Vitamin D Signaling Suppresses Early Prostate Carcinogenesis in TgAPT121 Mice

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Abstract

We tested whether lifelong modification of vitamin D signaling can alter the progression of early prostate carcinogenesis in studies using mice that develop high-grade prostatic intraepithelial neoplasia that is similar to humans. Two tissue-limited models showed that prostate vitamin D receptor (VDR) loss increased prostate carcinogenesis. In another study, we fed diets with three vitamin D3 levels (inadequate = 25 IU/kg diet, adequate for bone health = 150 IU/kg, or high = 1,000 IU/kg) and two calcium levels (adequate for bone health = 0.5% and high = 1.5%). Dietary vitamin D caused a dose-dependent increase in serum 25-hydroxyvitamin D levels and a reduction in the percentage of mice with adenocarcinoma but did not improve bone mass. In contrast, high calcium suppressed serum 1,25-dihydroxyvitamin D levels and improved bone mass but increased the incidence of adenocarcinoma. Analysis of the VDR cistrome in RWPE1 prostate epithelial cells revealed vitamin D-mediated regulation of multiple cancer-relevant pathways. Our data support the hypothesis that the loss of vitamin D signaling accelerates the early stages of prostate carcinogenesis, and our results suggest that different dietary requirements may be needed to support prostate health or maximize bone mass.

Significance: This work shows that disrupting vitamin D signaling through diet or genetic deletion increases early prostate carcinogenesis through multiple pathways. Higher-diet vitamin D levels are needed for cancer than bone.

Introduction

Serum 25-hydroxyvitamin D (25OHD) concentrations beyond those needed for bone health may reduce cancer risk (1). However, the evidence relating vitamin D status to prostate cancer risk in humans is mixed. Early association studies with small sample size were supportive (2), but larger population-based studies have shown protective (3), conditional (4, 5), and negative (6, 7) effects of increased vitamin D status on prostate cancer. More recently, the VITAL trial showed that 2,000 IU vitamin D3 per day over a median follow-up of 5.3 years reduced the HR for prostate cancer to 0.88 (8). However, the intervention was short relative to the process of human prostate carcinogenesis, and because few cases were documented, the comparison was underpowered and not significant (8). There is also evidence that high-dietary calcium (Ca) intake, a nutrient known to suppress renal production of the vitamin D hormone, 1,25(OH)2D, may increase prostate cancer risk (9). Thus, although the overall body of literature suggests a protective role for vitamin D on prostate cancer risk, the exact nature of the relationship, the protective serum vitamin D metabolite concentrations, and the mechanism of protective action have been elusive.

Animal and cell studies have provided essential proof for a direct causal relationship between vitamin D and prostate cancer development. In cultured prostate epithelial cells (PEC) or prostate cancer cells, 1,25(OH)2D suppresses proliferation, induces apoptosis, and promotes differentiation (10). Consistent with these data, vitamin D deficiency (11) and vitamin D receptor (VDR) gene deletion (12) increase, whereas injections with 1,25(OH)2D or vitamin
D analogs suppress (13) prostate tumor growth in various animal models. However, experiments linking prostate cancer development to human-relevant ranges of vitamin D status or Ca intake are limited (14–16).

Previously, we determined the vitamin D3 intake necessary to model human vitamin D status in mice (17) and found that intake as low as 100 IU vitamin D3/kg diet is sufficient to maintain the traditional vitamin D functions of bone growth and mineralization. Using this information, we showed that dietary vitamin D deficiency increased PEC proliferation, reduced PEC apoptosis, and increased the incidence of high-grade prostatic intraepithelial neoplasia (HGPIN) lesions in mice (18). Here, we report studies that extend our earlier work and directly address whether lifelong variation in the dietary levels of vitamin D and Ca can modify early-stage prostate cancer. In addition, we examine the importance of signaling through the VDR during early prostate carcinogenesis, and we identify potential candidate genes mediating the action of vitamin D on the PEC during carcinogenesis.

**Materials and Methods**

**Animals**

In our studies, we used TgAPT121 mice (TgAPT121+/−, C57BL/6 × DBA/2 background; ref. 19) bred to male B6D2F1 mice (Jackson Laboratories). This model has probasin promoter–driven, PEC-specific expression of a truncated SV40 Large T antigen protein that inactivates pRb family proteins. It has strong histologic similarities to the earlier stages of human prostate cancer (i.e., demonstrating hyperplasia, PIN, and adenocarcinoma over 6 months). Other mouse models used include mice with a floxed exon 2 in the Vdr gene (Vdrfl/fl, C57BL/6; ref. 18); mice with Cre recombinase expression driven by the rat probasin promoter (PB-Crefl/fl; C57BL/6; NCI Mouse Models of Human Cancer Consortium, # 01XF5); and Vdr knockout mice with intestine-specific transgenic expression of a hemagglutinin-tagged human VDR to generate mice lacking VDR in all cells in the prostate (HV2-VDR KO: TgAPT121+/−, HV2fl/fl, PB-Cre+/-; n = 32) and those with normal Vdr gene status (Cre-negative littermate controls: TgAPT121+/−, Vdrfl/fl, PB-Cre+/-; n = 33). At 28 weeks of age, mice were sacrificed and the prostate was harvested and prepared for histology.

**Experimental design**

**Experiment 1: Characterization of TgAPT121 mice.** For a microarray study, male TgAPT121+/− (n = 8) and TgAPT121−/− (n = 16) mice were used. After an overnight fast, 12-week-old mice were sacrificed, and prostate lobes were dissected on ice, snap-frozen in liquid nitrogen, and stored at −80°C. The anterior prostates from these mice were used for microarray analysis, whereas the dorsolateral prostate lobes were assessed for Vdr mRNA level by qPCR and VDR protein level by Western blot analysis. Prostates from a second 12-week-old cohort of 10 TgAPT121+/− and 4 TgAPT121−/− mice were used for histology and IHC.

**Experiment 2: PEC-specific Vdr gene deletion on prostate cancer.** The TgAPT121+/−, Vdrfl/fl, and PB-Cre+/- lines were crossed to mice with PEC-specific Vdr gene deletion (PEC-VDR KO: TgAPT121+/−, Vdrfl/fl, PB-Cre+/-; n = 32) and those with normal Vdr gene status (Cre-negative littermate controls: TgAPT121+/−, Vdrfl/fl, PB-Cre+/-; n = 33). At 28 weeks of age, mice were sacrificed and the prostate was harvested and prepared for histology.

**Experiment 3: Whole prostate Vdr gene deletion on prostate cancer.** TgAPT121 mice were crossed to Vdr knockout mice with intestine-specific transgenic expression of a hemagglutinin-tagged human VDR to generate mice lacking VDR in all cells in the prostate (HV2-VDR KO: TgAPT121+/−, HV2fl/fl, VDR−/-; n = 23) and littermate controls (TgAPT121+/−, HV2fl/fl, VDR+/-; n = 27). Transgenic expression of VDR in the intestine prevents abnormal Ca metabolism in Vdr knockout mice (20). At 26 weeks of age, mice were sacrificed and the prostate was harvested and prepared for histology.

**Experiment 4: Impact of diet on prostate tumorigenesis.** Male TgAPT121 transgenic mice were generated at Purdue University and then shipped to Ohio State University at weaning. Mice were randomly assigned to one of six AIN-76A–based diets with varying levels of dietary vitamin D3 (25, 150, and 1,000 IU/kg diet) and Ca (0.5, 1.5%) in a 2 × 3 factorial design (n = 34 mice per group). Diets and water were fed ad libitum. Mice were sacrificed at 28 weeks of age when blood, the right femur, and the prostate was harvested. Serum was prepared from blood, and a randomly selected subset of samples was analyzed for vitamin D metabolites (n = 10 per diet group). Intact femora were prepared for DEXA analysis (n = 25–27 per diet group). Prostates were processed for histology.

**Experiment 5: VDR chromatin immunoprecipitation sequencing analysis.** RWPE1 cells were cultured as we have described elsewhere (23). Cells were grown to 80% confluence and then treated with vehicle or 10 mmol/L 1,25 (OH)2D for 3 hours. Chromatin immunoprecipitation sequencing (ChiP-seq) and ChiP-qPCR analysis was conducted as described below.

**Methods**

**Prostate preparation, histologic grading, and scoring.** The bladder, prostate, and seminal vesicles were removed en bloc and processed for histology as described previously (18). Histologic examination of the anterior prostates was conducted using a modification of established...
guidelines (24) that better reflect the diversity of the early lesions in the TgAPT121 mouse prostate (see Supplementary Table S1 and Supplementary Fig. S1 for a more complete description of the grading criterion). Blinded assessment was conducted by one reviewer and was confirmed by a second reviewer.

Slides were digitized and the images were saved (ScanScope CS; Aperio). For experiments 2 and 3, lesions were marked on the digital images using the ImageScope Viewer (Aperio). The area covered by each lesion and the numbers of independent leukemic cells were determined, and the data were exported for further analysis. Total tissue area = the sum of the areas of all lesion foci plus the area covered by normal epithelium. Percent area = the proportion of total tissue area exhibiting the features of a lesion. Lesion count = the number of independent lesion foci. Incidence = the percentage of mice in a group with a particular lesion. Average lesion size = the ratio of the total area for a lesion type over the count of the lesion type.

For experiment 4, slides for the 204 mice were visually examined and scored for their highest histologic phenotype. Data were reported for the percentage of mice in each genotype as their most severe lesion.

**IHC analyses.** p53. Unstained sections were deparaffinized and rehydrated, followed by antigen retrieval for 30 minutes (Antigen Retrieval Citra Plus Solution, BioGenex). Endogenous peroxidase activity was quenched (DAKO Peroxidase Blocking Reagent, DAKO). Sections were incubated with rabbit polyclonal anti-mouse p53 antibody (NCL-p53-CM5p, 1:50; Leica Microsystems GmbH) for 1 hour at room temperature, followed by 30 minutes with DAKO EnVision+ Labeled Polymer-HRP anti-rabbit IgG secondary antibody. Antibody binding was visualized using the DAKO EnVision+ DAB+ Chromogen Solution, and slides were counterstained with hematoxylin.

**Apoptosis.** Unstained sections were evaluated by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining with the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit following the manufacturer’s instructions (Millipore Corp.). Slides were counterstained with methyl green.

**Quantification.** Three to five representative, nonoverlapping images without artifacts were captured by a high-resolution digital camera using bright field microscopy (Nikon ECLIPSE E800), and specific outcomes were analyzed using Image-Pro Plus 7.0 (Media Cybernetics, Inc.). A labeling index was calculated as the number of p53 or TUNEL-positive nuclei divided by total number of nuclei in the fields examined: (%Labeled nuclei) = I(L + C) × 100, where L = labeled nuclei and C = unlabeled nuclei.

**Measurement of prostate VDR protein level.** Frozen dorso-lateral prostates were homogenized on ice in lysis buffer [12 mmol/L NaCl, 0.5% Nonidet P-40, 0.02 mmol/L sodium orthovanadate, 5 mmol/L Tris-HCl (pH 8.0), and one Protease Inhibitor Cocktail tablet per 5 mL (Roche)]. Homogenates were centrifuged at 16,300 × g at 4°C for 15 minutes, and supernatants were harvested. Equal amounts of protein from 8 mice of each genotype were combined to generate two pooled samples. Samples (100 μg protein) were analyzed by Western blot analysis as previously described (23) and overnight incubation at 4°C with mouse anti-human VDR antibody (1:500; D-6, sc-13133; Santa Cruz Biotechnology) or mouse anti-β-actin antibody (1:5,000; AC-74; Sigma-Aldrich) followed by incubation with hors eradish peroxidase–conjugated goat-anti-mouse IgG light chain antibody (1:5,000; Jackson ImmunoResearch Laboratories) at room temperature for 1 hour.

**Prostate RNA preparation.** Frozen anterior prostates were crushed under liquid N2, and RNA was extracted with the SV Total RNA Isolation Kit (Promega). Genomic DNA was removed by treating RNA with RQ1 RNase-Free DNase (Promega) prior to repurification of RNA with the RNeasy Mini Kit (Qiagen). RNA was quantified, and RNA integrity was assessed visually from agarose gels.

**Analysis of VDR mRNA levels.** RNA was reverse transcribed into cDNA, and qPCR was conducted using primers and PCR conditions as we have previously reported (20).

**Microarray analysis.** RNA isolation. Equal amounts of RNA from 2 mice were pooled to yield 4 pooled TgAPT121+/− and 8 pooled TgAPT121−/− control samples. All samples used for microarray analysis had high RNA Integrity Number scores upon analysis on an Agilent Bioanalyzer. Gene expression profiling was conducted using the Genchip Mouse Gene 1.0 ST Array (ThermoFisher) and standard Affymetrix protocols at the Center for Medical Genomics at the Indiana University School of Medicine.

Microarray data were preprocessed using the Bioconductor package “xps,” and data were normalized by the Robust Multichip Average method. The data for this experiment are available in GEO (GSE50662). All arrays passed quality control tests and were included in the analyses. The preprocessed data were filtered using the detection above background algorithm (P ≤ 0.05: score = 1; 0.05 < P ≤ 0.07: score = 0.6; P > 0.07: score = 0), and a probeset was called “present” if sum score was ≥4.8 (n = 8, control) or 2.4 (n = 4, TgAPT121−/−). Probesets “present” in at least one of the two genotype groups and those with a coefficient of variation < 50 were included in subsequent steps. We removed probesets with no gene annotation, and only one probeset was used when multiple probesets were available. The final list contained 21,332 probesets. Differentially expressed transcripts were identified using the Significance Analysis of Microarray v3.09 (ref. 25; log2-transformed data, two class, unpaired analysis,
900 permutations). Significant transcripts (FDR < 5%) with fold change > 1.5 were analyzed using MetaCore (Clarivate Analytics).

Reanalysis of data from Tomlins and colleagues (26). Three normal PEC samples and 13 samples with HGPIN from GEO entry GSE6099 were examined for differential expression with GEO2R (15% FDR).

Reanalysis of Maund and colleagues (27) data. CEL files from 1,25(OH)2D-treated (±100 nmol/L; 6 or 48 hours) mouse prostate epithelial stem cells (ref. 27; GEO entry GSE18993) were processed using RMAexpress (http://rmaexpress.bmbolstad.com/). One array was abnormal and was excluded from the reanalysis (GSM469894). Differential gene expression was determined with Significance Analysis for Microarray (10% FDR) within BRB ArrayTools.

Serum vitamin D metabolite analysis. Serum 1,25(OH)2D and 25OHD were analyzed as previously described (20).

Bone analyses. The right femur from each mouse was stripped of muscle and analyzed for bone mineral density (BMD; g/cm²) using DEXA (20).

ChIP-seq analysis for VDR-binding sites. Sample preparation and ChIP-seq. ChIP was conducted as previously described (29) using normal rabbit IgG polyclonal antibody (12-370, Millipore Sigma) or anti-VDR polyclonal antibody (C-20, sc-1008, Santa Cruz Biotechnology, Inc.). ChIP-PCR (29) was used to identify three replicates with ligand-inducible VDR binding to the -252 to -51 bp region of the CYP24A1 gene promoter and no VDR binding to the GAPDH gene promoter. These were pooled by treatment to generate three samples (control treated/VDR IP, 1,25(OH)2D treated/VDR IP, and a pooled control + 1,25(OH)2D treated/IgG IP). ChIP was repeated on these samples and sent to the Michael Smith Genome Science Centre for sequencing (library construction on 100–300 bp fragments, 36 bp single-end reads, Illumina sequencing; ref. 30). The data for two lanes for each sample were pooled for analysis (GEO dataset GSE116843).

Bioinformatic analysis. ChIP-seq data were analyzed as reported elsewhere (31). We also reanalyzed ChIP-seq data from THP-1 cells (±100 nmol/L 1,25(OH)2D, 2 or 24 hours), LPS-treated THP-1 cells (±100 nmol/L 1,25(OH)2D, 24 hours), LS180 cells (100 nmol/L 1,25(OH)2D, 3 hours), and LX2 cells (100 nmol/L 1,25(OH)2D, 16 hours; ref. 32; and GEO entry GSE53041; multicell analysis upon request). ChIP-seq peaks were annotated to the nearest neighbor gene, and a search region of ±100 bp from the peak summit was examined for transcription factor–binding motifs with HOMER (33).

RWPE VDR ChIP-seq peaks were compared with 1,25(OH)2D-regulated genes from: (i) RWPE1 cells (10% FDR; ref. 34), (ii) mouse prostate stem cells (10% FDR), and (iii) 587 topologically associated domains (TAD) with vitamin D–regulated genes from THP-1 monocytes (35). Lists of genes with VDR-binding sites were examined for function and pathway enrichment using the IPA pathway analysis tool (Qiagen).

ChIP-PCR validation. Eighty percent confluent flasks of RWPE1 cells were treated with vehicle or 10 mmol/L 1,25(OH)2D for 3 hours (n = 3/treatment). ChIP-qPCR was conducted as described above for peaks associated with MAPK6; CTSD/SYT8; DDIT4; KLF4; CA9; ETNK2/REN; and CYP24A1 (Supplementary Fig. S2).

Statistical analysis of nonmicroarray data. Statistical analyses were conducted using SPSS for Windows-v.19.0 (IBM Corp.). In all experiments, differences were considered significant at P < 0.05, and a trend was defined as P < 0.10. Values are expressed as mean ± SEM of the non-transformed data. All sample size calculations were conducted for α = 0.05 and β = 0.8. In the tumor endpoint studies, 22 mice per group are necessary to detect 60% difference in the proportion of mice with adenocarcinoma using a χ² test. For serum vitamin D metabolites, 10 mice per group are necessary to detect a significant main effect of diet or genotype of 100% based on a variance = 75% of the difference between means (17). For bone, 17 mice are necessary to detect a significant main effect of diet or genotype of 4% based on a variance = difference between means (20).

The distribution of all continuous variables was examined by the Shapiro–Wilk test, and normal Q–Q plots and log- or square root transformations were used to achieve a normal distribution. If transformation did not normalize the distribution, the nonparametric Mann–Whitney U test was used. IHC and TUNEL data were analyzed by one-way ANOVA, followed by the Holm–Sidak step-down multiple comparison test to detect differences among lesion types. For the data from the mouse VDR deletion experiments and for Vdr mRNA levels, a Student t test was used to assess differences between genotype groups. For categorical variables in the cancer studies, the χ² test and Fisher exact tests were used to detect difference in frequencies. For the noncancer endpoints in the diet study, data were analyzed by two-way ANOVA to account for main effects and interactions between the dietary interventions.

Results

There were no observed adverse effects of the dietary interventions or genetic modifications on the growth, body weight, or appearance of the mice.

At 3 months of age, the anterior prostate of TgAPT121 mice is filled with a mix of low-grade (PIN I and II) and...
high-grade (PIN III and IV) lesions (18). We used micro-array analysis to determine whether the molecular phenotype of the anterior prostate in TgAPT121 mice adequately models features of human HGPIN, the precursor lesion to human prostate cancer (36). TgAPT121 mouse prostates had 3,440 upregulated and 2,087 downregulated genes compared with normal prostate (data available upon request). There were similarities in the TgAPT121 mouse prostate transcriptome compared with published studies comparing normal prostate epithelium with HGPIN in humans, i.e., 17 of the 21 genes reported individually in the literature, 12 of 72 genes from ref. 37, and 97 of 526 genes from ref. 26 (Supplementary Table S2). MetaCore analysis identified enriched pathways that link to prostate carcinogenesis within the TgAPT121 transcriptome including those regulating the DNA damage response, p53 signaling, apoptosis, TNF receptor signaling, TGFβ signaling, and signaling through the androgen receptor (Supplementary Table S3). Bioinformatic analysis also found transcription factor networks centered on proto-oncogenic transcription factors (e.g., Myc, Ap-1, Creb1, Nfkβ1, Ar, Egr1, Hnf4a) and Trp53 (encoding the protein p53). Increased p53 expression and apoptosis during the development of HGPIN lesions in TgAPT121 mouse prostate was confirmed by IHC (Fig. 1A and B). Collectively, these analyses support the use of the anterior prostate from TgAPT121 as a model of human HGPIN.

Vdr mRNA and protein levels were elevated in the dorsolateral lobe of 12-week-old TgAPT121+/− mice (Fig. 1C and D), and the microarray analysis shows that Vdr mRNA levels are also elevated in the anterior lobe (2.1-fold higher, Supplementary Table S2). To test the importance of VDR signaling in the progression of early stages of prostate cancer in TgAPT121+/− mice, we conducted two VDR gene deletion studies.

To test the role of VDR in the PEC, we used PEC-VDR KO mice. At 28 weeks of age, PIN III was the most prevalent lesion in the anterior prostate of TgAPT121 mice (60%–70% of total prostate tissue area), and the next most common lesion types were PIN II and PIN IV (Fig. 2). Compared with controls, the PEC-VDR KO mice had more independent adenocarcinoma foci (P = 0.003, Fig. 2A), the average size of the adenocarcinoma foci was larger...
P = 0.005, Fig. 2B), and the area covered by adenocarcinoma was larger (P = 0.004, Fig. 2C). In addition, a higher percentage of PEC-VDR KO mice developed focal adenocarcinoma than the control mice (73% vs. 38%; P = 0.004). Finally, although the percent area of the total prostate tissue occupied by adenocarcinoma was low, it was 2.8-fold higher in the PEC-VDR KO mice than the control mice (P = 0.003, Fig. 2D).

Mice with intestine-specific expression of VDR in VDR KO mice (HV2-VDR KO) have normal calcium homeostasis but no VDR within the entire prostate microenvironment. In these mice, the lesion count was higher for all of the advanced lesions compared with controls: PIN IV (P = 0.032), microinvasion (P = 0.004), and adenocarcinoma (P = 0.026; Fig. 3A). There was a trend toward larger adenocarcinoma lesion size (P = 0.062, Fig. 3B) and a greater area occupied by adenocarcinoma (P = 0.047, Fig. 3C) in the HV2-VDR KO mice. In addition, more HV2-VDR KO mice had adenocarcinoma than the control mice (48% vs. 19%; P = 0.027). Finally, HV2-VDR KO mice had higher percent area of adenocarcinoma and microinvasion (P = 0.001), lower percent area of the PIN II lesions (P = 0.024), and a trend toward higher percent area for the PIN IV lesions (P = 0.08, Fig. 3D).

Serum 25OHD levels were increased by raising dietary vitamin D₃ intake (P < 0.001, Fig. 4A). The levels in the...
25 IU/kg vitamin D3 group were slightly higher than we previously reported due to the contribution of residual vitamin D3 from casein in the diet. Dietary Ca did not alter serum 25OHD levels but reduced serum 1,25(OH)2D levels by more than 57% (Fig. 4B). There was a trend toward higher serum 1,25(OH)2D levels in mice fed the 1,000 IU/kg vitamin D3 diet (P = 0.074).

Low-dietary vitamin D intake increased the percentage of mice with adenocarcinoma as their most advanced lesion, such that mice fed the 25 IU/kg vitamin D3 diet had the highest incidence of this phenotype (93% vs. 68% incidence in the 1,000 IU/kg vitamin D3 group, P < 0.001, Fig. 4C). Increasing dietary Ca to 3 times greater than the rodent requirement caused a uniform 8.5% increase in adenocarcinoma incidence across the vitamin D diet groups (P < 0.05, Fig. 4C).

Although higher-dietary vitamin D suppressed the prostate cancer phenotype, higher vitamin D intake had no significant impact on BMD (Fig. 4D). However, increased dietary Ca increased BMD regardless of the dietary vitamin D intake level (P < 0.01).

VDR ChIP-seq analysis in RWPE1 cells was used to identify vitamin D target genes in prostate that may contribute to the protection of PECs against prostate cancer. VDR ChIP-seq analysis revealed 3,762 peaks in control cells and 3,445 peaks in 1,25(OH)2D-treated
cells with just 175 peaks common to both groups (Fig. 5A; Supplementary Table S4). DR3-type VDR-binding motifs that mediate VDR binding to DNA were found in 14.2% of the peaks, although more were found in the 1,25(OH)2D group (20%) and the overlap group (72.6%). VDR ChIP-seq sites were annotated to 3,390 protein coding genes, 682 long noncoding RNAs, and 472 miRNAs. This includes VDR-binding peaks in known vitamin D target genes like CYP24A1 (ref. 38; 5 peaks, 4 with DR3) and IGFBP3 (ref. 39; 2 peaks, 1 with DR3). The bulk of the peaks were intergenic (50.7%) or intronic (43.8%), reflecting long-distance regulation by enhancer elements. We confirmed the ChIP-seq results for 7 VDR-binding peaks by ChIP-PCR in the genes for: CYP24A1, MAPK6, CTSD/SYT8, DDIT4, KLF4, CA9, and ETNK2 (Supplementary Fig. S2). Note that 492 of the VDR sites in RWPE1 cells (associated with 461 genes) were found in ChIP-seq data from more than one cell type. Most of these peaks were from 1,25(OH)2D-treated cells or were peaks found in both treatment groups (n = 418, 73% with DR3 motifs). There was significant overlap between the VDR ChIP-seq peaks in RWPE1 cells and other datasets: (i) 1,573 peaks in 1,093 1,25(OH)2D-regulated genes from RWPE1 cells (ref. 34; Fig. 5B), (ii) 319 peaks in 193 1,25(OH)2D-regulated genes from mouse prostate epithelial stem cells (27), (iii) 263 genes containing a VDR peak within a TAD for vitamin D–regulated genes from human THP-1 cells (40).

Figure 4.
The effect of dietary Ca and vitamin D on TgAPTA121 mice. Diets were fed from weaning until 28 weeks of age (n = 34 per diet). A, Serum 25OHD and (B) serum 1,25(OH)2D are expressed as the mean ± SEM (n = 10/diet group). Values with different letter superscripts are significantly different (P < 0.05). C, Incidence of prostate adenocarcinoma in the anterior prostate (n = 34 per diet group). Values with different letter superscripts are significantly different between vitamin D groups (n = 60/group), while # signifies a significant effect of Ca (n = 90/diet Ca level, χ² test, P < 0.05). D, BMD of the femur. * signifies a significant effect of Ca (P < 0.01). Bone data are expressed as mean ± SEM (n = 50–54/diet Ca level).
The potential functional impact of vitamin D signaling on the PEC was broad. Enriched canonical pathways included those with cancer relevant functions (Table 1; Supplementary Table S5). Pathways regulating gene transcription included: one called "Transcriptional role for VDR in regulation of genes involved in osteoporosis" driven by genes like CYP24A1 and IGFBP3; four related to NF-κB signaling that included the genes NFKBIA and NFKBIZ; one for the oxidative stress response driven by the genes NFE2L2, TXNRD1, FOS, and JUNB; and several that included transcription factors whose genes contain VDR-binding sites, i.e., ESR1, CREB, HIF1A, and RBRJ. Note that 109 transcription factors were vitamin D-regulated in RWPE1 cells and contained a VDR peak. In addition, we found 259 interaction networks in the VDR cistrome that were centered on transcription factors, and 44 of these networks were centered on transcription factors whose genes had a DR3 motif below their VDR peak (Table 2).

**Discussion**

Vitamin D signaling through the VDR regulates cancer-relevant cellular events (10) and reduces prostate tumor growth (12, 13, 41), but definitive evidence for a beneficial effect of increasing vitamin D intake on prostate cancer risk has been lacking. Thus, specific recommendations for optimal vitamin D intake to reduce human prostate cancer risk are not yet possible. Rodent studies that manipulate dietary vitamin D intake have used either severe deficiency (11) or a large vitamin D excess (15, 16) and thus have modest translational potential. Meanwhile, human vitamin D3 interventions for cancer prevention (refs. 8, 42, 43; ClinicalTrials ID #NCT01463813) suffer from several limitations. First, they are typically conducted in older subjects with high baseline serum 25OHD levels (>75 nmol/L) and indolent cancer. Second, some studies use supplement levels that are too low for subjects who already have high vitamin D status (e.g., 800–2,000 IU/d; refs. 8, 43), whereas other studies use pharmacologic, bolus doses (e.g., monthly doses at ≥10,000 IU; ref. 42) that cause large swings in serum 25OHD and which may have adverse effects on other health outcomes (e.g., increased falls and fractures in older women; ref. 44). Finally, these studies are typically short even though targeting prostate carcinogenesis may require decades of intervention to capture effects of vitamin D. This may explain why the VITAL study results were promising (i.e., reduced cancer mortality only when the first 2 years of follow-up were excluded, nonsignificant reduction in HR for prostate cancer to 0.88) but reported as negative for the entire 5.3-year median follow-up period (8).

With the challenges of conducting human intervention studies in mind, we designed mouse studies that test the lifelong effects of manipulating vitamin D signaling on early stages of prostate carcinogenesis. Many animal models for prostate cancer exist (45), but we chose the TgAPT121 model because it models the early steps of...
prostate carcinogenesis, its prostate cancer phenotype develops gradually over 6 months (19), and because our data show that the HGPIN in TgAPT121 mice has a molecular phenotype similar to human HGPIN (46). Our findings in TgAPT121 mice consistently show that disrupting vitamin D signaling increases the progression of HGPIN lesions to focal adenocarcinoma. These studies extend our earlier report (18), and our new findings are similar to the effect of whole-body VDR gene deletion in the more aggressive LPB-Tag mouse (12). In addition, by using dietary vitamin D3 levels that allow us to model human relevant ranges of serum 25OHD [i.e., from adequate for bone health (50 nmol/L) to high natural levels seen in humans (125–150 nmol/L)], we can make two important conclusions. First, our data show that extreme deficiency that disrupts Ca homeostasis is not necessary to increase cancer risk. Also, we found that raising serum 25OHD to levels that are 50%–150% higher than those needed to protect bone can slow prostate cancer progression (Fig. 4). Still, before we can define the optimal vitamin D intake for prostate cancer prevention in humans, more research is needed to determine the life stages where increased vitamin D exposure is effective as well as to define the dose-response curve for the antiprostate cancer effects.

Although dietary vitamin D raises serum 25OHD levels, this metabolite is not a high-affinity ligand for the VDR. As such, the benefit of higher serum 25OHD levels is likely not direct but may be due to local conversion to 1,25(OH)2 D. Consistent with this hypothesis, cell studies have shown that primary PECs and prostate cancer cells can synthesize 1,25(OH)2 D from 25OHD (47, 48). In addition, Wagner and colleagues (49) found that supplementing preprostatectomy patients with 40,000 IU vitamin D3 a day for approximately 30 days increased prostate 1,25(OH)2 D levels. They also showed that prostate 1,25(OH)2 D levels were inversely associated with proliferation, suggesting protection resulting from local 1,25(OH)2 D production. Our study using higher-dietary Ca levels shows that the severity of prostate lesions in mice is also increased by suppressing serum 1,25(OH)2 D levels. However, although high-dietary Ca also increases prostate cancer, two other mouse models with slow-growing lesions (16) and low-serum 1,25(OH)2 D levels caused by high Ca diets did not accelerate prostate cancer in the more rapidly progressing LPB-Tag model (12). This suggests that the negative impact of high-dietary Ca on prostate carcinogenesis mediated through serum 1,25(OH)2 D levels may be limited to specific stages of cancer development; this hypothesis has not been formally tested. Some data also suggest that the benefit of higher-serum 25OHD on prostate cancer is modified by the level of dietary Ca (50), but we did not observe a significant Ca-by-vitamin D interaction in our study.

Our VDR ChIP-seq analysis demonstrates that activating vitamin D signaling affects multiple, complementary, cancer protective pathways in PECs, and this is consistent with our previous microarray reports (27, 34). The vitamin D–responsive gene signature in nontransformed PECs includes an antioxidant pathway signature that involves activation of NRF2, TXNRD1, FOS, and JUNB. A similar vitamin D effect in RWPE1 array Peak in multiple cell types

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<td>VD</td>
<td>–</td>
<td>48 h, –</td>
<td>N</td>
</tr>
<tr>
<td>EPAS1</td>
<td>Y</td>
<td>VD</td>
<td>–</td>
<td>24 h, +</td>
<td>N</td>
</tr>
<tr>
<td>FOS</td>
<td>Y</td>
<td>VD</td>
<td>+</td>
<td>all, +</td>
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<tr>
<td>GLI3</td>
<td>Y</td>
<td>VD</td>
<td>+</td>
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<td>Y</td>
</tr>
<tr>
<td>HIP1A</td>
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<td>VD</td>
<td>+</td>
<td>6, 48 h, +</td>
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<tr>
<td>KLF4</td>
<td>Y</td>
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<td>+</td>
<td>6 h, +</td>
<td>Y</td>
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<tr>
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<td>6, 24 h, +</td>
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<tr>
<td>RARA</td>
<td>Y</td>
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<td>N</td>
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<td>NRF</td>
<td>Y</td>
<td>VD</td>
<td>–</td>
<td>48 h, +</td>
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<tr>
<td>TP53</td>
<td>Y</td>
<td>VD</td>
<td>–</td>
<td>46 h, –</td>
<td>N</td>
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<tr>
<td>DEC1</td>
<td>Y</td>
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<td>Y</td>
<td>C</td>
<td>–</td>
<td>6, 48 h, –</td>
<td>Y</td>
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<tr>
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<td>Y</td>
<td>C</td>
<td>–</td>
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<td>N</td>
</tr>
<tr>
<td>MYC</td>
<td>Y</td>
<td>C</td>
<td>–</td>
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<tr>
<td>SMAD3</td>
<td>Y</td>
<td>C</td>
<td>–</td>
<td>24 h, –</td>
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<tr>
<td>AHR</td>
<td>Y</td>
<td>B</td>
<td>+</td>
<td>6 h, +</td>
<td>N</td>
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<tr>
<td>FOXO1</td>
<td>Y</td>
<td>B</td>
<td>+</td>
<td>6, 48 h, +</td>
<td>Y</td>
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<tr>
<td>RBPJ (CBP/1)</td>
<td>Y</td>
<td>B</td>
<td>+</td>
<td>48 h, –</td>
<td>Y</td>
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<tr>
<td>TBP</td>
<td>Y</td>
<td>B</td>
<td>+</td>
<td>6 h, –</td>
<td>Y</td>
</tr>
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</table>

*C, peak in ethanol-treated control; B, peak in both groups; VD, peak in 1,25(OH)2D-treated group.

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that vitamin D may amplify signals mediated through other transcription factors, e.g., by regulating genes for NFKBIA, an inhibitor of cancer-promoting NF-kB signaling (52), or FOXO1, whose loss promotes prostate carcinogenesis when TMPRSS2-ERG is overexpressed (53). Finally, the ChIP-seq data revealed VDR-binding peaks near genes that are involved in immune responses (e.g., IL1R2, IL20RB, IRAK2, and IRAK1BP1). These peaks may be responsible for the downregulation of immune/inflammatory mRNAs we reported earlier (34) and make PECs less responsive to proinflammatory, mitogenic signals from immune cells.

A final interesting finding from our mouse studies is that they suggest that the benefits of vitamin D signaling in the prostate may extend beyond the PEC. Although both prostate VDR deletion models had more advanced cancer, the impact of deletion was more dramatic in HV2-VDR KO mice that lack VDR in all prostate-associated cells than in the mice where VDR deletion was limited to PECs. Others have shown that prostate stromal cells are vitamin D target cells (54), and we previously showed that stromal cell proliferation is reduced in the prostates of PEC VDR KO mice (18). Collectively, these observations suggest that vitamin D signaling regulates multiple cells in the prostate, and it may modulate complex interactions among those cells. This hypothesis requires further testing.

In summary, our data provide solid support for the proof of principle that vitamin D signaling modulates progression through the early stages of prostate carcinogenesis and for the idea that early, life-long dietary manipulation of serum vitamin D metabolites can modify the course of early-stage prostate cancer. In addition, as Ames first suggested in his "triage theory" of nutrition and chronic disease (55), our data in mice demonstrate that there can be different dietary requirements for vitamin D and Ca based on the health outcome examined (i.e., higher vitamin D and lower Ca for prostate protection; lower vitamin D and higher Ca for bone).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J.C. Fleet, P.L. Kovalenko, S.K. Clinton Development of methodology: J.C. Fleet, P.L. Kovalenko, S.K. Clinton Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.C. Fleet, P.L. Kovalenko, S.K. Clinton Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.C. Fleet, P.L. Kovalenko, Y. Li, J. Smolinski, C. Spees, A. Neme, C. Carlberg, S.K. Clinton Writing, review, and/or revision of the manuscript (i.e., J.C. Fleet, P.L. Kovalenko, J. Smolinski, C. Spees, A. Neme, C. Carlberg, S.K. Clinton Administrative, technical, or material support (i.e., reviewing or organizing data, constructing databases): P.L. Kovalenko, M. Cui, S.K. Clinton Study supervision: J.C. Fleet, P.L. Kovalenko, S.K. Clinton

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