UNIVERSITY OF CALIFORNIA, SAN DIEGO

Vitamin D Regulates MUC17 Expression in Caco-2 Cells

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in

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by

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Professor Kim Barrett

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The Thesis of Sara Quraish Tabikh is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2010

My work is dedicated to my mother and father, who I am forever indebted to for their undying support, who have always motivated me to achieve of a higher level of education, and who have supported me to always strive for my dream of becoming a medical doctor since the age of three. It is also dedicated to those who suffer from gastrointestinal diseases; I hope this research proves promising towards the development of cures for GI diseases.

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LIST OF ABBREVIATIONS

Abbreviation	Where (page)	Definition
GI	xi	Gastrointestinal
VDR	xi	Vitamin D Receptor
MUC	xi	Mucin (human)
EGF	3	Epidermal Growth Factor
IBD	4	Inflammatory Bowel Disease
UC	4	Ulcerative Colitis
VNTR	4	Variable Number of Tandem Repeats
VDRE	5	Vitamin D Receptor Responsive Element
RXR	5	Retinoid X Receptor
RE	6	Response Element
CRD	8	Cysteine-Rich Domain
L	8	Linker
IBD	12	Inflammatory Bowel Disease
GC	14	Glucocorticoids
AMPK	16	AMP-Activated Protein Kinase

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ABSTRACT OF THE THESIS

Vitamin D Regulates MUC17 Expression in Caco-2 Cells

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Master of Science in Biology

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Professor Silvia Resta-Lenert, Chair
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Mucins are essential components of the gastrointestinal (GI) barrier and their downregulation contributes to the pathogenesis of chronic GI inflammation. Calcium plays an important role in stabilizing negatively charged mucins expressed by intestinal cells. Vitamin D regulates ion homeostasis and intestinal calcium absorption, through the nuclear vitamin D receptor (VDR). MUC17 gene regulator contains a VDR responsive element. We hypothesized that lack of VDR expression would disrupt mucin expression in intestinal epithelial cells. Methods: Caco-2 cells treated with siVDR or untreated control

were tested for MUC17 mRNA expression by PCR and for MUC17 levels by immunohistochemistry. Treatment with low calcium concentration by calcium switch and vitamin D_3 was also studied. Results: siVDR Caco-2 cells showed significant lower levels of immunohistochemically localizable MUC17 than the Caco-2 controls ($p<0.01$). MUC17 mRNA expression was also diminished by a factor of 2.5 in siVDR cells. When cells were exposed to low calcium, little MUC17 was detectable and thus the effect of gene silencing was intensified. Restoration of calcium levels in the siVDR Caco-2 evoked a rapid short-lived recovery of MUC17 mRNA expression. Vitamin D_3 treatment had no effect on both MUC17 mRNA and protein in siVDR Caco-2 cells. However, untreated controls, expressing normal levels of VDR, showed an increased MUC17 mRNA and protein expression after D_3 treatment. Our data underline the importance of calcium homeostasis in the intestinal epithelium and show that vitamin D promotes mucin expression and homeostasis.

INTRODUCTION

The Intestinal Epithelium

The intestinal tract is composed of three tissue layers arrayed in a concentric structure. The outer layer is several sheets of smooth muscle, the middle layer is filled with connective tissue, and the inner luminal surface has a single-cell layer of simple epithelium, termed the mucosa. The intestinal epithelium is the most rapidly self-renewing tissue in the adult mammal. It is ordered into crypts and villi; where crypts are tubular invaginations of the epithelium around the villi. Cells are newly generated in the crypts and cells are lost by apoptosis at the tips of villi. Mucins are expressed and secreted by epithelial cells.

Mucin Background

Mucins are a family of large extracellular O-glycoproteins with complex oligosaccharides, which forms high-ordered structures through the chemical linking of monomeric carbohydrate moieties to the hydroxyl l side of serine and threonine residues (Carlstedt, 1985)[1]. Mucins are expressed by various epithelial cells of the respiratory tract, the stomach, the intestinal tract, and in the secretory surfaces of the gallbladder, pancreas, liver, kidneys, salivary glands, lacrimal glands, and the eye (Forstner, 1978)[2]. Because these environments are subject to changes in its molecular composition, mucins have a role in promoting cell survival and maintaining homeostasis. Membrane-associated mucins communicate via signal transduction with the external environment and therefore serve as cell-surface receptors and sensors. It is believed that mucins might respond to external stimuli by conducting signals to secrete specialized cellular products, proliferate, differentiate, or cause apoptosis.

Mucins are essential components of the gastrointestinal barrier. This barrier is subdivided into an intrinsic and extrinsic barrier; the intrinsic barrier is composed of a layer of epithelial cells lining the GI tract, and the extrinsic barrier contains mucus produced by the epithelial cells. The mucus layer is comprised of membrane-bound mucins anchored at the surface of epithelial cells, which associate with cytoskeletal elements and participate in signal transduction, and by a viscous gel constituted of secreted mucins (Antalis, 1999)[3].

Thus far, a total of 21 mucin genes have been identified including MUC1, -2, -3A, -3B, -4, -5AC, -5B, MUC6-9, MUC11-13, MUC15-17, MUC19 -21 (9, 12, 14, 28, 37). These mucins are classified into two subgroups; membrane-bound and secreted mucins. Membrane-bound mucins are expressed at the apical cell surfaces of gastric pit cells, intestinal enterocytes, and colonic columnar cells. Secreted mucins are found in secretory cells such as gastric mucous cells and intestinal goblet cells. These mucin proteins are very large and typically contain a central domain of tandemly repeated units rich in serine and threonine residues, flanked by unique carboxyl and amino terminal domains. Membrane-bound mucins are characterized by an extracellular region with a short amino terminal domain followed by a large glycosylated tandem repeat domain, a cysteine-rich domain with similarity to epidermal growth factor (EGF)-like motifs, a transmembrane segment and a small cytoplasmic domain. In contrast, the secreted mucins have cysteine-rich amino and carboxyl terminal domains with similarity to Von Willebrand factor, allowing for polymerization through disulfide bonding and results in highly viscous mucous secretions (Ho, 2006)[4].

Mucin Expression and Regulation

Under normal physiological conditions, the production of mucins are optimally maintained by a host of elaborate and coordinated regulatory mechanisms, thereby affording a well-defined pattern of tissue-, time-, and developmental state-specific distribution. However, mucin homeostasis may be disrupted by the action of environmental and/or intrinsic factors that affect cellular integrity. This results in an altered cell behavior that often culminates into a variety of pathological conditions. Deregulated mucin production has indeed been associated with numerous types of cancers and inflammatory disorders. In inflammatory bowel disease (IBD), quantitative and qualitative changes in mucins have been reported. It has been shown that, in active ulcerative colitis (UC), MUC2 synthesis, secretion, and sulfation are all reduced, which makes the colon mucosa more vulnerable to toxic agents and pathogens. MUC2 knock out mice spontaneously develop UC and which can lead to adenocarcinoma. Further, the region of the membrane-bound mucin gene cluster (*MUC3A/B, MUC12,* and *MUC17*) has been implicated in genetic susceptibility to inflammatory bowel disease. The rare variable number of tandem repeat (VNTR) allele of the *MUC3A* mucin gene were more common in patients with ulcerative colitis compared with controls. In patients with a family history of Crohn's disease, a higher number of SNPs that replaces a tyrosine of the MUC3A cytoplasmic domain with an asparagine or histidine was observed compared to patients without a family history. Therefore, the mutated *MUC3* gene may result in a defective protein that would increase susceptibility to inflammatory bowel disease.

Mucin 17

MUC17 is a membrane-bound mucin found on chromosome 7 region q22.1. It transcribes into a 14.2-kb mRNA that contains 13 exons. Alternate splicing creates two variants of MUC 17; a membrane-bound and secreted form (MoniauxN., 2006)[5]. In 2002, Gum *et al.* identified a 59- amino acid tandem repeat, followed by two EGF-like domains, a hydrophobic transmembrane domain, and an 80-amino acid long cytoplasmic tail (Gum, 2002)[6]. Its N-terminal domain is followed by a short unique sequence of 34 amino acids that contains three cysteine residues and is not rich in serine, proline, and threonine[5]. Mucin downregulation contributes to the pathogenesis of chronic GI inflammation. The membrane-bound mucin MUC 17 is highly expressed on the apical surface of the intestinal epithelium and has been shown to maintain barrier integrity and promote healing.

Vitamin D Regulation

Calcium plays an important role in stabilizing negatively charged mucins expressed by intestinal cells. Vitamin D regulates ion homeostasis and intestinal calcium absorption by stimulating intestinal absorption of calcium and phosphate (Haussler, 1997)[7]. Vitamin D is modified by 25-hydroxylase in the liver to produce the active form of the metabolite (Nakane, 2007)[8]. The most biologically potent form of vitamin D is 1α,25-dihydroxyvitamin D3 ($1\alpha,25(OH)_2D_3$), and it plays an essential role for mineral homeostasis. Because of its importance in several bodily functions, the levels of $1\alpha,25(OH)_2D_3$ are tightly regulated by feedback regulation. D_3-24-hydroxylase is an enzyme encoded by the CYP24 gene and is the first step in

the pathway of degradation of $1\alpha,25(OH)_2D_3$. $1\alpha,25(OH)_2D_3$ itself is downregulates the CYP24 gene[8] (supplementary figure 1).

Vitamin D Receptor (VDR)

The effects of $1\alpha,25(OH)_2D_3$ is mediated through the nuclear vitamin D receptor (VDR). VDR is a ligand-activated nuclear receptor that controls gene expression in response to the binding of hydrophobic compounds. Responsiveness of a given gene to $1\alpha,25(OH)_2D_3$ requires that its regulatory regions contain a vitamin D responsive element(VDRE) (Vaisanen, 2007)[9]. The binding of $1\alpha,25(OH)_2D_3$ to VDR causes a conformational change in the ligand-binding domain of the VDR, which enhances its interaction with its heterodimeric partner, the retinoid X receptor (RXR), which is necessary in order for VDR to recognize VDREs in target genes. This change also enhances the activity of histone acetyltransferase, which leads to chromatin decondensation. The VDR-RXR heterodimer then selectively binds to VDREs in VDR target genes (Jurutka, 2007)[10]. The mechanism which these response elements work to regulate gene activity is still unclear (supplementary figure 2).

Vitamin D and Calcium Homeostasis

Vitamin D target organs include kidneys, intestine, bone, and the parathyroid glands. Vitamin D regulates calcium homeostasis. Low dietary calcium causes the release of parathyroid hormone, which subsequently stimulates the conversion of Vitamin D into its active form. The binding of $1\alpha,25(OH)_2D_3$ to VDR leads to the synthesis of calcium-binding proteins and increased transcellular calcium absorption (supplementary figure 3)[23] (Weaver, 2007)[23]. VDR KO mice are hypocalcemic and

exhibit decreased calbindin D_{9k} (a cytosolic calcium binding protein utilized in the facilitated diffusion of calcium) mRNA and protein expression in the duodenum (Song Y., 2002)[24].

Vitamin D and Mucin 17 expression

The MUC17 gene regulator contains a VDR responsive element. The distal VDREs regulate transcription by forming an enhancer via ligand-dependent, dynamic chromatin looping, which brings the distal elements in contact with the transcription start site[5]. We wanted to investigate if the VDR regulates MUC17 expression. We hypothesized that the VDRE on the MUC 17 gene regulator is a positive regulator of MUC 17, and therefore the lack of VDR expression would disrupt mucin expression in intestinal epithelial cells. We also wanted to investigate if other molecules involved with the VDR, such as D_3 and calcium, have an effect on MUC17 expression on silenced VDR cells.

METHODS

Cell Culture

Caco-2 cells, a line of heterogeneous human epithelial colorectal adenocarcinoma cells, were used in all experiments. This cell line was chosen for its particular mucin expression profile: low levels of MUC2 and MUC3, and normal levels of MUC17. This would limit interference of other mucin signaling. Caco-2 were grown as polarized cell monolayers on inserts. Cells were grown in McCoy 5A (Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum. We then treated the cells with medium alone or medium with 1, 25$(OH)_2$ D_3 (D3, 10nM) for 48 hours. For calcium switch experiments, the medium and FBS were previously dialyzed and the calcium concentration adjusted according to protocol (Zhang L., 2006)[22]

Silencing of VDR in cells

Transient VDR knockout cells were generated by gene silencing using electroporation; siRNA for human VDR was used (siRNA: hVDR [accession no. NM_000376], CCCACCUGGCUGAUCUUGUCAGUUA, AUGGCUUCAACCAGCUUAGCAUCC). A nonspecific control siRNA (scrambled, 100 pmol) was used as negative control (Santa Cruz Biotechnology). After silencing, the cells were treated with α-VDR antiserum and analyzed under reducing conditions by SDS-polyacrylamide gel electrophoresis and mRNA analysis.

Immunoblot Analysis

Total cell lysates were on ice using 200 μL of RIPA buffer [10 mM Tris-HCl (pH 8.0), containing 150 mM NaCl, 2mM phenylmethylsulfonyl fluoride (PMSF), 10

mM NaF, 1 mM sodium orthovanadate (Na_3VO_4), supplemented with Complete™ Protease Inhibitor Cocktail tablets (Antipain-dihydrochloride, Aprotinin, Bestatin, Chymostatin, E-64, EDTA-Na_2 , Leupeptin, Pefabloc SC, Pepstatin, Phosphoramidon; Roche Applied Science, Basel, Switzerland). Protein samples were quantified using a modified Lowry method (D_C Protein Assay, Bio-Rad, Hercules, CA). Seventy five µg of each protein sample were denatured for 10 min at 95 °C in the presence of 1× denaturing loading dye (6× loading dye: 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 1% SDS, 100 mM EDTA, pH 7.6) in a 36 µl volume. Horizontal agarose westerns were performed on 2% Tris-glycine agarose gels containing 0.1% sodium dodecyl sulfate (SDS) in SDS/Tris-glycine migration buffer (0.1% SDS, 25 mM Tris base, 115 mM glycine) at 100V for 6 h. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore, Boston, MA) by passive diffusion for 22 h using SDS/Tris-glycine buffer (containing 20% methanol, 0.025% SDS). Membranes were blocked for 2 h in PBS containing 5% Carnation® instant nonfat dry milk. Primary antibody (MUC17 rabbit polyclonal antibody (4)) was diluted in antibody diluent (PBS,1% in BSA, 0.1% in Tween 20) at 1:1000 and was incubated with membranes at 4 ºC overnight with gentle agitation (15 hr). Washes were carried in TBST (Tris-Buffered Saline, containing 0.05% Tween 20) for a total of 45 min and consisted of two quick rinses followed by three 15 min wash periods with agitation. Secondary antibody (anti-rabbit, GE Healthcare Bio-Sciences, Uppsala, Sweden) was diluted at 1:7500 in antibody diluent (PBS 1% in BSA, .1% in Tween 20) and were incubated with membranes for 1 h. Washing to remove secondary antibody was performed as

previously mentioned with a final rinse with PBS. Autoradiography was performed following ECL (Thermo Scientific, Pierce Biotechnology, Rockford, IL) incubation for 1 min. Protein band intensity was imaged using the Bio-Rad Gel Doc™ EQ system and quantified with Bio-Rad Quantity One® Software (version 4.1.1).

Calcium Switch

Calcium switch: Caco-2 cells were split in $CaCl_2$-free McCoy's with 10% dialyzed FCS. Cells were grown initially at a final Ca++ concentration of 50 μM by adding 2 M $CaCl_2$ (Ca++-switch cells) for 48 hours, then switched to 2mM calcium concentration after washings in calcium-free medium. Control cells were prepared in McCoy's/dialyzed FCS, with a final Ca++ concentration of the control cells of 2 mM.

RNA Isolation

The cell monolayers were used to isolate total RNA using the guanidine isothicyanate-cesium chloride cushion ultra centrifugation technique. The RNA was stored with 2.5 volumes of ethanol in 0.3 M sodium acetate at -70ºC (Ho, 2006)[4].

Reverse-Transcriptase-PCR

Caco-2 cells were seeded onto T25 flasks, and when cells reached approximately 85% confluency, TRI Reagent (Ambion, Austin, TX) was used to extract RNA. The RNA extract was then treated with TurboDNase (Ambion, Austin, TX), and the extract was reverse-transcribed with M-MLV (Invitrogen, Carlsbad, CA). This was

then screened for MUC17 and VDR with PCR. Forward primer: GGG CCA GCA TAG CTT CGA. Reverse primer: GCT ACA GGA ATT GTG GGA GTT CA. VDR sense primer: GCTATTGGCGGGAGTGGAC, Antisense: GCCGGGAGAGCTCATACAGA. GAPDH sense: TGATGACATCAAGAAGGTGGTGAAG, antisense: TCCTTGGAGGCCATGTGGGCCAT. GAPDH was used as a control: sense TGATGACATCAAGAAGGTGGTGAAG, antisense TCCTTGGAGGCCATGTGGGCCAT. The PCR products were viewed by gel electrophoresis. All blots were scanned for intensity of signal and analyzed using NIH Imager software.

Immunofluorescence and Confocal Fluorescence Microscopy

Caco-2 cells were incubated on inserts for 7 days in McCoy's 5A media. On the 7th day, they were removed and immediately fixed in a 5% Paraformaldehyde in PBS solution. Following washing, the cells were permealized with a 0.1% solution of Triton-X 100 in PBS, and washed again. The cells were then treated with primary antibody for Muc-17, rabbit species, and subsequently with the anti-GFP fluorescent protein rabbit conjugate IgG antibody Alexa Fluor® 488 (Invitrogen, Carlsbad, CA; excitation 495 nm, emission 519 nm). At this step, the cells were treated with the Dapi blue nuclear stain (Life technologies, Carlsbad, CA; excitation, 358 nm, emission, 461 nm). The cells were mounted on glass plates with the antifading mounting medium ProLong® (Invitrogen, Carlsbad, CA). The slides were visualized within 2 days.

Images were acquired using a Zeiss Light Sensitive Microscope (LSM) model 510 (Zeiss, Jena, Germany) with Lasers.

Statistical Analysis

The group data were expressed as means (SEM). The two-tailed unpaired Student t test was utilized and determined the analysis between the groups. One factor ANOVA with 95% confidence intervals was used for the analysis between multiple groups. SigmaPlot software (Systat, San Jose, CA) and Statview software (version 5.0.1; SAS Institute Inc., Car, North Carolina, USA) were used.

RESULTS

Expression profiling of VDR knockouts in Caco-2 cells

To investigate the ability of VDR and its ligand to regulate MUC17 expression, we chose to test this hypothesis in cells that were positive for the expression of VDR at both mRNA and protein levels, and that could effectively be silenced for VDR. To establish this model system in which to study the expression level and transcriptional responsiveness of MUC17 to VDR regulation, we have chosen the human epithelial colorectal adenocarcinoma cell line Caco-2, which is derived from colon carcinoma and expresses MUC17 at normal levels. Moreover, this cell line shows reduced expression of other mucins (MUC2 and MUC3), thus limiting interference of other mucin signaling and expression. Caco-2 also expresses normal levels of VDR, normal levels of all the calcium binding proteins and calcium transporters, thus representing an almost ideal model for our studies compared to other available human intestinal cell lines (Resta-Lenert, 2010)[25]. The results show that in silenced VDR cells, human VDR mRNA and protein expression have been effectively suppressed compared to controls (Figure 1). This VDR-silenced cell model was easily reproducible and the results were confirmed by multiple assays (N=6; VDR mRNA vs. controls, $p<0.01$).

Expression profiling of MUC17 in VDR knockouts-

Next we studied the characteristics of MUC17 expression in VDR knockout Caco-2 cells. The transient VDR knockout cells were assayed with α-MUC17 antiserum and specific nucleic acid probes by Western and Northern Blot analysis for protein and mRNA levels, respectively. Compared to controls, VDR-silenced Caco-2 cells

showed an approximate two-fold significant reduction in both MUC17 mRNA and MUC17 protein (Figure 2; N=4; MUC17 mRNA vs controls, $p<0.01$; MUC17 protein vs. controls, $p<0.05$).

Expression profiling of MUC17 after D_3 treatment in siVDR cells-

Since VDR is activated by interaction with its ligand, and together they initiate a complex cascade of gene regulation; we explored the effect of the treatment of both naïve Caco-2 and VDR silenced Caco-2 with active vitamin D (D3). Polarized monolayers of Caco-2 cells were treated with medium alone (control) or medium with $1\alpha,25(OH)_2D_3$ (D_3, 10nM) for 48 hours. As predicted, the experiments revealed that treatment with D_3 did not affect siVDR cells and MUC17 expression remained low in these cells. However, control cells showed enhanced expression of MUC17 at both the mRNA and protein levels (Figure 3). These results confirm the hypothesis that VDR is implicated in the regulation of MUC17 gene expression and that VDR signaling is activated by interaction with its ligand, vitamin D3, upon which interaction the receptor can bind and activate responsive elements in specific genes. Thus VDR and vitamin D may probably produce some of their protective effects on the intestinal epithelium through enhancing the mucin barrier between epithelial cells and luminal contents.

Expression profiling of MUC17 in low calcium concentrations in VDR knockouts-

We investigated the possibility that calcium may have some direct effect on the expression of MUC17 for several reasons. Both canonical (VDR plus ligand) and non-canonical (VDR alone) VDR signaling are implicated in calcium absorption by epithelial cells in the intestine (Resta-Lenert, 2007-2010)[25-31]. Also, our laboratory has shown that VDR activation elicits an immediate increase in TRPV6 calcium transporter and consequently an increase in active calcium transport across Caco-2 cells; and because calcium is considered crucial in mucins tertiary protein structure stabilization, we investigated this possiblity. The effect of normal versus low calcium concentration on MUC17 expression was then investigated. The transient VDR knockout cell controls were treated with McCoy's/dialyzed FCS with a final calcium concentration of 2mM. A calcium switch was performed on the transient VDR knockouts using $CaCl_2$-free medium with 10% dialyzed FCS. The final calcium concentration was raised to 50μM by adding 2M $CaCl_2$ for eight hours. The MUC17 protein levels were, in fact, significantly reduced in siVDR and control cells (Figure 4; N=4). Surprisingly, we also observed that low extracellular calcium concentration induced a small but significant decline of MUC17 mRNA only in siVDR cells, and this effect could be reversed by calcium switch for 8 hours (Figure 4). At this time we do not know why low calcium levels in the extracellular environment alters MUC17 expression. We can only speculate that this effect may results from feedback mechanisms involved in VDR signaling and more studies will be needed to clarify this point.

Expression profiling of MUC17 in siVDR-treated and untreated cells by confocal microscopy-

The distribution of MUC17 in siVDR-treated and untreated Caco-2 cells was investigated by immunofluorescent confocal microscopy. Caco-2 cells were incubated on inserts for 7 days in McCoy 5A media. On the 7th day, they were removed and immediately fixed in a 5% Paraformaldehyde in PBS solution. Following washing, the cells were permealized with a 0.1% solution of Triton-X 100 in PBS, and washed again. We found that the distribution and expression of MUC17 are significantly altered in siVDR-treated compared to control cells. Treatment with Vitamin D_3 produced a significant increase in the fluorescent signal for MUC17 in control Caco-2 cells, whereas siVDR cells showed no appreciable change. When siVDR cells were kept in low calcium conditions, we observed very low levels of MUC17. However, this effect was reversed by the switch to normal calcium concentrations in the growth medium. Vitamin D_3 had no effect on siVDR cells under low calcium concentration or after calcium switch. Low calcium and calcium switch had no significant effect on control Caco-2 cells, untreated or D_3-treated (Figure 5). These confocal studies confirmed the pattern of protein expression we had observed in the previous experiments, and added some insight on the disruption of MUC17 protein distribution on the surface of Caco-2 cells in the different experimental conditions adopted in this project. It is interesting to note that, again, low extracellular calcium significantly affected the levels of MUC17 in Caco-2 cells in which the VDR gene had been silenced compared to naïve cells (not shown).

DISCUSSION

Over the years, much interest in the study of mucins has increased due to their implications in numerous disorders for the development of early diagnostics (Yin, 2002)[14] and therapeutics (Pecher, 2002)[15] (Palmer, 2001)[16]. Epithelial cells maintain a dynamic mucus layer attracted by electrostatic forces. Mucins are thought to be important to the defense of epithelial surfaces, including digestive, ocular, reproductive, and respiratory surfaces.

Transmembrane mucins are thought to have important functions in the control of the mucosal surface environment. Goblet cells use calcium as the main element to compact mucins. Mucus is able to facilitate the migration of calcium from the lumen of the GI tract to inside the epithelial cells.

MUC 17 is a membrane-bound mucin highly expressed on the surface of intestinal cells and is thought to be cytoprotective. MUC 17 contains a large structural domain that contains >4000 amino acids followed by two cysteine-rich domains (CRD) separated by a linker (L) domain containing a sea-urchin sperm protein, enterokinase and agrin (SEA) module, followed by a cytoplasmic domain of 80 amino acids. It has been shown that MUC 17 is involved in the cell restitution process (Luu Y., 2010)[17]. In a high endogenous MUC17-expressing cell line, inhibition of the MUC17 mucin showed reduced cellular aggregation, increased susceptibility to apoptosis, and reduced spontaneous cell migration[17]. This may implicate the importance of membrane-bound mucins in cell to cell interactions and cell restitution[17]. Specifically, Luu *et al.* found that a recombinant protein containing

the two CRDs of MUC17 stimulated ERK phosphorylation, and resulted in enhanced cell migration, implicating that the CRDs of membrane-bound mucins may be the active site for extracting these processes[17].

Cell migration is an important process that allows for rapid cell restitution following intestinal injury. Cell migration allows for cell restitution to occur more rapidly than cell proliferation, and it has been shown to occur in response to intestinal injury (Feil W, 1987)[18]. The amount of epithelial damage that occurs in inflammatory conditions is correlated to the amount of apoptosis occuring (Borges HL, 2005)[19]. Agents that are anti-apoptotic and enhance cell migration are potentially therapeutic for inflammatory bowel disease (IBD), although they may enhance risk of cancer[17]. When MUC17-CRD1-L-CRD2 was administered in vitro to mouse models of colitis, stimulation of cell migration and inhibition of apoptosis was observed, overall demonstrating accelerated histologic healing[17]. Use of these recombinant mucin proteins may potentially serve as a therapy for mucosal diseases and IBD[17].

The vitamin D receptor (VDR) is a member of the nuclear receptor family of transcription factors. Vitamin D activates the VDR to form a heterodimer with the RXR, revealing the nuclear localization sequence which allows for import into the nucleus, which then binds to hormone response elements on DNA resulting in expression of specific gene products. We were interested in seeing if the VDR regulates MUC 17 expression, and our data demonstrates that the silencing of the VDR significantly reduces the expression of MUC17 mRNA and protein in Caco-2 cells. This may occur because vitamin D no longer has a target in the siVDR cells,

and thus there is no activation of the the VDR to form the VDR/RXR heterodimer to bind to the VDRE on the MUC17 gene regulator. Thus the lack of the heterodimer binding to the MUC17 VDRE may account for the decreased expression of both MUC17 mRNA and protein, implying that VDR plays an important role in the expression of MUC17.

Glucocorticoids (GCs) are involved in a feedback mechanism with the immune system to reduce inflammation. GCs bind to glucocorticoid receptors (GCR), and then the activated GCR complex then upregulates the expression of anti-inflammatory proteins and represses the expression of pro-inflammatory proteins. GCs are known to decrease the expression of VDRs, and because of this decrease therefore regulate the intestinal transport of calcium.

Under low calcium conditions, there is an increased synthesis of calcium-binding proteins mediated through the VDR, increasing trancellular calcium absorption. Vitamin D regulates the flux of calcium from the lumen to the basolateral side of the epithelium. Zhang *et al.* found that calcium plays an role in the activation of AMP-activated protein kinase (AMPK), which regulates the assembly of epithelial tight junctions (Zhang L., 2006)[22]. Cell-cell junctions in polarized epithelial cells are lost upon withdrawl of calcium in the medium, and the reinitiation of tight junctions occurs with the readdition of calcium (calcium switch)[22]. We were interested to see if molecules associated with VDRs, such as calcium and vitamin D, have any influence on the regulation of MUC17. After using cells with significantly reduced VDR, low calcium concentrations further reduced the expression of the MUC17 mRNA but the effect was reversed upon a calcium

switch of 12 hours. The small but significant decline of MUC17 mRNA in siVDR cells due to low extracellular calcium concentrations may suggest a possible role of calcium and its interaction with VDR to regulate MUC17 expression especially when VDR expression is low. Is the lack of VDR expression a limiting factor for active calcium transport via the transient receptor potential cation channel, subfamily V, member 6 (TRPV6)? TRPV6 regulates the process of active calcium absorption in the proximal small intestine by mediating the transfer of calcium across the intestinal apical membrane (Peleg S, 2010)[32]. D_3 increases the expression of TRPV6. (Lieben L, 2010)[33]. It has been demonstrated that in absence of active TRPV6, the level of calcium transport is markedly reduced (Resta-Lenert, 2010)[25]. However, passive calcium transport via diffusion and paracellular transport, obviate to limiting factors such as vitamin D presence and VDR activation (Wasserman, 2004)[34]. Our results did not resolve the implications precisely, and further investigation is required to delineate the mechanism by which D_3, VDR, TRPV6, and calcium affects VDRE target genes.

VDR is a phosphoprotein that binds the hormone $1,25(OH)_2D_3$ with high affinity and regulates the expression of genes via zinc finger-mediated DNA binding and protein-protein interactions[7]. Our results demonstrated control cells showed enhanced expression of MUC17 at both mRNA and protein levels after treatment of D_3. In 1997, Haussler *et al.* demonstrated that the $1,25(OH)_2D_3$ ligand promotes the heterodimerization of VDR-RXR, and specific high-affinity VDRE binding[7]. Taken together, the upregulation of MUC17 may occur through this pathway, where D_3 activates transcription of the MUC17 gene. Because silenced VDR cells did not alter

MUC17 levels upon addition of D_3, this further supports the notion that vitamin D enhances MUC17 mucin expression on the gut epithelial barrier.

Both quantitative and qualitative changes in mucins may contribute to impaired mucosal integrity, which is a feature of IBD (Buisine M.P., 2001)[20]. Our results demonstrated the significantly altered expression of MUC17 in siVDR-treated compared to control cells, where no appreciable change in MUC17 expression was observed in siVDR cells compared to control cells. The expression of MUC17 increased significantly with D_3 treatment, as expected from our results from the previous experiments. Overall, our data supports the hypothesis that the protective effect exerted by vitamin D on the gut epithelial barrier may be partially due to enhanced MUC17 mucin expression. This effect may contribute to the maintenance of gut homeostasis.

In conclusion, the data in this thesis demonstrates that vitamin D and endogenous VDR plays an important role in the regulation of the expression of MUC 17. MUC 17 plays an important role in cell restitution processes[17], and generally the altered expression of mucins may compromise the integrity of the mucosal epithelial barrier and the relevance of intestinal barrier functions to the cause of IBD has been reviewed (McGuckin MA, 2008)[21]. Further studies are required to explore the potential effects of the VDR *in vivo*, however a mouse model demonstrating silenced VDRs prove to be nonviable. The use of vitamin D and VDRs to enhance MUC17 expression can potentially be used as a therapeutic to augment the protective effect on the gut epithelial barrier.

TABLES AND FIGURES

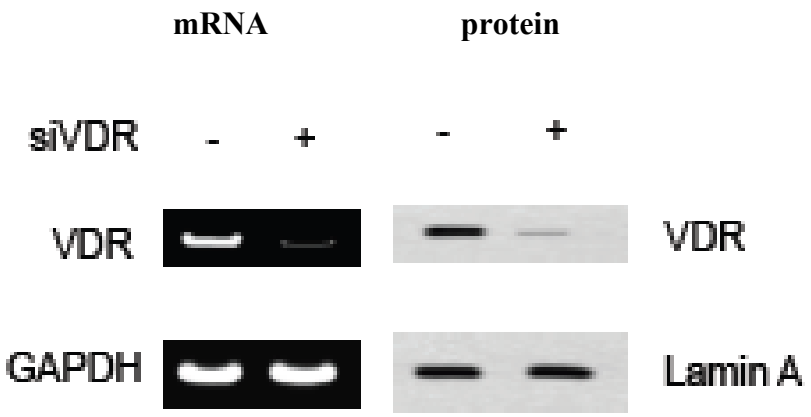
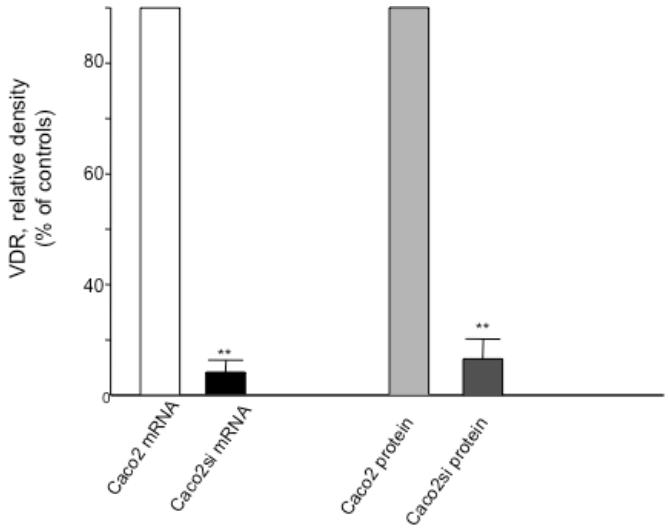


FIGURE 1 (a): ***Expression profiling of VDR knockouts in Caco-2 cells*** To test the role of VDR in regulating MUC17 expression, transiently-generated VDR knockout cells by gene silencing using electroporation was utilized. First panel shows Northern Blot results revealing significant suppression of hVDR in silenced cells compared to control. Right panel shows similar results for VDR protein expression; Western Blot reveals that in siVDR cells, hVDR protein expression have been effectively suppressed compared to controls.

FIGURE 1 (b): ***Quantification of expression profiling of VDR knockouts in Caco-2 cells*** Left bars show control Caco-2 VDR mRNA and Caco-2 silenced VDR mRNA. Right bars show VDR protein expression in control and silenced cells; and that cells have their VDR effectively suppressed compared to controls. (*Mean+SEM; N=4; *, p<0.05, **, p<0.01, silenced vs control; Student's t test).* Naïve cells had similar results as scrambled cells which are not shown for simplicity.



protein

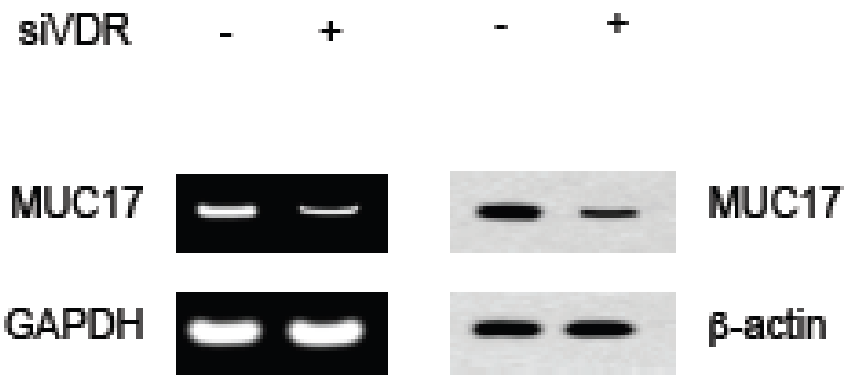
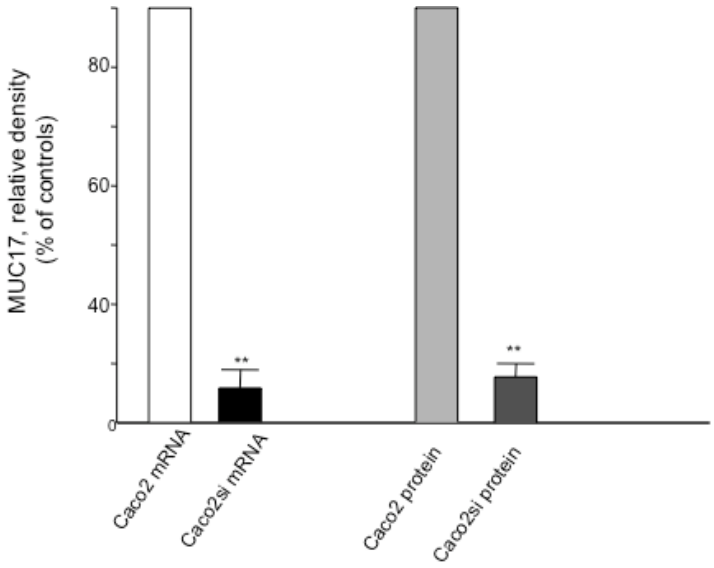


FIGURE 2 (a): ***Expression profiling of MUC17 in VDR knockouts*** We tested VDR-silenced cells and controls for MUC17 mRNA and protein expression. Northern Blot results on the left panel demonstrate that siVDR Caco-2 showed significant reduction of MUC17 mRNA. Right panel shows results from Western Blot and a significant reduction in MUC17 protein.

FIGURE 2 (b): ***Quantification of the expression profiling of MUC17 in VDR knockouts*** Left bars show control Caco-2 MUC17 mRNA and Caco-2 silenced VDR MUC17 mRNA is significantly reduced. Right bars show MUC17 protein expression in control and silenced cells; and that cells that have their VDR effectively suppressed show marked MUC17 protein downregulation compared to controls. *Mean+SEM; N=4; *, p<0.05, **, p<0.01, silenced vs control; Student's t test).* Naïve cells had similar results as scrambled cells which are not shown for simplicity.



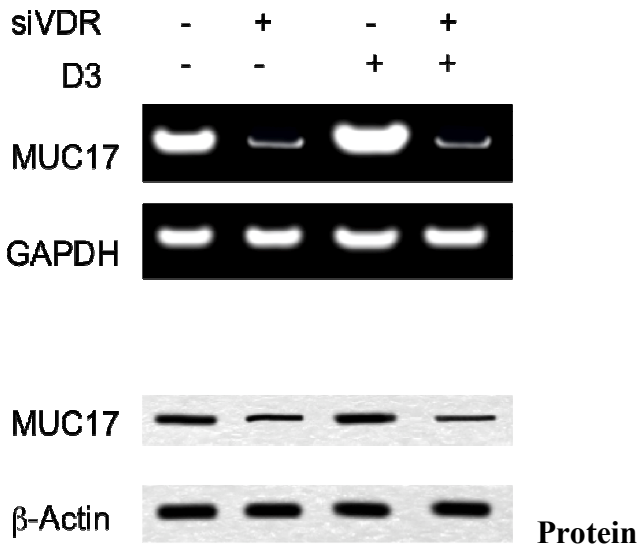
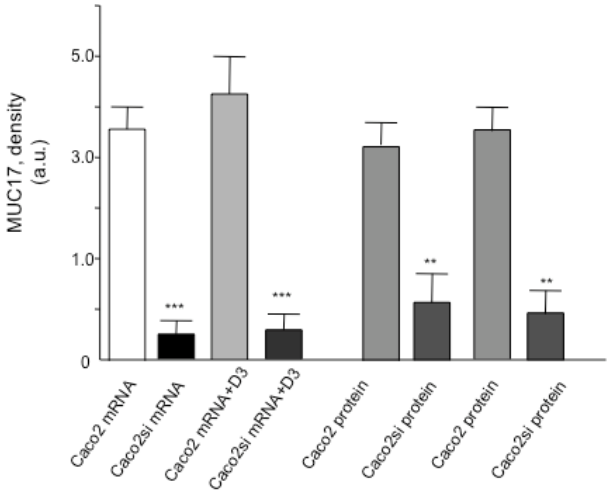


FIGURE 3 (a): ***Expression profiling of MUC17 after D_3 treatment in siVDR cells*** Upper panel shows MUC17 mRNA in silenced and control cells treated with D_3. Treatment with D3 did not affect siVDR cells and MUC17 mRNA expression remained low. Control cells showed enhanced expression of MUC17 mRNA. Lower panel shows similar results for MUC17 protein; control cells showed enhanced expression of MUC17 protein with D_3 treatment; siVDR cells were not affected at protein levels.

FIGURE 3 (b): ***Quantified* expression profiling of MUC17 after D_3 treatment in siVDR cells** Left bars show control Caco-2 MUC17 mRNA and siVDR cell MUC17 mRNA is significantly reduced. Treatment with D_3 had no significant effect on siVDR cells. Right bars show MUC17 protein expression in control and silenced cells; and D_3-treated cells show enhanced MUC17 protein expression in controls. MUC17 expression was not affected by D_3 treatment in siVDR cells. *Mean+SEM; N=4; *, p<0.05, **, p<0.01, silenced vs control; Student's t test).* Naïve cells had similar results as scrambled cells which are not shown for simplicity.



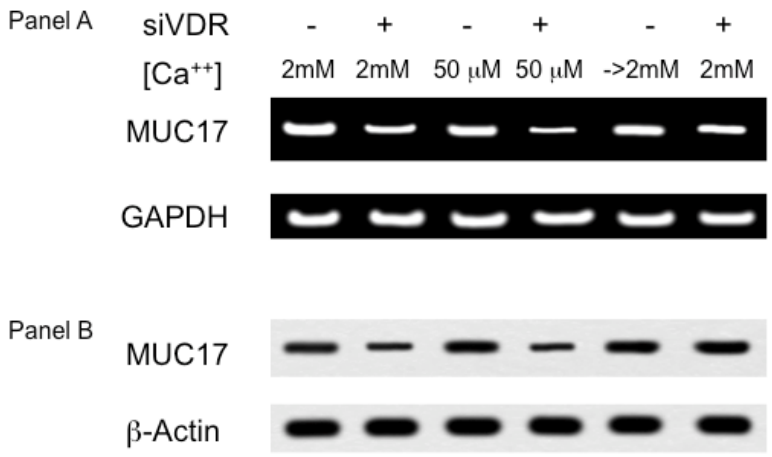
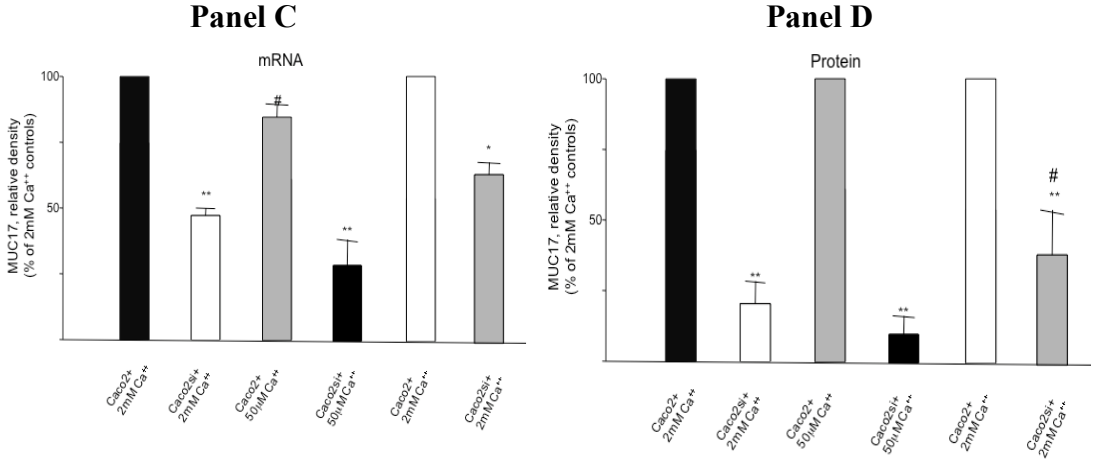


FIGURE 4 (a): ***Expression profiling of MUC17 in low calcium concentrations in VDR knockouts*** The effect of low versus normal concentrations of calcium on MUC17 expression was tested. Panel A demonstrates that a switch to low extracellular calcium concentration (50μM) induces a small but significant decline of MUC17 mRNA only in siVDR cells, and this effect could be reversed by a calcium switch (8h). Similar results were observed in panel B for MUC17 protein expression.

FIGURE 4 (b): ***Quantified expression profiling of MUC17 in low calcium concentrations in VDR knockouts*** The effect of low versus normal concentrations of calcium on MUC17 expression were quantified. Panel C corresponds to MUC17 mRNA expression, and Panel D shows MUC17 protein expression in response to a switch to low extracellular calcium concentration (50μM). *Mean+SEM; N=4; *, p<0.05, **, p<0.01, silenced vs control; Student's t test).* Naïve cells had similar results as scrambled cells which are not shown for simplicity.



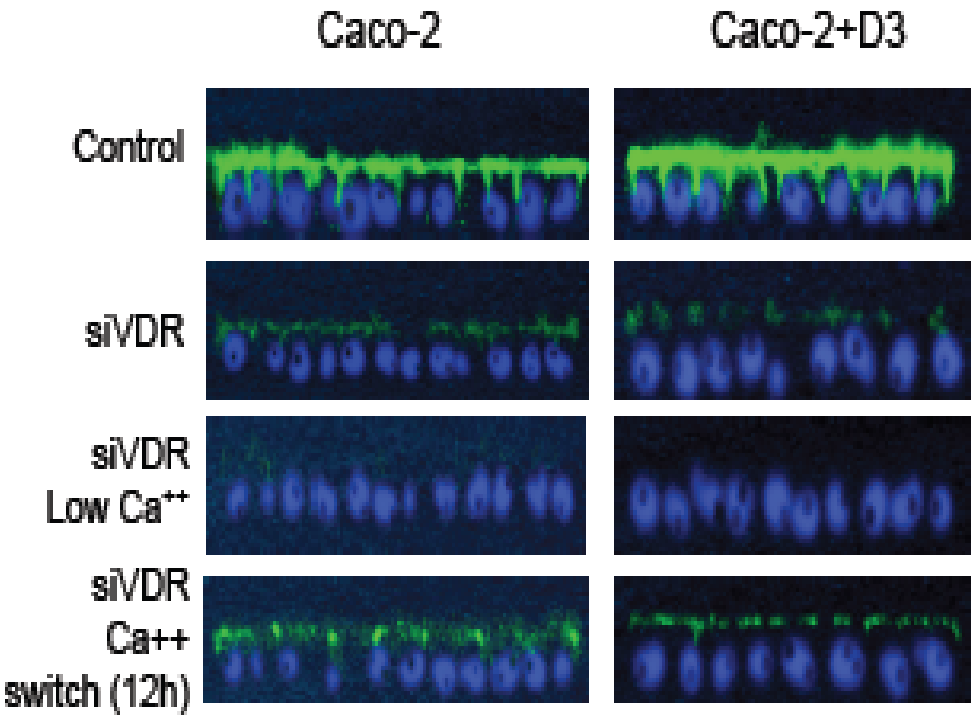
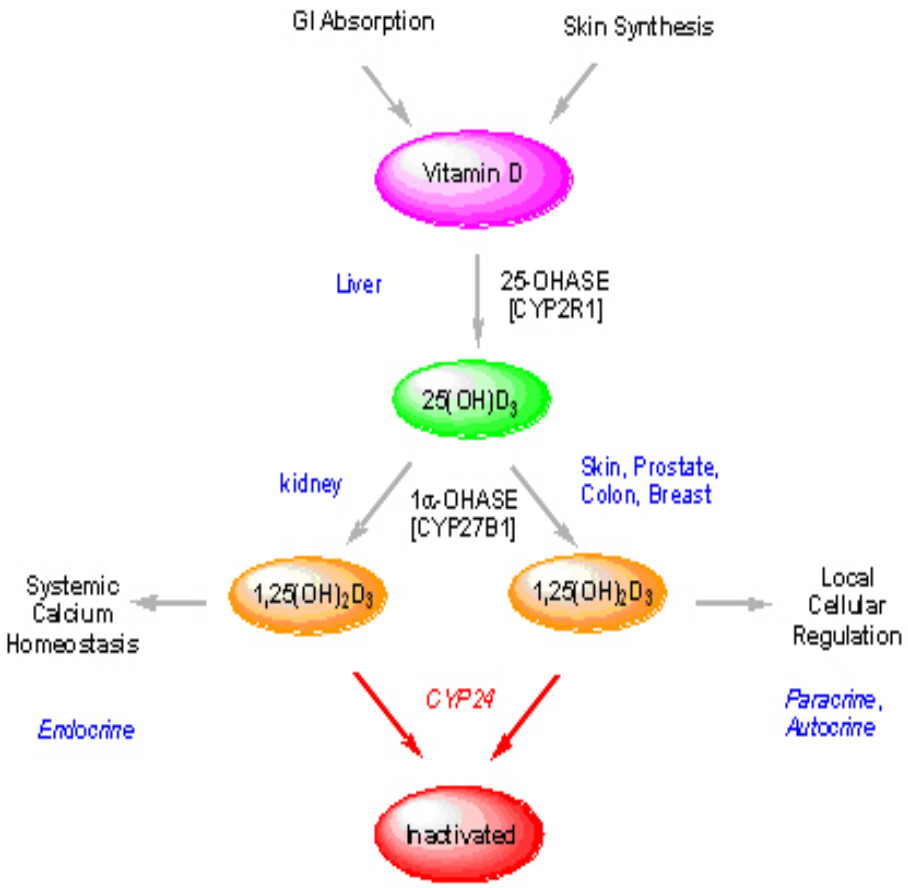
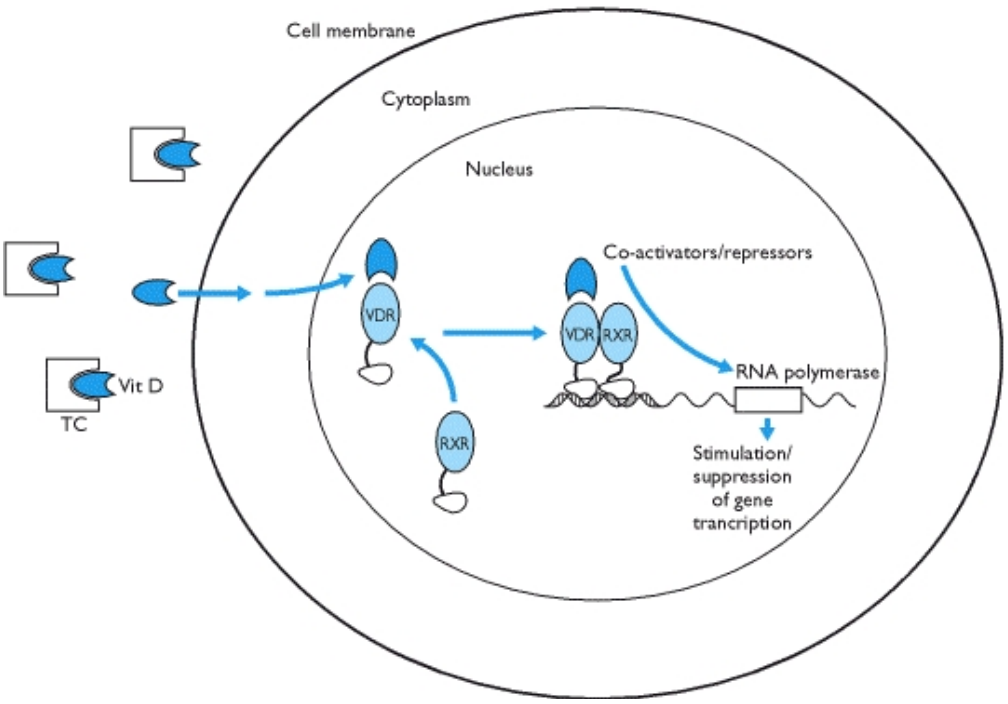


FIGURE 5: ***Expression profiling of MUC17 in siVDR-treated and untreated cells by immunoflluorescent confocal microscopy*** We then investigated the distribution of MUC17 in siVDR-treated and untreated Caco-2 cells by immunofluorescent confocal microscopy. We found that the distribution and expression of MUC17 are significantly altered in siVDR-treated compared to control cells. Treatment with Vitamin D3 produced a significant increase in the fluorescent signal for MUC17 in control Caco-2 cells, whereas siVDR cells showed no appreciable change. When siVDR cells were kept in low calcium conditions, we observed very low levels of MUC17. However, this effect was reversed by the switch to normal calcium concentrations in the growth medium. (Figure 4). Vitamin D3 had no effect on siVDR cells under low calcium concentration or after calcium switch. As shown in the data, low calcium and calcium switch had no significant effect on control Caco-2 cells, untreated or D3-treated.

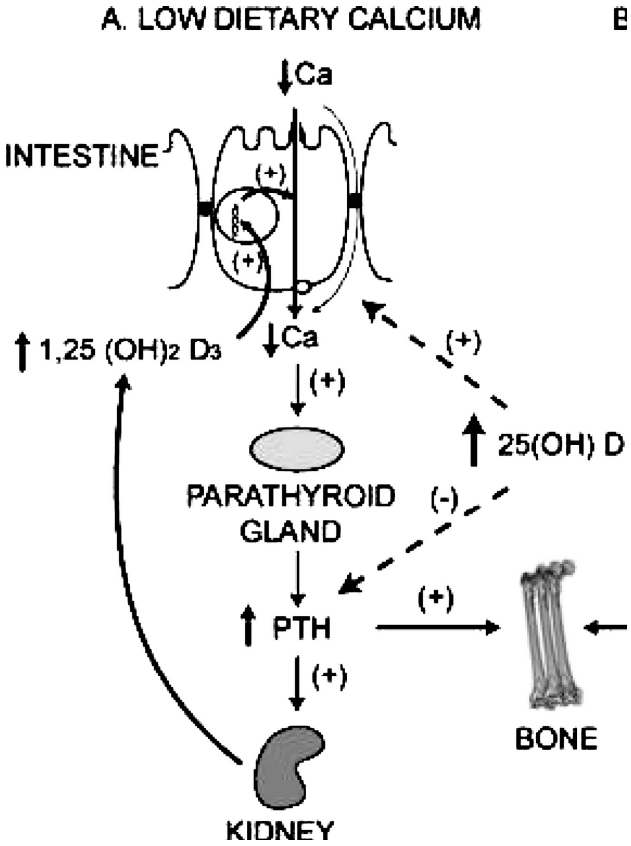
APPENDIX



Supplementary Figure 1: *Schematic of Vitamin D regulation*



Supplementary Figure 2: *Schematic of Vitamin D Receptor Activation*



Supplementary Figure 3: *Schematic of Vitamin D regulation of Low Dietary Calcium*

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