Tumor and Stem Cell Biology

Interleukin-1α Mediates the Antiproliferative Effects of 1,25-Dihydroxyvitamin D₃ in Prostate Progenitor/Stem Cells

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Abstract

Vitamin D₃ is a promising preventative and therapeutic agent for prostate cancer, but its implementation is hampered by a lack of understanding about its mechanism of action. Upon treatment with 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], the metabolically active form of vitamin D₃, adult prostate progenitor/stem cells (PrP/SC) undergo cell-cycle arrest, senescence, and differentiation to an androgen receptor–positive luminal epithelial cell fate. Microarray analyses of control- and vitamin D₃-treated PrP/SCs revealed global gene expression signatures consistent with induction of differentiation. Interestingly, one of the most highly upregulated genes by vitamin D₃ was the proinflammatory cytokine interleukin-1α (IL-1α). Studies biology analyses supported a central role for IL-1α in the vitamin D₃ response in PrP/SCs. siRNA-mediated knockdown of IL-1α abrogated vitamin D₃–induced growth suppression, establishing a requirement for IL-1α in the antiproliferative effects of vitamin D₃ in PrP/SCs. These studies establish a system to study the molecular profile of PrP/SC differentiation, proliferation, and senescence, and they point to an important new role for IL-1α in vitamin D₃ signaling in PrP/SCs. Cancer Res; 71(15); 1–1. ©2011 AACR.

Introduction

Prostate cancer is the second deadliest noncutaneous cancer in US men, accounting for an estimated 32,050 deaths in 2010 (1). The factors that lead to prostate cancer development and progression are poorly understood. Epidemiologic, genetic, and epigenetic studies contribute to the idea that prostate cancer development and progression are associated with vitamin D₃ deficiency. Clinical studies correlating circulating serum levels of 25-hydroxyvitamin D₃ [25(OH)D₃] and prostate cancer incidence have been inconclusive (2). However, epidemiologic and laboratory studies collectively point to a role for 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], the hormonally active form of vitamin D₃ in the prevention of prostate cancer (3). The "vitamin D hypothesis" in its essence states that vitamin D₃ maintains the differentiated phenotype of prostate cells and that vitamin D₃ deficiency allows prostate cancer to progress to clinical disease stages (4). The mechanistic action of 1,25(OH)₂D₃ in the prostate, however, remains largely undefined. 1,25(OH)₂D₃ induces cell-cycle arrest and differentiation in prostate epithelial cells and prostate cancer cells (5, 6). Upregulation of cyclin-dependent kinase (Cdk) inhibitors p21 and/or p27 is common and is implicated in 1,25(OH)₂D₃–mediated differentiation of LNCaP and PC-3 cells (7–10). Accumulation of prostate cells in G₁ as a result of p21 and/or p27 upregulation by 1,25(OH)₂D₃ may precede 1,25(OH)₂D₃-induced differentiation (11). In addition, 1,25(OH)₂D₃–induced differentiation of prostate cells is characterized by increased levels of prostate-specific antigen (PSA), kallikrein 2 (Klk2), E-cadherin, and androgen receptor (AR; refs. 10, 12–14). Pieces of the complex molecular mechanisms behind 1,25(OH)₂D₃ signaling in the cells of the prostate are starting to be identified, and we aim to add to this growing knowledge.

Recent studies including our own have identified putative adult prostate stem cells that undergo self-renewal and multilineage differentiation into the epithelial cell types of the prostate (15–19). The phenotypic attributes in common between normal stem cells and tumor cells, as well as the presence in the tumor of mutations in signaling pathways important for normal stem cell self-renewal, have led to the hypothesis that normal stem cells may be the target of mutagenesis leading to tumor formation (20). A major goal in prostate stem cell biology is to identify genes, pathways, and networks that control self-renewal and multilineage differentiation. Studies in this direction have been hampered by a lack of suitable models that allow for long-term maintenance of a stem cell population. Here, we present an in vitro system that overcomes these barriers and provides a model for studying
the molecular profile of prostate stem cell differentiation induced by 1,25(OH)2D3.

In accordance with the prostate cancer stem cell hypothesis, we believe that the prostate progenitor/stem cell (PrP/SC) is the most relevant target for chemoprevention. The PrP/SC has a nearly unlimited replicative capacity, but it can respond robustly to differentiation cues to enter a stage of limited or no replicative capacity. Agents that promote PrP/SC differentiation and limit replicative capacity are strong candidates for the development of a mechanism-based chemoprevention strategy for prostate cancer (21). We have confirmed the endogenous expression of I-α hydroxylase (1αOHase), the activating enzyme that converts 25(OH)D3 to 1,25(OH)2D3, in PrP/SCs by reverse transcriptase PCR (Supplementary Fig. S1), which supports our hypothesis that the PrP/SC is a suitable model for studying the mechanistic response to vitamin D. We hypothesize that 1,25(OH)2D3 regulates differentiation and limit replicative capacity are strong candidates for the functional role of vitamin D3 as a chemopreventative agent.

Materials and Methods

Culture of mouse PrP/SCs

Adult mouse PrP/SCs were isolated and maintained as described by Barclay and colleagues (15, 22). Experiments were carried out between passages 20 and 30.

Antibodies and reagents

Antibodies used were as follows: Interleukin-1 alpha (IL-1α; Santa Cruz Biotechnology Inc.), p21 and p27 (Cell Signaling Technology), β-actin (Sigma Aldrich), pRB (Pharmpingen), and Alexa Fluor 488 anti-rabbit (Invitrogen).

Reagents used were as follows: 25(OH)D3 and 1,25(OH)2D3 (BIOMOL International). When BIOMOL was integrated into Enzo Life Sciences, 1,25(OH)2D3 was purchased from Sigma Aldrich. IL-1α (R&D Systems; www.rndsystems.com).

Generation of Rb knockout PrP/SCs, pRBcre/cr cell lines, and Ink4A/Arf-null cells

Ink4A/Arf homozygous-null mice with deletion of exons 2 and 3 and Rb homozygous-floxed mice were from the National Cancer Institute (NCI) Mouse Models of Human Cancer Consortium (http://emice.nci.nih.gov/). Prostate epithelial cells from Ink4A/Arf-null and pRBcre/cr animals were harvested as described previously (15, 22). Late passage pRBcre/cr cells were infected with 5,000 plaque forming units (pfu) of adenovirus Cre recombinase vector (Ad-Cre; ref. 23) in naked DMEM/Ham’s F 12 supplemented with 2 μmol/L MgCl2, CaCl2 on a 60-mm dish. After 4 hours, complete medium was added to the infection medium and refreshed after 2 days. Seven sequential refections were necessary to generate pRBnull cells, as monitored by immunoblotting.

Isolation of VDR-null mouse prostatic epithelial cells

Animals were bred and mouse prostatic epithelial cells (MPEC) were isolated as described previously (22). Vitamin D receptor (VDR) knockout was confirmed using reverse transcriptase PCR (New England Biolabs).

Flow cytometry

A total of 1 × 105 cells were treated with vehicle (0.1% ethanol) or 100 nmol/L 1,25(OH)2D3 for the specified times (n = 3). Cells were harvested by trypsin digestion and collected by centrifugation. The pellet was washed with 1× PBS, fixed with 70% cold ethanol, and stored at 4°C for at least 24 hours. Fixed cells were centrifuged, washed in PBS, and incubated in 0.5 mg/mL ribonuclease A (RNase A; Sigma) at 37°C for 6 hours. Cells were collected by centrifugation, resuspended in 1 mL of 50 μg/mL propidium iodide (PI; Sigma) solution (0.6% NP-40 in water), and incubated overnight. Cells were analyzed using a FACStar Plus flow cytometer (Becton Dickinson), which acquired between 10,000 and 20,000 events for each sample. The results were analyzed using Cell Quest (Becton Dickinson), and the percent distribution of cells in G0–G1, S, and G2–M was determined using ModFit LT v.2.0 software (Verity Software House). Statistical evaluations were determined by ANOVA with post hoc analysis by Scheffe’s F test.

Immunoblotting

Procedures for immunoblotting protein lysates from cells grown in monolayer is described in detail elsewhere (22).

Microarray experiments

A total 1 × 105 PrP/SCs were grown to 70% confluence in 10-cm culture dishes before treatment with vehicle (0.1% ethanol) or 100 nmol/L 1,25(OH)2D3 in culture media [detailed in ref. 22; n = 3 or 4]. RNA was isolated at 6 and 48 hours by using the Chomczynski and Sacchi method (24). The RNA was used to probe Affymetrix 430A oligonucleotide arrays. The microarray data are publicly available in the Gene Expression Omnibus Database (www.ncbi.nlm.nih.gov/geo; accession number GSE18993). The data from all Affymetrix chips were normalized using the Robust Multichip Analysis (RMA) program (25, 26). Comparative analyses were carried out with tests for P value and B statistics for determination of significance (27). Data were further analyzed with the Ingenuity Pathways Analysis (IPA) program suite (www.ingenuity.com), GenMAPP 2.0 (www.genmapp.org), and DAVID (http://niaid.abcc.ncifcrf.gov).

Growth assays

Trypan blue exclusion assays were done as described elsewhere (8).

Clonogenic assays

Clonogenic assays were done as described elsewhere (15).

Quantitative real-time PCR analysis

RNA was isolated from triplicate from PrP/SCs treated with vehicle (0.1% ethanol) or 100 nmol/L 1,25(OH)2D3 for 24 hours, quantified and converted to cDNA using reverse transcriptase, and diluted at a 1:10 ratio in H2O. Quantitative real-time PCR (qPCR) was carried out using Bio-Rad iQ SYBR green supermix. The results were analyzed using ΔΔCt.
calculations and normalized to the control (error bars show the SDs). Statistical significance was determined by t test (critical value = 0.05; n = 3). Primer sequences are available upon request.

Short hairpin RNA targeting
Short hairpin RNA (shRNA) vectors were generated as described by Sui and colleagues (28). The IL-1α target sites are GGTAGTGAGACCGACCTCATT (shRNA1) and GACTGGCCCTCTATGACGACTT (shRNA2). After infection with ecotropic virus, single-cell clones were generated using cloning cylinders and the expression of IL-1α protein was evaluated by Western blotting after 24-hour treatments with 100 nmol/L 1,25(OH)2D3 or 0.1% ethanol. Viral infection efficiency was validated by positive green fluorescent protein (GFP) signal encoded by the virus.

ELISA
ELISA was conducted according to the manufacturer’s instructions in a kit from R&D Systems (catalogue number MLA00).

Immunofluorescence
Immunofluorescence was carried out as described elsewhere (29). Fluorescent signal images were captured using a Nikon DXM1200F digital camera on a Nikon Eclipse 50i microscope with an EXFO X-Cite 120 Fluorescence Illumination system.

Senescence-associated β-galactosidase assay
Senescence-associated β-galactosidase (SA-β-gal) activity was evaluated as described in Axanova and colleagues (30).

Results
1,25(OH)2D3 induces cell-cycle arrest and senescence in PrP/SCs
We first characterized the phenotypic effects of 1,25(OH)2D3 on proliferation and cell-cycle progression in PrP/SCs that were isolated and maintained in our laboratory (15, 22). Briefly, we defined a reproducible system for maintaining long-term culture of adult mouse PrP/SCs isolated from 10-week-old mice, termed WFU3 cells. A clonal population, WFU3 clone 3 (WFU3 cl.3), exhibited multilineage differentiation and self-renewal in vivo, and they expressed known progenitor cell markers Sca1 and CD49f as well as basal cell markers p63 and cytokeratins 5 and 14. A previous study verified 1,25(OH)2D3-mediated growth inhibition of the parental cell line WFU3, but the mechanism is unknown (31). In this study, we used the characterized WFU3 cl.3 cells, hereafter called PrP/SCs, to study the phenotypic and genotypic effects of 1,25(OH)2D3.

PrP/SCs underwent dose-dependent growth inhibition and clonogenic growth suppression in response to 1,25(OH)2D3 in trypan blue exclusion assays and clonogenic assays, respectively (Fig. 1A and B). Western blot analysis indicated that both p21 and p27 were induced by both 100 nmol/L 1,25(OH)2D3 and 1 µmol/L 25(OH)D3, respectively, compared with the control (0.1% ethanol; Fig. 1C). These data are consistent with the 1,25(OH)2D3 response in other cell lines (7, 8).

Figure 1. Vitamin D signaling inhibits PrP/SC growth. A, 5-day growth assay in PrP/SCs treated with vehicle control (0.1% ethanol) or 100 nmol/L 1,25(OH)2D3 (n = 3 or 4; *, P < 0.05, ANOVA). Error bars show SDs. B, clonogenic assay with PrP/SCs. Error bars show SDs (n = 4; *, P < 0.05, ANOVA). C, Western blotting with protein lysates from PrP/SCs treated with 0.1% ethanol (E), 1 µmol/L 25(OH)D3 (25), or 100 nmol/L 1,25(OH)2D3 (1,25) for the indicated times in hours. Note the 10-fold excess of 25(OH)D3 relative to 1,25(OH)2D3. D, growth assay data from cells of the indicated genetic backgrounds are normalized to the vehicle control. Bars represent mean ± SEM cell numbers (n = 3 or 4; *, P < 0.05, t test).
(OH)\(_2\)D\(_3\)-mediated antiproliferative response, we deleted exon 19 of the pRb locus (15). We previously reported the isolation and characterization of PrP/SCs from pRb\(_{\text{loxP/loxP}}\) animals (15, 22). These cells were infected in vitro with adenoviral Cre recombinase (Ad-Cre) and validated for loss of pRb by immunoblotting (Supplementary Fig. S2). pRb\(_{\text{null}}\) PrP/SCs were robustly growth inhibited by 100 nmol/L 1,25(OH)\(_2\)D\(_3\) to an extent similar to that in pRb\(_{\text{loxP/loxP}}\) PrP/SCs (Fig. 1D). Prostatic epithelial cells isolated from animals with deletion of exons 2 and 3 of the Ink4A\(_{\text{Arf}}\) locus (p16 and p19 null) were also robustly growth inhibited by 100 nmol/L 1,25(OH)\(_2\)D\(_3\) (Fig. 1D). As a positive control, we isolated MPECs from littermate-matched VDR wild-type (VDR\(_{\text{WT}}\)) and VDRnull animals (Supplementary Fig. S2; ref. 32). We confirmed that the antiproliferative effects of 1,25(OH)\(_2\)D\(_3\) are VDR dependent in our system; VDRnull cells were not growth inhibited by 1,25(OH)\(_2\)D\(_3\) (Fig. 1D).

We next analyzed cell-cycle progression in asynchronously dividing wild-type (WT) PrP/SCs, pRb\(_{\text{loxP/loxP}}\), and pRb\(_{\text{null}}\) PrP/SCs treated with vehicle control or 100 nmol/L 1,25(OH)\(_2\)D\(_3\) (Fig. 2A). Cell-cycle distribution was based on PI staining and was analyzed by flow cytometry. At 24 hours posttreatment, all 3 cell lines showed a significant increase in the G1 phase fraction, and the WT and pRb\(_{\text{loxP/loxP}}\) cells showed a significant decrease in the S-phase fraction. By 48 hours, all 3 cell lines had significantly reduced the fraction of S-phase cells. However, only the pRb\(_{\text{loxP/loxP}}\) cells exhibited a significant increase in the G1 phase fraction. Interestingly, they also exhibited a significantly greater fraction of cells in the G2–M phase and significantly fewer cells in S-phase. Taken together, these data suggest that 1,25(OH)\(_2\)D\(_3\) inhibits global cell-cycle progression of PrP/SCs by early effects at G1–S, followed by more delayed effects at G2–M.

**Figure 2.** 1,25(OH)\(_2\)D\(_3\) induces G1 and G2–M cell-cycle arrests and senescence in PrP/SCs. A, cells were treated with 0.1% ethanol (EtOH) or 100 nmol/L 1,25(OH)\(_2\)D\(_3\) for the indicated times. Each bar represents the mean ± SEM of 4 replicate samples. Statistical evaluations were determined by ANOVA with post hoc analysis by Scheffe’s F test (*, P < 0.05; **, P < 0.005). B, representative images of an SA-b-gal assay in cells treated with increasing doses of 1,25(OH)\(_2\)D\(_3\) every 48 hours for 96 hours. The positive control treatment was 100 nmol/L Dox. The negative control treatment was 100 nmol/L Dox at pH 7. Bars represent the percentage ± SEM of SA-b-gal-positive cells (n = 10; different letters indicate differences in means with P < 0.05, Fisher’s least significant difference test).
We recently discovered that 1,25(OH)₂D₃ induces senescence of prostate cancer cells (30). To test whether 1,25(OH)₂D₃ can also induce senescence of PrP/SCs, we assayed SA-β-gal activity in PrP/SCs treated with ethanol or 1,25(OH)₂D₃. The cells exhibiting SA-β-gal expression have a flattened, enlarged morphology characteristic of senescence, which was apparent upon treatment with the doxorubicin (Dox)-positive control (Fig. 2B). 1,25(OH)₂D₃ induced senescence in PrP/SCs in a dose-dependent manner (Fig. 2B), which likely contributed to the growth-suppressive effects of 1,25(OH)₂D₃.

1,25(OH)₂D₃ induces global gene expression changes in PrP/SCs

To identify novel targets of VDR transcriptional activity and to assess global gene expression changes, we probed Affymetrix gene expression arrays with RNA from 100 nmol/L 1,25(OH)₂D₃- or ethanol-treated PrP/SCs (Supplementary Fig. S3A for schema; Gene Expression Omnibus Database accession number GSE18993). At 6 hours, 263 genes were upregulated and 61 genes were downregulated by 1,25(OH)₂D₃ that were statistically significant relative to the control treatment. At 48 hours, 326 genes were upregulated and 205 genes were downregulated by 1,25(OH)₂D₃, also statistically significant (Supplementary Tables S1–4). The 6-hour time point is more likely to capture direct transcriptional targets of the VDR in a robust manner, whereas the 48-hour time point is likely to capture more secondary and tertiary targets. Supplementary Table S5 summarizes the top 20 up- and downregulated genes in PrP/SCs treated with 1,25(OH)₂D₃ for 6 and 48 hours, respectively. The most highly upregulated gene at 6 and 48 hours is Cyp24a1, which encodes 25(OH)D₃ 24-hydroxylase, the best-documented VDR transcriptional target that contributes to negative feedback of 1,25(OH)₂D₃ signaling. The similarities and differences in microarray profiles between 1,25(OH)₂D₃-treated PrP/SCs and other prostate cells, such as RWPE-1 cells, highlights the sensitivity of 1,25(OH)₂D₃ signaling to cellular context (33). Annotation of the genes according to function by the IPA program suite indicated that a number of the 1,25(OH)₂D₃-regulated genes are associated with the...
differentiated prostatic luminal epithelial cell, particularly among the genes upregulated at 48 hours (Supplementary Table S6).

Consistent with the cell-cycle analysis in Figure 2A, 100 nmol/L 1,25(OH)2D3 induced global regulation of genes involved in cell-cycle progression (Fig. 3A). These include genes encoding proteins important for G1–S progression, such as cyclin E2, Cks1b, proliferating cell nuclear antigen (Pcna), and multiple members of the E2F family of transcription factors. There was also the regulation of genes involved in DNA synthesis and replication fork loading, such as Cdc7, Orc2l, and Mcm6. Most notably, 1,25(OH)2D3 regulated numerous genes, the corresponding proteins of which directly contribute to or modulate spindle assembly and mitosis (Fig. 3A). Interestingly, neither p21 nor p27 was present in the gene lists at 6 hours, which is consistent with other studies in our laboratory that suggest that these proteins are regulated as secondary targets of 1,25(OH)2D3 in prostate cells (Supplementary Tables S1–S4; refs. 7, 8). Most notable among differentiation targets are AR and prostatic acid phosphatase (Acpp), which are both increased by 6 hours and exhibit further increases at 48 hours of 1,25(OH)2D3 treatment according to qPCR (Supplementary Table S6 and Fig. 3B). AR signaling is thought to be essential for the antiproliferative effects of 1,25(OH)2D3 in LNCaP cells, although there is no evidence that AR is a direct transcriptional target of 1,25(OH)2D3 (34–36). The regulation of numerous differentiation targets supports the hypothesis that 1,25(OH)2D3 promotes differentiation of PrP/SCs.

We confirmed a sample set of gene targets from the microarray results by qPCR, including AR, prostatic Acpp, Klk26, keratin 4 (Krt4), prostate stem cell antigen (Psca), stefin A1 (Stfa1), bone morphogenetic protein 4 (Bmp4), and bone morphogenetic protein receptor 1A (Bmpr1a; Fig. 3B). Psca is a misnomer for this gene/protein; Psca expression in the prostate stem cell is low, but levels increase when the cell undergoes differentiation into a transit-amplifying cell (37). The increase in Psca in response to 1,25(OH)2D3 supports the hypothesis that 1,25(OH)2D3 drives differentiation of the PrP/SC into a transit-amplifying cell. The other targets such as AR, Acpp, Krt4, and Klk4 suggest that the transit-amplifying cell population is progressing toward a luminal cell phenotype. We believe that this in vitro differentiation model will allow for in-depth analysis of the molecular programming behind PrP/SC differentiation.
IL-1α is a novel target for 1,25(OH)₂D₃ signaling in PrP/SCs

Our goal was to identify key pathways governing vitamin D₃-mediated effects so that we may better design rational combinatorial strategies for prostate cancer chemoprevention. Although the array data were informative, it was not clear which target(s) should be pursued based solely on fold induction; a systems biology approach was needed to make more informed decisions. To do this, we evaluated the microarray data using the IPA program suite, which identifies regulated networks based on signaling pathways, protein–gene and protein–protein interactions, biological functions, and diseases. Normalized and statistically significant array data were evaluated by IPA, and networks were generated using protocols provided with the software. Figure 4 shows the top-scoring network of annotated genes significantly regulated by 1,25(OH)₂D₃ at 6 hours in PrP/SCs. This network was focused around IL-1α signaling. When the top 8 most regulated networks were merged, IL-1α was centrally located, which suggests a central role for IL-1α in the gene and protein interactions in response to 1,25(OH)₂D₃ signaling (Supplementary Fig. S3B). The Affymetrix array data showed that IL-1α was upregulated 6.8-fold at 6 hours and 4.8-fold at 48 hours (Supplementary Tables S1 and 3). In addition, numerous previously defined targets of IL-1α signaling such as Mmp13, Cox2, and Nfkbia were upregulated at the 48-hour time point in the array data, suggesting that these are secondary targets of 1,25(OH)₂D₃ signaling mediated by IL-1α (Supplementary Fig. S4 and Supplementary Table S3).

IL-1α is one of a 3-member family of related cytokines that bind to the IL-1 receptor (IL-1R1) and have roles in inflammation, proliferation, and differentiation (38). IL-1α is synthesized as a 33-kDa pro-IL-1α. The amino-terminal propeptide contains a nuclear localization sequence sufficient to direct the 33-kDa form to the nucleus where it is thought to impact gene expression independently from the membrane-bound IL-1R1. The propeptide must be cleaved by calpain in order for the 17-kDa (mature) form to be tethered to the cell membrane and/or secreted by a nonclassical mechanism (39, 40). The effects of IL-1α are cell-type specific, and its potential role in 1,25(OH)₂D₃ signaling has previously been reported both in osteoclast differentiation and in modulation of keratinocyte inflammation (41, 42). Given the cell-specific effects of IL-1α, its putative role in the nucleus, and the dominant location of IL-1α in the array data and IPA analysis, we hypothesized that IL-1α mediates the antiproliferative effects of 1,25(OH)₂D₃ in PrP/SCs.

IL-1α mediates the antiproliferative effects of 1,25(OH)₂D₃ in PrP/SCs

We first validated the induction of IL-1α by 1,25(OH)₂D₃ in PrP/SCs. 1,25(OH)₂D₃ (100 nmol/L) induced IL-1α protein...
(33 kDa) and mRNA levels within 6 hours, and levels peaked at 24 hours of treatment in PrP/SCs (Fig. 5A and B). We also saw induction of IL-1α by 100 nmol/L 1,25(OH)2D3 in additional PrP/SC strains with different genetic backgrounds (Supplementary Fig. S5). Despite the strong induction of IL-1α by 1.25 (OH)2D3, IL-1α was not secreted into the medium beyond the minimum detection level at 24 hours, as measured by an ELISA, whereas at 48 hours, the levels of IL-1α in the medium reached only 9 pg/mL (Fig. 5C). Because IL-1α secretion was negligible, we used immunofluorescence to visualize IL-1α localization. IL-1α appeared to reside in the cytoplasmic and nuclear compartments of PrP/SCs upon treatment with 1,25(OH)2D3 (Fig. 6). In addition, Western blots did not detect a 17-kDa band for the membrane-associated form of IL-1α. Together, this suggests that IL-1α acts in a primarily intracellular (and not membrane-bound) manner in PrP/SCs, consistent with arguments for intracrine actions of IL-1α (38). However, high-dose exogenous IL-1α (100 ng/mL) elicited a 40% growth inhibition (Supplementary Fig. S6), suggesting the potential for receptor-mediated signaling to contribute to the observed effects.

To evaluate the role of IL-1α in the antiproliferative effects of 1,25(OH)2D3, we developed shRNA vectors that target IL-1α and a control vector. We verified by Western blotting that IL-1α expression was suppressed by the targeted shRNAs (Fig. 7A and B). We also verified that shRNA-infected cells maintained an intact VDR signaling pathway by assessment of Cyp24a1 mRNA expression (Supplementary Fig. S7). Figure 7A and B show that PrP/SCs infected with control shRNA (shRNA NC) were significantly growth inhibited in a dose-dependent manner by 1.25(OH)2D3 by 48 hours. In contrast, PrP/SC infected with IL-1α shRNAs were resistant to the antiproliferative effects of 1.25(OH)2D3. IL-1α suppression alone did not substantially alter PrP/SC proliferation (not shown), which is unsurprising given the low basal levels of IL-1α in PrP/SCs. To validate these results, we infected an additional strain of PrP/SCs from a different genetic background (pRbloxP/loxP) with control and IL-1α shRNA1 (Supplementary Fig. S8A). Treatment with 1.25(OH)2D3 validated our findings that IL-1α was necessary for the antiproliferative effects of 1.25(OH)2D3 in PrP/SCs (Supplementary Fig. S8B). TO test whether IL-1α is sufficient to restore growth inhibition by 1.25(OH)2D3, we treated IL-1α shRNA–infected PrP/SCs with a range of dose combinations of exogenous IL-1α and 1,25(OH)2D3 (Supplementary Fig. S9). IL-1α (10 ng/mL) was sufficient to rescue 1,25(OH)2D3-mediated growth suppression (Fig. 7C). However, 10 pg/mL IL-1α, the approximate concentration secreted from PrP/SCs upon 1,25(OH)2D3 treatment, was not sufficient to rescue growth inhibition by 1.25(OH)2D3 in IL-1α knockdown cells, suggesting a primarily intracellular role for IL-1α in 1,25(OH)2D3 signaling (Fig. 7C).

1,25(OH)2D3, the hormonal form of vitamin D3, is commonly used in vitro. However, nanomolar doses are superphysiologic. Prostatic epithelial cells, including PrP/SCs, express 1αOHase...
(Supplementary Fig. S1), which converts 25(OH)D3 to 1,25(OH)2D3. Physiologically relevant and safe levels of 25(OH)D3 can exceed 100 nmol/L. We treated control and IL-1α knockdown–infected PrP/SCs with physiologically relevant doses of 25(OH)D3 for 24 hours and observed induction of IL-1α in the control cells (Supplementary Fig. S8C). Furthermore, 25(OH)D3 induced dose-dependent growth inhibition of control PrP/SCs and not IL-1α shRNA PrP/SCs (Supplementary Fig. S8D). This further supports the necessity for IL-1α in the antiproliferative activity of vitamin D3 in PrP/SCs. In addition, because 25(OH)D3 was sufficient to suppress PrP/SC growth at doses readily achievable in vivo, it supports the relevance of vitamin D3 in the chemopreventative setting.

Discussion

Here we explore the role of 1,25(OH)2D3 as a modulator of prostate stem cell differentiation, proliferation, and senescence, and we present an in vitro model for studying the molecular program behind these actions. It is unclear whether the induction of senescence is coincident with or in addition to the effects of 1,25(OH)2D3 on cell-cycle arrest. The modest effects of vitamin D3 on the cell-cycle imply that additional mechanisms are important for overall growth regulation. We used superphysiologic levels of 1,25(OH)2D3 to induce senescence in vitro, and it will be important to test induction of senescence by vitamin D3 in vivo, given the disparate conditions. Senescence occurs in the prostate as a protective mechanism against prostate cancer progression (43). However, senescence has not been reported to occur naturally in the aged adult prostate. The finding that PrP/SCs undergo 1,25(OH)2D3-induced senescence suggests a possible mechanism for chemoprevention of prostate cancer by vitamin D3 that needs to be tested in vivo.

Cancer cells have many phenotypic parallels to stem cells, and an increasing number of genotypic parallels are being made as well that have led to the cancer stem cell hypothesis. These have best been characterized in the hematopoietic stem cell system, with emphasis on the roles of Wnt, Notch, and β-catenin signaling (20). Our array data revealed regulation of

Figure 7. IL-1α is necessary for 1,25(OH)2D3-induced growth inhibition. A and B, verification of IL-1α knockdown in PrP/SCs after 24 hours of 0.1% EtOH (E) or 100 nmol/L 1,25(OH)2D3 (D) and 48-hours trypan blue exclusion assays. Error bars indicate means ± SDs of multiple clones (n = 3 or 5; **, P < 0.005, ANOVA). NC, negative control. C, 48-hour trypan blue exclusion assay (n = 4). Control, 1% bovine serum albumin/PBS. Bars labeled "a" or "b" are significantly different according to ANOVA with post hoc Fisher’s least significant difference analysis (critical value = 0.05). NC, negative control.
genes involved in these pathways as well as BMP and TGFβ signaling pathways (Supplementary Tables S1–4 and S6). These networks likely play roles in prostate stem cell maintenance and differentiation, and we are beginning to interrogate the impact of 1,25(OH)2D3 signaling on these pathways. These experiments will provide insight into normal prostate development as well as the mechanism behind maintenance of prostate health by vitamin D, prompting rationale for an effective chemopreventive regimen.

These studies are among those that support a functional intersection between hormone and cytokine signaling. We found that IL-1α is a critical component for vitamin D3 signaling in PrP/SCs. A previous report has shown that IL-1α is not detected immunohistochemically in normal prostate cells in vivo, whereas IL-1α is detected in benign prostatic hyperplasia (BPH) and in prostate cancer cells (44). However, IL-1α was detected at the edges of the cell membranes, so it was perhaps derived from the proinflammatory microenvironment associated with prostate cancer. The roles of endogenous IL-1α and its signaling components in these cell types are unknown.

This is the first study reporting IL-1α in the normal adult PrP/SC, notably in response to 1,25(OH)2D3, and we have shown that IL-1α resides in the cytoplasm and nuclei of these cells. We have identified a putative vitamin D response element (VDRE) upstream of the IL-1α coding region that aligns with known consensus VDREs (refs. 45–48; Supplementary Table S7). Our microarray data also showed that 1,25(OH)2D3 decreased expression of IL-1 receptor antagonist (IL-1ra) and IL-6, a common downstream target of IL-1α associated with inflammation in the prostate (ref. 49; Supplementary Tables S1–4). Together, this leads us to hypothesize that IL-1α induced by 1,25(OH)2D3 does not act through its transmembrane receptor to promote inflammation in PrP/SCs. The actions of IL-1α are cell-type specific, and our data support an antiproliferative, intracellular role for IL-1α in the 1,25 (OH)2D3-induced growth inhibition of PrP/SCs. Overall, this work provides mechanistic support for the use of vitamin D3 as a chemopreventive agent for prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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