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**THE ROLE OF THE EGF RECEPTOR AND VITAMINS A AND D
IN THE DEVELOPMENT AND PROGRESSION OF BREAST CANCER
TO MORE MALIGNANT HORMONE-INDEPENDENT PHENOTYPES**

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THE ROLE OF THE EGF RECEPTOR AND VITAMINS A AND D IN THE DEVELOPMENT AND PROGRESSION OF BREAST CANCER TO MORE MALIGNANT HORMONE-INDEPENDENT PHENOTYPES

INTRODUCTION

Despite the fact that approximately 60% of human breast cancers contain estrogen receptor at the time of diagnosis, only about two thirds of these tumors will respond to anti-estrogen therapy, and many of those which do respond initially will develop resistance to anti-estrogen therapy over time. This progression to a more malignant form of the disease is often associated with an increase in substances known as growth factors or growth factor receptors that provide the tumor with an alternate signal telling the cells to grow. One such growth factor receptor whose presence in breast tumors strongly correlates with poor clinical prognosis and the lack of response to anti-estrogen therapy is the epidermal growth factor receptor (EGFR). This overall goal of this project is to understand how vitamins A and D control the level of this growth factor receptor that is functionally implicated in the development and progression of breast cancer.

BODY

Research Accomplishments (see attached manuscript for details)

We have investigated the effects of 1,25-dihydroxyvitamin D₃, analog C (which is a vitamin D analog), 9-cis RA, and all-trans RA on the growth and expression of EGFR in a panel of 4 breast cancer cell lines. We have chosen these cell lines based on their relative levels of EGFR. Since levels of EGFR and ER tend to be inversely correlated to one another in breast cancer cells, the lines we have selected represent a good approximation of the progression of breast cancer to a more aggressive, hormone independent phenotype characterized by a loss of ER and a gain in EGFR expression. The magnitude of the growth inhibitory effects that the vitamin D and retinoid compounds used in this study produce reflect this progression as those cell lines expressing the lowest levels of EGFR (MCF7 and T47D) had the greatest amount of growth inhibition while those with higher levels responded less significantly (BT474 and BT549).

The difference in the degree of growth inhibition produced by the vitamin D and the retinoid compounds may also in part be linked to their relative levels of VDR, RAR, and RXR expression. Specifically, those cell lines with the highest levels of VDR, RAR, or RXR would be predicted to have the largest degree of growth inhibition in response to vitamin D, all trans RA, or 9-cis RA treatment, respectively. This is especially recognizable in the case of RAR α which has an inverse correlation with EGFR and confers increased sensitivity to retinoid inhibition of growth when upregulated in ER negative breast cancer cells. In terms of vitamin D mediated growth inhibition, however, this is not what is observed as T47D and BT474 cells, which have relatively equivalent levels of VDR, have quite different growth profiles when treated with vitamin D. An explanation proposed for this variation in the degree of growth inhibition induced by vitamin D on cell lines with similar levels of VDR involves differential changes in the regulation of target genes which mediate its anti-proliferative effect. More specifically, it is speculated that vitamin D and retinoids exert their effects in controlling cell growth and differentiation through specific modulation of growth factor receptor expression.

In order to investigate the possibility that their growth inhibitory effects may be mediated by or linked to a down-regulation of EGFR, the expression of EGFR mRNA was examined in MCF7, T47D, BT474, and BT549 cell lines after vitamin D and retinoid treatments. The observation of a 72-hour-sustained 50% decrease in BT549 EGFR mRNA observed within 2 hours of 1,25-dihydroxyvitamin D₃ treatment allowed us to perform all subsequent experiments with 60-72 hour incubation times to maximize RNA and protein yields. By three days of vitamin D and retinoid treatments, MCF7, T47D, and BT549 cell lines demonstrated EGFR mRNA down-regulation while BT474 showed an upregulation, all in the context of a range of significant to insignificant growth inhibition. These changes in EGFR mRNA levels were found to translate into similarly altered levels of EGFR protein in the cell membrane for vitamin D, analog C, and 9-cis RA treated BT474 and BT549 cell lines.

The above data imply that growth inhibition and EGFR down-regulation by the retinoid and vitamin D compounds may be related effects in some breast cancer cells, but not in all. So as to better understand this issue of EGFR up- or down-regulation in the context of the growth inhibition induced by the retinoid and vitamin D compounds, we felt it first necessary to establish the molecular basis of EGFR regulation by the vitamin D, and subsequently, retinoid compounds in breast cancer cells. In so doing it is hoped that any mechanistic differences in the regulation of EGFR by vitamin D or retinoids will help in understanding the biologic and prognostic significance of the growth inhibition they produce in the cell lines examined.

It is generally accepted that vitamin D mediated regulation of gene expression is controlled at the level of transcription. Nevertheless, in order to rule out a contributing influence of altered mRNA stability, experiments were done on the BT474 and BT549 cell lines designed to measure EGFR mRNA half-life after a 3 day treatment with 1,25-dihydroxyvitamin D₃. Plotting the average decay of EGFR mRNA over a four hour period after addition of the RNA synthesis inhibitor, actinomycin D, no significant difference was observed between control (non-vitamin D treated) and vitamin D treated decay curves for BT474 cells, suggesting that EGFR mRNA half-life was not altered by vitamin D. Similar experiments that were performed on the BT549 cell line were uninformative since the actinomycin D failed to generate mRNA decay in both control and treatment groups. The prospect of performing the assay on T47D or MCF7 cells remains. However, given the low basal level of EGFR mRNA in these cell lines, the 50-70% reduction that is caused by vitamin D treatment, combined with further decay of the mRNA by actinomycin D, the limit of detection of the assay would most likely be exceeded. Therefore we directly asked if the vitamin D effect on EGFR mRNA expression has a transcriptional component by employing a reporter gene construct in transient transfection assays.

The 840 base pair portion of the EGFR promoter from 20 to 860 nucleotides upstream of the translation start site used in these assays contains multiple transcription start sites and sites for the binding of positive and negative regulatory factors. Transient transfections of this construct named pJFCAT 840 in MCF7, T47D, and BT549 cell lines followed by vitamin D or analog C treatment resulted in a down-regulation of CAT activity of similar magnitude as EGFR mRNA and protein down-regulation in these same cell lines. Transfection of pJFCAT 840 into BT474 cells demonstrated a low basal level of activity and a slight 1.3 fold induction in response to vitamin D. The magnitude of this induction is less than that which was seen with endogenous EGFR mRNA and

protein in response to vitamin D treatment. We speculate that either difficulties we observed in transfecting the BT474 cell line or additional EGFR sequences not represented within the pJFCAT 840 construct are responsible for the blunted reporter gene induction when compared to endogenous mRNA and protein changes. Both of these possibilities are currently being explored. Whatever the case, the fact that the BT474 cell line demonstrated an effect opposite that observed upon transfection of pJFCAT 840 into MCF7, T47D, and BT549 cells suggests that sequences within 840 bp of the EGFR promoter are important for mediating EGFR regulation by vitamin D. Since the same portion of the promoter is able to mediate different EGFR regulatory responses that mimic changes in endogenous EGFR mRNA and protein, it also indicates that there are probably cell specific factors involved.

We are currently delineating the minimal vitamin D responsive region of the EGFR promoter and characterizing the roles that the various factors involved play. To this end, we have identified by sequence analysis a region of the EGFR promoter between nucleotides 531 and 516 upstream of the translation start site that bears strong resemblance to the consensus DR3 PuG(G/T)TCA vitamin D response element, as well as known functional VDREs for the human and rat osteocalcin and mouse osteopontin genes. We hypothesize that this region of the EGFR promoter, by the binding of the VDR with one or more cell specific coregulatory factors, may mediate the differential vitamin D regulation of EGFR expression in BT474 versus MCF7, T47D, and BT549 cells. VDREs such as this are known to bind the ligand activated VDR as a homodimer or heterodimer in the context of a coregulatory molecule which often tends to be one of RXRs. Receptor binding subsequently mediates a positive or negative transcriptional response depending upon the gene considered and what other cell specific factors may be involved. Characterization of the factors and mechanisms involved in breast cancer cells will potentially provide us with an understanding of the varied effects that vitamin D has on cell growth and EGFR expression.

Training

This project is providing Mr. McGaffin with training in the areas of gene regulation and breast cancer. He has written one manuscript (see appendix), and is in the process of writing his thesis dissertation.

APPENDICES

KEY RESEARCH ACCOMPLISHMENTS

- 1,25-dihydroxyvitamin D₃, analog C (1,25-(OH)₂-16-en-23-yn-26,27-F₆-vitamin D₃), 9-cis retinoic acid, and all-trans retinoic acid inhibit the growth of MCF7, T47D, and BT474 breast cancer cells, not BT549 cells
- Three days treatment with vitamin D or retinoids results in a decrease in EGFR mRNA in MCF7, T47D, and BT549 cells, and an increase in BT474 cells; EGFR protein levels correlate with mRNA changes
- There is no change in EGFR mRNA half-life with vitamin D treatment, but reporter constructs indicate transcriptional regulation mediated through vitamin D responsive sequences in the EGFR promoter

REPORTABLE OUTCOMES

- Manuscript: McGaffin KR, Atkinson LE, Chrysogelos SA, Growth and EGFR Regulation in Breast Cancer Cells by Vitamin D and Retinoid Compounds. Manuscript submitted to Cell Growth and Differentiation.

MANUSCRIPTS AND ABSTRACTS ATTACHED

- Manuscript: McGaffin KR, Atkinson LE, Chrysogelos SA, Growth and EGFR Regulation in Breast Cancer Cells by Vitamin D and Retinoid Compounds. Manuscript submitted to Cell Growth and Differentiation.

GROWTH AND EGFR REGULATION IN BREAST CANCER CELLS BY VITAMIN D AND RETINOID COMPOUNDS¹

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Abstract

Vitamin D and retinoids mediate growth inhibition and EGFR regulation in a variety of breast cancer cells. In this study, the effect of 1,25-dihydroxyvitamin D₃, analog C (1,25-(OH)₂-16-en-23-yn-26,27-F₆-vitamin D₃), 9-cis retinoic acid, and all-trans retinoic acid on the growth and EGFR expression in MCF7, T47D, BT474, and BT549 breast cancer cells was examined. Significant growth inhibition by all 4 compounds was noted in MCF7, T47D, and BT474 cells by 8 days of treatment, while BT549 cells showed no significant growth inhibition. A 50% decrease in BT549 EGFR mRNA was observed by 2 hours and persisted through 72 hours post 1,25-dihydroxyvitamin D₃ treatment. Three days after administration of vitamin D or retinoids, EGFR mRNA showed a 20-70% reduction in MCF7, T47D, and BT549 cells, while BT474 cells demonstrated a 200-500% increase. EGFR protein levels correlated with these mRNA changes in BT474 and BT549 cells. Measurement of mRNA stability in vitamin D treated BT474 cells indicates that there is no change in EGFR mRNA half-life. Transfection of an EGFR promoter containing reporter plasmid demonstrated vitamin D induced changes in reporter gene activity that parallel the changes observed in EGFR mRNA and protein. These results indicate that the vitamin D effect on EGFR expression in breast cancer cells has a transcriptional component mediated through vitamin D responsive promoter sequences. They also suggest that growth inhibition and EGFR down-regulation by vitamin D and retinoids may be related events in some breast cancer cells, but not in all.

Running Title: Growth and EGFR Regulation in Breast Cancer Cells

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Introduction

It is hypothesized that the overexpression of growth factors or their receptors gives rise to a population of cells with a distinct growth advantage and tumorigenic potential (1). There are numerous reports in recent years implicating overexpression of the epidermal growth factor receptor (EGFR) in the development of breast and other neoplasia (1-6). In addition to its putative direct role in the pathogenesis of cancer, high level expression of EGFR in many cancerous sites has been repeatedly correlated with more malignant or advanced disease, poor prognosis, and/or likely patient failure on endocrine therapy with the appearance of hormone independent growth (7-12). One study in particular (12) found only lymph node status to be more powerful than EGFR status in predicting disease outcome. Given this, it has been suggested that EGFR can potentially serve as one in a series of important independent prognostic markers in terms of breast cancer treatment and survival (2,6-11).

EGFR is a member of the type I family of growth factor receptors whose gene is located on chromosome 7p12 (13) and encodes a 170 kilodalton transmembrane glycoprotein with tyrosine kinase activity (14). The cytoplasmic and transmembrane domains have a high degree of homology to the protein encoded by the avian erythroblastosis virus oncogene, *v-erbB* (15), suggesting that the EGFR gene corresponds to the human *c-erbB* oncogene. The intracellular region of the receptor contains an autophosphorylation site composed of three tyrosine residues and a tyrosine kinase domain (3). Binding of ligand to EGFR (i.e., EGF, TGF α , amphiregulin, etc.) promotes dimer and oligomer formation of the receptor which is critical in the regulation of its tyrosine kinase activity (14). Autophosphorylation of the receptor, phosphorylation of intracellular downstream effectors, and production and activation of many of the factors involved in normal cell growth then takes place. Additionally, activated EGFR is able to induce production and activation of nuclear proto-oncogenes and cyclins whose amplification is typically associated with dysregulation of EGFR and enhanced breast cancer proliferation (5).

Many reports note an inverse correlation between the presence of EGFR and the estrogen receptor (ER) in human breast cancers (3,16,17). This suggests that a complex series of

regulatory factors and/or pathways are involved in cell-type specific levels of EGFR expression. Elevated EGFR expression in breast cancer rarely (3% or less of all cases) results from gene amplification or rearrangement (16,18), but rather tends to be explained by transcriptional mechanisms (6,16,18). Experimentally, overexpression of EGFR by gene transfection in NIH3T3 cells leads to EGF dependent cellular transformation (19,20), and in ER positive MCF7 breast cancer cells leads to increased resistance to antiestrogen treatment (21). Further, ectopic expression of EGFR in ZR-75 breast cancer cells induces hormone independent growth (22). These data provide direct evidence that dysregulation of EGFR expression can be a contributing factor in breast cancer development and progression.

Understanding ways in which EGFR expression can be modulated to treat breast cancer is not a novel idea (23). EGFR levels have been shown to be influenced by its ligands, EGF and TGF α , in both breast and non-breast cells (24-26). However, EGF and TGF α have a stimulatory effect on cancer cell growth (9,27). Agents which have been shown to inhibit the proliferation of and incite differentiation in transformed cell lines are the retinoid and 1,25-dihydroxyvitamin D₃ compounds (28,29). Specifically, 1,25-dihydroxyvitamin D₃ and its analogs have been shown to induce apoptosis in (30), inhibit the invasive potential of (31), and reduce the growth of (32-38) human breast cancer cells *in vitro*. The retinoids have also been reported to cause growth inhibition and inhibit carcinogenesis in several breast cancer cell lines (32,39-42). Both groups of compounds demonstrate regulation of EGFR expression in a variety of cancer cells (27,43-46). However, the growth inhibitory effect of these differentiation agents only corresponds to a downregulation of EGFR in select cell lines (44,45). Some cancer cell lines demonstrate an opposite effect of EGFR upregulation accompanied by growth inhibition (27,43) or stimulation (45,46) depending upon the concentration of retinoid and 1,25-dihydroxyvitamin D₃ compounds used and/or the cell type considered. As the cited studies imply, only the growth reduction and degree of differentiation that the retinoid and 1,25-dihydroxyvitamin D₃ compounds produce seem to be interrelated, while their effects on EGFR expression and cell proliferation are not always associated.

This report documents the effect of 1,25-dihydroxyvitamin D₃, 1,25 dihydroxyvitamin D₃ analog C (chemical name: 1,25-(OH)₂-16-en-23-yn-26,27-F₆-D₃), 9-cis retinoic acid (RA), and all-trans RA treatment on the growth of MCF7, T47D, BT474, and BT549 breast cancer cells. We also demonstrate the effects of these compounds on EGFR mRNA and protein expression and present data indicating that this EGFR regulatory effect has a transcriptional mechanism that is mediated by EGFR promoter sequences containing a putative vitamin D response element (VDRE).

Results

The Vitamin D and Retinoid Compounds Cause Growth Inhibition in Select Cells. Retinoid and vitamin D compounds are known to exert their biologic effects in part by binding to and activating nuclear vitamin D (VD), retinoic acid (RA), and retinoid X (RX) receptors (47-50). The relative levels of these receptors in the MCF7, T47D, BT474, and BT549 breast cancer cell lines used in this study, along with their tissue source and tumor type, were taken from various published articles (34,51,52) and summarized on table 1. From this an inverse correlation between ER and EGFR levels in the different cell lines can be seen. An inverse correlation between RAR α and EGFR levels is also apparent. However, no other correlations among the receptors could be drawn.

Figure 1 demonstrates the chemical structures of the 1,25-dihydroxyvitamin D₃, analog C, 9-cis RA, and all trans RA ligands used. Analog C (1,25-(OH)₂-16-en-23-yn-26,27-F₆-D₃) is a vitamin D analog with greater potency (as measured by the ability to induce differentiation and inhibit cellular proliferation), less toxicity (as measured by the ability to stimulate intestinal calcium adsorption and bone calcium mobilization), and greater affinity binding to the vitamin D receptor than 1,25-dihydroxyvitamin D₃ (34,53). All ligands were used at a final concentration of 1 μ M. This concentration was chosen based on published dose response analyses for treatment with vitamin D and retinoid compounds (34,44,45,54).

Figure 2 panel a demonstrates the results of growth assays done with MCF7, T47D, BT474, and BT549 cell lines treated with 1,25-dihydroxyvitamin D₃, analog C, 9-cis RA, and all trans RA for 8 days. When compared to untreated control cells grown in fetal bovine serum (FBS) plus vehicle, significant growth inhibition was observed for MCF7 and T47D cells treated with any of the 4 ligands by the 6 day timepoint. By 8 days, the BT474 cell line showed significant growth inhibition for all 4 ligands. The BT549 cell line showed no significant growth inhibition at any point in the assay. Panel b summarizes the effect 1,25-dihydroxyvitamin D₃, analog C, 9-cis RA, and all trans RA after 8 days of treatment. It is interesting to note that the magnitude of growth inhibition elicited by the retinoid and vitamin D compounds administered

to the MCF7, T47D, BT474, and BT549 cell lines is inversely correlated with their relative levels of EGFR expression.

Vitamin D and Retinoids Can Regulate EGFR Expression. In order to assess the effect on EGFR expression after 1,25-dihydroxyvitamin D₃, analog C, 9-cis RA, and all trans RA treatment, RNase protection was employed to measure EGFR mRNA levels in MCF7, T47D, BT474, and BT549 cell lines. Shown in figure 3 is quantitated autoradiographic data from a timecourse experiment done using 1 μ M 1,25-dihydroxyvitamin D₃ and BT549 cells. Demonstrated is a 50% decrease in EGFR mRNA by 2 hours of treatment which is maintained through 72 hours. Data in these and subsequent RNase protection experiments were corrected for loading error by using the internal RNA control 36B4 (55).

Because the effect of 1,25-dihydroxyvitamin D₃ on BT549 EGFR mRNA levels was maximal by 2 hours post-treatment and sustained through 72 hours, all subsequent treatments were carried out for 60-72 hours to maximize RNA yields. Figure 4A represents a typical series of RNase protection gels showing the 1,25-dihydroxyvitamin D₃ effect on EGFR mRNA levels after 3 days of 1 μ M treatment when compared to untreated controls. Due to their lower expression of EGFR mRNA when compared to BT549 cells, the gels run for the MCF7, T47D, and BT474 cell lines were exposed to film for 2 weeks, 1 week, and 4 days, respectively. The 36B4 band, and the EGFR band in BT549 cells, was detected in all cells after a 6 hour exposure. Consequently, the raw data for EGFR and 36B4 signals in the MCF7, T47D, and BT474 cell lines is a representation of two different exposures. The data shown in panel 4A is represented in a histogram with similar data from at least three independent experiments in MCF7, T47D, BT474, and BT549 cell lines using the analog C, 9-cis RA, and all trans RA compounds (figure 4B-E). In each case, a similar pattern of EGFR mRNA expression was observed after treatment. Specifically, the MCF7, T47D, and BT549 cell lines demonstrated a significant 20-70% decrease in EGFR mRNA levels when treated with any of the ligands and compared to untreated controls. In contrast, the BT474 cell line demonstrated an opposite effect, with a significant 200-500%

increase in EGFR mRNA levels when treated with any of the ligands and compared to untreated controls.

In order to ascertain whether these effects on EGFR mRNA levels translated into a similar decrease or increase in EGFR protein depending upon the cell line, western analysis was employed. BT474 cells, which show an increase in EGFR mRNA, and BT549 cells, one of the three cell lines that show a decrease in EGFR mRNA, were treated with 1 μ M 1,25-dihydroxyvitamin D₃ or 1 μ M 9-cis RA for three days and the level of EGFR in their cell membranes was assessed. Figure 5A shows representative autoradiograms of western blotting done with a polyclonal antibody against EGFR and cell membrane protein isolated from the BT474 and BT549 cell lines after 1,25-dihydroxyvitamin D₃ treatment. On each film, a marker lane and the 170 kilodalton EGFR band are indicated. The histograms which accompany the autoradiograms in figure 5A are based on the results of data gained by densitometric scanning of EGFR immunoblotting performed in at least 3 independent experiments. Demonstrated is an average 700% increase in EGFR protein for BT474 cells and an average 45% decrease in EGFR protein for BT549 cells when compared to untreated controls. Figure 5B shows graphically the average results of densitometric scanning of autoradiograms obtained from western blotting performed in BT474 and BT549 cell lines after 1 μ M 9-cis RA treatment. Illustrated here is an average 250% increase in EGFR protein for BT474 cells and an average 30% decrease in EGFR protein for BT549 cells when compared to untreated controls. These changes in BT474 and BT549 EGFR protein levels after 9-cis RA, 1,25-dihydroxyvitamin D₃, or analog C (data not shown) treatments parallel both the direction and magnitude of those changes in EGFR mRNA levels observed in figure 4.

EGFR mRNA Stability is Not Altered in the BT474 Cell Line. To answer the question of whether or not the observed changes in EGFR mRNA and protein levels in response to 1,25-dihydroxyvitamin D₃ treatment are the result of an altered mRNA half life, the rate of EGFR mRNA decay was determined in BT474 cells treated with 1 μ M 1,25-dihydroxyvitamin D₃ and compared to that of untreated cells. After a three day incubation with 1 μ M 1,25-

dihydroxyvitamin D₃, BT474 cells were treated with the RNA synthesis inhibitor actinomycin D and RNA was isolated at times 0, 30, 60, 120, 180, and 240 minutes after actinomycin D administration. As the comparative control, a separate set of BT474 cells not pretreated with 1,25-dihydroxyvitamin D₃ were treated similarly with actinomycin D. The results of changes in EGFR mRNA levels as assessed by RNase protection experiments done in three independent experiments are shown graphically in figure 4. As shown, the decay curves for EGFR mRNA in 1,25-dihydroxyvitamin D₃ treated and untreated cells are nearly identical, indicating that the change in EGFR mRNA and protein in response to 1,25-dihydroxyvitamin D₃ treatment is not explained by altered mRNA half-life. Similar experiments were performed on the BT549 cell line, but were unsuccessful at generating mRNA decay in control and 1,25-dihydroxyvitamin D₃ treated cells, suggesting resistance to actinomycin D.

840 bp of the EGFR promoter mediate the 1,25-dihydroxyvitamin D₃ response.

Given the observations concerning EGFR mRNA half life in 1,25-dihydroxyvitamin D₃ treated versus untreated cells, and the increasing data in recent years demonstrating that genomic vitamin D responses are mediated through the VDR and a corresponding vitamin D response element (47-50), 840 bp of the EGFR promoter was placed in the context of the chloramphenicol acetyl-transferase (CAT) gene and used in transient transfections. Figure 7A shows pJFCAT, the parent CAT vector used in transfection studies. pJFCAT has a poly A trimer cassette inserted upstream of CAT and downstream of the plasmid backbone so as to block any read through transcription initiated on non-specific vector sequences (56). The promoter-less pJFCAT has minimal activity when transfected into any of the cell lines examined here, and displays no response to 1,25-dihydroxyvitamin D₃ treatment when compared to untreated controls (data not shown). Panel b of figure 7 illustrates the configuration of the native EGFR gene with BglII and SacI restriction sites at positions -860 and -20, respectively, relative to the start of translation. Panel 7C shows the pJFCAT 840 construct containing 840 bases of the EGFR promoter driving CAT gene expression.

Figure 8 shows the results of transient transfections of the pJFCAT 840 construct in MCF7, T47D, and BT549 cell lines. Autoradiograms are representative of the results obtained in three independent experiments. Shown graphically, MCF7, T47D, and BT549 cells transfected with pJFCAT 840 demonstrate a significant (25-50%) decrease in CAT activity upon 1,25-dihydroxyvitamin D₃ treatment as compared to CAT activity in untreated transfected controls. Similar results were obtained with analog C treatment (data not shown). This suggests that sequences important for mediating the repressive 1,25-dihydroxyvitamin D₃ effect in MCF7, T47D, and BT549 cells are located within 840 bases of the EGFR promoter. In contrast, transfection of BT474 cells with pJFCAT 840 resulted in very low basal activity and only a slight induction of that activity (1.3 fold) when treated with 1,25-dihydroxyvitamin D₃ (data not shown).

Discussion

1,25-dihydroxyvitamin D₃, 9-cis RA, and all-trans RA have established roles as differentiation and anti-proliferative agents in many transformed cell lines (28,29,32,33,35-42). Epidemiological studies have shown an inverse correlation between annual average sunlight exposure and the incidence of breast cancer in the United States, Canada, and the former Soviet Union (57-59). More specifically, studies have shown increases in the incidence and mortality of breast cancer in North America with increasing latitude (58,60). This suggests an association between the body's level of vitamin D production and breast cancer occurrence since it is estimated that sunlight provides for 70 or more percent of the vitamin D present in the blood of Caucasians (61). Similarly, it has been reported that low dietary or serum levels of β -carotene are associated with increased risk of a number of epithelial (62,63) and breast cancer (64) malignancies. Clinically, it has been shown that use of a topical vitamin D analog to treat 14 cutaneous breast cancer metastases resulted in a 50% decrease in the size of 3 lesions (65), and that those patients with VDR positive tumors have significantly longer disease free survival than those with receptor negative tumors (66). Experimentally, more than 80% of breast cancer specimens are VDR positive (66) irrespective of ER status. The potential significance of this has been explored by Koga and Sutherland (32) who demonstrate synergistic antiestrogen and vitamin D or retinoid inhibition of growth in T47D breast cancer cells. Further, it implies that those cells unresponsive to the growth inhibitory effect of antiestrogens may still be responsive to the anti-proliferative effects of vitamin D and/or retinoid compounds.

In the present study we have investigated the effects of 1,25-dihydroxyvitamin D₃, analog C (which is a vitamin D analog), 9-cis RA, and all-trans RA on the growth and expression of EGFR in a panel of 4 breast cancer cell lines. We have chosen these cell lines based on their relative levels of EGFR. Since levels of EGFR and ER tend to be inversely correlated to one another in breast cancer cells (3,16,17), the lines we have selected represent a good approximation of the progression of breast cancer to a more aggressive, hormone independent phenotype characterized by a loss of ER and a gain in EGFR expression. The magnitude of the

growth inhibitory effects that the vitamin D and retinoid compounds used in this study produce reflect this progression as those cell lines expressing the lowest levels of EGFR (MCF7 and T47D) had the greatest amount of growth inhibition while those with higher levels responded less significantly (BT474 and BT549).

The difference in the degree of growth inhibition produced by the vitamin D and the retinoid compounds may also in part be linked to their relative levels of VDR, RAR, and RXR expression (table 1). Specifically, those cell lines with the highest levels of VDR, RAR, or RXR would be predicted to have the largest degree of growth inhibition in response to vitamin D, all trans RA, or 9-cis RA treatment, respectively. This is especially recognizable in the case of RAR α which has an inverse correlation with EGFR and confers increased sensitivity to retinoid inhibition of growth when upregulated in ER negative breast cancer cells (67). In terms of vitamin D mediated growth inhibition, however, this is not what is observed as T47D and BT474 cells, which have relatively equivalent levels of VDR, have quite different growth profiles when treated with vitamin D (figure 2A). An explanation proposed for this variation in the degree of growth inhibition induced by vitamin D on cell lines with similar levels of VDR involves differential changes in the regulation of target genes which mediate its anti-proliferative effect. More specifically, it is speculated that vitamin D and retinoids exert their effects in controlling cell growth and differentiation through specific modulation of growth factor receptor expression (34).

In order to investigate the possibility that their growth inhibitory effects may be mediated by or linked to a down-regulation of EGFR, the expression of EGFR mRNA was examined in MCF7, T47D, BT474, and BT549 cell lines after vitamin D and retinoid treatments. The observation of a 72-hour-sustained 50% decrease in BT549 EGFR mRNA observed within 2 hours of 1,25-dihydroxyvitamin D₃ treatment allowed us to perform all subsequent experiments with 60-72 hour incubation times to maximize RNA and protein yields. By three days of vitamin D and retinoid treatments, MCF7, T47D, and BT549 cell lines demonstrated EGFR mRNA down-regulation while BT474 showed an upregulation, all in the context of a range of significant

to insignificant growth inhibition. These changes in EGFR mRNA levels were found to translate into similarly altered levels of EGFR protein in the cell membrane for vitamin D, analog C, and 9-cis RA treated BT474 and BT549 cell lines. Our present study, which notes a decrease in EGFR mRNA in MCF7 and T47D cells in response to vitamin D and analog treatment, supports the findings of Koga *et.al.* (44) who noted a decrease in [¹²⁵I]EGF binding to EGFR in MCF7 and T47D cells after 24 hours of vitamin D treatment. They also demonstrate that this effect is due to a direct decrease in EGFR number on the cell surface and not to altered EGFR internalization, degradation, or occupancy. Although not shown here, changes in EGFR protein levels after all trans RA treatment is hypothesized to correlate with similar fluctuations in EGFR mRNA. Support for this comes from the results of experiments done in the human epidermoid carcinoma cell line ME180 (45) and in NRK fibroblast cells (46). These two studies demonstrate a decrease and increase, respectively, in both EGFR mRNA and [¹²⁵I]EGF binding after all trans RA treatment, suggesting that the magnitude and direction of EGFR mRNA and protein changes correlate with the use of the all trans RA ligand as well.

The above data imply that growth inhibition and EGFR down-regulation by the retinoid and vitamin D compounds may be related effects in some breast cancer cells, but not in all. Studies by Koga *et.al.* (44) with MDA-MB-231 breast cancer cells and Desprez *et.al.* (27) and Falette *et.al.* (43) with BT-20 breast cancer cells demonstrated a similar discordance of growth inhibition in the face of EGFR up-regulation. In the Desprez *et.al.* (27) report it has been suggested that the up-regulation of EGFR mRNA by those differentiation agents that cause growth inhibition is restricted to undifferentiated malignant cell lines that do not respond to EGF by increased cell proliferation (27). While attractive, this hypothesis is refuted by other studies in our lab that demonstrate EGF mediated growth stimulation of BT474 cells (68), which in this study show EGFR up-regulation in the face of vitamin D and retinoid growth inhibition. So as to better understand this issue of EGFR up- or down-regulation in the context of the growth inhibition induced by the retinoid and vitamin D compounds, we felt it first necessary to establish the molecular basis of EGFR regulation by the vitamin D, and subsequently, retinoid

compounds in breast cancer cells. In so doing it is hoped that any mechanistic differences in the regulation of EGFR by vitamin D or retinoids will help in understanding the biologic and prognostic significance of the growth inhibition they produce in the cell lines examined.

It is generally accepted that vitamin D mediated regulation of gene expression is controlled at the level of transcription (69,70). Additionally, many reports have suggested that regulation of EGFR by the retinoids and vitamin D compounds is mediated at the level of transcription (43-46). Further, it is known that EGFR is most commonly regulated at the level of transcription in breast cancer cells (6,16,18). Nevertheless, in order to rule out a contributing influence of altered mRNA stability, experiments were done on the BT474 and BT549 cell lines designed to measure EGFR mRNA half-life after a 3 day treatment with 1,25-dihydroxyvitamin D₃. Plotting the average decay of EGFR mRNA over a four hour period after addition of the RNA synthesis inhibitor, actinomycin D, no significant difference was observed between control (non-vitamin D treated) and vitamin D treated decay curves for BT474 cells, suggesting that EGFR mRNA half-life was not altered by vitamin D. Similar experiments that were performed on the BT549 cell line were uninformative since the actinomycin D failed to generate mRNA decay in both control and treatment groups. The prospect of performing the assay on T47D or MCF7 cells remains. However, given the low basal level of EGFR mRNA in these cell lines, the 50-70% reduction that is caused by vitamin D treatment, combined with further decay of the mRNA by actinomycin D, the limit of detection of the assay would most likely be exceeded. Therefore we directly asked if the vitamin D effect on EGFR mRNA expression has a transcriptional component by employing a reporter gene construct in transient transfection assays.

The 840 base pair portion of the EGFR promoter from 20 to 860 nucleotides upstream of the translation start site used in these assays contains multiple transcription start sites (71) and sites for the binding of positive (72,73) and negative (74) regulatory factors. Transient transfections of this construct named pJFCAT 840 in MCF7, T47D, and BT549 cell lines followed by vitamin D or analog C treatment resulted in a down-regulation of CAT activity of

similar magnitude as EGFR mRNA and protein down-regulation in these same cell lines. Transfection of pJFCAT 840 into BT474 cells demonstrated a low basal level of activity and a slight 1.3 fold induction in response to vitamin D. The magnitude of this induction is less than that which was seen with endogenous EGFR mRNA and protein in response to vitamin D treatment. We speculate that either difficulties we observed in transfecting the BT474 cell line or additional EGFR sequences not represented within the pJFCAT 840 construct are responsible for the blunted reporter gene induction when compared to endogenous mRNA and protein changes. Both of these possibilities are currently being explored. Whatever the case, the fact that the BT474 cell line demonstrated an effect opposite that observed upon transfection of pJFCAT 840 into MCF7, T47D, and BT549 cells suggests that sequences within 840 bp of the EGFR promoter are important for mediating EGFR regulation by vitamin D. Since the same portion of the promoter is able to mediate different EGFR regulatory responses that mimic changes in endogenous EGFR mRNA and protein, it also indicates that there are probably cell specific factors involved.

We are currently delineating the minimal vitamin D responsive region of the EGFR promoter and characterizing the roles that the various factors involved play. To this end, we have identified by sequence analysis a region of the EGFR promoter between nucleotides 531 and 516 upstream of the translation start site that bears strong resemblance to the consensus DR3 PuG(G/T)TCA vitamin D response element (75), as well as known functional VDREs for the human (76) and rat (77) osteocalcin and mouse osteopontin (78) genes (figure 9). We hypothesize that this region of the EGFR promoter, by the binding of the VDR with one or more cell specific coregulatory factors, may mediate the differential vitamin D regulation of EGFR expression in BT474 versus MCF7, T47D, and BT549 cells. VDREs such as this are known to bind the ligand activated VDR as a homodimer (79,80) or heterodimer in the context of a coregulatory molecule which often tends to be one of RXRs (79-81). Receptor binding subsequently mediates a positive or negative transcriptional response depending upon the gene considered and what other cell specific factors may be involved. Characterization of the factors

and mechanisms involved in breast cancer cells will potentially provide us with an understanding of the varied effects that vitamin D has on cell growth and EGFR expression.

Materials and Methods

Cell Culture. Standard culture conditions consisted of Biofluids IMEM with phenol red (cat#105-500) supplemented with 10% heat-inactivated fetal bovine serum, 37°C humidified atmosphere of 95% air-5% CO₂, and feedings every 2-4 days. For ligand treatments, cells were grown in T75 flasks to 70% confluence and treated with either 1 μM of ligand or an equal volume of vehicle (100% ethanol) under standard culture conditions. 1,25-dihydroxyvitamin D₃ and analog C were obtained from Drs. Robert Buras and Moshen Shabahang (Georgetown University, Lombardi Cancer Center, Washington, DC). 9-cis RA was the kind gift of Dr. Robert Clarke (Georgetown University, Lombardi Cancer Center, Washington, DC). All-trans RA was purchased from Sigma (cat#R2625).

Growth Assays. Growth assays were performed by plating 2000 cells/well of MCF7, T47D, BT474, and BT549 breast cancer cells in 96 well microtiter plates which were treated with 1 μM of the appropriate ligand or equivalent volume of vehicle (100% ethanol) 24 hours later. All treatments were done as 6 replicates in phenol red IMEM supplemented with 10% FBS. Time-points were taken every 2 days and extended out to 8 days by crystal violet staining of adherent cells for 15 minutes in a solution of 0.1% crystal violet (Sigma) and 15% methanol, subsequently washed in distilled water, and allowed to air-dry for 24 hours. Numerical results were obtained on an ELISA reader at an absorbance of 540 nm after solubilization of crystal violet stained cells in a solution of 0.1 M sodium citrate and 50% ethanol. All absorbance readings were expressed as a percent change relative to the absorbance obtained on day 0. Data was analyzed for statical significance from untreated replicates.

RNAse Protection Assay. Cells were treated with either ligand or vehicle for 72 hours and total RNA was isolated by the one step acid-guanidinium method (82). In performing the timecourse on BT549 cells, treatments with 1,25-dihydroxyvitamin D₃ were carried out for 0, 1, 2, 4, 6, 8, 24, 48, and 72 hours followed by isolation of total RNA (82). 60 μg per sample of RNA was then added to *in vitro* synthesized (83) EGFR specific and internal ribosomal RNA

control 36B4 (55) riboprobes after denaturation at 90°C for 5 minutes. The EGFR template DNA was from obtained from Dr. Fran Kern (Georgetown University, Lombardi Cancer Center, Washington, DC), and required linearization with PvuII restriction endonuclease and incubation with SP6 RNA polymerase. The 36B4 template DNA was obtained from Dr. Mary Beth Martin (Georgetown University, Lombardi Cancer Center, Washington, DC), and required linearization with EcoRI restriction endonuclease and incubation with T7 RNA polymerase. Following incubation and hybridization at 50°C for 16-20 hours, samples were subject to RNase A digestion (60µg per sample) for 30 minutes at room temperature, extraction with phenol:chloroform, ethanol precipitation, and electrophoresis on a 6% denaturing polyacrylamide gel. Gels were subsequently dried under vacuum, exposed to film, and bands quantitated by densitometry. All data was corrected for loading error by comparison with the internal control 36B4. Specifically, the determined optical density value of EGFR band was divided by the optical density value of the corresponding 36B4 band. This EGFR:36B4 density ratio for untreated cells in each experiment was assigned a value of 100%, and the ratios of treated samples calculated as a percent of this control.

Preparation of Membrane Fractions. Membrane fractions were prepared by washing cells 2X with ice-cold 1X PBS and harvesting in an ice cold solution of 10mM HEPES pH 7.9, 1mM EDTA, 1mM DTT, 1µg/ml leupeptin, 1µg/ml aprotinin, 1µg/ml pepstatin, and 100µg/ml pefabloc. Cells were subsequently homogenized on ice with a Teflon-glass Potter-Elvehjem homogenizer by 30-40 strokes. Homogenates were centrifuged at 12000 x g, 4°C, for 30 minutes to pellet the cell membranes. The pellet was resuspended and stored at -80°C in a solution of 25% glycerol, 10mM HEPES pH 7.9, 1mM EDTA, 1mM DTT, 50 mM KCl, 1µg/ml leupeptin, 1µg/ml aprotinin, 1µg/ml pepstatin, and 100µg/ml pefabloc. Protein concentrations were determined by the Bradford assay (84).

Western Blot Analysis. Samples were electrophoresed (100µg of protein per sample) on a 7.5% SDS-polyacrylamide gel (85) along with Amersham's rainbow markers (cat#RPN-756) and electro-transferred (86,87) to a nitrocellulose (88,89) membrane. Membranes were stained

with Ponceau-S to check for equal loading (90). The blot was incubated with 1 µg/ml of the polyclonal antibody #1005 (Santa Cruz BioTechnology) against EGFR. Visualization of EGFR protein was done using a 1:1000 dilution of Amersham's horse radish peroxidase-conjugated anti-rabbit IgG, Amersham's ECL detection kit, and exposing to Amersham's Hyperfilm-ECL (91,92). Quantitation was performed by densitometric scanning of autoradiographs.

mRNA Stability Studies. For mRNA stability studies, cells were pre-treated with 1,25-dihydroxyvitamin D₃ or an equal volume of vehicle (100% ethanol) for 3 days. Vitamin D treated and untreated cells were then given the RNA synthesis inhibitor actinomycin D at a final concentration of 5 µg/ml. RNA was then isolated at times 0, 30, 60, 120, 180, and 240 minutes after administration of actinomycin D and subsequently analyzed by RNase protection as described above.

Transfections and CAT Assays. For each transfection of a 75mm² dish, cells were grown under standard culture conditions (described above) to 70% confluence. 30 minutes prior to transfection, 10 µg of CsCl banded pJFCAT DNA was incubated with 20 µl of lipofectamine (GIBCO/BRL) in 1 ml serum free IMEM at room temperature. 2 mls IMEM with 10% FBS and 1 ml serum free IMEM was then added per reaction mix, so that the final serum concentration was no more than 5%. Dishes were then incubated under standard conditions (described above) for 16-20 hours at which time cells were rinsed 2X with 1X PBS, trypsinized, pelleted, resuspended, mixed thoroughly, and replated in equal numbers to control for any differences in transfection efficiency from one plate to the next. Upon replating, cells were either given IMEM with 10% FBS and 1,25-dihydroxyvitamin D₃ or analog C, or IMEM with 10% FBS and an equivalent volume of vehicle (100% ethanol). Treatments were carried out for 48-60 hours under standard culture conditions, followed by harvesting. Cell pellets were lysed by freeze/thawing, and the concentration of the protein in the lysate determined by the Bradford assay (85). Equal amounts of protein (between 100-500 µg depending upon the cell line used) were then incubated at 37°C for 2 hours with 0.125 µCi ¹⁴C-chloramphenicol and 0.5mM acetyl CoA, followed by extraction with ethyl acetate. Samples were spotted onto thin layer chromatography plates, run

in a 95:5 chloroform:methanol tank, exposed to film, and quantitated by comparison of phosphorimager determination of percent conversion of chloramphenicol to acetylated forms in treated versus untreated samples.

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References

1. Rajkumar, T. and Gullick, W.J. The type I growth factor receptors in human breast cancer. *Breast Cancer Res. Treat.*, **29**:3-9, 1994.
2. Chrysogelos, S.A., Yarden, R.I., Lauber, A.H. and Murphy, J.M. Mechanisms of EGF receptor regulation in breast cancer cells. *Breast Cancer Res. Treat.*, **31**:227-36, 1994.
3. Jardines, L., Weiss, M., Fowble, B. and Green, M. Neu(c-erbB-2/HER2) and the epidermal growth factor receptor (EGFR) in breast cancer. *Pathobiology*, **61**:268-82, 1993.
4. Gullick, W.J. Prevalence of aberrant expression of the epidermal growth factor receptor in human cancers. *British Med. Bulletin*, **47**:87-98, 1991.
5. Chrysogelos, S.A. and Dickson, R.B. EGF receptor expression, regulation, and function in breast cancer. *Breast Cancer Res. Treat.*, **29**:29-40, 1994.
6. Harris, A.L. What is the biological, prognostic, and therapeutic role of the EGF receptor in human breast cancer? *Breast Cancer Res. Treat.*, **29**:1-2, 1994.
7. Koenders, P.G., Beex, L.V., Kienhuis, C.B., Kloppenborg, P.W. and Benraad, T.J. Epidermal growth factor receptor and prognosis in human breast cancer: a prospective study. *Breast Cancer Res. Treat.*, **25**:21-7, 1993.
8. Toi, M., Osaki, A., Yamada, H. and Toge, T. Epidermal growth factor receptor expression as a prognostic indicator in breast cancer. *Eur. J. Cancer*, **27**:977-80, 1991.
9. Toi, M., Nakamura, T., Mukaida, H., Wada, T., Osaki, A., Yamada, H., Toge, T., Niimoto, M. and Hattori, T. Relationship between epidermal growth factor receptor status and various prognostic factors in human breast cancer. *Cancer*, **65**:1980-4, 1990.
10. Harris, A.L., Nicholson, S., Sainsbury, J.R., Farnson, J. and Wright, C. Epidermal growth factor receptors in breast cancer: association with early relapse and death, poor response to hormones and interactions with neu. *J. Steroid Biochem.*, **34**:123-31, 1989.
11. Klijn, J.G.M., look, M.P., Portengen, H., Alexievafigusch, J., Vanputten, W.L.J. and Foeken, J.A. The prognostic value of epidermal growth factor receptor (EGF-R) in primary

breast cancer - results of a 10 year follow-up study. *Br. Cancer Res. Treat.*, **29**:73-83, 1994.

12. Sainsbury, J., Farndon, J., Needham, G., Malcolm, A. and Harris, A. Epidermal growth factor receptor status as a predictor of early recurrence of and death from breast cancer. *Lancet*, **i**:1398-1402, 1987.

13. Wang, Y., Minoshima, S. and Shimizu, N. Precise mapping of the EGF receptor gene on the human chromosome 7p12 using an improved FISH technique. *Jpn. J. Hum. Genet.*, **38**:399-406, 1993.

14. Ulrich, A. and Schlessinger, J. Signal transduction by receptors with tyrosine kinase activity. *Cell*, **61**:203-12, 1990.

15. Donward, J., Yarden, Y., Mayes, E., Scarce, G., Totty, N., Stockwell, P., Ulrich, A., Schlesinger, J. and Waterfield, M.D. Close similarity of epidermal growth factor receptor and v-erbB oncogene protein sequences. *Nature*, **307**:521-7, 1984.

16. Davidson, N.E., Gelmann, E.P., Lipmann, M.E. and Dickson, R.B. Epidermal growth factor receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. *Mol. Endocrinol.*, **1**:216-23, 1987.

17. Koenders, P.G., Beex, L.V.A.M., Geurts-Moespot, A., Heuvel, J.J.T.M., Kienhuis, C.B.M. and Benraad, T.J. Epidermal growth factor receptor-negative tumors are predominantly confined to the subgroup of estradiol receptor positive human primary breast cancers. *Cancer Res.*, **51**:4544-8, 1991.

18. King, C.R., Kraus, M.H., Williams, L.T., Merlino, G.T., Pastan, I.H. and Aaronson, S.A. Human tumor cell lines with egfr receptor gene amplification in the absence of aberrant sized mRNAs. *Nucl. Acids Res.*, **13**:8477-86, 1985.

19. DiFiore, P., Pierce, J., Fleming, T., Hazon, R., Ulrich, A., King, C.R., Schlessinger, J. and Aaronson, S. Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH3T3 cells. *Cell*, **51**:1063-70, 1987.

20. Velu, T.J, Bequinot, L., Vass, M.C., Willingham, M.C., Merlino, G.T., Pastan, I. and Lowy, D. Epidermal growth factor receptor-dependent transformation by a human EGF receptor proto-oncogene. *Science*, **238**:1408-10, 1987.
21. Liu, Y.E., Lupu, R. and Kern, F.G. Characterization of MCF7 breast carcinoma cells singly or doubly transfected with c-erbB2 and EGFR. *Proc. AACR*, **33**:368, 1992.
22. Agthoven, T.V., Agthoven, T.L.A.V., Portengen, H., Foekers, J.A. and Dorsse, L.C.J. Ectopic expression of epidermal growth factor receptors induces hormone independence in ZR-75-1 human breast cancer cells. *Cancer Res*, **52**:5082-8, 1992.
23. Baselga, J. and Medelsohn, J. The epidermal growth factor receptor as a target for therapy in breast carcinoma. *Breast Cancer Res. Treat.*, **29**:157-38, 1994.
24. Kudlow, J.E., Cheung, C.-Y.M. and Bjorge, J.D. Epidermal growth factor stimulates the synthesis of its own receptor. *J. Biol. Chem.*, **261**:4134-8, 1987.
25. Bjorge, J.D. and Kudlow, J.E. Epidermal growth factor receptor synthesis is stimulated by phorbol ester and epidermal growth factor. *J. Biol. Chem.*, **262**:6615-22, 1987.
26. Clark, A.J.L., Ishii, S., Richert, N., Merlino, G.T. and Pastan, I. Epidermal growth factor regulates the expression of its own receptor. *Proc. Natl. Acad. Sci. USA*, **82**:8374-8, 1985.
27. Desprez, P.Y., Poujol, D., Falette, N., Lefebvre, N.F. and Saez, S. 1, 25-dihydroxyvitamin D₃ increases epidermal growth factor gene expression in BT-20 breast carcinoma cells. *Biochem. Biophys. Res. Comm.*, **176**:1-6, 1991.
28. Prasad, K.N., Edwards-Prasad, J., Kumar, S. and Meyers, A. Vitamins regulate gene expression and induce differentiation and growth inhibition in cancer cells. Their relevance in cancer prevention. *Arch. Otolaryngol. Head Neck Surg.*, **119**:1133-40, 1993.
29. Bollag, W. Experimental basis of cancer combination chemotherapy with retinoids, cytokines, 1,25-dihydroxyvitamin D₃, and analogs. *J. Cell. Biochem.*, **56**:427-35, 1994.
30. Welsh, J. Induction of apoptosis in breast cancer cells in response to vitamin D and antiestrogens. *Biochem. Cell. Biol.*, **72**:537-45, 1994.

31. Hansen, C.M., Frandsen, T.L., Brunner, N. and Binderup, L. 1 alpha,25-dihydroxyvitamin D₃ inhibits the invasive potential of human breast cancer cells in vitro. *Clin. Exp. Metastasis*, **12**:195-202, 1994.
32. Koga, M and Sutherland, R.L. retinoic acid acts synergistically with 1,25-dihydroxyvitamin D₃ or antioestrogen to inhibit T-47D human breast cancer cell proliferation. *J. Steroid Biochem. Mol. Biol.*, **39**:455-60, 1991.
33. Brenner, R.V., Shabahang, M., Schumaker, L.M., Nauta, R.J., Uskokovic, M.R., Evans, S.R. and Buras, R.R. The antiproliferative effect of vitamin D analogs on MCF-7 human breast cancer cells. *Cancer Lett.*, **92**:77-82, 1995.
34. Buras, R.R., Schumaker, L.M., Davoodi, F., Brenner, R.V., Shabahang, M., Nauta, R.J and Evans, S.R. Vitamin D receptors in breast cancer cells. *Breast Cancer Res. Treat.*, **31**:191-202, 1994.
35. Chouvet, C., Vicard, E., Devonec, M. and Saez, S. 1,25-dihydroxyvitamin D₃ inhibitory effect on the growth of two human breast cancer cell lines (MCF, BT-20). *J. Steroid Biochem.*, **24**:373-6, 1986.
36. Colston, K.W., Chandler, S.K., MacKay, A.G. and Combes, R.C. Effects of synthetic vitamin D analogs on breast cancer cell proliferation *in vivo* and *in vitro*. *Biochem. Pharmacol.*, **44**:693-702, 1992.
36. Eisman, J.A., Koga, M., Sutherland, R.L., Barkla, D.H. and Tutton, P.J.M. 1,25-dihydroxyvitamin D₃ and the regulation of human cancer cell replication. *Soc. Expl. Biol. Med.*, **191**:221-6, 1989.
38. Frampton, R.J., Omond, S.A. and Eisman, J.A. Inhibition of human cancer cell growth by 1,25-dihydroxyvitamin D₃ metabolites. *Cancer. Res.*, **43**:4443-7, 1983.
39. Fontana, J.A. Interaction of retinoids and tamoxifen on the inhibition of human mammary carcinoma cell proliferation. *Exp. Cell. Biol.*, **55**:136-44, 1987.
40. Fontana, J.A., Hobbs, P.D. and Dawson, M.I. Inhibition of mammary carcinoma growth by retinoidal benzoic acid derivatives. *Exp. Cell. Biol.*, **56**:254-63, 1988.

41. Fontana, J.A., Mezu, A.B., Cooper, B.N. and Miranda, D. Retinoid modulation of estradiol-stimulated growth and of protein synthesis and secretion in human breast carcinoma cells. *Cancer Res.*, **50**:1997-2002, 1990.
42. Wetherall, N.T. and Taylor, C.M. The effects of retinoid treatment and antiestrogens on the growth of T47D human breast cancer cells. *Eur. J. Cancer. Clin. Oncol.*, **22**:53-9, 1986.
43. Falette, N., Frappart, L., Lefebvre, M.F. and Saez, S. Increased epidermal growth factor receptor level in breast cancer cells treated by 1,25-dihydroxyvitamin D₃. *Mol. Cell. Endo.*, **63**:189-98, 1989.
44. Koga, M., Eisman, J.A. and Sutherland, R.L. Regulation of epidermal growth factor receptor levels by 1,25-dihydroxyvitamin D₃ in human breast cancer cells. *Cancer Res.*, **48**:2734-9, 1988.
45. Zheng, Z.S., Polakowska, R., Johnson, A. and Goldsmith, L.A. Transcriptional control of epidermal growth factor receptor by retinoic acid. *Cell Grow. Diff.*, **3**:255-32, 1992.
46. Thompson, K.L. and Rosner, M.R. Regulation of epidermal growth factor gene expression by retinoic acid and epidermal growth factor. *J. Biol. Chem.*, **264**:3230-4, 1989.
47. Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. and Evans, R.M. The nuclear receptor superfamily: the second decade. *Cell*, **83**:835-9, 1995.
48. Power, R.F., O'Conneely, O.M. and O'Malley, B.W. New insights into activation of the steroid hormone receptor superfamily. *Trends Pharm. Science*, **13**, 318-23, 1992.
49. Minghetti, P.P. and Norman, A.W. 1,25-(OH)₂-vitamin D₃ receptors: gene regulation and genetic circuitry. *FASEB J.*, **2**:3043-53, 1988.
50. Pike, J.W. Intracellular receptors mediate the biologic action of 1,25-dihydroxyvitamin D₃. *Nutr. Rev.*, **43**:161-8, 1985.
51. Roman, S.D., Clarke, C.L., Hall, R.E., Alexander, I.E. and Sutherland, R.L. Expression and regulation of retinoic acid receptors in human breast cancer cells. *Cancer Res.*, **52**:2236-42, 1992.

52. Shao, Z.M., Sheikh, M.S., Chen, J.C., Kute, T., Aisner, S., Schnaper, L. and Fontana, J.A. Expression of the retinoic acid nuclear receptors (RARs) and retinoid X receptor genes in estrogen receptor positive and negative breast cancer. *Int. J. Oncol.*, 4:859-63, 1994.
53. Zhou, J.Y., Norman, A.W., Akashi, M., Chen, D.L., Uskokovic, M.R., Aurecoeha, J.M., Dauben, W.G., Okamura, W.H. and Koeffler, H.P. Development of a novel 1,25(OH)₂-vitamin D₃ analog with potent ability to induce HL-60 cell differentiation without modulating calcium metabolism. *Blood*, 78:75-82, 1991.
54. MacDonald, P.N., Dowd, D.R., Nakajima, S., Galligan, M.A., Reeder, M.C., Haussler, C.A., Ozato, K. and Haussler, M.R. Retinoid X receptors stimulate and 9-cis retinoic acid inhibits 1,25-dihydroxyvitamin D₃-activated expression of the rat osteocalcin gene. *Mol. Cell Biol.*, 13:5907-17, 1993.
55. Laborda, J. 36B4 cDNA used as an estradiol-independent mRNA control is the cDNA for human acidic ribosomal phosphoprotein PO. *Nucl. Acids Res.*, 19:3998, 1991.
56. Fridovich-Keil, J.L, Gudas, J.M., Bryan, I.B. and Pardee, A.B. Improved expression vectors for eukaryotic promoter/enhancer studies. *BioTechniques*, 11:572-9, 1991.
57. Garland, F.C., Garland, C.F., Gorham, E.D. and Young, J.F. Geographic variation in breast cancer mortality in the United States: a hypothesis involving exposure to solar radiation. *Prev. Med.*, 19:614-22, 1990.
58. Gorham, E.D., Garland, C.F. and Garland, F.C. Acid haze air pollution in breast and colon cancer mortality in 20 Canadian cities. *Can. J. Public Health*, 80:96-100, 1989.
59. Gorham, E.D., Garland, F.C. and Garland, C.F. Sunlight and breast cancer incidence in the USSR. *Int. J. Epidemiol.*, 19:820-24, 1990.
60. Blot, W.J., Fraumeni, J.F. and Stone, B.J. Geographic patterns of breast cancer in the United states. *JNCI*, 59:1407-11, 1977.
61. Haddad, J.G. and Hahn, T.J. Natural and synthetic sources of circulating 25-hydroxvitamin D in man. *Nature*, 244:515-17, 1973.

62. Lippman, S.M., Kessler, J.F. and Meyskens, F.L. Retinoids as preventive and therapeutic anticancer agents (part1). *Cancer Treat. Rep.*, **71**:391-405, 1987.
63. Sporn, M.B., Dunlop, N.M., Newton, D.L. and Smith, J.M. Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). *Fed. Proc.*, **35**:1332-8, 1976.
64. Hunter, D.J., Manson, J.E., Colditz, G.A., Stampfer, M.J., Rosner, B., Hennekens, C.H., Speizer, F.E. and Willett, W.C. A prospective study of the intake of vitamins C, E, and A and the risk of breast cancer. *N. Engl. J. Med.*, **329**:234-40, 1993.
65. Bower, M., Colston, K.W., Stein, R.C., Hedley, A., Gazet, J-C, Ford, H.T. and Coombes, R.C. Topical calcipotriol treatment in advanced breast cancer. *Lancet*, **337**:701-2, 1991.
66. Colston, K.W., Berger, U. and Coombes, R.C. Possible role for vitamin D in controlling breast cancer cell proliferation. *Lancet*, **1**(8631):188-91, 1989.
67. Sheikh, M.S., Shao, Z-M., Chen, J-C., Hussain, A., Jetten, A.M. and Fontana, J.A. Estrogen receptor-negative breast cancer cells transfected with the estrogen receptor exhibit increased RAR α gene expression and sensitivity to growth inhibition by retinoic acid. *J. Cell. Biochem.*, **53**:394-404, 1993.
68. Yarden, R.I., Wilson, M.A., Barth, M., and Chrysogelos, S.A. "The role of estrogen in the regulation of EGFR expression," In: Ernest Schering Research Foundation Workshop 19: EGF Receptor in Tumor Growth and Progression (R.B. Lichtner and R.N. Harkins, eds.), Springer Scientific Publishers, Berlin, pp. 129-54, 1996.
69. Darwish, H.M. and DeLuca, H.F. Recent advances in the molecular biology of vitamin D action (part III). *Prog. Nucl. Acid Res.*, **53**:329-32, 1996.
70. Pike, J.W. Vitamin D₃ receptors: structure and function in transcription. *Annu. Rev. Nutr.*, **11**:189-216, 1991.
71. Haley, J., Whittle, N., Bennet, P., Kinchington, D., Ulrich, A. and Waterfield, M. The human egf receptor gene: structure of the 110 kb locus and identification of sequences regulating its transcription. *Oncogene Res.*, **1**:375-96, 1987.

72. Kageyama, R., Merlino, G.T. and Pastan, I. A transcription factor active on the epidermal growth factor receptor gene. *Proc. Natl. Acad. Sci. USA*, **85**:5016-20, 1988.
73. Johnson, A.C., Ishii, S., Jinno, Y., Pastan, I., and Merlino, G.T. Epidermal growth factor gene promoter. Deletion analysis and identification of nuclear protein binding sites. *J. Biol. Chem.*, **263**:5693-9, 1988.
74. Kageyama, R. and Pastan, I. Molecular cloning and characterization of a human DNA binding factor that represses transcription. *Cell*, **59**:815-25, 1989.
75. Umesono, K., Murakami, K.K., Thompson, C.C. and Evans, R.M. Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D₃ receptors. *Cell*, **65**:1255-66, 1991.
76. Ozono, K., Liao, J., Kerner, S.A., Scott, R.A. and Pike, J.W. The vitamin D-responsive element in the human osteocalcin gene. *J. Biol. Chem.*, **265**:21881-8, 1990.
77. Demay, M.B., Gerardi, J.M., DeLuca, H.F. and Kronenberg, H.M. DNA sequences in the rat osteocalcin gene that bind the 1,25-dihydroxyvitamin D₃ receptor and confer responsiveness to 1,25-dihydroxyvitamin D₃. *Proc. Natl. Acad. Sci. USA*, **87**:369-73, 1990.
78. Noda, M., Vogel, R.L., Craig, A.M., Prah, J., DeLuca, H.F. and Denhardt, D.T. Identification of a DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D₃ receptor and 1,25-dihydroxyvitamin D₃ enhancement of mouse secreted phosphoprotein 1 (*Spp-1* or osteopontin.). *Proc. Natl. Acad. Sci. USA*, **87**:9995-9, 1990.
79. Mangelsdorf, D.J. and Evans, R.M. The RXR heterodimers and orphan receptors. *Cell*, **83**:841-50, 1995.
80. Kliewer, S.A., Umesono, K., Mangelsdorf, D.J. and Evans, R.M. Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D₃ signalling. *Nature*, **355**:446-9, 1992.
81. Yu, V.C., Delsert, C., Andersen, B., Holloway, J.M., Devary, O.V., Naar, A.M., Kim, S.Y., Boutin, J.M., Glass, C.K. and Rosenfeld, M.G. RXR β : a coregulator that enhances binding of

retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell*, **67**:1251-66, 1991.

82. Chomczynski, P. and Sacchi, N. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**:156-9, 1987.

83. Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucl. Acids Res.*, **12**:7045-56, 1984.

84. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.*, **72**:248-54, 1976.

85. Hames, B.D. and Rickwood, D. (eds.) *Gel Electrophoresis of Proteins*. IRL Press, Washington, DC, 1981.

86. Bjerrum, O.J. and Schafer-Nielsen, C. Buffer systems and transfer parameters for semidry electroblotting with a horizontal apparatus. In: *Electrophoresis '86*, pp.315-27. VCH Publishers, Deerfield Beach, Fla., 1986.

87. Harlow, E. and Lane, D. Immunoblotting. In: *Antibodies: A Laboratory Manual*, pp. 471-510. CSH Laboratory, Cold Spring Harbor, N.Y., 1988.

88. Burnette, W.N. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.*, **112**:195-203, 1981.

89. Towbin, H., Staehelin, T. and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels onto nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA*, **76**:4350-4, 1979.

90. Salinovich, O. and Montelaro, R.C. Reversible staining and peptide mapping of proteins transferred to nitrocellulose after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.*, **156**:341-7, 1986.

91. Sandhu, G.S., Eckloff, B.W. and Kline, B.C. Chemiluminescent substrates increase sensitivity of antigen detection in western blots. *BioTechniques*, **11**:14-6, 1991.

92. Schneppenheim, R., Budde, U., Dahlmann, N. and Rautenberg, P. Luminography - a new, highly sensitive visualization method for electrophoresis. *Electrophoresis*, **12**:367-82, 1984.

Fig 1. Chemical structures of 1,25-dihydroxyvitamin D₃, analog C (1,25-(OH)₂-16-en-23-yn-26,27-F₆-vitamin D₃), 9-cis retinoic acid (RA), and all-trans retinoic acid (RA).

Fig 2. Growth effects of 1 μ M 1,25-dihydroxyvitamin D₃, analog C, 9-cis RA, and all-trans RA in MCF7, T47D, BT474, and BT549 breast cancer cell lines. *A*, % change in growth of MCF7, T47D, BT474, and BT549 breast cancer cell lines treated with 1 μ M 1,25-dihydroxyvitamin D₃, analog C, 9-cis RA, and all-trans RA as compared to untreated fetal bovine serum (FBS) controls. Data is plotted over a course of 8 days with each data point representing the average of 6 replicates. Error bars are the standard deviation of the data. *B*, comparison of the % change in growth in the MCF7, T47D, BT474, and BT549 cell lines after 8 days of 1 μ M 1,25-dihydroxyvitamin D₃, analog C, 9-cis RA, and all-trans RA treatment. Error bars represent the standard deviation of the data.

Fig 3. Timecourse of EGFR mRNA regulation by 1 μ M 1,25-dihydroxyvitamin D₃ in BT549 cells. Demonstrated graphically is the rapid temporal onset of vitamin D regulation of EGFR mRNA in BT549 cells as determined by densitometric scanning of data obtained in a RNase protection analysis done on total RNA from treated and untreated (time 0) cells. Data was corrected for loading error using the internal control RNA 36B4 (55).

Fig 4. Effect of 1 μ M 1,25-dihydroxyvitamin D₃, analog C, 9-cis RA, and all-trans RA treatment on EGFR mRNA expression in MCF7, T47D, BT474, and BT549 breast cancer cells after 3 days of treatment. *A*, representative autoradiograms of RNase protection analyses done on total RNA from 1,25-dihydroxyvitamin D₃ (vit D) treated and untreated (FBS) MCF7, T47D, BT474, and BT549 cell lines. The free and protected forms of the EGFR and 36B4 probes are indicated, along with a tRNA control. The exposure time for

detection of protected EGFR and 36B4 signals in BT549 cells was 6 hours. The exposure time for detection of protected 36B4 signal in MCF7, T47D, and BT474 cells was 6 hours. The exposure time for detection of protected EGFR signal in MCF7, T47D, and BT474 cells was 2 weeks, 1 week, and 4 days, respectively. *B-E*, histograms demonstrating the relative change in EGFR mRNA in BT549 (*B*), MCF7 (*C*), T47D (*D*) and BT474 (*E*) cells after 3 days of 1 μ M of vitamin D and retinoid treatments as determined by densitometric scanning of RNase protection autoradiograms obtained in at least three independent experiments. Data was corrected for loading error using the internal control RNA 36B4 (55). Error bars represent the standard deviation of the data. Horizontal dashed line indicates the 100% activity level. (vit D = 1,25-dihydroxyvitamin D₃; ana C= analog C; 9-cis = 9-cis RA; and all-trans = all-trans RA)

Fig 5. Effect of 1 μ M 1,25-dihydroxyvitamin D₃ and 9-cis RA treatment on EGFR protein expression in BT474 and BT549 breast cancer cells after 3 days of treatment. *A*, representative autoradiograms of western analyses done on membrane protein from 1,25-dihydroxyvitamin D₃ (1 μ M D₃) treated and untreated BT474 and BT549 cells using a polyclonal anti-EGFR antibody. Arrowheads indicate the 170 kd EGFR band. Alignment of Amersham's rainbow marker is shown to the left of the film. The accompanying histograms represent the average change in EGFR protein in BT474 and BT549 cells as determined by densitometric scanning of western autoradiograms obtained in at least three independent experiments. The error bars illustrate the standard deviation of the data. *B*, histogram representing the average change in EGFR protein in BT474 and BT549 cells after 9-cis RA treatment as determined by densitometric scanning of western autoradiograms obtained in at least three independent experiments. The error bars demonstrate the standard deviation of the data.

Fig 6. EGFR mRNA stability in BT474 cells treated with 1 μ M 1,25-dihydroxyvitamin D₃ or equal volume of vehicle (100% ethanol) for 3 days prior to addition of actinomycin D at a final concentration of 5 μ g/ml. Data is plotted as % EGFR RNA remaining over the course of the assay and represents the densitometric scanning of RNase protection autoradiograms obtained in at least three independent experiments. Error bars represent the standard deviation of the data.

Fig 7. Gene constructs used for subcloning and transient transfections. *A*, diagram of the pJFCAT construct with poly A trimer cassette and polylinker regions indicated. *B*, partial restriction map and diagram of the native EGFR gene with the first major transcription start site indicated 264 nucleotides upstream of the translation start site. *C*, diagram of the pJFCAT construct after subcloning 840 nucleotides of the EGFR promoter between the BglIII and SacI sites into it.

Fig 8. Transient transfections of the EGFR promoter containing pJFCAT 840 construct into MCF7, T47D, and BT549 breast cancer cells followed by 1 μ M 1,25-dihydroxyvitamin D₃ treatment for 3 days. Histograms represent the average change in pJFCAT 840 reporter gene activity in response to 1 μ M 1,25-dihydroxyvitamin D₃ as determined by phosphorimaging of TLC plates generated in at least three independent experiments. Representative autoradiograms for each cell line are shown below each graph. Error bars represent the standard deviation of the data.

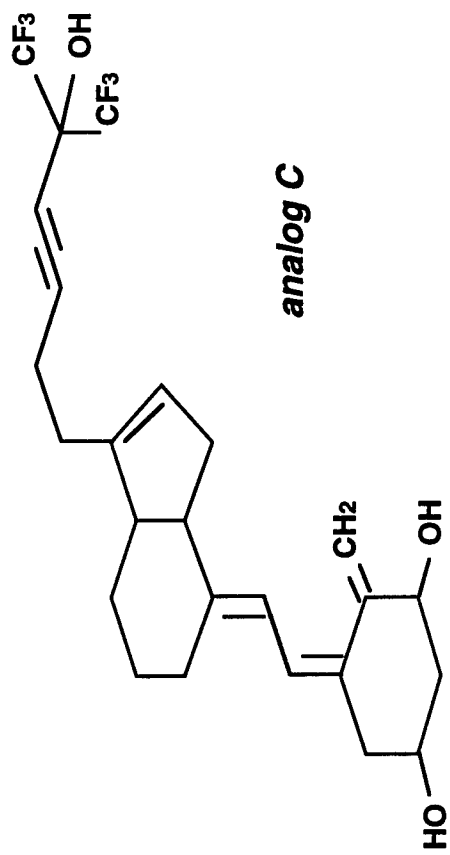
Fig 9. Schematic diagram of the EGFR promoter region contained within the pJFCAT 840 construct which demonstrated a vitamin D response in MCF7, T47D, BT474, and BT549 cell lines. The location of a putative VDRE within this stretch of the EGFR promoter is indicated along with a comparison of the sequence of the consensus

PuG(G/T)TCA DR3 VDRE (75), as well as known functional VDREs from the human (76) and rat (77) osteocalcin and mouse osteopontin (78) genes.

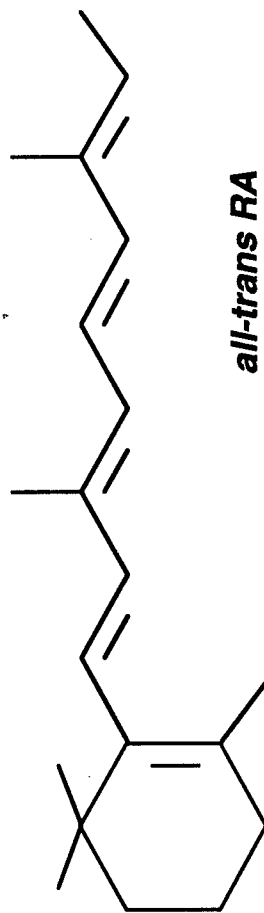
Table 1 Relative Expression of EGFR and Nuclear Receptors in Breast Cancer Cell Lines.^a

Cell line	Tumor type	Tissue source	ER	EGFR	VDR	RXR			RAR		
						α	β	γ	α	β	γ
MCF7	IDAC ^b	pl. effusion ^d	+++	+/-	++	+	?	-	++	-	?
T47D	IDAC	pl. effusion	++	+	+++	+	?	-	+	-	?
BT474	IDAC	primary	+	++	+++	+	?	-	+/-	-	?
BT549	PIDC ^c	primary	-	+++	+	+	?	-	-	-	?

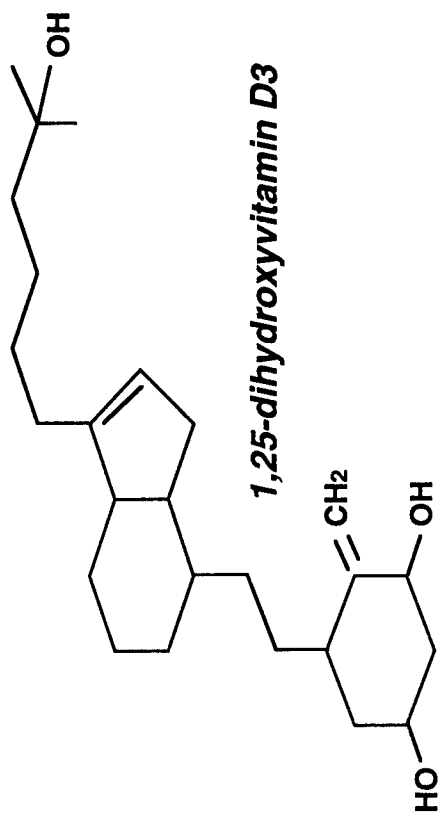
^afrom refs. 34,51,52; ^bIDAC-Infiltrating Ductal Adenocarcinoma; ^cPIDC-Papillary Infiltrating Ductal Carcinoma; ^dpl. effusion-pleural effusion. Relative levels of receptors are indicated by the "+" and "-" signs. The "?" is used where data is conflicting or unclear.



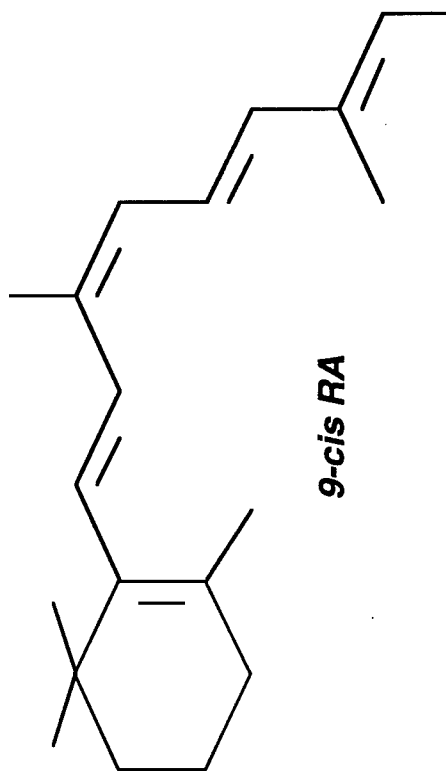
analog C



all-trans RA



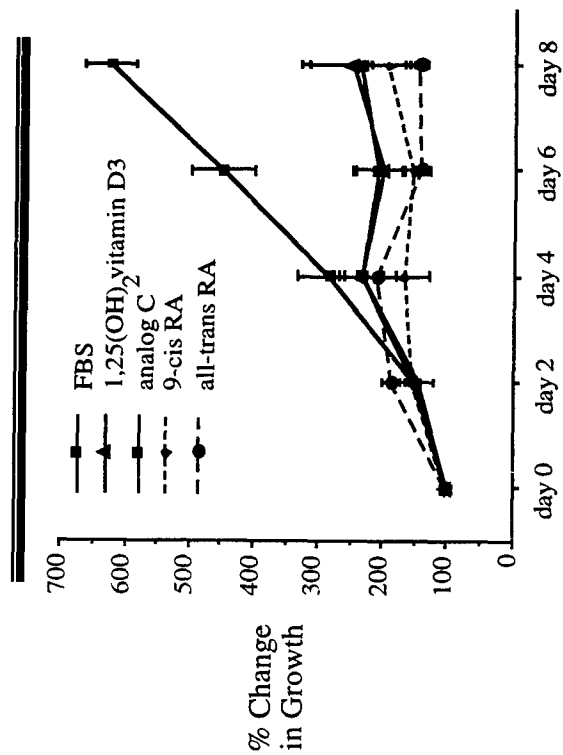
1,25-dihydroxyvitamin D₃



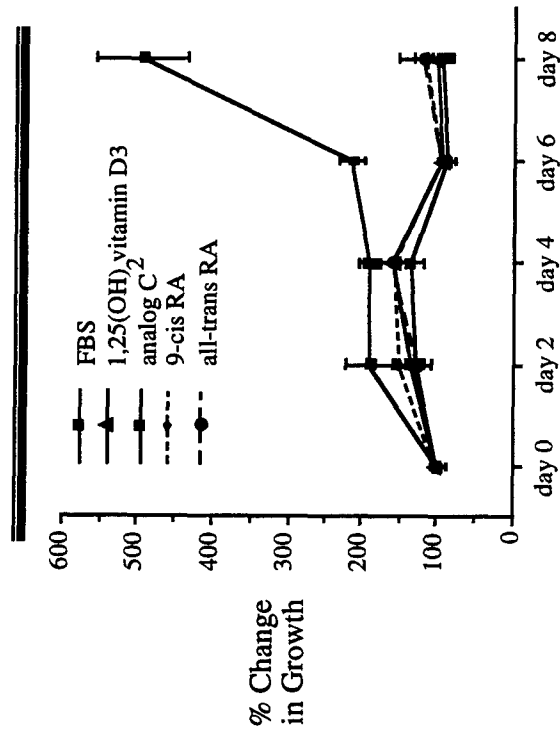
9-cis RA

A.

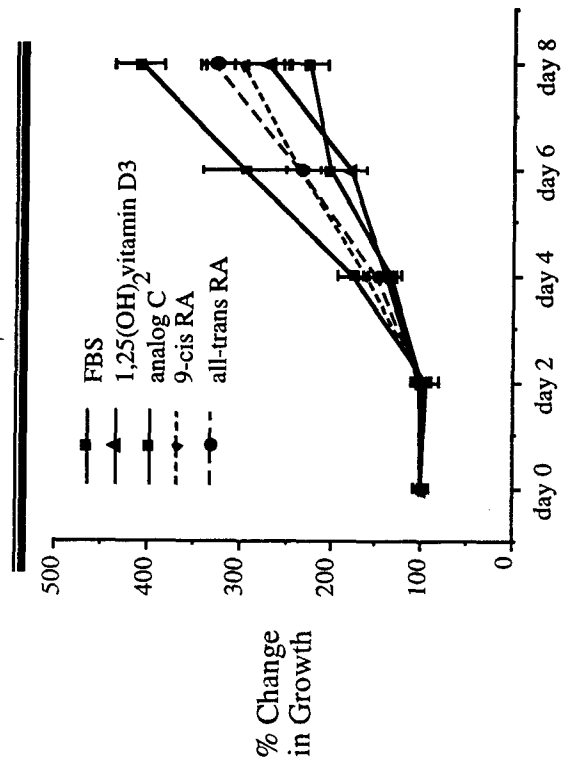
MCF7



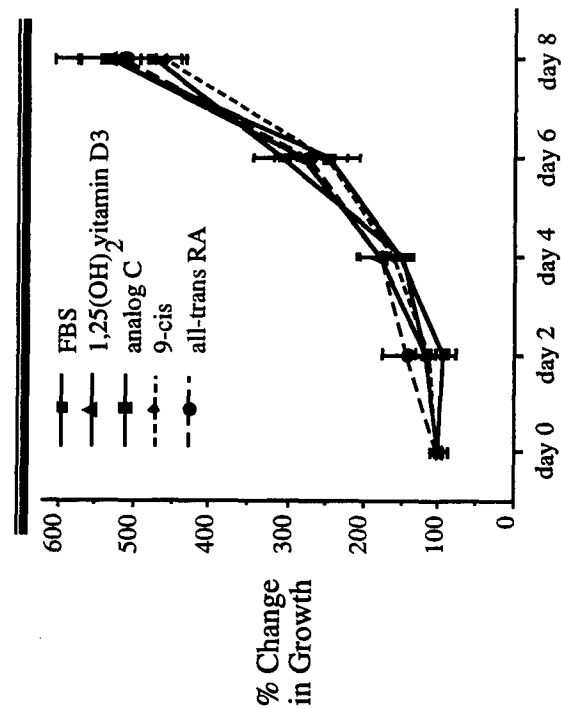
T47D



BT474

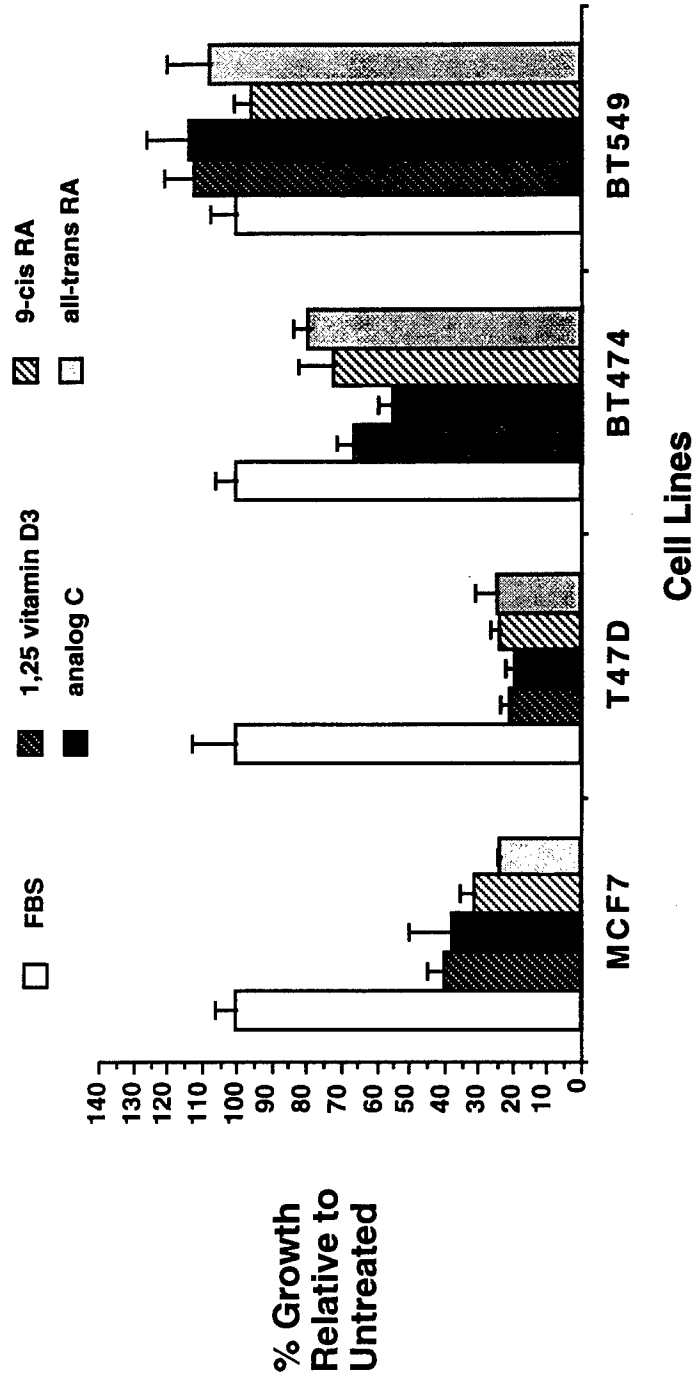


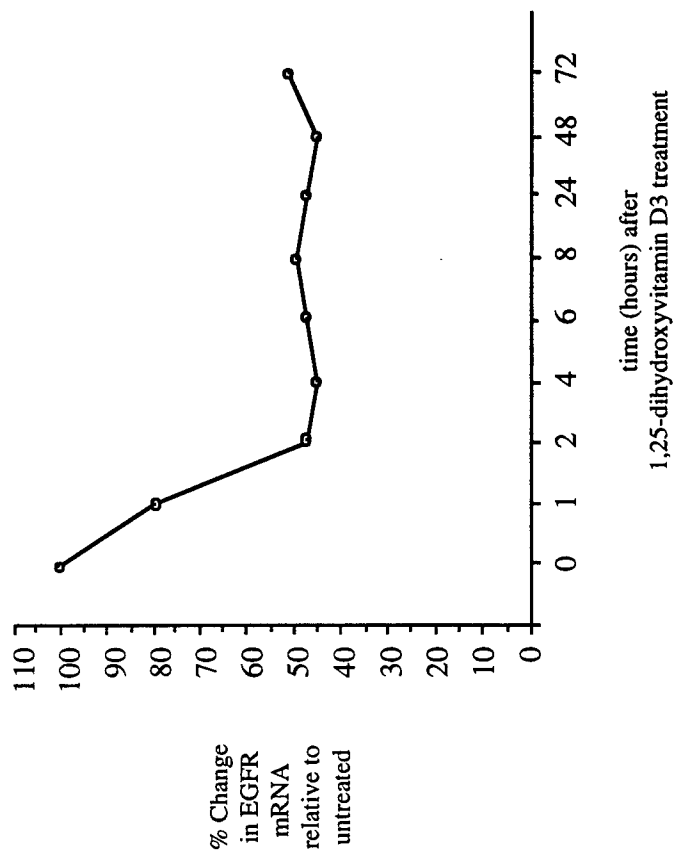
BT549



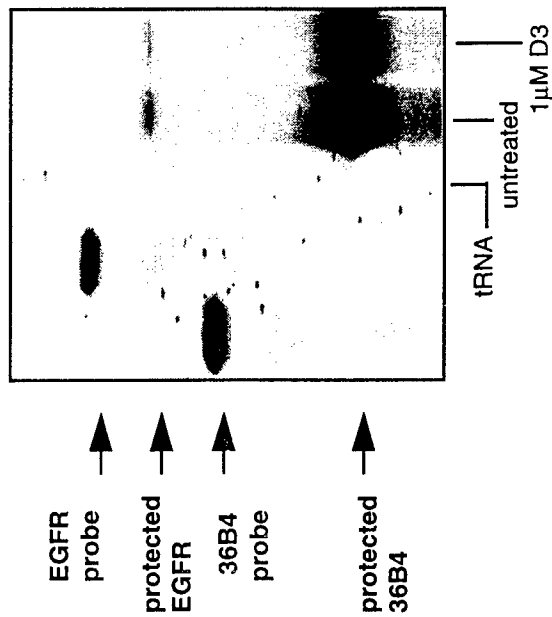
B.

Growth Effects After 8 Days at 1 μ M Treatment

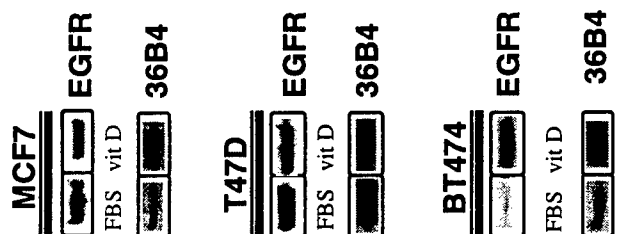
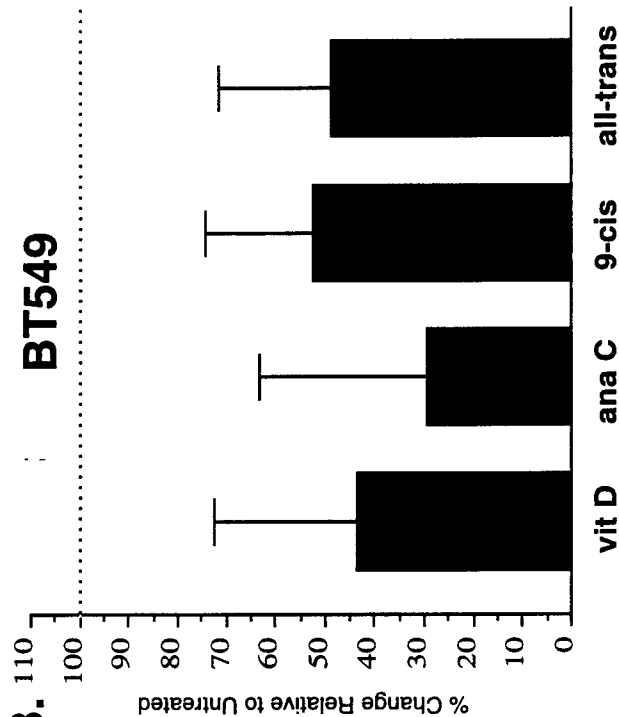




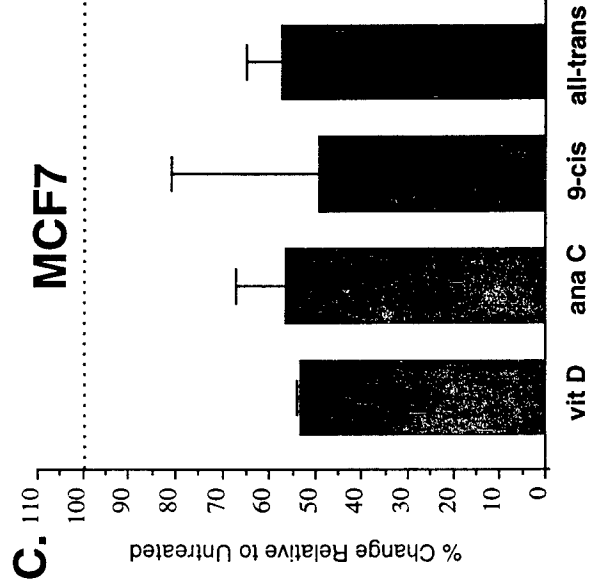
A.



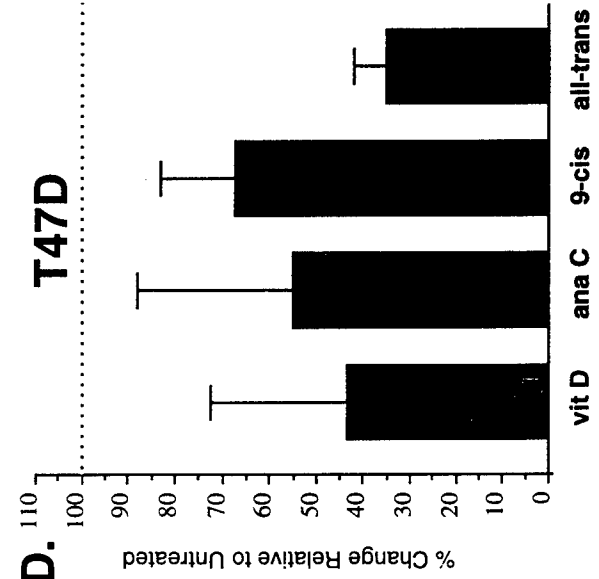
B.



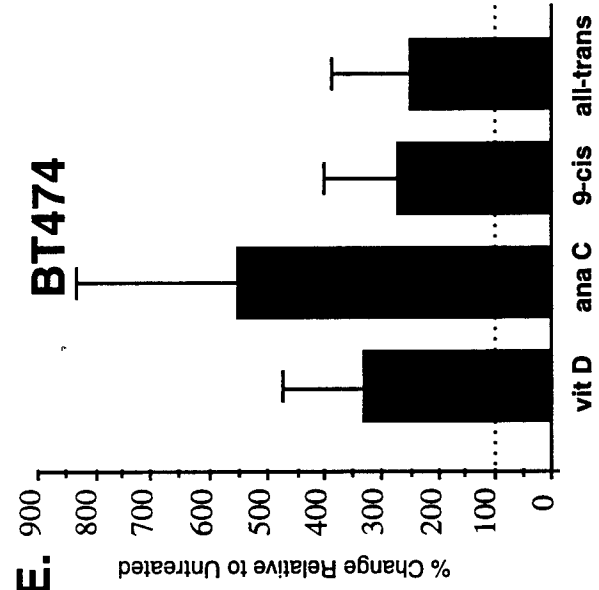
C.



D.

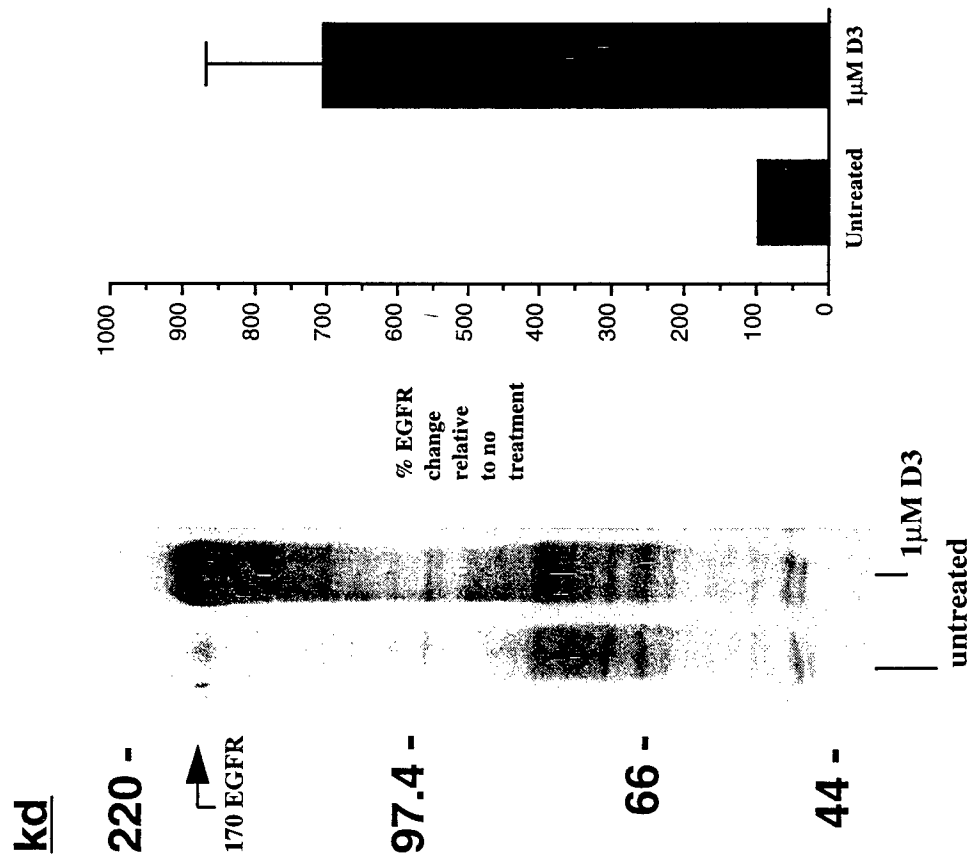


E.

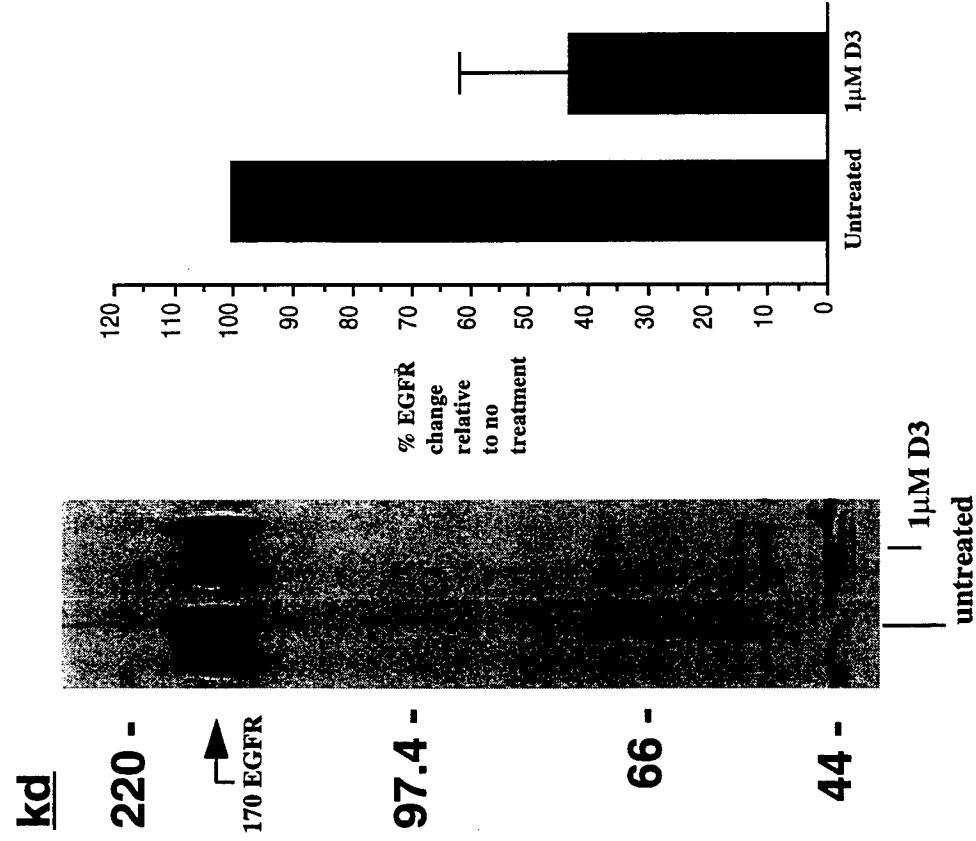


A.

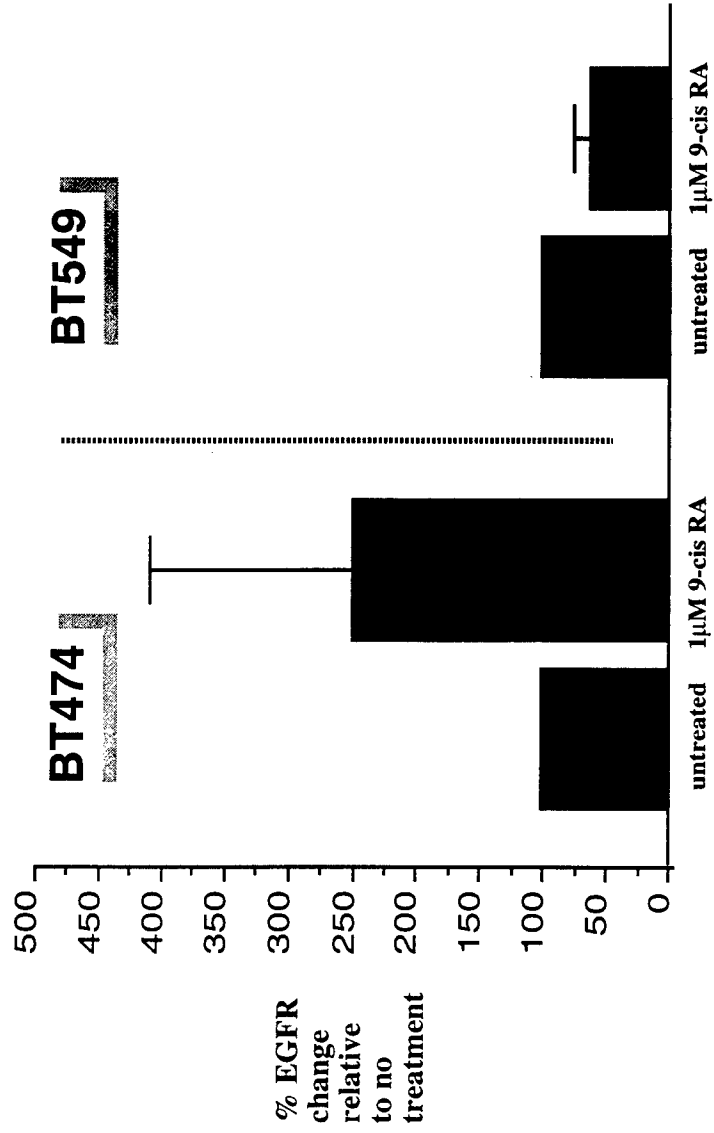
BT474

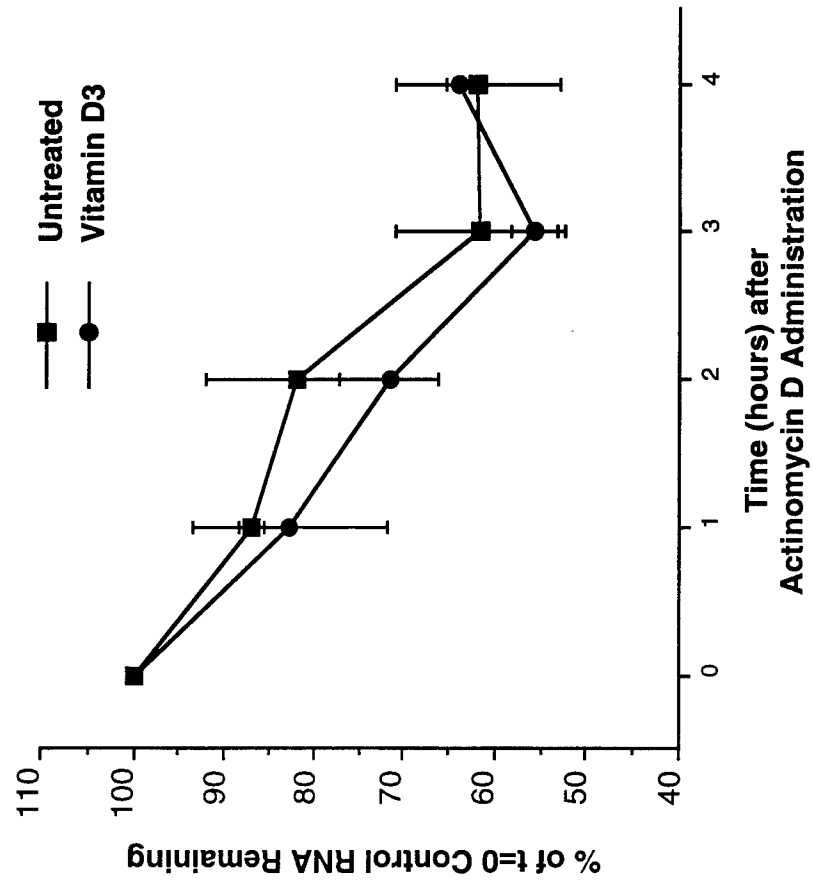


BT549



B.

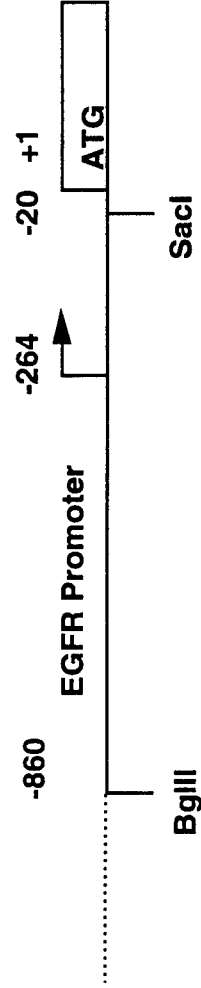




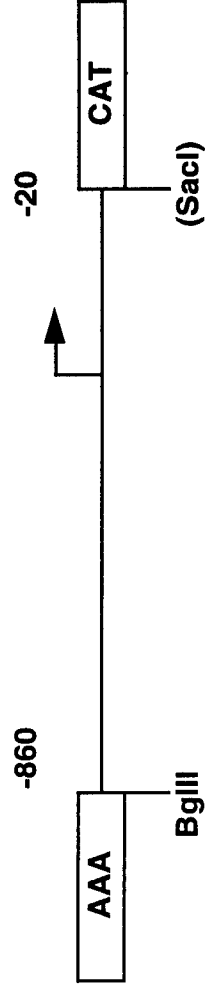
A. pJFCAT



B. Native EGFR



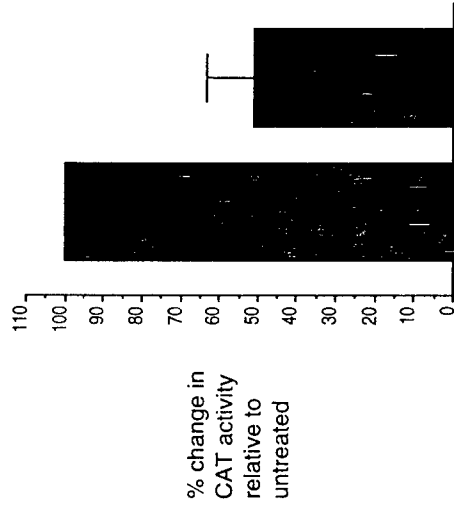
C. pJFCAT 840



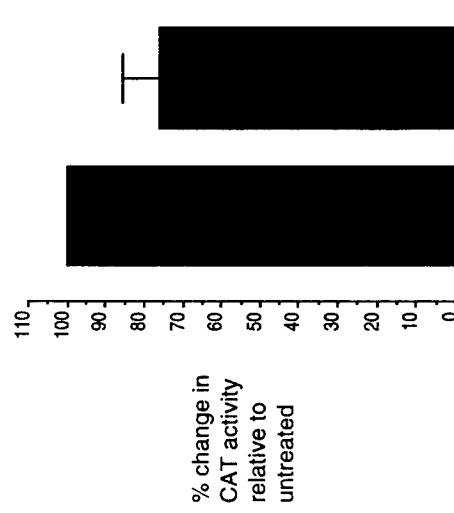
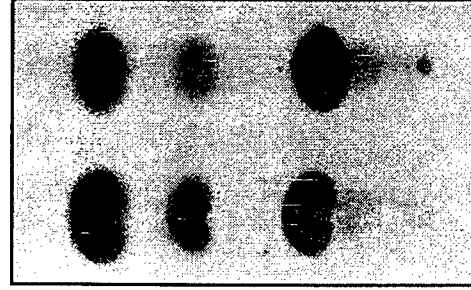
MCF7

T47D

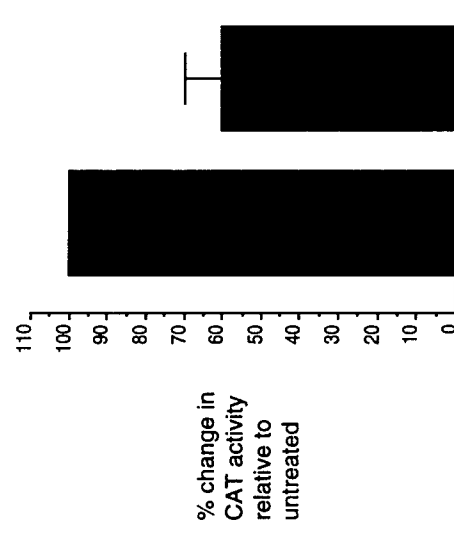
BT549



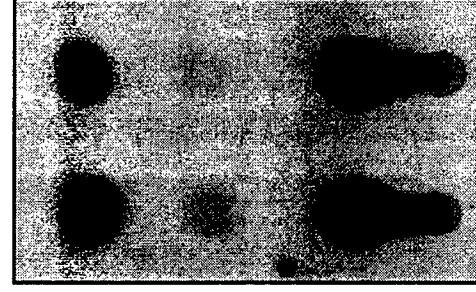
untreated vitamin D



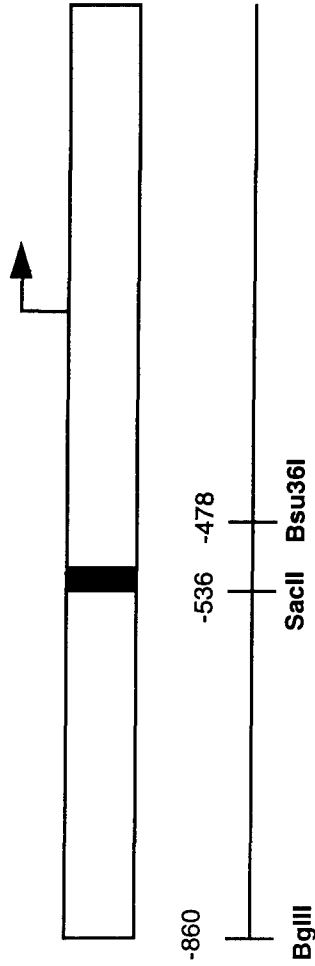
untreated vitamin D



untreated vitamin D



EGFR Promoter



putative VDRE

putative VDRE

position	sequence
-531 to -516	<u>GGGTCCAGAGGGGCA</u>

consensus VDRE

PuG(G/T)TCAnnnPuG(G/T)ICA

known functional VDREs

gene	sequence
human osteocalcin	<u>GGGTGAACGGGGCA</u>
rat osteocalcin	<u>GGGTGAATGAGACA</u>
mouse osteopontin	<u>GGTTCACGAGGTICA</u>



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

21 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
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1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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DAMD17-96-1-6020	ADB244256
DAMD17-96-1-6023	ADB231769
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DAMD17-99-1-9048	ADB258562
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DAMD17-96-1-6098	ADB239338
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DAMD17-94-J-4112	ADB222127
DAMD17-94-J-4391	ADB219964
DAMD17-94-J-4391	ADB233754