VITAMIN D-MEDIATED SUPPRESSION OF MAMMARY TUMORIGENESIS AND

MECHANISM OF ACTION

by

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ABSTRACT OF THE DISSERTATION

Vitamin D-Mediated Suppression of Mammary Tumorigenesis and Mechanism of Action

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Numerous preclinical, epidemiological and clinical studies with vitamin D and analogs have suggested the benefits of vitamin D and analogs for prevention and treatment of cancer. However, the hypercalcemic effects have limited the use of $1\alpha,25(OH)_2D_3$, the hormonally active form of vitamin D. Gemini vitamin D analogs with a unique structure of two six-carbon chains have shown activity in inhibiting tumor growth of colon cancer cells without inducing hypercalcemia. However, the molecular mechanism of Gemini vitamin D analogs has not been studied. Here, we have investigated the effects of novel Gemini vitamin D analogs on suppressing mammary tumorigenesis and the mechanism of action in breast cancer *in vitro* and *in vivo*.

We found that Gemini vitamin D analogs exhibited better inhibition of cell growth than $1\alpha,25(OH)_2D_3$ and regulated the cell proliferating related markers including the cyclin dependent kinase inhibitor, p21 and insulin-like growth factor binding protein 3 (IGFBP-3) in MCF10AT1 human breast epithelial cells. The transforming growth factor β (TGF-β) superfamily has been suggested to cross-talk with vitamin D signaling, and we determined that Gemini vitamin D analog Ro3582 activated Smads (Smad1/5), down-stream mediators of bone morphogenetic protein (BMP) signaling. In the study of upstream signaling pathways, we found that Ras/PKCα was involved in Smad activation and cell growth inhibition by Ro3582, suggesting that the Ras/PKCα-Smad signaling

pathway may mediate the inhibition of cell proliferation by Gemini vitamin D analog Ro3582 in MCF10 human breast epithelial cells.

Gemini vitamin D analogs exerted significant *in vivo* suppression of mammary tumorigenesis without inducing hypercalcemic toxicity in three different animal models: 1) N-methyl-N-nitrosourea (NMU)-induced estrogen receptor (ER) positive breast cancer, 2) ER-negative MCF10DCIS xenografts, and 3) an MMTV-her2/neu transgenic mouse model. These results suggest that Gemini vitamin D analogs be used as potent agents in the prevention and/or inhibition of different subtypes of breast cancers including luminal, Her2 positive and basal-like breast cancer.

In conclusion, Gemini vitamin D analogs regulate Smad signaling via the Ras/PKCα pathway, and may be potent agents for the prevention and treatment of breast cancer without calcemic toxicity.

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INTRODUCTION

I. VITAMIN D

In 1919, Edward Mellanby discovered vitamin D through his experiments with dogs raised indoors in the absence of sunlight or ultraviolet exposure. Mellanby suggested that deficiency of a trace fat soluble vitamin or food factor caused rickets [1]. Thereafter, McCollum *et al* recognized the differences between vitamin A, which was inactivated, and vitamin D, which was active during the preparation of the "fat-soluble vitamin" [2], and Hess *et al* discovered that ultraviolet irradiation could cure infantile rickets [3]. Further studies identified vitamin Ds as the constituents responsible for the antirachitic activity, and the chemical structure of vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) were determined as secosterols in 1932 [4] and 1936 [5], respectively (Fig.1). After the breakthrough studies identifying and characterizing the vitamin D receptor [6], as well as metabolites 25-hydroxyvitamin D_3 [7] and 1α,25-dihydroxyvitamin D_3 [8,9], vitamin D was found to play pivotal roles in the physiological regulation of calcium/phosphate homeostasis and bone mineralization, and great reviews of vitamin D are available [10-12].

A. Vitamin D sources and metabolism

Humans can obtain significant amounts of vitamin D from 1) dietary natural sources (salmon, cod liver oil, egg yolk etc.) 2) fortified foods (milk, juice, cheese etc) and dietary supplements [12]. Vitamin Ds from dietary sources enter the blood stream through the lymphatic system after the incorporation with chylomicrons [12]. Vitamin D_3 is also produced in the skin through the breakdown of 7-dehydrocholesterol into

previtamin D_3 by ultraviolet B energy from the sun light [13], and previtamin D_3 is then converted to vitamin D_3 by thermal isomerization [10]. The amount of vitamin D_3 produced from sunlight exposure is controlled via breakdown of over-produced previtamin D_3 or vitamin D_3 into inactive photoproducts by solar UVB irradiation [12].

A.1. 25-Hydroxylation of vitamin D

In the liver, vitamin D is metabolized into 25-hydroxyvitamin D (25(OH)D), the major circulating metabolite, by the hepatic cytochrome P-450 monooxygenase, 25-hydroxylase. Because the level of 25-hydroxyvitamin D is proportional to vitamin D intake [14], its plasma level is used as a common indicator for determining vitamin D status. Among the hepatic cytochrome P-450s having activity in 25-hydroxylation of vitamin D, the microsomal 25-hydroxylase, CYP3A4 is reported to hydroxylate vitamin D_2, but not vitamin D_3 [15]. In addition, the mitochondrial CYP27A1 can hydroxylate carbon 25 in vitamin D_3. However, CYP27 knockout mice (cyp27-/-) showed normal levels of vitamin D_3 metabolites [16]. Recently, CYP2R1 was reported to be mainly expressed in the liver and testis and hydroxylate both vitamin D_2 and vitamin D_3 [11,17,18]. Germline mutation of CYP2R1 have been identified in patients with low level of 25-hydroxyvitamin D and rickets [11,17,18]. These results suggest that CYP2R1 may encode the crucial 25-hydroxylase in vitamin D metabolism.

A.2. Renal 1α-hydroxylation of 25-hydroxyvitamin D

The second step for vitamin D activation is 1α-hydroxylation of 25-hydroxyvitamin D, mainly in the kidney [8]. The mitochondrial cytochrome P-450 enzyme, 25-hydroxyvitaminD_3-1α-hydroxylase (1α-hydroxylase) encoded by the gene CYP27B1 produces hormonally active vitamin D metabolite, 1α,25-dihydroxyvitamin D_3

$(1\alpha,25(OH)_2D_3)$ from $25(OH)D_3$ [19]. In mice experiment where 1α-hydroxylase is inactivated by homologous recombination or targeted ablation, those mice had undetectable level of serum $1\alpha,25(OH)_2D_3$ and developed hypocalcemia, hyperparathyroidism, retarded growth, rickets and osteomalacia [20,21], showing that 1α-hydroxylase and $1\alpha,25(OH)_2D_3$ produced by 1α-hydroxylase are critical mediators for calcium homeostasis.

A.3. Extrarenal 1α-hydroxylation of 25-hydroxyvitamin D

Extrarenal organs including colon, breast, prostate, lung, pancreas, monocytes, and skin are also known to express 1α-hydroxylase which converts $25(OH)D_3$ into $1\alpha,25(OH)_2D_3$ locally [22,23]. However, $1\alpha,25(OH)_2D_3$ produced in those organs functions as a tissue-specific autocrine/paracrine factor to mediate local actions of vitamin D in cell proliferation, differentiation, and immune regulation [11]. After completion of those tasks, $1\alpha,25(OH)_2D_3$ is inactivated by increasing 24-hydroxylase activity at the same sites (see below). This indicates that locally produced $1\alpha,25(OH)_2D_3$ does not enter the circulation to regulate calcium metabolism [12]. An overall diagram describing the metabolism of vitamin D is shown in Fig.2.

A. 4. Vitamin D catabolism

Vitamin D metabolites are catabolized mainly by oxidation of the side chain. The major enzyme responsible for the oxidation of $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ into $24,25(OH)_2D_3$ and $1\alpha,24,25(OH)_3D_3$, respectively, is the cytochrome P-450 25-hydroxyvitamin D 24-hydroxylase (24-hydroxylase, CYP24A1). 24-Hydroxylation is the rate-limiting step for vitamin D catabolism [24]. Further subsequent oxidative cleavages occur and the final oxidant of $1\alpha,25(OH)_2D_3$ is calcitroic acid which is biologically inactive

[10]. Mice with inactivated 24-hydroxylase by targeted ablation developed lethal hypercalcemia and poor mineralization of intramembranous bone because of high levels of $1\alpha,25(OH)_2D_3$, thus, demonstrating the critical roles of 24-hydroxylase in regulating and maintaining the level of vitamin D metabolites [24,25].

A.5. Regulation of $1\alpha,25(OH)_2D_3$ and 1α-hydroxylase

Due to the high potency of $1\alpha,25(OH)_2D_3$ in regulating serum calcium and phosphate levels, the activity of 1α-hydroxylase and the plasma level of $1\alpha,25(OH)_2D_3$ are tightly regulated for calcium homeostasis [11]. The major regulators are parathyroid hormone (PTH), calcium and $1\alpha,25(OH)_2D_3$ itself. When serum calcium level is low, secretion of PTH from parathyroid glands induces the expression of 1α-hydroxylase, which increases level of $1\alpha,25(OH)_2D_3$. The produced $1\alpha,25(OH)_2D_3$ enhances calcium absorption from the intestine and resorption from bone to increase the blood calcium level. Feedback inhibition of $1\alpha,25(OH)_2D_3$ leads to a decrease of $1\alpha,25(OH)_2D_3$ synthesis and PTH secretion. In addition, $1\alpha,25(OH)_2D_3$ increases the expression of 24-hydroxylase to induce the catabolism of $1\alpha,25(OH)_2D_3$ into inactive, water-soluble calcitroic acid [10,12].

B. Vitamin D Receptor (VDR)

Vitamin D receptor (VDR), a member of the nuclear receptor superfamily for the steroid hormones, was discovered in 1969 [6], and is known to be expressed in more than 30 tissues in man [26]. The crystal structure of the ligand binding domain (LBD) in VDR was first reported in 2000 [27]. A mouse model in which VDR is mutated by targeted gene ablation has demonstrated that the mice developed vitamin D deficiency rickets type II (VDDRII) with alopecia, osteomalacia, hypocalcemia, hyperparathyroidism,

impaired bone formation and female infertility. This demonstrates the critical roles of VDR in calcium homeostasis, bone formation and female reproduction [28,29].

B.1. Nuclear vitamin D receptor for genomic response

Similar to other nuclear receptors, the VDR acts as a transcription factor after ligand binding to regulate the expression of the target genes in a tissue-specific manner [11]. The VDR gene is located on chromosome 12q and the protein is composed of domains for ligand binding, heterodimerization with retinoid X receptor (RXR), binding to vitamin D receptor response elements (VDREs) and interacting with nuclear co-activators and co-repressors [11,19]. The ligand ($1\alpha,25(OH)_2D_3$) binding to the LBD at the C-terminus of VDR stabilizes VDR through the phosphorylation of serine 51 at the DNA binding domain and serine 208 at the hinge region by protein kinase C (PKC) and casein kinase II, respectively [24,30,31]. The liganded VDR selectively recruits the RXR for heterodimerization and induces conformational changes. This causes the dissociation from the co-repressor, silencing mediator for retinoid and thyroid hormone receptor (SMRT), and the association with stimulatory co-activators such as steroid receptor coactivators (SRC) through interaction with the VDR activation function 2 (AF2) domain, and then regulate the transactivating functions of VDR [19,24].

C. Transcriptional regulation by $1\alpha,25(OH)_2D_3$ and VDR

The regulation of target genes of $1\alpha,25(OH)_2D_3$ occurs through binding to the VDR. The $1\alpha,25(OH)_2D_3$ bound VDR heterodimerizes with RXR. In the nucleus, $1\alpha,25(OH)_2D_3$-VDR-RXR complex specifically interacts with vitamin D response element (VDRE), repeated pairs of hexanucleotide (GGTCCA, AGGTCA, or GGGTGA), where any kind of three nucleotides can connect them, in the promoter region of target genes

[32], and modulate the transcriptional activity. An overall diagram describing transcriptional regulation by $1\alpha,25(OH)_2D_3$ is shown in Fig. 3.

C.1. Transcriptional activation

For the enhancement of transcription of target genes by $1\alpha,25(OH)_2D_3$, different co-factors are involved [24,33,34]. The conformational change of VDR by $1\alpha,25(OH)_2D_3$ binding induces the VDR AF2 domain to bind coactivators including steroid receptor coactivators (SRCs), nuclear coactivator 62 kDa-SKI-interacting protein (NCoA62-SKIP), CREB binding protein (CBP)-p300, and polybromo-and SWI-2-related gene 1 associated factor (PABF), which has histone acetyltransferase activity to unfold and expose DNA for transcription [33,34]. After chromatin remodeling by histone acetylation, a second complex of coactivators, vitamin D receptor-interacting proteins (DRIPs) directly interacts with the AF2 domain of the VDR and recruits the basal transcriptional machinery for the gene expression such as 24-hydroxylase (CYP24A1) and p21 (CDKN1A) [24,33].

C.2. Transcriptional repression

VDR interacting repressor (VDIR) is known to specifically bind directly to E-box-type negative VDREs (nVDREs) sequences. For the ligand-induced repression of transcription of target genes such as 1α-hydroxylase (CYP27B1) and parathyroid hormone (PTH), the VDR-RXR heterodimer does not bind to nVDRE, but interacts with VDIR, which is followed by the dissociation of histone acetyltransferase (HAT) co-activators from VDIR and the association of histone deacetylase (HDAC) co-repressor complex [35,36]. In addition, it was reported that Williams syndrome transcription factor (WSTF) enhanced transcriptional repression through the physical interaction with ATP-dependent chromatin-remodeling complex (WINAC) and chromatin [24,37].

D. Non-genomic rapid action to 1α,25(OH)$_2$D$_3$

Rapid responses to 1α,25(OH)$_2$D$_3$ occurring from seconds to one hour have been shown to result in rapid calcium absorption in the intestine [38], insulin secretion in pancreatic β-cells [39], opening of voltage-gated calcium and chloride channels in osteoblasts [40] and smooth muscle cell migration in endothelial cells [41]. Norman's group have demonstrated that the flexibility of 1α,25(OH)$_2$D$_3$ in carbon 6 and 7 single bond determined genomic or nongenomic rapid responses, where 6-s-trans configuration of 1α,25(OH)$_2$D$_3$ binds to the VDR for genomic regulation and 6-s-cis-conformation is used by the VDR for rapid responses [42,43]. Those findings were based on structure-function studies showing that 6-s-cis locked 1α,25(OH)$_2$D$_3$-lumisterol (JN) exerted equipotent activity to that of 1α,25(OH)$_2$D$_3$ in initiating all rapid responses mentioned above, but 6-s-trans locked 1α,25(OH)$_2$D$_3$-dihydrotachysterol (JB) did not induce those responses [42,43]. Furthermore, the receptor ensemble model was used to identify an alternative ligand binding pocket in nuclear VDR depending on the positions of helix 12 of VDR, which suggests that VDR for rapid responses located in caveolae-enriched plasma membrane may prefer the alternative pocket for selective ligand accommodation [42-44].

D.1. Membrane-bound vitamin D receptor for nongenomic rapid response

After the finding rapid response by 1α,25(OH)$_2$D$_3$ in calcium absorption in vascularly perfused duodena of vitamin D-replete chicks [45], it has been demonstrated that rapid response (RR) is mediated by the receptor located in the plasma membrane or in the caveolae-rich environments, although the mechanism is not clearly understood (Fig. 3) [42]. The putative membrane binding protein, membrane-associated rapid

response steroid-binding protein (MARRS) rapidly responding to $1\alpha,25(OH)_2D_3$ for phosphate uptake in chick intestinal epithelial cells, was determined to be identical to ERp57 [46]. In addition, the membrane VDR (mVDR) in caveolae-enriched plasma membrane is suggested to be same as classic nuclear VDR (nVDR) based on the findings that 1) nVDR antibody immunoreacted with caveolae-enriched membrane fraction [47], 2) $[^3H]$- $1\alpha,25(OH)_2D_3$ binding in kidney tissue of nVDR knock-out mouse showed significant reduction in caveolae-enriched membrane fraction and nuclear fraction, compared to wild type mouse kidney tissue [47], and 3) rapid response was abrogated in VDR knock-out mice showing a functional nVDR is required for rapid modulation by $1\alpha,25(OH)_2D_3$ [48,49]. Although the rapid action of $1\alpha,25(OH)_2D_3$ works via non-genomic regulation through mVDR, it may affect gene transcription indirectly by secondary messengers inducing cross-talk with other signaling pathways such as the Ras/Raf/MAPK pathway [24,50]

II. BREAST CANCER

According to the Cancer Statistics 2008 from the American Cancer Society, breast cancer accounts for the second highest mortality rate (15%) after lung cancer among American women, and is the most frequently occurring cancer (26%) in women [51]. Human breast carcinogenesis has been shown to be highly heterogeneous, and it seems that there is no single dominant pathway playing a critical role during tumor progression [52,53]. This suggests the possibility that pre-existing differences between the cells composing the mammary gland significantly lead to different phenotypes of transformation into breast cancer [54].

The subtype of breast cancer and the risk for recurrence have been categorized and estimated based on the clinical and histological features including patient age, tumor size, lymph node status, hormone receptor or HER2 status to determine the appropriate adjuvant therapy such as tamoxifen therapy and/or chemotherapy for patients [55,56]. Berge *et al* summarized the population-based low-risk cohorts studies to select the optimal adjuvant therapy without overtreatment or undertreatment, and concluded that the patients having small tumors less than 20 mm in size, lymph node-negative, and estrogen receptor-positive are in low-risk group, and the majority of them may not need the additional chemotherapy [55,56]. However, the conventional predictive model still holds about 15% inaccuracy, which means 15% of recurrence in a low-risk cancer and 85% of recurrence in a high-risk cancer [55,56].

A. Molecular Subtypes in Breast Cancer

Recently, Perou and Sorlie proposed a novel classification of breast cancer based on distinct profiles of gene expression in human breast tumors which include luminal A, luminal B, basal-like, Her-2 positive (Her2+) and normal breast-like subtypes [57,58]. Here, molecular subtypes of breast cancer are summarized based on the reviews by Brenton *et al*. and Peppercorn *et al*. [56,59].

A.1. Luminal subtypes

Brenton *et al* summarized that luminal subtypes are (a) positive for estrogen/progesterone receptors, (b) express genes associated with ER activation and luminal cytokeratin 8/18, and (c) have a low frequency of *TP53* mutations [59]. Luminal breast cancers are the most common form of breast cancer representing two thirds of breast cancer and are usually treated with anti-estrogen therapy [59]. In luminal subtypes, there are at least two subtypes, luminal A and luminal B [60]. Luminal A

subtype expresses high level of ER-related genes and low level of cell proliferative genes, compared to luminal B subtype [59]. Luminal A is generally Her-2 negative, but luminal B is sometimes Her-2 positive by immunohistochemistry [60]. In addition, luminal A has a much better prognosis than luminal B in the study of disease outcome based on the data of time to development of distant metastasis and overall survival for the patients with advanced breast cancer [60].

A.2. Her-2 positive (Her2+) subtype

Brenton *et al* summarized that Her-2 positive subtype (Her2+) is characterized with ER negative, lower expression of ER related genes and overexpression of Her-2, and this subtype has higher mutation of TP53, higher grade than luminal subtypes, and poor differentiation [59]. The Her2+ subtype of breast cancers shows a poor prognosis which may be from higher risk of early recurrence [56]. Anti-Her-2 monoclonal antibody trastuzumab treatment combined with chemotherapy can be applied to control the metastatic breast cancer and to reduce the relapse in Her2+ subtype tumors, although some Her2+ tumors are resistant to trastuzumab treatment [59,61]. Recently, Alexe *et al* pointed that clustering largely based on estrogen pathway genes may misclassify Her2+ samples because they are divided into two groups, Her2+/ER- and Her2+/ER+, which are classified into Her2+ cluster and non-luminal A/ER+ cluster, respectively [62]. As an alternative approach, they first divided the tumor samples into Her2+ and Her2- and found two distinct Her2+ subtypes, showing different profiles in distant metastasis and recurrence rates [62].

A.3. Basal-like subtype

The basal-like subtype is characterized by ER negativity, lower expression of ER related genes and Her-2, high expression of basal stratified epithelial cytokeratins 5, 6,

and 17, expression of proliferation-related genes, as well as low expression of BRCA1 [59]. Basal-like tumors carry a poor prognosis, have frequent mutations in *TP53* and a higher grade than luminal breast cancers [56]. Moreover, patients having inherited BRCA1 mutations generally develop basal-like breast cancer [56,59]. In the Carolina Breast Cancer Study, basal-like tumors represented 20% of tumors, but more interestingly, basal-like subtype showed different incidence rates by race and age [63]. Premenopausal African American women developed more basal-like tumor (39%), compared to postmenopausal African American women (14%) and non-African American women (16%) [63]. In addition, tissue microarray analysis found that basal-like tumors commonly developed in younger patients of any ethnicity although the reason for this has not been determined [56,63].

III. VITAMIN D AND BREAST CANCER

Accumulating epidemiological, preclinical, and clinical studies have demonstrated that vitamin D and/or the metabolites exert anti-tumor activity by inhibiting cell proliferation, inducing apoptosis and cell differentiation, and inhibiting cell invasion, angiogenesis and metastasis [24,64,65]. However, in clinical trials, 20-30% of patients who were given a dose of 1.5-2.0 μg/day of $1\alpha,25(OH)_2D_3$ developed hypercalcemia, a potentially life threatening situation [66]. For this reason, different vitamin D analogs have been synthesized to overcome the hypercalcemic toxicity and to enhance the anti-tumorigenic activity.

A. Epidemiological evidence

Numerous epidemiological studies of the associations between vitamin D, ultraviolet irradiation and latitude and the risk of breast cancer have reported that

sufficient vitamin D status is associated with lower risk of cancer [64] (Summarized in Table 1).

A.1. Ultraviolet irradiation

Ultraviolet light absorption by sulfate pollutants (acid haze) exerted statistically significant positive relationship to age-adjusted mortality rates for breast cancer in Canadian cities [67]. Total average annual sunlight energy in US and USSR showed significantly negative association with age-adjusted breast cancer mortality rates [68,69]. In addition, residential exposure to solar UVB was inversely correlated with female breast cancer in incidence and mortality [70,71]. Based on the inverse correlation of vitamin D synthesis and serum level of $25(OH)D_3$ with latitude and the positive correlation with sunlight exposure, it has been hypothesized that the reduction of cancer risk by solar energy can be mediated through the increased serum level of vitamin D and/or vitamin D metabolites [64].

A.2. Serum level of vitamin D metabolite

Caucasian women having low level of $1\alpha,25(OH)_2D_3$ were reported to have a 5 times higher risk of breast cancer than matched controls [72]. Serum levels of $1\alpha,25(OH)_2D_3$ were also negatively correlated with the progression of breast cancer to bone metastases [73]. Individuals with a higher serum level of 25(OH)D (> 52 ng/ml), which is the major circulating metabolite of vitamin D and the marker for determination of a patient's vitamin D status [12], showed a 50% decrease of breast cancer risk compared to those with low levels of 25(OH)D (< 13 ng/ml) [74].

B. Anti-tumor activity of $1\alpha,25(OH)_2D_3$ and its analogs

$1\alpha,25(OH)_2D_3$ and its analogs have been investigated for their activity in cancer-chemoprevention and/or cancer therapy, and those effects have been demonstrated to be mediated through vitamin D receptor (VDR) to regulate cell growth, apoptosis, angiogenesis, invasion and metastasis (reviewed in reference [24]).

B.1. Inhibition of cell proliferation

Mechanisms of the anti-proliferative effect of $1\alpha,25(OH)_2D_3$ and its analogs on breast cancer include cell-cycle arrest by regulating cyclins, cyclin-dependent kinase (CDK), and CDK inhibitors (Summarized in Table 2). $1\alpha,25(OH)_2D_3$ and its analogs were reported to down-regulate cyclin D1/CDKs and up-regulate the cyclin-dependent kinase inhibitors p21 and p27, which leads to accumulation of the cells in the G1 phase in both estrogen receptor (ER)-positive and ER-negative breast cancer cells [75-80]. Although the gene *CDKN1A* encoding p21 is known to contain a functional VDRE in the promoter region, the cell cycle perturbation by vitamin D and its analogs can also be derived indirectly from the cross-talk with other signaling pathways including the epidermal growth factor (EGF) [81,82], the transforming growth factor-β (TGF-β) [83-85] and the MAPK-ERK1/2 or MAPK-p38 signaling pathway [85-87]. A vitamin D analog, $1\alpha(OH)D_5$, was also reported to inhibit cell proliferation by inducing cell differentiation via VDR [88,89]. In combination studies, $1\alpha,25(OH)_2D_3$ and its analogs enhanced the sensitivity to the treatment of adriamycin [90], melatonin [91], and the radiation [92].

B.2. Induction of apoptosis

Many studies have demonstrated that $1\alpha,25(OH)_2D_3$ and its analogs induced apoptosis by regulating the mediators including anti-apoptotic BCL-2, pro-apoptotic Bax in breast cancer cells (Table 2). In MCF-7 human breast cancer cells, $1\alpha,25(OH)_2D_3$ and

its analogs have been reported to induce apoptosis by inhibiting BCL-2 expression, translocating Bax, and inducing cytochrome c release [93-95] via the caspase-independent pathway [94,95]. In addition, mechanistic studies have suggested that $1\alpha,25(OH)_2D_3$ and its analogs down-regulate estrogen receptor [96], activate protein kinase C (PKC) [97], and regulate TGF-β and p38 MAPK signaling to induce apoptosis [85]. Furthermore, co-treatment of $1\alpha,25(OH)_2D_3$ or its analogs with other agents, such as tamoxifen [98], retinoids [99], adriamycin [90], and radiation [100,101] enhanced the induction of apoptosis (Table 2).

B.3. Effects on cell invasion, angiogenesis and metastasis

Studies of the role of $1\alpha,25(OH)_2D_3$ and its analogs on cell invasion have shown that $1\alpha,25(OH)_2D_3$ and its analogs inhibit cell invasion by decreasing invasion-related serine protease and metalloproteinase, MMP-9 [79,102,103]. Moreover, Mantell *et al* found that $1\alpha,25(OH)_2D_3$ inhibited the vascular endothelial growth factor (VEGF)-induced endothelial cell sprouting and elongation *in vitro* and also demonstrated *in vivo* suppression of vascularization in tumors [104]. In addition, there was significant decrease of tumor cell-induced angiogenesis by co-treatment of cells with $1\alpha,25(OH)_2D_3$ and retinoids [105]. The less calcemic vitamin D analog EB1089 was reported to suppress bone metastases by decreasing the number of bone metastases, surface area of osteolytic lesions, and tumor burden per animal after intracardiac injection of MDA-MB-231 human breast cancer cells [106].

C. Preclinical animal studies with $1\alpha,25(OH)_2D_3$ and its analogs

Mammary tumorigenesis is known to be a complicated and heterogenic process including diverse histopathologies, genomic variability and different clinical outcomes,

which indicates that a single animal model can not mimic all features of human breast carcinogenesis [53]. Therefore, different animal models have been tested to demonstrate the anti-tumor activity of $1\alpha,25(OH)_2D_3$ or its analogs (Table 3).

C.1. Carcinogen-induced breast cancer models

The effects of vitamin D analogs on tumor growth in chemical-induced mammary carcinogenesis model have been investigated that low level of calcium and vitamin D enhanced mammary tumorigenesis promoted by a high fat diet following initiation with 7,12-dimethylbenz(a)anthracene (DMBA) [107]. One of the most widely studied vitamin D analogs, EB1089, showed inhibition of tumor growth induced by N-methyl-n-nitrosourea (NMU) [108]. $1\alpha(OH)D_5$ also exerted inhibition of both DMBA-induced preneoplastic lesions and NMU-induced tumor incidence [109,110], and selectively protected against promotion or progression of mammary tumorigenesis [111].

C.2. Xenograft breast cancer models

In the ER-positive MCF-7 human breast cancer xenograft model, EB1089 treatment decreased the tumor volume and induced DNA fragmentation [112]. In addition, co-treatment of EB1089 with paclitaxel [113], all-trans-retinoic acid (ATRA) [114], and ionizing radiation [115] enhanced tumor growth inhibition and apoptosis. 22-Oxa-calcitriol alone and co-treatment with tamoxifen also suppressed the tumor growth of ER positive MCF-7 and ER-negative MX-1 xenografts [116]. Interestingly, $1\alpha(OH)D_5$-Her2 antibody conjugate (IMC) was reported to specifically bind to Her-2 overexpressing cells, and *in vivo* treatment of BT-474 cell xenografts in athymic mice with IMC significantly inhibited tumor growth, demonstrating the targeted delivery of $1\alpha(OH)D_5$ to

Her-2 positive breast cancer cells by immunoconjugation to the cell surface receptor antibody [117].

C.3. Genetically engineered mouse models

In a transgenic model of luteinizing hormone (LH) overexpressing mice, EB1089 was demonstrated to decrease the growth rate of hormone-induced mammary tumors [118].

D. Clinical trials with $1\alpha,25(OH)_2D_3$ and its analogs in breast cancer

The clinical trials of vitamin D and its analogs in breast cancer are summarized in Table 4. Gulliford *et al* conducted a phase I study of EB1089 to determine the calcemic effect in patients with advanced breast and colorectal cancer, and they found that EB1089 was less calcemic than $1\alpha,25(OH)_2D_3$ and approximately $7\mu g/m^2$ was tolerable for most patients [119]. The Women's Health Initiative (WHI) study group hypothesized that vitamin D (400 IU daily) and calcium (1000 mg daily) supplementation would lower the risk of breast cancer [120]. The association of calcium and vitamin D intake with breast cancer has not been reported yet [121]. A phase I/II clinical study in patients with metastatic breast cancer is planned at University of Illinois at Chicago to determine the safety and anti-cancer activity of a vitamin D analog, $1\alpha(OH)D_5$ [65].

IV. CROSS-TALK OF VITAMIN D SIGNALING WITH OTHER PATHWAYS

In addition to the gene regulation by vitamin D involved in calcium/phosphate homeostasis, $1\alpha,25(OH)_2D_3$ and its analogs have been demonstrated to activate or repress the transcription of different genes in normal and tumor tissues [122-128].

However, among those genes regulated by $1\alpha,25(OH)_2D_3$ and its analogs, especially the genes involved in carcinogenesis such as proto-oncogenes or tumor-suppressing genes, many do not have VDREs in their promoter regions (reviewed in Ref. [24]). This suggests that indirect modulation via cross-talk with other signaling pathways may be involved to derive different cellular responses in cell proliferation, differentiation and apoptosis [24,50].

A. The transforming growth factor-β (TGF-β) superfamily

TGF-βs are known to inhibit tumor formation at an early stage of carcinogenesis by suppressing cell proliferation and inducing apoptosis and differentiation. However, TGF-βs can promote tumorigenesis at later stage through loss of antiproliferative activity of TGF-βs, overexpression of TGF-βs in the microenvironment, induction of eptheilial-mesenchymal transitions (EMTs) and enhancement of invasion and metastasis [129]. The TGF-β superfamily, including TGF-βs, activins and bone morphogenetic proteins (BMPs), are multifunctional cytokines that affect inflammation, immune response, cell proliferation, differentiation and apoptosis [130]. TGF-β/activin ligands regulate their biological cell responses through binding to two types of serine/threonine kinase receptors. At first, the type II receptor kinase binds with TGF-β ligands and phosphorylates and activates the type I receptor [131]. The activated type I receptor triggers phosphorylation of the receptor-mediated Smads (R-Smads), Smad2 and Smad3 at the C-terminal of the MH2 domain. The phosphorylated R-Smads recruit the common Smad, Smad4, and translocate to the nucleus to mediate the transcription of the target genes [130]. The regulation of the downstream signaling by BMP ligands is similar to TGF-β/activin ligands except that BMPs have a higher affinity with BMP type I receptors and different R-Smads (Smad1, Samd5 and Smad8) are involved [132]. TGF-

β has also been demonstrated to activate the BMP-Smads in human keratinocytes [133], endothelial cells [134], and mammary carcinoma cells [135]. There is another group of Smad, inhibitory Smads including Smad6 and Smad7, which bind to type I receptor to inhibit phosphorylation of R-Smads [136]. An overall TGF-β signaling pathway is described in Fig. 4.

The nuclear receptor ligands, such as retinoic acid or vitamin D analogs, have been shown to induce the synthesis of ligands and receptors for TGF-βs and BMPs in different types of cells including epithelial and leukemia cells [83-85,91,137,138]. In addition, Mehta *et al* reported that the vitamin D analog, $1\alpha(OH)D_5$ significantly induced the expression of TGF-β1 and VDR in normal mouse mammary glands [109]. Among the studies with TGF-β/BMP signaling and nuclear receptors, $1\alpha,25(OH)_2D_3$ has been shown to induce an interaction between the intracellular Smad3 and the vitamin D receptor (VDR) in the nucleus, and to potentiate VDR-dependent transcription, suggesting that Smad3 may mediate cross-talk between the vitamin D and TGF-β signaling pathways, acting as a coactivator [139,140].

B. Protein kinase C (PKC)

The protein kinase C (PKC) family of serine/threonine kinases regulates cell growth, apoptosis, differentiation, cell migration and carcinogenesis in different types of cells [141,142]. The members of the PKC isozymes are mainly classified into three groups: classical (calcium and diacylglycerol dependent kinase; α, βI, βII, and γ), novel (calcium insensitive and diacylglycerol dependent kinase; δ, ε, μ, and θ) and atypical (calcium and diacylglycerol-insensitive kinase; η and λ/ι) PKC isozymes [141]. PKCs were originally thought to be pro-mitogenic kinases, but this effect may be PKC isoform-dependent and cell-type dependent, as many PKCs can also inhibit cell cycle

progression [142]. Among many different PKC isoforms, PKCα is known to inhibit cell proliferation via p21 induction and suppress tumor formation *in vivo* [143,144].

PKC has been shown to be regulated by 1α,25(OH)$_2$D$_3$ and several vitamin D analogs [145-147]. Boyan *et al* suggest that a caveolar environment may play an important role in mediating the PKC activation by 1α,25(OH)$_2$D$_3$ [146,147]. Studies investigating the effects of 1α,25(OH)$_2$D$_3$ and vitamin D analogs on the PKC family have demonstrated that regulation of the mitogen-activated protein kinase (MAPK) pathway by 1α,25(OH)$_2$D$_3$ and vitamin D analogs was mediated by the activation of RAS/RAF and/or PKC/RAF in muscle cells and myeloid leukemic cells [145,148]. Furthermore, Chen *et al* reported that 1α,25(OH)$_2$D$_3$ induced differentiation in Caco-2 human colon cancer cells through the PKCα/JNK and the activator protein-1 (AP-1) transcriptional activation [149].

C. Insulin-like growth factor binding proteins (IGFBPs)

Insulin-like growth factor binding proteins (IGFBPs), a group of six different proteins, sequester free ligands, insulin-like growth factors (IGFs), by binding with high affinity, thereby modulating the IGF signaling pathway, which has mitogenic and prosurvival activities [150]. After the study showing plasma levels of IGF-I were significantly associated with prostate cancer risk [151], the role of IGFBPs, especially IGFBP-3 in cell proliferation and tumor growth has been investigated. For example, induction of IGFBP-3 by androgens led to growth inhibition of LNCaP human prostate cancer cells [152,153], and prostate tumor growth was attenuated in IGFBP-3 overexpressing transgenic mice [154]. In breast cancer, several studies suggest that IGFBP-3 acts as a tumor suppressive factor [155,156] although the association of circulating IGFBP-3 with pre-menopausal breast cancer is still unsettled [157,158].

The expression of IGFBPs has been shown to be up-regulated by the hormonally active metabolite of vitamin D, $1\alpha,25(OH)_2D_3$ and its analogs in different cancer cells including prostate [159,160], colon [122,161] and breast [162]. Recently, a functional vitamin D response element (VDRE) has been identified in the promoter region of IGFBPs [163-165], suggesting that IGFBPs may be primary target genes of $1\alpha,25(OH)_2D_3$ and its analogs.

D. Epidermal growth factor receptor (EGFR)

Epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptors which are associated with cell surface-transmembrane glycoprotein containing tyrosine kinase activity. The EGFR family is composed of four closely related receptors, including EGFR (ErbB1), HER2/c-neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4) [166]. The downstream signaling includes MAPK, Akt and JNK pathways, which regulate cell growth, survival and cell differentiation [167]. Several studies have demonstrated that $1\alpha,25(OH)_2D_3$ and vitamin D analogs suppress cell proliferation by blocking the cell mitogenic signaling at the level of EGFR [24,81,82,168]. Treatment of breast cancer cells with $1\alpha,25(OH)_2D_3$ and vitamin D analogs reduced the specific binding of $[^{125}I]EGF$ by decreasing the number of receptors [81] and inhibited the mRNA synthesis of EGFR [82]. In addition, Tong *et al* reported that $1\alpha,25(OH)_2D_3$ specifically diminished the EGF-stimulation of tumor cell growth by down-regulating the EGFR mRNA and protein in colon cancer cells [168].

E. E- Cadherin/β-Catenin

β-Catenin, one of the intracellular anchoring proteins that bind to the E-cadherin cell adhesion receptor, plays critical roles in epithelial cell homeostasis [169]. Free β-

catenin forms a complex with axin, glycogen synthase kinase (GSK), and adenomatous polyposis coli (APC), which leads to degradation of β-catenin when Wnt signaling is not active. After the Wnt binding to Frizzled (Frz) receptors, β-catenin translocates to the nucleus, complexes with T cell factor (TCF), and regulates the transcription of target oncogenes such as Cyclin D1 and c-Myc, suggesting β-catenin as an oncoprotein [169,170].

Increases of E-cadherin expression by vitamin D analogs contributes to cell growth inhibition in prostate cancer cells [171]. Palmer *et al* demonstrated that $1\alpha,25(OH)_2D_3$ enhanced the differentiation of colon carcinoma cells by inducing the association of VDR with β-catenin, which prevented the formation of β-catenin/TCF complex and inhibited the Wnt-β-catenin signaling pathway [172]. Furthermore, $1\alpha,25(OH)_2D_3$ increased the expression of E-cadherin, rendering the β-catenin localized at the plasma membrane by binding to it [172]. Recently, a novel mechanism of $1\alpha,25(OH)_2D_3$ on Wnt signaling inhibition was reported that $1\alpha,25(OH)_2D_3$ increased both mRNA and protein expression of DICKKOPF-1 (DKK-1), extracellular Wnt inhibitor, which eventually promoted the cell differentiation in human colon cancer cells [173].

F. Other signaling pathways

Hmama *et al* reported that $1\alpha,25(OH)_2D_3$ induced monocytic cell differentiation through the rapid and transient activation of phosphatidylinositol-3-kinase (PI3K) in myeloid cells, suggesting an association between VDR and PI3K signaling [174].

V. GEMINI VITAMIN D ANALOGS

Gemini vitamin D analogs are a novel group of vitamin D analogs with a unique structure of two six-carbon chains with a C-20-normal and a C-20-epi side chain synthesized by Dr. Milan Uskokovic's group at Bioxell, Inc. (Nutley, NJ). (See the structures in Fig. 7)

A. Binding to vitamin D receptor (VDR)/conformational changes

The characteristics of Gemini vitamin D analogs and $1\alpha,25(OH)_2D_3$ in binding to VDR are summarized in Table 5. Compared to $1\alpha,25(OH)_2D_3$, Gemini analogs exert more structural flexibility, different conformational shape of the liganded VDR, more potent transcriptional activity through VDRE, although the binding affinity of Gemini analog to VDR is about 40% of $1\alpha,25(OH)_2D_3$ [175]. Binding of Gemini analogs to VDR expanded the volume of the ligand binding pocket (LBP) by the movements of the amino acids in the pocket, and Ala-303, Leu-309, and His-397 in LBP were confirmed to be critical for Gemini analogs [176]. In molecular dynamics (MD) simulations, the LBP was suggested to be flexible enough to fit the large Gemini analogs in, and one of the side chains (25-OH or 30-OH) in Gemini analogs was found to take the same position as the single side chain (25-OH) of $1\alpha,25(OH)_2D_3$, enabling the Gemini analogs to act as an agonist [176,177]. Recently, Ciesielski *et al* reported that the binding of first side chain of Gemini analogs caused the structural rearrangement by the rotation of the Leu-337, and opened the hydrophobic channels to accommodate the second side chain [178]. The conformational differences between $1\alpha,25(OH)_2D_3$-VDR and Gemini analog-VDR exhibited a different profile of VDR stabilization and transcriptional activity [175,179,180], suggesting that those differences may be from the alternative recruitment of co-factors induced by structural differences of the liganded VDR [179,180].

B. Gemini vitamin D analogs and cancer

Gemini vitamin D analog exerted significantly stronger activity in inhibiting the clonal growth of MCF-7 and HL-60 cells [175], and Gemini-23-yne-26,27-hexafluoro-D_3 suppressed Akt/mTOR signaling including the inhibition of the downstream effectors such as S6 kinase and 4E-BP1 in MCF-7 breast cancer cells, suggesting that Gemini-23-yne-26,27-hexafluoro-D_3 may suppress the clonal growth through the inhibition of the Akt/mTOR signaling pathway [181]. Several studies reported that novel Gemini vitamin D analogs showed prominent activity in preventing colorectal carcinogenesis [182-184]. In an animal study using implantation of mouse colorectal cancer cells (MC-26) in BALB/c mice, BXL-01-0072 (see the structure in Fig. 7) significantly reduced the tumor volume and weight at a 10-fold lower concentration than $1\alpha,25(OH)_2D_3$ without inducing hypercalcemia [182]. In addition, a deuterated Gemini vitamin D analog exerted more potent activity in reducing tumor size and preventing the invasive spread of tumor into the surrounding muscle compared to the $1\alpha,25(OH)_2D_3$ in the same model [183]. Recently, Gemini analogs were reported to inhibit mammary tumor growth in the NMU-induced rat mammary carcinogenesis model [184].

VI. *IN VITRO* AND *IN VIVO* BREAST CANCER MODELS

Studies that demonstrate the efficacy of the pharmaceutical agents using different and appropriate models are crucial before expensive and time-consuming clinical trials. Mammary tumorigenesis is a complicated and heterogenic process which includes diverse histopathologies, genomic variability and different clinical outcomes. This indicates that a single breast cancer model may not be enough to represent all human breast carcinogenesis [53].

A. *In vitro* MCF10 progressive human breast cancer model

The MCF10 progression model of human breast cancer was established by Dr. Fred Miller at Barbara Ann Karmanos Cancer Institute (Detroit, MI). The series of cell lines of the MCF10 model have the same origin of MCF10A, which was immortalized from normal cells originally derived from benign breast tissue from a woman with fibrocystic disease [185,186]. All established MCF10 breast epithelial cell lines were initiated after transfecting *Ha-ras* oncogene into MCF10A (designated as MCF10AT1), and further passaged in mice to select more aggressive and metastatic cell lines [185,186]. The series of MCF10 cell lines, namely MCF10AT1 (early premalignant), MCF10DCIS.com (invasive potential, isolated from MCF10AT1), MCF10CA1h (aggressive malignant, isolated from MCF10AT1) or MCF10CA1a (fully malignant and metastatic, isolated from MCF10AT1) provides a unique model of human breast cancer progression [187-189]. In xenograft assays, MCF10DCIS.com cells formed comedo type of ductal carcinoma in situ (DCIS) [186], and dynamically interacted with stroma during progression from *in situ* to invasive cancer [190]. In addition, Hu *et al* demonstrated that the tumors developed in MCF10DCIS.com xenograft model were closely relevant to human DCIS tumors in gene expression profiles [191]. Therefore, the MCF10 progression model can provide significant features of human breast cancer and be useful to identify the agents with potent activities in cancer chemoprevention during the progression of breast cancer [192].

B. Carcinogen-induced estrogen receptor (ER)-positive breast cancer model

Chemical carcinogen-induced mammary carcinogenesis in rats is a conventional model for the investigation of estrogen receptor (ER)-positive breast cancer and/or the determination of the effects of the pharmaceutical agents on ER-positive breast cancer

prevention. The most widely used carcinogens are N-methy-N-nitrosourea (NMU) and 7,12-dimethylbenz(a)anthracene (DMBA) [53]. The mammary tumors developed in these models are known to be hormone dependent adenocarcinoma [193], and the histological similarities between rat mammary tumors and human breast cancers have been reported in comparative studies [53,194]. In addition, Chan *et al* found that NMU-induced rat mammary tumors showed similar gene expression profiles to ER-positive, low to intermediate grade of human breast cancer [195]. As selective estrogen receptor modulators, tamoxifen and arzoxifene, have shown inhibitory effects in the NMU-induced mammary tumorigenesis model [196], it is considered as a useful model to evaluate the efficacy of the chemopreventive agents during the hormone dependent mammary tumorigenesis.

C. Xenograft model with human breast cancer cells

Xenograft models provide the *in vivo* environment for human originated cell lines [53,197]. This model has been used to understand the cancer biology through the studies that include the genetic alteration for tumor growth and the role of the microenvironment during the progression [53,197]. It is also used for determining tumor growth-inhibiting activity of pharmaceutical drugs [53,197]. This model can be useful for the investigation of human breast cancer because the orthotopic mammary fat pad injection can supply the similar tumor-stromal environment, although the stroma of the mouse mammary is different from that of human and there are limitations in reproducing the real complicated interaction between the epithelial and stromal cells [53,198]. Interestingly, however, Hu *et al* demonstrated that the tumors derived from subcutaneous injection of MCF10DCIS.com cells were closer to human high-grade comedo ductal carcinoma *in situ* (DCIS) than that from mammary fat pad injection [191], suggesting that local microenvironment is critical in tumor progression.

D. MMTV-Her2/neu transgenic mouse model

In about 30% of breast cancer, there is overexpression and amplification of the human epidermal growth factor receptor 2 (Her2)/neu, which is a transmembrane protein and a member of epidermal growth factor receptor family [199]. Genomic profile of Her2/neu positive tumors were reported to be different from the genes usually expressed in ER positive tumors [57]. In addition, Her2/neu is used as one of the molecular markers for predicting the breast cancer treatment [199]. Due to close association between overexpression and amplification of Her2/neu protooncogene and breast carcinogenesis, a transgenic mouse model overexpressing Her2/neu protein was generated and named mouse mammary tumor virus (MMTV)-Her2/neu model (also known as MMTV-Erbb2 model) [200,201]. The tumors developed in this model are ER negative and hormone-independent, and resemble solid or comedo forms of human ductal carcinoma in situ (DCIS), a specific type of human non-invasive breast cancer [201-203]. Therefore, MMTV-Her2/neu transgenic mouse model has been widely used for cancer prevention studies [201]. Interestingly, VDR is highly expressed in mammary tumors of MMTV-Her2/neu mice, and the mice heterozygous for VDR (neu/VDR$^{+/-}$) accelerated mammary tumor formation [204]. In addition, $1\alpha,25(OH)_2D_3$ was shown to reduce tyrosine phosphorylation of Her2, which led to growth inhibition of LNCaP-derived human prostate cancer cells [160]. These studies suggest that vitamin D signaling may play critical roles in mammary tumorigenesis in MMTV-Her2/neu model.

Numerous vitamin D analogs have been synthesized to overcome the toxicity and enhance the efficacy for the prevention and inhibition of cancers. However, EB1089, one of the most potent vitamin D analog, failed to show anti-tumor activity in clinical studies [119,205,206]. Therefore, vitamin D analogs having better efficacy and less

toxicity are still needed. The novel Gemini vitamin D analogs with a unique structure of two six-carbon chains have shown promising activity in inhibiting colon carcinogenesis without inducing hypercalcemia. However, Gemini vitamin D analogs have not been studied in breast cancer.

Here, we have investigated the effects of novel Gemini vitamin D analogs on suppressing mammary tumorigenesis and the mechanism of action in breast cancer progression *in vitro* and *in vivo*. The potency of the novel Gemini vitamin D analogs on a series of MCF10 breast epithelial cells in growth Inhibition was determined, and the changes of gene expression profiles by Gemini vitamin D analog Ro3582 was also studied. In a mechanistic study, the effect of a Gemini vitamin D analog Ro3582 on activating the BMP/Smad signaling pathway and the upstream signaling pathway were investigated in MCF10 human breast epithelial cells. In addition, to determine the *in vivo* efficacy of Gemini vitamin D analogs, we utilized three different mammary cancer models, 1) N-methtyl-N-nitrosourea (NMU)-induced breast cancer model in Sprague-Dawley rats, 2) MCF10DCIS.com xenograft model in immunodeficient mice, and 3) MMTV-Her2/neu transgenic mouse model.

MATERIALS AND METHODS

A. Reagents

The natural active metabolite, $1\alpha,25$-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$), classic vitamin D analog, Ro-26-2198 and novel Gemini vitamin D analogs including Ro-438-3582, Ro-438-3586, BXL-01-0072, BXL-01-0097, BXL-01-0088, and BXL-01-0084 (> 95% purity) were synthesized and provided by Dr. Milan Uskokovic at BioXell Inc. (Nutley, NJ). $1\alpha,25(OH)_2D_3$ and vitamin D analogs were dissolved in DMSO. For in vivo animal experiments, they were diluted in cremophore/PBS (1:8, v/v). Fugene6 was obtained from Roche Diagnostics (Indianapolis, IN), Trizol solution from Invitrogen (Carsbad, CA), BMP-2, BMP-6, Noggin, TGF-β1 and monoclonal anti-TGF-β1, β2, β3 (clone 1D11) from R & D systems (Minneapolis, MN), okadaic acid, TGF-β receptor I kinase inhibitor (SB431542) and PI3K inhibitor (LY294002•HCl) from Sigma (St. Louis, MO), PKC inhibitors (Go6976, Go6983 and PKCβ C2-4 inhibitor), MEK inhibitors (PD98059 and U0126), p38 inhibitor (SB203580), JNK inhibitor (SP600125), PKA inhibitor (H-89) and the AKT inhibitor (a phosphatidylinositol ether analog, 1L6-Hydroxymethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecyl-sn–glycerocarbonate) from Calbiochem (La Jolla, CA), Ras farnesyltransferase inhibitor (L-744832) from Biomol (Plymouth Meeting, PA). The powder-based chemicals were dissolved in appropriate solvent based on the recommendation from the company.

B. Cell culture

A series of human breast epithelial cell lines were developed and provided by Dr. Fred Miller's group at the Karmanos Cancer Institute (Detroit, MI). The cells (MCF10DCIS.com and MCF10CA1a) were maintained in DMEM/F12 medium

supplemented with 5% horse serum, 1% penicillin/streptomycin and 1% HEPES solution at 37°C, 5% CO_2. The culture medium for MCF10A and MCF10AT1 cells was the same as the medium for the above cells except it was supplemented with 10 µg/ml of insulin, 20 ng/ml of EGF, 0.5 µg/ml of hydrocortisone, and 100 ng/ml of cholera toxin. The cells were passed every 3-4 days.

C. [3H]Thymidine uptake assay

The cells (5,000 ~10,000 cells/well in 24-well plate) were incubated with compounds in DMEM/F12 medium supplemented with 5% horse serum, 1% penicillin/streptomycin and 1% HEPES solution for 3 days. One µCi of [3H]thymidine was added to each well 3 h before the harvest. The cells were washed with PBS and precipitated with 10% trichloroacetic acid for 10 min. The cells were solubilized with the NaOH solution containing the salmon sperm DNA and the radioactivity of [3H] thymidine incorporated to the cells was analyzed with a liquid scintillation counter (Beckman Coulter, Fullerton, CA).

D. Microarray analysis

MCF10 human breast epithelial cells (1×10^6 cells/100 mm dish) were treated with compounds for 4 and 12 hr. Total RNA was isolated using the Trizol according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). cDNA was synthesized with the Superscript Choice kit (Invitrogen, Carlsbad, CA). Biotin-labeled cRNA was synthesized by *in vitro* transcription. The cRNA was then fragmented and hybridized to a human HG-133A_2 chip (Affymetrix, Santa Clara, CA), which has 18,000 transcripts representing 14,500 genes and expressed sequence tags. The chips were washed, stained and scanned using an Affymetrix scanner. Scanned output files analyzed with the Affymetrix

Gene Chip Operating Software (ver 1.1.1.052 GCOS). Signal values were determined by a one-step Tukey's biweight algorithm and normalized to a mean value of 150. To determine significant changes between treatment and control groups, ratios were calculated by GCOS, and genes with a signal level of at least 200 and that were at least 2 fold higher than the control (p-value <0.003) were selected.

E. Quantitative RT-PCR analysis

Total RNA was isolated from cultured cells using the Trizol method from Invitrogen (Carlsbad, CA). One μg of total RNA is reverse-transcribed to cDNA using the random primers and Applied Biosystems High Capacity cDNA Archive Kit in a 96-well format Mastercycler Gradient from Eppendorf North America (Westbury, NY). Quantitative PCR was performed using Applied Biosystems Taqman Gene Expression Assay reagents on an ABI Prism 7000 Sequence Detection System (Foster City, CA). The thermal conditions were: 1 cycle of $50^{o}C$ for 2 min, 1 cycle of $95^{o}C$ for 10 min, 40 cycles of $95^{o}C$ for 15 second and $60^{o}C$ for 1 min. Labeled primers were obtained from Applied Biosystems (Foster City, CA) and GAPDH was used as an internal control. The relative changes in gene expression were calculated by the following formula: Fold change = $2^{(-\Delta\Delta Ct)} = 2^{-[\Delta Ct \text{ (treated samples)} - \Delta Ct \text{ (vehicle control)}]}$, where, $\Delta Ct = Ct$ (detected gene) - Ct (GAPDH) and Ct = threshold cycle number.

F. Western blot analysis

The cells were plated and starved for 24 hr in serum free DMEM/F12 medium. Cells were then incubated with compounds in 0.1% bovine serum albumin (BSA)/DMEM/F12 medium for 24 hr. The proteins were extracted by cell lysis with RIPA buffer (10 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium

deoxycholate, 0.1% sodium dodecyl sulfate, 0.1mM Na_3VO_4, 1% PMSF, 1% aprotinin and 0.1% leupeptin). After determining the protein concentration, the same amount of protein was run in 4-15% gradient gel (Biorad, Hercules, CA) and transferred to the PVDF membrane (PALL, Ann Arbor, MI). The primary and secondary antibodies used in this study were summarized in Table 6.

G. Plasmids and transient transfection assay

For Luciferase assay pCMV-β-gal was provided by Dr. David Mangelsdorf (University of Texas Southwestern Medical School at Dallas, Texas) and 3GC2-Lux was from Dr. Kohei Miyazono (The University of Tokyo, Japan). For transient transfection assays, cells were plated and transfected with DNA vectors 3GC2-Lux or pCMV-β-gal. Cells were transfected using Fugene6 in serum-free medium for 6 hr, and then replaced with fresh medium (0.1% BSA/DMEM) with test compounds. Twenty-four hr later, cells were washed with PBS and lysed with 100 µl of 1X reporter lysis buffer (Promega Corp., Madison, WI). Luciferase values were analyzed using Veritas Microplate Luminometer (Turner Biosystems, CA) and normalized for β-galactosidase activity.

For the overexpression of PKC isoforms The vectors containing PKCα, δ, ϵ or ζ isoforms linked to HA were kindly provided by Drs. Bernard Weinstein and Jae-Won Soh [207], and we sub-cloned the fusion protein of GFP-PKCα using pEGFP-C1 vector (BD Bioscience clontech, Palo Alto, CA). For the transient transfection of PKCα, DNA was incubated with jetPEI transfecting agent (Poly-plus transfection, San Marcos, CA) in 150 mM NaCl for 20 min, and this was directly added to the cells in MCF10AT1 culture medium. After 24 hr, the cells were starved in serum free DMEM/F12 overnight and then treated with experimental test compounds in 0.1% BSA/DMEM/F12.

For the transient transfection of siRNA against PKCα and VDR The siRNAs against PKCα and VDR were purchased from Thermo Scientific (Chicago, IL). MCF10AT1 cells (1×10^5 cells/well in 6-well plate) were plated and incubated overnight. Twenty five μl of Accell Non-targeting siRNA and Accell siRNAs against PKCα and VDR (100 μM stock in siRNA buffer) was mixed with 2 ml of Accell siRNA delivery media and treated into the cells. After 3 days, Gemini vitamin D analog Ro3582 (10 nM) was treated in 0.1%BSA/DMEM/F12 media for 24 hrs, and protein was harvested for the Western blotting.

H. Immunoassay for BMP-2 and BMP-6

MCF10AT1 cells (1×10^6 cells) were plated in 100 mm dish and starved for 24 hr in serum-free DMEM/F-12 medium. Then, cells were incubated with the vitamin D analog Ro3582 (1 and 10 nM) in 0.1% BSA/DMEM/F-12 medium for 48 hr. The supernatant was collected and stored as aliquots at -20°C. The supernatant was concentrated by using Amicon Bioseparations Centricon YM-10 from Millipore Corporation (Bedford, MA) for 90 min. The BMP-2 and BMP-6 proteins secreted into cell culture supernates were detected by Quantikine® BMP-2 immunoassay and DuoSet® Human BMP-6 from R & D systems (Minneapolis, MN), according to the manufacturer's instruction.

I. Phosphatase Assay

MCF10AT1 cells were grown in growth medium, collected, centrifuged, and homogenized in cell extraction buffer (50 mM Tris-HCl, pH 7.0, 0.1% (v/v) β-mercaptoethanol, 0.1 mM EDTA, 0.1 mM EGTA, 25 μg/ml leupeptin, and 25 mg/ml aprotinin) on ice for 30 seconds. The homogenized lysate was centrifuged at 100,000 x

g at 4°C for 1 hr, and endogenous inorganic phosphate from the supernatant was removed using a Sephadex G-25 resin column. Test compounds were then added to the cell lysate, incubated for 10 min at 30°C, mixed with molybdate dye/additive solution, and inorganic phosphate was determined by measuring the absorbance at 595 nm.

J. Fluorescence microscopy

Plates were coated with poly-D-lysine (0.1 mg/ml) overnight at 37°C. After cells were plated and incubated with compounds, cells were fixed in 4% paraformaldehyde (1x PBS, pH 7.4) at room temperature. Cells were then washed and blocked with 10% bovine serum albumin/0.5% Triton-X/1x PBS solution. The primary antibody solution (1:100 dilution for phospho-Smad1/5 and PKCα) was added to the plates, and the cells were incubated at 4°C overnight. Fluorophore-conjugated secondary antibody (1:200 dilution, Alexa Fluor® 488 goal anti-rabbit IgG, Molecular Probes, OR) against the species of the primary antibody was added and incubated after washing. The cells were irradiated with a green laser (488 nm). For DAPI staining, plain UV light (364 nm) was used.

K. Animal experiment of NMU-induced mammary tumorigenesis in rats

Female Sprague-Dawley rats (21 ± 1 days old) were purchased from Taconic Farms (Germantown, NY). Rats were treated at 23 ± 1 days of age with a single intraperitoneal injection of the carcinogen N-methyl-N-nitrosourea (NMU, 50 mg/kg body weight). One week after NMU injection, rats were treated with DMSO (0.1 %, control), 1α,25(OH)$_2$D$_3$, or Gemini vitamin D analogs via intraperitoneal injection daily except weekends. The rat weight and tumor size of each animal were measured weekly. Nine weeks after NMU injection, rats were sacrificed and tumors were weighed at autopsy.

Tumor and blood samples were collected for further analysis. Serum was collected after centrifugation of blood samples. All animal studies were performed in accordance with an institutionally approved protocol.

L. Immunohistochemistry

Tumor samples from each group were harvested at autopsy and fixed in 10% formalin for 24 hr. They were sectioned into 8 to 10 segments, paraffin embedded, and microtomed into 4 μm thick tissue sections. In H&E staining, the slides were stained with Harris Hematoxylin solution and Eosin Y solution (Sigma, St. Louis, MO). To evaluate the expression level in tumor tissues, the slides were incubated overnight at room temperature with antibody to proliferating cell nuclear antigen (PCNA) (1:1000 diluted, BD Pharmingen, San Diego, CA), or caspase-3 (1:200 diluted, Cell Signaling Technology, Danvers, MA). The slides were incubated with biotinylated secondary antibody, and then with avidin/biotinylated peroxidase complex for 30 min at room temperature (Vector Labs, Burlingame, CA), and were then incubated with 3'-diaminobenzamine (DAB) substrate. The sections were then counterstained with Modified Harris Hematoxylin. The images were taken randomly at 400X using Zeiss AxioCam HRc camera fitted to a Zeiss Axioskope 2 Plus microscope.

M. Determination of serum calcium level

The concentration of calcium in serum samples was determined using the calcium reagent set (POINTE Scientific, INC. Canton, MI) and we followed the procedure provided by the manufacturer. Briefly, serum (5 μl) was mixed with the appropriately diluted reagent (200 μl) in 96 well-plate and after incubating for 1 min, the plate was read at 550 nm using a spectrophotometer. To adjust for differences in hemolysis among

samples, serum blanks (serum in water) were prepared and the absorbance reading was subtracted from the test reading. The calcium concentration was calculated from calcium standards provided by the manufacturer.

N. Animal experiment in xenograft model

MCF10DCIS.com cells were trypsinized and prepared in Hanks' Balanced Salt Solution (Invitrogen, Carlsbad, CA). After the midline incision around second teat in immonodeficient nu/nu mice or severe combined immunodeficiency (SCID) mice, 1×10^6 cells were injected in the mammary fat pad. The incision was closed by wound clips and the clips were removed after 4 days. DMSO (control) and Gemini vitamin D analog 0097 were injected intraperitoneally daily from Day 4 until the termination of the experiment. Body weight and tumor size were measured every week. Six weeks after cell injection, tumors were weighed and collected at autopsy for further analysis. All animal studies were performed in accordance with an institutionally approved protocol.

O. Animal experiment in MMTV-Her2/neu model

MMTV-Her-2/neu mice were received at 6-7 weeks old from Jackson Laboratory (Bar Harbor, ME). When they were 20 weeks old, vehicle (DMSO, n=16) and Gemini vitamin D analog BXL0124 (0.3 μg/kg body weight, n=8) were given via intraperitoneal injection three times a week. Body weight and tumor size were measured once a week. The mice were sacrificed and tumors were weighed at autopsy. Tumor and blood samples were collected for further analysis. Serum was collected after centrifugation of blood samples. All animal studies were performed in accordance with an institutionally approved protocol.

P. Analysis of tumor tissues

Tumor samples were homogenized in RIPA buffer (10 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.1mM Na_3VO_4, 1% PMSF, 1% aprotinin and 0.1% leupeptin) using a Dounce homogenizer (Wheaton, Millville, NJ) to extract proteins, and then we followed the regular Western Blot procedure as mentioned above. Total RNA was isolated from the tumors or kidneys by homogenizing in the Trizol solution from Invitrogen (Carlsbad, CA), extracting RNA using chloroform, and precipitating using isopropyl alcohol. One μg of total RNA was reverse-transcribed to cDNA and quantitative PCR was performed as mentioned above.

Q. Statistical analysis

Statistical significance between control and treatment groups was evaluated using the Student's *t*-test.

PART I: Gene Expression Profiling and Protein Changes Induced by a Novel Gemini Vitamin D Analog during the Progression of Breast Cancer

I.1. INTRODUCTION

The series of MCF10 cell lines developed by Dr. Fred Miller group at Barbara Ann Karmanos Cancer Institute (Detroit, MI) is a tumor progression model of cell lines representing different stages of breast carcinogenesis following *Ha-ras* transfection [192]. Although it is known that Ras mutation is found only in 5% of breast cancer, von Lintig *et al* reported that Ras is highly activated in breast cancers, especially in cancers expressing epidermal growth factor receptor (EGFR) and/or ErbB2 (Her-2) [208]. This finding suggests that Ras play a significant role in breast carcinogenesis overexpressing EGFR and/or Her-2. In this part, we first characterized four cell lines, namely MCF10A, MCF10AT1, MCF10DCIS.com and MCF10CA1a, by determining the basal level of well-known signaling molecules in carcinogenesis such as the MAPK and Akt signaling pathway, as well as the level of Her-2. In addition, the *in vivo* tumor growth rate in *Ha-ras* transfected cell lines was studied using xenograft model in immunodeficient mice. Using microarray analysis, we compared the basal level of mRNA in MCF10AT1 cells (premalignant early stage) to that in MCF10CA1a cells (metastatic later stage) to determine the up-regulated and down-regulated genes during progression of breast cancer in MCF10 model.

Gemini vitamin D analogs with two six-carbon side chains exerted inhibitory effects on colon carcinogenesis without hypercalcemic toxicity [182,183]. The less calcemic effect of Gemini vitamin D analogs have been suggested to be from the conformational differences between liganded VDR with $1\alpha,25(OH)_2D_3$ and Gemini vitamin D analogs as well as different profiles of VDR stabilization and transcription

activity [175,179,180]. However, Gemini vitamin D analogs have not been studied in breast cancer models. Therefore, we first investigated the effects of Gemini vitamin D analogs on growth inhibition, mRNA regulation and protein expression in MCF10AT1 and MCF10CA1a human breast epithelial cells.

I.2. RESULTS

A. Comparison of basal level in MCF10 progressive human breast epithelial cell lines

A.1. Basal level of proteins, Her-2, pErk, pAkt and VDR in MCF10 progressive breast cancer cell lines.

We first compared the basal level of proteins among four different cell lines, MCF10A (immortalized normal cells), MCF10AT1 (early premalignant), MCF10DCIS.com (invasive potential) and MCF10CA1a (aggressive with metastatic potential). As shown in Fig.5, the basal level of phospho-Erk, downstream of Ras-Raf-MAPK signaling involved in cell division in tumor cells [209], phospho-Akt, also known to control cell proliferation and apoptosis [210], and Her-2, frequently overexpressed in human breast cancer, were gradually increased as the cells become more aggressive and malignant.

A.2. Xenograft tumor growth rate in MCF10 progressive breast cancer cell lines in immunodeficient mice.

In vivo tumor growth rate in immunodeficient mice was compared among MCF10AT1, MCF10DCIS.com and MCF10CA1a cells. While MCF10AT1 cells did not form any tumors until 60 days after cell implantation via mammary fat pad injection,

MCF10DCIS.com and MCF10CA1a cells developed tumors within 2 weeks and 1 week after cell injection, respectively (Fig. 6). Tumor growth of MCF10CA1a cells was much faster than that of MCF10DCIS.com cells (Fig. 6). The differences of *in vivo* tumor growth in MCF10 breast epithelial cell lines at different stage of breast cancers may be from different level of MAPK and Akt signaling and Her-2 expression.

A.3. Basal mRNA levels in MCF10AT1 and MCF10CA1a cells.

We used the comparative analysis algorithm provided in Affymetrix GCOS software to compare the basal mRNA level between Ha-ras transfected premalignant MCF10AT1 and aggressive metastatic MCF10CA1a human breast cancer cells. We found that there were at least 200 genes with different basal expression profiles between MCF10AT1 and MCF10CA1a cells (p value < 0.05 by ANOVA test, data not shown). Among them, genes that have a low level of expression in premalignant MCF10AT1 and a higher level in the aggressive MCF10CA1a cell line were: osteonectin, bone marrow stromal cell antigen 2, tumor protein D52, 7-dehydrocholesterol reductase, protein phosphatase 1, lanosterol synthase, cathepsin H, cytochrome p450 51A1, stanniocalcin 1, CD44, RAS guanyl releasing protein 1, ubiquitin-activating enzyme E1C, BMP-7, and tubulin α3. Genes that have a high level of expression in premalignant MCF10AT1 and a lower level in aggressive MCF10CA1a cell line were: p21, tissue inhibitor of metalloproteinase 3 (TIMP-3), SHC transforming protein 1, TGF-β-induced, calcineurin Aα, IGFBP6, IGFBP7, mucin 1, keratins, collagenase 3 (MMP-13), proteasome 26S subunit, interleukin 1α, and HMG-box transcription factor, and metallothioneins (1H, 1X, 1f, 2A).

B. Effect of Gemini vitamin D analogs in MCF10 progressive human breast epithelial cell lines

B.1. Growth inhibitory effect of 1α,25(OH)$_2$D$_3$ and the Gemini vitamin D analogs in MCF10AT1, MCF10DCIS.com and MCF10CA1a cells.

We tested more than 60 different Gemini vitamin D analogs for their ability to inhibit cell proliferation of MCF10 breast epithelial cells including MCF10AT1, MCF10DCIS.com and MCF10CA1a cells. Among the Gemini vitamin D analogs we tested for growth inhibitory activity (see the structures in Fig. 7), Ro3582 showed stronger activity than 1α,25(OH)$_2$D$_3$ or the other analogs in cultured breast epithelial cells, when determined by the [^{3}H]thymidine incorporation assay (Fig. 8). We selected Ro3582 for studies to determine its ability to regulate gene expression in MCF10AT1 and MCF10CA1a cells.

B.2. Effect of Ro3582 on gene expression in MCF10AT1 and MCF10CA1a cells.

We investigated the ability of Ro3582 to regulate gene expression in MCF10AT1 and MCF10CA1a cells. In our study, both cell lines were treated with Ro3582 (1 nM), and total RNA was obtained after 4 hr and 12 hr and processed for microarray analysis. The genes regulated by Ro3582 were determined with a 2-fold change cut-off value. In MCF10AT1 cells, as shown in Fig. 9, we determined that 100 and 271 genes were up-regulated by Ro3582 (1 nM) at 4 hr and 12 hr, respectively, and 35 and 120 genes were down-regulated by Ro3582 at 4 hr and 12 hr, respectively. In MCF10CA1a cells, we determined that 37 and 115 genes were up-regulated by Ro3582 (1 nM) at 4 hr and 12 hr, respectively and 7 and 41 genes were down-regulated by Ro3582 at 4 hr and 12 hr, respectively. Overall, the Gemini vitamin D analog Ro3582 induced more significant

gene changes in the early premalignant MCF10AT1 cells than in the malignant and metastatic MCF10CA1a cells. In addition, small number of genes was commonly regulated by Ro3582 in both cell lines although they have the same origin, MCF10A cells, indicating that the gene expression profiles by Ro3582 were different between those two cell lines.

B.3. Effect of Ro3582 on gene expression in MCF10AT1 and MCF10CA1a cells as measured by quantitative RT-PCR.

To confirm the gene expression changes by Ro3582 obtained from the microarray study (Tables 7 and 8), we used the same RNA samples and performed quantitative RT-PCR analyses. The results are summarized in Table 9.

B.3.a. Vitamin D target genes

We first confirmed the microarray data with the vitamin D target genes, such as CYP24A1, osteocalcin, and osteopontin in these cells. Vitamin D 24-hydroxylase (CYP24A1) was by far the strongest responsive gene induced by Ro3582 (Tables 7 and 8). Osteocalcin and osteopontin were also significantly induced by Ro3582.

B.3.b. Cell adhesion, invasion, angiogenesis, and metastasis

We found that Ro3582 regulated cell adhesion molecules, such as collagens, laminins, thrombospondin, keratin 16, and zyxin (Tables 7 and 8). Among them, we confirmed that collagen type XVIα1 and laminin β3 were up-regulated, while thrombospondin-1 and laminin β1 were down-regulated by Ro3582 (Table 9). Interestingly, one of the MMPs, MMP-2, which is known to be involved in invasion was down regulated by Ro3582 in both MCF10AT1 and MCF10CA1a cells, while the

expression of MMP-1 was induced in MCF10AT1 cells (Table 9). The expression of an angiogenic marker, connective tissue growth factor (CTGF), was inhibited by Ro3582 in both MCF10AT1 and MCF10CA1a cell lines (Table 9).

B.3.c. Cell growth, apoptosis and signal transduction

IGFBP-3 (Insulin-like growth factor binding protein-3) has been shown to play a key role in the growth arrest induced by $1\alpha,25(OH)_2D_3$ [163]. We found that insulin receptor substrate 1 and IGFBP-3 were both up-regulated by Ro3582 (Table 9). Furthermore, dual specificity phosphatase 10, negatively regulating members of the MAPK superfamily, was highly up-regulated by Ro3582 in MCF10AT1 and MCF10CA1a cells. The gene expression changes of p21, p27 or BCL-2 induced by Ro3582 were less than 2-fold when determined by quantitative RT-PCR (Table 9).

B.3.d. The TGF-β superfamily

In the microarray analysis, Ro3582 induced the expression of BMP-2, BMP-6 and activin A in MCF10AT1 cells, while BMP-2 and BMP-6 were down regulated by Ro3582 in MCF10CA1a cells (Table 7). However, using quantitative RT-PCR, we confirmed that BMP-2, BMP-6 and activin A were up-regulated in both cell lines. Furthermore, Smad6, an inhibitory Smad of BMP signaling, was down-regulated by Ro3582 (Table 9). However, the levels of TGF-β2, TGF-β receptor II, Smad7 and Smurf1 were not changed significantly by Ro3582.

B.3.e. Effect of Ro3582 on protein levels in MCF10 breast epithelial cells

Because the regulation at the mRNA level does not always predict regulation at the protein level, we investigated the effects of Ro3582 on several key proteins by

Western Blot analysis (Fig. 10). First, we determined the induction of the VDR protein expression by Ro3582. Although Ro3582 induced the VDR protein expression in both cell lines, induction of the VDR protein in MCF10AT1 cells was much stronger than in MCF10CA1a. In addition, we analyzed several key proteins that are known to regulate cell growth and apoptosis, such as IGFBP-3, p21, p27, and BCL-2, in both cell lines. We found that the level of IGFBP-3 was increased substantially by Ro3582 in both cell lines. Interestingly, Ro3582 induced the expression of p21 protein in MCF10AT1 cells, while the expression of p21 protein is lost in MCF10CA1a cells. In contrast, MCF10CA1a cells have a much higher basal level of p27 than MCF10AT1 cells, but Ro3582 did not change the protein level of p27 in either cell line. In addition, the level of anti-apoptotic protein, BCL-2, was decreased by Ro3582 in MCF10AT1, but it was not detected in MCF10CA1a cells (Fig. 10). Since Ro3582 regulated the expression of the members of the TGF-β/BMP signaling pathway, we also determined the level of phosphorylation of Smad1/5, which is known to be phosphorylated by active BMP-receptor kinase. The level of phospho-Smad1/5 was strongly increased by the treatment with Ro3582 in MCF10AT1 cells, while it was not changed in MCF10CA1a cells (Fig. 10).

C. Effects of BMPs in MCF10AT1 and MCF10CA1a human breast epithelial cells

C.1. Activation of Smad signaling and inhibition of cell growth by BMP-2 and BMP-6 in premalignant MCF10AT1 cells and aggressive MCF10CA1a cells.

A Gemini vitamin D analog Ro3582 inhibited cell growth in both MCF10AT1 and MCF10CA1a cells, but induced phosphorylation of Smad1/5 only in MCF10AT1 cells. Here, we tested whether BMP/Smad signaling is involved in cell proliferation of those cell lines. As shown in Fig. 11, BMP ligands, BMP-2 and BMP-6, induced phosphorylation of R-Smad (Smad1/5) and sustained for 24 hrs after treatment in

MCF10AT1 cells. However, Smad activation by BMPs in MCF10CA1a cells was relatively much weaker than MCF10AT1 cells and disappeared within 6 hrs. In growth inhibition assay, BMP-2 (100 ng/ml) and BMP-6 (20 ng/ml) started to inhibit cell proliferation after 12 hrs treatment and showed about 60% and 80% inhibition of cell growth in MCF10AT1 cells after 3 days treatment, respectively (Fig. 12). However, BMPs did not show the inhibitory effects on MCF10CA1a cell growth (Fig. 12).

I.3. DISCUSSION

In this part, we first compared the basal level of key proteins playing critical roles in carcinogenesis as well as *in vivo* tumor growth rate in immunodeficient mice using 4 different MCF10 series of breast epithelial cell lines. Her-2, which is overexpressed in about 30% of human breast cancer [53], was highly expressed as the cells became aggressive (Fig.5). In addition, MAPK and Akt signaling involved in regulating cell proliferation and apoptosis were gradually activated as the cells became more malignant (Fig. 5), and it is associated with shorter latency and more rapid tumor growth *in vivo* (Fig. 6). Although we showed high expression of Her-2 protein in MCF10DCIS.com cells, Hu *et al* reported that MCF10DCIS.com xenograft model reflected basal-like features of human breast cancer and suggested strong similarity to human basal-like DCIS [191] among different molecular subtypes of breast cancers [56,57].

We analyzed the differential gene expression profiles among different stages of breast cancer cells, from MCF10AT1 to MCF10CA1a, and found that there were at least 200 genes with different basal expression profiles between MCF10AT1 and MCF10CA1a cells. Among these genes, metallothioneins are known to constitute the majority of intracellular protein thiols and act as cell survival factors. Therefore, lower

levels of metallothioneins in MCF10CA1a cells may be correlated with the higher levels of spontaneous apoptosis observed in these cells (unpublished). In accord with these observations, the level of anti-apoptotic protein BCL-2 was not detectable in MCF10CA1a cells, while MCF10AT1 cells have higher basal level of BCL-2 protein (Fig. 10). Interestingly, when we compared p21 in both cell lines, the expression of p21 was not detectable in MCF10CA1a cells. The opposite was shown with p27 protein expression that there was a much higher level of p27 in MCF10CA1a cells than in MCF10AT1 cells. It is possible that the loss of p21 in aggressive metastatic MCF10CA1a cells may, in part, contribute to its malignant phenotype.

Several studies have shown gene expression profile changes after treatment with $1\alpha,25(OH)_2D_3$ in prostate, colon, ovarian, and breast cancer cells [122-127,162,163,211]. In this part, we studied the effect of a novel Gemini vitamin D analog, Ro3582, on gene expression in breast cancer progression model including MCF10AT1 (early premalignant cell line) and MCF10CA1a (aggressive metastatic cell line). In both breast epithelial cell lines, the most responsive gene identified was human CYP24 (24-hydroxylase). CYP24 is an enzyme involved in the degradation of $1\alpha,25(OH)_2D_3$ to an inactive metabolite by adding a hydroxyl group to the C24 position. This induction was confirmed by quantitative RT-PCR. Other known vitamin D target genes such as osteopontin, osteocalcin and CD14 were also highly induced by Gemini vitamin D analog Ro3582. Both our study with Ro3582 and previously reported studies with $1\alpha,25(OH)_2D_3$ show that many genes related to cell growth and cell adhesion/matrix proteins are regulated by treatment with $1\alpha,25(OH)_2D_3$ or a Gemini analog [122-126,212]. Those genes are insulin-like growth factor binding protein 3 (IGFBP-3), thioredoxin reductase 1, zyxin, kallikrein 6, G-protein-coupled receptor kinase 5, bone morphogenetic protein 6 (BMP-6), and dual specificity phosphatase 10 [122-127,212].

It was previously reported that Smad3, a downstream mediator in the TGF-β signaling pathway, acts as a co-activator for the vitamin D receptor in the nucleus [140]. Here, we also found that Ro3582 strongly induced mRNA level of BMP-2 and BMP-6, which are members of the TGF-β superfamily, and enhanced the level of phospho-Smad1/5 in early premalignant MCF10AT1 cells. However, Ro3582 did not affect phospho-Smad1/5 levels in malignant MCF10CA1a cells. Interestingly, BMP ligands showed different profiles in Smad1/5 activation and cell growth inhibition between MCF10AT1 and MCF10CA1a cells. BMP-2 and BMP-6 strongly induced phosphorylation of Smad1/5 and inhibited cell proliferation in MCF10AT1, while BMPs induced phospho-Smad1/5 weakly and did not inhibit cell growth in MCF10CA1a cells (Figs. 11 and 12), suggesting an association of the activation of BMP/Smad signaling with inhibition of cell proliferation.

Interestingly, the mRNA of v-fos FBJ murine osteosarcoma viral oncogene homolog (also known as c-FOS, AP-1) was more strongly regulated in MCF10CA1a cells than MCF10AT1 cells (Table 7). Because it is suggested that c-FOS is involved in tumor necrosis factor related apoptosis inducing ligand (TRAIL)-induced apoptosis [213], Gemini vitamin D analogs may induce apoptosis more significantly in MCF10CA1a cells. In addition, IGFBP-3, the marker related to the cell proliferation, was upregulated by a Gemini vitamin D analog Ro3582 in MCF10CA1a cells (Fig. 10). These results may explain the finding that Gemini vitamin D analogs showed better growth inhibitory effect in MCF10CA1a cells than MCF10AT1 cells (Fig. 8), although further studies are needed.

In summary, we investigated the effects of novel Gemini vitamin D analogs in a unique human breast cancer progression model. We found that MCF10AT1 cells were more responsive to the Gemini vitamin D analog Ro3582 in inducing gene expression changes than MCF10CA1a cells. In addition, there were distinct differences of gene

expression profile and protein expression between the early premalignant MCF10AT1 and the aggressive/metastatic MCF10CA1a cell lines.

PART II: Novel Vitamin D Analogs Activate Bone Morphogenetic Protein Signaling in MCF10 Breast Epithelial Cells

II.1. INTRODUCTION

BMPs are members of the transforming growth factor-β (TGF-β) superfamily which are now generally considered as multifunctional cytokines that affect inflammation, immune response, cell proliferation, differentiation and apoptosis [130]. Like the TGF-βs, BMPs regulate their biological cell responses through binding to two types of serine/threonine kinase receptors, which trigger phosphorylation of the receptor-mediated Smad (R-Smad) at the C-terminus of the MH2 domain [132]. The activated R-Smads recruit the common Smad, Smad4, and translocate to the nucleus to mediate the transcription of BMP-dependent target genes, primarily during embryonic development and bone formation [132].

Although a potential role of the BMP/Smad pathway in the progression of breast cancer has been reported [214,215], there is an accumulation of data suggesting the possible role of BMP as a tumor suppressor [216-221]. For example, activation of the BMP signaling pathway inhibited the growth of breast cancer cells by inducing cyclin dependent kinase inhibitor, p21 [216,217], and *in vivo* tumor growth of androgen-insensitive prostate carcinoma cells was suppressed by BMP signals [218]. In addition, cancer-associated stromal cells expressed the BMP antagonist, gremlin 1, which can promote tumor cell growth [219]. Mutation or loss of expression of molecules in the BMP signaling pathway led to the enhancement of tumor progression [220,221], indicating that BMP signaling may be important for inhibition of tumorigenesis. Therefore,

modulating BMP signaling during the formation of breast cancer may be important for the prevention and treatment of breast cancer.

As summarized in Introduction, many studies have demonstrated that vitamin D and its analogs may trigger cross-talk with TGF-β/BMP signaling by interaction of these intracellular mediators with the vitamin D receptor, or by inducing the synthesis of ligands and receptors for TGFβs and BMPs in different type of cells [83-85,91,137-140]. In the previous study in Part I, we also found that the Gemini vitamin D analog, Ro3582, increased the mRNA level of BMP-2 and BMP-6, and induced the phosphorylation of Smad1/5 in MCF10AT1 cells. Here, we determined whether Smad activation determined by phosphorylation of Smad1/5 is BMP-specific, and we further investigated the mechanism of BMP/Smad signaling activation by Gemini vitamin D analog Ro3582 in MCF10AT1 human breast epithelial cells.

II.2. RESULTS

A. Activation of BMP/Smad signaling by Gemini vitamin D analogs in MCF10AT1 human breast epithelial cells

A.1. Phosphorylation of Smad1/5 is increased by Gemini vitamin D analogs in MCF10AT1 breast epithelial cells.

In studies described in Part I, we found that the Gemini vitamin D analog Ro3582 induced phosphorylation of Smad1/5 using a single antibody that recognizes pSmad1(Ser463/465), pSmad5(Ser463/465), and pSmad8(Ser426/428), indicating the activation of the BMP signaling pathway. We confirmed that treatment of MCF10AT1 cells with a classic vitamin D analog, Ro2198 and a Gemini vitamin D analog, Ro3582,

caused a strong induction of phospho-Smad1/5 in a dose dependent manner (0.1, 1, and 10 nM), while the naturally occurring active metabolite, $1\alpha,25(OH)_2D_3$, had a small increase at 10 nM (Fig. 13). Furthermore, Ro3582 began to increase phosphorylation of Smad1/5 at 12 hr and the phosphorylation was much stronger at 24 hr (Fig. 13). Other Gemini vitamin D analogs, BXL0097 and BXL0072, showed the same level of induction of Smad1/5 phosphorylation, compared to Ro3582 (Fig.13).

A.2. Upregulation of BMP signaling by the Gemini vitamin D analog Ro3582 is not blocked by TGF-β neutralizing antibody but by a BMP antagonist Noggin.

We used the TGF-β antibody (1D-11) to determine whether the effects of Ro3582 are mediated by an increase in TGF-β synthesis in MCF10AT1 breast epithelial cells. The antibody to phospho-Smad3 used (Cell Signaling Technology Inc.) recognizes both phospho-Smad3 (lower band) and phospho-Smad1 (upper band). The phosphorylation of Smad3 induced by TGF-β_1 (1 ng/ml) was blocked by TGF-β neutralizing antibody (1D11, 50 µg/ml). However, Smad1/5 phosphorylation by the Gemini vitamin D analog Ro3582 (10 nM) or BMP-2 (100 ng/ml) was not blocked by the neutralizing antibody to TGF-β (Fig. 14A). More importantly, we evaluated the effect of a specific antagonist of BMP, Noggin, to determine whether a BMP antagonist blocks the action of Ro3582. As shown in Fig. 14B, the phosphorylation of Smad1/5 induced by Ro3582 (10 nM) or BMP-2 (100 ng/ml) was inhibited by the antagonist, Noggin [+, 60 ng/ml or ++, 300 ng/ml], while the induction of phospho-Smad3 (the lower band) by TGF-β_1 (1 ng/ml) was not blocked by Noggin. These results indicate that induction of R-Smads by the Gemini vitamin D analog Ro3582 is mediated by BMP signaling.

A.3. Nuclear localization of phospho-Smad1/5 is induced by the Gemini vitamin D analog Ro3582 in MCF10 breast epithelial cells.

R-Smads are known to be translocalized to the nucleus after the phosphorylation. Therefore, we identified the cellular localization of phospho-Smad1/5 activated by the Gemini vitamin D analog Ro3582 in MCF10AT1 breast epithelial cells. As shown in Fig. 15, the phosphorylation of Smad1/5 and its localization in the nucleus was markedly increased by treatment with the Gemini vitamin D analog Ro3582 (1 nM) or BMP (100 ng/ml) in MCF10AT1 cells. Furthermore, when cells were treated with the BMP antagonist Noggin, the accumulation of phospho-Smad1/5 in the nucleus induced by Ro3582 or BMP-2 was blocked, confirming that Smad1/5 activation by the Gemini vitamin D analog Ro3582 is BMP mediated. DAPI staining was used to recognize the nuclear morphology in cells.

A.4. The Gemini vitamin D analog Ro3582 enhances BMP transcriptional activation in MCF10AT1 breast epithelial cells.

We determined whether phospho-Smad1/5 induced by the Gemini vitamin D analog Ro3582 can lead to activation of BMP-mediated transcription, using a transfection assay with a GC binding element linked to luciferase. This construct, 3GC2-Lux, contains three repeats of the GC-rich sequence derived from the proximal BMP response element in the Smad6 promoter [222] and is specific for response to BMPs. After transient transfection 3GC2-Lux and pCMV-β-gal, either DMSO or the Gemini vitamin D analog Ro3582 (1, 0.1, or 0.01 nM) was treated for 24 hr in MCF10AT1 cells. The BMP-dependent response to Ro3582 was determined by assaying for luciferase activity. As shown in Fig. 16, the Gemini vitamin D analog Ro3582 increased this activity by 3-fold at 1 nM.

B. Induction of BMPs and inhibition of Smad6 by the Gemini vitamin D analog Ro3582 in MCF10AT1 human breast epithelial cells

B.1. Vitamin D-induced increase in phospho-Smad1/5 is not due to the inhibition of phosphatase activity.

Since it was reported that vitamin D analogs might regulate phosphatase activity [223], we determined whether the increased amount of phospho-Smad1/5 is due to inhibition of phosphatase activity. A serine/threonine phosphatase enzyme inhibitor, okadaic acid, is known to suppress the activity of protein phosphatases, mostly isotype PP2A, which accounts for most of the cellular activity. It has been shown that treatment of HL-60 leukemia cells with okadaic acid increased the level of phospho-Smad2 [224]. In this study, okadaic acid did not significantly increase the phosphorylation of Smad2, Smad3, or Smad1/5 in MCF10AT1 cells, while the phosphorylation of Smad1/5 was induced by the Gemini vitamin D analog Ro3582 (10 nM) or BMP-2 (100 ng/ml) (Fig. 17A). We performed *in vitro* serine/threonine phosphatase enzyme assays to determine whether the Gemini vitamin D analog Ro3582 inhibits phosphatase activity in MCF10AT1 cells. Using MCF10AT1 cell lysate as the source for the enzyme, Ro3582 (10, 100, and 1000 nM) or okadaic acid (OA, 10, 100, and 1000 nM) was treated to the cell lysate, and non-radioactive specific phosphopeptide RRA(pT)VA was used as a peptide substrate for serine/threonine protein phosphatases 2A, 2B and 2C. Okadaic acid inhibited the phosphatase activity dose dependently, whereas the Gemini vitamin D analog Ro3582 did not inhibit the activity even at 1 µM (Fig. 17B).

B.2. The Gemini vitamin D analog Ro3582 not only induces the synthesis of mRNA and protein of both BMP-2 and BMP-6, but also inhibits the expression of Smad6 mRNA.

In Fig. 18, MCF10AT1 cells were treated with the Gemini vitamin D analog Ro3582 (1 nM) for 4 or 12 hr, and BMP-2, BMP-6 and Smad6 mRNA production was measured by quantitative PCR. The Gemini vitamin D analog Ro3582 (1 nM) increased the synthesis of BMP-2 and BMP-6 mRNA, and down-regulated the mRNA of Smad6, an inhibitor of BMP-mediated R-Smads, compared to DMSO control (Fig. 18A). We also determined that the Gemini vitamin D analog Ro3582 induced the synthesis of BMP-2 and BMP-6 protein in MCF10AT1 cells, as shown by immunoassay for BMP-2 and BMP-6 (Fig.18B). Approximately 130 and 360 picogram of BMP-2 protein, and 360 and 400 picogram of BMP-6 protein were synthesized from MCF10AT1 (5×10^5 cells) treated with the Gemini vitamin D analog Ro3582 at 1 and 10 nM for 48 hr, respectively (Fig. 18B). In addition, BMP antagonist, Noggin, significantly inhibited the synthesis of BMP-2 and BMP-6 mRNA induced by Ro3582 (Fig. 19).

II.3. DISCUSSION

BMPs, members of the TGF-β superfamily, have been identified as multifunctional regulators of development, bone formation and tissue remodeling [132,225]. Recently, the role of BMPs in proliferation, differentiation, apoptosis and angiogenesis has drawn much attention [216-221,226,227], although the role of BMPs as tumor suppressors in breast cancer is still unsettled [214,228]. In this part, we investigated the action of vitamin D analogs on Smad signaling proteins, which are known to be important cytoplasmic mediators of signals from the TGF-β/activin/BMP receptor serine/threonine kinases. We showed that the Gemini vitamin D analog Ro-3582 activated the BMP-specific Smad signaling in MCF10 breast epithelial cells. The vitamin D analog increased phosphorylation of receptor-regulated Smad1/5 (Fig. 13),

translocated phosphorylated Smad1/5 into the nucleus (Fig. 15), and enhanced Smad1/5-dependent activation of BMP-mediated gene transcription (Fig. 16). The vitamin D analog also induced the synthesis of mRNA and protein of BMP-2 and BMP-6, and reduced the expression of Smad6 mRNA (Fig. 18). Noggin, a BMP antagonist, reversed the phosphorylation of Smad1/5 induced by Ro3582 (Fig. 14), and inhibited the nuclear localization of phospho-Smad1/5 by the vitamin D analog Ro3582 (Fig. 15), suggesting that Smad activation by Gemini vitamin D analog Ro3582 is BMP-specific.

Interactions between the nuclear receptor family and the TGF-β superfamily have been investigated [137,229-231]. Steroids, such as vitamin D analogs and retinoids, are known to enhance the response to TGF-β/BMP by inducing the synthesis of more TGF-β/BMP ligands and their receptors [137,229,231,232]. Furthermore, there are many studies on the interaction of steroids and their nuclear receptors with Smads [139,140,230]. Cao *et al* showed that certain vitamin D analogs induced phospho-Smad2 in myeloid leukemia cells, and that phosphorylation of Smad2 was a critical sensor for the differentiation of these cells [224]. In our study, we have shown that Gemini vitamin D analogs activated the BMP/Smad signaling by selectively phosphorylating Smad1/5 in MCF10AT1 breast epithelial cells.

Because it has been shown that C-terminal phosphorylation of R-Smads by the TGF-β type I receptor is a key event in the activation of Smads [136], we have utilized the antibody against Ser433/435 phosphorylation at the C-terminal domain of Smad3 and the antibody to pSmad1(Ser463/465)/ pSmad5(Ser463/465)/ pSmad8(Ser426/428) to determine the effects of Gemini vitamin D analogs on the TGF-β/BMP signaling system in MCF10 epithelial cells. Using these antibodies to the phospho-Smads, we found that the Gemini vitamin D analog Ro3582 increased the phosphorylation of Smad1/5 responsible for activating the BMP pathway but not Smad2 and Smad3 of the

TGF-β/activin pathways. Furthermore, the phosphorylation of Smad1/5 by Ro3582 was abrogated by the BMP antagonist, Noggin, suggesting that the activation of R-Smads by a vitamin D derivative was specific to BMP signaling. In a time course study, the vitamin D analog started to increase Smad1/5 phosphorylation at 12 hr, and increased the level of phosphorylation markedly by 24 hr. These results indicate that activation of Smad1/5 by the vitamin D analogs may not be caused by a direct action on Smad1/5 molecules, but it may require time for the activation or synthesis of the kinase that phosphorylates Smad1/5.

The possible mechanism of activation of Smad1/5 is that the vitamin D analog functions by inhibiting phosphatases that dephosphorylate phospho-Smad1/5. However, as shown in Fig. 17, vitamin D analog Ro3582 did not regulate the phosphatase activity. It is more likely that vitamin D analogs activate BMP-specific signaling by increasing the production of BMPs and/or by down-regulating the inhibitory system of the signaling such as Smad6. Smad6 is identified as an inhibitory Smad by inhibiting the BMP/Smad signaling. It binds to BMP receptor type I and blocks the phosphorylation of Smad1/5 [233,234], and it also inhibits the complex formation of Smad1 and Smad4 by binding to activated Smad1 [235]. The activity of Smad ubiquitin regulatory factor 1 (Smurf1) is enhanced through interacting with Smad6 [236]. The results in Fig. 18 show that Ro3582 not only increased mRNA expression and protein synthesis of BMP-2 and BMP-6, but also reduced the Smad6 mRNA level. We also determined the expression level of BMP-7, but the basal level was too low to be detected by quantitative real time RT-PCR in MCF10AT1 cells. These results suggest that Gemini vitamin D analogs activate BMP-specific signaling by increasing the synthesis of BMP-2 and BMP-6, and down-regulating the inhibitory Smad6.

A potential role for the BMP/Smad pathway during the progression of estrogen receptor-positive breast cancer has been recently reported [214]. However, the anti-

proliferating and pro-apoptotic effects of BMPs are reported in breast cancer cell lines, and significantly decreased expression of BMP-2 has been shown in non-invasive, invasive, and liver metastatic breast tumor tissue compared to normal breast tissue [225], suggesting a possible role of the BMP-2 as a tumor suppressor. Since we have shown that the vitamin D system interacts with BMP signaling, it will be interesting to determine whether vitamin D analogs induce growth suppression in breast cancer by the activation of BMP signaling.

In summary, the present study demonstrated the effects of $1\alpha,25(OH)_2D_3$ and vitamin D analogs on enhancing BMP/Smad signaling. This effect occurs by inducing the phosphorylation of Smad1/5 in the cytoplasm followed by its translocation to the nucleus where it may interact with the VDR or other molecules for transcription.

PART III: Activation of BMP Signaling by a Gemini Vitamin D Analog is Mediated by Ras/Protein Kinase Cα

III.1. INTRODUCTION

In the previous studies, we found that Gemini vitamin D analog Ro3582 activated the BMP signaling pathway in MCF10 human breast epithelial cells [237,238]. We have further explored the upstream kinase signaling pathways responsible for activation of BMP signaling by Ro3582 and for its role in growth inhibition of breast epithelial cells. The effects of $1\alpha,25(OH)_2D_3$ and its analogs are mainly mediated through the vitamin D receptor (VDR) or through the membrane-associated signaling pathway [11,42]. Membrane-associated responses to $1\alpha,25(OH)_2D_3$ (non-genomic, rapid response to $1\alpha,25(OH)_2D_3$), where the mechanism is still unclear, is now considered an essential type of action involved in calcium/phosphate transport, activation of protein kinase C (PKC) and/or the mitogen activated protein kinase (MAPK) cascade [42,46,145,148].

PKC, a group of serine/threonine kinases known to regulate cell growth, apoptosis, differentiation, cell migration and carcinogenesis, has been shown to be regulated by $1\alpha,25(OH)_2D_3$ and certain several vitamin D analogs [141,142,145-147]. Several studies have suggested that PKC interacts with the TGF-β/BMP signaling pathway [239-241]. PKC-dependent phosphorylation of the MH1 domain of TGF-β specific Smads (Smad2/3) led to down-regulation of the growth inhibitory and apoptotic action of TGF-β [239]. In contrast, BMP-2 enhances apoptosis by using the PKC-dependent signaling pathway in human osteoblasts [240]. It was also reported that Smad6 regulates TGF-β and plasminogen activator inhibitor-1 through a PKC-β-

dependent mechanism [241], and Smad3 and PKCδ mediate TGF-β1-induced collagen I expression in human mesangial cells [242]. These studies suggest the mechanism for the regulation of PKC by $1\alpha,25(OH)_2D_3$ and vitamin D analogs that may affect the TGF-β/BMP signaling pathway.

Therefore, in this part, we investigated whether PKC is involved in the activation of BMP/Smad signaling by a Gemini vitamin D analog Ro3582, and whether this is important for the inhibition of cell proliferation of breast epithelial cells.

III.2. RESULTS

A. Effects of PKCα/βI inhibitor on Smad activation by Ro3582 in MCF10AT1 human breast epithelial cells

A.1. The activation of Smad signaling by Ro3582 is blocked by PKCα/βI inhibitor Go6976 in MCF10AT1 breast epithelial cells.

In previous studies Part I and II, we showed that $1\alpha,25(OH)_2D_3$ and Ro3582 inhibited the proliferation and activated Smad signaling, and Ro3582 exerted a much stronger effect than $1\alpha,25(OH)_2D_3$ [237,238], as determined by the phosphorylation of Smad1/5 in MCF10AT1 breast epithelial cells. Here, we tested 7 different serine/threonine kinase and phosphoinositide-3 kinase (PI3K) inhibitors to investigate the upstream cell signaling pathways that may be responsible for the activation of Smad1/5 signaling. Among the inhibitors tested, the PKC inhibitor (Go6976, an inhibitor of the classical Ca^{2+}-dependent PKCα,βI isoforms) blocked the phosphorylation of Smad1/5 induced by Ro3582, whereas the PI3-kinase inhibitor (LY294002•HCl), the

MEK inhibitors (PD98059 and U0126), the p38 inhibitor (SB203580), the JNK inhibitor (SP600125), the PKA inhibitor (H-89), the AKT inhibitor (a phosphatidylinositol ether analog), and the TGF-β type I receptor inhibitor (SB431542) showed little or no effect on the level of phospho-Smad1/5 induced by Ro3582 (Fig. 20).

A.2. PKCα/βI inhibitor Go6976 partly inhibits BMP2/6 and CYP24A1 mRNA synthesis induced by Ro3582.

We tested whether the PKCα/βI inhibitor Go6976 regulates the target genes of BMP and VDR signaling induced by Ro3582. After treatment with Ro3582 with or without Go6976 in MCF10 AT1 cells, mRNA level of BMPs and CYP24A1 were measured by quantitative PCR. CYP24A1 (vitamin D 24-hydroxylase) is a prominent vitamin D response gene and is known to inactivate $1\alpha,25(OH)_2D_3$ by hydroxylation in the 24-position. As shown in Fig. 21, mRNA levels for BMP-2, BMP-6 and CYP24A1 were increased by Ro3582 at 12 hr and more markedly at 24 hr confirming our earlier studies [237,238] and the induction was significantly inhibited by Go6976.

A.3. Growth inhibition by Ro3582 is partly reversed by PKCα/βI inhibitor Go6976 in MCF10AT1 breast epithelial cells.

We previously reported that Ro3582 activates Smad signaling and inhibits cell growth in MCF10AT1 cells [237,238]. Here, we used the PKCα/βI inhibitor Go6976 to determine whether blocking Smad signaling by Go6976 would affect growth inhibition by Ro3582 in MCF10AT1 cells. As shown in Fig. 22, Ro3582 exerted about 60% growth inhibition at 1 nM, whereas Go6976 itself has little effect on growth inhibition at 0.1 μM. When cells were treated with Ro3582 together with Go6976, the PKCα/βI inhibitor Go6976 partly reversed the growth inhibition induced by Ro3582.

B. PKCα activation and its effects on Smad activation by Ro3582 in MCF10AT1 human breast epithelial cells

B.1. PKCα is involved in the phosphorylation of Smad1/5 by Ro3582.

Since the PKC family has multiple isoforms, we next determined which PKC isoforms may be involved in the activation of Smad signaling by Ro3582. We first tested the different types of PKC inhibitors, such as Go6976 (PKC α, βI inhibitor), Go6983 (PKC α, β, γ, δ, ζ inhibitor) and PKCβ C2-4 inhibitor (PKC α, β inhibitor) at several concentrations. The phosphorylation of Smad1/5 induced by Ro3582 was inhibited by all of these PKC inhibitors, suggesting that PKCα and/or β might be involved in Smad activation by Ro3582 (Fig. 23A). In addition, we tested 4 different PKC isoforms: PKCα (a classic PKC isoform), PKCε and δ (novel PKC isoforms), and PKCζ (an atypical PKC isoform). These vectors were linked to an HA tag and the transfection of vectors was confirmed by expression of HA (Fig. 23B). Overexpression of PKCα enhanced the phosphorylation of Smad1/5 induced by Ro3582. However, overexpression of PKCε, PKCδ or PKCζ showed little or no effect on the Ro3582-mediated increase in the level of pSmad1/5 (Fig. 23B).

B.2. Ro3582 activates PKCα in MCF10AT1 breast epithelial cells.

PKCα is known to be translocated to the plasma membrane after calcium and diacylglycerol binding to C2 and C1 domain respectively, which can lead to enzymatic activation [142]. Using confocal microscopy, the cellular distribution of PKCα was determined after treatment with Ro3582 or 12-O-tetradecanoylphorbol-13-acetate (TPA),

a well-known PKC activator, in MCF10AT1 cells. DAPI staining was used to recognize the nuclear morphology in cells. First, we used PKCα antibody to detect the location of endogenous PKCα in MCF10AT1 cells. When the cells were treated with TPA (10 nM), PKCα translocated to the membrane within 1 hr. However, Ro3582 (10 nM) did not change the cellular location of PKCα at 1 hr. Interestingly, 24 hr after Ro3582 treatment, PKCα was markedly translocated to the membrane (Fig. 24A). Furthermore, GFP-PKCα vector (PKCα linked to a fluorescent marker, GFP) was transfected and PKCα was detected directly using green fluorescence (GFP) at 488 nm. We confirmed that both Ro3582 and TPA triggered the translocation of PKCα to the membrane (Fig. 24B).

B.3. Vitamin D receptor (VDR) is required for the induction of pSmad1/5, but knock-down of PKCα does not affect the phosphorylation of Smad1/5 by Ro3582.

Since we found that Ro3582 activated PKCα and overexpression of PKCα enhanced phosphorylation of Smad1/5, we tested siRNA against PKCα to see the effect of the decreased level of PKCα protein on phospho-Smad1/5 induced by Ro3582. As shown in Fig. 25, siRNA against PKCα significantly decreased the protein level of PKCα. However, phosphorylation of Smad1/5 induced by Ro3582 was not affected by knock-down of PKCα, suggesting that PKCα may not be the only mediator involved in Smad1/5 phosphorylation by Ro3582. In case of VDR, pSmad1/5 induced by Ro3582 was significantly blocked by knock-down of VDR protein using siRNA against VDR, indicating that VDR is required for Smad activation by Ro3582 (Fig. 25).

C. Involvement of Ras in Smad activation by Ro3582 in MCF10AT1 human breast epithelial cells.

C.1. Ras is necessary for the induction of pSmad1/5 and inhibition of cell growth by Ro3582.

Because studies of the inter-regulation between Ras and TGF-β/BMP signaling were reported earlier [243-247], we investigated whether the transfected Ras in MCF10AT1 cells may affect the regulation of Smad signaling induced by Ro3582. MCF10AT1 cells were established by transfecting Ha-ras into MCF10A normal breast epithelial cells and passaging in animals [188,248]. As shown in Fig. 26, both MCF10A and MCF10AT1 cell lines showed the same level of response to TGF-β or BMP-2 treatment, determined by the phosphorylation of Smad3 by TGF-β and the phosphorylation of Smad1/5 by BMP-2. However, Ro3582 increased pSmad1/5 in Ha-ras transfected MCF10AT1 cells, but not in the parent MCF10A cells. In growth inhibition assay, Ro3582 exerted about 60% inhibition at 1 nM in Ha-ras transfected MCF10AT1 cells, but did not show any inhibitory effect in MCF10A cells (Fig. 27), suggesting the critical role of Ras in Smad activation and growth inhibition by Ro3582. In addition, inhibiting Ras activity by a Ras farnesyltransferase inhibitor (Ras inhibitor, L-744832) blocked the phosphorylation of Smad1/5 induced by Ro3582 in MCF10AT1 cells (Fig. 28A), although the Ras inhibitor did not reverse growth inhibition induced by Ro3582 (Fig. 28B), which may be due to the growth inhibitory effect of the Ras inhibitor by itself (Fig. 28C) in MCF10AT1 cells.

C.2. PKCα activation by Ro3582 is blocked by a Ras farnesyltransferase inhibitor in MCF10AT1 cells.

The integration between Ras/MAPK and PKC has been reported [248-250] and we found that both PKCα and Ha-ras regulated the phosphorylation of Smad1/5 that was induced by Ro3582. Therefore, we examined whether Ras signaling is necessary to

regulate PKCα, which in turn enhances Smad signaling by Ro3582. Here, the treatment with a Ras farnesyltransferase inhibitor (L-744832) did not affect PKCα distribution in MCF10AT1 cells. However, Ro3582 induced the cellular translocation of PKCα to the membrane, and when treated together with the Ras inhibitor, the translocation of PKCα to the membrane induced by Ro3582 was markedly inhibited (Fig. 29).

III.3. DISCUSSION

In our previous studies, we found that a novel Gemini vitamin D analog Ro3582 strongly inhibited the growth of human MCF10AT1 breast epithelial cells [237,238]. Mechanistic studies indicated that this compound activated BMP/Smad signaling, as determined by increased phosphorylation of Smad1/5, translocation of pSmad1/5 to the nucleus, and enhancement of the BMP/Smad transcriptional activity [238]. In this part, we identified key upstream signaling pathways responsible for BMP/Smad signaling that are activated by Ro3582. We found that the vitamin D analog Ro3582 induced the phosphorylation of Smad1/5 through vitamin D receptor, VDR. Ro3582 also activated PKCα, which may be indirectly involved in phosphorylation of Smad1/5 and inhibition of the growth of MCF10AT1 cells. Furthermore, Ras was involved in the activation of PKCα and Smad signaling by Ro3582. These results suggest that VDR, Ras and PKCα may act as crucial mediators of Ro3582 effects on MCF10AT1 breast epithelial cells.

Many studies of cross-talk between Ras and TGF-β/Smad signaling have been described [244-247,251]. Yue *et al* reported that TGF-β activated the Ras/MAPK pathway required for the autocrine TGF-β production and Smad1 regulation [251]. Ras was also suggested as a mediator of pleiotropic TGF-β1 signaling in developing neurons

[244]. The activation of Ras/MAPK or the presence of oncogenic Ras was shown to enhance TGF-β-induced epithelial-mesenchymal transition [245,246]. More importantly, it has been shown that $1\alpha,25(OH)_2D_3$ regulates the MAP kinase pathway by activating Ras/RAF-1 signaling in muscle cells and in myeloid leukemic cells [145,148]. We demonstrated that the vitamin D analog Ro3582 increased phosphorylation of Smad1/5 and inhibited cell proliferation in MCF10AT1 cells transfected with Ha-ras, but not in the parent MCF10A cells that lack Ras (Figs. 26-28). This suggests that Ras is critical for the activation of Smad signaling and growth inhibition by Ro3582.

The kinase pathways, such as Ras/Erk/MAPK, MEKK, JNK, p38 MAP kinase, CDK and PKC, have been shown to regulate Smad signaling [252-255]. MEKK1 or JNK enhanced Smad phosphorylation, nuclear localization and Smad-mediated transcription [253,254]. In contrast, the phosphorylation of R-Smads at the linker domain or MH-1 domain induced by Ras/Erk/MAPK, CDK2/4 and PKC inhibited the activation of Smad signaling [239,252,255,256]. Yakymovych *et al* reported that PKC activation resulted in the phosphorylation of the MH1 domains of Smad2 and Smad3 to abrogate DNA binding of Smad3 [239]. Although this study suggests that PKC may play a negative regulatory role in TGF-β/Smad-mediated transcription [239], our previous studies indicated that Ro3582 induced the phosphorylation of Smad1/5 in the MH2 domain (Ser463/465) and enhanced the BMP-specific signaling pathway [237,238]. In addition, our present study demonstrated that this was blocked by PKC inhibitors (Figs. 20 and 23), suggesting a role of PKC for the activation of BMP/Smad signaling by Ro3582.

Although PKCs have been suggested to be pro-mitogenic kinases, it may be PKC isoform-dependent and cell-type dependent, as many PKCs can also inhibit cell cycle progression [142]. Among PKC isoforms, PKCα inhibited cell growth via p21 induction and suppress *in vivo* tumor formation [144,257]. Several studies have

previously shown that $1\alpha,25(OH)_2D_3$ activates PKCα, and activation of this isoform acts as an anti-proliferative signal [144-146,257]. Our results also indicate that PKCα overexpression enhanced phosphorylation of Smad1/5 and inhibiting PKC activity reversed growth inhibition induced by Ro3582 (Figs. 22, 23), suggesting PKCα mediates the activation of Smad signaling. However, when we knocked-down the protein level of PKCα using siRNA against PKCα, it did not block the phosphorylation of Smad1/5 induced by Ro3582. This suggests the possibilities that 1) Smad1/5 may not be direct target of PKCα, 2) other isoforms of PKC may be also involved in Smad activation because the kinase inhibitors we tested are not specific to single PKC isoform, or 3) other kinases may contribute together with PKCα to Smad phosphorylation.

It was reported that PKCα activation by $1\alpha,25(OH)_2D_3$ was through the $1\alpha,25(OH)_2D_3$-membrane associated rapid response steroid binding protein [46,146]. The classic VDR, which is known to translocate to the nucleus after ligand binding, has been proposed to be associated with caveolae in the plasma membrane and is responsible for the rapid response to vitamin D ligands [42,43]. The structural flexibility of $1\alpha,25(OH)_2D_3$ and vitamin D analogs may determine their preferences for binding to different locations on the VDR and for selective responses between genomic and membrane-mediated effects [42,43]. In this study, we knocked-down VDR using siRNA against VDR, which significantly blocked the enhancement of Smad1/5 phosphorylation induced by Ro3582 in MCF10AT1 cells (Fig. 25), suggesting that VDR is necessary for the activation of Smad1/5 by Gemini vitamin D analog. However, it still needs to be determined whether Ro3582 activate BMP/Smad signaling through rapid response using membrane VDR (mVDR) or through genomic response using nuclear VDR (nVDR), although the mVDR in caveolae-enriched plasma membrane is speculated to be same as nVDR [47-49].

In summary, Gemini vitamin D analog, Ro3582, activates the BMP/Smad signaling through a Ras/PKCα pathway, which may lead to the inhibition of cell proliferation in MCF10 human breast epithelial cells. Further investigations are needed to understand the interactions between the Ras/PKCα pathway and the regulation of BMP/Smad signaling by vitamin D analogs, and their interactions with the nuclear or membrane-bound VDR.

PART IV: Animal Studies: Gemini Vitamin D Analogs Inhibit Estrogen Receptor Positive and Estrogen Receptor Negative Tumorigenesis without Hypercalcemic Toxicity

IV.1. INTRODUCTION

In the United States, breast cancer remains the most frequently diagnosed cancer and the second leading cause of cancer deaths in women according to Cancer Statistics from the American Cancer Society [51]. Because of the complexity and heterogeneity of mammary carcinogenesis [53], many pharmacological agents have been studied for their effects on the prevention of breast cancer. For example, selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene have achieved significant reduction of breast cancer incidence in women at high risk [258,259]. However, SERMs are not effective in preventing estrogen receptor (ER)-negative breast cancer, which corresponds to at least one third of the breast cancer cases [260].

The vitamin D receptor (VDR), a member of the nuclear receptor superfamily, has been suggested as a target for both ER-positive and ER-negative breast cancer prevention [24,261] because it is present in most breast tumors [260], and *VDR* ablation in mice was reported to enhance carcinogen-induced formation of mammary tumors [262]. These results suggest a role of vitamin D signaling in the regulation of mammary tumorigenesis. Based on that, numerous vitamin D analogs have been synthesized and tested in preclinical and clinical studies to demonstrate the benefits of vitamin D analogs for the prevention and treatment of cancer (Tables 2, 3 and 4). However, one of the most potent and widely studied vitamin D analogs, EB1089, failed to show the anti-tumor activity and caused hypercalcemia in patients with breast cancer, colorectal cancer and

hepatocellular carcinoma [119,205,206], suggesting we still need better vitamin D analogs.

The novel Gemini vitamin D analogs we have tested *in vitro* have considerably less toxicity than $1\alpha,25(OH)_2D_3$ in animals [182,183], and certain Gemini vitamin D analogs had a greater inhibitory effect on cell proliferation than $1\alpha,25(OH)_2D_3$ in MCF10 breast epithelial cells [237]. In animal studies, Gemini vitamin D analogs have shown promising activity in inhibiting colon carcinogenesis, as summarized in Introduction. In this part, we tested a classic vitamin D analog, Ro-26-2198, and several Gemini vitamin D analogs (Fig. 7) for their effects on NMU-induced ER-positive mammary carcinogenesis. An ER-negative xenograft model with implanted MCF10DCIS.com cells and MMTV-Her2/neu transgenic mouse model were also employed to investigate the effects of Gemini vitamin D analogs on the growth of ER-negative mammary tumors *in vivo*. Molecular mechanisms of action of Gemini vitamin D analogs that inhibited the growth of both ER-positive and ER-negative tumors were studied.

IV.2. RESULTS

A. Effects of Gemini vitamin D analogs in NMU-induced breast cancer model

A.1. Gemini vitamin D analogs inhibit NMU-induced mammary tumorigenesis in rats.

The efficacy of one classic vitamin D analog and 6 different Gemini vitamin D analogs on NMU-induced mammary tumorigenesis were tested in Sprague-Dawley female rats. The tumors formed with this model are ER-positive [195]. The doses of vitamin D analogs in Table 10 were determined based on the maximum tolerated dose in

CD-1 mice and our preliminary data (data not shown). As summarized in Table 10, body weights of animals treated with the vitamin D analogs were not significantly different from those in the control group throughout the study. Among 7 different vitamin D analogs, administration of BXL0072 and BXL0097 resulted in approximately a 60% reduction of the average tumor burden per rat. These compounds had stronger activity than Ro3586 (44% reduction) and BXL0084 (29% reduction). Other analogs, Ro2198, Ro3582, and BXL0088 had little or no effect on tumor burden at the dose tested (Table 10).

A.2. Gemini vitamin D analog BXL0097 inhibits tumor growth without hypercalcemia toxicity.

Hypercalcemic toxicity has limited the use of naturally occurring $1\alpha,25(OH)_2D_3$ as a chemopreventive agent although it has been shown to have a potent anti-proliferating activity in different cell types [24,97,263,264]. Among the vitamin D analogs we tested (Table 10), BXL0097 exerted the strongest activity in inhibiting tumor growth, and it was selected for a further dose dependent study. In Table 11, we compared the efficacy and toxicity of BXL0097 to those of $1\alpha,25(OH)_2D_3$. Table 11 shows that body weight in $1\alpha,25(OH)_2D_3$ treated groups were comparable to those in the control group, and tumor growth was inhibited 20% and 34% at doses of 0.1 μg/kg body weight and 0.3 μg/kg body weight, respectively. However, we observed that the average level of calcium in serum was increased to 10.9 mg/dl and 11.7 mg/dl by $1\alpha,25(OH)_2D_3$ at tumor inhibitory doses (Table 11). The normal range of calcium in serum is 8.8–10.4 mg/dL [182]. As shown in Table 11, doses of BXL0097 up to 0.3 μg/kg body weight inhibited tumor burden per rat but did not show any significant changes in body weight or serum calcium

level compared to the control group, indicating that BXL0097 had a better efficacy/toxicity profile than $1\alpha,25(OH)_2D_3$.

A.3. Gemini vitamin D analogs increase markers of cell cycle arrest and apoptosis in NMU-induced mammary tumors.

In Western blot analysis, we showed that administration of BXL0072 and BXL0097 upregulated the VDR and increased the level of the CDK inhibitor p21 in NMU-induced mammary tumors (Fig. 30). Another CDK inhibitor, p27, was not affected. In addition, administration of 0072 and 0097 increased the level of cleaved PARP and caspase-3, which are markers of apoptosis. These results suggest that administration of BXL0072 and BXL0097 inhibit tumor growth by arresting the cell cycle and inducing apoptosis.

A.4. Evaluation of proliferating cell nuclear antigen (PCNA) and caspase-3 expression of mammary tumors.

Tumors from control, BXL0072 or BXL0097 treated groups shown in Fig. 31 were evaluated as adenocarcinoma. The tumor type, differentiation/grade and stromal responses among the control group and BXL0072 and BXL0097 treated groups were not different in H & E staining (Fig.31). However, as shown in Fig. 31, the proliferating cell nuclear antigen (PCNA) staining of the tumor tissue was much stronger in control group (vehicle) than in the BXL0072 or BXL0097 treated groups. The percentage (± S.E.) of PCNA positive cells of the tumor tissue in the control group were 62.6 ± 8.5%, whereas the percentage of PCNA positive cells from BXL0072 and BXL0097 treated group were 32.5 ± 5.1% (significantly different from control group, p = 0.02) and 45.3 ± 6.7%, respectively. In addition, the expression of cleaved caspase-3 was evaluated in five to

six different sections and a representative section of mammary tumor samples from the control group or Gemini vitamin D analogs treated groups are shown (Fig. 31). The percentage (± S.E.) of cleaved caspase-3 positive cells in tumors from the control group were 1.8 ± 0.4, whereas the percentage of cleaved caspase-3 positive cells from BXL0072 and BXL0097 treated group were 13.1 ± 2.7 and 15.2 ± 4.3, respectively (both significantly different from control group, $p < 0.01$), indicating that Gemini vitamin D analogs induce apoptosis in mammary tumors.

A.5. Effect of administration of BXL0097 on the expression of CYP24A1, IGFBP-3 and p21 in NMU-induced mammary tumors.

CYP24A1 is a well-known target gene of vitamin D and it has been shown to be induced by $1\alpha,25(OH)_2D_3$ or its analogs in different cells [24]. In this study, the mRNA level for CYP24A1 determined by quantitative PCR was increased by BXL0097 in tumor tissue dose dependently (Fig. 32A). The protein expression of insulin-like growth factor binding protein 3 (IGFBP-3), and CDK inhibitor p21 were enhanced by BXL0097, indicating that BXL0097 may regulate the IGF pathway to inhibit ER-positive mammary tumorigenesis in rats (Fig. 32).

B. Effects of Gemini vitamin D analogs in xenograft model

B.1. Inhibitory effect of administration of Gemini vitamin D analogs on the growth of MCF10DCIS.com tumors in a xenograft model.

Because NMU-induced mammary tumors in rats are ER-positive [195], we investigated the anti-tumor effect of Gemini vitamin D analogs on the growth of ER-negative MCF10DCIS.com cells. In an ER-negative MCF10DCIS.com xenograft model,

administration of BXL0097 inhibited the tumor weight by 50% without significant changes in body weight (Fig. 33A). Interestingly, the expression levels of IGFBP-3 and p21 protein were upregulated by BXL0097 in MCF10DCIS.com tumors (Fig. 33C), suggesting that IGFBP-3 and p21 may act as common mediators of the effects of Gemini vitamin D analogs to inhibit the growth of both ER-positive and ER-negative mammary tumors. In previous study, we found that Gemini vitamin D analogs activate Smad signaling by enhancing the phosphorylation of Smad1/5 in MCF10AT1 human breast epithelial cells [237,238]. Here, we confirmed that levels of phospho-Smad1/5 in tumors were enhanced by BXL0097 treatment *in vivo* (Fig. 33B). Furthermore, another Gemini vitamin D analog, BXL0124, showed a significant reduction of tumor volume (Fig. 34B) and tumor weight (67% inhibition, Fig. 34C), compared to control group without affecting the body weight (Fig. 34A).

C. Effects of a Gemini vitamin D analog in MMTV-Her2/neu transgenic mouse model

C.1. The Gemini vitamin D analog BXL0124 inhibits Her2/neu overexpressed mammary tumorigenesis in MMTV-Her2/neu transgenic mouse model.

Because of the close association between Her-2 and human breast cancer, MMTV/Her2/neu transgenic mouse model is one of the most widely used hormone independent prevention models for breast carcinogenesis [53]. Here, we tested a Gemini vitamin D analog BXL0124 to determine the efficacies on Her-2 overexpressed mammary tumors. The mice were treated with vehicle or BXL0124 (0.3 μg/kg body weight) by i.p. injection three times a week from 20 weeks old until 54 weeks old. As shown in Fig. 35, BXL0124 significantly inhibited tumor growth, reduced tumor

multiplicity (45% decrease) and tumor weight (54% deduction) without body weight changes.

VI.3. DISCUSSION

Breast cancer in humans is a complex disease involving different histopathologies, genetic changes, and clinical outcomes, and a single animal model may not mimic all features of human breast carcinogenesis [53]. In this part, we evaluated the inhibitory effects of Gemini vitamin D analogs on tumorigenesis in three different preclinical models, and demonstrated that certain Gemini vitamin D analogs significantly suppressed tumor development in an ER-positive NMU-induced breast cancer model in rats, tumor growth in an ER-negative MCF10DCIS.com xenograft model in immunodeficient mice, and tumor development in a MMTV-Her2/neu transgenic mouse model without hypercalcemic toxicity (Table 11, Figs. 33-35). Hypercalcemia is a well-established toxicity induced by $1\alpha,25(OH)_2D_3$ and for many classical vitamin D derivatives. We found that $1\alpha,25(OH)_2D_3$ significantly increased the serum calcium level at doses showing tumor growth inhibition (Table 11). In contrast, BXL0097 exerted potent efficacy in preventing mammary tumor growth without causing hypercalcemia (Table 11). These results are in agreement with previous reports using a colon cancer model [182,183].

The lack of hypercalcemic effects of Gemini vitamin D analogs at anticancer doses may be because of the flexibility of the ligand binding pocket in the vitamin D receptor [175]. Recently, it was reported that structural rearrangement of the ligand binding pocket provides space to accommodate the second side chain of Gemini vitamin D analogs, which leads to expansion of the ligand binding pocket volume [178]. This conformational flexibility of the ligand binding pocket to accommodate different ligands

may result in different ligand-specific cofactor binding and/or selectivity of transcription of target genes [175,180], thus suggesting that Gemini vitamin D analogs may show different regulation of vitamin D target genes when compared with $1\alpha,25(OH)_2D_3$.

In our mechanistic study, the expression levels of cyclin dependent kinase (CDK) inhibitor p21 and insulin-like growth factor binding protein-3 (IGFBP-3) in tumor tissues from two different models were enhanced by BXL0097 treatment (Figs. 30-33). Interestingly, however, the markers for apoptosis including cleaved PARP and caspase-3 were regulated by BXL0072 and BXL0097 in ER-positive mammary tumors induced by carcinogen (Fig. 30), but not in ER-negative tumors from the xenograft model (data not shown). It has been shown in several studies that $1\alpha,25(OH)_2D_3$ and its analogs suppress the mRNA and protein synthesis of ER through the negative vitamin D response element (nVDRE) in the ER promoter, which eventually leads to inhibition of cell proliferation through cell cycle arrest and apoptosis [96,265,266]. The involvement of ER in inducing apoptosis by Gemini vitamin D analogs in NMU-induced mammary tumors needs to be further investigated.

Insulin-like growth factor binding proteins (IGFBPs) which inhibit IGF signaling having mitogenic activity by binding to free insulin-like growth factors (IGFs) have been shown to be up-regulated by $1\alpha,25(OH)_2D_3$ and certain of its analogs in different cancer cells including prostate [159,267], colon [122,161], and breast cancer [237]. In breast cancer, several studies suggest that IGFBP-3 acts as a tumor suppressive factor [155,156]. Recently, in the promoter region of the IGFBPs, the functional vitamin D response element (VDRE) has been identified [163-165] and we previously reported Gemini vitamin D analogs increased the level of mRNA and protein of IGFBP3 in MCF10 progressive human breast epithelial cell lines [237,238]. This indicate that IGFBPs may be the primary target genes of $1\alpha,25(OH)_2D_3$ and its analogs. In the present study, we

demonstrated that Gemini vitamin D analog treatment increased the expression of IGFBP-3 in breast tumors from both the carcinogen-induced model and the xenograft model, suggesting that IGFBP-3 induction may be mediated via direct transcriptional regulation. Interestingly, IGFBPs were shown to be involved in the regulation of the CDK inhibitor, p21 [153,268]. IGFBP-3 knockdown by siRNA reversed the induction of p21 and growth inhibition by synthetic androgen R1881 in LNCaP human prostate cancer cells, indicating that IGFBP-3 was upstream of p21 [153]. These studies support our hypothesis that the Gemini vitamin D analog BXL0097 inhibits tumor growth, at least in part, through the regulation of the link between IGFBP-3 and p21.

In several published studies, vitamin D and its analogs have been involved in the regulation of transforming growth factor-β (TGF-β) signaling, which is extensively reviewed by Deeb *et al* [24]. We have also found that Gemini vitamin D analogs were shown to activate the bone morphogenetic protein (BMP)/Smad signaling pathway via Ras/PKCα, which may mediate the proliferation of the MCF10 breast epithelial cells [238,269]. Here, we demonstrated that the Gemini vitamin D analog BXL0097 activates Smad1/5 signaling *in vivo* in MCF10DCIS.com transplanted tumor tissues (Fig. 33C).

In summary, we investigated the *in vivo* effect of certain Gemini vitamin D analogs on mammary tumor growth in a chemically induced breast cancer model, in a xenograft model, and in a MMTV-her2/neu transgenic mouse model. We found that Gemini vitamin D analogs significantly inhibit ER-positive (NMU-induced) and ER-negative (MCF10DCIS.com xenograft and MMTV-her2/neu) mammary tumor growth without increasing serum calcium levels. Mechanistic studies showed that the inhibitory activity was associated with the induction of IGFBP-3 and the CDK inhibitor p21 in ER-positive NMU-induced and ER-negative xenograft breast cancer models, although we need to confirm the mechanism by Gemini vitamin D analogs in tumors developed in

MMTV-her2/neu transgenic mouse model. Taken together, these results suggest that Gemini vitamin D analogs may be promising agents for the prevention and treatment of breast cancer without hypercalcemic toxicity.

CONCLUSION

We have investigated tumor suppressive activities and mechanism of action of Gemini vitamin D analogs in MCF10 progressive human breast epithelial cell lines and mammary tumorigenesis models. Gemini vitamin D analogs showed different profiles of gene and protein regulation during the progression of breast cancer, more significantly in early stage of breast epithelial cells. They also exerted better activity in inhibiting cell proliferation than naturally occurring $1\alpha,25(OH)_2D_3$, and regulated the protein expression of the markers related to cell proliferation such as cyclin dependent kinase inhibitor p21 and insulin-like growth factor binding protein 3 (IGFBP-3). To determine the mechanism involved in inhibiting cell proliferation by Gemini vitamin D analogs, we investigated the mRNA regulation and found that the Gemini vitamin D analog Ro3582 not only increased the synthesis BMP ligands, namely BMP-2 and BMP-6, but also down-regulated the inhibitory Smad, Smad6. In line with these findings, we confirmed that Gemini vitamin D analogs activated BMP-specific Smad signaling by increase of Smad1/5 phosphorylation, translocation of phospho-Smad1/5 to the nucleus, and enhancement of transcriptional responses. In the study of upstream signaling pathways for Smad activation by Gemini vitamin D analogs, VDR, Ras, and PKCα were found to be involved in Smad activation. In *in vivo* experiments, Gemini vitamin D analogs inhibited estrogen receptor (ER) positive (NMU-induced breast cancer model) and ER negative (MCF10DCIS.com xenograft model and MMTV-Her2/neu transgenic mouse model) mammary tumorigenesis without hypercalcemic toxicity. These results suggest that Gemini vitamin D analogs should be considered as potent anticancer agents having better efficacy with less calcemic toxicity for the prevention and treatment of breast cancer.

REFERENCES

1. Mellanby, E. and Cantag, M.D. (1919) Experimental investigation on rickets. *Lancet*, **193**, 407-412.
2. McCollum, E.V., Simmonds, N., Becker, J.E. and Shipley, P.G. (1922) Studies on experimental rickets. XXI. An experimental demonstration of the existence of a vitamin which promotes calcium deposition. *J Biol Chem*, **53**, 293-312.
3. Hess, A.F. and Unger, L.J. (1921) Cure of infantile rickets by sunlight. *Jama*, **77**, 39.
4. Windaus, A., Linsert, O., Luttringhaus, A. and Weidlinch, G. (1932) Uber das krystallistierte vitamin D2. *Justis. Liebigs. Ann. Chem.*, **492**, 226-231.
5. Brockmann, H. (1936) Die Isolierung des antirachitischen vitamins aus thunfischleberol. *H.-S.Zeit. Physiol. Chem.*, **241**, 104-115.
6. Haussler, M.R. and Norman, A.W. (1969) Chromosomal receptor for a vitamin D metabolite. *Proc Natl Acad Sci U S A*, **62**, 155-62.
7. Blunt, J.W., DeLuca, H.F. and Schnoes, H.K. (1968) 25-hydroxycholecalciferol. A biologically active metabolite of vitamin D3. *Biochemistry*, **7**, 3317-22.
8. Fraser, D.R. and Kodicek, E. (1970) Unique biosynthesis by kidney of a biological active vitamin D metabolite. *Nature*, **228**, 764-6.
9. Lawson, D.E., Fraser, D.R., Kodicek, E., Morris, H.R. and Williams, D.H. (1971) Identification of 1,25-dihydroxycholecalciferol, a new kidney hormone controlling calcium metabolism. *Nature*, **230**, 228-30.
10. Brown, A.J., Dusso, A. and Slatopolsky, E. (1999) Vitamin D. *Am J Physiol*, **277**, F157-75.
11. Dusso, A.S., Brown, A.J. and Slatopolsky, E. (2005) Vitamin D. *Am J Physiol Renal Physiol*, **289**, F8-28.
12. Holick, M.F. (2007) Vitamin D deficiency. *N Engl J Med*, **357**, 266-81.
13. Holick, M.F., Frommer, J.E., McNeill, S.C., Richtand, N.M., Henley, J.W. and Potts, J.T., Jr. (1977) Photometabolism of 7-dehydrocholesterol to previtamin D3 in skin. *Biochem Biophys Res Commun*, **76**, 107-14.
14. Holick, M.F. (1981) The cutaneous photosynthesis of previtamin D3: a unique photoendocrine system. *J Invest Dermatol*, **77**, 51-8.
15. Gupta, R.P., Hollis, B.W., Patel, S.B., Patrick, K.S. and Bell, N.H. (2004) CYP3A4 is a human microsomal vitamin D 25-hydroxylase. *J Bone Miner Res*, **19**, 680-8.
16. Rosen, H., Reshef, A., Maeda, N., Lippoldt, A., Shpizen, S., Triger, L., Eggertsen, G., Bjorkhem, I. and Leitersdorf, E. (1998) Markedly reduced bile acid synthesis but maintained levels of cholesterol and vitamin D metabolites in mice with disrupted sterol 27-hydroxylase gene. *J Biol Chem*, **273**, 14805-12.
17. Cheng, J.B., Motola, D.L., Mangelsdorf, D.J. and Russell, D.W. (2003) De-orphanization of cytochrome P450 2R1: a microsomal vitamin D 25-hydroxilase. *J Biol Chem*, **278**, 38084-93.
18. Cheng, J.B., Levine, M.A., Bell, N.H., Mangelsdorf, D.J. and Russell, D.W. (2004) Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase. *Proc Natl Acad Sci U S A*, **101**, 7711-5.
19. Haussler, M.R., Whitfield, G.K., Haussler, C.A., Hsieh, J.C., Thompson, P.D., Selznick, S.H., Dominguez, C.E. and Jurutka, P.W. (1998) The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. *J Bone Miner Res*, **13**, 325-49.

20. Dardenne, O., Prud'homme, J., Arabian, A., Glorieux, F.H. and St-Arnaud, R. (2001) Targeted inactivation of the 25-hydroxyvitamin D(3)-1(alpha)-hydroxylase gene (CYP27B1) creates an animal model of pseudovitamin D-deficiency rickets. *Endocrinology*, **142**, 3135-41.

21. Panda, D.K., Miao, D., Tremblay, M.L., Sirois, J., Farookhi, R., Hendy, G.N. and Goltzman, D. (2001) Targeted ablation of the 25-hydroxyvitamin D 1alpha - hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction. *Proc Natl Acad Sci U S A*, **98**, 7498-503.

22. Zehnder, D., Bland, R., Williams, M.C., McNinch, R.W., Howie, A.J., Stewart, P.M. and Hewison, M. (2001) Extrarenal expression of 25-hydroxyvitamin d(3)-1 alpha-hydroxylase. *J Clin Endocrinol Metab*, **86**, 888-94.

23. Hewison, M., Zehnder, D., Chakraverty, R. and Adams, J.S. (2004) Vitamin D and barrier function: a novel role for extra-renal 1 alpha-hydroxylase. *Mol Cell Endocrinol*, **215**, 31-8.

24. Deeb, K.K., Trump, D.L. and Johnson, C.S. (2007) Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. *Nat Rev Cancer*, **7**, 684-700.

25. St-Arnaud, R., Arabian, A., Travers, R., Barletta, F., Raval-Pandya, M., Chapin, K., Depovere, J., Mathieu, C., Christakos, S., Demay, M.B. and Glorieux, F.H. (2000) Deficient mineralization of intramembranous bone in vitamin D-24-hydroxylase-ablated mice is due to elevated 1,25-dihydroxyvitamin D and not to the absence of 24,25-dihydroxyvitamin D. *Endocrinology*, **141**, 2658-66.

26. Reichel, H., Koeffler, H.P. and Norman, A.W. (1989) The role of the vitamin D endocrine system in health and disease. *N Engl J Med*, **320**, 980-91.

27. Rochel, N., Wurtz, J.M., Mitschler, A., Klaholz, B. and Moras, D. (2000) The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. *Mol Cell*, **5**, 173-9.

28. Li, Y.C., Pirro, A.E., Amling, M., Delling, G., Baron, R., Bronson, R. and Demay, M.B. (1997) Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia. *Proc Natl Acad Sci U S A*, **94**, 9831-5.

29. Yoshizawa, T., Handa, Y., Uematsu, Y., Takeda, S., Sekine, K., Yoshihara, Y., Kawakami, T., Arioka, K., Sato, H., Uchiyama, Y., Masushige, S., Fukamizu, A., Matsumoto, T. and Kato, S. (1997) Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. *Nat Genet*, **16**, 391-6.

30. Hsieh, J.C., Jurutka, P.W., Galligan, M.A., Terpening, C.M., Haussler, C.A., Samuels, D.S., Shimizu, Y., Shimizu, N. and Haussler, M.R. (1991) Human vitamin D receptor is selectively phosphorylated by protein kinase C on serine 51, a residue crucial to its trans-activation function. *Proc Natl Acad Sci U S A*, **88**, 9315-9.

31. Jurutka, P.W., Hsieh, J.C., MacDonald, P.N., Terpening, C.M., Haussler, C.A., Haussler, M.R. and Whitfield, G.K. (1993) Phosphorylation of serine 208 in the human vitamin D receptor. The predominant amino acid phosphorylated by casein kinase II, in vitro, and identification as a significant phosphorylation site in intact cells. *J Biol Chem*, **268**, 6791-9.

32. Carlberg, C., Bendik, I., Wyss, A., Meier, E., Sturzenbecker, L.J., Grippo, J.F. and Hunziker, W. (1993) Two nuclear signalling pathways for vitamin D. *Nature*, **361**, 657-60.

33. Rachez, C. and Freedman, L.P. (2000) Mechanisms of gene regulation by vitamin D(3) receptor: a network of coactivator interactions. *Gene*, **246**, 9-21.

34. Li, B., Carey, M. and Workman, J.L. (2007) The role of chromatin during transcription. *Cell*, **128**, 707-19.

35. Murayama, A., Kim, M.S., Yanagisawa, J., Takeyama, K. and Kato, S. (2004) Transrepression by a liganded nuclear receptor via a bHLH activator through co-regulator switching. *Embo J*, **23**, 1598-608.

36. Kim, M.S., Fujiki, R., Murayama, A., Kitagawa, H., Yamaoka, K., Yamamoto, Y., Mihara, M., Takeyama, K. and Kato, S. (2007) 1Alpha,25(OH)2D3-induced transrepression by vitamin D receptor through E-box-type elements in the human parathyroid hormone gene promoter. *Mol Endocrinol*, **21**, 334-42.

37. Fujiki, R., Kim, M.S., Sasaki, Y., Yoshimura, K., Kitagawa, H. and Kato, S. (2005) Ligand-induced transrepression by VDR through association of WSTF with acetylated histones. *Embo J*, **24**, 3881-94.

38. Norman, A.W., Okamura, W.H., Hammond, M.W., Bishop, J.E., Dormanen, M.C., Bouillon, R., van Baelen, H., Ridall, A.L., Daane, E., Khoury, R. and Farach-Carson, M.C. (1997) Comparison of 6-s-cis- and 6-s-trans-locked analogs of 1alpha,25-dihydroxyvitamin D3 indicates that the 6-s-cis conformation is preferred for rapid nongenomic biological responses and that neither 6-s-cis- nor 6-s-trans-locked analogs are preferred for genomic biological responses. *Mol Endocrinol*, **11**, 1518-31.

39. Zanello, L.P. and Norman, A.W. (1997) Stimulation by 1alpha,25(OH)2-vitamin D3 of whole cell chloride currents in osteoblastic ROS 17/2.8 cells. A structure-function study. *J Biol Chem*, **272**, 22617-22.

40. Kajikawa, M., Ishida, H., Fujimoto, S., Mukai, E., Nishimura, M., Fujita, J., Tsuura, Y., Okamoto, Y., Norman, A.W. and Seino, Y. (1999) An insulinotropic effect of vitamin D analog with increasing intracellular Ca2+ concentration in pancreatic beta-cells through nongenomic signal transduction. *Endocrinology*, **140**, 4706-12.

41. Rebsamen, M.C., Sun, J., Norman, A.W. and Liao, J.K. (2002) 1alpha,25-dihydroxyvitamin D3 induces vascular smooth muscle cell migration via activation of phosphatidylinositol 3-kinase. *Circ Res*, **91**, 17-24.

42. Norman, A.W., Mizwicki, M.T. and Norman, D.P. (2004) Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. *Nat Rev Drug Discov*, **3**, 27-41.

43. Norman, A.W. (2006) Minireview: vitamin D receptor: new assignments for an already busy receptor. *Endocrinology*, **147**, 5542-8.

44. Mizwicki, M.T., Keidel, D., Bula, C.M., Bishop, J.E., Zanello, L.P., Wurtz, J.M., Moras, D. and Norman, A.W. (2004) Identification of an alternative ligand-binding pocket in the nuclear vitamin D receptor and its functional importance in 1alpha,25(OH)2-vitamin D3 signaling. *Proc Natl Acad Sci U S A*, **101**, 12876-81.

45. Nemere, I., Yoshimoto, Y. and Norman, A.W. (1984) Calcium transport in perfused duodena from normal chicks: enhancement within fourteen minutes of exposure to 1,25-dihydroxyvitamin D3. *Endocrinology*, **115**, 1476-83.

46. Nemere, I., Farach-Carson, M.C., Rohe, B., Sterling, T.M., Norman, A.W., Boyan, B.D. and Safford, S.E. (2004) Ribozyme knockdown functionally links a 1,25(OH)2D3 membrane binding protein (1,25D3-MARRS) and phosphate uptake in intestinal cells. *Proc Natl Acad Sci U S A*, **101**, 7392-7.

47. Huhtakangas, J.A., Olivera, C.J., Bishop, J.E., Zanello, L.P. and Norman, A.W. (2004) The vitamin D receptor is present in caveolae-enriched plasma membranes and binds 1 alpha,25(OH)2-vitamin D3 in vivo and in vitro. *Mol Endocrinol*, **18**, 2660-71.

48.	Nguyen, T.M., Lieberherr, M., Fritsch, J., Guillozo, H., Alvarez, M.L., Fitouri, Z., Jehan, F. and Garabedian, M. (2004) The rapid effects of 1,25-dihydroxyvitamin D3 require the vitamin D receptor and influence 24-hydroxylase activity: studies in human skin fibroblasts bearing vitamin D receptor mutations. *J Biol Chem*, **279**, 7591-7.

49.	Zanello, L.P. and Norman, A.W. (2004) Rapid modulation of osteoblast ion channel responses by 1alpha,25(OH)2-vitamin D3 requires the presence of a functional vitamin D nuclear receptor. *Proc Natl Acad Sci U S A*, **101**, 1589-94.

50.	Losel, R. and Wehling, M. (2003) Nongenomic actions of steroid hormones. *Nat Rev Mol Cell Biol*, **4**, 46-56.

51.	Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Murray, T. and Thun, M.J. (2008) Cancer statistics, 2008. *CA Cancer J Clin*, **58**, 71-96.

52.	Stingl, J. and Caldas, C. (2007) Molecular heterogeneity of breast carcinomas and the cancer stem cell hypothesis. *Nat Rev Cancer*, **7**, 791-9.

53.	Vargo-Gogola, T. and Rosen, J.M. (2007) Modelling breast cancer: one size does not fit all. *Nat Rev Cancer*, **7**, 659-72.

54.	Ince, T.A., Richardson, A.L., Bell, G.W., Saitoh, M., Godar, S., Karnoub, A.E., Iglehart, J.D. and Weinberg, R.A. (2007) Transformation of different human breast epithelial cell types leads to distinct tumor phenotypes. *Cancer Cell*, **12**, 160-70.

55.	Bergh, J. and Holmquist, M. (2001) Who should not receive adjuvant chemotherapy? International databases. *J Natl Cancer Inst Monogr*, 103-8.

56.	Peppercorn, J., Perou, C.M. and Carey, L.A. (2008) Molecular subtypes in breast cancer evaluation and management: divide and conquer. *Cancer Invest*, **26**, 1-10.

57.	Perou, C.M., Sorlie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Rees, C.A., Pollack, J.R., Ross, D.T., Johnsen, H., Akslen, L.A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S.X., Lonning, P.E., Borresen-Dale, A.L., Brown, P.O. and Botstein, D. (2000) Molecular portraits of human breast tumours. *Nature*, **406**, 747-52.

58.	Sorlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Thorsen, T., Quist, H., Matese, J.C., Brown, P.O., Botstein, D., Eystein Lonning, P. and Borresen-Dale, A.L. (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A*, **98**, 10869-74.

59.	Brenton, J.D., Carey, L.A., Ahmed, A.A. and Caldas, C. (2005) Molecular classification and molecular forecasting of breast cancer: ready for clinical application? *J Clin Oncol*, **23**, 7350-60.

60.	Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J.S., Nobel, A., Deng, S., Johnsen, H., Pesich, R., Geisler, S., Demeter, J., Perou, C.M., Lonning, P.E., Brown, P.O., Borresen-Dale, A.L. and Botstein, D. (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A*, **100**, 8418-23.

61.	Nagata, Y., Lan, K.H., Zhou, X., Tan, M., Esteva, F.J., Sahin, A.A., Klos, K.S., Li, P., Monia, B.P., Nguyen, N.T., Hortobagyi, G.N., Hung, M.C. and Yu, D. (2004) PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell*, **6**, 117-27.

62.	Alexe, G., Dalgin, G.S., Scanfeld, D., Tamayo, P., Mesirov, J.P., DeLisi, C., Harris, L., Barnard, N., Martel, M., Levine, A.J., Ganesan, S. and Bhanot, G. (2007) High expression of lymphocyte-associated genes in node-negative HER2+ breast cancers correlates with lower recurrence rates. *Cancer Res*, **67**, 10669-76.

63. Carey, L.A., Perou, C.M., Livasy, C.A., Dressler, L.G., Cowan, D., Conway, K., Karaca, G., Troester, M.A., Tse, C.K., Edmiston, S., Deming, S.L., Geradts, J., Cheang, M.C., Nielsen, T.O., Moorman, P.G., Earp, H.S. and Millikan, R.C. (2006) Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. *Jama*, **295**, 2492-502.

64. Garland, C.F., Garland, F.C., Gorham, E.D., Lipkin, M., Newmark, H., Mohr, S.B. and Holick, M.F. (2006) The role of vitamin D in cancer prevention. *Am J Public Health*, **96**, 252-61.

65. Vijayakumar, S., Boerner, P.S., Mehta, R.R., Packianathan, S., Mehta, R.G. and Das Gupta, T.K. (2006) Clinical trials using chemopreventive vitamin D analogs in breast cancer. *Cancer J*, **12**, 445-50.

66. Osborn, J.L., Schwartz, G.G., Smith, D.C., Bahnson, R., Daye, R. and Trump, D.L. (1995) Phase II trial of oral 1,25-dihydroxyvitamin D (calcitriol) in hormone refractory prostate cancer. *Urol Oncol*, **1**, 195-198.

67. Gorham, E.D., Garland, C.F. and Garland, F.C. (1989) Acid haze air pollution and breast and colon cancer mortality in 20 Canadian cities. *Can J Public Health*, **80**, 96-100.

68. Garland, F.C., Garland, C.F., Gorham, E.D. and Young, J.F. (1990) Geographic variation in breast cancer mortality in the United States: a hypothesis involving exposure to solar radiation. *Prev Med*, **19**, 614-22.

69. Gorham, E.D., Garland, F.C. and Garland, C.F. (1990) Sunlight and breast cancer incidence in the USSR. *Int J Epidemiol*, **19**, 820-4.

70. Freedman, D.M., Dosemeci, M. and McGlynn, K. (2002) Sunlight and mortality from breast, ovarian, colon, prostate, and non-melanoma skin cancer: a composite death certificate based case-control study. *Occup Environ Med*, **59**, 257-62.

71. Grant, W.B. (2003) Ecologic studies of solar UV-B radiation and cancer mortality rates. *Recent Results Cancer Res*, **164**, 371-7.

72. Janowsky, E.C., Lester, G.E., Weinberg, C.R., Millikan, R.C., Schildkraut, J.M., Garrett, P.A. and Hulka, B.S. (1999) Association between low levels of 1,25-dihydroxyvitamin D and breast cancer risk. *Public Health Nutr*, **2**, 283-91.

73. Mawer, E.B., Walls, J., Howell, A., Davies, M., Ratcliffe, W.A. and Bundred, N.J. (1997) Serum 1,25-dihydroxyvitamin D may be related inversely to disease activity in breast cancer patients with bone metastases. *J Clin Endocrinol Metab*, **82**, 118-22.

74. Garland, C.F., Gorham, E.D., Mohr, S.B., Grant, W.B., Giovannucci, E.L., Lipkin, M., Newmark, H., Holick, M.F. and Garland, F.C. (2007) Vitamin D and prevention of breast cancer: pooled analysis. *J Steroid Biochem Mol Biol*, **103**, 708-11.

75. Wu, G., Fan, R.S., Li, W., Ko, T.C. and Brattain, M.G. (1997) Modulation of cell cycle control by vitamin D3 and its analogue, EB1089, in human breast cancer cells. *Oncogene*, **15**, 1555-63.

76. Verlinden, L., Verstuyf, A., Convents, R., Marcelis, S., Van Camp, M. and Bouillon, R. (1998) Action of 1,25(OH)2D3 on the cell cycle genes, cyclin D1, p21 and p27 in MCF-7 cells. *Mol Cell Endocrinol*, **142**, 57-65.

77. Verlinden, L., Verstuyf, A., Van Camp, M., Marcelis, S., Sabbe, K., Zhao, X.Y., De Clercq, P., Vandewalle, M. and Bouillon, R. (2000) Two novel 14-Epi-analogues of 1,25-dihydroxyvitamin D3 inhibit the growth of human breast cancer cells in vitro and in vivo. *Cancer Res*, **60**, 2673-9.

78. Jensen, S.S., Madsen, M.W., Lukas, J., Binderup, L. and Bartek, J. (2001) Inhibitory effects of 1alpha,25-dihydroxyvitamin D(3) on the G(1)-S phase-controlling machinery. *Mol Endocrinol*, **15**, 1370-80.

79. Flanagan, L., Packman, K., Juba, B., O'Neill, S., Tenniswood, M. and Welsh, J. (2003) Efficacy of Vitamin D compounds to modulate estrogen receptor negative breast cancer growth and invasion. *J Steroid Biochem Mol Biol*, **84**, 181-92.

80. Hussain-Hakimjee, E.A., Peng, X., Mehta, R.R. and Mehta, R.G. (2006) Growth inhibition of carcinogen-transformed MCF-12F breast epithelial cells and hormone-sensitive BT-474 breast cancer cells by 1alpha-hydroxyvitamin D5. *Carcinogenesis*, **27**, 551-9.

81. Koga, M., Eisman, J.A. and Sutherland, R.L. (1988) Regulation of epidermal growth factor receptor levels by 1,25-dihydroxyvitamin D3 in human breast cancer cells. *Cancer Res*, **48**, 2734-9.

82. McGaffin, K.R., Acktinson, L.E. and Chrysogelos, S.A. (2004) Growth and EGFR regulation in breast cancer cells by vitamin D and retinoid compounds. *Breast Cancer Res Treat*, **86**, 55-73.

83. Wu, G., Fan, R.S., Li, W., Srinivas, V. and Brattain, M.G. (1998) Regulation of transforming growth factor-beta type II receptor expression in human breast cancer MCF-7 cells by vitamin D3 and its analogues. *J Biol Chem*, **273**, 7749-56.

84. Yang, L., Yang, J., Venkateswarlu, S., Ko, T. and Brattain, M.G. (2001) Autocrine TGFbeta signaling mediates vitamin D3 analog-induced growth inhibition in breast cells. *J Cell Physiol*, **188**, 383-93.

85. Li, F., Ling, X., Huang, H., Brattain, L., Apontes, P., Wu, J., Binderup, L. and Brattain, M.G. (2005) Differential regulation of survivin expression and apoptosis by vitamin D3 compounds in two isogenic MCF-7 breast cancer cell sublines. *Oncogene*, **24**, 1385-95.

86. Capiati, D.A., Rossi, A.M., Picotto, G., Benassati, S. and Boland, R.L. (2004) Inhibition of serum-stimulated mitogen activated protein kinase by 1alpha,25(OH)2-vitamin D3 in MCF-7 breast cancer cells. *J Cell Biochem*, **93**, 384-97.

87. Rossi, A.M., Capiati, D.A., Picotto, G., Benassati, S. and Boland, R.L. (2004) MAPK inhibition by 1alpha,25(OH)2-Vitamin D3 in breast cancer cells. Evidence on the participation of the VDR and Src. *J Steroid Biochem Mol Biol*, **89-90**, 287-90.

88. Lazzaro, G., Agadir, A., Qing, W., Poria, M., Mehta, R.R., Moriarty, R.M., Das Gupta, T.K., Zhang, X.K. and Mehta, R.G. (2000) Induction of differentiation by 1alpha-hydroxyvitamin D(5) in T47D human breast cancer cells and its interaction with vitamin D receptors. *Eur J Cancer*, **36**, 780-6.

89. Mehta, R.R., Bratescu, L., Graves, J.M., Green, A. and Mehta, R.G. (2000) Differentiation of human breast carcinoma cells by a novel vitamin D analog: 1alpha-hydroxyvitamin D5. *Int J Oncol*, **16**, 65-73.

90. Sundaram, S., Chaudhry, M., Reardon, D., Gupta, M. and Gewirtz, D.A. (2000) The vitamin D3 analog EB 1089 enhances the antiproliferative and apoptotic effects of adriamycin in MCF-7 breast tumor cells. *Breast Cancer Res Treat*, **63**, 1-10.

91. Bizzarri, M., Cucina, A., Valente, M.G., Tagliaferri, F., Borrelli, V., Stipa, F. and Cavallaro, A. (2003) Melatonin and vitamin D3 increase TGF-beta1 release and induce growth inhibition in breast cancer cell cultures. *J Surg Res*, **110**, 332-7.

92. Chaudhry, M., Sundaram, S., Gennings, C., Carter, H. and Gewirtz, D.A. (2001) The vitamin D3 analog, ILX-23-7553, enhances the response to adriamycin and

irradiation in MCF-7 breast tumor cells. *Cancer Chemother Pharmacol*, **47**, 429-36.

93. James, S.Y., Mackay, A.G. and Colston, K.W. (1996) Effects of 1,25 dihydroxyvitamin D3 and its analogues on induction of apoptosis in breast cancer cells. *J Steroid Biochem Mol Biol*, **58**, 395-401.

94. Mathiasen, I.S., Lademann, U. and Jaattela, M. (1999) Apoptosis induced by vitamin D compounds in breast cancer cells is inhibited by Bcl-2 but does not involve known caspases or p53. *Cancer Res*, **59**, 4848-56.

95. Narvaez, C.J. and Welsh, J. (2001) Role of mitochondria and caspases in vitamin D-mediated apoptosis of MCF-7 breast cancer cells. *J Biol Chem*, **276**, 9101-7.

96. Simboli-Campbell, M., Narvaez, C.J., van Weelden, K., Tenniswood, M. and Welsh, J. (1997) Comparative effects of 1,25(OH)2D3 and EB1089 on cell cycle kinetics and apoptosis in MCF-7 breast cancer cells. *Breast Cancer Res Treat*, **42**, 31-41.

97. Narvaez, C.J., Byrne, B.M., Romu, S., Valrance, M. and Welsh, J. (2003) Induction of apoptosis by 1,25-dihydroxyvitamin D3 in MCF-7 Vitamin D3-resistant variant can be sensitized by TPA. *J Steroid Biochem Mol Biol*, **84**, 199-209.

98. Welsh, J. (1994) Induction of apoptosis in breast cancer cells in response to vitamin D and antiestrogens. *Biochem Cell Biol*, **72**, 537-45.

99. James, S.Y., Mackay, A.G. and Colston, K.W. (1995) Vitamin D derivatives in combination with 9-cis retinoic acid promote active cell death in breast cancer cells. *J Mol Endocrinol*, **14**, 391-4.

100. Sundaram, S. and Gewirtz, D.A. (1999) The vitamin D3 analog EB 1089 enhances the response of human breast tumor cells to radiation. *Radiat Res*, **152**, 479-86.

101. Polar, M.K., Gennings, C., Park, M., Gupta, M.S. and Gewirtz, D.A. (2003) Effect of the vitamin D3 analog ILX 23-7553 on apoptosis and sensitivity to fractionated radiation in breast tumor cells and normal human fibroblasts. *Cancer Chemother Pharmacol*, **51**, 415-21.

102. Koli, K. and Keski-Oja, J. (2000) 1alpha,25-dihydroxyvitamin D3 and its analogues down-regulate cell invasion-associated proteases in cultured malignant cells. *Cell Growth Differ*, **11**, 221-9.

103. Sundaram, S., Beckman, M.J., Bajwa, A., Wei, J., Smith, K.M., Posner, G.H. and Gewirtz, D.A. (2006) QW-1624F2-2, a synthetic analogue of 1,25-dihydroxyvitamin D3, enhances the response to other deltanoids and suppresses the invasiveness of human metastatic breast tumor cells. *Mol Cancer Ther*, **5**, 2806-14.

104. Mantell, D.J., Owens, P.E., Bundred, N.J., Mawer, E.B. and Canfield, A.E. (2000) 1 alpha,25-dihydroxyvitamin D(3) inhibits angiogenesis in vitro and in vivo. *Circ Res*, **87**, 214-20.

105. Majewski, S., Marczak, M., Szmurlo, A., Jablonska, S. and Bollag, W. (1995) Retinoids, interferon alpha, 1,25-dihydroxyvitamin D3 and their combination inhibit angiogenesis induced by non-HPV-harboring tumor cell lines. RAR alpha mediates the antiangiogenic effect of retinoids. *Cancer Lett*, **89**, 117-24.

106. El Abdaimi, K., Dion, N., Papavasiliou, V., Cardinal, P.E., Binderup, L., Goltzman, D., Ste-Marie, L.G. and Kremer, R. (2000) The vitamin D analogue EB 1089 prevents skeletal metastasis and prolongs survival time in nude mice transplanted with human breast cancer cells. *Cancer Res*, **60**, 4412-8.

107. Jacobson, E.A., James, K.A., Newmark, H.L. and Carroll, K.K. (1989) Effects of dietary fat, calcium, and vitamin D on growth and mammary tumorigenesis

induced by 7,12-dimethylbenz(a)anthracene in female Sprague-Dawley rats. *Cancer Res*, **49**, 6300-3.

108. Colston, K.W., Mackay, A.G., James, S.Y., Binderup, L., Chander, S. and Coombes, R.C. (1992) EB1089: a new vitamin D analogue that inhibits the growth of breast cancer cells in vivo and in vitro. *Biochem Pharmacol*, **44**, 2273-80.

109. Mehta, R.G., Moriarty, R.M., Mehta, R.R., Penmasta, R., Lazzaro, G., Constantinou, A. and Guo, L. (1997) Prevention of preneoplastic mammary lesion development by a novel vitamin D analogue, 1alpha-hydroxyvitamin D5. *J Natl Cancer Inst*, **89**, 212-8.

110. Mehta, R., Hawthorne, M., Uselding, L., Albinescu, D., Moriarty, R., Christov, K. and Mehta, R. (2000) Prevention of N-methyl-N-nitrosourea-induced mammary carcinogenesis in rats by 1alpha-hydroxyvitamin D(5). *J Natl Cancer Inst*, **92**, 1836-40.

111. Mehta, R.G. (2004) Stage-specific inhibition of mammary carcinogenesis by 1alpha-hydroxyvitamin D5. *Eur J Cancer*, **40**, 2331-7.

112. VanWeelden, K., Flanagan, L., Binderup, L., Tenniswood, M. and Welsh, J. (1998) Apoptotic regression of MCF-7 xenografts in nude mice treated with the vitamin D3 analog, EB1089. *Endocrinology*, **139**, 2102-10.

113. Koshizuka, K., Koike, M., Asou, H., Cho, S.K., Stephen, T., Rude, R.K., Binderup, L., Uskokovic, M. and Koeffler, H.P. (1999) Combined effect of vitamin D3 analogs and paclitaxel on the growth of MCF-7 breast cancer cells in vivo. *Breast Cancer Res Treat*, **53**, 113-20.

114. Koshizuka, K., Kubota, T., Said, J., Koike, M., Binderup, L., Uskokovic, M. and Koeffler, H.P. (1999) Combination therapy of a vitamin D3 analog and all-trans-retinoic acid: effect on human breast cancer in nude mice. *Anticancer Res*, **19**, 519-24.

115. Sundaram, S., Sea, A., Feldman, S., Strawbridge, R., Hoopes, P.J., Demidenko, E., Binderup, L. and Gewirtz, D.A. (2003) The combination of a potent vitamin D3 analog, EB 1089, with ionizing radiation reduces tumor growth and induces apoptosis of MCF-7 breast tumor xenografts in nude mice. *Clin Cancer Res*, **9**, 2350-6.

116. Abe-Hashimoto, J., Kikuchi, T., Matsumoto, T., Nishii, Y., Ogata, E. and Ikeda, K. (1993) Antitumor effect of 22-oxa-calcitriol, a noncalcemic analogue of calcitriol, in athymic mice implanted with human breast carcinoma and its synergism with tamoxifen. *Cancer Res*, **53**, 2534-7.

117. Punj, V., Graves, J.M. and Mehta, R.R. (2004) Effect of vitamin D analog (1alpha hydroxy D5) immunoconjugated to Her-2 antibody on breast cancer. *Int J Cancer*, **108**, 922-9.

118. Milliken, E.L., Zhang, X., Flask, C., Duerk, J.L., MacDonald, P.N. and Keri, R.A. (2005) EB1089, a vitamin D receptor agonist, reduces proliferation and decreases tumor growth rate in a mouse model of hormone-induced mammary cancer. *Cancer Lett*, **229**, 205-15.

119. Gulliford, T., English, J., Colston, K.W., Menday, P., Moller, S. and Coombes, R.C. (1998) A phase I study of the vitamin D analogue EB 1089 in patients with advanced breast and colorectal cancer. *Br J Cancer*, **78**, 6-13.

120. Group, T.W.s.H.I.S. (1998) Design of the Women's Health Initiative clinical trial and observational study. The Women's Health Initiative Study Group. *Control Clin Trials*, **19**, 61-109.

121. Prentice, R.L. and Anderson, G.L. (2008) The women's health initiative: lessons learned. *Annu Rev Public Health*, **29**, 131-50.

122. Palmer, H.G., Sanchez-Carbayo, M., Ordonez-Moran, P., Larriba, M.J., Cordon-Cardo, C. and Munoz, A. (2003) Genetic signatures of differentiation induced by 1alpha,25-dihydroxyvitamin D3 in human colon cancer cells. *Cancer Res*, **63**, 7799-806.

123. Katayama, M.L., Pasini, F.S., Folgueira, M.A., Snitcovsky, I.M. and Brentani, M.M. (2003) Molecular targets of 1,25(OH)2D3 in HC11 normal mouse mammary cell line. *J Steroid Biochem Mol Biol*, **84**, 57-69.

124. Guzey, M., Luo, J. and Getzenberg, R.H. (2004) Vitamin D3 modulated gene expression patterns in human primary normal and cancer prostate cells. *J Cell Biochem*, **93**, 271-85.

125. Krishnan, A.V., Shinghal, R., Raghavachari, N., Brooks, J.D., Peehl, D.M. and Feldman, D. (2004) Analysis of vitamin D-regulated gene expression in LNCaP human prostate cancer cells using cDNA microarrays. *Prostate*, **59**, 243-51.

126. Peehl, D.M., Shinghal, R., Nonn, L., Seto, E., Krishnan, A.V., Brooks, J.D. and Feldman, D. (2004) Molecular activity of 1,25-dihydroxyvitamin D3 in primary cultures of human prostatic epithelial cells revealed by cDNA microarray analysis. *J Steroid Biochem Mol Biol*, **92**, 131-41.

127. Zhang, X., Li, P., Bao, J., Nicosia, S.V., Wang, H., Enkemann, S.A. and Bai, W. (2005) Suppression of death receptor-mediated apoptosis by 1,25-dihydroxyvitamin D3 revealed by microarray analysis. *J Biol Chem*, **280**, 35458-68.

128. Nagpal, S., Na, S. and Rathnachalam, R. (2005) Noncalcemic actions of vitamin D receptor ligands. *Endocr Rev*, **26**, 662-87.

129. Bierie, B. and Moses, H.L. (2006) Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. *Nat Rev Cancer*, **6**, 506-20.

130. Siegel, P.M. and Massague, J. (2003) Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer*, **3**, 807-21.

131. Massague, J. (1998) TGF-beta signal transduction. *Annu Rev Biochem*, **67**, 753-91.

132. Miyazono, K., Maeda, S. and Imamura, T. (2005) BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. *Cytokine Growth Factor Rev*, **16**, 251-63.

133. Bharathy, S., Xie, W., Yingling, J.M. and Reiss, M. (2008) Cancer-associated transforming growth factor beta type II receptor gene mutant causes activation of bone morphogenic protein-Smads and invasive phenotype. *Cancer Res*, **68**, 1656-66.

134. Goumans, M.J., Valdimarsdottir, G., Itoh, S., Rosendahl, A., Sideras, P. and ten Dijke, P. (2002) Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *Embo J*, **21**, 1743-53.

135. Liu, X., Yue, J., Frey, R.S., Zhu, Q. and Mulder, K.M. (1998) Transforming growth factor beta signaling through Smad1 in human breast cancer cells. *Cancer Res*, **58**, 4752-7.

136. Derynck, R. and Zhang, Y.E. (2003) Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature*, **425**, 577-84.

137. Hatakeyama, S., Ohara-Nemoto, Y., Kyakumoto, S. and Satoh, M. (1996) Retinoic acid enhances expression of bone morphogenetic protein-2 in human adenocarcinoma cell line (HSG-S8). *Biochem Mol Biol Int*, **38**, 1235-43.

138. Jung, C.W., Kim, E.S., Seol, J.G., Park, W.H., Lee, S.J., Kim, B.K. and Lee, Y.Y. (1999) Antiproliferative effect of a vitamin D3 analog, EB1089, on HL-60 cells by the induction of TGF-beta receptor. *Leuk Res*, **23**, 1105-12.

139. Yanagi, Y., Suzawa, M., Kawabata, M., Miyazono, K., Yanagisawa, J. and Kato, S. (1999) Positive and negative modulation of vitamin D receptor function by transforming growth factor-beta signaling through smad proteins. *J Biol Chem*, **274**, 12971-4.

140. Yanagisawa, J., Yanagi, Y., Masuhiro, Y., Suzawa, M., Watanabe, M., Kashiwagi, K., Toriyabe, T., Kawabata, M., Miyazono, K. and Kato, S. (1999) Convergence of transforming growth factor-beta and vitamin D signaling pathways on SMAD transcriptional coactivators. *Science*, **283**, 1317-21.

141. Teicher, B.A. (2006) Protein kinase C as a therapeutic target. *Clin Cancer Res*, **12**, 5336-45.

142. Griner, E.M. and Kazanietz, M.G. (2007) Protein kinase C and other diacylglycerol effectors in cancer. *Nat Rev Cancer*, **7**, 281-94.

143. Detjen, K.M., Brembeck, F.H., Welzel, M., Kaiser, A., Haller, H., Wiedenmann, B. and Rosewicz, S. (2000) Activation of protein kinase Calpha inhibits growth of pancreatic cancer cells via p21(cip)-mediated G(1) arrest. *J Cell Sci*, **113 (Pt 17)**, 3025-35.

144. Oster, H. and Leitges, M. (2006) Protein kinase C alpha but not PKCzeta suppresses intestinal tumor formation in ApcMin/+ mice. *Cancer Res*, **66**, 6955-63.

145. Buitrago, C.G., Pardo, V.G., de Boland, A.R. and Boland, R. (2003) Activation of RAF-1 through Ras and protein kinase Calpha mediates 1alpha,25(OH)2-vitamin D3 regulation of the mitogen-activated protein kinase pathway in muscle cells. *J Biol Chem*, **278**, 2199-205.

146. Boyan, B.D., Wang, L., Wong, K.L., Jo, H. and Schwartz, Z. (2006) Plasma membrane requirements for 1alpha,25(OH)2D3 dependent PKC signaling in chondrocytes and osteoblasts. *Steroids*, **71**, 286-90.

147. Boyan, B.D., Wong, K.L., Wang, L., Yao, H., Guldberg, R.E., Drab, M., Jo, H. and Schwartz, Z. (2006) Regulation of growth plate chondrocytes by 1,25-dihydroxyvitamin D3 requires caveolae and caveolin-1. *J Bone Miner Res*, **21**, 1637-47.

148. Hughes, P.J. and Brown, G. (2006) 1Alpha,25-dihydroxyvitamin D3-mediated stimulation of steroid sulphatase activity in myeloid leukaemic cell lines requires VDRnuc-mediated activation of the RAS/RAF/ERK-MAP kinase signalling pathway. *J Cell Biochem*, **98**, 590-617.

149. Chen, A., Davis, B.H., Bissonnette, M., Scaglione-Sewell, B. and Brasitus, T.A. (1999) 1,25-Dihydroxyvitamin D(3) stimulates activator protein-1-dependent Caco-2 cell differentiation. *J Biol Chem*, **274**, 35505-13.

150. Grimberg, A. and Cohen, P. (2000) Role of insulin-like growth factors and their binding proteins in growth control and carcinogenesis. *J Cell Physiol*, **183**, 1-9.

151. Chan, J.M., Stampfer, M.J., Giovannucci, E., Gann, P.H., Ma, J., Wilkinson, P., Hennekens, C.H. and Pollak, M. (1998) Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. *Science*, **279**, 563-6.

152. Peng, L., Malloy, P.J., Wang, J. and Feldman, D. (2006) Growth inhibitory concentrations of androgens up-regulate insulin-like growth factor binding protein-3 expression via an androgen response element in LNCaP human prostate cancer cells. *Endocrinology*, **147**, 4599-607.

153. Peng, L., Wang, J., Malloy, P.J. and Feldman, D. (2007) The role of insulin-like growth factor binding protein-3 in the growth inhibitory actions of androgens in LNCaP human prostate cancer cells. *Int J Cancer*, **122**, 558-566.

154. Silha, J.V., Sheppard, P.C., Mishra, S., Gui, Y., Schwartz, J., Dodd, J.G. and Murphy, L.J. (2006) Insulin-like growth factor (IGF) binding protein-3 attenuates

prostate tumor growth by IGF-dependent and IGF-independent mechanisms. *Endocrinology*, **147**, 2112-21.

155. Pazaitou-Panayiotou, K., Kelesidis, T., Kelesidis, I., Kaprara, A., Blakeman, J., Vainas, I., Mpousoulegas, A., Williams, C.J. and Mantzoros, C. (2007) Growth hormone-binding protein is directly and IGFBP-3 is inversely associated with risk of female breast cancer. *Eur J Endocrinol*, **156**, 187-94.

156. Tomii, K., Tsukuda, K., Toyooka, S., Dote, H., Hanafusa, T., Asano, H., Naitou, M., Doihara, H., Kisimoto, T., Katayama, H., Pass, H.I., Date, H. and Shimizu, N. (2007) Aberrant promoter methylation of insulin-like growth factor binding protein-3 gene in human cancers. *Int J Cancer*, **120**, 566-73.

157. Renehan, A.G., Harvie, M. and Howell, A. (2006) Insulin-like growth factor (IGF)-I, IGF binding protein-3, and breast cancer risk: eight years on. *Endocr Relat Cancer*, **13**, 273-8.

158. Schernhammer, E.S., Holly, J.M., Hunter, D.J., Pollak, M.N. and Hankinson, S.E. (2006) Insulin-like growth factor-I, its binding proteins (IGFBP-1 and IGFBP-3), and growth hormone and breast cancer risk in The Nurses Health Study II. *Endocr Relat Cancer*, **13**, 583-92.

159. Boyle, B.J., Zhao, X.Y., Cohen, P. and Feldman, D. (2001) Insulin-like growth factor binding protein-3 mediates 1 alpha,25-dihydroxyvitamin d(3) growth inhibition in the LNCaP prostate cancer cell line through p21/WAF1. *J Urol*, **165**, 1319-24.

160. Stewart, L.V., Lyles, B., Lin, M.F. and Weigel, N.L. (2005) Vitamin D receptor agonists induce prostatic acid phosphatase to reduce cell growth and HER-2 signaling in LNCaP-derived human prostate cancer cells. *J Steroid Biochem Mol Biol*, **97**, 37-46.

161. Oh, Y.S., Kim, E.J., Schaffer, B.S., Kang, Y.H., Binderup, L., MacDonald, R.G. and Park, J.H. (2001) Synthetic low-calcaemic vitamin D(3) analogues inhibit secretion of insulin-like growth factor II and stimulate production of insulin-like growth factor-binding protein-6 in conjunction with growth suppression of HT-29 colon cancer cells. *Mol Cell Endocrinol*, **183**, 141-9.

162. Swami, S., Raghavachari, N., Muller, U.R., Bao, Y.P. and Feldman, D. (2003) Vitamin D growth inhibition of breast cancer cells: gene expression patterns assessed by cDNA microarray. *Breast Cancer Res Treat*, **80**, 49-62.

163. Peng, L., Malloy, P.J. and Feldman, D. (2004) Identification of a functional vitamin D response element in the human insulin-like growth factor binding protein-3 promoter. *Mol Endocrinol*, **18**, 1109-19.

164. Matilainen, M., Malinen, M., Saavalainen, K. and Carlberg, C. (2005) Regulation of multiple insulin-like growth factor binding protein genes by 1alpha,25-dihydroxyvitamin D3. *Nucleic Acids Res*, **33**, 5521-32.

165. Carlberg, C., Dunlop, T.W., Saramaki, A., Sinkkonen, L., Matilainen, M. and Vaisanen, S. (2007) Controlling the chromatin organization of vitamin D target genes by multiple vitamin D receptor binding sites. *J Steroid Biochem Mol Biol*, **103**, 338-43.

166. Hackel, P.O., Zwick, E., Prenzel, N. and Ullrich, A. (1999) Epidermal growth factor receptors: critical mediators of multiple receptor pathways. *Curr Opin Cell Biol*, **11**, 184-9.

167. Oda, K., Matsuoka, Y., Funahashi, A. and Kitano, H. (2005) A comprehensive pathway map of epidermal growth factor receptor signaling. *Mol Syst Biol*, **1**, 2005 0010.

168. Tong, W.M., Hofer, H., Ellinger, A., Peterlik, M. and Cross, H.S. (1999) Mechanism of antimitogenic action of vitamin D in human colon carcinoma cells:

relevance for suppression of epidermal growth factor-stimulated cell growth. *Oncol Res*, **11**, 77-84.

169. Conacci-Sorrell, M., Zhurinsky, J. and Ben-Ze'ev, A. (2002) The cadherin-catenin adhesion system in signaling and cancer. *J Clin Invest*, **109**, 987-91.

170. Sellin, J.H., Umar, S., Xiao, J. and Morris, A.P. (2001) Increased beta-catenin expression and nuclear translocation accompany cellular hyperproliferation in vivo. *Cancer Res*, **61**, 2899-906.

171. Campbell, M.J., Elstner, E., Holden, S., Uskokovic, M. and Koeffler, H.P. (1997) Inhibition of proliferation of prostate cancer cells by a 19-nor-hexafluoride vitamin D3 analogue involves the induction of p21waf1, p27kip1 and E-cadherin. *J Mol Endocrinol*, **19**, 15-27.

172. Palmer, H.G., Gonzalez-Sancho, J.M., Espada, J., Berciano, M.T., Puig, I., Baulida, J., Quintanilla, M., Cano, A., de Herreros, A.G., Lafarga, M. and Munoz, A. (2001) Vitamin D(3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of beta-catenin signaling. *J Cell Biol*, **154**, 369-87.

173. Aguilera, O., Pena, C., Garcia, J.M., Larriba, M.J., Ordonez-Moran, P., Navarro, D., Barbachano, A., Lopez de Silanes, I., Ballestar, E., Fraga, M.F., Esteller, M., Gamallo, C., Bonilla, F., Gonzalez-Sancho, J.M. and Munoz, A. (2007) The Wnt antagonist DICKKOPF-1 gene is induced by 1alpha,25-dihydroxyvitamin D3 associated to the differentiation of human colon cancer cells. *Carcinogenesis*, **28**, 1877-84.

174. Hmama, Z., Nandan, D., Sly, L., Knutson, K.L., Herrera-Velit, P. and Reiner, N.E. (1999) 1alpha,25-dihydroxyvitamin D(3)-induced myeloid cell differentiation is regulated by a vitamin D receptor-phosphatidylinositol 3-kinase signaling complex. *J Exp Med*, **190**, 1583-94.

175. Norman, A.W., Manchand, P.S., Uskokovic, M.R., Okamura, W.H., Takeuchi, J.A., Bishop, J.E., Hisatake, J.I., Koeffler, H.P. and Peleg, S. (2000) Characterization of a novel analogue of 1alpha,25(OH)(2)-vitamin D(3) with two side chains: interaction with its nuclear receptor and cellular actions. *J Med Chem*, **43**, 2719-30.

176. Molnar, F., Perakyla, M. and Carlberg, C. (2006) Vitamin D receptor agonists specifically modulate the volume of the ligand-binding pocket. *J Biol Chem*, **281**, 10516-26.

177. Vaisanen, S., Perakyla, M., Karkkainen, J.I., Uskokovic, M.R. and Carlberg, C. (2003) Structural evaluation of the agonistic action of a vitamin D analog with two side chains binding to the nuclear vitamin D receptor. *Mol Pharmacol*, **63**, 1230-7.

178. Ciesielski, F., Rochel, N. and Moras, D. (2007) Adaptability of the Vitamin D nuclear receptor to the synthetic ligand Gemini: remodelling the LBP with one side chain rotation. *J Steroid Biochem Mol Biol*, **103**, 235-42.

179. Bury, Y., Herdick, M., Uskokovic, M.R. and Carlberg, C. (2001) Gene regulatory potential of 1alpha,25-dihydroxyvitamin D(3) analogues with two side chains. *J Cell Biochem*, **81**, 179-190.

180. Weyts, F.A., Dhawan, P., Zhang, X., Bishop, J.E., Uskokovic, M.R., Ji, Y., Studzinski, G.P., Norman, A.W. and Christakos, S. (2004) Novel Gemini analogs of 1alpha,25-dihydroxyvitamin D(3) with enhanced transcriptional activity. *Biochem Pharmacol*, **67**, 1327-36.

181. O'Kelly, J., Uskokovic, M., Lemp, N., Vadgama, J. and Koeffler, H.P. (2006) Novel Gemini-vitamin D3 analog inhibits tumor cell growth and modulates the Akt/mTOR signaling pathway. *J Steroid Biochem Mol Biol*, **100**, 107-16.

182. Spina, C., Tangpricha, V., Yao, M., Zhou, W., Wolfe, M.M., Maehr, H., Uskokovic, M., Adorini, L. and Holick, M.F. (2005) Colon cancer and solar ultraviolet B radiation and prevention and treatment of colon cancer in mice with vitamin D and its Gemini analogs. *J Steroid Biochem Mol Biol*, **97**, 111-20.

183. Spina, C.S., Ton, L., Yao, M., Maehr, H., Wolfe, M.M., Uskokovic, M., Adorini, L. and Holick, M.F. (2007) Selective vitamin D receptor modulators and their effects on colorectal tumor growth. *J Steroid Biochem Mol Biol*, **103**, 757-62.

184. Maehr, H., Uskokovic, M., Adorini, L., Penna, G., Mariani, R., Panina, P., Passini, N., Bono, E., Perego, S., Biffi, M., Holick, M., Spina, C. and Suh, N. (2007) Calcitriol derivatives with two different side chains at C-20 III. An epimeric pair of the gemini family with unprecedented antiproliferative effects on tumor cells and renin mRNA expression inhibition. *J Steroid Biochem Mol Biol*, **103**, 277-81.

185. Miller, F.R., Soule, H.D., Tait, L., Pauley, R.J., Wolman, S.R., Dawson, P.J. and Heppner, G.H. (1993) Xenograft model of progressive human proliferative breast disease. *J Natl Cancer Inst*, **85**, 1725-32.

186. Miller, F.R., Santner, S.J., Tait, L. and Dawson, P.J. (2000) MCF10DCIS.com xenograft model of human comedo ductal carcinoma in situ. *J Natl Cancer Inst*, **92**, 1185-6.

187. Strickland, L.B., Dawson, P.J., Santner, S.J. and Miller, F.R. (2000) Progression of premalignant MCF10AT generates heterogeneous malignant variants with characteristic histologic types and immunohistochemical markers. *Breast Cancer Res Treat*, **64**, 235-40.

188. Santner, S.J., Dawson, P.J., Tait, L., Soule, H.D., Eliason, J., Mohamed, A.N., Wolman, S.R., Heppner, G.H. and Miller, F.R. (2001) Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. *Breast Cancer Res Treat*, **65**, 101-10.

189. Tang, B., Vu, M., Booker, T., Santner, S.J., Miller, F.R., Anver, M.R. and Wakefield, L.M. (2003) TGF-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *J Clin Invest*, **112**, 1116-24.

190. Tait, L.R., Pauley, R.J., Santner, S.J., Heppner, G.H., Heng, H.H., Rak, J.W. and Miller, F.R. (2007) Dynamic stromal-epithelial interactions during progression of MCF10DCIS.com xenografts. *Int J Cancer*, **120**, 2127-34.

191. Hu, M., Yao, J., Carroll, D.K., Weremowicz, S., Chen, H., Carrasco, D., Richardson, A., Violette, S., Nikolskaya, T., Nikolsky, Y., Bauerlein, E.L., Hahn, W.C., Gelman, R.S., Allred, C., Bissell, M.J., Schnitt, S. and Polyak, K. (2008) Regulation of in situ to invasive breast carcinoma transition. *Cancer Cell*, **13**, 394-406.

192. Miller, F.R. (2000) Xenograft models of premalignant breast disease. *J Mammary Gland Biol Neoplasia*, **5**, 379-91.

193. Russo, J. and Russo, I.H. (2000) Atlas and histologic classification of tumors of the rat mammary gland. *J Mammary Gland Biol Neoplasia*, **5**, 187-200.

194. Thompson, H.J. and Singh, M. (2000) Rat models of premalignant breast disease. *J Mammary Gland Biol Neoplasia*, **5**, 409-20.

195. Chan, M.M., Lu, X., Merchant, F.M., Iglehart, J.D. and Miron, P.L. (2005) Gene expression profiling of NMU-induced rat mammary tumors: cross species comparison with human breast cancer. *Carcinogenesis*, **26**, 1343-53.

196. Suh, N., Glasebrook, A.L., Palkowitz, A.D., Bryant, H.U., Burris, L.L., Starling, J.J., Pearce, H.L., Williams, C., Peer, C., Wang, Y. and Sporn, M.B. (2001) Arzoxifene, a new selective estrogen receptor modulator for chemoprevention of experimental breast cancer. *Cancer Res*, **61**, 8412-5.

197. Heppner, G.H., Miller, F.R. and Shekhar, P.M. (2000) Nontransgenic models of breast cancer. *Breast Cancer Res*, **2**, 331-4.
198. Hovey, R.C., McFadden, T.B. and Akers, R.M. (1999) Regulation of mammary gland growth and morphogenesis by the mammary fat pad: a species comparison. *J Mammary Gland Biol Neoplasia*, **4**, 53-68.
199. Winston, J.S., Ramanaryanan, J. and Levine, E. (2004) HER-2/neu evaluation in breast cancer are we there yet? *Am J Clin Pathol*, **121 Suppl**, S33-49.
200. Guy, C.T., Webster, M.A., Schaller, M., Parsons, T.J., Cardiff, R.D. and Muller, W.J. (1992) Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci U S A*, **89**, 10578-82.
201. Green, J.E. and Hudson, T. (2005) The promise of genetically engineered mice for cancer prevention studies. *Nat Rev Cancer*, **5**, 184-98.
202. Cardiff, R.D. and Wellings, S.R. (1999) The comparative pathology of human and mouse mammary glands. *J Mammary Gland Biol Neoplasia*, **4**, 105-22.
203. Cardiff, R.D., Anver, M.R., Gusterson, B.A., Hennighausen, L., Jensen, R.A., Merino, M.J., Rehm, S., Russo, J., Tavassoli, F.A., Wakefield, L.M., Ward, J.M. and Green, J.E. (2000) The mammary pathology of genetically engineered mice: the consensus report and recommendations from the Annapolis meeting. *Oncogene*, **19**, 968-88.
204. Zinser, G.M. and Welsh, J. (2004) Vitamin D receptor status alters mammary gland morphology and tumorigenesis in MMTV-neu mice. *Carcinogenesis*, **25**, 2361-72.
205. Evans, T.R., Colston, K.W., Lofts, F.J., Cunningham, D., Anthoney, D.A., Gogas, H., de Bono, J.S., Hamberg, K.J., Skov, T. and Mansi, J.L. (2002) A phase II trial of the vitamin D analogue Seocalcitol (EB1089) in patients with inoperable pancreatic cancer. *Br J Cancer*, **86**, 680-5.
206. Dalhoff, K., Dancey, J., Astrup, L., Skovsgaard, T., Hamberg, K.J., Lofts, F.J., Rosmorduc, O., Erlinger, S., Bach Hansen, J., Steward, W.P., Skov, T., Burcharth, F. and Evans, T.R. (2003) A phase II study of the vitamin D analogue Seocalcitol in patients with inoperable hepatocellular carcinoma. *Br J Cancer*, **89**, 252-7.
207. Soh, J.W., Lee, E.H., Prywes, R. and Weinstein, I.B. (1999) Novel roles of specific isoforms of protein kinase C in activation of the c-fos serum response element. *Mol Cell Biol*, **19**, 1313-24.
208. von Lintig, F.C., Dreilinger, A.D., Varki, N.M., Wallace, A.M., Casteel, D.E. and Boss, G.R. (2000) Ras activation in human breast cancer. *Breast Cancer Res Treat*, **62**, 51-62.
209. Johnson, G.L. and Lapadat, R. (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science*, **298**, 1911-2.
210. Vivanco, I. and Sawyers, C.L. (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer*, **2**, 489-501.
211. Eelen, G., Verlinden, L., Van Camp, M., Mathieu, C., Carmeliet, G., Bouillon, R. and Verstuyf, A. (2004) Microarray analysis of 1alpha,25-dihydroxyvitamin D3-treated MC3T3-E1 cells. *J Steroid Biochem Mol Biol*, **89-90**, 405-7.
212. Swami, S., Krishnan, A.V., Peehl, D.M. and Feldman, D. (2005) Genistein potentiates the growth inhibitory effects of 1,25-dihydroxyvitamin D3 in DU145 human prostate cancer cells: role of the direct inhibition of CYP24 enzyme activity. *Mol Cell Endocrinol*, **241**, 49-61.

213. Zhang, X., Li, W. and Olumi, A.F. (2008) Chapter 20 Overcoming Resistance to Trail-Induced Apoptosis in Prostate Cancer by Regulation of c-FLIP. *Methods Enzymol*, **446**, 333-49.

214. Helms, M.W., Packeisen, J., August, C., Schittek, B., Boecker, W., Brandt, B.H. and Buerger, H. (2005) First evidence supporting a potential role for the BMP/SMAD pathway in the progression of oestrogen receptor-positive breast cancer. *J Pathol*, **206**, 366-76.

215. Montesano, R. (2007) Bone morphogenetic protein-4 abrogates lumen formation by mammary epithelial cells and promotes invasive growth. *Biochem Biophys Res Commun*, **353**, 817-22.

216. Pouliot, F. and Labrie, C. (2002) Role of Smad1 and Smad4 proteins in the induction of p21WAF1,Cip1 during bone morphogenetic protein-induced growth arrest in human breast cancer cells. *J Endocrinol*, **172**, 187-98.

217. Pardali, K., Kowanetz, M., Heldin, C.H. and Moustakas, A. (2005) Smad pathway-specific transcriptional regulation of the cell cycle inhibitor p21(WAF1/Cip1). *J Cell Physiol*, **204**, 260-72.

218. Miyazaki, H., Watabe, T., Kitamura, T. and Miyazono, K. (2004) BMP signals inhibit proliferation and in vivo tumor growth of androgen-insensitive prostate carcinoma cells. *Oncogene*, **23**, 9326-35.

219. Sneddon, J.B., Zhen, H.H., Montgomery, K., van de Rijn, M., Tward, A.D., West, R., Gladstone, H., Chang, H.Y., Morganroth, G.S., Oro, A.E. and Brown, P.O. (2006) Bone morphogenetic protein antagonist gremlin 1 is widely expressed by cancer-associated stromal cells and can promote tumor cell proliferation. *Proc Natl Acad Sci U S A*, **103**, 14842-7.

220. Horvath, L.G., Henshall, S.M., Kench, J.G., Turner, J.J., Golovsky, D., Brenner, P.C., O'Neill, G.F., Kooner, R., Stricker, P.D., Grygiel, J.J. and Sutherland, R.L. (2004) Loss of BMP2, Smad8, and Smad4 expression in prostate cancer progression. *Prostate*, **59**, 234-42.

221. Haramis, A.P., Begthel, H., van den Born, M., van Es, J., Jonkheer, S., Offerhaus, G.J. and Clevers, H. (2004) De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. *Science*, **303**, 1684-6.

222. Ishida, W., Hamamoto, T., Kusanagi, K., Yagi, K., Kawabata, M., Takehara, K., Sampath, T.K., Kato, M. and Miyazono, K. (2000) Smad6 is a Smad1/5-induced smad inhibitor. Characterization of bone morphogenetic protein-responsive element in the mouse Smad6 promoter. *J Biol Chem*, **275**, 6075-9.

223. Bettoun, D.J., Lu, J., Khalifa, B., Yee, Y., Chin, W.W. and Nagpal, S. (2004) Ligand modulates VDR-Ser/Thr protein phosphatase interaction and p70S6 kinase phosphorylation in a cell-context-dependent manner. *J Steroid Biochem Mol Biol*, **89-90**, 195-8.

224. Cao, Z., Flanders, K.C., Bertolette, D., Lyakh, L.A., Wurthner, J.U., Parks, W.T., Letterio, J.J., Ruscetti, F.W. and Roberts, A.B. (2003) Levels of phospho-Smad2/3 are sensors of the interplay between effects of TGF-beta and retinoic acid on monocytic and granulocytic differentiation of HL-60 cells. *Blood*, **101**, 498-507.

225. Reinholz, M.M., Iturria, S.J., Ingle, J.N. and Roche, P.C. (2002) Differential gene expression of TGF-beta family members and osteopontin in breast tumor tissue: analysis by real-time quantitative PCR. *Breast Cancer Res Treat*, **74**, 255-69.

226. Fukuda, N., Saitoh, M., Kobayashi, N. and Miyazono, K. (2006) Execution of BMP-4-induced apoptosis by p53-dependent ER dysfunction in myeloma and B-cell hybridoma cells. *Oncogene*, **25**, 3509-17.

227. Stabile, H., Mitola, S., Moroni, E., Belleri, M., Nicoli, S., Coltrini, D., Peri, F., Pessi, A., Orsatti, L., Talamo, F., Castronovo, V., Waltregny, D., Cotelli, F., Ribatti, D. and Presta, M. (2007) Bone morphogenic protein antagonist Drm/gremlin is a novel proangiogenic factor. *Blood*, **109**, 1834-40.

228. Alarmo, E.L., Kuukasjarvi, T., Karhu, R. and Kallioniemi, A. (2007) A comprehensive expression survey of bone morphogenetic proteins in breast cancer highlights the importance of BMP4 and BMP7. *Breast Cancer Res Treat*, **103**, 239-46.

229. Falk, L.A., De Benedetti, F., Lohrey, N., Birchenall-Roberts, M.C., Ellingsworth, L.W., Faltynek, C.R. and Ruscetti, F.W. (1991) Induction of transforming growth factor-beta 1 (TGF-beta 1), receptor expression and TGF-beta 1 protein production in retinoic acid-treated HL-60 cells: possible TGF-beta 1-mediated autocrine inhibition. *Blood*, **77**, 1248-55.

230. Matsuda, T., Yamamoto, T., Muraguchi, A. and Saatcioglu, F. (2001) Cross-talk between transforming growth factor-beta and estrogen receptor signaling through Smad3. *J Biol Chem*, **276**, 42908-14.

231. Hallahan, A.R., Pritchard, J.I., Chandraratna, R.A., Ellenbogen, R.G., Geyer, J.R., Overland, R.P., Strand, A.D., Tapscott, S.J. and Olson, J.M. (2003) BMP-2 mediates retinoid-induced apoptosis in medulloblastoma cells through a paracrine effect. *Nat Med*, **9**, 1033-8.

232. Koli, K. and Keski-Oja, J. (1995) 1,25-Dihydroxyvitamin D3 enhances the expression of transforming growth factor beta 1 and its latent form binding protein in cultured breast carcinoma cells. *Cancer Res*, **55**, 1540-6.

233. Imamura, T., Takase, M., Nishihara, A., Oeda, E., Hanai, J., Kawabata, M. and Miyazono, K. (1997) Smad6 inhibits signalling by the TGF-beta superfamily. *Nature*, **389**, 622-6.

234. Chen, D., Zhao, M. and Mundy, G.R. (2004) Bone morphogenetic proteins. *Growth Factors*, **22**, 233-41.

235. Hata, A., Lagna, G., Massague, J. and Hemmati-Brivanlou, A. (1998) Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. *Genes Dev*, **12**, 186-97.

236. Murakami, G., Watabe, T., Takaoka, K., Miyazono, K. and Imamura, T. (2003) Cooperative inhibition of bone morphogenetic protein signaling by Smurf1 and inhibitory Smads. *Mol Biol Cell*, **14**, 2809-17.

237. Lee, H.J., Liu, H., Goodman, C., Ji, Y., Maehr, H., Uskokovic, M., Notterman, D., Reiss, M. and Suh, N. (2006) Gene expression profiling changes induced by a novel Gemini Vitamin D derivative during the progression of breast cancer. *Biochem Pharmacol*, **72**, 332-43.

238. Lee, H.J., Wislocki, A., Goodman, C., Ji, Y., Ge, R., Maehr, H., Uskokovic, M., Reiss, M. and Suh, N. (2006) A novel vitamin D derivative activates bone morphogenetic protein signaling in MCF10 breast epithelial cells. *Mol Pharmacol*, **69**, 1840-8.

239. Yakymovych, I., Ten Dijke, P., Heldin, C.H. and Souchelnytskyi, S. (2001) Regulation of Smad signaling by protein kinase C. *Faseb J*, **15**, 553-5.

240. Hay, E., Lemonnier, J., Fromigue, O. and Marie, P.J. (2001) Bone morphogenetic protein-2 promotes osteoblast apoptosis through a Smad-independent, protein kinase C-dependent signaling pathway. *J Biol Chem*, **276**, 29028-36.

241. Berg, D.T., Myers, L.J., Richardson, M.A., Sandusky, G. and Grinnell, B.W. (2005) Smad6s regulates plasminogen activator inhibitor-1 through a protein kinase C-beta-dependent up-regulation of transforming growth factor-beta. *J Biol Chem*, **280**, 14943-7.

242. Runyan, C.E., Schnaper, H.W. and Poncelet, A.C. (2003) Smad3 and PKCdelta mediate TGF-beta1-induced collagen I expression in human mesangial cells. *Am J Physiol Renal Physiol*, **285**, F413-22.

243. Yue, J. and Mulder, K.M. (2000) Requirement of Ras/MAPK pathway activation by transforming growth factor beta for transforming growth factor beta 1 production in a smad-dependent pathway. *J Biol Chem*, **275**, 35656.

244. Lhuillier, L. and Dryer, S.E. (2003) Ras is a mediator of TGFbeta1 signaling in developing chick ciliary ganglion neurons. *Brain Res*, **982**, 119-24.

245. Xie, L., Law, B.K., Chytil, A.M., Brown, K.A., Aakre, M.E. and Moses, H.L. (2004) Activation of the Erk pathway is required for TGF-beta1-induced EMT in vitro. *Neoplasia*, **6**, 603-10.

246. Davies, M., Robinson, M., Smith, E., Huntley, S., Prime, S. and Paterson, I. (2005) Induction of an epithelial to mesenchymal transition in human immortal and malignant keratinocytes by TGF-beta1 involves MAPK, Smad and AP-1 signalling pathways. *J Cell Biochem*, **95**, 918-31.

247. Cordenonsi, M., Montagner, M., Adorno, M., Zacchigna, L., Martello, G., Mamidi, A., Soligo, S., Dupont, S. and Piccolo, S. (2007) Integration of TGF-beta and Ras/MAPK signaling through p53 phosphorylation. *Science*, **315**, 840-3.

248. Zhao, Y., Zhang, L. and Longo, L.D. (2005) PKC-induced ERK1/2 interactions and downstream effectors in ovine cerebral arteries. *Am J Physiol Regul Integr Comp Physiol*, **289**, R164-71.

249. Bivona, T.G., Quatela, S.E., Bodemann, B.O., Ahearn, I.M., Soskis, M.J., Mor, A., Miura, J., Wiener, H.H., Wright, L., Saba, S.G., Yim, D., Fein, A., Perez de Castro, I., Li, C., Thompson, C.B., Cox, A.D. and Philips, M.R. (2006) PKC regulates a farnesyl-electrostatic switch on K-Ras that promotes its association with Bcl-XL on mitochondria and induces apoptosis. *Mol Cell*, **21**, 481-93.

250. Chaturvedi, K. and Sarkar, D.K. (2005) Role of protein kinase C-Ras-MAPK p44/42 in ethanol and transforming growth factor-beta3-induced basic fibroblast growth factor release from folliculostellate cells. *J Pharmacol Exp Ther*, **314**, 1346-52.

251. Yue, J. and Mulder, K.M. (2000) Requirement of Ras/MAPK pathway activation by transforming growth factor beta for transforming growth factor beta 1 production in a smad-dependent pathway. *J Biol Chem*, **275**, 30765-73.

252. Matsuura, I., Denissova, N.G., Wang, G., He, D., Long, J. and Liu, F. (2004) Cyclin-dependent kinases regulate the antiproliferative function of Smads. *Nature*, **430**, 226-31.

253. Engel, M.E., McDonnell, M.A., Law, B.K. and Moses, H.L. (1999) Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. *J Biol Chem*, **274**, 37413-20.

254. Brown, J.D., DiChiara, M.R., Anderson, K.R., Gimbrone, M.A., Jr. and Topper, J.N. (1999) MEKK-1, a component of the stress (stress-activated protein kinase/c-Jun N-terminal kinase) pathway, can selectively activate Smad2-mediated transcriptional activation in endothelial cells. *J Biol Chem*, **274**, 8797-805.

255. Yamagata, H., Matsuzaki, K., Mori, S., Yoshida, K., Tahashi, Y., Furukawa, F., Sekimoto, G., Watanabe, T., Uemura, Y., Sakaida, N., Yoshioka, K., Kamiyama, Y., Seki, T. and Okazaki, K. (2005) Acceleration of Smad2 and Smad3 phosphorylation via c-Jun NH(2)-terminal kinase during human colorectal carcinogenesis. *Cancer Res*, **65**, 157-65.

256. Kretzschmar, M., Doody, J., Timokhina, I. and Massague, J. (1999) A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras. *Genes Dev*, **13**, 804-16.

257. Detjen, K.M., Brembeck, F.H., Welzel, M., Kaiser, A., Haller, H., Wiedenmann, B. and Rosewicz, S. (2000) Activation of protein kinase Calpha inhibits growth of pancreatic cancer cells via p21(cip)-mediated G(1) arrest. *J Cell Sci*, **113**, 3025-35.

258. Cuzick, J., Powles, T., Veronesi, U., Forbes, J., Edwards, R., Ashley, S. and Boyle, P. (2003) Overview of the main outcomes in breast-cancer prevention trials. *Lancet*, **361**, 296-300.

259. Vogel, V.G., Costantino, J.P., Wickerham, D.L., Cronin, W.M., Cecchini, R.S., Atkins, J.N., Bevers, T.B., Fehrenbacher, L., Pajon, E.R., Jr., Wade, J.L., 3rd, Robidoux, A., Margolese, R.G., James, J., Lippman, S.M., Runowicz, C.D., Ganz, P.A., Reis, S.E., McCaskill-Stevens, W., Ford, L.G., Jordan, V.C. and Wolmark, N. (2006) Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *Jama*, **295**, 2727-41.

260. Shen, Q. and Brown, P.H. (2003) Novel agents for the prevention of breast cancer: targeting transcription factors and signal transduction pathways. *J Mammary Gland Biol Neoplasia*, **8**, 45-73.

261. Welsh, J., Wietzke, J.A., Zinser, G.M., Byrne, B., Smith, K. and Narvaez, C.J. (2003) Vitamin D-3 receptor as a target for breast cancer prevention. *J Nutr*, **133**, 2425S-2433S.

262. Zinser, G.M., Suckow, M. and Welsh, J. (2005) Vitamin D receptor (VDR) ablation alters carcinogen-induced tumorigenesis in mammary gland, epidermis and lymphoid tissues. *J Steroid Biochem Mol Biol*, **97**, 153-64.

263. Chung, I., Wong, M.K., Flynn, G., Yu, W.D., Johnson, C.S. and Trump, D.L. (2006) Differential antiproliferative effects of calcitriol on tumor-derived and matrigel-derived endothelial cells. *Cancer Res*, **66**, 8565-73.

264. Bouillon, R., Eelen, G., Verlinden, L., Mathieu, C., Carmeliet, G. and Verstuyf, A. (2006) Vitamin D and cancer. *J Steroid Biochem Mol Biol*, **102**, 156-62.

265. Stoica, A., Saceda, M., Fakhro, A., Solomon, H.B., Fenster, B.D. and Martin, M.B. (1999) Regulation of estrogen receptor-alpha gene expression by 1, 25-dihydroxyvitamin D in MCF-7 cells. *J Cell Biochem*, **75**, 640-51.

266. Swami, S., Krishnan, A.V. and Feldman, D. (2000) 1alpha,25-Dihydroxyvitamin D3 down-regulates estrogen receptor abundance and suppresses estrogen actions in MCF-7 human breast cancer cells. *Clin Cancer Res*, **6**, 3371-9.

267. Stewart, L.V. and Weigel, N.L. (2005) Role of insulin-like growth factor binding proteins in 1alpha,25-dihydroxyvitamin D(3)-induced growth inhibition of human prostate cancer cells. *Prostate*, **64**, 9-19.

268. Terrien, X., Bonvin, E., Corroyer, S., Tabary, O., Clement, A. and Henrion Caude, A. (2005) Intracellular colocalization and interaction of IGF-binding protein-2 with the cyclin-dependent kinase inhibitor p21CIP1/WAF1 during growth inhibition. *Biochem J*, **392**, 457-65.

269. Lee, H.J., Ji, Y., Paul, S., Maehr, H., Uskokovic, M. and Suh, N. (2007) Activation of bone morphogenetic protein signaling by a Gemini vitamin D3 analogue is mediated by Ras/protein kinase C alpha. *Cancer Res*, **67**, 11840-7.

270. Grant, W.B. (2002) An ecologic study of dietary and solar ultraviolet-B links to breast carcinoma mortality rates. *Cancer*, **94**, 272-81.

271. John, E.M., Schwartz, G.G., Dreon, D.M. and Koo, J. (1999) Vitamin D and breast cancer risk: the NHANES I Epidemiologic follow-up study, 1971-1975 to

1992. National Health and Nutrition Examination Survey. *Cancer Epidemiol Biomarkers Prev*, **8**, 399-406.

272. Shin, M.H., Holmes, M.D., Hankinson, S.E., Wu, K., Colditz, G.A. and Willett, W.C. (2002) Intake of dairy products, calcium, and vitamin d and risk of breast cancer. *J Natl Cancer Inst*, **94**, 1301-11.

273. Hiatt, R.A., Krieger, N., Lobaugh, B., Drezner, M.K., Vogelman, J.H. and Orentreich, N. (1998) Prediagnostic serum vitamin D and breast cancer. *J Natl Cancer Inst*, **90**, 461-3.

274. Simard, A., Vobecky, J. and Vobecky, J.S. (1991) Vitamin D deficiency and cancer of the breast: an unprovocative ecological hypothesis. *Can J Public Health*, **82**, 300-3.

275. Robsahm, T.E., Tretli, S., Dahlback, A. and Moan, J. (2004) Vitamin D3 from sunlight may improve the prognosis of breast-, colon- and prostate cancer (Norway). *Cancer Causes Control*, **15**, 149-58.

276. Campbell, M.J., Gombart, A.F., Kwok, S.H., Park, S. and Koeffler, H.P. (2000) The anti-proliferative effects of 1alpha,25(OH)2D3 on breast and prostate cancer cells are associated with induction of BRCA1 gene expression. *Oncogene*, **19**, 5091-7.

277. Jensen, S.S., Madsen, M.W., Lukas, J., Bartek, J. and Binderup, L. (2002) Sensitivity to growth suppression by 1alpha,25-dihydroxyvitamin D(3) among MCF-7 clones correlates with Vitamin D receptor protein induction. *J Steroid Biochem Mol Biol*, **81**, 123-33.

278. Gonzalez-Sancho, J.M., Alvarez-Dolado, M. and Munoz, A. (1998) 1,25-Dihydroxyvitamin D3 inhibits tenascin-C expression in mammary epithelial cells. *FEBS Lett*, **426**, 225-8.

279. Anzano, M.A., Smith, J.M., Uskokovic, M.R., Peer, C.W., Mullen, L.T., Letterio, J.J., Welsh, M.C., Shrader, M.W., Logsdon, D.L., Driver, C.L. and et al. (1994) 1 alpha,25-Dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol (Ro24-5531), a new deltanoid (vitamin D analogue) for prevention of breast cancer in the rat. *Cancer Res*, **54**, 1653-6.

280. Cope, M.B., Steele, V.E., Eto, I., Juliana, M.M., Hill, D.L. and Grubbs, C.J. (2002) Prevention of methylnitrosourea-induced mammary cancers by 9-cis-retinoic acid and/or vitamin D3. *Oncol Rep*, **9**, 533-7.

281. Koshizuka, K., Koike, M., Kubota, T., Said, J., Binderup, L. and Koeffler, H.P. (1998) Novel vitamin D3 analog (CB1093) when combined with paclitaxel and cisplatin inhibit growth of MCF-7 human breast cancer cells in vivo. *Int J Oncol*, **13**, 421-8.

282. Bower, M., Colston, K.W., Stein, R.C., Hedley, A., Gazet, J.C., Ford, H.T. and Combes, R.C. (1991) Topical calcipotriol treatment in advanced breast cancer. *Lancet*, **337**, 701-2.

283. Wactawski-Wende, J., Kotchen, J.M., Anderson, G.L., Assaf, A.R., Brunner, R.L., O'Sullivan, M.J., Margolis, K.L., Ockene, J.K., Phillips, L., Pottern, L., Prentice, R.L., Robbins, J., Rohan, T.E., Sarto, G.E., Sharma, S., Stefanick, M.L., Van Horn, L., Wallace, R.B., Whitlock, E., Bassford, T., Beresford, S.A., Black, H.R., Bonds, D.E., Brzyski, R.G., Caan, B., Chlebowski, R.T., Cochrane, B., Garland, C., Gass, M., Hays, J., Heiss, G., Hendrix, S.L., Howard, B.V., Hsia, J., Hubbell, F.A., Jackson, R.D., Johnson, K.C., Judd, H., Kooperberg, C.L., Kuller, L.H., LaCroix, A.Z., Lane, D.S., Langer, R.D., Lasser, N.L., Lewis, C.E., Limacher, M.C. and Manson, J.E. (2006) Calcium plus vitamin D supplementation and the risk of colorectal cancer. *N Engl J Med*, **354**, 684-96.

284. Jackson, R.D., LaCroix, A.Z., Gass, M., Wallace, R.B., Robbins, J., Lewis, C.E., Bassford, T., Beresford, S.A., Black, H.R., Blanchette, P., Bonds, D.E., Brunner, R.L., Brzyski, R.G., Caan, B., Cauley, J.A., Chlebowski, R.T., Cummings, S.R., Granek, I., Hays, J., Heiss, G., Hendrix, S.L., Howard, B.V., Hsia, J., Hubbell, F.A., Johnson, K.C., Judd, H., Kotchen, J.M., Kuller, L.H., Langer, R.D., Lasser, N.L., Limacher, M.C., Ludlam, S., Manson, J.E., Margolis, K.L., McGowan, J., Ockene, J.K., O'Sullivan, M.J., Phillips, L., Prentice, R.L., Sarto, G.E., Stefanick, M.L., Van Horn, L., Wactawski-Wende, J., Whitlock, E., Anderson, G.L., Assaf, A.R. and Barad, D. (2006) Calcium plus vitamin D supplementation and the risk of fractures. *N Engl J Med*, **354**, 669-83.

285. Lappe, J.M., Travers-Gustafson, D., Davies, K.M., Recker, R.R. and Heaney, R.P. (2007) Vitamin D and calcium supplementation reduces cancer risk: results of a randomized trial. *Am J Clin Nutr*, **85**, 1586-91.

Table 1. Epidemiological evidence

Favorable association of sunlight and/or vitamin D intake		
Epidemiological studies	**Results**	**Ref.**
UV light blocking air pollution (Sulfur dioxide) vs. age-adjusted breast cancer mortality rates in women	Significant positive association	[67]
Total average annual sunlight energy vs. age-adjusted breast cancer mortality rates in US	Inverse relationship	[68]
Total average annual sunlight energy vs. age-adjusted breast cancer mortality rates in USSR	Significant negative association	[69]
Residential exposure to sunlight vs. female breast cancer mortality	Significant negative association	[70,71,270]
Vitamin D from sunlight exposure, diet and supplements vs. the risk of breast cancer	Risk reduction of breast cancer for women living in high solar radiation area in US	[271]
Intake of vitamin D vs. the risk of breast cancer	Inverse association in premenopausal women	[272]
Level of $1\alpha,25(OH)_2D_3$ vs. bone metastasis of breast cancer	Inverse association in breast cancer patients	[73]
Level of $1\alpha,25(OH)_2D_3$ vs. the risk of breast cancer	Significant lower level of $1\alpha,25(OH)_2D_3$ in Caucasian women with breast cancer than the controls without breast cancer	[72]
Serum level of 25(OH)D vs. the risk of breast cancer	50% risk reduction of breast cancer in individuals with serum 25(OH)D (>52 ng/ml), compared to those with 25(OH)D (< 13 ng/ml)	[74]

No significant association
Epidemiological studies/Results
No relationship between the level of $1\alpha,25(OH)_2D_3$ and later breast cancer incidence [273]
No association between low vitamin D consumption and breast cancer development in a Canadian case control study [274]
No geographical variation between UV radiation and cancer fatality (breast, colon and prostate cancer) in Norway [275]

Table 2. Activity of vitamin D and its analogs in cell proliferation, apoptosis, invasion, angiogenesis, and metastasis

Effects on proliferation and differentiation			
Cell Line	**Vitamin D treatment**	**Involved signaling pathway or mediators**	**Ref**
MCF7/T47D/ BT549	$1\alpha,25(OH)_2D_3$	Regulation of EGF receptor level	[81,82]
MCF7	$1\alpha,25(OH)_2D_3$/EB1089	Induction of p21 and p27, Inactivation of Cdk2 and G1 arrest	[75]
	$1\alpha,25(OH)_2D_3$/EB1089	Regulation of TGF-β receptor II expression	[83]
	$1\alpha,25(OH)_2D_3$	G1 arrest by inhibiting cyclin D1/cdk4, increasing cdk inhibitor p21, down-regulating c-Myc	[76,78]
	$1\alpha,25(OH)_2D_3$	Down-regulation of estrogen receptor (ER) transcription	[265,266]
	$1\alpha,25(OH)_2D_3$	Induction of BRCA1 mRNA and protein by transcriptional activation through VDR	[276]
	14-epi-analogs of $1\alpha,25(OH)_2D_3$ (TX522 and TX527)	Down-regulation of cyclin D1 and cyclin C Increase of p21 and p27	[77]
	$1\alpha,25(OH)_2D_3$	Relationship between growth inhibition and induced level of VDR protein	[277]
	$1\alpha,25(OH)_2D_3$	Serum induced MAPK (ERK1/2) inhibition by inactivating Src tyrosine kinase	[86,87]
	$1\alpha,25(OH)_2D_3$ /EB1089/CB1093	Down-regulation of surviving through TGF-β and p38 MAPK signaling	[85]
T47D	$1\alpha(OH)D_5$	Induction of differentiation via VDR	[88]
UISO-BCA-4	$1\alpha(OH)D_5$	Induction of cell differentiation. Induction of VDR and TGF-β1 proteins	[89]
184A1 MCF-7	EB1089	Induction of autocrine TGFβ activity PI3-Kinase dependent	[84]
MCF-12F/BT-474	$1\alpha(OH)D_5$	G1 arrest, p21 and p27 induction. Inhibition of cell growth in transformed and breast cancer cells, but not in normal-like breast epithelial cells	[80]
SUM-159PT (ER negative)	$1\alpha,25(OH)_2D_3$/EB1089	Increase of p21 and p27	[79]

Table 2. *Continued*

Effects on apoptosis			
Cell Line	**Vitamin D treatment**	**Involved signaling pathway or mediators**	**Ref**
MCF-7	$1\alpha,25(OH)_2D_3$/EB1089	Down-regulation of estrogen receptor	[96]
	$1\alpha,25(OH)_2D_3$/EB1089	Reduction of BCL-2, induction of clusterin and DNA fragmentation	[93]
	$1\alpha,25(OH)_2D_3$ /EB1089/CB1093	Inhibition of BCL-2 Caspase- and p53-independent pathway	[94]
	$1\alpha,25(OH)_2D_3$	Bax translocation, cytochrome c release and production of ROS in a caspase-independent manner	[95]
	$1\alpha,25(OH)_2D_3$	Sensitization of mitochondrial disruption via PKC activation	[97]
	$1\alpha,25(OH)_2D_3$ /EB1089/CB1093	Down-regulation of surviving through TGF-β and p38 MAPK signaling	[85]
SUM-159PT (ER negative)	$1\alpha,25(OH)_2D_3$/EB1089	Increase of bax, cytochrome c release, PARP cleavage	[79]
Effects on invasion, angiogenesis, and metastasis			
Cell line	**Vitamin D treatment**	**Involved signaling pathway or mediators**	**Ref**
EpH4	$1\alpha,25(OH)_2D_3$	Inhibition of Tenascin-C related to growth, invasion and angiogenesis promotion	[278]
MDA-MB-231	$1\alpha,25(OH)_2D_3$/ Deltanoids	Decrease of invasion related serine protease, urokinase plasminogen activator, and metalloproteinase, MMP-9. Induction of their inhibitors	[102]
MDA-MB-231	EB1089	Decrease of total number of bone metastasis, surface area of osteolytic lesions, and tumor burden per animal	[106]
MCF-7 /MDA435S	$1\alpha,25(OH)_2D_3$	Inhibition of VEGF-induced endothelial cell sprouting and elongation, and network formation of elongated endothelial cells. Suppression of vascularization in tumors	[104]
SUM-159PT(ER-)	$1\alpha,25(OH)_2D_3$/EB1089	Inhibition of cell invasion in Matrigel invasion assay	[79]
MKL-4	$1\alpha,25(OH)_2D_3$/EB1089 + QW	Blockage of CYP24 expression induced by $1\alpha,25(OH)_2D_3$ or EB1089 Inhibition of the invasiveness	[103]

Table 2. *Continued*

Synergistic activity of vitamin D and its analogs				
Cell Line	**Vitamin D treatment**	**Responsive activity**	**Involved signaling pathway or mediators**	**Ref.**
MCF-7	$1\alpha,25(OH)_2D_3$ + 4-hydrotamoxifen	Apoptosis	Enhancement of apoptosis. Induction of clusterin	[98]
T47D	Retinoids + $1\alpha,25(OH)_2D_3$ or Interferon α	Anti-angiogenesis	Significant decrease of tumor cell-induced angiogenesis (TIA)	[105]
MCF-7	EB1089 + 9-cis-retinoic acid	Apoptosis	Enhanced down-regulation of BCL-2, up-regulation of p53 and Induction of DNA fragmentation	[99]
MCF-7	EB1089 + Radiation	Apoptosis	Increase of DNA fragmentation	[100]
MCF-7	EB1089 + adriamycin	Anti-proliferation, Apoptosis	Enhancement of sensitivity to adriamycin. Increase of insulin-like growth factor binding protein (IGFBP)	[90]
MCF-7	ILX 23-7553 + radiation/ILX 23-7553 + adriamycin	Anti-proliferation, Apoptosis	Enhancement of apoptotic and anti-proliferative effects	[92] [101]
RM4	$1\alpha,25(OH)_2D_3$ + Melatonin (MEL)	Anti-proliferation	Increase of sensitivity to $1\alpha,25(OH)_2D_3$ by MEL Enhanced secretion of TGFβ1	[91]

Table 3. Pre-clinical *in vivo* studies with vitamin D and analogs using different animal models

Carcinogen-induced breast cancer model			
Carcinogen	**Vitamin D treatment**	**Effects of treatment/Comments**	**Ref.**
DMBA	Low level of calcium and vitamin D in high fat diet	Increase of the mammary lesion incidence and total number of lesions Promoting effects of high fat diet on mammary tumorigenesis	[107]
NMU	EB1089	Inhibition of tumor growth	[108]
NMU	Ro24-5531 Ro24-5531 + tamoxifen	Significant extension of tumor latency and decrease of tumor incidence Enhancement of tumor burden reduction by tamoxifen	[279]
DMBA	$1\alpha(OH)D_5$	Inhibition of DMBA-induced preneoplastic lesions. Induction of VDR and TGFβ1	[109]
NMU	$1\alpha(OH)D_5$	Reduction of tumor incidence and tumor multiplicity	[110]
NMU	Vitamin D_3 + 9-cis-retinoic acid	Significant decrease of mammary tumor number	[280]
DMBA	$1\alpha(OH)D_5$	Selective protection of vitamin D during promotion or progression of carcinogenesis	[111]
Xenograft breast cancer model			
Cell line	**Vitamin D treatment**	**Effects of treatment/Comments**	**Ref.**
MCF-7	22-oxa-calcitriol (OCT) + Tamoxifen	Suppression of tumor growth synergistically	[116]
MX-1 (ER negative)	22-oxa-calcitriol (OCT)	Suppression of tumor growth	[116]
MCF-7	EB1089 + paclitaxel	Better inhibition of EB1089 than paclitaxel. Additive effect in combination (4-fold decrease of tumor mass)	[113]
MCF-7	EB1089 + all-trans-retinoic acid (ATRA)	Better inhibition of ATRA than EB1089 Additive effect in combination (3-fold decrease of tumor mass)	[114]
MCF-7	CB1089 + paclitaxel + cisplatin	About 83% inhibition of tumor weight by triple combination	[281]

Table 3. *Continued*

MCF-7	EB1089	Decrease of tumor volume (4-fold), increase of DNA fragmentation (6-fold), and reduction of cell proliferation (2-fold)	[112]
MCF-7	EB1089 + Radiation	Higher rate of decline in tumor volume (finally 50 % lower than that in only radiation treatment) Marked reduction of cell proliferation and enhancement of apoptosis	[115]
UISO-BCA-4	$1\alpha(OH)D_5$	Inhibition of tumor growth in athymic mice	[89]
BT-474	$1\alpha(OH)D_5$-Her2 antibody conjugate (IMC)	Specific binding to Her-2 expressing cells in vitro Inhibition of tumor growth in athymic mice	[117]
Genetically engineered mouse model			
Model	**Vitamin D treatment**	**Effects of treatment/Comments**	**Ref.**
Luteinizing hormone (LH)-overexpressing mice	EB1089	Inhibition of tumor growth rate	[118]

Table 4. Clinical trials using vitamin D analogs in breast cancer

Study (# of patient)	Treatment	Dose/Frequency	Response	Ref.
Bower et al (19)	Calcipotriol (MC903)	100 μg, daily for 6 weeks	16 patients completed 6 wk treatment 3 patients showed 50% reduction of lesions 2 patients became hypercalcemic	[282]
Gulliford et al (36)	EB1089	0.15-17.0 μg/m^2 daily for 1.5-33.5 weeks	Dose-dependent hypercalcemia 6 patients on treatment over 90 days showed stabilized disease ~ 7 μg/m^2 was tolerable for most patients	[119]
Women's Health Initiative	Vitamin D$_3$ and calcium	1000 mg calcium 400 IU vitamin D$_3$ for 8 years	No effect on colorectal cancer incidence Significant improvement in hip bone density Effect on breast cancer is not reported yet.	[120,121, 283,284]
Lappe et al	Vitamin D$_3$ and calcium	1400-1500 mg calcium 1100 IU vitamin D$_3$ for 4 years	Double-blind, randomized placebo-controlled trial Significant reduction of cancer risk in postmenopausal women	[285]
Planned study at UIC	1α(OH)D$_5$	Phase I/II trial in patients with metastatic breast cancer		[65]

Table 5. Characteristics of 1α,25(OH)$_2$D$_3$ and Gemini vitamin D analogs

Ligands	Ligands Volume(%)[a]	Liganded LBP Volume (%)[b]	Binding Affinity[c]		Transcriptional Activation (ED$_{50}$)[d]
			DBP (%)	VDR (%)	
1α,25(OH)$_2$D$_3$	100	100	100.0	100.0	10
Gemini Analog	120 – 125	103 – 119	2.5	38	1

[a] and [b] The volumes of ligands and their respective ligand binding pocket (LBP) were calculated using Sybyl and Voidoo software based on the molecular dynamics (MD) simulations (Ref. 177)

[c] The binding affinity of human vitamin D binding protein (DBP) and chick intestinal vitamin D receptor (VDR) were determined by steroid competition assay (Ref. 176).

[d] Transcriptional activity was determined transfecting the plasmid containing the human osteopontin VDRE into rat osteosarcoma cells (Ref. 176).

Table 6. Antibodies used in Western Blotting

Primary Antibody	Source	Molecular weight (kDa)	Company (Catalog#)	Dilution factor
β-Actin (Monoclonal)	Mouse	42	Sigma (A 1978)	1:2000
Bcl-2 (Polyclonal)	Rabbit	28	Santa Cruz (sc-783)	1:200
Cleaved Caspase-3 (Polyclonal)	Rabbit	17,19	Cell Signaling (9661)	1:1000
Her-2 (Polyclonal)	Rabbit	185	Thermo Scientic (RB-103-P0)	1:1000
HA (Monoclonal)	Mouse	-	COVANCE	1:1000
IGFBP-3	Rabbit	40	Upstate (06-108)	1:1000
Phospho-Akt (Polyclonal)	Rabbit	60	Cell Signaling (9271)	1:1000
Phospho-p44/42 (Polyclonal)	Rabbit	42,44	Cell Signaling (9101)	1:1000
p21 (Monoclonal)	Mouse	21	Santa Cruz (sc-6246)	1:200
p27 (Monoclonal)	Mouse	27	Santa Cruz (sc-1641)	1:200
PARP (Polyclonal)	Rabbit	24, 89, 116	Cell Signaling (9542)	1:1000
Cleaved PARP (Polyclonal)	Rabbit	89	Cell Signaling (9541)	1:1000
PKCα (Monoclonal)	Mouse	80	Santa Cruz (sc-8393)	1:200
PKCα (Polyclonal)	Rabbit	80	Cell Signaling (2056)	1:1000
Phospho-Smad1/5 (Monoclonal)	Rabbit	60	Cell Signaling (9516)	1:1000
Phospho-Smad1/5/8 (Polyclonal)	Rabbit	60	Cell Signaling (9511)	1:1000
Phospho-Smad2 (Polyclonal)	Rabbit	58	Chemicon International (AB3849)	1:1000
Phospho-Smad3 (Polyclonal)	Rabbit	58	Cell Signaling (9514)	1:1000
VDR (Monoclonal)	Rat	54	ABR (MA1-710)	1:1000

Secondary Antibody	Company	Dilution
Anti-Mouse	Santa Cruz (sc-2005)	1:2000
Anti-Rabbit	Santa Cruz (sc-2030)	1:2000
Anti-Rat	Santa Cruz (sc-2006)	1:2000

Table 7. Up-regulated gene expression by the Gemini vitamin D analog Ro3582 in MCF10 breast epithelial cells (fold change)

Gene Bank ID	Gene description	AT1		CA1a	
		4 hr	12hr	4 hr	12 hr
Vitamin D, bone, calcium metabolism					
NM_000711	Bone gamma-carboxyglutamate (gla) protein (osteocalcin) (BGLAP)	1.7	**7.0**	1.1	**2.1**
NM_001740	Calbindin 2, 29Kda (calretinin)	1.5	**3.7**	1.1	1.4
AI093569	Calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma	**2.0**	**2.0**	1.5	**2.0**
NM_001216	Carbonic anhydrase IX	-1.3	**3.5**	1.2	**3.5**
NM_000782	Cytochrome P450 family 24, subfamily A, polypeptide 1	**194.0**	**724.0**	**104.0**	**588.0**
D12620	Cytochrome P450 family 4, subfamily F, polypeptide 3	**4.0**	**10.6**	1.5	**2.0**
NM_019885	Cytochrome P450, family 26, subfamily B, polypeptide 1	**4.9**	**5.7**	**2.8**	**9.8**
NM_002963	S100 calcium binding protein A7 (psoriasin 1)	**13.0**	**34.3**	1.3	**4.9**
NM_003330	Thioredoxin reductase 1	**2.0**	**2.8**	1.5	1.5
AI023317	Thyroid hormone receptor associated protein 4	**2.1**	**3.2**	1.2	1.5
Lipid metabolism					
NM_022977	Acyl-CoA synthetase long-chain family member 4	1.9	**3.5**	1.4	1.3
AL512760	Fatty acid desaturase 1	1.1	**2.1**	1.2	**2.0**
NM_003645	Soluble carrier family 27 (fatty acid transporter), member 2	1.1	1.9	-1.1	**2.0**
Cell adhesion, extracellular matrix, cytoskeleton					
NM_003474	A disintegrin and metalloproteinase domain 12 (meltrin a)	1.1	**2.6**	1.3	**2.0**
NM_001856	Collagen, type XVI, a1	**2.3**	**3.7**	1.5	**3.0**
AF061812	Keratin 16 (focal non-epidermolytic palmoplantar keratoderma)	1.7	**3.0**	1.4	**3.7**
L25541	Laminin b3	**2.1**	**2.0**	**2.1**	**2.8**
NM_003461	Zyxin	1.9	**2.1**	1.6	**2.0**
Cell Proliferation, apoptosis, signal transduction					
NM_001945	Diphtheria toxin receptor	**2.0**	**2.5**	1.5	**2.3**
N36770	Dual specificity phosphatase 10	**6.5**	**8.0**	**4.3**	**7.5**
NM_024530	FOS-like antigen 2	**2.8**	**2.3**	**2.1**	**2.1**
NM_005308	G protein-coupled receptor kinase 5	**2.1**	**6.1**	1.4	**3.5**
NM_001924	Growth arrest and DNA-damage-inducible a	1.7	**2.1**	1.3	1.6
NM_005261	GTP binding protein overexpressed in skeletal muscle	**4.0**	**4.6**	**4.0**	**2.1**
NM_004838	Homer homolog 3	1.5	**2.6**	1.3	**2.6**
NM_005544	Insulin receptor substrate 1	1.6	**3.7**	1.5	**6.5**

Table 7. *Continued*

M31159	Insulin-like growth factor binding protein 3	1.6	1.9	**2.6**	**2.8**
NM_002774	Kallikrein 6 (neurosin, zyme)	**6.1**	**18.4**	**2.6**	**11.3**
NM_014751	Metastasis suppressor 1	**3.5**	**6.1**	1.1	1.7
NM_013982	Neuregulin 2	**2.5**	1.4	1.4	**2.0**
NM_006096	N-myc downstream regulated gene 1	1.1	**2.1**	1.0	1.7
NM_020529	Nuclear factor of kappa light polypeptide gene enhancer, IkB-like activity	**2.6**	**2.3**	**2.6**	**3.0**
NM_014456	Programmed cell death 4 (neoplastic transformation inhibitor)	1.3	**2.5**	1.4	**2.5**
NM_004878	Prostaglandin E synthase	1.2	1.6	1.2	**2.5**
NM_002564	Purinergic receptor P2Y, G-protein coupled, 2	**5.3**	**5.3**	**2.1**	**2.3**
NM_003028	SHB (Src homology 2 domain containing) adaptor protein B	**2.3**	1.6	1.3	1.6
BC004490	v-Fos FBJ murine osteosarcoma viral oncogene homolog	**5.3**	**3.2**	**14.9**	**39.4**

Immune response, growth factors, cytokines, inflammation

NM_000591	CD14 antigen	**26.0**	**52.0**	**6.1**	**27.9**
NM_001784	CD97 antigen	**3.0**	**4.6**	**2.0**	**2.3**
NM_000882	Interleukin 12A	1.2	**19.7**	**2.5**	**3.5**
NM_003266	Toll-like receptor 4	1.9	**2.5**	**2.3**	1.7
NM_018643	Triggering receptor expressed on myeloid cells 1	1.4	**2.5**	1.1	**7.5**
NM_003596	Tyrosylprotein sulfotransferase 1	**2.8**	**3.7**	**2.3**	**2.3**

Transcription

D13889	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	1.3	1.5	1.6	**2.1**
NM_002167	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	1.4	1.7	1.7	**2.1**
NM_002229	Jun B proto-oncogene	1.7	**2.1**	**2.0**	**2.6**
AI824012	Nuclear receptor interacting protein 1	1.6	1.9	**2.0**	**2.1**
NM_003195	Transcription elongation factor A (SII), 2	**3.0**	**16.0**	**-4.6**	**3.2**
NM_003447	Zinc finger protein 165	1.7	**2.1**	1.5	1.9
NM_003407	Zinc finger protein 36, C3H type homolog	**2.1**	**2.1**	1.2	1.5

TGF-β, BMP, activin, smad signaling

AB046845	E3 ubiquitin ligase SMURF1	1.6	**2.0**	1.1	1.4
M13436	Inhibin, beta A (activin A, activin AB alpha polypeptide)	1.3	**2.1**	1.9	**4.9**
NM_001200.1	BMP-2	1.1	**2.3**	1.9	-1.2
NM_001718	BMP-6	**2.5**	**4.0**	-3.5	-1.2
M19154.1	TGF-β2	1.3	1.4	1.5	**2.1**

Transporters, channels

Table 7. *Continued*

AI769688	ATPase, aminophospholipid transporter (APLT), Class I, type 8A, member 1	1.4	**9.1**	**3.7**	**4.6**
NM_014211	Gamma-aminobutyric acid (GABA) A receptor, pi	1.2	1.7	1.4	**2.6**
L38019	Inositol 1,4,5-triphosphate receptor, type 1	1.1	**2.0**	1.3	**2.8**
AF053755	Solute carrier family 4, member 7	**2.5**	**7.0**	1.7	**3.5**
NM_016354	Solute carrier organic anion transporter family, member 4A1	1.4	**2.3**	1.5	**2.1**
NM_015993	Transmembrane 4 superfamily member 11 (plasmolipin)	1.6	**4.3**	-1.1	**2.5**
Others					
NM_024734	Calmin (calponin-like, transmembrane)	**2.5**	**4.6**	1.9	**2.8**
NM_001873	Carboxypeptidase E	1.5	**7.0**	-1.1	**4.3**
U19970	Cathelicidin antimicrobial peptide (LPS binding protein)	**9.2**	**19.7**	**7.0**	**22.6**
BF514079	Kruppel-like factor 4 (gut)	**2.5**	**3.7**	1.5	**2.8**
NM_016233	Peptidyl arginine deiminase, type III	**8.6**	**45.3**	**2.0**	**4.0**
AI554300	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1	**4.6**	**12.1**	**2.3**	**9.2**
U90902	T-cell lymphoma invasion and metastasis 1	**2.6**	**2.8**	**2.0**	**2.6**
BE676218	Three prime repair exonuclease 2	1.4	**2.0**	1.3	**2.3**
NM_000930	Tissue plasminogen activator (tPA)	**2.0**	**2.0**	**2.0**	**2.3**
NM_003282	Troponin I, skeletal, fast	1.4	1.5	**2.1**	**9.8**

Bold indicates more than 2-fold changes

Table 8. Down-regulated gene expression by the Gemini vitamin D analog Ro3582 in MCF10 breast epithelial cells (fold change)

Gene Bank ID	Gene description	AT1		CA1a	
		4 hr	12hr	4 hr	12 hr
Cell adhesion, extracellular matrix, cytoskeleton					
NM_003246	Thrombospondin 1	-1.4	**-2.0**	-1.3	-1.6
NM_002291	Laminin b1	-1.5	**-2.3**	-1.2	-1.9
X16354	Carcinoembryonic antigen-related cell adhesion molecule 1	-1.3	**-3.5**	-1.1	**-2.5**
NM_001723	Bullous pemphigoid antigen 1	-1.5	**-2.0**	-1.3	**-2.8**
BF196457	Desmocollin 2	-1.6	**-2.0**	-1.4	-1.5
AF307080	Latrophilin 3	-1.1	**-3.2**	-1.1	-1.9
Signal transduction, apoptosis, cell cycle					
NM_003507	Frizzled homolog 7	-1.3	**-2.5**	-1.6	**-2.3**
NM_002048	Growth arrest-specific 1	**-3.2**	**-3.5**	-1.9	**-2.8**
NM_003897	Immediate early response 3	-1.5	**-2.5**	-1.1	-1.3
NM_000676	Adenosine A2b receptor	-1.4	**-2.3**	-1.2	-1.3
Growth factors, cytokines, immune responses, inflammation					
NM_005582	Lymphocyte antigen 64 homolog, radioprotective	-1.8	**-4.9**	-1.8	**-6.5**
M92934	Connective tissue growth factor (CTGF)	-1.9	**-2.6**	2.3	**-3.0**
Transcription					
NM_005375	v-myb myeloblastosis viral oncogene homolog	**-2.3**	-1.9	-1.5	-1.7
AI572079	Snail homolog 2 (SNAI2)	-1.2	-1.5	**-2.3**	**-4.0**
Others					
NM_005077	Transducin-like enhancer of split 1	-1.6	**-2.3**	-1.4	**-2.0**
NM_001218	Carbonic anhydrase XII	1.0	-1.7	-1.2	**-2.8**
NM_004385	Chondroitin sulfate proteoglycan 2 (versican)	-1.5	**-2.5**	-1.1	-1.7

Bold indicates more than 2-fold changes

Table 9. Validation of microarray analyses by quantitative RT-PCR (fold change)

Gene description	AT1		CA1a	
	4 hr	**12hr**	**4 hr**	**12 hr**
Vitamin D target genes				
CYP24A1	**6028 ± 310**	**7523 ± 162**	**462 ± 54**	**4144 ± 95**
Osteocalcin	**80.7 ± 2.5**	**279 ± 71**	**9.2 ± 1.8**	**56.2 ± 5.8**
Osteopontin	**7.0 ± 1.2**	**30.1 ± 10.1**	1.5 ± 0.8	**6.6 ± 0.4**
Vitamin D Receptor (VDR)	1.0 ± 0.3	1.4 ± 0.2	1.1 ± 0.2	1.3 ± 0.1
Cell adhesion, Invasion, angiogenesis, metastasis				
Collagen, type XVI, α1	**2.5 ± 0.0**	**3.9 ± 0.1**	1.4 ± 0.1	**2.2 ± 0.5**
Laminin β3	**2.7 ± 0.5**	**3.0 ± 0.2**	**2.2 ± 0.2**	**3.7 ± 0.1**
Thrombospondin 1	-1.7 ± 0.3	**-2.4 ± 0.8**	-1.5 ± 0.1	-1.7 ± 0.0
Laminin β1	-1.3 ± 0.2	**-2.3 ± 0.5**	-1.4 ± 0.1	**-2.4 ± 0.1**
MMP-1	**2.1 ± 0.6**	**9.4 ± 2.8**	0.8 ± 0.0	1.8 ± 0.7
MMP-2	-1.1 ± 0.1	**-3.0 ± 0.1**	-1.3 ± 0.5	**-2.5 ± 0.7**
Connective tissue growth factor (CTGF)	**-2.1 ± 0.1**	**-2.2 ± 0.0**	1.0 ± 0.2	**-5.0 ± 0.3**
Cell growth, apoptosis, signal transduction				
Insulin receptor substrate 1	1.9 ± 0.1	**3.6 ± 0.7**	1.3 ± 0.1	**6.4 ± 1.6**
Insulin-like growth factor binding protein 3	1.5 ± 0.1	**2.2 ± 0.2**	**2.7 ± 0.3**	**3.0 ± 0.2**
Dual specificity phosphatase 10	**10.3 ± 2.8**	**10.4 ± 0.5**	**4.1 ± 0.0**	**8.8 ± 1.4**
P21	1.2 ± 0.6	1.3 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
P27	0.8 ± 0.2	1.0 ± 0.0	0.9 ± 0.1	0.7 ± 0.0
BCL-2	-0.9 ± 0.0	-1.3 ± 0.1	-1.6 ± 0.1	-1.2 ± 0.2
Transcription				
Snail homolog 2 (SNAI2)	-1.3 ± 0.3	**-2.0 ± 0.1**	**-2.1 ± 0.8**	**-4.5 ± 1.2**
TGF-β/BMP signaling				
BMP-2	**2.0 ± 0.2**	**2.6 ± 0.0**	1.5 ± 0.1	**2.3 ± 0.7**
BMP-6	**4.6 ± 1.0**	**8.0 ± 2.7**	1.1 ± 0.1	1.8 ± 0.3
BMP-7	-	-	0.8 ± 0.0	0.7 ± 0.1
Inhibin, beta A (activin A)	1.4 ± 0.1	1.9 ± 0.2	**2.0 ± 0.6**	**4.1 ± 0.4**
TGF-β2	1.5 ± 0.1	0.9 ± 0.2	1.7 ± 0.8	1.3 ± 0.1
TGF-β Receptor II	1.3 ± 0.3	1.4 ± 0.1	1.1 ± 0.1	0.9 ± 0.1
Smad-6	**-3.3 ± 0.0**	**-2.1 ± 0.0**	**-2.4 ± 0.3**	**-4.4 ± 0.7**
Smad-7	1.0 ± 0.3	1.4 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
E3 ubiquitin ligase SMURF1	1.3 ± 0.3	1.4 ± 0.4	0.9 ± 0.2	1.2 ± 0.2

Bold indicates more than 2-fold changes

Table 10. Prevention of NMU-induced breast cancer by vitamin D analogs

Treatment*	Dose (µg/kg body weight)	No. animals	Body weight at autopsy (grams) (mean ± S.E.)	Tumor burden[†] (grams) (mean ± S.E.)
Control (Vehicle)	-	12	242.7 ± 6.5	9.0 ± 2.8
Ro2198	0.03	6	224.3 ± 16.0	8.4 ± 4.1
Ro3582	0.1	6	248.3 ± 10.6	9.2 ± 2.3
Ro3586	0.3	6	235.0 ± 2.6	5.0 ± 2.0
BXL0084	0.03	6	247.6 ± 8.1	6.4 ± 3.0
BXL0088	0.1	6	255.7 ± 9.9	8.9 ± 2.9
BXL0072	0.3	6	250.8 ± 9.5	3.6 ± 1.7 (p=0.06[‡])
BXL0097	0.3	6	262.7 ± 7.0	3.4 ± 1.7 (p=0.05[‡])

*All rats (21 days old) received NMU (50 mg /kg body weight, i.p.) 1 week before injecting vitamin D analogs i.p. once a day, 5 days a week for 9 weeks.
[†] Tumor burden; average tumor weight at autopsy.
[‡] p is the value for the comparison of tumor burden in rats treated with vitamin D analogs when compared with control rats treated with vehicle alone.

Table 11. Effect of different doses of 1α,25(OH)₂D₃ (calcitriol) and Gemini vitamin D analog, BXL0097, on NMU-induced breast cancer.

Treatment*	Dose (µg/kg body weight)	No. animals	Body weight at autopsy (grams) (mean ± S.E.)	Tumor burden[†] (grams) (mean ± S.E.)	Serum calcium level (mg/dl) (mean ± S.E.)
Control (Vehicle)	0	30	241.3 ± 4.0	8.5 ± 1.7	9.3 ± 0.9
1α,25(OH)₂D₃	0.03	15	238.3 ± 4.2	8.8 ± 1.8	9.3 ± 0.8
1α,25(OH)₂D₃	0.1	15	241.9 ± 6.6	6.8 ± 1.5	10.9 ± 1.5**
1α,25(OH)₂D₃	0.3	14	225.7 ± 9.9	5.6 ± 1.5	11.7 ± 1.4**
BXL0097	0.03	15	251.2 ± 6.1	9.0 ± 2.6	9.5 ± 1.0
BXL0097	0.1	15	248.5 ± 6.1	4.2 ± 1.2 (p=0.02[‡])	9.6 ± 1.5
BXL0097	0.3	15	252.5 ± 5.4	4.2 ± 1.4 (p=0.03[‡])	9.9 ± 1.1

*All rats (21 days old) received NMU (50 mg /kg body weight, i.p.) 1 week before injecting vitamin D analogs i.p. once a day, 5 days a week for 9 weeks.
[†] Tumor burden; average tumor weight at autopsy.
[‡] p is the value for the comparison of tumor burden in rats treated with calcitriol or Gemini 0097 when compared with control rats treated with vehicle alone.
**Hypercalcemic. The normal range of calcium in serum is 8.8–10.4 mg/dL [176].

Vitamin D₂
(ergocalciferol)

Vitamin D₃
(cholecalciferol)
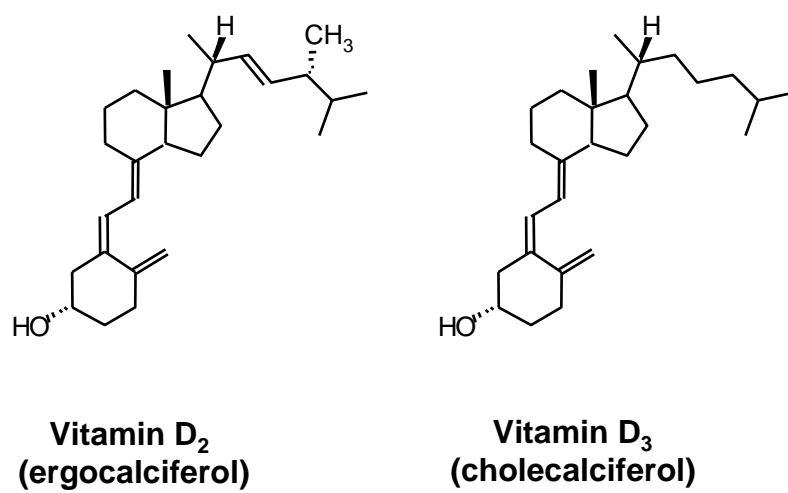
Fig. 1. Structures of vitamin D$_2$ (ergocalciferol) and vitamin D$_3$ (cholecalciferol)
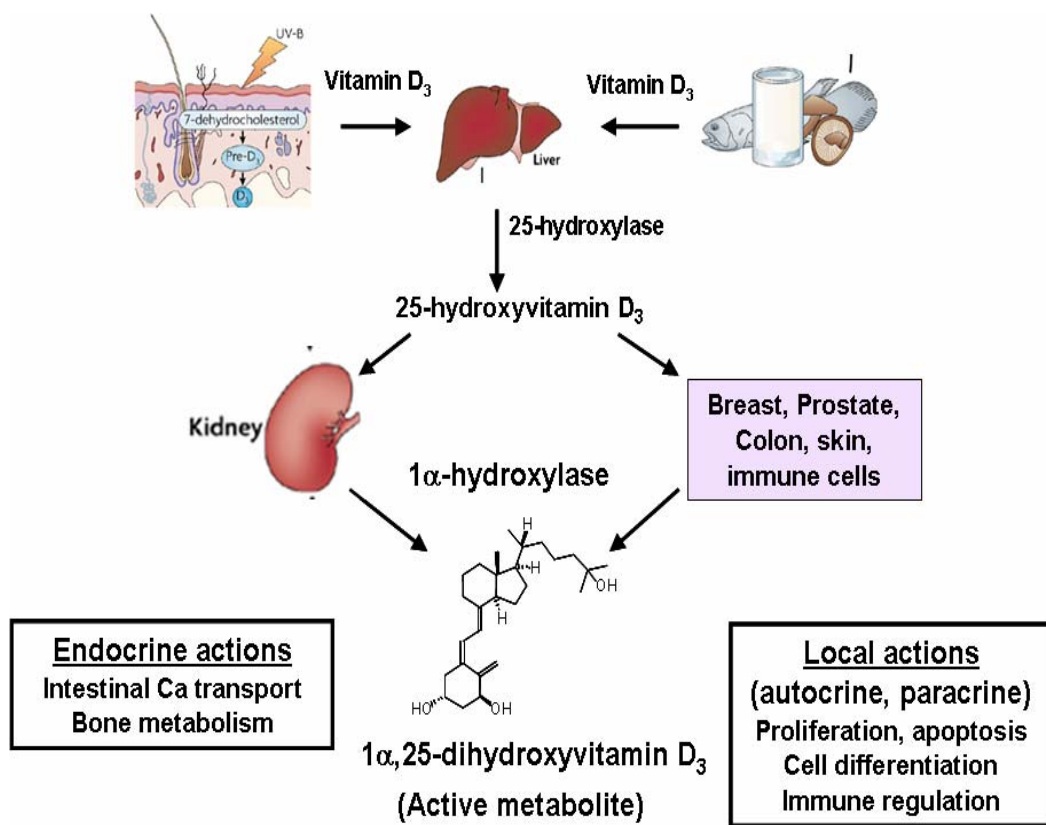
Fig. 2. Metabolism of vitamin D$_3$ (Ref. 24)
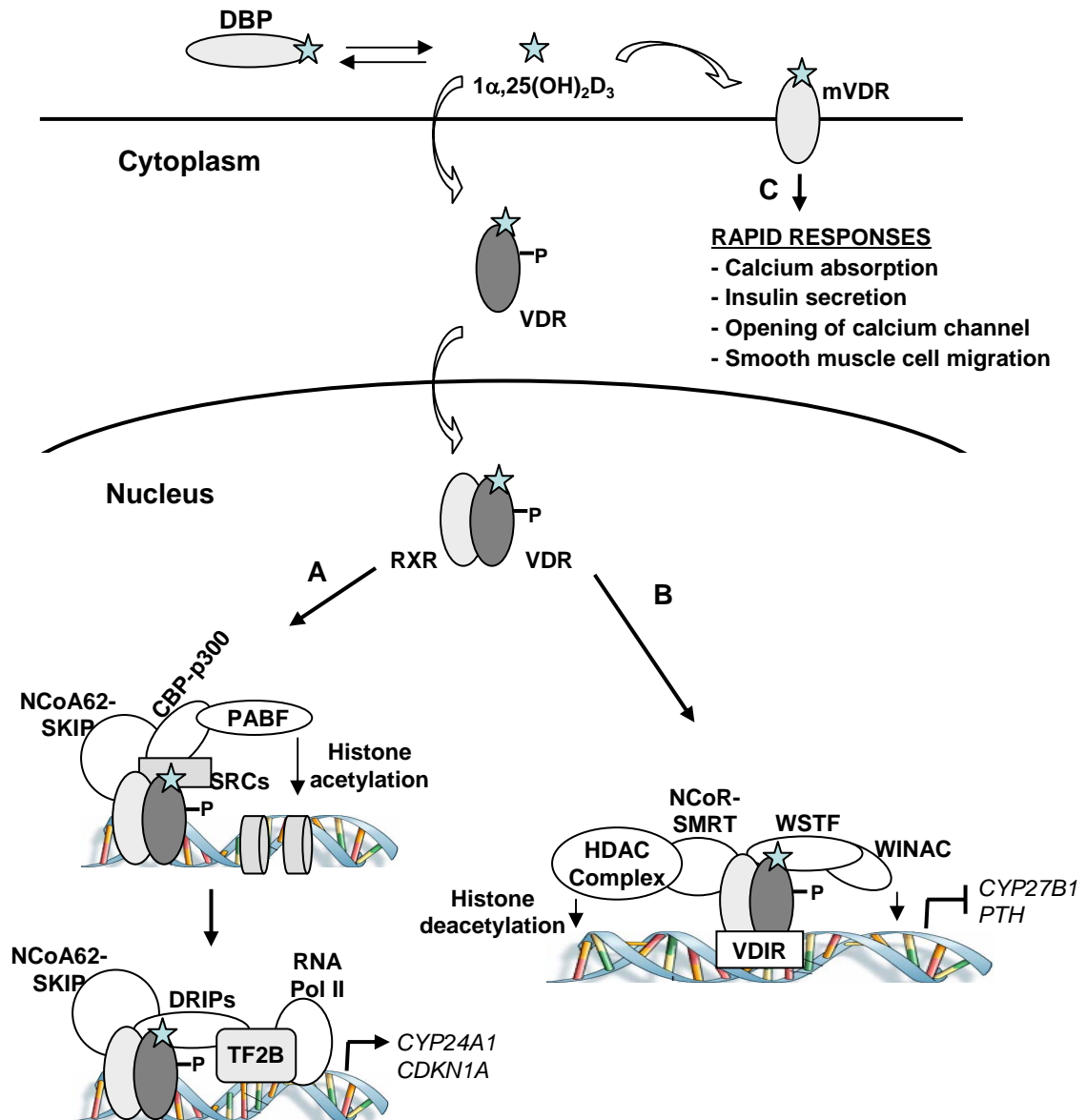
Fig. 3. Transcriptional regulations and rapid responses by 1α,25(OH)₂D₃ (Ref. 11 and 24)

A. Transcriptional activation. RXR: retinoid X receptor, NCoA62-SKIP: nuclear coactivator 62 kDA-SKI-interacting protein, SRCs: steroid receptor coactivators, CBP-p300: CREB binding protein-p300, PBAF: polybromo-and SWI-2-related gene 1 associated factor, DRIPs: vitamin D receptor-interacting proteins. **B.** Transcriptional repression. VDIR: VDR interacting repressor, WSTF: Williams syndrome transcription factor, WINAC: ATP-dependent chromatin-remodeling complex, NCoR-SMRT: nuclear receptor corepressors-silencing mediator for retinoid and thyroid hormone receptors. **C.** Non-genomic rapid responses through the membrane-bound vitamin D receptor (mVDR).
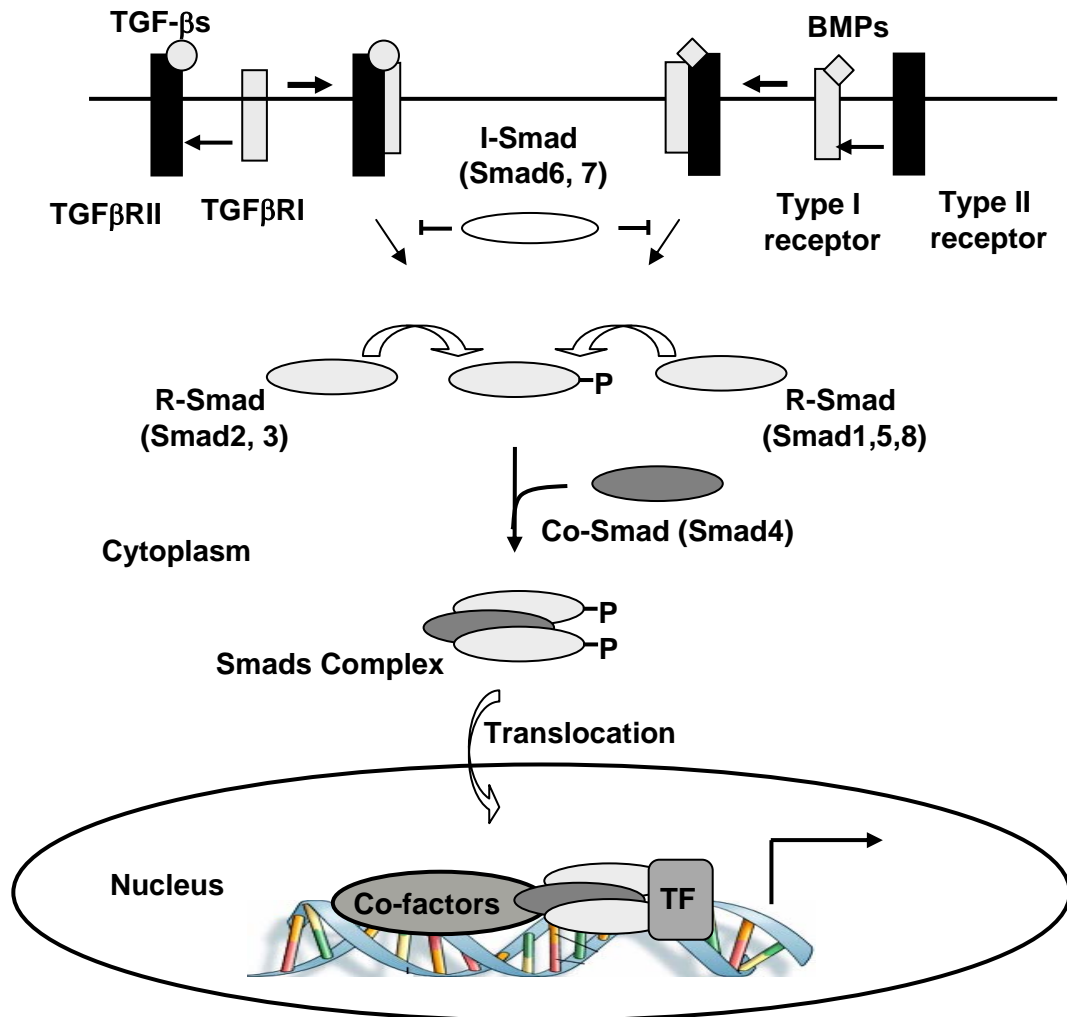
Fig. 4. Overview of the TGF-β signaling pathway (Ref. 130-132)
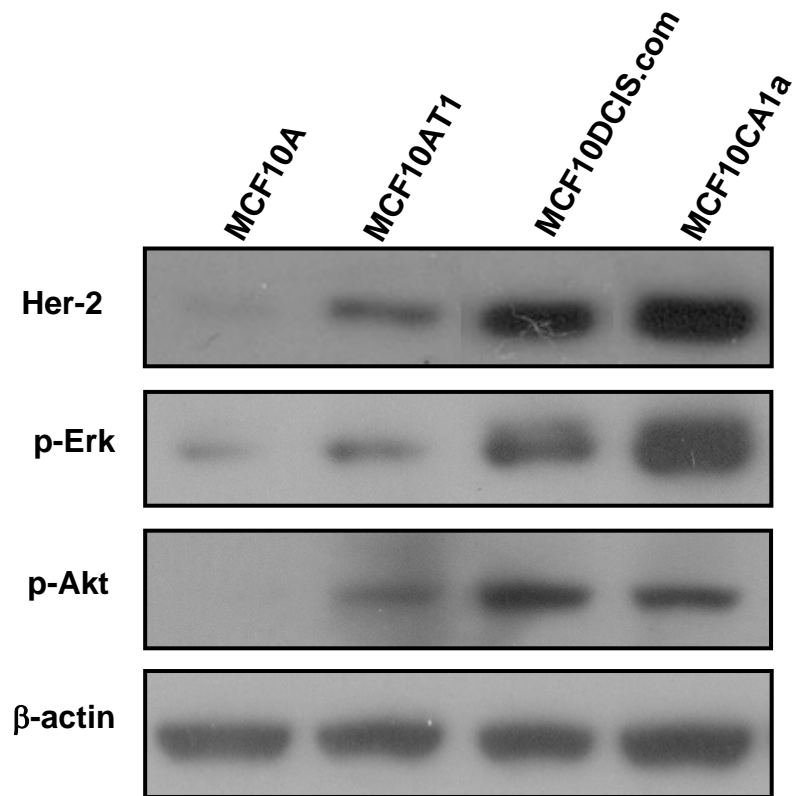
Fig. 5. Comparison of basal level of protein expression in the series of MCF10 human breast epithelial cells

The level of Her-2, p-Erk, p-Akt protein expression is gradually increased in MCF10 progressive human breast epithelial cell lines (MCF10A, MCF10AT1, MCF10DCIS.com and MCF10CA1a). The cells (1×10^6 cells/100 mm dish) were incubated in the growth media as described in Materials and Methods and harvested for western blotting.
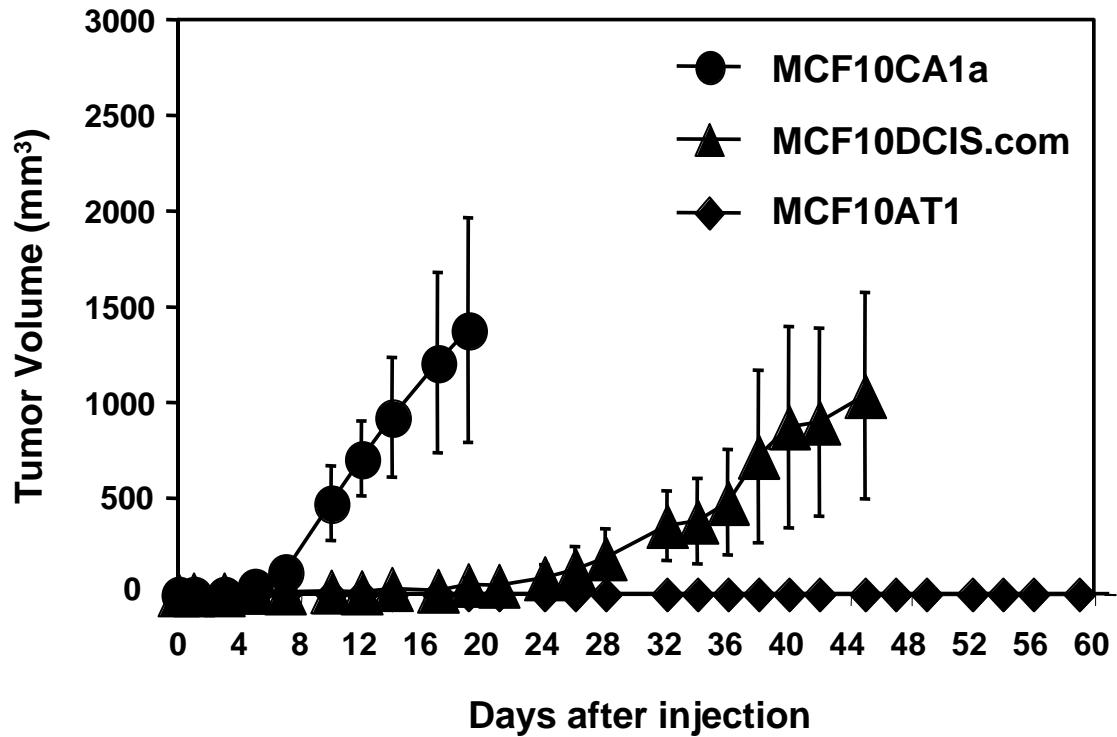
Fig. 6. Comparison of tumor growth in xenograft model with the series of MCF10 human breast epithelial cells

Xenograft tumors from aggressive MCF10CA1a cells grow faster than those from MCF10DCIS.com cells. The cells (MCF10AT1, MCF10DCIS.com and MCF10CA1a) were trypsinized and prepared in Hanks' Balanced Salt Solution. One million cells (1×10^6 cells) were injected in the mammary fat pad as described in Materials and Methods.
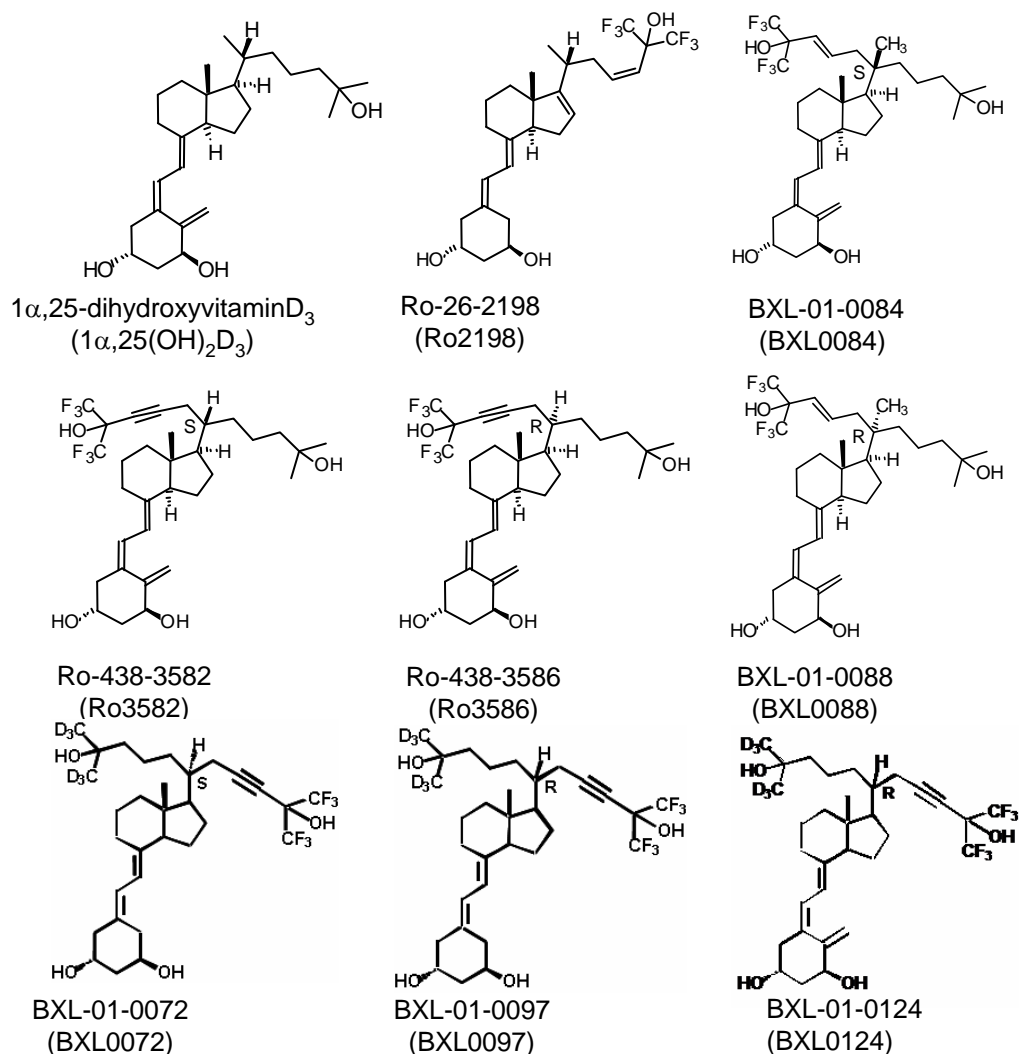
Fig. 7. Structures of 1α,25(OH)₂D₃ and Gemini vitamin D analogs

1α,25-dihydroxyvitamin D3 (active vitamin D_3 metabolite, $1\alpha,25(OH)_2D_3$), **Ro-26-2198** (1α,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-19-nor-cholecalciferol), **BXL-01-0084** (1α,25-dihydroxy-20-methyl-20S-(4-hydroxy-4-methyl-pentyl)-23E-ene-26,27-hexafluoro-cholecalciferol, BXL0084), **Ro-438-3582** (1α,25-dihydroxy-20S-21(3-hydroxy-3-methyl-butyl)-23-yne-26,27-hexafluoro-cholecalciferol, Ro3582), **Ro-438-3586** (1α,25-dihydroxy-20R-21(3-hydroxy-3-methyl-butyl)-23-yne-26,27-hexafluoro-cholecalciferol, Ro3586), **BXL-01-0088** (1α,25-dihydroxy-20-methyl-20R-(4-hydroxy-4-methyl-pentyl)-23E-ene-26,27-hexafluoro-cholecalciferol, BXL0088), **BXL-01-0072** (1α,25-dihydroxy-20S-21(3-hydroxy-3-trideuteromethyl-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-19-nor-cholecalciferol, BXL0072), **BXL-01-0097** (1α,25-dihydroxy-20R-21(3-hydroxy-3-trideuteromethyl-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-19-nor-cholecalciferol, BXL0097), **BXL-01-0124** (1α,25-dihydroxy-20R-21-(3-hydroxy-3-deuteromethyl-4,4,4-trideuterobutyl)-23-yne-26,27-hexafluoro-cholecalciferol, BXL0124)
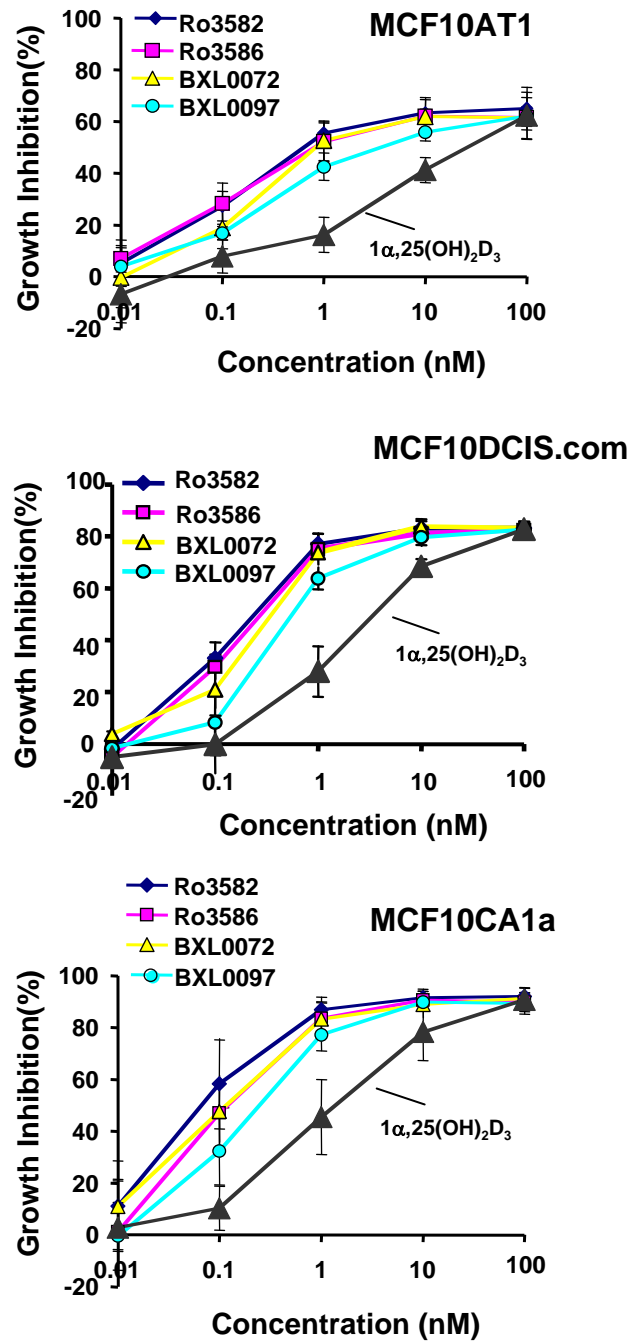
Fig. 8. Growth inhibitory effect of Gemini vitamin D analogs in MCF10AT1, MCF10DCIS.com, and MCF10CA1a cells

Gemini vitamin D analogs show better inhibition of cell growth in MCF10AT1, MCF10DCIS.com and MCF10CA1a cells than $1\alpha,25(OH)_2D_3$. Cells were incubated with vitamin D analogs in 5% horse serum/DMEM/F-12 for 3 days. Dose-response curves of $1\alpha,25(OH)_2D_3$ and Gemini vitamin D analogs by the $[^3H]$thymidine incorporation assay are shown. Three separate experiments were performed and combined.
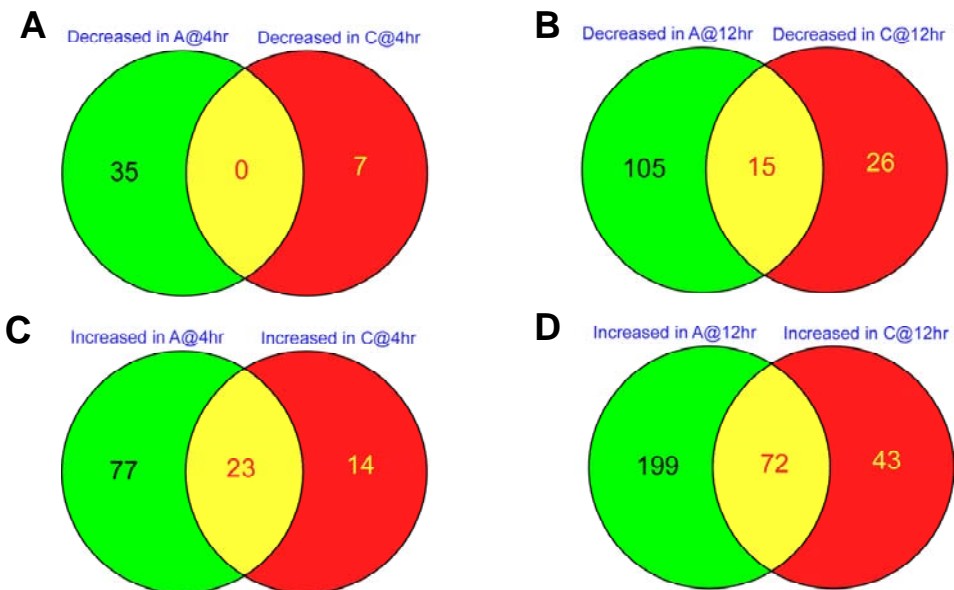
Fig. 9. Overlap of gene profiling regulated by Gemini vitamin D analog Ro3582 in MCF10AT1 and MCF10CA1a cells

Venn Diagram shows the overlap of gene profiling regulated by Gemini vitamin D analog Ro3582 (1 nM) between MCF10AT1 and MCF10CA1a cells at two time points (4hr and 12hr). A and B diagrams show the number of down-regulated gene by Ro3582 at 4hr and 12hr treatment, respectively. C and D diagrams show the number of up-regulated genes. Green indicate probesets uniquely regulated in MCF10AT1 cells and red indicate probesets regulated in MCF10CA1a cells, while the yellow indicate commonly regulated genes.
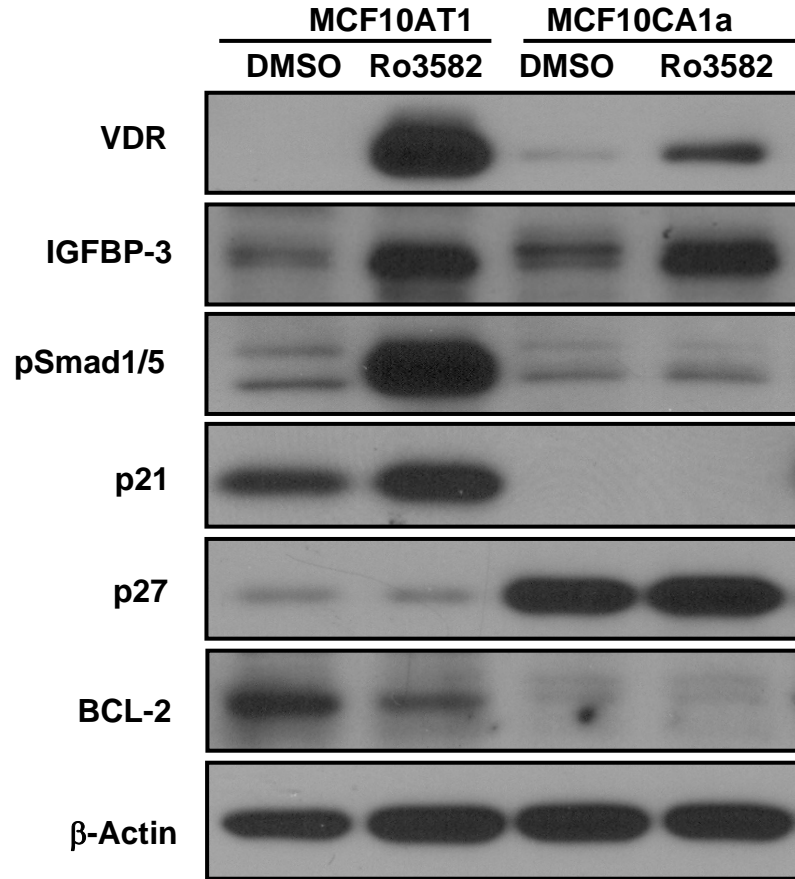
Fig. 10. Regulation of protein expression by Gemini vitamin D analog Ro3582 in MCF10AT1 and MCF10CA1a cells.

MCF10AT1 and MCF10CA1a cells (5 X 10^5 cells/well in 6-well plate) were starved for 24 hr in serum-free DMEM/F-12 medium. Then, cells were incubated with DMSO or Ro3582 (10 nM) in 0.1% BSA/DMEM/F-12 medium for 24 hr before protein harvest.
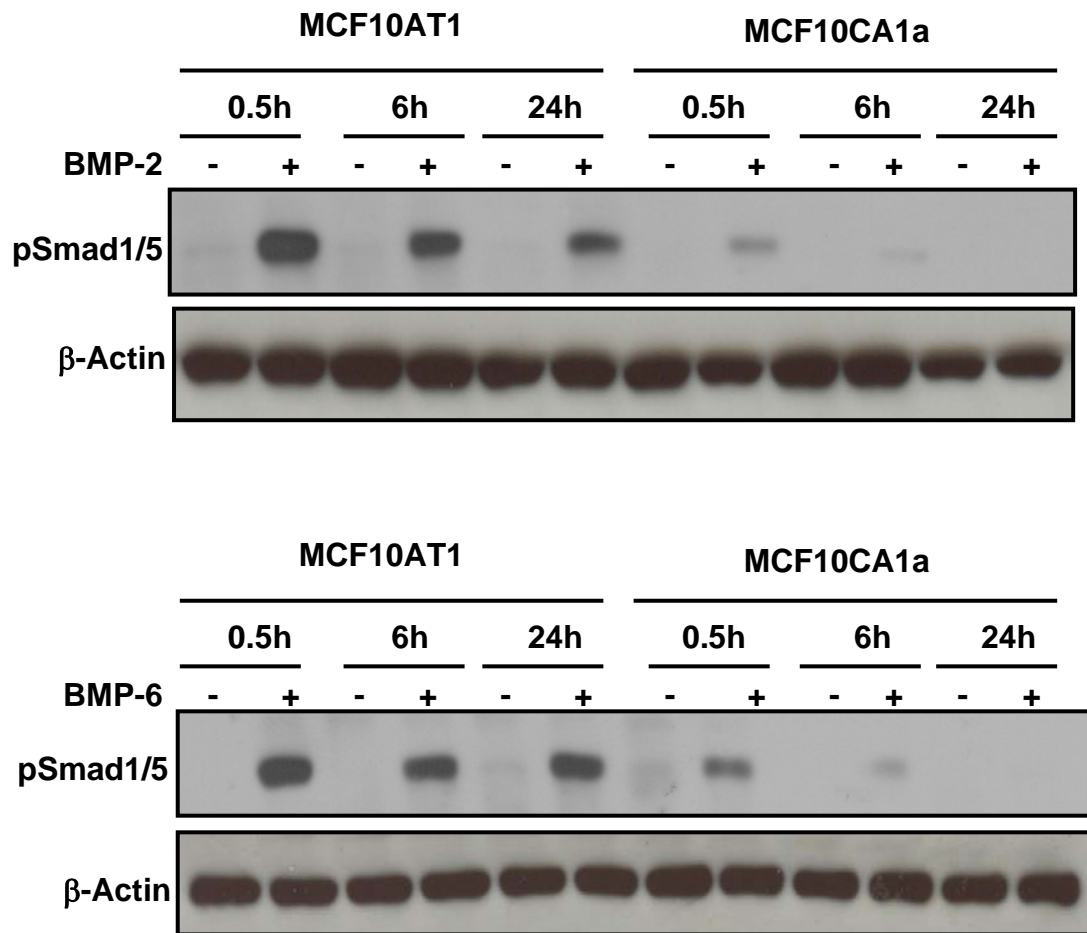
Fig. 11. Regulation of Smad responses by BMP ligands in MCF10AT1 and MCF10CA1a cells

BMP-2 and BMP-6 activate Smad signaling more significantly in MCF10AT1 than MCF10CA1a cells. MCF10AT1 and MCF10CA1a cells (5 X 10^5 cells/6-well plate) were starved for 24 hr in serum-free DMEM/F-12 medium. Then, cells were incubated with DMSO, BMP-2 (100 ng/ml) or BMP-6 (10 ng/ml) in 0.1% BSA/DMEM/F-12 medium for 30 min (0.5h), 6h, and 24 h before protein harvest.
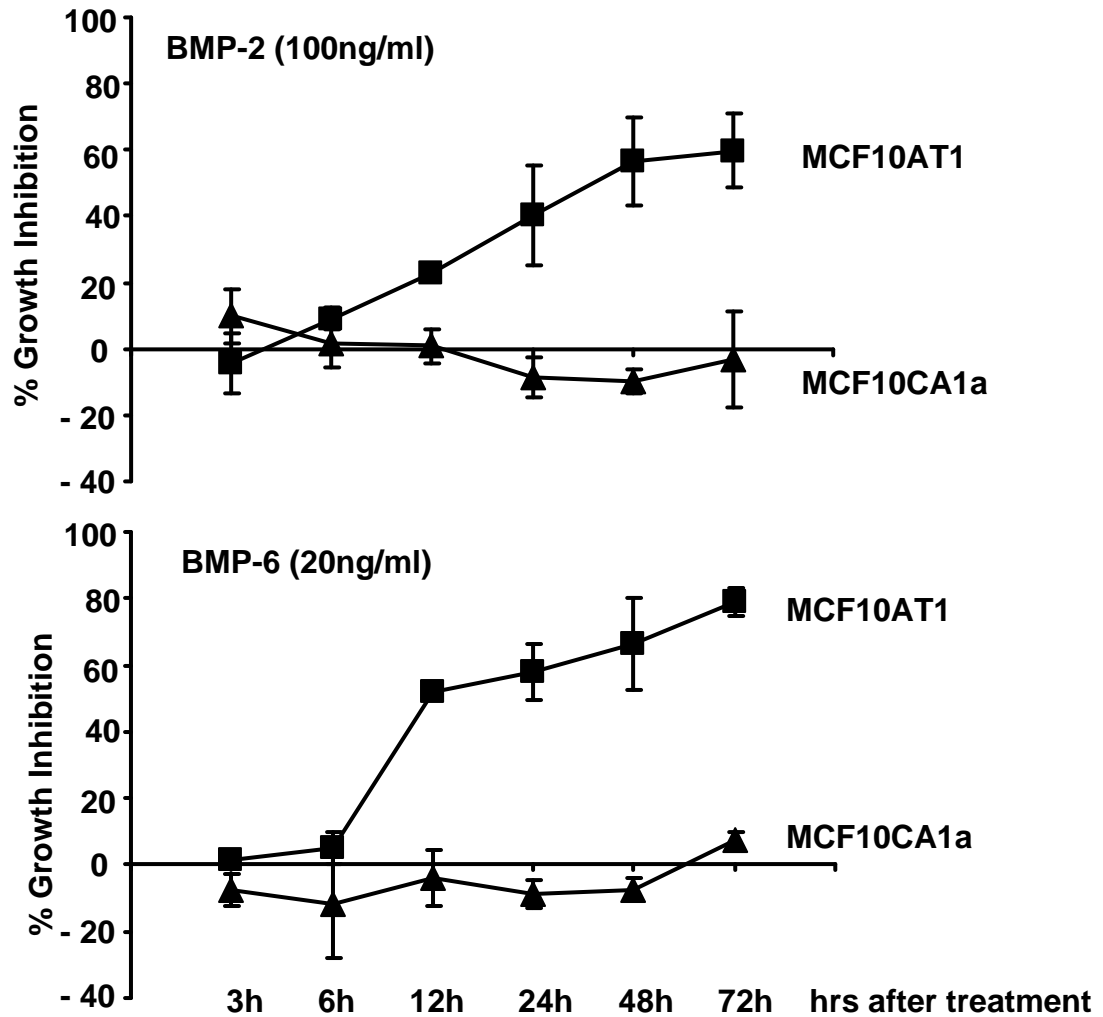
Fig. 12. Growth inhibition by BMPs in MCF10AT1 and MCF10CA1a cells

BMP-2 and BMP-6 inhibit cell growth in MCF10AT1 cells but not in MCF10CA1a cells. The cells (8,000 cells/well of MCF10AT1 and MCF10CA1a) were incubated with BMP-2 (100 ng/ml) and BMP-6 (20 ng/ml) in 5% horse serum/DMEM/F-12 for 3h, 6h, 12h, 24h, 48h and 72 h. Time-response curves of BMP-2 and BMP-6 by the [^{3}H]thymidine incorporation assay are shown. Two separate experiments were performed and combined.
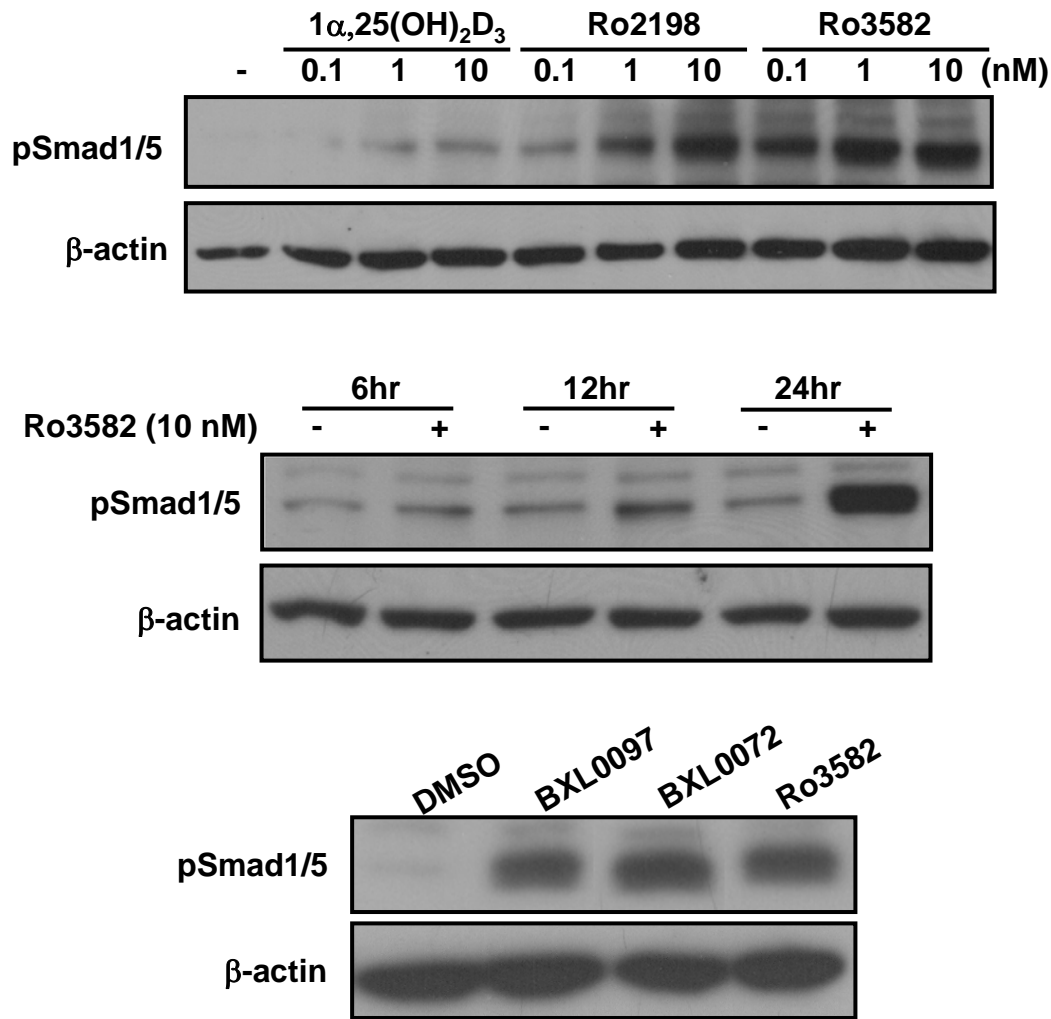
Fig. 13. Activation of Smad signaling by Gemini vitamin D analogs in MCF10AT1 cells.

Gemini vitamin D analogs induce more Smad1/5 phosphorylation than $1\alpha,25(OH)_2D_3$ in a dose dependent and time dependent manner. MCF10AT1 cells (5 X 10^5 cells/6-well plate) were starved for 24 hr in serum-free DMEM/F-12 medium. Then, cells were incubated with DMSO, $1\alpha,25(OH)_2D_3$, Ro2198, Ro3582 (0.1, 1, 10 nM) or Ro3582 (10 nM), BXL0097 (10 nM) and BXL0072 (10 nM) in 0.1% BSA/DMEM/F-12 medium. MCF10AT1 cells were treated for 24 hr if not mentioned. Upregulation of the phospho-Smad1/5 by vitamin D3 analogs is shown.
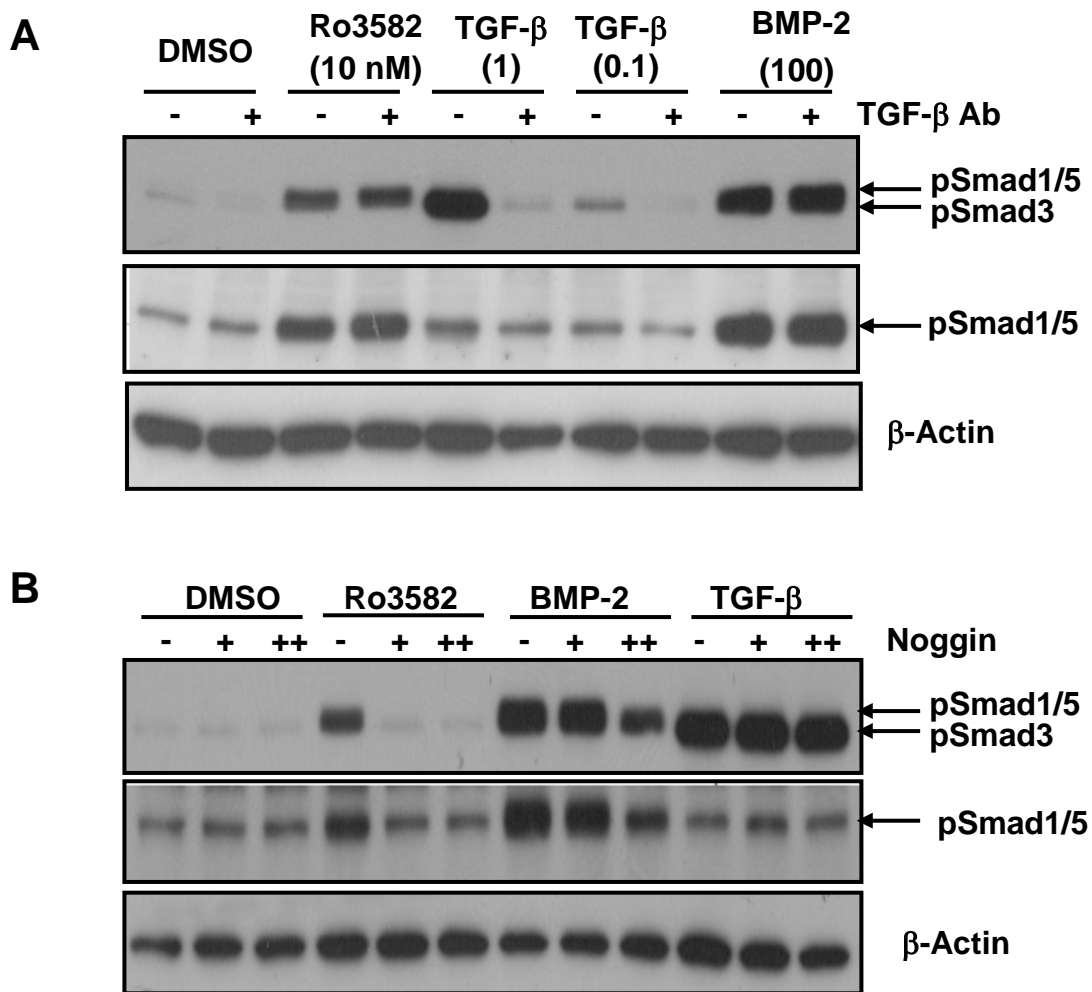
Fig. 14. BMP-specific Smad activation by Ro3582 in MCF10AT1 cells.

The phosphorylation of Smad1/5 induced by vitamin D analog Ro3582 is not blocked by TGF-β neutralizing antibody, but by a BMP antagonist, Noggin. **A.** MCF10AT1 cells (5 X 10^5 cells/6-well plate) were starved for 24 hr in serum-free DMEM/F-12 medium. Then, cells were incubated with the indicated compounds in 0.1% BSA/DMEM/F-12 medium for 24 hr. The phosphorylation of Smad3 induced by TGF-β1 (0.1 and 1 ng/ml) was blocked by TGF-β neutralizing antibody (TGF-β Ab, 1D11, 50 μg/ml). The induction of the phospho-Smad1/5 by Ro3582 (10 nM) or BMP-2 (100 ng/ml) was not blocked by the neutralizing antibody to TGF-β. **B.** MCF10AT1 cells (5 X 10^5 cells/6-well plate) were starved for 24 hr in serum-free DMEM/F-12 medium. Then, cells were incubated with the indicated compounds in 0.1% BSA/DMEM/F-12 medium for 24 hr. The phosphorylation of Smad1/5 induced by the vitamin D analog Ro3582 (10 nM) or BMP-2 (100 ng/ml) was blocked by treatment with Noggin, while the induction of the phospho-Smad3 by TGF-β1 (1 ng/ml) was not abolished by Noggin (+, 60 ng/ml, ++, 300 ng/ml).
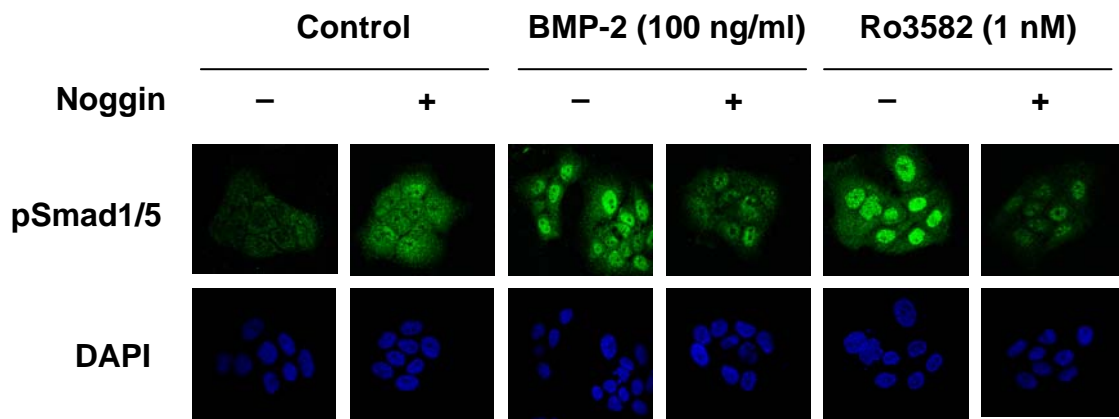
Fig. 15. Nuclear localization of pSmad1/5 by Ro3582 in MCF10AT1 cells.

Gemini vitamin D analog Ro3582 induces translocation of pSmad1/5 in MCF10AT1 cells. MCF10AT1 cells (30,000/chamber) were incubated with Ro3582 (1 nM) or BMP (100 ng/ml) in the presence and absence of BMP antagonist, Noggin (300 ng/ml), in 0.1% BSA/DMEM/F-12 medium for 24 hr. The staining for phospho-Smad1/5 was shown as green and DAPI staining for the nucleus was shown as blue. Immunofluorescence microscopy was shown (63 X magnification).
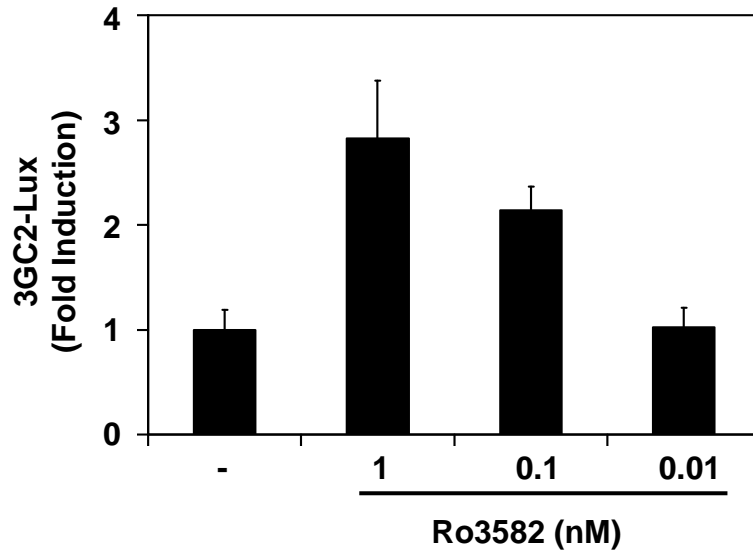
Fig. 16. BMP-dependent transcriptional activation by Ro3582 in MCF10AT1 cells.

Gemini vitamin D analog Ro3582 enhances BMP-dependent transcriptional activation. MCF10AT1 cells (40,000/well in 24 well plates) were plated and transfected with 3GC2-Lux vector (100 ng/well) for 6 hr and further treated with Ro3582 at concentrations of 1, 0.1, and 0.01 nM for 24 hr. Luciferase values were normalized for β-galactosidase activity.

A

B
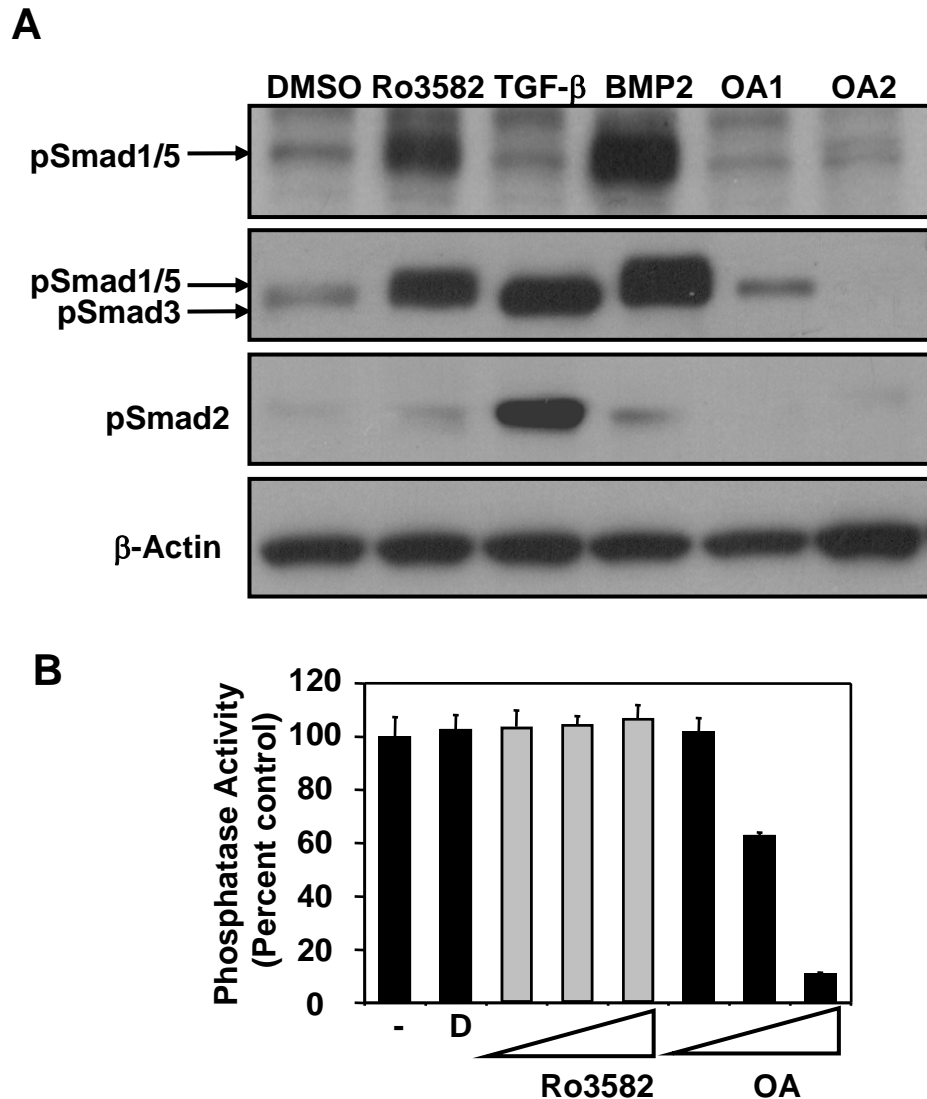
Fig. 17. Effect of Ro3582 on phosphatase activity and effect of phosphatase inhibition on Smad activation in MCF10AT1 cells.

Increased phospho-Smad1/5 is not due to the inhibition of phosphatase activity. A. MCF10AT1 cells (1 X 10⁶ cells/dish) were starved for 24 hr in serum-free DMEM/F-12 medium. Then, cells were incubated with the indicated compounds in 0.1% BSA/DMEM/F-12 medium for 24 hr. The phosphorylation of Smad1/5 induced by the Gemini vitamin D analog Ro3582 (10 nM) or BMP-2 (100 ng/ml) was shown, whereas TGF-β1 (1 ng/ml) or OA1 and OA2 (Okadaic acid, 10 and 100 nM) did not increase phospho-Smad1/5 in these cells. **B.** *In vitro* phosphatase assay was carried out using MCF10AT1 cell lysate. Ro3582 (10, 100, and 1000 nM) or okadaic acid (OA, 10, 100, and 1000 nM) was added to the cell lysate and inhibition of phosphatase enzyme activity was determined using non-radioactive specific phosphopeptide RRA(pT)VA, a peptide substrate for serine/threonine protein phosphatases 2A, 2B and 2C. D: DMSO
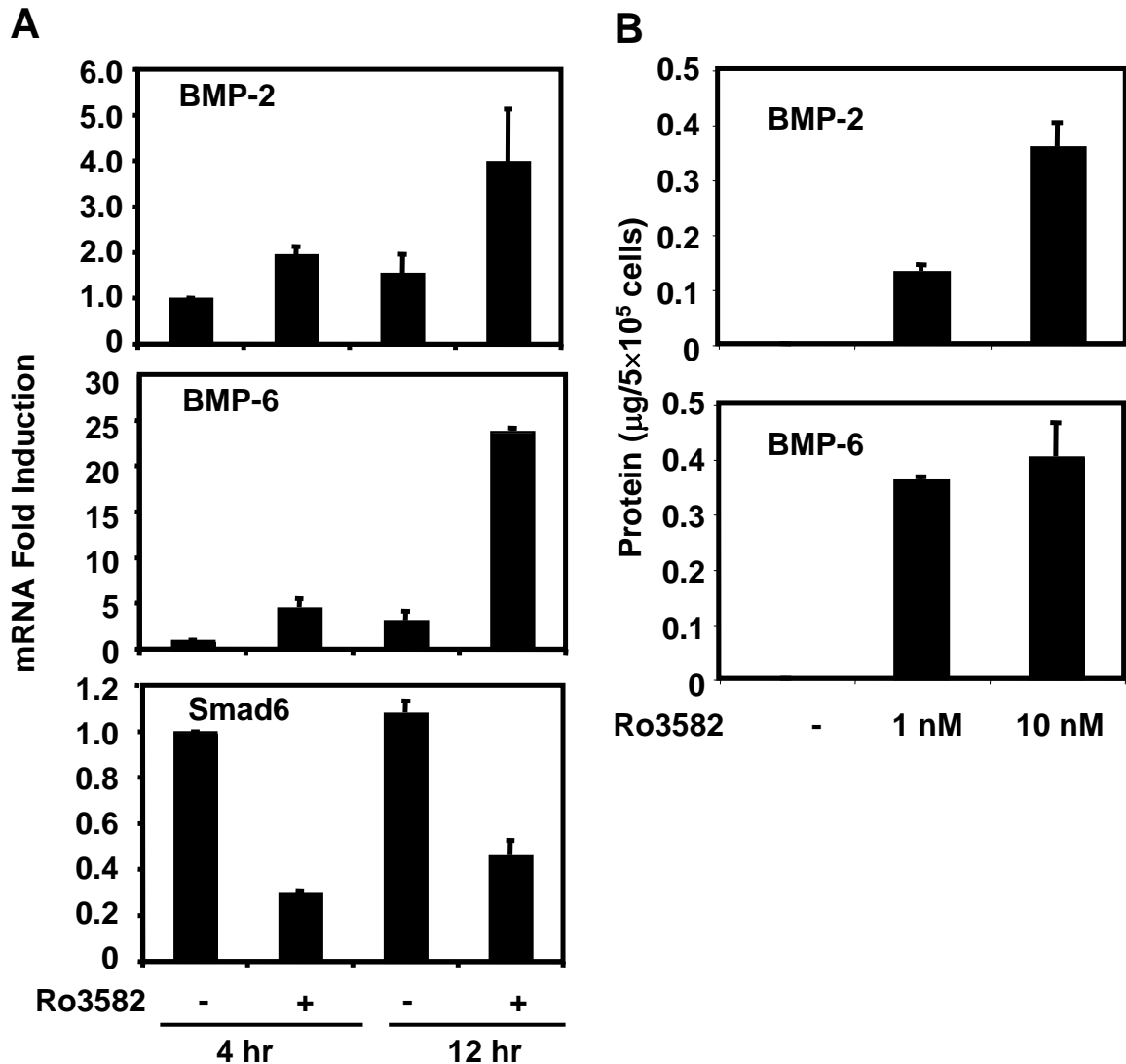
Fig. 18. Production of BMP ligands and down-regulation of inhibitory Smad6 by Ro3582 in MCF10AT1 cells.

Gemini vitamin D analog Ro3582 induces the mRNA and protein synthesis of BMP-2 and BMP-6, and also inhibits the mRNA expression of Smad6. A. MCF10AT1 cells (1 X 10^6 cells/100 mm dish) were incubated with the Gemini vitamin D analog Ro3582 (1 nM) in DMEM/F-12 medium supplemented with 5% horse serum for 4 or 12 hr. Total RNA was isolated, and the measurement of BMP-2, BMP-6 and Smad6 mRNA was performed. GAPDH values were used to normalize the production of the mRNA. Two separate experiments were performed and combined. **B.** MCF10AT1 cells (5 X 10^5 cells/100 mm dish) were starved for 24 hr in serum-free DMEM/F-12 medium. Then, cells were incubated with Ro3582 (1 and 10 nM) in 0.1% BSA/DMEM/F-12 medium for 48 hr. The supernatant was collected and stored at –20°C until assayed. The BMP-2 and BMP-6 protein synthesized and secreted into the medium were measured by the immunoassay. Representative data are shown from two similar experiments.
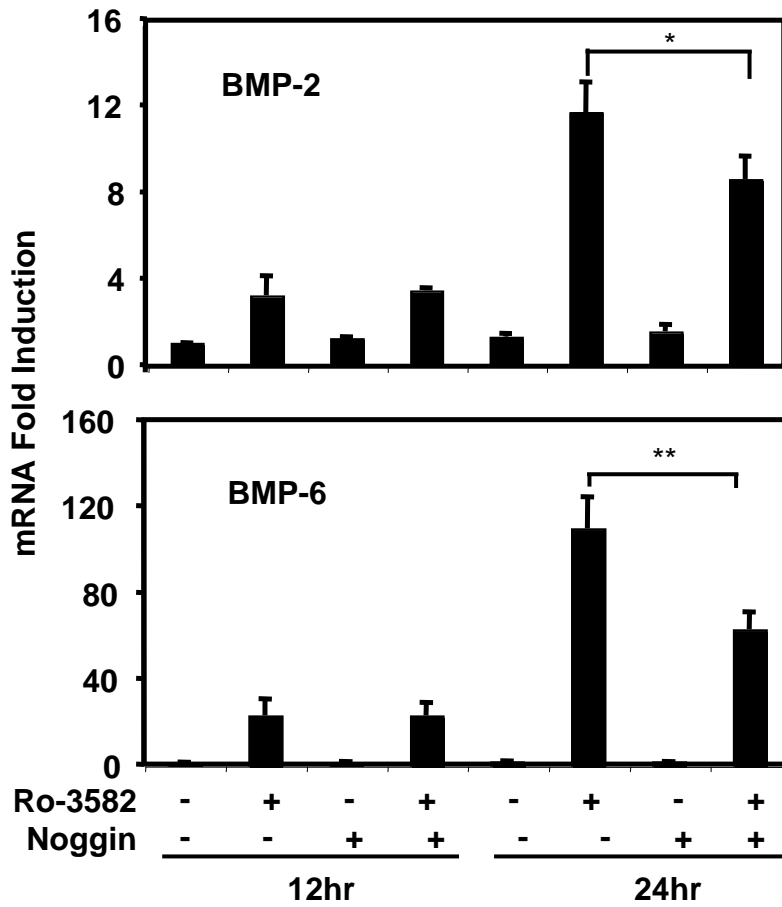
Fig. 19. Down-regulation of Ro3582-induced mRNA level of BMPs by Noggin in MCF10AT1 cells.

The BMP antagonist, Noggin, blocks the mRNA synthesis of BMP-2 and BMP-6 induced by Ro3582. MCF10AT1 cells (1 X 10^6 cells/100 mm dish) were starved for 24 hr in serum-free DMEM/F-12 medium. Then cells were incubated with Ro3582 (10nM) with or without Noggin (300 ng/ml) in DMEM/F-12 medium supplemented with 0.1% BSA for 12 or 24 hr. Total RNA was isolated, and the measurement of BMP-2 and BMP-6 mRNA was performed. GAPDH values were used to normalize the production of the mRNA. Two separate experiments were performed and combined (* p<0.05, ** p<0.01).
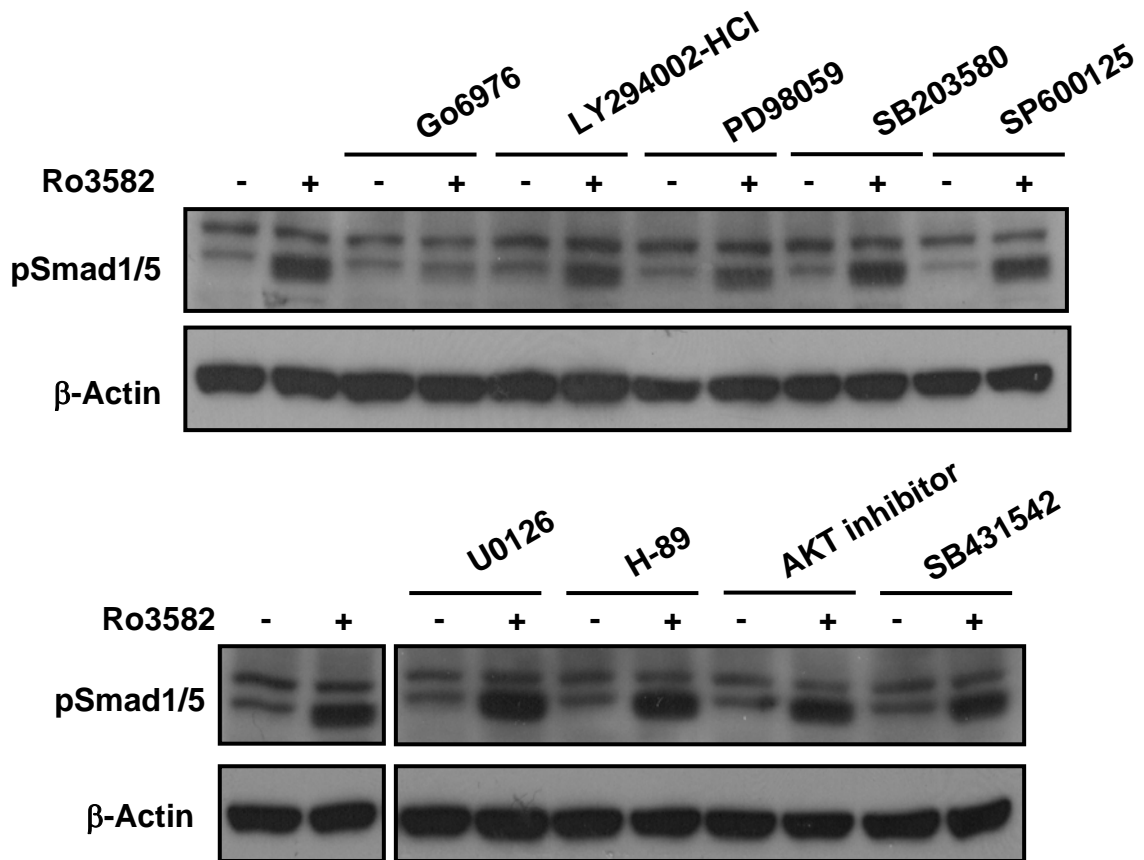
Fig. 20. Blockage of Ro3582-induced pSmad1/5 by PKCα/βI inhibitor in MCF10AT1 cells.

PKCα/βI inhibitor Go6976 blocks the phosphorylation of Smad1/5 induced by Gemini vitamin D analog Ro3582. MCF10AT1 cells (1 × 10^6 cells/100 mm dish) were starved in serum-free DMEM/F12 medium for 24 hr, and then incubated with Ro3582 (10 nM) and/or the following different types of kinase inhibitors: PKCα/βI inhibitor (Go6976, 0.5 μM), PI3K inhibitor (LY294002•HCl, 10 μM), MEK inhibitors (PD98059, 10 μM and U0126, 20 μM), p38 kinase inhibitor (SB203580, 10 μM), a JNK inhibitor (SP600125, 20 μM), a PKA inhibitor (H-89, 10 μM), an Akt inhibitor (a phosphatidylinositol ether analog, 10 μM), and a TGF-β type I receptor inhibitor (SB431542, 10 μM) in 0.1% BSA/DMEM/F12 medium for 24 hr. The level of phospho-Smad1/5 and β-actin was shown by Western blotting. Induction of the phosphorylation of Smad1/5 by the Gemini vitamin D analog Ro3582 was abolished by the PKCα/βI inhibitor Go6976.
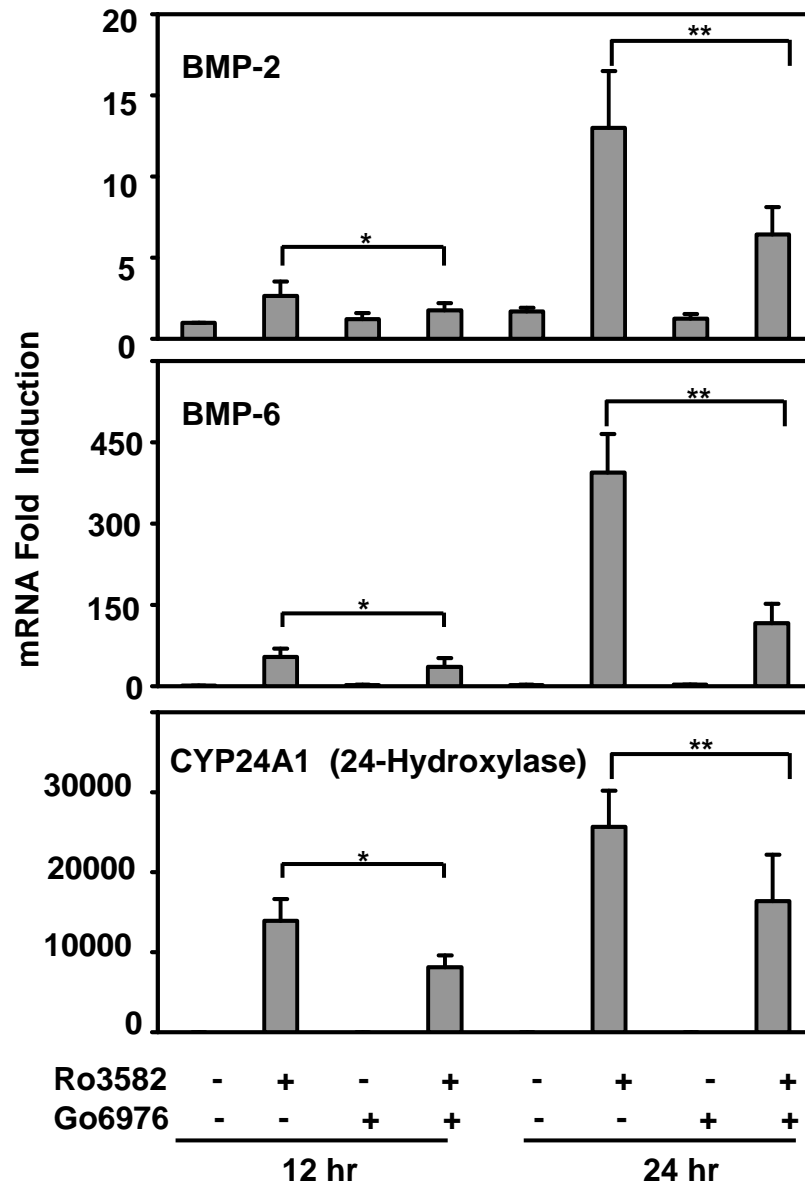
Fig. 21. Blockage of Ro3582-induced mRNA of BMPs and CYP24A1 by PKCα/βI inhibitor in MCF10AT1 cells.

PKCα/βI inhibitor Go6976 inhibits BMP-2, BMP-6 and CYP24A1 mRNA synthesis induced by Gemini vitamin D analog Ro3582. MCF10AT1 cells (1×10^6 cells/100 mm dish) were starved in serum free DMEM/F12 medium and then treated with the Gemini vitamin D analog Ro3582 (10 nM) and/or the PKCα/βI inhibitor Go6976 (0.5 μM) in 0.1% BSA/DMEM/F-12 medium for 12 or 24 hr. Total RNA was isolated, and the measurement of BMP-2, BMP-6 or CYP24A1 mRNA was performed as described in Materials and Methods. GAPDH values were used to normalize the production of the mRNA. Two separate experiments were performed and combined (the statistical significance is shown with *p<0.05, **p<0.01).
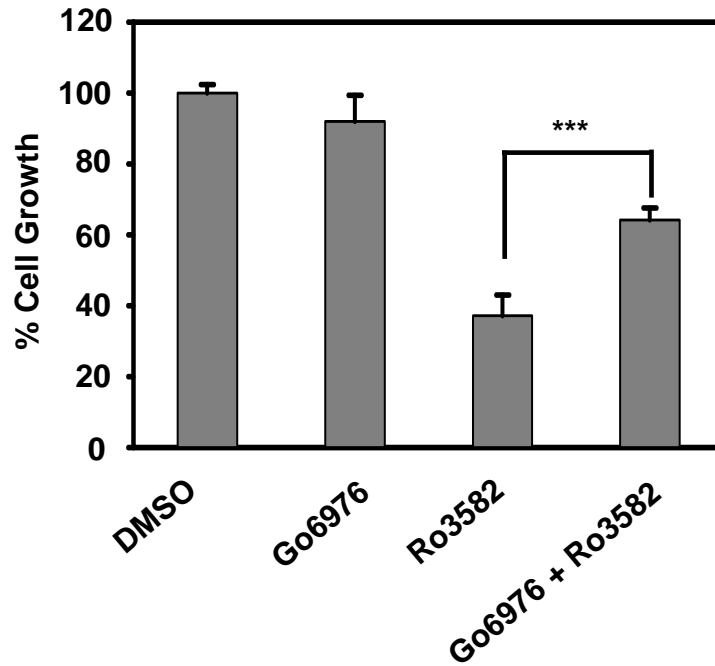
Fig. 22. Reversal of Ro3582-induced growth inhibition by PKCα/βI inhibitor in MCF10AT1 cells.

PKCα/βI inhibitor Go6976 reverses growth inhibition induced by Gemini vitamin D analog Ro3582. MCF10AT1 cells (5,000 cells/well in a 24-well plate) were treated with Ro3582 (1 nM) and/or PKCα/βI inhibitor Go6976 (0.1 μM) for 3 days in DMEM/F12 medium supplemented with 5% horse serum and 1% penicillin/streptomycin. One μCi of [3H]-thymidine was added 3 hr before the harvest and radioactivity in total DNA was measured using a liquid scintillation counter. Three separate experiments were performed and combined (the statistical significance is shown with ***$p < 0.001$).

A

B
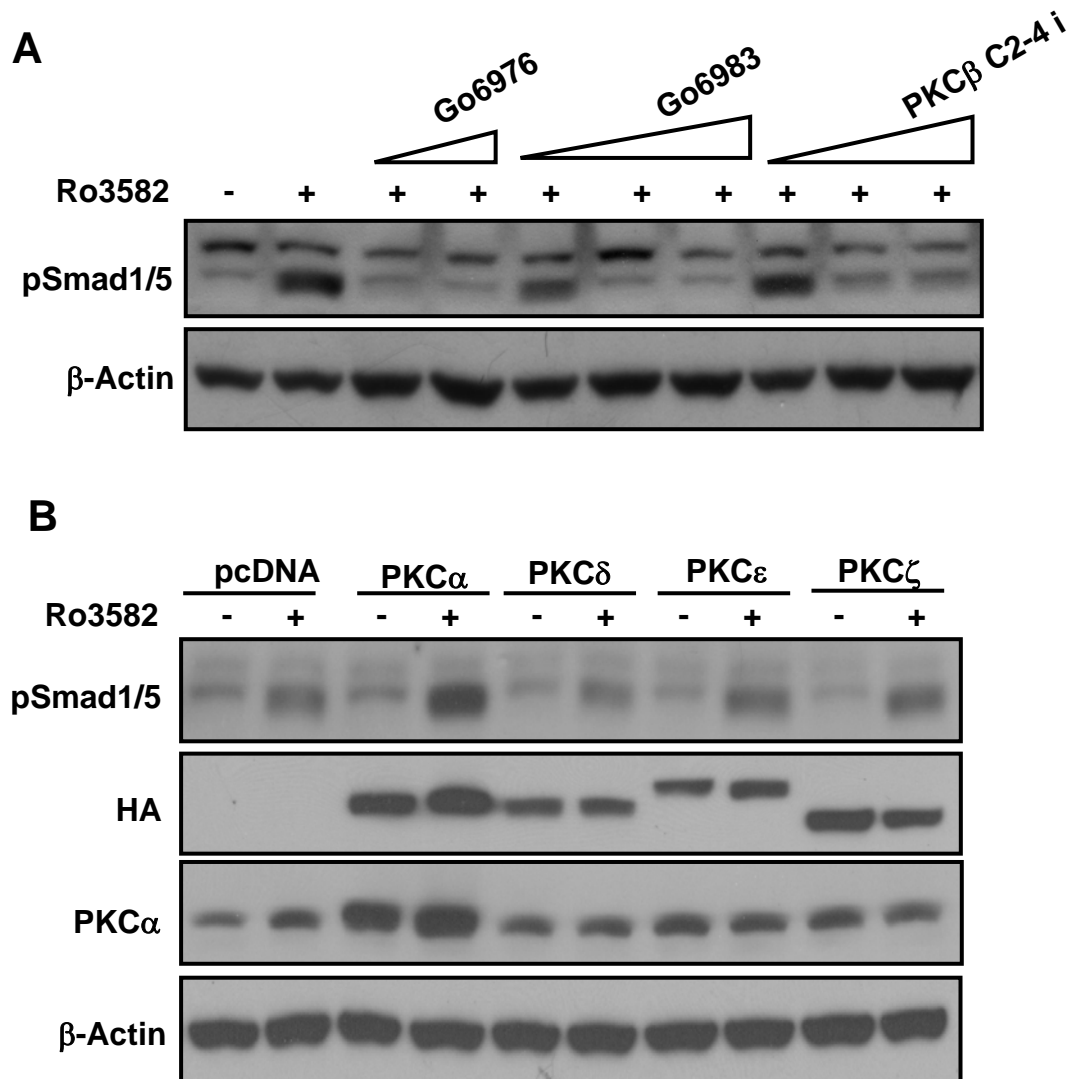
Fig. 23. Involvement of PKCα in Smad activation by Ro3582 in MCF10AT1 cells.

PKCα is involved in Smad activation induced by Gemini vitamin D analog Ro3582.
A. MCF10AT1 cells (1 × 10^6 cells/100 mm dish) were starved in serum free DMEM/F12 medium and then incubated with Ro3582 (10 nM) and/or three different PKC inhibitors, PKCα/βl inhibitor (Go6976, 0.5 μM and 5 μM), PKCα/β/γ/δ/ζ inhibitor (Go6983, 1 μM, 5 μM and 10 μM), and PKCβ C2-4 inhibitor (PKCα, β inhibitor, 1 μM, 5 μM and 10 μM) for 24 hr. **B.** MCF10AT1 cells (2 × 10^5 cells/well in a 6-well plate) were transfected with the vectors containing PKC isoforms (α, δ, ε or ζ) linked to HA under the condition described in Materials and Methods. After starvation in serum-free DMEM/F12 medium, cells were treated with Ro3582 (10 nM) for 24 hr. The level of phospho-Smad1/5, HA, PKCα and β-actin was shown by Western blotting.
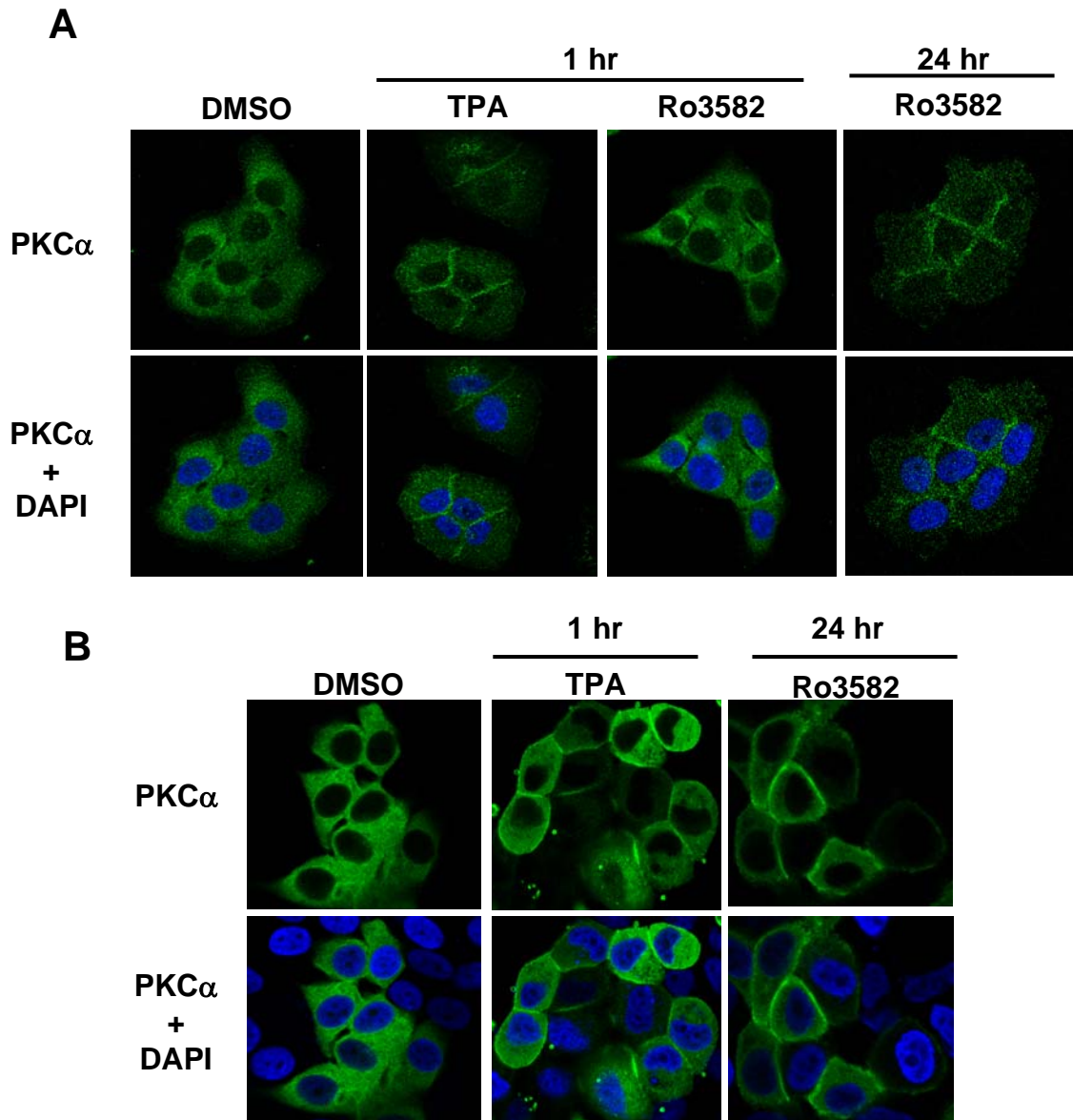
Fig. 24. Activation of PKC α/βI inhibitor by Ro3582 in MCF10AT1 cells

Gemini vitamin D analog Ro3582 activates PKCα. A. MCF10AT1 cells (20,000 cells/chamber in a 4-well chamber slide) were starved in serum-free DMEM/F12 medium, and then incubated with Ro3582 (10 nM) for 1 hr or 24 hr in 0.1% BSA/DMEM/F12 medium. TPA (10 nM) treatment for 1 hr was used as a positive control for PKCα activation. The staining for PKCα is shown as green and DAPI staining for the nucleus is shown as blue (63 X magnification). **B.** MCF10AT1 cells (2 × 10⁵ cells/glass bottom dish) were transfected transiently with the vector expressing GFP-PKCα fusion protein for 24 h. After starvation, TPA (10 nM) and Ro3582 (10 nM) were treated for 1 hr and 24 hr, respectively. GFP was detected at 488 nm as green and DAPI staining for the nucleus is shown as blue (63 X magnification).
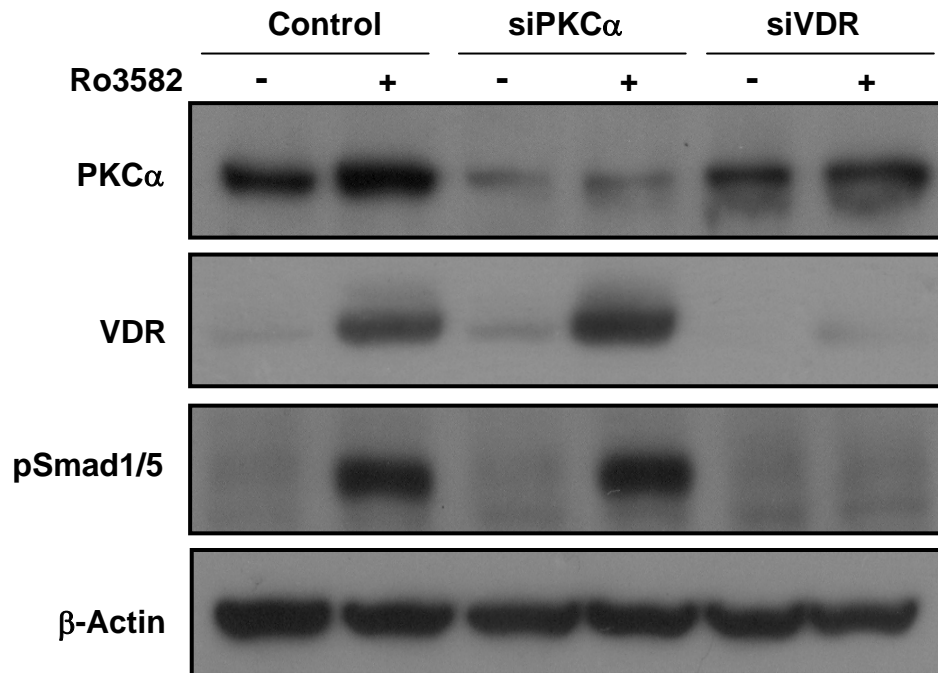
	Control		siPKCα		siVDR	
Ro3582	-	+	-	+	-	+
PKCα						
VDR						
pSmad1/5						
β-Actin						

Fig. 25. Effects of knock-down of PKCα and VDR on Smad activation by Ro3582 in MCF10AT1 cells.

Vitamin D receptor (VDR) is required to activate Smad signaling by Ro3582, but PKCα does not directly activate Smad1/5 induced by Gemini vitamin D analog Ro3582 in MCF10AT1 cells. MCF10AT1 cells (1 X 10^5 cells/6-well plate) were transfected with siRNA of VDR and PKCα for 72 hrs and treated with Ro3582 for 24 hr 0.1%BSA/DMEM/F-12 medium. Knock-down of PKCα and VDR protein and the level of phosphorylation of Smad1/5 were determined by Western blotting.
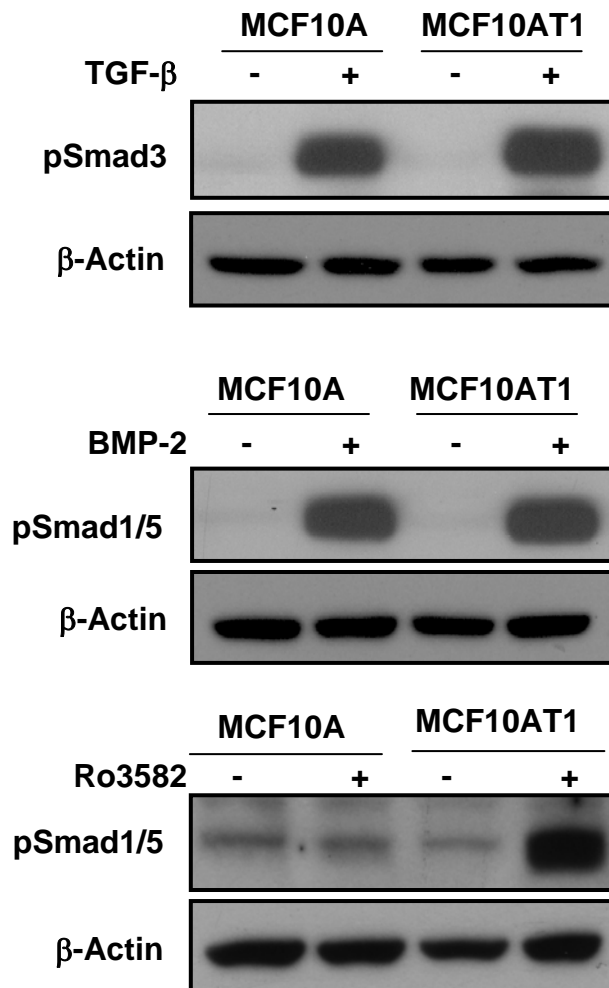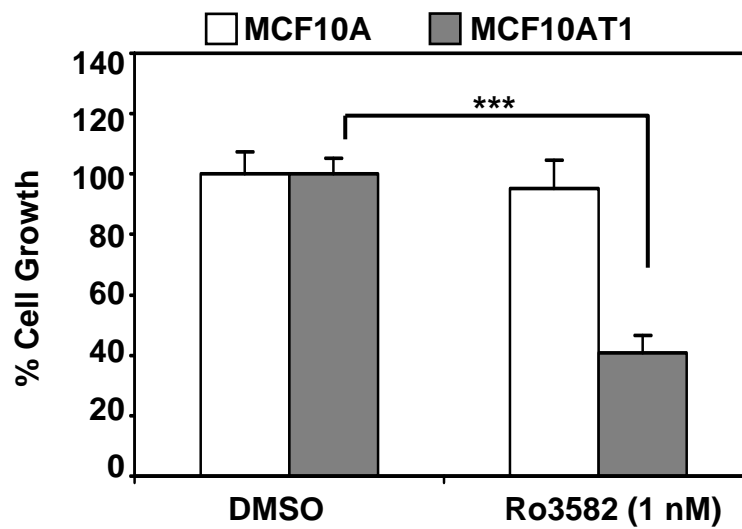
Fig. 26. Comparison of Smad activation in Ha-Ras transfected MCF10AT1 cells to that in the parent MCF10A cells

Gemini vitamin D analog Ro3582 activates Smad signaling in Ha-Ras transfected MCF10AT1 cells, but not in the parent MCF10A immortalized human breast epithelial cells. MCF10A and MCF10AT1 cells (5 X 10⁵ cells/6-well plate) were starved for 24 hr in serum-free DMEM/F-12 medium. Then, cells were incubated with TGF-β (1 ng/ml) and BMP-2 (100 ng/ml) for 30 min, and Ro3582 (10 nM) for 24 hr in 0.1% BSA/DMEM/F-12 medium. The phospho-Smad protein induced by TGF-β1, BMP-2 or Ro3582 is shown.

Fig. 27. Comparison of growth inhibition by Ro3582 in Ha-Ras transfected MCF10AT1 cells to that in the parent MCF10A cells

Gemini vitamin D analog Ro3582 inhibits cell growth in Ha-Ras transfected MCF10AT1 cells, but not in the parent MCF10A cells. MCF10A and MCF10AT1 cells (5,000 cells/well in a 24-well plate) were treated with Ro3582 (1 nM) for 3 days in DMEM/F12 medium supplemented with 5% horse serum and 1% penicillin/streptomycin. One μCi of [^{3}H]-thymidine was added 3 hr before the harvest and radioactivity in total DNA was measured using a liquid scintillation counter (the statistical significance is shown with ***$p < 0.001$).
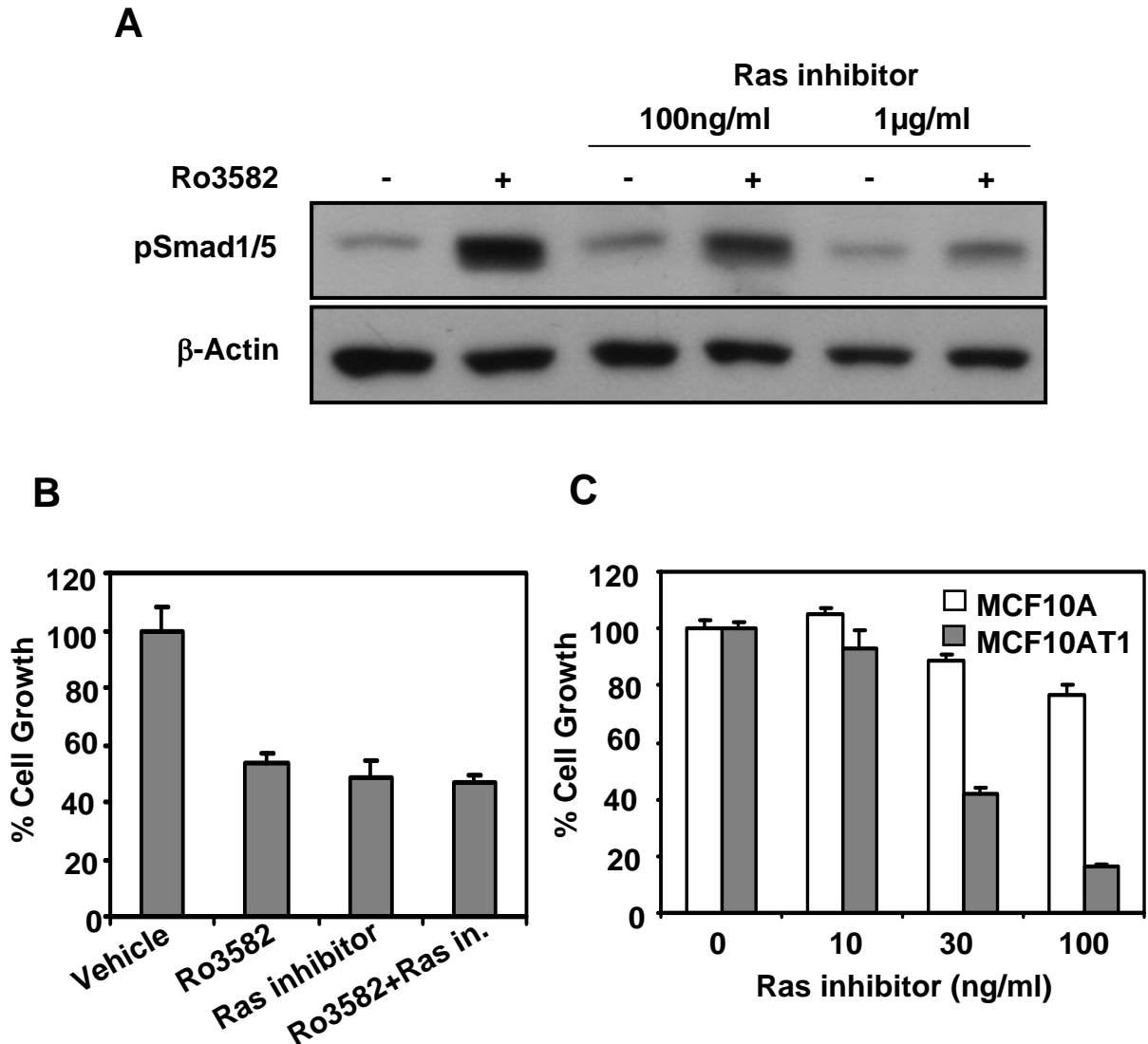
A

B

C

Fig. 28. Involvement of Ras in Smad activation by Ro3582 in MCF10AT1 cells.

Ras farnesyltransferase inhibitor (Ras inhibitor) inhibits cell growth more specifically in MCF10AT1 cells and blocks the phosphorylation induced by Ro3582. A. MCF10AT1 cells (5 X 10^5 cells/6-well plate) were starved for 24 hr in serum-free DMEM/F-12 medium. Then, cells were incubated with Ro3582 and/or Ras inhibitor in 0.1% BSA/DMEM/F-12 medium for 24 hr. The level of phospho-Smad1/5 and β-actin was shown by Western blotting. **B and C.** MCF10A and MCF10AT1 cells (5,000 cells/well in a 24-well plate) were treated with Ro3582 (1 nM) and/or Ras inhibitor (30 ng/ml in **B**, and 10, 30, 100 ng/ml in **C**) for 3 days in DMEM/F12 medium supplemented with 5% horse serum and 1% penicillin/streptomycin. One μCi of [^{3}H]-thymidine was added 3 hr before the harvest and radioactivity in total DNA was measured using a liquid scintillation counter.
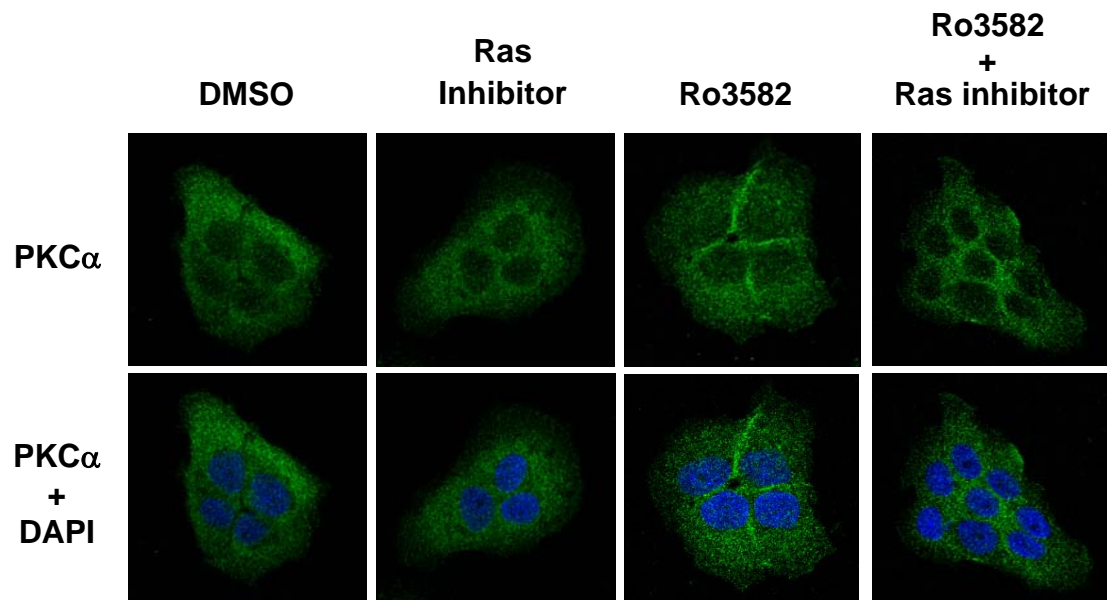
Fig. 29. Blockage of PKCα activation by a Ras farnesyltransferase inhibitor in MCF10AT1 cells.

PKCα activation by Ro3582 is blocked by a Ras farnesyltransferase inhibitor in MCF10AT1 cells. MCF10AT1 cells (20,000 cells/chamber in a 4-well chamber slide) were starved in serum-free DMEM/F12 medium, and then incubated with Ro3582 (10 nM) and/or the Ras inhibitor (1 μg/ml) in 0.1% BSA/DMEM/F12 medium for 24 h. The staining for PKCα is shown as green and the nucleus stained with DAPI is shown as blue (63 X magnification).
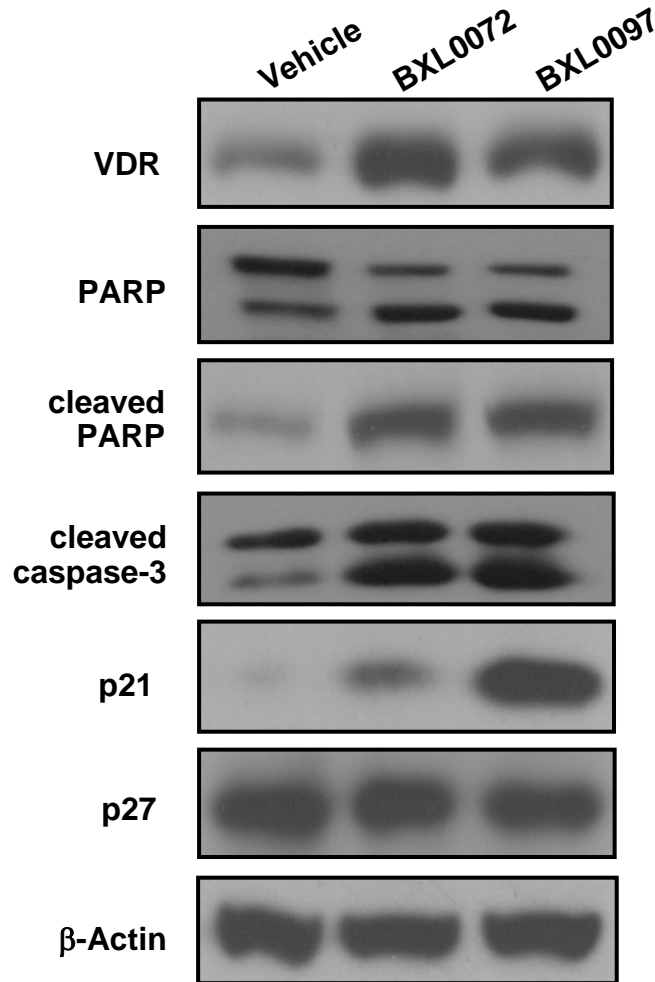
Fig. 30. Protein regulation by Gemini vitamin D analogs in NMU-induced mammary tumors.

Gemini vitamin D analogs, BXL0072 and BXL0097, inhibit cell proliferation and induce apoptosis in NMU-induced tumors. NMU-treated rats were injected with BXL0072 and BXL0097 and tumor samples were analyzed by Western blotting. Gemini vitamin D analogs BXL0072 and BXL0097 (0.3 μg/kg body weight/day) increased the expression of VDR, apoptosis and a cell cycle arrest-related proteins in mammary tumors induced by NMU in rats.
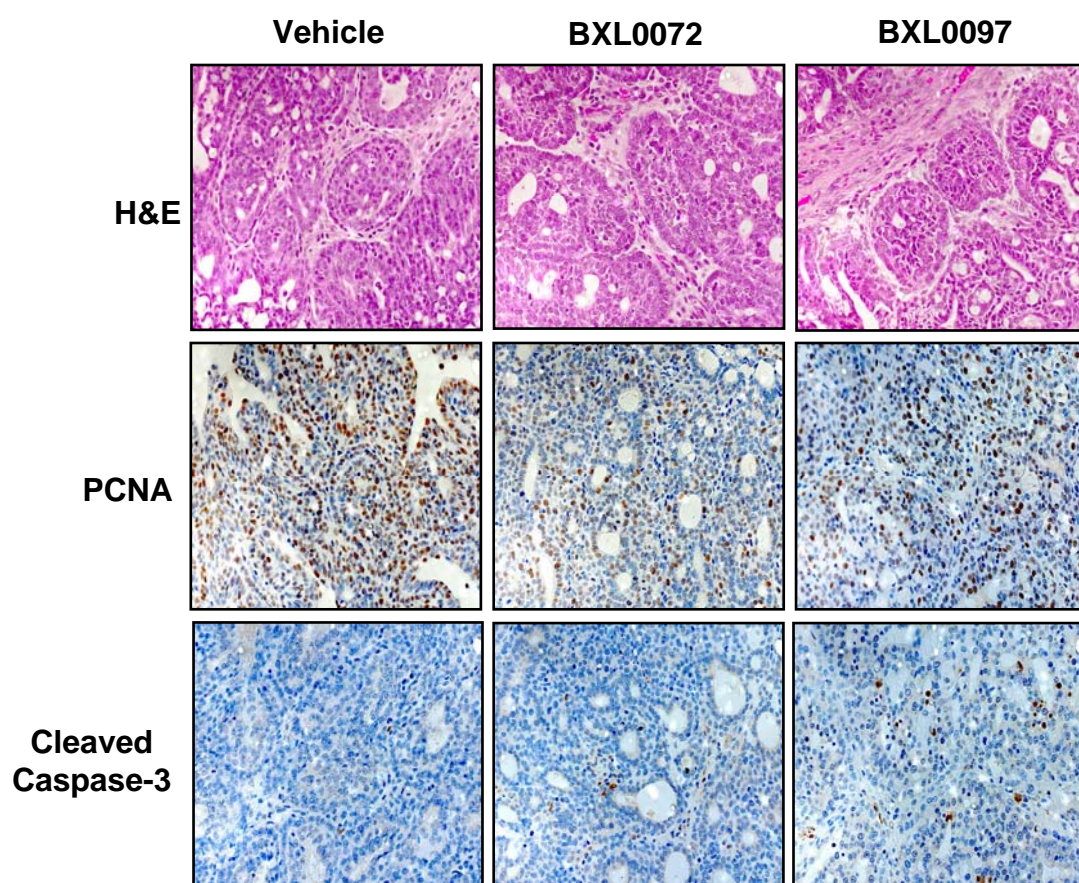
	Vehicle	BXL0072	BXL0097
H&E			
PCNA			
Cleaved Caspase-3			

Fig. 31. Immunohistochemistry of NMU-induced mammary tumors treated with Gemini vitamin D analogs.

Gemini vitamin D analogs, BXL0072 and BXL0097, do not affect on the tumor type, differentiation/grade and stromal responses in H & E staining, but inhibit PCNA expression and increase the cleaved caspase-3 expression in NMU-induced tumors. NMU-treated rats were injected with BXL0072 and BXL0097 (0.3 μg/kg body weight/day) and tumor samples were analyzed by immunohistochemistry. Hematoxylin and eosin (H&E), PCNA and cleaved caspase-3 staining of tumors are shown. Positive reactions for PCNA and cleaved caspase-3 are noted by a reddish brown precipitate in the cells.
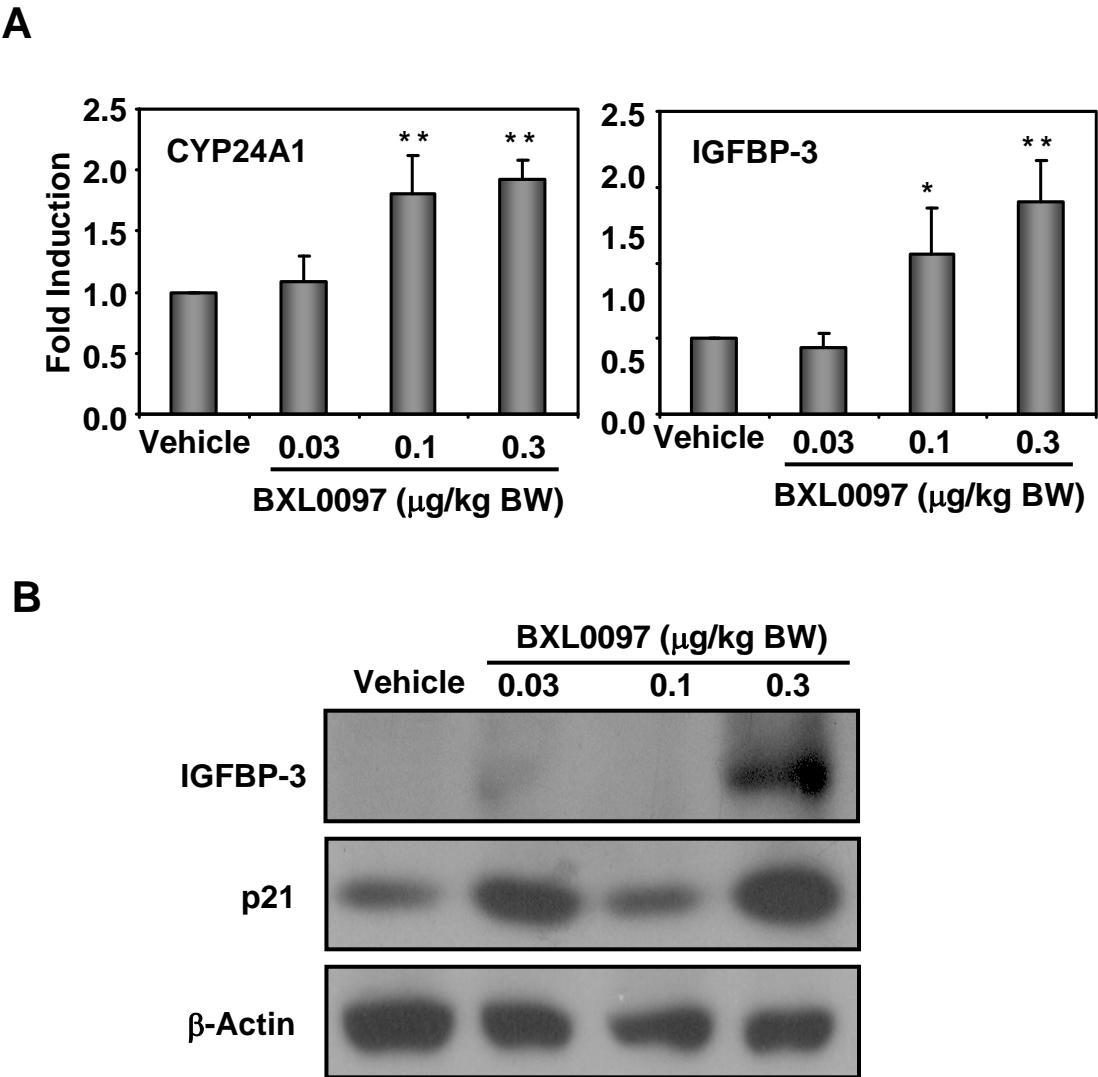
A

B

Fig. 32. Effect of Gemini vitamin D analog BXL0097 on mRNA and protein regulation in NMU-induced mammary tumors.

Gemini vitamin D analog BXL0097 induces mRNA of CYP24A1 and IGFBP-3, as well as protein expression of IGFBP-3 and p21 in NMU-induced mammary tumors. NMU-treated rats were injected with BXL0097 and tumor samples were analyzed by quantitative RT-PCR and Western blotting. **A.** The Gemini vitamin D analog BXL0097 increases the mRNA level of CYP24A1 encoding 24-hydroxylase and insulin-like growth factor binding protein-3 (IGFBP-3) in NMU-induced mammary tumors. Two separate analyses were performed and combined (statistical significance: *$p<0.01$, **$p<0.001$). **B.** The Gemini vitamin D analog BXL0097 increases the protein level of IGFBP-3 and p21 in NMU-induced mammary tumors.

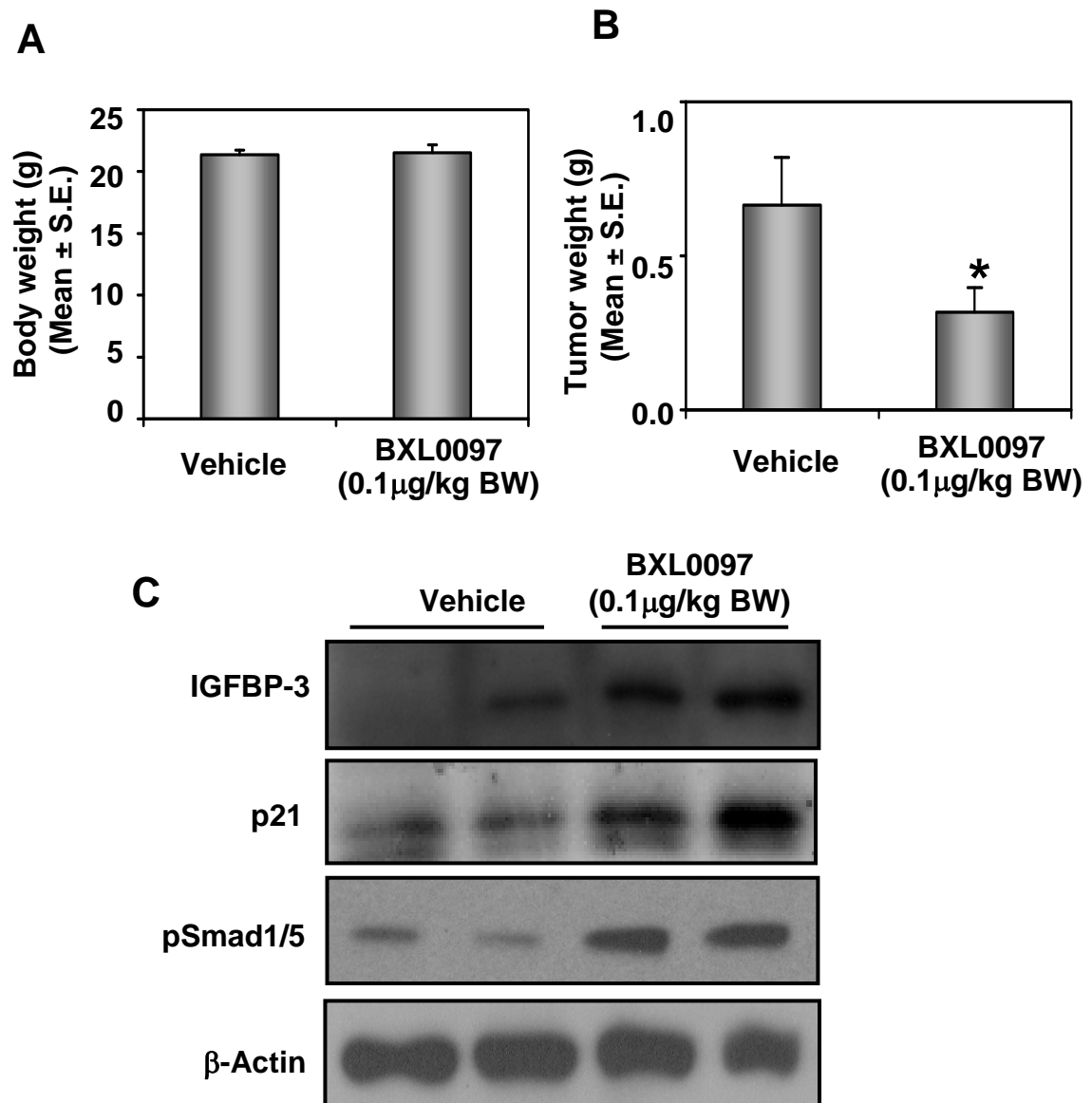**Fig. 33. Effect of BXL0097 on MCF10DCIS.com xenograft tumor growth**

Gemini vitamin D analog BXL0097 significantly inhibits tumor growth and regulates the level of IGFBP-3, the CDK inhibitor p21 and pSmad1/5 in ER-negative MCF10DCIS.com xenograft tumors in SCID mice. MCF10DCIS.com cells (1×10^6 cells) were injected into a mammary fat pad. Four days later, the mice received either vehicle or Gemini vitamin D analog BXL0097 (0.1 µg/kg body weight) intraperitoneally daily. **A.** The body weight was not affected by Gemini vitamin D analog treatment. **B.** The average tumor weight per mouse were shown. The number of mice in vehicle control and BXL0097 treated group was 7 and 5, respectively (statistical significance: *, p= 0.04). **C.** The proteins from tumor samples were extracted with RIPA buffer and the expression level of IGFBP-3, CDK inhibitor p21, pSmad1/5 and β-actin was determined by Western blotting.
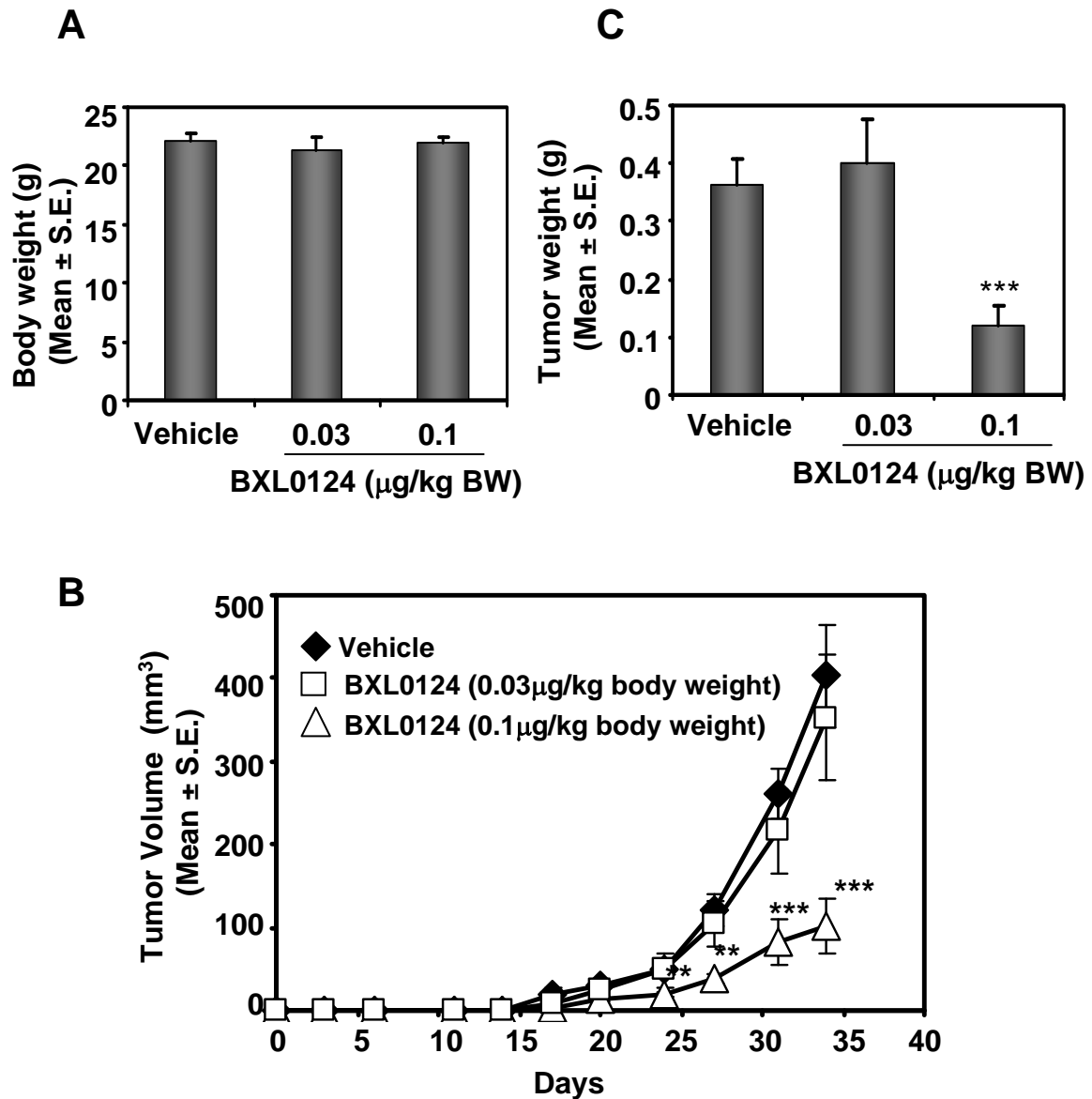
Fig. 34. Effect of Gemini vitamin D analog BXL0124 on MCF10DCIS.com xenograft tumor growth.

The Gemini vitamin D analog BXL0124 significantly inhibits tumor growth in MCF10DCIS.com xenograft tumors in SCID mice. Experimental design is same as shown in Fig. 33. Vehicle or Gemini vitamin D analog BXL0124 (0.03 and 0.1 μg/kg body weight) were injected intraperitoneally daily. Body weight and tumor volume were measured twice a week. Mice were sacrificed at day 34. Data are expressed as mean ± S.E. (*p<0.05, **p<0.01, ***p<0.001) (N = 7). **A.** The body weight was not affected by Gemini vitamin D analog BXL-0124 treatment in SCID mice. **B.** MCF10DCIS.com xenograft tumor growth was significantly inhibited at high dose of BXL0124. **C.** Tumor weight was significantly reduced by the high dose of BXL0124 in MCF10DCIS.com xenograft model.
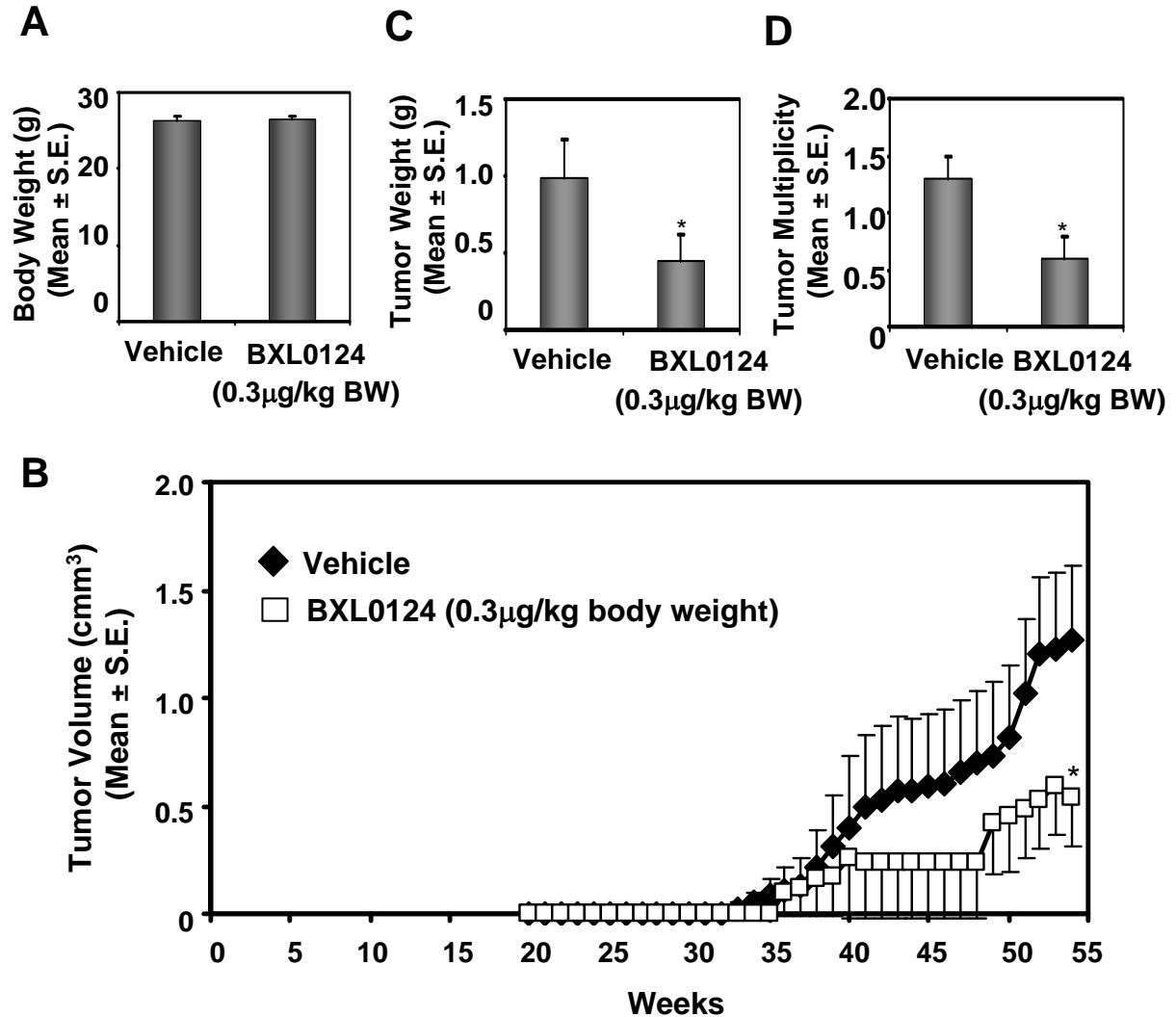
Fig. 35. Effect of BXL0124 on tumor growth in MMTV-Her2/neu transgenic mouse model.

The Gemini vitamin D analog BXL0124 significantly inhibits tumor growth in ER-negative MMTV-Her2/neu transgenic mouse model. When MMTV-Her2/neu mice were 20 weeks old, vehicle (DMSO, n=16) and Gemini vitamin D analog BXL0124 (0.3 µg/kg body weight, n=8) were given intraperitoneally three times a week. Body weight and tumor volume were measured weekly. Mice were sacrificed at 54 weeks of age. **A.** The body weight was not affected by Gemini vitamin D analog BXL0124 treatment. **B and C.** Tumor growth and tumor weight were significantly inhibited by BXL0124 treatment. **D.** Tumor multiplicity per mouse was also significantly reduced by BXL0124 treatment. Data are expressed as mean ± S.E. (*p<0.05).

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RESEARCH PUBLICATIONS:

1. Liu,Y., Xiao, H., Tian, Y., Nekrasova, T., Hao, X., **Lee, H.J.**, Suh, N., Yang, C.S., Minden, A. The Pak4 protein kinase plays a key role in cell survival and tumorigenesis in athymic mice*. Mol Cancer Res.* 6(7): 1215-1224, **2008**.
2. **Lee, H.J.**, Ji, Y., Paul, S., Maehr, H., Uskokovic, M., and Suh, N. Activation of bone morphogenetic protein signaling by a Gemini vitamin D_3 analog is mediated by the Ras/protein kinase Cα pathway. *Cancer Res.* 67(24): 11840-11847, **2007**.
3. Suh, N., Paul, S., **Lee, H.J.**, Ji, Y., Lee, M.J., Yang, C.S., Reddy, B.S., and Newmark, H. Mixed tocopehrols inhibit N-methyl-N-nitrosourea-induced mammary tumor growth in rats. *Nutr Cancer.* 59(1): 76-81, **2007**.
4. **Lee, H.J.**, Wislocki, A., Goodman, C., Ji, Y., Ge, R., Maehr, H., Uskokovic, M., Reiss, M., and Suh, N., A novel vitamin D derivative activates bone morphogenetic protein signaling in MCF10 breast epithelial cells. *Mol Pharmacol.* 69(6): 1840-1848, **2006**.
5. Ji, Y., **Lee, H.J.**, Goodman, C., Uskokovic, M., Liby, K., Sporn, M.B., and Suh, N. The synthetic triterpenoid CDDO-imidazolide induces monocytic differentiation by activating the Smad and ERK signaling pathways in HL60 leukemia cells. *Mol Cancer Ther.* 5: 1452-1458, **2006**.
6. **Lee, H.J.**, Liu, H., Goodman, C., Ji, Y., Maehr, H., Uskokovic, M., Notterman, D., Reiss, M., and Suh, N. Gene expression profiling changes induced by a novel Gemini vitamin D derivative during the progression of breast cancer. *Biochem Pharmacol.* 72: 332-343, **2006**. (Equal contribution)
7. **Lee, H.J.**, Lee, K.W., Kim, K.H., Kim, H.K., and Lee, H.J. Antitumor activity of peptide fraction from Korean traditional soy sauce. *J Microbiol Biotechnol.* 14: 628-630, **2004**.
8. Yi, H.J., Kim, J.Y., Kim, K.H., **Lee, H.J.,** and Lee, H.J. Anticancer activity of peptide fractions from egg white hydrolysate against mouse lymphoma cells*. Food Sci Biotechnol.* 12: 224-227, **2003**.