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Award Number: DAMD17-99-1-9337

TITLE: Regulation of Growth and Metastases in an Estrogen Independent Breast Cancer Cell by Vitamin D Compounds

PRINCIPAL INVESTIGATOR: Louise A. Flanagan, B.Sc.
Dr. Jo-Ellen Welsh

CONTRACTING ORGANIZATION: University of Notre Dame
Notre Dame, Indiana 46556

REPORT DATE: August 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2001	Annual Summary (1 Aug 99 - 30 Jul 01)

4. TITLE AND SUBTITLE
Regulation of Growth and Metastases in an Estrogen Independent Breast Cancer Cell by Vitamin D Compounds

5. FUNDING NUMBERS
DAMD17-99-1-9337

6. AUTHOR(S)
Louise A. Flanagan, B.Sc.
Dr. Jo-Ellen Welsh

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Notre Dame
Notre Dame, Indiana 46556

E-mail: Louise.Flanagan.25@nd.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
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12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*
Metastatic spread of cancer continues to be the greatest barrier to cancer cure. In ER-positive breast cancer cells, the effects of $1,25(OH)_2D_3$ are similar to those induced by anti-estrogens, where $1,25(OH)_2D_3$ transcriptionally down-regulates ER expression. If so, then sensitivity to $1,25(OH)_2D_3$ mediated apoptosis could be reduced in estrogen independent breast cancer cells. We focused on the role of vitamin D_3 compounds to activate the apoptotic pathway in ER- negative SUM-159PT cells and tumors and whether vitamin D compounds were capable of inhibiting the invasion of SUM-159PT cells. Our major findings are that $1,25(OH)_2D_3$ induces apoptosis in the ER-negative SUM-159PT cells by disruption of mitochondrial function which is associated with Bax translocation to mitochondria and cytochrome c release. Downstream events of the mitochondria included PARP(poly(ADP-ribose) polymerase) cleavage. Our *in vivo* data is the first to show apoptotic tumor regression of an ER-independent breast cancer model system by the vitamin D analogue EB1089. Our *in vitro* invasion assays demonstrate a dramatic reduction in invasive potential of SUM-159PT cells upon exposure to $1,25(OH)_2D_3$ and EB1089 and we are currently testing the effects of EB1089 on metastatic spread *in vivo*. Utilizing our SUM-159PT^{GFP} model system *in vivo* we can more efficiently examine the mechanisms of invasion and metastasis and the efficacy of vitamin D compounds to modulate these processes.

14. SUBJECT TERMS
Breast Cancer
$1,25(OH)_2D_3$, EB1089, estrogen independent, apoptosis, invasion, metastasis

15. NUMBER OF PAGES
47

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Metastatic spread of cancer continues to be the greatest barrier to cancer cure. It is ultimately the metastatic process that causes mortality of breast cancer since uncontrolled growth of malignant cells in other tissues continues at the expense of the normal function of that tissue. Understanding the molecular mechanisms of metastasis is crucial for the design and effective use of novel therapeutic strategies to combat cancer. In estrogen dependent mammary epithelial cells, estrogen ablation or anti-estrogen treatments such as Tamoxifen activate apoptosis secondary to disruption of estrogen survival signals. Anti-estrogen therapies can induce remission in patients whose tumors contain estrogen dependent cells, however prolonged treatment with anti-hormonal therapies often leads to the emergence of hormone independent cells, which is frequently associated with increased metastatic capacity and reduced survival time (Beckman, Niederacher et al. 1997). Therefore, apoptosis in breast tumors consisting of estrogen independent cells would require use of agents that exploit signaling pathways which are independent from those triggered by disruption of estrogen receptor (ER) function. Studies have revealed that the Vitamin D Receptor (VDR) is present in ER- negative/independent cell lines and tumors and $1,25(OH)_2D_3$ and the vitamin D analogue EB1089 has been shown to inhibit the growth of ER-negative cells lines such as the BT-20 and MDA-MB-231 (Buras, Schumaker et al. 1994). This may indicate that vitamin D compounds and anti-estrogens trigger apoptosis by two distinct mechanisms, and that breast cancer sensitivity to $1,25(OH)_2D_3$ is retained in cells that have progressed to estrogen independence. There is however no data available on modulation of breast cancer invasion and metastases by vitamin D compounds *in vivo*. The aim of these studies is to examine the effects of vitamin D compounds in an estrogen independent cell line (SUM-159PT) that is invasive both *in vitro* and *in vivo*. Activation of apoptosis at any point in the metastatic cascade would lead to elimination of the potentially metastatic cell and retard the process of tumor spread. The scope of these studies will determine whether the effects of EB1089 on SUM-159PT tumor volume is related to inhibition of proliferation, activation of apoptosis or both and whether vitamin D compounds are capable of inhibiting the invasion of SUM-159PT cells. Inhibition of metastatic spread by vitamin D compounds would suggest these compounds could offer significant therapeutic advantage over current therapies for advanced breast cancer. The scope of these studies will also seek to understand the mechanism of breast cancer invasion and metastasis. To determine if metastasis involves co-operative intravasation and/or extravasation of invasive and non-invasive epithelial cell types, we have also isolated sub-lines of the non-invasive MCF-7 and invasive SUM-159PT cell lines that have been stably transfected with different genetic markers. These studies offer a straight forward approach for tracking metastatic cells *in vivo* using fluorescent microscopy, while also enabling enhanced sensitivity by PCR detection in various organs.

ANNUAL SUMMARY-BODY

The scope of this research has primarily been to investigate whether vitamin D compounds can induce apoptosis in ER- negative breast cancer cells and whether vitamin D compounds can inhibit the invasion of these cells. The cell line used in these studies (SUM-159PT) was obtained from the University of Michigan cell and Tissue bank. Characterization of the SUM-159PT cell line is described in the attached manuscript entitled "SUM-159PT cells: A novel estrogen independent human breast cancer model system" located in the Appendix section.

In ER-positive breast cancer cells, the effects of $1,25(OH)_2D_3$ are similar to those induced by anti-estrogens, where $1,25(OH)_2D_3$ transcriptionally down-regulates ER expression. These finding have led to the hypothesis that, at least in MCF-7 cells, the effects of $1,25(OH)_2D_3$ may be related to disruption of estrogen mediated survival signals. If so, then sensitivity to $1,25(OH)_2D_3$ mediated growth arrest and apoptosis could be reduced in estrogen independent breast cancer cells. Previously, in the grant proposal, our preliminary results indicated that $1,25(OH)_2D_3$ inhibited proliferation in the ER-independent SUM-159PT cells and were therefore not consistent with this hypothesis. The growth-inhibitory effect of $1,25(OH)_2D_3$ or its analogue EB1089 in SUM-159PT cells was associated with cell cycle arrest in G1 and/or G2; upregulation of p21 and p27 and downregulation of cdc2p34. Like MCF-7 cells, SUM-159PT cells also underwent $1,25(OH)_2D_3$ mediated apoptosis (Welsh, Simboli-Campbell et al. 1995). Both $1,25(OH)_2D_3$ and EB1089 induced apoptosis in SUM-159PT cells *in vitro*, as detected by the TUNEL method. Other proteins linked to apoptosis which are modulated in breast cancer cells by $1,25(OH)_2D_3$ include bcl-2, p53, clusterin and cathepsin B. Our previous studies have shown that treatment of SUM-159PT cells with $1,25(OH)_2D_3$ or EB1089 increases the mature form of cathepsin B, suggesting that apoptosis in SUM-159PT cells may be associated with increased lysosomal activity. This suggestion is consistent with previous work describing the induction of lysosomal enzymes during apoptosis associated with mammary gland regression (Guennette, Mooibroek et al. 1994).

My current research has expanded these findings by further examining the effects of vitamin D compounds on the apoptotic pathway in SUM-159PT cells *in vitro*. The bcl-2 family of anti-apoptotic and pro-apoptotic proteins determines the release of cytochrome c, which activates death proteases called caspases, resulting in cleavage and degradation of substrates such as PARP (Gross, McDonnell et al. 1999). Cytochrome c release from the mitochondria is thus central to apoptotic signal transduction (Tsujimoto and Shimizu 2000). The pro-apoptotic protein, Bax has been reported to translocate to the mitochondria following exposure of cells to apoptotic stress including cytokine withdrawal and treatment of cytotoxic drugs such as etoposide (Green and Reed 1998; Tsujimoto and Shimizu 2000). These observations, coupled with reports that Bax causes the release of mitochondrial cytochrome c, implicate Bax as a central mediator of the apoptotic process (Green and Reed 1998) (Finucane, Bossy-Wetzel et al. 1999). In SUM-159PT cells, using subcellular fractionation we demonstrated a shift in Bax localization from cytosol to non-nuclear membranes following exposure to $1,25(OH)_2D_3$ or etoposide. Concominant release of cytochrome c from the non-nuclear membrane to the cytosol was also observed in $1,25(OH)_2D_3$ or etoposide treated cells (Figure 1a). Bax translocation and cytochrome c release appeared to act in concert with activation of downstream caspases as determined by cleavage/degradation of PARP, an indication of caspase activation (Figure 1b), as PARP is cleaved by caspase 3 and 7. Our work is the first to describe intracellular signalling events leading to apoptosis in ER-negative breast cancer cells. Further studies to

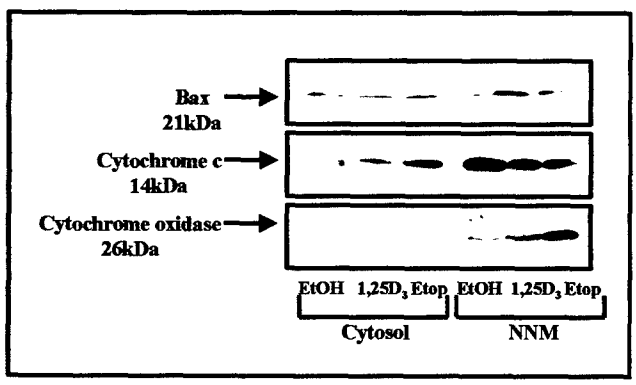

Figure 1a: Effects of $1,25(OH)_2D_3$ and etoposide on expression of apoptosis related proteins in SUM-159PT cells. Cytosol and non-nuclear membrane (NMN) fractions prepared from SUM-159PT cells treated with EtOH vehicle, 100nM $1,25(OH)_2D_3$ or 10μM etoposide for 96 were separated on 12.5% SDS-PAGE gels and immunoblotted with either a rabbit polyclonal antibody directed against Bax or mouse monoclonal antibodies directed against Cytochrome c or Cytochrome oxidase. Blots are representative of two independent experiments

characterize the activation of specific caspases in the SUM-159PT cells upon $1,25(OH)_2D_3$ treatment would also clearly be of interest.

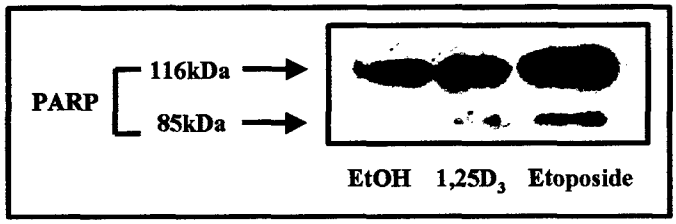

Figure 1b: Effects of $1,25(OH)_2D_3$ and etopsoside on expression and cleavage of PARP in SUM-159PT cells. Nuclear fractions prepared from SUM-159PT cells treated with ethanol vehicle, 100nM $1,25(OH)_2D_3$ or 10μM etoposide for 96 h were separated on a 7.5% SDS-PAGE gel and immunoblotted with a mouse monoclonal antibody directed against PARP. Blot is representative of two independent experiments.

Thus, while the downregulation of the ER observed in MCF-7 cells may be a contributing factor to the apoptotic and growth inhibitory effects of $1,25(OH)_2D_3$, the effects of $1,25(OH)_2D_3$ are not dependent upon estradiol signaling, as both the growth inhibitory and apoptotic effects of $1,25(OH)_2D_3$ and EB1089 were observed in the ER negative SUM-159PT cells.

Previous results from the grant proposal indicated that tumor volumes from nude mice with SUM-159PT tumors inoculated orthotopically treated with sustained release pellets that released 120pmole/day EB1089 were approximately 65-70% smaller than that of control treated tumors after five weeks of treatment. These data indicated that EB1089 was effective in reducing growth even when SUM-159PT tumors were growing in the mammary fat pad where they exhibited an aggressive, metastatic potential.

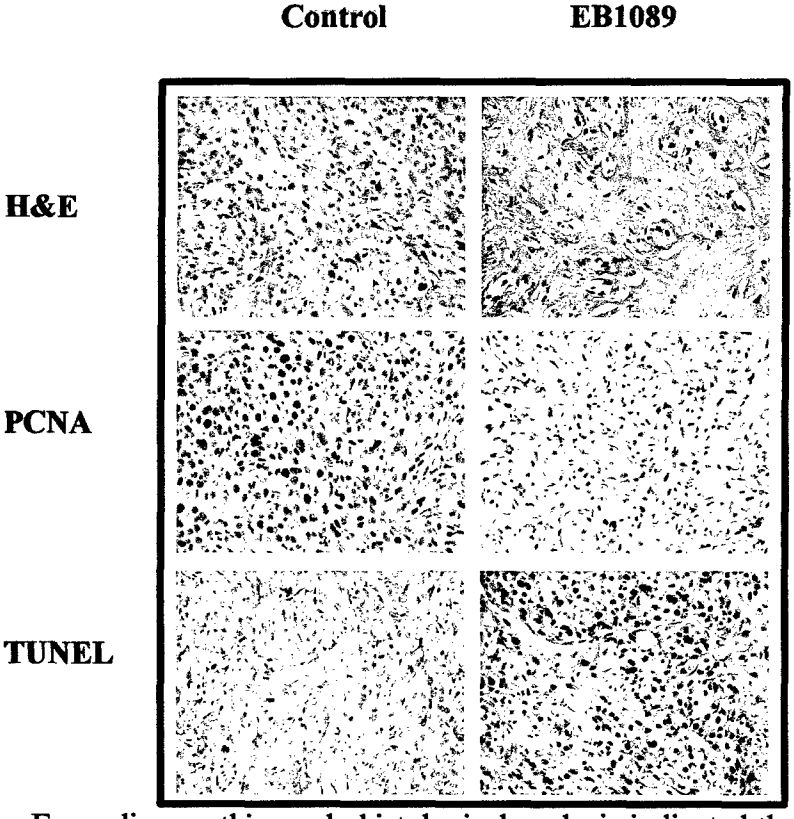

Figure 2: Effect of EB1089 on morphology and proliferation of SUM-159PT orthotopic tumors.
Representative tumor sections from mice treated with placebo control or 120 pmole/day EB1089 pellets for 5 weeks were processed for Hematoxylin and Eosin staining or processed for PCNA or TUNEL. Staining for PCNA or TUNEL is indicated by the brown nuclear staining. 40x magnification.

Expanding on this work, histological analysis indicated that the decreased size of the tumors from EB1089-treated mice, compared with that in control mice, was associated with a reduction in the epithelial component and an

increase in the stroma. The reduction of tumor volume in mice treated with 120pmole/day EB1089 for five weeks could reflect a decreased rate of cell proliferation or an increased rate of cell death or both. Our analyses confirmed that EB1089 mediates tumor regression by modulation of both apoptosis and proliferation of tumor epithelial cells (Figure 2).

Semi-Quantitative analyses of DNA fragmentation indicated that tumors from EB1089 treated mice exhibited apoptotic morphology and an increase in the amount of TUNEL-positive cells compared with tumors from control mice (Table 1). Our data demonstrating induction of apoptosis in SUM-159PT tumors correlates with our *in vitro* data that $1,25(OH)_2D_3$ and EB1089 induce apoptosis in SUM-159PT cells, consistent with data in MCF-7 cells (VanWeelden, Flanagan et al. 1998). In addition to induction of apoptosis, EB1089-treated tumors exhibited a decrease in proliferation after five weeks of treatment, as measured by PCNA. These data are again consistent with *in vitro* work indicative that $1,25(OH)_2D_3$ and EB1089 increase levels of p21 and p27, thereby inducing a block in the cell cycle, in many breast cancer cell lines.

	PCNA EXPRESSION	TUNEL EXPRESSION
CONTROL	++/+++	+/++
EB1089	+	++/+++

Table 1: Semiquantitation of PCNA expression and DNA fragmentation in SUM-159PT tumors. Tumor sections from mice treated with placebo or 120pmoles EB1089 for 5 weeks were processed for PCNA or TUNEL immunohistochemistry. After photoghraphing, 2-4 fields of view were counted on each section, with 6-10 samples evaluated for each treatment.

Our data are the first to show apoptotic tumor regression of an ER-independent breast cancer model system by the vitamin D analogue EB1089 and are consistent with previous reports of tumor regression and apoptosis in the ER-dependent MCF-7 tumors. Our work also shows that sensitivity of breast tumors to the vitamin D analogue EB1089 is not diminished in tumors that have progressed to estrogen independence.

For the tumor suppressive activity of vitamin D compounds *in vivo*, additional aspects may be involved. Our current data suggests that vitamin D compounds also inhibit invasion and metastasis. In the *in vitro* invasion assay, $1,25(OH)_2D_3$ and EB1089 reduced invasion of SUM-159PT cells by 65 and 80% respectively, independent of the anti-proliferative effects of these compounds (Figure 3). Our results have further demonstrated a reduction in the numbers of mice exhibiting secondary tumors after treatment with EB1089, indicating a direct effect of EB1089 on invasion and metastases. This data show that vitamin D compounds may play an inhibitory effect on breast cancer metastasis and may be useful in future studies in the clinical setting, alone or in conjunction with other agents, for the treatment of metastatic cancer.

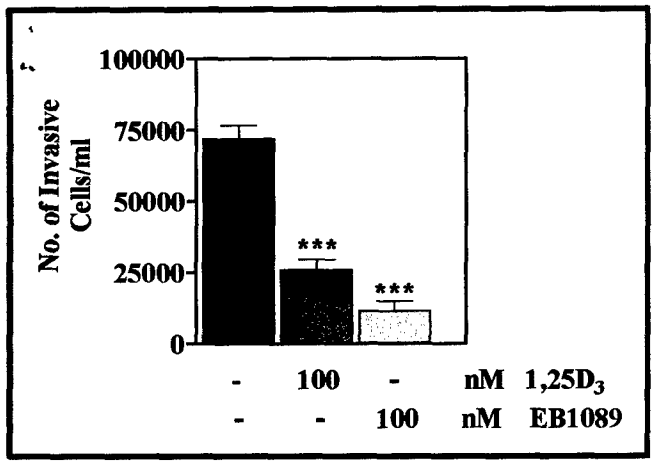

Figure 3: Effects of $1,25(OH)_2D_3$ or EB1089 on SUM-159PT invasion *in vitro*. SUM-159PT cells treated with EtOH (-), $1,25(OH)_2D_3$ or EB1089 were analyzed in the Boyden chamber invasion assay. Cells that invaded the 8μM Matrigel membrane and attached to the bottom of the well were trypsinized and counted. The data represent the mean±SEM from three wells. These data are representative of two independent experiments.

To summarize this set of experiments, the VDR is present in both estrogen-dependent and estrogen-independent breast cancer cell lines and sensitivity to vitamin D compounds is not diminished or lost in breast cancer cells that have progressed to estrogen independence. Preclinical animal studies demonstrate significant effects on the growth of estrogen-independent tumors, indicating that vitamin D compounds trigger growth arrest and apoptosis by a mechanism that is independent of an estrogen-regulated pathway.

The early stages of tumor progression and micrometastasis formation are difficult to analyze due to the inability to identify small numbers of tumor cells against a background of many host cells. In our own metastatic model, we have previously demonstrated metastatic spread of SUM-159PT cells to lymph nodes, muscle and liver. However the extent of metastases detected is likely an underestimate since small metastatic foci cannot be detected with standard hematoxylin and eosin staining. In addition sectioning and staining sections for the detection of micrometastases from tumors and multiple organs is a long and arduous process. A method to visualize tumor invasion and micrometastasis formation in viable fresh tissue would greatly benefit studies on tumor progression. To develop a model with enhanced resolution of micrometastases in the nude mouse, we used the EGFP fluorescent marker gene, cloned from the bioluminescent jellyfish *Aequorea Victoria* (previously described in original grant proposal). Orthotopic implantation of SUM-159PT^{GFP} was carried out to allow for the metastatic expression of the inoculated cells in nude mice. We previously reported that use of SUM-159PT cells expressing GFP enabled visualization of cancer cells in the mammary fat pad as well as distant micrometastases in nude mice including the lymph nodes, lung, liver and in to a lesser extent, the brain and kidney. Resolution of SUM-159PT^{GFP} cells was down to the single cell level, and high-level GFP expression was maintained in primary tumors for the duration (12 weeks) of this study. This model is the first to demonstrate the capability of GFP for detection and visualization of human breast cancer metastases in a clinically relevant model. Metastases were detected in fresh tissue *in situ* in host organs as early as two weeks after inoculation of SUM-159TP^{GFP} cells. This novel method is thus far superior than routine hematoxylin and eosin staining for the detection and study of patterns of tumor invasion and metastasis *in vivo*.

Our current results have also shown that PCR and southern blot analysis could further enhance the sensitivity and quantitation of GFP detection in primary tumors, lymph nodes, lungs, liver, brain and kidney of nude mice inoculated with SUM-159PT^{GFP} cells, confirming the results we obtained by examining fresh tissue microscopically (Figure 4). Our work complements the work of other groups indicating that the use of PCR for the detection of micro- and macro- metastases is superior to conventional immunohistochemistry techniques (Schoenfeld, Luqmani et al. 1996; Mori, Mimori et al. 1998).

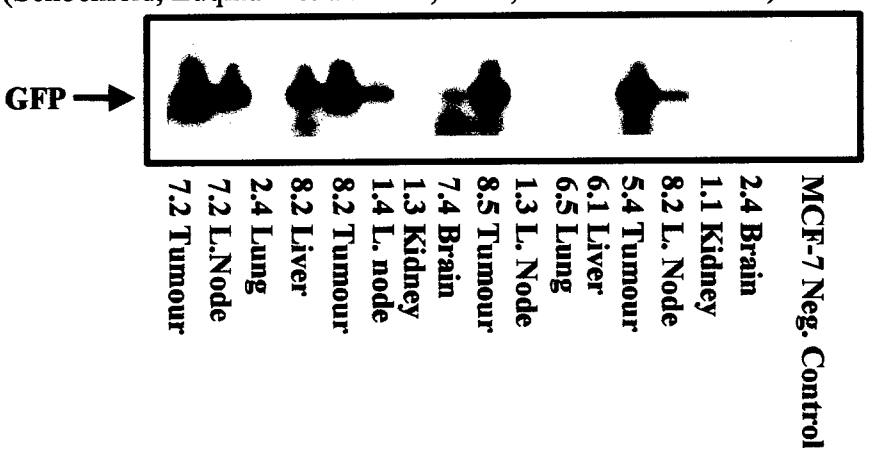

Figure 4: Southern blot analysis of GFP in tumors and organs derived from mice inoculated with SUM-159PT^{GFP} cells. PCR analysis was carried out on DNA derived from tumors and organs from mice inoculated with SUM-159PT^{GFP} cells using primers directed against a 410 bp segment of the GFP gene. The samples were electrophoresed on an agarose gel and transferred to nitrocellulose. The nitrocellulose was then hybridized with a ^{32}P labelled 410bp GFP probe.

The results presented here provide significant, new opportunities to understand and develop treatments that prevent and possibly eliminate metastasis. Having previously shown that $1,25(OH)_2D_3$ and EB1089 can inhibit the invasion of SUM-159PT cells *in vitro* and possibly reduce the numbers of mice developing secondary tumors, it would clearly be of interest to further investigate the effects of vitamin D compounds in this SUM-159PT^{GFP} model system. We are currently carrying out these experiments to accurately measure the preventative effects of EB1089 on invasion and metastases and to study the direct effect of vitamin D on established metastases using our SUM-159PT^{GFP} model system. In summary we have developed the first model system utilizing GFP for sensitive detection and visualization of human breast cancer metastases *in vivo*. We anticiptate that this model will provide a powerful approach to study the effects of genetic manipulations and therapeutic strategies of vitamin D compounds on the biology of breast cancer metastasis.

Our studies have also sought to determine if metastasis involves the co-operative intravasation and/or extravasation of invasive and non-invasive epithelial cell types, we have also isolated sub-lines of the non-invasive MCF-7 and invasive SUM-159PT cell lines that have been stably transfected with different genetic markers (pEGFP-C1 and pCDNA6-V5/His A).

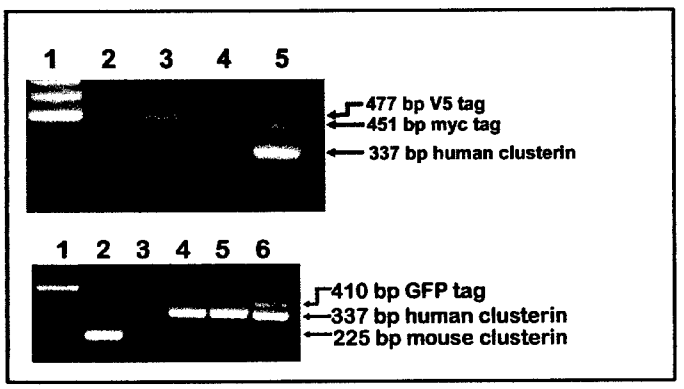

Figure 5: PCR analysis was carried out on DNA derived from SUM-159PT$^{V5/HisA}$ and MCF-7^{GFP} cells with primers directed against a 477bp segment of the V5/HisA gene and a 410bp segment of the GFP gene. Top Panel. Lanes 1: 100 bp ladder; **2**: SUM-159PTV5/HisA with human clusterin primers; **3**: SUM-159PTV5/HisA with human clusterin and V5 primers; **4**: SUM-159PT with human clusterin and V5 primers; **5**. SUM-159PT-myc/HisA with human clusterin and myc primers. **Bottom panel. Lanes 1**: 100 bp ladder; **2**: mouse liver with mouse clusterin and GFP primers; **3**: mouse liver with human clusterin and GFP primers; **4**: MCF-7 with human clusterin and GFP primers; **5**: SUM-159PT with human clusterin and GFP primers; **6**:MCF-7GFP with human clusterin and GFP primers.

We have orthotopically inoculated nude mice with a mixture of MCF-7^{GFP} and SUM-159PT-V5/His A cells and have followed the progression of metastasis using fluorescent microscopy and PCR to determine whether the non-invasive MCF-7^{GFP} can be detected in distant metastases associated with V5/His tagged SUM-159PT cells. These experiments will determine whether the co-operative interaction between ER-positive and ER-negative cells during intravasation and extravasation may contribute to the established heterogeneity of metastatic breast cancer.

Using laser capture microscopy, specific cells of interest from mice inoculated with non-invasive MCF-7GFP and invasive SUM-159PT V5/His tagged cells were captured and further analysed for specific markers using PCR analyses.

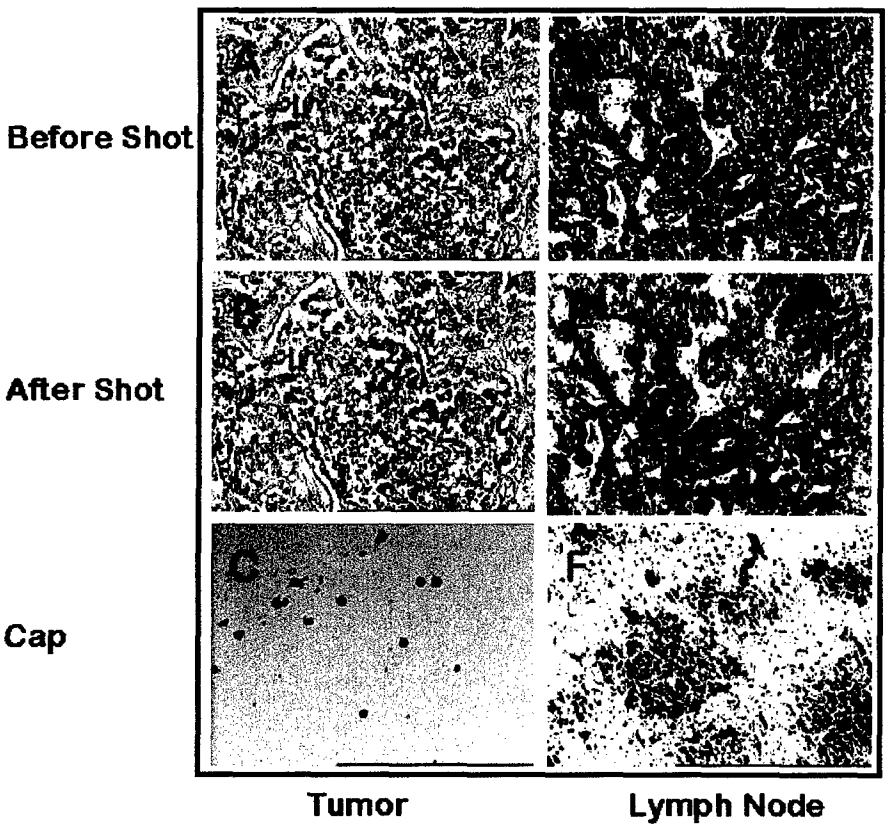

Figure 6: Hematoxylin and eosin staining of tumors and lymph nodes from nude mice inoculated with SUM-159PT$^{V5/HisA}$ and MCF-7^{GFP} cells.
Primary tumors (**Panels A and B**) and lymph nodes (**D and E**) from mice inoculated with a mixture of SUM-159PT$^{V5/HisA}$ and MCF-7^{GFP} cells were formalin fixed, paraffin embedded, sectioned and stained with hematoxylin and eosin. Photographed at 10x magnification. Cells from tumor and lymph node sections were captured using a laser capture microscope.

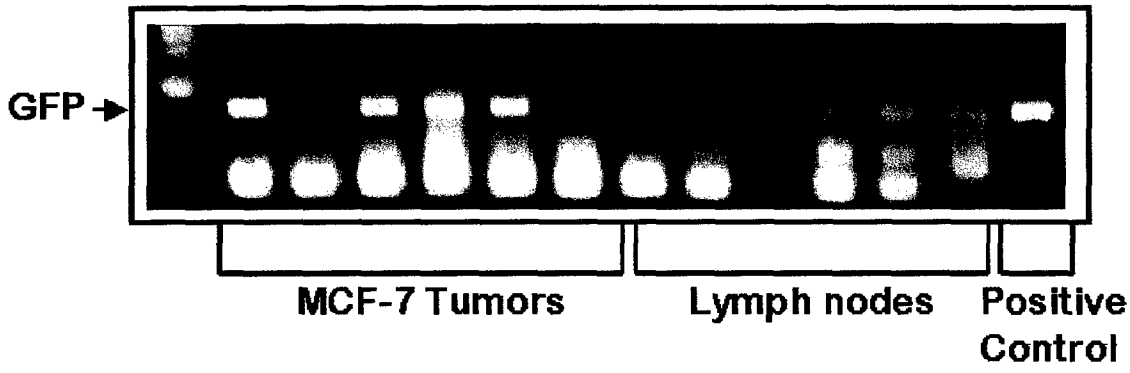

Figure 7: PCR analyses of GFP in tumors and Lymph nodes from mice inoculated with SUM-159PTV5/HisA and MCF-7GFP cells. PCR analyses was carried out on DNA derived from cells isolated from tumors and lymph nodes from mice inoculated with SUM-159PT$^{V5/HisA}$ and MCF-7^{GFP} cells with primers directed against a 410bp segment of the GFP gene.

Using the laser capture assay, DNA from several cells were isolated and analyzed by PCR for the expression of GFP. Our preliminary data indicates migration of MCF-7^{GFP} cells to the lymph nodes of mice that were co-inoculated with the invasive SUM-159PT$^{V5/HisA}$ cells, indicating a co-operative interaction between these cells during intravasation and extravasation may contribute to the established heterogeneity of metastatic breast cancer.

Our future experiments will utilize fluorescently labeled invasive SUM-159PT and non-invasive MCF-7 cells to confirm whether co-operative interaction between ER-positive non-invasive and ER-negative invasive cells during intravasation and extravasation may contribute to the established heterogeneity of metastatic breast cancer.

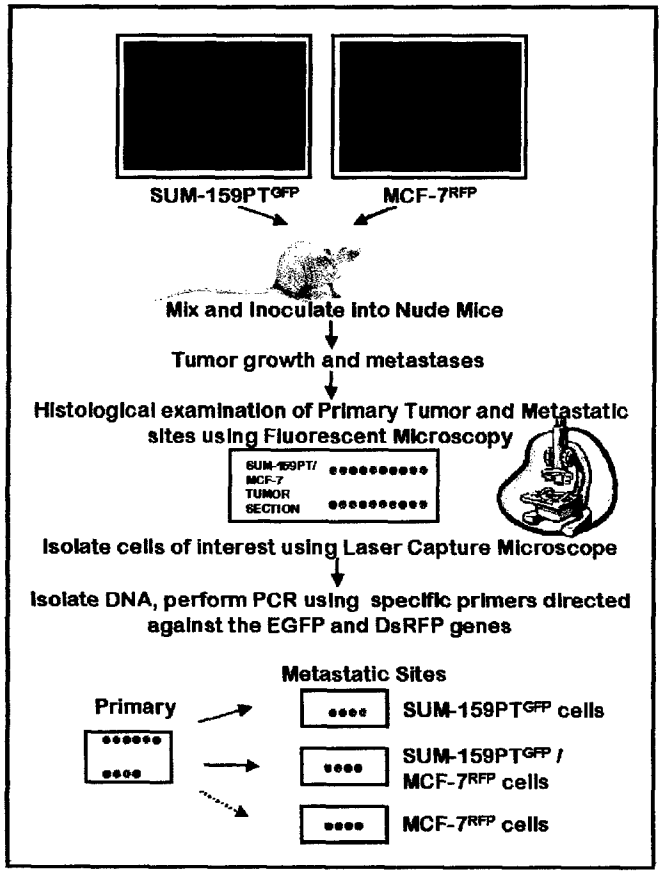

Figure 8: Schematic diagram of future experiments using SUM-159PT^{GFP} and MCF-7^{RFP} cells.

In conclusion we hope that this later set of experiments will provide us with valuable information as to the mechanism of invasion and metastases and provide in the future new insights into alternatives for the treatment of metastatic breast cancer.

Statement of Work:

Task 1: Inoculation of nude mice with SUM-159PT^{GFP} cells, establishment of tumors and treatment of nude mice with EB1089 or placebo pellets. Although my studies so far have not included the inoculation of SUM-159PT^{GFP} cells orthotopically into nude mice and treatment of established tumors with EB1089 and placebo via sustained release pellets, I have as stated above examined the effects of EB1089 and placebo treatment on SUM-159PT tumors that were inoculated orthotopically. We have as stated above also been utilizing the GFP model system described in the grant proposal to more efficiently track the metastatic spread of SUM-159PT cells to various organs by utilizing PCR. By using PCR we should be able to more effectively examine the effects of EB1089 on tumor growth and metastatic spread. Using the data compiled from both of the above studies we have decided not to fully complete Task 1 but to continue on with Tasks 3-9.

Task 2: Tumor histopathology and western blotting to quantitate the relative effects of EB1089 on apoptosis and mitosis of SUM-159PT^{GFP} tumors. Although I have not examined the effects of EB1089 on apoptosis and mitosis of SUM-159PT^{GFP} tumors, I have however examined these effects on SUM-159PT tumor samples taken from a previous experiment that were treated via sustained release pellets with placebo or 120 pmoles/day EB1089. Our current work did examine the relative effects of EB1089 on apoptosis and mitosis in SUM-159PT tumors as shown above. Our work has also further concentrated on examining the effects the effects of vitamin D compounds on the apoptotic signalling pathway in SUM-159PT cells *in vitro*.

Tasks 3 and 6: Reagents and mice have been ordered. SUM-159PT^{GFP} cells were expanded *in vitro* for inoculation into nude mice. Inoculation of nude mice has been carried out. We are carrying out Tasks 3 and 6 simultaneously.

Tasks 4 and 7: We will implant placebo or EB1089 pellets after 2 weeks of tumor growth (before metastases are present- Task 4) and after 6 weeks of tumor growth (to ensure that metastatic foci have developed- Task 6).

Tasks 5 and 8 will be completed once Tasks 4 and 7 have been successfully completed.

KEY RESEARCH ACCOMPLISHMENTS

- The observation that upon exposure of ER-negative SUM-159PT cells to $1,25(OH)_2D_3$, a shift in Bax localization from cytosol to non-nuclear membranes occurs. Concominant release of cytochrome c from the non-nuclear membrane to the cytosol was also observed in $1,25(OH)_2D_3$ treated cells. Bax translocation and cytochrome c release acted in concert with activation of downstream caspases as determined by cleavage/degradation of PARP. The *in vitro* data indicate that sensitivity cells to vitamin D is not diminished in the ER-negative SUM-159PT cells.

- Our data are the first to show apoptotic tumor regression of an ER-independent breast cancer model system by the vitamin D analogue EB1089. Our work also shows that sensitivity of breast tumors to the vitamin D analogue EB1089 is not diminished in tumors which have progressed to estrogen independence.

- Our data suggests that vitamin D compounds also inhibit invasion and metastasis. In the *in vitro* invasion assay, $1,25(OH)_2D_3$ and EB1089 reduced invasion of SUM-159PT cells by 65 and 80% respectively, independent of the anti-proliferative effects of these compounds.

- Our current results have also shown that PCR and southern blot analysis could further enhance the sensitivity and quantitation of GFP detection in primary tumors, lymph nodes, lungs, liver, brain and kidney of nude mice inoculated with SUM-159PT^{GFP} cells, confirming the results we obtained by examining fresh tissue microscopically, indicating that the use of PCR for the detection of micro- and macro- metastases is superior to conventional immunohistochemistry techniques.

- Our studies have also sought to determine if metastasis involves the co-operative intravasation and/or extravasation of invasive and non-invasive epithelial cell types, we have also isolated sub-lines of the non-invasive MCF-7 and invasive SUM-159PT cell lines that have been stably transfected with different genetic markers (pEGFP-C1 and pCDNA6-V5/His A). Our preliminary data indicates migration of MCF-7^{GFP} cells to the lymph nodes of mice that were co-inoculated with the invasive SUM-159PT$^{V5/HisA}$ cells, indicating a co-operative interaction between these cells during intravasation and extravasation may contribute to the established heterogeneity of metastatic breast cancer.

REPORTABLE OUTCOMES FOR ARMY GRANT PERIOD 1999-2201

- Completion of Ph.D. Thesis written and accepted. Defense passed.
- Travel Award to the Walther Cancer Annual Retreat at University of Michigan, Ann Arbor, August 23-25.
- **Flanagan L,** Wang Y, Welsh JE and Tenniswood M. Detection and mechanisms of human breast cancer metastases *in vivo*. Presented at the Walther Cancer Scientific Retreat, Ann Arbor, Michigan, Aug 23-25, 2001
- **Flanagan L,** Wang Y, Welsh JE and Tenniswood M. Detection and mechanisms of human breast cancer metastases *in vivo*. Presented at the ENDO 2001 meeting in Denver, CO, June 2001
- **Flanagan L**, Juba B and Welsh JE. Vitamin D and Metastatic Breast Cancer. *Vitamin D Endocrine System: Structural, Biological, Genetic and Clinical Aspects.* (Norman AW, Bouillon R and Thomasett M, Eds.) University of California Riverside Press, pp511- 514.
- Packman K, **Flanagan L**, Zinser G, Mitsch R, Tenniswood M and Welsh JE. Combination Treatment of MCF-7 xenografts with the vitamin D_3 analog EB1089 and antiestrogens. (2000) *Vitamin D Endocrine System: Structural, Biological, Genetic and Clinical Aspects.* (Norman AW, Bouillon R and Thomasett M, Eds.) University of California Riverside Press, pp.515-518.
- Abstract promoted to a 15 min presentation at the Eleventh Worshop on Vitamin D in Nashville TN, May 27-June 1, 2000. Abstract Title: "Vitamin D and Metastatic Breast Cancer."
- **Flanagan L,** Van Weelden K, Ammermam C, Ethier SP and Welsh JE (1999) SUM-159PT cells: a novel estrogen independent human breast cancer model system. Breast Cancer Research and Treatment 58:193-204
- Byrne I, **Flanagan L**, Tenniswood M and Welsh JE (2000) Identification of a hormone responsive promoter immediately upstream of exon 1c in the human vitamin D receptor gene. Endocrinology 141(8): 2829-2835
- **Flanagan L**, Packman K, Juba B, Tenniswood M and Welsh JE Regulation of growth, apoptosis and invasion of estrogen independent SUM-159PT human breast cancer cells and tumors by 1,25-Dihydroxyvitamin D_3 and its analog EB1089. To be submitted to Endocrinology.
- Packman K, Zinser G, **Flanagan L**, Tenniswood M and Welsh JE Combination Treatment of MCF-7 Xenografts with EB1089 and Antiestrogens. To be submitted to Endocrinology
- **Flanagan L**, Byrne I, Wang Y, Tenniswood M and Welsh JE. Utilization of green fluorescent protein for the identification of metastasis in an *in vivo* breast cancer model system. In Preparation.

REPRINTS OF ALL MANUSCRIPTS WILL BE SENT UPON ACCEPTANCE

CONCLUSION

Our data has shown that the VDR is present in estrogen-dependent and estrogen-independent breast cancer cell lines and sensitivity to vitamin D compounds is not diminished or lost in breast cancer cells that have progressed to estrogen independence. Our preclinical animal studies demonstrate significant effects of the vitamin D analogue EB1089 on the growth of estrogen-independent tumors, indicating that vitamin D compounds trigger growth arrest and apoptosis by a mechanism that is independent of an estrogen-regulated pathway.

Our data also indicates that we have developed the first model system utilizing GFP for sensitive detection and visualization of human breast cancer metastases *in vivo*. We anticiptate that this model will provide a powerful approach to study the effects of genetic manipulations and therapeutic strategies of vitamin D compounds on the biology of breast cancer metastasis.

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VITAMIN D AND METASTATIC BREAST CANCER

Louise Flanagan [1,2], Brian Juba [2] and JoEllen Welsh [2], University College Dublin, Belfield, Ireland [1]; University of Notre Dame, IN 46556 USA [2].

Breast cancer is one of the leading causes of death among women in North America and Western Europe despite intense efforts to develop more effective chemo- and hormonal therapies. Although five-year survival rates for localized and regionally spread breast cancer have improved, the survival rate for women with distant metastases is only 20%. The development of a metastatic, hormone independent, and drug resistant phenotype is largely responsible for the high percentage of treatment failures among breast cancer patients. Although understanding the molecular mechanisms of cancer is crucial for the design of effective, novel therapeutic strategies, progress has been limited by the lack of appropriate experimental models for the study of metastatic breast cancer. In these studies, we describe and characterize, *in vitro* and *in vivo*, the growth of a novel estrogen independent human breast cancer cell line, SUM-159PT and examine the effects of vitamin D_3 compounds on its growth and invasion.

Our studies have shown that ER protein was not detected in SUM-159PT cells or tumors, correlating with lack of estradiol binding. Furthermore SUM-159PT cells were tumorigenic in ovariectomized nude mice in the absence of estradiol supplementation, consistent with estrogen independence. Our work has shown that the SUM-159PT cell line is epithelial in origin, as determined by expression of cytokeratin 18 by western blot. However, the levels of cytokeratin 18 expression were considerably lower (10-fold) than those observed in the estrogen-dependent MCF-7 cells. We demonstrated that approximately 45% of SUM-159PT cells are capable of invading through an 8μM Matrigel invasion chamber whilst exhibiting a stellate morphology in the Matrigel outgrowth assay. In contrast, the non-invasive MCF-7 cells formed smooth spherical colonies in Matrigel and were minimally invasive *in vitro*. These results correlate well with previous data indicating that poorly differentiated cell lines express cytokeratins at low levels, and are highly invasive in *in vitro* chemoinvasion assays.

To investigate metastatic markers in SUM-159PT cells, we examined the expression of vimentin and E-cadherin, proteins that have been strongly implicated in the transition of carcinoma cells to a metastatic phenotype. SUM-159PT cells and xenografts were found to express vimentin, which has been associated with poor prognosis, low differentiation, lack of ER, high invasive capacity and shorter disease-free interval. In contrast, the well-differentiated MCF-7 cells did not express vimentin. In breast cancer, loss of E-cadherin expression has been observed in high-grade tumors and correlates with increased invasiveness and significantly shorter disease-free interval. E-cadherin was expressed in the MCF-7 cells, but no expression was observed in the invasive, vimentin-positive SUM-159PT cells. We also report an inverse relationship between the levels of the putative anti-metastatic protein nm23 and tumor aggressiveness. Little or no nm23

expression was observed in the invasive SUM-159PT xenografts, in contrast to high expression detected in well-differentiated MCF-7 tumors.

Thus SUM-159PT cells can be categorized with other poorly-differentiated fibroblastic-like breast cancer cell lines (1). Use of SUM-159PT cells as an *in vitro* model of estrogen-independent human breast cancer will be useful for molecular studies on the mechanisms of invasion and for testing the effectiveness of potential therapeutics in advanced breast cancer cells.

Our lab and others have shown that the vitamin D metabolite $1,25(OH)_2D_3$ induces growth arrest and apoptosis in MCF-7 cells *in vitro* (2). In ER-positive breast cancer cells, the effects of $1,25(OH)_2D_3$ are similar to those induced by anti-estrogens in that $1,25(OH)_2D_3$ mediated cell death in MCF-7 cells is preceded by down regulation of the ER. These finding have led to the hypothesis that, at least in MCF-7 cells, the effects of $1,25(OH)_2D_3$ may in part be related to disruption of estrogen related survival signals. If so, then sensitivity to $1,25(OH)_2D_3$ mediated growth arrest and apoptosis could be reduced in estrogen independent breast cancer cells. Our studies have demonstrated that both $1,25(OH)_2D_3$ and its analog EB1089 caused a significant 60-70% reduction in SUM-159PT cell number after a six day time course relative to that of EtOH control. We have shown that SUM-159PT cells express the VDR which is capable of binding $1,25(OH)_2D_3$ and VDREs and its protein expression is upregulated or stabilized upon treatment with $1,25(OH)_2D_3$ or EB1089. Because previous work has demonstrated that MCF-7 cells treated with $1,25(OH)_2D_3$ or EB1089 arrest in G_0/G_1, we examined the effects of these agents on cell cycle regulatory proteins (p21 and p27) and the M phase cdc2p34 protein kinase in the estrogen independent SUM-159PT cell line. SUM-159PT cells treated with $1,25(OH)_2D_3$ or EB1089 exhibited an early increase (48 h) in both p27 and p21 expression compared to EtOH controls. It has been previously shown that a block in the M-phase is imposed by inhibiting the cdc2p34 protein kinase. In SUM-159PT cells treated with $1,25(OH)_2D_3$ little or no downregulation of cdc2p34 was observed. In contrast, a significant downregulation of cdc2p34 was observed when SUM-159PT cells were treated with EB1089 for 72 h. We also examined expression of the lysosomal protein, cathepsin B thought to be involved in the apoptotic pathway, and found an increase in the processed, mature form of cathepsin B in SUM-159PT cells treated with $1,25(OH)_2D_3$ or EB1089. Furthermore, using the TUNEL method, positive nuclear staining indicative of DNA fragmentation was detected in SUM-159PT cells treated with $1,25(OH)_2D_3$ or EB1089 for four days, similar to that observed in MCF-7 cells.

To further elucidate the apoptotic pathway in the SUM-159PT cells we analyzed the expression and subcellular distribution of several proteins that have recently been implicated in apoptotic signaling (3). In SUM-159PT cells, the proapoptotic protein bax was translocated to the mitochondrial fraction upon treatment with $1,25(OH)_2D_3$ or etoposide. This translocation of bax to the mitochondria was concominant with the release of cytochrome c from the mitochondria into the cytosol after 96 h treatment with $1,25(OH)_2D_3$ or 48 h with etoposide. To exclude

mitochondrial contamination in the cytosol fractions, cytochrome oxidase expression was assessed and found to be present only in the mitochondrial fractions. To determine whether caspase activation was involved in apoptosis of SUM-159PT cells, we assessed the cleavage of PARP, a substrate of caspase 3 and 7. SUM-159PT cells treated with $1,25(OH)_2D_3$ for 96 h or etoposide for 48h were analyzed with an antibody that recognizes both intact PARP (116 kDa) and the apoptotic cleavage product (85 kDa). SUM-159PT cells treated with $1,25(OH)_2D_3$ or etoposide exhibited cleavage of PARP to generate the 85kDa fragment, whereas no PARP cleavage was detected in EtOH treated cells.

Thus, while the downregulation of the ER observed in MCF-7 cells may be a contributing factor to the apoptotic and growth inhibitory effects of $1,25(OH)_2D_3$, the effects of $1,25(OH)_2D_3$ are not dependent upon estradiol signaling, as both the growth inhibitory and apoptotic effects of $1,25(OH)_2D_3$ and EB1089 were observed in the ER-negative SUM-159PT cells.

To determine whether the vitamin D analog EB1089 could modulate growth and/or apoptosis of estrogen independent breast tumors, ovariectomized nude mice bearing either subcutaneous or orthotopic tumors were implanted with pellets designed to release 120 pmoles EB1089 per day. A similar reduction in tumor volume was observed upon treatment with EB1089 in either subcutaneous or orthotopic tumors. In mice implanted with EB1089 pellets, tumor volumes decreased gradually over the four weeks of treatment. Tumor volumes after four weeks was significantly ($P<0.001$) lower in the EB1089 treated groups (99.7 ± 61.3 mm^3) than in the control placebo group (656.5 ± 321.05 mm^3). In addition, several tumors completely regressed in response to EB1089 treatment. Morphological analysis of SUM-159PT tumors from mice treated with 120 pmole EB1089 or placebo was accomplished after staining with hematoxylin and eosin. Tumors from placebo treated mice were primarily composed of dense tumor epithelial cells, with small amounts of mouse-derived stroma, frequent blood cells and numerous mitotic figures. In contrast, tumors from mice treated with 120pmoles EB1089/day displayed an increased percentage of stroma, loss of epithelial cells, few mitotic figures and in many areas, epithelial cells with classic apoptotic morphology were observed. To determine whether the changes in SUM-159PT tumor morphology induced by EB1089 were associated with alterations in mitotic or apoptotic index, we examined expression of PCNA as a marker of proliferation and DNA fragmentation (assessed as TUNEL-positive cells) as a marker of apoptosis. Analyses of DNA fragmentation indicated that tumors from EB1089 treated mice exhibited apoptotic morphology and an increased number of TUNEL-positive cells compared with tumors from control mice. In addition to induction of apoptosis, EB1089-treated tumors exhibited a decrease in proliferation, as measured by PCNA. Our data demonstrating induction of apoptosis and decrease in proliferation in EB1089 treated SUM-159PT tumors correlates with our *in vitro* data that $1,25(OH)_2D_3$ and EB1089 induce apoptosis and cell cycle arrest in SUM-159PT cells.

Our data are the first to show apoptotic tumor regression of ER-independent breast tumors by the vitamin D analog EB1089, and are consistent with previous reports of tumor regression and apoptosis in the ER-dependent MCF-7 tumors (4). Our work also shows that sensitivity of breast tumors to the vitamin D analog EB1089 *in vivo* is not diminished during progression to estrogen independence.

Our *in vivo* studies further demonstrated that SUM-159PT cells were metastatic after inoculation into the mammary fat pad of ovariectomized nude mice, consistent with the expression of metastatic markers and *in vitro* invasiveness. Micro-metastases were observed in the lymph nodes, muscle area surrounding the mammary fat pad, lungs and liver, Metastatic spread was not observed when SUM-159PT cells were inoculated subcutaneously. Therefore, implantation of tumor cells into anatomically appropriate (orthotopic) sites, rather than subcutaneously, allowed for the metastatic potential of SUM-159PT cells.

Very few studies have addressed the potential effects of vitamin D compounds on invasion and metastases in any cancer type but in the few studies that have been carried out, vitamin D compounds have been implicated in the regulation of angiogenesis, invasion and metastasis (5). In SUM-159PT cells $1,25(OH)_2D_3$ and EB1089 induced 65% and 80% reductions respectively in invasive potential compared to EtOH treated cells. These effects were independent of the anti-proliferative effects of the compounds. Our results have further demonstrated a reduction in the numbers of mice exhibiting secondary tumors after treatment with EB1089, indicating a direct effect of EB1089 on metastases. Further investigation on the effects of EB1089 in preventing SUM-159PT metastases *in vivo* would clearly be of interest.

These data demonstrate that vitamin D compounds may inhibit both growth of primary tumors and metastasis of breast cancer cells, and may in the future prove to be highly useful in the clinical setting, alone or in conjunction with other agents, in the treatment of metastatic breast cancer.

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VITAMIN D AND METASTATIC BREAST CANCER. L. Flanagan, B. Juba and J. Welsh, University College Dublin, Belfield, Ireland; University of Notre Dame, IN 46556.

Breast cancer remains one of the most common malignant diseases in women in both North America and Western Europe. Therapies for the most aggressive of breast cancers, estrogen independent tumors, are severely limited and the development of novel therapies is hindered by the lack of available model systems. In these studies we present a potential model system for the investigation of estrogen independent breast cancer. SUM-159PT cells are a human breast cancer cell line, epithelial in origin that lack ER expression as detected by western blot and ligand binding assays. Furthermore, SUM-159PT cells injected subcutaneously or orthotopically are tumorigenic in ovariectomized nude mice. We have demonstrated that SUM-159PT cells are capable of invading through an 8μM Matrigel membrane, exhibit a stellate morphology in Matrigel, express high levels of vimentin, do not express E-cadherin, all of which are the hallmarks of a metastatic phenotype. Utilizing the SUM-159PT cell line stably transfected with pEGFP-C1 (enhanced green fluorescent protein) we have been able to successfully track the metastatic spread of SUM-159PT cells *in vivo*. Metastatic spread has been detected in the lymph nodes, lungs, liver, kidney and brain of athymic nude mice by PCR analysis and fluorescence microscopy. Thus SUM-159PT cells represent an excellent model for the investigation of human ER independent breast cancer. Both $1,25(OH)_2D_3$ and its analog EB1089 significantly reduce SUM-159PT cell number, increase the number of cells in the G_0/G_1 phase of the cell cycle, upregulate p21, p27 and cathepsin B. The inhibition of cell cycle is followed by induction of the apoptotic pathway as detected by *in situ* end labeling and translocation of bax from the cytosol to the mitochondria upon treatment with $1,25(OH)_2$ D_3. When SUM-159PT tumor bearing nude mice are implanted with pellets designed to release 120pmoles EB1089 per day, a significant reduction in tumor volume is observed after 5 weeks of treatment with several mice showing complete tumor regression. The reduced growth of tumors from EB1089 treated mice is associated with characteristic apoptotic morphology, an increase in DNA fragmentation (measured by *in situ* end labeling), and a reduction in proliferation (measured by PCNA) relative to contol tumors. Of particular interest in the invasive SUM-159PT tumors, EB1089 not only affects the size of the primary tumor but also reduces the numbers of mice with secondary tumors. These studies provide preliminary evidence that $1,25(OH)_2D_3$ and its analogs may diminish the metastatic capability of human breast cancers. (Support: AICR-98A100; DAMD-17-99-1-9337)

Presented at the ENDO 2001 meeting, Denver and at the Walther Cancer Scientific Retreat, Michigan

Detection and Mechanisms of Human Breast Cancer Metastases *in Vivo*

Louise Flanagan, Yao Wang, JoEllen Welsh and Martin Tenniswood, Department of Biological Sciences, University of Notre Dame, IN 46556

The early stages of tumor progression and micrometastasis formation are difficult to analyze due to the inability to identify small numbers of tumor cells against a background of many host cells. We have developed an orthotopic xenograft model in estrogen receptor (ER) negative breast cancer using the human SUM-159PT cell line, which metastasizes to the lymph nodes, muscle and liver of nude mice. The extent of metastasis detected by histochemical methods is likely an underestimate since small metastatic foci cannot be accurately detected. To develop a model with enhanced resolution of micrometastases we created a stable cell line expressing green fluorescent protein (SUM-159PT^{GFP}). After orthotopic implantation, SUM-159PT^{GFP} cells can be visualized by fluorescent microscopy in the mammary fat pad as well as distant micrometastases in nude mice including the lymph nodes, lungs, liver and to a lesser extent, in the brain and kidney, without fixation. Metastases are detectable as early as 2 weeks after inoculation of SUM-159PT^{GFP} cells. We have also shown that PCR and Southern blot analysis further enhance the sensitivity of GFP detection in primary tumors and metastatic sites. To determine if metastasis involves the co-operative intravasation and/or extravasation of invasive and non-invasive epithelial cell types, we have also isolated sub-lines of the non-invasive MCF-7 and invasive SUM-159PT cell lines that have been stably transfected with different genetic markers (pEGFP-C1, pCDNA6-V5/His A, pCDNA6-myc/His A). We have orthotopically inoculated nude mice with a mixture of MCF-7^{GFP} and SUM-159PT-V5/His A cells and we are following the progression of metastasis using fluorescent microscopy and PCR to determine whether the non-invasive MCF-7^{GFP} can be detected in distant metastases associated with V5/His tagged SUM-159PT cells. These experiments will determine whether the co-operative interaction between ER-positive and ER-negative cells during intravasation and extravasation may contribute to the established heterogeneity of metastatic breast cancer.

COMBINATION TREATMENT OF MCF-7 XENOGRAFTS WITH THE VITAMIN D_3 ANALOG EB1089 AND ANTIESTROGENS.

K. Packman, L. Flanagan, G. Zinser, R. Mitsch, M. Tenniswood, and J. Welsh
Department of Biological Sciences, University of Notre Dame, Notre Dame, IN

Introduction Our lab has shown antiestrogens have an additive effect on EB1089 induced apoptosis in MCF-7 cells *in vitro*. In the present studies, the ability of combined therapy with antiestrogens to potentiate the anti-tumor effects of EB1089 *in vivo* was investigated. A breast cancer model was utilized with established, rapidly growing tumors in the continuous presence estradiol throughout treatment. Combined treatment of MCF-7 xenografts with EB1089 and either the partial ER agonist tamoxifen or the pure ER antagonist ICI182,780 was compared. Previous studies with xenografts derived from MCF-7^{D3Res} cells demonstrated the tumors were resistant to the growth inhibitory properties of EB1089. Studies of MCF-7^{D3Res} cells *in vitro* demonstrated that although MCF-7^{D3Res} cells were resistant to EB1089, they were sensitive to antiestrogens, and antiestrogen induced apoptosis was potentiated by co-incubation of cells with EB1089. The current studies examined whether tumors derived MCF-7^{D3Res} cells exhibited comparable sensitivity to antiestrogen induced apoptosis *in vivo*, and whether combined treatment of MCF-7^{D3Res} xenografts with EB1089 and tamoxifen or ICI 182,780 would produce additional anti-tumor effects.

Materials and Methods In all studies, ovariectomized NCr nu/nu mice were implanted sc with 1.7 mg 17ß-estradiol sustained release pellets (Innovative Research). Mice were inoculated sc in the flank region with approximately 5×10^6 MCF-7 or MCF-7^{D3Res} cells suspended in 0.3 ml Matrigel/ αMEM. After three weeks, mice bearing tumors with volumes averaging approximately 200 mm^3 were randomized for treatment. Treatments and duration for each experiment are located in the corresponding figures or figure legends.

After treatment was completed, tumors were removed from mice, formalin-fixed, paraffin embedded, and sectioned at 5 μM. Mitotic index and apoptotic index were assessed by quantitative morphometric analysis of proliferating cell nuclear antigen (PCNA) expression and in situ terminal transferase mediated fluorescein dUTP nick end labeling (TUNEL), established markers of proliferation and apoptosis. PCNA expression and TUNEL were quantitated by viewing and photographing four random fields of each tissue section with a brightfield microscope and 40x objective. Photographs were taken with a digital camera, and were analyzed with the Zeiss KS 300 Imaging Analysis software. To detect vitamin D receptor (VDR) expression tissue sections were incubated with an anti-VDR (clone 9A7) monoclonal antibody. To detect estrogen receptor (ER) or progesterone receptor (PR) expression, sections were incubated with an anti-ER (clone 6F11) or anti-PR (clone 1A6) monoclonal antibody. The ABC technique was used to develop VDR, ER, or PR staining followed by the substrate DAB. ER, PR, and VDR expression were evaluated by utilizing a qualitative scale with sections receiving a score from (++++) for highest staining, to (-) for no staining.

Tumor growth data are expressed as the mean ± SE. Differences between means were considered significant when $p<0.05$. Statistical comparisons were performed using the Kruskal-Wallis ANOVA. Post-test comparisons between groups were made using the Dunn test.

Results

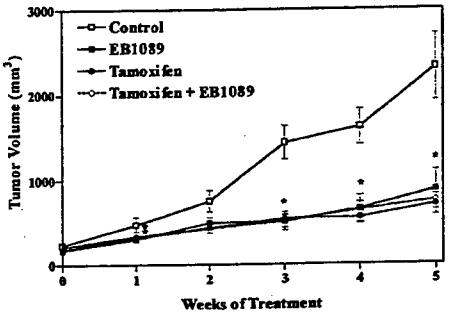

Figure 1. *Effect of EB1089, tamoxifen, or combination treatment, on MCF-7 tumor volume.* Tumor volume of MCF-7 xenografts treated with either vehicle + placebo (control) (n=8) 45 pmol EB1089 3x/ week (n=10), 15 mg/ 35 days tamoxifen (n=7), or both EB1089 and tamoxifen (n=10), *p<0.05 for EB1089, tamoxifen, and EB1089 + tamoxifen, compared to control.

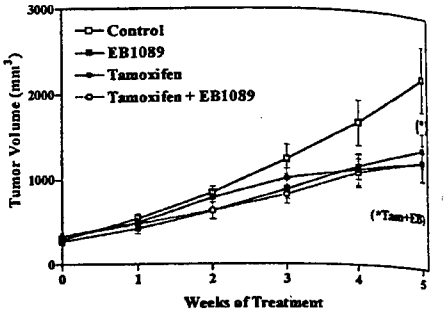

Figure 2. *Effect of EB1089, tamoxifen, or combination treatment, on MCF-7 tumor volume.* Tumor volume of MCF-7 xenografts treated with either vehicle + placebo (control) (n=9) 25 pmol EB1089 3x/ week (n=11), 7.5 mg/ 42 days tamoxifen (n=10), or both EB1089 and tamoxifen (n=12), *p<0.05 for EB1089 + tamoxifen, compared to control.

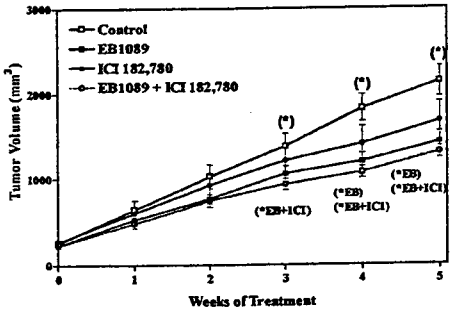

Figure 3. *Effect of EB1089, ICI 182,780, or combination treatment, on MCF-7 tumor volume.* Tumor volume of MCF-7 xenografts treated with either vehicle + placebo (control) (n=11) 25 pmol EB1089 3x/ week (n=11), 10 mg/ 35 days ICI 182,780 (n=8), or EB1089+ ICI 182,780 (n=14), *p<0.05 for EB1089, and EB1089 + ICI 182,780, compared to control.

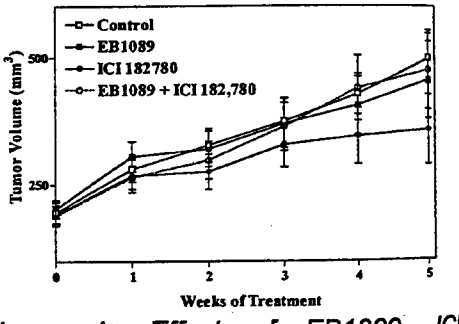

Figure 4. *Effect of EB1089, ICI 182,780, or combination treatment, on MCF-7^{D3Res} tumor volume.* Tumor volume of MCF-7^{D3Res} xenografts treated with either vehicle + placebo (control) (n=13) 25 pmol EB1089 3x/ week (n=13), 10 mg/ 35 days ICI 182,780 (n=13), or both EB1089 and ICI 182,780 (n=12).

% Cells	Control	EB1089	Tamoxifen	EB + Tam
A. PCNA	66.11 ± 3.26 n=6	62.97 ± 1.26 n=8	61.54 ± 2.71 n=9	45.08 ± 4.38* n=10
TUNEL	2.35 ± .40 n=9	29.74 ± 4.9* n=10	15.89 ± 3.12* n=9	23.70 ± 4.13* n=10
% Cells	Control	EB1089	ICI 182,780	EB + ICI
B. PCNA	67.15 ± 2.91 n=10	55.65 ± 3.06 n=10	54.63 ± 3.22 n=10	35.97 ± 2.35*[a] n=10
TUNEL	3.31 ± 1.09 n=10	21.37 ± 6.33* n=10	25.48 ± 4.09* n=10	15.16 ± 4.08* n=10

Table 1. *Quantitation of PCNA expression and DNA fragmentation in MCF-7 tumors.* Data are expressed as percentage of cells positive for PCNA or DNA fragmentation. *A.* Tumor sections from mice treated with vehicle, 25 pmol EB1089 3x/wk, 7.5 mg/ 42 days tamoxifen, or EB1089 + tamoxifen for 6 weeks. *B.* Tumor sections from mice treated with vehicle, 25 pmol EB1089 3x/wk, 10 mg/ 35 days ICI 182,780, or EB1089 + ICI 182,780 for 5 weeks. *$p<0.05$ compared to control. [a]$p<0.05$ compared to EB1089 and ICI 182,780.

VDR	++++	+++	++	VDR	++++	+++	++
A. Control	22%	67%	11%	B. Control	31%	54%	15%
EB1089	55%	36%	9%	EB1089	45%	36%	18%
Tamoxifen	50%	40%	10%	ICI182,780	30%	50%	20%
EB1089 + Tamoxifen	27%	64%	9%	EB1089 + ICI182,780	29%	36%	36%

Table 2. *Effect of EB1089, antiestrogen, or combination treatment, on relative VDR expression in MCF-7 xenografts.* Data are expressed as percentage of tumors with relative VDR expression. *A.* MCF-7 xenografts were treated for 6 weeks with either vehicle (n=9), 25 pmol EB1089 3x/ week (n=11), 7.5 mg/ 42 days tamoxifen (n=10), or both EB1089 and tamoxifen (11). *B.* MCF-7 xenografts were treated for 5 weeks with either vehicle (n=13), 25 pmol EB1089 3x/ week (n=11), 10 mg/ 35 days ICI 182,780 (n=10), or both EB1089 and ICI 182,780 (n=14).

ER	+++	++	+	PR	+++	++	+	+/-	-
A. Control	56%	22%	22%	A. Control	56%	22%	22%	0%	0%
EB1089	36%	45%	18%	EB1089	36%	18%	9%	9%	27%
Tamoxifen	50%	40%	10%	Tamoxifen	30%	40%	10%	10%	10%
EB1089 + Tamoxifen	50%	42%	8%	EB1089 + Tamoxifen	42%	33%	8%	0	17%
B. Control	64%	21%	14%	B. Control	43%	21%	14%	14%	7%
EB1089	42%	42%	17%	EB1089	33%	25%	8%	17%	17%
ICI182,780	46%	39%	15%	ICI182,780	31%	31%	8%	8%	23%
EB1089 + ICI182,780	36%	43%	21%	EB1089 + ICI182,780	21%	21%	21%	0%	36%

Table 3. *Effect of EB1089, antiestrogen, or combination treatment, on relative ER and PR expression in MCF-7 xenografts.* Data are expressed as percentage of tumors with relative ER or PR expression. *A.* MCF-7 xenografts were treated for 6

weeks with either vehicle (n=9), 25 pmol EB1089 3x/ week (n=11), 7.5 mg/ 42 days tamoxifen (n=10), or both EB1089 and tamoxifen (n=12). *B.* MCF-7 xenografts were treated for 5 weeks with either vehicle (n=14), 25 pmol EB1089 3x/ week (n=12), 10 mg/ 35 days ICI 182,780 (n=13), or both EB1089 and ICI 182,780 (n=14).

Discussion Our previous studies have shown that both the partial agonist tamoxifen and the pure antagonist ICI 182,780 can potentiate EB1089 induced apoptosis in MCF-7 and MCF-7^{D3Res} cells *in vitro*. We have also demonstrated that EB1089 exerts anti-tumor effects in MCF-7 tumors by inducing apoptosis. In the current studies, we demonstrated that EB1089 is as effective as antiestrogens, the endocrine therapy of choice, in MCF-7 tumor growth inhibition. In contrast to *in vitro* studies however, results of combination studies *in vivo* indicated that additive effects on MCF-7 and MCF-7^{D3Res} tumors could not be observed with EB1089 in combination with either tamoxifen or ICI 182,780. Mitotic index demonstrated some enhancement of anti-tumor effect with EB1089 + antiestrogens over single treatments alone in MCF-7 tumors however, the relevance of these small differences is questionable, since overall changes in tumor volume and weight were minimal. Regardless of how these small differences are interpreted, a much larger effect on anti-tumor activity was anticipated with combination treatment based on *in vitro* studies.

Studies of ER and PR expression in MCF-7 xenografts after treatment with EB1089 and antiestrogens implicated down-regulation of the ER growth regulatory pathway as a mechanism of action for EB1089. Comparison of ER and VDR regulation with combination treatment *in vitro* and *in vivo* indicates that ER down-regulation is maintained *in vivo*, whereas VDR up-regulation is absent *in vivo*. Thus, upregulation of VDR with combination treatment may be necessary for additive anti-tumor effects. *Supported by AICR 98A100 and DAMD17-97-1-726.*

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Breast Cancer Research and Treatment **58:** 193–204, 2000.

Report

SUM-159PT cells: a novel estrogen independent human breast cancer model system

Louise Flanagan[1,2], Kathryn Van Weelden[2], Cheryl Ammerman[3], Stephen P. Ethier[3], and JoEllen Welsh[2]

[1]*University College Dublin, Belfield, Ireland;* [2]*University of Notre Dame, Notre Dame, IN;* [3]*The University of Michigan Medical School, Ann Arbor, MI, USA*

Key words: breast cancer, estrogen independent, MCF-7 cells, metastases, SUM-159PT cells

Summary

Breast cancer remains one of the most common malignant diseases in women in North America and Western Europe, yet therapies for the more aggressive estrogen independent tumors are limited and few model systems are available for the study of this type of breast cancer. In these studies, we characterized a novel estrogen independent breast cancer cell line, SUM-159PT. SUM-159PT cells are epithelial in origin, demonstrated by expression of cytokeratin 18. SUM-159PT cells are estrogen independent, demonstrated by lack of estrogen receptor (ER) protein and ER ligand binding studies. Furthermore, SUM-159PT cells injected subcutaneously or orthotopically are tumorigenic in ovariectomized athymic nude mice in the absence of estradiol supplementation. SUM-159PT cells are capable of invading through an 8 μm Matrigel membrane and display a stellate morphology in Matrigel, indicative of a metastatic phenotype. Correlating with this phenotype, we have detected secondary tumors upon inoculation of SUM-159PT cells into the mammary fat pad. To further investigate the metastatic potential of the SUM-159PT cells, we examined the expression of two proteins, vimentin and E-cadherin, implicated in the transition of carcinoma cells to a metastatic phenotype. Western blot and immunohistochemical analysis demonstrated that both SUM-159PT cells and xenografts express vimentin. No expression of E-cadherin was detected in SUM-159PT cells. Our data indicate that despite estrogen independence, SUM-159PT cells are growth inhibited *in vitro* by compounds such as $1,25(OH)_2D_3$, transforming growth factor β (TGF-β), and the phorbol ester TPA. These studies indicate that SUM-159PT cells represent a good model system for the study of late stage estrogen independent, invasive breast cancer.

Introduction

Breast cancer is one of the leading causes of death among women in North America and Western Europe, despite intense efforts to develop more effective chemo- and hormonal therapies. Although five-year survival rates for localized and regionally spread breast cancer has improved, the survival rate for women with distant metastases is only 20% [1]. Many breast tumors appear to follow a predictable pattern, being confined initially to local structures, responsive to endocrine manipulation and/or sensitive to cytotoxic chemotherapy. However, many of these tumors progress to a more malignant phenotype, characterized by invasive/metastatic foci, and become resistant to endocrine manipulation and chemotherapeutic intervention [2–4]. The development of a metastatic, hormone independent, and drug-resistant phenotype is largely responsible for the high percentage of treatment failures among breast cancer patients.

Recent studies have linked vimentin expression, an intermediate filament protein normally present in cells of mesenchymal origin, to poor prognosis, high growth fraction, and poor differentiation grade [5–7]. There has also been a strong correlation between vimentin expression, low levels of keratins, and invasiveness in both the Matrigel outgrowth assay and the Boyden chamber invasion assay [8, 9]. Loss of

cell–cell adhesion in carcinoma cells may be an important step in the acquisition of an invasive metastatic phenotype, and the expression of some cell adhesion molecules such as E-cadherin is frequently reduced in invasive cell lines [10, 11]. Indeed fibroblastic, highly invasive vimentin-expressing breast cancer cell lines do not express E-cadherin and express a stellate morphology in Matrigel [9, 12].

Probably because investigators have wanted to work with cells with a clear phenotype, breast cancers showing high vimentin expression and low keratin expression have been less intensively studied and characterized. Hence, to date, there are few good models for the study of ER-negative, hormone independent breast cancer.

In this report, we describe and characterize, *in vitro* and *in vivo*, the growth of a novel estrogen independent human breast carcinoma cell line, SUM-159PT. SUM-159PT cells along with 11 other human breast cancer cell lines were developed at the University of Michigan cell/tissue bank, where extensive information about the phenotypic and genotypic properties of these cell lines is available. This resource of well-characterized cell lines is likely to be useful in the search for novel breast cancer associated genes [13, 14]. In contrast to most ER-negative breast cancer cell lines, which were isolated from pleural effusions and have been in continuous culture for many years, SUM-159PT cells were developed from a primary anaplastic breast tumor, cultured in a selective defined growth medium, and were available at early passage. In this report, we demonstrate that SUM-159PT cells represent an invasive metastatic cell type which expresses vimentin but not E-cadherin and displays a stellate morphology in Matrigel. The SUM-159PT cell line is sensitive to a number of growth inhibitory compounds such as $1,25(OH)_2D_3$, TPA, and TGF-β. We postulate that SUM-159PT cells represent an excellent model of estrogen independent, metastatic human breast cancer.

Materials and methods

Cell culture, viability, Matrigel outgrowth and invasion assays

The SUM-159PT human breast cancer cells used in this study were isolated from an anaplastic primary carcinoma using published procedures, and are available at early passage through the University of Michigan Human Breast Cancer Cell/Tissue Bank [15]. SUM-159PT cells were cultured in Ham's F-12 medium (Life Technologies Inc, Gaithersburg, MD) containing 10 mM HEPES, 5 μg/ml insulin, 1 mg/ml hydrocortisone (Sigma, St. Louis, MO), and 5% charcoal-stripped serum (Hyclone, Logan, Utah). MCF-7 cells, which were used as a well-characterized ER positive control cell line, were cultured in α-MEM medium (Life Technologies) containing 25 mM HEPES and 5% fetal bovine serum (Life Technologies). MDA-MB-231 cells, which were used as an ER negative cell line, were cultured in α-MEM medium containing 25 mM HEPES and 5% fetal bovine serum. Cells were routinely passaged every 4–5 days.

For SUM-159PT cell proliferation experiments, cells were plated at a density of either 2 or 4 × 10^3 cells ml per in medium containing 5% charcoal stripped serum. Treatments with $1,25(OH)_2D_3$ (Biomol, Plymouth Meeting, PA), TPA, TGF-β1 (Sigma), or ethanol vehicle were initiated on the day following plating. For quantitation of total cell number, cells were fixed with 1% gluteraldehyde for 15 min, incubated with 0.1% crystal violet for 30 min, destained with H_20, and solubilized with Triton X-100. Absorbance at 600 nm was determined on a microplate reader.

The Matrigel outgrowth assays were performed as described in [6]. Briefly, cells were harvested, pelleted, and resuspended in cold Matrigel (Collaborative Biomedical, Waltham, MA) (1 × 10^5 cells per 0.2 ml), and then quickly added to a preset Matrigel layer in a 24-well dish. The top layer was gelled for 30 min in the incubator, and then covered with culture medium (0.5 ml), incubated, and fed as for monolayer cultures. Cells were allowed to grow for approximately 10 days in culture and then photographed.

For the biocoat Matrigel invasion assay (Fisher Scientific, Pittsburgh, PA) both MCF-7 and SUM-159PT cells were plated at 100,000 cells per ml in each 8 μm Matrigel chamber and allowed to grow for 24 h. Assays were carried out in triplicate and control inserts (without Matrigel) were used for each cell line. After 24 h growth, the Matrigel was removed from the inserts and the cells at the base of the membrane were fixed using 1% gluteraldehyde for 15 min, stained with 0.1% crystal violet for 30 min, and destained with H_2O. Membranes were allowed to dry and numbers of invasive cells were quantitated by viewing random fields of each membrane using a Nikon Optiphot-2 microscope. The percentage of cells invading was calculated by dividing the number of invasive cells in the

Matrigel membranes by the number of cells invading in the control inserts.

Estrogen receptor function

Binding of ^{3}H-17β-estradiol was measured in triplicate in nuclear extracts from untreated SUM-159PT and MCF-7 cells prepared in KTED buffer (10 mM Tris–HCl, 300 mM KCl, 1 mM EDTA, 10 mM Na-molybdate, and protease/phosphatase inhibitors). After centrifugation at $105{,}000 \times g$ for 45 min, 1.5 mg/ml of supernatant protein was incubated with increasing concentrations of ^{3}H-17β-estradiol (0.5–2 nM) (Amersham Life Science, Buckinghamshire, England) for 21 h at 4°C. Nonspecific binding was determined using a 250-fold excess of cold diethylstilbestrol (DES) (Sigma). Bound and free hormone were separated by addition of dextran-coated charcoal, incubation for 15 min, and centrifugation at $3500 \times g$ for 15 min. Bound ^{3}H-17β-estradiol in the supernatant was counted in a Beckman scintillation counter. Data are expressed as femtomoles ^{3}H-17β-estradiol bound per milligram protein and represent the means of triplicate determinations after subtraction of nonspecific binding from total binding.

Western blot analysis of VDR, ER, cytokeratin 18, vimentin, and E-cadherin

For analysis of cytokeratin 18, vimentin, and E-cadherin, cells were plated in 150 cm^2 flasks at 4×10^3 cells per ml and allowed to grow for approximately 96 h. Equal amounts of protein from SUM-159PT and MCF-7 cell lysates (approximately 50–75 μg) were separated on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with a monoclonal antibody to cytokeratin 18 (Sigma), vimentin (Novocastra Laboratories), or E-cadherin (Transduction Laboratories, Lexington, KY). After incubation with secondary antibody (horseradish peroxidase-conjugated antimouse Ig), specific bands were detected by enhanced chemiluminescence using products from Pierce (Rockford, IL). For VDR, proteins were precipitated from high salt nuclear extracts derived from SUM-159PT cells treated with ethanol or 100 nM $1,25(OH)_2D_3$ for 48, 72, or 96 h. Proteins were separated on SDS-PAGE, transferred to nitrocellulose, and blotted with monoclonal anti-VDR (Neomarkers, Fremont, CA), followed by horseradish peroxidase conjugated anti-rat IgG. For ER, proteins were precipitated from high salt nuclear extracts derived from untreated MCF-7, SUM-159PT, and MDA-MB-231 cells. Proteins were separated on SDS-PAGE, transferred to nitrocellulose, and blotted with monoclonal anti-ER (Novocastra Laboratories, NewCastle Upon Tyne, UK), followed by horseradish peroxidase-conjugated antimouse Ig (Amersham). The cytokeratin 18 blot was quantitated by scanning on a laser densitometer, and data are given as the fold difference of MCF-7 samples over SUM-159PT samples.

Nude mouse xenograft model

Six-week-old ovariectomized female NCr-*nu* mice (Taconic Farms, Germantown, NY) were used as hosts for SUM-159PT xenografts. SUM-159PT cells were trypsinized and resuspended in medium. After centrifugation, cells were resuspended in Matrigel/Ham's F-12 media (4:1). SUM-159PT cells were either injected subcutaneously (4×10^6 cells per injection) or orthotopically (1×10^6 cells per injection) in the mammary fat pad. At both sites, the tumor take rate was approximately 90%. Tumor volumes were monitored weekly by caliper measurements of the length, width, and height and were calculated using the formula for a semi-ellipsoid: $(4/3)\pi r^3/2$. After 11 weeks, mice were euthanized and tumor samples were removed and processed for general morphology and immunohistochemistry.

Histological analysis of cells and tumors

For vimentin localization, 5 μM formalin fixed paraffin-embedded sections were incubated with mouse monoclonal anti-vimentin (Novocastra Laboratories) at a 1:50 dilution for 2 h in 1% BSA-PBS. A biotin-conjugated antibody to mouse IgG (Vector Laboratories, Burlingame, CA) was applied at a 1:200 dilution for 30 min in 1% BSA-PBS. The ABC technique was used (avidin and biotinylated horseradish peroxidase complex, Vector), followed by diaminobenzidine (Sigma) to localize peroxidase in the sections, and the sections were counterstained with hematoxylin (Harris modified, Fisher). Tumors were viewed and photographed using a Nikon Optiphot-2 microscope and Nikon Microflex UFX-IIA photomicrographic attachment (Nikon Corp., Melville, NY).

Statistical evaluation

Data are expressed as the mean ± SE, with the number of replicates indicated in the figure legends. One way analysis of variance was used to assess statistical significance between means. Differences between

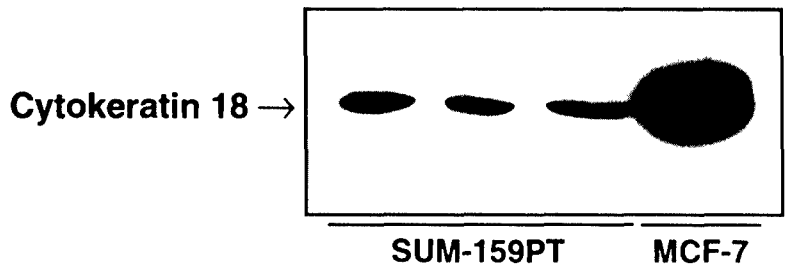

Figure 1. Expression of cytokeratin 18 in SUM-159PT cells. Cell lysates derived from untreated SUM-159PT and MCF-7 cells were separated on 12.5% SDS-PAGE, transferred to nitrocellulose, and blotted with a mouse monoclonal antibody against cytokeratin 18. Horseradish peroxidase (HRP)-conjugated secondary antibody was detected by chemiluminescence. Blot is representative of three independent experiments ($n \geq 3$).

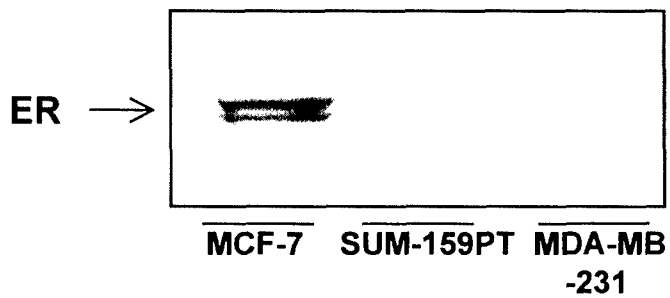

Figure 2. Estrogen receptor status in SUM-159PT cells. High salt nuclear extracts prepared from untreated MCF-7, SUM-159PT, and MDA-MB-231 cells were solubilized in Laemlli buffer, separated on 10% SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with a mouse monoclonal antibody directed against the estrogen receptor (66 kDa). HRP-conjugated secondary antibody was detected by chemiluminescence. Blot is representative of two independent experiments ($n \geq 2$).

samples were considered significant at $P < 0.05$ (*) and $P < 0.001$ (**) using the GraphPad Instat computer program (Intuitive Software for Science, San Diego, CA).

Results

Origin and ER status of SUM-159PT cells

Cytokeratin 18

Cytokeratins are specific markers of epithelial cell differentiation and cytokeratin 18 is the primary type I keratin expressed in simple epithelial cells. It has been previously described that many undifferentiated carcinoma cells exhibit undetectable or reduced levels of cytokeratins [9, 12, 16]. We thus compared the expression of cytokeratin 18 in the SUM-159PT cells to the well differentiated, epithelial MCF-7 cells. On western blots of cell lysates we observed that SUM-159PT cells express cytokeratin 18 at the expected molecular weight of 45 kDa; however, the level of expression is approximately nine fold lower than that of the well differentiated MCF-7 cells (Figure 1). This data confirms that the SUM-159PT cell line is epithelial in origin.

Estrogen receptor

SUM-159PT cells were isolated from an anaplastic carcinoma of the breast, which generally predicts a poor prognosis for the patient. To determine whether this cell line was estrogen dependent, we examined the ER status of SUM-159PT cells in comparison to the ER-positive MCF-7 cells and the ER-negative MDA-MB-231 cells. Using western blot analysis, we demonstrated that the SUM-159PT cells lack the ER protein, which corresponds with lack of ER expression in the ER-negative MDA-MB-231 cells. In contrast, the MCF-7 ER-positive cells express the ER protein at the expected molecular weight of 66 kDa (Figure 2). Furthermore, ligand binding studies performed in triplicate, using MCF-7 cells as positive control, demonstrated no estradiol binding in SUM-159PT cells over a range of ^{3}H-17β-estradiol concentrations of 0.5–2 nM (Table 1).

Vimentin and E-cadherin expression in SUM-159PT/MCF-7 cells

Vimentin

Vimentin filaments have been detected in primary human breast tumors by immunohistochemistry, and are

Table 1. Estradiol binding in SUM-159PT and MCF-7 cells over a range of ^{3}H-17β-estradiol concentrations (0.5–2 nM)

	0.5 nM	1 nM	2 nM
SUM-159PT	0	0	0
MCF-7	43.4 ± 2.1	43.9 ± 1	46.2 ± 0.7

High salt extracts were derived from SUM-159PT and MCF-7 cells, and the ligand binding assay was carried out as described in Materials and methods. Data are expressed as fmol ligand bound per milligram nuclear extract protein. These data are representative of two independent trials, each assayed in triplicate.

expressed more frequently in ER-negative tumors than ER-positive tumors [17]. Overexpression of vimentin has also been associated with enhanced motility and invasiveness *in vitro* [6, 8, 18]. On western blots of cell lysates derived from untreated cells, we observed abundant expression of vimentin at the expected size of 57 kDa in the SUM-159PT cell lysates, but no expression was observed in the ER-dependent MCF-7 cells (Figure 3, top). In addition, vimentin was detected by immunohistochemistry in SUM-159PT xenografts, localized to the membrane and cytosol (Figure 3, bottom). In contrast, no vimentin expression was detected by immunohistochemistry in the MCF-7 xenografts (data not shown).

E-cadherin

In a study of invasive ductal carcinomas, downregulation or functional inactivity of E-cadherin at the cell surface was associated with high histologic grade, increased invasiveness, and negative ER status [10]. Hence we assessed the expression of E-cadherin in SUM-159PT, MCF-7, and A431 (positive control received from Transduction Laboratories) cell lysates. As indicated in Figure 4, no expression of E-cadherin was observed in SUM-159PT cell lysates. In contrast, the MCF-7 cells express E-cadherin at the expected molecular weight of 120 kDa, consistent with a non-invasive phenotype.

Invasive capacity of SUM-159PT cells and tumors

In vitro chemoinvasion assay

To test invasiveness *in vitro*, both MCF-7 and SUM-159PT cells were analyzed in the Boyden chamber chemoinvasion assay, in which the cells must penetrate a uniform Matrigel barrier and transverse a porous polycarbonate filter. SUM-159PT cells were tested in two experiments, each containing MCF-7 cells as a negative control. As depicted in Table 2, 45% of the SUM-159PT cells were capable of transversing the Matrigel membrane after 24 h, in contrast to the low invasiveness of MCF-7 cells.

Growth of SUM-159PT and MCF-7 cells in Matrigel

Previous studies have correlated a stellate, invasive morphology of cancer cells embedded in Matrigel with their metastatic potential [19]. As depicted in Figure 5, SUM-159PT and MCF-7 cells exhibit distinct morphological responses when grown in Matrigel. The invasive, stellate morphology characteristic of invasive cells was observed in Matrigel cultures of the ER independent, vimentin-positive SUM-159PT cells. In contrast the ER-dependent, vimentin-negative MCF-7 cells formed smooth spherical colonies, characteristic of non-invasive cells.

Growth kinetics of SUM-159PT tumors

Several groups have now shown that human breast cancer cells implanted orthotopically in the mammary fat pad of nude mice exhibit enhanced tumorigenicity and metastatic potential compared to cells inoculated subcutaneously [20–22]. There is also extensive literature showing that the mammary fat pad exerts growth-modulating effects in normal, preneoplastic, and neoplastic mammary epithelium [4, 23]. In this study, growth kinetics of orthotopic SUM-159PT tumors were measured in ovariectomized nude mice in the absence of estradiol supplementation. As depicted in Figure 6, SUM-159PT cells grew as tumors in the mammary fat pad of athymic nude mice in the absence of estradiol, indicative of their estrogen independence. The estimated doubling time for SUM-159PT tumors in this experiment was 27 ± 7.5 days, which is comparable to what would be observed in a rapidly growing human breast tumor. SUM-159PT tumor growth kinetics were similar to those observed in the ER-negative MDA-MB-231 and MDA-MB-435 breast tumors [20]. In a separate study where SUM-159PT cells were inoculated subcutaneously, similar growth kinetics were observed (data not shown), although a higher initial cell count per injection was required (see Materials and methods). In contrast to subcutaneous cell injections, we only observed secondary tumors upon injection of SUM-159PT cells into the orthotopic site. Preliminary analysis of tissue sections from peripheral organs indicated micro-metastases of SUM-159PT cells in the liver, lung, and lymph nodes (data not shown). Collectively, these studies have provided

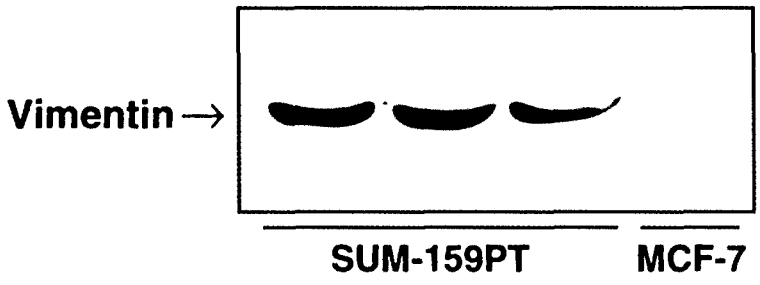

Figure 3. Expression of vimentin in SUM-159PT cells. Top: Cell lysates derived from SUM-159PT and MCF-7 cells were separated on 10% SDS-PAGE, transferred to nitrocellulose, and blotted with a mouse monoclonal antibody directed against human vimentin (57 kDa). HRP-conjugated secondary antibody was detected by chemiluminescence. Blot is representative of three independent experiments ($n \geq 3$). Bottom: Immunohistochemical expression of vimentin in SUM-159PT tumors. Sections were incubated with a mouse monoclonal antibody directed against human vimentin and developed with the ABC technique as described in Materials and methods. Vimentin expression appears as brown membrane and cytoplasmic staining.

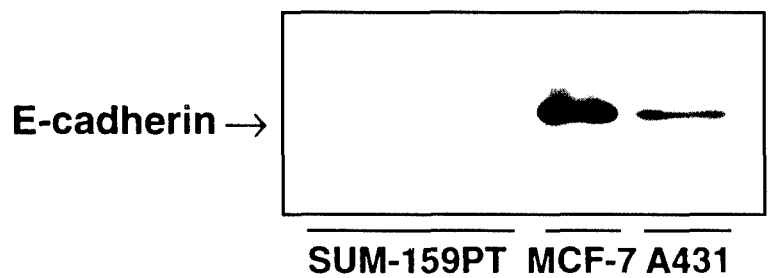

Figure 4. Expression of E-cadherin in SUM-159PT cells. Cell lysates derived from SUM-159PT, MCF-7, and A431 cells were separated on 7.5% SDS-PAGE, transferred to nitrocellulose, and blotted with a mouse monoclonal directed against human E-cadherin (120 kDa). HRP-conjugated secondary antibody was detected by chemiluminescence. Blot is representative of two independent experiments which yielded similar results ($n \geq 2$).

Table 2. Invasive activity of SUM-159PT and MCF-7 cells *in vitro*

	SUM-159PT	MCF-7
# Invasive cells	70.8 ± 2.3	3 ± 0.6
# Invasive cells (control insert)	158 ± 4.6	57.3 ± 3
Percentage of invasion	44.8	5.3

SUM-159PT and MCF-7 cells were analyzed in the Boyden chamber invasion assay. Assays were carried out in triplicate and control inserts (without Matrigel) were used for each cell line. Numbers of invasive cells were quantitated by viewing random fields of each membrane and the percentage of cells invading was calculated by dividing the number of invasive cells in the Matrigel membranes by the number of cells invading in the control inserts. The data represent mean ± SEM from five representative fields from each of three replicate filters. Both cell lines were tested in two independent experiments which yielded similar results.

evidence that SUM-159PT tumors are invasive and metastatic *in vivo*.

Effects of growth inhibitory compounds on SUM-159PT cells

VDR pathway

Most breast cancer cell lines and more than 80% of breast tumors contain high affinity intracellular receptors for 1,25-dihydroxyvitamin D_3, the active hormonal form of vitamin D_3 [24]. Unlike estrogen, which stimulates tumor growth, vitamin D_3 and its analogs have been shown to inhibit proliferation of breast cancer cells *in vitro* [25–26]. We therefore wanted to test if SUM-159PT cells contained the vitamin D receptor (VDR). Using western blot analysis on nuclear extracts derived from SUM-159PT cells, treated with ethanol or 100nM $1,25(OH)_2D_3$ for 48, 72, and 96 h (Figure 7), we have demonstrated that the VDR is expressed in SUM-159PT cells at the expected size of 53 kDa and that this receptor is upregulated upon treatment with $1,25(OH)_2D_3$. Furthermore, using ligand binding studies we have demonstrated that VDR in SUM-159PT cells is capable of binding its ligand, $1,25(OH)_2D_3$ (data not shown). These data suggest that the VDR in SUM-159PT cells is functional.

Effects of $1,25(OH)_2D_3$, TGFβ1, and TPA on growth of SUM-159PT cells

Since the SUM-159PT cell line represents an estrogen-independent invasive cell type, we examined its sensitivity to known growth inhibitory compounds, namely $1,25(OH)_2D_3$, TGF-β1, and TPA. Treatment of SUM-159PT cells with 100 nM $1,25(OH)_2D_3$ for 7 days resulted in a 70% reduction in viable cell number relative to that of ethanol treated-control cells (Figure 8). Treatment of SUM-159PT cells with 10 ng/ml TGF-β1 caused an approximate 25% decrease in cell number, whereas treatment with 100 nM $1,25(OH)_2D_3$ and TGF-β1 resulted in a 90% decrease in cell number as compared to ethanol control after 7 days of treatment (Figure 8).

Treatment of SUM-159PT cells for 72 h with 0.1–100 nM TPA significantly reduced viable cell number relative to ethanol treated controls (Figure 9A). Cotreatment of TPA with 100 nM $1,25(OH)_2D_3$ resulted in an approximately 85% reduction in viable cell number within 72 h of treatment at any of the TPA concentrations (0.1–100 nM) (Figure 9B). This data confirms that although SUM-159PT cells are an invasive estrogen independent cell type, they retain sensitivity to a number of growth inhibitory compounds.

Discussion

Metastatic spread of cancer continues to be the greatest barrier to cancer cure. Understanding the molecular mechanisms of metastases is crucial for the design and effective use of novel therapeutic strategies to combat cancer. Therefore, it is important to have good experimental models for the study of metastatic breast cancer. This report describes the characterization of a novel ER-independent model system for the study of metastatic breast cancer. Our studies have shown that no ER protein was detected in SUM-159PT cells, correlating with lack of estradiol binding. Furthermore, SUM-159PT cells are tumorigenic in ovariectomized nude mice in the absence of estradiol supplementation, consistent with their estrogen independence. Since altered regulation of a specific subset or network of estrogen-related genes – progesterone receptor (PgR), pS2, and cathepsin D – in the malignant progression of human breast cancer has been frequently observed, further assessment of these proteins in SUM-159PT cells would be of interest [27–28].

Our work has shown that the SUM-159PT cell line is epithelial in origin, as determined by the expression of cytokeratin 18. However, the levels of cytokeratin 18 expression were considerably lower than those observed in the estrogen-dependent MCF-7 cells. Our results demonstrate that approximately 45% of SUM-

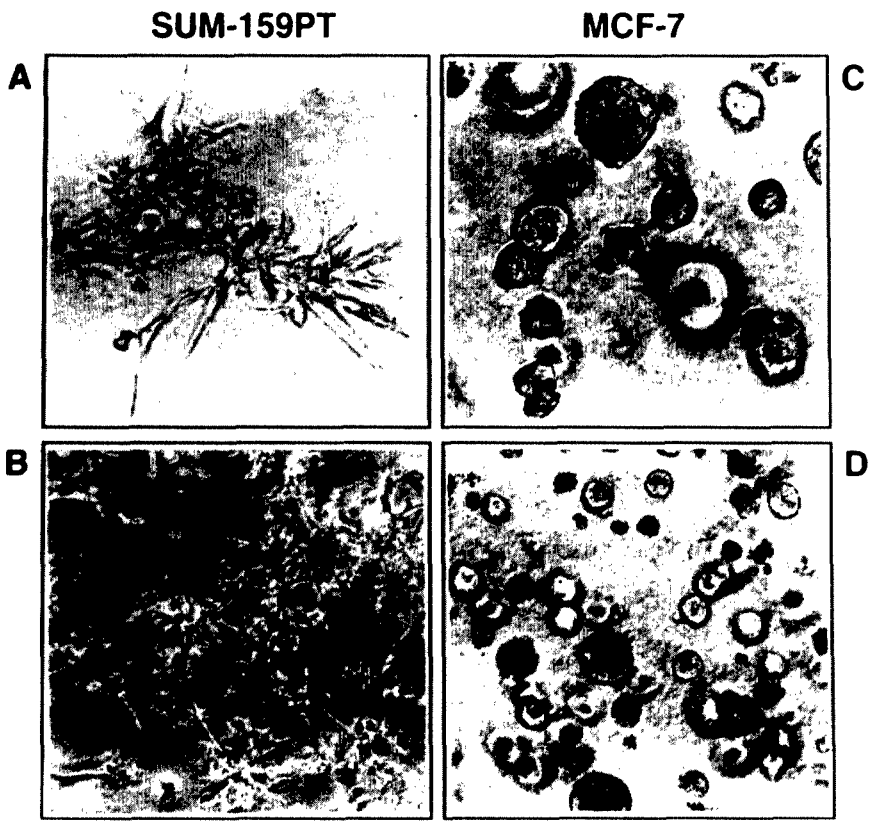

Figure 5. SUM-159PT cell growth in Matrigel matrix. SUM-159PT and MCF-7 cells were cultured, harvested, and plated as described in Materials and methods. Cells were photographed after approximately 10 days in Matrigel. Panels A and B: SUM-159PT cells at X40 and X20 magnification. Panels C and D: MCF-7 cells at X40 and X20 magnification.

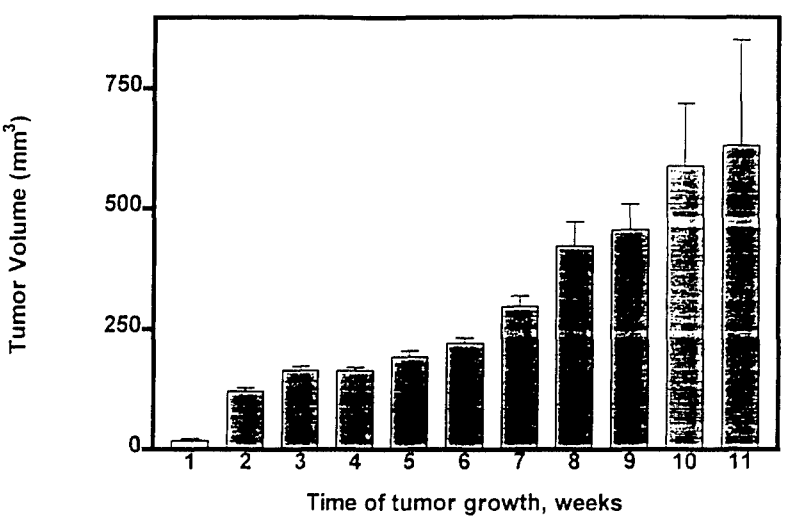

Figure 6. Growth of SUM-159PT cells in the mammary fat pad of athymic nude mice. Ovariectomized nude mice without estradiol supplementation were inoculated with 1×10^6 cells per injection in the mammary fat pad and grown for 11 weeks. Tumor volumes were monitored weekly as described in Materials and methods. Each bar represents the mean ± SEM of tumors from 25 mice.

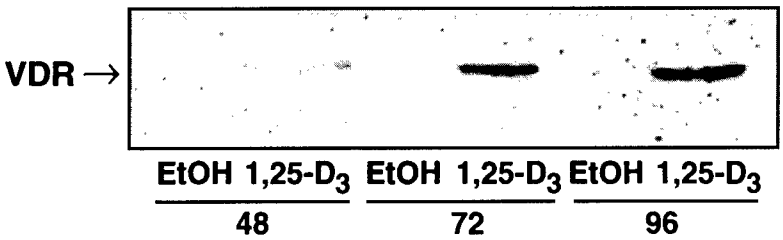

Figure 7. Effects of $1,25(OH)_2D_3$ on VDR protein expression in SUM-159PT cells. SUM-159PT cells were treated with ethanol or 100 nM $1,25(OH)_2D_3$ for the indicated times. Nuclear extracts were separated on 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an antibody against the VDR (53 kDa). HRP-conjugated secondary antibody was detected by chemiluminescence. Blot is representative of at least three independent experiments that yielded similar results ($n \geq 3$).

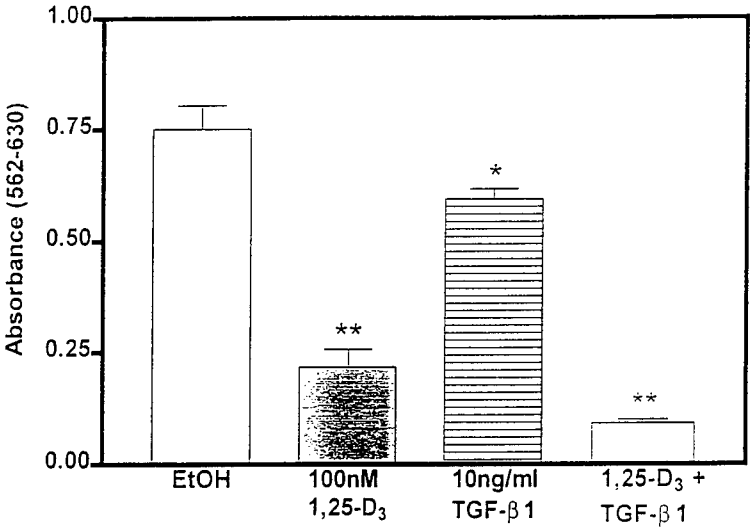

Figure 8. Effects of $1,25(OH)_2D_3$ and TGF-β1 on SUM-159PT cell growth. Cells were plated at a density of 2,000 cells per well in 24-well dishes. On the day after plating, cells were treated with ethanol vehicle, 100 nM $1,25(OH)_2D_3$, or 10 ng/ml TGF-β1 ± 100 nM $1,25(OH)_2D_3$ for up to 7 days. Total cell number was determined by crystal violet as described in Materials and methods. Data represent mean ± SEM of four values per time point. Similar results were obtained from at least two other independent experiments. *, $P < 0.05$; **, $P < 0.001$; ethanol control vs treated.

159PT cells are capable of invading through an 8 μm Matrigel invasion chamber after 24 h whilst exhibiting a stellate invasive morphology in the Matrigel outgrowth assay. In contrast, the non-invasive MCF-7 cells formed smooth spherical colonies in Matrigel cultures and were minimally invasive *in vitro*. These results correlated well with previously published data indicating that poorly differentiated cell lines expressed cytokeratins at much lower levels, and were highly invasive in the *in vitro* chemoinvasion assay [9, 12].

To investigate metastatic markers in SUM-159PT cells, we examined the expression of vimentin and E-cadherin, two proteins that have been strongly implicated in the transition of carcinoma cells to a metastatic phenotype. SUM-159PT cells were found to express vimentin, which has been associated with poor prognosis, low differentiation, lack of ER, and high invasive capacity [6, 8, 18]. In contrast, the well differentiated MCF-7 cells did not express vimentin. Clinical data suggests that the mean survival time is highest in patients bearing tumors negative for vimentin and positive for hormone receptor (ER and PgR) expression [17]. The data from the above suggests that human breast cancer progression is associated with loss of estrogen dependence and subsequently with vimentin acquisition. The cell adhesion molecule E-cadherin was expressed in MCF-7 cells, but no expression was observed in SUM-159PT cells. Several agents, including tamoxifen and retinoic acid, have been shown to upregulate E-cadherin resulting in an increase in cell aggregation and an inhibition of invasion *in vitro* [29–30]. In addition, the restoration of E-cadherin expression via gene transfer has

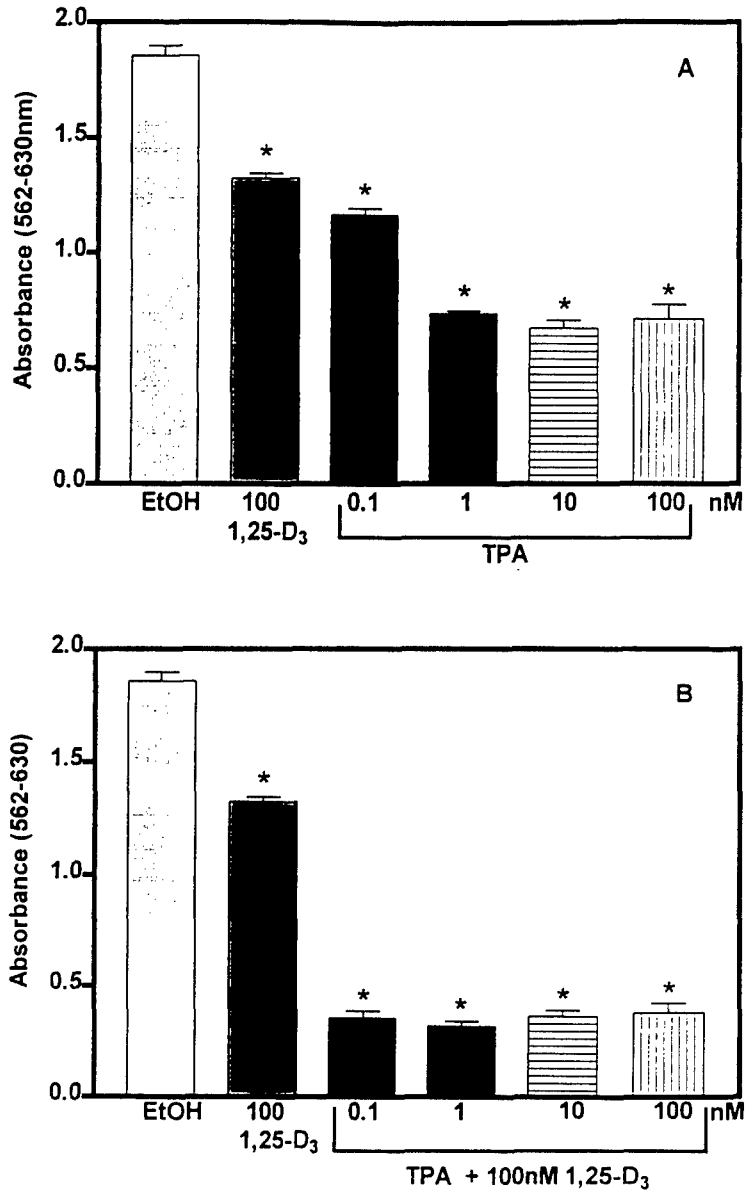

Figure 9. Effects of $1,25(OH)_2D_3$ and TPA on SUM-159PT cell growth. Cells were plated at a density of 4,000 cells per well in 24-well dishes. On the day after plating, cells were treated with ethanol vehicle, or 0.1–100 nM TPA ± $1,25(OH)_2D_3$ for 72 h. Total cell number was determined by crystal violet as described in Materials and methods. Data represent mean ± SEM of four values per time point. Similar results were obtained from at least two other independent experiments. *, $P < 0.001$; ethanol control vs treated.

been shown to inhibit the invasive and metastatic properties of cells *in vitro* and in animal model systems [31]. It would therefore be of interest to determine if upregulation of E-cadherin in SUM-1599PT cells would alter their invasive properties *in vitro* and *in vivo*. Thus, SUM-159PT cells can be categorized along with other poorly-differentiated fibroblastic-like breast cancer cell lines, previously reported to be E-cadherin-negative, vimentin-positive, invasive in the Boyden chamber chemoinvasion assay, and capable of forming branching structures in Matrigel [9–12].

Consistent with expression of metastatic markers and *in vitro* invasiveness, preliminary studies have shown that SUM-159PT cells were metastatic after inoculation into the mammary fat pad of ovariectomized nude mice. Metastatic spread was not observed when SUM-159PT cells were inoculated subcutaneously, although primary tumors developed. Therefore, im-

plantation of tumor cells into anatomically appropriate (orthotopic) sites, rather than subcutaneously, allowed for the expression of the metastatic phenotype of SUM-159PT cells. The present work correlates with the work of many other groups, indicating that the mammary fat pad is a more favorable site than the subcutis for the metastasis of mammary tumors [20–22].

Our studies further demonstrate that despite expression of the poorly differentiated, metastatic phenotype, SUM-159PT cells retain sensitivity to a number of growth inhibitory compounds. The phorbol ester TPA, a known potent growth regulator of breast cancer cells, significantly reduced SUM-159PT cell number within 72 h of treatment. Co-treatment of TPA with $1,25(OH)_2D_3$ caused a more than additive effect – 85% reduction in viable cell number after only 72 h treatment. TGF-β has been shown to strongly inhibit the growth of ER negative breast cancer cell lines at doses that were ineffective in ER-dependent cell lines [32]. In SUM-159PT cells, TGF-β1 caused a 25% decrease in cell number after 7 days treatment; however, co-treatment with $1,25(OH)_2D_3$ caused an additive sustained effect on cell viability after 7 days treatment.

We and others have shown that the vitamin D metabolite $1,25(OH)_2D_3$ induces growth arrest and apoptosis in MCF-7 cells *in vitro* [33]. Subsequently it has been reported that apoptosis in breast cancer cells treated with $1,25(OH)_2D_3$ is associated with chromatin condensation, nuclear matrix degradation, up-regulation of proteins linked to apoptosis in the mammary gland such as clusterin and cathepsin B, and down-regulation of the anti-apoptotic protein Bcl-2 [25, 26, 34].

The current studies demonstrate that $1,25(OH)_2D_3$ significantly reduced viable SUM-159PT cell number over 7 days of treatment. The presence of the VDR and its upregulation by $1,25(OH)_2D_3$ in SUM-159PT cells indicates that the VDR is capable of transcriptional activation in SUM-159PT cells. This indicates that the growth inhibitory effects of $1,25(OH)_2D_3$ are not limited to estrogen-dependent cells and that breast cancer sensitivity to $1,25(OH)_2D_3$ is not diminished in cells that have progressed to estrogen independence. The detection of the VDR in more than 80% of tumors suggests that $1,25(OH)_2D_3$ or vitamin D analogs may prove useful as a component of combination therapy in breast cancer [24, 35].

In summary, our studies show that the SUM-159PT cell line is an estrogen independent, vimentin-positive, E-cadherin-negative breast cancer cell line that is invasive *in vitro*. In the absence of estrogen, SUM-159PT cells are tumorigenic and metastatic after orthotopic inoculation into the mammary fat pad of athymic nude mice. Thus, SUM-159PT and MCF-7 cells, which were both developed from human mammary gland carcinomas (primary tumor and pleural effusion, respectively), represent cancer cells from two distinct stages of tumor progression. Use of SUM-159PT cells as an *in vitro / in vivo* model of estrogen-independent human breast cancer will be useful for molecular studies on the mechanisms of metastases, and for studying the effectiveness of vitamin D_3 and other potential therapeutics in advanced breast cancer.

Acknowledgements

This work was funded by The American Institute for Cancer Research (#95B068 and #98A100). We thank Carol Spierto, Pamuela Murphy, and Kenneth Jones for animal care. Special thanks to Marina LaDuke for the expert assistance with the preparation of the figures.

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Address for offprints and correspondence: Dr. JoEllen Welsh, Associate Professor, Biology, University of Notre Dame, Notre Dame, IN 46556; *Tel:* (219) 631-3371; *Fax:* (219) 631-7413; *E-mail:* j.welsh.19@nd.edu

0013-7227/00/$03.00/0
Endocrinology

Vol. 141, No. 8
Printed in U.S.A.

Identification of a Hormone-Responsive Promoter Immediately Upstream of Exon 1c in the Human Vitamin D Receptor Gene*

IAN M. BYRNE, LOUISE FLANAGAN, MARTIN P. R. TENNISWOOD, AND JoELLEN WELSH

Department of Biological Sciences (I.M.B., L.F., M.P.R.T., J.W.), University of Notre Dame, Notre Dame, Indiana 46556; and Departments of Zoology (I.M.B.), and Botany (L.F.), University College Dublin, Belfield, Ireland

ABSTRACT

To gain insight into the molecular regulation of the human vitamin D_3 receptor (hVDR), we have cloned and sequenced the 5′ flanking region of exon 1c and examined promoter activity of this region in breast cancer cells. Sequence analysis of the first 1300 bp upstream of exon 1c reveals several characteristics of a class II promoter, including GC-rich regions and the presence of a TATA box at −29 bp. Putative transcription factor binding sites identified in this potential hVDR promoter include AP-2, Sp-1, and glucocorticoid response elements. No consensus vitamin D_3 (VDRE) or estrogen (ERE) responsive elements were identified in the promoter sequence. Primer extension analysis performed with a primer specific for exon 1c confirms that transcription initiated in the 5′ flanking region of exon 1c occurs in MCF-7 cells. Transient transfection of MCF-7 cells with this putative promoter region cloned into the pRLnull luciferase reporter vector generates significant reporter gene activity that is enhanced by treatment with forskolin, retinoic acid, and 17β-estradiol. The enhancement of exon 1c promoter activity by 17β-estradiol is blocked by the selective estrogen response modifier (SERM) tamoxifen and is not observed in estrogen receptor-negative breast cancer cells. In summary, we have cloned and characterized a TATA containing promoter upstream of exon 1c of the hVDR and provide evidence that this region represents a hormonally regulated hVDR promoter. (*Endocrinology* **141:** 2829–2836, 2000)

1,25DIHYDROXYCHOLECALIFEROL [$1,25(OH)_2D_3$] is the hormonal form of vitamin D_3 that regulates calcium homeostasis, immune responses, and cell growth. The growth regulatory effects of $1,25(OH)_2D_3$, which include inhibition of proliferation, induction of differentiation, and activation of apoptosis, have been demonstrated in numerous cancer cell lines and tumors, including those derived from breast (1, 2), and are mediated through the vitamin D receptor (VDR), a ligand dependent transcription factor with similarity to other nuclear receptors. Because the genomic actions of $1,25(OH)_2D_3$ are dependent on the presence of the VDR, receptor abundance is an important determinant of cellular sensitivity to $1,25(OH)_2D_3$ (3). VDR abundance is affected by many physiological factors and is likely achieved through a variety of mechanisms, including transcriptional regulation, messenger RNA (mRNA) stability, posttranslational modifications, and ligand induced stabilization of the receptor protein (4). Specific regulators of the VDR include $1,25(OH)_2D_3$ itself and other steroids such as 17β-estradiol, dexamethasone (Dex), and retinoic acid, all of which have been shown to up-regulate the VDR protein and/or mRNA *in vitro* (4). Despite these observations, characterization of the relative contributions of transcriptional and posttranscriptional mechanisms in regulation of VDR levels and activity has been difficult due to the scarcity of information available on the VDR promoter region and its regulation.

Elucidation of the transcriptional regulation of the human VDR (hVDR) gene has primarily been hampered by the complexity of its promoter region. The hVDR gene has a similar intron/exon structure to other members of the steroid receptor superfamily with the exception of the untranslated exon 1, which is present in multiple copies and is associated with at least two and probably three differentially used promoters (5, 6). Attempts to demonstrate hormone regulation of these promoter regions, using reporter gene assays in numerous cell lines, have been largely unsuccessful, with one notable exception—the demonstration of a retinoic acid-responsive region in the intronic region located between exon 1c and exon 2 (5); however, no further characterization of this region has been reported.

We show that VDR transcripts containing exon 1c alone are present in MCF-7 human breast cancer cells and are regulated by a promoter immediately upstream of this exon that has the characteristics of a TATA containing promoter. Luciferase reporter constructs containing either 1300 bp or 800 bp of the region immediately upstream of the transcription start site show significant activity following transient transfection in MCF-7 cells. Agents known to up-regulate the VDR, including 17β-estradiol (E_2), forskolin, all *trans*-retinoic acid (ATRA) and Dex, enhance activity of both promoter constructs in breast cancer cells. 17β-Estradiol treatment enhances hVDR promoter activity in estrogen receptor positive MCF-7 cells, but not in estrogen receptor-negative SUM 159PT breast cancer cells. These studies provide evidence of a hormonally responsive promoter region upstream of exon

Received February 1, 2000.
Address all correspondence and requests for reprints to: Dr. JoEllen Welsh, Department of Biology, University of Notre Dame, Notre Dame, Indiana 46556. E-mail: jwelsh3@nd.edu.
* Portions of this work were presented at the Keystone Symposium on Programmed Cell Death, Breckenridge, Colorado, January 1999. This work was supported by NIH NCI Grant No. RO1-CA069700 (to J.W.).

1c in the hVDR gene and support the hypothesis that estrogen, and possibly other hormones, regulates breast cancer cell sensitivity to $1,25(OH)_2D_3$ via transcriptional regulation of the VDR promoter.

Materials and Methods

Cell culture

MCF-7 and SUM159PT human breast cancer cell lines were cultured in phenol red free Ham's F12 media (Life Technologies, Inc., Gaithersburg, MD) containing 10 mM HEPES, 5 μg/ml insulin, 1 mg/ml hydrocortisone and supplemented with 5% FBS (Life Technologies, Inc.) or 5% charcoal-stripped serum (CSS, HyClone Laboratories, Inc., Logan, UT). The estrogen-responsive MCF-7 cells were obtained from ATCC, and the estrogen independent SUM159PT cells were obtained from the University of Michigan Human Breast Cancer Cell/Tissue Bank (Ann Arbor, MI). Both cell lines endogenously express VDR (7, 8).

XL-PCR amplification of the 5′ flanking region of exon 1c

The region upstream of exon 1c of the hVDR gene was amplified by PCR using the Human Genome Walker Kit (CLONTECH Laboratories, Inc., Palo Alto, CA). A nested set of primers specific for exon 1c of the hVDR gene was designed (DNAstar Software) and synthesized. The primer located furthest 3′ of the start of exon 1c was designated E1C1 (5′-ACTTCCTCGTCCCCCGTCCATTCACC-3′; +55/+80). The nested primer was designated E1C2 (5′-TCGGGTCCCCACGAGAAGACACTCCAG - 3′; +28/+54). Each of the five libraries supplied by the manufacturer was subjected to two rounds of PCR as described in the manufacturer's protocol with the exception of the substitution of rtTH XL polymerase Retrotherm reverse transcriptase (Perkin-Elmer Corp., Foster City, CA). The first round of PCR included the adapter primer supplied with the kit and the E1C1 primer. The cycling conditions for the primary PCR amplification were as follows: 7 cycles of denaturation at 94 C for 25 sec followed by annealing and elongation at 72 C for 4 min and 32 cycles of 94 C for 25 sec followed by 67 C for 4 min. One microliter of the primary PCR reaction was diluted into 49 μl of ddH_2O and used in the secondary PCR reaction. The primers employed in the secondary PCR reaction were a nested adapter primer (supplied) and the primer E1C2. The cycling conditions for the secondary PCR reaction were as follows: 5 cycles of 94 C for 25 sec followed by 72 C for 4 min and 22 cycles of 94 C for 25 sec and 67 C for 4 min, followed by an additional cycle of 64 C for 4 min. Forty microliters of the secondary PCR reactions were analyzed on a 1.2% agarose gel. Amplification products of 1300 bp and 800 bp were cloned into TA cloning vectors and sequenced at least twice on both strands using automated DNA sequencing.

Construction of luciferase reporter plasmids and transient transfections

The 800- and 1300-bp products were subcloned into the promoterless pRL null vector (Promega Corp., Madison, WI) which contains the renilla luciferase reporter gene. Transient transfections with the pRL constructs were performed in MCF-7 and SUM159PT cells plated in six-well plates at a density of 1.5×10^5 cells per well. The cells were incubated at 37 C overnight, then cotransfected with 0.75 μg of the designated pRL construct and 0.25 μg of pGL-3 SV40 (total of 1 μg of DNA per well) in 1 ml of serum free Ham's F12 media. After incubation at 37 C for 1 h, 1 ml of Ham's F12 media containing either 5% FBS or 5% CSS supplemented with the appropriate treatment, or an equal volume of ethanol vehicle, was added to each well. After 18 h, cells were lysed and luciferase activity was determined with the Dual Luciferase Assay Kit (Promega Corp.). Transfection efficiency was normalized using the pGL-3 SV40 construct, and data are expressed as relative luciferase units (RLUs). Each experiment was performed in triplicate and replicated between three and six times. The luciferase assay data were statistically analyzed by ANOVA and either Dunnett's or Tukey's posthoc tests, as appropriate, with Graph Pad Instat Software (San Diego, CA). Means were considered significantly different if P values of 0.05 or less were obtained.

Isolation of total RNA and primer extension

RNA isolated from MCF-7 cells growing in 5% FBS was used for primer extension to identify transcripts originating from the promoter upstream of exon 1c of the hVDR. Primer extension was performed using ^{32}P-end labeled primer E1C2 (5′-TCGGGTCCCCACGAGAAGACATCCAG-3″; +28/54) and polyA mRNA isolated with Ultraspec RNA isolation reagent (Biotecx Laboratories Inc., Houston, TX). Primer E1C2 (1 fmol) was hybridized to 10 μg of polyA mRNA in a volume of 10 μl ddH_2O at 66 C for 25 min using reagents from Epicentre Technologies (Madison, WI). After addition of 1 μl of Retrotherm reverse transcriptase, reactions were incubated at 66 C for 40 min, heated to 95 C for 10 min and products were separated on a denaturing polyacrylamide gel containing 8% acrylamide, 7 M urea, and 1× TBE. ^{32}P-end labeled Ø *Hinf* 1 DNA markers (Promega Corp.) were electrophoresed on the gel to serve as molecular weight markers. The gel was electrophoresed at 250 V for 2.5 h, vacuum dried, and exposed to x-ray film for 3 days.

Results

At the time the studies reported here were initiated, there was no evidence that the hVDR gene contained multiple promoters and exon 1 variants. Consequently, we concentrated on the region immediately upstream of the extant exon 1, now referred to as exon 1c (see Fig. 1 for location of this region). The region immediately upstream of exon 1c of the hVDR gene was amplified using the Human Genome Walker kit, and products of 1300 bp and 800 bp were obtained. These products were cloned into TA cloning vectors and sequenced. The sequence of the 800- and 1300-bp products indicates that the 5′ flanking region of exon 1c displays an organization reminiscent of a typical TATA containing promoter (Fig. 1). A consensus TATA sequence (GATAAAA) is present 29 bp upstream from the transcription start site. A number of putative regulatory regions, identified using the Transfac online transcription factor database, are present in the 1300-bp region immediately upstream of exon 1c. These include several SP1 and AP-2 sites upstream of the TATA box in addition to consensus sequences corresponding to AP-1, p53, c-myb, and glucocorticoid receptor (GR) response elements. Notably, no sequences corresponding to consensus direct repeat vitamin D_3 response elements (VDRE) or estrogen response elements (ERE) are present in this region.

To establish that the promoter region upstream of exon 1c is used in a cellular context, primer extension analysis was used to identify transcripts initiated immediately upstream of the predicted start site of exon 1c. As can be seen in Fig. 2, a 79-bp extension product was identified in MCF-7 cells, consistent with a VDR transcript containing only exon 1c. Several larger primer extension products, ranging from approximately 140 bp up to 450 bp, are also present in MCF-7 cells. These transcripts appear to represent splice variants containing exon 1c and several of the alternative exons identified further upstream. Based on reported exon sizes (5, 6), we have tentatively identified these transcripts as splice products containing exons 1f, 1e, and 1c (447 bp) and 1f, 1a, and 1c (365 bp). Another primer extension product containing exons 1a and 1c, was detected upon longer exposure of the film (not shown). While confirmation of these assignments will require cloning and sequencing of these products, the primer extension analysis indicates that the putative promoter upstream of exon 1c of the hVDR gene is active in MCF-7 human breast cancer cells.

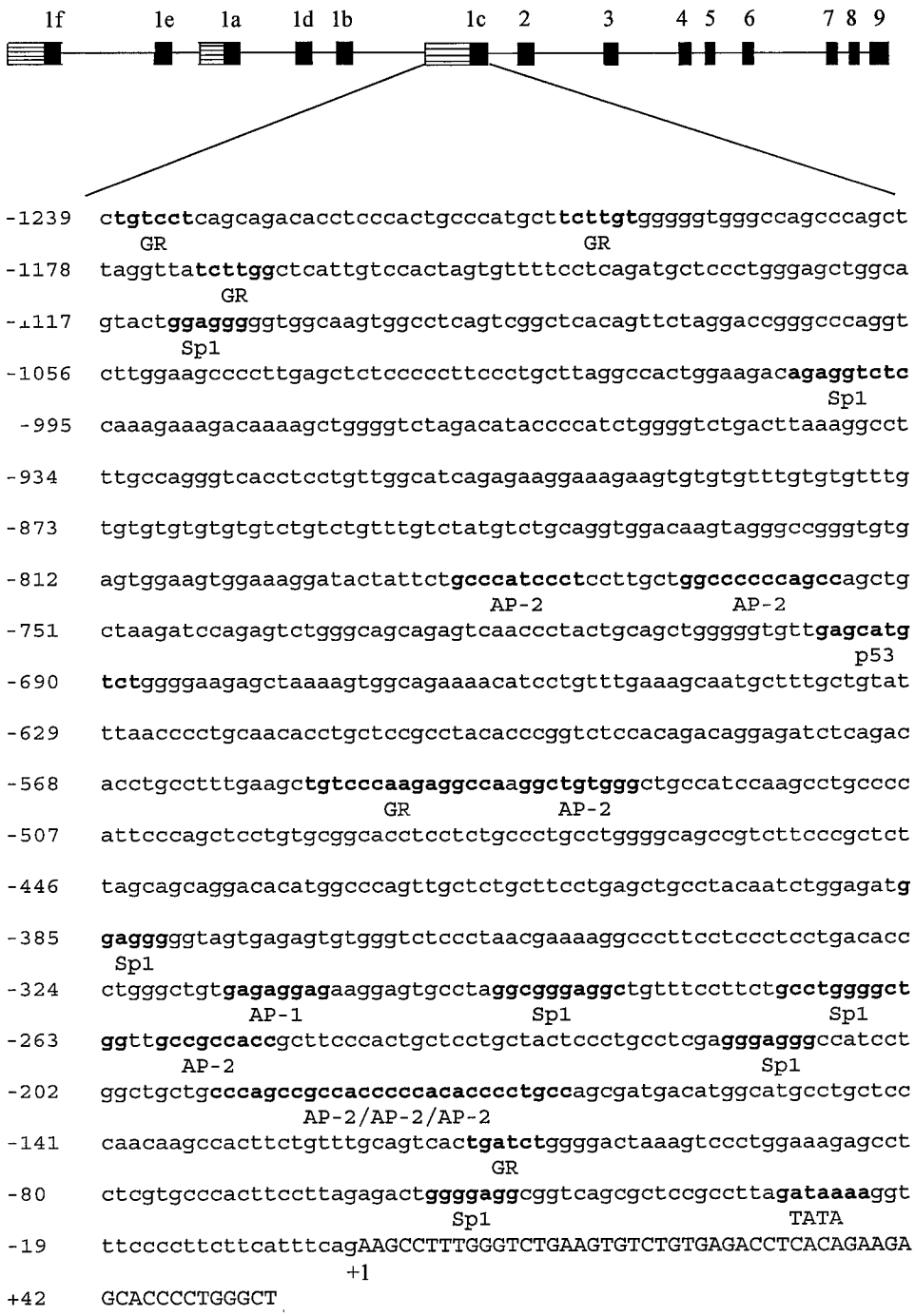

FIG. 1. Map of the human vitamin D receptor (hVDR) gene. *Top*, Intron/exon organization of hVDR, showing exons 2 through 9 (coding region) and untranslated exons 1a through 1f. Putative promoter regions 1a, 1f, and 1c are indicated in the *striped boxes*. *Bottom*, The first 1300 bp upstream of exon 1c was sequenced and analyzed with the Transfac online transcription factor database. Potential regulatory regions are denoted in *bold* with the name(s) of the consensus binding site(s) indicated *below*.

To determine whether the exon 1c promoter region is capable of regulating transcription, the 800- and 1300-bp sequences were cloned into the promoterless renilla luciferase plasmid, pRLnull, and transiently transfected into MCF-7 cells. As shown in Fig. 3a, there is significant promoter activity in MCF-7 cells transfected with either the pRL 800 or the pRL 1300 construct, compared with cells transfected with the pRL null control vector. After correction for transfection efficiency, promoter activity for these constructs is elevated up to 80-fold above baseline activity detected with the pRL null vector. Basal activity of this promoter is also significantly enhanced when MCF-7 cells are stimulated with FBS, indicating that the promoter may be regulated by hormones and growth factors present in the serum (data not shown).

To investigate the potential relevance of the promoter region upstream of exon 1c, we examined whether agents known to regulate VDR expression in breast cancer cells could modulate activity of the pRL800 and pRL1300 promoter constructs. It is well accepted that $1,25(OH)_2D_3$ upregulates the VDR protein; however, the mechanism of this effect is controversial, with evidence supporting both transcriptional regulation and ligand induced stabilization (4). Thus, we examined the possibility that $1,25(OH)_2D_3$ could

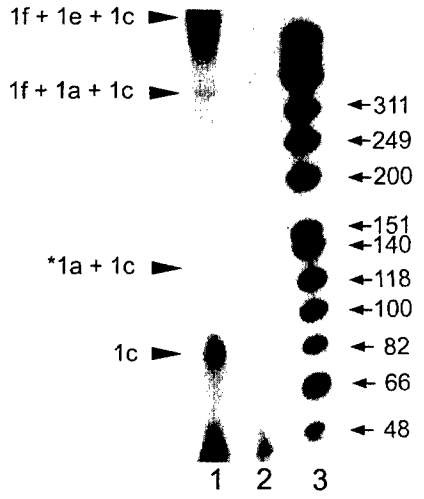

FIG. 2. Primer extension of the 5'flanking region of exon 1c of the hVDR. Poly A mRNA (10 μg) isolated from MCF-7 cells grown in FBS was incubated with ^{32}P-endlabeled primer E1C2 specific for exon 1c and then with Retrotherm reverse transcriptase as described in *Materials and Methods*. Reaction products were separated on 8% acrylamide gels and visualized by autoradiography. Lane 1, Complete reaction; lane 2, reaction minus mRNA. MW, ϕX174 *Hinf* molecular weight markers. *Arrows* indicate position of primer extension products with tentative assignment of exon usage for each transcription. *, Position of band detected upon longer exposure of the gel that likely corresponds to a transcript containing exons 1a and 1c.

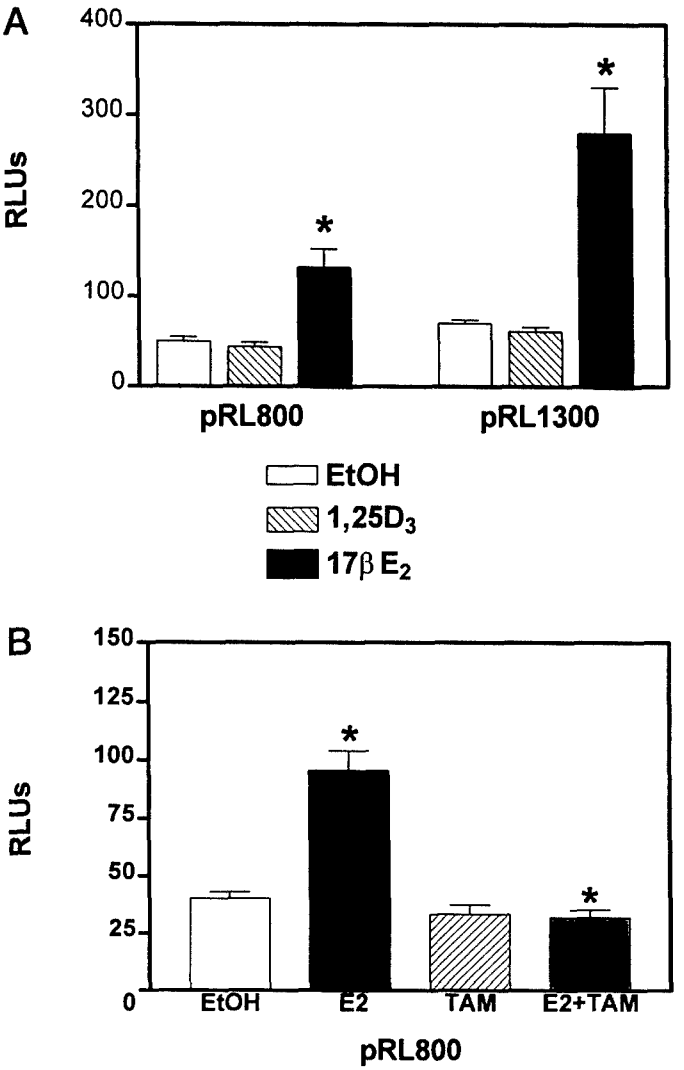

FIG. 3. Effects of 1,25 $(OH)_2D_3$ and 17β-estradiol on hVDR exon 1c promoter constructs in MCF-7 cells. A, MCF-7 cells were transfected with the pRL800 or pRL1300 renilla luciferase constructs or the pRLnull empty vector and treated with ethanol vehicle (*open bars*), 100 nM 1,25 $(OH)_2D_3$ (*hatched bars*) or 10 nM 17β-estradiol (*filled bars*) in phenol red-free media containing charcoal-stripped serum. RLUs have been corrected for transfection efficiency with cotransfected pGL3 SV40 using the dual luciferase assay and expressed relative to values obtained with pRLnull, which were normalized to 1. Data reflect the mean ± SEM of three wells and are representative of three or more independent trials. *, Significantly different from ethanol control ($P < 0.01$). B, MCF-7 cells transfected with the pRL800 construct were treated with ethanol vehicle (*open bars*), 1 nM 17β-estradiol (*filled bars*), 1 μM 4-hydroxytamoxifen (*hatched bars*), or 1 nM 17β-estradiol plus 1 μM 4-hydroxytamoxifen (*gray bars*) for 24 h in phenol red-free media containing charcoal-stripped serum. RLUs have been corrected for transfection efficiency with cotransfected pGL3 SV40 using the dual luciferase assay and expressed relative to values obtained with pRLnull, which were normalized to 1. *, Significantly different from ethanol control ($P < 0.001$); **, significantly different from 17β-estradiol treated ($P < 0.001$).

modulate reporter gene activity in MCF-7 cells, a cell line in which up-regulation of the VDR by 1,25$(OH)_2D_3$ has been demonstrated (7). As shown in Fig. 3a, no induction of either hVDR promoter construct was observed after treatment of MCF-7 cells with 100 nM 1,25$(OH)_2D_3$ for 18 h. Under the same conditions, 100 nM 1,25$(OH)_2D_3$ induced reporter gene activity of the rat 24-hydroxylase luciferase promoter (a know vitamin D responsive promoter) more than 15-fold in MCF-7 cells (data not shown). Even with extended treatment time (up to 72 h) under various culture conditions, no induction by 1,25$(OH)_2D_3$ of these promoter constructs is demonstrable in MCF-7 cells (data not shown).

In estrogen-responsive breast cancer cells, estrogens and antiestrogens have been shown to alter VDR expression and sensitivity to 1,25$(OH)_2D_3$ (4, 9). Although the sequence upstream of exon 1c does not contain any classical consensus ERE binding sites, numerous AP-1 and SP-1 binding sites, which can also mediate estrogen receptor transactivation (10–12), are present in the sequence. To establish whether up-regulation of the VDR by estrogen in breast cancer cells is correlated with transactivation of the region upstream of exon 1c, we examined reporter gene activity in MCF-7 cells treated with 17β-estradiol. As demonstrated in Fig. 3a, 10 nM 17β-estradiol significantly ($P < 0.01$) up-regulated the activity of both the pRL1300 and pRL800 constructs, by 4- and 2-fold, respectively, relative to ethanol vehicle-treated control cells. To determine whether the effects of 17β-estradiol on the hVDR constructs are mediated directly by the estrogen receptor, the activity of the pRL800 reporter construct was measured in MCF-7 cells treated with 4-hydroxytamoxifen, the biologically active form of the SERM tamoxifen. At the dose of 1 nM, 17β-estradiol significantly increase the activity of the pRL800 promoter activity (235% relative to ethanol-treated cells: $P < 0.001$) as shown in Fig. 3b. Treatment of MCF-7 cells with 1 μM 4-hydroxytamoxifen completely blocked the stimulation of the pRL800 reporter construct by 17β-estradiol ($P < 0.001$; 17β-estradiol *vs.* 17β-estradiol plus 4-hydroxytamoxifen). Similar results were obtained in MCF-7 cells transfected

with the pRL1300 construct and treated with 17β-estradiol with and without 4-hydroxytamoxifen (not shown). Collectively, these data suggest that the effect of 17β-estradiol on transcription initiated in the promoter region immediately upstream of exon 1c is directly mediated via the estrogen receptor.

To determine whether additional agents known to regulate VDR expression modulate reporter gene activity of the exon 1c constructs, similar experiments were conducted in MCF-7 cells treated for 18 h with ATRA, Dex, the phorbol ester TPA, and forskolin. As shown in Fig. 4, ATRA (1 nM) and forskolin (1 μM) up-regulate the promoter activity of both the pRL800 and pRL1300 constructs in MCF-7 cells, whereas neither TPA (1 nM) or the synthetic glucocorticoid Dex (10 nM) induce transcription above the basal levels seen in vehicle-treated controls cells. Forskolin induces the promoter activity by approximately 5-fold with both the pRL1300 and pRL800 constructs, suggesting that the sequences responsible for this regulation are localized in the first 800 bp upstream of the promoter. On the other hand, the induction by ATRA is greater when the longer construct is utilized, suggesting that the retinoid-mediated regulation of the promoter lies, at least in part, in the more distal region of the pRL1300 construct.

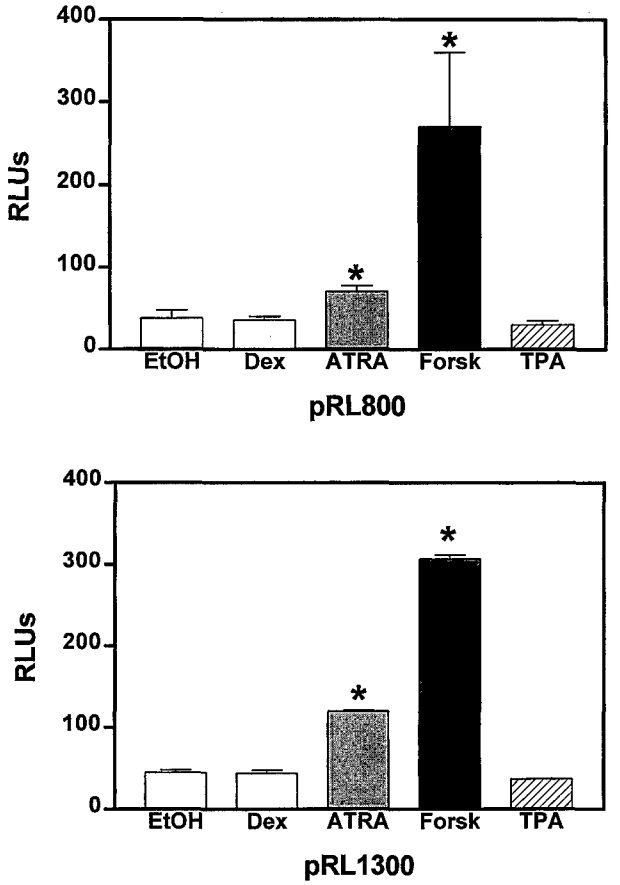

FIG. 4. Effects of retinoic acid, Dex, forskolin, and TPA on hVDR exon 1c promoter activity in MCF-7 cells. MCF-7 cells were transfected with the pRL800 or pRL1300 hVDR constructs or the empty vector pRLnull and treated with ethanol vehicle (EtOH), Dex (10 nM), ATRA (1 nM), forskolin (Forsk, 1 μM), or TPA (1 nM) in phenol red-free media containing charcoal-stripped serum. After 18 h, RLUs were measured by dual luciferase assay and corrected for transfection efficiency using cotransfected pGL3 SV40. RLU values of the pRL constructs are expressed relative to pRLnull, which was normalized to 1, and represent mean ± SEM of three wells. Similar results were obtained in six independent experiments. *, Significantly different from ethanol value, $P < 0.01$.

Because progression of breast cancer is associated with loss of estrogen responsiveness, we examined regulation of the hVDR promoter constructs in an estrogen receptor-negative human breast cancer cell line, SUM159PT (8). This human breast cancer cell line lacks the estrogen receptor and thus is insensitive to estrogens and antiestrogens. Expression of the VDR in SUM159PT cells is lower than that in MCF-7 cells, and consequently, SUM159PT cells are less sensitive to the growth inhibitory effects of $1,2(OH)_2D_3$ than MCF-7 cells (8). Transient transfection experiments under the same conditions as those used for MCF-7 cells demonstrated that the basal level of transcription for both the pRL800 and pRL1300 constructs is significantly lower in SUM159PT cells than in MCF-7 cells, with activities ranging between 5- and 10-fold over those of the pRLnull vector alone (Fig. 5). Notably, 10 nM 17β-estradiol treatment of SUM159PT cells does not induce transcription of the reporter gene from either construct, consistent with the lack of estrogen receptor expression in these cells (8). Similar to MCF-7 cells, treatment with ATRA and forskolin significantly ($P < 0.01$) enhanced reporter activity in SUM159PT cells. In contrast to MCF-7 cells, Dex significantly enhanced pRL800 activity in SUM159PT cells ($P < 0.05$), suggesting differential regulation of the hVDR exon 1c reporter constructs in estrogen-dependent *vs.* estrogen-independent breast cancer cells.

Discussion

In this paper, we describe a TATA-containing promoter immediately upstream of exon 1c of the hVDR and demonstrate that this region represents a hormonally regulated hVDR promoter active in breast cancer cells. Exon 1 of the hVDR gene is present in multiple copies (exons 1a through 1f), and the existence of at least three differentially utilized promoters (exons 1a, 1d, and 1f) has been proposed (5, 6). In the studies reported here, primer extension using RNA isolated from untreated MCF-7 cells identifies transcripts containing only exon 1c, as well as additional transcripts initiated from other promoters further upstream that contain exon 1c. These data are consistent with earlier studies that demonstrated numerous transcripts containing exon 1c by 5'RACE (5, 6). Miyamoto *et al.* (5) identified transcripts originating upstream of exon 1a that contained exon 1c using human kidney RNA as a template. Crofts *et al.* (6) identified transcripts initiated upstream of exons 1a, 1d, and 1f that contained exon 1c in a panel of 15 cell lines but did not utilize primers that would identify transcripts initiated immediately upstream of exon 1c. Notably, transcripts originating upstream of exon 1f were restricted to kidney, parathyroid, and intestinal cell lines, whereas transcripts originating upstream of exons 1a and 1d were equally expressed in all 15 cell lines examined, including the estrogen-responsive breast cancer cell line T47D (6). Positive identification of the primer extension products generated in MCF-7 cells will be neces-

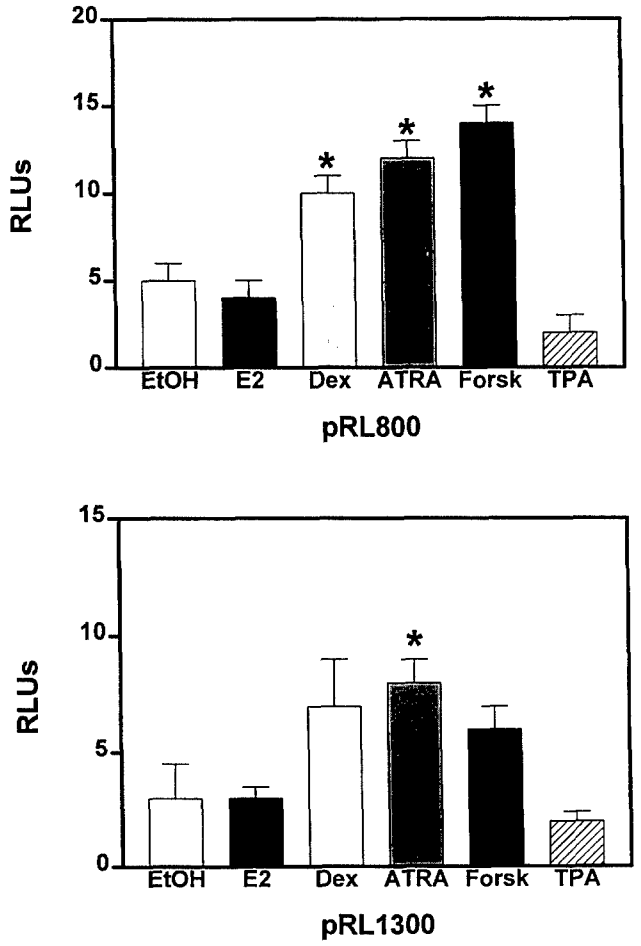

FIG. 5. Activity and regulation of hVDR exon 1c promoter in estrogen independent SUM159PT breast cancer cells. SUM159-PT cells were transfected with the pRL800 or pRL1300 hVDR constructs or the empty vector pRLnull and treated with ethanol vehicle (EtOH), Dex (10 nM), ATRA (1 nM), forskolin (Forsk, 1 μM), or TPA (1 nM) in phenol red-free media containing charcoal-stripped serum. After 18 h, RLUs were measured by dual luciferase assay and corrected for transfection efficiency using cotransfected pGL3 SV40. RLU values of the pRL constructs are expressed relative to pRLnull, which was normalized to 1, and represent mean ± SEM of three wells. Similar results were obtained in two independent experiments. *, Significantly different from ethanol value, $P < 0.05$ (*); $P < 0.01$ (**).

sary to determine the extent to which transcription is initiated from the various promoter regions, and if promoter usage is altered by hormonal treatments.

Consistent with the primer extension data demonstrating transcription initiation upstream of exon 1c, 5′ flanking sequences of exon 1c were active in reporter gene assays in MCF-7 cells. Similar studies have demonstrated that the promoters upstream of exons 1a and 1f can direct reporter gene activity in mammalian cell lines (COS 7, NIH 3T3, HeLa), whereas the 5′ flanking region of exon 1d is inactive in reporter gene assays (5, 6). At the present time, there is no evidence to suggest that the promoter regions upstream of exons 1a, 1d, or 1f are hormonally regulated. A major finding of this paper, therefore, is that the promoter immediately upstream of exon 1c is regulated by hormones and other agents in breast cancer cells. In these studies, we report that exon 1c promoter activity is up-regulated by 17β-estradiol, retinoic acid, and forskolin in MCF-7 estrogen-responsive breast cancer cells, but only the latter two agents enhance promoter activity in SUM159PT cells, which do not express the estrogen receptor (8). All of the agents that up-regulate hVDR promoter activity in the studies presented here have previously been shown to up-regulate VDR expression in numerous cell lines (4). Our data suggest that the mechanism by which these agents up-regulate VDR expression may involve, at least partially, direct regulation of VDR transcription.

In MCF-7 cells, 17β-estradiol up-regulates hVDR promoter activity through both the pRL800 and pRL1300 constructs, with a peak induction of 6-fold relative to ethanol-treated control cells. The magnitude of this effect is comparable to the induction of other estrogen-responsive reporter genes known to be regulated by the endogenous estrogen receptor in MCF-7 cells (10, 11). The data presented here indicate that the up-regulation of hVDR promoter activity by 17β-estradiol is mediated by the estrogen receptor because it is blunted by 4-hydroxytamoxifen and is not observed in the estrogen receptor negative SUM159PT breast cancer cells. Although these data indicate that a functional estrogen receptor is necessary for induction of hVDR promoter activity, no consensus ERE (GGTCAnnnTGACC) is present in either the pRL800 or pRL1300 constructs. This suggests that the 17β-estradiol-estrogen receptor complex mediates its effects via an alternative pathway, such as through interactions with AP-1 or Sp1 transcription factors. The role of AP-1 in induction of estrogen-responsive genes lacking ERE has been well documented (12), and more recently, evidence that 17β-estradiol mediates effects through interactions with Sp1 transcription factors has emerged (10). The presence of AP-1 and Sp1 sites in the promoter region upstream of exon 1c suggests that 17β-estradiol might enhance hVDR promoter activity through one or both of these alternative pathways. In particular, six Sp1 sites in the exon 1c promoter are identical to GC/GA-rich sequences recently shown to confer 17β-estradiol responsiveness to the bcl-2 promoter in MCF-7 cells (10). These sites (GGAGG at −1112, −386, −291, −215 and −55; GGGCTGG at −268) will be the initial focus of further studies to map the estrogen responsive region of the hVDR promoter. Because the GGAGG site located at −1112 is the only Sp1 site not present in the pRL800 construct, it is possible that the increased responsiveness to 17β-estradiol of the larger construct is mediated through this region.

Our data are consistent with previous reports that 17β-estradiol up-regulates VDR protein in estrogen-responsive human breast cancer cells (9) and VDR mRNA levels in human osteoblast-like cells (13, 14). Conversely, 4-hydroxytamoxifen down-regulates the VDR protein (7) and down-regulates the promoter activity in MCF-7 cells. In addition, VDR expression tends to be higher in estrogen receptor-positive than in estrogen receptor-negative breast cancer cells (15). Collectively, these data support the concept that estrogen is an important physiological regulator of VDR expression in breast cancer cells which mediates its effects via transcriptional regulation of the promoter region immediately upstream of exon 1c.

Regulation of the hVDR promoter by 17β-estradiol has numerous clinical implications arising from the potential use

of SERMs and vitamin D_3 analogs for prevention and/or treatment of breast cancer and osteoporosis (16–18). The efficacy and toxicity of vitamin D_3 analogs is determined, in part, by the level of VDR in target tissues, and our data suggest that estrogen status influences VDR abundance. In this respect, it will be important to determine whether novel SERMs such as raloxifene act as estrogen agonists or antagonists in regulation of hVDR promoter activity in different $1,25(OH)_2D_3$ target cells. Recent data indicate that transcriptional activation by SERMs is cell type specific, promoter dependent, and different for the two estrogen receptor subtypes, ERα and ERβ (11).

Despite data demonstrating ligand-dependent regulation of VDR expression in MCF-7 and other human derived cell lines (7, 19, 20), treatment with $1,25(OH)_2D_3$ has no measurable effect on activity of the promoter immediately upstream of exon 1c. Similarly, the 5′ flanking sequence of exon 1a was unresponsive to $1,25(OH)_2D_3$ when tested in reporter gene assays (5). While it remains possible that $1,25(OH)_2D_3$ may induce hVDR transcription via one or more of the newly identified, or as yet unidentified, promoter regions, up-regulation of the VDR protein by $1,25(OH)_2D_3$ may also result from enhanced mRNA stability (19), ligand induced stabilization (21, 22), or reduced proteosomal degradation (23) rather than transcriptional activation.

The up-regulation of the pRL800 and pRL1300 constructs, which contain promoter sequence upstream of exon 1c, by retinoic acid in MCF-7 cells is particularly interesting in light of previous reporter gene assays in ROS17/2.8 rat osteosarcoma cells, which also identified a retinoid-responsive region in the hVDR gene. In those studies, however, retinoic acid regulation was attributed to a region *downstream* of exon 1c because a construct including the region upstream of exon 1c was not retinoid responsive (5). These discrepancies in retinoic acid regulation of the region surrounding exon 1c may reflect species (rat *vs.* human) or cell type (osteosarcoma *vs.* breast cancer) differences, which can only be resolved by further investigations. Despite these discrepancies, it is likely that the well established ability of retinoic acid to up-regulate VDR is mediated, at least in part, at the transcriptional level.

Forskolin, an activator of adenylate cyclase, is a potent enhancer of the promoter immediately upstream of exon 1c in both estrogen receptor positive and negative breast cancer cell lines. There has been a recent report of a forskolin-responsive region upstream of exon 1a in the mouse VDR promoter (24), although it is not clear if a similar forskolin responsive region is present in the 5′ flanking region of exon 1a in the hVDR. These data are intriguing because forskolin has been shown to up-regulate VDR mRNA and responsiveness to $1,25(OH)_2D_3$ in human cells (25). While no consensus cAMP response element has been identified in the exon 1c promoter sequence, recent studies have implicated AP-2 elements in mediating cAMP responsiveness of other gene promoters (26, 27). Thus, the effect of forskolin may be mediated through AP-2 sites present in the 1300 bp upstream of exon 1c. Further studies are necessary to examine the role of AP-2, and to determine whether other hormones, such as PTH, which activate adenylate cyclase and enhance VDR expression (4), also modulate this promoter activity.

The presence of consensus glucocorticoid response elements in the promoter immediately upstream of exon 1c suggests that this promoter activity should be responsive to the synthetic glucocorticoid, Dex. However, Dex fails to induce promoter activity in MCF-7 cells, despite previous reports that Dex enhances the growth inhibitory effects of $1,25(OH)_2D_3$ in MCF-7 cells (28). In contrast to MCF-7 cells, Dex up-regulates the hVDR exon 1c reporter constructs in SUM159PT cells. These discrepancies are consistent with literature indicating that effects of glucocorticoids on VDR abundance may be species and tissue specific (4). The limited studies conducted in human-derived cell lines indicate up-regulation, down-regulation, or no change in VDR expression after treatment with Dex (19, 29, 30). Further studies to examine the molecular basis of glucocorticoid regulation of the VDR in estrogen-dependent *vs.* estrogen-independent breast cancer cells will be necessary to resolve these differences.

Although TPA has been shown to alter cellular responsiveness to $1,25(OH)_2D_3$ and up-regulate VDR protein expression in MCF-7 cells (31), TPA does not up-regulate exon 1c promoter activity. This suggests that TPA modulates VDR expression in MCF-7 cells via posttranscriptional rather than transcriptional mechanisms. Posttranslational effects of TPA are consistent with data indicating that the VDR protein is phosphorylated by PKC at several sites (32).

In summary, we have used the region immediately upstream of exon 1c of the hVDR to demonstrate hVDR promoter activity, which is regulated by 17β-estradiol, retinoic acid, and forskolin in MCF-7 cells. Because the promoters upstream of exon 1a and 1f do not appear to be hormone responsive, the data presented here suggest that the promoter region upstream of exon 1c is responsible for the hormone-regulated transcription of the hVDR gene, at least in MCF-7 breast cancer cells, and possibly other hormone-responsive tissues and tumors. Examination of this possibility will require comparison of promoter usage in MCF-7 and other cells after hormonal treatment. Furthermore, additional studies with the exon 1c promoter region are necessary to determine the relative importance of transcriptional, post-transcriptional, and translational mechanisms in overall VDR regulation, and the cell type specificity of such mechanisms. These data suggest that estrogen, and possibly other hormones, regulates breast cancer cell sensitivity to $1,25(OH)_2D_3$ via transcriptional regulation of the hVDR promoter.

Acknowledgments

The authors would like to thank Pamela Adams for technical assistance with sequencing, Leo Pharmaceuticals (Ballerup, Denmark) for supplying $1,25(OH)_2D_3$, Dr. J. Omdahl for the vitamin D_3-responsive 24-hydroxylase luciferase construct, and Dr. S. Ethier for the SUM159PT cell line.

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

REPLY TO
ATTENTION OF

MCMI-IMI-S (70-1y) 15 May 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers 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. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
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