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

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Support: J.D.S., M.D.H., T.T.L., and D.B.D. received support from NIH R01 DK078394. J.H.B. and R.A.P. received support from award NIH P30 CA047904. D.P.N. received support from NIH P30 CA047904. D.A.L. and G.B. received support from the Cancer Foundation of Australia (GB, PG2210) and Cancer Australia (GB, APP1032970).

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Running title: Differential effects of Hic-5 on VDR in prostate cells

Keywords: prostate cancer, vitamin D, calcitriol, Hic-5, TGF-b, microenvironment, fibroblasts, stroma

Disclosure: The authors have nothing to disclose.

Word count: 6497
8 figures, 3 supplementary tables, 6 supplementary figures
Abstract

Prostate cancer patients 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 anti-proliferative 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 CRPC patients has not been assessed. Transforming growth factor-β (TGF-β), which is associated with the development of tumorigenic "reactive stroma" in prostate cancer, induced VDR expression in the human WPMY-1 prostate stromal cell line. Similarly, TGF-β enhanced 1,25D3-induced up-regulation of CYP24A1, which metabolizes 1,25D3 and thereby limits VDR activity. Ablation of Hic-5, a TGF-β-inducible nuclear receptor co-regulator, inhibited basal VDR expression, 1,25D3-induced CYP24A1 expression and metabolism of 1,25D3 and TGF-β-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 co-culture with Hic-5-ablated WPMY-1 cells. Therefore, these findings indicate that the search for mechanisms to sensitize prostate cancer cells to the anti-proliferative effects of VDR ligands needs to account for the impact of VDR activity in the tumor microenvironment.

Implications: Hic-5 acts as a co-regulator 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.
Introduction

Prostate cancer is one of the leading causes of cancer-related death among men in the US. 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 US 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 (SNPs) 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 prostate cancer patients 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 anti-proliferative and pro-apoptotic 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 (IL-8) and nuclear factor-κB (NF-κB), and confer quiescence (20-22). One molecular mechanism that could contribute to the anti-tumor effects of 1,25D₃ was revealed in a study showing an inhibition of TGF-β-induced epithelial-to-mesenchymal transition (EMT) and fibrosis mediated by an interaction between ligand-bound VDR and the TGF-β-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 TGF-β 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 overhealing 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 TGF-β 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 TGF-β (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 co-regulator (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 (keratinocyte growth factor), 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 co-repressor (NCoR) complex (45, 46). In addition to binding to nuclear receptors, Hic-5 also binds to other transcription factors, feeding back onto TGF-β 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 co-activator 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 anti-proliferative 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 TGF-β1 was purchased from R&D Systems (Minneapolis, MN) and reconstituted in 4.0 mM HCl, according to the manufacturer’s protocol. 1,25D₃ was purchased from Cayman Chemical (Ann Arbor, MI) and reconstituted to 20 μM in cell culture-grade DMSO. Mock treatments with 1,25D₃ used equivalent volume of DMSO vehicle. EB1089 was purchased from Tocris Bioscience (Minneapolis, MN). A mouse monoclonal anti-Hic-5 antibody (clone 34/Hic-5) was purchased from BD Biosciences (San Jose, CA). The mouse monoclonal anti-VDR antibody (clone D-6), rabbit polyclonal anti-VDR antibody (clone C-20X), and rabbit polyclonal anti-α-tubulin antibody (clone H-300) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary HRP-conjugated antibodies were purchased from Sigma (St. Louis, MO).

Cell culture

WPMY-1 cells were purchased from American Type Culture Collection (Rockville, MD). 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, Los Angeles, CA) (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 pHR 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 x 10^5 per well for 24 hrs before infection. Lentivirus infection media containing polybrene (8 μg/ml) was used to infect the cells for 24 hrs. The next day, the media was changed, and the cells were cultured for an additional 48 hrs, 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, transferred 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 x 10^5 cells per well in a 6-well plate and cultured overnight. The cells were then cultured in serum-free medium for 24 hrs. The following day, they were treated with TGF-β1 (0, 2.0 ng/mL) and incubated at 37°C for 10 hrs. The medium was then removed, and cells were lysed in 500 µL cold TRIzol (Life Technologies, Grand Island, NY). RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA). 300 ng of total RNA extracted from each of 5 replicate treatments were analyzed using Affymetrix Human Gene 1.0 ST Arrays (Santa Clara, CA). Bioinformatics were performed in R Statistical Software using Bioconductor and the Limma package. Briefly, array data was 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 data sets were deposited in GEO (Accession # GSE47354).

**Western blot**

5.0 x 10^5 Scr or shHic-5 cells were plated on 65-mm plates and grown overnight. The following day, they were cultured in serum-free medium for ~2 hrs, then treated with TGF-β1 (0, 10 ng/mL) and incubated at 37°C for 24 hrs. At the conclusion of the treatment, whole-cell lysates
were obtained by resuspending cells in RIPA buffer (140 mM NaCl, 0.1% sodium deoxycholate, 10 mM Tris buffered to pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton-X, 0.1% SDS, 1 mM NaF, 1 mM PMSF, 100 μM sodium orthovanadate, protease inhibitor cocktail (Sigma)), and protein concentration was quantified by the Lowry assay. 15-20 μg of total protein were electrophoresed in a 10% acrylamide gel and transferred to a PVDF membrane (Millipore, Billerica, MA) using a Transblot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA). 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 (RT-qPCR)

Scr and shHic-5 cells were plated in 6-well plates at a density of 1.75 x 10^5 cells per well and grown overnight. The next day, they were cultured in serum-free medium for ~2 hr. TGF-β1 (0, 3.5 ng/mL) and 1,25D_3 (0, 100 nM) were added to fresh serum-free medium to add to the cells. The cells were incubated at 37˚C for 6 hrs. RNA extraction was performed as described, with the resulting RNA quantified using the NanoDrop ND-1000 spectrophotometer (Wilmington, DE). Complementary DNA (cDNA) was generated using the High Capacity RNA-to-cDNA kit (Applied Biosciences, Carlsbad, CA) according to the kit protocol. The resulting samples were then diluted to 100 μL with nuclease-free water. RT-qPCR was then performed on the samples with the iTaq Sybr Green Supermix with ROX (Bio-Rad) on the Stratagene Mx3000P thermocycler (Cedar Creek, TX) 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 $3.0 \times 10^5$ cells per well and cultured overnight. The next day, the cells were treated with 1,25D$_3$ (0, 100 nM) for 6 hrs. 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 $5.0 \times 10^5$ cells per 65-mm dish and were grown overnight. The next day, the cells were cultured in serum-free medium for ~2 hrs. Cells were treated in duplicate with 1,25D$_3$ (0, 100 nM) for 0 or 24 hrs at 37°C. Reference treatments in cell-free dishes were included to account for spontaneous degradation of 1,25D$_3$. Cells were scraped into medium at each time-point, flash-frozen in liquid nitrogen, and stored at -80°C prior to analysis by 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) (51). Scr and shHic-5 cells were plated at a density of $7.5 \times 10^4$ 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, Indianapolis, IN) were incubated in OPTIMEM (100 μL/well) for 1 hr. 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 hrs. They were then
treated in triplicate with TGF-β1 (0, 3.5 ng/mL) and 1,25D₃ (0, 100 nM) and incubated for 6 hr at 37°C. Cells were lysed and freeze-fractured overnight in the passive lysis buffer contained in the Dual-Luciferase Reporter Assay system (Promega, Madison, WI). 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 tranfections were performed with the plasmid p(VDRE)₄-TATA-luc, obtained from the laboratory of Nancy Weigel (Baylor College of Medicine) (52). Scr and shHic-5 cells were plated at a density of 3.5 x 10⁵ 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 30-60 minutes. Cells were then transfected with 100 μL of the mixture and incubated overnight prior to lysate preparation and luciferase assay.

**Chromatin Immunoprecipitation (ChIP) Assay**

Scr and shHic-5 cells were plated at 0.5 x 10⁶ cells and 2 days after plating were treated for 4 hr with 1,25D₃ (0, 100 nM) in serum-free media. Experiment was performed as described previously (53). Lysates were briefly sonicated in 4 x 30-second bursts on high (Diagenode Inc, Denville, NJ). Samples were immunoprecipitated using 4 μg of either anti-VDR C-20 antibody or non-specific 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 represents the average of three independent 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/rsg/), and a search for putative transcription factors was performed using the Transcription Element Search System (TESS) (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 x 10³ cells per well in a 96-well plate for at least 18 hr. The cells were carefully treated in triplicate with 1,25D₃ (0, 10, 100 nM) or EB1089 (0, 10, 100 nM) in RPMI 1640 containing 10% FBS for 0 and 72 hr 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, Sunnyvale, CA). Data at 72 hr was normalized to baseline at 0 hr.

**Co-culture proliferation assay**

25-mm circular coverslips were made suitable for co-culture 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 hr. The coverslips were then placed in 6-well dishes and incubated in poly-D-lysine (10 µg/mL) for either 2 hrs 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 x 10^5 cells per well and grown overnight. Simultaneously, Scr and shHic-5 cells were plated at 2.0 x 10^5 per well in two 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 two untreated coverslips from each epithelial line were washed in PBS and fixed to the coverslips immediately in 4% paraformaldehyde (PFA) at 0 hr to establish a baseline. The co-cultures were then treated with 1,25D_3 (0, 100 nM) or EB1089 (0, 1.0 nM) in RPMI 1640 medium containing 10% FBS for 72 hr. 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, Burlingame, CA). The slides were visualized and photographed under epifluorescence at 200X using the Olympus IX-81 microscope (Center Valley, PA). Counts were averaged from 6 fields per coverslip and normalized to the 0-hr time-point for each respective epithelial line.

**Co-culture viability assay**

18 mm x 18 mm coverslips were prepared as above. LNCaP/Hic-5, Scr, and shHic-5 cells were plated and grown as above. The co-cultures were treated with 1,25D_3 (0, 10 nM) in RPMI-1640 medium supplemented with 10% FBS and incubated at 37°C for 72 hr. At the end of the incubation, the coverslips were moved to another 6-well plate and trypsinized in 1.0 mL trypsin for ~1 hr at 37°C. The supernatants from the co-cultures were collected in 2.0-mL microcentrifuge tubes and centrifuged at 1.0 x 10^3 X g for 10 min. The trypsinized LNCaP/Hic-5 cells were added to their respective supernatant pellets and centrifuged again at 1.0 x 10^3 g and 4°C for 10 min. 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, Grand Island, NY). 10 µL of the sample were 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-way or three-way mixed-models ANOVA with Satterthwaite approximation, followed by cell-means post-hoc test in SAS (55). Interval data proportion data, Western blot data that contained results below detectable levels were left untransformed, while 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 TGF-β and Hic-5 in WPMY-1 prostate stromal cells

Hic-5 functions as a co-activator of androgen receptor in WPMY-1 prostate stromal myofibroblast cells and is an established component of the TGF-β signaling pathway. (42, 47, 48, 57, 58) To evaluate the impact of Hic-5 on the TGF-β 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 TGF-β was assessed by gene expression microarray 10 hours after treatment with 2.0 ng/mL TGF-β1 (Supplementary Figure S1). Of particular relevance here, VDR was identified in the microarray as a TGF-β1 target gene whose basal expression was reduced upon Hic-5 knockdown.

In order to validate the microarray data, TGF-β1 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. Likewise, basal expression of VDR mRNA (Figure 1B, Supplementary Figure S2) and protein (Figures 1C, D) was reduced upon Hic-5 ablation. However, TGF-β1 induction of VDR mRNA (Figure 1A) and protein (Figures 1C, 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 TGF-β1-regulated genes (Supplementary Figure S1A). For example, JAG1 is a TGF-β target gene whose induction requires Hic-5 as revealed by microarray data and validated by RT-qPCR (Supplementary Figure S1C). The fact that Hic-5 ablation can lead to enhancement or repression of TGF-β1 responsiveness is consistent with previous results that established Hic-5's
impact on the activity of both transcriptional co-activators and co-repressors (45, 59). Finally, TGF-β1 induction of Hic-5 protein (Figure 1C) may trigger a feed-forward pathway that further modulates TGF-β1 responsiveness.

**Hic-5 regulates 1,25D₃- and TGF-β₁-induced expression of CYP24A1 in prostate stromal cells**

In osteoblasts and resting-zone chondrocytes, TGF-β 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 1,24,25D₃, 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. In order to discern the effect of TGF-β on *CYP24A1* gene expression, Scr cells were co-treated with 3.5 ng/mL TGF-β₁ and 100 nM 1,25D₃ and mRNA expression analyzed by RT-qPCR. Whereas treating with TGF-β₁ alone minimally induced expression of *CYP24A1*, co-treatment with 1,25D₃ enhanced *CYP24A1* expression 300-fold above baseline and 10-fold above treatment with 1,25D₃ alone (Figure 2B, Supplementary Figure S4B). One source of this enhanced gene expression may be the increased induction of VDR itself upon TGF-β₁ treatment.

In shHic-5 cells, which have reduced Hic-5 expression that was not influenced by TGF-β₁ or 1,25D₃ (Figure 2A, Supplementary Figure S3A), similar dynamics were revealed with minimal induction of *CYP24A1* by TGF-β₁, greater induction with 1,25D₃, and enhanced expression upon co-treatment of TGF-β₁ and 1,25D₃ (Figure 2B, Supplementary Figure 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 TGF-β₁. Together, these results indicate that Hic-5 is required for maximal induction of *CYP24A1*
by 1,25D₃ alone or in combination with TGF-β1. Although CYP24A1 retains some capacity to respond to TGF-β1 and 1,25D₃ in Hic-5-ablated WPMY-1 cells, its reduced response has metabolic and biological consequences, as will be highlighted below.

Reduced CYP24A1 mRNA expression upon Hic-5 knockdown may limit the auto-inhibitory effects of 1,25D₃ on its own accumulation. Liquid chromatography-tandem mass spectrometry (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% (Figure 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 appears 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 bp and 5 bp upstream of the transcription start site (TSS) included two known Vitamin D response elements (VDREs) located 293 bp 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 TGF-β1. As expected, 1,25D₃ induced CYP24A1 promoter-driven luciferase reporter activity in Scr cells, while treatment with TGF-β1 alone did not induce activity (Figure 4A). Co-treatment of 1,25D₃ and TGF-β1 in Scr cells enhanced luciferase expression above reporter activity when treated with 1,25D₃ treatment alone, revealing a
permissive effect when TGF-β1 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 co-treatment with TGF-β1 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 Figure 4B, knockdown of Hic-5 inhibited 1,25D₃ induction from the VDRE-TATA promoter, although co-treatment with 1,25D₃ and TGF-β1 generated minimal induction. The mechanistic basis for the TGF-β1 effect is not clear but our data clearly demonstrate the Hic-5 dependence of two 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 bp 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 co-treatment of TGF-β and 1,25D₃, supporting results obtained from the pCYP24-537-luc construct (Figure 4C). However, treatment of 1,25D₃ alone or together with TGF-β failed to induce minimal luciferase expression above baseline from p392-luc above baseline. The ability to both induce luciferase upon 1,25D₃ treatment above minimal levels and enhance its expression upon TGF-β co-treatment 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/TGF-β1 co-treatment). Therefore, a Hic-5-responsive sequence was identified at 392-451 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 (Figure 4D).
Overexpression of Hic-5 sensitizes epithelial LNCaP cells to the anti-proliferative effects of 1,25D_3

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 prostate cancer patients. 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 1,25D_3-induced growth inhibition. We therefore examined the effects of 1,25D_3 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 nM 1,25D_3 for 72 hours. As shown in Figure 5B, growth of LNCaP cells was significant reduced in response to 100 nM 1,25D_3, but only minimally at 10 nM. In contrast, LNCaP/Hic-5 cells experienced growth inhibition at a significantly enhanced magnitude in the presence of 10 nM 1,25D_3 than in LNCaP cells. Furthermore, treatment at 100 nM 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 (Figure 5A). Additionally, LNCaP/Hic-5 cells were also sensitized to the growth inhibitory effects of EB1089, a non-metabolizable analogue of 1,25D_3 (Figure 5C). Therefore the sensitization of LNCaP cells overexpressing Hic-5 to the anti-proliferative effects of VDR are not due to alterations in VDR ligand metabolism or alterations of VDR protein expression.

CYP24A1 expression is correlated with cellular resistance to 1,25D_3 treatment (62). Given our observation that Hic-5 is required for efficient 1,25D_3-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 (Figure 5B) and EB1089 (Figure 5C), a non-metabolizable analogue, suggested that the Hic-5 effect might be independent of CYP24A1 activity. 100 nM 1,25D₃ had no effect on Hic-5 mRNA expression (Figure 6A) but induced expression of CYP24A1 mRNA to the same extent in LNCaP and LNCaP/Hic-5 cells (Figure 6B, Supplementary Figures S5-S6). Therefore, the sensitization of LNCaP/Hic-5 cells to the anti-proliferative 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 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 two-compartment model system would limit inactivating metabolic activity of CYP24A1, further enhancing growth-inhibitory effects of 1,25D₃ on LNCaP/Hic-5 cells (Figure 7A, B). An in vitro co-culture 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 Figure 8A, proliferation of LNCaP cells was not significantly affected by a 72-hr treatment of 100 nM 1,25D₃ in co-culture with Scr stromal cells. However, when co-cultured with shHic-5 cells, the growth inhibitory effects of 1,25D₃ in LNCaP cells was enhanced compared to LNCaP cells co-cultured with Scr cells (Figure 8A). The growth-inhibitory effects of EB1089 in LNCaP cells were unaffected by the Hic-5 status of co-cultured WPMY-1 cells (Figure 8B), confirming that the sensitization to anti-proliferative effects of 1,25D₃ conferred Hic-5-ablated
WPMY-1 cells was likely due to reduced metabolism of 1,25D₃ (Figure 3). In contrast, while LNCaP/Hic-5 cells were sensitive to the growth-inhibitory effects of 1,25D₃ in co-culture with Scr and shHic-5 stromal cells, the inhibition was significantly amplified in co-culture with shHic-5 cells (Figure 8A). More importantly, after treatment there were fewer viable LNCaP/Hic-5 cells in co-culture with shHic-5 stromal cells than were present at 0 hr.

The reduction in number of LNCaP/Hic-5 cells following treatment with 1,25D₃ in co-culture with shHic-5 cells suggested a cytotoxic effect. In order to examine this possibility, we used Trypan blue to count viable LNCaP/Hic-5 cells upon co-culture with Scr or shHic-5 cells and 1,25D₃ treatment. As shown in Figure 8C, 1,25D₃ treatment reduced the proportion of viable LNCaP/Hic-5 cells when co-cultured with shHic-5 stroma, whereas treatment in co-culture with Scr stroma did not produce a statistically significant decrease in viability. Therefore the combination of reduced paracrine metabolism of 1,25D₃ (i.e. due to limited induction of CYP24A1 in Hic-5 ablated stromal cells) and enhanced anti-proliferative 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 (CAFs). 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 TGF-β induced VDR expression (65). This presented a dichotomy, as prostate cancer cells typically express high expression of TGF-β, which in turn induces stromal fibroblasts to undergo transdifferentiation to myofibroblasts, a characteristic of reactive stroma (30). We found that TGF-β treatment of the WPMY-1 stromal cell line induced VDR expression. An interesting consequence of TGF-β treatment on WPMY-1 cells is a permissive enhancement of 1,25D$_3$-induced CYP24A1 transcription. TGF-β was previously found to induce CYP24A1 expression in resting-zone chondrocytes and osteoblast-like cell lines, but its effect on enhancement on 1,25D$_3$-induced transcription was only observed upon pre-incubation of TGF-β (60, 61). An increase in CYP24A1 expression in the tumor microenvironment would increase metabolism of 1,25D$_3$ and therefore decrease its availability to the tumor (66). Therefore, high tumor and stromal expression of TGF-β may negatively impact 1,25D$_3$ therapy. The consequences of stromal VDR expression, especially under the influence of TGF-β, 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 ASCENT (Androgen-independent prostate cancer Study of Calcitriol ENhancing Taxotere), which yielded mixed results in Phase II and III trials.(11)

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

In addition to affecting VDR expression, Hic-5 also impacts 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) (42, 45, 68). In the WPMY-1 prostate stromal cell line, Hic-5 acts as an AR co-activator 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 unmetabolized 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 TGF-β co-treatment 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 two 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 1,25D$_3$-induced CYP24A1 expression, and Hic-5 itself is a co-activator of Sp1 (47, 71). Additionally, 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 cytosolic function of Hic-5 as a scaffolding protein may extend to its nuclear receptor co-factor 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 1,25D$_3$-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 co-activator. 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 co-activation of VDR targets by Hic-5.

The distinct consequences of Hic-5 co-activation of VDR in prostate cancer (reduced proliferation) versus stromal (increased 1,25D$_3$ metabolism) cells provide the context for
examining cancer cell/stromal cell co-cultures as an *in vitro* mimic of the tumor microenvironment. As we demonstrated above, the most potent anti-proliferative effects of 1,25D$_3$ on LNCaP cells occurs when they ectopically express Hic-5 and are co-cultured with stromal cells ablated of Hic-5. Treatment with EB1089, which cannot be metabolized by CYP24A1, restored sensitivity to LNCaP cells co-cultured with CYP24A1-expressing Scr stromal cells. Therefore, we demonstrated that reduced proliferation is due to the reduced stroma-mediated CYP24A1 activity, reducing metabolism of 1,25D$_3$, coupled with enhanced VDR regulation of anti-proliferation genes upon co-activation by ectopic Hic-5 expression. Regulation of Bax and Bcl-2, two target genes associated with apoptosis, may account for the enhanced 1,25-D$_3$-induced cytotoxic activity observed in LNCaP cells ectopically expressing Hic-5 and co-cultured 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 anti-proliferative 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 co-regulator 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. Prostate cancer patients 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).
Acknowledgements

We would like to thank Pamela Hershberger, Steffi Oesterreich, David Callen, Nancy Weigel, and Neil Bhowmick for materials important to the preparation of this paper. This project used the UPCI Biostatistics, Chemical Biology and Viral Vector cores that were supported in part by awards P30 CA047904 and P30 CA047904. This work was also funded by the Prostate Cancer Foundation of Australia (GB, PG2210) and Cancer Australia (GB, APP1032970). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health.

Conflict of Interest

The authors declare no conflicts of interest.

References


Figure legends

Figure 1: Hic-5 knockdown reduces basal and TGF-β-induced VDR mRNA and protein expression. A, B. Scr and shHic-5 cells were serum-starved for 2 hr, then treated for 6 hr with 0 or 3.5 ng/mL TGF-β1. mRNA was extracted for cDNA synthesis and RT-qPCR probing for Hic-5 (A), VDR (B), and GAPDH expression. Comparisons were made using the comparative Ct (ddCt) method. TGF-β1 induced VDR transcription in both Scr and shHic-5 cells. Data were analyzed with two-way ANOVA with mixed models, followed by cell-means post-test. Bars represent mean±SEM of six independent experiments normalized to basal expression in Scr cells. * p < 0.05, ** p < 0.01. C. Scr and shHic-5 cells were serum-starved for 2 hr, then treated for 24 hr with 0 or 10 ng/mL TGF-β1. Blot is representative of three independent experiments. D. Western blot results were analyzed by densitometry in ImageJ. TGF-β1 induced VDR expression in both Scr and shHic-5 cells. Basal expression of VDR in shHic-5 was below detectable levels, so the data was not transformed. A two-way ANOVA with mixed models followed by cell-means post-test were performed. Bars represent mean±SEM of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 2: Hic-5 knockdown in WPMY-1 cells reduced TGF-β-mediated enhancement of 1,25D₃-induced CYP24A1 expression. Scr and shHic-5 cells were serum-starved for 2 hr prior to a 6-hr treatment with TGF-β1 (0, 3.5 ng/mL) and 1,25D₃ (0, 100 nM). mRNA was extracted for cDNA synthesis and RT-qPCR analysis for expression of Hic-5 (A), CYP24A1 (B), and GAPDH. Comparisons were made using the ddCt method. Results were analyzed with three-way ANOVA with mixed models and cell-means post-test. Bars represent mean±SEM of three independent experiments normalized to the basal condition in Scr cells. * p < 0.05, *** p < 0.001.

Figure 3: Hic-5 knockdown reduced CYP24A1 activity in shHic-5 cells. Cells were serum-starved for 2 hr prior to treatment with 100 nM 1,25D₃ and were harvested at 0 and 24 hr. Two cell-free control plates were set up to control for spontaneous degradation. Cells were scraped into their respective medium and flash-frozen in liquid nitrogen prior to
mass spectrometry analysis. Boxplots represent data from three independent experiments. Data was analyzed by two-way ANOVA with mixed models followed by cell-means post-tests. * p < 0.05, *** p < 0.001.

**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)₄-TATA (B) overnight, serum-starved for 2 hr, and treated with TGF-b1 (0, 3.5 ng/mL) and/or 1,25D₃ (0, 100 nM) for 6 hr. Cells were lysed in Passive Lysis Buffer and freeze-fractured at -20°C overnight. Samples from lysates were analyzed for luciferase activity. Boxplots represent data from two independent experiments performed in triplicate. Data was analyzed using three-way ANOVA with mixed models and cell-means post-tests. * p < 0.05, *** 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 two (p392-luc) or three (p451-luc, p470-luc, p496-luc) independent experiments performed in triplicate. Three-way ANOVA followed by cell-means post-tests were performed, *** p<0.001. D. VDR binding to DNA in Scr and shHic-5 cells treated with 100 nM 1,25D₃ was measured via ChIP using anti VDR C-20X or nonspecific IgG antibody. Data represents fraction input enrichment at a 120 bp region of chromatin between 370 bp and 491 bp proximal to the CYP24A1 transcriptional start site. Data represents the average of three independent ChIP experiments + SEM with significance measured using two-way ANOVA with mixed models and pairwise cell-means post-tests. *** p < 0.001.

**Figure 5: Overexpression of Hic-5 sensitized LNCaP cells to growth inhibition upon treatment of 1,25D₃ or EB1089 independently of VDR expression.** A. LNCaP and LNCaP/Hic-5 cells were plated overnight prior to lysis in RIPA buffer. Blot is representative of three independent experiments. Western blot results were analyzed by densitometry in ImageJ. Bars represent mean±SEM normalized to α-tubulin from the three independent experiments. B and C. LNCaP and LNCap/Hic-5 cells were treated in triplicate in 96-well plates with 0, 10, and 100 nM 1,25D₃ (B) or EB1089 (C) at 0 and 72 hr. Upon removal of medium, the plates were frozen overnight at -80°C. After thawing, cells were
lysed and stained in CyQuant assay buffer and read on a fluorimeter. Boxes represent results of five independent experiments performed in triplicate. Two-way mixed-models ANOVA followed by cell-means post-tests were performed on the log-transformed data. *p < 0.05, ***p < 0.001.

**Figure 6: Overexpression of Hic-5 did not increase expression of CYP24A1 in LNCaP cells.** LNCaP and LNCaP/Hic-5 cells were treated with 1,25D₃ (0, 100 nM) for 6 hr. mRNA was extracted for cDNA synthesis and RT-qPCR to measure Hic-5 (A) and CYP24A1 (B) mRNA levels. Comparisons were made using the ddCt method. Data were analyzed with two-way ANOVA with mixed models and cell-means post-test. Bars represent mean±SEM from four independent experiments normalized to basal expression in WT LNCaP cells. NS = not significant, ***p < 0.001.

**Figure 7: Proposed model of Hic-5 impact on VDR action in the tumor microenvironment.** 1,25D₃-induced expression of CYP24A1 in Hic-5-expressing stromal cells (A) metabolizes 1,25D₃ to an inactive form, inhibiting its anti-proliferative effects on the tumor. If Hic-5 is depleted in the stromal layer (B), CYP24A1 expression is inhibited, and more active 1,25D₃ is available to the tumor to induce growth inhibition.

**Figure 8: Knockdown of Hic-5 in WPMY-1 cells sensitizes LNCaP and LNCaP/Hic-5 cells to 1,25D₃-induced inhibition in co-cultures.** A. LNCaP and LNCaP/Hic-5 cells were cultured on coverslips separately from Scr (Scr) and shHic-5 (SH) cells, then co-cultured in the absence (-) or presence (+) of 1,25D₃ (100 nM) for 72 hr, fixed to the coverslip, and stained with DAPI. Cells were counted at six random fields per coverslip, averaged, and compared against cell numbers obtained at 0 hr. Boxplots represent data from five independent experiments. A three-way ANOVA with mixed models followed by cell-means post-tests were performed on the log-transformed data. NS = not significant, *p < 0.05, **p < 0.01, ***p < 0.001. B. Proliferation of LNCaP cells was assessed as described above upon co-culture with Scr and shHic-5 and EB1089 treatment. The above experiment was repeated with 1 nM EB1089 using only LNCaP cells. C. Viability of LNCaP/Hic-5 cells was assessed upon co-culture with Scr and shHic-5 and 1,25D₃ treatment. Cell pellets from trypsinized coverslips were combined with floating cells in the medium, resuspended, and
stained with Trypan blue to assess viability. Boxplots represent four independent experiments. A two-way ANOVA with mixed models followed by cell-means post-test were performed. * < 0.05, ** < 0.01.
VDR Activity is Differentially Affected by Hic-5 in Prostate Cancer and Stromal Cells

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Mol Cancer Res Published OnlineFirst May 13, 2014.

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Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-13-0395

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