Published in final edited form as:

J Steroid Biochem Mol Biol. 2014 October ; 144PA: 65–73. doi:10.1016/j.jsbmb.2013.10.022.

Modeling vitamin D actions in triple negative/basal-like breast cancer

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Abstract

Breast cancer is a heterogeneous disease with six molecularly defined subtypes, the most aggressive of which are triple negative breast cancers that lack expression of estrogen receptor (ER) and progesterone receptor (PR) and do not exhibit amplification of the growth factor receptor HER2. Triple negative breast cancers often exhibit basal-like gene signatures and are enriched for CD44+ cancer stem cells. In this report we have characterized the molecular actions of the VDR in a model of triple negative breast cancer. Estrogen independent, invasive mammary tumor cell lines established from wild-type (WT) and VDR knockout (VDRKO) mice were used to demonstrate that VDR is necessary for 1,25-dihydroxyvitamin D₃ (1,25D) mediated anti-cancer actions *in vitro* and to identify novel targets of this receptor. Western blotting confirmed differential VDR expression and demonstrated the lack of ER, PR and Her2 in these cell lines. Re-introduction of human VDR (hVDR) into VDRKO cells restored the anti-proliferative actions of 1,25D. Genomic profiling demonstrated that 1,25D failed to alter gene expression in KO240 cells whereas major changes were observed in WT145 cells and in KO clones stably expressing hVDR (KO^{hVDR} cells). With a 2-fold cutoff, 117 transcripts in WT145 cells and 197 transcripts in the KO^{hVDR} clones were significantly altered by 1,25D. Thirty-five genes were found to be commonly regulated by 1,25D in all VDR-positive cell lines. Of these, we identified a cohort of four genes (*Plau*, *Hbegf*, *Postn*, *Has2*) that are known to drive breast cancer invasion and metastasis whose expression was markedly down regulated by 1,25D. These data support a model whereby 1,25D coordinately suppresses multiple proteins that are required for survival of triple-negative/basal-like breast cancer cells. Since studies have demonstrated a high prevalence of vitamin D deficiency in women with basal-like breast cancer, correction of vitamin D deficiency in these women represents a reasonable, but as yet untested, strategy to delay recurrence and extend survival.

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Introduction

The heterogeneity of breast cancer makes the identification of intervention strategies for this disease particularly challenging. Although many trials have assessed nutrient status in relation to breast cancer, rarely are results stratified based on molecular sub-type. Of the >200,000 women diagnosed with breast cancer annually, between 15–25% are diagnosed with “triple negative” breast cancers which lack estrogen and progesterone receptors and do not exhibit amplification of HER2. These tumors often have basal-like gene expression signatures and are enriched for CD44+ breast cancer stem cells [1]. Women with triple negative cancers have poor prognosis and few treatment options, therefore new therapeutic targets for these aggressive tumors are critically needed. Data from the Cancer Genome Atlas [2] indicates that more than 95% of basal-like breast tumors express the vitamin D receptor (VDR). Furthermore, 1,25-dihydroxyvitamin D₃ (1,25D, the active form of vitamin D₃) and other VDR agonists induce cell cycle arrest, trigger apoptosis and inhibit invasion in basal-like breast cancer cell lines *in vitro* and reduce metastasis in nude mouse models [3–10]. Despite VDR expression, a high prevalence of vitamin D deficiency has been noted in women with triple negative/basal-like breast cancer [11,12]. Sub-optimal serum vitamin D levels would reduce availability of vitamin D to tumor VDR, limiting its activity. Correction of vitamin D deficiency or provision of supplemental vitamin D in women living with this sub-type of breast cancer would be predicted to delay recurrence and extend survival.

Although population studies have often demonstrated inverse correlations between vitamin D status and breast cancer risk/progression, the data are inconsistent and rarely account for the known molecular heterogeneity of the disease. Mechanistic insight into vitamin D actions in triple negative/basal-like breast cancer cells is sorely lacking. To further evaluate the effects of 1,25D in triple negative/basal-like breast cancers, we developed an *in vitro* model of murine breast cancer cell lines with differential VDR expression. In this report we have characterized the 1,25D/VDR dependent genomic profiles of these cells and demonstrate that 1,25D down-regulates a cohort of genes that are frequently up-regulated in aggressive breast cancers. Our data defines novel pathways that are targeted by vitamin D in human breast cancer and provide insight into mechanisms by which VDR signaling reduces invasion and metastasis.

Methods

Generation and culture of stable cell lines

WT145 and KO240 cells were originally established in our lab from tumors induced by DMBA treatment of wild-type and VDRKO mice [13]. For the studies described here, WT145 and KO240 cells were adapted to growth in DMEM/F12 medium supplemented with 5% charcoal-stripped FBS to eliminate exposure to serum vitamin D metabolites. In previous work [14] we confirmed that stable expression of hVDR into KO240 cells using a plasmid vector (pSG5-hVDR-hygro) recapitulated 1,25D mediated anti-cancer signaling, however, sensitivity to 1,25D was lost over time in these clones. To overcome this problem, we created a retroviral hVDR vector (pBABE-puro-hVDR, Fig 1) and generated a new series of hVDR expressing cells which we characterized in the present studies. Briefly, KO240 cells were infected with pBABE-puro-hVDR or pBABE-puro empty vector (EV)

retroviral particles and selected with 5 μ g/ml puromycin to generate mass cultures from which individual clones were isolated and characterized. Once selected, KO^{EV} and all KO^{hVDR} stable cell lines were maintained in DMEM/F12 medium supplemented with 5% charcoal-stripped FBS. The KO^{hVDR} clones maintained sensitivity to 1,25D for extended passages in the absence of puromycin.

Growth assays and microscopy

To determine the effect of 1,25D on culture growth, cells were treated on the day after plating with 100nM 1,25D or vehicle. After 96 hours, adherent cells were fixed in glutaraldehyde, stained with crystal violet and dried overnight. Stain was resuspended in 0.2% Triton X-100 and absorbance was read at 590 nm as an indication of adherent cell density. To visualize cell morphology, cells were treated with vehicle or 10nM 1,25D for 96h and photographed live on a Nikon Eclipse TS100 phase contrast microscope. For VDR immunofluorescence, KO^{hVDR} cells growing in chamber slides were fixed and permeabilized with ice-cold methanol, blocked overnight in PBS/1% BSA and incubated with antibody against VDR (clone D6, Santa Cruz Biotechnology, Santa Cruz, CA) followed by anti-mouse Alexa Fluor 488 secondary antibody (Life Technologies, Grand Island, NY). Coverslips were applied with ProLong Gold Antifade reagent with DAPI (Life Technologies, Grand Island, NY) and imaged on a Leica DMI6000 microscope with TCS SP5 confocal laser scanner using Leica Application Suite AF version 2.6.0.7266 software.

Western blotting

Whole cell lysates were prepared by scraping confluent monolayers in Laemmli buffer containing protease and phosphatase inhibitors. Lysates were sonicated and protein concentration was determined with the BCA protein assay (Pierce Biotechnology, Rockford, IL). Cell lysates (50 μ g per lane) were separated via SDS-PAGE, transferred to nitrocellulose or PVDF membranes, blocked in skim milk, and incubated overnight with primary antibodies including VDR clone D6 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal antibody against ER (6F11, Vector Laboratories, Burlingame, CA), mouse monoclonal antibody against PR (PR-1, Thermo Scientific, Fremont, CA), or rabbit monoclonal antibody against HER2 (Cell Signaling, Danvers, MA) followed by appropriate HRP-conjugated secondary antibodies. Detection was with ECL Plus (Pierce Biotechnology, Rockford, IL) on a Storm 860 Molecular Imaging system. Blots were then stripped using acetonitrile, 0.1M glycine and 1M Tris base (pH 7.5) and re-probed with mouse monoclonal antibodies against GAPDH or actin.

Microarray screening, pathway analysis and quantitative PCR

Three KO^{hVDR} clones that were confirmed to express functional VDR (KO^{hVDR1}, KO^{hVDR2}, and KO^{hVDR4}), along with WT145 and KO240 cells were treated with ethanol (EtOH) or 100nM 1,25D for 24h. RNA was isolated and hybridized to Affymetrix mouse gene 1.0 ST arrays comprising >28,000 annotated genes and datasets were analyzed with Genespring v10 software (Agilent, Santa Clara, CA). Briefly the data was filtered to remove probe sets with low signal across all samples. The resulting list was filtered to exclude genes with >25% CV across all replicates within a condition. The filtered entities were subjected to a moderated t-test (p<0.05) with a Benjamini-Hochberg false discovery rate to correct for

multiple comparisons included. These were further filtered to identify entities that were differentially expressed in either WT145 or all three hVDR-expressing KO clones in the presence of 1,25D at a 2-fold or higher level. A venn diagram was used to identify a subset of 35 genes that were commonly altered by 1,25D in WT145 cells and in all three KO^{hVDR} clones. Pathway analysis on this 35-gene set was performed using Babelomics 4 gene expression and functional profiling analysis suite (<http://babelomics.bioinfo.cipf.es>).

For real-time PCR, mRNA was harvested with RNEasy Mini Kits (Qiagen, Valencia, CA) according to manufacturer's instructions, and two-three independent cDNA stocks were prepared from each sample using random hexamer primers (Applied Biosystems, Foster City, CA). cDNA preparations were independently analyzed in duplicate with mouse specific primers for *Cyp24a1*, *Cib2*, *Enpp1*, *Prepl*, *Has2*, *Hbegf*, *Plau* and *Postn* (Origene, Rockville, MD) using SYBR Green PCR Master Mix (ABgene, Thermo Scientific) on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Data were calculated by the Ct method, normalized against 18S and expressed relative to values obtained for vehicle treated cells.

Statistics

Data are expressed as mean±standard error, and statistical evaluation was by Students t test using GraphPad Prism software (La Jolla, CA). Comparisons were routinely made on the effect of treatment within (rather than between) each cell line. In all cases $p < 0.05$ was considered to be significant and is indicated by an asterisk above data bars.

Results

Development of mammary tumor cells with differential VDR expression

To study the VDR dependent effects of 1,25D on breast cancer, we previously established a series of cell lines from DMBA-induced mammary tumors that were generated in wild-type (WT) and VDR knockout (KO) mice [13]. Cells derived from WT mice (termed WT145 cells) abundantly expressed VDR protein which was transcriptionally activated by 1,25D as measured by a vitamin D responsive luciferase reporter assay [13]. Furthermore, we showed that growth of WT145 cells was dose and time dependently inhibited by 1,25D. In contrast to WT145 cells, KO240 cells derived from tumors induced in VDRKO mice did not express detectable VDR and failed to respond to 1,25D. We also demonstrated that treatment of mice with a VDR agonist (EB1089) retarded growth of xenografts composed of WT145 cells, but not those composed of KO240 cells [15].

Recently we provided proof of principle that expression of VDR in KO240 cells using a plasmid vector recapitulated 1,25D mediated anti-cancer signaling [14] however, these VDR expressing cells lost sensitivity over time. For these follow-up studies, we created a retroviral hVDR expression vector (pBABE-hVDR-Puro, Fig 1A) and selected multiple KO240 clonal cell lines with stable expression of hVDR protein. Immunofluorescent staining (Fig 1B) confirmed that KO^{hVDR} cells expressed VDR protein in the nucleus as evident by co-localization with the nuclear stain DAPI. Four clones were originally characterized, three of which (clones 1, 2 and 4) expressed detectable VDR by western

blotting (Fig 2A) and underwent growth arrest and apoptosis when treated with 1,25D (data from clone 4 is shown in Fig 2B and 2C). The magnitude of growth inhibition in response to 1,25D treatment in hVDR-expressing clones #1, #2 and #4 was similar to or greater than that observed in WT145 cells (Fig 2B and data not shown). Similar to KO240 cells, growth of in KO^{EV} cells and KO^{hVDR} clone #3 (which failed to express detectable VDR protein by western blotting) was unaffected by 1,25D (not shown).

WT145 and KO240 cell lines are triple negative

Western blotting was used to assess expression of estrogen receptor α (ER), progesterone receptor (PR) and Her2 in WT145 and KO240 cells adapted to charcoal stripped FBS. As shown in Fig 3A, neither WT145 or KO240 cells grown in charcoal stripped FBS express ER, and there was no significant effect of the ER antagonist tamoxifen on growth of either cell line (Fig 3B). Since our previous data indicated that WT145 and KO240 cells grown in complete FBS express ER [13], adaptation to charcoal stripped FBS apparently selected for cells lacking this receptor. These results are consistent with our previous data indicating that xenografts derived from WT145 and KO240 cells grew robustly in ovariectomized mice indicating that the parental mass cultures contain estrogen independent cells [13]. No expression of either PR or HER2 was detected in WT145, KO240 or KO^{hVDR4} cell lysates (Fig 3C, 3D). These data indicate that the WT145 cells as well as the KO240 mass cultures and stable hVDR expressing clones can be categorized as triple negative breast cancer cell lines. Further studies are needed to determine whether these murine triple negative cells inherently express gene signatures similar to that of human basal-like breast cancers.

Genomic Profiling

Microarray profiling was conducted in this model system to identify common 1,25D responsive gene targets in cells expressing mouse and human VDRs since both could trigger growth arrest in response to 1,25D. Cells were treated for 24h with 100nM 1,25D in an effort to identify gene changes that preceded growth inhibition (which is not evident for at least 48h after treatment). As shown in Fig 4A, and consistent with the lack of functional VDR, global gene expression was not significantly altered by 1,25D in KO240 cells. In contrast, major changes in gene expression were observed in WT145 cells and in all three KO^{hVDR} clones (#1, #2, and #4). Interestingly, as evident from the heat map in Fig 4A, the genomic profiles of the three hVDR-expressing clones were similar to each other but quite distinct from the WT145 cells. After statistical analysis and 2-fold cutoff, 117 transcripts in WT145 cells and 197 transcripts in the KO^{hVDR} clones were found to be significantly ($p < 0.05$) altered by 1,25D. In both cell lines approximately 70% of altered transcripts were up-regulated (85/117 in WT145 cells and 146/197 in KO^{hVDR} cells).

Through comparison of these distinct gene profiles, we identified a cohort of 35 genes that were similarly altered in WT145 cells and all three KO^{hVDR} clones (Fig 4B). Gene ontology analysis (Fig 4C) indicated that these common transcripts are enriched in pathways related to polysaccharide and proteoglycan binding. The list of 1,25D responsive genes and the expression data as calculated from the microarray analysis for each cell line is shown in Table 1. Of these 35 commonly altered genes, the vast majority (31) were up-regulated, with

only 4 genes down-regulated in WT145 cells and all three KO^{hVDR} clones treated with 1,25D.

Confirmation of selected VDR modulated genes by PCR

We chose to follow-up a subset of 8 genes identified in the microarray datasets by PCR analysis of independent samples. In addition to *Cyp24a1* (a canonical VDR target gene [16]), three previously uncharacterized 1,25D up-regulated genes were selected: *Cib2* (calcium and integrin binding protein2), *Prelp* (proline/arginine-rich end leucine-rich repeat protein) and *Enpp1* (ectonucleotide pyrophosphate phosphodiesterase-1). All four genes were validated to be 1,25D responsive in a VDR dependent manner (Fig 5). *Cyp24a1* was dramatically induced in all VDR positive cell lines, with the highest response in WT145 cells (this may indicate that the murine VDR is more effective at inducing the murine *Cyp24a1* target gene than is hVDR). *Cib2* was the second most highly induced gene, with significant increases of 50–100 fold above basal in WT145 cells and all KO^{hVDR} clones in response to 1,25D. Under the same conditions the *Prelp* gene was up-regulated 10–20 fold and the *Enpp1* gene was increased 5–15 fold in these cell lines. With the exception of *Cyp24a1*, there were no consistent differences in the magnitude of target gene induction by 1,25D in cells expressing mVDR or hVDR.

All four genes that were found to be down-regulated upon 1,25D treatment of WT145 and KO^{hVDR} cells in the microarray dataset were examined by PCR of independent samples (Fig 6). *Plau* (plasminogen activator, urokinase), *Has2* (hyaluronan synthase 2) and *Postn* (periostin) were down-regulated by 1,25D from 50–75% in all VDR positive cell lines but unaffected in parental KO240 cells. *Hbegf* (heparin-binding EGF-like growth factor) was strongly down-regulated by 1,25D in KO^{hVDR} cells but was not consistently altered in WT145 cells.

Discussion

In this report we have characterized a murine cellular model of triple negative breast cancer, an aggressive subtype of the disease which often exhibits the basal-like gene signature [2]. Similar to human triple negative breast tumors, the cell lines described here (which were derived from DMBA induced mammary tumors generated in C57Bl/6 mice) lack ER, PR and HER2 expression. This is not surprising since comparative genomic analysis of mouse tumor models and human breast cancers has demonstrated that most DMBA-induced mouse tumors show basal-like cell lineage features [17]. Since human triple negative breast cancers have been further sub-divided into six genomic clusters [18], further data mining will be needed to determine which subtype of triple negative human breast cancer these cells most resemble.

To characterize the actions of 1,25D, we isolated triple-negative cell lines from mammary tumors generated in wild-type and VDRKO mice and demonstrated that 1,25D mediates growth inhibition only in VDR expressing cells. We also engineered cells from VDRKO mice that stably expressed human VDR and showed that 1,25D exerted similar anti-proliferative and genomic effects in cells expressing murine or human receptors. Our data are thus consistent with reports that 1,25D exerts anti-proliferative effects in cell lines

derived from human triple negative breast tumors such as Hs578T, SUM159PT and DCIS.com cells [4–6,19]. The expression of VDR in human triple negative breast cancers suggests that optimizing the activity of this receptor (either through maintenance of adequate vitamin D status or via administration of synthetic agonists) may be useful therapeutically. Our data also raise concerns about the high prevalence of vitamin D deficiency reported in patients with triple negative/basal-like breast cancers [11,12].

We have utilized our model system to comprehensively profile the genomic actions of 1,25D with the goal of identifying specific pathways targeted by VDR signaling that contribute to growth inhibition. Microarray screening was used to compare genomic changes induced by 24h treatment with 100nM 1,25D in cells expressing either murine VDR (WT145 cells) or human VDR (KO^{hVDR} cells). Despite similar origins of the WT145 and KO^{hVDR} cells and identical side-by-side experimental protocols for the microarray, genetic background (and possibly differences between human and mouse VDR) markedly affect the cellular responses to 1,25D. Therefore we chose to focus on the 35 transcripts (Table 1) that were commonly altered by 1,25D in all VDR expressing cell lines reasoning that these would be most relevant to the common endpoint of growth inhibition. Since arrays were conducted after 24h of treatment, the cohort of genes we identified likely include both primary and secondary targets of the 1,25D-VDR complex.

Of the 35 altered genes we identified in all VDR positive cell lines, 13 (*Apcdd1*, *C3*, *Cib2*, *Ckb*, *Crabp2*, *Cyp24a1*, *Enpp1*, *Has2*, *Prelp*, *Rassf2*, *Serpinb9b*, *Sulf2* and *Tgm2*) overlap with an array dataset of 1,25D regulated genes associated with induction of cell-cycle arrest, senescence, and differentiation in progenitor cells isolated from the murine prostate gland [20]. Four of the 35 common genes (*Ckb*, *Rassf2*, *Sulf2* and *Hbegf*) contain VDR consensus binding sites as identified by *in silico* screening for DR3 or ER6 sequences within –10 to +5 kb of transcription start sites in the mouse genome [21] suggesting that these might represent direct VDR targets. However, since it is clear that VDR binding is not limited to proximal promoter regions or known consensus binding sites [22–25], and that indirect mechanisms involving other transcription factors or regulation of micro RNAs contribute to 1,25D actions [26–29], future mechanistic studies will likely uncover novel mechanisms for 1,25D regulation of these genes.

In this study, the majority (31 out of 35) of genes that were altered upon 1,25D treatment were up-regulated. Using real time PCR, we confirmed that *Cyp24a1*, *Cib2*, *Enpp1* and *Prelp* were up-regulated, whereas *Has2*, *Plau* and *Postn* were down-regulated, in mammary tumor cells expressing either murine or human VDR. *Hbegf* was strongly repressed in all three KO^{hVDR} clones but, despite concordance in the array dataset, PCR follow-up showed inconsistent down-regulation of *Hbegf* in WT145 cells. Further studies will be needed to determine if there are differences between human and mouse VDR signaling that account for these discrepancies, or whether *Hbegf* expression is deregulated in WT145 cells. PCR analysis did confirm that 1,25D had no significant effects on the expression of these genes in VDR negative KO240 cells (Figs 5, 6) or KO^{EV} cells (data not shown).

Although further studies will be necessary to determine the significance of these genes to the anti-tumor actions of 1,25D, several of them have known roles in mammary gland biology

or cancer progression. The *Cib2* gene codes for a 22kDa, EF hand containing calcium binding protein with sequence similarity to calcineurin B and calmodulin. We chose to follow-up *Cib2* since it was the second most highly up-regulated gene in both WT and KO^{hVDR} cells (after *Cyp24a1*) and it is highly expressed in normal human breast tissue (<http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.129867>). In addition, the function of *Cib2* in regulation of calcium signaling may be particularly relevant since 1,25D mediated growth inhibition of MCF-7 breast cancer cells has been linked to changes in intracellular calcium [30]. The *Enpp1* gene may also contribute to the anti-proliferative actions of 1,25D since it codes for a 105kDa type II transmembrane glycoprotein that inhibits the tyrosine kinase activity of the insulin receptor. Insulin signaling, which promotes breast tumorigenesis and likely contributes to the link between obesity and breast cancer risk, is attenuated by 1,25D in breast cancer cells [19,31]. In addition, high Enpp1 protein expression was associated with increased progression-free survival [32], suggesting its importance in human breast cancer. In contrast to these findings, however, a recent report demonstrated that forced expression of *Enpp1* enhanced the ability of triple negative MDA-MB-231 cells to form bone metastasis, suggesting a complex relationship between *Enpp1* and breast cancer progression [33]. The fourth protein confirmed to be up-regulated by 1,25D, *Prelp*, codes for a heparin binding extracellular matrix protein that has been shown to reduce growth and spread of breast cancer to bone in xenograft models. Collectively, our data demonstrating up-regulation of *Cib2*, *Enpp1* and *Prelp* by 1,25D identifies additional candidate mediators and mechanisms by which VDR signaling may act to inhibit breast cancer.

With respect to the functional significance of the genes that were down regulated by 1,25D in our model system, all four are known to be elevated during cancer progression. *Plau* codes for urokinase-type plasminogen activator (uPA), a serine protease that has consistently been linked to tumor cell migration and the invasive phenotype [34]. Binding of uPA to its receptor uPAR triggers the cleavage of plasminogen to plasmin with subsequent activation of metalloproteinases, favoring tumor cell invasion and metastasis. The *Postn* gene codes for periostin, a secreted heparin binding protein normally expressed in bone where it mediates cell adhesion and mineralization, however, it too has consistently been identified as a marker of invasion and metastasis [35–39]. Periostin is increased in breast cancer compared with benign and normal breast tissue and positively correlates with clinical stage by immunohistochemistry [36,40]. Mechanistically, periostin interacts with multiple cell-surface receptors to promote cancer cell survival, epithelial-mesenchymal transition, invasion, and metastasis. *Has2* codes for hyaluronan synthase, the enzyme that synthesizes the extracellular proteoglycan hyaluronic acid (HA). HA binds to the breast cancer stem cell receptor CD44 and fosters complexes between CD44 and growth factor receptors/ligands, promoting survival [41,42]. Forced expression of *Has2* in triple negative breast cancer cells drives bone metastasis while inhibition or silencing of *Has2* reduces the malignant phenotype [41,43–45]. We have confirmed that 1,25D reduces *Has2* expression and HA secretion in WT145 and KO^{hVDR} cells and that exogenous HA partially rescues 1,25D mediated growth inhibition (data not shown), thus functionally linking *Has2* to the anti-tumor actions of 1,25D. These newly discovered links between 1,25D, *Has2* and HA complement data showing down-regulation of CD44 mediated survival signaling by 1,25D

in the human DCIS.com cell line, a distinct model of triple-negative breast cancer [4,5]. The fourth 1,25D-repressed gene we identified, *Hbegf*, was one of three genes found to promote metastasis of breast cancer to brain [46]. Interestingly, *Hbegf* has been shown to signal through the EGF receptor, HA and CD44 in the epidermis during wound healing, epidermal hyperplasia and hair follicle morphogenesis [47–50], processes which are known to be modulated by VDR signaling. Thus, the four 1,25D repressed genes identified in this model system all function in the extracellular matrix to suppress distinct stages in cancer progression: proliferation, epithelial-mesenchymal transition, invasion and metastasis.

In summary, our studies have identified a number of novel target genes that are modulated by the 1,25D/VDR complex in aggressive triple negative breast cancer cells. Challenges for the future will be to complete the validation of the 35 gene signature common to all VDR positive cell lines and to functionally characterize their role(s) in breast cancer invasion and metastasis. Such studies may identify new opportunities for targeting the vitamin D pathway in the treatment of human breast cancer.

Acknowledgments

We greatly appreciate the assistance of Meggan Keith, PhD, Gennifer Goode and Teresa Lloyd-Coronado with initial cell line characterization and James Keith, PhD with creation of the hVDR retroviral expression vector.

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Highlights

- A model of triple negative breast cancer with differential VDR expression was created
- The growth regulatory and genomic effects of 1,25-dihydroxyvitamin D were profiled
- 1,25D failed to alter growth or gene expression in cells lacking VDR
- 1,25D regulated genes included *Cib2*, *Enpp1*, *Prep*, *Has2*, *Plau*, *Postn* and *Hbegf*

Figure 1. Expression of hVDR in murine VDRKO cells

A, Retroviral expression vector for hVDR was created in pBABE-puro. B, Localization of VDR in KO^{hVDR} cells. Cells were stained with DAPI for nuclear chromatin (top) and monoclonal VDR antibody Clone D6 (bottom) and visualized by confocal microscopy.

Figure 2. Sensitivity of murine cell lines to 1,25D

A, Western blot for VDR in WT145 cells, KO240 cells and four independent clones isolated from KO^{hVDR} mass cultures. B, Adherent cell density was measured by crystal violet absorbance after 96h treatment with 100nM 1,25D or ethanol (EtOH) vehicle. Bars represent mean±standard error, n = 4. *p< .05, 1,25D vs control for each cell line, as evaluated by Student's t test. C, Phase contrast images of KO240 cells expressing empty vector (KO^{EV}) or hVDR (KO^{hVDR4}) after 96h treatment with ethanol vehicle (Control) or 10nM 1,25D.

Figure 3. WT145 and KO240 cells are triple negative

A, Western blot for ERα (top) in lysates from WT145 and KO240 cells. MCF7 breast cancer cell lysate was used as positive control. Blots were stripped and re-probed with actin antibody to verify protein loading. B. Effect of tamoxifen on WT145 and KO240 cell density. Cells were treated with ethanol vehicle control (Con) or 100nM tamoxifen (Tam) (bottom) for 96h and analyzed by crystal violet staining for adherent cell density. C. Western blot for progesterone receptor (PR) in WT145, KO240 and KO^{hVDR} cell lysates; homogenate from pregnant mouse mammary gland (MG) was used as positive control. Blots were stripped and re-probed with Gapdh antibody to confirm protein loading. D. Western blot for Her2 in WT145, KO240 and KO^{hVDR} cell lysates; lysate of known HER2+ human

breast cancer cell line SUM190 was used as positive control. Blots were stripped and re-probed with Gapdh antibody to confirm protein loading.

Figure 4. Heat map of 1,25D regulated genomic profiles in murine cells with differential VDR expression

A, Heat maps of genomic profiles obtained from WT145 cells, KO240 cells and three independently derived KO^{hVDR} clones (# 1, 2, 4) after 24h treatment with ethanol vehicle control (Con) or 100nM 1,25D. Cells were processed for Affymetrix microarray screening and analyzed by Genespring as described in Methods. B, Venn diagram of 1,25D regulated genes identified a cohort of 35 genes that were similarly regulated by 1,25D in both WT145 and KO^{hVDR} cells. C, Pathway analysis of the 35 genes commonly regulated by 1,25D in WT145 and KO^{hVDR} cells.

Figure 5. PCR analysis of select VDR up-regulated target genes in murine cell lines with differential VDR expression

WT145 cells, KO240 cells and three clones of KO240 cells stably expressing hVDR (KO^{hVDR}1, KO^{hVDR}2, KO^{hVDR}4) were treated with 100nM 1,25D or ethanol vehicle for 24h. RNA was isolated and used for real-time PCR evaluation of *Cyp24*, *Cib2*, *Prepl* and *Enpp1*. Data are normalized against 18S and expressed as fold change (1,25D treated vs control) for each cell line. Each bar represents mean $\pm$ standard error of 2–3 independent samples analyzed in duplicate. *p < .05, control vs 1,25D treated for each cell line.

Figure 6. PCR analysis of select VDR down-regulated target genes in murine cell lines with differential VDR expression

KO240 cells, WT145 cells, and three clones of KO240 cells stably expressing hVDR (KO^{hVDR1}, KO^{hVDR2}, KO^{hVDR4}) were treated with 100nM 1,25D or ethanol vehicle for 24h. RNA was isolated and used for real-time PCR evaluation of *Plau*, *Postn*, *Has2* and *Hbegf*. Data are normalized against 18S and expressed as 1,25D treated relative to control for each cell line. Each bar represents mean $\pm$ standard error of 2–3 independent samples analyzed in duplicate. * $p < .05$, control vs 1,25D treated for each cell line.

Table 1

Microarray data

List of genes commonly regulated by 1,25D (100nM/24h) in all VDR positive cells (WT145 cells and three KO^{hVDR} clones). None of these genes were significantly altered by 1,25D treatment in KO240 cells. Shown are fold change for each cell line (treated/control) calculated from the array dataset. Validation of a subset of these genes by real time PCR is shown in Figures 5 and 6.

Gene	Fold Change (100nM 1,25D/Con)				
	KO240	WT145	KO ^{hVDR1}	KO ^{hVDR2}	KO ^{hVDR4}
A930018M24RK	1.2	5.5	7.4	6.0	6.5
Abcd2	1.0	2.2	2.1	1.8	2.2
Apcdd1	1.1	3.8	2.1	2.6	1.9
C3	1.2	2.1	2.7	2.0	1.7
Cih2	1.0	4.6	9.9	8.0	9.5
Ckb	1.3	2.2	3.4	3.2	4.0
Crabp2	1.0	4.0	2.6	2.6	4.3
Cyp24a1	1.1	50.3	19.4	15.9	16.0
Elov17	1.0	4.3	4.5	3.4	4.4
Enpp1	1.0	6.7	3.8	4.3	4.5
Fam210b	1.0	2.1	2.6	2.7	1.9
Fbn1	1.0	2.2	2.0	2.0	2.1
Fst	1.0	2.6	2.2	1.9	2.1
Gpc4	1.1	2.3	2.5	2.1	2.7
Grk5	1.0	3.0	3.2	3.2	3.7
Has2	0.9	0.5	0.3	0.4	0.3
Hbegf	1.0	0.5	0.4	0.5	0.4
Igfbp4	1.0	2.9	3.1	3.2	6.4
Islr	0.8	4.3	5.2	2.2	3.5
Kltl	1.0	2.5	2.8	2.8	3.0
Kl19	0.9	2.3	3.4	2.3	2.7
Npr3	1.0	2.2	5.3	5.8	6.3
Plau	1.0	0.4	0.3	0.3	0.3

Gene	Fold Change (100nM 1,25D/Con)					
	KO240	WT145	KO ^{bVDR1}	KO ^{bVDR2}	KO ^{bVDR4}	
Pap1	1.0	2.1	2.3	2.0	2.4	
Postn	1.0	0.2	0.3	0.3	0.4	
Prep1	0.9	5.2	6.3	6.3	8.8	
Rassf2	1.0	3.4	5.0	3.3	4.1	
Relt	1.0	2.4	2.5	2.5	2.6	
Rgl1	0.9	2.5	1.8	2.5	2.3	
Sepp1	1.0	3.4	5.7	4.0	6.1	
Serp1b9b	0.9	2.0	2.3	3.0	3.0	
Sfn2	0.9	2.4	2.4	1.8	2.3	
Sulf2	1.0	2.9	3.3	2.5	2.6	
Tgm2	1.0	2.0	4.9	4.9	5.3	
Tspan2	1.0	2.3	4.9	3.6	4.1	