

Ultraviolet Radiation Inhibits Mammary Carcinogenesis in an ER-Negative Murine Model by a Mechanism Independent of Vitamin D₃



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Abstract

Three decades ago, the Garlands postulated that vitamin D₃ produced in the skin by ultraviolet radiation (UVR)-induced conversion of 7-dehydrocholesterol to pre-D₃ has anticancer effects, thus triggering more than 9,500 publications on D₃ and cancer. Here, we report that UVR treatment of transgenic mice of the well-established C3(1)/SV40 Tag mammary cancer model significantly inhibits both autochthonous carcinogenesis and allograft tumor growth, but in contrast neither dietary nor topical D₃ influences mammary carcinogenesis in this specific mouse model. Furthermore, UVR's

inhibitory effects occur irrespective of whether or not the treatment increases circulating D₃ in the mice. The inhibitory effect of UVR on autochthonous tumors occurs at or before the stage of ductal carcinoma in situ. Our studies indicate clearly that UVR can exert D₃-independent anticancer effects in C3(1)/SV40 Tag mice. Therefore, supplemental D₃ may not mimic all possible beneficial effects of UVR, and uncovering non-D₃-mediated mechanisms of UVR tumor inhibition may lead to novel strategies for cancer prevention. *Cancer Prev Res*; 11(7); 383–92. ©2018 AACR.

Introduction

Seven decades ago, Apperly postulated that the decreased incidence and decreased mortality of colorectal, breast, and several other cancers at lower latitudes are due to increased ambient sun light exposure (1), which the Garlands postulated acts by producing D₃ (2). This idea has contributed to the widespread use of oral D₃ supplementation in the hope of protecting against various maladies beyond abnormalities of bone mineralization (3). However, recent publications indicate increasing doubt about the range of action of D₃ (4, 5). Despite the reporting of numerous careful studies in this field, few have assessed ultraviolet radiation (UVR)'s effect on autochthonous murine carcinogenesis. We report here that, consistent with Apperly's hypothesis, UVR does inhibit murine mammary

carcinogenesis in the well-established C3(1)/SV40 Tag model (6) but that, in this specific model the inhibitory effect is independent of D₃, thus suggesting the existence of a D₃-independent anticancer effect of UVR.

Materials and Methods

Mice

All mouse studies were done as per the protocol approved by the CHORI IACUC. FVB/NJ and NOD/SCID mice were from The Jackson Laboratory (JAX, Sacramento, stock nos. 001800 and 001303, respectively). C3(1)/SV40 Tag transgenic mice provided by J. Green (NCI NIH, Bethesda, MD; ref. 7) were maintained by breeding with FVB/NJ nontransgenic mice. Transgenic animals were identified by PCR analyses of genomic DNA extracted from ear punches using a mouse genotyping kit (KAPA Biosystems) and primer sequences and PCR conditions as previously described (7). Mice from each litter were assigned randomly to different treatment groups. We enrolled 6-week-old mice in all studies unless otherwise specified. All NOD/SCID mice and C3(1)/SV40 Tag not enrolled in dietary studies were maintained on a normal D₃/normal minerals diet—D₃ 1,500 IU/kg, Ca 1%, phosphate 0.7%; TD2018; Harlan. Mice were housed under standard conditions (fluorescent lighting 12 hours per day, room

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temperature 23°C–25°C, and relative humidity 45%–55%). Animal care and use were in compliance with protocols 197 and 201 approved by the Institutional Animal Care and Use Committee (IACUC) of Children's Hospital Oakland Research Institute (CHORI).

D₃ dietary study

C3(1)/SV40 Tag transgenic mothers and pups were weaned onto and maintained on a D₃-depleted diet. At age 6 weeks, mice were enrolled randomly in one of four dietary groups: D₃-depleted, D₃ normal, D₃ rescue, or a high D₃ diet (Table 2).

UVR light source and treatment

We used a UVR irradiation unit (R2025-bx-0012; Daavlin Co.) with a bank of F40-T12 sunlamps arranged in parallel, emitting UVB (280–315 nm), and equipped with an electronic controller to regulate dosage. The dose was calibrated with an independent external UV light meter (925GOX96; Daavlin Co.). UVR treatments were delivered consistently as described (8). In brief, mice were shaved weekly and treated at the same time of the day in a cage without bedding placed below the same areas of the device with a distance between light sources and mouse skin of 20 cm.

Transgenic mice were treated 3×/week with 350 mJ/cm² UVR (i) starting at age 6 weeks until euthanasia or until the age when the first tumor became palpable or (ii) starting at the time of appearance of the first palpable tumor until euthanasia. NOD/SCID mice were treated 3×/week with 350 mJ/cm² UVR starting from the age when the longest tumor diameter reached 1 cm until euthanasia. To study UVR-induced cyclopyrimidine dimers, we exposed 4- to 5-month-old NOD/SCID mice to a single dose of UVR (350 mJ/cm²), euthanized mice 30 minutes later, and collected skin and allograft tumors for histologic examination.

Topical D₃ treatment

Transgenic D₃-depleted 6-week-old mice were randomly assigned to groups treated with either topical D₃ or acetone vehicle control (*n* = 9 mice/group). We treated mice in the D₃ topical group by applying cholecalciferol (0.38 µg/day, Sigma-Aldrich) to a small shaved area of the dorsal neck, 5 days/week in 20 µL of acetone, age 6 to 12 weeks. Control mice received topical acetone 5 days/week for the same duration. We confirmed that mice do not lick the application site by monitoring the persistence of a water-soluble ink at this site and its disappearance at a lickable site. NOD/SCID mice received 3.8 µg/day of cholecalciferol starting from the age when the longest tumor diameter reached 1 cm until euthanasia. All enrolled mice were housed singly and were shaved dorsally weekly.

Subcutaneous allografts in NOD/SCID mice

M28 and M6 mouse mammary carcinoma cell lines derived from C3 (1)/SV40 Tag mice (9) were cultured in

Dulbecco's modified Eagle Medium supplemented with 10% (v/v) FBS (UCSF Cell Culture Facility) and penicillin/streptomycin. At 70% confluence, 1×10^6 cells or 2×10^6 cells (100 µL; as indicated on the figures) were resuspended in DMEM/Matrigel (1/1, v/v) and injected (100 µL/per injection) subcutaneously into each flank of 6-week-old shaved NOD/SCID mice fed a D₃ normal diet (TD2018, Harlan Teklad). Twenty-six mice received M28 cells, and 6 received M6 cells. When a single tumor had reached 1 cm in longest diameter (after approximately 4 weeks, i.e., at ~10 weeks of age), NOD/SCID mice were assigned randomly to one of the four treatment groups: UVR or untreated control and topical D₃ or topical acetone. Individual body weights and tumor sizes were measured weekly as described below.

Microscopic tumors assessment

C3(1)/SV40 Tag mice develop DCIS as early as age 9 weeks and palpable mammary tumors at approximately age 16 weeks. To analyze the effect of UVR on progression during the early stage of carcinogenesis, we euthanized C3(1)/SV40 Tag mice at ages 9, 11, 13, 14, and 15 weeks and collected all 10 mammary glands from each mouse (*n* = 3–5 mice/group). Histologic analysis of H&E-stained sections was performed blindly by the breast cancer pathologist (P. Du), and the percentages of mammary glands with atypical hyperplasia, DCIS, and invasive carcinoma were determined.

Tumor monitoring

In studies of the autochthonous model, we recorded body weights weekly as well as time to first palpable tumor (subsequently confirmed histologically as a mammary tumor), numbers of palpable tumors, and sizes of tumors starting at age 10 weeks. In studies of mice bearing allograft tumors, we recorded mouse body weights and tumor sizes weekly starting at age 6 weeks (i.e., at the time of allograft placement). We used an electronic caliper (10) to measure the length, width, and depth consistently, and we calculated tumor volume using the following formula: length × width × depth × 0.52 (11). Mice were euthanized when a single tumor reached a longest diameter of 2 cm or the sum of multiple tumors approached 9% of body weight, as per our IACUC-approved protocol. At euthanasia, blood was taken by cardiac puncture for D₃ analysis; tumors, mammary glands, and skin samples were collected for histology, flow cytometry, and cell sorting.

Immunohistochemistry

Collected tissues were fixed in paraformaldehyde 4% overnight at 4°C. Prior to commercial automated paraffin processing (Redwood Dermatopathology Laboratory), tissues were washed twice with phosphate-buffered saline and transferred to isopropanol 70%. Four-micrometer sections were subjected to standard immunostaining

procedures as described previously (12). The following primary antibodies (Ab) were used: anti-SV40 TAG mouse monoclonal Ab (1:100, clone PAb 101, #554149; BD Pharmingen), anti-Ki-67 rabbit monoclonal Ab (RM-9106-R7; Thermo Fisher Scientific), and anti-CPD Ab (MC-062; Kamiya Biomedical Company).

Serum D₃ and 25 (OH)D₃ analyses

D₃ and 25(OH)D₃ levels were measured commercially (Heartland Assays LLC & Metabolic Technologies, Inc.). Radioimmunoassay (RIA) was used to quantify 25(OH)

D₃, while D₃ was measured by LC/MS/MS using an Agilent 1290/6460 Series Triple Quadrupole System and a deuterated internal standard.

Flow-cytometry analysis of apoptosis

We detected apoptosis and necrosis in M28 allografts using Alexa Fluor 488 labeled Annexin V and propidium iodide (PI) staining by flow-cytometric analysis, following the manufacturer's protocols (Invitrogen). Briefly, collected allograft tumors were digested in 0.1% collagenase IV (C5138, Sigma-Aldrich) to obtain single-cell suspensions.

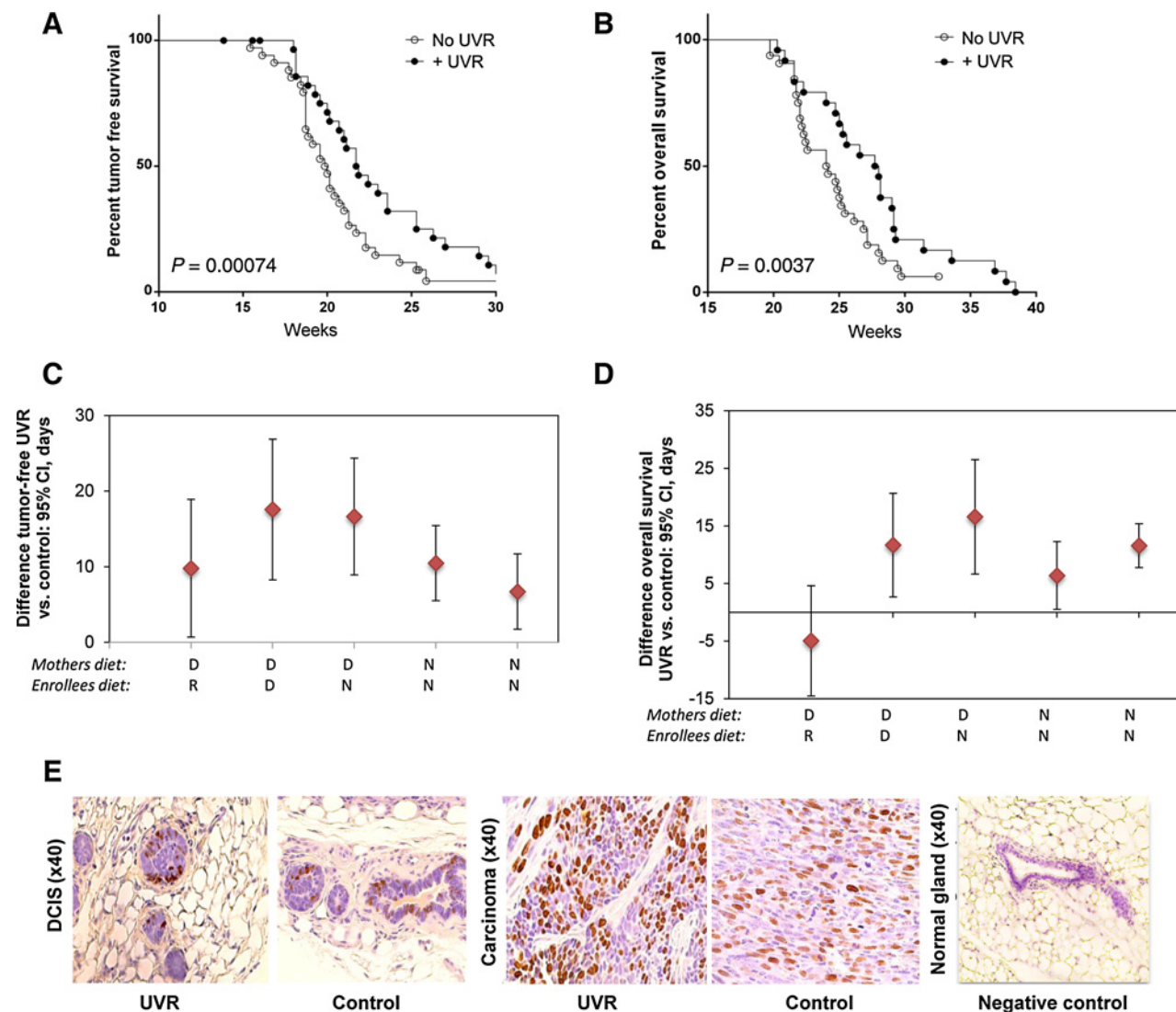


Figure 1.

UVR treatment consistently delays mammary carcinogenesis in FVB/NJ-Tag mice. FVB/NJ-Tag dams were weaned onto and maintained on either a D₃-depleted or D₃ normal diet (see Table 2). At age 6 weeks, pups were assigned randomly to either D₃ rescue (R), D₃ depleted (D), or D₃ normal (N) diets. UVR (350 mJ/cm²) was continuously administered 3×/week from age 6 to 25 weeks. **A** and **B**, UVR significantly increased tumor-free ($P = 0.023$, log-rank test) and overall survival ($P = 0.03$, log-rank test) of FVB/NJ-Tag mice on a D₃ normal diet born from D₃ normal mothers, $n = 30$ mice per group. **C** and **D**, UVR increased tumor-free (combined $P = 0.00074$, t test) and overall survival (combined $P = 0.0037$, t test) in multiple experiments. Data are presented as difference with 95% confidence interval (CI), $n = 30$ mice per group for each experiment. **E**, Similar T antigen expression in DCIS and mammary carcinoma lesions in autochthonous mammary glands from UVR-treated FVB/NJ-Tag mice, untreated control FVB/NJ-Tag mice, FVB/NJ mice (negative control). Magnification, ×40.

Cells were washed in PBS and resuspended in buffer (50 mmol/L HEPES, 700 mmol/L NaCl, 12.5 mmol/L CaCl_2 , pH 7.4). After addition of 5 μL Annexin V and 1 μL PI, cells were incubated at 37°C in light-protected vials for 20 minutes. Samples were diluted with binding buffer and were analyzed using the BD LSR-Fortessa (BD Bioscience). Results were analyzed using FlowJo single-cell analysis software 8.5.2 (TreeStar).

Statistical analyses

Kaplan–Meier survival analyses, determination of significance of difference between groups using log-rank test,

Gehan–Wilcoxon test and Student *t* tests were carried out using GraphPad Prism 6 software. Values are presented as mean $\pm$ SD or mean $\pm$ SEM; those with $P \leq 0.05$ were considered to be statistically significant. All studies were designed to yield at least $n = 30$ for each group of transgenic mice and $n \geq 3$ for each group of NOD/SCID. Data were collected from multiple litters; mice from each litter were randomized into different treatment groups. Each data point represents a biological replicate. The investigators were not blinded to allocation during experiments and outcome assessment. For all experiments presented in this study, the sample size was large enough to measure the

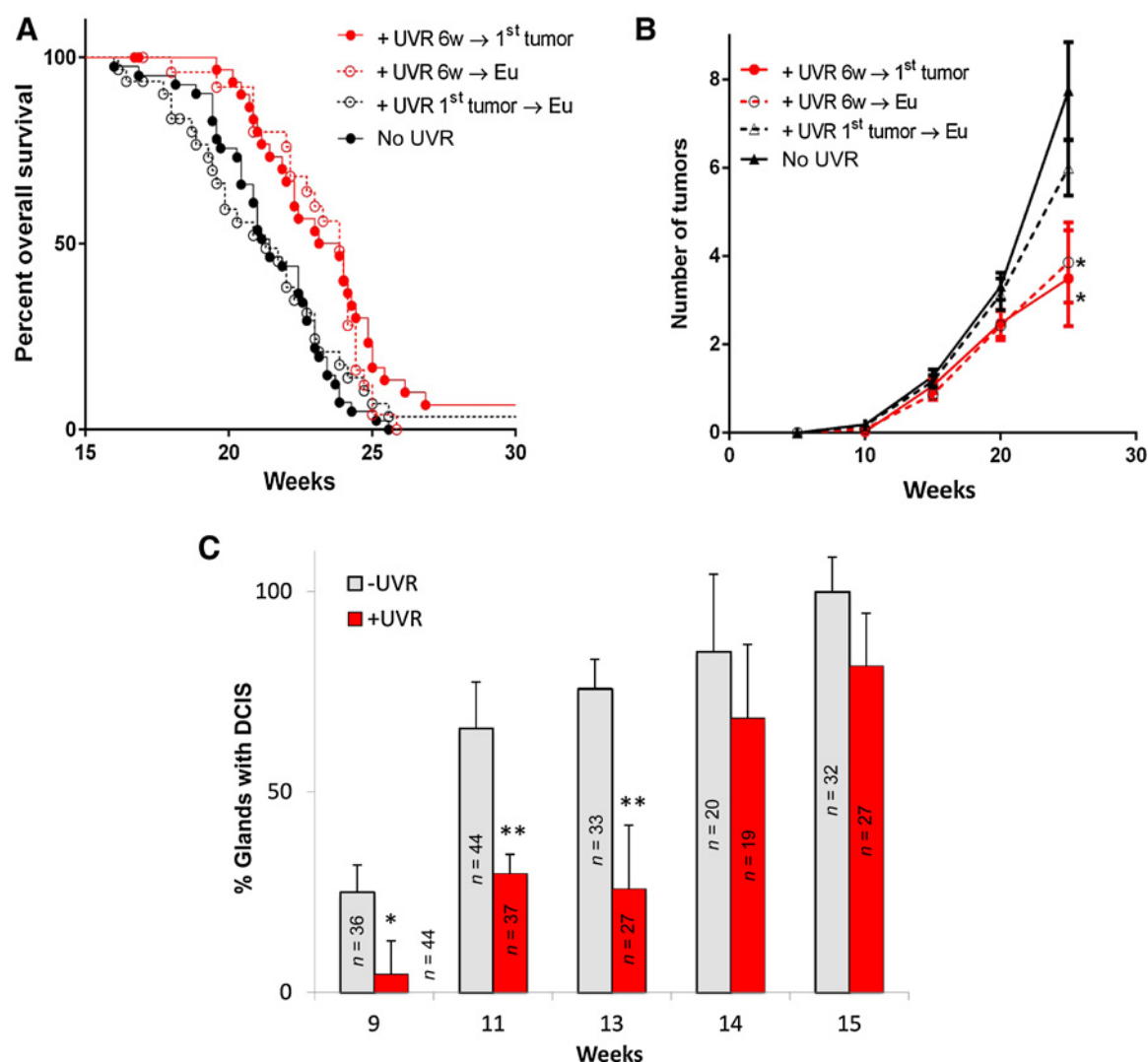


Figure 2.

The inhibitory effect of UVR occurs early during carcinogenesis. **A**, UVR significantly ($P = 0.0007$, log-rank test) increased overall survival of FVB/NJ-Tag mice fed a D_3 normal diet born to mothers fed a D_3 normal diet when given from age 6 weeks until the first palpable tumor as well as when given from age 6 weeks until euthanasia (Eu) but has no effect when given from the appearance of the first palpable tumor until Eu. Pairwise comparisons: UVR 6 weeks to the first tumor vs. no UVR, $P = 0.0017$; UVR 6 weeks to Eu vs. no UVR, $P = 0.0016$; UVR 6 weeks to the first tumor vs. UVR 6 weeks to Eu, $P = 0.83$; UVR first tumor to Eu vs. no UVR, $P = 0.57$, Gehan–Wilcoxon test. $n = 30$ mice/group. **B**, UVR decreased the number of palpable tumors when given from age 6 weeks until the first palpable tumor as well as when given from age 6 weeks until Eu in mice on D_3 normal diet born from D_3 normal mothers; *, $P \leq 0.05$ vs. no UVR, $n = 30$ mice/group, mean $\pm$ SEM. **C**, UVR when given from age 6 weeks until age 15 weeks to mice on D_3 normal diet born from D_3 normal mothers reduced the percentage of mammary glands with DCIS at early ages 9–13 weeks, but only minimally at later ages 14 and 15 weeks as indicated histologically; *, $P \leq 0.05$; **, $P \leq 0.01$.

effect size. All statistics were done in consultation with the CHORI statistician G. Gildengorin.

Results

UVR inhibits autochthonous Tag mouse mammary carcinogenesis

We have tested the hypotheses of Apperly (1) and of the Garlands (2) using C3(1)/SV40 Tag mice (6), in which a promoter active in the mammary gland drives expression of the SV40 T antigen, which inactivates both p53 and RB proteins. Mammary carcinogenesis in these mice appears most closely to resemble the triple negative, basal form of human breast cancer (13–16). We exposed the shaved dorsal skin of these mice to UVR (350 mJ/cm²) three times per week starting from age 6 weeks. We found in multiple independent experiments that UVR treatment significantly delayed the appearance of the first palpable mammary tumor (age 18.9 vs. 16.8 weeks, combined $P = 0.00074$; Fig. 1A and C), increased overall survival (age 24.8 vs. 22.8 weeks, combined $P = 0.0037$; Fig. 1B and D), and reduced the number of palpable mammary tumors per mouse at age 25 weeks (4.3 ± 2.1 vs. 6.7 ± 1.0 tumors, $P = 0.048$; Fig. 2B, red dashed vs. black solid lines). UVR's inhibitory effect occurred in mice fed either a D₃-depleted/high minerals diet or a diet with normal amounts of D₃ and minerals (Tables 1 and 2). In mice fed a diet with normal D₃/high minerals (D₃ rescue diet), UVR delayed the development of palpable tumors (Fig. 1C) but, inexplicably, had no significant effect on either overall survival (Fig. 1D) or tumor multiplicity. UVR did not change expression of the oncogenic T antigen (Fig. 1E). As expected (8), this UVR dose produced skin tumors of the squamous cell carcinoma (SCC) lineage in these mice starting at age 25 weeks.

The antitumor effect of UVR occurred early during carcinogenesis: (i) UVR started at age 6 weeks prolonged overall survival and reduced the number of palpable mammary tumors equally well if continued until death

or if stopped at the time when the first tumor became palpable (Fig. 2A and B in red; Table 1); (ii) UVR treatment started after the first tumor became palpable had little to no effect on overall survival ($P = 0.5$) or on the number of tumors (Fig. 2A and B in black); and (iii) UVR started at age 6 weeks reduced the percentage of mammary glands with DCIS at ages 9 to 13 weeks but not at ages 14 weeks and later (Fig. 2C). UVR significantly decreased proliferation in histological samples with DCIS at ages 10 to 15 weeks as measured by Ki67 protein expression.

D3 administration fails to inhibit Tag mouse mammary carcinogenesis

We next compared the effects of UVR with those of orally ingested D₃. We weaned female pups from mothers fed D₃-depleted chow onto diets containing D₃ in amounts varying from none to a level 20-fold higher than normal (Tables 1 and 2). In mice not exposed to UVR, these dietary changes failed to affect significantly the number of palpable mammary tumors that formed (Fig. 3A), the age at first palpable tumor (Fig. 3B), or the age at which IACUC guidelines mandated euthanasia. This lack of phenotypic effect occurred despite elevation of circulating 25(OH)D₃ caused by oral D₃ ingestion to levels similar to those induced by UVR treatment (D₃ normal diet; 31 ± 8 vs. 40 ± 9 ng/mL, respectively; $P = 0.33$) or to levels 4-fold higher (high D₃ diet; 31 ± 8 vs. 112 ± 31 , $P \leq 0.01$; Fig. 3C).

To more closely mimic the effects of D₃ produced in the skin, we applied 15.2 IU (0.38 µg)/day D₃ (cholecalciferol) to the shaved dorsal skin of the neck (a site that mice cannot self-lick) of singly housed mice that were fed a D₃-depleted diet. This normalized circulating 25(OH)D₃ and increased circulating D₃ to levels similar to those achieved by UVR treatment (Fig. 3D). Nonetheless, topical D₃ failed to affect early-stage mammary carcinogenesis as assessed by histologic analysis of mammary glands from 12-week-old C3(1)/SV40 Tag mice (Fig. 3E). Of note, the same dose of topical D₃ applied to the back of the neck robustly delayed

Table 1. Experimental treatment groups

Study	Figure	Diet (abbrev.)		Treatments and timeline	Controls
		Mothers	Enrollees		
Dietary I	1	D	D	UVR 6~35 wks	No UVR
		D	R		
		D	N		
		N	N		
Dietary II	3A–C	D	D	UVR 6~35 wks	No UVR
		D	N		
		D	High D		
Timing	2A and B	N	N	UVR 6~35 wks UVR 6 wks – 1 st tumor UVR 1 st tumor ~35 wks	No UVR
DCIS	2C	N	N	UVR 6–15 wks	No UVR
	3D and E	D	D	Cholecalciferol D ₃ (15.2 IU/day) 6–12 wks	Acetone
Allografts	4A–E	N	N	UVR (~10~25 wks)	No UVR
	4C and F	N	N	Cholecalciferol D ₃ (152 IU/day) (~10~25 wks)	Acetone

Abbreviations: D, D₃ depleted; N, D₃ normal; R, D₃ rescue; High D, High D₃ diet.

¹UVR was given at a dose of 350 mJ/cm² 3 times/week.

²Mice were euthanized at experimental/humane endpoints ~35 weeks of age.

Table 2. Animal diets used in the study

Diet	Abbrev.	Source	Diet composition			
			D ₃ , IU/kg	Ca, %	P, %	Lactose, %
D ₃ depleted	D	TD87095 Harlan	0	2	1.25	20
D ₃ normal	N	TD2018 Harlan	1,500	1	0.7	—
D ₃ rescue	R	TD09031 Harlan	1,000	2	1.25	20
High D ₃ diet	High D	D10012G Res. Diets Inc	20,000	0.5	0.3	—

the development of visible BCCs in *Ptch1*^{+/-} mice (17), thus indicating its successful absorption and distribution to other tissues.

UVR but not D3 inhibits Tag mouse mammary cell line autograft growth

Finally, we produced mammary tumor allografts on NOD/SCID mice using cell lines derived from a histolog-

ically normal mammary gland from an 8-week-old FVB/NJ-Tag transgenic mouse (M28) and from an invasive mammary carcinoma from an older mouse (M6; ref. 9). NOD/SCID mice themselves and their mothers were fed diets containing normal amounts of D₃ (Table 1). The M28 allograft tumors from the early-stage cell line were sensitive to inhibition of growth by UVR (Fig. 4A). By contrast, M6 allograft tumors from the

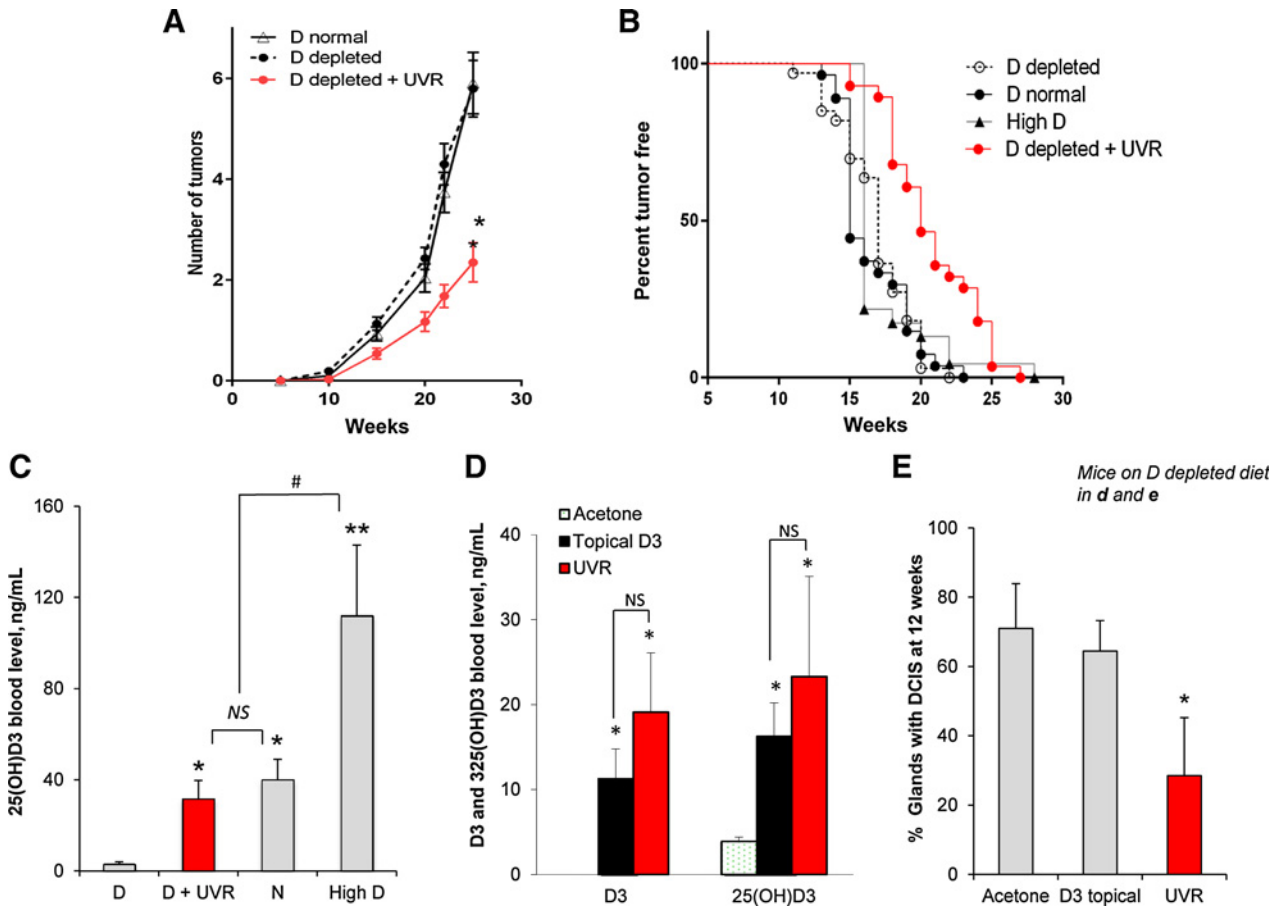


Figure 3. Neither oral nor topical D₃ treatment affected mammary carcinogenesis. **A**, Ingestion of normal amounts of D₃ (solid black line) failed to reduce the numbers of palpable tumors compared with D₃-depleted mice (dashed black line) and unlike UVR treatment (solid red line), *n* = 30 mice/group; *, *P* ≤ 0.05, compared with D₃-depleted mice; mean ± SEM. **B**, Dietary D₃ (given either as N or high D diet), unlike UVR, had no effect on tumor-free survival compared with D₃-depleted mice. Pairwise comparisons: D-depleted vs. D normal diet, *P* = 0.46, D-depleted vs. high D diet, *P* = 0.59, D-depleted vs. D-depleted + UVR, *P* < 0.0001, Gehan-Wilcoxon test. **C**, Circulating 25(OH)D₃ levels were undetectable in mice ingesting a D₃-depleted diet, normal in mice ingesting a D₃ normal diet, and 3-fold higher than normal in mice ingesting a high D₃ diet, *n* = 3 mice/group; *, *P* ≤ 0.01; **, *P* ≤ 0.005 compared with D depleted control; #, *P* ≤ 0.01, NS = 0.33; mean ± SD. **D**, Topical D₃ (cholecalciferol 15.2 IU/day) normalized circulating 25(OH)D₃ and increased circulating D₃ to levels similar to those achieved by UVR, *n* = 3 mice/group; *, *P* ≤ 0.05 vs. untreated control; mean ± SD. **E**, Unlike UVR, topical D₃ (cholecalciferol 15.2 IU/day) failed to affect early-stage mammary carcinogenesis in singly housed mice on D-depleted diet; *n* = 9 mice/group; mean ± SD; *P* ≤ 0.001.

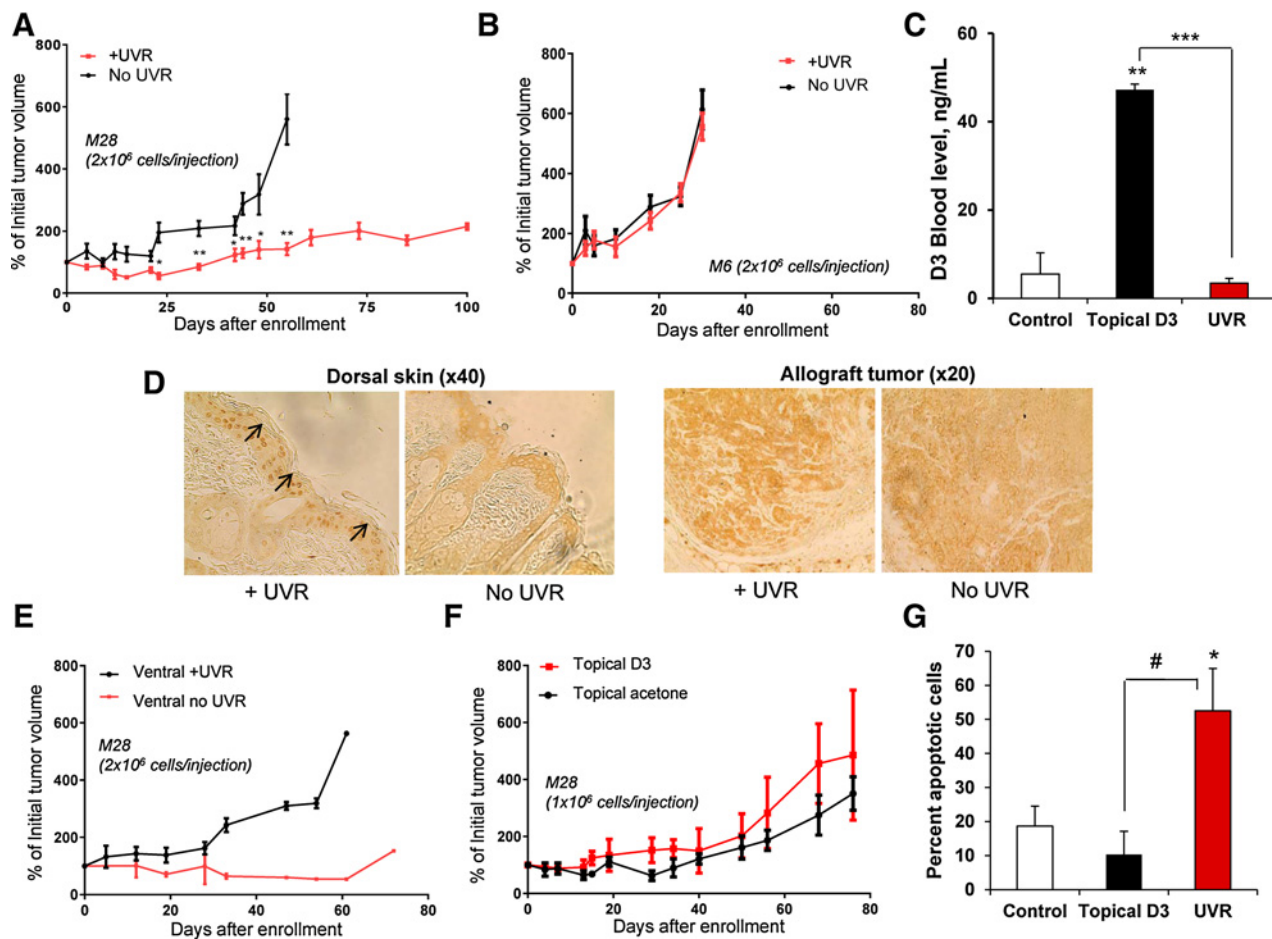


Figure 4.

UVR but not topical D₃ inhibited the growth of allografts derived from a histologically normal C3(1)/SV40 Tag mammary gland cell line (M28), but not allografts derived from a C3(1)/SV40 Tag invasive carcinoma cell line (M6). **A**, UVR treatment significantly reduced the allograft tumor growth (assessed as tumor volumes as a percentage of initial tumor volume) of M28 cell line allografts in NOD/SCID mice compared with untreated controls. Cells were injected into 6-week-old mice, UVR started (enrollment) when tumor reached 1 cm, and continued until humane endpoints were reached. $N = 24$ allograft tumors/group; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; mean $\pm$ SEM. **B**, UVR had no effect on the tumor growth of M6 cell line allografts in NOD/SCID mice, $n = 6$ allograft tumors/group; mean $\pm$ SEM. **C**, Unlike topical D₃ (cholecalciferol), UVR treatment failed to increase the circulating D₃ (cholecalciferol) level in female NOD/SCID mice; $n = 3$ mice/group. **, $P \leq 0.01$, compared with control; ***, $P \leq 0.001$, mean $\pm$ SEM. **D**, Neither dorsal skin dermis nor underlying allograft tumors had evidence of DNA damage from UVR as shown by IHC for UVR-induced CPD DNA damage (brown nuclei indicated with arrows) in dorsal skin and underlying allograft tumors from NOD/SCID mice exposed to a single dose of UVR (350 mJ/cm²) and control untreated mice. **E**, M28 allografts were placed on the ventral body. UVR inhibited the growth of allografts even not directly exposed to UVR, but underlying the ventral skin, $n = 4$ allografts/group. **F**, Topical D₃ (cholecalciferol) applied to the back of the neck of the NOD/SCID mice at 152 IU/day (3.8 μ g) in acetone failed to blunt the rate of growth of the M28 cell line allografts; $n = 4$ allografts/group; mean $\pm$ SEM. **G**, UVR but not topical D₃ (152 IU/day) treatment increased the percentage of apoptotic cells (as assessed by flow-cytometry analysis) in M28 cell line allograft tumors excised from NOD/SCID host mice at Eu; $n = 3$ allografts/group; *, $P \leq 0.001$, compared with control; #, $P \leq 0.001$; UVR vs. D₃; mean $\pm$ SEM.

invasive carcinoma cell line were resistant to UVR (Fig. 4B), mirroring the stage-specific effects on autochthonous tumors in UVR-treated mice outlined above. UVR inhibition of the growth of the M28 allograft tumors occurred despite UVR's failure to increase serum D₃ in host NOD/SCID female mice (Fig. 4C), confirming that UVR has a D₃-independent effect on tumorigenesis. The tumor inhibitory effect of UVR was not due to direct irradiation of the allografts because (i) UVR produced abundant cyclobutane pyrimidine dimers (CPD), classic indicators of UV-induced DNA damage, in the overlying epidermis

but none in the tumor allografts or in the dermis (Fig. 4D) and (ii) UVR inhibited the growth of the allograft tumors underlying the ventral (unexposed; Fig. 4E) as well as the dorsal (exposed; Fig. 4A) skin.

In marked contrast to UVR-induced inhibition, topical application of D₃ (cholecalciferol) to the back of the neck of NOD/SCID host mice at a dose of 152 IU/day (3.8 μ g/day), a 10-fold higher dose than used in our autochthonous tumor studies, failed to blunt the rate of growth of the tumor allografts (Fig. 4F), despite raising circulating D₃ levels (Fig. 4C). Correspondingly, UVR

significantly increased apoptosis in allograft tumors ($P = 0.0001$), whereas topical D_3 had no effect on apoptosis (Fig. 4G).

Discussion

In consonance with our findings, a very rarely cited paper from Apperly (18) reported in 1945 that UVR, but not orally administered D_3 , reduced mammary cancer in a spontaneously cancer-prone strain of A/J mice. Thus, the anti-mammary cancer activity of UVR in these two mouse models is consistent despite the passage of seven decades and the use of different sources of radiation and different strains of mice. These data, moreover, are consistent with the beneficial effects of UVR on experimental murine autoimmune encephalomyelitis, a model of human multiple sclerosis in which the effect is also unrelated to vitamin D_3 levels (19). UVR's inhibitory effect on murine mammary (extracutaneous) cancer differs markedly from its stimulation of cutaneous SCC carcinogenesis, an effect that is due, at least in part, to immune inhibition (20). The maintenance of efficacy of UVR versus the Tag mammary carcinoma allografts in host NOD/SCID mice significantly depleted in T and B cells, suggests that UVR's anti-carcinogenesis mechanism is independent of the adaptive immune system. These data identify the FVB/NJ C3 (1)/SV40 Tag mouse as a tractable model for the elucidation of a D_3 -independent mechanism of UVR's anti-cancer effect but do not in any way conflict with the sizable previous body of data indicating that, in other preclinical cancer models, vitamin D_3 has significant anticancer effects.

To the degree that these murine findings reflect the human condition, they may be highly significant for public health—they support the idea that sun exposure may reduce the incidence of cancers of the breast and perhaps of the colon and other organs (21–23). Indeed, some data suggest that more sun exposure is associated with reduced overall mortality (24, 25). However, unlike the majority of past studies, one recent epidemiologic study failed to find any effect of exposure to sunlight on human breast cancer development (26). Our findings indicate that although sun protection to prevent skin cancers might increase extracutaneous cancer risk,

neither oral nor topical D_3 replacement will reverse whatever portion of the increased risk is mediated by non- D_3 mechanisms. However, more happily, our findings suggest that elucidation of the full mechanism of UVR's anticancer effect could uncover new preventive approaches to reduce the incidence of breast cancer in women living at higher latitudes to that of women living closer to the equator and perhaps even further in women everywhere (27).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.M. Makarova, F. Frascari, P. Davari, F. Gorouhi, L. Wang, G. Wang, J.E. Green, E.H. Epstein Jr

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.M. Makarova, F. Frascari, F. Gorouhi, P. Dutt, A. Dhawan, J.E. Green, E.H. Epstein Jr

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Wang, A. Dhawan, G. Wang, E.H. Epstein Jr

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