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Constitutively active RAS signaling reduces 1,25 dihydroxyvitamin D-mediated gene transcription in intestinal epithelial cells by reducing vitamin D receptor expression

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Abstract

High vitamin D status is associated with reduced colon cancer risk but these studies ignore the diversity in the molecular etiology of colon cancer. RAS activating mutations are common in colon cancer and they activate pro-proliferative signaling pathways. We examined the impact of RAS activating mutations on 1,25 dihydroxyvitamin D ($1,25(OH)_2D$)-mediated gene expression in cultured colon and intestinal cell lines. Transient transfection of Caco-2 cells with a constitutively active mutant K-RAS (G12V) significantly reduced $1,25(OH)_2D$-induced activity of both a human 25-hydroxyvitamin D, 24 hydroxyase (CYP24A1) promoter-luciferase and an artificial 3X vitamin D response element (VDRE) promoter-luciferase reporter gene. Young Adult Mouse Colon (YAMC) and Rat Intestinal Epithelial (RIE) cell lines with stable expression of mutant H-RAS had suppressed $1,25(OH)_2D$-mediated induction of CYP24A1 mRNA. The RAS effects were associated with lower Vitamin D receptor (VDR) mRNA and protein levels in YAMC and RIE cells and they could be partially reversed by VDR overexpression. RAS-mediated suppression of VDR levels was not due to either reduced VDR mRNA stability or increased VDR gene methylation. However, chromatin accessibility to the VDR gene at the proximal promoter (−300 bp), an enhancer region at −6 kb, and an enhancer region located in exon 3 was significantly reduced in RAS transformed YAMC cells (YAMC-RAS). These data show that constitutively active RAS signaling suppresses $1,25(OH)_2D$-mediated gene transcription in colon epithelial cells

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Declaration of Interests

The authors have no conflicts of interest to declare.

Submission Declaration

This work is not being considered, nor will it be submitted, for publication at any other journal.

by reducing VDR gene transcription but the mechanism for this suppression is not yet known. These data suggest that cancers with RAS-activating mutations may be less responsive to vitamin D mediated treatment or chemoprevention.

Keywords

Vitamin D Receptor; gene expression; MAPK; colon cancer

Introduction

High vitamin D status has been proposed as protective against colon cancer by exerting anti-cancer mechanisms through the biologically active form of vitamin D, 1α,25-dihydroxyvitamin D (1,25$(OH)_2$D) [(1)]. For example, in Caco-2 colon cancer cells, 1,25$(OH)_2$D inhibited cell proliferation by decreasing EGF receptor expression, activating the TGF-β pathway, and increasing p21 expression [(2–4)]. 1,25$(OH)_2$D regulates transcription of anti-cancer genes by binding to the vitamin D receptor (VDR), a member of the nuclear receptor superfamily that heterodimerizes with one of the three retinoid X receptor (RXR) isoforms (α, β, and γ) and binds to vitamin D response elements (VDREs) in the promoters of vitamin D responsive genes [(5)]. When the VDR-RXR complex is bound to the VDRE, coactivators such as SRC, CBP/p300, and the mediator complex induce chromatin remodeling and recruit RNA polymerase II to initiate gene transcription.

Although there is evidence that 1,25$(OH)_2$D action may protect against colon cancer, some studies have shown that VDR levels fall in the early stages of colon cancer [(6,7)] and that 1,25$(OH)_2$D resistance may develop during cancer progression [(8)]. This suggests that the molecular mutations that develop during colon cancer can regulate cellular events that antagonize signaling through the VDR. KRAS activating mutations are found in 40% of sporadic colorectal cancers and are one of the early mutations found during colon cancer development [(9)]. We and others have previously shown that activated RAS signaling suppresses 1,25$(OH)_2$D action in prostate epithelial cells [(10)], mammary epithelial cells [(11)], non—small cell lung cancer [(12)], and keratinocytes [(13)]. Here we examined the effect of activated RAS signaling on 1,25$(OH)_2$D-mediated regulation of CYP24A1 gene expression as a sensitive marker of transcriptional events mediated through the VDR. We found that activated RAS signaling suppressed 1,25$(OH)_2$D action in human colon cancer cells (Caco-2), mouse colon epithelial cells (YAMC), and rat intestinal epithelial cells (RIE) through suppression of VDR gene expression. These results suggest that an early molecular event in colorectal cancer, RAS activation, may suppress 1,25$(OH)_2$D-mediated chemopreventative effects.

Material and Methods

Reagents

Cell culture reagents were obtained from Cambrex (Rockland, ME) and Invitrogen (Carlsbad, CA). Cell culture plasticware was obtained from BD Biosciences (Sparks, MD). 1,25$(OH)_2$D was purchased from Enzo Life Science (Farmingdale, NY) and dissolved in

ethanol. 5-aza-2′-deoxycytidine was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in DMSO.

Cell culture

The Caco-2 human colonic adenocarcinoma cell line was obtained from American Type Culture Collection (Rockville, MD) and cultured as previously described [14]. YAMC (young adult mouse colon) and H-Ras-transformed YAMC cells (YAMC-RAS) were provided by Dr. Robert H. Whitehead (Royal Melbourne Hospital) [15] and cultured in RPMI 1640 media supplemented with 1% penicillin/streptomycin, 1% insulin/transferrin/selenite, 5% fetal bovine serum (FBS), and 1 IU/μl IFN-γ (Gibco, Grand Island, NY). Cells were maintained at 33 °C in a 5% CO_2 – 95% air atmosphere and grown in collagen I-coated flasks (BD Bioscience; Bedford, MA). Immortalized rat intestinal epithelial cells (RIE) and RIE cells transformed with an inducible version of H-RAS (RIE-iRAS) were provided by Dr. Daniel Beauchamp (Vanderbilt University) [16]. RIE and RIE-iRAS cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) containing 10% FBS supplemented with L-glutamine, 1% penicillin/streptomycin, 400 μg/ml G418 sulfate and 150 μg/ml of Hygromycin B. Cells were maintained at 37 °C in a 5% CO_2 – 95% air atmosphere. Although the RIE-iRAS cells were designed to have inducible expression of H-RAS, over time the promoter driving RAS expression has become leaky leading to constitutive activation of MAPK signaling.

Plasmids

The −298 to +74 bp rat CYP24A1 promoter-luciferase reporter gene construct containing two functional VDREs (rCYP24-luc) was a gift from Dr. John L. Omdahl (University of New Mexico, Albuquerque, NM) [17]. A luciferase reporter gene driven by an artificial promoter with three copies of the osteopontin VDRE linked to a minimal thymidine kinase promoter (3XVDRE-luc) was a gift from Dr. Sunil Nagpal (Lilly Research Laboratory, IN) [18]. The expression vector for the constitutively active MEK1 mutant (MEK1RAF) was a gift from Dr. Melanie Cobb (University of Texas Southwest Medical Center, Dallas, Texas) [19]. The expression vector for the constitutively active KRAS mutant (KRAS12V) was purchased from Addgene (Cambridge, MA, #12544).

Reporter gene assay and VDR gene overexpression

Proliferating Caco-2 cells were seeded in 24-well plates and grown to 40–50% confluence. Cells were transfected using Lipofectamine Plus (Invitrogen, Carlsbad, California) following the protocol provided by the manufacturer using a 1:5:5 ratio of DNA/Plus/Lipofectamine. Caco-2 were transfected with 300 ng (per well) rCYP24A1-luc or 3XVDRE-luc, 500 ng of MEK1RAF or KRAS12V plasmids, and 2 ng of pRL CMV-Renilla or pRL-null Renilla vector (Promega, Madison, WI). YAMC and YAMC-RAS cells were seeded in 24-well plates and grown to 50–70% confluence and RIE and RIE-iRAS cells were seeded in 24-well plates and grown to 20–50% confluence. Cells were transfected using the Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, California) according to manufacturer's protocol. For VDR protein over expression, 5 ng of human VDR expression vector, pCR3.1-hVDR [20], was transfected into cells. For reporter gene studies, 24 h after transfection, cells were treated with media containing vehicle, 10 or 100 nM $1,25(OH)_2D$ for

24 h in medium plus 5% FBS. At the end of the treatment period cells were harvested and analyzed for firefly and renilla luciferase using methods we have described previously [14]. For measurement of VDR protein, Western blot analysis was conducted on cell extracts isolated 24 h after the transfection and samples were analyzed as described below.

RNA isolation and analysis

Cells were plated in either six or twelve-well dishes. Cells were treated 0 (0.1% ethanol), 10nM, or 100nM 1,25$(OH)_2$D for different time points in medium plus 5% FBS. Cells were harvest into TriReagent (Molecular Research Center, Inc., Cincinnati, OH) and RNA was isolated following manufacturer's instructions. Total RNA was converted to cDNA and gene expression was analyzed using real-time PCR with primers and conditions previously described [14]. PCR primers for detecting mouse Snail1 were 5′-TCTCTAGGCCCTGGCTGCTT-3′ and 5′-AAAGCACGGTTGCAGTGG-3′, and for detecting mouse Snail2 were 5′-CTCACCTCGGGAGCATACAG and 5′-GAGAAAGGCCACTGGGTAAAG-3′. Gene expression data was normalized to RPLP0 mRNA expression.

Western blot analysis

Cells were harvested in lysis buffer containing 120 mM NaCl, 0.5% Nonidet P-40, 0.2 mM sodium orthovanadate, 50 mM Tris–HCl pH 8.0, 1 Protease Inhibitor Cocktail Tablet per 50 ml (Roche Ltd, Basel, Switzerland). Extracts were centrifuged at 16,300 X g at 4°C for 15 min. Total protein content of samples was determined using the BioRad Protein Assay (BioRad Laboratories, Hercules, CA). Specific proteins were detected by Western blot analysis using methods described previously [14] and the following antibodies: rabbit anti-ERK1/2 (Cell Signaling Technology, Danvers, MA), rabbit anti-phospho ERK1/2 (Cell Signaling Technology), rabbit anti-CREB (06-863 Millipore Billerica, MA), rabbit anti-phospho-CREB (Ser133) (06-519 Millipore Billerica, MA) rabbit anti-C/EBPβ (sc-150 Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phospho-C/EBPβ (Thr235) (Cell Signaling Technology), mouse anti-β-actin (AC-74 Sigma-Aldrich St. Louis, MO), and mouse-anti-human VDR (sc-13133 Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were incubated overnight with 1:1000 dilutions of the primary antibody prepared in blocking solution followed by a 1:5,000 dilution of horseradish-peroxidase-conjugated goat-anti-mouse IgG (Invitrogen, Carlsbad, CA) or mouse anti-rabbit IgG light chain specific secondary antibody (West Grove, PA).

Chromatin accessibility analysis

YAMC and YAMC-RAS cells were seeded in 48-well plates and grown to 50–60% confluence. Chromatin analysis was conducted using the EpiQ Chromatin Analysis Kit from Bio-Rad (Hercules, CA). Chromatin accessibility at the GAPDH gene was measured as a positive control while chromatin accessibility at the Rhodopsin gene was used as the reference gene that is not susceptible to nuclease digestion. The primers for the positive control and reference genes were provided by the kit. Primers for the mouse VDR regulatory regions are: S1: forward: $^{5'}$ggttttctgggtcacaggccatgct$^{3'}$, reverse: $^{5'}$ggctcccgtggaatgagaaggctct$^{3'}$; S2: forward: $^{5'}$ccactccccgtgttcctggactctc$^{3'}$, reverse: $^{5'}$tgtggattggtggtgtggctcaatg$^{3'}$; S3: forward: $^{5'}$gtttcaccccgggtcactggagatg$^{3'}$,

reverse: $^{5'}$ggagatccccgatgagaaggcactg$^{3'}$; S4: forward: $^{5'}$ggaacgccccatgtcttcaatttgc$^{3'}$, reverse: $^{5'}$ctccaggtacacggtgatggggtga$^{3'}$: S5: forward: $^{5'}$tggctccttttccctgtggcaacttc$^{3'}$, reverse: $^{5'}$ggggaaagcacacgtttcccttgaa$^{3'}$; PP: forward: $^{5'}$gcccctcaaccacgcccactaagtt$^{3'}$, reverse: $^{5'}$gcccctcaaccacgcccactaagtt$^{3'}$; U1: forward: $^{5'}$cagcagcccaggaatgcgttaaaca$^{3'}$, reverse: $^{5'}$ttgaggtcacttctcggtggcagga$^{3'}$;. Chromatin accessibility was measured using the calculation from the EpiQ Chromatin Kit Data Analysis Tool as (1 - (amplification efficiency of the target gene) $^{\text{Ct undigested – Ct digested target gene sample}}$/ (amplification efficiency of the reference gene) $^{\text{Ct undigested – Ct digested reference gene sample}}$) X 100.

Statistical analysis

All quantitative data are expressed as the mean the standard error of the mean (SEM). Statistical analysis of the data was performed by a two-way t-test or by ANOVA followed by Turkey's HSD ($P < 0.05$). Data not normally distributed, was natural log or square-root transformed prior to analysis. P-Values less than 0.05 were considered statistically significant.

Results

RAS activation suppresses 1,25(OH)$_2$D-mediated CYP24A1 reporter gene and mRNA induction

In proliferating Caco-2 cells transient transfection with expression vectors for constitutively active KRAS or MEK1 mutants reduced 1,25(OH)$_2$D-induced activity of the rCYP24-luc (−60% by each mutant) (Fig. 1A). A minimal 3X VDRE-luc construct was less responsive to 1,25(OH)$_2$ D treatment compared to the rCYP24-luc construct (> 60% lower). However, both the KRAS and MEK1 mutants significantly suppressed vitamin D-mediated expression of the 3XVDRE-luc gene by 30% (Fig. 1B).

YAMC-RAS (Fig. 1C) and RIE-iRAS (Fig 1D) cells had elevated phospho-ERK levels reflecting activation of the RAS-MEK-ERK signaling pathway. Compared to their non-transformed parental lines, the 1,25(OH)$_2$D-induced expression of the natural CYP24A1 mRNA (10 nM, 8 h) was reduced by 70% in YAMC-RAS cells ($p<0.05$, Fig. 1E) and was essentially eliminated in RIE-RAS cells ($p<0.05$, Fig. 1F). Similar reductions in 1,25(OH)$_2$D-induced expression of the rCYP24-luc promoter were also observed in YAMC-RAS (−60%, Fig. 3A) and RIE-iRAS cells (−80%, Fig. 3B).

VDR levels partially account for impaired 1,25(OH)$_2$D-induced gene expression in RAS-transformed cells

VDR mRNA (Fig 2A, 2B) and protein levels (Fig 3A, 3B) were significantly reduced in both YAMC-RAS and RIE-iRAS cells compared to their parental lines. VDR gene overexpression increased VDR protein levels to 100% above the normal wild-type level (Fig 3A, B). Elevated VDR expression resulted in a significant increase in the reporter gene response to 1,25(OH)$_2$D treatment in all cell types and it partially reversed the blunted induction of the rCYP24-luc promoter by 1,25(OH)$_2$D seen in YAMC-RAS (Fig. 3A) and RIE-iRAS cells (Fig. 3B).

VDR mRNA half-life and VDR gene methylation are not affected by RAS activation in YAMC cells

To determine the mechanism for reduced VDR levels in cells with constitutively active RAS signaling we examined several possibilities. First, the impact of RAS signaling on VDR mRNA half-life was assessed in YAMC and YAMC-RAS cells treated with the transcriptional inhibitor actinomycin D. VDR mRNA half-life was similar between the two cell lines (approximately 8.9 h in YAMC, 8.2 h in YAMC-RAS; Fig. 4A), indicating the 80% reduction in VDR mRNA between these two cell lines does not result from a large difference in the rate of VDR mRNA degradation.

Recently Marik et al. [21] showed that the VDR gene promoter is hypermethylated in breast cancer cell lines. Inhibiting methylation in these cell lines increased VDR levels and increased 1,25$(OH)_2$D-mediated growth arrest suggesting the existence of methylation-dependent transcriptional silencing for the VDR gene. In contrast to this hypothesis, when YAMC and YAMC-RAS were treated with an inhibitor of DNA methylation (5′-aza-2′-deoxycytidine) VDR mRNA levels decreased in YAMC cells but did not increase in YAMC-RAS cells (Fig. 4B). This was confirmed using a variety of conditions (e.g. 1 or 5 μM of 5′-aza-2′-deoxycytidine for 48, 72, or 96 h; data not shown).

Chromatin accessibility at regulatory regions in the mouse VDR gene is suppressed in YAMC-RAS cells

Zella et al. [22] found that transcription of the mouse VDR gene is regulated at seven promoter and enhancer regions (Fig. 5A) in the mouse osteoblast cell line MC3T3-E1. In addition, the ENCODE project has identified DNAse I hypersensitive sites in the mouse large intestine that overlap with these regions (http://mouseencode.org/) [23] (Fig. 5A). When we examined the chromatin accessibility within these regions, four had very low chromatin accessibility in both YAMC and YAMC-RAS cell lines: the regulatory regions around exons 1 and 2 (S5 and S4) and between exons 3 and 4 (S2 and S1). In contrast, chromatin accessibility on the S3 region (located around exon 3), the proximal promoter (PP), and the U1 region (6 kb upstream of the PP) was moderate to high in YAMC cells and accessibility of these regions was significantly reduced in the YAMC-RAS cells (Fig. 5B).

Several transcription factors can bind to the known regulatory regions in the VDR gene and regulate VDR gene transcription: e.g. the negative regulators Snail1 and Snail2 [24] (PP region), and the positive regulators CREB (cAMP response element-binding) and C/EBPβ (Ccaat-enhancer-binding-protein) [22] (U1, PP or S3 regions). There was a trend towards increased Snail1 mRNA expression ($p=0.057$), and significantly lower Snail2 mRNA levels ($p<0.05$) in YAMC-RAS cells compared to YAMC cells (Fig. 6A). C/EBPβ protein is expressed in the YAMC and RIE cell lines and there was no difference in expression in their RAS-transformed counterparts (Fig. 6B). Others have demonstrated that phosphorylation of C/EBPβ (threonine 235 in human, Thr189 in rat, Thr188 in mouse) regulates transcriptional activation of this transcription factor [25,26]. However, C/EBPβ phosphorylation levels were not different between the YAMC-RAS and YAMC cell lines or between RIE and RIE-iRAS cells (Fig. 6B). CREB levels were higher in YAMC-RAS and the RIE-iRAS cells compared to YAMC and RIE cells, respectively (Fig. 6B). MAPK induced phosphorylation of CREB

occurs on serine 133 [27] leading to increased VDR gene expression [22]. After adjusting for differences in CREB levels, CREB phosphorylation levels were not increased in the YAMC-RAS and RIE-iRAS cells compared to the YAMC and RIE-iRAS cells (Figure 6B).

Discussion

Vitamin D has been proposed as a chemotherapeutic or chemopreventative agent against cancer but the process of carcinogenesis may impair vitamin D signaling in a variety of ways [1]. For example, others have found that VDR mRNA or protein levels are reduced in human colon tumors [7,28] and other cancers [29–31]. Forty percent of colon tumors have mutations in the K-RAS gene [9] and previous research has shown that RAS activating mutations suppress $1,25(OH)_2D$ action in some cells [10–12,32–34] but enhance $1,25(OH)_2D$ action in MG-63, HeLa, and COS-1 kidney cells [33,35].

Here we found that constitutively active RAS signaling suppresses the transcriptional activity of $1,25(OH)_2D$ in several intestinal cell models. Although RAS transformation inhibits $1,25(OH)_2D$ action by phosphorylation of the VDR heterodimeric partner RXRα in prostate epithelial cells and keratinocytes [10,34,36], our new data suggest that the primary mechanism for RAS-mediated inhibition of $1,25(OH)_2D$ action in intestinal cells is due to a 70% reduction of VDR gene expression. This is consistent with other groups that have reported that Ras-activating mutations reduce VDR mRNA and/or protein levels in NIH3T3 cells (H-Ras [37]), non-small cell lung cancer cell lines (K-Ras [12], and mouse mammary epithelial cells (H-Ras [11,32]). Thus, VDR loss may be a critical contributor to Ras-mediated transformation and VDR loss may reduce the beneficial effects of high vitamin D status on cancer progression.

Adequate VDR levels are necessary for optimal $1,25(OH)_2$ D-action in Caco-2 cells [20] and in the mouse small intestine [38] while VDR over-expression in H-Ras transformed NIH3T3 cells reduces their ability to form cellular foci [37]. Consistent with a role for low VDR levels as a mechanism accounting for reduced vitamin D responses in YAMC-RAS and RIE-RAS cells, VDR transfection increased $1,25(OH)_2D$-induced rCYP24A1 promoter luciferase activity in the RAS transformed cells (Fig. 3B and D). While rCYP24-luc reporter gene activation was normalized by VDR transfection in YAMC-RAS cells, it was still significantly blunted in VDR transfected RIE-RAS cells. This is similar to what we previously reported in RAS-transformed prostate epithelial cell line, RWPE2, where MAPK-mediated phosphorylation impaired recruitment of co-activators to RXRα in the VDR-RXR transcriptional complex [10]. Additional studies will be necessary to determine whether phosphorylation of RXR or other proteins in the transcriptional complex accounts for the reduced induction of CYP24 gene expression seen in RIE-RAS cells after normalizing VDR levels.

There are two ways that constitutively active RAS signaling could reduce VDR mRNA levels: by increasing degradation of VDR mRNA or by suppressing VDR gene expression. Although H-Ras transformation increased VDR mRNA degradation modestly in HC11 mammary epithelial cells [39], VDR mRNA half-life was not significantly different between

the YAMC and YAMC-RAS cells (Fig. 4), indicating that activated RAS signaling did not influence VDR mRNA stability.

Epigenetic modifications like histone deacetylation and DNA methylation have recently been identified as important regulators of VDR gene transcription. The proximal promoter region of the VDR gene is hypermethylated in breast cancer cell lines [21] and some adrenocortical carcinomas [40]. In addition, inhibition of DNA methylation using 5′-aza-2′-deoxycytidine increased VDR expression and 1,25$(OH)_2$D-mediated growth arrest in breast cancer cell lines [21] and VDR expression in HT-29 colon cancer cells [7]. In contrast, Habano et al. [41] reported that inhibition of DNA methylation had no effect on VDR mRNA expression in several colon cancer cell models. Consistent with Habano et al. we found that inhibition of methylation had no impact on VDR mRNA levels in the YAMC-RAS cell line and actually reduced VDR mRNA levels in the YAMC parental cells (Fig. 4B). Although DNA methylation is an important mechanism regulating basal VDR gene expression in colon epithelial cells, our data suggest that the reduction of VDR expression in the YAMC-RAS cells is mediated by a different mechanism, as 5′-aza-2′-deoxycytidine was not able to induce VDR expression, but rather reduced it.

We next hypothesized that activated RAS signaling was directly influencing transcription of the VDR gene. Using ChIP-chip, Zella et al. [22] identified 7 regulatory regions in the mouse VDR gene using the osteoblast cell line MC3T3–E1 (Fig. 5A). These 7 regions bind multiple transcription factors in the presence of 1,25$(OH)_2$D and under basal conditions [22]. In YAMC cells, three of these regulatory regions are active and each of these had reduced chromatin accessibility in YAMC-RAS cells: the U1 region 6 kb upstream of the transcriptional start site, the proximal promoter (PP) region, and the S3 region at the translation start site in Exon 3 (Fig. 5). Several transcription factors regulate the VDR gene and can bind to one or more of the three regulatory regions we identified as active in intestinal epithelial cells. Snail1 and Snail2 (also known as SLUG) overexpression in colon cancer suppresses VDR gene expression [42–44], while activated RAS signaling increases Snail2 and SnaiI1 mRNA levels in HCT116 and SW480 cells [45]. In contrast, we found that Snail1 mRNA was only modestly increased in YAMC-RAS cells (1.4 fold increase, p= 0.06) while Snail2 expression was significantly lower (Fig. 6A). C/EBPβ is a bZIP transcription factor that enhances VDR expression in MC3T3-E1 cells [22]. In addition, RAS transformation increases C/EBPβ phosphorylation and this alters C/EBPβ transcriptional activation [46,47]. However, neither C/EBPβ protein (Fig. 6B) nor C/EBPβ phosphorylation were altered in either YAMC-RAS or RIE-iRAS cells (Fig. 6B), suggesting that changes in C/EBPβ do not account for reduced VDR gene expression in RAS transformed intestinal cells. Finally, we examined CREB, a transcription factor that binds to cAMP response elements (CRE) in the VDR gene promoter following phosphorylation at serine 133 [22,27]. In contrast to our observation that constitutively active RAS reduces VDR gene transcription, total CREB and serine 133 phosphorylated CREB levels were higher in the RAS transformed cells compared to the parental cell lines (Fig. 6B). DNase-seq data on mouse large intestine from the ENCODE project (www.encodeproject.org/) indicate that there may be additional, uncharacterized regulatory regions upstream from U1 that are active in the transcription of the VDR gene (Fig 5A). Future studies will be necessary to explore the effect of Ras signaling on these regions.

In summary, our data demonstrate that constitutive activation of RAS signaling impairs 1,25$(OH)_2$D action in colonic and intestinal epithelial cells. This effect is due in part to reduced transcription of the VDR gene and reduced levels of VDR mRNA and protein. Although we found that accessibility to 3 regulatory regions in the VDR gene is reduced by constitutive activation of RAS signaling in YAMC cells, future studies are needed to determine whether these findings are generalizable to multiple cell types as well as to determine which transcription factors are mediating the suppressive effect of constitutive RAS activity on VDR gene expression. Nonetheless, our studies provide additional evidence that colon cancer-causing mutations alter vitamin D signaling through inhibition of VDR gene expression.

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Abbreviations

1, 25$(OH)_2$D	1α, 25-dihydroxyvitamin D
C/EBPβ	Ccaat-enhancer-binding protein beta
CREB	cAMP response element-binding
CYP24	24-hydroxvitamin D, 24-hydroxylase
ERK	extracellular regulated kinase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HSS	DNAse I hypersensitivity site
MAPK	mitogen activated protein kinase
MEK	MAPK kinase/ERK kinase kinase
Pit-1	pituitary-specific positive transcription factor 1
RAS	rat sarcoma
RPLP0	60S acidic ribosomal protein P0
RUNX2	runt related transcription factor 2
VDR	vitamin D receptor

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Highlights

- Activated RAS signaling reduces vitamin D mediated gene expression in intestinal epithelial cells
- Activated RAS signaling reduced Vitamin D Receptor (VDR) level in intestinal epithelial cells
- Chromatin accessibility at multiple regulatory sites in the VDR gene is reduced by RAS activation in intestinal epithelial cells

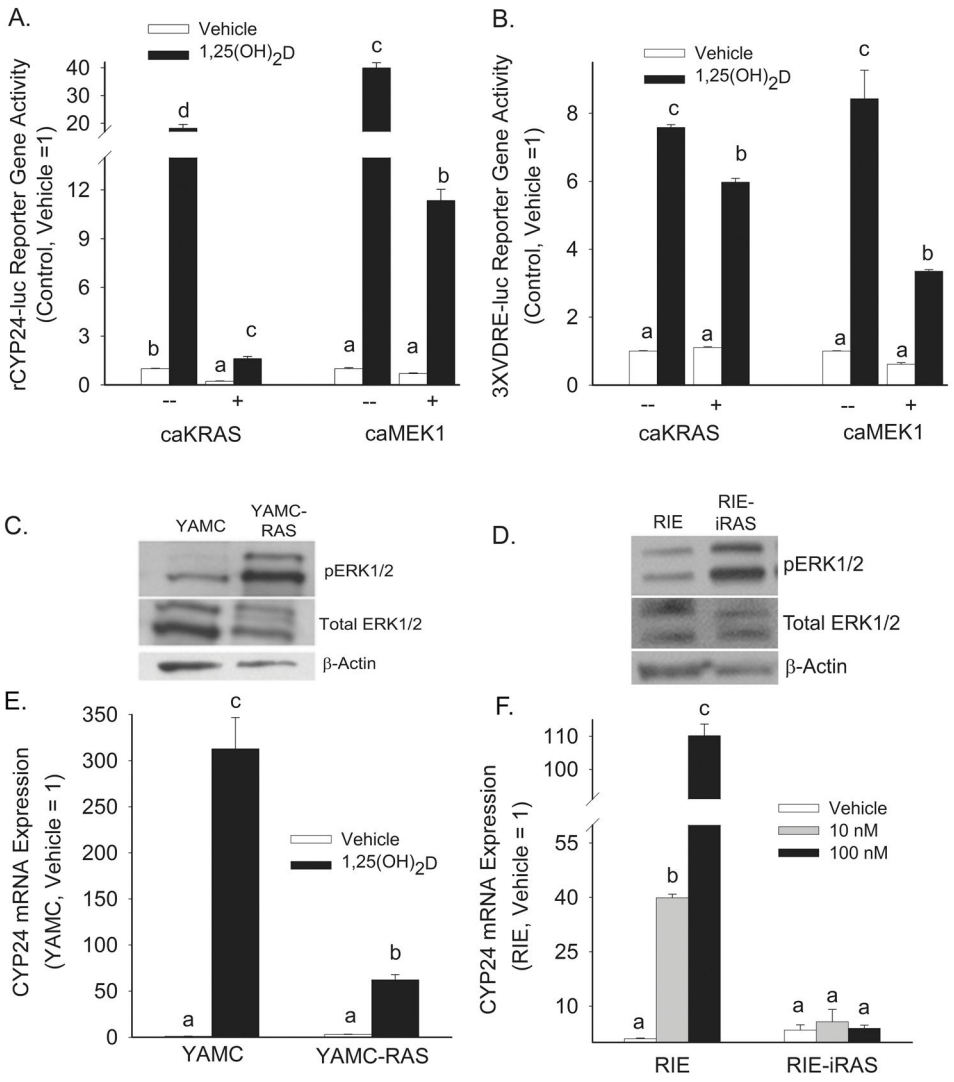

Figure 1.

Constitutive activation of RAS/MAPK signaling decreases 1,25(OH)$_2$D-mediated gene expression. 1,25(OH)$_2$D-mediated induction of a rat −298 CYP24A1 promoter (A) or a minimal 3XVDRE promoter (B) was examined in proliferating Caco-2 cells after cells were transfected with expression vectors encoding constitutively active (ca) versions of KRAS or MEK1. Demonstration that constitutively active RAS increases the level of phosphorylated ERK1/2 (reflective of increased RAS activity) in (C) YAMC/YAMC-RAS and (D) RIE/RIE-iRAS cells. The impact of constitutive RAS activation on 1,25(OH)$_2$D-induced accumulation of CYP24A1 mRNA was examined in (E) YAMC and YAMC-RAS or (F) RIE and RIE-iRAS cells. Cells were treated with 0, 10, or 100 nM 1,25(OH)$_2$D for 8 h and CYP24A1 mRNA levels were measured and normalized to RPLP0 expression. Western blots showing representative data on the impact of RAS transformation on total and phospho-ERK 1 and 2 levels in YAMC or RIE cells are shown above bar graphs in (C) and (D). Bars (mean SEM, n=3) with different letter superscripts are significantly different from one another ($p < 0.05$, Tukey's HSD).

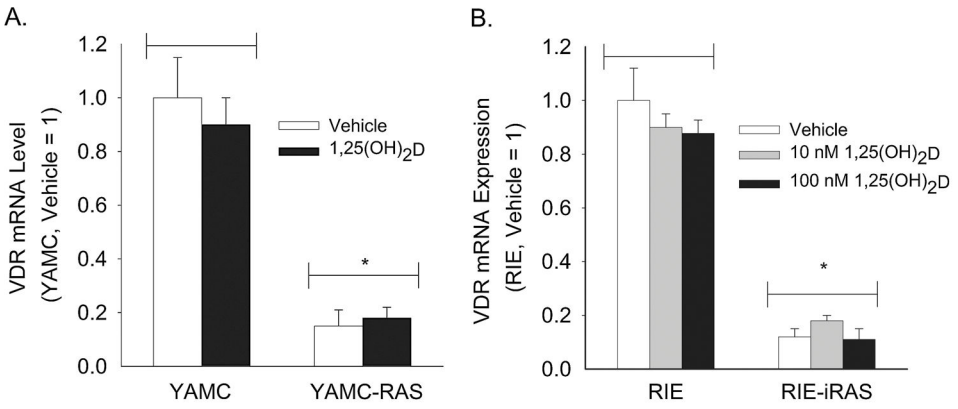

Figure 2.

VDR mRNA levels are significantly reduced in intestinal cells with constitutively activated RAS signaling. Cells were treated with $1,25(OH)_2D$ for 8 h (panel A, 0 or 100 nM; panel B, 0, 10, or 100 nM) and VDR mRNA levels were measured and normalized to RPLP0 expression. (A) Parental YAMC and H-Ras transformed YAMC (YAMC-RAS) cells. (B) Parental RIE and H-Ras transformed RIE (RIE-iRAS) cells. Data are expressed mean SEM, n=3 per group. * Significantly lower than the parental cell line ($p < 0.01$, Tukey's HSD).

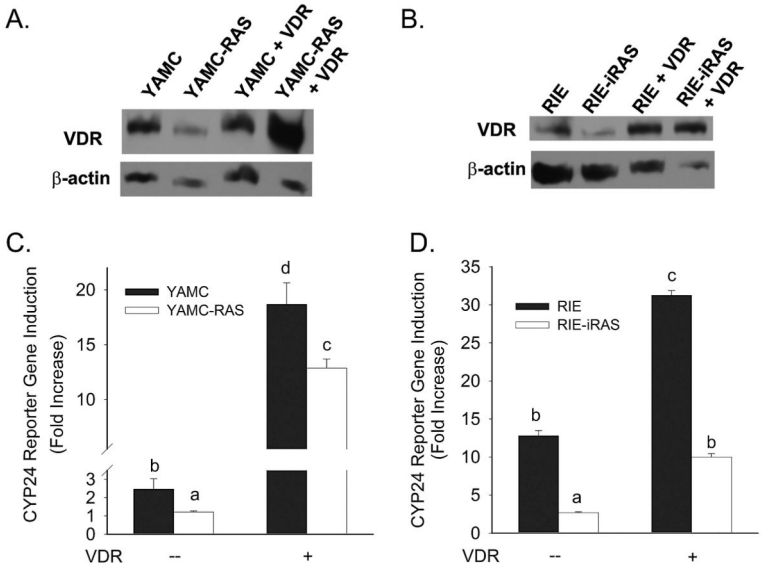

Figure 3.

VDR overexpression partially overcomes the RAS induced suppression of $1,25(OH)_2D$ action. Cells were transfected with a VDR expression vector and a rCYP24-luciferase reporter gene and 24 h later cells were treated with vehicle or 100 nM $1,25(OH)_2D$ for another 24 h. (A,B) The impact of Ras status and VDR transfection on VDR protein levels in (A) YAMC, and (B) RIE cells. Vitamin D mediated regulation of rCYP24-luciferase reporter gene activity was measured in (C) Parental YAMC and H-Ras transformed YAMC (YAMC-RAS) cells or (D) Parental RIE and H-Ras transformed RIE (RIE-iRAS) cells. Data are expressed as $1,25(OH)_2D$ induced fold-change (mean SEM, n=6). Bars with different letter superscripts are significantly different from each other ($p < 0.05$, Tukey's HSD).

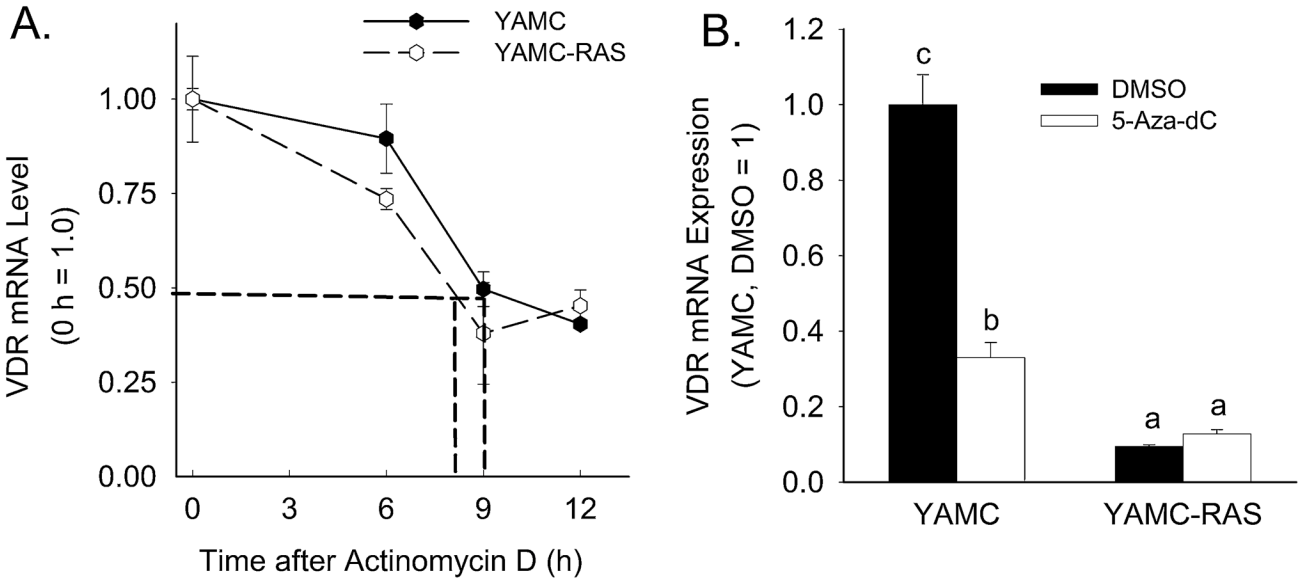

Figure 4.

Neither VDR mRNA half-life is nor VDR gene methylation explain the impact of RAS on VDR mRNA level. (A) VDR mRNA half-life in YAMC and YAMC-RAS cells. Cells were treated with actinomycin D (4 μg/ml) for 0, 6, 9, and 12 h after which RNA was isolated and analyzed for VDR mRNA level and normalized to RPLP0 expression. Data are expressed relative to 0 h = 1.0 (mean SEM, n=3 per group). (B) VDR gene methylation in YAMC and YAMC-RAS cells. Cells were treated with vehicle or 1 μM 5′-aza-2′-deoxycytidine for 72 h. Afterwards RNA was isolated, analyzed for VDR mRNA level and normalized to RPLP0 expression. Data are expressed relative to the vehicle-treated YAMC cells (mean SEM, n=6). Bars with different letter superscripts are significantly different from each other ($P < 0.05$, Tukey's HSD).

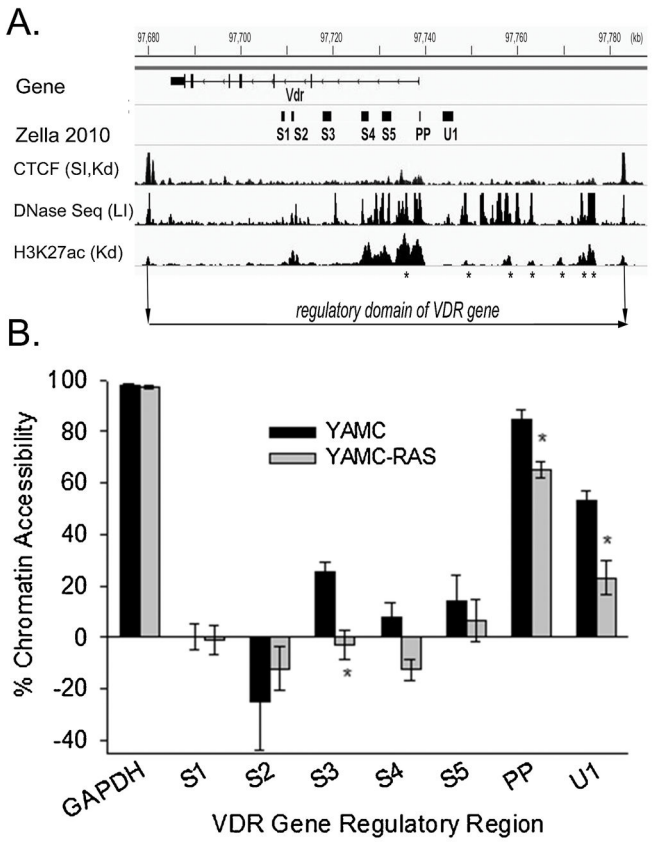

Figure 5.

Activated RAS signaling decreases chromatin accessibility at the mouse VDR gene. (A) Regulatory regions in the mouse VDR gene. Promoter and enhancer regions controlling VDR gene regulation in osteoblasts (Zella 2010 [(22)]). ENCODE data was used to identify CTCF sites reflecting the regulatory domain of the VDR gene in small intestine (SI) and kidney, DNAse I hypersensitive sites (HSS) in large intestine (DNase Seq(LI)), and markings of active enhancers from kidney (H2K27ac (Kd)). (B) Chromatin accessibility was measured at the 7 osteoblast regions in the VDR gene using DNA isolated from YAMC and YAMC-RAS cells. TSS = the transcriptional start site. Data are expressed as % chromatin accessibility (mean+SEM, n=3 per cell line). * Significantly different from YAMC value ($P < 0.05$, Tukey's HSD).

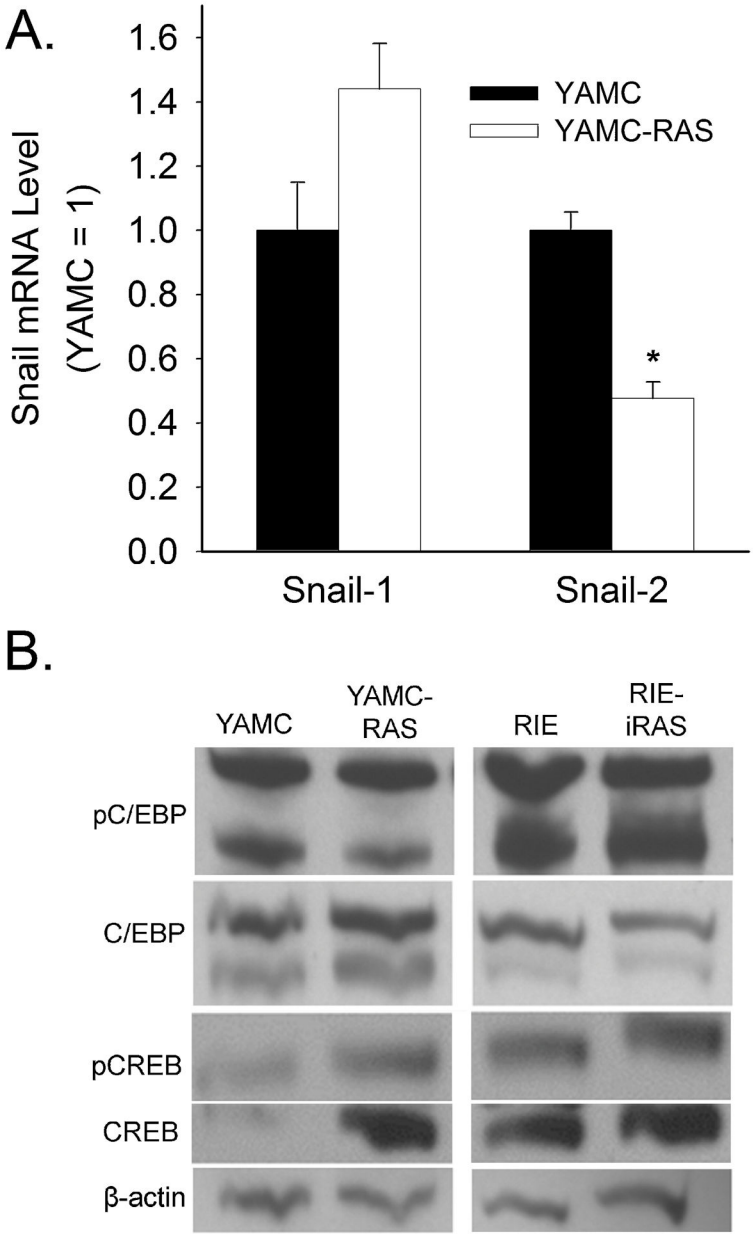

Figure 6.

Expression of transcription factors known to bind to VDR gene regulatory regions. (A) Snail1 and Snail2 mRNA Data are expressed as mean SEM, n=6 per group and normalized to RPLP0 expression. * Significantly different from YAMC value (t-test, $p < 0.05$). (B) Representative Western blots of C/EBPβ, Thr188 phospho-C/EBPβ, CREB, and Ser133 phospho-CREB protein levels. β-actin was used as a loading control.