Molecular Cancer Therapeutics

Inhibition of Mouse Breast Tumor-Initiating Cells by Calcitriol and Dietary Vitamin D

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Abstract

The anticancer actions of vitamin D and its hormonally active form, calcitriol, have been extensively documented in clinical and preclinical studies. However, the mechanisms underlying these actions have not been completely elucidated. Here, we examined the effect of dietary vitamin D and calcitriol on mouse breast tumor–initiating cells (TICs, also known as cancer stem cells). We focused on MMTV-*Wnt1* mammary tumors, for which markers for isolating TICs have previously been validated. We confirmed that these tumors expressed functional vitamin D receptors and estrogen receptors (ER) and exhibited calcitriol-induced molecular responses including ER downregulation. Following orthotopic implantation of MMTV-*Wnt1* mammary tumor cells into mice, calcitriol injections or a vitamin D–supplemented diet caused a striking delay in tumor appearance and growth, whereas a vitamin D–deficient diet accelerated tumor appearance and growth. Calcitriol inhibited TIC tumor spheroid formation in a dose-dependent manner in primary cultures and inhibited TIC self-renewal in secondary passages. A combination of calcitriol and ionizing radiation inhibited spheroid formation more than either treatment alone. Further, calcitriol significantly decreased TIC frequency as evaluated by *in vivo* limiting dilution analyses. Calcitriol inhibition of TIC spheroid formation could be overcome by the overexpression of β-catenin, suggesting that the inhibition of Wnt/β-catenin pathway is an important mechanism mediating the TIC inhibitory activity of calcitriol in this tumor model. Our findings indicate that vitamin D compounds target breast TICs reducing tumor-initiating activity. Our data also suggest that combining vitamin D compounds with standard therapies may enhance anticancer activity and improve therapeutic outcomes. *Mol Cancer Ther; 14(8); 1951–61. ©2015 AACR.*

Introduction

Breast cancer is the most common cancer and the second most common cause of cancer death in women. Breast tumors are composed of heterogeneous subpopulations of cells and are maintained by tumor-initiating cells [TIC; also called cancer stem cells (CSC)] that can initiate new tumors, which recapitulate the heterogeneity of the original mass (1). TICs can self-renew to generate more TICs or can differentiate into progeny called nontumorigenic cells (NTC), which often make up the bulk of a tumor (2). Because TICs maintain tumors, elimination of TICs is critical for achieving cure. Previous studies have found that cells resembling TICs are relatively resistant to radiotherapy and chemotherapy compared to NTCs (3, 4), suggesting that improved outcomes for breast cancer patients will require direct targeting of TICs or overcoming their resistance mechanisms.

Calcitriol (1,25-dihydroxyvitamin D_3), the hormonally active form of vitamin D, is a potent steroid hormone that acts through its cognate nuclear receptor, the vitamin D receptor (VDR), and regulates the expression of target genes in most tissues of the body in a context specific manner (5). In addition to its classical actions on calcium homeostasis and bone mineralization, calcitriol has multiple nonskeletal actions, and exhibits direct antiproliferative, pro-differentiating and anti-inflammatory activities in multiple cancer cells including breast cancer cells (6–11). A recent study of human pancreatic tumors shows that calcitriol exerts inhibitory and anti-inflammatory activities on the cancer-associated fibroblast-like cells in the tumor stroma that promote the initiation and progression of pancreatic cancer, revealing another important mechanism of the anticancer activity of calcitriol (12). In preclinical mouse studies, calcitriol also inhibits estrogen synthesis as well as estrogen receptor (ER) expression and estrogen signaling, and shows beneficial effects especially in the treatment of ER-positive breast cancer (13). Dietary vitamin D supplementation has been shown to exhibit equivalent anticancer actions to calcitriol in a mouse breast cancer model because of intra-tumoral conversion of circulating 25-hydroxyvitamin D [25(OH)D] to calcitriol (14, 15). However, the mechanisms by which vitamin D and calcitriol inhibit the initiation and progression of breast cancer have not been completely elucidated.

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Recent studies have suggested that calcitriol regulates the self-renewal and differentiation of several types of normal stem cells as well as TIC-like cells found in established cell lines. In one study, calcitriol treatment promoted cell-cycle arrest, senescence, and the differentiation of a cell line model of normal prostate progenitor/stem cells into luminal cell fate through IL1α (16). Previous studies of TIC-like cells found that the vitamin D analog BXL0124 repressed the expression of CD44, a TIC marker, in a breast cancer cell line (17). Silencing of the *VDR* gene increased the expression of genes related to epithelial–mesenchymal transition (EMT) and mammosphere formation in triple negative and SKBR3 cells (18). Another study reported the repression of markers associated with stem cell–like phenotype as well as pluripotency markers in MCF10DCIS cell line treated with calcitriol or a vitamin D analog (19). These data suggest that vitamin D may inhibit normal stem cell function and may target TIC-like cells.

Although these findings using TIC-like cells are intriguing, significant uncertainty remains regarding how well these cells approximate TICs from primary tumors. We therefore hypothesized that vitamin D and calcitriol target primary breast cancer TICs and set out to test this hypothesis using TICs from MMTV-*Wnt1* tumors, for which markers for isolating TICs have previously been validated (14, 20). We discovered that vitamin D and calcitriol inhibited the growth of MMTV-*Wnt1* mammary tumors in mice and calcitriol decreased TIC proliferation and self-renewal, measured both *ex vivo* and *in vivo*. Calcitriol treatment inhibited *Wnt*-target gene expression in the tumors as well as in primary cultures of the TICs. Constitutive activation of the Wnt/β-catenin pathway in the TICs abrogated calcitriol's inhibitory effect on TIC proliferation *ex vivo*. Further, a combination of calcitriol and ionizing radiation inhibited TIC proliferation more than either treatment alone. Our findings suggest that vitamin D compounds target breast TICs and therefore have potential therapeutic utility in breast cancer prevention and treatment.

Materials and Methods

Materials

1,25-Dihydroxyvitamin D_3 (calcitriol) was a kind gift from Milan Uskokovic (BioXell Company). The rodent diets were from Research Diets Inc. [H^3]-labeled 17-β estradiol (E_2) and [H^3]-1,25-dihydroxyvitamin D_3 were obtained from Amersham – GE Healthcare Life Sciences. Tissue culture media, supplements, and FBS were obtained from GIBCO BRL and Mediatech Inc. Matrigel was obtained from BD Biosciences. Female FVB/NJ female mice used for tumor implantation and testing the effects of the vitamin D compounds were obtained from The Jackson Laboratory.

Isolation of breast tumor-initiating cells

Mammary tumors from FVB/NJ female mice bearing MMTV-*Wnt1* tumor orthografts (FVB.Cg-Tg(Wnt1)1Hev/J; 21) were minced with a razor blade and suspended in 10 mL of L-15 Leibovitz medium (Thermo Fisher Scientific Inc.) supplemented with 0.5 mL of collagenase/hyaluronidase (Stem Cell Technologies). Tumors were digested to completion for 1.5 to 2 hours at 37°C and 5% CO_2 with manual dissociation by pipetting every 30 minutes. Once digested, 20 mL of Hank's balanced salt solution (HBSS) with 2% bovine calf serum (BCS) was added and tumor cells were collected by centrifugation. Tumor cells were resuspended in 5 mL of trypsin/0.05% Ethylenediaminetetraacetic acid (EDTA) for 5 minutes and centrifuged. The cell pellet was resuspended in HBSS with 2% BCS and incubated with 100 Kunitz units of DNase I (Sigma) and Dispase (Stem Cell Technologies) for 5 minutes at 37°C and centrifuged again with the addition of HBSS with 2% BCS. Once digested, tumor cells were treated with ammonium–chloride–potassium (ACK) lysis buffer to lyse the red blood cells and filtered through a 40 μm cell strainer (BD Biosciences). After centrifugation, tumor cells were resuspended in HBSS with 2% BCS, blocked with rat IgG for 10 minutes, and stained with rat anti-mouse CD31 (Biolegend), anti-mouse CD45 (Biolegend), anti-mouse CD140a (eBioscience), rat anti-mouse EpCAM (Biolegend), and rat anti-human/mouse CD49f (BD Biosciences). Lineage negative, viable, $EpCAM^{+}CD49f^{high}$ cells were sorted for further analysis. A minimum three tumors from different mice were used to generate the tumor spheroid assay results described below and the numbers of replicates are indicated in each figure legend.

Ex vivo tissue slice culture assays

Sections of 300 μm were precision cut from MMTV-*Wnt1* tumor orthografts to generate tissue slices. The slices were transferred in a sterile manner to titanium mesh inserts in sterile six-well plates containing culture media mounted on a rotating platform set at a 30° angle in a tissue culture incubator at 37°C with 95% air and 5% CO_2 as described before (22, 23). The tumor tissue slices were incubated in phenol-red–free DMEM-F12 media containing 5% charcoal-stripped FBS containing vehicle, calcitriol (100 nmol/L), E_2 (10 nmol/L), or a combination of both for 5 hours following which RNA was isolated from the tissue slices for the measurement of estrogen receptor α (*Erα*), progesterone receptor (*Pr*), 25-hydroxyvitamin D_3-24 hydroxylase (*Cyp24*), 25-hydroxyvitamin D_3-1a hydroxylase (*Cyp27B1*), *Vdr*, and aromatase (*Cyp19*) mRNA expression. At least three tumors from different mice were used to generate the tissue slices.

[3H]-Estradiol and [3H]-1,25$(OH)_2D_3$ binding assays

The expression of ER and VDR in high salt homogenates of MMTV-*Wnt1* tumor tissue was determined by [^{3}H]-estradiol and [^{3}H]-1,25$(OH)_2D_3$ binding respectively, as described before (24, 25). Protein concentrations of tissue homogenates were measured by the method of Bradford (26).

Mouse studies

All animal procedures were performed in compliance with the guidelines approved by Stanford University Administrative Panels on Laboratory Animal Care (APLAC). Five- to six-week-old female FVB/N mice were obtained from Harlan Laboratories. Mice were housed in a designated pathogen-free area in a facility at Stanford University School of Medicine accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

Dietary manipulations and calcitriol treatment

The mice were randomly assigned to different experimental groups and fed various diets for a period of 12 weeks prior to tumor inoculation. The mice on the standard diet received vehicle or calcitriol injections 8 weeks prior to tumor inoculation. The four experimental groups were as follows: (i) mice fed a standard diet (AIN76, Research Diets Inc., 1000 IU of vitamin D_3/kg diet) receiving injections of vehicle, (ii) mice fed standard diet receiving injections of calcitriol (50 ng/mouse three times/week), (iii) mice

fed a vitamin D–supplemented diet (5000 IU of vitamin D_3/kg of diet), and (iv) mice fed a vitamin D–deficient diet (100 IU of vitamin D_3/kg of diet). Stock solutions of calcitriol were made in 100% ethanol and stored at −20°C. Appropriate dilutions were made in sterile PBS and were administered by i.p. injections three times a week (on Mondays, Wednesdays, and Fridays). The mice in the control group received i.p. injections of 0.1% ethanol in PBS (vehicle). The dosage of calcitriol and the intermittent administration regimen were based on our prior work (15, 27).

Establishment and growth of MMTV-Wnt1 orthotopic tumors

Tumors were established in FVB/NJ female mice by injecting MMTV-*Wnt1* tumor cell suspensions (~150,000 cells suspended in 100 μL of a 1:1 mixture of the culture medium and Matrigel). Orthotopic implantation was done using a midline abdominal skin incision to visualize the fourth mammary fat pads (inguinal glands) and cell suspensions were injected directly into the mammary fat pad on the left side. For tumor cell implantation, mice were manipulated in surgical aseptic conditions under isoflurane anesthesia and received carprofen (5 mg/kg) for analgesia. The experimental diets and calcitriol injections were continued over the next 7 weeks. Body weights and tumor sizes were measured weekly after tumor inoculation and continued throughout the experiment. Tumor volumes were calculated from two tumor diameter measurements using a Vernier caliper and using the formula: tumor volume = (length × width^2)/2 (15, 27). At the end of the study, 14 hours after the final calcitriol injections, mice were euthanized according to APLAC guidelines using CO_2. Blood samples were collected by cardiac puncture while under CO_2 anesthesia causing exsanguinations and serum samples were prepared and frozen. Tumors were harvested, measured, weighed, and snap frozen in liquid nitrogen for subsequent analysis.

Tumor spheroid culture assay

FACS-sorted MMTV-*Wnt1* tumor cells were cultured in the presence of vehicle or calcitriol in DMEM/F12 medium with 2% B27, 20 ng/mL mouse EGF, 20 ng/mL human FGF (BD, #354060), and 1% antibiotics, and plated on top of solidified Matrigel (28) for 10 to 14 days. Spherical colonies (>50 μm in diameter) were counted using Clono-counter or manually. For the secondary colony formation assay, spheroids were dissociated with 1 mg/mL dispase (Invitrogen, #17105-041) for 30 minutes, digested with trypsin/0.05% EDTA for 5 minutes, and passaged through a 27-G needle five times to dissociate into single cells (29). After centrifugation, cells were resuspended and cultured as described above. For radiosensitization experiments, sorted TICs were irradiated by 2 Gy and cultured in media containing vehicle or calcitriol for 10 to 14 days.

Transplantation and limiting dilution analysis

Dissociated spheroid cells were resuspended in culture medium with 25% Matrigel and injected subcutaneously into mice in the vicinity of the mammary fat pads. After injections, mice were examined weekly for up to 4 to 5 months for tumor formation. TIC frequencies were calculated using L-Calc (http://www.stemcell.com).

Statistical analysis

Statistical analyses were performed in GraphPad Prism using a Student *t*-test for single comparisons and ANOVA for multiple comparisons, with Bonferroni correction for post hoc analyses. The limiting dilution analysis experiments were analyzed using L-Calc (http://www.stemcell.com). Data are presented as mean ± SEM using at least three independent experiments. $P < 0.05$ was considered significant. Detailed methods are available in Supplementary Materials and Methods.

Results

Estrogen- and calcitriol-mediated bio-responses in MMTV-*Wnt1* tumors

To explore the effect of vitamin D or calcitriol treatment on breast TICs, we focused on MMTV-*Wnt1* mammary tumors, because they have well-characterized TIC subpopulation (14, 20, 30). In initial experiments, we used tissue slice cultures that provide realistic preclinical models of diverse tissues and organs. The use of tissue slice cultures to investigate hormone dependence and cancer-specific responses has been validated in studies of benign and malignant human prostate tissue (23). [^{3}H]-labeled ligand binding assays in *ex vivo* cultures of MMTV-*Wnt1* tumor tissue slices demonstrated that these tumors expressed functional ER and VDR proteins (Fig. 1A). Calcitriol treatment of tissue slices induced *Cyp24* mRNA expression (Fig. 1B) and repressed that of *Cyp27B1* (Fig. 1C), whereas *Vdr* mRNA showed no changes (Fig. 1D). Calcitriol also repressed the expression of *Erα* (Fig. 1E) and aromatase (*Cyp19*) mRNA (Fig. 1F). Estradiol (E_2) treatment of tissue slice cultures caused a significant induction of *Pr* mRNA expression, confirming ER function (Fig. 1G) and cotreatment with calcitriol attenuated PR induction by E_2 (Fig. 1G). These data demonstrate calcitriol inhibition of estrogen synthesis and signaling as previously described in a different breast cancer model (13). These data also demonstrate the presence of both ER and VDR in MMTV-*Wnt1* tumor cells and their functional responses to hormone.

Dietary vitamin D_3 status and calcitriol effect on tumor appearance and growth

We next investigated the *in vivo* effects of diets supplemented with vitamin D_3 and calcitriol injections on the growth of MMTV-*Wnt1* mammary tumors. Mice in the various experimental groups were monitored for the appearance of palpable tumors. As shown in Fig. 2A, within a week of tumor cell implantation (experimental week 13), palpable tumors appeared in 20% of the mice fed the vitamin D–deficient diet whereas no tumors were noted in the mice in other experimental groups. Palpable tumors appeared in 20% of the mice fed the standard diet 4 weeks after tumor inoculation (experimental week 16) at which time point approximately 66% of the mice on the vitamin D–deficient diet exhibited tumors. However, in calcitriol-treated mice and in mice fed the vitamin D–supplemented diet, tumor appearance was first noted at 5 and 6 weeks after tumor inoculation (experimental weeks 17 and 18), respectively (Fig. 2A). The time to appearance of tumors in 50% of mice in the vitamin D–deficient group (3.5 weeks after tumor inoculation) was significantly shorter ($P = 0.05$) than in the control group receiving the standard diet (5.5 weeks after tumor inoculation). In contrast, the time to appearance of tumors in 50% of mice in the vitamin D–supplemented group (7 weeks after tumor inoculation) was significantly delayed ($P = 0.016$) compared to the group fed the standard diet. There were no significant differences between the standard diet group and the calcitriol-treated group in the time taken for the appearance of

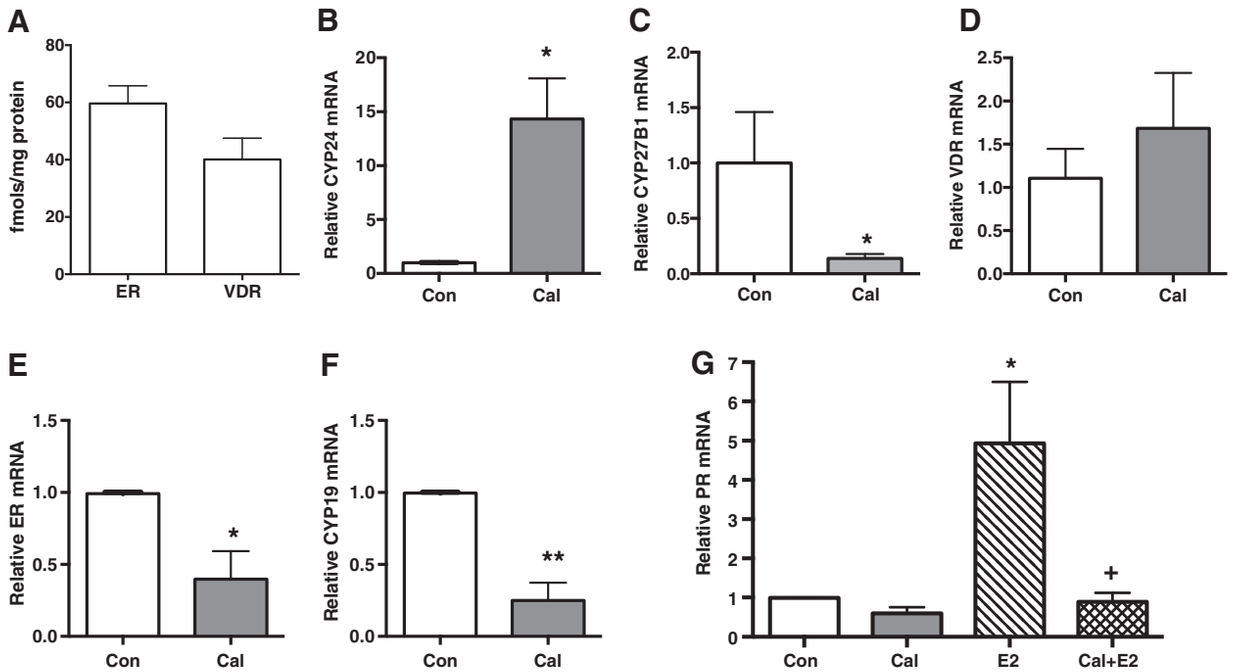

Figure 1.

Presence and functional activity of ER and VDR in tissue slice cultures of MMTV-*Wnt1* tumors. MMTV-*Wnt1* tumor orthografts were used to generate tissue slices. The slices were cultured and the expression of ER and VDR and their functional responses were determined as described in Materials and Methods. A, basal ER and VDR levels. [^{3}H]-labeled ligand binding assays revealed the expression of ER and VDR proteins in the tumor slices. B–F, VDR functional responses. Tissue slice cultures were treated with 0.1% ethanol vehicle or 100 nmol/L calcitriol (Cal) for 5 hours and the mRNA levels of the *Cyp24*, *Cyp27B1*, *Vdr*, *Erα*, and *Cyp19* were determined by qRT-PCR ($n = 4$; *, $P < 0.05$ and **, $P < 0.01$ as compared to the Std group). G, ER functional response. Tissue slice cultures in phenol red–free culture media were exposed to 0.2% ethanol vehicle (control, Con), 100 nmol/L calcitriol (Cal), 10 nmol/L E_2 (E2), or a combination of both (Cal+E2) for 5 hours and PR mRNA levels were determined by qRT-PCR ($n = 4$; *, $P < 0.05$ as compared to Con; +, $P < 0.05$ as compared to E2). At least three different orthografts were used to generate the tissue slices and each experiment was conducted in duplicate. Values represent mean ± SEM.

tumors in 50% of the mice but calcitriol treatment reduced the total number of tumors that appeared. At the end of the study, 20% of the mice were tumor-free in the vitamin D–deficient and the standard diet groups, whereas 40% of the mice were tumor-free in the calcitriol and vitamin D–supplemented groups.

We further observed that the mean tumor volume in mice receiving calcitriol injections or fed the vitamin D_3-supplemented diet was significantly decreased compared to mice on the standard or vitamin D–deficient diet at the endpoint of the study (experimental week 19; Fig. 2B). In the group that received the standard diet, the tumors achieved a mean volume of approximately 1,150 mm^3, whereas in mice receiving either calcitriol injections or the vitamin D_3-supplemented diet mean endpoint tumor volumes were approximately 66% and 67% lower ($P < 0.05$ to $P < 0.01$). Although a significant acceleration in the time of appearance of tumors was seen in mice fed the vitamin D–deficient diet, the mean endpoint tumor volume in these mice was similar to that seen in the mice fed the standard diet. Thus, vitamin D deficiency accelerated tumor appearance and dietary vitamin D supplementation and calcitriol treatment inhibited both tumor appearance and the growth of tumors.

Effects of dietary D_3 and calcitriol on body weight and serum calcium and vitamin D metabolites

Blood samples were drawn at the time of sacrifice 14 hours after the final calcitriol injections for the measurement of serum calcium and vitamin D metabolites. As expected, administration of the vitamin D–supplemented diet caused a significant elevation (~72% increase, $P < 0.001$) and the vitamin D–deficient diet caused a significant decrease (~63% decrease, $P < 0.001$) in the mean serum levels of 25(OH)D, in comparison to the mice fed the standard diet (Table 1). In the mice receiving calcitriol, the mean serum 25(OH)D level was significantly decreased (Table 1) as expected after calcitriol treatment because of the induction of CYP24 and degradation of 25(OH)D (5). Also, in the mice receiving calcitriol injections, the mean serum $1,25(OH)_2D$ level showed an expected decrease because of induction of *Cyp 24* (Table 1). It is important to note that the blood samples were drawn 14 hours after the final calcitriol injections and the data from mice receiving calcitriol therefore represent the nadir values of serum $1,25(OH)_2D$ concentrations that peak between 1 and 3 hours after injections (31). The mice receiving the vitamin D–deficient diet also registered a significant decrease in the mean serum $1,25(OH)_2$ D level. Interestingly, mice ingesting the vitamin D–supplemented diet displayed a substantial and statistically significant elevation in the mean serum $1,25(OH)_2D$ level, a phenomenon we previously described in tumor-bearing mice in other mouse models of breast and prostate cancers and is thought to be because of intra-tumoral synthesis of $1,25(OH)_2D$ and its release into the circulation (15). Although the mean serum calcium level in the calcitriol-treated group was slightly higher than the mean serum calcium in the group receiving the standard diet, this increase was not statistically significant (Table 1). There

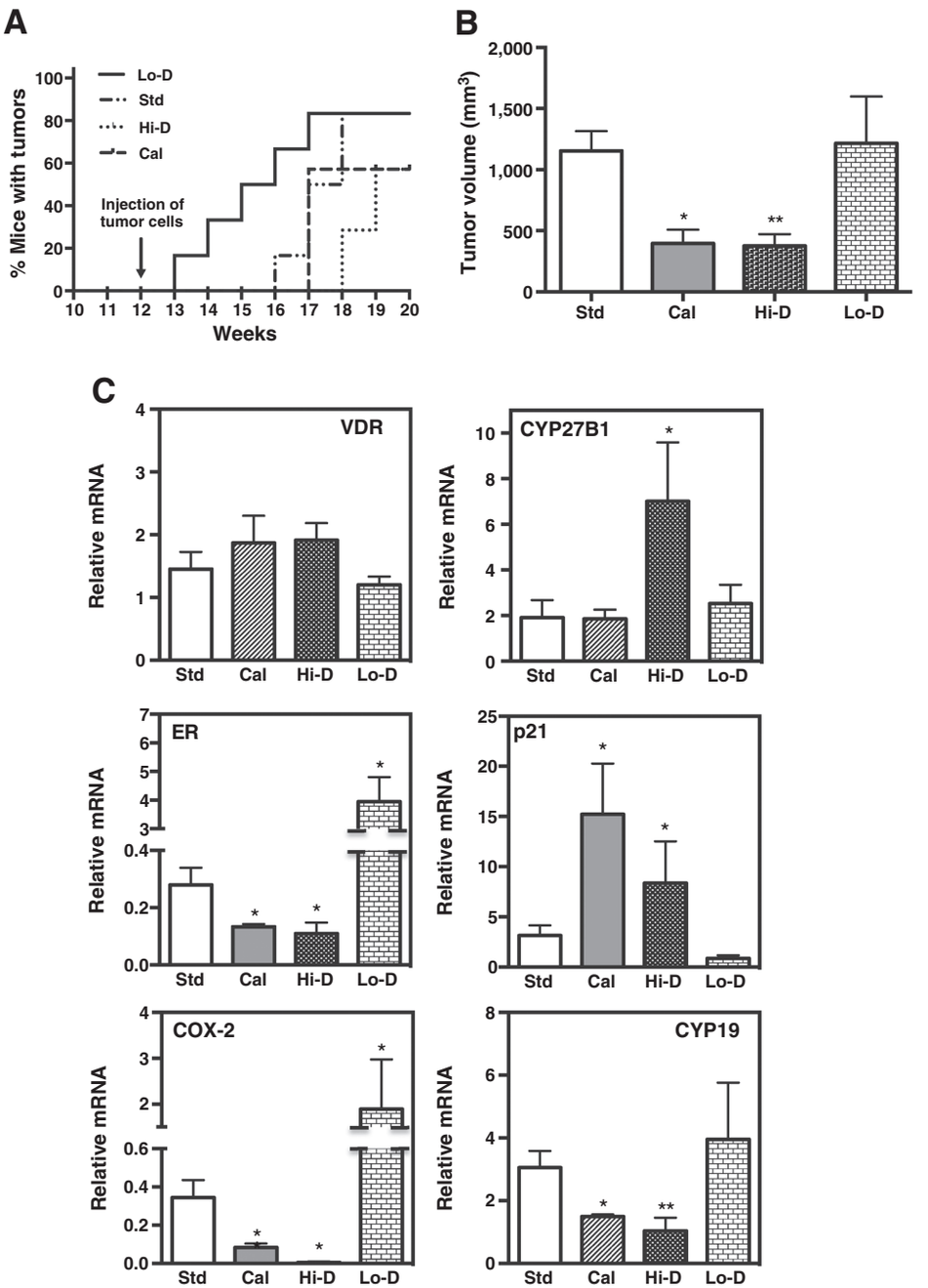

Figure 2.
Effect of calcitriol and dietary vitamin D_3 levels on the appearance and growth of MMTV-*Wnt1* tumors. FVB/N mice were fed the standard diet (Std), a vitamin D–deficient diet (Lo-D), or a vitamin D–supplemented diet (Hi-D) for 12 weeks (experimental weeks 0–12). A parallel experimental group consisted of mice on the standard diet receiving calcitriol injections (Cal) in the last 7 weeks of the 12-week period (experimental weeks 6–12). On experimental week 12, MMTV-*Wnt1* tumor cell suspensions were implanted in the left inguinal mammary fat pads of the mice. Diets and calcitriol treatments were continued for the next 7 weeks (experimental weeks 12–19) and tumor appearance and growth were monitored. A, tumor appearance. Kaplan–Meier analysis of the occurrence of palpable tumors in mice in the various experimental groups: Std, Lo-D, Cal, and Hi-D. B, endpoint tumor volumes. Tumor volumes in the various experimental groups at the end of the study are shown. (The experiment was performed twice with half the number of mice each time. Total number of mice in each group was as follows: Std diet = 10; Cal = 6; Hi-D = 6; and Lo-D = 6; *, $P < 0.05$ and **, $P < 0.001$ as compared to the Std group.) C, changes in mRNA expression of *Vdr*, *CYP27B1*, *Erα*, *p21*, *Cox-2*, and aromatase (*Cyp19*) in MMTV-*Wnt1* tumors because of calcitriol and dietary vitamin D status. Relative mRNA expression of each gene is shown with the expression in tumors from mice on the standard diet (Std) set at 1. (n = 6–10 determinations; *, $P < 0.05$ as compared to Std.) Values represent mean ± SEM.

were no statistically significant differences in mean serum calcium levels between the other experimental groups (Table 1). There were no significant differences in body weights of the mice in the various experimental groups (Table 1).

Changes in gene expression due to dietary vitamin D_3 and calcitriol in MMTV-*Wnt1* tumors

We next examined the effect of dietary vitamin D supplementation and calcitriol administration on gene expression in

Table 1. Body weights and serum measurements

Groups	Body weight calcium (g)	Serum 25-hydroxy-vitamin D (ng/mL)	Serum vitamin D (pg/mL)	Serum 1,25-dihydroxy-vitamin D (mg/dL)
Std	23 ± 0.8	47 ± 3	91 ± 10	10.5 ± 2
Cal	21 ± 0.8	35 ± 11	46 ± 8[a]	11.2 ± 3
Hi-D	22 ± 0.7	74 ± 5[a]	197 ± 33[a]	10.5 ± 1
Lo-D	24 ± 0.6	10 ± 4[a]	43 ± 3[b]	9.5 ± 3

Abbreviations: Cal, standard diet+calcitriol; Hi-D, high vitamin D diet; Lo-D, vitamin D-deficient diet; Std, standard diet.
[a]$P < 0.05$.
[b]$P < 0.01$ when compared with Std diet. Values are represented as mean ± SEM of 5 to 8 determinations for each group.

orthotopic MMTV-*Wnt1* tumors as a measure of their abilities to inhibit estrogen synthesis (*Aromatase*) and signaling (*Erα*) as well as to exert anti-inflammatory (*Cox-2*) and antiproliferative (*p21*) activities. As shown in Fig. 2C, MMTV-*Wnt1* tumors expressed *Vdr* and *CYP27B1* mRNA at levels that were not altered by calcitriol or dietary vitamin D except for the increase seen in *CYP27B1* mRNA in mice receiving the vitamin D–supplemented diet, a phenomenon we have described previously in other mouse models of breast cancer (15). Calcitriol and dietary vitamin D administration resulted in significantly decreased tumor mRNA expression of *Erα*, aromatase (*Cyp19*) and *Cox-2* compared to mice fed the standard diet, whereas the mRNA levels of the cell-cycle inhibitor *p21* was increased by calcitriol and dietary vitamin D supplementation (Fig. 2C). Dietary vitamin D deficiency had the opposite effects causing increased expression of *Erα*, aromatase and *Cox-2* mRNA and decreased *p21* mRNA in the tumors. Thus, the dietary content of vitamin D and the dose of calcitriol that we used were sufficient to lead to vitamin D signaling-related gene expression changes in the tumors, whereas the vitamin D deficient diet caused a reversal of these expression patterns.

Calcitriol targets TICs

Given the ability of dietary vitamin D and calcitriol to inhibit tumor growth and previous observations of effects of vitamin D compounds on normal stem cells and TIC-like cells from cell lines (16–19), we hypothesized that these agents target TICs in MMTV-*Wnt1* mammary tumors. To functionally test this hypothesis, first we sorted the CD49f^{high}Epcam^{low} population of cells, that has been shown to be significantly enriched for TICs (14, 20), from freshly dissociated tumors (Fig. 3A) and treated them with calcitriol *in vitro* overnight. Although calcitriol treatment did not change *Vdr* gene expression, it decreased the expression of *Cyp27b1* mRNA and increased the expression of *Cyp24a1* mRNA, demonstrating that TICs in MMTV-*Wnt1* mammary tumors express VDR, which is functionally responsive to calcitriol (Fig. 3B–D).

To further examine whether vitamin D compounds functionally target TICs, we plated TICs from MMTV-*Wnt1* mammary tumors in a previously described three-dimensional tumor spheroid culture assay (14). Treatment with calcitriol decreased TIC spheroid formation in a dose-dependent manner (Fig. 4A) and calcitriol-treated TICs formed fewer spheroids than vehicle-treated TICs in secondary passage without further calcitriol addition, suggesting that calcitriol inhibits both TIC-initiated spheroid formation and the ability of TICs to self-renew (Fig. 4B).

Previous studies from several labs, including ours, have shown that TICs are more resistant to ionizing radiation and chemotherapy than nontumorigenic cancer cells (3, 4). Because calcitriol was able to target TICs and inhibit their proliferation, we tested whether calcitriol could also enhance the inhibitory effect of ionizing radiation on TICs. As shown in Fig. 4C, calcitriol in combination with a clinically relevant dose of ionizing irradiation (2 Gy) showed an additive effect in inhibiting TIC spheroid formation.

To demonstrate that calcitriol treatment inhibits not only the proliferation of TICs but also their tumor initiating activity, we tested whether calcitriol could suppress *in vivo* tumor forming ability of TICs. Purified CD49f^{high}Epcam^{low} TIC-enriched cells were treated with vehicle (ethanol) or calcitriol, dissociated into single cell suspensions, and orthotopically implanted into syngeneic mice in limiting dilution fashion. As shown in Table 2, calcitriol treatment decreased the frequency of TICs by greater

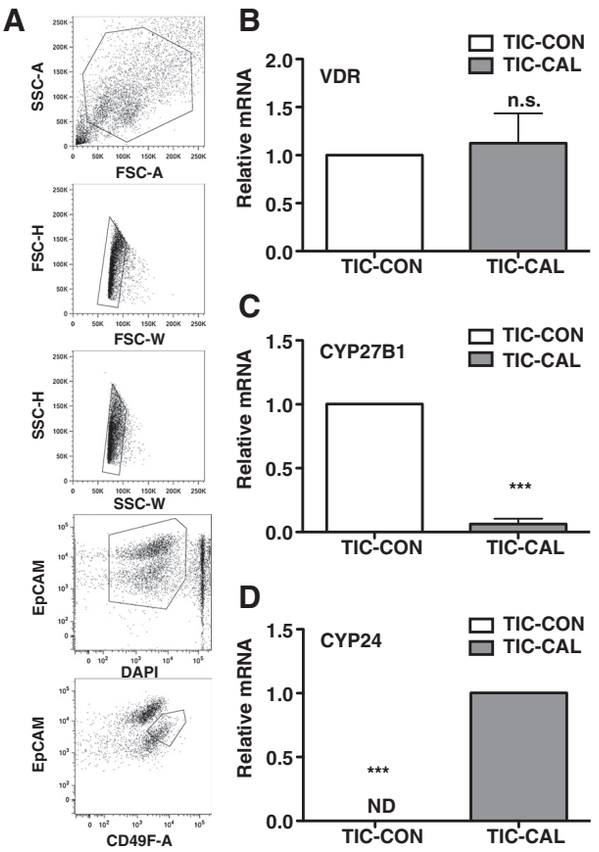

Figure 3.
Breast cancer TICs express functionally active vitamin D receptor. A, FACS sorting schema for purification of CD49f^{hi}EpCam^{low} MMTV-Wnt-1 TIC-enriched cells. B–D, VDR functional responses in breast cancer TICs. Sorted CD49f^{hi}EpCam^{low} TIC cells were treated with 1 nmol/L calcitriol (Cal) for 16 hours and mRNA levels of *Vdr*, *Cyp24*, and *Cyp27b1* were determined by qRT-PCR ($n = 3$; ***, $P < 0.01$ as compared to the CON group; n.s., not significant; ND, not detected). Values represent mean ± SEM.

than three-fold [control group: 1/98 (1/66–1/147), calcitriol-treated group: 1/320 (1/207–1/495), $P < 0.0046$]. In addition, the appearance of palpable tumors was significantly delayed by calcitriol pretreatment (Fig. 5). Taken together, these data demonstrate that calcitriol targets TICs and inhibits their tumor initiating ability.

Inhibition of Wnt/β-catenin signaling pathway contributes to the anti-TIC effect of calcitriol and vitamin D

Wnt/β-catenin signaling plays an important role in normal and cancer stem cell function (32, 33). It is activated in triple negative breast cancers, and has been shown to be associated with poor survival in these patients (34, 35). We therefore hypothesized that calcitriol inhibits the Wnt/β-catenin pathway in TICs from MMTV-*Wnt1* tumors. We first examined the effect of calcitriol *in vivo* on the expression of the *Wnt* target genes (*Lef1*, *Axin2*, *Cdh1*, *Ccnd*, and *Tcf4*) in MMTV-*Wnt1* tumors. As shown in Fig. 6A, calcitriol administration to mice significantly reduced expression of the *Wnt* target genes in MMTV-*Wnt1* orthotopic tumors compared to the mice on the standard diet. Consistently, *in vitro* treatment of TIC cultures with calcitriol for 16 hours also

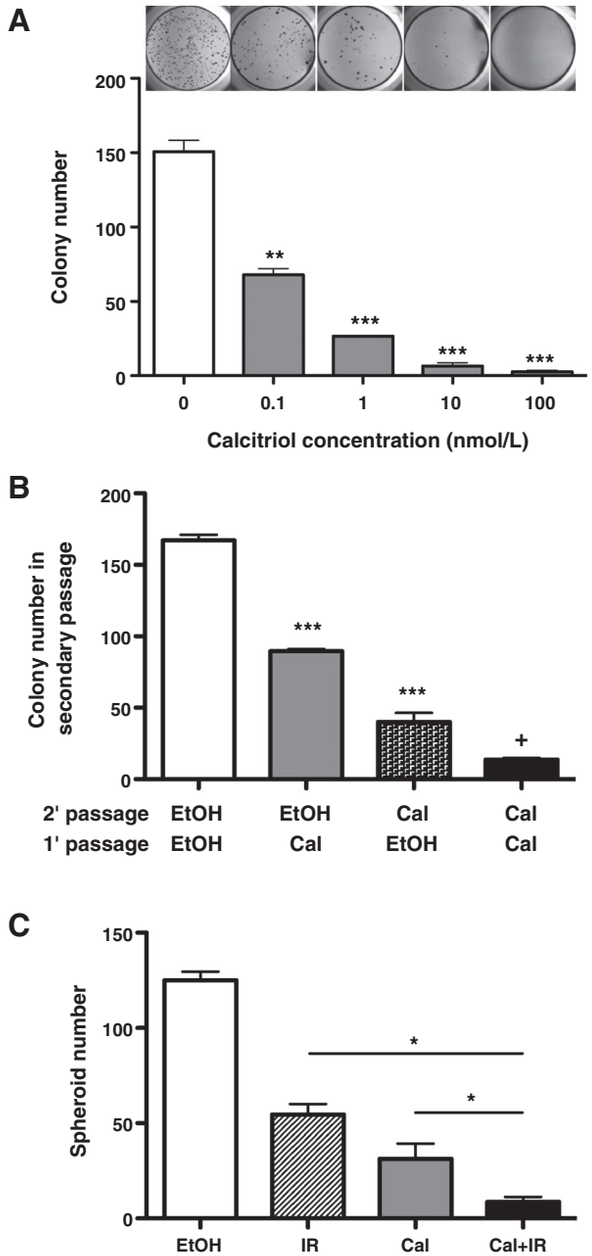

Figure 4.
Calcitriol inhibits the self-renewal of breast cancer TICs. A, representative images and bar graphs showing that calcitriol treatment (vehicle or 0.1, 1, 10, and 100 nmol/L) decreased the spheroid-forming abilities of TICs in a dose-dependent manner ($n = 3$; **, $P < 0.01$; ***, $P < 0.001$ as compared to the control). B, calcitriol treatment (at 1 nmol/L) in primary culture reduced the spheroid formation in secondary culture ($n = 3$; ***, $P < 0.001$ as compared to white bar; +, $P < 0.001$ as compared to dotted bar). C, analysis of combined treatment of TICs with calcitriol (10 nmol/L) and 2 Gy of ionizing radiation ($n = 3$; *, $P < 0.05$). Values represent mean ± SEM.

decreased the expression of these *Wnt* target genes (Fig. 6B). However, *Wnt1* gene expression was not significantly affected by calcitriol treatment (Supplementary Fig. S1), showing that calcitriol inhibits Wnt/β-catenin pathway without inhibiting expression of *Wnt1*.

Table 2. Limiting dilution analysis

Cell number implanted	Vehicle treatment	Calcitriol treatment
500	5/6	4/5
100	5/5	1/5
50	4/5	1/5
TIC frequency ($P < 0.01$)	1/98 (1/66–1/147)	1/320 (1/207–1/495)

NOTE: Number of tumors formed/number of injections. Limiting dilution analysis of purified CD49f^{high}EpCam^{low} TIC-enriched cells from MMTV-*Wnt*1 mammary tumors. TICs were cultured in the presence of calcitriol (1 nmol/L) or vehicle (ethanol). After 2 weeks, breast tumor spheroids were dissociated into single cells, and varying numbers of viable tumor cells were orthotopically implanted into the mammary fat pad and assayed for tumor initiation. TIC frequencies and *P* value were calculated using L-Calc.

Finally, to examine the role of the repression of Wnt signaling in calcitriol's inhibitory effect on TICs, we tested whether the constitutive expression of β-catenin could overcome calcitriol's inhibitory effect on TIC spheroid formation. A constitutively active β-catenin expression plasmid was introduced into CD49f^{high} Epcam^{low} TIC-enriched cells using lentiviral transduction (36). As shown in Fig. 6C, the overexpression of β-catenin completely abrogated the inhibitory effect of calcitriol on TIC spheroid formation. These data indicate that inhibition of the Wnt/β-catenin signaling pathway is essential for calcitriol regulation of TICs.

Discussion

Intriguing but inconsistent epidemiological data have generally supported the hypothesis that vitamin D may improve breast cancer risk and prognosis (7, 37). Consistent and compelling data from cultured cell experiments have demonstrated a substantial inhibitory effect of calcitriol on breast cancer cell growth, suggesting the epidemiologic benefits result from a direct causative effect of vitamin D signaling on breast cancer (7, 10, 11). *In vivo* experiments using a variety of mouse tumor models have likewise shown tumor regression in response to calcitriol and vitamin D (6–11). In addition, *Vdr*-null mice show increased susceptibility to carcinogen-induced breast cancer (11). At a mechanistic level, many genes relevant to cancer progression are regulated by calcitriol and a number of molecular pathways appear to be involved in the anticancer effects of calcitriol (5–7). However, the essential molecular and cellular mechanisms underlying the effects of vitamin D signaling on breast cancer remain incompletely understood.

New insight into cancer biology has identified a subpopulation of cells in certain tumors, including breast cancer, as competent to regenerate entire tumors and therefore targeting these cells is critical for curing patients. Identification of existing and novel agents that target breast TICs promises to reduce breast cancer incidence and improve disease outcomes. If calcitriol inhibited breast TIC activity as a part of its effect on breast cancer, its potential for breast cancer prevention and treatment would be more promising. Recently, vitamin D and calcitriol were shown to regulate a cell line model of prostate progenitor/stem cells and mesenchymal stem cells (16, 38, 39), suggesting the possibility that vitamin D could also inhibit breast TICs. Furthermore, treatment of breast cancer cell lines with calcitriol or an analogue suppressed CD44 expression (17) and repressed pluripotency markers and markers associated with stem cell–like phenotypes (19), whereas *VDR* silencing enhanced mammosphere formation and expression of genes involved in EMT (18). However, these studies focused on TIC-like cells in long-term cultured breast cancer cell lines whose

relationship to TICs in primary tumors remains unclear. In addition, these studies did not directly examine whether a change in TIC frequency occurs after calcitriol treatment.

In our study, we have demonstrated that calcitriol treatment of MMTV-*Wnt1* tumor tissue slices resulted in increased expression of *Cyp24* mRNA, a marker for the actions of calcitriol. This was accompanied by decreases in *Erα* and *Cyp19* mRNA suggesting decreased estrogen synthesis and estrogen signaling. Tissue slices contain an unsorted population of cells representing a mixture of both TICs and non-TICs and it is not possible to conclude whether the calcitriol responsiveness was because of TICs, non-TICs, or both from these experiments alone. We therefore purified TICs from tumor orthografts in order to specifically analyze effects of calcitriol on this subpopulation. We demonstrated that calcitriol treatment not only suppressed TIC-initiated tumor spheroid formation in primary culture but also decreased spheroid formation in the secondary passage, even without further calcitriol treatment of cells in the secondary passage. Furthermore, we corroborated these findings by limiting dilution analysis in mice. These results suggest that calcitriol targets the ability of breast cancer TICs to self-renew. In addition, we found that the combination of calcitriol and a clinically relevant dose of ionizing radiation eliminated more TICs than either treatment alone. Finally, we provide evidence that the mechanism of action is at least partly because of inhibition of the *Wnt* pathway and that constitutive *Wnt* pathway activation can overcome the TIC inhibitory effect of calcitriol.

Combinations of vitamin D compounds and anticancer drugs have been evaluated in experimental models of a variety of cancers as well as in a few clinical trials (7, 40). The rationale behind these studies is that the combination approach could reduce toxicity by allowing the use of lower doses of the individual agents to still elicit significant anticancer activity. Combinations of calcitriol and cytotoxic chemotherapy or aromatase inhibitors have been shown to elicit enhanced antiproliferative and apoptotic effects in breast cancer cell lines (41, 42) and enhanced antitumor effects in mouse models (27, 43, 44). However, a phase II clinical trial evaluating the combination of a high dose of calcitriol with docetaxel in advanced prostate cancer patients yielded negative findings (45). These results have been criticized as having been impacted by trial design issues and the advanced stage of cancer in the

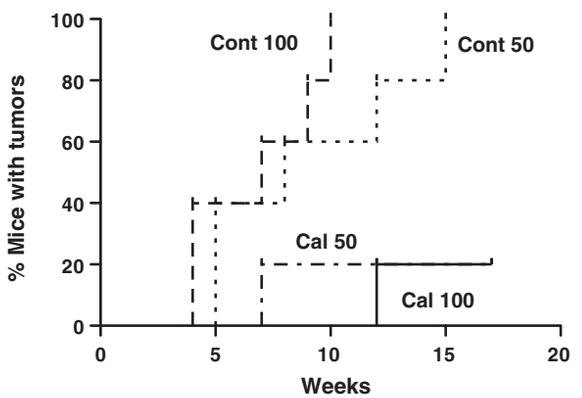

Figure 5.
Analysis of time to tumor appearance. Kaplan-Meier plot depicting time to appearance of gross tumors after orthotopic implantation of 50 or 100 control (vehicle-treated; Cont) or calcitriol-treated (Cal) cells.

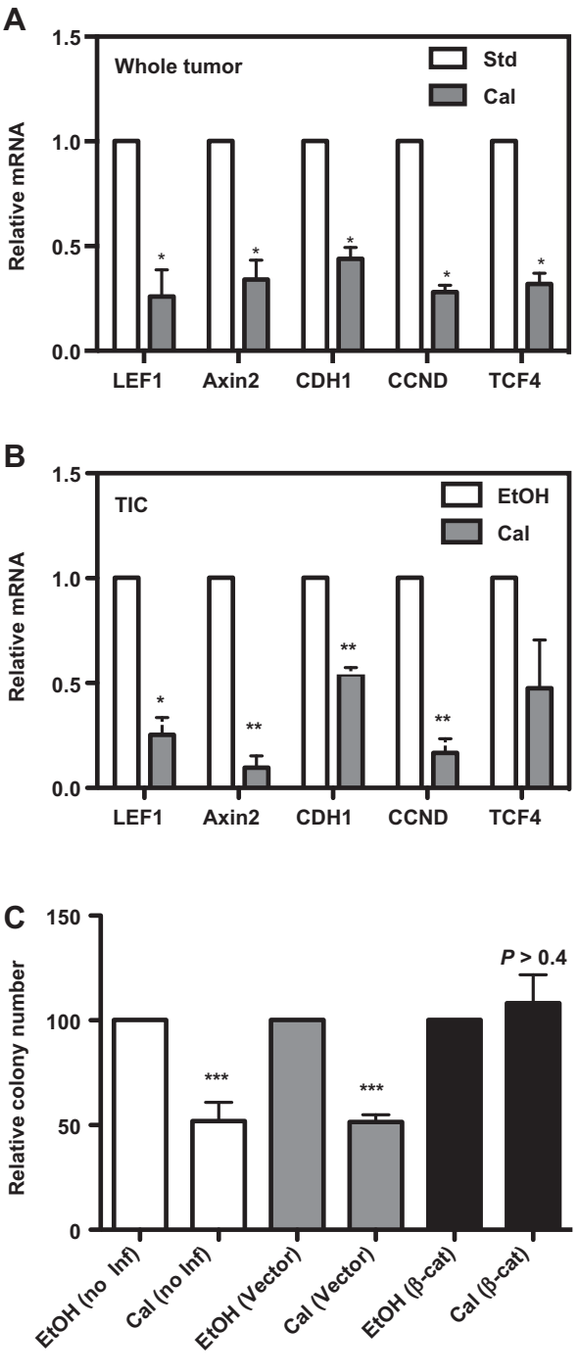

Figure 6.
Calcitriol inhibits *Wnt* signaling in TICs. A, changes in the expression of *Wnt* target genes in MMTV-*Wnt1* mammary tumors harvested from mice on the standard diet (Std) and those receiving calcitriol (Cal). Relative mRNA expression of each gene is shown with the expression in tumors from mice on the standard diet (Std) normalized to 1 ($n = 6$–10; *, $P < 0.05$). B, changes in the expression of *Wnt* target genes in CD49f^{hi}EpCAM^{low} TIC-enriched cells treated with ethanol or calcitriol (1 nmol/L) for 16 hours. Relative mRNA expression of each gene is shown ($n = 3$; *, $P < 0.05$; **, $P < 0.01$). C, effect of constitutively active β-catenin on calcitriol sensitivity of TICs. Spheroid formation by CD49f^{hi}EpCam^{low} cells was assayed after treatment with vehicle (ethanol) or calcitriol (1 nmol/L) following transduction with control or constitutively active β-catenin expressing lentiviruses ($n = 5$–6; ***, $P < 0.001$). Values represent mean ± SEM.

study population (40). Importantly, our observation that vitamin D compounds target TICs suggests that future clinical studies should incorporate analysis of TICs as endpoints since therapeutic effects on TICs could be missed if only tumor size–based response metrics are considered.

Our study also provides evidence that the addition of calcitriol may be a useful strategy for enhancing anti-TIC effects of ionizing radiation. We, and others, have previously shown that TICs are relatively resistant to radiotherapy and chemotherapy compared to NTCs (3, 46) and this likely contributes to treatment failure. Our data revealed that calcitriol in combination with irradiation demonstrated an additive effect on the suppression of TIC-initiated spheroid formation, suggesting that these therapies likely target different pathways and provide a rationale for considering combining the two agents in clinical trials. Our findings are in agreement with previous reports documenting a sensitizing or additive effect of calcitriol with ionizing radiation (47–49). It will be interesting to further explore the molecular effects of combined treatment with calcitriol and radiation and whether calcitriol can also enhance the anti-TIC effects of chemotherapy.

Recent studies show that vitamin D compounds exert inhibitory effects on TIC signaling pathways such as Notch, Hedgehog, Wnt and TGF-β. Our data indicate that the mechanism of the inhibition of TICs from MMTV-*Wnt1* tumors by calcitriol is at least in part mediated by the inhibition of the Wnt/β-catenin signaling pathway. Wnt/β-catenin signaling has previously been implicated in various cancers, including colorectal cancer, hepatocellular carcinoma, acute myeloid leukemia, and breast cancer and plays an important role in the self-renewal and maintenance of TICs in breast and colorectal cancers (4, 32, 50, 51). β-Catenin signaling has been shown to promote EMT (52) and is implicated in stem/progenitor cell survival (2, 53). Furthermore, recent studies have provided evidence for the activation of β-catenin signaling in triple negative breast cancers and shown that β-catenin activation portends poor survival (34, 35). Similar to our findings in breast TICs, studies in colon cancer cells have shown that calcitriol represses the Wnt/β-catenin signaling pathway (9). *Vdr*-null colon cancers show higher expression of Wnt/β-catenin target genes and enhanced nuclear translocation of β-catenin (54). Calcitriol has also been shown to induce the expression of the Wnt antagonist Dickkopf-1 (55). In our experiments, calcitriol decreased the expression of Wnt target genes in breast TICs both *in vivo* and *in vitro* while not affecting the expression of Wnt1 itself. Importantly, constitutive activation of β-catenin counteracted calcitriol's TIC inhibitory effect, indicating that vitamin D compounds can inhibit TICs by suppressing Wnt/β-catenin. Further studies are needed to elucidate the mechanisms underlying calcitriol inhibition of Wnt/β-catenin signaling in TICs. Future studies also should evaluate if this mechanism is active in other breast cancer models as well as patient-derived xenografts and whether measurement of baseline Wnt/β-catenin activity may allow selection of patients who will be most likely to benefit from calcitriol treatment.

In conclusion, we have demonstrated that vitamin D compounds, both vitamin D dietary supplements and calcitriol, inhibit MMTV-*Wnt1* tumor growth and that vitamin D deficiency accelerates tumor growth. In mechanistic studies, we showed that calcitriol targets breast cancer TICs by suppressing the Wnt/β-catenin signaling pathway. Developing a deeper understanding of vitamin D signaling in TICs will likely elucidate additional pathways that may be important in self-renewal and survival of these cells and could thus lead to new strategies for their elimination. Furthermore, our findings suggest that vitamin D compounds represent potential therapeutic agents for targeting breast cancer TICs and that avoidance of vitamin D deficiency might improve breast cancer risk and prognosis. Future clinical trials employing vitamin D compounds in breast cancer will likely benefit from combining these agents with other therapies and incorporating translational endpoints focused on quantifying TICs.

Disclosure of Potential Conflicts of Interest

R.L. Horst has ownership interest in Heartland Assays, LLC. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and provided facilities, etc.): Y. Jeong, S. Swami, A.V. Krishnan, J.D. Williams, S. Martin, M.A. Albertelli, M. Diehn,

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Jeong, S. Swami, A.V. Krishnan, S. Martin, R.L. Horst, B.J. Feldman, D. Feldman, M. Diehn

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Study supervision: Y. Jeong, S. Swami, D. Feldman, M. Diehn

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