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microRNAs and DICER1 are regulated by 1,25-dihydroxyvitamin D in prostate stroma

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Abstract

Vitamin D deficiency increases the risk of lethal prostate adenocarcinomas (PCa) and the majority of older men are deficient. Although PCa arises from the epithelium, the surrounding stroma has hormonal regulatory control over the epithelium and contributes to carcinogenesis. Herein, we describe regulation of microRNAs (miRs) by the active hormone dihydroxyvitamin D (1,25(OH)₂D) in human prostate stroma. 1,25(OH)₂D binds the vitamin D receptor (VDR) transcription factor to regulate gene expression, including miRs, which have emerged as potent regulators of protein expression. 1,25(OH)₂D-regulated miRs were identified by profiling in primary human prostatic stromal cells (PrS) and three miRs, miR-126-3p, miR-154-5p and miR-21-5p were subsequently validated in laser-capture micro-dissected prostate stromal tissue from a vitamin D3 clinical trial (N=45). Regulation of these miRs by 1,25(OH)₂D was VDR-dependent. Network analysis of known and putative mRNA targets of these miRs was enriched with cancer and inflammation pathways, consistent with known roles of stroma and of vitamin D in carcinogenesis. Expression of the miR processing ribonuclease, DICER1, positively correlated with vitamin D metabolite levels in the clinical trial specimens. High epithelial/stromal ratios of DICER1 were significantly associated biochemical recurrence (OR 3.1, p=0.03) in a tissue microarray of 170 matched PCa patients. In summary, these results underscore the role of the prostate stroma in regulating responses to the hormone 1,25(OH)₂D and identified miRs and DICER1 as being regulated in human prostate stroma. Regulation of stromal DICER1 by 1,25(OH)₂D may also have clinical relevance in protection against aggressive PCa.

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5.1. Declaration of interest

The authors do not have any financial or personal relationships to disclose.

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Keywords

Vitamin D; Prostate Cancer; microRNA; Prostate Stroma

1. INTRODUCTION

In addition to the classical role of vitamin D as a regulator of calcium homeostasis and bone health [1], this hormone promotes cell differentiation and inhibits proliferation of prostate cancer (PCa) cells [2, 3]. There is substantial *in vitro* and *in vivo* evidence that 1,25-dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}$), the active metabolite of vitamin D, suppresses PCa cell growth, reduces tumor volume and metastasis *in vivo*, and alters phenotypes consistent with cancer prevention in benign prostate cells [3–6]. $1,25(\text{OH})_2\text{D}$ acts genomically via binding to the vitamin D receptor (VDR), which is a classical steroid receptor and transcription factor. Prostate cells are not solely dependent on circulating $1,25(\text{OH})_2\text{D}$ as they express the enzyme CYP27B1, which generates $1,25(\text{OH})_2\text{D}$ from its circulating precursor, 25-hydroxyvitamin D ($25(\text{OH})\text{D}$) [3]. Clinical trials further support the beneficial action of vitamin D in the prostate. Vitamin D3 supplementation in PCa patients lowered the serum prostate specific antigen (PSA) levels [7], reduced the number of PCa positive cores on repeat biopsies [8], and decreased the number of proliferating cells within the prostate gland [9].

Although the *in vitro*, *in vivo* and clinical trial data are significant, epidemiological studies have reported mixed results in the relationship between vitamin D status, as measured by serum levels of metabolites and overall PCa risk [10, 11]. A recent meta-analysis of 14 studies calculated an insignificant relative risk of 1.04 (95% CI: 0.98, 1.09) for the association between serum $25(\text{OH})\text{D}$ and PCa risk [11]. However, when PCa prognosis or aggressiveness is examined instead of overall PCa risk, vitamin D deficiency is associated with an increased risk of PCa mortality [12–14]. In two nested case-control studies, Ahonen *et al.* and Fang *et al.* concluded that men with deficient serum $25(\text{OH})\text{D}$ ($<16\text{--}18\text{ ng/mL}$) had a 1.5 to 3-fold increased risk of aggressive PCa compared to men with replete serum $25(\text{OH})\text{D}$ [12, 13]. Taken together, these reports support an association between vitamin D status and aggressive PCa, but also demonstrate the need to define vitamin D status and to consider genetic variations to fully elucidate the beneficial anti-tumor roles of vitamin D in the prostate.

PCa is an adenocarcinoma that originates from the glandular luminal epithelial cells. However, the bulk of the prostate gland is made up of a fibromuscular stroma, which has paracrine control over the epithelium via growth factors and cytokines [15]. Disruption of crosstalk between the prostate stroma and epithelium contributes to the development of prostate carcinogenesis [16]. Whereas, the role of other stromal steroid hormones and their receptors, such as androgen and estrogen, in regulating stromal-epithelial interactions in the prostate are well-studied [15], there are limited studies on vitamin D in the prostate stroma. Our lab recently showed that primary prostate stromal cells (PrS) and patient stromal tissues have a functional vitamin D signaling axis and that the stromal cells contribute to the anti-inflammatory effects of $1,25(\text{OH})_2\text{D}$ in primary prostate epithelial cells [17].

The genomic actions of vitamin D, via 1,25(OH)₂D binding to VDR, are not restricted to regulation of coding genes. miR expression is altered by 1,25(OH)₂D in many cell types including benign prostate epithelium, PCa, colon cancer, melanoma and human leukemia cells [18]. Previous work in our lab identified miR-100 and miR-125b, tumor suppressive miRs, as regulated by 1,25(OH)₂D in primary prostate epithelial (PrE) cultures. A positive correlation was also observed between vitamin D metabolites and these miRs in laser-capture microdissected (LCM) epithelial tissue from PCa patients from a vitamin D clinical trial [19].

In the current study, regulation of miRs and the miR processing ribonuclease DICER1 by vitamin D was examined in the prostate stroma, which may provide insight into the actions of this hormone in the prostate tumor microenvironment. miRs were identified by array profiling in 1,25(OH)₂D -treated benign PrS cells and were subsequently validated in LCM-collected benign prostate stromal tissue from a vitamin D3 clinical trial (10). Given that global miR expression is dysregulated in cancers and that coordinated expression of miRs have been implicated in alterations of hundreds of proteins, our novel findings suggest that vitamin D-mediated regulation of miRs in prostate stroma may contribute to paracrine regulation of epithelial differentiation.

2. MATERIALS AND METHODS

2.1. Primary Cell culture

Primary human stromal (PrS) cells were isolated from radical prostatectomy tissue from PCa patients at the University of Illinois at Chicago Medical Center according to an Institutional Review Board-approved protocol previously described [17]. Briefly, fresh tissue from the peripheral zone was excised with a 5 mm punch from transverse sections of radical prostatectomy specimens by a pathologist. To determine the pathology of the punch, a thin slice was removed for H&E histological examination by a board certified pathologist. Tissues were disrupted mechanically then digested overnight with collagenase at 37°C. PrS cells were plated in MCDB-105 media (Sigma–Aldrich, St. Louis, MO) with 10% FBS. Following passage, no epithelial cells remained and stromal cell identity was confirmed by expression of vimentin, smooth muscle actin and TIMP3. PrS cells for all experiments were between passage 2 and 6. Based on the pathology of the H&E, cells were named PrS for 100% benign areas and PrS-Ca for >90% PCa.

2.2. RNA isolation

RNA was isolated from cell cultures with Trizol reagent (Thermo Fisher, Waltham, MA) using a modified protocol for small RNA recovery; 2X volume of 100% isopropanol instead of 1X for RNA precipitation. RNA quality and quantity was determined by absorbance at 260 and 280 nm on the NanoDrop ND-1000 spectrophotometer (Thermo Fisher, Waltham, MA).

2.3. MicroRNA PCR array

PrS cells were treated with 10 and 50 nM 1,25(OH)₂D for 8 or 16 hours (hrs) using ethanol (EtOH) as a vehicle control. RNA was extracted (described above) and cDNA generated

from 40ng RNA with the Exiqon Universal cDNA Synthesis kit (Exiqon, Denmark). Exiqon® qPCR array panels I+II (768 miRs) were run according to the manufacturer's protocol. miR-27b-3p was selected as the optimal reference gene using the normfinder GenX software (Figure S1). Per Exiqon guidelines, Ct values greater than 37 were omitted. The full array Ct values are available in Table S1. **Analysis.** Relative quantities (RQ) were determined by the 2^{-Ct} method. logRQ was used to calculate student's t-test for each miR. miRs with $p < 0.05$ were selected for further analysis. **Heat map.** RQ values were calculated relative to the EtOH control for each cell line. miRs with $p < 0.10$ (t-test) were imported into GenePattern (Broad Institute, Cambridge, MA, USA) [20], log2 transformed and a heatmap created sorted by logRQ at 16h-50nM.

2.4. Vitamin D3 Clinical trial

Details of the phase II clinical trial at University Health Network in Toronto, Canada, from which we obtained benign Laser Capture Microdissected (LCM) stroma have been previously described [9]. The randomized trial, registered with www.clinicaltrials.gov (NCT00741364), involved 66 patients, who gave signed consent, given oral vitamin D3 (cholecalciferol) at doses 400, 10,000, or 40,000 IU/day for 3–8 weeks prior to prostatectomy at which time fresh tissue was collected. Paraffin blocks of prostatectomy specimens and serum were obtained from 15 randomly selected patients from each treatment group. Blinded specimens were demarcated by a pathologist for LCM. LCM was used to separately collect benign stroma via a procedure previously described for these samples [9]. Serum samples were obtained at entry and at the final visit before as previously described [9].

2.5. Laser Capture Microdissection

Prostate stroma from benign areas was collected by LCM as described earlier by Nonn and colleagues [17, 19, 21]. Briefly, two 8 μ M formalin-fixed paraffin-embedded sections from each prostatectomy specimen were mounted on RNase-free PEN slides (Leica, Buffalo Grove, IL). Specimens were deparaffinized, fixed, and stained with 0.5% toluidine blue. Using a Leica LMD-6000 LCM instrument (Leica, Buffalo Grove, IL) stroma surrounding benign regions were collected into Eppendorf caps containing digestion buffer (Thermo Fisher, Waltham, MA). RNA was isolated from the tissues as previously described [17, 19].

2.6. Vitamin D metabolite measurement in prostate tissue

Full details of statistical analysis of serum and tissue levels of vitamin D metabolites were reported elsewhere [9]. Briefly, vitamin D metabolites were pre-extracted from fresh frozen prostate tissue and 1,25(OH)₂D levels were determined by enzyme immunoassay (Immunodiagnostic Systems, Scottsdale, AZ). Tissue was from the same area of the prostatectomy specimen, but not the identical piece used for RNA isolation. Tissue vitamin D metabolites were reported as the mean of two samples of fresh frozen tissue, which included normal and benign regions.

2.7. RT-qPCR in prostate cells and clinical samples

2.7.1. Cell cultures—For miR analysis, cDNA was generated from 150 ng of RNA was using miRCURY LNA™ Universal RT kit (Exiqon, Woburn, MA). PCR was run using LNA™ PCR primers sets. For mRNA analysis, cDNA was generated from 355 ng of RNA with the High Capacity Kit (Thermo Scientific, Waltham, MA). qPCR was run with SYBR green using the QuanStudio6 (Thermo Scientific, Waltham, MA). A complete list of primers is included in Table S2. Ct values were normalized to the reference genes HPRT and GAPDH for mRNA and miR-27b-3p and U66 for miR using Ct method.

2.7.2. Tissues—For miR analysis 50ng of RNA was reverse-transcribed using Exiqon Universal cDNA kit. Fold-changes were calculated using C_T method and normalized to the expression of miR-27b-3p and miR-125b. Since patient samples were run on four separate 384 plates, the following interplate calibration equation was used for C_T data correction:

$$Cq_i^{corrected} = Cq_i^{uncorrected} - Cq_i^{IPC} + \frac{1}{\# \text{ of plates}} \sum_{i=1}^{\# \text{ of plates}} Cq_i^{IPC}$$

2.8. Statistical analysis of gene expression

RT-qPCR gene expression data as relative quantities (RQs) was analyzed by Wilcoxon signed-rank test or Kruskal–Wallis test as the data were not distributed normally. For analysis of miR expression in patient tissue by tertile of prostatic 1,25(OH)₂D (**Tertiles: 15–24, 25–34, 35–79 pmol/kg of 1,25(OH)₂D**), ANOVA was used with subsequent Bonferonni post-hoc test for comparison of each tertile and post hoc linear trend for dose response. Correlation analyses (Spearman's rank rho) were used to test for relationships between tertiles of miR expression levels in patient tissue and prostate or serum 1,25(OH)₂D or 25(OH)D levels. The two-tailed test was performed for the correlation analyses. All data points were included in the analyses with the exception of miR-21, for which correlation analysis is shown with and without 3 outlier points.

2.9. Network analysis for 1,25(OH)₂D regulated miR mRNA targets

A list of predicted and validated mRNA targets was obtained from Targetscan [22], PicTar [23] and Diana Tools (TarBase v.6.0) [24] and imported into Cytoscape 3.1's ClueGo application [25]. The KEGG pathway database was used for pathway enrichment [26].

2.10. Knockdown of VDR

siRNA control or a cocktail of three siRNAs to VDR (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) was transfected into three different PrS cell lines with Dharmafect (Dharmacon, Lafayette, CO, USA). The three PrS cells were derived from benign areas of the prostate from three different patients including PrS-1 from the original profiling. Cells were seeded at 5×10^5 in 60mm dishes in MCBD-105/10% FBS and transfected with 5nM of the siRNAs. RNA was isolated the RT-qPCR carried out as described above.

2.11. Prostate cancer tissues and DICER1 protein immunohistochemistry

The Outcome tissue microarray (TMA) was obtained from the Cooperative Prostate Cancer Tissue Resource (CPCTR) study [27]. This TMA contains cores from radical prostatectomy specimens; 4 cores per patient and each patient with PSA recurrence was matched (by age, grade and stage) to a patient without recurrence [27]. Slides 1 and 2 of the array were used and contained 170 total patients, 85 matched pairs. Five micron sections were deparaffinized, subjected to antigen retrieval, stained with anti-DICER1 1:100 overnight at 4°C (Prestige antibody HPA000694, Sigma-Aldrich, Hamburg, Germany) and visualized with mouse DAB Avidin/Biotin Complex Kit (VectorLabs, Peterborough, UK). **Analysis.** Areas of epithelium and stroma were marked manually by a pathologist, then segmented and quantified with the Vectra Tissue Imaging System (PerkinElmer, Waltham, MA). Cores that were incomplete, damaged or did not contain cancer epithelium and stroma were excluded. Areas of inflammation were not included. Using the Vectra system, the total DAB intensities for the epithelium and the stroma was determined from the area and pixel intensity. To control for core-to-core staining differences, the epithelial/stromal ratio per core was determined and analyzed by conditional logistic regression using the outcome of biochemical PSA recurrence (BCR). A total of 170 patients were analyzed; N=85 non-recurrent, N=85 for recurrent disease (BCR).

3. RESULTS

3.1. 1,25(OH)₂D regulated miRs in primary prostatic stromal cultures

To identify miRs regulated by 1,25(OH)₂D in prostate stroma, miRs were profiled in primary human prostate stromal cells (PrS) from three patients, followed by prioritization of leads and validation in prostate tissues from a clinical trial (Figure 1A). 732 miRs were profiled by RT-qPCR arrays in three different patient-derived benign primary prostatic stromal cultures (PrS) from three patients after treatment with 1,25(OH)₂D; control vehicle (0.01% EtOH), 10 nM for 16 hr, 50 nM for 16 hr or 50 nM for 8 hr. Cell proliferation was not altered at these concentrations of 1,25(OH)₂D (**data not shown**). Approximately one third of the miRs on the arrays (253 of 732) were detected at robust levels in the PrS cells. Differential expression of miRs between the benign prostate stromal and primary prostatic epithelial cells (PrE) was examined by comparison to our previous data set [19]. miRs were ranked by Ct and the top 50 miRs were compared between the PrS and PrE cultures. About half of the miRs were common between PrS and PrE cells (27 miRs), whereas 23 miRs were unique to PrE or PrS cells (Table S3).

Significant 1,25(OH)₂D-regulated miRs were determined by paired student's t-test of the log₂ relative quantity (RQ) values between EtOH and each of the treatment groups. miR-27b-3p was selected for normalization based on NormFinder (Figure S1A). Clustering analysis of all miRs with p < 0.10 showed miRs increased and decreased by 1,25(OH)₂D in the PrS cells (Figure 1B).

3.2. miRs in prostate stroma correlated with vitamin D metabolites in prostate tissues from a vitamin D3 clinical trial

miRs were prioritized for validation based on significance ($p < 0.05$), magnitude of the fold changes and previously published evidence of activity in stromal cells [28–30] (Table S4). Eight miRs (miR-299-5p, miR-154-5p, miR-132-5p, miR-126-3p, miR-485-3p, miR-654-5p, miR-212-3p and miR-21-5p) were selected for further validation in LCM benign prostate stromal tissue.

The tissue samples used were from a clinical trial, the details of which have been previously reported [9]. Briefly, pre-radical prostatectomy patients had oral supplementation of cholecalciferol (metabolite precursor to 25(OH)D) at 400IU, 10000IU and 40000IU, which resulted in increased 25(OH)D and 1,25(OH)₂D metabolite levels in the serum and prostate [9]. The stroma was isolated by LCM and the RNA did not express the epithelial marker NKX3.1 and PCa marker AMACR [17]. Fifteen patients from each group were included for this study (N=45). miRs expression was quantified by RT-qPCR and normalized to miR-27b-3p. miRs with a Ct > 37 were discarded. The miRs-126-3p, -132-5p, -154-5p and -21-5p were detected in the majority of the patients (Figure 2), whereas miRs-181c-3p, -299-5p, -485-3p and -654-5p were detected in less than 50% of the patients (**data not shown**).

The expression of the miRs was compared to the prostatic concentration of the active hormone 1,25(OH)₂D by tertiles. Both miR-154-5p (ptrend = 0.0145) and miR-126-3p (ptrend = 0.0301) were significantly associated and increased dose dependently with prostatic 1,25(OH)₂D tertiles (Figure 2A). ANOVA analyses between tertiles showed miR-132-5p also increased in the highest tertile (Figure 2A). In addition to prostatic measurements of 1,25(OH)₂D, the concentrations of prostatic 25(OH)D, serum 1,25(OH)₂D and serum 25(OH)D were also known for the study patients [9]. The miR levels were compared to serum and prostatic levels of vitamin D metabolites by correlation analysis (Table 1). miR-154-5p levels positively and significantly correlated to prostatic 1,25(OH)₂D levels (spearman $r = 0.4123$, $p = 0.0326$). Correlation analyses revealed other associations between miR-212-3p and prostatic 25(OH)D levels ($p = 0.0421$), miR-132-3p with serum 1,25(OH)₂D metabolite levels ($p = 0.0105$) (Table 1).

A recent study reported that elevated expression of prostate stromal miR-21-5p in Gleason 6 patients correlated with poor biochemical recurrence-free survival after radical prostatectomy surgery [29]. Here, tertile analysis revealed a significant decrease in the mean stromal miR-21 expression in tertile two compared with tertiles one and three (Figure 2B), which is suggestive of a U-shaped relationship. Graphical analysis of the correlation between miR-21-5p with prostatic 1,25(OH)₂D levels was also suggestive of a U-shaped association (Figure 2B), but there were only 3 points at the highest levels of prostatic 1,25(OH)₂D drove the U-shape. Removal of the outlier data points resulted in a consistent decrease of miR-21-5p with higher 1,25(OH)₂D (Figure 2B). Taken together, these results show that vitamin D treatment regulates the expression of miRs relevant to prostate stroma and prostate cancer in clinical trial patient tissue.

3.3. Network analysis of messenger RNA targets of 1,25(OH)₂D-regulated miRs in the prostate stroma

Based on the most robust validation in the clinical trial tissues, mRNA targets of miRs-126-3p, -154-5p and -21-5p were examined to identify pathways that may be regulated by these miRs in the prostate stroma. These miRs were regulated in a VDR-dependent manner in PrS cells as shRNA to VDR blocked regulation by 1,25(OH)₂D (Figure S3). Network analysis was performed with predicted and validated targets for these miRs using the ClueGo application in Cytoscape [25, 31](Figure 3, Table S5). The most significantly enriched signaling pathway was the FoxO pathway ($p < 4.45 \times 10^{-8}$), accounting for 37 target genes. Several inflammation-related signaling pathways, including TNF- α , JAK-STAT and chemokine signaling, were also enriched. These results emulate the known actions of both vitamin D and stroma in the prostate [3, 17, 32].

3.4. Regulation of miRs by 1,25D was VDR-dependent

1,25D is a steroid hormone that regulates gene expression by binding to the VDR, a classical steroid receptor transcription factor. To confirm the role of VDR in the mechanism of action for regulation of miRs by 1,25D, VDR levels were knocked down in PrS cells. Suppression of VDR activity by the siRNA was demonstrated by attenuation of CYP24A1 induction (Figure 4A), well-characterized VDR target which contains multiple VDREs in the promotor. The effect of 1,25D on miR-154, miR-126-3p and miR-21 was suppressed in the siVDR cells, confirming that VDR is required for regulation by 1,25D. (Figure 4B–D).

3.5. DICER1 expression correlated with prostatic 1,25(OH)₂D in prostate stroma

To extend the survey of miR regulated by vitamin D in prostate stroma, DICER1 expression was analyzed. DICER1 is a cytosolic RNase that processes pre-microRNA hairpins into mature miRs and it has previously been reported to be aberrantly expressed in PCa lesions [33–35]. RT-qPCR analysis of DICER1 in the clinical trial samples showed a dose-dependent increase of DICER1 in prostate stroma for prostatic 1,25(OH)₂D both by Spearman correlation and by tertile ANOVA (Figure 5A). In comparison to prostate epithelium from normal (benign) and cancer areas, DICER1 had a lower expression in the stroma (Figure 5B). However, the prostate epithelium lacked a relationship between 1,25(OH)₂D and DICER1 expression (**data not shown**). 1,25(OH)₂D did not regulate DICER1 in PrS cell lines (**data not shown**).

3.6. DICER1 protein levels in the stroma associated with biochemical recurrence of PCa

Expression of DICER1 protein in prostate stroma was reported once previously [35], but has not been widely examined in PCa tissues. Immunohistochemical staining of PCa tissues showed abundant expression of DICER1 in PCa epithelium, as well as staining in the stromal areas (Figure 5C). Given the recent finding that miR-21-5p levels in stroma predicted disease recurrence in Gleason 3+3 PCa patients [29], stromal and epithelial DICER1 protein levels were examined in slides 1 and 2 of the CPCTR Outcome TMA which contain tissues from 170 patients (85 matched pairs of recurrence versus non-recurrence) [27]. The DICER1 staining intensity was determined for each compartment and per person ratios of epithelial to stromal DICER1 were calculated. The epi/stroma ratio

analysis controls for core-to-core variation in staining and an increase in the ratio may reflect increased epithelial DICER1, decreased stromal DICER1, or a combination of both. In conditional logistic regression analysis for outcome, the highest quartile of epi/stroma DICER1 was a significant predictor of BCR. (Table 2, Figure 5D). Regression analysis of the individual compartments showed that the highest quartile of stromal DICER1 approached significance for a reduced OR. This association was not observed in the epithelial compartment. Representative DICER1-stained cores and ratios are shown in Figure 5C. In summary, prostatic 1,25(OH)₂D positively correlated with *DICER1* mRNA and low stromal DICER1 (as reflected in a high epi/stroma ratio) was associated with BCR, demonstrating a potential connection between 1,25(OH)₂D and BCR, via DICER1.

4. DISCUSSION

Despite the well-known regulatory function of prostate stroma over epithelial growth and differentiation, this is the first report of miR and DICER1 regulation by 1,25(OH)₂D in the stroma from prostate cancer patients from a vitamin D clinical trial. These miRs may contribute to the anti-tumor activity of vitamin D in the prostate by directly or indirectly targeting genes involved in stromal-epithelial crosstalk during prostate carcinogenesis.

In this study miRs 126-3p, 132-5p, 212-3p, 154-5p and 21-5p were associated with the concentrations of vitamin D metabolites (either by correlation or differences between tertiles) in patient prostate stroma and were regulated by 1,25(OH)₂D in *in vitro* PrS cells. Our previous analysis of miRs in prostate epithelium showed that the majority of miRs were upregulated by 1,25(OH)₂D [19], but this was not observed in the stroma. Independent of vitamin D, there are only a few reports on the expression of specific miRs in prostate stroma and functional studies have not been reported. Ren *et al.* showed that let-7c expression was significantly lower in tumor associated stroma tissue and this decrease was associated with extraprostatic extension [36]. Walter *et al.* suggested that miRs in the benign stroma may influence the adjacent epithelium as they identified an 11 miR signature (miR-let7, miR-1, miR-98, miR-126, miR-132, miR-142, miR-143, miR-144, miR-205, miR-210) that discriminated between the tumor and benign prostate epithelium [30]. Two of the miRs in the Walter *et al.* signature, miR-132-5p and miR-126-3p, were examined in our study and showed regulation by 1,25(OH)₂D *in vitro* and in patient tissues. miR-132-5p and miR-212-3p are part of a miR cluster [37], and expression of both these miRs is low in PCa [30, 38]. Formosa *et al.* showed that miR-132-5p expression was inversely correlated with metastatic events in PCa specimens and was transcriptionally inactivated by DNA methylation in PCa [38]. miR-126-3p was lower in patient-derived xenografts (PDXs) derived from PCa metastasis compared to non-metastatic PDX [39]. These findings support that vitamin D may mediate stromal-epithelial crosstalk and PCa metastasis via regulation of these miRs.

miRs -299-5p, -154-5p and -485-3p were included in our validation set because they belong to the DLK-DIO3 miR cluster, which is dysregulated in several malignancies including PCa [28]. In our study, only miR-154-5p was robustly expressed in the patient stroma and positively correlated with 1,25(OH)₂D, although miR-299-5p was close to significant correlation ($r=0.436$, $p=0.07$). In PCa, miR-154-5p is considered a tumor suppressor as re-

expression inhibited epithelial-to-mesenchymal transition in PC-3 and DU-145 PCa cells [40].

miR-21-5p is widely known as an “oncomiR” in many cancers including PCa [41], but a recent report shifted the paradigm on the role of this miR in PCa, where it has not consistently functioned as an oncomiR experimentally [42]. Melbo-Jorgensen *et al* showed that it was expression of miR-21-5p in the prostate *stroma*, not the tumor epithelium, associated with BCR in PCa patients with Gleason score 6 [29]. miR-21 is regulated by androgen signaling and overall levels of miR-21-5p in whole tissues were higher in tumor samples compared to matched benign samples [41]. In our study miR-21 was modestly, yet significantly, regulated by 1,25(OH)₂D in PrS culture. In the patients from the vitamin D clinical trial, there was significantly lower stromal miR-21-5p expression in tertile 2 (25–34 pmol/kg) of prostatic 1,25(OH)₂D, supporting the *in vitro* findings. However, we also observed a compelling suggestion of a U-shaped association. Although our study was too small to definitively identify or rule out a U-shaped association, it is intriguing because it emulates the association of vitamin D with observed PCa risk in the SELECT trial PCa patients [43]. In SELECT, plasma 25(OH)D levels ranging from 40–70nmol/L for overall PCa risk were optimally protective [43]. Given the published reports of miR-21-5p as an oncomiR, our novel observation of potential miR-21 regulation by vitamin D in the stroma warrants further studies as it could have prognostic implications on PCa risk and outcome.

The network analysis on the predicted and validated target genes for significantly regulated miRs, miR-21-5p, miR-154-5p and miR-126-3p, which were regulated *in vitro* in PrS cultures by 1,25(OH)₂D in a VDR-dependent manner, showed that several inflammation-related pathways were enriched. These inflammatory pathways are dysregulated in PCa [44, 45] and prostatitis [46]. Since 1,25(OH)₂D suppresses inflammatory pathways via inhibition of prostaglandin, NF- κ B and MAPK signaling [4–6], the results of the network analysis are consistent with the anti-inflammatory and anti-proliferative actions of vitamin D in the prostate. Our network analyses also implicate that genes involved in the FOXO pathway are targets of miRs-21-5p, -154-5p and -126-3p. This finding complements a recent study by Georgescu *et. al*, which found that stromal FOXO1 expression discriminated Gleason 4 from Gleason 3 tumors [28], suggesting an emerging role of FOXO1 in the prostate stroma. Lastly, miR-21-5p targets were enriched in the TGF- β pathway via SMAD7 and TGFB2, which is consistent with a previous report that miR-21 increased TGF- β signaling in cancer associated fibroblasts (CAFs) by inhibiting translation of SMAD7 [47]. Increased TGF- β pathway signaling, in general, is known to alter the tumor microenvironment and promote prostate carcinogenesis [48], suggesting that vitamin D may regulate CAF formation during prostate carcinogenesis.

Our study revealed a novel correlation between stromal DICER1 and 1,25(OH)₂D as well as an previously unknown association between low stromal DICER1 and increased risk of BCR. Our data complement the relationship between stromal miR-21-5p and BCR, described above [29], and together demonstrate a central role for the prostate stroma in the microenvironment of disease progression. Correlation between stromal DICER1 and BCR and vitamin D does not imply a causal relationship and this was not examined in our study. However, in several cancers, including PCa, DICER1 is considered a haploinsufficient tumor

suppressor [49]. Although DICER1 is higher in the PCa epithelium [35], within PCa cases, low DICER1 mRNA in PCa cases has been associated with early BCR [49]. A recent study by Zhang *et al.* demonstrated a dosage-dependent role for Dicer in the *Pten*^{-/-} mouse model, in which complete Dicer loss inhibited PCa development, but hemizygous loss of Dicer increased invasion in the model [49]. Given that miRs can be packaged into exosomes and the regulatory crosstalk between the epithelium and stroma, there may be link between stromal DICER1 and the global reduction of mature miRs that is a hallmark of most cancers, including prostate [50].

Despite the novelty of this study and its results, there are certain limitations to this work. First, the sample size of our clinical study was limited to 47 patients, which may have been a reason why statistical significance was not reached for some miRs in our tertile analysis. Additionally, our study could not take into account genotypic variations in vitamin metabolism enzymes in our clinical trial patient cohort, which might have affected our findings. Our data support that there is heterogeneity in vitamin D metabolism and distribution in that our correlation data between the miRs and the vitamin D metabolites (Table 1) revealed interesting associations between miRs with one metabolite or another, but not with all. This is consistent with previous reports from this study [9, 17, 19]. These findings may be explained by inherent differences in serum vitamin D binding protein [51], internalization of 25(OH)D into the prostate by megalin[52] and/or metabolism of vitamin D within the prostate by the hydroxylases [17, 53].

In conclusion, the prostate stroma expressed miRs differently than the epithelium and 1,25(OH)₂D regulated several miRs with known effects in PCa. These miRs also correlated with vitamin D metabolite concentrations in clinical trial specimens. 1,25(OH)₂D-regulated miRs target pathways known to be regulated by vitamin D and the stroma in the prostate. There are several key points to emphasize from our study; 1) though 1,25(OH)₂D does not produce dramatic changes in the expression levels of individual miRs, the sum of the 1,25(OH)₂D-mediated miR expression changes may exerts a greater effect towards the protein landscape than individual miRs expression. 2) the fact that 1,25(OH)₂D regulates miR clusters, which may possess overlapping miR targets due to high sequence homology[54], suggests that the this hormone may control certain cellular processes by design as further emphasized by our network analyses, and 3) up-regulation of stromal DICER1 by 1,25(OH)₂D may contribute to decreased risk of BCR as low stromal expression of DICER1 was associated with future BCR in PCa patients. Further studies are needed to elucidate the role of miRs, DICER1, and vitamin D in regulating prostate carcinogenesis via alterations in stromal-epithelial crosstalk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- The active hormone 1,25(OH)₂D regulates miRs in prostate stroma.
- Inflammation-related pathways were enriched in the putative targets for the 1,25(OH)₂D-regulated miRs.
- The miR-processing enzyme DICER1 positively correlated with 1,25(OH)₂D in the stroma.
- A high epithelial:stromal ratio of DICER1 protein levels associated with future biochemical recurrence.

Figure 1. Study design to identify 1,25(OH)₂D -regulated miRNAs in prostate stroma
A, Diagram of study design to incorporate primary human prostate stromal cells (PrS) and patient tissues to identify miRNAs regulated by 1,25(OH)₂D in prostate stroma. *Trial details and metabolites levels previously published by Wagner et al [9]. **B**, Heatmap of 1,25(OH)₂D regulated microRNAs with p<0.1 in primary human prostate stromal cells (PrS) from 3 patients. The log Relative Quantity values (RQ), compared to vehicle (ethanol) are shown. MiR selected for further validation are bolded and marked with a star. Further details on selection of miRNAs in Table S3.

Figure 2. Expression of 1,25(OH)₂D -regulated miRs in LCM-collected stroma from vitamin D3 clinical trial patients

A, analysis of miR expression (RT-qPCR) by tertiles of prostatic 1,25(OH)₂D levels (pmol/kg wet weight). (Tertiles: 15–24, 25–34, 35–79 pmol/kg of 1,25(OH)₂D). ANOVA differences between tertiles shown in grey if significant (<0.05) and post hoc linear trend (P_{Trend}) analysis shown for all miRs-126-3p, -132-5p, -154-5p, -212-3p, -299-5p, 485-3p. **B**, tertile (left panel) and Spearman's rank correlation analysis (right panel) of miR-21-5p and prostatic 1,25(OH)₂D levels. Correlation analysis is shown with the full data set and with removal of outlier points (open circles removed).

Figure 4. Regulation of miRs by 1,25D was VDR-dependent

siRNA cocktail to VDR was transfected into PrS cells for 24 hours followed by treatment with 50 nM 1,25D for 24 hours. **A**, Suppression of VDR activity was assessed by CYP24A1 mRNA induction via RT-qPCR. **B–D**, miR levels were quantified by RT-qPCR for miR-154, miR-126-3p and miR-21, Mean data from three experiments with three different PrS cultures is shown $\pm$ standard deviation. * $p < 0.05$ by paired student's t-test.

Figure 5. The expression of DICER1 associated with 1,25(OH)₂D and BCR in prostate stroma RT-qPCR gene expression of *DICER* in, **A**, prostate stroma by tertile of prostatic 1,25(OH)₂D (P-trend across tertiles) and, **B**, in stroma, normal (benign) prostate epithelium and cancer epithelium. ***p<0.05, 1 way ANOVA, Dunn's Multiple Comparison's test. **C**, IHC staining of DICER1 in representative PCa tissues from non-recurred and recurred patients with epithelial/stroma ratio calculated. **D**, Graphs of log odds ratio for BCR based on conditional logistic regression of ordinal quartiles, PSA included in the model. P value from Wald Chi-square.

Table 1

Correlations between miR levels in prostate stroma with vitamin D metabolite levels in the prostate and serum.

miRNA	Tissue			Serum		
	1,25D	25D	25D (Vf)	1,25D (Vf)	25D (Vf)	
miR-126-3p	N*	33	33	34	41	
	r [†]	0.098	0.027	0.187	-0.075	
	P [‡]	0.59	0.88	0.29	0.64	
miR-132-5p	N	27	27	27	31	
	r	0.231	-0.075	0.484	0.051	
	P	0.24	0.71	0.01*	0.79	
miR-212-3p	N	29	29	30	35	
	r	0.308	0.380	0.126	0.211	
	P	0.10	0.04*	0.51	0.22	
miR-154-5p	N	27	27	27	31	
	r	0.4123	0.086	0.218	0.175	
	P	0.03*	0.70	0.28	0.35	
miR-299-5p	N	18	18	18	22	
	r	0.436	0.087	0.101	0.027	
	P	0.07	0.73	0.69	0.91	
miR-485-3p	N	26	26	26	28	
	r	0.099	0.353	0.154	0.158	
	P	0.63	0.08	0.45	0.42	
miR-21-5p	N	33	33	34	41	
	r	-0.143	-0.080	-0.127	-0.17	
	P	0.43	0.66	0.47	0.29	

Vf= serum concentrations at the final visit from the trial

* N may differ between miRs due to undetectable levels of either the miRs, the vitamin D metabolite, or both.

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Spearman's rank correlation (r) with 2-tailed Gaussian approximation p-value
P<0.05 indicated by italics and *
P<0.1 indicated by italics only biochemical recurrence on the CPCTR Outcome TMA

Table 2

Conditional logistic regression for DICER immunohistochemistry to predict biochemical recurrence on the CPCTR Outcome TMA

Parameter	Odds ratio (-/+ 95%CI) [*]	P-value [†]	P-Trend
Epi/Stroma ratio	-	-	
Quartile 1 (ref)			
Quartile 2	0.928 (0.346,2.489)	0.88	
Quartile 3	2.283 (0.742,7.02)	0.15	0.03
Quartile 4	3.068 (1.055,8.93)	0.04	
PSA	1.050 (0.995,1.107)	0.07	

Wald Chi-squared

^{*} Confidence Limits and

[†] Pr>ChiSq