Tumor Autonomous Effects of Vitamin D Deficiency Promote Breast Cancer Metastasis

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Patients with breast cancer (BCa) frequently have preexisting vitamin D deficiency (low serum 25-hydroxyvitamin D) when their cancer develops. A number of epidemiological studies show an inverse association between BCa risk and vitamin D status in humans, although some studies have failed to find an association. In addition, several studies have reported that BCa patients with vitamin D deficiency have a more aggressive molecular phenotype and worse prognostic indicators. However, it is unknown whether this association is mechanistically causative and, if so, whether it results from systemic or tumor autonomous effects of vitamin D signaling. We found that ablation of vitamin D receptor expression within BCa cells accelerates primary tumor growth and enables the development of metastases, demonstrating a tumor autonomous effect of vitamin D signaling to suppress BCa metastases. We show that vitamin D signaling inhibits the expression of the tumor progression gene *Id1*, and this pathway is abrogated in vitamin D deficiency in vivo in 2 murine models of BCa. These findings are relevant to humans, because we discovered that the mechanism of VDR regulation of Inhibitor of differentiation 1 (ID1) is conserved in human BCa cells, and there is a negative correlation between serum 25-hydroxyvitamin D levels and the level of *ID1* in primary tumors from patients with BCa. **(*Endocrinology* 157: 0000–0000, 2016)**

Vitamin D, derived from diet or sunlight driven synthesis in the skin, is converted by the liver to 25-hydroxyvitamin D (25(OH)D), the circulating form of the hormone. 25(OH)D is subsequently converted to 1α,25-dihydroxyvitamin D_3 (calcitriol) primarily in the kidney but also in many target tissues, including breast cancer (BCa) cells (1). Calcitriol is the active hormone, binds to the vitamin D receptor (VDR), a member of the nuclear receptor family, and regulates gene transcription by binding to vitamin D-response elements in target genes (1–3).

A number of epidemiological studies (but not all) (4) indicate that there is an inverse correlation between the risk of developing BCa and serum 25(OH)D levels in humans (5–7). Further, biological studies suggest that pharmacologic levels of calcitriol inhibit cancer growth, particularly in tissue culture models (1, 8). However, treatment with high-dose vitamin D likely has pharmacological effects that are distinct from the pathophysiology of vitamin D deficiency.

Provocatively, low vitamin D levels are associated with worse prognostic indicators and outcomes in patients with BCa (9–11). Because vitamin D deficiency is common in patients diagnosed with BCa (1, 10, 12–14), elucidating the cause of the association between poor outcomes and vitamin D deficiency promises to have a significant impact on improving care for patients with BCa, including enabling the development of novel therapeutic approaches. Metastatic disease is the primary cause of treatment failure and poor prognosis in patients with BCa. However, although there are intriguing data suggesting that systemic vitamin D levels affect the growth of BCa cells that are already metastatic (15, 16) and BCa cells that are directly seeded into distal sites (17), the effect of vitamin D deficiency on the critical step in tumor progression from non-

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Abbreviations: BCa, breast cancer; BLI, bioluminescent imaging; ID1, Inhibitor of differentiation 1; KD, knockdown; nVDRE, vitamin D repressive-response element; 25(OH)D, 25-hydroxyvitamin D; VDR, vitamin D receptor.

metastatic to metastatic is unknown. Finally, whether the mechanisms by which vitamin D deficiency and VDR signaling modulates BCa growth and metastasis are systemic or tumor autonomous is also unclear. Therefore, in this study, we tested the impact of vitamin D deficiency on BCa growth and investigated the tumor autonomous effect of VDR signaling on BCa progression and metastasis.

Materials and Methods

Study approvals

All animal studies were approved by the Stanford Institutional Animal Use and Use Committee.

All clinical samples were obtained in accordance with a Stanford Institutional Review Board approved protocol, which included informed consent.

Mice

Five to 6-week-old female wild-type BALB/c mice were purchased from The Jackson Laboratory. AIN76 diets containing 500-IU vitamin D/kg were used as standard diets and compared with vitamin D-deficient diets which had 25-IU/kg diet. All diets were obtained from Research Diets, Inc. Tumors were inoculated into the left inguinal mammary fat pad using 1×10^5 cells suspended 50:50 matrigel (Thermo Fisher) and PBS. Bioluminescent imaging (BLI) was performed once a week using the IVIS 200 (Caliper) imaging system at the Stanford Small Animal Imaging Facility as described (18, 19). Tumor volume was estimated by measuring 2 tumor diameters using a vernier calipers. Lungs, liver, and spleen were subjected to ex vivo BLI to map metastatic lesions and histopathological confirmation.

Measurement of 25(OH)D

Mouse serum 25(OH)D levels were measured as described (20). Human serum 25(OH)D levels were measured by mass spectroscopy in the Stanford Clinical Laboratories.

Generation of stable *Vdr* knockdown (KD) and Rescue cell lines

Short hairpin RNAs directed against mouse *Vdr* (Sigma-Aldrich) or control vector were stably transfected into 168FARN-luc cells (a gift from Fred Miller, Wayne State University) and selected for puromycin resistance clonal selection from a single cell. The cells were then transfected with a VDR expression plasmid or empty vector (pEF6; Thermo Fisher) control and selected with puromycin and blasticidin.

Statistical analyses

Student's *t* test was computed to determine statistically significant differences between 2 groups. For comparisons between more than 2 groups, ANOVA was performed followed by Tukey's post hoc test. Spearman's test was performed to compute correlation analysis. $P < .05$ was considered statistically significant. GraphPad Prism (GraphPad Software, Inc) was used to perform statistical calculations and for plotting graphs.

For antibodies, please see Table 1. Additional Materials and Methods are detailed in the Supplemental Materials and Methods.

Results and Discussion

Diet-induced vitamin D deficiency promotes BCa growth in vivo

To understand the impact of systemic vitamin D deficiency on BCa progression, we generated a mouse model of vitamin D deficiency by placing wild-type mice on a standard chow diet (control, 500-IU vitamin D_3/kg diet) or on a low vitamin D diet (LD25, 25 IU/kg) for 10 weeks. Ingestion of the LD25 diet caused a statistically significant decrease in serum 25(OH)D levels compared with control mice (Supplemental Figure 1). We orthotopically seeded the vitamin D-deficient and control cohorts with primary MMTV-*Wnt1* tumor cells (21) into the fourth mammary fat pad of the mice. We found that vitamin D-deficient mice developed mammary tumors that were palpable an average of 7 days earlier than control mice. Importantly, vitamin D deficiency also accelerated tumor growth, resulting in a significant increase in the mean tumor volume after 6 weeks compared with tumors grown in mice fed the control diet (Figure 1A).

Tumor autonomous effects of vitamin D/VDR signaling

We next explored whether the impact of vitamin D signaling on tumor growth was tumor autonomous or resulted from systemic effects. First, we examined 2 BCa cell lines that stably express luciferase, 168FARN and 4T1 (22), for their in vivo growth characteristics and VDR expression levels. As previously reported (23), we found that the tumors generated in vivo from 4T1 cells grew

Table 1. Antibody Table

Peptide/ Protein Target	Antigen Sequence (if Known)	Name of Antibody	Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody	Species Raised in; Monoclonal or Polyclonal	Dilution Used
VDR		sc-1008	Santa Cruz Biotechnology, Inc	Rabbit; polyclonal	1:1000
ID1		BCH-1/27-2	Biocheck	Rabbit; monoclonal	1:500
β-Actin		AB8227	Abcam	Rabbit; polyclonal	1:10 000
β-Tubulin		A01717	Genescript	Mouse; monoclonal	1:10 000

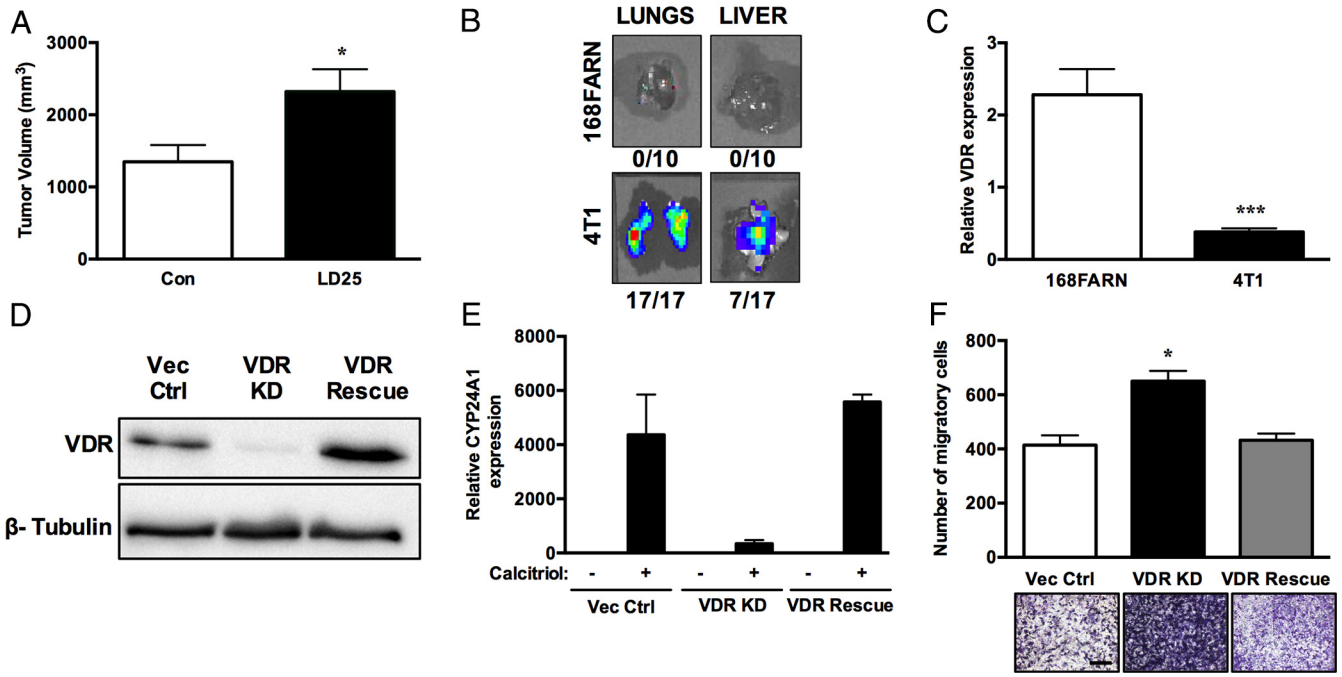

Figure 1. Vitamin D and VDR deficiency promotes tumor aggressiveness. A, Volumes of MMTV-Wnt tumors grown in vitamin D sufficient (Con) (n = 10) and deficient (LD25) mice (n = 10). B, BLI of lungs and liver from mice with 168-FARN (n = 10) or 4T1 tumors (n = 17). C, RT-qPCR quantifying VDR mRNA expression levels in 168FARN and 4T1 cells. D, Western blottings measuring VDR levels in 168FARN cells with an empty vector control (Vec Ctrl) compared with 168FARN cells with VDR KD or VDR KD with reexpression of VDR (VDR Rescue). E, RT-qPCR quantifying the level of CYP24A1 mRNA in response to calcitriol treatment in Vec Ctrl compared with VDR KD and VDR Rescue cells. F, Transwell assays (upper) quantifying the average number of cells that migrated for each cell line (n = 3) and (lower) phase contrast images of stained cells from examples of each cell line (scale bar, 200 μm). *, $P < .05$; ***, $P < .001$. Error bars represent SEM.

more rapidly and developed distal metastases, whereas the 168FARN tumors grew more slowly and did not macrometastasize outside of the primary tumor site (Figure 1B). Intriguingly, we discovered that the highly aggressive and metastatic 4T1 cell line has significantly lower levels of VDR expression compared with the less aggressive, metastatic-deficient 168FARN cell line (Figure 1C).

In order to test whether the observed difference in aggressiveness between the cell lines is regulated by the differences in vitamin D/VDR signaling in the tumor cells, we generated a 168FARN cell line with stable *Vdr* knockdown (VDR KD). We confirmed that *Vdr* KD results in a substantial decrease in VDR levels compared with the vector control cells selected in parallel (Figure 1D). To validate that this level of *Vdr* KD is functionally relevant, we treated the VDR KD and vector control cell lines with calcitriol. A robust induction in mRNA expression of the classic calcitriol target gene, *Cyp24A1*, was observed in control cells, whereas this functional response was significantly diminished in VDR KD cells, demonstrating the attenuation of VDR signaling (Figure 1E). To establish that any functional differences between the vector control and VDR KD cell lines were specifically due to inhibition of *Vdr* expression, we rescued *Vdr* expression in the VDR KD line by stably integrating a *Vdr* expression vector into the VDR KD cell line (VDR Rescue), which rescued both VDR levels and function (Figure 1, D and E).

To test the effect of *Vdr* KD on cell migration, an early step in BCa progression, we used a transwell migration assay (24, 25). We discovered that VDR KD cells exhibit a notable increase in the number of migratory cells compared with control cells and this phenotype was rescued in the VDR Rescue cells (Figure 1F). These data indicate that VDR signaling is functionally relevant to regulating BCa migration ex vivo and therefore may impact BCa progression and metastasis.

Loss of vitamin D/VDR signaling promotes BCa metastasis in vivo

We next examined whether there are tumor autonomous effects of vitamin D signaling in vivo using the VDR KD cells. We established mammary tumors in BALB/c mice by orthotopic injections of the luc-labeled control, VDR KD, and VDR Rescue cell lines and followed tumor appearance and growth over 4 weeks by BLI (26). We found that VDR KD tumors grew significantly faster than control or VDR Rescue cells in vivo (Figure 2A). This striking pattern remained consistent until the endpoint of the study when the mice with VDR KD tumors needed to be euthanized for morbidity (Figure 2B). Primary tumors

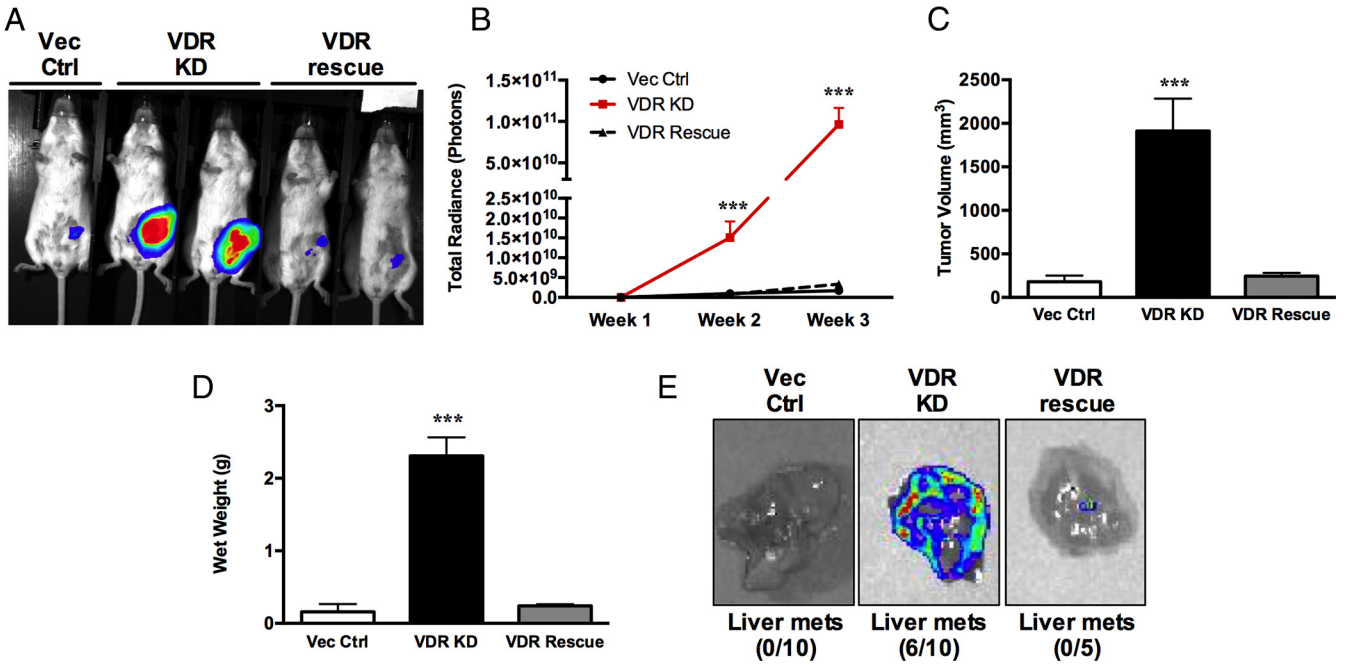

Figure 2. VDR deficiency promotes in vivo tumor autonomous growth and metastasis. A, Examples of in vivo BLI of mice with tumors generated from 168FARN cells with vector control (Vec Ctrl) or VDR KD or VDR KD with VDR reexpressed (VDR Rescue). B, Serial quantifications of BLI intensity from all mice (Vec Ctrl, n = 10; VDR KD, n = 10; VDR Rescue, n = 5) during in vivo tumor growth. Average tumor volumes (C) and tumor wet weights (D) from all mice. E, Examples and totals of BLIs of livers harvested from mice with tumors. ***, $P < .001$. Error bars represent SEM.

were harvested, measured, weighed, and imaged (Figure 2, C and D, and Supplemental Figure 2). These results confirmed the in vivo BLI signal data that the VDR KD tumors were substantially larger than control and VDR Rescue tumors. Remarkably, ex vivo BLI imaging (Figure 2E) and direct histology (Supplemental Figure 3) revealed that 60% of the mice with VDR KD tumors had metastatic spread to the liver. This represents a particularly significant finding as no evidence of macrometastatic tumor spread was found in the control or rescue groups (Figure 2E and Supplemental Figure 3). Our results indicate that loss of vitamin D/VDR signaling is sufficient to convert the cells from nonmetastatic to metastatic.

Vitamin D/VDR regulates *Id1* expression in BCa

In order to elucidate the mechanisms by which vitamin D/VDR signaling exerts an inhibitory effect on tumor progression, we performed expression profiling using PCR arrays comparing tumors that developed in the diet-induced vitamin D-deficient mice with tumors that developed in mice fed the standard chow. In addition, we compared the expression profile of the VDR KD cells with control cells. We then compared the differentially (>2-fold) expressed genes to identify genes with similarly altered expression profiles in both tumors from vitamin D-deficient mice and VDR KD cells (Supplemental Table 1). These studies revealed *Id1* as significantly up-regulated in both the tumors that developed in vitamin D-deficient mice and the VDR KD cells, suggesting that *Id1* is repressed by VDR signaling in BCa. This 'hit' was of particular interest because of the role of *Id1* in BCa progression (27) and most intriguing because calcitriol was found to induce, rather than repress, *ID1* expression in a colon carcinoma cell line (28). We validated that *Id1* expression was up-regulated in both tumors from vitamin D-deficient mice as well as in the VDR KD cells (Figure 3, A and B).

To test whether the regulation of *Id1* by VDR is ligand dependent in BCa cells, we treated 168FARN cells with calcitriol and showed that this treatment represses the expression of *Id1* (Figure 3C). We also found that calcitriol treatment significantly decreases Inhibitor of differentiation 1 (ID1) protein levels in a dose-dependent manner (Figure 3D). This suppression of *Id1* by calcitriol treatment was substantially abrogated in the VDR KD cells (Figure 3D). Interestingly, basal levels of ID1 protein expression were significantly increased in the VDR KD cell line relative to control cells (Figure 3D) and the VDR Rescue cell line had lower levels of ID1 (Figure 3E; reprobed Western blot from Figure 1D). These results suggest that VDR might directly regulate *Id1* in BCa cells and that basal endogenous levels of VDR suppress *Id1* expression. Therefore, we investigated the promoter and proximal region of the *Id1* gene for a negative vitamin D response element (nVDRE) that is functional in BCa cells. We transfected cells with a construct containing an approximately

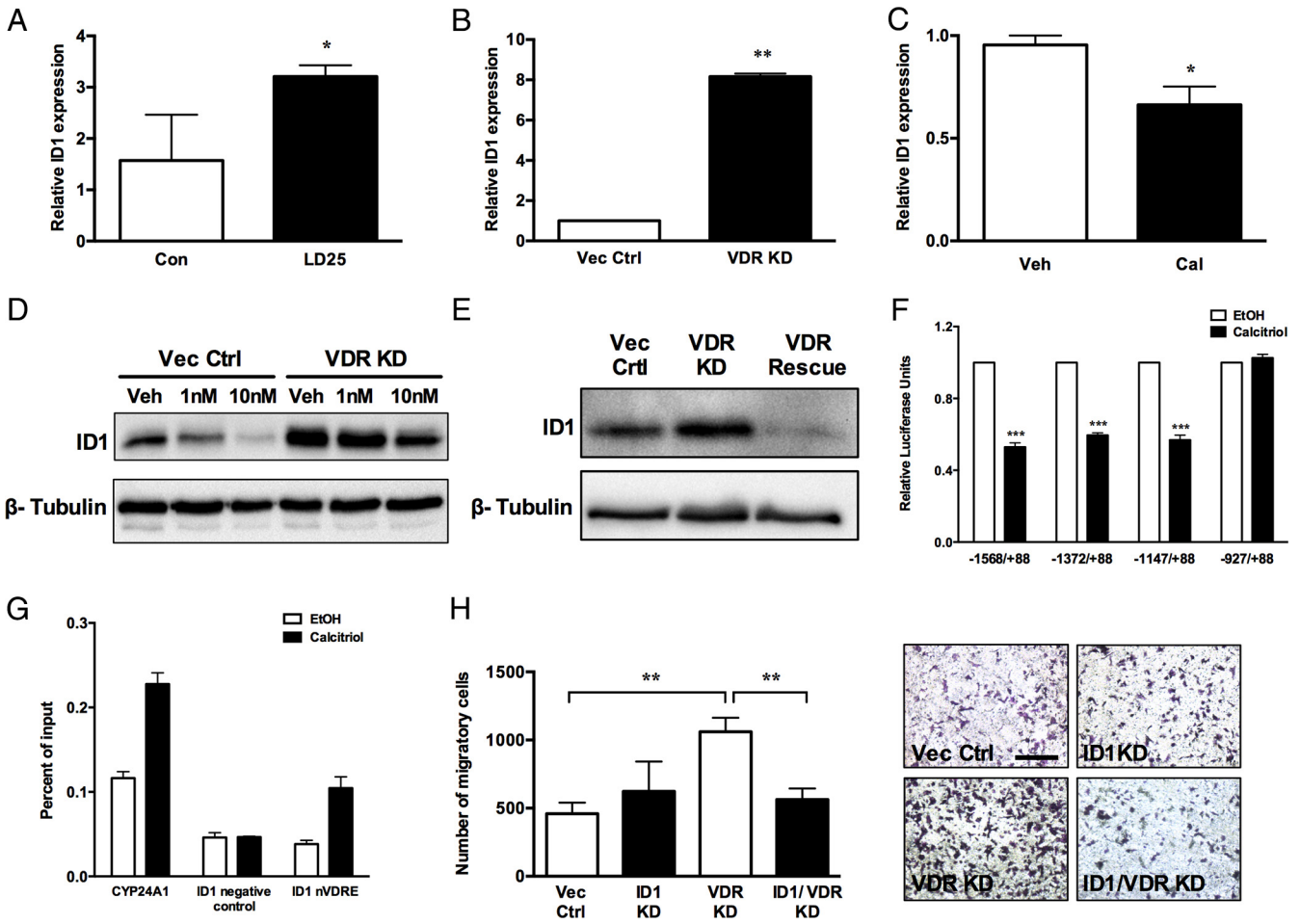

Figure 3. Vitamin D/VDR regulates *Id1* expression in BCa. A, RT-qPCR measuring *Id1* expression levels in tumors isolated from mice with vitamin D deficiency (LD25) (n = 5) compared with vitamin D-sufficient mice (Con) (n = 3). B, RT-qPCR measuring *Id1* expression levels in 168FARN cells with VDR KD compared with 168FARN cells with vector control (Vec Ctrl). C, RT-qPCR quantifying the level of *Id1* expression in response to treating 168FARN cells with 10nM calcitriol (Cal) or vehicle control (Veh). D, Western blottings measuring ID1 and β-tubulin protein levels in Vec Ctrl and VDR KD cells in response to Cal treatment or Veh. E, Reprobe of Western blotting (Figure 1D) for ID1 protein levels in Vec Ctrl, VDR KD, and VDR Rescue cells. F, Luciferase assays quantifying the effect of 1nM Cal treatment on the transcriptional activity of the *Id1* promoter and proximal elements. G, Chromatin immunoprecipitation (ChIP) of VDR in 168FARN cells showing treatment with Cal enhances occupancy of a known VDRE in the CYP24A1 gene (positive control) and there is no enrichment of VDR in the DNA region proximal to the nVDRE in ID1 (negative control). Cal increases occupancy of the nVDRE in ID1. H left, Transwell assays quantifying the number of migrating cells in Vec Ctrl (n = 3) or cells with Id1 KD (ID1 KD) (n = 3) compared with VDR KD (n = 3) and cells with both VDR and ID1 KD (VDR KD/ID1 KD) (n = 3). H right, Representative examples of phase contrast images of each of migrating cells from each condition (scale bar, 200 μm). *, $P < .05$; **, $P < .01$; ***, $P < .001$. Error bars represent SEM.

1.5-kb fragment of the *Id1* promoter and proximal region cloned into a luciferase reporter vector and found that calcitriol treatment of the transfected cells results in decreased luciferase activity (Figure 3F), whereas VDR KD increased luciferase levels (Supplemental Figure 4). Using luciferase reporter vectors with sequential deletions, we mapped a nVDRE to approximately 1 kb upstream of the *Id1* transcriptional start site as functionally relevant in BCa cells (Figure 3F), the same nVDRE that is active in murine osteoblasts (29). Using chromatin immunoprecipitation of VDR on the endogenous nVDRE, we confirmed that calcitriol treatment enriches VDR occupancy of this element in 168FARN cells (Figure 3G).

We next tested whether the regulation of *Id1* is the mechanism underlying the effect of vitamin D/VDR signaling to inhibit tumor cell migration and metastasis. In the transwell migration assay, KD of *Vdr* expression increased cell migration, as we showed previously in Figure 1F. Importantly, we discovered that KD of *Id1* in VDR KD cells abolished the increase in cell migration seen in VDR KD cells, reducing the number of cells migrating back to control levels (Figure 3H).

Vitamin D/VDR regulation of ID1 is conserved in human BCa

To determine whether the findings from our mouse models are relevant to human BCa, we first tested whether *ID1* expression is modulated by calcitriol treatment in the widely studied human breast adenocarcinoma cell line MDA-MB-231 (30). We found that calcitriol robustly

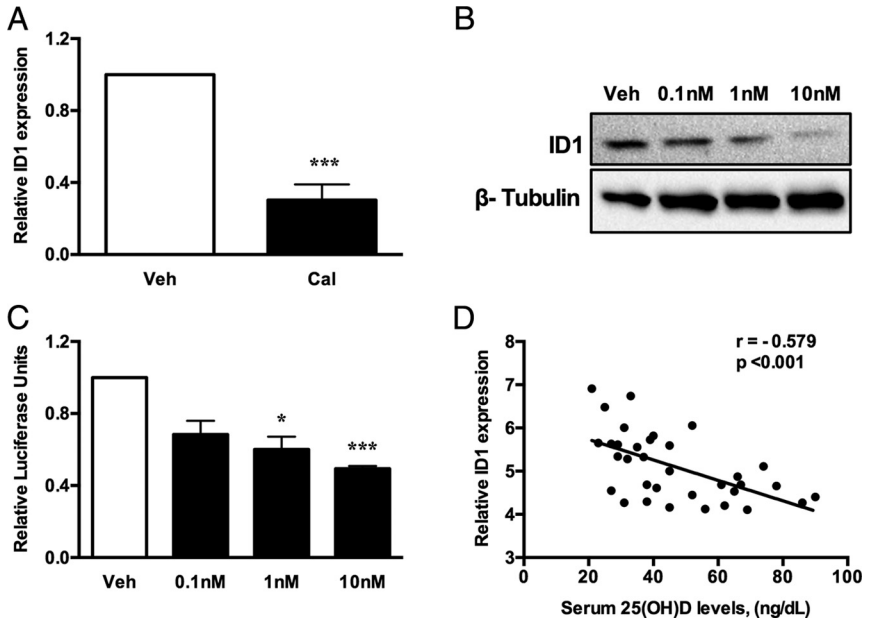

Figure 4. Regulation of *ID1* is conserved in human BCa. A, RT-qPCR quantifying the level of *ID1* expression in response to treating MDA-MD-231 cells with 10nM calcitriol or vehicle control (Veh). B, Western blottings measuring ID1 and β-tubulin protein levels in MDA-MD-231 cells in response to a titration of calcitriol doses or Veh. C, Luciferase assays quantifying the effect of a titration of calcitriol doses or Veh on the transcriptional activity of the human *ID1* promoter and proximal elements. D, Correlation between patient circulating serum 25(OH)D levels and the ID1 expression levels in their tumors. *, $P < .05$; ***, $P < .001$. Error bars represent SEM.

down-regulated *ID1* mRNA (Figure 4A) and protein levels (Figure 4B). Next, we searched the sequence of the human *ID1* gene in silico and identified a DNA element approximately 1 kb upstream of the *ID1* transcriptional start site with 98% nucleotide identity with the region containing the murine nVDRE (Supplemental Figure 5). Using a luciferase reporter vector containing the human *ID1* promoter and 1.3 kb of the upstream proximal region, we confirmed that the conserved nVDRE regulatory element in *ID1* is functional in human BCa cells (Figure 4C).

In order to test whether our findings are relevant to patients with BCa, we obtained permission from our IRB and were generously granted access by the independent investigators of a recently completed clinical trial (NCT01472445) to deidentified data in order to examine the relationship between circulating 25(OH)D levels and tumor *ID1* expression levels in patients undergoing BCa resections. This study was a randomized, double blind clinical trial in women with newly diagnosed BCa. Vitamin D_3 was administered orally in the neo-adjuvant period to women scheduled for surgical resection of their tumors, with the intervention occurring during the 1- to 6-week neo-adjuvant interval before surgery. Subjects were randomly assigned to receive either 400 or 10 000 IU/d of vitamin D_3 in a 1:2 ratio, respectively. This enabled us to analyze the relationship between a patient's circulating 24(OH)D level and *ID1* expression levels in their tumor at the same point in time (at surgical resection).

We found that serum 25(OH)D levels at the time of surgery were negatively associated with *ID1* expression levels in the corresponding tumors (Spearman correlation coefficient $r = -0.579$, $P < .001$), with every 10-ng increase in circulating 25(OH)D levels being associated with a 20% reduction in *ID1* expression levels in the tumor (Figure 4D). These results indicate that the relationship between *Id1* expression and vitamin D status/VDR signaling that we identified in our mouse studies is conserved in humans and is relevant to patients with BCa.

Although many studies have investigated the effects of the administration of calcitriol or vitamin D analogs as a therapeutic approach in BCa (1), relatively less attention has been focused on specifically evaluating the detrimental effects of vitamin D deficiency on BCa progression and to metastasis. Our results show that dietary vitamin D deficiency accelerates the growth of BCa tumors. Further, we discovered that attenuated vitamin D signaling due to *Vdr* ablation enhances tumor progression and, importantly, is sufficient to enable metastases, which are the primary cause of treatment failure and death for patients with BCa.

We also discovered that, although circulating 25(OH)D has many systemic effects (1), a critical mechanism for vitamin D signaling to inhibit BCa progression is a tumor autonomous activity that suppresses *Id1* expression. Interestingly, Sherman et al (31) found a tumor cell nonautonomous mechanism for VDR signaling to inhibit pancreatic ductal adenocarcinoma progression and Fernandez-Garcia et al (28) found that calcitriol induces, rather than inhibits, *Id1* expression in a colon carcinoma cell line. Lungchukiet et al found that VDR tumor autonomously inhibits ovarian cancer invasion and metastasis through both ligand dependent and independent mechanisms (32). These distinctions with our study indicate that vitamin D signaling is highly context specific.

Significantly, our results demonstrate that, in BCa cells, VDR regulation of *ID1* expression is mechanistically conserved in humans and that there is a negative association between circulating 25(OH)D levels and the expression of *ID1* in primary tumors from BCa patients. We believe these findings contribute to the cause of the epidemiological association between low circulating 25(OH)D levels

and poor prognosis in patients with BCa. In addition, our data suggest that correcting vitamin D deficiency in BCa patients may have beneficial effects to inhibit BCa progression and improve prognosis.

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