VDR Activity Is Differentially Affected by Hic-5 in Prostate Cancer and Stromal Cells

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Abstract

Patients with prostate cancer treated with androgen deprivation therapy (ADT) eventually develop castrate-resistant prostate cancer (CRPC). 1,25-Dihydroxyvitamin D3 (1,25D3/calcitriol) is a potential adjuvant therapy that confers antiproliferative and pro-differentiation effects in vitro, but has had mixed results in clinical trials. The impact of the tumor microenvironment on 1,25D3 therapy in patients with CRPC has not been assessed. Transforming growth factor β (TGFβ), which is associated with the development of tumorigenic "reactive stroma" in prostate cancer, induced vitamin D3 receptor (VDR) expression in the human WPMY-1 prostate stromal cell line. Similarly, TGFβ enhanced 1,25D3-induced upregulation of CYP24A1, which metabolizes 1,25D3 and thereby limits VDR activity. Ablation of Hic-5, a TGFβ-inducible nuclear receptor coregulator, inhibited basal VDR expression, 1,25D3-induced CYP24A1 expression and metabolism of 1,25D3 and TGFβ-enhanced CYP24A1 expression. A Hic-5-responsive sequence was identified upstream (392–451 bp) of the CYP24A1 transcription start site that is occupied by VDR only in the presence of Hic-5. Ectopic expression of Hic-5 sensitized LNCaP prostate tumor cells to growth-inhibitory effects of 1,25D3 independent of CYP24A1. The sensitivity of Hic-5–expressing LNCaP cells to 1,25D3-induced growth inhibition was accentuated in coculture with Hic-5–ablated WPMY-1 cells. Therefore, these findings indicate that the search for mechanisms to sensitize prostate cancer cells to the antiproliferative effects of VDR ligands needs to account for the impact of VDR activity in the tumor microenvironment.

Implications: Hic-5 acts as a coregulator with distinct effects on VDR transactivation, in prostate cancer and stromal cells, and may exert diverse effects on adjuvant therapy designed to exploit VDR activity in prostate cancer.

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Introduction

Prostate cancer is one of the leading causes of cancer-related death among men in the United States. It was the most diagnosed cancer among men in 2011, affecting 241,740 new patients and leading directly to 28,170 deaths, second only to lung cancer (1). Approximately 1 in 6 males in the United States can expect to develop prostate cancer in their lifetimes. Currently, therapies for metastatic prostate cancer are limited. Androgen deprivation therapy (ADT) and hormone therapies are standard, but the cancer eventually becomes castrate resistant, and chemotherapy offering little more than palliative care (2). There is a need to develop new therapies or regimes to increase the quality of life and to extend prognosis for those patients undergoing ADT.

Epidemiological data reveal an inverse correlation between the incidence of lethal prostate cancer and resident latitude, which is taken to be indicative of chronic, lifetime exposure to sunlight (3, 4). Indeed, serum levels of 25-hydroxyvitamin D3 (25D3) are induced by exposure to sunlight, expression of VDR in tumors, and single nucleotide polymorphisms (SNP) in the VDR gene or its associated metabolizing enzymes, such as 25-hydroxylase (CYP27A1), are inversely correlated with incidence of the lethal disease (5–8). As such, treatment with Vitamin D3 or its metabolites—25D3 or 1,25D3—present promising opportunities for adjuvant therapy. Nonetheless, serum levels of 25D3 do not correlate with overall prostate cancer risk, and dietary supplementation of Vitamin D3 does not influence the risk of developing prostate, breast, or colorectal cancers (9). Moreover, clinical trials with 1,25D3 in patients with prostate cancer have generated mixed results. In a phase II trial, high-dose 1,25D3 treatment improved the prognosis of patients receiving docetaxel therapy, but disappointingly, the phase III trial did not yield the same results (10, 11).
1,25D₃ has been examined for effects on growth and survival of many cancer cells in vitro and in vivo. For example, it exerts antiproliferative and proapoptotic responses on the LNCaP prostate cancer cell line and on prostate cancer xenografts in immunocompromised mice (12–19). Treatment with 1,25D₃ or its analogues can also inhibit inflammatory mediators, such as interleukin-8 (IL8) and nuclear factor-κB (NF-κB), and confer quiescence (20–22). One molecular mechanism that could contribute to the antitumor effects of 1,25D₃ was revealed in a study showing an inhibition of TGFβ–induced epithelial-to-mesenchymal transition (EMT) and fibrosis mediated by an interaction between ligand-bound VDR and the TGFβ-activated transcription factor Smad3 (23). However, not all VDR/Smad3 interactions are universally inhibitory, as Smad3 can potentiate VDR-induced transactivation (24).

To date, the effects of the tumor microenvironment on 1,25D₃ activity in prostate cancer are virtually unknown. The prostate stroma comprises a heterogeneous mixture of fibromuscular tissue, including fibroblasts, smooth muscle cells, and a collagen-rich extracellular matrix (25). Under normal conditions, paracrine factors, such as keratinocyte growth factor (KGF), which is produced by stromal cells upon androgen stimulation, promote epithelial development (26, 27). Paradoxically, high circulating levels of testosterone can maintain adult stromal cells in a quiescent state (28). This homeostasis is regulated by divergent actions of specific cell types within the stroma, as evident by the stimulation of epithelial proliferation by stromal fibroblasts but reduced epithelial response to androgen-stimulated stromal smooth-muscle cells (29). A disruption in this tissue homeostasis is symptomatic of prostate cancer. Epithelial tumors secrete TGFβ that "activates" surrounding stromal fibroblasts from their resting state (30, 31). This activation process is similar to the fibroblast response during wound healing, which is characterized by the appearance of myofibroblastic cells that possess properties of both smooth muscle and fibroblastic cells (32, 33). This "reactive stroma," which resembles an over-healing wound, confers a fertile environment for the tumor through secretion of growth factors and remodeling of the extracellular matrix (34–39).

Numerous transcriptional regulators modulate stromal response to TGFβ and the ensuing reactive-stroma phenotype. One such important cofactor is Hic-5, which is expressed predominantly within the prostate stroma under normal and malignant conditions and upregulated by TGFβ (40–42). Hic-5, a member of the paxillin family of group III LIM domain-containing proteins, is localized either at focal adhesions in the cytoplasm, where it regulates cell migration, or within the nucleus, where it functions as a transcriptional coregulator (42–44). Hic-5 has no intrinsic chromatin-modifying enzymatic activity on its own, but rather acts as a scaffolding protein to assemble multi-subunit complexes containing regulated transcription factors, including most notably, nuclear receptors (45). In prostate stromal cells, for example, Hic-5 is necessary for full transactivation of the androgen receptor (AR) target gene KGF, influencing the recruitment of various transactivators (42). Alternatively, in the absence of steroid ligands, Hic-5 functions in transcriptional repression through the gene-specific recruitment of the nuclear receptor corepressor (NCoR) complex (45, 46). In addition to binding to nuclear receptors, Hic-5 also binds to other transcription factors, feeding back onto TGFβ signaling through interaction with Smad3. However, this interaction yields conflicting effects, being enhancing or inhibitory depending on cellular context (47, 48).

In this study, we extend the previous analysis of Hic-5 regulation of steroid receptors and establish its role as a coactivator for VDR in the WPMY-1 human myofibroblastic prostate stromal cell line. In that context, Hic-5 is essential for VDR-mediated transcriptional activation of CYP24A1, which encodes the 1,25D₃-metabolizing enzyme 24-hydroxylase, thereby contributing to a negative feedback loop that limits VDR activity. Hic-5 also acts in prostate cancer cells to enhance the antiproliferative effect of 1,25D₃. The recent demonstration of Hic-5 expression in prostate epithelial cancer cells upon androgen ablation suggests that compartment specific-effects of Hic-5 could generate diverse responses of prostate tumors to 1,25D₃, particularly during ADT (49). Our results indicate the importance of assessing expression and activity of Hic-5 in both cancer cells and the tumor microenvironment in order to better understand and predict patient response to 1,25D₃ therapy.

Materials and Methods
Chemicals and reagents
Recombinant TGFβ1 was purchased from R&D Systems and reconstituted in 4.0 mmol/L HCl, according to the manufacturer’s protocol. 1,25D₃ was purchased from Cayman Chemical and reconstituted to 20 μmol/L in cell culture-grade DMSO. Mock treatments with 1,25D₃ used equivalent volume of DMSO vehicle. EB1089 was purchased from Tocris Bioscience. A mouse monoclonal anti-Hic-5 antibody (clone 34/Hic-5) was purchased from BD Biosciences. The mouse monoclonal anti-VDR antibody (clone D-6), rabbit polyclonal anti-VDR antibody (clone C-20X), and rabbit polyclonal anti-β-tubulin antibody (clone H-300) were purchased from Santa Cruz Biotechnology. Secondary horseradish peroxidase (HRP)–conjugated antibodies were purchased from Sigma.

Cell culture
WPMY-1 cells were purchased from American Type Culture Collection. Scr and shHic-5 cells were generated as described below. LNCaP cells transfected with control lentivirus and lentivirus containing the gene coding for murine Hic-5 (LNCaP/Hic-5) were obtained from the laboratory of Neil Bhowmick (Cedars-Sinai Medical Center; ref. 49). Cells were maintained as monolayers in RPMI 1640 medium containing 5% fetal bovine serum (FBS; for WPMY-1 cells) or 10% FBS (for PS30, LNCaP, and LNCaP/Hic-5 cells) and 1% penicillin/streptomycin at 37°C and 5% CO₂. Cells were passaged at ~90% confluence.
Generation of stable knockdown cells

Short-hairpin RNA (shRNA) sequences were designed against Hic-5 (SH1-4) or a scrambled (Scr) control (Supplementary Table S1). The oligonucleotides were annealed to form dsDNA and inserted into the pHRI CMV PURO WsIN18 plasmid after enzymatic digestion by SpeI and PstI. The plasmids were packaged into lentiviral vectors. WPMY-1 cells were seeded on 6-well cell culture dishes at a density of 2.5 × 10^5 per well for 24 hours before infection. Lentivirus infection media containing polybrene (8 µg/mL) was used to infect the cells for 24 hours. The next day, the media was changed, and the cells were cultured for an additional 48 hours, trypsinized, and passed to new tissue culture dishes. Cells were then treated with medium containing puromycin (1 µg/mL) for 3 days. The resulting pooled colonies were selected, transfected to 96-well dishes, and maintained in puromycin selection medium. Pooled colonies were expanded in 30-mm dishes. The line generating the most efficient knockdown (SH2) was renamed shHic-5.

RNA microarray

Scr and shHic-5 cells were plated at a density of 2.5 × 10^5 cells per well in a 6-well plate and cultured overnight. The cells were then cultured in serum-free medium for 24 hours. The following day, they were treated with TGFβ1 (0 and 2.0 ng/mL) and incubated at 37°C for 10 hours. The medium was then removed, and cells were lysed in 500 µL cold TRIzol (Life Technologies). RNA was extracted using the RNeasy Mini Kit (Qiagen). Three hundred nanograms of total RNA extracted from each of 5 replicate treatments were analyzed using Affymetrix Human Gene 1.0 ST Arrays. Bioinformatics were performed in R Statistical Software using Bioconductor and the Limma package. Briefly, array data were normalized using RMA, filtered by mapped probes and an arbitrary minimum expression threshold, and genes different between groups called by Benjamini–Hochberg adjusted P values determined from Bayesian linear regression modeling. Microarray datasets were deposited in GEO (Accession No. GSE47354).

Western blot analysis

5.0 × 10^5 Scr or shHic-5 cells were plated on 65-mm plates and grown overnight. The following day, the cells were cultured in serum-free medium for ~2 hours, then treated with TGFβ1 (0 and 10 ng/mL) and incubated at 37°C for 24 hours. At the conclusion of the treatment, whole-cell lysates were obtained by resuspending cells in RIPA buffer [140 mmol/L NaCl, 0.1% sodium deoxycholate, 10 mmol/L Tris-buffered to pH 8.0, 1 mmol/L, EDTA, 0.5 mmol/L EGTA, 1% Triton-X, 0.1% SDS, 1 mmol/L NaF, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), 100 µmol/L sodium orthovanadate, protease inhibitor cocktail (Sigma)], and protein concentration was quantified by the Lowry assay. Fifteen to twenty micrograms of total protein were electrophoresed in a 10% acrylamide gel and transferred to a PVDF membrane (Millipore) using a Transblot SD Semi-Dry Transfer Cell (Bio-Rad). Membranes were probed for expression of VDR, Hic-5, and α-tubulin. Between each primary antibody incubation, blots were stripped in Re-Blot Plus Strong solution following the manufacturer’s instructions (Millipore). Densitometry was analyzed using ImageJ (NIH). The results were analyzed by two-way mixed-models ANOVA, followed by cell-means posttest on untransformed data.

RNA isolation, reverse transcription, and reverse transcription quantitative PCR

Scr and shHic-5 cells were plated in 6-well plates at a density of 1.75 × 10^5 cells per well and grown overnight. The next day, they were cultured in serum-free medium for ~2 hours. TGFβ1 (0 and 3.5 ng/mL) and 1,25D₃ (0 and 100 nmol/L) were added to fresh serum-free medium to add to the cells. The cells were incubated at 37°C for 6 hours. RNA extraction was performed as described, with the resulting RNA quantified using the NanoDrop ND-1000 spectrophotometer. Complementary DNA (cDNA) was generated using the High Capacity RNA-to-cDNA Kit (Applied Biosciences) according to the kit protocol. The resulting samples were then diluted to 100 µL with nuclease-free water. Reverse transcription-quantitative PCR (RT-qPCR) was then performed on the samples with the iTaq Sybr Green Supermix with ROX (Bio-Rad) on the Stratagene Mx3000P thermocycler with primers directed toward GAPDH, VDR, CYP24A1, JAG1, and Hic-5 (Supplementary Table S2). Relative expression was quantified using the comparative Ct (ddCt) method.

In a similar experiment, LNCaP and LNCaP/Hic-5 cells were seeded on a 6-well plate at a density of 3.0 × 10^5 cells per well and cultured overnight. The next day, the cells were treated with 1,25D₃ (0 and 100 nmol/L) for 6 hours. RNA extraction and cDNA synthesis were performed as described. RT-qPCR was performed with primers directed toward GAPDH, CYP24A1, and human (WPMY-1 cells) or murine (LNCaP cells) Hic-5 (Supplementary Table S2).

Metabolism assay

Scr and shHic-5 cells were plated at a density of 5.0 × 10^5 cells per 65-mm dish and were grown overnight. The next day, the cells were cultured in serum-free medium for ~2 hours. Cells were treated in duplicate with 1,25D₃ (0 and 100 nmol/L) for 0 or 24 hours at 37°C. Reference treatments in cell-free dishes were included to account for spontaneous degradation of 1,25D₃. Cells were scraped into medium at each time point, flash-frozen in liquid nitrogen, and stored at −80°C before analysis by liquid chromatography/tandem mass spectrometry (LC/MS-MS) at the University of Pittsburgh Cancer Institute (UPCI) Clinical Pharmacology Analytical Facility. LC/MS-MS was performed as previously described (50).

Luciferase expression assay

Plasmid pCYP24-537-luc was obtained from the laboratory of Pamela Hershberger (Roswell Park Cancer Institute) and plasmids p392, p451, p470, and p496 were obtained from the laboratory of David Callen (University of Adelaide; ref. 51). Scr and shHic-5 cells were plated at a density of...
7.5 × 10⁴ cells per well in a 12-well plate and were grown overnight in antibiotic-free RPMI medium containing 5% FBS. The following day, the indicated plasmid containing a firefly luciferase reporter (0.5 μg/well; Supplementary Table S3), a Renilla luciferase plasmid containing a CMV reporter (0.1 μg/well), and X-tremeGENE lipophilic transfection reagent (5.0 μL/well; Roche Applied Science) were incubated in OPTIMEM (100 μL/well) for 1 hour. Cells were then transfected with 100 μL of the mixture and incubated overnight. The following day, the transfection medium was removed, and the cells were cultured in serum-free medium for ~2 hours. They were then treated in triplicate with TGFβ1 (0, 3.5 ng/mL) and 1,25D₃ (0 and 100 nmol/L) and incubated for 6 hours at 37°C. Cells were lysed and freeze-fractured overnight in the passive lysis buffer contained in the Dual-Luciferase Reporter Assay system (Promega). Lysates were analyzed in the Veritas Microplate Luminometer (Promega) using the Dual-Luciferase Kit to record firefly and Renilla readings in relative luminescence units (RLU). Firefly values were normalized to Renilla values.

Transient transfections were performed with the plasmid p(VDRE)₄-TATA-luc, obtained from the laboratory of Nancy Weigel (Baylor College of Medicine; ref. 52). Scr and shHic-5 cells were plated at a density of 3.5 × 10⁴ cells per well in a 24-well plate and were grown overnight in antibiotic-free RPMI medium containing 5% FBS. The following day, transfections were performed using the Lipofectamine LTX-PLUS kit (Life Technologies). p(VDRE)₄-TATA (700 μg/well), the Renilla luciferase plasmid (100 μg/well), and PLUS reagent (2.0 μg/well) were incubated in OPTIMEM medium (100 μL/well) for 10 minutes, then incubated with Lipofectamine LTX (1.5 μL/well) for 30 to 60 minutes. Cells were then transfected with 100 μL of the mixture and incubated overnight before lysis preparation and luciferase assay.

**Chromatin immunoprecipitation assay**
Scr and shHic-5 cells were plated at 0.5 × 10⁶ cells per well and 2 days after plating were treated for 4 hours with 1,25D₃ (0 and 100 nmol/L) in serum-free media. Experiment was performed as described previously (53). Lysates were briefly sonicated in 4 × 30-second bursts on high (Diagenode Inc.). Samples were immunoprecipitated using 4 μg of either anti-VDR C-20 antibody or nonspecific rabbit IgG (Santa Cruz Biotechnology) as control. DNA was purified using phenol-chloroform extraction and resulting DNA samples were quantified using RT-qPCR against primers stated in Supplementary Table S2, using iQ SYBR Green Supermix (Bio-Rad) on a CFX96 thermocycler (Bio-Rad). Data represent the average of 3 independent chromatin immunoprecipitation (ChiP) experiments ± SEM.

**In silico analysis of transcription factors**
The sequence of the human CYP24A1 promoter from region −496 to −392 bp was obtained from RefSeqGene (code number NG_008334.1 from www.ncbi.nlm.nih.gov/refseq/), and a search for putative transcription factors was performed using the Transcription Element Search System (TESS; ref. 54). Unique sites were analyzed in the literature for previously reported interactions of the target transcription factor with Hic-5.

**Proliferation assay**
LNCaP and LNCaP/Hic-5 cells were plated at 2.5 × 10³ cells per well in a 96-well plate for at least 18 hours. The cells were carefully treated in triplicate with 1,25D₃ (0, 10, and 100 nmol/L) or EB1089 (0, 10, and 100 nmol/L) in RPMI 1640 containing 10% FBS for 0 and 72 hours at 37°C. At each time point, the plate was aspirated and frozen overnight at −80°C. The next day, the plate was thawed to room temperature. The CyQuant Cell Proliferation Assay Kit (Invitrogen) was used to measure nuclear staining. Each well was incubated with the prepared dye mixture (100 μL/well) in the dark for 10 minutes. Fluorescence was read at excitation wavelength of 480 nm and an emission wavelength of 520 nm on a SpectraMax Gemini EM plate reader (Molecular Devices). Data at 72 hours were normalized to baseline at 0 hour.

**Coculture proliferation assay**
Twenty-five-millimeter circular coverslips were made suitable for coculture using nail polish to create pedestals. Four drops of nail polish were added to each coverslip and then allowed to dry under an ultraviolet lamp for additional sterilization for 1 hour. The coverslips were then placed in 6-well dishes and incubated in poly-D-lysine (10 μg/mL) for either 2 hours at 37°C or overnight at 4°C. The coverslips were then washed twice in water. LNCaP and LNCaP/Hic-5 cells were plated at 1.5 × 10⁵ cells per well and grown overnight. Simultaneously, Scr and shHic-5 cells were plated at 2.0 × 10⁵ per well in 2 other 6-well plates and grown overnight. The next day, the coverslips were moved to the 6-well plates containing the stromal cultures. Cells from 2 untreated coverslips from each epithelial line were washed in PBS and fixed to the coverslips immediately in 4% paraformaldehyde (PFA) at 0 hour to establish a baseline. The cocultures were then treated with 1,25D₃ (0 and 100 nmol/L) or EB1089 (0 and 1.0 nmol/L) in RPMI 1640 medium containing 10% FBS for 72 hours. The cells were washed once in PBS and then fixed to the coverslips in 4% PFA. The cells were then permeabilized in PBS containing 0.1% Triton-X. The cells were washed twice in PBS and then incubated with DAPI (1.0 mg/mL) at room temperature. The cells were washed twice in PBS, and the coverslips were mounted on slides using Vectashield medium (Vector Laboratories). The slides were visualized and photographed under epifluorescence at 200× using the Olympus IX-81 microscope. Counts were averaged from 6 fields per coverslip and normalized to the 0-hour time point for each respective epithelial line.

**Coculture viability assay**
18 mm × 18 mm coverslips were prepared as above. LNCaP/Hic-5, Scr, and shHic-5 cells were plated and grown as above. The cocultures were treated with 1,25D₃ (0 and 10 nmol/L) in RPMI 1640 medium supplemented with 10%
FBS and incubated at 37°C for 72 hours. At the end of the incubation, the coverslips were moved to another 6-well plate and trypsinized in 1.0 mL trypsin for ~1 hour at 37°C. The supernatants from the cocultures were collected in 2.0-mL microcentrifuge tubes and centrifuged at 1.0 × 10^3 g for 10 minutes. The trypsinized LNCaP/Hic-5 cells were added to their respective supernatant pellets and centrifuged again at 1.0 × 10^3 g and 4°C for 10 minutes. The pellets were resuspended in 100 µL of medium and stored on ice until counting. Each sample was diluted with 100 µL of Trypan blue ( Gibco). Ten microliters of the sample was loaded into both sides of a hemocytometer. Dead cells were counted in both sides of the hemocytometer under a light microscope by blue stain, whereas live cells were counted by dye exclusion. Three counts of the sample were taken and added together. Viability was determined by dividing the total number of live cells by the total number of cells counted.

Statistical analysis
Multiple comparisons were performed with the two- or three-way mixed-models ANOVA with Satterthwaite approximation, followed by cell-means post hoc test in SAS (55). Interval data, proportion data, and Western blot data that contained results below detectable levels were left untransformed, whereas ratio data that did not skew close to 0 were log-transformed (56). RT-qPCR analysis was performed on the cycles scale, and confidence intervals were transformed to the concentration scale as estimates.

Results
Regulation of VDR expression by TGFβ and Hic-5 in WPMY-1 prostate stromal cells
Hic-5 functions as a coactivator of androgen receptor in WPMY-1 prostate stromal myofibroblast cells and is an established component of the TGFβ signaling pathway (42, 47, 48, 57, 58). To evaluate the impact of Hic-5 on the TGFβ response of WPMY-1 cells, we generated a stable knockdown of Hic-5 using a specific lentivirally encoded shRNA (shHic-5). Specifically, WPMY-1 cells were stably infected with lentivirus encoding either scrambled shRNA (Scr) or shHic-5. The response of Scr and shHic-5 WPMY-1 cell lines to TGFβ was assessed by gene expression microarray 10 hours after treatment with 2.0 ng/mL TGFβ1 (Supplementary Fig. S1). Of particular relevance here, VDR was identified in the microarray as a TGFβ1 target gene whose basal expression was reduced upon Hic-5 knockdown.

To validate the microarray data, TGFβ1 and Hic-5 effects on VDR mRNA and protein expression were analyzed respectively using RT-qPCR and Western blotting in independent biological samples from Scr and shHic-5 cells. Figure 1A shows that expression of Hic-5 mRNA was indeed significantly reduced in shHic-5 cells. Similarly, basal expression of VDR mRNA (Fig. 1B and Supplementary Fig. S2) and protein (Fig. 1C and D) was reduced upon Hic-5 ablation. However, TGFβ1 induction of VDR mRNA (Fig. 1A) and protein (Fig. 1C and D) was not sensitive to Hic-5 ablation. In fact, microarray analysis of Scr and shHic-5 cells revealed both Hic-5-dependent and -independent TGFβ1-regulated genes (Supplementary Fig. S1A). For example, JAG1 is a TGFβ target gene whose induction requires Hic-5 as revealed by microarray data and validated by RT-qPCR (Supplementary Fig. S1C). The fact that Hic-5 ablation can lead to enhancement or repression of TGFβ1 responsiveness is consistent with previous results that established Hic-5's impact on the activity of both transcriptional coactivators and corepressors (45, 59). Finally, TGFβ1 induction of Hic-5 protein (Fig. 1C) may trigger a feed-forward pathway that further modulates TGFβ1 responsiveness.

Hic-5 regulates 1,25D3- and TGFβ1-induced expression of CYP24A1 in prostate stromal cells
In osteoblasts and resting-zone chondrocytes, TGFβ regulates the activity of CYP24A1, a target of VDR that metabolizes active 1,25D3 to its inactive form (60, 61). CYP24A1 expression is a major barrier to effective 1,25D3 treatment in prostate cancer, metabolizing 1,25D3 to the inactive form 1,24,25D4, which is further metabolized to calcitroic acid, which is then excreted in urine (62). However, the contribution of stromal cells to 1,25D3 metabolism is not currently known, nor have compartment-specific regulators of CYP24A1 expression been identified. To discern the effect of TGFβ on CYP24A1 gene expression, Scr cells were cotreated with 3.5 ng/mL TGFβ1 and 100 nmol/ L 1,25D3 and mRNA expression analyzed by RT-qPCR. Although treating with TGFβ1 alone minimally induced expression of CYP24A1, cotreatment with 1,25D3 enhanced CYP24A1 expression 300-fold above baseline and 10-fold above treatment with 1,25D3 alone (Fig. 2B and Supplementary Fig. S4B). One source of this enhanced gene expression may be the increased induction of VDR itself upon TGFβ1 treatment.

In shHic-5 cells, which have reduced Hic-5 expression that was not influenced by TGFβ1 or 1,25D3 (Fig. 2A and Supplementary Fig. S3A), similar dynamics were revealed with minimal induction of CYP24A1 by TGFβ1, greater induction with 1,25D3, and enhanced expression upon cotreatment of TGFβ1 and 1,25D3 (Fig. 2B and Supplementary Fig. S4B). Direct comparison of the magnitude of these effects between Scr and shHic-5 cells identified a significant reduction in CYP24A1 basal expression and a reduced 1,25D3-induced response alone or in combination with TGFβ1. Together, these results indicate that Hic-5 is required for maximal induction of CYP24A1 by 1,25D3 alone or in combination with TGFβ1. Although CYP24A1 retains some capacity to respond to TGFβ1 and 1,25D3 in Hic-5–ablated WPMY-1 cells, its reduced response has metabolic and biological consequences, as will be highlight below.

Reduced CYP24A1 mRNA expression upon Hic-5 knockdown may limit the auto-inhibitory effects of 1,25D3 on its own accumulation. LC/MS-MS was performed to analyze 1,25D3 concentration using medium and cellular content from Scr and shHic-5 cultures. Upon 24 hours of treatment, the 1,25D3 concentration in Scr cells and medium was reduced by 50% (Fig. 3). In contrast, the 1,25D3 concentration in shHic-5 cells and medium was only
reduced by about 15%. No spontaneous degradation occurred within the control medium, so the reduction of 1,25D3 in the Scr-associated medium was due solely to CYP24A1 activity. It thus seems that Hic-5 is necessary for optimal induction of a negative feedback loop on VDR activity through CYP24A1-mediated metabolism of 1,25D3.

Identification of a Hic-5–responsive element within the CYP24A1 promoter that is occupied by VDR

Transfection experiments using luciferase reporter constructs were used to determine whether Hic-5 affects transcriptional activation from the proximal promoter of CYP24A1. A luciferase reporter construct (pCYP24-537-luc) containing a 532-bp promoter sequence between 537 and 5 bp upstream of the transcription start site (TSS) included 2 known Vitamin D response elements (VDRE) located 293 and 172 bp upstream of the TSS (63). pCYP24-537-luc was transfected into Scr and shHic-5 cells and luciferase reporter activity measured upon induction by 1,25D3 alone or in combination with TGFβ1. As expected, 1,25D3 induced CYP24A1 promoter–driven luciferase reporter activity in Scr cells, whereas treatment with TGFβ1 alone did not induce activity (Fig. 4A). Cotreatment of 1,25D3 and TGFβ1 in Scr cells enhanced luciferase expression above reporter activity when treated with 1,25D3 treatment alone, revealing a permissive effect when TGFβ1 is combined with 1,25D3. However, in shHic-5 cells transfected with pCYP24-537-luc, treatment with 1,25D3 failed to induce luciferase expression above the baseline, and cotreatment with TGFβ1 only triggered a small induction that was significantly lower than for Scr cells. To analyze the effect of the VDRE alone, 1,25D3 effects were examined on a transiently transfected plasmid containing the rat osteocalcin VDRE attached to a TATA sequence [p(VDRE)4-TATA]. As shown in Fig. 4B, knockdown of Hic-5 inhibited 1,25D3 induction from the VDRE-TATA promoter, although cotreatment with 1,25D3 and TGFβ1 generated minimal induction. The mechanistic basis for the TGFβ1 effect is not clear but our data clearly demonstrate the Hic-5 dependence of 2 distinct VDR responsive promoters, at least in transiently transfected cells.

The Callen laboratory demonstrated that 1,25D3-induced expression of a luciferase reporter containing portions of the CYP24A1 promoter is dependent on a sequence 392 to 470 bp upstream of the TSS (51). Several deletion
luciferase constructs that include the proximal promoter regions from 496 bp (p496-luc), 470 bp (p470-luc), 451 bp (p451-luc), and 392 bp (p392-luc) upstream of the TSS were examined to identify potential sites required for Hic-5 co-activation in WPMY-1 cells. Luciferase expression from p496-luc in Scr cells demonstrated enhanced expression upon cotreatment of TGFβ and 1,25D3, supporting results obtained from the pCYP24-537-luc construct (Fig. 4C). However, treatment of 1,25D3 alone or together with TGFβ failed to induce minimal luciferase expression above baseline. The ability to both induce luciferase upon 1,25D3 treatment above minimal levels and enhance its expression upon TGFβ cotreatment was restored in p451-luc, which demonstrated significant increases in expression above levels seen in p392-luc (P = 0.0002 for contrast of 1,25D3 treatment alone, P < 0.0001 for contrast of 1,25D3/TGFβ cotreatment). Therefore, a Hic-5–responsive sequence was identified at 392 to 451 bp upstream of the TSS. ChIP assays performed with Scr and the shHic-5 cells showed that the chromatin occupancy of VDR at this unique VDR response element requires Hic-5 (Fig. 4D).

Overexpression of Hic-5 sensitizes epithelial LNCaP cells to the antiproliferative effects of 1,25D3

A previously unknown function of Hic-5 has been revealed in prostate epithelial cells (49). Within 7 days of castration, Hic-5 expression was de-repressed in both endogenous mouse prostate epithelium and tumor xenografts derived from patients with prostate cancer. Furthermore, aggressive tumor growth of mixed xenografts generated with Tgfbr2 knockout of prostate stroma mixed with LNCaP cells was inhibited upon overexpression of murine Hic-5 in LNCaP cells. Thus, ectopic expression of Hic-5 in prostate epithelium was associated with reduced tumor growth and was uncovered following short-term castration.

Given our demonstration of Hic-5–mediated enhancement of VDR activity in WPMY-1 cells, we sought to determine whether ectopic expression of Hic-5 would sensitize LNCaP cells to 1,25D3–induced growth inhibition. We therefore examined the effects of 1,25D3 treatment on proliferation of LNCaP and LNCaP/Hic-5 cells using the CyQuant nuclear dye assay. LNCaP and LNCaP/Hic-5 cells were treated with 0, 10, and 100 nmol/L 1,25D3 for 72 hours. As shown in Fig. 5B, growth of LNCaP cells was significant reduced in response to 100 nmol/L 1,25D3, but only minimally at 10 nmol/L. In contrast, LNCaP/Hic-5 cells experienced growth inhibition at a significantly enhanced magnitude in the presence of 10 nmol/L 1,25D3 than in LNCaP cells. Furthermore, treatment at 100 nmol/L also significantly inhibited growth in LNCaP/
Hic-5 cells to a greater extent than in LNCaP cells. Interestingly, Western blot analysis did not detect a change in VDR expression levels in LNCaP/Hic-5 cells (Fig. 5A). In addition, LNCaP/Hic-5 cells were also sensitized to the growth inhibitory effects of EB1089, a nonmetabolizable analogue of 1,25D3 (Fig. 5C). Therefore, the sensitization of LNCaP cells overexpressing Hic-5 to the antiproliferative effects of VDR are not because of alterations in VDR ligand metabolism or alterations of VDR protein expression. CYP24A1 expression is correlated with cellular resistance to 1,25D3 treatment (62). Given our observation that Hic-5 is required for efficient 1,25D3-induced transcription of CYP24A1 in WPMY-1 cells, we might expect ectopic Hic-5 expression in LNCaP cells to generate more potent induction of CYP24A1. However, the sensitization of LNCaP/Hic-5 cells to growth inhibitory effects of both 1,25D3 (Fig. 5B) and EB1089 (Fig. 5C), a nonmetabolizable analogue, suggested that the Hic-5 effect might be independent of CYP24A1 activity. One hundred nmol/L 1,25D3 had no effect on Hic-5 mRNA expression (Fig. 6A) but induced expression of CYP24A1 mRNA to the same extent in LNCaP and LNCaP/Hic-5 cells (Fig. 6B and Supplementary Figs. S5 and S6). Therefore, the sensitization of LNCaP/Hic-5 cells to the antiproliferative effect of 1,25D3 treatment is independent of CYP24A1 activity.

Compartment-specific effects of Hic-5 enhance growth-inhibitory effects of 1,25D3 treatment on LNCaP cells As shown above, ectopic Hic-5 expression sensitizes LNCaP cells to enhanced 1,25D3-induced growth inhibitory effects of both 1,25D3 and EB1089. Given our observation that Hic-5 is required for efficient 1,25D3-induced transcription of CYP24A1 in WPMY-1 cells, we might expect ectopic Hic-5 expression in LNCaP cells to generate more potent induction of CYP24A1. However, the sensitization of LNCaP/Hic-5 cells to growth inhibitory effects of both 1,25D3 (Fig. 5B) and EB1089 (Fig. 5C), a nonmetabolizable analogue, suggested that the Hic-5 effect might be independent of CYP24A1 activity. One hundred nmol/L of 1,25D3 had no effect on Hic-5 mRNA expression (Fig. 6A) but induced expression of CYP24A1 mRNA to the same extent in LNCaP and LNCaP/Hic-5 cells (Fig. 6B and Supplementary Figs. S5 and S6). Therefore, the sensitization of LNCaP/Hic-5 cells to the antiproliferative effect of 1,25D3 treatment is independent of CYP24A1 activity.

**Figure 4.** Hic-5 knockdown blocks VDR recruitment to a functional VDR response element located within the CYP24A1 promoter. A and B, Scr and shHic-5 cells were transfected with pCYP24-537-luc (A) or p(VDRE)-TATA (B) overnight, serum-starved for 2 hours, and treated with TGFβ1 (0 and 3.5 ng/mL) and/or 1,25D3 (0 and 100 nmol/L) for 6 hours. Cells were lysed in passive lysis buffer and freeze-fractured at -80°C overnight. Samples from lysates were analyzed for luciferase activity. Boxplots represent data from 2 independent experiments performed in triplicate. Data were analyzed using three-way ANOVA with mixed models and cell-means posttests. ***P < 0.001. C, transfections and treatments were performed as above with p392-luc, p451-luc, p470-luc, and p496-luc in Scr cells. Boxes represent 2 (p392-luc) or 3 (p451-luc, p470-luc, p496-luc) independent experiments performed in triplicate. Three-way ANOVA followed by cell-means posttests were performed. ***P < 0.001. D, VDR binding to DNA in Scr and shHic-5 cells treated with 100 nmol/L 1,25D3 was measured via ChIP using anti VDR C-20X or nonspecific IgG antibody. Data represent fraction input enrichment at a 120 bp region of chromatin between 370 and 491 bp proximal to the CYP24A1 transcriptional start site. Data represent the average of 3 independent ChIP experiments ± SEM with significance measured using two-way ANOVA with mixed models and pairwise cell-means posttests. ***P < 0.001.
inhibition by a mechanism independent of CYP24A1 activity. However, knockdown of Hic-5 in WPMY-1 cells reduced the transcriptional activity of VDR, thus reducing CYP24A1 expression and metabolism of 1,25D3. These findings suggest that reduced stromal Hic-5 expression in a 2-compartment model system would limit inactivating metabolic activity of CYP24A1, further enhancing growth-inhibitory effects of 1,25D3 on LNCaP/Hic-5 cells (Fig. 7A and B). An in vitro coculture experiment was therefore designed to test whether expression of Hic-5 in stromal and/or epithelial cells affected 1,25D3 inhibition of LNCaP and LNCaP/Hic-5 cell proliferation. As shown in Fig. 8A,
proliferation of LNCaP cells was not significantly affected by a 72-hour treatment of 100 nmol/L \(1,25\text{D}_3\) in coculture with Scr stromal cells. However, when cocultured with shHic-5 cells, the growth inhibitory effects of \(1,25\text{D}_3\) in LNCaP cells was enhanced compared with LNCaP cells cocultured with Scr cells (Fig. 8A). The growth-inhibitory effects of EB1089 in LNCaP cells were unaffected by the Hic-5 status of cocultured WPMY-1 cells (Fig. 8B), confirming that the sensitization to antiproliferative effects of \(1,25\text{D}_3\) conferred Hic-5-ablated WPMY-1 cells was likely because of reduced metabolism of \(1,25\text{D}_3\) (Fig. 3). In contrast, although LNCaP/Hic-5 cells were sensitive to the growth-inhibitory effects of \(1,25\text{D}_3\) in coculture with Scr and shHic-5 stromal cells, the inhibition was significantly amplified in coculture with shHic-5 cells (Fig. 8A). More importantly, after treatment there were fewer viable LNCaP/Hic-5 cells in coculture with shHic-5 stromal cells than were present at 0 hour.

The reduction in number of LNCaP/Hic-5 cells following treatment with \(1,25\text{D}_3\) in coculture with shHic-5 cells suggested a cytotoxic effect. To examine this possibility, we used Trypan blue to count viable LNCaP/Hic-5 cells upon coculture with Scr or shHic-5 cells and \(1,25\text{D}_3\) treatment. As shown in Fig. 8C, \(1,25\text{D}_3\) treatment reduced the proportion

**Figure 7.** Proposed model of Hic-5 impact on VDR action in the tumor microenvironment. \(1,25\text{D}_3\)-induced expression of CYP24A1 in Hic-5-expressing stromal cells (A) metabolizes \(1,25\text{D}_3\) to an inactive form, inhibiting its antiproliferative effects on the tumor. If Hic-5 is depleted in the stromal layer (B), CYP24A1 expression is inhibited, and more active \(1,25\text{D}_3\) is available to the tumor to induce growth inhibition.
of viable LNCaP/Hic-5 cells when cocultured with shHic-5 stroma, whereas treatment in coculture with Scr stroma did not produce a statistically significant decrease in viability. Therefore, the combination of reduced paracrine metabolism of 1,25D3 (i.e., because of limited induction of CYP24A1 in Hic-5–ablated stromal cells) and enhanced antiproliferative action of VDR (i.e., upon ectopic expression of Hic-5) can also limit the viability of 1,25D3-treated LNCaP cells.

Discussion

Previous studies examining VDR expression in prostate stromal cells had focused on comparing normal stroma-associated fibroblasts with cancer-associated fibroblasts (CAF). Those studies had concluded that VDR expression was comparable in CAFs and normal stroma-associated fibroblasts (64). However, examination of dendritic-cell differentiation to Langerhans cells demonstrated that treatment of myeloid cells with TGFβ induced VDR expression (65). This presented a dichotomy, as prostate cancer cells typically express high expression of TGFβ, which in turn induces stromal fibroblasts to undergo transdifferentiation to myofibroblasts, a characteristic of reactive stroma (30). We found that TGFβ treatment of the WPMY-1 stromal cell line induced VDR expression. An interesting consequence of TGFβ treatment on WPMY-1 cells is a permissive enhancement of 1,25D3-induced CYP24A1 transcription. TGFβ was previously found to induce CYP24A1 expression in resting-zone chondrocytes and osteoblast-like cell lines, but its effect on enhancement on 1,25D3-induced transcription was only observed upon preincubation of TGFβ (60, 61). An increase in CYP24A1 expression in the tumor microenvironment would increase metabolism of 1,25D3 and therefore decrease its availability to the tumor (66). Therefore, high tumor and stromal expression of TGFβ may negatively affect 1,25D3 therapy. The consequences of stromal VDR expression, especially under the influence of TGFβ, have not been previously examined in conjunction with clinical trials, and it may be a contributing factor influencing the outcomes of clinical trials, such as
androgen-independent prostate cancer Study of Calcitriol ENhancing Taxotere (ASCENT), which yielded mixed results in phase II and III trials (11).

Despite playing an active role in mediating transactivation of nuclear receptor targets, the coregulator Hic-5 has not yet been found to regulate nuclear receptor expression. Our study provides the first evidence of Hic-5 as a coregulator of VDR expression as well as its activity. Hic-5 knockdown reduced basal expression of VDR, but did not inhibit TGFβ-mediated induction. However, induction of JAG1, a Smad3-dependent target of TGFβ, was inhibited. Moreover, treatment of osteoblasts with 1,25D₃ actually decreased expression of Smad2 in osteoblasts (67). Thus, although Smad-dependent targets may require functional Hic-5, which has been previously shown to interact with Smad proteins, TGFβ-induced expression of VDR and Hic-5– influenced basal expression seem to be Smad independent (47, 48). Further studies may therefore be focused on potential Smad-independent TGFβ signaling pathways that may affect Hic-5 regulation of VDR expression.

In addition to affecting VDR expression, Hic-5 also affects 1,25D₃-induced transactivation of the CYP24A1 promoter, reducing CYP24A1 expression. This extends the range of Hic-5 targets within the nuclear receptor superfamily beyond AR, glucocorticoid receptor (GR), and progesterone receptor (PR; refs. 42, 45, and 68). In the WPMy-1 prostate stromal cell line, Hic-5 acts as an AR coactivator that influences expression of paracrine factors, such as KGF, which in turn affect the neighboring tumor (42). The consequence of decreased 1,25D₃-induced CYP24A1 expression upon Hic-5 knockdown is increased accumulation of unmethylated 1,25D₃ in the culture medium. If this mechanism were applied to a clinical condition, reduced stromal Hic-5 expression may enhance therapeutic benefit of 1,25D₃ for patients with prostate cancer by prolonging its bioavailability (69, 70). Although Hic-5 knockdown completely inhibited VDR transactivation of the proximal CYP24A1 promoter in transiently transfected cells, induction of endogenous CYP24A1 was significantly reduced, but not completely inhibited upon Hic-5 knockdown upon TGFβ cotreatment with 1,25D₃. Nonetheless, the reduction of CYP24A1 expression was functionally significant, as 1,25D₃ metabolism was dramatically reduced in shHic-5 cells.

Hic-5 does not interact directly with DNA, but it may bind to multiple transcription factors in complex, thus acting as a bridge between transcription factors binding at multiple sites throughout the promoter. In the U2OS human osteosarcoma cell line, Hic-5 depletion does not affect recruitment of the glucocorticoid receptor protein onto glucocorticoid-regulated genes whose hormone-regulated transcription is modulated by Hic-5 (59). Thus, Hic-5 may modulate nuclear receptor regulated transcription via enhanced recruitment of transcriptional coregulators (59) or the nuclear receptor itself, as we observed on the functional VDR binding site of the CYP24A1 promoter in prostate stromal cells. In silico analysis of the functional VDR binding site located −392 to −451 bp upstream of the CYP24A1 promoter using TESS did not reveal a traditional VDRE, but instead indicated 2 potential Hic-5 targets (51, 63). One proposed site at −445 to −452 bp showed high homology with the AP-2/Sp1 binding sequence. Sp1 knockdown is associated with reduction in 1,25D₃-induced CYP24A1 expression, and Hic-5 itself is a coactivator of Sp1 (47, 71). In addition, a putative Sp1 site has been previously reported (72). Another site identified at −420 to −424 bp shares high homology with the TCF/LEF consensus binding sequence. Hic-5 has previously been demonstrated to interact with TCF4, but in this case it functions to repress TCF4 transcriptional activation (49, 73). Thus, the cysrotic function of Hic-5 as a scaffolding protein may extend to its nuclear receptor cofactor function, bridging VDR binding within target genes to other transcription factors.

Although Hic-5 expression is mainly confined to the stromal compartment in the prostate, it is de-repressed upon short-term castration in mouse prostate epithelium and human prostate xenografts in mice (42, 49). Furthermore, as shown here, ectopic Hic-5 expression also enhances VDR activity in the LNCaP human prostate cancer cell line, leading to enhanced sensitivity to 1,25D₃-induced growth inhibition. This enhanced sensitivity may also be influenced by the lack of Hic-5 effects on CYP24A1 expression in LNCaP cells, highlighting the cell-specific effects of Hic-5 as a VDR coactivator. Genome-wide analysis comparing the VDR cistrome and transcriptome in prostate cancer and stromal cells with altered Hic-5 expression will enhance our understanding of the seemingly paradoxical cell-specific transcriptional coactivation of VDR targets by Hic-5.

The distinct consequences of Hic-5 coactivation of VDR in prostate cancer (reduced proliferation) versus stromal (increased 1,25D₃ metabolism) cells provide the context for examining cancer cell/stromal cell cocultures as an in vivo mimic of the tumor microenvironment. As we demonstrated above, the most potent antiproliferative effects of 1,25D₃ on LNCaP cells occurs when they ectopically express Hic-5 and are cocultured with stromal cells ablated of Hic-5. Treatment with EB1089, which cannot be metabolized by CYP24A1, restored sensitivity to LNCaP cells cocultured with CYP24A1-expressing Scr stromal cells. Therefore, we demonstrated that reduced proliferation is because of the reduced stroma-mediated CYP24A1 activity, reducing metabolism of 1,25D₃, coupled with enhanced VDR regulation of antiproliferation genes upon coactivation by ectopic Hic-5 expression. Regulation of Bax and Bcl-2, 2 target genes associated with apoptosis, may account for the enhanced 1,25-D₃– induced cytotoxic activity observed in LNCaP cells ectopically expressing Hic-5 and cocultured with stromal cells ablated of Hic-5 (16, 74).

In summary, our findings suggest that the search for mechanisms to sensitize prostate cancer cells to the antiproliferative effects of VDR ligands needs to account for the impact of VDR activity in the tumor microenvironment as well as in cancer cells. By acting as a coregulator with distinct effects on VDR transactivation in prostate cancer and stromal cells, Hic-5 could exert diverse effects on adjuvant therapy designed to exploit VDR activity in prostate cancer.
Patients with prostate cancer with low expression of Hic-5 in the tumor microenvironment and de-repressed expression of Hic-5 in the tumor might be predicted to receive maximal benefit from therapy with VDR ligands. Furthermore, if Hic-5 de-repression is restricted to a specific phase of therapy, such as during ADT, VDR ligand therapy may need to be initiated at distinct periods during the therapy (49).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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