

# Chemoprevention Activity of 25-Hydroxyvitamin D in the MMTV-PyMT Mouse Model of Breast Cancer

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## Abstract

Development of oncologic conditions is often accompanied by inadequate vitamin D status. The chemoprevention ability of this molecule is of high interest for breast cancer, the most common malignancy in women worldwide. Because current effective vitamin D analogues, including the naturally occurring active metabolite 1,25-dihydroxycholecalciferol (1,25(OH)<sub>2</sub>D), frequently cause hypercalcemia at pharmacologic doses, the development of safer molecules for clinical chemopreventive use is essential. This study examines whether exogenously supplied prohormone 25-hydroxycholecalciferol (25(OH)D) can delay tumor progression *in vivo* without hypercalcemic effects. A low vitamin D diet (25 IU/kg) in the non-immunodeficient MMTV-PyMT mouse model of metastatic breast cancer revealed a significant acceleration of mammary neoplasia compared with normal diet (1,000 IU/kg). Systemic

perfusion of MMTV-PyMT mice with 25(OH)D or 1,25(OH)<sub>2</sub>D delayed tumor appearance and significantly decreased lung metastasis, and both metabolites reduced Ki-67, cyclin D1, and ErbB2 levels in tumors. Perfusion with 25(OH)D caused a 50% raise in tumor 1,25(OH)<sub>2</sub>D levels, indicating good tumor penetration and effective activation. Importantly, in contrast with 1,25(OH)<sub>2</sub>D, perfusion with 25(OH)D did not cause hypercalcemia. *In vitro* treatment of cultured MMTV-PyMT mammary tumor cells with 25(OH)D inhibited proliferation, confirming local activation of the prohormone in this system. This study provides an *in vivo* demonstration in a non-immunodeficient model of spontaneous breast cancer that exogenous 25(OH)D delays neoplasia, tumor growth, and metastasis, and that its chemoprevention efficacy is not accompanied by hypercalcemia. *Cancer Prev Res*; 8(2); 120–8. ©2014 AACR.

## Introduction

Breast cancer is the most common malignancy in women worldwide, with more than 220,000 new cases reported in the United States alone in 2012, and is the second leading cause of female cancer-related death (1). The discovery of novel and effective chemopreventive agents for people at higher risk of developing mammary malignancies could help reduce cancer appearance or delay its progression. Among several agents under study, the biologically active form and analogues of vitamin D appear promising due to their antiproliferative, prodifferentiating, anti-inflammatory, and immunomodulatory activities (2).

Vitamin D (cholecalciferol) is the essential precursor to the potent steroid hormone calcitriol that has effects in almost every cell in the body and influences proliferation, differentiation, and apoptosis events (3–6). Humans obtain vitamin D in their diet but the largest input occurs in the skin through sunlight conversion of 7-dihydrocholesterol to previtamin D<sub>3</sub> and cholecalciferol. Cholecalciferol is activated after hydroxylation into calcidiol (25(OH)D) by liver CYP27A1 hydroxylase, and further hydroxylation by renal CYP27B1 into the biologically active 1,25-dihydroxycholecalciferol (1,25(OH)<sub>2</sub>D). Renal CYP27B1 is tightly regulated by calcium and parathyroid hormone to maintain optimal circulating levels of 1,25(OH)<sub>2</sub>D. Kidney was originally believed to be the only site of 1,25(OH)<sub>2</sub>D production. However, elevated 1,25(OH)<sub>2</sub>D levels observed after bilateral nephrectomy (7) suggested the existence of extra-renal hydroxylation, and CYP27B1 was subsequently confirmed in several tissues among which breast where tissue-specific signals control its activity, and its product 1,25(OH)<sub>2</sub>D is not secreted but mostly displays autocrine/paracrine effects (8–14). To exert its biologic activity, 1,25(OH)<sub>2</sub>D binds the vitamin D receptor that heterodimerizes with the retinoid X receptor and interacts with discrete vitamin D-responsive elements in DNA as a ligand-activated transcription factor and influences the expression of hundreds of genes (6, 15).

Despite a large body of evidence supporting an inverse association between vitamin D levels and cancer in general (5, 16), and breast cancer in particular (17–20), the epidemiologic evidence remains controversial (21–24) and a 20,000 subject 5-year primary cancer and cardiovascular disease prevention trial for

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vitamin D (VITAL) is ongoing (25). Because tissues do not respond to vitamin D identically, further studies are needed to determine the dose–response relation between vitamin D status and cancer risk, optimal treatment duration, time of life when exposure is most relevant, and optimal metabolite to use.

Various vitamin D metabolites present different absorption and transformation rates; cholecalciferol metabolism, for example, depends on hepatic health (26) and its circulating half-life is short (27). Consequently, other natural and synthetic vitamin D metabolites are investigated for clinical use. Apart from its classical role in calcium and phosphate homeostasis, 1,25(OH)<sub>2</sub>D displays antineoplastic activity (28), which proceeds through growth arrest and differentiation, induction of apoptosis, inhibition of invasion, metastasis, and angiogenesis, as well as anti-inflammatory effects (29). Most anticancer trials have been conducted with 1,25(OH)<sub>2</sub>D; however, a major drawback of this molecule is the possibility of toxic hypercalcemic side effects (28). An intermittent administration protocol must be followed to avoid hypercalcemia, and important benefits on tumor outcome is rarely seen (29). Novel 1,25(OH)<sub>2</sub>D analogues with low calcemic capacity are widely used in treatment of psoriasis, secondary hyperparathyroidism, and parathyroid hyperplasia (15), but still cause hypercalcemia in cancer therapy where high doses must be used for long periods and produce inconsistent antitumor results (29).

The immediate metabolic precursor to the biologically active 1,25(OH)<sub>2</sub>D is prohormone 25(OH)D. Several epidemiologic studies suggest an inverse relationship between levels of circulating 25(OH)D and cancer survival (16, 19, 30). The presence of CYP27B1 in many target tissues, including breast (31, 32), allows local transformation of prohormone 25(OH)D into 1,25(OH)<sub>2</sub>D, pointing to possible local activation and tumor growth repression. 25(OH)D can inhibit chemically induced mammary alveolar lesions in *ex-vivo* mouse organ culture (33), and *in vivo* evidence that exogenous 25(OH)D can delay the disease or prolong survival would be of high interest for chemoprevention protocols. Consequently, this study investigates the anticancer chemoprevention potential for 25(OH)D using the mouse mammary tumor virus promoter-driven polyoma middle T oncoprotein (MMTV-PyMT) mouse, an oncogene-driven model of highly aggressive spontaneous mammary tumors that closely mimics the human disease and is widely used to model estrogen receptor (ER)–negative breast cancer that metastasizes to lung (34–37). The *in vivo* model allows follow-up of both primary tumor development and lung metastasis without discontinuity, and provides the advantage of an intact immune system, an important part of the cancer equation missing in xenograft models. Using the MMTV-PyMT model, we show that that exogenous 25(OH)D activated into 1,25(OH)<sub>2</sub>D within breast tumor cells delays neoplasia, tumor growth, and metastasis without inducing detrimental hypercalcemic effects.

## Materials and Methods

### Animals

MMTV-polyoma middle T antigen (PyMT) transgenic mice (strain #634) on an FVB background were obtained from Dr W. Muller (McGill University, Montréal, QC, Canada). In this model, all mammary glands display tumors by 14 to 16 weeks (37). Male homozygous PyMT mice were randomly bred with FVB females lacking the PyMT transgene to obtain female mice het-

erozygous for the PyMT transgene that were crossed to obtain homozygous female MMTV-PyMT 634. All mice analyzed in this study were homozygous for the PyMT transgene on FVB background.

### Low vitamin D diet

Female FVB MMTV-PyMT mice were housed in individual cages in a UVB light-free environment (Clear UV Tube Guards, Pegasus Lighting) on a 12-hour light–dark cycle and were randomized to AIN93M diets with low (25 IU: 0.625 µg) or normal (1,000 IU: 25 µg) levels of vitamin D<sub>3</sub>/kg (Harlan) from weaning (3 weeks) until sacrifice (*n* = 15 mice/group).

### Hyperplasia measurements

Tissues were fixed, embedded, sliced, and photomicrographs of hematoxylin and eosin (H&E)–stained slides of breast tissue at 6 weeks were analyzed with the ImageJ software (<http://rsbweb.nih.gov/ij/index.html>).

### Cell culture and proliferation assays

Spontaneous primary mammary tumors were harvested from 12-week-old MMTV-PyMT animals, minced and incubated in DMEM (without FBS) containing 2.4 mg/mL collagenase B and dispase II (Roche) at 37°C for 2 hours. Floating cells were collected and propagated in DMEM (10% FBS), passaged three times, and aliquots were frozen. Cells were tested for viability and population uniformity by flow cytometry (next section). For proliferation assays, 24-well plates were seeded with 5,000 cells per well, incubated for 24 hours in complete DMEM, and then serum-starved for 6 hours. The cells were treated with either 1,25(OH)<sub>2</sub>D (10<sup>−7</sup> mol/L) or 25(OH)D (10<sup>−7</sup> mol/L) in complete DMEM 24 hours, trypsinized, and counted on a Z1 Coulter Counter (Beckman). 1,25(OH)<sub>2</sub>D and 25(OH)D were from Sigma-Aldrich.

### Flow cytometry

Cultured MMTV-PyMT tumor cells were assessed for population uniformity by flow cytometry using CK8 markers (anti-CK8-AF647; Novus Biologicals). CK8 is a cytokeratin indicator of cells of epithelial origin and a modulator of cell adhesion/growth-dependent signal transduction in breast tumor cells (38). The cells were tested for viability by Fixable Viability Dye-efluor 506 staining (Affymetrix eBioscience). Cells (1 × 10<sup>6</sup>) were stained on ice for 30 minutes with 1-µL dye in 1-mL PBS, washed twice with PBS, and resuspended in 100-µL PBS with 5-µL of one of the fluor-linked antibodies on ice 30 minutes. Fluorescence-activated cell sorting analysis was conducted using a BD LSR Fortessa Cell Analyzer (BD Biosciences).

### Perfusion conditions

Four-week-old female MMTV-PyMT mice under light anesthesia (ketamine 100 mg/kg, xylazine 10 mg/kg, and acepromazine 3 mg/kg in 0.9% NaCl) were implanted subcutaneously with osmotic minipumps (Alzet model 2004; Alza Corporation). Each minipump contained either 1,25(OH)<sub>2</sub>D, 25(OH)D, or vehicle dissolved in 1 mL of 1:4 ethanol:saline solution, and delivered a continuous dose for 4 weeks at a rate of 0.25 µL/h. Animals received 1,25(OH)<sub>2</sub>D (12 pmol/24 h) or 25(OH)D (2,000 pmol/24 h). Pumps were reimplanted after 4 weeks and the same continuous doses delivered for another 4 weeks until sacrifice (12-week-old animals, total treatment duration: 8 weeks).

### Tumor palpation

For the Kaplan–Meier analysis, mammary glands of female mice (genotype-blinded) were palpated twice-weekly from 4 weeks (treatment beginning) until sacrifice. Tumor diameter long axis ( $L$ ) and mean mid-axis width ( $W$ ) were measured with calipers to estimate tumor volume using:

$$V = \frac{4}{3}\pi\left(\frac{LW}{2}\right)$$

Growth curves were generated by plotting mean tumor volume beginning at 12 weeks. Female mice were sacrificed before tumor diameters reached 1.5 cm. All mammary tumors were excised and weighed. Random selections of mammary tumor carcinomas were used for whole mount preparation.

### Histology

Mammary tumor paraffin-embedded tissues sections (5  $\mu\text{m}$ ) were stained with H&E. Immunofluorescence (IF) staining was conducted on deparaffinized sections using goat anti-total Ki-67, mouse cyclin D1 and ErbB2, Alexa fluor 555- and 488-conjugated anti-mouse, or goat IgG (InVitrogen) antibodies. Results were analyzed with an LSM 510 Metaconfocal microscope (Carl Zeiss Microimaging).

### Metastases quantification

Female mice were sacrificed at 12 weeks. Exposed lungs were injected with 2 mL of 10% neutral-buffered formalin by tracheal cannulation to fix inner air spaces and inflate the lung lobes, then excised and formalin-fixed for 48 hours. Representative lungs were paraffin-embedded and processed for histologic analysis. Care was taken so that any evident metastases dissected during sectioning were only represented once in the H&E-stained slides. Lung metastases surfaces were scored in a genotype-blinded fashion with a Nikon SMZ-1500 stereomicroscope, and areas were calculated with BioQuant software (R&M Biometrics). Total area of metastatic tissue was compared between groups and percentage of metastatic area in treated animals expressed as percentage of vehicle-treated mice.

### Measurement of 1,25(OH)<sub>2</sub>D, 25(OH)D, and calcemia levels

Tumors and kidneys were homogenized in 95% ethanol (100 mg tissue in 900  $\mu\text{L}$  ethanol), centrifuged (10,000  $\times$  g; 10 minutes), and the supernatant was frozen until assay. Blood was collected at sacrifice, serum was separated and frozen until radioimmunoassays for 1,25(OH)<sub>2</sub>D and 25(OH)D (39, 40). Briefly, extracts and standards were dried in tubes, mixed with water:acetonitrile (1:1), centrifuged, and the supernatant transferred to a new tube. One sample volume of 12 mmol/L sodium metaperiodate was added and incubated 30 to 60 minutes at room temperature. The metaperiodate destroys metabolites with vicinal diols [1,24,25(OH)<sub>3</sub>D and 24,25(OH)<sub>2</sub>D]. The 1,25(OH)<sub>2</sub>D was further purified from remaining metabolites on Bond-Elut C18-OH columns. Analysis of serum 1,25(OH)<sub>2</sub>D was conducted as described in ref. (39). Calculated assay precision for within and between assay variation was 6% and 16%, respectively, for 25(OH)D assays, and 8% and 18% for 1,25(OH)<sub>2</sub>D assays. The goat anti-25(OH)D antibody was a gift from Dr. Bruce Hollis, Department of Pediatrics, Medical University of South Carolina, Charleston, SC. <sup>125</sup>I-25(OH)D<sub>3</sub> and donkey anti-goat secondary antibody were from Diasorin. Serum calcium was determined with a Synchron 67 autoanalyzer (Beckman). Corrected plasma calcium was calculated using the

formula: plasma total calcium + [(40 – plasma albumin)]  $\times$  0.02. Note that animals were treated with vitamin D<sub>3</sub> metabolites by perfusion but all assays detect vitamin D<sub>2</sub> and D<sub>3</sub> metabolites.

### Western blot analyses

Proteins were extracted from tissues in RIPA buffer and 30 to 50  $\mu\text{g}$  fractionated by SDS-PAGE electrophoresis, blotted, and reacted with primary mouse cyclin D1 and ErbB2 antibodies (Santa Cruz Biotechnology) and anti-mouse horseradish peroxidase (HRP)-linked secondary antibody, and developed by enhanced chemiluminescence.

### Statistical analysis

All results are expressed as mean  $\pm$  SE. Statistical comparisons were made using the unpaired Student  $t$  test ( $P < 0.05$  was considered significant). The statistical difference of tumor onset rate of the animals was determined by the Kaplan–Meier analysis.

### Study approval

These animal studies were approved the McGill University Animal Compliance Office. All experiments were carried out in compliance with regulations of the McGill University Institutional Animal Care Committee. All animal surgeries were conducted in accordance with principles and procedures dictated by the highest standards of humane animal care.

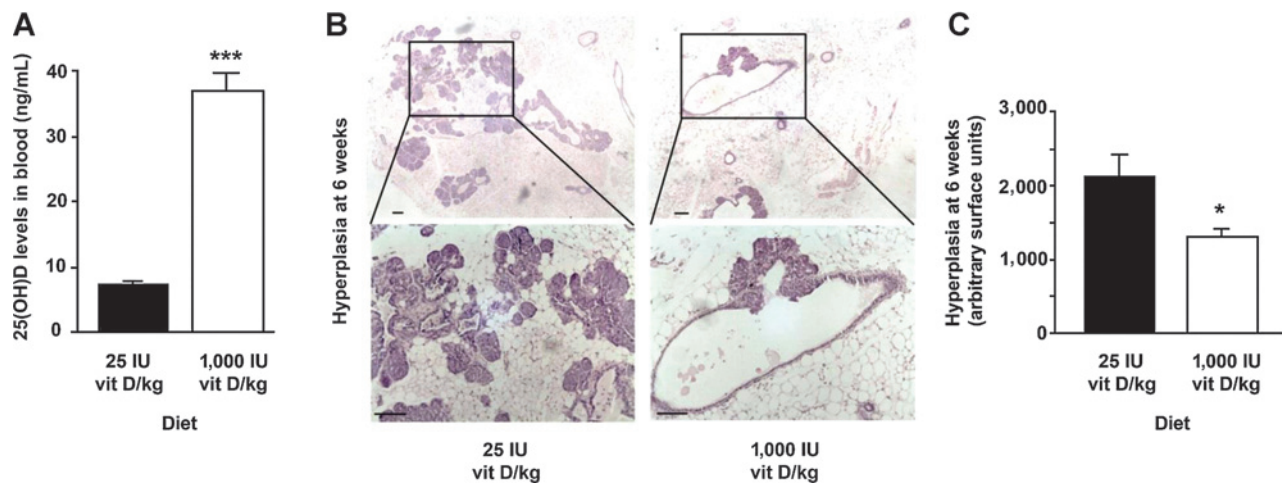
## Results

### Low vitamin D diet accelerates mammary hyperplasia and tumor growth in MMTV-PyMT mice

Vitamin D nutritional input as a cancer-delaying approach was tested in female wild-type MMTV-PyMT mice by feeding the animals a normal (1,000 IU/kg) or low (25 IU/kg) vitamin D<sub>3</sub> diet to achieve either normal or low circulating 25(OH)D levels, as described previously (41). After 6 weeks of treatment, blood 25(OH)D levels in low vitamin D mice were only 18% of those in vitamin D-replete mice (Fig. 1A) and animals on the low vitamin D diet exhibited a significantly faster onset of spontaneous mammary gland hyperplasia (Fig. 1B and C). These data show that dietary vitamin D insufficiency accelerates the appearance and progression of mammary tumors in this immunocompetent oncogene-driven breast cancer model.

### Continuous perfusion treatment with 25(OH)D or 1,25(OH)<sub>2</sub>D delays spontaneous primary mammary tumor onset and slows tumor growth in female MMTV-PyMT mice

Female MMTV-PyMT mice were treated with 1,25(OH)<sub>2</sub>D (12 pmol/24 h) or 25(OH)D (2,000 pmol/24 h) or vehicle by systemic perfusion starting at 4 weeks and the animals were monitored for 7 weeks. The Kaplan–Meier analysis for mice with palpable primary breast tumors indicates a substantial delay in the appearance of tumors in mice treated with vitamin D metabolites. Tumors were detectable at 42 days in control mice, compared with 48 to 50 days for metabolite-treated animals. All controls presented palpable tumors at 52 days of age, compared with 58 and 62 days for 1,25(OH)<sub>2</sub>D- and 25(OH)D-treated animals, respectively (Fig. 2A). The average number of tumors/animal at sacrifice was decreased by 40% by vitamin D metabolites, with respect to controls (Fig. 2B and C). Palpable primary breast tumor growth rate was reduced by 61% and 75% by 1,25(OH)<sub>2</sub>D and 25(OH)D, respectively (Fig. 2D). Growth inhibition



**Figure 1.**

Low vitamin D diet accelerates mammary hyperplasia and tumor growth in MMTV-PyMT mice. A, blood 25(OH)D levels in mice (6 weeks) with low- or normal-vitamin D diets ( $P < 0.0001$ ). B, H&E staining of breast tissue at 6 weeks. Low-vitamin D diet (25 IU/kg, left), normal-vitamin D diet (1,000 IU/kg, right). Bottom, magnifications of insets at top. C, quantification of hyperplasia surface in breast tissue of mice with low- or normal-vitamin D diets ( $n = 7/\text{group}$ ). \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ . Scale bars, 50  $\mu\text{m}$  (B).

capacity of the metabolites was confirmed *in vitro*, where a 24-hour  $10^{-7}$  mol/L administration of 25(OH)D or 1,25(OH)<sub>2</sub>D inhibited proliferation of cultured MMTV-PyMT breast tumor cells by 25% and 49% with respect to untreated cells (Fig. 2E and F). Viability and cellular homogeneity of tumor-derived cells tested with Fixable Viability Dye and CK8 markers (38, 42) showed near-100% viability, exclusion of connective tissue, and invasive potential of the cultured tumor cells (Supplementary Fig. S1). These data show that continuous infusion with vitamin D<sub>3</sub> metabolites delays primary breast cancer in PyMT mice, and that at the present dosages, 25(OH)D presents a slightly higher efficacy than 1,25(OH)<sub>2</sub>D. The results also confirm that isolated MMTV-PyMT breast tumor cells can transform exogenous 25(OH)D into biologically active 1,25(OH)<sub>2</sub>D.

#### 25(OH)D and 1,25(OH)<sub>2</sub>D perfusion significantly decreases lung metastasis

In MMTV-PyMT mice, microscopic lung metastases spontaneously develop in animals by 12 to 13 weeks of age (43). At sacrifice (12 weeks), lung sections from all mice revealed invasion (Table 1). However, the lung area with metastases was 25% of vehicle-treated controls in the 25(OH)D ( $P < 0.002$ ) and 35% in the 1,25(OH)<sub>2</sub>D-treated group ( $P < 0.05$ ). The mean number of metastases/mouse in controls was  $12.9 \pm 5.4$ , while animals treated with 1,25(OH)<sub>2</sub>D displayed a  $5.14 \pm 1.67$  reduction (60%;  $P < 0.05$ ), and 25(OH)D-treated mice showed a  $3.42 \pm 1.22$  reduction (73.4%,  $P < 0.002$ ). These data show that systemic perfusion of MMTV-PyMT female mice with 1,25(OH)<sub>2</sub>D or 25(OH)D starting at 4 weeks does not prevent appearance of lung metastases but significantly reduces their size and numbers.

#### 25(OH)D and 1,25(OH)<sub>2</sub>D perfusion effect on cell proliferation and cancer-related markers

Continuous perfusion treatment with either vitamin D metabolite reduced expression of cell proliferation markers Ki-67, ErbB2, and cell-cycle progression marker cyclin D1 (Fig. 3A–D). MMTV-PyMT tumors are initially ER- $\alpha$ -positive but eventu-

ally progress to ER-independent adenocarcinomas, while expression of cyclin D1 and HER2 persist (37, 44). This confirms that exogenous 25(OH)D and 1,25(OH)<sub>2</sub>D act through inhibition of cell proliferation and cancer-related markers in this oncogene-driven breast cancer model.

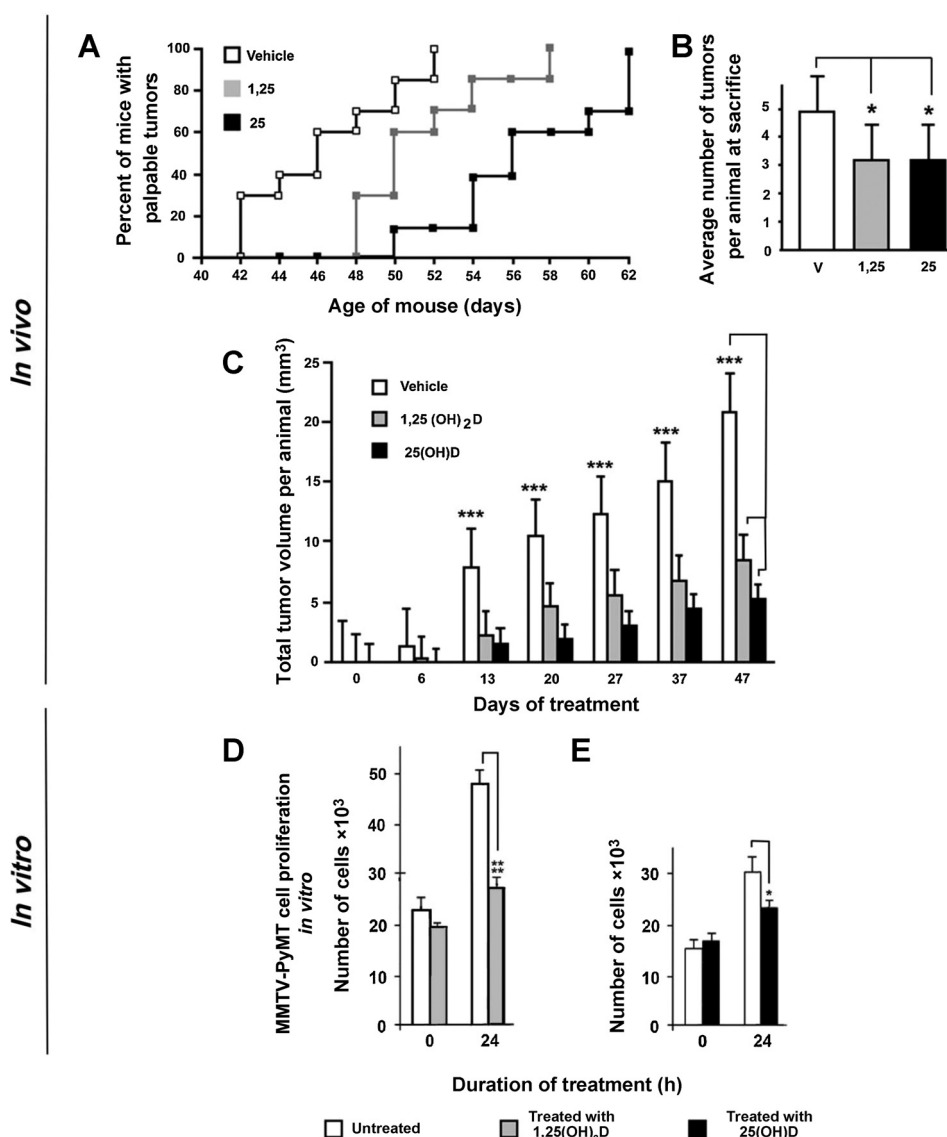
#### 25(OH)D perfusion increases local production of 1,25(OH)<sub>2</sub>D in breast tumors without increasing blood calcemia

Continuous systemic perfusion with 25(OH)D caused a significant elevation of breast tumor, kidney, and serum levels of this metabolite (Fig. 4A and B), and substantially raised local 1,25(OH)<sub>2</sub>D concentration in breast tumor tissues but not in normal kidneys where 1,25(OH)<sub>2</sub>D synthesis is tightly regulated (Fig. 4C; ref. 45). For the same reason, 1,25(OH)<sub>2</sub>D did not modify renal 1,25(OH)<sub>2</sub>D levels (Fig. 4C) but did, however, increase blood calcemia, whereas 25(OH)D did not (Fig. 4D; Supplementary Table S1 and Supplementary Fig. S2).

These data indicate that in MMTV-PyMT breast tumors *in vivo*, exogenous 25(OH)D causes significant local accumulation of 1,25(OH)<sub>2</sub>D. In contrast, perfusion with 1,25(OH)<sub>2</sub>D causes no 1,25(OH)<sub>2</sub>D kidney accumulation because of intra-renal regulation (46). Although both 25(OH)D and 1,25(OH)<sub>2</sub>D raise local levels of 1,25(OH)<sub>2</sub>D in breast tumor, only 25(OH)D can be perfused without causing hypercalcemia (Fig. 5).

## Discussion

The vitamin D pathway has long been suspected of involvement in carcinogenesis prevention. Vitamin D deficiency is not only widespread in patients with cancer, but correlates with advanced-stage disease independently of age, sex, and body mass index (47). Vitamin D deficiency also enhances human breast cancer cell lines growth and metastasis in xenograft models (48–50) and increases the incidence of chemically induced mammary lesions in rats (51). However, the immune response is a crucial component of cancer progression, and carcinogen induction is a confounding factor, so we used here the MMTV-PyMT model of spontaneous oncogene-driven



**Figure 2.** Continuous perfusion treatment with 25(OH)D or 1,25(OH)<sub>2</sub>D delays spontaneous primary mammary tumor onset and slows tumor growth in MMTV-PyMT mice. A, the Kaplan-Meier analysis of spontaneous primary breast tumor occurrence in mice perfused with vehicle (open squares), 1,25-(OH)<sub>2</sub>D (gray), or 25-(OH)D (black). B, average number of primary tumors at sacrifice (12 weeks) after 8 weeks of perfusion [25(OH)D, *n* = 12; 1,25(OH)<sub>2</sub>D, *n* = 9; controls, *n* = 10; *P* < 0.05]. C, total mammary tumor volume per animal with vehicle perfusion (white), 1,25-(OH)<sub>2</sub>D (gray), or 25-(OH)D (black) treatment. D, *in vitro* proliferation of cultured MMTV-PyMT mammary tumor cells treated for 24 hours with 10<sup>-7</sup> mol/L 1,25 (OH)<sub>2</sub>D. White, control; gray, 1,25 (OH)<sub>2</sub>D-treated. E, *in vitro* proliferation of cultured MMTV-PyMT mammary tumor cells treated for 24 hours with 10<sup>-7</sup> mol/L 25(OH)D. White, control; black, 25(OH)D-treated; *P* < 0.001. \*, *P* < 0.05; \*\*\*, *P* < 0.001; \*\*\*\*, *P* < 0.0001.

breast cancer that closely recapitulates the main features of aggressive human disease including distal metastasis (34, 36, 37). Although PyMT is not a human mammary oncogene, it activates c-Src/PI3K/Akt and Shc/ras/MAPK pathways like HER2 does (34, 44). In the immunocompetent MMTV-PyMT mouse, we show that a vitamin D-deficient diet accelerates spontaneous neoplasia, although a catching-up in tumor growth occurs in later stages. This agrees with *in vitro* observations that the Ro3582 vitamin D analogue induces

more significant gene changes in early premalignant MCF10AT1 cells than in malignant metastatic MCF10CA1a cells (52), and suggests a better efficacy for vitamin D metabolites at earlier rather than later stages of breast cancer.

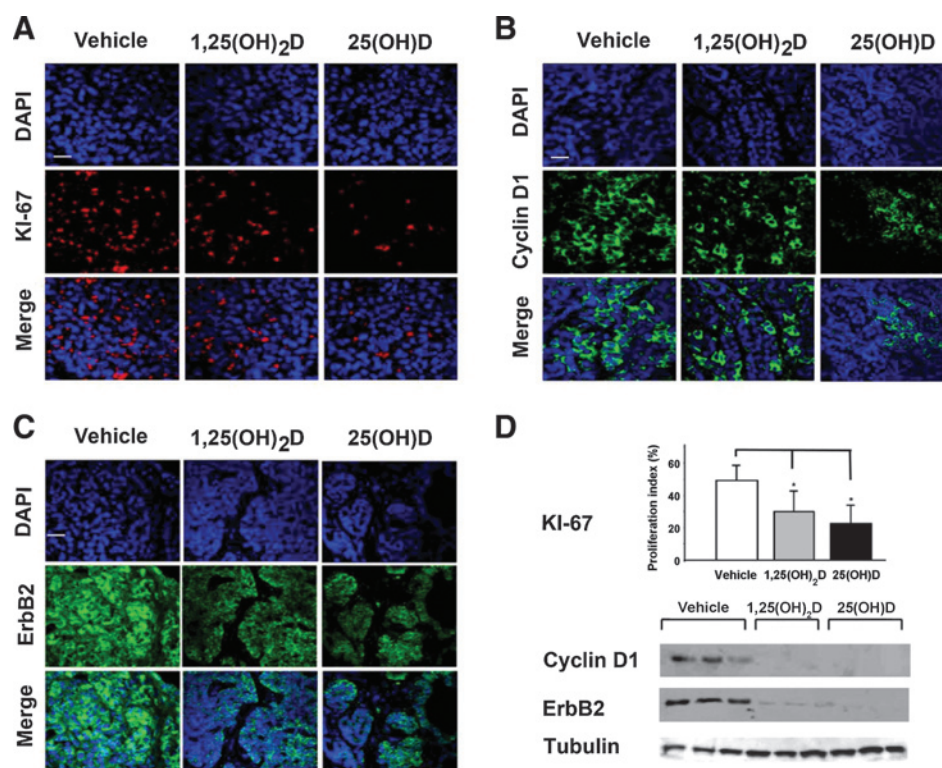
We also demonstrate here the feasibility of using 25(OH)D *in vivo* in a chemopreventive approach to delay breast cancer appearance and significantly decrease the extent of lung metastases (the preferred distal invasion site in the MMTV-PyMT mouse). 25(OH)D can be hydroxylated to 1,25(OH)<sub>2</sub>D locally in normal human

**Table 1.** Effect of 1,25(OH)<sub>2</sub>D and 25(OH)D perfusion on metastatic spread to lungs at 12 weeks

|  | Vehicle    | 1,25(OH) <sub>2</sub>          | 25(OH)                          |
|--|------------|--------------------------------|---------------------------------|
| Percentage of animals with metastases                          | 100%       | 100%                           | 100%                            |
| Average number of metastases per animal                        | 12.9 ± 5.4 | 5.14 ± 1.67 ( <i>P</i> < 0.05) | 3.42 ± 1.22 ( <i>P</i> < 0.002) |
| Relative area of metastases compared with vehicle-treated mice | 100%       | 35% ± 4% ( <i>P</i> < 0.05)    | 25% ± 7% ( <i>P</i> < 0.002)    |

NOTE: Systemic perfusion of female MMTV-PyMT mice starting at 4 weeks with 1,25(OH)<sub>2</sub>D or 25(OH)D did not prevent appearance of tumors in lungs but reduced the size and numbers of metastases.

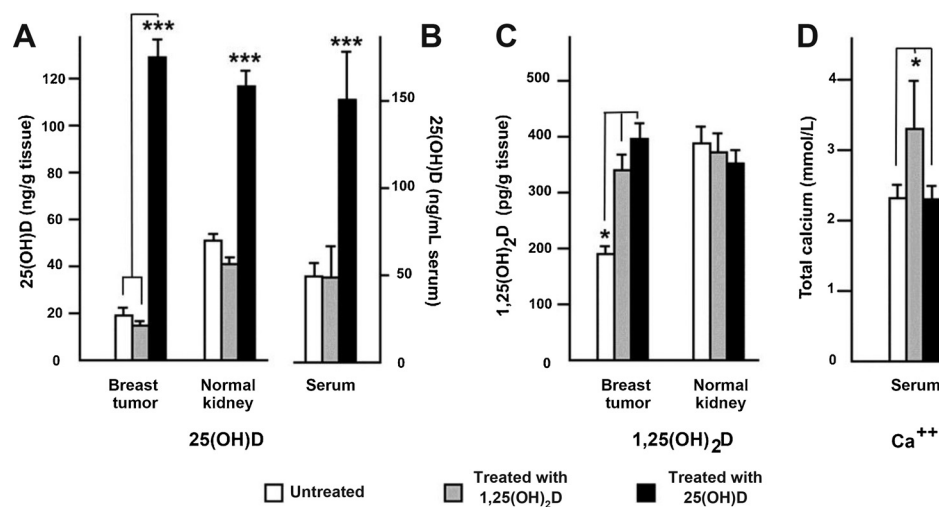
**Figure 3.** 25(OH)D and 1,25(OH)<sub>2</sub>D perfusion treatment effect on cancer-related markers: IF stains illustrating expression levels for Ki-67 (A), cyclin D1 (B), ErbB-2 (C) proto-oncogene in mammary tumor tissue at sacrifice after vehicle, 1,25(OH)<sub>2</sub>D or 25(OH)D perfusion treatments. D, quantitation by positive cell count or Western blot analysis. \*, *P* < 0.05. Scale bar, 200 μm.

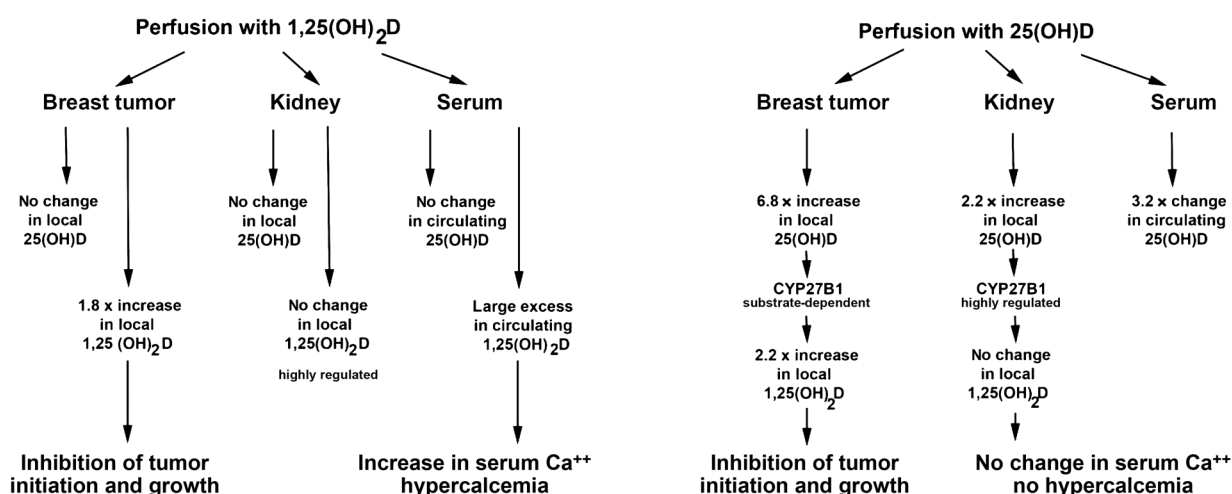


breast tissue and breast tumors (53). Similarly, 25(OH)D perfusion of MMTV-PyMT mice on a normal diet indicates tumoral activation to 1,25(OH)<sub>2</sub>D, an observation confirmed in MMTV-PyMT breast tumor cells in culture. Exogenous 25(OH)D has good tumor penetration as indicated by the sharp increase in 1,25(OH)<sub>2</sub>D in breast tumors after 25(OH)D perfusion. Most importantly, 25(OH)D causes no hypercalcemic side effect and displays high efficacy in delaying spontaneous tumor appearance, suggesting that it undergoes little degradation by tumoral CYP24A1. 24-Hydroxylation of 25(OH)D by the near-ubiquitous CYP24A1

hydroxylase catalyzes an inactivation process resulting in truncated molecules that prevents excess precursor 25(OH)D in target cells (15). It must be noted that perfusion with 25(OH)D raises concentration of this inactive precursor in both breast tumors and kidney. Consequently, a local increase in 1,25(OH)<sub>2</sub>D is observed in breast cancer cells because extra-renal CYP27B1 is substrate dependent (54). In contrast, the same 25(OH)D treatment does not affect kidney 1,25(OH)<sub>2</sub>D production because, as the main contributor to circulating 1,25(OH)<sub>2</sub>D, renal CYP27B1 is subjected to tight regulation (45, 55).

**Figure 4.** 25(OH)D perfusion increases local production of 1,25(OH)<sub>2</sub>D in breast tumor tissue without increasing blood calcemia. A, levels of 25(OH)D in breast tumors, normal kidney and B, serum after vehicle (white), 1,25(OH)<sub>2</sub>D (gray), or 25(OH)D (black) perfusion. C, levels of 1,25(OH)<sub>2</sub>D in breast tumors and normal kidney after vehicle (white), 1,25(OH)<sub>2</sub>D (gray), or 25(OH)D (black) perfusion. D, calcium levels in serum after vehicle (white), 1,25(OH)<sub>2</sub>D (gray), or 25(OH)D (black) perfusion. \*, *P* < 0.05; \*\*\*, *P* < 0.001.





**Figure 5.** Effect of systemic perfusion of 1,25(OH)<sub>2</sub>D or 25(OH)D on tumor growth and blood calcemia: systemic perfusion of 1,25(OH)<sub>2</sub>D (left) and 25(OH)D (right) both increase 1,25(OH)<sub>2</sub>D tumoral concentrations. In the case of 25(OH), the increase in tumoral 1,25(OH)<sub>2</sub>D results from CYP27B1 action. Both treatments inhibit breast tumor initiation and growth. Excess of circulating exogenous 1,25(OH)<sub>2</sub>D due to 1,25(OH)<sub>2</sub>D perfusion causes hypercalcemia. In contrast, exogenous 25(OH)D elevates 25(OH)D kidney levels but does not increase 1,25(OH)<sub>2</sub>D synthesis due to kidney CYP27B1 regulation and no hypercalcemia ensues. (See absolute values in Supplementary Table S1). It must be noted that intermittent 1,25(OH)<sub>2</sub>D treatment may avoid hypercalcemia but rarely presents therapeutic benefit (29).

Perfusion of cancer-prone animals with 1,25(OH)<sub>2</sub>D or 25(OH)D is accompanied here by a reduction in proliferation (Ki-67) and cell-cycle progression (cyclin D1) markers and a decrease in ErbB2/HER2/neu oncogene expression. In parallel, the stimulation in ER $\alpha$  expression by vitamin D metabolites (not shown) is interesting as breast carcinomas that lack ER $\alpha$  expression often display more aggressive phenotypes (56, 57) and confirms *in vitro* results with breast cancer SUM 229 cell line (58).

The use of 1,25(OH)<sub>2</sub>D in patients bypasses kidney control and has been associated with increased serum and urine calcium concentrations consequent to increased intestinal calcium absorption, limitation of renal calcium elimination, and calcium-releasing action on bone cells (59). The increased serum calcium can cause hypercalcemia, a dangerous condition leading to renal and extra-renal calcifications (46). Antimitotic structural analogues with reduced calcemic effects have been developed (60); however, anticancer approaches require high-dose intermittent administration that can still cause hypercalcemia. Furthermore, synthetic analogues can exhibit reduced affinity for vitamin D transport protein, resulting in rapid liver clearance or accelerated destruction due to CYP24A1 upregulation (15). Therapeutic efficacy of vitamin D itself depends on the individual's hepatic health (26) and cholecalciferol presents a short circulating half-life (27). In contrast, the natural 25(OH)D metabolite appears to circumvent these problems. 25(OH)D is FDA-approved and marketed as Calderol for use against vitamin D deficiency, vitamin D-resistant rickets, familial hypophosphatemia and hypoparathyroidism, hypocalcemia and renal osteodystrophy, as well as osteoporosis prevention (61). 25(OH)D also presents a long half-life in circulation ( $t_{1/2}$  = 3 weeks) compared with 1,25(OH)<sub>2</sub>D ( $t_{1/2}$  = 4–6 hours; ref. 28). The improved effects of 25(OH)D over 1,25(OH)<sub>2</sub>D observed here are likely dose-dependent, indicating potential for better efficacy without hypercalcemic limitations.

Our observations on the inhibitory effect of vitamin D metabolites treatments on breast tumor growth support epidemiologic studies demonstrating an association between vitamin D status and breast cancer mortality (62, 63). *In vitro*, 25(OH)D displays antiproliferation efficacy in cultured colon cancer cells (64), primary human mammary epithelial cells (10), and against 7,12 dimethylbenz(a)anthracene (DMBA)-induced carcinogenesis in *ex vivo* mammary organ culture (33). The 25(OH)D derivative 25(OH)D-3-bromoacetate also has growth-inhibitory activity in a human prostate cancer cell line (65). *In vivo*, we previously showed that 25(OH)D perfusion inhibits tumor growth from injected ras-transformed keratinocytes in severely immunodeficient mice (66). Here, we demonstrate chemopreventive efficacy of exogenous 25(OH)D through an effect consequent to autocrine synthesis of 1,25(OH)<sub>2</sub>D in an immunocompetent model that closely mimics human breast cancer pathology. In view of the still controversial epidemiologic data, there needs to be further evidence from clinical trials for the efficacy of 25(OH)D in human pathology, optimal dosage, and mode of delivery, as well as potential side effects. However, the absence of hypercalcemia during 25(OH)D treatment, combined with its serum stability are promising factors to consider when designing a therapeutic protocol. The innocuity and good pharmacokinetics of 25(OH)D suggests the metabolite could be envisioned for cancer chemoprevention use in view of its efficacy to provoke local 1,25(OH)<sub>2</sub>D synthesis in tumors.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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