Vitamin D Inhibits the Formation of Prostatic Intraepithelial Neoplasia in *Nkx3.1; Pten* Mutant Mice

Whitney Banach-Petrosky,[1,2] Xuesong Ouyang,[1,2,3] Hui Gao,[1,2] Kamyar Nader,[1,2] Yan Ji,[1,4] Nanjoo Suh,[1,4] Robert S. DiPaola,[1,3] and Cory Abate-Shen[1,2,3]

Abstract

Purpose: Epidemiologic studies have shown that reduced levels of vitamin D represent a major risk factor for prostate cancer. In this report, we have examined the efficacy of 1α,25-dihydroxy-vitamin D_3 (1,25 D_3) as a chemopreventive agent using *Nkx3.1; Pten* mutant mice, which recapitulate stages of prostate carcinogenesis from prostate intraepithelial neoplasia (PIN) to adenocarcinoma.

Experimental Design: 1,25 D_3 (or vehicle) was delivered continuously to *Nkx3.1; Pten* mutant or control mice for a 4-month period beginning before (precancerous cohort) or after (cancerous cohort) these mice developed PIN. At the conclusion of the study, the mice were analyzed for the occurrence of PIN and/or cancer phenotypes by histologic analyses and immunostaining using known markers of cancer progression in these mice.

Results: We found that sustained delivery of 1,25 D_3 to the *Nkx3.1; Pten* mutant mice resulted in a significant reduction in the formation of PIN while having no apparent effect on the control mice. Furthermore, 1,25 D_3 was maximally effective when delivered before, rather than subsequent to, the initial occurrence of PIN. We further show that this 1,25 D_3–mediated inhibition of PIN was coincident with up-regulation of vitamin D receptor expression in the prostatic epithelium of the mutant mice, as well as in CASP prostate epithelial cell lines developed from these mice, while having no effect on androgen receptor expression or androgen receptor signaling.

Conclusion: Our findings show the value of chemoprevention studies using *Nkx3.1; Pten* mutant mice, particularly for evaluating the efficacy and underlying mechanisms of potential agents and to gain insights about the optimal timing of their delivery. In particular, our study predicts that vitamin D may have differential effects during early-stage versus late-stage disease and that it is more likely to be beneficial if delivered either before the overt manifestation of clinically detectable disease or during the earliest disease stages, rather than in advanced disease. Thus, our findings support the assessment of vitamin D analogues for chemoprevention in clinical trials targeting patients with early-stage disease and also establish molecular markers that can be used in such trials to determine biological activity and to optimize further clinical trials.

Authors' Affiliations: [1]The Cancer Institute of New Jersey, New Brunswick, New Jersey; [2]Center for Advanced Biotechnology and Medicine; [3]Department of Medicine, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School; and [4]Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey

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Note: W. Banach-Petrosky and X. Ouyang contributed equally to this work.

Requests for reprints: Cory Abate-Shen, Center for Advanced Biotechnology and Medicine, 679 Hoes Lane, Piscataway, NJ 08854. Phone: 732-235-5161; Fax: 732-235-5789; E-mail: abate@cabm.rutgers.edu or Robert S. DiPaola, The Cancer Institute of New Jersey, 195 Little Albany Street, New Brunswick, NJ 0890. Phone: 732-235-7469; Fax: 732-235-7493; E-mail: dipaolrs@umdnj.edu.

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Prostate cancer is the most common cancer among American men, resulting in significant morbidity and ~30,000 deaths annually. Whereas the diagnosis and treatment of patients with early-stage disease have markedly improved, the prognosis for men diagnosed with advanced stage disease remains poor. Undoubtedly, the most effective means of eradicating prostate cancer or the morbidity associated with its treatment is to establish effective approaches to prevent, reduce, or delay its occurrence (1, 2). Accordingly, several promising compounds are now under investigation as potential chemoprotective agents for prostate cancer, including natural derivatives, such as phytoestrogens, lycopene, selenium, and vitamins A, E, and D, and pharmaceutical agents, such as 5α-reductase inhibitors and nonsteroidal anti-inflammatory drugs (1, 3, 4).

The actions of vitamin D are presumed to be mediated by its interaction with vitamin D receptor, a member of the nuclear hormone receptor superfamily, which is known to regulate the expression of genes that control the proliferation, apoptosis, and/or differentiation of prostate epithelial cells (5, 6). The

potential efficacy of vitamin D as a chemopreventive agent for prostate cancer has attracted considerable interest because a large body of epidemiologic, cell culture, and clinical studies have suggested its antitumor activities for prostate cancer (e.g., refs. 7–14). However, unlike colon cancer for which the benefits of vitamin D in chemoprevention have been well established (reviewed in ref. 15), many critical issues remain unresolved about the effectiveness of vitamin D for prostate cancer prevention, despite numerous studies in animal models and human clinical trials that have been primarily designed to assess its effects in advanced prostate cancer (13, 16–19).

In the current study, we have examined the efficacy of 1α,25-dihydroxyvitamin D_3 (1,25 D_3) for prevention of prostate cancer using a mouse model based on the combinatorial loss-of-function of the *Nkx3.1* homeobox and *Pten* tumor suppressor genes (20, 21), which are known to be relevant for human prostate cancer (22, 23). Among their advantages for chemoprevention studies, *Nkx3.1; Pten* mutant mice recapitulate many aspects of human prostate cancer, including its critical dependence on aging for cancer progression (20, 21). Moreover, because they represent an autochthonous (indigenous) model of prostate cancer, *Nkx3.1; Pten* mutant mice enable the evaluation of 1,25 D_3, or other potential chemopreventive agents in the context of the prostate microenvironment and an intact host immune system, which are likely to have a significant effect on the efficacy of such agents *in vivo* (24).

We now report that 1,25 D_3 results in a significant reduction in the incidence and severity of the prostate intraepithelial neoplasia (PIN) phenotypes when delivered to *Nkx3.1; Pten* mutant mice before, but not subsequent to, the occurrence of PIN. We further show that the chemopreventive effects of 1,25 D_3 were coincident with increased expression of vitamin D receptor in the prostatic epithelium of these mutant mice and their derivative cell lines. Our studies show the value of the *Nkx3.1; Pten* mouse model for chemoprevention studies and provide new insights about the design of clinical trials to assess the role of vitamin D for chemoprevention of prostate cancer.

Materials and Methods

Mutant mice and analyses. The *Nkx3.1; Pten* mutant mice have previously been described (20, 21). Analyses were done on a hybrid 129/SvImJ and C57BL/65 background. Cohort groups were composed of age-matched littermates of wild-type mice ($Nkx3.1^{+/+}$; $Pten^{+/+}$) or mutant mice ($Nkx3.1^{+/-}$; $Pten^{+/-}$ or $Nkx3.1^{-/-}$; $Pten^{+/-}$). Note that the $Nkx3.1^{+/-}$; $Pten^{+/-}$ and $Nkx3.1^{-/-}$; $Pten^{+/-}$ mice have a similar phenotype (20, 21). Data presented show the results for the $Nkx3.1^{-/-}$; $Pten^{+/-}$ mutant mice; similar results were obtained for the $Nkx3.1^{+/-}$; $Pten^{+/-}$ mutant mice (data not shown).

Osmotic pumps (Alzet minipumps model 2004; 0.25 μL/h) were used for sustained delivery of 1,25 D_3 (Sigma) or vehicle (propylene glycol; Sigma) for a period of 1 to 4 months; pumps were replaced monthly as needed. The data shown were obtained with 46 ng/kg/d of 1,25 D_3; to confirm that the compound was active during the duration of the experiment, calcium levels were measured from serum by a colorimetric method with a Calcium Reagent Set (Teco Diagnostics, Anaheim, CA). Aside from the delivery of 1,25 D_3, all other variables, such as diet and environmental factors, were maintained under controlled conditions for the duration of the experiment.

At the time of sacrifice, the prostatic lobes were dissected individually, visually inspected, and fixed in formalin for histopathologic analyses. Histologic analyses was done on paraffin-embedded tissues by H&E staining as described (20, 21). Criteria for grading PIN phenotypes in *Nkx3.1; Pten* mutant mice, and specifically for distinguishing low-grade versus high-grade PIN, are well established and have previously been described in detail (25). Immunohistochemistry was done as described (21) with the following antibodies: Ki67 (Novocastra, Newcastle, United Kingdom; 1:1,000), androgen receptor (Sigma; 1:2,000), smooth muscle actin (Sigma; 1:2,000), p-Akt (Ser^{473}) (Cell signaling Technology, Beverly, MA; 1:200), and vitamin D receptor (Affinity BioReagents, Inc., Golden, CO; 1:250). Terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling assays were done with an *in situ* cell death detection kit (Roche Diagnostics, Mannheim, Germany).

Evaluation of the phenotype. To evaluate the consequences of 1,25 D_3 treatment, the experimental (mutant and wild-type) mice were examined to assess their histologic phenotype and to evaluate the status of markers of cancer progression by immunohistochemical staining. In particular, for each experimental mouse, multiple H&E-stained sections from at least six distinct regions of the anterior and dorsolateral prostatic lobes were evaluated for the occurrence of PIN; the slides were evaluated blinded and independently by three of us (H.G., W.B-P., and C.A.S). Data are shown for the anterior lobe; similar results were obtained for the dorsolateral prostate (data not shown). To further verify the phenotypic assessment, immunohistochemical analyses was done on sections from the anterior prostate of eight independent mice per experimental group using known markers of cancer progression in these mutant mice (i.e., actin, which is attenuated, and phospho-Akt, which is up-regulated during cancer progression; refs. 20, 21). Thus, the histologic grading for each experimental animal presented in Table 1 represents the sum of the histologic grading (done by three of us independently) plus the immunohistochemical staining for actin and Akt.

Table 1. Summary of the consequences of sustained delivery of 1,25 D_3

Experimental group	*N*	Normal	Low-grade PIN*	High-grade PIN with invasion	*P*†
Wild-type—vehicle	4	4	0	0	
Wild-type—1,25 D_3	8	8	0	0	
Precancerous mutant—vehicle	8	0	0	8	
Precancerous mutant—1,25 D_3	12	0	10	2	<0.001
Cancerous mutant—vehicle	8	0	0	8	
Cancerous mutant—1,25 D_3	8	0	1	7	

*Criteria for designating low-grade and high-grade PIN are described in ref. 25; see Materials and Methods for details about how the phenotype of the mice is evaluated.

†The *P* value shown compares the precancerous mutant mice in the vehicle-treated or 1,25 D_3–treated groups; none of the other groups showed a significant difference in phenotypic outcome.

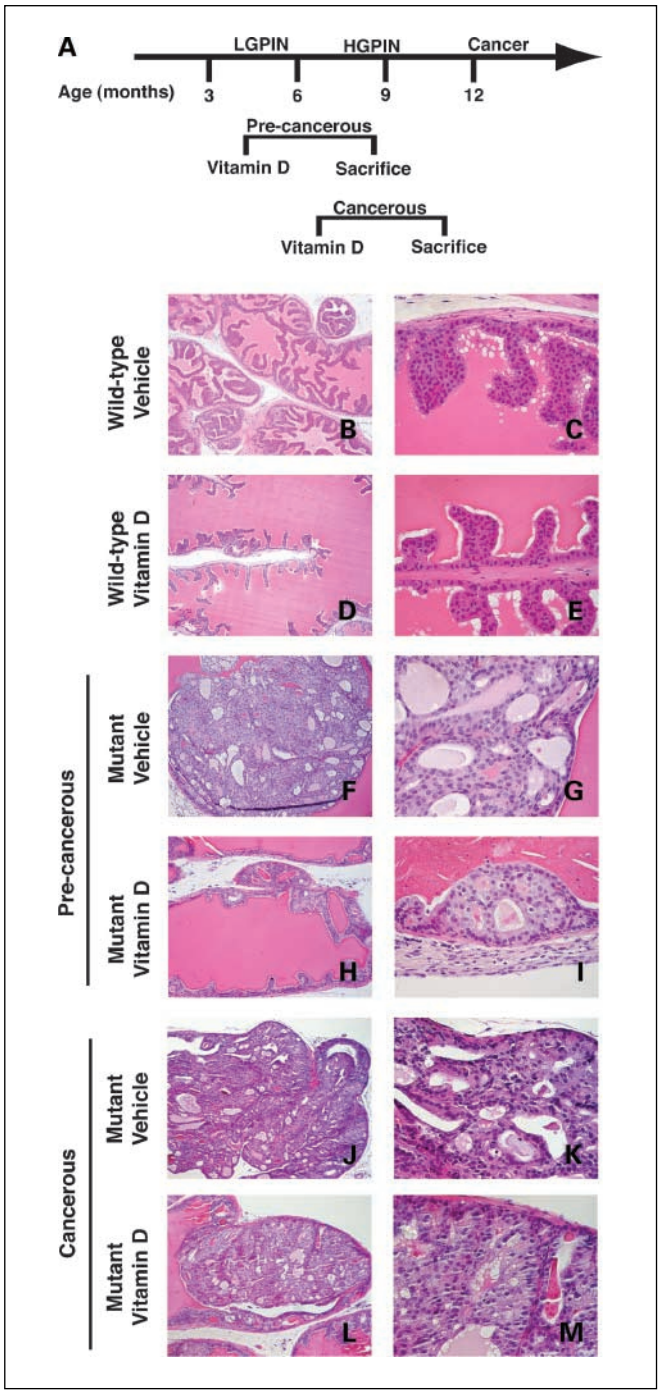

Fig. 1. 1,25 D_3 inhibits PIN formation in *Nkx3.1; Pten* mutant mice. *A,* diagram of the experimental variables. Top, characteristic time course of prostate cancer progression in *Nkx3.1; Pten* mutant mice from low-grade PIN (*LGPIN*) to high-grade PIN (*HGPIN*) and cancer. Bottom, experimental variables of the current study. For the precancerous group, cohorts of *Nkx3.1; Pten* mutant or wild-type control mice at 4 to 5 months of age received 1,25 D_3 (*Vitamin D*) continuously for a 4-month period and were sacrificed at 8 to 9 months. For the cancerous group, cohorts of mice at 6 to 7 months of age received 1,25 D_3 continuously for a 4-month period and were sacrificed at 10 to 11 months. *B* to *M,* histologic phenotype. H&E-stained sections of the anterior prostate of wild-type (*Nkx3.1*$^{+/+}$; *Pten*$^{+/+}$) or mutant (*Nkx3.1*$^{-/-}$; *Pten*$^{+/-}$) mice in the precancerous or cancerous group, as indicated. In the precancerous group, the vehicle-treated mutants display high-grade PIN lesions that encompass the lumen of the prostate (*F* and *G*), whereas the 1,25 D_3–treated mutants (vitamin D) display focal regions of low-grade PIN (*H* and *I*). In contrast, in the cancerous group, both the vehicle- and 1,25 D_3–treated mutants display extensive high-grade PIN that encompasses the lumen of the prostate.

Cell culture studies. The CASP cell lines, which were generated from *Nkx3.1; Pten* mutant mice, have characteristics of prostate epithelial cells, were derived from primary tumors, retain wild-type androgen receptor, and express androgen receptor at levels that are comparable to those expressed in the endogenous prostate epithelium (26, 27). We used one CASP cell line that is dependent on androgens for growth in culture (CASP 2-1) and its companion cell line (CASP 1-1), which has similar growth and tumorigenic properties but is not androgen responsive (27). CASP cells were grown in the presence of 1,25 D_3 or vehicle at a concentration of 1×10^7 or 1×10^9. Media contained 1% charcoal/dextran–treated fetal bovine serum with or without dihydrotestosterone as indicated. Proliferation assays were done as described (28) using 1×10^5 CASP cells per 35-mm dish. To measure androgen receptor responsivity, CASP cells (2×10^5 per 35-mm dish) were treated with 1,25 D_3 or vehicle for 24 hours, followed by transfection with an androgen receptor–responsive promoter, mouse mammary tumor virus-luciferase. For analyes of expression levels of androgen receptor or vitamin D receptor (or other genes), RNA was isolated from CASP cells treated with 1,25 D_3 or vehicle for 24 hours and quantitative reverse transcription-PCR analyses of androgen receptor were done using the Mx4000 Multiplex Quantitative PCR system (Stratagene, La Jolla, CA).

Results

Evaluating the consequences of 1,25 D_3 in precancerous versus cancerous Nkx3.1; Pten mutant mice. The *Nkx3.1; Pten* mutant mice display an age-dependent and highly penetrant (>85%) time course of disease progression (refs. 20, 21; Fig. 1A). Specifically, by 4 months of age, these mice develop focal low-grade PIN, which, by 6 months of age, progresses to high-grade PIN. As these mice approach 9 months of age, the high-grade PIN lesions are extensive and completely encompass the prostatic lobe; by 12 months, these mice display regions of overt adenocarcinoma, as well as extensive high-grade PIN. In this study, we refer to *Nkx3.1; Pten* mutant mice younger than 6 months as the "precancerous" cohort and those older than 6 months as the "cancerous" cohort.

To investigate its consequences for cancer progression, we delivered 1,25 D_3 (or vehicle) continuously for a 4-month period to cohorts of precancerous or cancerous mutant (*Nkx3.1*$^{+/-}$; *Pten*$^{+/-}$ or *Nkx3.1*$^{-/-}$; *Pten*$^{+/-}$) mice and age-matched wild-type (*Nkx3.1*$^{+/+}$; *Pten*$^{+/+}$) controls (Fig. 1A; Table 1). In initial studies, we determined that delivery of 23 or 46 ng/kg/d was effective for inhibiting the PIN/cancer phenotypes in these mice;[5] we used the latter dosage for subsequent studies. To ensure delivery of a constant amount of 1,25 D_3 for the duration of the experiment (up to 4 months), we used osmotic pumps containing vehicle or 1,25 D_3. The pumps were implanted into the back of the experimental mice and replaced monthly. Notably, we confirmed that the levels of calcium in the serum of 1,25 D_3–treated mice were much higher than those in the control mice at the conclusion of the experiment.

1,25 D_3 inhibits PIN in precancerous but not cancerous mutant mice. Using this experimental paradigm, we found that sustained delivery of 1,25 D_3 to the precancerous cohort of *Nkx3.1; Pten* mutant mice resulted in a significant inhibition ($P < 0.001$) in the occurrence of high-grade PIN, whereas it had no obvious effects on the histologic phenotype of the wild-type

[5] W. Banach-Petrosky and C. Abate-Shen, unpublished data.

mice (Table 1; Fig. 1B-I). Specifically, at the conclusion of the 4-month study, the prostatic epithelium of these 1,25 D_3–treated mutant mice (ages 8-9 months) contained large regions of relatively normal epithelium (Fig. 1H). Moreover, even when PIN lesions occurred in these mice, they were typically small and focal (Fig. 1I), similar to the characteristic phenotype of young (<6 months) *Nkx3.1; Pten* mutants (21, 25). This phenotype contrasts strikingly with that of the age-matched vehicle-treated mutants, which display extensive high-grade PIN lesions that typically fill the lumen of the prostate (Fig. 1F and G), as is characteristic of these mutant mice by 8 to 9 months of age (20, 21).

In marked contrast to its profound effects on the precancerous cohort, 1,25 D_3 was minimally effective for inhibiting prostate cancer progression when delivered to the cancerous cohort (Fig. 1J-M; Table 1). In particular, this group of *Nkx3.1; Pten* mutant mice displayed high-grade PIN lesions regardless of whether they received 1,25 D_3 or vehicle (Fig. 1J-M). Moreover, 1,25 D_3 also had no effect on the histologic phenotypes of the age-matched wild-type mice (data not shown). These findings suggest that vitamin D may be effective for preventing or delaying the initial occurrence of prostate cancer, but less so for inhibiting its progression.

The decreased formation of PIN following delivery of 1,25 D_3 to the precancerous, but not the cancerous, cohort of mutant mice was further evident by inspection of histologic markers of prostate cancer progression (Fig. 2). In particular, one characteristic feature of cancer progression in the *Nkx3.1;*

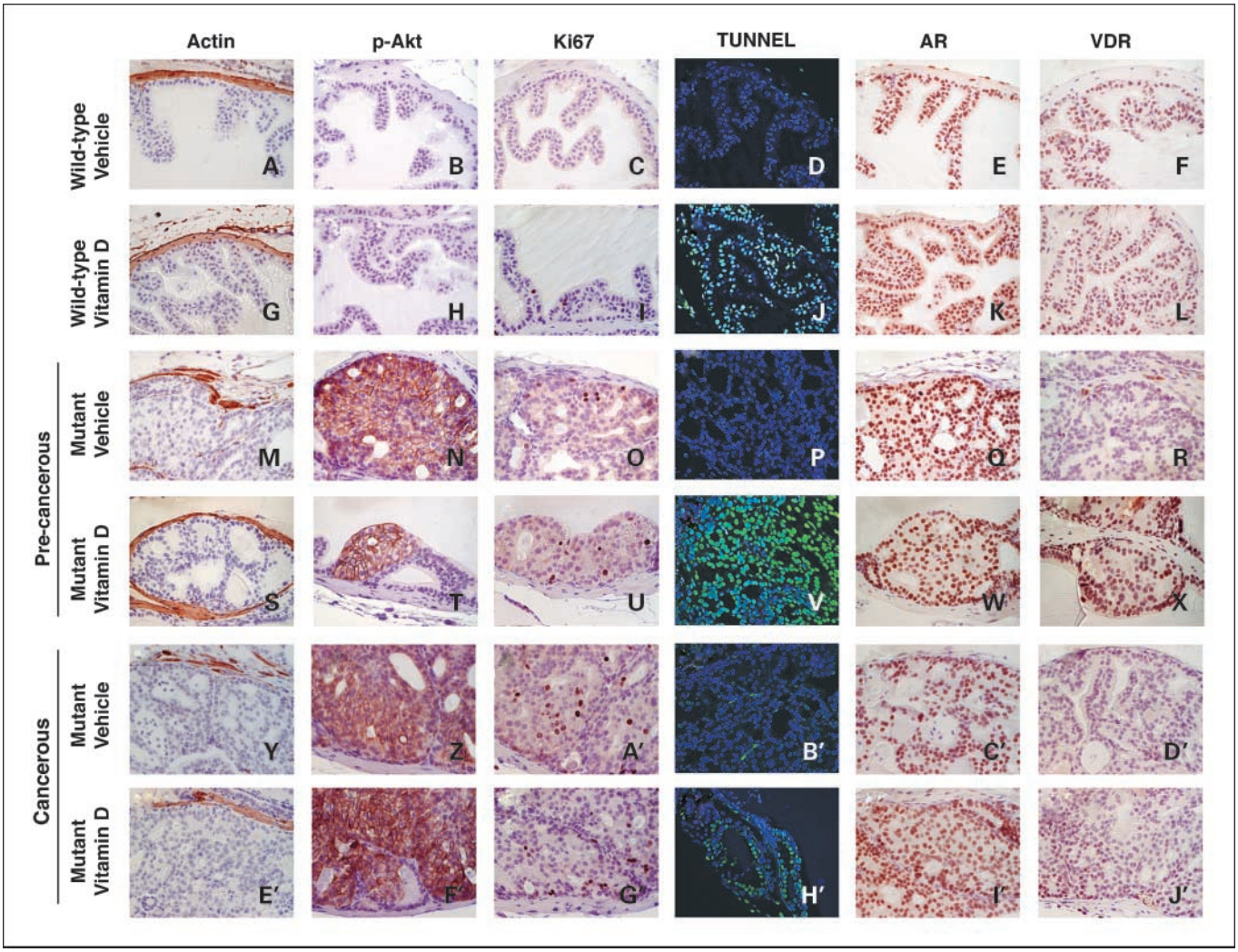

Fig. 2. Immunohistochemical analyses show inhibition of cancer progression following delivery of 1,25 D_3 to precancerous mutant mice. Immunohistochemical analyses of anterior prostate of wild-type mice (*Nkx3.1*$^{+/+}$; *Pten*$^{+/+}$; *A-L*), precancerous mutant cohort (*Nkx3.1*$^{-/-}$; *Pten*$^{+/-}$; *M-X*), or cancerous mutant cohort (*Nkx3.1*$^{-/-}$; *Pten*$^{+/-}$; *Y-J′*) treated with vehicle or 1,25 D_3. *A, G, M, S, Y,* and *E′,* staining for smooth muscle actin (*actin*) shows that in the precancerous cohort the stroma of the 1,25 D_3–treated mutant (*S*) is similar to that of the wild-type (*A* and *G*), whereas the vehicle-treated mutants and the cancerous cohort (vehicle and 1,25 D_3 treated) display attenuated stroma (*M, Y,* and *E′*). *B, H, N, T, Z,* and *F′,* staining for activated Akt kinase (p-Akt) shows its reduced activation in the precancerous 1,25 D_3–treated mutant (*T*) compared with the precancerous vehicle-treated group and the cancerous group treated with vehicle or 1,25 D_3 (*N, Z,* and *F′*). *C, I, O, U, A′,* and *G′,* staining for Ki67 shows that 1,25 D_3 does not have a significant effect on cellular proliferation *in vivo*. *D, J, P, V, B′,* and *H′,* terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling assays show profound apoptosis in the 1,25 D_3–treated wild-type and precancerous mutants (*J* and *V*). These sections were taken from 1,25 D_3–treated (or vehicle-treated) mice 1 week after delivery of the compound. *E, K, Q, W, C′,* and *I′,* staining for androgen receptor (*AR*) shows that its expression level is relatively unchanged in the prostate epithelium of the various experimental groups. *F, L, R, X, D′,* and *J′,* staining for vitamin D receptor (*VDR*) shows reduced expression in the vehicle-treated mutant mice (*R* and *D′*), whereas its expression is robustly elevated in the precancerous 1,25 D_3–treated mutants but only modestly elevated in the cancerous 1,25 D_3–treated mutants (*R* and *J′*).

Pten mutant mice is a marked attenuation of the stroma, which is evident by staining for smooth muscle actin (20, 21). However, in the precancerous cohort, the stromal layer of 1,25 D_3–treated mutant mice was largely intact and more similar to that of the wild-type prostate than to the attenuated stroma of the vehicle-treated mutants (N = 8 per group; Fig. 2A, G, M, and S). In contrast, in the cancerous cohort, the stroma was attenuated in both the vehicle- and 1,25 D_3–treated groups (N = 8 per group; Fig. 2Y and E′).

Another characteristic marker of cancer progression in *Nkx3.1; Pten* mutant mice is activation of the Akt kinase, which is a consequence of *Pten* loss-of-function (20, 21). Notably, the precancerous cohort of 1,25 D_3–treated mutant mice displayed minimal activation of Akt, in contrast to their vehicle-treated counterparts, which displayed robust levels of activated Akt (N = 8 per group; Fig. 2N and T). In the cancerous group, however, the levels of Akt activation were similar for both the vehicle- and 1,25 D_3–treated groups (N = 8 per group; Fig. 2Z and F′). Taken together, these findings further suggest that 1,25 D_3 may be beneficial for inhibiting cancer initiation, although it may not be as effective for inhibiting cancer progression if initiation had already occurred.

1,25 D_3 promotes apoptosis* in vivo *in the context of the prostate microenvironment, whereas it inhibits cellular proliferation in culture. Interestingly, the 1,25 D_3–treated mutant mice did not display a significant reduction in cellular proliferation, as evidenced by staining for Ki67 (N = 8; Fig. 2I and U), although they displayed a marked elevation in apoptosis particularly in the precancerous cohort, as evidenced by terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling staining (N = 8; Fig. 2J and V). This contrasts with the reported effects of vitamin D in cell culture where it results in reduced proliferation of prostatic epithelial cells (e.g., refs. 29, 30).

To reconcile this apparent discrepancy, we examined the consequences of 1,25 D_3 for growth in culture using CASP cells, which were derived from the *Nkx3.1; Pten* mutant mice (refs. 26, 27; Fig. 3A). Notably, the CASP cells were derived from primary tumors, rather than metastases, and they retain wild-type androgen receptor function and, in general, are less highly transformed than most of the human prostate cancer cell lines, including LNCaP (26, 27).[6] For these studies, we used two CASP cell lines, one that is androgen responsive (CASP 2-1) and one that is androgen nonresponsive, and we tested the consequences of 1,25 D_3 in these cell lines in the presence or absence of dihydrotestosterone. We found that 1,25 D_3 inhibited the growth of both CASP cell lines (CASP 2-1 and CASP 1-1) to a similar extent regardless of whether cells were grown in the presence or absence of dihydrotestosterone (Fig. 3A). These findings show that 1,25 D_3 inhibits the growth of prostate epithelial cells in culture more significantly than *in vivo*, analogous to our previous study in which we observed a differential response of these cells to androgen ablation in culture versus *in vivo* (27). Importantly, these studies suggest that the actions of chemopreventive agents may differ depending on the context in which they are tested, and emphasize the significance of the evaluating such agents in the context of the prostate microenvironment *in vivo*.

[6] H. Gao and C. Abate-Shen, unpublished data.

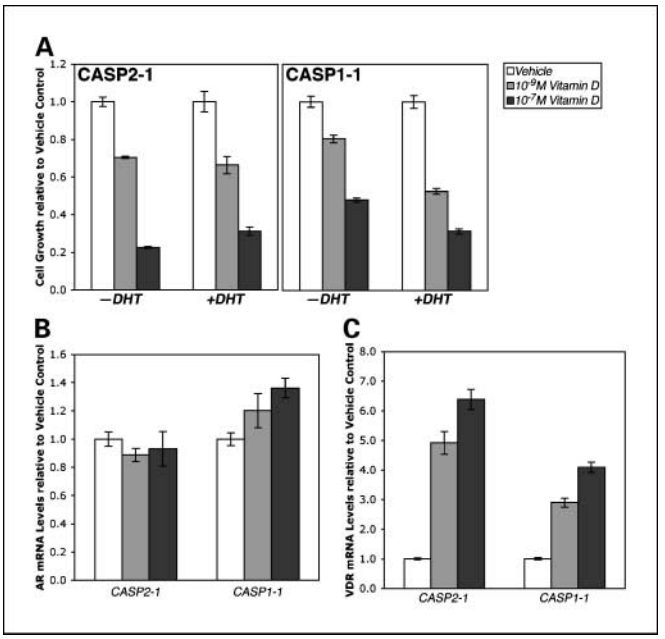

Fig. 3. 1,25 D_3 inhibits growth in culture and promotes expression of vitamin D receptor, but not androgen receptor. *A,* proliferation assays were done in androgen-responsive CASP 2-1 and androgen-nonresponsive cell line CASP 1-1 grown in the indicated concentrations of 1,25 D_3 (vitamin D) in media depleted of androgens (*−DHT*) or with androgens (*+DHT*). Representative assays done in triplicate; bars, 1 SE. *B* and *C,* real-time PCR was done using RNA made from CASP 2-1 or CASP 1-1 cells grown in the indicated concentrations of 1,25 D_3. The levels of androgen receptor (*B*) or vitamin D receptor (*C*), normalized to glyceraldehyde-3-phosphate dehydrogenase, are expressed relative to the wild-type control. The results shown are from cells grown in the presence of dihydrotestosterone; similar results were obtained using cells grown in the absence of dihydrotestosterone (data not shown).

1,25 D_3 promotes expression of vitamin D receptor while having no effect on expression of androgen receptor or androgen receptor signaling. Finally, we examined the consequences of sustained delivery of 1,25 D_3 for the expression levels of androgen receptor and vitamin D receptor in wild-type and mutant mice. In the wild-type mice, we observed that androgen receptor and vitamin D receptor were expressed in the prostatic epithelium and their expression levels were not appreciably changed by sustained delivery of 1,25 D_3 (N = 8 per group; Fig. 2E, F, K, and L). In contrast, expression of vitamin D receptor in the prostatic epithelium of vehicle-treated mutant mice was barely detectable in the PIN lesions both in the precancerous and cancerous cohorts (N = 8 per group; Fig. 2R and D′). In comparison, the levels of vitamin D receptor expression were significantly higher in the precancerous cohorts of 1,25 D_3–treated mutant mice whereas these were only modestly elevated in the cancerous cohorts of 1,25 D_3–treated mutants (N = 8; Fig. 2X and J′). This was specific for vitamin D receptor because expression of androgen receptor was similar in the vehicle- and 1,25 D_3–treated mutant mice (N = 8; Fig. 2Q, W, C′, and I′). These findings were further verified in the CASP cells, in which we observed using real-time PCR analyses that vitamin D receptor, but not androgen receptor, was up-regulated by 1,25 D_3 in both the androgen-responsive (CASP 2-1) and androgen-nonresponsive (CASP 1-1) cells regardless of whether the cells were grown in the presence or absence of dihydrotestosterone (Fig. 3B and C, and data not shown). Moreover, 1,25 D_3 treatment also did not affect the androgen receptor responsivity of the cells, as shown by luciferase reporter gene assay (data not shown).

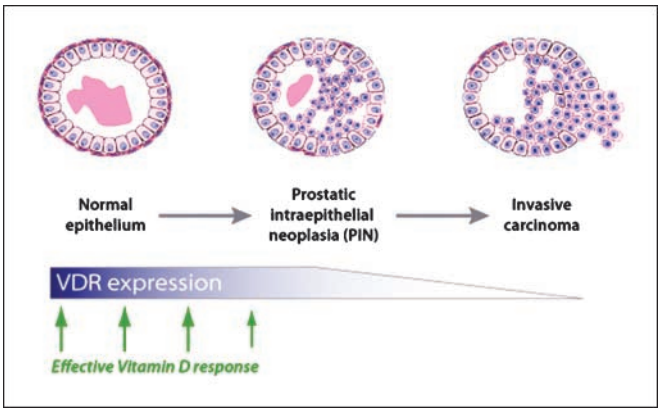

Fig. 4. A model of chemoprevention of prostate cancer with vitamin D. Our findings predict that the chemopreventive actions of vitamin D will be optimally beneficial if delivered during early stages of prostate carcinogenesis, during which time vitamin D receptor is expressed in the prostatic epithelium. Our study further predicts that delivery of vitamin D subsequent to cancer initiation may not be effective for preventing its progression and may have alternative effects on vitamin D receptor and androgen receptor than in the precancerous group.

Therefore, inhibition of cancer progression by 1,25 D_3 in the precancerous, but not cancerous, *Nkx3.1; Pten* mutant mice is coincident with the up-regulation of vitamin D receptor expression with no effect on androgen receptor expression or androgen receptor responsivity. Notably, our findings in the precancerous mutant mice and their derivative cell lines contrast with studies in the more highly transformed prostate cancer cell lines, such as LNCaP (14, 30), which further emphasizes the differential activity of 1,25 D_3 in early-stage versus late-stage disease. Indeed, our observations about the differential activity of 1,25 D_3 for vitamin D receptor expression in precancerous versus cancerous cohorts may explain why delivery of calcitriol (1,25 D_3) to patients with clinically overt disease resulted in a reduction (rather than an increase) in expression of vitamin D receptor in the prostate epithelium (31).

Discussion

Whereas prevention is undoubtedly the most effective means of eradicating prostate cancer, there are many inherent challenges to investigating potential chemopreventive agents in the human population. Chemoprevention studies in mouse models can help to overcome these challenges and guide studies in humans (24). Our current analyses in *Nkx3.1; Pten* mice, as well as previous reports using the TRAMP model of prostate cancer (32, 33), emphasize the importance of investigating chemopreventive agents in autochthonous models, which enable investigations in the context of the prostate microenvironment.

Our current findings provide new insights about the use of vitamin D for chemoprevention of prostate cancer (Fig. 4). Notably, we find that 1,25 D_3 is effective for inhibiting the formation of PIN in *Nkx3.1; Pten* mutant mice, whereas it does not have any apparent affects on the prostatic epithelium of wild-type mice. The implication is that vitamin D may be administered without adverse consequences to a wide spectrum of the population for prevention of prostate cancer. Whereas the levels of 1,25 D_3 used in this study are relatively high and may ultimately lead to hypercalcemia, derivatives of vitamin D (34) or modified schedules of its administration may be effective without the consequences of hypercalcemia. Indeed, it has been shown that intermittent doses of high-level calcitriol reduce the risk of hypercalcemia in humans (35).

Our findings leave open the question about whether 1,25 D_3 prevents the occurrence of PIN in the *Nkx3.1; Pten* mutant mice or simply delays it. We favor the latter interpretation because the low-grade PIN lesions that do occur in the 1,25 D_3–treated mutant mice are virtually identical to those seen in young (<6 months) *Nkx3.1; Pten* mutant mice. Nonetheless, considering the characteristic age-dependent slow onset of prostate cancer in humans, a significant delay in the occurrence of PIN may still have significant benefits for cancer prevention.

Finally, these data support the further use of the *Nkx3.1; Pten* mutant mice to design and optimize therapeutic clinical trials in patients at risk for prostate cancer, with PIN, and/or with prostate cancer. The clinical behavior of prostate cancer and the outcome of any clinical trial depend greatly on the point of progression in which patients present as well as any therapy, such as androgen ablation, which may have been instituted; analysis of chemopreventive agents in the context of these conditions makes clinical trial design difficult. Indeed, one important conclusion of our study is that the timing of the delivery of 1,25 D_3 relative to cancer initiation may determine its effectiveness for chemoprevention (Fig. 4) because we have observed that 1,25 D_3 is maximally effective when delivered before, rather than subsequent to, the occurrence of PIN or invasive cancer. Furthermore, our findings suggest that the consequences of vitamin D and its effects on vitamin D receptor and/or androgen receptor signaling may be different in patients with androgen-dependent disease versus those with androgen-independent disease. Considering that most clinical trials done to investigate the efficacy of vitamin D for prostate cancer have been conducted with patients with advanced disease (18), our findings support the assessment of vitamin D analogues in clinical trials of patients diagnosed with PIN or before the development of PIN. These data also provide insights on tumor marker assessments that can be used in clinical trials to determine the biological effects of these agents.

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