1-1-2015

Desensitization Of Hacat Keratinocytes To Vitamin D3 Occurs Via Loss Of Redd1 Regulation Of The Mtor Pathway

Michelle Leigh Jones
Wayne State University,

DESENSITIZATION OF HACAT KERATINOCYTES TO VITAMIN D_3 OCCURS VIA LOSS OF REDD1 REGULATION OF THE MTOR PATHWAY

by

MICHELLE LEIGH JONES

THESIS

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

2015

MAJOR: NUTRITION AND FOOD SCIENCE

Approved By:

_____

Advisor Date

DEDICATION

I dedicate this work to my loving husband, Chuck, whose support and encouragement enabled me to go back to school. You have built me up and given me the confidence to achieve my dreams. Thank you for allowing me to see what you see.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Ahmad Heydari, for his guidance and support of this work. He inspired in me the confidence to develop myself as a researcher and a teacher. My experience in his laboratory enabled me learn and refine essential skills that will benefit me both personally and professionally for years to come.

I would like to thank my committee, Drs. Diane Cabelof and Pramod Khosla, for sharing their invaluable time to oversee my defense.

I would also like to thank Ali Fardous, Safa Fardous, Tom Prychitko, and Dr. Rawia Khasawneh for their support in the lab. A special thank you goes to Ali for troubleshooting new western blot techniques and sharing his knowledge with me.

Last, I would like to thank Debbie Zebari for assuring I met all of my requirements for graduation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: Introduction

Skin cancer is the most common form of cancer in the world.[1,2,3] It is generally split between two main categories: melanoma from melanocytes, and non-melanoma skin cancer (NMSC), which consists of basal cell and squamous cell carcinomas. Melanoma is the least common, but the most malignant. Basal cell carcinoma (BCC) is the most benign and also the most prevalent. It accounts for 80% of all skin cancers, making it the most common form of any cancer in the world.[1] BCC presents as a "flat, firm, pale areas" or "small, raised, pink or red bumps that may bleed after a minor injury."[1,2] Treatment often involves surgical removal of the affected area and can leave disfiguring scars, especially in cases of large tumors.[1] Additionally, scarring from tumors located in regions around the eyes, ears, and mouth may be structurally compromising and aesthetically displeasing.[4] Instances of BCC are increasing, and because of its prevalence and recurrence, the cost to healthcare is tremendous. Over the last decade or so the cost of treating skin cancer has jumped from $3.6 billion to $8.1 billion annually, an increase of 126.6%. Of that, NMSC accounted for an increase from $2.7 to $4.8 billion.[3] For these reasons, non-invasive outpatient treatments for basal cell carcinoma are of great interest.[4]

Vitamin D_3 supplementation is often considered in the treatment of cancer, including BCC, due to its well-documented anti-proliferative effects.[5,6] In the absence of vitamin D signaling, mice exposed to ultraviolet (UV) light develop skin cancer, underscoring its importance.[7,8] Indeed, the regulatory role of vitamin D most likely evolved from a necessity to protect against the harmful effects of UV light.[6] However, while adequate levels may be protective, supplementation may not necessarily add additional benefit. Animal and cell culture studies have in fact found protective effects of vitamin D in several forms of cancer models, and clinical

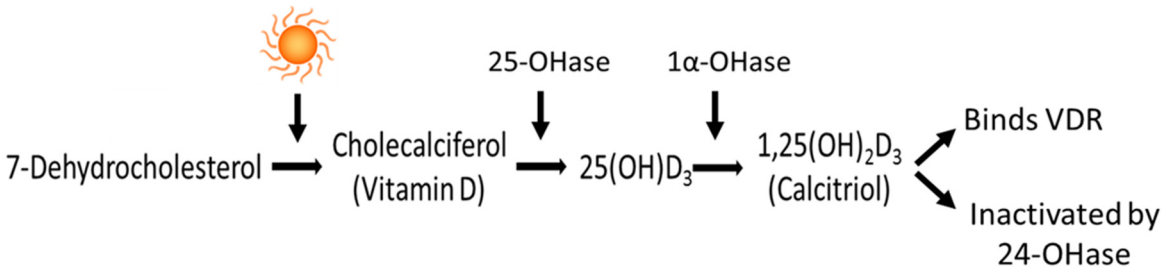
studies of supplementation have been positive in cases of breast and colorectal cancer.[8,9] Unfortunately, human clinical studies of BCC have been rare and inconclusive as to its actual benefit, and intervention studies are essentially non-existent. Analysis of two prospective cohort studies found no association between dietary vitamin D intake and BCC occurrence in men or women.[10,11] Another study found an inverse relationship between serum levels of the circulating form of vitamin D and NMSC in elderly men.[12] On the other hand, studies have also shown that high serum levels actually increase the risk of developing BCC.[13,14] The data is further confounded because of the varied impact of vitamin D_3 on multiple signaling pathways and the mutagenic nature of the cancer. More data is needed to elucidate the actual effects of vitamin D treatment in BCC.

Vitamin D is a general term for a group of fat soluble vitamins that are most notably responsible for calcium homeostasis in the body.[15] The most common forms are vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol), and it is D_3 that shows promise in the treatment of cancer. The metabolism of vitamin D_3 involves several steps that occur in different organs (Figure 1.1). The body is capable of synthesizing 7-dehydrocholesterol in the skin, however, this form of the vitamin requires UV light to convert it to cholecalciferol. Cholecalciferol can also be provided in the diet by a limited number of foods, such as eggs and fortified milk.[6,16] It is this form that is referred to as vitamin D_3, although it still requires the addition of two hydroxyl groups before it is biologically active. Cholecalciferol first must travel to the liver, where the enzyme vitamin D 25-hydroxylase (25-OHase) converts it to 25-hydroxycholecalciferol, also known as calcidiol. Next, the vitamin precursor travels to the kidneys, where the enzyme 25-hydroxyvitamin D_3 1-alpha-hydroxylase (1α-OHase) converts it to 1,25-dihydroxycholecalciferol, or calcitriol.[6,16] This

is the active form, which is capable of binding the vitamin D receptor (VDR), a transcription factor that controls the expression of over 900 genes, many of which are involved in cell cycle regulation, differentiation, and apoptosis.[17] Therefore, vitamin D_3 and its receptor may play an important role in the treatment of cancer, possibly by way of controlling cellular proliferation.

One theory behind vitamin D_3 supplementation in cases of BCC is that by providing additional vitamin D_3 one can prevent uncontrolled cell growth by encouraging VDR-induced inhibition of cellular proliferation. Also present in the kidneys is an enzyme called 25-hydroxyvitamin D3-24-hydroxylase (24-OHase), which adds a third hydroxyl group to vitamin D_3. This enzyme is up-regulated in response to calcitriol, and results in its inactivation and degradation to calcitroic acid.[6,16] High expression of 24-hydroxylase was associated with poor survival in lung cancer patients.[17] Interestingly, all three of these enzymes are also expressed in the skin, demonstrating the importance of a reliable balance of vitamin D_3 in this organ, perhaps as an immediate defense mechanism against the harmful effects of UV radiation.[18]

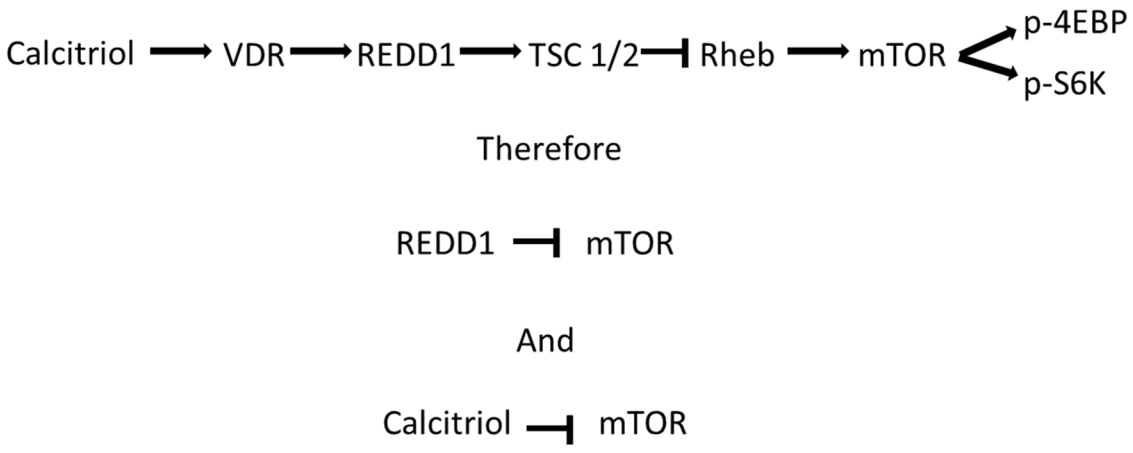
Figure 1.1 Vitamin D_3 metabolism



One of the signaling cascades that VDR regulates is the mTOR pathway (Figure 1.2). The mammalian target of rapaycin (mTOR) is a major control switch involved in cell survival, growth,

and proliferation. mTOR phosphorylates its targets, S6K and 4EBP, which results in the activation of protein synthesis.[19] This pathway is often up-regulated in cancer, whereas down-regulation is associated with differentiation and apoptosis. Therefore, compounds that can negatively regulate the mTOR pathway have the potential to be powerful anti-cancer drugs. When calcitriol binds to VDR, one of the genes that is up-regulated is DNA-damage-inducible transcript 4 (DDIT4), also known as regulated in development and DNA damage response 1 (REDD1).[19] REDD1 activates TSC1 and TSC2. These proteins are responsible for inhibiting Rheb, which in turn is an activator of mTOR. By up-regulating REDD1 via VDR, vitamin D_3 is thought to inhibit mTOR and thus its ability to phosphorylate S6K and 4EBP.[19] In fact, Lisse *et al.* saw a 50% reduction in cellular proliferation of osteoblasts in response to calcitriol treatment.[20] They determined this to be an effect of the demonstrated increase in REDD1 protein, resulting in a decrease in phosphorylation of S6K.

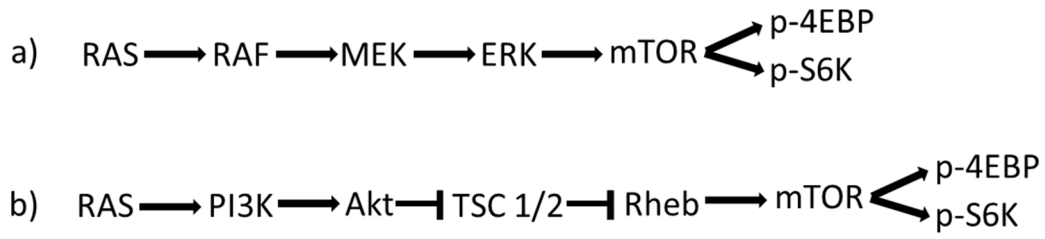
Figure 1.2 Vitamin D_3 and the mTOR pathway



The described vitamin D – mTOR pathway is just a small segment of a much larger signal transduction picture. This pathway shares downstream targets of the RAS-RAF-MEK-ERK and

PI3K/AKT pathways (Figure 1.3).[21] Ras is a GTPase that is active when bound to GTP, and inactive when bound to GDP. When activated, Ras induces translocation and phosphorylation of RAF, resulting in its activation. Activated RAF then phosphorylates MEK, which phosphorylates extracellular signal-regulated kinases 1 and 2 (ERK1/2). ERK1/2 can then activate mTOR. Ras is also capable of activating phosphinositide-3 kinase (PI3K). PI3K phosphorylates phosphatidylinositol bisphosphate (PIP_2), forming PIP_3, which activates protein kinase B (AKT). AKT is a kinase that is responsible for phosphorylating several proteins, including TSC 2. This phosphorylation inhibits TSC1 and TSC2, preventing them from down-regulating Rheb, which can potentially lead to the activation of mTOR. Therefore, Ras can indirectly activate mTOR through both of these pathways, contributing to increased cell growth and proliferation.

Figure 1.3 RAS activation of mTOR via RAF/MEK/ERK (a) and PI3K/AKT (b)



Indeed, both pathways were shown to be involved synergistically in the development of esophageal cancer. Wei and Xu showed that phosphorylation of both AKT and ERK was increased in cancer patients.[22] Interestingly, in many cancers, and as observed in our laboratory in human BCC tissue, a gain of function mutation is often seen in Ras.[23] Our lab also reported an increase in expression of Ras mRNA in cancer tissue. Over-activity of Ras signaling pathways may

overwhelm any inhibition from vitamin D_3 supplementation via VDR/REDD1/mTOR signaling. To make matters worse, oncogenic Ras may also reduce the expression of VDR. Rozenchran et al. found that over-expression of Ras led to reduced levels of VDR due to mRNA instability.[24] Considering all of these factors collectively may help explain why the effectiveness of vitamin D_3 treatment is so inconsistent in clinical studies.

Another pathway involved in BCC progression is the Hedgehog (HH) pathway (Figure 1.4). Patched1 (PTCH1) is a membrane receptor that binds to and inhibits Smoothened (SMO). Sonic hedgehog protein (HH) disrupts this inhibition, allowing SMO to activate Gli proteins. These proteins are transcription factors that then up-regulate genes involved in cellular proliferation. Mutations in this pathway, such as those that destroy the ability of PTCH1 to inhibit SMO, are the most common found in basal cell carcinoma.[25]

Figure 1.4 The Hedgehog pathway

In a second mechanism of the anti-cancer effects of vitamin D_3, Uhmann *et al.* showed that calcitriol inhibits the HH pathway in a *VDR-independent fashion* by inhibiting SMO directly.[26] Tang, *et al.* disagree with this finding, on the basis of the form of vitamin D_3 that is responsible for the inhibition of SMO. They showed that only the non-hydroxylated vitamin D_3,

cholecalciferol, could inhibit cellular proliferation.[27] They also showed that cholecalciferol could reduce Gli1 mRNA, but the hydroxylated forms could not.

In contrast to the studies by Uhmann and Tang, Teichert *et al.* found that calcitriol is involved in the *VDR-dependent* gene down-regulation of several of the hedgehog signaling pathway proteins.[8] They also demonstrated that in the absence of VDR several of these genes were over-expressed. Despite their disagreement on the mechanism involved, these studies make a strong argument for the inclusion of vitamin D_3 in the treatment of basal cell carcinoma.

Complicating the picture further, Wang et al. showed that activation of the mTOR pathway is capable of activating Gli proteins.[27] This activation was shown to come from S6K and occurred in the absence of SMO, signifying the complex inter-relationship of the hedgehog and RAS/PI3K pathways. This data was supported by another study in which a RAS/RAF pathway inhibitor was able to decrease Gli1 mRNA and protein expression.[28] Furthermore, in a study published in 2013, researchers demonstrated that mice with RAS mutations required Gli for the formation of pancreatic cancer.[29] They also found that RAS up-regulated the expression of HH, thus resulting in the activation of Gli.

Mutations resulting in dysfunction of the HH pathway are the most common in BCC. However, as mentioned, they are often not the only mutations present. Up-regulation of RAS or other oncogenes may negate the protective effect of vitamin D_3 by overwhelming it and/or circumventing it. This complex relationship stresses the importance of dual-pathway inhibitors in the treatment of cancer. A compound that is capable of down-regulating aspects of both pathways could have tremendous benefit in the treatment of this form of cancer.

Despite its promise *in vitro*, vitamin D_3 has yet to be proven effective in the clinical treatment of basal cell carcinoma. In a personal communication between clinical practitioners and our laboratory, the need for more research into the molecular mechanisms involved in this treatment was discussed. If supplementation is not beneficial perhaps it should no longer be prescribed. Previous work in our laboratory involved comparison of gene expression in cancer, proximal, and distal tissue taken from the head and neck regions of human patients. Our research indicated that genes involved in both the metabolism and signaling of vitamin D_3 were up-regulated in cancer tissue. Whether this is an innate defense against cancer, an effect of the cancer itself, or a combination of the two is yet to be determined.

Specific Aim

In addition to its role in bone mineralization and calcium homeostasis, Vitamin D_3 has been implicated as a major regulator of cellular proliferation and differentiation. The anti-proliferative impact of Vitamin D_3 is observed in clinical studies of breast and colon cancer, where vitamin D_3 treatment is generally positive. However, clinical data on vitamin D_3 treatment of basal cell carcinoma (BCC) is lacking and inconclusive at best. Because the majority of the *in vitro* studies in support of the protective effects of vitamin D_3 in onset and progression of cancer were not performed in skin cells these findings may not be applicable to BCC. Unfortunately, the non-metastatic nature of BCC makes cell lines difficult to grow in culture.[30] The goal of this study is to determine the impact of vitamin D_3 on proliferation of an immortalized human keratinocyte cell (HaCaT), a model for the basal layer of the skin. The HaCaT cells were spontaneously immortalized *in vitro* without transformation. Their name references their **H**uman **A**dult origin,

and their immortalization treatment of low **Ca**lcium and high **T**emperature. The HaCaT cells exhibit normal differentiation markers and are non-tumorigenic.[31]

Previous data in our laboratory indicate that the Hedgehog and mTOR-signaling pathways are activated in BCC tissue, despite increased expression of active vitamin D_3.[23] Therefore, I hypothesize that vitamin D_3 treatment will increase proliferation of HaCaT cells by modulating the mTOR-signaling pathway and failing to inhibit the Hedgehog pathway. Thus, vitamin D_3 treatment of skin cancer is ineffective and potentially detrimental. This hypothesis will be tested by the following specific aims:

1. **To determine the impact of calcitriol and cholecalciferol on proliferation of HaCaT cells**
2. **To dissect the molecular mechanism by which vitamin D_3 supplementation alters the proliferation of HaCaT cells.**

CHAPTER 2: Materials and Methods

Cell Culture

Human keratinocyte (HaCaT) cells were obtained from AddexBio. Cultures were grown in Gibco DMEM High Glucose media supplemented with 10% FBS and antibiotics. Cells were subcultured approximately twice weekly. Media was purchased from Life Technologies.

Treatment

Calcitriol was purchased from Sigma Aldrich. A 10^{-5} M stock solution was prepared in ethanol. Cholecalciferol was purchased from Fisher Scientific. A 1 M stock solution was prepared in ethanol. Ten-fold serial dilutions were made from each stock. Treatments were prepared 1% v/v in complete media. Concentrations tested were 10^{-8} M and 10^{-7} M calcitriol, and $5x10^{-6}$ M and 10^{-5} M cholecalciferol, as found in the literature.[12]

5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was purchased from Toronto Research Chemicals. This molecule phosphorylates AMPK, mimicking low energy status ([ATP]/[AMP]), which then results in inhibition of proliferation.[32] It will be used as a positive control, as vitamin D_3 inhibits proliferation independently of AMPK. A 1 M stock solution was prepared in DMSO. Ten-fold serial dilutions were made. Treatments were prepared 1% v/v in complete media. Concentrations tested were 10^{-4} M and 10^{-3} M, as found in the literature.[32]

WST-1 Cell Proliferation Assay

WST-1 cell proliferation reagent was purchased from Roche. Viable, proliferating cells express active mitochondrial dehydrogenases. This assay exploits the activity of these enzymes to compare proliferation of cells following different treatment conditions. The enzymes cleave tetrazolium salts in the WST-1 reagent to produce formazan, a dye that absorbs at 420-480 nm. The absorbance directly correlates to the number of viable cells in the sample.

Cells were plated in 96-well tissue culture-treated plates at a concentration of 4000 cells per well and allowed to recover overnight. The next day, media was replaced with the treatments listed in Table 2.1. After 24 or 48 hours, treatment media was replaced with complete media containing 10% WST-1 reagent. Three wells were reserved for blanks, in which no cells had been plated. Following four hours of incubation at 37°C the absorbance was read at 450 nm. Average blank absorbance was subtracted from all samples. Changes in proliferation following vitamin or AICAR treatment were compared to controls treated with vehicle alone. Results are given as fold-change versus controls, with controls normalized to 1.

Table 2.1 Treatment of HaCaT keratinocyte cells for MTT cell proliferation assay.

EtOH Vehicle	1 µL EtOH	100 µL Media
10 nM Calcitriol	1µL 1 µM Stock	100 µL Media
100 nM Calcitriol	1 µL 10 µM Stock	100 µL Media
5 µM Cholecalciferol	1 µL 500 µM Stock	100 µL Media
10 µM Cholecalciferol	1 µL 1 mM Stock	100 µL Media
DMSO Vehicle	1 µL DMSO	100 µL Media
0.1 mM AICAR	1 µL 10 mM Stock	100 µL Media
1 mM AICAR	1 µL 100 mM Stock	100 µL Media

RNA Isolation and cDNA Synthesis

Cells were plated in 6-well tissue culture-treated plates at a concentration of $5x10^4$ cells per well and allowed to recover overnight. The next day, media was replaced with the treatments listed in Table 2.2. After 24 or 48 hours of treatment, cells were rinsed with PBS, and RNA was extracted using the TRIzol method (Life Technologies). RNA concentrations were measured with the Nanodrop UV/Vis spectrophotometer. Next, 1 µg of RNA was used to synthesize cDNA using the Promega ImProm-II™ Reverse Transcription System. RNA was first incubated with random primers in a volume of 10 µL at 70°C for five minutes. Next, master mix was added to each tube, which contained 4 µL 5x buffer, 4 µL $MgCl_2$, 1 µL dNTPs, and 1 µL reverse transcriptase. Samples were placed in an Eppendorf thermocycler. The reverse transcription (RT-PCR) program consisted of the following steps: 25°C for five minutes, 42°C for one hour, 70°C for 15 minutes, and a 4°C hold. The reaction products were then purified with the Qiagen QIAquick® PCR Purification Kit. From the four samples from each treatment group, the three with the highest concentrations were chosen to use for RealTime PCR.

Table 2.2 Treatment of HaCaT keratinocyte cells for RNA isolation

EtOH Vehicle	**20 µL EtOH**	**2 mL Media**
100 nM Calcitriol	**20 µL 10 µM Stock**	**2 mL Media**
10 µM Cholecalciferol	**20 µL 1 mM Stock**	**2 mL Media**

Real-Time PCR

Primers were purchased from Sigma Aldrich and reconstituted as 100 µM Stocks. Working stocks consisted of 5 µM forward plus 5 µM reverse primer. Primer sequences are listed in Table 2.3. The LightCycler® 480 SYBR Green I Master kit from Roche was used to prepare

samples for qPCR. Samples consisted of 12.5 µL MasterMix from the kit, 1 µL primer mix (0.75 µL for 25OHase), 3 µL cDNA (30.3 ng for 24 hour samples, 41.55 ng for 48 hour samples) and DNAse-free water to 23 µL. Reactions were performed in a Stratagene thermocycler, and consisted of a five minute 95°C pre-incubation step, followed fifty cycles of amplification (ten seconds at 95°C followed by 30-60 seconds at the annealing temperature listed in Table 2.3) after which SYBR Green fluorescence was measured. A third segment generated dissociation curve data, and consisted of heating the samples to 95°C for one minute, cooling to the proper annealing temperature for 30-60 seconds, and then heating to 95°C again, while constantly measuring fluorescence. Threshold cycle (Ct) values were collected. First, data was compared to the average of the controls (ΔCt) and then calculated as fold-change versus controls ($2^{-\Delta Ct}$). Data is presented with controls normalized to 1.

Table 2.3 Real-Time PCR Primer Sequences and Annealing Temperatures

Target	Sequence	Tm	Annealing Temp
VDR fwd	CCAGTTCGTGTGAATGATGG	64.1	57.0
VDR rev	GTCGTCCATGGTGAAGGA	62.3	57.0
25OHase fwd	GGCAAGTACCCAGTACGG	60.6	55.0
25OHase rev	AGCAAATAGCTTCCAAGG	57.0	55.0
1αOHase fwd	TGTTTGCATTTGCTCAGA	59.0	59.0
1αOHase rev	CCGGGAGAGCTCATACAG	61.0	59.0
24OHase fwd	GCAGCCTAGTGCAGATTT	58.1	55.0
24OHase rev	ATTCACCCAGAACTGTTG	55.7	55.0
Gli1 fwd	CTCCCGAAGGACAGGTATGTAAC	64.5	57.0
Gli1 rev	CCCTACTCTTTAGGCACTAGAGTTG	62.3	57.0
REDD1 fwd	GGTCACTGAGCAGCTCGAA	64.6	63.0
REDD1 rev	CCTGGACAGCAGCAACAGT	64.3	63.0
KRAS fwd	AAACTTGTGGTAGTTGGAGCTGG	55.3	50.0
KRAS rev	TGATTCTGAATTAGCTGTATCGTCAA	53.2	50.0

Protein Isolation and Quantification

Cells were plated in 100 mm tissue culture-treated dishes at a concentration of $5x10^5$ cells per dish and allowed to recover overnight. The next day, media was replaced with the treatments listed in Table 2.4. After 24 or 48 hours of treatment, plates were rinsed with PBS, and cells were incubated in 700 µL lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 0.1% SDS, 0.5% sodium deoxycholate, 1x protease inhibitor) for 30 minutes at 4°C. Following incubation, cells were collected with a cell scraper and placed at -80°C overnight. Following freeze-thaw, lysates were spun to remove pellet and then concentrated to <100 µL in 10 kDa spin column concentration units. Concentrations were determined using the Pierce BCA Assay Kit. Samples were prepared in Laemmli buffer and heated to 70°C for 5 min before running SDS PAGE.

Table 2.4 Treatment of HaCaT keratinocyte cells for protein isolation.

EtOH Vehicle	100 µL EtOH	10 mL Media
100 nM Calcitriol	100 µL 10 µM Stock	10 mL Media
10 µM Cholecalciferol	100 µL 1 mM Stock	10 mL Media
DMSO Vehicle	100 µL DMSO	10 mL Media
1 mM AICAR	100 µL 100 mM Stock	10 mL Media

SDS PAGE and Western Blot

50 µg of whole cell extract samples were separated by SDS polyacrylamide gel electrophoreses (PAGE) using 4-15% or 10% Criterion™ TGX Stain-Free™ Precast gels (Bio-Rad). These gels were run at 250 V until the dye front reached the bottom of the gel (approximately thirty minutes). They were then activated in the Bio-Rad Gel Doc™ EZ Imager, using the Image Lab™ software prior to being transferred onto nitrocellulose membrane at 75 V for 15 minutes.

The activation step involves the UV linkage of trihalo compounds in the precast gels to tryptophan residues in the proteins. This produces fluorescence that can be detected by the imaging software. The membranes were then imaged post-transfer to observe transfer efficiency. After transfer membranes were blocked in 5% milk for 45-60 minutes. Membranes were then incubated overnight in primary antibody solutions of TBS-Tween containing 5% BSA and sodium azide (Table 2.5). HRP-conjugated secondary antibody solutions were prepared in TBS-Tween containing 5% BSA. Blots were first probed with phospho-antibodies, followed by a stripping step (Restore™ Thermo Scientific) before probing with their total protein-specific antibody counterparts. Blots were incubated with SuperSignal® West Pico or Femto chemiluminescent substrate (Thermo Scientific) before detection in the gel imager using the Quantity One® 1-D Analysis software. Bands were detected and quantified with the Image Lab™ software. The change in phosphorylation of three proteins of interest was calculated. Results are given as fold-change versus controls, with controls normalized to 1.

Table 2.5 Antibodies used in Western Blot

Target	Vendor	Catalog #	Dilution	Species
phospho-4EBP, Thr 37/46	Cell Signaling	236B4	1:1000	Rabbit
Total 4EBP	Cell Signaling	9644	1:1000	Rabbit
phospho-p70-S6K, Thr 389	Cell Signaling	9206	1:1000	Mouse
Total p70-S6K	Cell Signaling	2708	1:1000	Rabbit
phospho-mTOR, Ser 2448	Cell Signaling	5536	1:1000	Rabbit
Total mTOR	Cell Signaling	2983	1:1000	Rabbit
Anti-rabbit	Santa Cruz	Sc-2301	1-10,000	Goat
Anti-mouse	Santa Cruz	Sc-2302	1:10,000	Goat

Statistical Analysis

A *t* test was performed to analyze all data. A *p* value of less than 0.05 was considered significant.

CHAPTER 3: Results

WST-1 CELL PROLIFERATION ASSAY

HaCaT cells were treated for 24 or 48 hours with two forms of vitamin D_3, calcitriol and cholecalciferol. Results presented are from three independent experiments. Following 24 hours of treatment 100 nM calcitriol increased cellular proliferation significantly (p = 0.03*)*. The absorbance at 450 nm increased by 19%, which directly correlates to a 19% increase in viable cells. The other three treatment groups at this time point were not significantly different from controls (Figure 3.1). Following 48 hours of treatment, all four groups had a significant increase in proliferation versus controls ($p < 10^{-5}$). 10 nM and 100 nM Calcitriol increased proliferation by 23% and 25%, respectively. 5 µM and 10 µM cholecalciferol increased proliferation by 29% and 32%, respectively (Figure 3.2).

HaCaT cells were treated for 24 or 48 hours with AICAR. Results presented are from three independent experiments. Following 24 hours of treatment 1 mM AICAR decreased cellular proliferation significantly by 32% ($p < 10^{-5}$) (Figure 3.3). After 48 hours of treatment, both treatment groups had a significant decrease in proliferation versus controls; 0.1 mM AICAR decreased proliferation by 14% (p = 0.004), and 1 mM AICAR decreased proliferation by 42% ($p < 10^{-10}$) (Figure 3.4).

REAL-TIME PCR

HaCaT cells were treated in with 100 nM calcitriol, 10 µM cholecalciferol, or EtOH vehicle for 24 or 48 hours. Isolated RNA was used to create cDNA, which was then used to perform Real-time analysis of several genes of interest. Dissociation curves were analyzed for all genes to

exclude amplification results attributed to the presence of primer dimers. The 25OHase reaction was repeated with 25% less primer to remove primer dimer amplification. Genes tested were the vitamin D_3 metabolizing genes (25OHase, 1αOHase, and 24OHase), three genes hypothesized to be affected by vitamin D_3 treatment (VDR, REDD1, and Gli1), and Ras. Ras was chosen because previous data from our laboratory showed an increased in Ras expression alongside increases in vitamin D activating enzymes in BCC tissue. We wanted to determine whether or not this was directly caused by an increase in active vitamin D. The samples called 24h Calcitriol-3 and 48h Calcitriol-2 both appear to be outliers, and each increases the average CT values for their groups. They have not been excluded, but will be addressed for each gene when necessary.

25-OHase) Calcitriol treatment did not have an effect on expression of this gene following 24 hours of treatment. On average, the 48 hour treatment samples did not have an effect on this gene. One sample, Calcitriol-3, had a two-fold increase, which brought the average close to that of controls. When this sample is excluded the group has a reduction in 25OHase expression, although the data is not significant (p = 0.1). Cholecalciferol appeared to increase expression following 24 hours, however the data is not significant. Following 48 hours of treatment with cholecalciferol, 25OHase cDNA is significantly decreased compared to controls (p = 0.01) (Figure 3.5).

1α-OHase) Calcitriol treatment did not have an effect on expression of this gene. Removal of both the 24 and 48 hour outliers brought the averages of these groups closer to that of controls. Cholecalciferol did not have an effect on expression following 24 hours. However, following 48

hours of treatment with cholecalciferol, 1αOHase cDNA was significantly elevated above controls ($p = 0.04$) (Figure 3.6).

24-OHase) Both calcitriol and cholecalciferol treatment increased the expression of this gene. The 24 hour data is not significant due to the variation within the groups, even when the outlier is excluded. However, the trend is clearly evident and is supported by the several thousand-fold scale of the y-axis. Following 48 hours of treatment the increase in expression with calcitriol and cholecalciferol treatment is significant ($p < 0.015$ and 10^{-5}, respectively). Excluding the outliers in the calcitriol treatment groups does not eliminate the significance (Figure 3.7).

VDR) Calcitriol treatment did not have an effect on this gene. Average fold-change was similar to controls, and exclusion of outliers brought the data even closer to controls. Cholecalciferol treatment significantly increased VDR gene expression ($p = 0.04$). This increase was gone after 48 hours (Figure 3.8).

REDD1) 24 hour treatment with calcitriol did not affect the expression of this gene, especially when the outlier was removed. 48 hour treatment indicated a trend of increased expression ($p = 0.08$), however the variation within the data prevented it from being significant, even with exclusion of the outlier. Cholecalciferol treatment had a trend of increasing expression at 24 hours. This increase was significant following 48 hours of treatment ($p = 0.01$) (Figure 3.9).

Gli1) Neither calcitriol nor cholecalciferol treatment had an effect on the expression of this gene following 24 or 48 hours of treatment (Figure 3.10).

RAS) Neither calcitriol nor cholecalciferol treatment had an effect on the expression of this gene following 24 or 48 hours of treatment (Figure 3.11).

WESTERN BLOT

HaCaT cells were treated with Calcitriol, Cholecalciferol, or AICAR for 24 or 48 hours. Whole cell extract was isolated and analyzed by western blot. Membranes were probed with antibodies for phosphorylated mTOR and total mTOR; phosphorylated S6K and total S6K; and phosphorylated 4EBP and total 4EB.

mTOR) Both calcitriol and cholecalciferol 24 hour treatment resulted in a significant increase in phosphorylated mTOR (p = 0.04 and 0.02, respectively). Calcitriol increased levels by 2.8 fold, while cholecalciferol increased levels by 2.3-fold. Phosphorylation of mTOR returned to control levels after 48 hours (Figure 3.12).

P70-S6K) Both calcitriol and cholecalciferol treatment appeared to increase phosphorylation of S6K following 24 hours of treatment, however the data was not significant (p = 0.14 and 0.17, respectively). The trend is gone following 48 hours of treatment (Figure 3.13). Data are a combination of two western blots with the same samples.

4EBP) Neither calcitriol nor cholecalciferol treatment significantly increased phosphorylation of 4EBP following 24 hour treatment, however, a trend is evident. After 48 hours of treatment, both forms of the vitamin significantly increased 4EBP phosphorylation status (p = 0.0008 and 0.005, respectively) (Figure 3.14). Calcitriol treatment resulted in a two-fold increase in phosphorylated 4EBP, while cholecalcitriol increased the levels by 2.5-fold. Data are a combination of two western blots with the same samples.

AICAR Treatment) As a control, cells were treated with 1 mM AICAR for 48 hour and whole cell extract was analyzed via western blot. The same proteins were probed for as the vitamin D_3 treatment blots. Treatment with AICAR resulted in a significant decrease in phosphorylation of all three proteins tested (p_{mTOR} = 0.03, p_{S6K} = 0.02, p_{4EBP} = 0.04) (Figure 3.15). The decrease is phosphorylation of mTOR was by 91%. Phosphorylation of P70-S6K decreased by 55%. Phosphorylation of 4EBP decreased by 45%. The data for 4EBP were a combination of two western blots with the same samples.

Figure 3.1. Cellular proliferation following 24 hour treatment with vitamin D_3. HaCaT cells were treated for 24 hours with calcitriol (10 nM and 100 nM), cholecalciferol (5 µM and 10 µM), or vehicle. Cells were then incubated with WST-1 reagent for four hours at 37°C. Absorbance was read at 450 nM. Results are presented as percentage versus controls. A p-value of less than 0.05 is considered significant. Bars with different symbols are significantly different from each other.

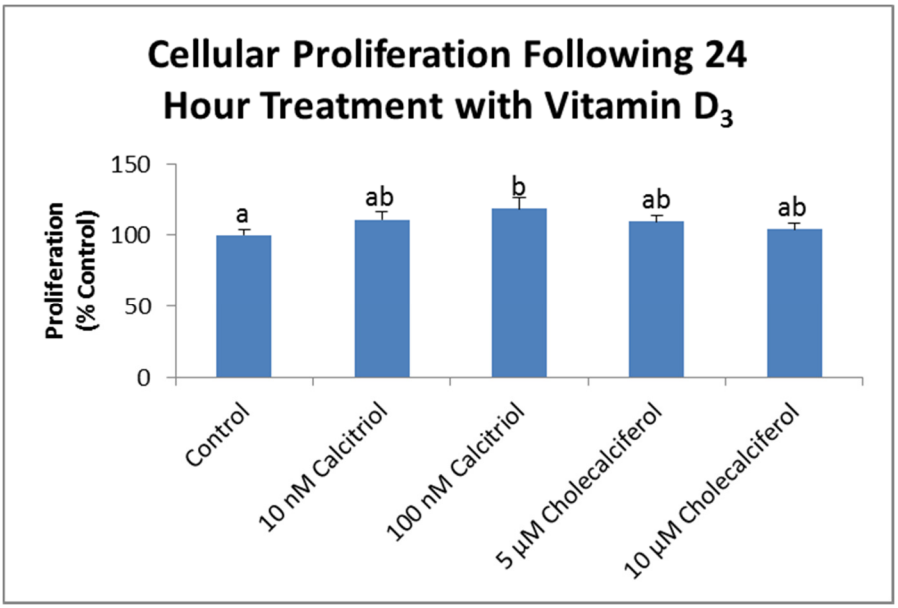


Figure 3.2. Cellular proliferation following 48 hour treatment with vitamin D_3. HaCaT cells were treated for 48 hours with calcitriol (10 nM and 100 nM), cholecalciferol (5 µM and 10 µM), or vehicle. Cells were then incubated with WST-1 reagent for four hours at 37°C. Absorbance was read at 450 nM. Results are presented as percentage versus controls. A p-value of less than 0.05 is considered significant. Bars with different symbols are significantly different from each other.

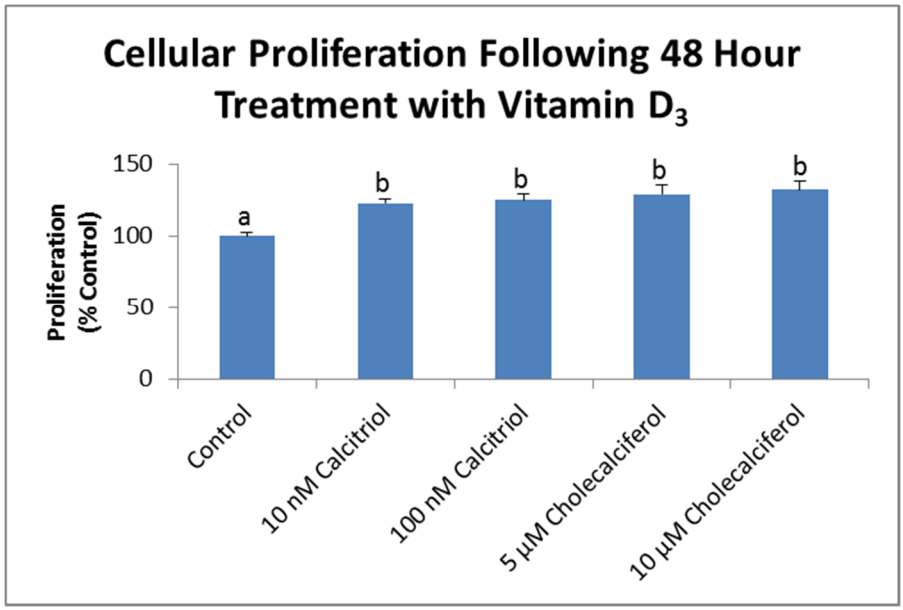


Figure 3.3. Cellular proliferation following 24 hour treatment with AICAR. HaCaT cells were treated for 24 hours with AICAR (0.1 mM and 1 mM) or vehicle. Cells were then incubated with WST-1 reagent for four hours at 37°C. Absorbance was read at 450 nM. Results are presented as percentage versus controls. A p-value of less than 0.05 is considered significant. Bars with different symbols are significantly different from each other.

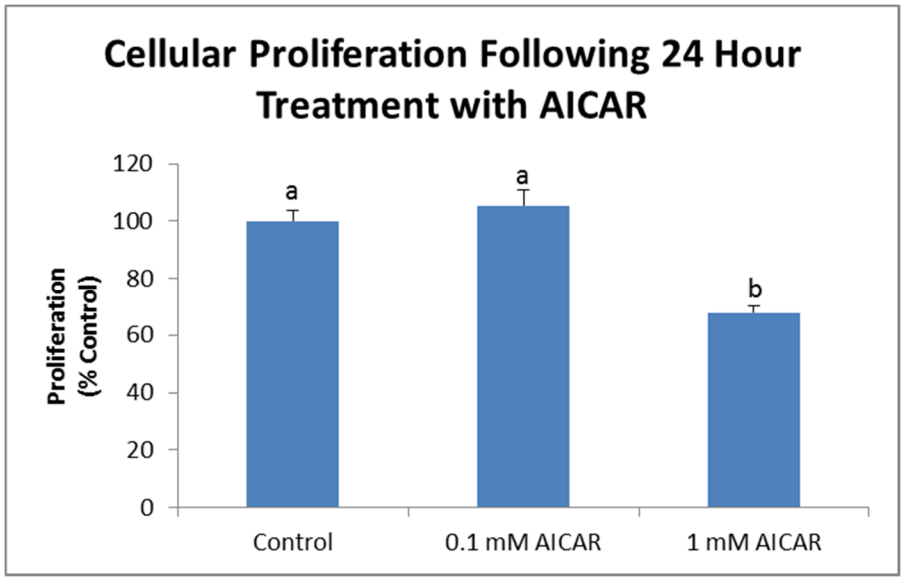


Figure 3.4. Cellular proliferation following 48 hour treatment with AICAR. HaCaT cells were treated for 48 hours with AICAR (0.1 mM and 1 mM) or vehicle. Cells were then incubated with WST-1 reagent for four hours at 37°C. Absorbance was read at 450 nM. Results are presented as percentage versus controls. A p-value of less than 0.05 is considered significant. Bars with different symbols are significantly different from each other.

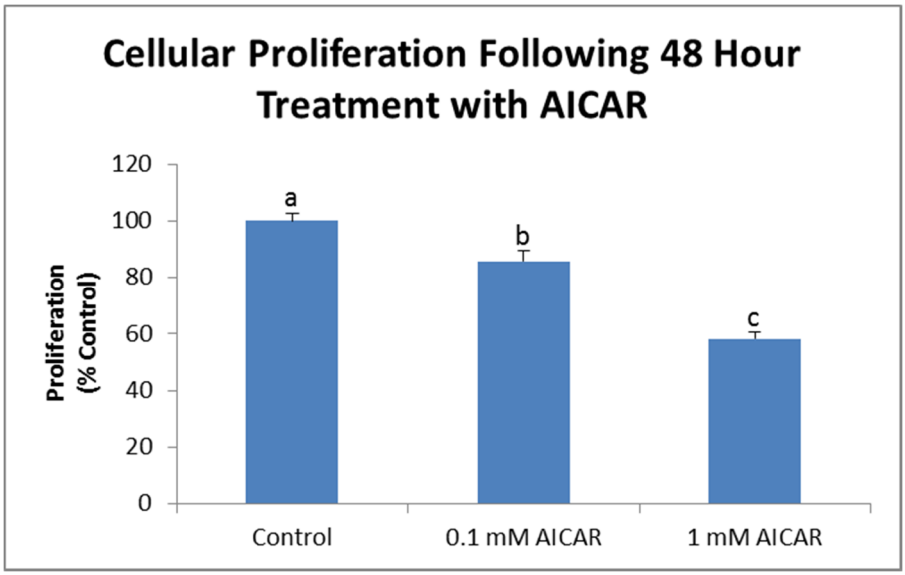


Figure 3.5. 25-OHase transcript fold change following treatment with vitamin D_3. HaCaT cells were treated with 100 nM calcitriol, 10 µM cholecalciferol, or vehichle for 24 hours (a,b) or 48 hours (c,d). Real-time PCR was performed using primers specific for 25-hydroxylase. Changes in expression of this gene are presented as fold change relative to controls. A p-value of less than 0.05 is considered significant. Bars with different symbols are significantly different from each other.

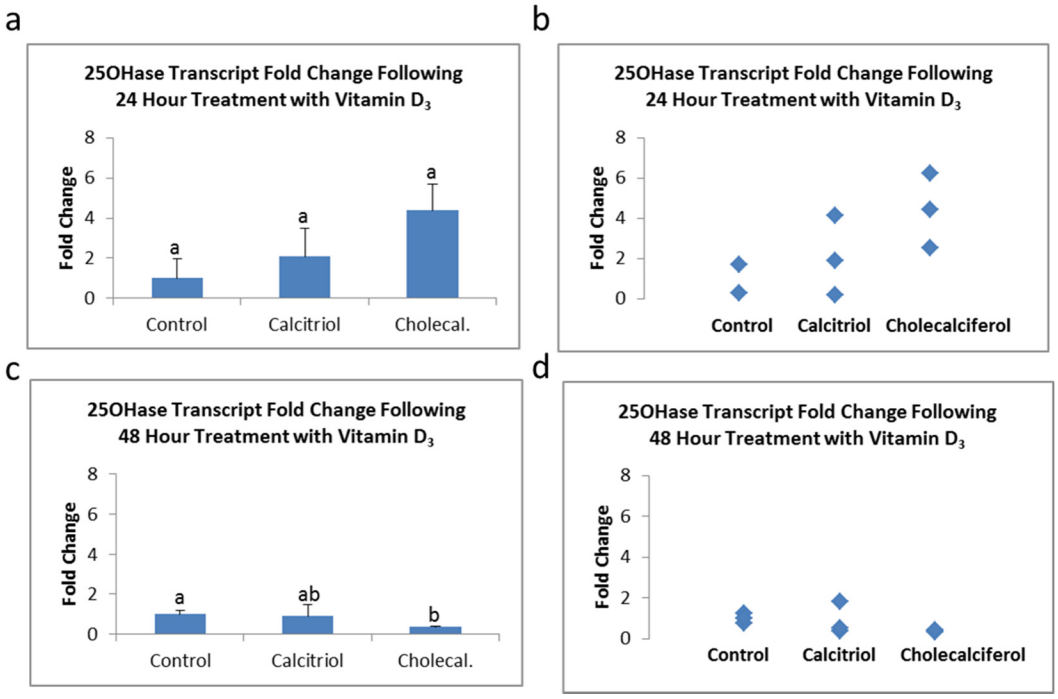


Figure 3.6. 1α-OHase transcript fold change following treatment with vitamin D_3. HaCaT cells were treated with 100 nM calcitriol, 10 µM cholecalciferol, or vehichle for 24 hours (a,b) or 48 hours (c,d). Real-time PCR was performed using primers specific for 1α-hydroxylase. Changes in expression of this gene are presented as fold change relative to controls. A p-value of less than 0.05 is considered significant. Bars with different symbols are significantly different from each other.

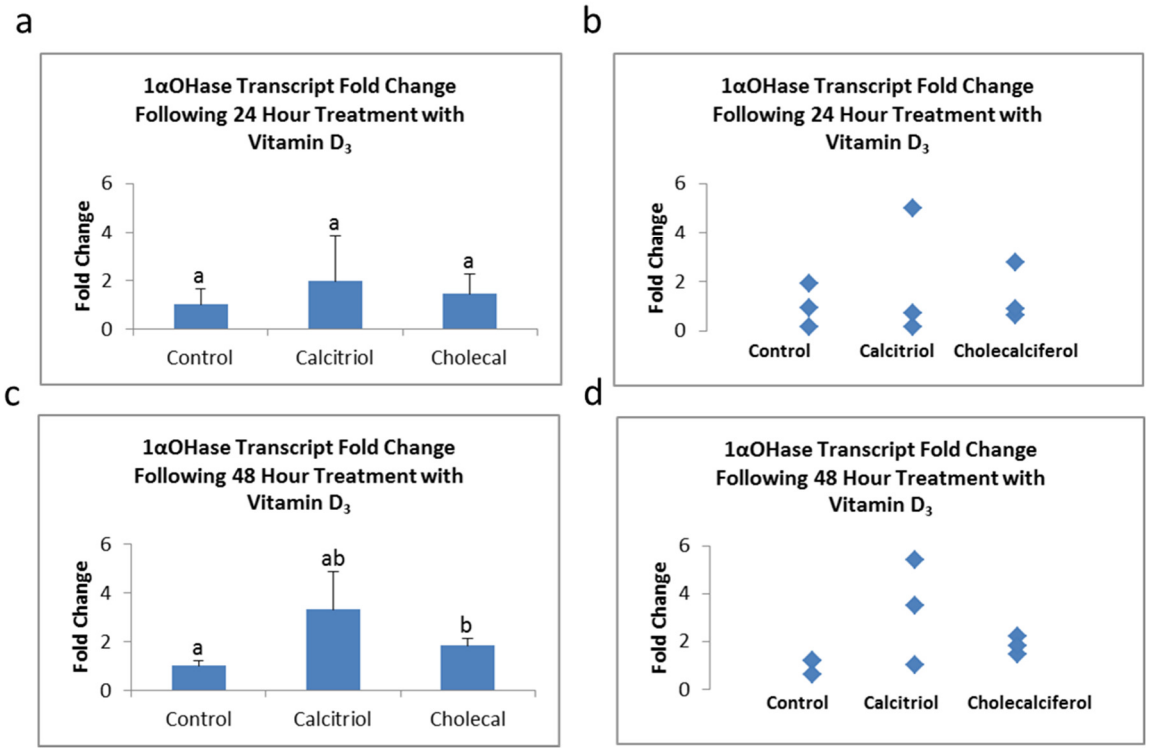


Figure 3.7. 24-OHase transcript fold change following treatment with vitamin D_3. HaCaT cells were treated with 100 nM calcitriol, 10 µM cholecalciferol, or vehichle for 24 hours (a,b) or 48 hours (c,d). Real-time PCR was performed using primers specific for 24-hydroxylase. Changes in expression of this gene are presented as fold change relative to controls. A p-value of less than 0.05 is considered significant. Bars with different symbols are significantly different from each other.

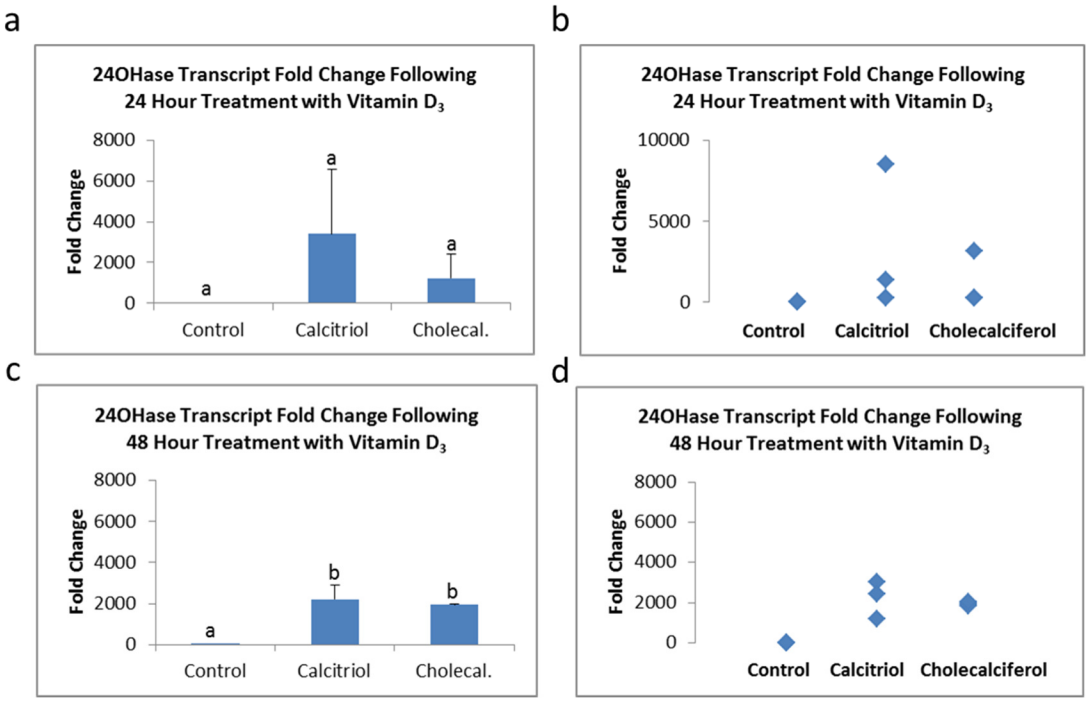


Figure 3.8. VDR transcript fold change following treatment with vitamin D_3. HaCaT cells were treated with 100 nM calcitriol, 10 µM cholecalciferol, or vehichle for 24 hours (a,b) or 48 hours (c,d). Real-time PCR was performed using primers specific for the vitamin D receptor. Changes in expression of this gene are presented as fold change relative to controls. A p-value of less than 0.05 is considered significant. Bars with different symbols are significantly different from each other.

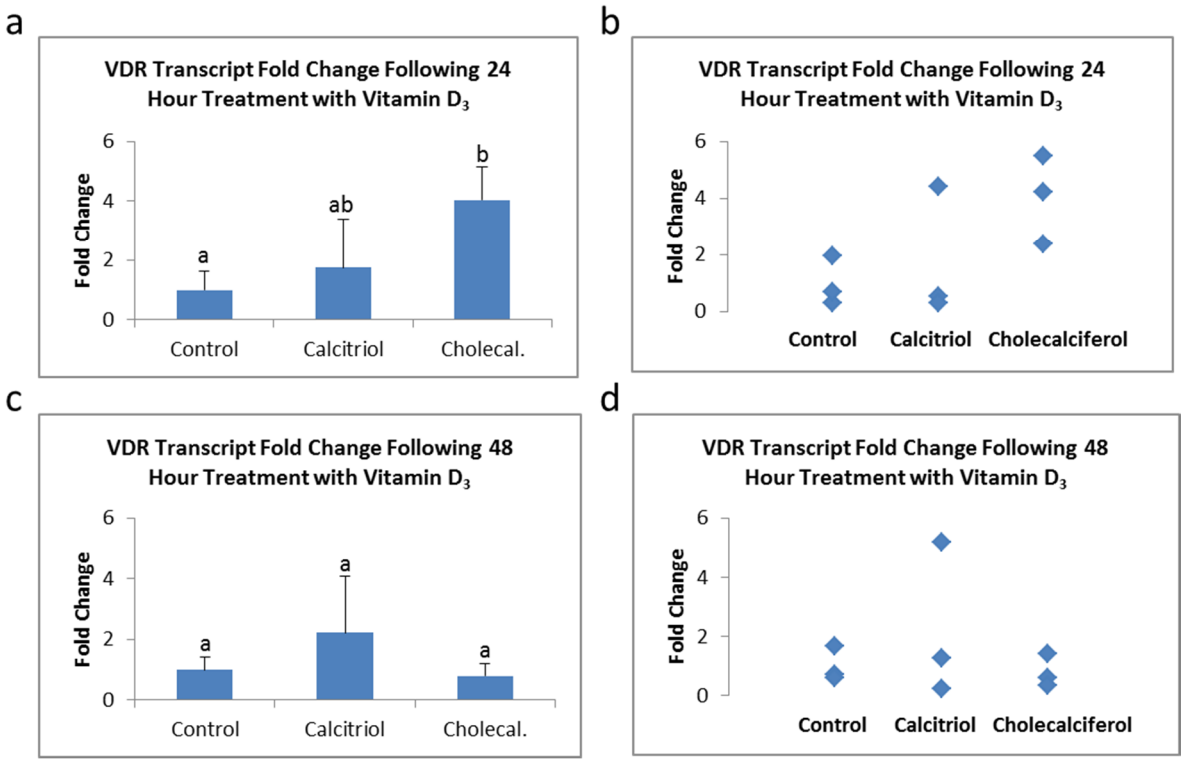


Figure 3.9. REDD1 transcript fold change following treatment with vitamin D_3. HaCaT cells were treated with 100 nM calcitriol, 10 µM cholecalciferol, or vehichle for 24 hours (a,b) or 48 hours (c,d). Real-time PCR was performed using primers specific for REDD1. Changes in expression of this gene are presented as fold change relative to controls. A p-value of less than 0.05 is considered significant. Bars with different symbols are significantly different from each other.

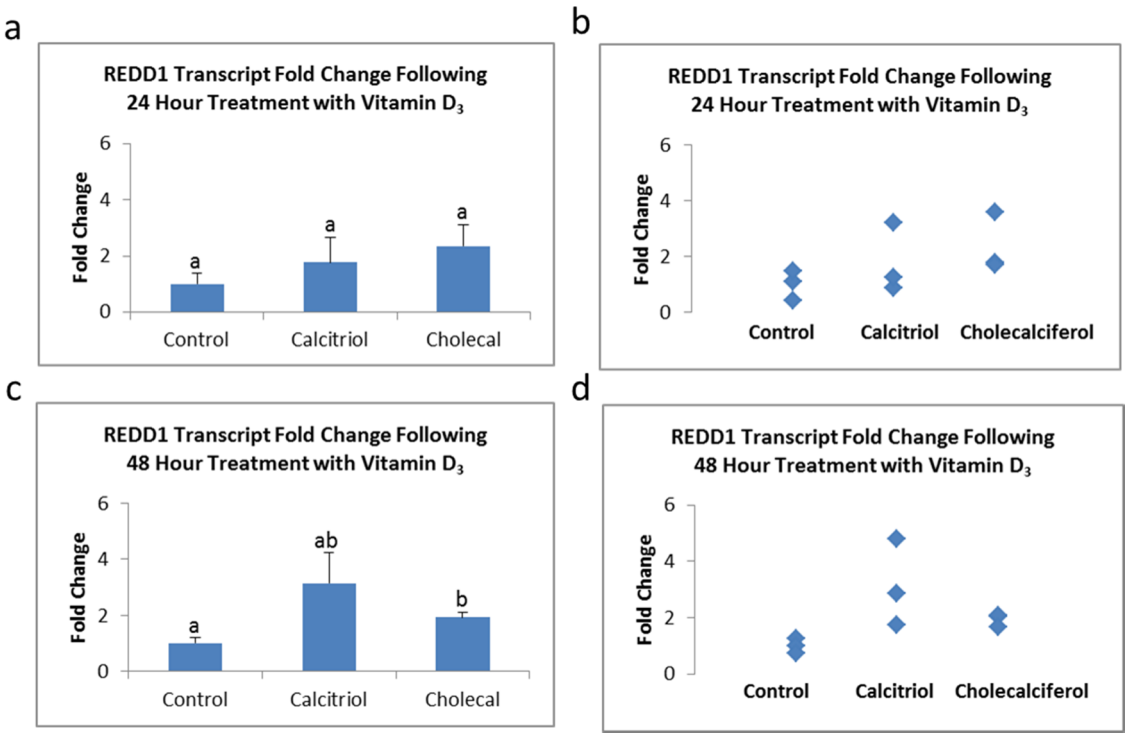


Figure 3.10. Gli1 transcript fold change following treatment with vitamin D_3. HaCaT cells were treated with 100 nM calcitriol, 10 µM cholecalciferol, or vehichle for 24 hours (a,b) or 48 hours (c,d). Real-time PCR was performed using primers specific for Gli1. Changes in expression of this gene are presented as fold change relative to controls. A p-value of less than 0.05 is considered significant. Bars with different symbols are significantly different from each other.

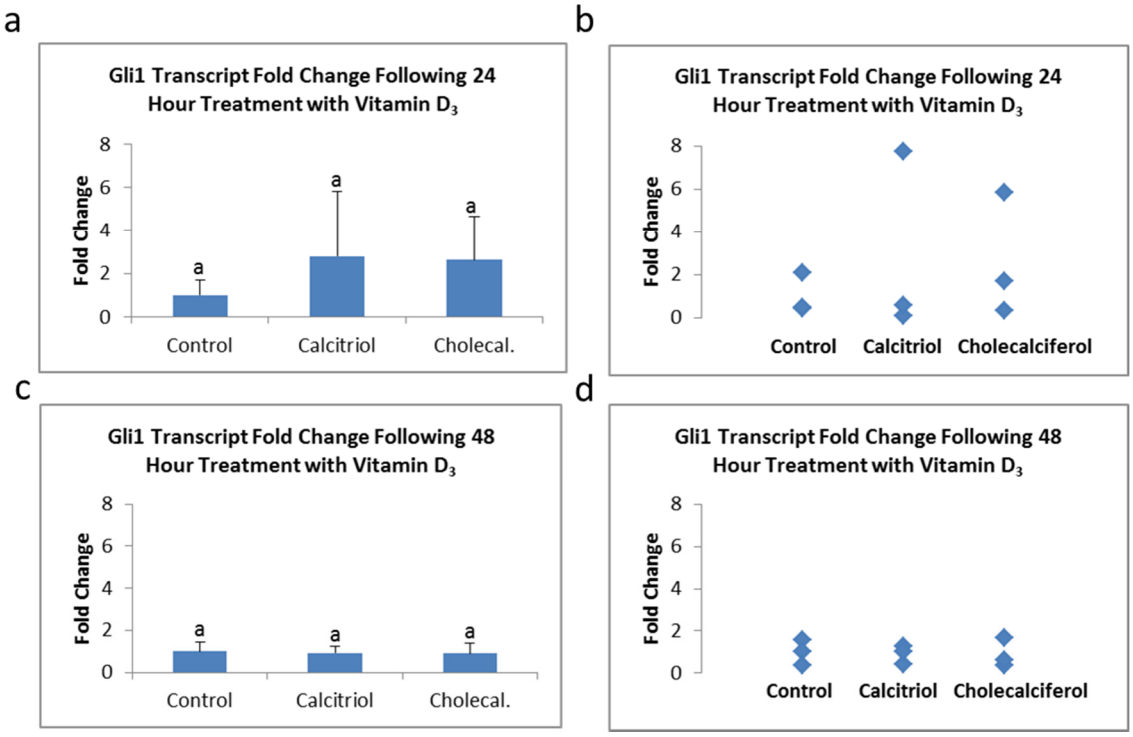


Figure 3.11. Ras transcript fold change following treatment with vitamin D_3. HaCaT cells were treated with 100 nM calcitriol, 10 µM cholecalciferol, or vehichle for 24 hours (a,b) or 48 hours (c,d). Real-time PCR was performed using primers specific for Ras. Changes in expression of this gene are presented as fold change relative to controls. A p-value of less than 0.05 is considered significant. Bars with different symbols are significantly different from each other.

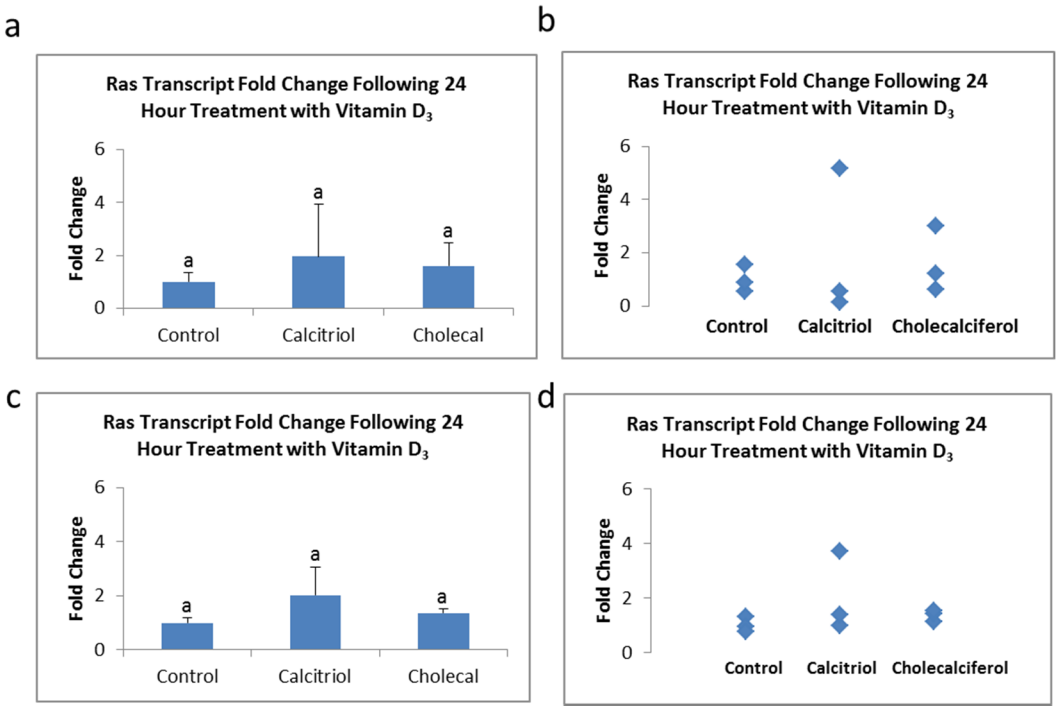


Figure 3.12. Change in phosphorylation of mTOR following treatment with vitamin D_3. HaCaT cells were treated with 100 nM calcitriol, 10 µM cholecalciferol, or vehichle for 24 hours or 48 hours. Whole cell extract (50µg per sample) was compared by western blot analysis. Blots were probed with antibody specific for phosphorylated mTOR (Ser 2448) and total mTOR. Changes in phosphorylation of this gene are presented as fold change relative to controls. A p-value of less than 0.05 is considered significant. Bars with different symbols are significantly different from each other.

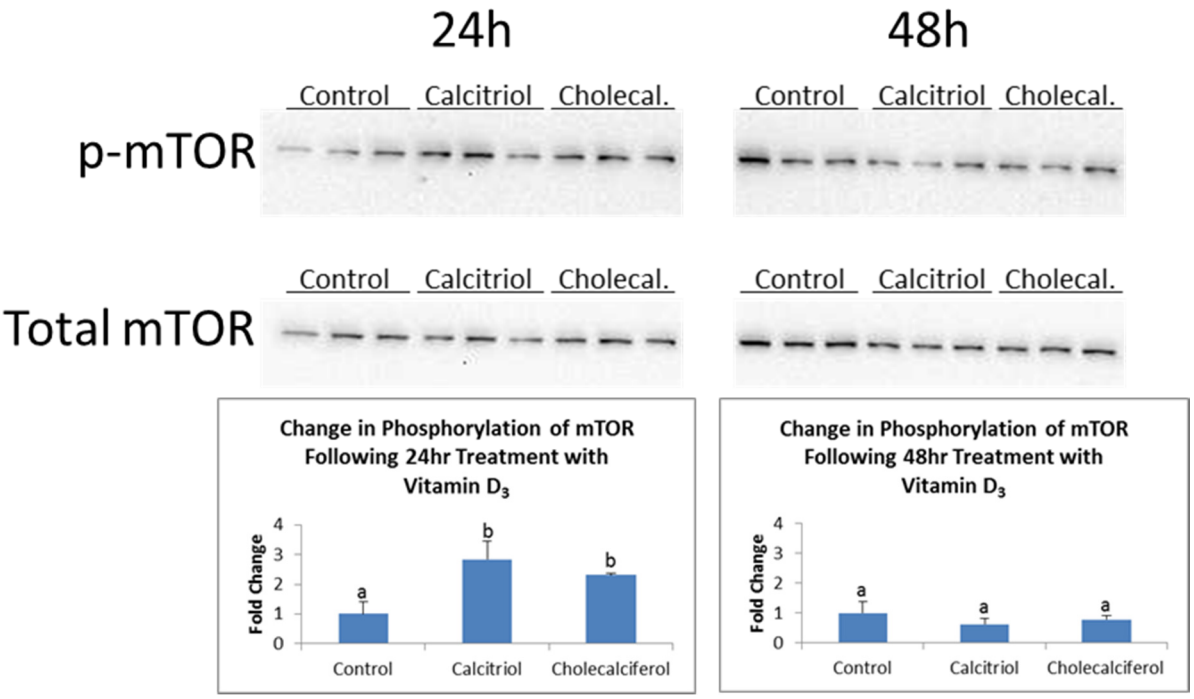


Figure 3.13. Change in phosphorylation of P70-S6K following treatment with vitamin D_3. HaCaT cells were treated with 100 nM calcitriol, 10 µM cholecalciferol, or vehichle for 24 hours or 48 hours. Whole cell extract (50µg per sample) was compared by western blot analysis. Blots were probed with antibody specific for phosphorylated P70-S6K (Thr 389) and total P70-S6K. Changes in phosphorylation of this gene are presented as fold change relative to controls. A p-value of less than 0.05 is considered significant. Bars with different symbols are significantly different from each other.

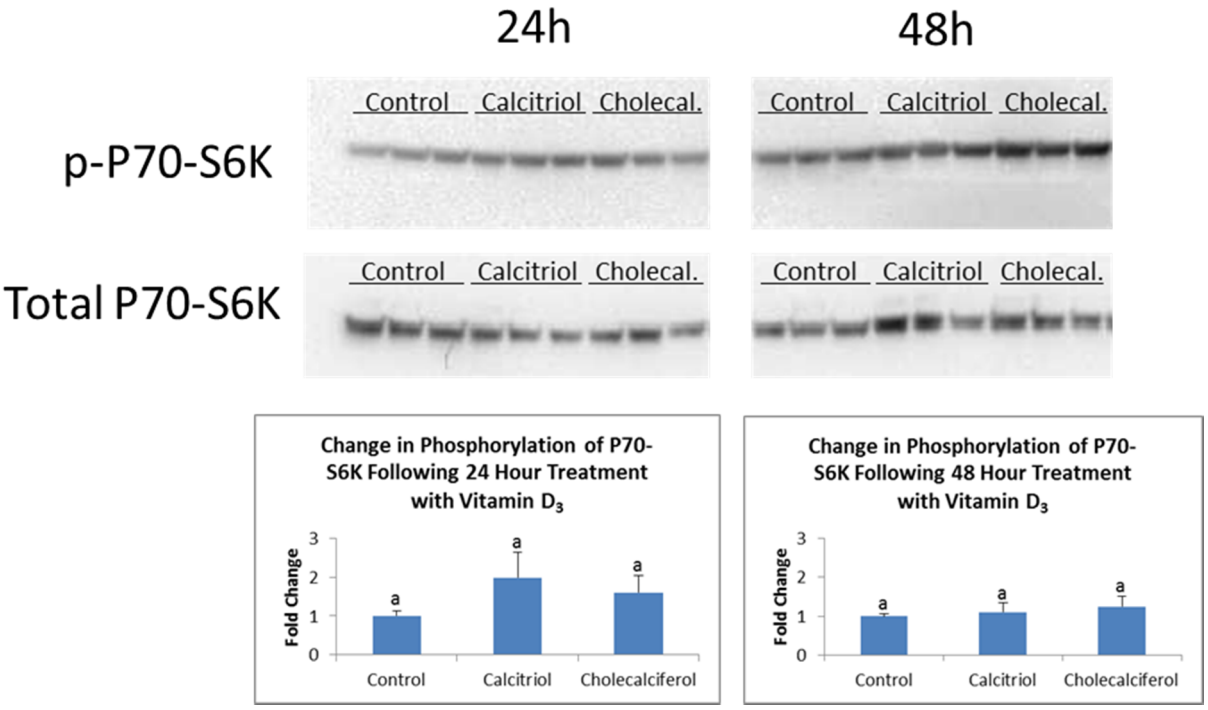


Figure 3.14. Change in phosphorylation of 4EBP following treatment with vitamin D_3. HaCaT cells were treated with 100 nM calcitriol, 10 µM cholecalciferol, or vehichle for 24 hours or 48 hours. Whole cell extract (50µg per sample) was compared by western blot analysis. Blots were probed with antibody specific for phosphorylated 4EBP (Thr 37/46) and total 4EBP. Changes in phosphorylation of this gene are presented as fold change relative to controls. A p-value of less than 0.05 is considered significant. Bars with different symbols are significantly different from each other.

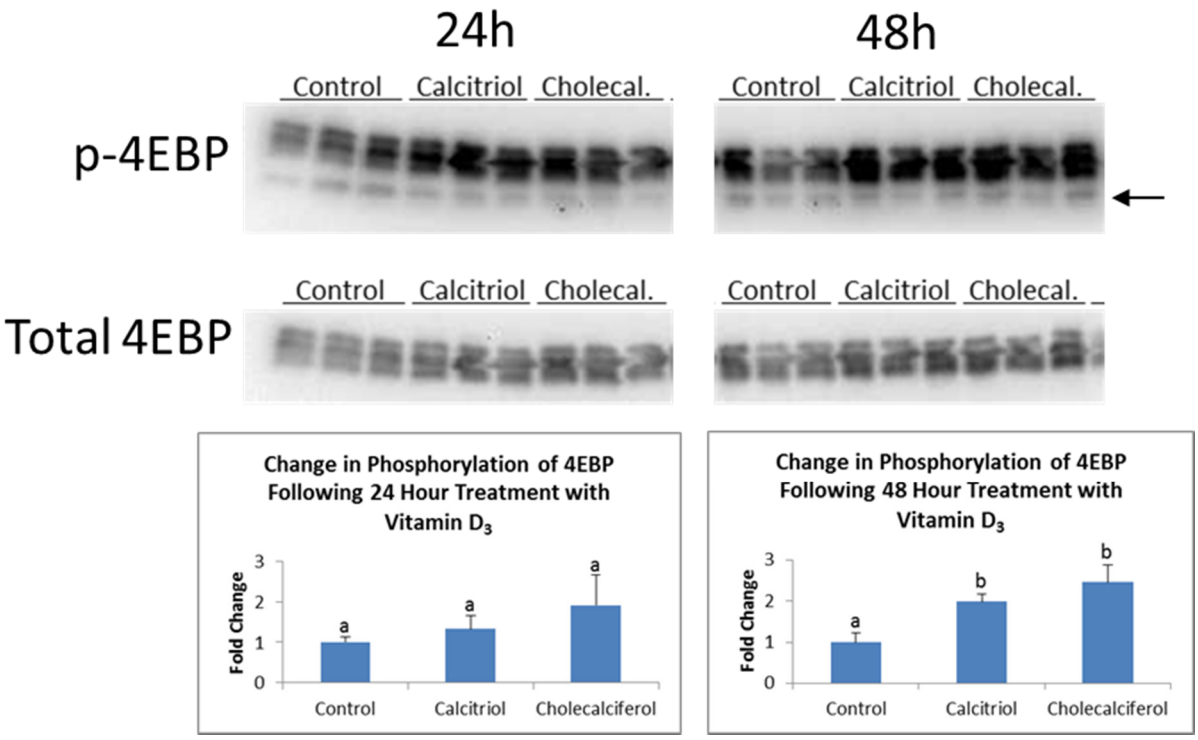
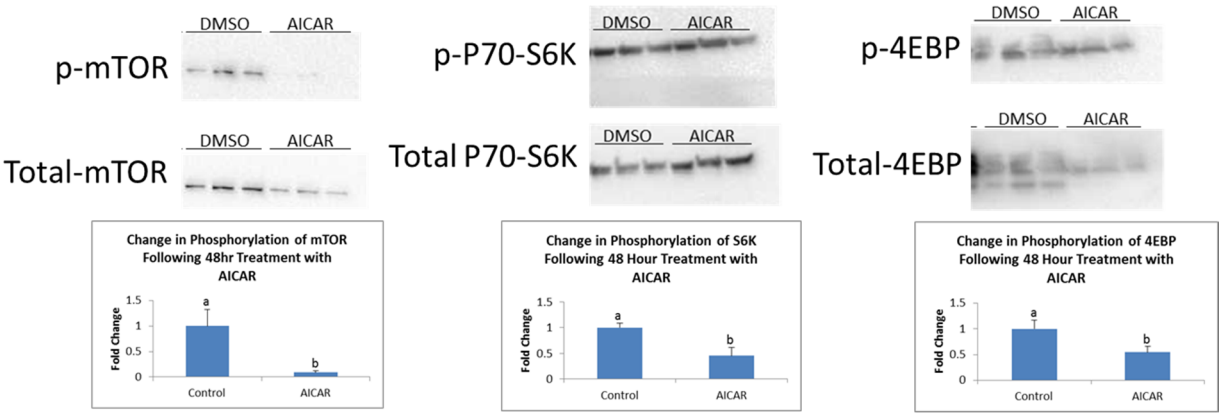


Figure 3.15. Change in phosphorylation of mTOR, P70-S6K, and 4EBP following treatment with AICAR. HaCaT cells were treated with 1 mM AICAR or vehichle for 48 hours. Whole cell extract (50µg per sample) was compared by western blot analysis. Blots were probed with antibody specific for phosphorylated mTOR (Ser 2448) and total mTOR (a), phosphorylated P70-S6K (Thr 389) and total P70-S6K (b), and phosphorylated 4EBP (Thr 37/46) and total 4EBP (c). Changes in phosphorylation of this gene are presented as fold change relative to controls. A p-value of less than 0.05 is considered significant. Bars with different symbols are significantly different from each other.



CHAPTER 4: Discussion

The vast majority of research indicates that vitamin D_3 has anti-proliferative effects *in vitro*. These effects have even been shown in clinical studies of breast and colon cancer, and the results of vitamin D_3 treatment are generally positive for these cancers. Unfortunately, data is lacking and inconclusive in the treatment of basal cell carcinoma. Perhaps one reason for the varied response to treatment for different tissues is the difference in exposure to UV light combined with the availability of vitamin D_3 metabolizing enzymes. The skin is unique because it has all of these, and thus it has the potential to synthesize active vitamin D_3 *de novo*, as well as inactivate it when high levels are no longer desirable. Because of this, it is necessary to perform cell culture experiments in skin cell models of basal cell carcinoma, and not cells from other tissues. Unfortunately, experiments are difficult because BCC cells do not proliferate well in culture. The HaCaT keratinocyte cell line, therefore, is a useful model for skin tissue because it is immortalized and grows rapidly in culture.

In the present study, HaCaT cells were treated with various concentrations of calcitriol or cholecalciferol for 24 or 48 hours. As a positive control, cells were also treated with AICAR for 24 or 48 hours, which significantly decreased proliferation relative to controls. The AICAR results indicate that proliferation of these cells is capable of being inhibited despite high levels of growth factors in the media. Interestingly, not only did treatments with both calcitriol and cholecalciferol fail to inhibit proliferation, they actually *increased* it. Early research involving these cells indicated that high calcium concentrations may actually render calcitriol treatment proliferative, however current research rarely considers this.[33,34] Calcium concentration in the skin is stratified and corresponds to the level of differentiation of the cells, with low levels in the stratum basale,

and high levels in the stratum granulosum.[35] If high calcium correlates with a more differentiated phenotype, it seems counter-intuitive that high levels would also render calcitriol treatment proliferative, as differentiation is usually associated with growth cessation. Analysis of the DMEM used in our experiments showed that our cell culture media contained 1.8 mM calcium, which corresponded to the high concentrations tested in these early experiments. It should be noted that this is a common formulation used in many cell culture experiments, including those relevant to the current study. Other early studies suggest that low, physiological concentrations of calcitriol promoted growth, however, the concentrations tested in the present study were higher than these and were expected to be inhibitory.[36] Taking these older studies into consideration may make it feasible that vitamin D_3 treatment may not inhibit growth under all conditions. Based on these results, we decided to investigate key effectors in the mTOR and Hedgehog pathways to understand how their status may impact proliferation in our treatment groups.

Real time PCR was performed on cDNA from cells that were treated with calcitriol, cholecalciferol, and ethanol vehicle. Calcitriol treatment had no effect on expression of the metabolizing 25-hydroxylase and 1α-hydroxylase genes, as it is an end product of their sequential reactions. Cholecalciferol appeared to increase 25-hydroxylase mRNA following 24 hours of treatment ($p = 0.1$), and then significantly decrease it after 48 hours. A significant increase in 1α-hydroxylase was seen after 48 hours. Taken together, the expression of these enzymes may correlate with sequential hydroxylation of cholecalciferol to form calcitriol.

Both calcitriol and cholecalciferol treatment significantly increased 24-hydroxylase (the vitamin D_3 inactivating enzyme) mRNA after 48 hours of treatment. The 24-hydroxylase

promoter contains two vitamin D response elements (VDREs) that bind the vitamin D receptor.[37] The increase seen here verifies that both calcitriol and cholecalciferol successfully entered the cells and were capable of exerting an effect on gene expression via the vitamin D receptor (VDR). Additionally, this verifies that cholecalciferol was successfully hydroxylated by 25-hydroxylase and 1α-hydroxylase, as only calcitriol binds VDR.[38] The thousand-fold increase in expression of this gene is too substantial to be ignored. 24-hydroxylase is often considered an oncogene because it results in the degradation of vitamin D_3.[39] Indeed, inhibition of 24-hydroxylase enhanced the anti-proliferative properties of calcitriol in prostate cancer cells.[40] Perhaps the major up-regulation of this gene is also somehow responsible for the increase in proliferation seen here. Another point to consider is that any effects seen in this study may be an artifact of the limitations of cell culture. 24-hydroxylase inactivation of calcitriol and cholecalciferol *in vivo* results in their clearance from the body, while *in vivo* these products accumulate in the media.

While both forms of vitamin D_3 were able to regulate 24-hydroxylase gene expression, only cholecalciferol had an effect on the VDR transcript. Levels increased significantly with 24 hours of treatment, and then returned to control levels after 48 hours (as protein concentration was most likely sufficient). However, it cannot be directly inferred that the difference in expression of VDR mRNA between calcitriol and cholecalciferol treatment groups affected the activity of VDR protein. REDD1 (a target of VDR transcription regulation) mRNA was significantly increased by cholecalciferol treatment after 48 hours. However, the trend is also evident for calcitriol treatment ($p = 0.08$), so it is most likely that REDD1 mRNA is also increased in this group. Perhaps cholecalciferol treatment increased VDR expression, which allowed for a faster accumulation of REDD1, however, this conclusion needs further investigation.

The most common mutations found in BCC are those affecting the Hedgehog pathway. Vitamin D_3 is perhaps so compelling in the treatment of BCC because multiple studies suggest that calcitriol and/or cholecalciferol can inhibit activation of this pathway. While some suggest that the inhibition comes at the level of blocking Smo, independent of the VDR, others say that calcitriol, via VDR, controls the expression of several of the genes involved. Ultimately, all suggested means of inhibition result in a decrease in Gli1 mRNA. Analysis of Real Time PCR data showed that neither calcitriol nor cholecalciferol treatment had any effect on the expression of Gli1 transcript in the current study. One possible explanation for this is that both forms of vitamin D_3 are incapable of inhibiting this pathway due to the cell culturing conditions. It could be that the presence of growth factors override the ability of VDR to control the expression of genes in the pathway. It is possible that another nutrient in the media is blocking the ability of calcitriol and/or cholecalciferol to bind to and inhibit Smo. A second explanation may be that Gli1 mRNA can only be reduced when it is over-expressed in the first place. No studies suggest any mutations in this pathway in HaCaT cells, so it can be assumed that this pathway is not over-active. Testing this theory might involve the introduction of a deregulating mutation to Ptch or Smo, and then observation of the effects of vitamin D_3 treatment on Gli1 mRNA expression. Regardless of the cause, the absence of an effect on Gli1 transcript expression in the present study provides two important pieces of information. First, it supports our findings that vitamin D_3 treatment does not always inhibit proliferation of HaCaT cells. Second, it shows that vitamin D_3 treatment does not upregulate this pathway, and therefore it is not the cause of the increase in proliferation seen in our WST-1 assay.

We also looked at the expression of Ras in response to calcitriol and cholecalciferol. Earlier research in our laboratory showed that Ras was up-regulated in cancer tissue, along with up-regulation of 1α-hydroxylase. We wanted to confirm that the increase in Ras transcript was due to a malfunction of the cancer cells, and not somehow caused by increasing concentrations of calcitriol. Real Time PCR data showed that calcitriol and cholecalciferol treatments had no effect of Ras expression.

Taken together, the results from Real Time PCR indicate that cholecalciferol is converted to calcitriol by 25-hydroxylase and 1α-hydroxylase. Calcitriol is then able to bind to VDR, resulting in up-regulation of REDD1 and 24-hydroxylase. REDD1 inhibits the mTOR pathway, so the increase in REDD1 we see should lead to inhibition of proliferation. Neither calcitriol nor cholecalciferol inhibited the Hedgehog pathway, as determined by the lack of effect on Gli1 expression. While this may be partially responsible for the lack of inhibition on cell proliferation, it does not explain the increase in proliferation seen. To further probe the cause of the dysfunction, we decided to investigate what is occurring downstream of REDD1. To do so required the utilization of western blotting, as the signaling involves the phosphorylation status of key proteins, including mTOR, P70-S6K, and 4EBP.

HaCaT cells were treated with calcitriol, cholecalciferol, or ethanol vehicle for 24 or 48 hours. As a control, cells were also treated with AICAR or DMSO vehicle for 48 hours. The increase in REDD1 was expected to result in a decrease in phosphorylation of mTOR, which would inhibit proliferation. After 24 hours of treatment with both calcitriol and cholecalciferol we see a significant *increase* in phosphorylation of mTOR (Figure 3.12). After 48 hours the phosphorylation of mTOR returns to control levels. Phosphorylation of mTOR results in its

activation, which leads to downstream signaling that encourages cellular proliferation. This helps explain the increase in proliferation seen in the WST-1 assay.

To probe whether the activation of mTOR had effects downstream, we also looked at the phosphorylation of its targets, P70-S6K and 4EBP. Neither treatment had a significant effect on P70-S6K phosphorylation, however an increasing trend is seen at 24 hours ($p < 0.2$). The treatment groups return to levels of controls after 48 hours (Figure 3.13). Next we looked at 4EBP. Neither treatment had an effect on phosphorylation of this protein after 24 hours. However, after 48 hours of treatment both calcitriol and cholecalciferol had a significant increase in 4EBP phosphorylation. This clearly helps to explain the increase in cellular proliferation we see in response to treatment with both forms of vitamin D_3. Phosphorylation of 4EBP results in its inactivation, and removes its inhibition of the translation initiation factor, 4E. This results in the recruitment of the translation machinery and assembly of the ribosome. Therefore, 4EBP is essential in controlling the rate of translation in the cell, and its inactivation removes inhibition of protein synthesis. This, in turn, can contribute to an increase in cellular proliferation.

As mentioned, we also treated cells with AICAR as a positive control. AICAR phosphorylates AMPK, which is a sensor of cellular energy status. An increase in p-AMPK signals a decrease in ATP/ADP ratio, and results in inhibition of proliferation. One way this happens is by direct control of mTOR activation. Protein analysis via western blot yielded a significant decrease in phosphorylation of mTOR, P70-S6K, and 4EBP following 48 hours of AICAR treatment (Figure 3.15). This is essential to explain the decrease in proliferation due to AICAR, but not Vitamin D_3, treatment seen in the WST-1 assay. When the mTOR pathway is inhibited

proliferation can also be inhibited. However, more research is needed in order to prove causality, because AICAR may control other pathways as well.

Under conditions in our laboratory, vitamin D_3 treatment increased the proliferation of HaCaT cells. AICAR treatment showed that cell proliferation was capable of being inhibited in the media formulation used. Neither form of Vitamin D_3 decreased Gli1 transcript levels, indicating that they do not inhibit the Hedgehog pathway in these conditions. This helped us understand why proliferation was not inhibited, but to explain why it was increased required analysis of the mTOR pathway. AICAR treatment also showed that the mTOR pathway was intact, and inhibition of this pathway may inhibit proliferation. Real-time PCR data showed that REDD1 transcript was increased by cholecalciferol, and most likely by calcitriol as well (p = 0.08). An increase in REDD1 should result in a decrease in mTOR phosphorylation, but we saw the opposite effect. Therefore, the dysfunction seen in the inhibition of proliferation by Vitamin D_3 must be presenting at the level of the inhibition of mTOR by REDD1. Somehow calcitriol and cholecalciferol treatment either block the negative regulation of REDD1 on mTOR, or they impact another pathway that activates mTOR, and this activity overwhelms the negative regulation coming from REDD1. Either way, the negative regulation of vitamin D_3 on cellular proliferation is reversed due to insensitivity of mTOR to REDD1, and activation of the mTOR pathway.

The results presented here suggest that vitamin D_3 treatment increases proliferation of HaCaT cells under conditions in our laboratory. This goes against most current research, which generally supports the anti-proliferative activity of vitamin D_3. Early research from the 1990's suggested that high calcium concentrations may render calcitriol proliferative in these cells. Our media contains 1.8 mM calcium, which corresponds to these high concentrations. Therefore, the

dysregulation seen in the current study may be occurring due to an interaction between vitamin D_3 and extracellular calcium. However, several current studies culture cells in media containing 1.8 mM calcium, yet still show that vitamin D_3 has anti-proliferative properties.[12,25]

It should be noted that this research does not condemn the use of vitamin D_3 in cancer treatment or nutritional supplementation. The effects vitamin D_3 has on bone remodeling, immune function, and cell proliferation are vast and varied. Normal, physiological concentrations of vitamin D_3 are essential for proper functioning of cells, tissues, organs, and organ systems. The ability to increase concentrations of vitamin D_3 are also essential to protect against stress from the environment, such as UV rays. However, this research shows that over-supplementation is not always beneficial. Under HaCaT cell culture conditions in our laboratory, too much of a good thing turned out to be bad.

Future Directions

In order to determine whether these results are affected by the formulation of the media, more experiments are required. The next logical experiments would include serum deprivation to control for the effects of growth factors in the media. Also desirable are experiments performed in the presence of lower concentrations of calcium.

One interesting question that arose from this project is why treatment had no effect on control of the Hedgehog pathway. I hypothesize that vitamin D_3 only inhibits Gli1 expression when it is over-expressed. Testing this would involve either mutating PTCH or Smo, or transgenically over-expressing Gli1. Doing so would help explain why our data disagree with what so many other studies suggest.

The main concern of the current study is that vitamin D_3 treatment increased proliferation of these cells, when we expected to see a decrease. I hypothesize that this is caused by the extreme increase in 24-hydroxylase gene expression. The skin is unique in its ability to synthesize and activate vitamin D_3, because it is directly exposed to the UV light and it possesses all of the enzymes required for its conversion to calcitriol. This can lead to a rapid accumulation of calcitriol, which other organs do not experience. Other organs gradually obtain calcitriol from the blood, after it has been activated in the liver and kidneys. For this reason, the skin also needs to be able to inactivate calcitriol rapidly. Treating skin cells with pharmacologic doses of vitamin D_3 resulted in a several thousand-fold increase in 24-hydroxylase. I propose that in addition to inhibition of calcitriol, 24-hydroxylase has other oncogenic effects. I hypothesize that 24-hydroxylase or its product, $1\alpha,24,25(OH)_3$ D_3, is involved in one or more signal transduction pathways that lead to increased proliferation. Testing this would first involve silencing 24-hydroxylase and looking for proliferation inhibition following vitamin D_3 treatment. Determining its direct effect would involve the analysis of the status of several signaling pathways, including the RAS/RAF/MEK/ERK and PI3K/AKT pathways.

The present study needs to be repeated in primary cells to confirm that our results are true under even more accurate physiological conditions. While HaCaT cells are a good model for the skin, they are not the best. Unfortunately, however strong an *in vitro* model may be, the results obtained may never truly replicate the influence of organ systems *in vivo*. Due to the limitations of cell culture, the only true way to test our results is to sample human tissue.

REFERENCES

1. “Skin Cancer: Basal and Squamous Cell.” *Cancer.Org.* The American Cancer Society, 20 Feb 2014. Web. 24 Feb 2015.

2. American Cancer Society. *Cancer Facts and Figures 2014.* Atlanta: American Chemical Society; 2014.

3. Guy GP Jr., Machlin SR, Ekwueme DU, Yabroff KR. Prevalence and Costs of Skin Cancer Treatment in the U.S., 2002-2006, and 2007-2011. *Am J Prev Med* 2015; 48(2):183-187.

4. Nitzki F, Becker M, Frommhold A, Schulz-Schaeffer W, Hahn H. Patched knockout mouse models of basal cell carcinoma. *Journal of Skin Cancer* 2012; vol. 2012; Article ID 907543, 11 pages.

5. Bikle DD. Vitamin D and cancer: the promise not yet fulfilled. Endocrine 2014; 46:29-38.

6. Bikle DD, Elalieh, H, Welsh J, Oh D, Cleaver J, Teichert A. Protective role of vitamin D signaling in skin cancer formation. *J Steroid Biochem* 2013; 136:271-279.

7. Ellison TI, Smith MK, Gilliam, AC, MacDonald PN. Inactivation of the vitamin D receptor enhances susceptibility of murine skin to UV-induced tumorigenesis. *J Invest Dermatol* 2008 October; 128(10):2508-2517.

8. Teichert AE, Elalieh H, Elias PM, Welsh J, Bikle D. Overexpression of hedgehog signaling is associated with epidermal tumor formation in vitamin D receptor-null mice. *J Invest Dermatol* 2011; 131:2289-2297.

9. Deeb KK, Trump DL, Johnson, CS. Vitamin D Signaling pathways in cancer: potential for anticancer therapeutics. *Nat Rev Cancer* 2007; 7:684-700.

10. Hunter DJ, Colditz GA, Stampfer MJ, Rosner B, Willett WC, Speizer FE. Diet and risk of basal cell carcinoma of the skin in a prospective cohort of women. *Ann Epidemiol* 1992; 2(3):231-239.

11. van Dam RM, Huang Z, Giovannucci E, Rimm EB, Hunter DJ, Colditz GA, Stampfer MJ, Willett WC. Diet and basal cell carcinoma of the skin in a prospective cohort of men. *Am J Clin Nutr* 2000; 71(1):135-141.

12. Tang JY, Parimi N, Wu A, Boscardin WJ, Shikany JM, Chren MM, Cummings SR, Epstein EH Jr., Bauer DC. Inverse association between serum 25(OH) vitamin D levels and non-melanoma skin cancer in elderly men. *Cancer Cause Control* 2010; 21:387-391.

13. Asgari MM, Tang J, Warton EM, Chren MM, Quesenberry CP Jr., Bikle D, Horst RL, Orentreich N, Vogelman JH, Friedman GD. Association of prediagnostic serum vitamin D levels with the development of basal cell carcinoma. *J Invest Dermatol* 2010; 130(5):1438-1443.

14. Eide MJ, Johnson DA, Jacobsen GR, Krajenta RJ, Rao DS, Lim HW, Johnson CC. Vitamin D and nonmelanoma skin cancer in a health maintenance organization cohort. *Arch. Dermatol* 2011; 147(12):1379-1384.

15. Vitamin D. (n.d.) in Encyclopedia Brittanica online. Retrieved from http://www.britannica.com/EBchecked/topic/631106/vitamin-D.

16. Christakos S, Ajibade DV, Dhawan P, Fechner AJ, Mady LJ. Vitamin D: Metabolism. *Endocrin Metab Clin* 2010; 39(2):243-253.

17. Nemazannikova N, Antonas K, Dass CR. Vitamin D: Metabolism, molecular mechanisms, and mutations to malignancies. *Mol Carcinogen* 2014; 53:421-431.

18. Schuessler M, Astecker N, Herzig G, Vorisek G, Schuster I. Skin is an autonomous organ in synthesis, two-step activation and degradation of vitamin D_3: CYP27 in epidermis completes the set of essential vitamin D_3-hydroxylases. *Steroids* 2001; 66:399-408.

19. Lisse TS. and Hewison M. Vitamin D: A new player in the world of mTOR signaling. *Cell Cycle* 2011; 10(12):1888-1889.

20. Lisse TS, Liu T, Irmler M, Beckers J, Chen H, Adams JS, Hewison M. Gene targeting by the vitamin D response element binding protein reveals a role for vitamin D in osteoblast mTOR signaling. *FASEB J* 2011; 25:937-947.

21. Castellano E and Downward J. RAS interaction with PI3K: More than just another effector pathway. *Genes Cancer* 2011; 2(3):261-274.

22. Wei L and Xu Z. Cross-signaling among phosphinositide-2 kinase, mitogen-activated protein kinase and sonic hedgehog pathways exists in esophageal cancer. *Int J Cancer* 2011; 129:275-284.

23. Khasawneh R. Desensitization of basal cell carcinoma to the anti-tumoral effect of vitamin D. Diss. Wayne State University. 2013.

24. Rozenchan PB, Folgueira MAAK, Katayama MLH, Snitcovsky IML, Brentani MM. Ras activation is associated with vitamin D receptor mRNA instability in HC11 mammary cells. *J Steroid Biochem* 2004; 92:89-95.

25. Uhmann A, Niemann H, Lammering B, Henkel C, Heb I, Nitzki F, Fritsch A, Prüfer N, Rosenberger A, Dullin C, Schraepler A, Reifenberger J, Schweyeer S, Pietsch T, Strutz F, Schulz-Schaeffer W, Hahn H. Antitumoral effects of calcitriol in basal cell carcinomas

involve inhibition of hedgehog signaling and induction of vitamin D receptor signaling differentiation. *Mol Cancer Ther* 2011; 10:2179-2188.

26. Tang JY, Xiao TZ, Oda Y, Chang KS, Shpall E, Wu A, So P, Hebert J, Bikle D, Epstein Jr EH. Vitamin D3 inhibits hedgehog signaling and proliferation in murine basal cell carcinomas. *Cancer Prev Res* 2011; 4:744-751.

27. Wang Y, Ding Q, Yen C, Xia W, Izzo JG, Lang J, Li C, Hsu J, Miller SA, Wang X, Lee D, Hsu J, Huo L, LaBaff AM, Liu D, Huang T, Lai C, Tsai F, Chang W, Chen C, Wu T, Buttar NS, Wang K, Wu Y, Wang H, Ajani J. The crosstalk of mTOR/S6K1 and hedgehog pathways. *Cancer Cell* 2012; 21:374-387.

28. Mazumdar T, DeVecchio J, Agyeman A, Shi T, Houghton JA. The GLI genes as the molecular switch in disrupting hedgehog signaling in colon cancer. *Oncotarget* 2011; 2:638-645.

29. Mills LD, Zhang Y, Marler RJ, Herreros-Villanueva M, Zhang L, Almada LL, Couch F, Wetmore C, Pasca di Magliano M, Fernandez-Zapico ME. Loss of the transcription factor GLI1 identifies a signaling network in the tumor microenvironment mediating KRAS oncogene-induced transformation. *J Biol Chem* 2013; 288:11786-11794.

30. Philpott MP, Bergamaschi D, Storey A. Models for Skin Cancer. The Cancer Handbook. 2007.

31. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploidy human keratinocyte cell line. *J Cell Biol* 1988; 106:761-771.

32. Saha AK, Persons K, Safer JD, Luo Z, Holick MF, Ruderman NB. AMPK regulation of the growth of cultured human keratinocytes. *Biochem Bioph Res Co* 2006; 349:519-524.

33. Gniakecki R. Stimulation versus inhibition of keratinocyte growth by 1,25-dihydroxyvitamin D_3 : dependence on cell culture conditions. *J Invest Dermatol* 1996; 106:510-516.

34. Garach-Jehoshua O, Ravid A, Liberman U, Koren R. 1,25-Dihydroxyvitamin D_3 increases the growth-promoting activity of autocrine epidermal growth factor receptor ligands in keratinocytes. *Endocrinology* 1999; 140(2):713-721.

35. Bikle DD, Ng D, Tu C-L, Oda Y, Xie Z. Calcium- and vitamin D-regulated keratinocyte differentiation. *Mol Cell Endocrinol* 2001; 177:161-171.

36. Bollag WB, Ducote J, Harmon CS. Biphasic effect of 1,25-dihydroxyvitamin D_3 on primary mouse epidermal keratinocyte proliferation. *J Cell Physiol* 1995; 163:248-256.

37. Chen K-S, DeLuca HF. Cloning of the human 1 α, 25-dihydroxyvitamin D-3 24-hydroxylase gene promoter and identification of two vitamin D-response elements. *Biochim Biophys ACTA* 1995; 1263:1-9.

38. Kato S. The function of the vitamin D receptor in vitamin D action. *J Biochem* 2000; 127:717-722.

39. Luo W, Hershberger PA, Trump DL, Johnson CS. 24-hydroxylase in cancer: Impact of vitamin D-based anticancer therapeutics. *J Steroid Biochem* 2013; 136:252-257.

40. Ly LH, Zhao X-Y, Holloway L, Feldman D. Liarozole acts synergistically with 1α, 25-dihydroxyvitamin D_3 to inhibit growth of DU 145 human prostate cancer cells by blocking 24-hydroxylase activity. *Endocrinology* 1999; 140(5):2071-2076.

ABSTRACT

DESENSITIZATION OF HACAT KERATINOCYTES TO VITAMIN D_3 OCCURS VIA LOSS OF REDD1 REGULATION OF THE MTOR PATHWAY

by

MICHELLE LEIGH JONES

May 2015

Advisor: Dr. Ahmad R. Heydari

Major: Nutrition and Food Science

Degree: Master of Science

Vitamin D_3 treatment has long been considered in the treatment of cancer due to its well-documented anti-proliferative effects *in vitro*. While clinical studies have been positive in other cancers, results are inconclusive in cases of basal cell carcinoma. To better understand the reasons underlying this disconnect, this study employs an immortalized human keratinocyte cell line (HaCaT) to observe the effects of vitamin D_3 treatment on cellular proliferation. The results show that both activate vitamin D_3 (calcitriol) and its precursor (cholecalciferol) increase proliferation of these cells. Real-time PCR and western blot data indicate that the mTOR pathway becomes activated despite increased VDR signaling and expression of REDD1. Treatment was also unable to inhibit the Hedgehog signaling pathway.

AUTOBIOGRAPHICAL STATEMENT

I earned a Bachelor of Science in Microbiology from The Pennsylvania State University in 2006. Following graduation I began work as a research assistant at the Fraunhofer Institute of Cell and Molecular Biotechnology in Newark, Delaware. There I worked on multiple vaccine development projects, and gained a tremendous amount of skills. I worked at Fraunhofer for four years, but the learning experience was the equivalent of many more. In 2010 I moved to Detroit, Michigan and began working as a research assistant in the Emergency Medicine Basic Research Laboratory in the Wayne State University School of Medicine. While there, I took advantage of the free education offered to me and began working on a Master of Science degree in Nutrition and Food Science. In September of 2014 I earned the Graduate Professional Scholarship, and was able to join the department as a full-time student.

By returning to school for this degree, I have developed considerably more than I anticipated. I am much more independent, and immensely more confident in my abilities than ever before. I have a new passion for teaching that makes the career ahead of me ever more exciting. The decision to go back to school was not an easy one, but it will surely be remembered as one of my best.