Tumor Suppressor microRNAs, miR-100 and -125b, Are Regulated by 1,25-dihydroxyvitamin D in Primary Prostate Cells and in Patient Tissue

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Abstract

MiR-100 and miR-125b are lost in many cancers and have potential function as tumor suppressors. Using both primary prostatic epithelial cultures and laser capture-microdissected prostate epithelium from 45 patients enrolled in a vitamin D3 randomized trial, we identified miR-100 and -125b as targets of 1,25-dihydroxyvitamin D3 (1,25D). In patients, miR-100 and -125b levels were significantly lower in tumor tissue than in benign prostate. Similarly, miR-100 and -125b were lower in primary prostate cancer cells than in cells derived from benign prostate. Prostatic concentrations of 1,25D positively correlated with these miRNA levels in both prostate cancer and benign epithelium, showing that patients with prostate cancer may still benefit from vitamin D3. In cell assays, upregulation of these miRNAs by 1,25D was vitamin D receptor dependent. Transfection of pre-miR-100 and pre-miR-125b in the presence or absence of 1,25D decreased invasiveness of cancer cell, RWPE-2. Pre-miR-100 and pre-miR-125b decreased proliferation in primary cells and cancer cells respectively. Pre-miR-125b transfection suppressed migration and clonal growth of prostate cancer cells, whereas knockdown of miR-125b in normal cells increased migration indicates a tumor suppressor function. 1,25D suppressed expression of previously bona fide mRNA targets of these miRNAs, E2F3 and Plk1, in a miRNA-dependent manner. Together, these findings show that vitamin D3 supplementation augments tumor suppressive miRNAs in patient prostate tissue, providing evidence that miRNAs could be key physiologic mediators of vitamin D3 activity in prevention and early treatment of prostate cancer. Cancer Prev Res; 6(5); 483–94. ©2013 AACR.

Introduction

In the past decade, new research has revealed health benefits of vitamin D that extend beyond its role in calcium homeostasis. Preclinical, epidemiologic, and clinical studies show that maintaining vitamin D status has potential benefits for several conditions including cancer, diabetes, multiple sclerosis, infection, depression, pain, and cardiovascular diseases (1). Recent reports show widespread deficiency of vitamin D in adults (2). In prostate cancer, the same factors that associate with deficient vitamin D levels (age, African-American ethnicity, and residence at northern latitudes) also associate with increased prostate cancer risk (3); suggesting vitamin D status alters prostate cancer risk. The slow growing nature of prostate cancer provides a long window of opportunity for chemopreventive agents, such as vitamin D (4). Please note that we use "vitamin D" in general discussion, whereas the specific form of vitamin D3/metabolites will be used when describing specific results or experiments.

Several decades of studies support a chemopreventive role for vitamin D in prostate cancer. A recent report showed that men supplemented with 4,000 IU/day vitamin D3 for one year had a decrease in positive cores at repeat biopsy compared to a control population (5). In prostate cancer cell culture and in vivo studies, 1,25-dihydroxyvitamin D3 (1,25D) regulates proliferation (6), apoptosis (7), inflammation (8), and differentiation (8) through binding to the vitamin D receptor (VDR), a transcription factor (9, 10). In prostate cancer tissues, high tumor VDR protein was correlated with low prostate-specific antigen (PSA), Gleason, and less advanced tumor stage (11). Shui and colleagues recently reported that men in the highest quartile of serum 25-hydroxyvitamin D3 (25D) regulates proliferation (6), apoptosis (7), inflammation (8), and differentiation (8) through binding to the vitamin D receptor (VDR), a transcription factor (9, 10). In prostate cancer tissues, high tumor VDR protein was correlated with low prostate-specific antigen (PSA), Gleason, and less advanced tumor stage (11). Shui and colleagues recently reported that men in the highest quartile of serum 25-hydroxyvitamin D3 (25D) had a 57% decrease in risk of lethal prostate cancer (12) which is consistent with other studies showing that high serum 25D levels were associated with decreased prostate cancer incidence and mortality (3, 13). However, epidemiologic studies do not consistently

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find association between vitamin D status and prostate cancer risk (14–17). 25D is the major circulating metabolite of vitamin D and precursor to the active 1,25D. The prostate expresses the VDR protein (18) and CYP27B1, the enzyme that converts 25D into 1,25D (19), showing that local production of 1,25D occurs in the prostate. Therefore, local prostatic levels of 1,25D may be an important factor in determination of vitamin D status and prostate cancer risk.

Given the genomic activity of vitamin D, via VDR binding to DNA and regulating gene transcription, it is likely that both coding genes and noncoding RNAs are regulated by vitamin D. MicroRNAs (miRNA) are small (~22 nucleotide) noncoding RNAs that can function via binding to the 3’ untranslated region of target mRNA resulting in mRNA degradation and/or translational repression (20, 21). Aberrant expression of miRNAs is observed in human cancer tissues/cells and may promote carcinogenesis and progression (22–26). miRNA signatures unique to prostate cancer have been identified (22–25) and various oncomiR and tumor suppressor miRNAs characterized. One recent study examined miRNAs regulated by 1,25D with testosterone in LNCaP cells (27). To date, no studies have investigated miRNAs regulated by vitamin D or vitamin D metabolites in human primary prostatic epithelial cells or in patients with prostate cancer.

In the current study, we identified and characterized miRNAs that are regulated by vitamin D. miRNA expression was profiled in normal human prostatic epithelial cells that were treated with a nongrowth inhibitory dose of 1,25D. Candidate miRNAs were validated in laser capture microdissected epithelium from patient prostate tissue from a clinical trial in which the men were given various doses of vitamin D3 prior to radical prostatectomy. Targets of validated miRNAs and their effects on cell phenotype were further characterized in vitro. This translational study found that the chemopreventive activity of vitamin D in the prostate involves upregulation of tumor suppressor miRNAs.

Materials and Methods

Cell cultures

As described previously (28), primary prostatic epithelial cells (PrE) were established from radical prostatectomy tissue at the University of Illinois at Chicago Medical Center (Chicago, IL). Fresh tissue from the peripheral zone was selected by a pathologist according to an Institutional Review Board-approved protocol. Briefly, the tissue was digested in collagenase, and plated on collagen-coated dishes in PrEGM media (Lonza) for epithelial cell outgrowth. Epithelial cells are abbreviated PrE cells (from benign prostate peripheral zone) or PrE-Ca (from an area of >80% cancer). The PrE cells were authenticated after primary culture via gene expression known basal epithelial cells markers, cytokeratin 5 and p63 and lack of androgen receptor. All cells were used at secondary passage and approximately 70% confluency (cell density). LNCaP, DU145, and PC3 cell lines (obtained directly from American Type Culture Collection) at various times since 2006 and frozen into multiple aliquots) are grown in RPMI media (Life Technologies)/10% FBS. RWPE-1 (normal)/RWPE-2 (cancer) cells (cell stocks were obtained from Dr. Muka Webber, Prof. Emeritus, Michigan State, MI, USA; ref. 29), via Dr. Michael Waalkes, (National Institute of Environmental Health, NC, USA) were grown in keratinocyte serum-free media (Life Technologies). 1,25D was dosed at 50 nmol/L unless otherwise noted.

Clinical samples

Paraffin blocks of prostatectomy specimens from 45 patients in the clinical trial were used. In this trial, 66 patients (age 42–67 years) were randomized into 3 dose groups of vitamin D3 (cholecalciferol); 400, 10,000, or 40,000 IU/day given orally in the time interval between randomization and prostatectomy (3–8 weeks). The trial was registered with www.clinicaltrials.gov (NCT00741364). The specimens were obtained from Dr. T. van der Kwast (University Health Network, Toronto, Canada) who, blinded by treatment group, demarcated benign and prostate cancer areas for laser capture microdissection. Benign: glands in the peripheral zone without signs of atrophy or inflammation and without high-grade prostatic intraepithelial neoplasia. Prostate cancer: highest density of Gleason grade 3 glands.

Laser Capture Microdissection

Two 8 µm formalin-fixed paraffin-embedded sections of each patient prostatectomy specimen were mounted on RNase-free PEN slides (Leica). As previously described by Nonn and colleagues (30), specimens were deparaffinized, fixed, and stained with 0.5% toluidine blue. Using the Leica LMD-6000 (Leica) 100 to 150 acini of benign and prostate cancer epithelium were collected into Eppendorf caps containing digestion buffer (Life Technologies).

RNA isolation

For cell cultures, total RNA was isolated with Trizol (Life Technologies). For patient tissue samples, RNA was extracted with the RecoverAll kit using a modified protocol as previously described (30). RNA quality and quantity were evaluated by measuring absorbance at 260 and 280 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

RT-qPCR of prostate cells and clinical samples

Cells. For miRNA analysis, stem-loop cDNAs were generated on 10 ng of RNA using a pool of 5X RT primers and the TaqMan MicroRNA RT Kit (Applied Biosystems) or on 20 ng of RNA using LNA PCR primers sets and the miRCURY LNA Universal RT kit (Exiqon). For mRNA analysis, cDNA was generated from 500 ng of RNA with the High Capacity Kit (Applied Biosystems). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was run with either SYBR Green or TaqMan Gene Expression Master Mix (miRNA; Applied Biosystems) using the StepOne Plus or HT7900 machines (Applied Biosystems). Cg values were normalized to housekeeping genes TBP and/or B2M for mRNA and RNU144 and/or RNU48 for miRNA.
Tissue. For miRNA analysis, 45 ng of RNA was used with Megaplex Pool A RT Primers and the TaqMan miRNA RT kit. For mRNAs, RNA (50 ng) was reverse-transcribed using the Vilo cDNA Kit (Invitrogen). cDNAs were preamplified according to the manufacturer’s protocol using TaqMan PreAmp Master Mix and Specific TaqMan-designed assays (Supplementary Table S1). For miRNAs, the Megaplex Pool A Pre-Amp primers were used in the preamplification reaction. Fold-changes were calculated using $2^{-\Delta\Delta C_t}$ method and normalized to the expression of RNU44 and RNU48 or mRNA housekeeping genes (B2M, HPRT, Actin, GAPDH, and/or CK18).

Vitamin D metabolite measurement in serum and prostate tissue

Serum and tissue 25D levels were measured by liquid chromatography-tandem mass spectrometry as described previously (31). Serum and tissue 1,25D levels were determined by enzyme immunoassay (Immunodiagnostic Systems). In tissues, vitamin D metabolite concentrations were measured by an initial preextracted procedure as previously described (31). Tissue vitamin D metabolites were reported as the mean of 2 samples (transitional and peripheral zone) of fresh frozen tissue. Full statistical analysis of serum and tissue levels of vitamin D metabolites are reported elsewhere (32).

miRNA profiling by TaqMan low-density array

PrE cells (70% confluency) were treated with 1,25D or ethanol for 24 hours and RNA was extracted. Megaplex pool of RT primers (Applied Biosystems) specific to either TaqMan low-density array (TLDA) plate A/B and 350 ng RNA was used for the cDNA reaction and PCR was run according to the manufacturer’s protocol. Results were analyzed with RQ Manager, Data Assist Software (Applied Biosystems), and manual $2^{-\Delta\Delta C_t}$ method. G values more than 33 were omitted, and RNU44 and RNU48 were analyzed as endogenous controls.

Pre-miR/anti-miR/siRNA transfection

PrE or LNCaP cells were transfected using NeoFx reverse transfection kit (Ambion) with a hsa-pre-miR, hsa-anti-miR to miR-100 or miR-125b (Applied Biosystems), or siRNA-VDR or negative siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) at a final concentration of 5 to 50 nmol/L.

Cell proliferation assay

PrE cells were transfected with a hsa-pre-miR or with hsa-anti-miR at a final concentration 10 nmol/L and plated in triplicate in a 24-well plate at a density of $2.5 \times 10^5$ cells per well. 24 hours after transfection, cells were treated in the presence or absence of 1,25D and allowed to grow for another 48 hours. Cell number was counted with the Cellometer Auto T4 (Nexcelom Bioscience).

Migration (scratch) assay

PrE cells ($3.5 \times 10^3$) were scratched 24 hours after transfection with 50 nmol/L hsa-pre-miR. Pictures were taken at times 0 to 24 hours using the EVOS Digital Microscope (Advanced Microscopy Group). Migration was analyzed by percent open of the area of the scratch with ImageJ software.

Clonogenic assay

LNCaP cells were transfected in triplicate with 50 nmol/L hsa-pre-miR at a density of 500 cells per 60 mm dish. After 10 days, the colonies were stained with 0.5% crystal violet and counted.

Invasion assay

RWPE-2 cells were transfected with hsa-pre-miRs (50 nmol/L). Twenty-four hours following transfection, cells were counted and plated on Matrigel-coated 8 μm diameter pore inserts (BD Biosciences) in media containing ethanol or 1,25D (50 nmol/L). 10% FBS was added to the bottom chamber as a chemoattractant. After 48 hours, noninvaded cells were removed and invasive cells on the underside of the insert were fixed and stained with 0.5% crystal violet. Four fields per insert were photographed and counted at $\times 20$.

Immunoblot

Proteins were isolated in Cell Lysis buffer (Cell Signaling) followed by centrifugation. Protein (10 μg) was loaded onto a 10% Bis-Tris NuPAGE gel (Life Technologies) and transferred to polyvinylidene difluoride membrane. The membranes were probed with anti-VDR 1:500 (Santa Cruz Biotechnology), anti-β-tubulin 1:1,000, anti-actin 1:1,000, anti-PLK1 1:500 (Cell Signaling), or anti-E2F3 1:1,000 (AbCam). Bands were visualized using LumiGLO reagent (Cell Signaling).

Statistical analysis

Unsupervised hierarchical clustering was done with DataAssist 3.0 (Applied Biosystems). For in vitro experiments the difference between 2 groups was analyzed with Student t test. In patient samples, the correlation between miRNA and vitamin D metabolites was determined by Spearman correlation. A paired samples t test was used to analyze differences in paired benign and prostate cancer tissues. ANOVA analyzed the difference between vitamin D3 treatment groups. Differences were considered significant at $P < 0.01$ and $P < 0.05$.

Results

1,25D alters miRNA expression profiles in primary human epithelial cells

miRNA expression was analyzed in PrE cells after 24 hours of 50 nmol/L 1,25D or vehicle (0.01% ethanol) treatment. To control for cell density-induced changes in miRNAs (33), PrE cells were treated at 70% confluence with a nongrowth inhibitory dose of 1,25D (Supplementary Fig. S1). Expression of 667 miRNAs was profiled by miRNA TLDA in 5 different patient-derived primary PrE cells. Of the miRNAs profiled, approximately 30% of
miRNAs on array A and 15% on array B were detected ($C_t < 33$) in PrE cells (Supplementary Table S2). Up- or downregulated miRNAs were identified by a more than 1.5-fold increase or less than 0.5 decrease and a significance of $P < 0.05$ across 3 patients. Paired t test identified miR-100, miR-125b, and 29 other miRNAs that were increased by 1,25D and only one downregulated miRNA, miR-196b (Supplementary Table S3).

MiRNAs were selected for validation by magnitude of change and relevance to cancer/prostate cancer from published studies (22–24, 26). MiR-100, miR-125b, miR-200c, miR-197, miR-196b, miR-106b, miR-141, miR-103, miR-146a, miR-301a, miR-331-3p, and let-7b were analyzed by individual PCR in 3 of the original PrE cells and 3 additional PrE cells. Unsupervised hierarchical clustering of the 3 patients showed that intrapatient miRNA expression for these miRNAs was more similar than 1,25D-induced expression changes (Fig. 1A). However, all 12 miRNAs were significantly regulated by 1,25D in the 3 original PrE cell lines in the TLDA array (Supplementary Table S3). Of the 12 miRNAs analyzed, individual qRT-PCR validation confirmed that miR-100 and miR-125b were the most consistently and significantly upregulated by 1,25D (1.5–2.5-fold) across the 6 total PrE cells (Fig. 1B). Further validation of miR-100 and miR-125b in other prostate cells lines RWPE-1, RWPE-2 (Fig. 1B), and the commercially available PrEC cells (Supplementary Fig. S2) showed similar 1,25D-mediated changes in the miRNAs. In contrast, no significant regulation was observed in LNCaP (Fig. 1B), DU145, and PC3 cells (Figure S2).

Suppression of miR-100 and miR-125b targets by 1,25D

MiR-100 and miR-125b were further characterized, as they have known tumor suppressor activity (34, 35) and are located on the same chromosomal region 11q13 (36). We examined functional activity of miR-100 and miR-125b by measuring previously bona fide mRNA targets of the miRNAs; Plk1 for miR-100 and E2F3 for miR-125b (37, 38). MiR-125b was inversely correlated with its target E2F3 ($r = -0.52, p = 0.03$) and miR-100 to its target PLK1 ($r = -0.5; P = 0.04$) in 1,25D-treated PrE cells from 9 patients, supporting regulation of these genes by the miRs by 1,25D (Supplementary Fig. S3). Transfection with anti-
miR-100 or miR-125b in PrE cells showed that miR-100 abrogated regulation of PLK1 by 1,25D, showing that the miRNAs are required for its target regulation by 1,25D (Fig. 1C). 1,25D also dose dependently decreased E2F3 and PLK1 protein levels in PrE cells (Fig. 1D).

**MiR-100, miR-125b, E2F3, and PLK1 are differentially expressed in prostate cancer cells**

We examined miR-100 and miR-125b expression in a matched pair of PrE and PrE-Ca cells that were derived from the same patient and in the prostate cell line RWPE. The PrE-Ca cells [alpha methylacyl CoA racemase (AMACR) positive; Fig. 2A], had lower miR-125b and higher E2F3 in basal conditions compared with the normal PrE cells (Fig. 2B). We did not observe a reduction in basal levels of miR-100; however, its target Plk1 was increased in PrE-Ca cells compared with normal cells (Fig. 2C). 1,25D treatment increased miR-100 and miR-125b and decreased their targets in both the normal PrE cells and PrE-Ca cells compared with their control further suggesting that 1,25D augments these miRNAs in normal and prostate cancer cells (Fig. 2B and C). 1,25D also inversely regulated the miRNAs and their targets in the RWPE cells, but there were no significant differences in the response between the RWPE 1 (normal) and RWPE-2 (cancer) cells (Supplementary Fig. S4). Overall, these data suggest that miR-100 and miR-125b levels are lower in prostate cancer and their suppression inversely correlates with PLK1 and E2F3 in PrE cells.

**MiR-100 and -125b suppress cancer-associated phenotypes**

*In vitro* phenotypes consistent with tumor suppressive functions of miR-100 and miR-125b (34, 39) were investigated by the analysis of invasion, cell growth, migration, and clonogenicity, following modulation of miR-100 and miR-125b levels in RWPE-2, PrE, and LnCaP cells. In RWPE-2 cells, pre-miR-125b significantly reduced invasion through Matrigel and 1,25D further reduced invasiveness of the pre-miR-100 and pre-miR-125b–transfected cells (Fig. 3A). Cell proliferation was quantified in PrE, RWPE-2, and LnCaP cells 72 hours after transfection with pre-miRs or anti-miRs. Pre-miR-100 significantly reduced growth of PrE cells compared with control, whereas pre-miR-125b decreased growth in LnCaP and RWPE-2 cancer cells (Fig. 3B). Anti-miR-100 transfection in PrE cells in the presence or absence of 1,25D resulted in a small but significant 7% increase in growth (Supplementary Table S4). Pre-miR-100 and pre-miR-125b in RWPE-2 cells showed a 8% and 24% decrease in growth, respectively, and miR-125b decreased growth of LnCaP 16% (ethanol) and 18% (1,25D-treated) (Supplementary Table S4). In addition, in LnCaP cells, pre-miR-125b decreased colony formation compared with the control (Fig. 3C) supporting the tumor-suppressive activity of miR-125b as clonogenicity correlates with tumorigenesis in animals (40). Cell migration by scratch assay showed that pre-miR-125b decreased migration (more open) of RWPE-2 (Fig. 3D) and PrE cells.

**Figure 2.** Differential expression of miR-100, miR-125b and their targets in 1,25D-treated PrE cells. qRT-PCR shows the expression of AMACR, A, miR-125b and E2F3, B, miR-100 and PLK1, in 1,25D-treated paired PrE and PrE-Ca cells (C) derived from one patient. Error bars = SD of experimental replicates and experiments were repeated twice.
Anti-miR transfection of the miRNAs in the presence or absence of 1,25D showed an increase in closure (less open) in 1,25D-treated PrE cells at 24 hours. Modulating miRNA levels in LNCaPs did not show any changes in migration (Fig. 3D). These data indicate that miR-100 and miR-125b have cell-specific antimigratory, antiproliferative, and/or anticolonogenic properties in normal and cancerous prostate cells, which is consistent with tumor suppressor actions.

Regulation of miR-100 and miR-125b and their targets by 1,25D is VDR-dependent

VDR expression was analyzed in PrE, LNCaP, and RWPE cells. Primary PrE and RWPE cells had comparable basal levels of VDR expression (Supplementary Fig. S6). Knockdown of VDR in PrE cells by siRNA reduced VDR protein levels by approximately 50%, which was sufficient to blunt VDR activity as shown by reduced VDR-responsive CYP24A1 expression (Fig. 4A and B). The reduction in VDR abrogated upregulation of miR-100 and miR-125b by 1,25D. VDR knockdown with siRNA also confirmed that regulation of E2F3 and PLK1 expression was VDR dependent (Fig. 4C). These results show that upregulation of miR-100 and miR-125 and downregulation of their targets E2F3 and PLK1 by 1,25D occurs in a VDR-dependent manner.

miR-100, miR-125b, E2F3, and PLK1 are differentially expressed in tissue

The in vitro findings were validated in prostate tissue from a Phase II clinical trial of vitamin D3 in patients with prostate cancer. This trial was run at the University Health Network and Sunnybrook Hospital (Toronto, Ontario, Canada). Patients (N = 66) were randomized to 3 treatment groups; 1 = 400 IU/day, 2 = 10,000 IU/day, and 3 = 40,000 IU/day of oral vitamin D3 (cholecalciferol) for 3 to 8 weeks before surgery (Principal investigator: Reinhold Vieth). Tumor and benign prostate epithelium from 15 patients per treatment group was collected by LCM (Fig. 5A) and the expression of 12 miRNAs (miR-100, miR-125b, miR-103, miR-331-3p, miR-146a, miR-155, miR-197, miR-106b, miR-141, miR-301a, let-7a, and let-7b) was quantified by qRT-PCR. Areas of prostate cancer were AMACR positive (Fig. 5B) confirming that tumor was sampled. Both miR-100 and miR-125b were decreased in tumor compared with benign epithelium, P < 0.001 (Fig. 5B). In addition, the other 9 miRNAs that we analyzed in patient tissue (miR-103, miR-331-3p, miR-146a, miR-155, miR-197, miR-106b, miR-141, miR-301a, let-7a, and let-7b) were significantly lower in prostate cancer tissue (Supplementary Table S5). These results are consistent with previous findings that miR-100 and miR-125b are downregulated in prostate cancer (26). E2F3 and PLK1 expression were measured in...
a subset of the vitamin D₃-treated clinical trial samples. E2F3 was slightly increased in prostate cancer versus benign epithelium ($P = 0.09$) and PLK1 was unchanged (Fig. 5C). Overall these data suggest that miR-100 and miR-125b levels are lower in prostate cancer and suppression of miR-125b may upregulate E2F3.

**miR-100 and miR-125b are upregulated by dietary vitamin D₃ in clinical trial specimens**

Wagner and colleagues found that serum and tissue from the high dose of vitamin D₃, significantly increased 25D and 1,25D over baseline (32). The mean serum levels (±95% CI) of vitamin D metabolites for the 400, 10,000, and 40,000 IU/day, respectively, were as follows: 1,25D (115 ± 26.4, 136 ± 20, 171 ± 18.5 pmol/L); 25D (71 ± 8.08, 122 ± 14, 288 ± 41.8 nmol/L). Mean tissue levels (±95% CI) of vitamin D metabolites for the 400, 10,000, and 40,000 IU/day, respectively, were as follows: 1,25D (29 ± 6.8, 30 ± 5.0, 40 ± 8.5 pmol/kg); 25D (90 ± 7.6, 117 ± 12.2, 178 ± 34.2 nmol/kg; ref. 32). Analysis by group showed a trending increase in miRNA levels with vitamin D dose (Fig. 5D). However, when analyzed irrespective of treatment groups, prostatic 1,25D concentrations (available post intervention only) positively correlated with miR-100 and miR-125b in both benign and prostate cancer epithelium (Fig. 5E, Table 1). miR-100 and miR-125b levels correlated with serum levels of 1,25D or 25D, but the results varied between benign and prostate cancer tissue (Table 1). Six of the other 10 miRNAs analyzed (miR-106b, miR-141, miR-103, miR-331-3p, let-7a, and let-7b) also positively correlated with prostatic 1,25D in either benign or prostate cancer epithelium (Supplementary Table S6). When correlating miRNA expression with the change in serum 1,2D and 25D levels from baseline, we also found positive correlations in benign or prostate cancer epithelium. These results fully substantiate the in vitro data and show that miR-100 and miR-125b and other miRNAs are regulated by vitamin D₃ in the prostate.

In addition, the correlation between miRNA and target levels were measured in a subset of tissue ($N = 15$) from the clinical trial, where we saw a trend toward PLK1 and E2F3 being correlated to their targets in normal and/or prostate cancer [miR-125b (normal; $r = 0.36$, $P = 0.09$; cancer; $r = 0.30$, $P = 0.15$); miR-100 (normal; $r = 0.08$, $P = 0.40$; cancer; $r = -0.35$, $P = 0.09$)].
a robust upregulation of miRNA expression globally (33). Cell density was carefully controlled in our study and we used nongrowth inhibitory dose of 1,25D in PrE cells. By using both in vitro and clinical approaches, we were able to identify, validate, and characterize miR-100 and miR-125b as targets of 1,25D in both cells and patient
miR-100 and miR-125b are upregulated by 1,25-dihydroxyvitamin D3

Table 1. Correlations between miRNAs and vitamin D metabolites

<table>
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<tr>
<th>MiRNA</th>
<th>Tissue 1,25D</th>
<th>Tissue 25D</th>
<th>Serum 1,25D</th>
<th>Serum 25D</th>
<th>(VF-V1) 1,25D</th>
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<td></td>
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<td></td>
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*P<0.05, bolded.

Prostate tissue. Levels of miR-100, miR-125b, and 9 other miRNAs were suppressed in prostate cancer tissue compared with benign tissue, which support previous studies that found widespread downregulation of miRNAs in prostate cancer (26). The fact that dietary vitamin D3 is correlated with the miRNAs in both benign and prostate cancer tissue, and 1,25D upregulated the miRNAs in PrE-Ca cells, in the presence of a significant suppression of miRNA expression, implicates an overall benefit of vitamin D in benign and prostate cancer tissue. We observed that serum 25D correlated with the miRNAs only in the prostate cancer areas (Supplementary Table S6), suggesting that cancerous tissue may respond to vitamin D3 supplementation faster than the benign areas.

MiR-100 and miR-125b have been shown to have both tumor-suppressive and oncogenic properties depending on the cell type (22, 24, 34–36, 46, 47). In prostate cancer, miR-100 levels are lower in tumor compared with normal tissue (34) and in the early stages of hepatocarcinoma suggesting involvement in carcinogenesis (47). miR-125b is hypermethylated in breast cancer (35), has oncogenic properties in prostate cancer, and supresses malignant phenotypes in vitro and in xenografts (48). However, overexpression of miR-125b decreased antiapoptotic proteins in prostate cancer xenografts (49), high levels of miR-100 increased the risk of prostate cancer recurrence (50), and miR-100 was oncogenic in acute myeloblastic leukemia (46). The seemingly discrepant roles for these miRNAs in cancer may be a result of specific model systems or overexpression methodologies. Our findings were consistent with tumor suppressor activities of miR-100 and 125b as that both miRNAs were significantly lower in prostate cancer compared with benign epithelium and in vitro overexpression of the miRNAs decreased cancer-associated phenotypes.

There are several characterized targets for miR-100 and miR-125b, 2 of which we examined. Previously, others have validated the interaction between miR-100 and Plk1 and miR-125b and E2F3 with luciferase assays (37, 38). Consistent with these reports, we observed a negative correlation between miR-100 and Plk1 and between miR-125b and E2F3 in vitro. The small number of patients may contribute to the weaker association between the miRNAs and their targets in the clinical trial specimens. Plk1 and E2F3 are cell-cycle proteins that are overexpressed in a variety of cancers including prostate cancer (22, 37, 38, 47). Previous studies of E2F3 and PLK1 protein expression in prostate cancer patient tissue arrays showed a clear correlation between high nuclear expression of the E2F3 protein and poor overall survival (51) and a positive correlation between PLK1 and Gleason grade (52). In prostate cancer, E2F3 positivity correlated with the Enhancer of Zeste Homolog gene 2 (EZH2), a well studied prostate cancer marker and oncogene (53) indicating that E2F3-EZH2 contribute to prostate cancer aggressiveness (51). In our study, EZH2 expression, measured in a small subset of patients (N = 20), was increased in prostate cancer compared with benign tissue (P = 0.05; Supplementary Fig. S7), but was not correlated with 1,25D or 25D (data not shown).

Let-7a is located on chromosome 11q13 (36) adjacent to miR-100 and miR-125b-1. A recent systematic review identified let-7, miR-100, and miR-125 as 3 of the 4 most frequently selected miRNA classifiers of cancer patient outcome across 46 publications (54). In our study, let-7a was not regulated by 1,25D in our array therefore not included in the original validation. Because of its chromosomal location, we later quantified let-7a in a subset of tissues and found that let-7a was downregulated in prostate cancer versus benign epithelium and also positively correlated with prostatic 1,25D in both benign (P = 0.047) and prostate...
cancer epithelium (P = 0.033; Supplementary Fig. S8). The let-7 family is well studied as tumor suppressive, and let-7a suppressed prostate cancer cell proliferation and tumor growth in vivo (55).

In the clinical trial samples, there was heterogeneity in prostatic 1,25D levels within each treatment group, as a result there were no significant differences in miRNA levels when analyzed by treatment group. The variation in prostatic 1,25D may be a result of interpatient variability in calcium levels and/or vitamin D metabolism. Importantly, when we analyzed prostatic 1,25D concentration, there was a strong correlation between prostatic 1,25D and miRNAs, which fully validated our in vitro findings.

There is a controversial epidemiologic relationship between vitamin D status and prostate cancer risk that has been based upon serum levels of 25D or 1,25D. Although serum 25D measures overall vitamin D status, the results of the clinical trial suggest that although serum levels do correlate to tissue levels, there are patient-to-patient differences in prostate tissue levels of vitamin D within intervention groups (32). Similar to our miRNA findings, Wagner and colleagues showed that Ki67 staining in the prostate was dependent on the prostatic 1,25D levels rather than the dose of the vitamin D3 intervention (32). Wagner and colleagues also showed that PSA decreased in the 10,000 IU/day and 40,000 IU/day groups compared with the 400 IU/day groups. Future studies of this nature are important in identifying whether prostatic levels of vitamin D are correlative to prostate cancer outcome and survival. In our data, miR-100 and miR-125b correlate better with prostatic 1,25D than with serum 1,25D or 25D or with the change in serum 1,2D and 25D levels from baseline and there was no correlation to tissue 25D. The positive correlation between the miRNAs and tissue 1,25D supports the hypothesis that local tissue levels of 1,25D are important in prostate cancer pathogenesis, as prostate cells express CYP27B1, (19) locally producing 1,25D. Therefore, not only is there an effect of local prostatic 1,25D, but also measurement of prostatic 1,25D may be superior to serum in epidemiologic studies on prostate cancer prognosis.

We show that vitamin D regulates known tumor suppressive miRNAs, miR-100 and miR-125b in both prostate cancer and benign prostate tissue cells and tissue. The tumor-suppressive activities of these miRNAs support a chemopreventive role for vitamin D. Our data also indicate that vitamin D3 and 1,25D may upregulate miRNAs globally to counteract the downregulation of miRNAs observed in cancer. In addition, the fact that a seemingly short duration of treatment with vitamin D3 in prostate cancer patients resulted in differences in the expression of these miRNAs suggests that further long-term studies with vitamin D3 could be useful in examining global regulation of miRNAs in prostate. It is crucial that future research determine the clinical importance of the regulation of miRNAs to determine if global changes alter cancer progression and/or outcome in patients with prostate cancer.

Disclosure of Potential Conflicts of Interest

R. Vieth has honoraria from speakers’ bureau from Ortho Clinical Diagnostics. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Vaishnav, D. Wagner, A. Finelli, N. Fleshner, T. van der Kwast, R. Vieth, L. Nonn

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