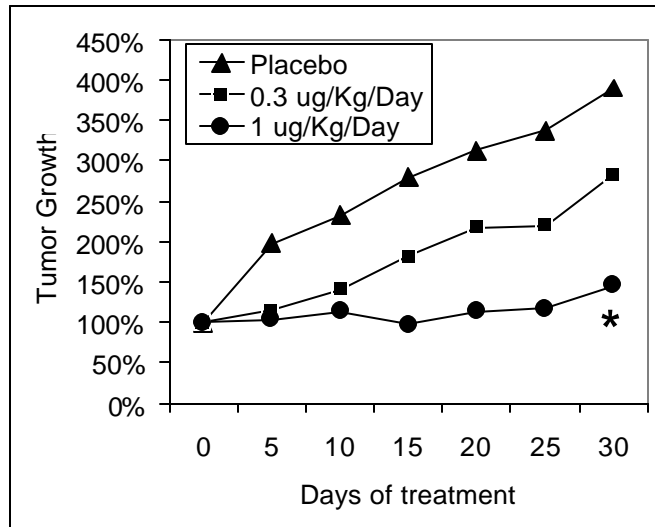
**Fig. 27. EB1089 is more effective than 1,25VD in inducing GADD45 reporter activity.** OVCAR3 cells were transfected with 0.2  $\mu\text{g}$  GADDLuc, 0.05  $\mu\text{g}$  pCMVgal, 0.05  $\mu\text{g}$  p91023B-VDR and 0.05  $\mu\text{g}$  pCMX-RXRb and treated with ETOH, 1,25VD or EB1089 at the indicated concentrations. Luciferase activity was determined and normalized with cognate  $\beta$ -gal activity.

at concentrations 10 times lower than 1,25VD (Fig. 27), suggesting a mechanism of EB1089 action in OCa cells.

To determine whether the effect of 1,25VD and EB1089 on OCa cell growth can be directly translated into tumor suppression in the whole animal, we tested the effect of EB1089 in OVCAR3 tumor xenografts. As shown in Figure 28A, EB1089 at 1 $\mu$ g/day/kg totally inhibited the growth of tumors while a dose of 0.3  $\mu$ g/day/kg partially inhibited the growth. Based on statistical analysis of independent samples with the T-test, the difference between placebo control (6 tumors) and 1.0  $\mu$ g/day/kg (9 tumors) is significant ( $p=0.003$ ). The difference between the 0.3 (6 tumors) and 1.0  $\mu$ g/kg/day group is also significant ( $p=0.04$ ). The difference between placebo control and 0.3  $\mu$ g/kg was not significant ( $p=0.281$ ). This is probably due to the fact that the number of groups was small and the size of tumors was heterogeneous at the start of the study. In our experiments, each individual mouse was marked and the tumor volume was recorded separately; the difference between 0.3  $\mu$ g/kg/day and placebo is obvious when the growth of each individual tumor was examined separately.

To determine if EB1089 is less calcemic, serum calcium levels were determined in treated nude mice (Fig. 28B). Blood tests at 30 days did not reveal significant increase in blood calcium. The levels in placebo (~9 mg/dl) and both of EB 1089 groups (9~10.5 mg/dl) were within the normal range (7-11 mg/dl). As expected, 1,25VD treatment for 1.0  $\mu$ g/kg/day induced higher levels of serum calcium (11~12 mg/dl at day-15 and day-30), which is outside the normal range. Importantly, no abnormality was seen in mice treated with EB1089 at 0.3 or 1.0  $\mu$ g/kg/day for 4 weeks and normal body weight was maintained (data not shown).

A



B

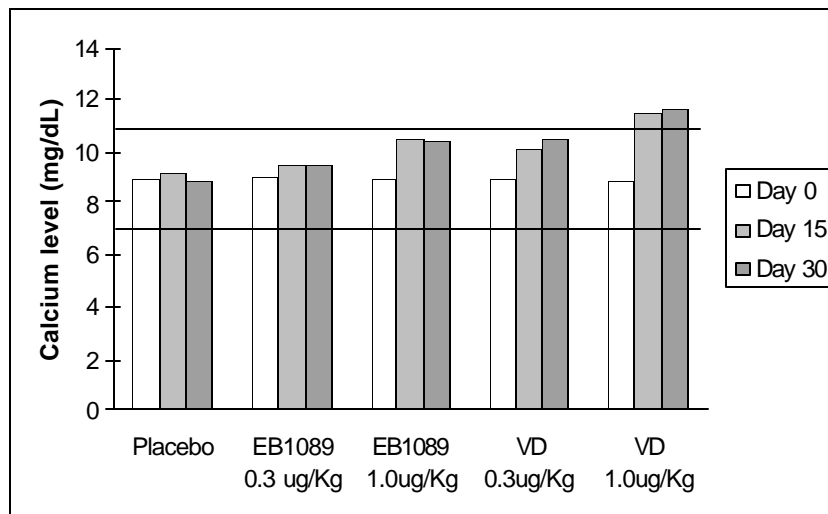


Fig. 28. EB1089 inhibits OCa xenograft growth.

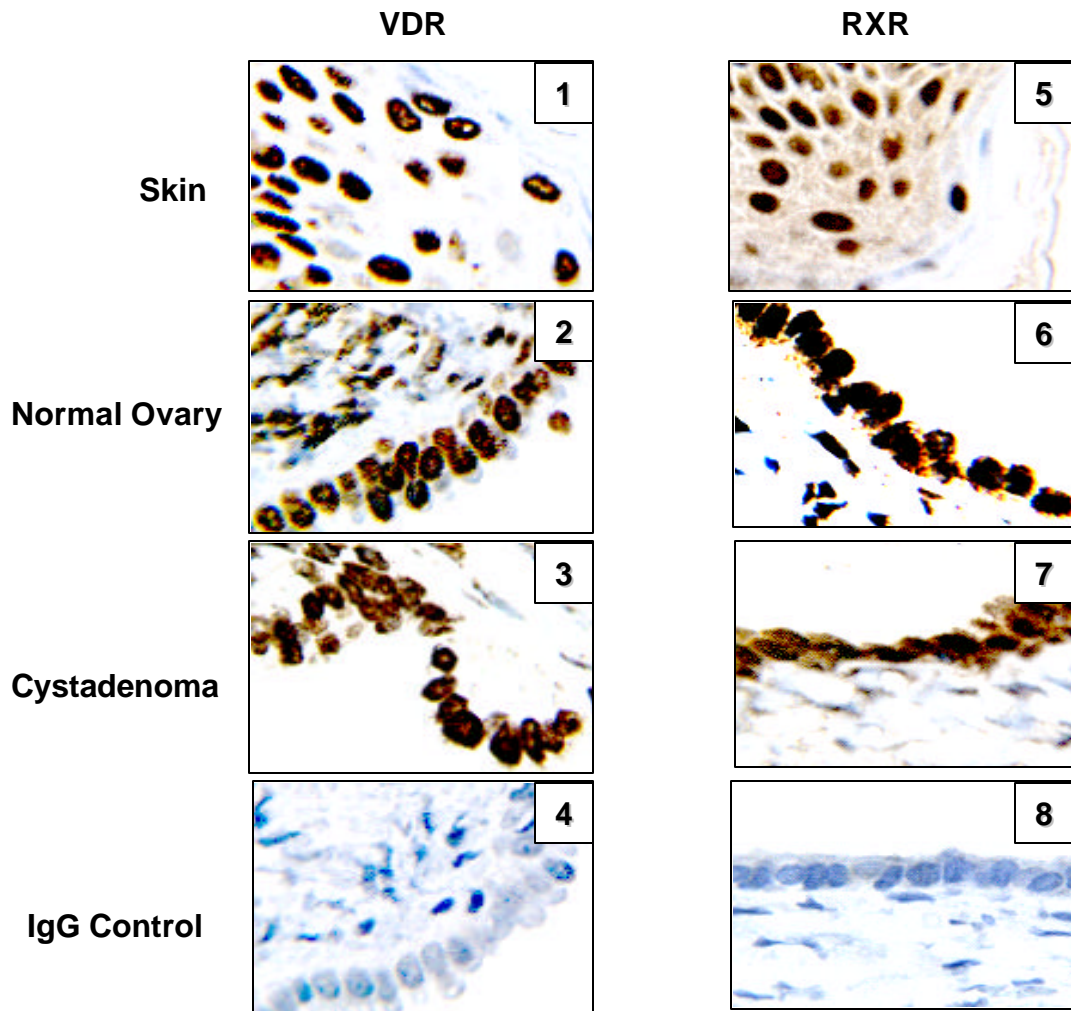
**Fig. 28. EB1089 inhibits OCa xenograft growth.** (A)  $2 \times 10^6$  OVCAR3 cells were injected s.c. into one site in the dorsal side of nude mice. After the tumors grew to  $150 \text{ nm}^3$ , mice were randomly divided into three groups and treated with placebo or EB1089 at indicated doses daily as described in Materials and Methods. The tumor size was measured every five days and the growth status of each tumor was recorded separately. \*,  $p < 0.01$  compared with placebo group. (B) Average serum calcium levels for each treatment group.

**VDR and RXR are expressed in precursors for epithelial OCa and normal ovarian surface epithelial cells are responsive to 1,25VD.**

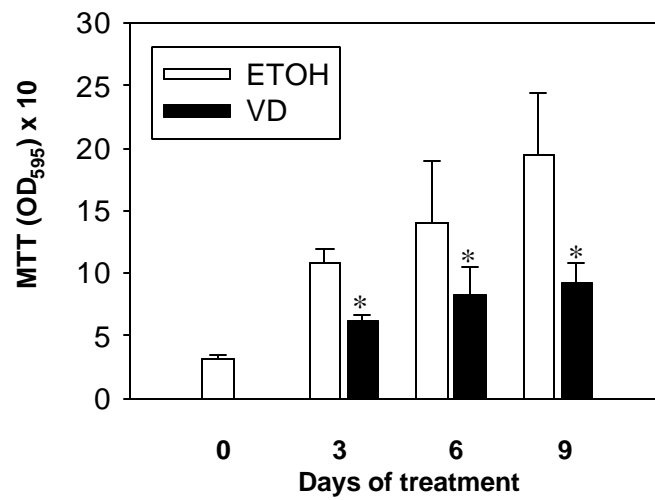
If 1,25VD is useful for OCa prevention, its receptors should be expressed in OSE cells and benign ovarian tumors, the precursors for malignant epithelial OCa, which gives rise to more than 90% of human OCa. Indeed, our immunohistochemical studies showed that both VDR and RXR are expressed strongly in human OSE cells and in benign tumors. Skin tissues which express VDR and RXR were used as positive control and the primary antibody was substituted with IgG as negative control (Fig. 29).

To test whether normal ovarian surface epithelial (OSE) cells respond to 1,25VD's growth suppression, primary human OSE cell cultures were treated with ETOH or 1,25VD and their growth was analyzed by the MTT assays. As shown in Figure 30, the growth of human OSE cells was suppressed by 1,25VD in a similar way as seen with OVCAR3 cells. These data clearly demonstrate that human primary ovarian cells are sensitive to 1,25VD.

Collectively, the expression of VDR in human ovary and the growth inhibition of human primary OSE by 1,25VD indicate that 1,25VD analogue could also be used for chemoprevention.



**Fig. 29. Normal ovarian epithelial cells and benign adenomas express VDR and RXR.** Paraffin sections of tissues were stained with anti-VDR (rat monoclonal 9A7) or anti-RXR (rabbit polyclonal) antibodies. The anti-RXR antibody was generated against RXR $\alpha$ , but also cross reacts with RXR $\beta$  and RXR $\gamma$ . IgG controls were performed with normal ovarian sections (rat IgG for VDR and rabbit serum for RXR).



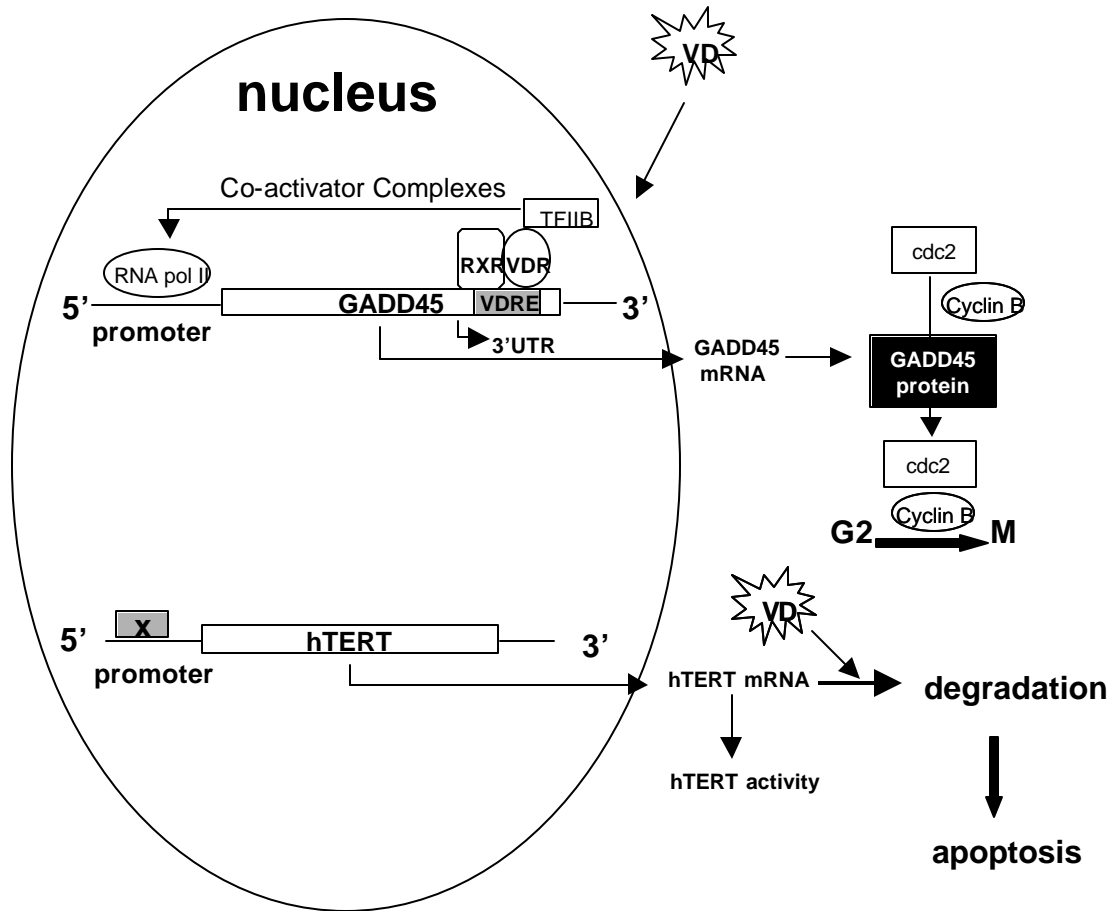
**Fig. 30. 1,25VD suppresses human primary ovarian cell growth.** Human primary ovarian epithelial cells were plated in 96-well plates and treated with  $10^{-7}$  M 1,25VD (VD) or ETOH. Cell numbers were determined at the indicated times by the MTT assays. Eight samples were analyzed for each data point. \*  $P < 0.05$  (versus ETOH treatment).



## DISCUSSION

To demonstrate the role of 1,25VD and its analogues in OCa treatment and prevention, the mechanism by which 1,25VD mediated its antiproliferative activity was explored using a VD sensitive OCa cell line (OVCAR3) as a model system. The effect of the 1,25VD analogue EB1089 on OCa cell growth was also investigated *in vitro* and *in vivo*. Cell cycle studies showed that 1,25VD increased the proportion of OVCAR3 cells in the G0/G1 and G2/M phase and decreased those in the S phase. 1,25VD also induced apoptosis in OVCAR3 cells.

Furthermore, we have identified GADD45 and telomerase as target genes for 1,25VD and suggest a model of VD action in OCa cells (Fig. 31). 1,25VD induces binding of the VDR/RXR heterodimer to the VDRE located in the fourth exon of GADD45 gene at a position downstream from the termination codon for protein translation. Presumably through the recruitment of co-activator complexes, the activated VDR/RXR interacts with the Pol II complex bound to the promoter and increases the rate of GADD45 transcription. This leads to an increase in the amount of GADD45 protein that, through a yet unknown undefined mechanism, decreases the level of cyclin B, the regulatory subunit of cdc2 kinase. The resulting decrease in cdc2 activity is then responsible for disturbed cell cycle progression to M phase. In addition, 1,25VD inhibits



**Fig. 31. Model for the integrated cellular pathways of 1,25VD action in OCa cells (See text for details).**

telomerase activity by decreasing the stability of hTERT mRNA, not by the putative VDRE in the hTERT promoter which is not functional *in vivo*. Importantly, hTERT clones are more resistant to 1,25VD-induced apoptosis and growth inhibition. In contrast to parental cells that recover slowly from prolonged treatment with 1,25VD, hTERT clones re-grow quickly after 1,25VD withdrawal. Lastly, our investigation of the antiproliferative effects of EB1089 on OVCAR3 cells and xenografts without inducing hypercalcemia may indicate a novel preventive and therapeutic option for the treatment of OCa.

### **Upregulation of GADD45 by 1,25VD is mediated through a novel exonic enhancer**

Induction of GADD45 can be detected in cells within 2 hrs of treatment with 1,25VD (Fig. 8B). With the lack of a VD effect on mRNA stability (Fig. 8C) and the identification of functional VDREs in the genome (Figs. 9-12), our data establish GADD45 as a primary and immediately early response gene for 1,25VD. Furthermore, our data suggest that GADD45 regulation is mediated through a VDR/RXR heterodimer, instead of a VDR/VDR homodimer. This conclusion is reached based on the inability of VDR to bind to all putative VDREs in EMSAs until the addition of RXR and the upshifting of the complexes by RXR antibody (Fig. 9). At variance with the belief that nuclear receptors forming heterodimers with RXRs bind DNA in the absence of ligand, our ChIP assays (Fig. 12B) clearly show that *in vivo* binding of VDR to the exonic VDRE is ligand-dependent. Recently, Yamamoto *et al.* (2003) reported a similar observation on VDRE located in the promoter of the OPN gene, suggesting that the

ligand dependency is common to VDREs located inside and outside the promoter regions.

Since p53 in OVCAR3 cells is mutated (Yaginuma, 1992), the regulation of GADD45 transcription by 1,25VD obviously occurs independently of p53 activity. This is consistent with the identification of the VDRE in the fourth exon that is distant from the p53 binding site which is located in the third intron (Hollander, 1993). This is also consistent with the conclusion reached by a study of squamous cell carcinoma (Prudencio, 2001). Furthermore, the VD regulation of GADD45 is also likely to be independent of BRCA1 since the DNA element for this tumor suppressor is located in the promoter region (Jin, 2000). It is striking that four of the five putative VDREs bound VDR/RXR equally well in EMSAs, but only VDRE-E mediated the up-regulation of GADD45 reporter by 1,25VD. The other putative VDREs are either nonfunctional or may act in a negative way. Overall, the data suggest that the regulation of GADD45 by 1,25VD is a complex process that may involve the interaction of the receptor bound to the VDRE and other transcription factors. The different VDREs may also function in a cell-specific manner to mediate the regulation of GADD45 expression by 1,25VD.

**GADD45 protein upregulation by 1,25VD is required for the hormone-induced cell cycle arrest at the G2/M but not the G1/S checkpoint**

Our studies link GADD45 induction specifically to the inhibition by 1,25VD of cell cycle progression through the G2/M checkpoint. This linkage was established using cells in which GADD45 expression was compromised by the anti-sense approach or genetic knock out. It is important to determine the effect of GADD45 anti-sense on 1,25VD-induced growth inhibition in the stably transfected cells, which would reveal

whether G2/M arrest is a major or minor factor in the overall growth inhibition. However, our analysis with MTT assay revealed little difference in the overall response to 1,25VD between anti-sense GADD45 and control clones (data not shown). This may be due to cell cycle arrest being shifted from G2/M to G1/S checkpoint in anti-sense clones.

Previous studies (Akutsu, 2001) have shown that GADD45 induction in squamous cell carcinoma by 1,25VD is associated with an increased interaction with PCNA. Although we have not examined the association of GADD45 with PCNA, this seems unlikely in OVCAR3 cells. PCNA is required for DNA replication in S phase but our data show that G1/S arrest still occurs in OVCAR3 cells expressing the anti-sense cDNA of GADD45 (Fig. 13C and 13D). Instead of PCNA, our studies show a 1,25VD-induced decrease in cdc2 activity, which is associated with a decrease in the level of cyclin B1 protein. The decrease was not observed in the stable clones expressing GADD45 anti-sense cDNA (Fig. 15). The data suggest that GADD45 mediates the effect of 1,25VD on cdc2 activity. Our data concur with the proposed role of GADD45 in G2/M arrest induced by certain types of DNA damaging agents (Wang, 1999; Zhan, 1999). Zhan *et al.* (1999) have shown that GADD45 inhibits the interaction between cyclin B and cdc2. Later, it was shown that GADD45-induced cell cycle arrest at G2/M is associated with an altered cellular distribution of cyclin B1 (Jin, 2002), which seems to require a functional p53. Our conclusions, however, differ from the above studies since we detected a decrease in the level of cyclin B1 protein (Fig. 15). As mentioned earlier, OVCAR3 cells contain a mutant p53 (Yaginuma, 1992). Obviously, GADD45 exerts its effect on cdc2 activity and the G2/M transition in the OCa cells independently of p53. It remains to be determined whether the effect of 1,25VD on G2/M in OCa cells is exerted

through GADD45 alone or in combination with another protein that functions similarly to p53.

Besides its role in regulating G2/M transition, GADD45 plays an essential role in DNA repair and in the maintenance of genomic stability. MEFs derived from GADD45-null mice exhibit aneuploidy, chromosomal abnormalities, gene amplification and centrosomal amplification (Hollander, 1999). GADD45-null mice display increased sensitivity to dimethylbenzanthracene-induced carcinogenesis (Hollander, 2001). It is intriguing that dimethylbenzanthracene increased female ovarian tumors more efficiently in GADD45-null mice than wild type (Hollander, 2001). GADD45 induction by 1,25VD through VDR suggests that VDR may act in a p53 independent tumor-suppressing pathway to affects ovarian genomic stability. Along this line, VDR has been shown to act as a bile acid sensor for the secondary bile acid lithocholic acid (LCA) (Makishima, 2002), a potential enteric carcinogen that induces DNA strand breaks, forms DNA adducts and inhibits DNA repair enzymes. It will be interesting to show whether ovarian carcinogens or DNA-damaging processes (e.g. ovulation) activate VDR in ovarian epithelial cells and, if yes, whether the VD-independent VDR activation functions in DNA repair.

**Down-regulation of telomerase by 1,25VD is mediated through destabilization of hTERT mRNA**

Hormones regulate the expression of telomerase in hormone-responsive cell systems. In some cases, induction or repression of telomerase has been considered a consequence of maturation and/or growth arrest, rather than a direct hormonal effect. For example, androgens stimulate telomerase indirectly since the hTERT promoter construct

was not activated by androgen and transcription of the endogenous gene was not stimulated early enough in cultured cells to be considered a direct target of androgens. However, it has also been reported that inhibition of telomerase activity is an early event of the differentiation process in leukemia cells rather than its consequence (Albanell, 1996; Savoysky, 1996). Retinoids and VD negatively regulate telomerase that is associated with differentiation in leukemia cells. However, it is not yet clear at which level of gene expression this down-regulation occurs. In addition, numerous studies showed that hormones directly regulate telomerase via different mechanisms. For example, estrogens activates telomerase through the direct interaction of ligand-activated ER with the ERE in the hTERT promoter. Progesterone regulates hTERT transcription via the MAP kinase cascade.

There was a significant correlation of telomerase activity with hTERT mRNA expression but not with TP1 or hTR. hTERT is the major determinant of telomerase activity (Counter, 1998). In our study, the repression of telomerase activity by 1,25VD accompanied down-regulation of hTERT mRNA as shown by RT-PCR and confirmed by Real-time PCR.

The present study demonstrates that 1,25VD down-regulates telomerase activity mediated through repression of hTERT mRNA (Fig. 17-20). The stability of hTERT mRNA was decreased by 1,25VD (Fig. 20), demonstrating that the regulation was posttranscriptional. Our findings provide direct evidence that hTERT is a target gene for 1,25VD.

Although EMSA revealed that the putative VDRE bound specifically to the VDR/RXR heterodimer, as shown by Ikeda *et al*, 2003, it is worth noting that in their

study, this VDRE only mediated the down-regulation of reporter activity by cotreatment of 1,25VD and 9-cis RA, but not 1,25VD alone. The limitation of this VDRE function may be due to different cellular context. Transcriptional assays using luciferase reporter plasmids containing full length of hTERT promoter showed no down-regulation by 1,25VD or by combination of 1,25VD and 9-cis RA in OCa cells. Furthermore, CHIP analysis showed that no detectable binding of VDR to this VDRE *in vivo*. All these data indicate that this putative VDRE is not functional in OVCAR3 cells. Real-time PCR analysis revealed that 1,25VD decreased the stability of hTERT mRNA.

Real-time PCR showed that hTERT mRNA was degraded to  $\leq 50\%$  within ~10 hr by treatment of 1,25VD, while no detectable increase in the number of apoptotic cells within this timeframe (data not shown). The time course of destabilization of hTERT mRNA by 1,25VD precedes induction of apoptosis by 1,25VD, suggesting that decreases in hTERT mRNA are not a result of the induction of apoptosis in these cells.

To our knowledge, this is the first study incident showing that the stability of hTERT mRNA was regulated. This may open new aspects to study the regulation of hTERT, especially in the repression of telomerase.

Sequence analysis of the hTERT promoter revealed binding sites for several transcription factors suggesting hTERT could be regulated by different factors in different cellular contexts (Cong, 2002). The oncogene *c-myc* has been shown to activate telomerase through two *myc* binding sites in hTERT promoter (Wu, 1999). Since 1,25VD have been shown to inhibit *c-myc* (Saunders, 1993) and *c-myc* binding sites are present in full-length hTERT reporter construct, this oncogene could mediate the repression of telomerase by 1,25VD. However, the persistent inhibition of hTERT mRNA in the



presence of cycloheximide and the lack of inhibition in reporter assays rule out the indirect effect of 1,25VD mediated through *c-myc*.

**Over-expression of hTERT allows the recovery of OCa cells after 1,25VD treatment and partially relieves 1,25VD-induced apoptosis**

Treatment of 1,25VD in parental OCa cells causes massive apoptosis leading to the loss of the entire population. In addition to the decrease of telomerase activity, apoptosis was also induced by 1,25VD and the recovery from 1,25VD treatment was slow in parental OVCAR3 cells. By contrast, the hTERT-overexpressing cells maintained telomerase activity at higher levels and telomere length at a size much longer than parental cells over time, 1,25VD-induced apoptosis and growth inhibition were partially rescued, leading to cell recovery and growth (Fig 21-25). This indicates that a certain level of telomerase activity may play an important role in protecting cells from apoptosis in OCa cells. Our results provide the first direct evidence that telomerase may affect 1,25VD-induced apoptosis in OCa cells.

It is well known that there is strong correlation between telomere function and senescence (Kolquist, 1998). Telomerase activation is shown as one of the three steps needed to overcome senescence and transform normal human epithelial cells to cancer cells (Hahn, 1999). Cells undergoing senescence have a large and flat morphology, express acidic beta-galactosidase ( $\beta$ -gal) and show a permanent G0/G1 arrest, whereas apoptotic cells show evidence of DNA fragmentation (Biroccio, 2003). Many studies have linked telomerase inhibition to apoptosis. For example, hTERT-expressing cells were more resistant to apoptosis induced by UV and  $\gamma$ -irradiation (Gorbunova, 2002). Following ectopic expression of dominant-negative TERT into transformed cells, growth

inhibition and apoptosis was induced (Hahn, 1999; Zhang, 1999). In our model system, 1,25VD-induced decrease in telomerase activity is correlated with 1,25VD-induced apoptosis, not senescence. This is supported by our data that the senescence  $\beta$ -gal, a marker for senescence, was not induced by 1,25VD treatment in OCa cells (data not shown). Instead, extensive cell death was observed. Our data strongly suggested that telomerase plays a critical role in cellular resistance to apoptosis.

Little is known about the signaling pathways mediating VD-induced apoptosis. Several factors involving mitochondrial and caspase changes were investigated. Bcl-2 is down-regulated in VD-induced apoptosis in breast and prostate cancer cells (Mathiasen, 1999; Blutt, 2000), pro-apoptotic Bak is induced in colon cancer cells (Diaz, 2000). VD induced-apoptosis is caspase-dependent in prostate cancer cells, while in breast cancer cells, calpain may replace caspases as a key mediator (Mathiasen, 1999 and 2002).

It is assumed that some anti-apoptotic pathways are activated by telomerase or that the apoptotic pathway induced by 1,25VD is blocked by telomerase overexpression. As suggested in a recent report, overexpression of wild-type hTERT in HeLa cells increases their resistance to apoptosis, induced by the DNA damaging agent etoposide, and TERT suppresses apoptosis at a premitochondrial step by a mechanism requiring reverse transcriptase activity and 14-3-3 protein-binding (Zhang, 2003). Overexpression of Bcl-2 and the caspase inhibitor zVAD-fmk protected cells against apoptosis in the presence of telomerase inhibitors in pheochromocytoma cells (Fu, 1999). All these data suggest that the telomerase function is upstream of caspase activation and mitochondrial dysfunction. But different from those DNA damaging reagents-induced apoptosis, 1,25VD induced apoptosis could mediate through different pathway. It will be

worthwhile to study how telomerase inserts anti-apoptotic function to block 1,25VD-induced apoptosis.

Transformed human cells enter crisis once TRF reach a length of ~4 kb. Telomeres that have been shortened to this degree may no longer protect chromosome ends, and in turn, may lead to the genomic instability and cell death (Counter, 1998). This is consistent with the fact that many cancers have mean telomere lengths well below normal and often close to the threshold required for cell survival. Tumor cells, such as OVCAR3 cells, have very short telomere and appear to require telomerase activity even for short-term viability. In OVCAR3 cells, the short telomere length of 3 kb is close to the threshold level and was maintained by high telomerase activity. It is postulated that in these cells telomerase activity is required at nearly every division of cells replicating with critically short telomeres and inhibition of telomerase activity in these cells causes loss of viability through activation of apoptotic pathways (Zhang, 1999).

It is known that telomerase inhibition may lead to a phenotypic lag in which cells would continue to divide until the point at which the telomeres became critically short. This lag phase varies depending on the initial telomere length. In terms of telomerase as anti-cancer target for VD, OCa cells, which have short telomeres, could have short lag phase and thus be efficiently inhibited by VD.

**1,25VD analogue EB1089 has potent anti-tumor activity *in vitro* and *in vivo*, and has strong implications to cancer treatment and prevention**

Since the pharmacological levels of 1,25VD ( $10^{-7}$  M) induce hypercalcemia, the goal of our research is to establish synthetic 1,25VD analogues, such as less calcemic EB1089, as chemotherapeutic treatment for OCa.

Our study is the first to demonstrate EB1089 inhibits growth of OCa cells and tumor xenografts in nude mice. Importantly, the tumor inhibition *in vivo* occurred without physical symptoms of hypercalcemia, and particularly promising is the minimal effect on serum calcium (Fig. 28). It demonstrates that EB1089 may be suitable for long-term treatment and/or chemoprevention.

EB1089 could also be useful for treating VD-resistant cancers. A recent study demonstrated that down-regulation of 24-hydroxylase enhanced 1,25VD levels and improved mitotic control of tumor cells (Cross, 2003). The rapid breakdown of 1,25VD was suspected to be the cause of the resistance of DU145, a prostate cancer cell line, to 1,25VD. In the presence of the 24-hydroxylase inhibitor, growth of DU145 cells was inhibited by 1,25VD and as much as in LNCaP cells (Zhao, 2001). EB1089 has 50-fold lower affinity than 1,25VD for 24-hydroxylase, and it is more slowly inactivated by 24-hydroxylation (Roy, 1995), supporting the potential effect of EB1089 in the treatment of VD-resistant cancers.

The pathogenesis of epithelial OCa is not completely understood, but it is believed that the process of recurrent ovulation (incessant ovulation) causes genetic damage to ovarian epithelial cells and that sufficient genetic damage can lead to OCa in susceptible individuals (Rodriguez, 2003). According to this model, reproductive and hormonal factors, including VD, retinoids, and non-steroidal anti-inflammatory drugs, may decrease OCa risk via their inhibitory effects on ovulation, leading to the biologic effects on the ovarian epithelium that are cancer preventive. Along this line, VDR expression is required for 1,25VD-mediated growth effects. We have shown the expression of VDR and its heterodimer partner, RXR, in human ovary and the response

of primary human ovarian cells to 1,25VD. These data indicate that the human ovary is a VD responsive organ and can be impacted strongly by “environmental” VD, leading to decreased OCa risk.

Most tumors have compromised p53 function and half of OCa are estimated to have lost p53 function, thus becoming refractory to drugs targeting p53 pathways. Examination of p53-independent response in p53-mutant OVCAR3 cells warrants closer attention. These tumors may however still be sensitive to synthetic VD since 1,25VD induces GADD45 and inhibit telomerase in a p53-independent manner. Better understanding of the molecular mechanism of VD is critical for determining the ultimate utility of 1,25VD analogues in the clinic.

In conclusion, molecular studies show that 1,25VD inhibits OCa cell growth and is mediated through regulation of specific genes including GADD45 and telomerase. Our preclinical data show that 1,25VD analogue reduces OCa development in animals. Data from normal human ovarian tissues and cells demonstrate the presence of VDR and a response to 1,25VD. Studies from cellular, molecular and animal levels strongly suggest that VD represents a molecular target for chemotherapy and chemoprevention of OCa.

## **SUMMARY AND THE PERSPECTIVES OF FUTURE STUDY**

OCa is a deadly disease and its etiology is largely unknown. Lack of both reliable detection methods and early symptoms results in poor prognosis for patients with OCa. Relapse and resistance to current treatment necessitate the development of new therapeutic methods to fight this deadly disease. Our studies on VD action in OCa suggest that 1,25VD and its synthetic analogues may be effective therapeutic treatments for OCa.

GADD45 is identified as one of the primary target genes by 1,25VD in OCa cells. Based on the similarity to consensus sequences, several putative VDREs identified by EMSA are localized in GADD45 introns or exons but not in the promoter region. Receptor response elements usually lie in the promoter region of regulated gene and all known VDRE have been identified in the promoter. Of particular interest to our study is VDRE in the 3' untranslated region of exon 4 plays critical role in mediating the transcriptional regulation of GADD45. To our knowledge, this is the first study showing that a vitamin D enhancer element is localized in the exon region. Our study can not exclude that other VDREs act in a negative way and that different VDREs in the GADD45 genome may function in a cell-specific manner to mediate the regulation of GADD45 expression by 1,25VD.

Since the specific VDRE is located in an exon and falls into the 3' untranslated region of GADD45, it provides an excellent model system to study how DNA response elements in the 3' end of the coding sequence may regulate the transcription of a target gene via the Pol II complex which bound to the promoter at the 5' end. Site-specific transcription factors (Agalioti, 2002), including nuclear hormone receptors (Louie, 2003), recruit components of the Pol II complex to the enhancer sequences and, after co-activator mediated chromatin remodeling, the adjacent nucleosome slides downstream to initiate transcription. It remains to be seen whether DNA response elements at the 3' end of the coding sequence recruit Pol II components and, if so, how the modified nucleosome moves to the correct position to permit initiation of transcription since the nucleosome has to either slide upstream by a significant distance or jump across the coding region to initiate transcription.

VD-dependent apoptosis and cell cycle arrest could engage activation of the p38 MAPK pathway and induction of GADD45 (Sutter, 2003). Recent data showed that GADD45 is required for p38 activation. Disruption of GADD45 abrogates H-ras induced cell-cycle arrest and p38 activation (Bulavin, 2003). It is quite likely that upregulation of GADD45 by 1,25VD is required for activation of p38, that may mediate 1,25VD-induced growth inhibition. In support this hypothesis, it is known that most biological actions of VD are mediated through the nuclear VDR-mediated expression of target genes. The study of VDRKO mice showed that nongenomic effects of VD in osteoblasts are abrogated in the absence of nuclear VDR (Erben, 2002) and suggest that some nongenomic responses require a functional nuclear VDR. Using GADD45 as a model

system, future work may decipher the cooperation of nongenomic and genomic effects of VD.

Given that telomerase activity is regulated at multiple levels by different stimuli, including hormones, the mechanisms involved in telomerase regulation are far from established. Our data addressed a novel mechanism of down-regulation of telomerase by 1,25VD. Better understanding of the regulation of telomerase by 1,25VD will provide the basis for telomerase activity in VD-targeted therapy.

Although hTERT is regulated tightly at the promoter machinery and a putative VDRE in the hTERT promoter responds to a combination of 1,25VD and 9-cis RA in certain prostate cancer cells, it fails to respond to 1,25VD alone (Ikeda, 2003). It is not unusual that sequences match *in vitro* but do not provide a functional VDRE (Colnot, 2000; Gonzalez, 2002). We provide strong evidence that VD increase the degradation of hTERT mRNA by real-time PCR analysis using 2 probes. Our study is the first to demonstrate down-regulation of telomerase mediated by decreasing the hTERT mRNA stability.

Given that upregulation of hTERT mRNA and telomerase activity induced by multiple oncogenetic factors in cancer cells, degradation of hTERT mRNA by 1,25VD could be an effective way to suppress the effect of multiple oncogenic pathways in OCa.

Recent studies showed that double-stranded RNA, including small interfering RNA (siRNA) and microRNA (miRNA) can induce the degradation of homologous RNAs in organisms as diverse as protozoa, animals, plants and fungi, resulting in posttranscriptional gene silencing (Lewis, 2003). MiRNA are endogenous ~22nt RNAs that arise from larger precursors transcribed from non-protein-coding genes. SiRNA arise



by cleavage of long, double-stranded RNAs. Despite the differences in origin, miRNA and siRNA are functionally interchangeable (Carrington, 2003; Nelson, 2003). 1,25VD induced degradation of hTERT mRNA may provide a model to study the posttranscriptional repression of hTERT mRNA by double-stranded RNA. hTERT is not included in those ~400 targets of mammalian miRNA identified in the present database, perhaps due to the limited sensitivity of current bioinformatic methods. The actual number of target genes regulated by each miRNA is likely to be substantially higher (Lewis, 2003). It remains to be seen if hTERT would be a target for miRNA; if so, it will be important to establish how 1,25VD mediates down-regulation of hTERT mRNA through MiRNA.

The ability of 1,25VD to induce cell cycle arrest and apoptosis without the involvement of p53 may prove useful in therapy. It is known that p53 stimulates the activities of p21/WAF1, gadd45 and bax genes to enhance their expression as a transcriptional factor resulting in cell cycle arrest, DNA repair and apoptosis (Bargonetti, 2002). p53 is mutated in 50% of human malignancies, including ovarian cancer and tumors with mutant p53 resist conventional p53-target therapy. In our model system, 1,25VD stimulates GADD45 and down-regulates telomerase in a p53-independent manner. 1,25VD therapy might compensate or substitute for part of the p53 function to induce cell cycle arrest and apoptosis. 1,25VD may prove valuable candidates for treating OCa, especially those OCa that have acquired resistance to other apoptosis-inducing agents due to a mutation in p53.

VD decreases the generation of single-strand DNA induced by diethylnitrosamine (DEN), a mutagen that induces chromosome aberrations (Basak, 2000). DNA double

strand breaks are generated from mutagen-induced DNA lesions in the S-phase of the cell cycle. It is reasonable to assume that double strand breaks are repaired in the G2 phase by post-replicative repair mechanism. Therefore, VD-mediated suppression of double strand breaks could be mediated through GADD45, which has been shown to play role in DNA repair. GADD45 modified DNA accessibility on damaged chromatin (Carrier, 1999) and affects chromatin remodeling of templates concurrent with DNA repair. Slower nucleotide excision repair was found in GADD45 deficient keratinocytes exposed to UV (Maeda, 2002). Based on these data GADD45 may participate in the coupling between chromatin assembly and DNA repair. Since telomerase maintains the telomere length and contributes to chromosome stability (Cech, 2004), it will be worthwhile to investigate the role of GADD45 and telomerase in VD-protected chromosome stability and induced DNA repair. These two pathways will provide useful targets for DNA-damaging chemotherapeutics against p53-defective OCa, which have decreased ability to repair chemotherapeutic damage.

We were first to provide preclinical data on the 1,25VD analogue EB1089 in OCa. The goal of future studies is to test EB1089 or other promising 1,25VD analogues in clinical trial of OCa. To increase the antiproliferative potency without increasing side effects, use of less calcemic analogues appears to be the most reasonable approach. Several promising new synthetic VD analogs are also under development, such as KH-1060, LG190119, deltanoids, 1-alpha-hydroxyvitamin D5, vitamin D2, QW-1624F2-2, etc.

EB1089 has been widely used in breast and colon cancer patients and stabilization of disease was observed in a phase I trial (Gulliford, 1998). In a phase II study of EB1089

in patients with inoperable hepatocellular carcinoma, strikingly, out of 33 patients, two had complete response, 12 stable diseases. Complete regression appeared after 6 and 24 months of treatment and lasted 29 to 36 months (Dalhoff, 2003). EB1089 was also well tolerated. Most patients tolerated a daily dose of 10  $\mu$ g of EB1089. A Phase III trial in hepatoma are currently ongoing. These studies showed EB1089 response by a reduction of solid tumor size and provide the rationale that EB1089 can be used for treatment. The effectiveness of 1,25VD analogues in slowing the progression of prostate cancer, was shown in a study of an oral 1,25VD analogue (Rocaltrol) to treat early recurrent prostate cancer. PSA doubling time was significantly prolonged by the treatment in all 7 cases (Zhao, 2001).

Administration of 1,25VD analogues through dietary supplementation suggests that oral ingestion of VD-based chemotherapy is an effective and feasible approach. Oral administration and long-term safety of 1,25VD analogues clearly has the advantage in terms of feasibility of cancer chemotherapy and chemopreventive agents (Welsh, 2003).

Our study showed human OSE growth was inhibited by 1,25VD and that VDR, RXR are expressed in normal human ovary tissues, which support the suspected role of VD in OCa initiation. Our data suggest that VD may have a cancer preventative effect. While numerous issues remain to be addressed, it is essential to investigate the anti-tumor activity in the OCa “prevention” model after defining the downstream targets of VDR in the normal ovary.

In summary, we link the specific molecular pathway to 1,25VD’s biological function in this study. We provide the first molecular evidence that G2/M arrest by 1,25VD in OCa cells is mediated through the induction of GADD45 via a novel exonic

enhancer. We are also first to show that 1,25VD induced-apoptosis is mediated by destabilization of hTERT mRNA and a decrease in telomerase activity. In addition, our study is the first to demonstrate that 1,25VD analogue EB1089 inhibits OCa xenograft *in vivo*. With the ubiquitous expression of VDR and RXR in ovarian tissues and response of primary OSE to 1,25VD, our data strongly suggest further investigation of less calcemic synthetic 1,25VD analogues as chemopreventive and chemotherapeutic agents against OCa. Furthermore, half of OCa is estimated to lose p53 function, thus becoming refractory to drugs targeting p53 pathways. These tumors may however still be sensitive to synthetic VD since 1,25VD induces GADD45 and inhibits telomerase in a p53-independent manner.

## MATERIALS AND METHODS

### Materials

pHG45-HC containing the 8 kb genomic sequence of human GADD45 (Hollander, 1993), pCMV45 containing the open reading frame of human GADD45 cDNA and pCMVAS45 containing human GADD45 cDNA in the anti-sense orientation (Zhan, 1994), pCMVgal (Li, 2003), p91023B-VDR (Baker, 1988; Hilliard, 1994), pCMX-RXR $\beta$  (Mangelsdorf, 1992), p23 containing rat 24-hydroxylase promoter in pMAMMneoLuc (Arbour, 1998) and pBabhTERT (Vaziri, 1998) hTERT reporter construct pGL3-3328Luc (Kyo, 1999) have been described previously. pGL3-promoter, pGL3-basic and pGL3-control vectors were from Promega (Madison, WI). MEFs from wild type and GADD45-null mice have been described (Hollander, 1999). 1,25VD was from Calbiochem (La Jolla, CA). EB1089 was kindly provided by Dr. Binderup of Leo Pharmaceuticals Products (Ballerup, Denmark). Baculovirus-expressed human VDR protein, human RXR $\beta$  protein and anti-RXR $\beta$  antibody were from Affinity BioReagents Inc. (Golden, CO). Anti-VDR antibody was from Chemicon International (Temecula, CA). Anti-Flag M2 antibody and anti- $\beta$ -actin antibody were from Sigma (St. Louis, MO). Anti-GADD45 antibody (C-20), anti-cdc2 antibody and anti-cyclin B1 antibody (D11) were from Santa Cruz Biotech (Santa Cruz, CA). All oligonucleotides were synthesized by Invitrogen (Carlsbad, CA). The sequence of primers used for the construction of

GADDLuc reporter by polymerase chain reaction (PCR) is: 5' - GGTGGTACGCGTCCCGAACTTCTCTTACCTACC-3' (forward) and 5' - GGTGGTAGATCTACCCAAACTATGGCTGCACAC-3' (reverse). The sequence of the oligonucleotides in sense orientation for producing complementary double strand oligoes for EMSA and site-mutagenesis is listed below: human OC VDRE 5' - ttggtgactcaccGGGTGAacgGGGGCAtt- 3'; putative GADD45 VDRE-A 5' - ttggcgtgcagGGGTCAtgGGGGTGacg- 3'; putative GADD45 VDRE-B 5' - taggtggGGGTCAggaGGGTGGctgcctttgt- 3'; putative GADD45 VDRE-C 5' - aactGTTTCActcAGGTCAgggtacaagt- 3'; putative GADD45 VDRE-D 5' - cagcttgGGTTGCatgGGTTCAgactttgc- 3'; putative GADD45 VDRE-E 5' - gccaaggGGCTGAgtgAGTTCAactacatg- 3'; putative hTERT VDRE 5' - caccactgtaaggAGTTCAtgAGTTCAat- 3'; primer for the mutation of VDRE-A 5' - gtgcagGGGTCAtgGGGtTtacggggccgcggga- 3'; primer for the mutation of VDRE-B 5' -GGGTCAggaGGGTttctgcctttgtccgactagagtg- 3'; primer for the mutation of VDRE-D 5' -cagcttgGGTTGCatgGGTTtgactttgcaatgtgtag- 3'; primer for the mutation of VDRE-E 5' -gccaaggGGCTGAgtgAGTTttactacatgttctggg- 3'. Capital letters are used to indicate the hexameric core binding motifs in the VDRE primers and bold letters in lowercase are used to indicate the nucleotides in the mutagenesis primers that are different from the wild type sequence. The sequence of primers for chromatin immunoprecipitation (ChIP) assays is listed below: VDRE-E region (2565/2767) -CTGAACGGTGATGGCATCTG-3' (forward) and 5' -CTGTTTCAACACAGCTTCCTTC- 3' (reverse); promoter region-1 (-1492/-1241) 5' -GTTGTCCATGGCTGACAACA-3' (forward) and 5' - GCTCCCACATGCTTGCATTC-3' (reverse); promoter region-2 (-505/-310) 5' -

CACTTCTGAGGTAAACTTTGC-3' (forward) and 5'-  
GAAGCAGGCTGCCAAGTGTT-3' (reverse).

#### **Colorimetric methylthiazole tetrazolium (MTT) assays and statistical analysis**

To measure cell growth, OVCAR3 cells were plated at  $2 \times 10^3$  cells/well in 96-well plates and treated with VD or vehicle. MTT assays were performed as described (Li, 2001). OD<sub>595</sub> was read on a MRX microplate reader (DYNEX Technologies, Chantilly, VA).

For cell growth and cell cycle analyses, statistical analysis was performed using the independent-samples *t* test.  $P < 0.05$  was considered to be statistically significant.

#### **Cell cycle and apoptosis analysis by flow cytometry**

To determine the cell cycle distribution, cells were harvested by trypsin digestion and fixed with 70% ethanol in PBS for 12 hours at 4 °C. Fixed cells were incubated overnight with 100 µg/ml RNase, stained with 50 µg/ml propidium iodide at 4 °C and subjected to cell cycle analysis on a FACScan (Becton Dickinson, Mountain View, CA). To determine the apoptosis, cells were harvested by trypsin digestion and washed with PBS. Cell suspension in 1x assay buffer was added annexin V-FITC and propidium iodide and incubated for 15min, then subjected to flow cytometry.

#### **Northern blot analysis**

To determine the level of GADD45 mRNA, Northern blot was performed as described (Zhang, 2003). Briefly, OVCAR3 cells were incubated with 1,25VD or vehicle for the indicated times. Total cellular RNA was isolated by TRIzol (Invitrogen) method following manufacture's instruction. Samples containing 20 µg RNA were run on a 1%

agarose gel in denaturing gel buffer (Ambion, Austin, TX) and transferred onto a nylon membrane. The membrane was pre-hybridized at 65 °C for 4 h and hybridized with the GADD45 or GAPDH probe at 65 °C for overnight. Washes were performed in high stringency buffers. To prepare GADD45 probe, full-length GADD45 cDNA was released from pCMV45 vector with HindIII/XbaI digestion, separated and recovered from agarose gel. GAPDH probe was from Ambion. The probes were labeled with <sup>32</sup>P using random-primed DNA labeling kit (Ambion). Signal densities were analyzed with scion Image software (Scion Corp., Frederick, MD).

### **Gel mobility shift assay (EMSA)**

EMSA was performed as described (Li, 2003; Gonzalez, 2002) with modifications. Briefly, double-stranded oligonucleotides were end-labeled with <sup>32</sup>P using a T4 polynucleotide kinase labeling system (Life Technologies, Rockville, MD). 1 µl of radiolabeled probe (roughly 50,000 cpm) was mixed with 19 µl DNA binding reaction mixture that contains 250 ng VDR, 250 ng RXR, 10 mM Tris-Cl (pH7.9), 100 mM KCl, 0.1 mM EDTA, 15% glycerol, 100 µg/ml poly(dI:dC), 0.1 µg/µl bovine serum albumin, 1 mM DTT and 10<sup>-7</sup> M 1,25VD. The mixture was incubated at room temperature for 30 minutes. For competition experiments, VDR/RXR was pre-incubated with 2 µg anti-RXRβ, anti-Flag M2 antibody or excess amount of cold probes on ice for 20 minutes before the EMSA reaction. The reaction mixture was resolved in a 5% non-denaturing polyarylamide gel and protein-oligo complexes were revealed by autoradiography.

### **Construction of luciferase reporter plasmids, deletion and site-directed mutagenesis**



To construct GADDLuc, GADD45 genomic DNA fragment from +366 to +2926 was amplified by PCR using primers described in the Material. The forward primer contains a MluI and the reverse primer a BglII site. The amplified PCR fragment was cloned into the MluI and BglII sites of pGL3-promoter vector.

Luc1 was generated by digesting the GADDLuc with KpnI and religation. Luc2 was generated by digesting GADDLuc with MluI and EcoRI, filling with Klenow fragments and religation. Luc3 was generated by sub-cloning into BglII site of pGL3-promoter vector a 440 bp DNA fragment released from GADDLuc with BamHI and BglII. Luc4 was generated by digesting GADDLuc with BglII and EcoRI, filling with Klenow fragments and religation. Luc5 was generated by sub-cloning into pGL3-promoter vector at KpnI and BglII sites a 777 bp fragment released from Luc2 with KpnI and BamHI.

Site-directed mutagenesis was performed as described (Lee, 2002) using QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). The sequence of all mutant constructs was verified by DNA sequencing.

### **Transcriptional analysis**

For transfection studies, OVCAR3 cells were plated in 15% FBS RPMI 1640 medium at  $1 \times 10^5$  cells/well and HeLa cells in 10% FBS DMEM at  $5 \times 10^4$  cells/well in 12-well plates. On the next day, OVCAR3 cells were transfected by Lipofectamine Plus and HeLa cells by Lipofectamine following the protocol from Invitrogen. 4 h post-transfection, cells were treated with 1,25VD or vehicle in fresh medium for 36 h. Cells

were harvested and luciferase and  $\beta$ -galactosidase ( $\beta$ -gal) assays were performed as described (Lee, 2000 and 2002).

### **Chromatin immunoprecipitation (ChIP) assays**

For ChIP assays, OVCAR3 cells were treated with EOH or  $10^{-7}$  M VD for 60 min and cross-linked with 1% formaldehyde at room temperature for 10 min. Then, the cells were incubated with 0.125 M glycine for 5 min, washed, scraped in ice-cold PBS containing protease inhibitor cocktail (Roche) and lysed in buffer (pH 8.0) containing 5 mM PIPES, 85 mM KCl, 0.5 % NP 40 and protease inhibitor cocktail. Cell nuclei were re-suspended in lysis buffer containing 50 mM Tris-Cl (pH. 8.1), 10 mM EDTA, 1% SDS and protease inhibitor cocktail. Soluble chromatin was prepared by sonication and diluted in buffer containing 16.7 mM Tris-Cl (pH 8.1), 0.01 % SDS, 1.1% Triton X 100, 1.2 mM EDTA, 167 mM NaCl and protease inhibitor cocktail. The diluted chromatin solution was pre-cleared with pre-immune serum and protein G agarose (Santa Cruz) pre-coated with sheared sperm DNA (Ambion). Immunoprecipitations were carried for overnight at 4 °C with rat anti-VDR antibody or Rat IgG (Sigma) followed by incubation with pre-coated protein G agarose for 2 h at 4 °C. The beads were sequentially washed at room temperature for two times (10 min/wash) in each of the following buffers: the dilution buffer, TSE-500 wash buffer containing 20 mM Tris-Cl (pH 8.1), 0.1% SDS, 1% Triton X 100, 2 mM EDTA, 500 mM NaCl) and LiCl/detergent wash buffer containing 100 mM Tris-Cl (pH 8.1), 1% NP40, 1% deoxycholic acid, 500 mM LiCl). After the final wash in TE buffer containing 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA, the immunocomplexes were eluted from the beads with 50 mM NaHCO<sub>3</sub> and 1% SDS for two times. The immunocomplexes were pooled and heated at 65 °C overnight to reverse the cross-

linking. DNA was extracted from the immunocomplexes using a QIAquick Spin Kit (Qiagen, CA). 2  $\mu$ l out of 30  $\mu$ l DNA extract was used for PCR.

### **Immunoblotting analysis**

Immunoblotting analysis of GADD45 protein was performed as previously described (Li, 2001) with modification. Briefly, cells were harvested in lysis buffer containing 50 mM Tris-Cl (pH 7.5), 1% NP-40, 0.25% deoxycholic acid, 400 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM NaF, and protease inhibitor cocktail. The protein concentration of the cell lysate was assayed using Bio-Rad kit. Extracts containing 50  $\mu$ g of protein were separated on a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane. GADD45 was detected using ECL Kit following the manufacture's instruction (Amersham-Pharmacia Biotech, Piscataway, NJ). The immunoblotting analysis of cdc2, cyclinB1 and VDR was performed similarly as for GADD45 except that the cell extracts were prepared in buffer containing 20 mM Tris-Cl (pH 7.5), 300 mM NaCl, 3 mM EDTA, 3 mM EGTA, 100  $\mu$ M  $\text{Na}_3\text{VO}_4$ , 1% NP-40, and protease inhibitor cocktail (Zhang, 2003).

### **Establishment of stable clones from OVCAR3 cells**

OVCAR3 cells were transfected with 10  $\mu$ g pCMVAS45 or hTERT plasmid together with 0.5  $\mu$ g pcDNA3 for the establishment of GADD45 anti-sense, telomerase stable clones. pcDNA3 alone transfected into OVCAR3 cells for the Vector-OVCAR3 controls. For the establishment of stable clones with GADDLuc reporter, OVCAR3 cells were transfected with 10  $\mu$ g GADDLuc plasmid and 0.5  $\mu$ g pcDNA3. All stable clones

were obtained through selection with 100  $\mu\text{g/ml}$  G418 for a period of about 4 weeks and isolated by cloning with glass cylinders.

### ***In vitro* immunocomplex kinase assays**

*In vitro* immunocomplex kinase assays were performed as described (Lee, 2000) with minor modifications. In brief, cells were washed with ice-cold PBS and cellular extracts are prepared in buffer containing 20 mM Tris-Cl (pH 7.5), 300 mM NaCl, 3 mM EDTA, 3 mM EGTA, 100  $\mu\text{M}$   $\text{Na}_3\text{VO}_4$ , 1% NP-40, protease inhibitor cocktail. Cellular extracts containing 200  $\mu\text{g}$  protein were immunoprecipitated with anti-cyclin B1 antibody. Kinase assays were performed at 30  $^\circ\text{C}$  in 20  $\mu\text{l}$  reaction buffer containing 20 mM HEPES (pH7.5), 5 mM  $\text{MgCl}_2$ , 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 20  $\mu\text{M}$  ATP, 3  $\mu\text{g}$  histone H1, and 10  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ] ATP. Reactions were terminated by adding 2  $\times$  SDS-PAGE sample buffer, analyzed on a SDS-PAGE and visualized by autoradiography.

### **Telomerase activity assay**

Telomerase activity was measured with the telomerase PCR- enzyme-linked immunosorbent assay (ELISA) kit (Roche) base on the telomeric repeat amplification protocol (TRAP) assay. Cells were suspended in lysis buffer (Roche), incubated on ice for 30 min, and then centrifuged at 14000  $\times$  g for 20 min. Supernatants were used for the detection of telomerase or flash frozen and stored at -80  $^\circ\text{C}$ . According to the manufacturer's instructions, 200 ng of protein extract were assayed for telomerase activity after 30 cycles of amplification by PCR. The resulting PCR product (5  $\mu\text{l}$ ) was quantified by ELISA. Telomerase activity was expressed as absorbance values (OD) measured using a microtiter reader at 450 nm with a reference wavelength of 595 nm. All

assays were performed in duplicate and a dilution series of control telomerase extracts was always examined in parallel to give titration curve for normalizing experimental variations.

### **RT-PCR analysis**

Analysis of the expression of hTERT and GAPDH were performed by reverse transcription-PCR amplification as previously described (Nakamura, 1997). RNA was isolated with Trizol (Invitrogen) according to the manufacturer. 1 µg of total RNA was reverse-transcribed using the RNA PCR kit version 2 (TaKaRa, Ohtsu, Japan) with oligo-dT primers. To amplify the cDNA, 1 aliquots of the reverse-transcribed cDNA (20ul) from 2 µg of RNA were subjected to PCR in 25 ul of 1x buffer (10mM Tris-Cl (pH8.3), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl), containing 1 mM each of the dNTP; 1.25 U of Taq DNA polymerase (TaKaRa); and 0.2 µM of specific primers. Primer sequence was chosen to amplify a 145-bp region presenting in all transcripts of hTERT mRNA 1784 (5'-cggaagagtgtctggagcaa- 3') and 1910 (5'-ggatgaagcggagtctgga- 3') for 30 cycles (94°C for 30s, 60 °C for 30s, 72 °C for 90s). As a positive control, GAPDH mRNA was amplified in parallel using primers 5'-ctcagacaccatggggaaggtga- 3' and 5'-atgatcttgaggctgtgtcata-3' for 16 cycles (94°C for 30s, 55 °C for 30s, 72 °C for 45s). PCR products were electrophoresed in 15% polyacrylamide gel and stained with SYBR green. For semi-quantification of hTERT mRNA expression, serially diluted cDNA reverse-transcribed from 2 µg RNA (corresponding to 50 ng to 1 µg RNA) was subjected to RT-PCR.

### **Primers, probes for Real-time PCR**

Primers and probes for the hTERT gene were chosen with the assistance of the Primer Express (Perkin-Elmer Applied Biosystems, Foster city, CA). To avoid

amplification of contaminating genomic DNA, either upstream or downstream primer was placed in a different exon. Forward primer of hTERT1 (1909F) and probe1 was placed in exon 4, whereas reverse primer (2017R) was spanning exon 4 and 5 junction. Forward primer of hTERT2 (3081F) was spanning exon 13 and 14 junction, probe2 and reverse primer (3162R) in exon 14. The nucleotide sequences are as follows:

hTERT1: 1909F: 5'-gtccagactccgcttcataa-3'; 2017R: 5'-gagacgctcggcctctt-3';

FAM/TAMRAprobe1: 5'-ttctggctcccacgacgtagtcctg-3'; PCR product size: 109bp.

hTERT2: 3081F: 5'-cgtacaggttcacgcatgtg-3'; 3162R: 5'-atgacgcgcaggaaaaatg-3';

TAM/TAMRAprobe2: 5'-agctcccatttcacgcaagtttgaag-3'. PCR product size: 82bp.

### **Real-time PCR**

RNA was prepared using the RNasy RNA isolation kit (Qiagen) and DNase digested on-column using RNase-free DNase set (Qiagen) and then reverse-transcribed. An RNA pool was generated by mixing aliquots of RNA from cells treated with vehicle or 1,25VD for various times. Concentrations of the pooled RNA ranging from 0 (buffer alone) to 50 ng/ $\mu$ l were used in the PCR analysis to generate the standard curve for each gene. The Ct value was generated by the ABI PRISM 7700 SDS software version 1.7 and then exported to an Excel spreadsheet where equations from the standard curve were generated. Using the Ct values, concentrations of the hTERT and GAPDH mRNAs were calculated from the equations. Each sample was analyzed at two different concentrations (50 ng/ $\mu$ l and 2 ng/ $\mu$ l) with only results in the most sensitive region of the standard curve presented. Samples at each concentration were analyzed in triplicates. hTERT levels were normalized to the corresponding input total RNA, base on quantitation of GAPDH.

### **Telomere length analysis**

Telomere length was measured using a non-radioactive chemiluminescent assay developed by Roche Diagnostics. In brief, genomic DNA was isolated using a Roche DNA isolation kit. 5 µg DNA was digested overnight with 20 U of Hinf I and Rsa I, fractionalized on a 0.8% agarose gel. Gels were denatured for 30 min and then neutralized for 30 min. DNA was transferred to a nylon membrane, hybridized with digoxigenin 3'-end tailing – labeled (Roche) (TTAGGG)<sub>4</sub> oligonucleotides overnight at 50 °C in PerfectHyb solution (Sigma). Membranes were washed twice in 2 x SSC, 0.1% SDS for 15 min at RT and twice in 0.1 x SSC, 0.1% SDS for 15 min at 50 °C. The probe was detected by the digoxigenin luminescence detection and processed by Southern blotting and chemiluminescent detection. The average telomere length can be determined by comparing the signals relative to a molecular weight standard.

#### **Immuno histochemical analysis**

To confirm the VDR and RXR expression in normal human tissue, paraffin-embedded tissues were immunostained with anti-VDR antibody and anti-RXR antibody. The signal was detected with the avidin-biotin complex (ABC) immunoperoxidase kit (Vectastain Elite ABC, Vector Laboratories, CA). The cell nucleus was counterstained with hematoxylin. Positive (skin tissue) and negative controls (pre-immune serum) were included in all immuno-reactions.

#### **Nude mouse tumor studies**

The studies were performed as described (Blutt, 2000) with little modification. Briefly, OVCAR3 cells were trypsinized and resuspended in 50% Matrigel Matrix (Becton Dickinson, San Jose, CA) at a concentration of  $2 \times 10^6$  cells/100ul matrix. Female athymic nude mice, ~6 weeks of age, on a vitamin D-deficient diet supplemented with

1% calcium, received injections s.c. with  $2 \times 10^6$  cells on the dorsal surface. After tumors grow to a volume of about  $150 \text{mm}^3$ , the mice were randomly divided to three groups treated with placebo or EB1089 at 0.3 or  $1.0 \mu\text{g}/\text{kg}/\text{day}$ . EB1089 was delivered daily by gavage. The size of the tumor was determined by measuring the tumor diameters with a Vernier caliper every 5 days and using the formula tumor volume =  $\frac{d \times D \times D}{2}$  (where d and D represent the shortest and the longest diameter, respectively).



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### **About the Author**

Feng Jiang received her M.D. degree in Xi'an Medical University, China in 1994 and a M.S. in Pathology from Immunopathology Institute of Xi'an Medical University in 1997. She was a resident in the Department of Ophthalmology of the Second Affiliated Hospital of Xi'an Medical University from 1997-1999.

She entered the Ph.D. program at Dr. Bai's laboratory at the University of South Florida in 1999. She has several publications and participated in the 94<sup>th</sup> and 95<sup>th</sup> Annual Meetings of the American Association of Cancer Research in 2003 and 2004. She received an "Outstanding graduate student award" in 2001 and "Superior presenter's award" at the Health Science Center Research Day in 2003 and 2004.