Calcipotriol Targets LRP6 to Inhibit Wnt Signaling in Pancreatic Cancer

Michael D. Arensman¹, Phillip Nguyen¹, Kathleen M. Kershaw¹, Anna R. Lay¹, Claire A. Ostertag-Hill², Mara H. Sherman², Michael Downes², Christopher Liddle³, Ronald M. Evans²,⁴, and David W. Dawson¹,⁵

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy in need of more effective treatment approaches. One potential therapeutic target is Wnt/β-catenin signaling, which plays important roles in PDAC tumor initiation and progression. Among Wnt inhibitors with suitable in vivo biologic activity is vitamin D, which is known to antagonize Wnt/β-catenin signaling in colorectal cancer and have antitumor activity in PDAC. For this study, the relationship between vitamin D signaling, Wnt/β-catenin activity, and tumor cell growth in PDAC was investigated through the use of calcipotriol, a potent non-hypercalcemic vitamin D analogue. PDAC tumor cell growth inhibition by calcipotriol was positively correlated with vitamin D receptor expression and Wnt/β-catenin activity. Furthermore, vitamin D and Wnt signaling activity were found to be reciprocally linked through feedback regulation. Calcipotriol inhibited autocrine Wnt/β-catenin signaling in PDAC cell lines in parallel with decreased protein levels of the low-density lipoprotein receptor-related protein 6 (LRP6), a requisite coreceptor for ligand-dependent canonical Wnt signaling. Decrease in LRP6 protein seen with calcipotriol was mediated through a novel mechanism involving transcriptional upregulation of low-density lipoprotein receptor adaptor protein 1 (LDLRAP1). Finally, changes in LRP6 or LDLRAP1 expression directly altered Wnt reporter activity, supporting their roles as regulators of ligand-dependent Wnt/β-catenin signaling.

Implications: This study provides a novel biochemical target through which vitamin D signaling exerts inhibitory effects on Wnt/β-catenin signaling, as well as potential biomarkers for predicting and following tumor response to vitamin D–based therapy. Mol Cancer Res; 13(11); 1509–19. ©2015 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly recalcitrant malignancy with an overall 5-year survival rate of 6% (1). Its poor prognosis is linked to its typically advanced clinical stage at diagnosis, rapid disease progression, and resistance to cytotoxic or targeted molecular therapies (2). Improved therapeutic approaches based on a detailed understanding of the molecular and cellular basis of PDAC are needed. One therapeutic target now under consideration is Wnt/β-catenin signaling, one of 12 core pathways which is known to antagonize Wnt/β-catenin signaling in colorectal cancer and have antitumor activity in PDAC. For this study, the relationship between vitamin D signaling, Wnt/β-catenin activity, and tumor cell growth in PDAC was investigated through the use of calcipotriol, a potent non-hypercalcemic vitamin D analogue. PDAC tumor cell growth inhibition by calcipotriol was positively correlated with vitamin D receptor expression and Wnt/β-catenin activity. Furthermore, vitamin D and Wnt signaling activity were found to be reciprocally linked through feedback regulation. Calcipotriol inhibited autocrine Wnt/β-catenin signaling in PDAC cell lines in parallel with decreased protein levels of the low-density lipoprotein receptor-related protein 6 (LRP6), a requisite coreceptor for ligand-dependent canonical Wnt signaling. Decrease in LRP6 protein seen with calcipotriol was mediated through a novel mechanism involving transcriptional upregulation of low-density lipoprotein receptor adaptor protein 1 (LDLRAP1). Finally, changes in LRP6 or LDLRAP1 expression directly altered Wnt reporter activity, supporting their roles as regulators of ligand-dependent Wnt/β-catenin signaling.

Implications: This study provides a novel biochemical target through which vitamin D signaling exerts inhibitory effects on Wnt/β-catenin signaling, as well as potential biomarkers for predicting and following tumor response to vitamin D–based therapy. Mol Cancer Res; 13(11); 1509–19. ©2015 AACR.

¹Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, Los Angeles, California. ²Gene Expression Laboratory, Salk Institute, La Jolla, California. ³The Storr Liver Unit, Westmead Millennium Institute and University of Sydney, Westmead Hospital, Westmead, New South Wales, Australia. ⁴Howard Hughes Medical Institute, Salk Institute, La Jolla, California. ⁵Jonsson Comprehensive Cancer Center, David Geffen School of Medicine at UCLA, Los Angeles, California.

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

Corresponding Author: David W. Dawson, David Geffen School of Medicine at UCLA, Mail Code 17291E, 10833 Le Conte Avenue, Los Angeles, CA 90095-1732. Phone: 310-267-2799; Fax: 310-267-2058; E-mail: ddawson@mednet.ucla.edu
doi: 10.1158/1541-7786.MCR-15-0204
©2015 American Association for Cancer Research.
Vitamin D is acquired through dietary intake and ultraviolet light–mediated metabolism of its precursors in the skin. Vitamin D metabolites bind to VDR, a transcription factor that functions as an obligate heterodimer with the retinoid X receptor (RXR) to regulate the expression of target genes with vitamin D response elements. In addition to regulating calcium homeostasis, vitamin D exhibits anticancer action through a variety of antiproliferative, proapoptotic, and differentiation effects. Accordingly, vitamin D and its analogues are promising chemopreventative and chemotherapeutic agents for numerous malignancies. In relation to PDAC, some epidemiologic studies show higher intake or increased plasma levels of vitamin D correlate with decreased incidence of PDAC (12, 13). Although a published meta-analysis of vitamin D levels and PDAC risk (14), a high prevalence of vitamin D insufficiency has been observed in patients with advanced pancreatic cancer (15). Published experimental studies show that vitamin D and its synthetic analogues inhibit the in vitro and in vivo growth of some but not all PDAC cell lines through proapoptotic and antiproliferative actions (16–18). A recent study by Sherman and colleagues finds that VDR also functions as a master transcriptional regulator of pancreatic stellate cell quiescence and that the vitamin D analogue calcipotriol can suppress pancreatitis and mediate stromal remodeling to improve chemotherapeutic drug delivery and survival in a Kras-driven mouse model of PDAC (19).

Here, we examine the potential interplay between Wnt/β-catenin and vitamin D signaling in PDAC. We show that the ability of calcipotriol to inhibit PDAC cell growth strongly correlates with expression of VDR, which is correlated with and regulated by Wnt signaling. We demonstrate that calcipotriol induces expression of low-density lipoprotein receptor adaptor protein 1 (LDLRAP1), which in turn mediates rapid reduction in LRP6 protein levels and corresponding inhibition of Wnt signaling, providing an additional novel mechanism by which vitamin D can inhibit Wnt signaling activity. Our findings suggest that, in addition to potential use as stromal therapy, vitamin D analogues are suitable agents for directly targeting tumor cells in the subset of pancreatic cancers and precursor lesions defined by Wnt-dependent growth activity and higher VDR expression.

Materials and Methods

Cell lines and reagents

All cell lines were cultured as previously described (8). AsPC-1, HPAF-2, MiaPaCa-2, and PANC-1 were purchased from the American Type Culture Collection in 2005. YAPC, Suit, and HCG25 were kindly provided by Dr. Eric Collisson (University of California, San Francisco) in 2012. Cell lines have not been further authenticated since initial receipt. Calcipotriol, BaLomy (sc-5546; Santa Cruz Biotechnology), VDR (sc-13133), E-cadherin (sc-21791), DKK-1 (GTX62902; GeneTex), LRP6 (CST3395; Cell Signaling Technology), RXRα (CST3085), RXRβ (CST8715), LDLRAP1 (C20125; LSBio), or LC3B (ab48394; Abcam). For LR6P and LC3B immunoblots, lysates were resolved on 4% to 20% gradient gels. Coimmunoprecipitations were performed as previously described (21). Briefly, AsPC-1 lysates were precleared with A/G-PLUS agarose beads (Santa Cruz Biotechnology; sc-1008), or control isotype-matched IgG (Santa Cruz Biotechnology; sc-2025 or Abcam; ab46540). After multiple washes, immune complexes were boiled in 6× SDS-load dye, resolved by SDS-PAGE, and transferred to nitrocellulose membranes for immunoblotting.

Cell growth assays

MTT assays (ATCC) were carried out as per the manufacturer’s instructions in 96-well plates with initial plating of 500 (Suit2), 1,000 (Tu8988t), or 2,000 (HPAF-2, YAPC, MiaPaCa-2, AsPC-1, and PANC-1) cells per well. Cells were allowed to adhere overnight and then treated with calcipotriol. Soft-agar assays were performed as previously described (20).

Wnt reporter assays

Baseline Wnt reporter activity was measured in dual luciferase assays (Promega) as previously described (8) by transient cotransfection with control plasmid with constitutive EF1α promoter driving Renilla expression (serving as a normalization control) and either BAR (β-catenin–activated reporter, 12 TCF response elements) or fuBAR (found unresponsive BAR, contains mutated TCF response elements). In other experiments, Wnt reporter activity was measured in AsPC-1 stably transduced with both BAR reporter and Renilla control as previously described (20).

LRP6 overexpression

LRP6 or GFP (control) expression constructs in pcDNA vector have been previously described (22) and were kindly provided by Edward De Robertis (University of California, Los Angeles). Transfections were performed with X-tremeGENE9 (Roche) as per the manufacturer’s instructions.

RNA isolation, library generation, sequencing, and analysis

Total RNA was isolated from human pancreatic cancer cell lines as previously described (19). Sequencing libraries were prepared from 100 to 500 ng of total RNA using the TruSeq RNA sample preparation kit v2 (Illumina) according to the manufacturer’s protocol. Briefly, mRNA was purified, fragmented, and used for first- and second-strand cDNA synthesis followed by adenylation of 3’ ends. Samples were ligated to unique adapters and subjected to PCR amplification. Libraries were validated using the 2100 BioAnalyzer (Agilent), normalized, and pooled for sequencing.
RNA-seq libraries from three biologic replicates for each experimental condition were sequenced on the Illumina HiSeq 2000 using barcoded multiplexing and a 100-bp read length. Image analysis and base calling were done with Illumina CASAVA 1.8.2. This yielded a median of 12.4 million usable reads per sample. Short read sequences were mapped to a UCSC hg19 reference sequence using the RNA-seq aligner STAR (23). Known splice junctions from hg19 were supplied to the aligner, and de novo junction discovery was also permitted. Differential gene expression analysis, statistical testing, and annotation were performed using Cuffdiff 2 (24). Transcript expression was calculated as gene-level relative abundance in fragments per kilobase of exon model per million mapped fragments and employed correction for transcript abundance bias (25). RNA-seq data are deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA accession number SRP057571). Functional enrichment analysis was limited to gene ontology terms and examined using default parameters in the web-based Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.7; http://david.abcc.ncifcrf.gov; ref. 26).

Microarray data analysis
Gene set enrichment analysis (GSEA) method (http://www.broadinstitute.org/gsea; ref. 27) was used to determine whether a defined pancreatic-specific Wnt/β-catenin target gene set showed statistically significant concordance with VDR expression in a previously published gene expression microarray dataset of 25 primary human PDAC samples (deposited in NCBI GEO under accession number GSE32688). GSEA default parameter settings and the Pearson metric for ranking genes were used with VDR expression serving as a continuous phenotypic variable. The defined pancreatic-specific Wnt/β-catenin target gene set was the previously published (20) overlap of downregulated transcripts observed in AsPC-1 in response to either Wnt inhibitor ICG-001 or CTNNB1 siRNA transfection.

Statistical methods
Statistical analyses were performed in GraphPad Prism (ver 5.04). Student t tests were used to compare continuous variables. The level of significance for all statistical tests was defined as α = 0.05.

Results
PDAC growth inhibition by calcipotriol is linked to VDR expression and Wnt signaling
In previous work, we described a distinct subset of PDAC cell lines with higher levels of autocrine Wnt/β-catenin signaling and an anchorage-independent growth phenotype responsive to genetic or pharmacologic inhibition of Wnt (8). Given that vitamin D inhibits Wnt signaling in colon cancer (10, 11), we hypothesized that the growth-inhibitory effects of the vitamin D on PDAC tumor cells might depend on their baseline autocrine Wnt/β-catenin signaling activity. Autocrine Wnt/β-catenin activity was measured across a panel of PDAC cell lines by dual luciferase promoter-reporter assays to determine the ratio of BAR (beta-catenin–activated reporter, 12 TCF response elements driving luciferase expression) to fuBAR (found unresponsive BAR, contains mutated TCF response elements; ref. 28). AsPC1, HPAF-2, YAPC, and Suit2 each had higher levels of autocrine Wnt activity (hereafter designated as high-BAR lines), whereas MiaPaCa-2, PANC-1, and HCG25 each had lower or absent levels of autocrine Wnt activity (hereafter designated as low-BAR lines; Fig. 1A). Levels of autocrine Wnt activity were observed to closely parallel VDR protein expression (Fig. 1A) with Western blots showing higher VDR expression (high-VDR) in high-BAR lines and low or undetectable VDR expression (low-VDR) in low-BAR lines. Furthermore, calcipotriol inhibited anchorage-dependent growth of high-BAR/high-VDR cell lines in a dose-dependent manner at concentrations ranging from 10 nmol/L to 1 μmol/L, but was unable to inhibit the growth of low-BAR/low-VDR cell lines even at concentrations up to 10 μmol/L (Fig. 1B). Calcipotriol inhibited anchorage-independent growth of high-BAR/high-VDR cell lines in soft-agar assays (Fig. 1C), a phenotype we previously showed to be dependent on Wnt signaling in high-BAR PDAC lines (8). These results demonstrate that PDAC growth inhibition by calcipotriol correlates with VDR expression status and might be functionally linked to underlying differences in Wnt activity, whereby Wnt/β-catenin signaling serves as a mediator and/or target of vitamin D action.

To more directly address mechanistic links between vitamin D and Wnt signaling in PDAC, AsPC-1 cells stably transduced with BAR were treated with increasing concentrations of calciptiol. Calcipotriol significantly inhibited Wnt/β-catenin activity in a dose-dependent manner as measured by BAR reporter (Fig. 2A). Calcipotriol also significantly inhibited expression of the endogenous Wnt/β-catenin transcriptional target AXIN2 across all high-BAR/high-VDR cell lines (Fig. 2B). To confirm that calcipotriol inhibited Wnt signaling in a VDR-dependent manner, cells were treated in the context of siRNA-mediated VDR knockdown. VDR knockdown fully rescued inhibition of BAR reporter seen with calcipotriol (Fig. 2C and D), indicating that Wnt inhibition by calcipotriol was VDR-dependent and did not occur through off-target or other receptor-independent effects.

We next explored the mechanism by which calcipotriol inhibits Wnt activity in PDAC. In colon cancer cell lines, ligand-activated VDR has been shown to directly interact with β-catenin, leading to cytoplasmic sequestration of β-catenin and inhibition of β-catenin/TCF-mediated transcription (10). Addressing this possibility in PDAC, cell extracts of AsPC-1 treated with either vehicle or calcipotriol were immunoprecipitated with anti–β-catenin antibody. Although E-cadherin coimmunoprecipitated with β-catenin as expected, VDR failed to coimmunoprecipitate with β-catenin either in the presence or absence of calcipotriol (Supplementary Fig. S1A). Reverse coimmunoprecipitation with anti-VDR antibody also failed to demonstrate an interaction between VDR and β-catenin (data not shown), suggesting Wnt inhibition by calcipotriol does not occur through sequestration of β-catenin by ligand-activated VDR. Calcitriol has been shown to increase expression of the secreted Wnt inhibitor DKK1 through an indirect transcriptional mechanism (11). Although calcipotriol increased DKK1 protein levels in AsPC-1, this increase was functionally decoupled from inhibition of Wnt signaling by calcipotriol, as inhibition of BAR activity by calcipotriol was not rescued by concurrent siRNA-mediated knockdown of DKK1 (Supplementary Fig. S1B and S1C). Furthermore, siRNA knockdown of DKK1 paradoxically reduced BAR activity in the absence of calcipotriol, indicating DKK1 does not functionally inhibit Wnt signaling in AsPC-1 cells. Together, these results suggest that calcipotriol inhibits Wnt signaling in PDAC through an alternative mechanism.
Calcipotriol reduces LRP6 protein levels

LRP6 is a requisite coreceptor for Wnt ligand–initiated canonical signaling. We noted that LRP6 protein levels decreased within 6 hours of calcipotriol treatment in all high-BAR/high-VDR PDAC lines (Fig. 3A and B), but not in low-BAR/low-VDR PDAC lines (Supplementary Fig. S2A). Knockdown of VDR by siRNA blocked the decrease in LRP6 protein observed with calcipotriol (Fig. 2C), indicating that the decrease in LRP6 protein by calcipotriol is VDR-dependent. Transient overexpression of wild-type LRP6 increased BAR activity compared with GFP control vector in both vehicle- and calcipotriol-treated cells (Fig. 3C), indicating changes in LRP6 expression directly alter Wnt reporter activity in AsPC-1.

When normalized to vehicle control, calcipotriol inhibited BAR activity in control GFP- or LRP6-transfected cells to a similar extent (31% and 28% relative change, respectively; Fig. 3C), suggesting calcipotriol may inhibit BAR activity through an LRP6-independent mechanism. Alternatively, overexpressed LRP6 might remain subject to relative downregulation by calcipotriol via the mechanism further detailed below.

To discriminate between genomic versus potential nongenomic mechanisms of action of VDR on LRP6 levels, the VDR transcriptional coactivators RXRa and RXRb were knocked down in tandem by siRNA transfection. Combined RXRa/RXRb knockdown blocked the decrease in LRP6 seen with calcipotriol, arguing again in favor of a VDR/RXR-dependent transcriptional mechanism (Fig. 3D). However, although calcipotriol decreased LRP6 protein levels as early as 6 hours in each of the high-BAR/high-VDR PDAC lines, LRP6 transcript levels only differed between calcipotriol and vehicle control treatments after 24 hours in AsPC-1, HPAF-2, and YAPC (Fig. 3E and F) and not at all in Suit2 (Fig. 3F). This difference in transcript levels at 24 hours specifically reflected an increase in LRP6 expression in vehicle-treated cells that did not occur in calcipotriol-treated cells. Given LRP6 protein decreases prior to any effect on LRP6 transcript levels, we speculated that calcipotriol inhibits Wnt signaling in PDAC by regulating LRP6 protein levels indirectly through an VDR/RXR-dependent transcriptional mechanism.
LDLRAP1 mediates reduced LRP6 protein levels in response to calcipotriol

To further explore the manner in which LRP6 protein was decreased in response to calcipotriol, RNA sequencing (RNA-seq) was performed on AsPC-1 cells. Comparison of transcriptomes following calcipotriol treatment confirmed expression of known vitamin D transcriptional targets, including CYP24A1, as the most highly upregulated gene (Fig. 4A and Supplementary Table S2). A total of 325 genes (220 upregulated and 105 downregulated) showed at least 1.5-fold change in expression at 6 hours ($P < 0.00001$). Top gene ontology terms enriched in the subset of the top 220 upregulated genes included regulation of transcription (0.00001). Top gene ontology terms enriched in the subset of the top 220 upregulated genes included regulation of transcription (0.00001).

Expression of the most enriched categories such as the endomembrane system (i.e., LRP6, LDLRAP1) showed at least 1.5-fold change in expression at 6 hours ($P < 0.00001$). Top gene ontology terms enriched in the subset of the top 220 upregulated genes included regulation of transcription from RNA polymerase II promoter, osteoblast differentiation, cell adhesion molecules (CAMs), and cell junctions (i.e., CYP24A1, C/EBPβ, PDGFA, SHH, THBD), and cellular homeostasis (i.e., CLDN1 and CSF1), as well as multiple genes with established roles in BMP and Wnt signaling (Fig. 4A).

In relation to the endomembrane system, the cytoplasmic adapter protein LDLRAP1 was significantly upregulated at 2 and 6 hours after calcipotriol treatment (Fig. 4A). LDLRAP1 is required for efficient endocytosis of LDL receptors in polarized cells and is able to interact with LRP1 and LRP2 to facilitate LDL receptor clustering into clathrin-coated pits (29–31). Notably, internalization of LRP6 via clathrin antagonizes Wnt signaling (32, 33) and promotes LRP6 degradation through endosomal trafficking to lysosomes (34–36). We speculated that LDLRAP1 might also decrease LRP6 protein levels through a similar mechanism involving receptor internalization and degradation. To address this, we first assessed whether lysosomal activity was necessary to decrease LRP6 protein levels in response to calcipotriol treatment. Treatment with Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase that blocks acidification and protein degradation in lysosomes, blocked the decrease in LRP6 protein levels seen with calcipotriol treatment (Fig. 4B). Bafilomycin also increased LRP6 protein levels in the absence of calcipotriol, indicating lysosomal protein degradation plays a role in the regulation of steady-state levels of LRP6 in PDAC lines. RNA-seq data were validated by qPCR in separate experiments with calcipotriol, confirming LDLRAP1 gene expression was significantly upregulated in AsPC-1 cells within 4 hours of calcipotriol treatment (Fig. 4C). Western blot analysis also confirmed that LDLRAP1 protein was also increased with calcipotriol (Fig. 4D). Further in silico analysis of the LDLRAP1 locus identified a predicted vitamin D response element (VDRE) at the 3′ end of the LDLRAP1 gene. Evaluation of published ChIP-seq data generated from hepatic stellate cells (37) revealed VDR occupancy of the site encompassing this VDRE, indicating VDR can directly bind the LDLRAP1 gene. To further link calcipotriol-induced LDLRAP1 expression to changes in LRP6 protein and Wnt signaling, siRNA-mediated knockdown of LDLRAP1 was performed. Knockdown of LDLRAP1 significantly abrogated the reduction in LRP6 protein seen with calcipotriol treatment (Fig. 4D). Concurrent evaluation of Wnt reporter activity for AsPC-1 transfected with either siVDR or control showed at least 1.5-fold change in expression at 6 hours ($P < 0.00001$). In addition, comparison of transcriptomes following calcipotriol treatment confirmed expression of known vitamin D transcriptional targets, including CYP24A1, as the most highly upregulated gene (Fig. 4A and Supplementary Table S2). A total of 325 genes (220 upregulated and 105 downregulated) showed at least 1.5-fold change in expression at 6 hours ($P < 0.00001$). Top gene ontology terms enriched in the subset of the top 220 upregulated genes included regulation of transcription from RNA polymerase II promoter, osteoblast differentiation, cell adhesion molecules (CAMs), and cell junctions (i.e., CYP24A1, C/EBPβ, PDGFA, SHH, THBD), and cellular homeostasis (i.e., CLDN1 and CSF1), as well as multiple genes with established roles in BMP and Wnt signaling (Fig. 4A).

In relation to the endomembrane system, the cytoplasmic adapter protein LDLRAP1 was significantly upregulated at 2 and 6 hours after calcipotriol treatment (Fig. 4A). LDLRAP1 is required for efficient endocytosis of LDL receptors in polarized cells and is able to interact with LRP1 and LRP2 to facilitate LDL receptor clustering into clathrin-coated pits (29–31). Notably, internalization of LRP6 via clathrin antagonizes Wnt signaling (32, 33) and promotes LRP6 degradation through endosomal trafficking to lysosomes (34–36). We speculated that LDLRAP1 might also decrease LRP6 protein levels through a similar mechanism involving receptor internalization and degradation. To address this, we first assessed whether lysosomal activity was necessary to decrease LRP6 protein levels in response to calcipotriol treatment. Treatment with Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase that blocks acidification and protein degradation in lysosomes, blocked the decrease in LRP6 protein levels seen with calcipotriol treatment (Fig. 4B). Bafilomycin also increased LRP6 protein levels in the absence of calcipotriol, indicating lysosomal protein degradation plays a role in the regulation of steady-state levels of LRP6 in PDAC lines. RNA-seq data were validated by qPCR in separate experiments with calcipotriol, confirming LDLRAP1 gene expression was significantly upregulated in AsPC-1 cells within 4 hours of calcipotriol treatment (Fig. 4C). Western blot analysis also confirmed that LDLRAP1 protein was also increased with calcipotriol (Fig. 4D). Further in silico analysis of the LDLRAP1 locus identified a predicted vitamin D response element (VDRE) at the 3′ end of the LDLRAP1 gene. Evaluation of published ChIP-seq data generated from hepatic stellate cells (37) revealed VDR occupancy of the site encompassing this VDRE, indicating VDR can directly bind the LDLRAP1 gene. To further link calcipotriol-induced LDLRAP1 expression to changes in LRP6 protein and Wnt signaling, siRNA-mediated knockdown of LDLRAP1 was performed. Knockdown of LDLRAP1 significantly abrogated the reduction in LRP6 protein seen with calcipotriol treatment (Fig. 4D). Concurrent evaluation of Wnt reporter activity for AsPC-1 transfected with either siVDR or control showed at least 1.5-fold change in expression at 6 hours ($P < 0.00001$).
relative reduction in BAR activity observed with calcipotriol treatment in control versus LDLRAP1 siRNA-transfected cells was similar (30% and 31%, respectively), changes in the absolute level of BAR activity (Fig. 4E) paralleled the observed changes in LRP6 expression (Fig. 4D), potentially implicating a link between regulation of LRP6 expression and Wnt reporter activity by calcipotriol through LDLRAP1. In summary, LDLRAP1 is a direct transcriptional target of ligand-activated VDR that mediates reduction in LRP6 protein in coordination with the inhibition of Wnt signaling observed with calcipotriol.

VDR is regulated by Wnt signaling

Extending upon the observed correlation between VDR expression and autocrine Wnt signaling activity across a panel of PDAC cell lines (Fig. 1A), we determined whether a similar correlation exists in clinical specimens. GSEA of a previously published cohort of 25 primary PDAC tumors (38) confirmed positive enrichment of a PDAC-specific Wnt/β-catenin target gene set in tumors with higher VDR expression (Fig. 5A, normalized enrichment score = 1.39, false discovery rate q value = 0.08). To determine whether VDR is directly regulated by Wnt/β-catenin signaling in PDAC, AsPC-1 cells with stable BAR reporter were transfected with siRNA against WNT7B, a Wnt ligand previously shown to be essential for autocrine Wnt signaling activity in this cell line (8). WNT7B knockdown significantly inhibited BAR activity with a concomitant ~70% decrease in VDR expression (Fig. 5B). Addressing reciprocal activation of Wnt/β-catenin signaling, high-BAR/high-VDR and low-BAR/low-VDR
Calciptiol Targets LRP6

Figure 4. Calciptiol induces LDLRAP1 to regulate LRP6 protein levels. A, heat map of selected genes from RNA-seq after 2 or 6 hours treatment of AsPC-1 cells with vehicle or 100 nmol/L calciptiol. B, Western blot for LC3B, LRP6, and tubulin (loading control) after AsPC-1 cells were treated with vehicle, 100 nmol/L Calciptiol, 100 nmol/L Bafilomycin A1, or a combination of the two for 6 hours. C, LDLRAP1 expression by qPCR after treatments described in Fig. 3E. D, Western blot for LDLRAP1, LRP6, and tubulin (loading control). AsPC-1 cells were transfected with control or LDLRAP1 siRNA for 48 hours and then treated with vehicle or 100 nmol/L calciptiol for 24 hours. Relative densitometry values for LDLRAP1 and LRP6 expression normalized to tubulin loading control are shown beneath corresponding blots. E, BAR-luciferase activity in AsPC-1 cells treated as in D. Values are shown normalized to respective controls, with BAR reported as mean ± SD and qPCR reported as mean ± SEM. One representative experiment of 3 biologic repeats is shown. *, P < 0.05; **, P < 0.01; ***. P < 0.001.

cell lines were treated with the selective GSK3β inhibitor CHIR99021. CHIR99021 activated Wnt reporter as expected with a concomitant increase in VDR expression (Fig. S3C), indicating Wnt/β-catenin signaling positively regulates VDR expression in PDAC. These data suggest a context-dependent Wnt feedback mechanism such that VDR may inhibit Wnt signaling in situations when sufficient levels of vitamin D are available to activate VDR and its downstream transcriptional targets.

Having demonstrated ligand-activated VDR inhibits Wnt/β-catenin signaling and Wnt/β-catenin positively regulates VDR transcript, we next examined whether calciptiol might inhibit VDR gene expression through downregulation of Wnt/β-catenin signaling. Time course with AsPC-1 showed that VDR message was significantly decreased at 6 and 24 hours following calciptiol treatment (Supplementary Fig. S3A), paralleling effects on LRP6 protein expression and thus compatible with a potential mechanism for feedback inhibition at the level of VDR message (Fig. 3A). Conversely, however, VDR protein levels were significantly increased within 4 to 6 hours and up to 24 hours following calciptiol treatment (Supplementary Fig. S3B). This paradoxical increase in VDR protein may be due to a conformational change and resulting stabilization of VDR protein that has been previously shown to occur when VDR is bound by vitamin D or vitamin D analogues (39).

Discussion

Diverse antitumorigenic actions of vitamin D have been shown in vitro and in vivo preclinical studies of pancreatic cancer (16–19). In particular, nonhypercalcemic analogues of vitamin D circumventing dose-limiting side effects of hypercalcemia and hypercalciuria represent especially promising chemotherapeutic agents for PDAC and other malignancies. To date, there are only limited published data addressing the clinical efficacy of vitamin D in PDAC. In a nonrandomized phase II clinical trial of advanced stage, PDAC single-agent vitamin D analogue seocalcitol (EB1089) was well tolerated but did not improve patient survival (40). This study involved patients with advanced disease and did not address biomarkers such as VDR expression levels, Wnt signaling activity, or other biochemical changes to determine their potential roles in predicting biologic response to seocalcitol. Another phase II trial of advanced PDAC involving oral vitamin D (calciptiol) in combination with docetaxel demonstrated prolonged time to disease progression relative to docetaxel alone (41). Offering a potential explanation, VDR was recently shown to be a master regulator of PDAC stromal activation with calciptiol functioning as a stromal targeting agent capable of enhancing delivery of gemcitabine chemotherapy in a genetically engineered mouse model of PDAC (19). Clinical trials focused on this exciting result are now under way utilizing the vitamin D analogue paricalcitol (i.e., NCT#02030860). Importantly, newer synthetic
vitamin D analogues (i.e., calcipotriol and paricalcitol) may be more efficacious as they are resistant to degradation by the product of the VDR target gene CYP24A1, which is rapidly induced by liganded VDR and is responsible for rapid feedback inhibition of naturally occurring vitamin D (i.e., calcitriol). Although tumor stroma is an important target for evaluating the effects of vitamin D–based therapy in PDAC, such stromal effects also need to be cautiously interpreted in relation to any further direct actions of vitamin D on PDAC tumor cells themselves. For instance, calcitriol directly potentiates the cytotoxic activity of gemcitabine in PDAC cell lines both in vitro and in vivo (17). Furthermore, our results here demonstrate functional cross-talk between VDR and Wnt/β-catenin signaling in PDAC tumor cells that may have significant bearing on the clinical and biologic response of subsets of PDAC tumors (i.e., those with higher autocrine Wnt signaling) to vitamin D analogues. Pancreatic stromal cells are also influenced by paracrine Wnt signaling (42), offering a viable signaling pathway through which vitamin D could exert its antistromal effects.

Our calcipotriol results are in line with previous studies showing that vitamin D and its analogues inhibit the growth of certain PDAC cell lines (i.e., AsPC-1) but not others (i.e., MiaPaCa-2 and PANC-1; ref. 18). Our study expands this observation to additional PDAC cell lines and indicates that growth-inhibitory activity is dependent on levels of VDR expression, which in turn is positively regulated by Wnt/β-catenin signaling. This bidirectional relationship between Wnt and vitamin D signaling could have significant bearing on identifying patients most likely to respond to vitamin D–based therapy and raises a potential confounding issue related to the use of vitamin D analogues either alone or in combination with other therapeutic Wnt inhibitors. Namely, inhibition of Wnt may reduce Wnt/β-catenin–regulated expression of VDR message, with the potential to blunt any further VDR-dependent vitamin D response. Although other mechanisms (i.e.,
VDR protein level paradoxically increased in AsPC-1 at early time points after calcipotriol treatment) may compensate for loss of Wnt-mediated VDR expression, careful consideration should be given to the combination of and order in which various treatments (i.e., a second Wnt inhibitor drug) are used in combination with vitamin D analogues.

Calcipotriol inhibited anchorage-independent growth in cell lines with high endogenous Wnt activity, a phenotype we previously attributed to autocrine Wnt signaling in these cell lines (8). However, calcipotriol also inhibited anchorage-dependent growth, a phenotype we previously showed to be decoupled from Wnt signaling in these cell lines (8). These divergent results are not surprising given known pleiotropic antitumor effects attributed to vitamin D extending beyond its actions as an inhibitor of Wnt signaling. Vitamin D–mediated growth-inhibitory effects in PDAC have previously been attributed to cell-cycle arrest occurring via upregulation of key regulators of cell cycle such as p21 and p27 (16, 18). Indeed, our RNA-seq analysis of calcipotriol-treated cell lines revealed altered expression of several key mediators of cell cycle (i.e., SKP2 and CDKN1A) as well as apoptosis (i.e., BIRC5 and BCL10).

Calcipotriol specifically inhibited Wnt/β-catenin transcriptional activity in PDAC cell lines characterized by higher levels of Wnt ligand–mediated autocrine signaling and VDR expression. Vitamin D was previously shown to inhibit Wnt/β-catenin signaling in colon cancer cell lines by mediating an interaction between VDR and β-catenin that disrupts β-catenin/TCP-mediated transcription (10) and by indirectly increasing expression of the secreted Wnt antagonist DKK1 (11). We were unable to detect a physical interaction between VDR and β-catenin in PDAC lines either in the presence or absence of calcipotriol. While calcipotriol did induce the expression of DKK1 in AsPC-1 cells, this could not be functionally linked to its inhibition of Wnt signaling, as DKK1 knockdown did not rescue the inhibition of Wnt signaling seen with calcipotriol. Nevertheless, our results do not exclude other proposed mechanisms of VDR and β-catenin cross-regulation, such as possible re-distribution of β-catenin across the genome in response to calcipotriol treatment (43, 44).

This study provides a novel mechanism through which vitamin D could negatively regulate Wnt signaling. Protein levels of the Wnt coreceptor LRP6 were reduced as early as 6 hours after calcipotriol treatment. This reduction was dependent upon VDR transcriptional activity because knockdown of the VDR cotranscriptional activator RXR rescued the reduction of LRP6 protein seen with calcipotriol treatment. Rapid reduction of LRP6 protein was not the result of reduced LRP6 transcript levels, which were only altered 24 hours after calcipotriol treatment when LRP6 transcript levels actually increased in vehicle control and not in calcipotriol-treated cells. We speculate that the dramatic increase in LRP6 transcript seen at 24 hours in control cells may be tied to cell-cycle–dependent changes in LRP6 expression (45, 46), whereas calcipotriol-treated cells under cell-cycle arrest would be expected to retain lower levels of LRP6 transcript.

LRP6 is a critical mediator of ligand-mediated Wnt/β-catenin signaling. Although we show overexpression of LRP6 increased Wnt reporter activity in the absence or presence of calcipotriol (Fig. 3C), this cannot be concluded to represent specific rescue of the mechanism by which calcipotriol directly inhibits the pathway. RNA-seq analysis presented here offers several additional potential regulators of Wnt signaling that could mediate calcipotriol effects, including BAMBI (BMP and activin membrane bound inhibitor) that was significantly decreased within 2 hours of calcipotriol treatment. BAMBI is a known Wnt/β-catenin agonist that promotes the interaction between Fzd and Dvl (47). We are now investigating BAMBI and other alternative targets through which vitamin D may directly inhibit Wnt signaling in pancreatic can.

This study also further focused on induction of LDLRAP1 message and protein by calcipotriol and the role of LDLRAP1 in regulating LRP6 protein levels. LDLRAP1 is an adapter protein that binds to tyrosine motifs in the cytoplasmic tail of LDL receptors, which couples them to the endocytic machinery of the clathrin coated pit (31, 48). LDLRAP1 promotes clathrin-mediated endocytosis of LRP1 and LRP2 (29–31). Tyrosine motifs in the cytoplasmic tail of LDL receptor family member LRP6 are critical for its clathrin-mediated internalization (49). Our data establish an additional role for LDLRAP1 in regulating steady-state levels of LRP6 and lead us to speculate that LDLRAP1 facilitates endocytosis and lysosome-mediated degradation of LRP6.

In summary, we have elucidated a bidirectional feedback interaction between Wnt and vitamin D signaling in PDAC. Upon activation, Wnt signaling induces expression of VDR. Furthermore, ligand-activated VDR positively regulates the transcription of LDLRAP1. LDLRAP1 contributes to a rapid reduction in proteins levels of LRP6, a critical coreceptor for ligand-dependent Wnt signaling. Activation of Wnt signaling induces the expression of VDR in PDAC tumor cells, which renders those cells susceptible to vitamin D antitumor effects. In addressing the reciprocal relationship between vitamin D and Wnt signaling, this study offers new insights into the possible use of vitamin D as a therapeutic Wnt inhibitor, as well as possible biomarkers for predicting or following response to calcipotriol and other vitamin D analogues.

Disclosure of Potential Conflicts of Interest
R.M. Evans has ownership interest (including patents) in XTel Pharmaceuticals, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: M.D. Arensman, K.M. Kershaw, M. Downes, D.W. Dawson
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.D. Arensman, P. Nguyen, K.M. Kershaw, A.R. Lay, C.A. Ostertag-Hill, M.H. Sherman, D.W. Dawson
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.D. Arensman, P. Nguyen, K.M. Kershaw, A.R. Lay, M.H. Sherman, C. Liddle, D.W. Dawson
Writing, review, and/or revision of the manuscript: M.D. Arensman, P. Nguyen, M.H. Sherman, M. Downes, R.M. Evans, D.W. Dawson
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.D. Arensman, A.R. Lay, D.W. Dawson
Study supervision: M.D. Arensman, M. Downes, D.W. Dawson

Grant Support
M.D. Arensman was supported by U54NS080523 (NIH, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Department of Health and Human Services), U54CA212345 (National Cancer Institute, National Institutes of Health), P01CA180201 (National Cancer Institute, National Institutes of Health), and National Institutes of Health (National Cancer Institute) U54CA212345 (Center Grant). D.W. Dawson was supported by grants from the American Cancer Society (RSG-12-083-01-TBG), NIH (PO1 CA163200 and P01 CA180201), and the Hirshberg Foundation for Pancreatic Cancer Research. R.M. Evans is an Investigator of the Howard Hughes Medical Institute (HHMI) at the Salk Institute and March of Dimes Chair in Molecular and Developmental Biology and is supported by NIH grants (DK057978, DK090962, HL088093, 1517)
References


Molecular Cancer Research

Calcipotriol Targets LRP6 to Inhibit Wnt Signaling in Pancreatic Cancer


Updated version
Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-15-0204

Supplementary Material
Access the most recent supplemental material at:
http://mcr.aacrjournals.org/content/suppl/2015/08/08/1541-7786.MCR-15-0204.DC1

Cited articles
This article cites 48 articles, 22 of which you can access for free at:
http://mcr.aacrjournals.org/content/13/11/1509.full.html#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
/content/13/11/1509.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.